INTEGRATED CONTROL OF ENDOTHELIAL VASOREACTIVITY

Y.J.H.J. Taverne

Integrated control of endothelial vasoreactivity

Cover: Y.J. Taverne

Printing: Optima Grafische Communicatie, Rotterdam, the Netherlands

ISBN: 978-94-6361-012-4

Thesis Erasmus Medical Center, Rotterdam.

Integrated Control of Endothelial Vasoreactivity

Geïntegreerde Controle van Endotheliale Vaatreactiviteit

Proefschrift

ter verkrijging van de graad van doctor aan

de Erasmus Universiteit Rotterdam

op gezag van de rector magnificus

Prof. dr. H. Pols

en volgens besluit van het College van Promoties.

De openbare verdediging zal plaatsvinden op

vrijdag 1 december 2017 om 11.30

door

Yannick J.H.J. Taverne

geboren te Roeselare, België

Erasmus University
Rotterdam

Promotiecommissie

Promotor:	Prof. dr. D.J.G.M. Duncker
	Prof. dr. A.J.J.C. Bogers
Leescommissie:	Prof. dr. J.J.M. Takkenberg
	Dr. E.G. Mik
	Dr. S. Verheule
Copromotor:	Dr. D. Merkus

The studies in this thesis have been conducted at the Laboratory for Experimental Cardiology and at the Department of Cardiothoracic Surgery, Thorax Center, Rotterdam, The Netherlands.

The studies described in this thesis were supported by a grant of the Dutch Heart Foundation (DHF 2000T042). The publication of this thesis is further financially supported by the Dutch Heart Foundation and gratefully acknowledged.

To my mother – in memoriam.

Table of Contents

1

GENERAL INTRODUCTION AND OUTLINE OF THIS THESIS

THE CIRCULATORY SYSTEM

The endothelium forms an essential part of the vasculature and upholds an "atheroprotective" environment through complementary actions of endothelial cell-derived vasoactive factors and its environment. Endothelial cells are involved in many aspects of vascular biology and impaired function is seen as a hallmark for a wide variety of cardiovascular diseases. The overall vascular, but also myocardial response, is the result of a complex interaction of vasoactive pathways which vary dependent on the type of vascular system and cardiovascular pathophysiology. However, before we can discuss the necessity of this dynamic organ lining the vessel wall and elucidate its integrated control in cardiovascular biology, we need to address the necessity for a closed vascular system and the development of a discrete barrier between blood and vessel wall in complex life.

Evolution

Larger body size in animal evolution mandates changes in structural design [1]. In small organisms, metabolic demands can be met by the process of diffusion to supply oxygen (O_2) and nutrients, and to remove carbon dioxide. Despite the fact that diffusion is energetically inexpensive, it is a very slow process and works only over small distances (diffusion path <1 mm) [2]. However, with increasing complexity and size, the diffusional process is compromised. Therefore, the possibility of growth is limited by the pathway of O_2 and nutrient delivery. It has been well established that a change in body size disproportionally decreases the ratio of surface area to volume as surface area increases in proportion to the radius squared, whereas its volume increases more rapidly [1]. Thus, at some point, organisms will reach their maximum size above which their metabolic demands can no longer be met by diffusion. One possibility to overcome the diffusional distance, and thus still be a simple multicellular organism, is to increase surface area (by incorporating folded areas) to assume optimal diffusional body geometry (e.g. diploblastic animals) or by minimizing metabolic demands [3].

With increased size, development of an internal transport and exchange system that reaches every cell of the body (i.e. a circulatory system) was mandatory. Evidence suggests that the vascular system first appeared, as a means to overcome the time-distance constraints of diffusion, in an ancestor of the triploblasts over 600 million years ago [1]. A circulatory system is any system of moving fluids that reduces the functional diffusion distance that nutrients, gases, and metabolic waste products must traverse regardless of its embryological origin or its design [1,3]. It consists of

blood-filled (or endo-lymph in insects) spaces (e.g. vessels, sinuses, hemocoels, and/or pumping organs) within the connective tissue compartment, which is continuous around and between all tissue layers in the body [2]. Blood is pumped around by the heart in this circulatory system which not only occurs in all vertebrates, but also in a wide variety of invertebrates (e.g. annelids, cephalopods) [4]. Blood is confined to a space that is physically separated from the intercellular fluid or body cells. In invertebrates, these blood-filled spaces are lined only by a matrix. However, within evolution, a true vertebrate hallmark was the development of a secondary (endothelial) cell lining in blood vessels and appeared some 540-510 million years ago. These endothelial cells express baso-apical polarity and contribute to optimize flow dynamics, to barrier function and to localize immune and coagulation functions [1, 4]. Exchange takes place in specialized areas such as capillary beds (low-flow) and surface contact is maximized to optimize the possibility of diffusion.

Vascular anatomy

Blood vessels provide the main link between the heart and tissues (Figure 1). There are three major types of blood vessels: the arteries, carrying blood away from the heart; the capillaries, which enable the actual exchange of water and nutrients between the blood and tissues; and the veins, carrying blood back towards the heart. All parts of the circulatory system that are in direct contact with blood, including the inner lining of the heart, are paved with endothelium. The internal layer of all blood vessels, the tunica intima, is a single layer of simple squamous endothelial cells glued by a polysaccharide intercellular matrix, surrounded by a thin layer of sub-endothelial connective tissue, interlaced with a number of circularly arranged elastic bands, called the internal elastic lamina. The middle layer, which is absent in capillaries, is the thickest layer in arteries and called the tunica media. It consists of circularly arranged elastic fibers, connective tissue, polysaccharide substances and vascular smooth muscle. The tunica media is separated from the outer layer, the tunica externa, by an external elastic lamina. The tunica externa is entirely made of connective tissue and contains nerves and vasa vasorum in the larger blood vessels.

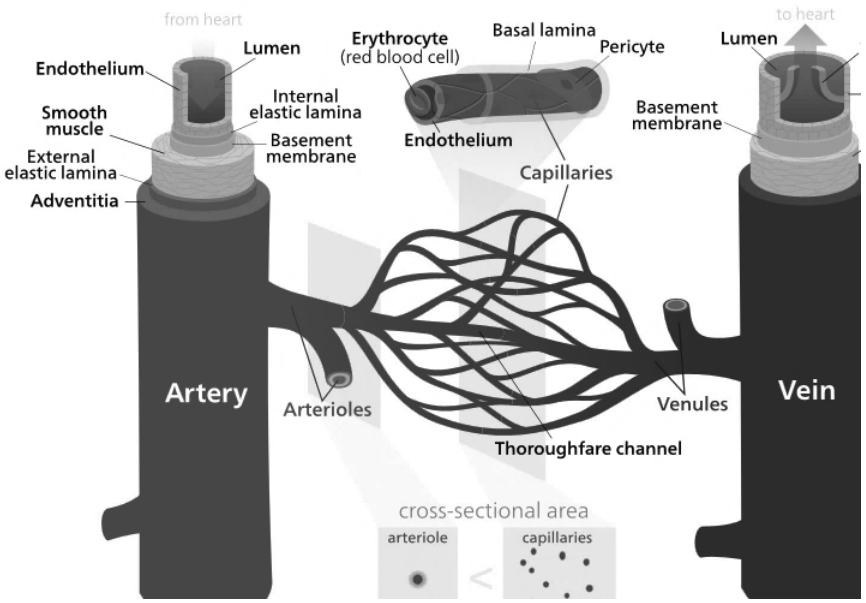


Figure 1: The circulatory system.

Vascular Physiology

The physiology of a closed circuit, and more important the separation between a systemic and pulmonary circulation, as found in mammals, was first correctly described by William Harvey (1578-1657) [5]. Both systems receive the same input from the heart with each beat (stroke volume), however, resistance of the pulmonary and systemic vascular circuits are very different, resulting in markedly lower blood pressures in the pulmonary as compared to the systemic vasculature. In order to pump blood through the vasculature, the heart pumps approximately once every second without interruption. This continuous action requires a lot of energy and the heart thus consumes a large amount of O_2 and nutrients. These are supplied through the coronary circulation. The importance of this circulation is underlined by the fact that 5% of the cardiac output flows through the coronary vasculature and 60-80% of O_2 is extracted from the blood during a single passage through the heart; contrary to the 30-40% extraction in the body as a whole [6, 7]. The fact that O_2 and nutrient extraction varies throughout the body and must meet metabolic demands, which change with external factors mandating higher delivery (such as exercise), emphasizes the importance of the vascular system to be able to alter blood flow via

O_2 induced electromechanical coupling of the smooth musculature through the endothelial mediator [8, 9].

HEMODYNAMICS

Regulation of vascular tone, thus alterations of resistance by changes in diameter, is the key step in regulation of blood flow. Blood flow is a function of pressure and resistance which is determined by two factors, namely pressure difference (ΔP) between two ends of a vessel and resistance to blood flow (R). Blood flow can be described by Darcy's law or Ohm's law (as used for current: I = V/R)

$$F = \Delta P / R$$

Where F (sometimes depicted as Q) is the blood flow, ΔP the pressure gradient (or perfusion pressure) and R the resistance to flow offered by the blood vessel and its interactions with the flowing blood. Importantly, ΔP is the cause of flow; without pressure difference there would be no flow, while resistance is the cause of the pressure drop over the course of the vessel.

Resistance causes pressure drops across the vascular system and resistance is relatively small in large arteries. Small arteries have moderate resistance to flow, while being highest in the arterioles [10]. Therefore, pressure drop is highest across the terminal part of small arteries and arterioles (Figure 2).

Resistance vessels make pressure drops from ~100 to 30 mmHg and based on Darcy's law, high resistance in these vessels increases the arterial pressure and decreases capillary pressure [10]. Both systems are physiologically imperative as high arterial pressure makes sure blood reaches all parts of the body (e.g. the head), while low capillary pressure prevents damage to capillaries and makes transport across the vessel wall feasible.

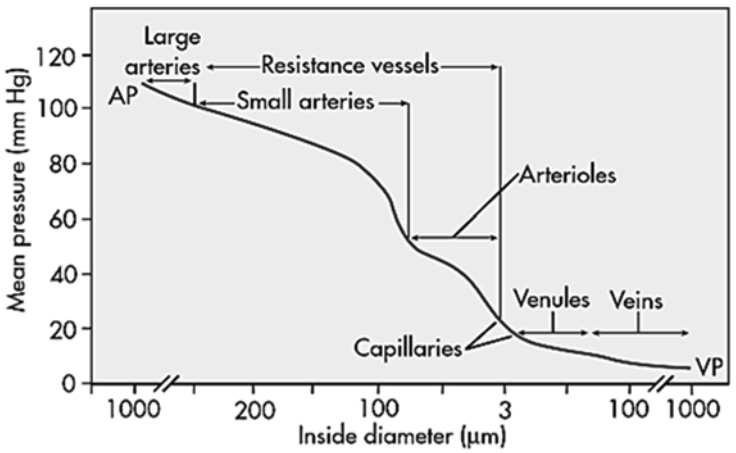


Figure 2. Pressure drop across the vascular system in the hamster cheek pouch. AP, mean arterial pressure; VP, venous pressure. From Berne and Levy [11].

A key feature of vessel physiology is the ability to contract with changes of pressure. To be able to change its diameter, and thus increase volume with increased transmural pressure ($P_{inside} - P_{outside}$), is called vascular compliance and can be quantified by the change in volume (ΔV) divided by the change in pressure (ΔP):

$$C = \Delta V / \Delta P$$

Slight changes in diameter of a blood vessel cause tremendous changes in the ability of vessels to conduct blood. Conductance (being the reciprocal of resistance) is often used in cardiovascular research and can be seen as the measure of blood flow through a vessel for a given pressure difference. The relationship between conductance and blood vessel diameter can be described by Pouiseuille's law, where Q is the rate of blood flow, ΔP the pressure difference between the ends of the vessel, r the radius of the vessel, l the length of the vessel and ŋ the viscosity of the blood:

$$Q = (\pi \Delta P r^4) / 8 \eta l$$

For all biological systems, pressure-volume relation curves towards the volume axis meaning that by increasing volume or pressure, stiffness increases (Figure 3).

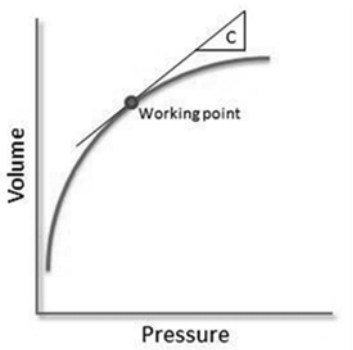


Figure 3. Pressure-volume relationship. The change of volume per one unit change of pressure is called compliance C = ΔV / ΔP.

However, when analyzing the relationship between pressure and volume, the slope is not linear mostly due to the heterogeneous tissue in the vessel wall (Figure 3 & 4) [12]. Thus, compliance decreases with higher pressures and volumes. As evidenced in veins, with compliance being 20-fold that of arteries (Figure 4), large volume changes can be can accommodated with a small change in pressure. Nevertheless, this effect disappears at high pressures and volumes.

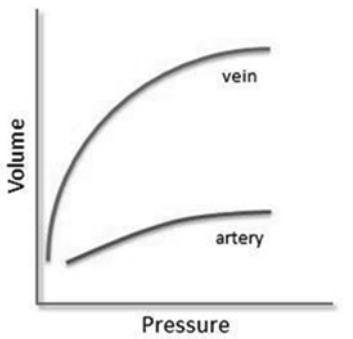


Figure 4. Compliance curve for artery and vein. The slope of the curve represents the compliance. At low pressures, venous compliance is 10-20 times greater than arterial compliance, however, both compliances are similar at high pressures.

Compliance increases with declines in vascular tone, nevertheless, when interpreting pressure-volume relationship, it is important to realize that compliance

is depicted as static compliance and therefore does not show the initial changes in pressure. In fact, when a constant volume is added, there is a so called 'stress relaxation' depending on the viscous properties of biological tissues. Thus, compliance of vessels is also dependent upon the rate of volume change constituting the dynamic component of compliance [12].

Vascular tone

Vessel diameter is set by the contractile state (tone) of the smooth muscle cells (SMCs) in the vascular wall. In general, vasodilation occurs if the basal tone or resting vasoconstrictor tone (if present) decreases, and/or vasodilator nerves (if present) are activated (Figure 5 and 10). On the other hand, vasoconstriction follows an increase in basal or reflex vasoconstrictor tone.

Basal vascular tone differs among organs with a large vasodilatory capacity (e.g., myocardium, skeletal muscle, skin, splanchnic circulation) which have a high vascular tone, and organs with relatively low vasodilatory capacity (e.g., cerebral and renal circulations) which have a low vascular tone. Vascular tone is determined by many different competing vasoconstrictor and vasodilator influences acting on the blood vessel (Figure 5 & 6) [10,12].

These influences can be devided into extrinsic and intrinsic factors with main regulation of the arterial blood pressure (altering systemic vascular resistance) and local blood flow within an organ respectively. The intricate balance between vasoconstrictor and vasodilator influences dictates vascular tone.

One important extrinsic mechanism regulating vascular diameter operates through autonomic regulation of blood vessels. In general, sympathetic adrenergic influences cause resistance vessels (both arteries and veins) to partially constrict under basal conditions. Also, a myriad of circulating vasoactive substances are responsible for the adaptation of vascular tone to the metabolic demands (Figure 5).

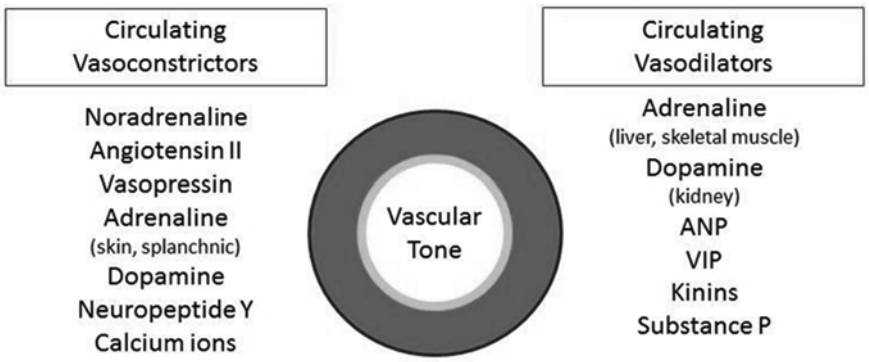


Figure 5. Circulating vasodilators and vasoconstrictors regulating vascular tone. ANP, atrial natriuretic peptide; VIP, vasoactive intestinal peptide.

On the other hand, organs are able to regulate their own blood flow which is mediated by vasoactive substances released from the surrounding tissue and vascular endothelium controlling local blood flow (intrinsic mechanism) (Figure 6).

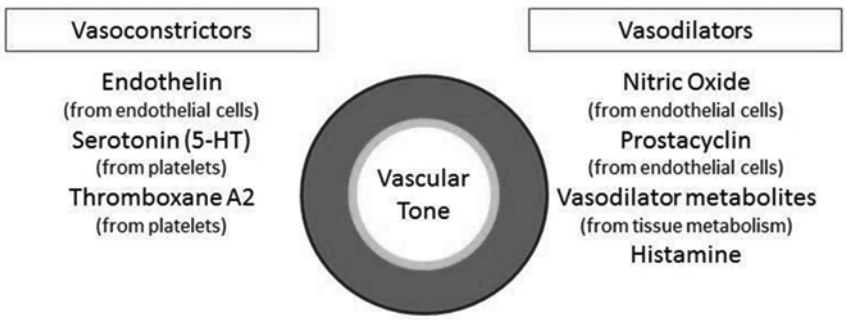


Figure 6. Intrinsic regulatory mechanism of the vascular tone.

CIRCULATION

Systemic circulation is defined by high pressures and high resistance, while the pulmonary circulation constitutes a low pressurized system with low resistance. This means that the capacity of the vascular bed lying in between is very compliant with

a high capacity for blood volume. Changes in capacity will reflect in pressure changes on both sides of the heart. Since evolutionary pressure favored a closed pressurized system, based on the law of Pascal, pressures are propagated thereby linking, for example, pulmonary to left atrial pressure (e.g. Swan Ganz measurements).

Systemic circulation

Blood is pumped into the aorta under a mean arterial pressure of about 80-90mmHg and returns to the right atrium at a pressure of about -5 mmHg. Given that flow remains the same, this enormous pressure drop requires a systemic vascular resistance which is about ten times higher than the pulmonary circulation (see below) [7]. Within the systemic circulation, greater arteries function as blood conduits. However, upon division into microcirculation, vascular resistance increases containing up to 80% of the total resistance. The increase in cardiac output during exercise, together with the large pressure drop, reflects a much higher vascular resistance as compared to the pulmonary vascular bed.

The systemic circulation ensures supply of O_2 and nutrients, and the removal of CO_2 and other waste products. The actual exchange takes place in the capillary bed and at rest about 45% of O_2 is extracted from the body to accommodate the body's O_2 demand [13]. Blood is distributed throughout the body according to local needs. By adjusting their resistance locally through physical factors, the chemical environment and neurohumoral effectors, all organs and muscles can be adequately supplied with O_2. In other words, distribution of blood flow is stratified to each organ/muscle so not all organs increase their perfusion simultaneously. Cardiac output and its distribution is mainly regulated by balancing the activity of the sympathetic and parasympathetic nervous system. With exercise, sympathetic tone increases shifting blood flow from abdominal organs towards active musculature [14,15]. However, the significant increase in muscular O_2 demand during exercise, cannot be met by redistribution alone. In order to meet these high metabolic demands, myocardial blood flow must increase dramatically. So, besides the increase in cardiac output leading to increased muscular perfusion (up to 50 times) [16], a marked vasodilation in skeletal muscle appears accounting for 70-80% of the decrease in systemic vascular resistance during exercise [17]. Moreover, skeletal muscle O_2 extraction increases from ~30% to ~70% during severe exercise [18]. Consequently, whole body O_2 extraction increases from the basal 45% to up to ~70% [13].

Pulmonary circulation

The most important function of the pulmonary circulation is to exchange CO_2 for O_2 in the blood. Blood is pumped into the lungs from the right side of the heart. It encompasses a low pressure system and in order for the entire cardiac output to flow through the lungs it only requires a pressure drop of 7 mmHg. Ergo, the pulmonary vascular bed is a highly compliant system and comprises low vascular resistance. Exercise causes a significant increase in mean pulmonary pressure (up to ~40 mmHg) [19] resulting in part from increased left arterial pressure which is transmitted back to the lungs as a result of the closed nature of the system. Exercise also results in increased cardiac output which contributes further to the increase in pulmonary artery pressure. In order to limit increased pulmonary pressures, in humans pulmonary vascular resistance can drop by up to 50% during exercise [17].

Coronary circulation

Coronary circulation can be seen as an evolutionary 'trade-off' among vertebrates, where mammals, reptiles, and avian all have complete coronary systems [20]. There are two ways of myocardial oxygenation; one by removing O_2 from blood passing through the lumen of the heart; the other by supplying blood directly to the cardiac muscle from the outside [21]. Mammals and adult birds have evolved such dependence to the coronary circulation that interruption of blood flow for even a few minutes leads to permanent cardiac damage or even death [20]. This 'special' circulation originates from the aortic root through the right and left coronary artery and thus constitutes a high pressure system. It receives about 5% of the resting cardiac output, even though the heart constitutes less than 0.5% of the total body weight [10, 12]. The continuous rhythmic activity of the heart and the ensuing high metabolic demand require a more or less continuous supply of O_2 to the cardiac muscle [7]. Cardiac metabolism highly depends on oxidative energy production, thus upon higher metabolic needs, O_2 must be readily available. At rest, O_2 consumption, normalized per gram of myocardium, is already 20-fold higher than that of skeletal muscle [7]. As an adaptation to the high O_2 demands, the heart maintains a very high level of O_2 extraction so that 70–80% of the arterially delivered O_2 is extracted, compared with 30–40% in skeletal muscle [7,15,22]. Regulation of coronary vascular resistance is the result of a balance between a myriad of vasodilator and vasoconstrictor signals exerted by neurohormonal influences, the endothelium, and metabolic signals from the myocardium (Figure 5 & 6). Because of the high level of O_2 extraction by the myocardium during resting conditions, increases in O_2 demand

are mediated principally by an increase in coronary blood flow (active hyperemia) thereby matching coronary blood supply to the requirement of oxygen and nutrients [7].

Matching coronary vasomotor tone to myocardial metabolism is best studied by examining the relationship between coronary venous O_2 tension and myocardial O_2 consumption [23,26]. For example, an increase in coronary resistance vessel tone will limit coronary blood flow and hence the O_2 supply, thereby forcing the myocardium to increase O_2 extraction, with a consequent reduction in coronary venous O_2 levels. Conversely, a decrease in coronary tone will increase blood flow and O_2 supply to the heart; if O_2 consumption remains constant, O_2 extraction will decrease and coronary venous O_2 levels will increase. Thus coronary venous oxygen tension is an index of tissue oxygenation that reflects the balance between the oxygen supply and demand of the heart and is ultimately determined by the coronary resistance vessel tone [7,25].

In this thesis, focus lies on endothelial factors that control vasoreactivity and will be discussed below.

THE ENDOTHELIUM

Endothelial anatomy

The endothelium originates from the mesoderm, which is the middle layer of embryological cells, inserted between the ectoderm and endoderm. Blood cells and vessels, connective tissue and muscles all arise from the mesodermal lineage. Being an intricate part of the vessel wall, the endothelial lining consists of a thin layer of simple squamous cells aligning the inner surface of all blood and lymphatic vessels, but also the inner surface of the heart. The endothelial layer is separated from the surrounding outer layers by a basal lamina (Figure 7 & 8).

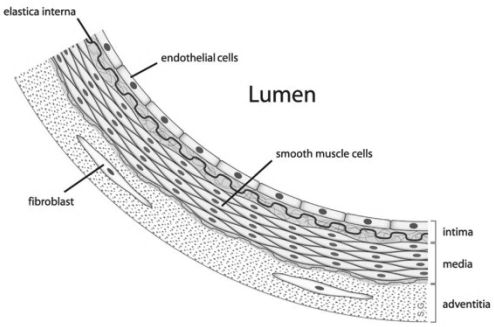


Figure 7. Cross-section of a vessel wall. The inner lining constitutes the endothelium.

Blood vessel diameter differs throughout the body with altered density of connective and smooth muscle, however, always with a continuous endothelial lining. For example, capillaries and sinusoids are nothing more than endothelial cells with a basal lamina (Figure 7 and 8) and some functionally important pericytes. These are cells of the connective-tissue family, related to vascular smooth muscle cells, which wrap themselves around small vessels. In its entirety, the endothelium is composed of ~1-6x10^{13} endothelial cells covering a surface of more than a 1000 square meter [4,6,17].

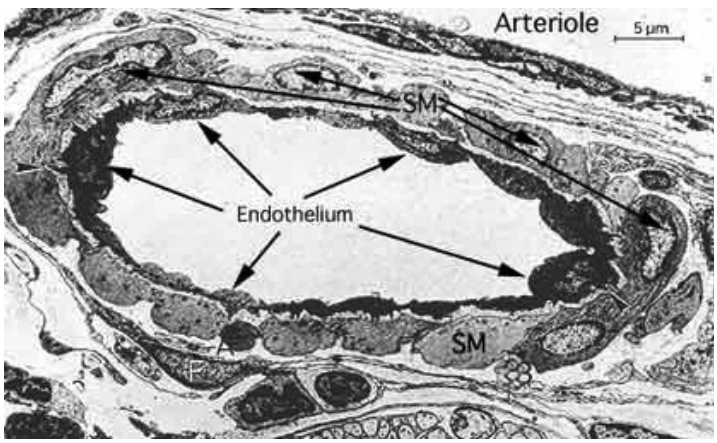


Figure 8. Electron micrograph of a cross section of arteriole. The wall is formed by a single endothelial cell surrounded by a basal lamina.

Endothelial function

Endothelial cells are involved in many aspects of vascular biology. First, they act as a semi-selective barrier between the vessel lumen and the surrounding tissue, thereby controlling passage of molecules and transit of white blood cells in and out the blood stream [27]. Consequently, they are involved in inflammation, where the excessive permeability leads to edema. Also, the endothelium aligns all surfaces that come in contact with blood, thereby providing a non-thrombotic surface. Under physiological conditions, endothelial cells are involved in the modulation of metabolic homeostasis (trophic function), vascular hemodynamics (tonic function), vascular permeability, coagulation and cell extravasation (trafficking). In a quiescent state, endothelial cells balance the release of various vasodilating (e.g. nitric oxide (NO)) or vasoconstricting factors (e.g. endothelin (ET), reactive oxygen species (ROS)) to maintain vascular tone, blood flow and blood pressure [28,29]. Due to its intricate role in vasomotor function, endothelial dysfunction forms the hallmark for vascular disease.

Endothelial dysfunction is characterized by a shift of endothelial reactivity towards reduced vasodilation and a pro-inflammatory / prothrombic state [29]. Endothelial dysfunction can result from and/or contribute to several disease processes, such as hypertension, coronary artery disease, chronic heart failure, peripheral artery disease and diabetes (Figure 9) [29]. Of importance is that every injury to the arterial endothelium represents a primary event in atherogenesis.

One of the main hallmarks of endothelial dysfunction is reduced NO-production, often due to high levels of asymmetric dimethylarginine (an endogenous NO inhibitor), interfering with normal L-arginine-stimulated NO synthesis [30,31]. Mechanisms that contribute to the reduced vasodilatory responses in endothelial dysfunction include reduced NO generation, oxidative excess, and reduced production of the hyperpolarizing factor [30, 31]. Also, up-regulation of adhesion molecules, generation of chemokines such as macrophage chemoattractant peptide-1, and production of plasminogen activator inhibitor-1 participate in the inflammatory response and contribute to a prothrombic state [30,31]. Further, vasoactive peptides such as angiotensin II (Ang II) and ET-1, but also the accumulation of asymmetric dimethylarginine and the diminished NO concentration – thus no more natural antagonism for ET-1- all contribute to this downwards spiral of impaired vasomotor biology.

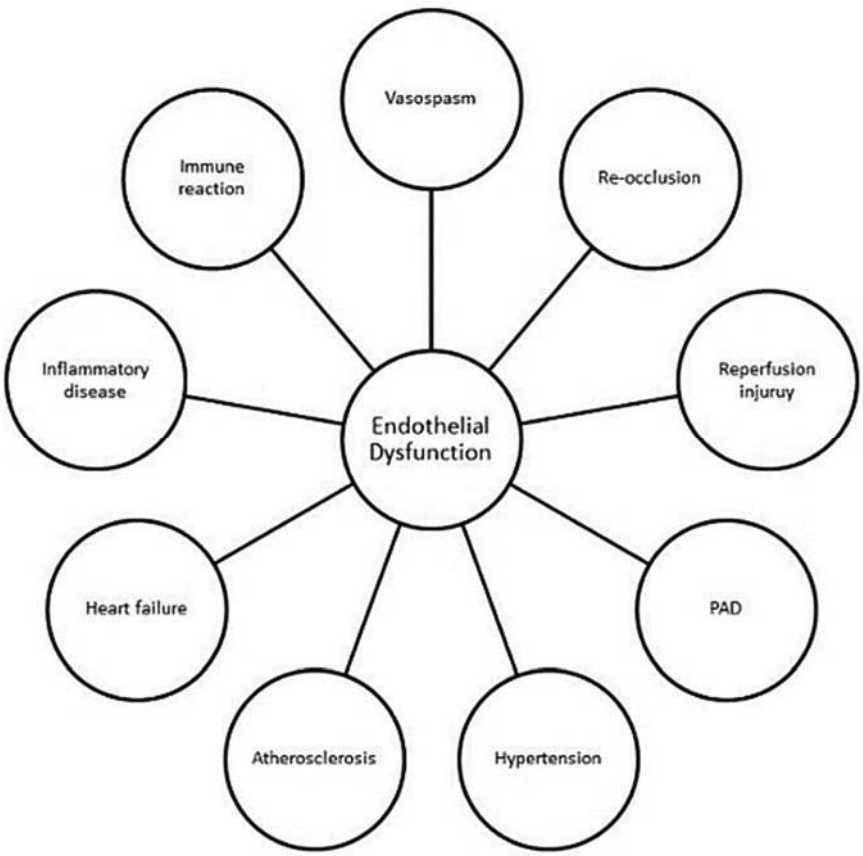


Figure 9. Overview of pathological conditions associated with endothelial dysfunction. PAD, peripheral artery disease.

In light of this thesis, we will focus on the main endothelial derived vasodilator and vasoconstricting factors (marked by the inner rectangle in figure 10).

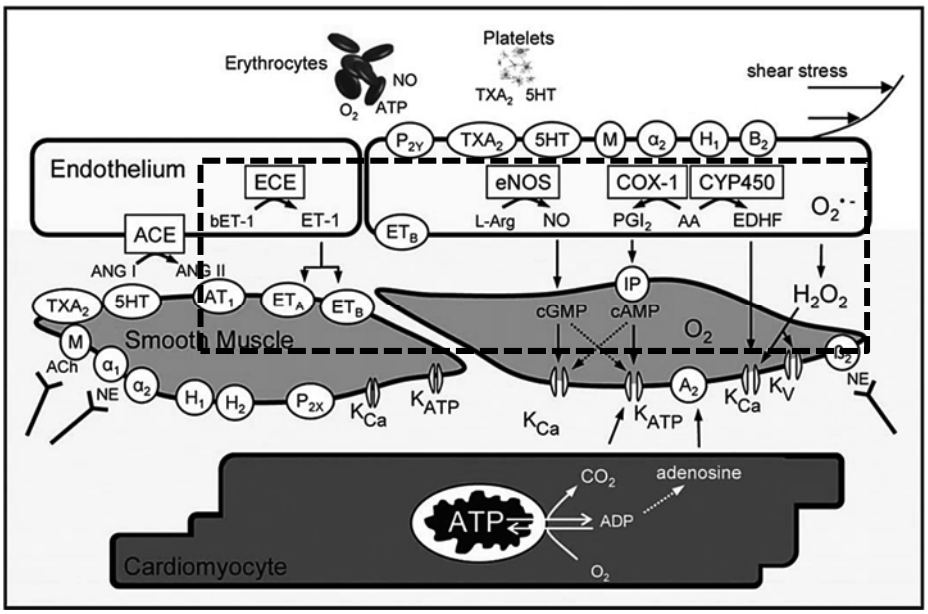


Figure 10. Schematic drawing of a coronary arteriole and the various influences that determine coronary vasomotor tone and diameter. Po_2, oxygen tension; TxA_2, thromboxane A_2 (receptor); 5HT, serotonin or 5-hydroxytryptamine (receptor); P_{2X} and P_{2Y}, purinergic receptor subtypes 2X and 2Y that mediate ATP-induced vasoconstriction and vasodilation, respectively; ACh, acetylcholine; M, muscarinic receptor; H_1 and H_2, histamine receptors type 1 and 2; B_2, bradykinin receptor subtype 2; ANG I and ANG II, angiotensin I and II; AT_1, angiotensin II receptor subtype 1; ET, endothelin; ET_A and ET_B, endothelin receptor subtypes A and B; A_2, adenosine receptor subtype 2; ß$_2$, ß$_2$-adrenergic receptor; α_1 and α_2, α-adrenergic receptors; NO, nitric oxide; eNOS, endothelial NO synthase; PGI_2, prostacyclin; IP, prostacyclin receptor; COX-1, cyclooxygenase-1; EDHF, endothelium-derived hyperpolarizing factor; CYP_{450}, cytochrome P_{450} 2C9; K_{Ca}, calcium-sensitive K^+ channel; K_{ATP}, ATP-sensitive K^+ channel; K_V, voltage-sensitive K^+ channel; AA, arachidonic acid; l-Arg, l-arginine; O2·-, superoxide. Receptors, enzymes, and channels are indicated by an oval or rectangle, respectively. With permission from Duncker et al [7].

Key Endothelial Players

Under normal circumstances, healthy endothelium promotes vascular smooth muscle cell relaxation (vasodilation) through elaboration of substances such as NO and prostacyclin, predominating over endothelial vasoconstrictors. Dysfunctional endothelium (e.g. atherosclerotic vessels) secretes reduced amounts of vasodilators causing the balance to shift toward vasoconstriction (Figure 10).

Endothelin-1

ET-1 is a 21 amino acid peptide and formed from a 39 amino acid precursor; big ET [32]. This potent vasoconstrictor is produced through actions of the endothelin converting enzyme (ECE) which is found on the endothelial cell membrane [33] and is expressed in the body in three isoforms. Endothelial cells only release ET-1 by converting Big-ET-1 to ET-1 [34]. ET-1 production is stimulated by a number of molecules, such as AngII, antidiuretic hormone, thrombin, cytokines, ROS, and inflammation [16,33,35]. Shear stress initially promotes ET-1 production, however, eventually leading to decreased ET-1 production [31]. Inhibition of ET-1 release is provided through prostacyclin, atrial natriuretic peptide and NO. Upon release, ET-1 binds to receptors (ET_A and $ET_{B1\ and\ B2}$) on the target tissue to exert its function [31]. Distribution of ET-1 receptors is dependent on the type of vascular bed with ET_A and ET_{B2} predominantly identified on smooth muscle cells and ET_{B1} on endothelial cells [31,36]. Stimulation of ET_A and ET_{B2} receptors increases inositol trisphosphate (IP_3) concentrations which release Ca^{2+} from the sarcoplasmic reticulum with consequent smooth muscle contraction [31,32]. Activation of ET_{B1} receptors on the endothelial surface leads to vasodilation by releasing NO and PGI_2 [31,37] (Figure 11).

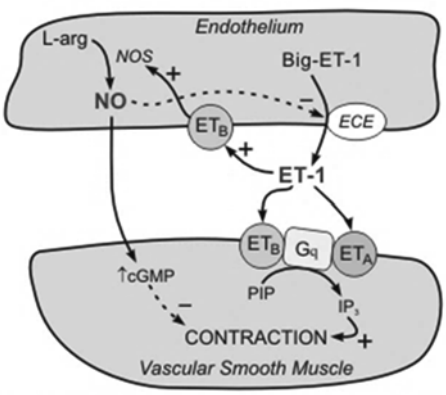


Figure 11. Endothelin-1 formation and secondary messenger pathways. PIP, phosphatidylinositol 4,5-bisphosphate; IP3, Inositol trisphosphate; cGMP, Cyclic guanosine monophosphate; ECE, Endothelin-converting enzyme. From Klabunde 2012 [32].

Besides receptor mediated reactions, ET-1 can also directly affect cardiovascular function by stimulating aldosterone secretion, thereby decreasing renal blood flow and glomerular filtration rate, and releasing atrial natriuretic peptide [16].

Addition to being a potent vasoconstrictor, studies have shown that ET-1 is also a potent stimulator of oxidative stress [38,39] with production of vascular superoxide ($O_2^{\cdot-}$) and consequential decrease in NO concentration [40,41]. More specific, ET_A-receptor stimulation causes production of $O_2^{\cdot-}$ by NADPH oxidase-dependent and –independent mechanisms [38, 42, 43]. $O_2^{\cdot-}$ binds rapidly to NO to form peroxynitrite ($ONOO^-$), thereby reducing bioavailability of the endothelium-derived NO. In addition, it adversely affects the activity of soluble guanylyl cyclase, one of the main targets of NO in the vascular wall [44]. Thus, ET-1 can, through the production of superoxide anions, silence its inhibition by endothelium-derived NO [45].

Nitric oxide

In the late 1970s, Furchgott and Zawadzki found that acetylcholine released a vasodilating substance in vessels with an intact endothelium. They initially called this substance endothelium-derived relaxing factor (EDRF), however, by 1986 Furchgott had worked out EDRF's nature and mode of action and determined that EDRF was in fact NO [32]. His research eventually led to the Nobel prize for physiology in 1998 [46].

Under normal, basal conditions, blood vessels continually produce NO. There are three isoforms of the nitric oxide synthase (NOS) enzyme: endothelial (eNOS), neuronal (nNOS) and inducible (iNOS). The neuronal and endothelial isoform are both calcium- and calmodulin-dependent and, when stimulated, involve release of calcium ions from subsarcolemmal storage sites [29]. In order to form NO, the NOS enzyme oxidizes the guanidine group of L-arginine thereby consuming five electrons and resulting in the creation of NO with the stoichiometric formation of L-citrulline. The process involves oxidation of NADPH and reduction of molecular oxygen and transformation occurs at a catalytic site adjacent to a specific binding site of L-arginine. Activation of these enzymes can take place through two different mechanisms. First, there is receptor-stimulated NO formation where, for example, acetylcholine, bradykinin, substance-P and adenosine, can stimulate Ca^{2+} release with subsequent NO production. On the other hand, increases in blood flow also activate eNOS ensuing NO production (flow-dependent NO formation) [31]. It is important to recognize this Ca^{2+} dependency as a reduction of Ca^{2+} causes the

calcium-calmodulin complex to dissociate from eNOS, which in turn will bind to caveolin turning eNOS into its inactivated state [31,47].

Upon formation, NO quickly reacts with $O_2^{\cdot-}$ (as both molecules have unpaired electrons making them highly reactive) thereby diminishing its half-life to a few seconds and making $O_2^{\cdot-}$ able to reduce NO bioavailability [46,48]. NO also avidly binds to the heme moiety of hemoglobin where it is subsequently broken down. The remaining NO diffuses across the endothelium into the adjacent vascular smooth muscle cells where it binds to and thus activates guanylyl cyclase (Figure 12). This enzyme increases the conversion rate guanosine triphosphate (GTP) to cGMP [31,32]. cGMP reduces Ca^{2+} release from the sarcoplasmic reticulum but also helps to restore Ca^{2+} to the sarcoplasmic reticulum so both actions lead to relaxation [12] (Figure 12). cGMP also activates K^+ channels, leading to hyperpolarization and relaxation of vascular smooth muscle cells [12].

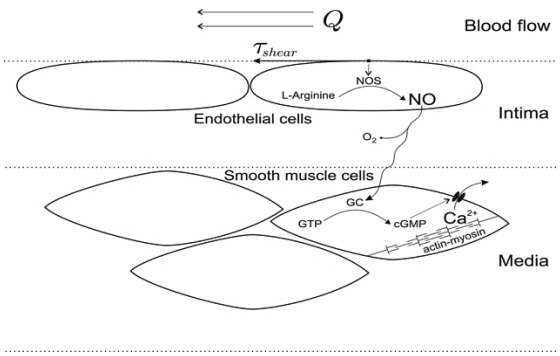


Figure 12. NO formation and secondary messenger pathways. NO, nitric oxide; NOS, nitric oxidide synthase; GTP, guanosine triphosphate; GC, Guanylate cyclase; cGMP, cyclic guanosine monophosphate.

In sum, through different pathways, NO has many vascular actions such as a direct vasodilation (flow dependent and receptor mediated), an indirect vasodilation by inhibiting vasoconstrictor influences (e.g., inhibits angiotensin II and sympathetic vasoconstriction), an anti-thrombotic effect (NO inhibits platelet adhesion to the vascular endothelium), an anti-inflammatory effect (NO inhibits leukocyte adhesion to vascular endothelium and scavenges superoxide anion), and an anti-proliferative effect (NO inhibits smooth muscle hyperplasia) [12,32].

Oxidative and nitrosative stress

ROS are chemically reactive molecules derived from molecular oxygen and formed as a natural by-product of aerobic metabolism [28]. During energy conversion, ROS are produced as a by-product of oxidative phosphorylation, which is presumed to be the major source of $O_2^{\cdot-}$ production [49,50]. ROS can also be produced through a variety of enzymes including xanthine oxidase and NAD(P)H oxidase [51]. ROS encompass free radicals, oxygen ions, and peroxides, both organic and inorganic, but all derived from molecular oxygen. They are formed as necessary intermediates in a variety of normal biochemical reactions [28,51]. Only when produced in excess or not appropriately controlled, can they inflict damage within the body.

Reactive nitrogen species (RNS) are a family of molecules derived from $^{\bullet}NO$ and $O_2^{\cdot-}$ causing nitrosative stress [52-54]. Under normal circumstances, ROS/RNS concentrations are tightly controlled by anti-oxidants, keeping them in the picomolar range [51]. These low concentrations of ROS enable their role as messengers in signal transduction for vascular homeostasis and cell signaling [29]. When excessively produced, or when antioxidants are depleted, ROS/RNS can inflict damage onto lipids, proteins, and DNA. This intracellular reduction-oxidation imbalance, called oxidative and nitrosative stress, can subsequently contribute to the development and/or progression of cardiovascular diseases such as atherosclerosis, ischemia-reperfusion injury, chronic ischemic heart disease, cardiomyopathy, congestive heart failure, and even ensuing arrhythmias [48,52-55].

AIM AND OUTLINE OF THIS THESIS

Aim

As outlined above, there is intricate interplay between NO, ET and ROS, that together form an important regulatory system for vascular function. Disruption of this balance is thought to play a major role in development and progression of vascular dysfunction and consequently in many forms of cardiovascular disease. Despite great progress in the field of free radical biology and advances in cardiovascular medicine, we still do not have a complete understanding of the underlying mechanisms of cardiovascular disease and consequences of pathophysiologically elevated ROS in cardiovascular tissue.

Consequently, the aim of this thesis was to investigate the interrelationship of these pathways under different conditions of endothelial (dys)function in animal models and patients (Figure 13).

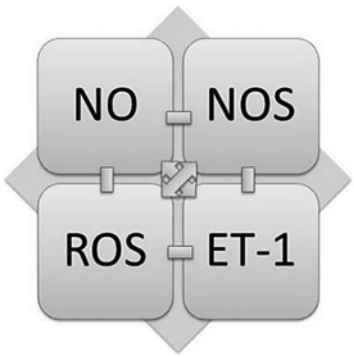


Figure 13. Interrelationship of different vasodilator and vasoconstrictor pathways presenting the scope of this thesis. NO, nitric oxide; NOS, nitric oxide synthase; ROS, reactive oxygen species; ET-1, endothelin-1.

It is very hard to study these systems in vivo since most pathways are working in the picomolar range, molecules have a very short half-life and many balancing systems exist in order to maintain vascular homeostasis. Therefore, with every experiment, we focused on the diagram above and tried to analyze one or more pathways under different conditions. In order to amplify the effects, we either changed the condition or the outcome, thereby mimicking loss of endothelial integrity or creating a change in endothelial function. By doing so, we aimed to determine the effects in health and disease in animal studies and extrapolate and implement these results in a clinical setting.

In order to amplify the "endothelial derived signature", all animal experiments were also performed during incremental levels of exercise. Exercise is the most important physiological stimulus for increasing myocardial O_2 demand. The requirement of exercising skeletal muscle for increased blood flow is met by vasodilation of resistance vessels in the skeletal muscle, which requires an increase in cardiac output, and is facilitated by an increase in arterial pressure. The hemodynamic adjustments, resulting in increased cardiac output and arterial pressure during exercise, cause increases in each of the major determinants of myocardial oxygen demands: heart rate, contractility, and ventricular work [7]. It can be hypothesized that when O_2 demand is high, oxidative and nitrosative stress are apparent and many pathways are activated in order to maintain cellular homeostasis.

The general aim of this thesis was to study the endothelial vasomotor effects when either mimicking or creating (temporary) endothelial dysfunction. In order to test the effects of oxidative and nitrosative stress and their relationship with NO and ET-1, a number of experimental settings were created, as summarized in figure 2.

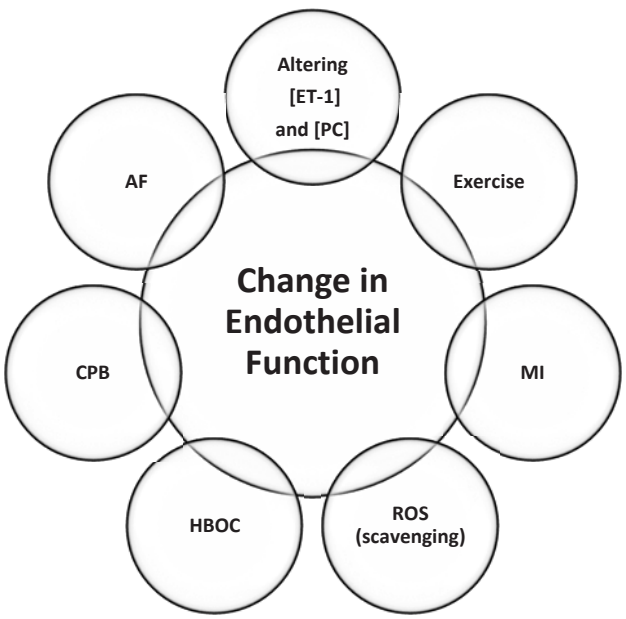


Figure 14. Summary of this thesis. All experiments were designed to either change endothelial function or study the effects of endothelial dysfunction. AF, atrial fibrillation; CPB, cardiopulmonary bypass; HBOC, hemoglobin-based oxygen carrier; ROS, reactive oxygen species; MI, myocardial infarction; ET-1, endothelin1; PC, prostacyclins.

Outline

Chapter 2 starts off with a more philosophical outline beginning with the question that if ROS are so deleterious, why did complex life adapt to an aerobic state for energy household, rather than using alternative means of energy transfer? It is an imperative timeline transcending the convergent focus on ROS, but also implementing broader knowledge in order to fully comprehend the vastness of oxygenic life and its perks.

In Chapter 3, this overall aspect of an evolved mitochondrial energy transfer system was implemented in cardiovascular physiology and pathophysiology. Here we tried to summarize different pathways used in cellular homeostasis, but also when endothelial dysfunction is apparent. More specific, we addressed the question whether altering oxidative states could be beneficial in the clinical setting.

ET-1 and NO are natural antagonists and key players in vascular basal tone. With exercise, O_2 consumption increases and vasomotor control must respond accordingly by enhancing blood flow. We previously demonstrated that ET mediated coronary vasoconstriction wanes with increasing exercise intensity via NO- and prostacyclin dependent mechanism in healthy subjects (57). Therefore, in Chapter 4, we investigated weather waning of ET coronary vasoconstriction is the result of decreased production of ET and/or decreased ET receptor sensitivity and tried to analyze whether other mechanisms, like oxidative stress and alteration in NO-ET balance, could lead to enhanced ET production.

Another aspect of vasomotor physiology is the presence of ROS. It is well established for many years that ROS play an important part in vasomotor tone. ROS are formed in mammalian cells as a consequence of aerobic respiration. Despite multiple conserved redox modulating systems, a given proportion of ROS continuously escape from the mitochondrial respiratory chain, being sufficiently potent to damage cells in various ways. Consequently, we hypothesized that, during exercise, this naturally occurring mitochondrial electron leakage and possibly other ROS sources, would increase oxidative stress thereby possibly hampering vasomotor action. So, in Chapter 5, we designed a study, in reaction to different statements, whether using AO is beneficial (see Chapter 3), to determine the involvement of ROS in exercise hyperemia in healthy swine.

In Chapter 6, we implement a cell free oxygen carrier (HBOC-201) to mimic transient endothelial dysfunction. HBOC's are presumed to be the holy grail of emergency medicine, however, clinical use has been hampered by the subsequent pressor effects. HBOC's would have certain advantages over human red cells, including rapid

and widespread availability, fewer requirements with regard to storage, transport, and compatibility testing, a longer shelf life, and a more consistent supply. An ideal substitute would be less antigenic than allogenic red cells, and would have less risk of disease transmission. Ideally, a HBOC would provide vital tissues and organs with oxygen transport after major hemorrhage in the field, and before typed and cross-matched blood is available for transfusion. However, a major drawback of administrating HBOC, is the apparent transient hypertension mediated by scavenging NO (58, 59). In Chapter 6, by using such an agent, we tried to determine interactions of the quadripartite ET-NO-ROS-RNS and possibly augment the effects due to increments of exercise. We mimic endothelial dysfunction by introducing an NO scavenger and thereby possibly disrupting the NO-ET homeostasis in healthy swine.

Previous results from our laboratory already suggested that in diseased endothelium, vasomotor reactions are not solely due to increased ET release, but also due to altered release of NO. In light of these observations, in Chapter 7 we investigated whether blunting of this ET-mediated constriction was attributed to NO or possibly other molecules, such as prostanoids. Also, after myocardial infarction, oxidative stress increases, favoring the imbalance and resulting in coronary vasoconstriction. Consequently, we hypothesized that ROS scavenging results in coronary vasodilation, particularly after MI, and ROS production is enhanced after inhibition of NO production (Chapter 8).

Assuming nitroso-redox balance is of major importance in pathophysiology, we extended this rationale to the clinical setting and postulated that the same vasoactive substances could be accounted responsible for a major post-operative co-morbidity, such as atrial fibrillation. 40-60% of post-cardiac surgery patients develop atrial fibrillation, where endothelial and myocardial nitroso-redox balance is assumed to play a major role. Disrupting this delicate endothelial-derived state due to the use of cardiopulmonary bypass, superposed on pre-existing (cardio)vascular disease, could possibly lead to a pro-arythmogenic state and consequent upregulation of ET (Chapter 9).

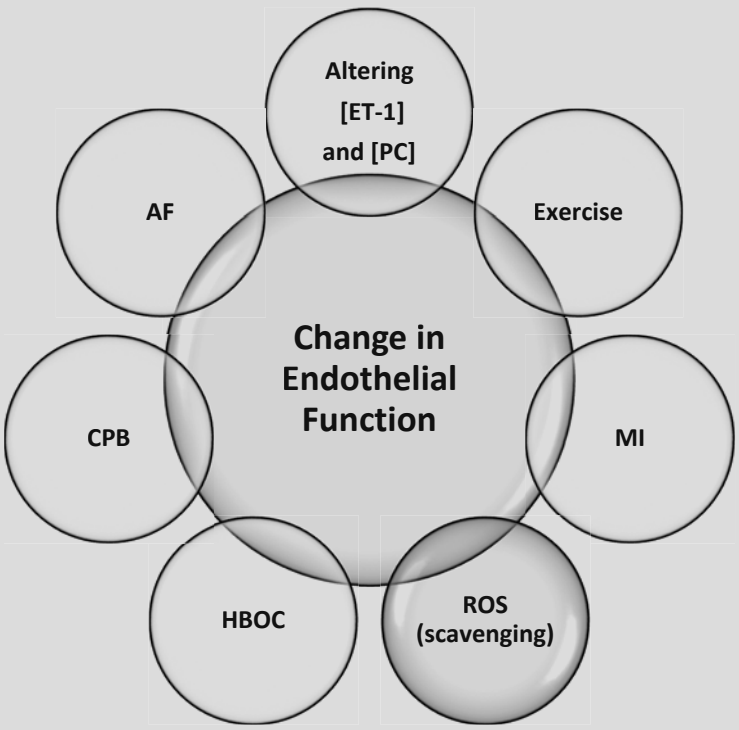
REFERENCES

1. Monahan-Earley R, Dvorak AM, Aird WC. Evolutionary origins of the blood vascular system and endothelium. J Thromb Haemost. 2013;11 Suppl 1:46-66.
2. EE Ruppert KC. Morphology of metazoan circulatory systems. Zoomorphology. 1983;103(3):193-208.
3. Banavar JR, Cooke TJ, Rinaldo A, Maritan A. Form, function, and evolution of living organisms. Proc Natl Acad Sci U S A. 2014;111(9):3332-7.
4. W. Burggren AFaHL. Vertebrate Cardiovascular Systems. Library WO, editor2011.
5. Ribatti D. William Harvey and the discovery of the circulation of the blood. J Angiogenes Res. 2009;1:3.
6. Barrett Kim E. BS, Barman Susan M. and Brooks Heddwen L. Ganong's Review of Medical Physiology: The McGraw-Hill Companies, New York; 2010.
7. Duncker DJ, Bache RJ. Regulation of coronary blood flow during exercise. Physiological reviews. 2008;88(3):1009-86.
8. Jackson WF. Ion channels and vascular tone. Hypertension. 2000;35(1 Pt 2):173-8.
9. Filosa JA. Vascular tone and neurovascular coupling: considerations toward an improved in vitro model. Front Neuroenergetics. 2010;2.
10. Lilly LS. Pathophysiology of heart disease : a collaborative project of medical students and faculty. 5th ed. Philadelphia, Pa. ; London: Lippincott Williams & Wilkins; 2010.
11. M.N. L, B.M. K, B.A. S. Principles of Physiology: Elsevier; 2007.
12. Opie LH. Heart physiology : from cell to circulation. 4th ed. Philadelphia: Lippincott Williams & Wilkins; 2004. xix, 648 p. p.
13. Haitsma DB, Bac D, Raja N, Boomsma F, Verdouw PD, Duncker DJ. Minimal impairment of myocardial blood flow responses to exercise in the remodeled left ventricle early after myocardial infarction, despite significant hemodynamic and neurohumoral alterations. Cardiovasc Res. 2001;52(3):417-28.
14. Stubenitsky R, Verdouw PD, Duncker DJ. Autonomic control of cardiovascular performance and whole body O2 delivery and utilization in swine during treadmill exercise. Cardiovasc Res. 1998;39(2):459-74.
15. Laughlin MH. Cardiovascular response to exercise. The American journal of physiology. 1999;277(6 Pt 2):S244-59.
16. MH Laughlin RK, DJ Duncker and RJ Bache. Handbook of Physiology, Exercise: Regulation and Integration of Multiple Systems.: Wiley; 2011.
17. Taylor JRaA. Handbook of Physiology. Exercise: regulation and integration of multiple systems. Shepherd LRaJ, editor: Am Phys Soc; 1996.

18. Andersen P, Saltin B. Maximal perfusion of skeletal muscle in man. J Physiol. 1985;366:233-49.
19. Merkus D, de Beer VJ, Houweling B, Duncker DJ. Control of pulmonary vascular tone during exercise in health and pulmonary hypertension. Pharmacol Ther. 2008;119(3):242-63.
20. Reese DE, Mikawa T, Bader DM. Development of the coronary vessel system. Circ Res. 2002;91(9):761-8.
21. D S, T W. Ontogeny and Phylogeny of the Vertebrate Heart: Springer; 2012.
22. Feigl EO. Coronary physiology. Physiological reviews. 1983;63(1):1-205.
23. Bache RJ, Dai XZ, Herzog CA, Schwartz JS. Effects of nonselective and selective alpha 1-adrenergic blockade on coronary blood flow during exercise. Circ Res. 1987;61(5 Pt 2):II36-41.
24. Duncker DJ, Merkus D. Acute adaptations of the coronary circulation to exercise. Cell Biochem Biophys. 2005;43(1):17-35.
25. Tune JD, Gorman MW, Feigl EO. Matching coronary blood flow to myocardial oxygen consumption. J Appl Physiol (1985). 2004;97(1):404-15.
26. Tune JD, Richmond KN, Gorman MW, Feigl EO. Control of coronary blood flow during exercise. Exp Biol Med (Maywood). 2002;227(4):238-50.
27. Alberts B, Johnson A, Lewis J. The Cell: Garland Science; 2002.
28. Halliwell B. Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life. Plant Physiol. 2006;141(2):312-22.
29. Taverne YJ, Bogers AJ, Duncker DJ, Merkus D. Reactive oxygen species and the cardiovascular system. Oxid Med Cell Longev. 2013;2013:862423.
30. Deanfield JE, Halcox JP, Rabelink TJ. Endothelial function and dysfunction: testing and clinical relevance. Circulation. 2007;115(10):1285-95.
31. Sandoo A, van Zanten JJ, Metsios GS, Carroll D, Kitas GD. The endothelium and its role in regulating vascular tone. Open Cardiovasc Med J. 2010;4:302-12.
32. Klabunde RE. Cardiovascular Physiology Concepts: Lippincott Williams & Wilkins; 2012.
33. Aagaard P, Simonsen EB, Andersen JL, Magnusson SP, Halkjaer-Kristensen J, Dyhre-Poulsen P. Neural inhibition during maximal eccentric and concentric quadriceps contraction: effects of resistance training. J Appl Physiol (1985). 2000;89(6):2249-57.
34. Alonso D, Radomski MW. The nitric oxide-endothelin-1 connection. Heart Fail Rev. 2003;8(1):107-15.
35. Luscher TF, Barton M. Endothelins and endothelin receptor antagonists: therapeutic considerations for a novel class of cardiovascular drugs. Circulation. 2000;102(19):2434-40.
36. Bacon CR, Cary NR, Davenport AP. Distribution of endothelin receptors in atherosclerotic human coronary arteries. J Cardiovasc Pharmacol. 1995;26 Suppl 3:S439-41.

37. Cardillo C, Kilcoyne CM, Cannon RO, 3rd, Panza JA. Interactions between nitric oxide and endothelin in the regulation of vascular tone of human resistance vessels in vivo. Hypertension. 2000;35(6):1237-41.
38. Callera GE, Tostes RC, Yogi A, Montezano AC, Touyz RM. Endothelin-1-induced oxidative stress in DOCA-salt hypertension involves NADPH-oxidase-independent mechanisms. Clin Sci (Lond). 2006;110(2):243-53.
39. Li L, Fink GD, Watts SW, Northcott CA, Galligan JJ, Pagano PJ, et al. Endothelin-1 increases vascular superoxide via endothelin(A)-NADPH oxidase pathway in low-renin hypertension. Circulation. 2003;107(7):1053-8.
40. Laplante MA, Wu R, Moreau P, de Champlain J. Endothelin mediates superoxide production in angiotensin II-induced hypertension in rats. Free Radic Biol Med. 2005;38(5):589-96.
41. Yao L, Kobori H, Rahman M, Seth DM, Shokoji T, Fan Y, et al. Olmesartan improves endothelin-induced hypertension and oxidative stress in rats. Hypertens Res. 2004;27(7):493-500.
42. Li L, Chu Y, Fink GD, Engelhardt JF, Heistad DD, Chen AF. Endothelin-1 stimulates arterial VCAM-1 expression via NADPH oxidase-derived superoxide in mineralocorticoid hypertension. Hypertension. 2003;42(5):997-1003.
43. Viel EC, Benkirane K, Javeshghani D, Touyz RM, Schiffrin EL. Xanthine oxidase and mitochondria contribute to vascular superoxide anion generation in DOCA-salt hypertensive rats. Am J Physiol Heart Circ Physiol. 2008;295(1):H281-8.
44. Stasch JP, Pacher P, Evgenov OV. Soluble guanylate cyclase as an emerging therapeutic target in cardiopulmonary disease. Circulation. 2011;123(20):2263-73.
45. De Mey JG, Vanhoutte PM. End o' the line revisited: moving on from nitric oxide to CGRP. Life Sci. 2014;118(2):120-8.
46. Furchgott RF. Endothelium-derived relaxing factor: discovery, early studies, and identification as nitric oxide. Biosci Rep. 1999;19(4):235-51.
47. Fleming I, Busse R. Signal transduction of eNOS activation. Cardiovasc Res. 1999;43(3):532-41.
48. Griendling KK, FitzGerald GA. Oxidative stress and cardiovascular injury: Part I: basic mechanisms and in vivo monitoring of ROS. Circulation. 2003;108(16):1912-6.
49. Chen Q, Vazquez EJ, Moghaddas S, Hoppel CL, Lesnefsky EJ. Production of reactive oxygen species by mitochondria: central role of complex III. J Biol Chem. 2003;278(38):36027-31.
50. Turrens JF. Mitochondrial formation of reactive oxygen species. J Physiol. 2003;552(Pt 2):335-44.
51. Droge W. Free radicals in the physiological control of cell function. Physiological reviews. 2002;82(1):47-95.

52. Papaharalambus CA, Griendling KK. Basic mechanisms of oxidative stress and reactive oxygen species in cardiovascular injury. Trends Cardiovasc Med. 2007;17(2):48-54.
53. Charles RL, Eaton P. Redox signalling in cardiovascular disease. Proteomics Clin Appl. 2008;2(6):823-36.
54. Vermeer F, Simoons MC, Lubsen J. Reduced frequency of ventricular fibrillation after early thrombolysis in myocardial infarction. Lancet. 1986;1(8490):1147-8.
55. Griendling KK, FitzGerald GA. Oxidative stress and cardiovascular injury: Part II: animal and human studies. Circulation. 2003;108(17):2034-40.
56. Sies H. Oxidative stress: oxidants and antioxidants. Exp Physiol. 1997;82(2):291-5.
57. Merkus D, Sorop O, Houweling B, Boomsma F, van den Meiracker AH, Duncker DJ. NO and prostanoids blunt endothelin-mediated coronary vasoconstrictor influence in exercising swine. Am J Physiol Heart Circ Physiol. 2006;291(5):H2075-81.
58. Yu B, Shahid M, Egorina EM, Sovershaev MA, Raher MJ, Lei C, et al. Endothelial dysfunction enhances vasoconstriction due to scavenging of nitric oxide by a hemoglobin-based oxygen carrier. Anesthesiology. 2010;112(3):586-94.
59. Yu B, Bloch KD, Zapol WM. Hemoglobin-based red blood cell substitutes and nitric oxide. Trends Cardiovasc Med. 2009;19(3):103-7.


Altering
[ET-1]
and [PC]
AF
Exercise
Change in
Endothelial
Function
CPB
MI
HBOC
ROS
(scavenging)

2

THE BIOLOGICAL IMPACT OF OXYGEN AND REACTIVE OXYGEN SPECIES FROM EARTH'S EARLY HISTORY TO THE ORIGINS OF COMPLEX LIFE

Y.J. Taverne, D. Merkus, A.J.Bogers, B. Halliwell, D.J. Duncker and T.W. Lyons

Submitted 2017

ABSTRACT

Introduction of O_2 to Earth's early biosphere stimulated remarkable evolutionary adaptations, however, also presented major challenges. Cellular respiration had to evolve early out of necessity to produce energy and a wide range of electron acceptors allowed diverse, energy-yielding metabolic pathways. Enzymatic reduction of O_2 yielded a several-fold increase in energy production, enabling evolution of multi-cellular animal life. However, O_2 and its free radicals are highly toxic, and evolution had to generate an arsenal of coping mechanisms possibly halting multicellular evolution after the Great Oxidation Event. Although the harnessing of chemical and thermo-dynamic properties of O_2 for aerobic metabolism was an evolutionary milestone, the ability to use reactive oxygen species (ROS) for cell signaling and regulation may have been similarly consequential. Moreover, ROS, and especially hydrogen peroxide, played a major part in the development of oxygenic pathways, thus becoming an intricate part of evolution of complex animal life.

INTRODUCTION

Oxygen (O_2) makes up nearly 21% of Earth's atmosphere today and, in terms of mass, is the third most abundant molecule in the Universe [1]. Anaerobic conditions of early Earth limited the opportunity for complex life to evolve. The unique chemical and thermodynamic properties of O_2 facilitate rapid reaction with molecules capable of one-electron transfer [2], and evolution of aerobic respiration enabled cells to produce many times more ATP than their ancestors that relied on anaerobic respiration [3]. However, oxidative phosphorylation resulted in the production of reactive oxygen species (ROS), free radicals (FRs), and reactive molecules derived from O_2. ROS are capable of producing oxidative stress (OS), leading to cell death and genetic damage [4, 5]. In response, anti-oxidant defense mechanisms evolved to reduce OS, but even more striking is the suggestion that ROS appear to have been adopted for cell signaling and regulation processes [6]. Moreover, hydrogen peroxide (H_2O_2) may have played a major role in evolution of life on Earth, and over the past ten years researchers have begun to embrace the concept that cellular redox state is as necessary in normal physiology as protein phosphorylation [2, 4, 5].

In order to fully understand their roles and overall importance in life, we must look at evolution and how ROS were generated early in Earth's history. With this goal in mind, we provide an overview of O_2-derived FRs, including details about their discoveries, chemistry, and physiological roles in the evolution of complex life. Our specific hope for this synthesis is to distill wide-ranging research and advances in the area of biology and physiology into a deeper understanding of the vital role of ROS in the development and diversification of oxidative metabolism.

DISCOVERY OF FREE RADICAL SPECIES

FRs were initially recognized in the first half of the 20th century as intermediates of chemical reactions and considered irrelevant to biology. Later, however, pioneers such as Otto Warburg and Leonor Michaelis suggested that O_2 is reduced during respiration—thereby creating radical intermediates [7, 8]. Despite these and other [9] reports, such advances were largely ignored until 1970 when Fridovich, McCord, and colleagues discovered superoxide dismutase (SOD) in mammals [10]. More specifically, these researchers showed for the first time that an enzyme for the dismutation of the superoxide radical ($O_2^{\cdot-}$) persisted through evolution, converting it to H_2O_2 in the process. The evolutionary preservation of dismutation enzymes suggests that radicals are constantly produced, even during normal physiology [4, 5].

Discovery of related intermediates followed quickly, including the hydroxyl radical ($OH^{\bullet}$) and hypochlorous acid, and ROS were shown to be produced during mitochondrial respiration for phagocytary killing and for xenobiotic metabolism [11]. The discovery of Fenton chemistry and OS were of particular importance in understanding the role of ROS in cellular (patho)physiology. Fenton chemistry is defined by reactions catalyzed by transition metals to produce $OH^{\bullet}$ (an extremely potent oxidant) from H_2O_2 [12]. The term OS, proposed by Helmut Sies in the 1980s [13], is defined by an imbalance between FRs and antioxidants and has been viewed as a purely pathological process for many years. ROS concentrations must be maintained in the picomolar range. Otherwise, DNA, lipids, and proteins are damaged, leading to cellular injury [4, 5]. This concept placed FRs, and hence an impaired antioxidant state, at the center of human pathophysiology, igniting a large number of studies exploring the protective effect of antioxidants in a variety of human diseases. Unfortunately, however, results of these studies are inconclusive [4], demanding a more careful look at the detrimental influence of ROS.

The notion that highly reactive intermediate products could act as signaling molecules was first accepted when nitric oxide ($NO^{\bullet}$) was discovered as a mediator of vascular regulation [4]. This discovery initiated a new area of research focused on radical intermediates—not just as toxic waste products but also as essential mediators of cellular physiology [11, 14]. For example, by acting as second messengers, oxidants have the power to regulate cellular pathways [2, 4]. Nevertheless, cellular redox chemistry remains incompletely understood.

CHEMISTRY OF O_2

In addition to its essential role in aerobic energetic metabolism, O_2 is involved in pathogenesis of many diseases due to the formation of ROS [4, 15, 16]. After almost complete transformation and elimination of O_2, in the form of CO_2 and H_2O, a small leakage of ROS remains under normal physiological conditions [5].

The term ‘oxygen’, first coined by Antoine Lavoisier, derives from the Greek ‘oxy’ and ‘genes’, which, when combined, translate to ‘acid forming’. The molecule O_2 was first discovered in 1772 by the chemist Carl Wilhelm Scheele, and as early as the late 18^{th} century, scientists reported on the dual beneficial and toxic effect of O_2 on living organisms. This duality is the result of the combination of two atoms in covalent bond to form O_2. The relatively high 402 kJ/mol of energy required to break the covalent bond underlies the beneficial effects of O_2, as it is stable marked by slow reaction rates with most other molecules. Because O_2 has two unpaired

electrons in its ground state (making it a triplet molecule and a FR), reactions of molecular oxygen are thermodynamically but not kinetically favored (Figure 1) [2].

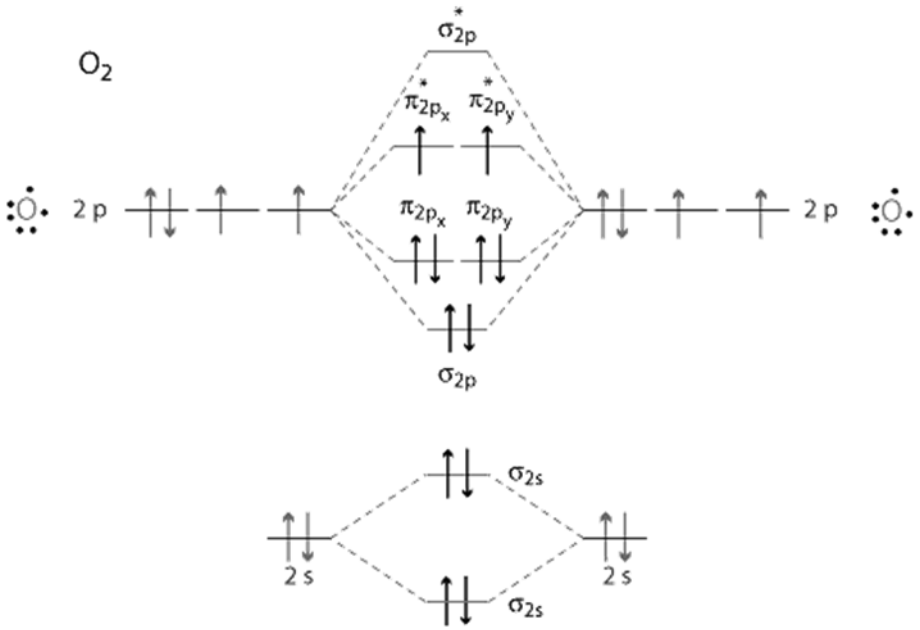


Figure 1. The valence orbitals of molecular oxygen. In the ground state the electrons in the π orbitals have parallel spins.

However, once a reaction with O_2 is initiated (catalyzed), it releases great amounts of energy, contributing to its toxic effects. The triplet state is prone to one-electron transfers, thus creating radical species. On the other hand, most of the electrons in organic molecules are paired and thus are singlet in the ground state, making a reaction with O_2 unlikely. In order for O_2 to react with these organic molecules, it must receive a pair of electrons, which requires a spin reversal prohibited by the spin conversion rule (Figure 2).

Instead, O_2 receives electrons by one-electron steps, favoring rapid reaction with molecules that are capable of one-electron transfer. Important examples include biomolecules, like flavin enzymes and coenzymes, and species holding unpaired electrons, such as other FRs and transition metals. It is therefore not surprising that most bio-enzymes catalyzing oxidation reactions contain transition metal ions and/or flavin co-enzymes in their active sites [2].

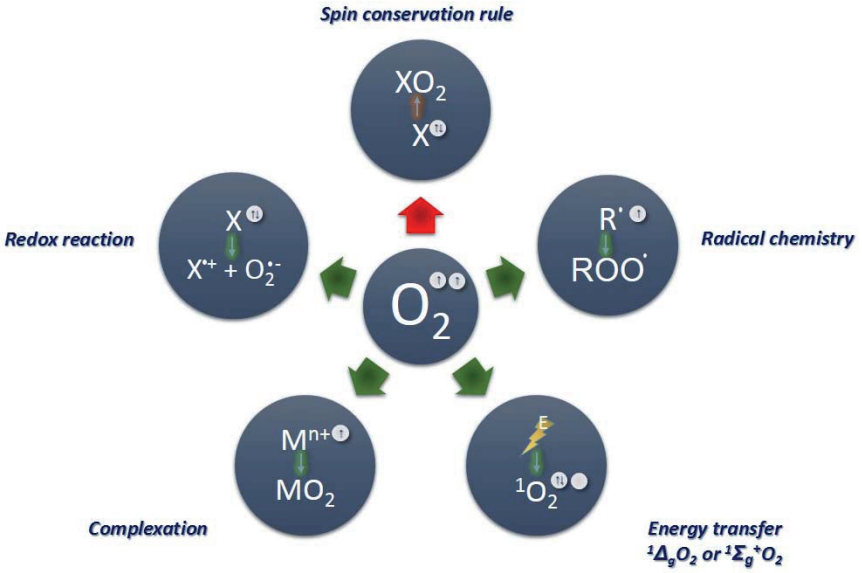


Figure 2. Main reactions of O_2. Due to the spin conservation rule, O_2 reactions with other molecules would be extremely slow. One pathway for O_2 to react with organic molecules requires bombarding O_2 with energy from light, for example, and excited molecules to produce singlet oxygen. This form of oxygen is very reactive with biomolecules, as spin restriction no longer applies. In contrast to reactions with transition metal ions (Mn+), molecules that create stable radicals by one-electron transfer (X.+) or free radicals (R.), are very fast.

FORMATION AND EARLY HISTORY OF OUR PLANET

During its formation and earliest geological history, our ca. 4.5-billion-year-old planet offered many challenges to life. Those obstacles included frequent impactors, including the Mars-sized object that spawned our moon [17]. Over the ensuring hundreds of millions of years, large amounts of carbon dioxide (CO_2) and methane (CH_4), among other key gases, were released into the primitive atmosphere from biotic and abiotic sources such that greenhouse conditions compensated for the lower solar output at that time [18].

Accumulating CO_2, principally from volcanic sources, was buffered by chemical weathering of crustal rocks as continents first began to form and emerge above the oceans through the Archean. The sources and sinks for these essential gases—including biologically produced CH_4—remain hot topics of research. Among the

farthest-reaching questions are those centered on the timing and styles of early plate tectonics and their relationships with the evolving atmosphere.

Given the absence of any record of sedimentary rocks older than ca. 3.8 billion years ago, it remains unknown when precisely conditions conducive to the origin and evolution of life were initially established. However, recent results are suggesting a Hadean Earth (the first 500 million years) that was far less hellish than the name implies [19]. Developments favoring the early origin and sustainability of life include the possibility of oceans extending back as far as 4.4 billion years ago [20, 21], an early life-favoring magnetic field [22], and the likelihood that the late heavy bombardment and its capacity to sterilize surface life about 4.1 to 3.8 billion years ago, was far less heavy than imagined [23]. Nevertheless, the atmosphere and ocean system remained virtually devoid of O_2, for about two billion years, until 2.3 to 2.4 billion years ago at the so-called Great Oxidation Event (GOE) (Figure 3).

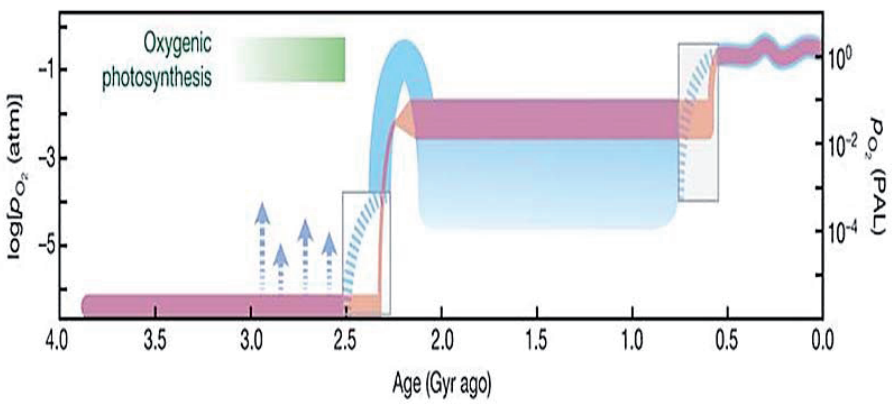


Figure 3. Evolution of Earth's atmospheric O_2 content through time. The faded blue and red curve shows a 'classical, two-step' view of atmospheric evolution, while the blue dotted lines and gray boxes convey remaining uncertainties surrounding the key transitions in our oxygenation history. pO_2 is the atmospheric partial pressure of O_2. On the right axis, pO_2 is presented relative to the present atmospheric level (PAL). The left axis shows log pO_2 in atmospheres. Arrows denote possible transient 'whiffs' of O_2 late in the Archaean; their duration and magnitude are poorly understood. From Lyons et al [35], used with permission.

The GOE is defined by the first permanent accumulation of appreciable O_2 in the atmosphere [24, 25]. Several processes have been proposed to explain the earliest production of O_2 on Earth. Photolysis of H_2O with hydrogen escape is one way to

produce traces of O_2 abiotically. Ultimately, however, appreciable levels of O_2 production and eventual accumulation in the atmosphere became possible only after the evolution of oxygenic photosynthesis. Burial and long-term sequestration of the co-produced organic carbon over geological time scales results in a net release and build-up of biospheric O_2 (35). The beginnings of biological O_2 production were traditionally constrained by organic biomarker evidence for cyanobacteria at 2.7 billion years ago [26], now recognized to be spurious as a consequence of contamination [27]. New suggestions, however, via inorganic geochemical fingerprints have provided alternative windows to early biological oxygenesis now placed as far back as 3.0 or more billion years ago [28-30]. In the hundreds of millions of years between life's first production of O_2 and its first permanent accumulation in the atmosphere at the GOE, the biosphere was marked by O_2 enrichments limited to the surface ocean [31] and transient 'whiffs' in the atmosphere [32-34]. Others have argued, for a much later advent of oxygenic photosynthesis generally coincident with the GOE, although this is not a widely held view [35].

Multiple geochemical proxies leave little doubt that O_2 irreversibly rose at the GOE to levels greater than at least 0.001% of the present atmosphere level (PAL) and likely much more [25, 36-40] (Figure 3). More recent evidence, including the isotopic composition of sedimentary chromium, indicates that the O_2 content of the atmosphere mostly remained low, from less than 0.001 to perhaps 0.1 to 1.0 % PAL, for more than the first 80% of Earth history until about 800 million years ago near the end of the Proterozoic Eon [41]. Although the specifics are debated, it seems likely that O_2 increased during the late Proterozoic and that this rise supported the respiration requirements of animals. Increasing oxygen may have triggered the initial appearance of animals and certainly favored their subsequent taxonomic and ecological diversification [41, 42], although the cause and effect relationship between this rise of oxygen and animal evolution remains poorly understood [43].

PRIMITIVE ORGANISMS AND THE O_2 BURST

Respiration must have evolved early and out of necessity to produce energy [44, 45], and early anaerobic processes included methanogenesis during which electrons from hydrogen are transmitted to CO_2. However, as evolution progressed, a wide range of electron acceptors allowed diverse, energy-yielding metabolic pathways [46]. The shift toward these other acceptors was possible without alteration of membrane structure. Hence, substitution of alternative acceptors in the chemi-osmotic coupling (discussed below) was merely a matter of time [14, 44, 47].

Furthermore, hydrogen sulphide (H_2S), nitric oxide (NO), and reduced and even oxidized iron were plentiful on Earth's early surface and may have been involved in the first forms of prokaryote specialization, including important roles during and as a consequence of early anoxygenic photosynthesis [2, 47].

Photosynthesis, like respiration, can use different sources of electrons. During our early history, H_2S and iron were likely major electron donors (as they are for many bacteria today). By using solar energy, chlorophyll transforms into an oxidant and harvests electrons from a variety of molecules and passes them along to CO_2—constituting the hallmark of photosynthesis [2] (equation 2), which is the reciprocal reaction of aerobic respiration (equation 3):

$$CO_2 + H_2O \rightarrow CH_2O + O_2 \qquad \textit{Oxygenic photosynthesis (2)}$$

$$CH_2O + O_2 \rightarrow CO_2 + H_2O \qquad \textit{Aerobic respiration (3)}$$

O_2 production through photosynthesis was, as it is today, the dominant source of atmospheric and oceanic O_2. Use of water instead of reductants sourced primarily from hydrothermal vents, such as H_2S, ultimately transformed the redox state of our planet with concomitant increases in biomass [48]. This evolutionary milestone released O_2 into the ocean and atmosphere and, after initial buffering reactions with reduced species such as sulphur, ferrous iron, and CH_4, eventually accumulated by the GOE [25, 49]. Recent examples of related mass balance arguments for biospheric oxygenation are presented in Shields and Mill [50] and Krissansen et al [51]. This landmark in Earth history, for both the bio- and geosphere, eventually paved the way for evolution of complex animal life [40, 45, 52]. However, while photosynthesis is the primal source of O_2, there are other environmental factors to consider. For the build-up of biosphere O_2 to occur, the net production of O_2 must have exceeded the delivery of reduced inorganic species, including those sourced from deep within the Earth by geological processes. These reduced species, particularly CH_4, were also sourced by biological processes modulated by the evolving ocean chemistry [53].

Evolution of oxygenic life

The impact of O_2

Oxygenic photosynthesis and the eventual buildup of O_2 in the biosphere led to the evolution of aerobic respiration, thereby enabling cells to produce many times more ATP than their ancestors that relied entirely on anaerobic respiration. Interestingly,

O_2 had little effect on prokaryote physiology; even in the presence of O_2, no complex prokaryotic organisms evolved [54]. Perhaps even more important than the evolution of aerobic respiration was the juxtaposition of O_2 with the electron transport chain of bacteria [3]. These chains are branched, inducible, and modular in that they can 'select' their electron transport chains from a DNA library [3, 55]. In contrast, eukaryotic electron transport resides with redox reactions in the mitochondria through movement of electrons from an electron donor (NADH or QH_2) to a terminal electron acceptor (O_2). In each cell, mitochondria typically account for more than 90% of the O_2 consumed and ATP produced. Because of its pivotal roles in generating ATP and ROS, mitochondrial dysfunction can cause a variety of diseases [56].

O_2 utilization to produce ATP was a major revolution for early life forms as it allowed for energy-efficient metabolic pathways. O_2 has favorable thermodynamic properties and is well suited to be an electron acceptor for many reasons. For example, reduction of O_2 delivers the highest free energy per electron transfer (except for fluorine). Also, O_2 can diffuse through biological membranes and bind to heme moieties (e.g., hemoglobin), thereby facilitating O_2 delivery. Finally, biochemical symmetry (the $H_2O \rightarrow O_2 \rightarrow H_2O$ cycle) of photosynthesis and aerobic respiration maintains atmospheric homeostasis [3].

Benefits of mitochondria

A puzzling question is why eukaryotes have mitochondria. Specifically, prokaryotes can yield a maximum of 38 ATP molecules, while eukaryotes derive a maximum of 36 due to the loss of two molecules to NADH passing through the mitochondrial membrane [3, 57]. If we take into account that mitochondrial genetic function operates in strict connection with the nucleocytoplasm, than their own duplication (with specific DNA and RNA polymerases) seems inefficient. Also, the rate of evolutionary change is limited by the 'asexual' reproduction of mitochondria and is thus mostly neutral or reductive [56]. The fact that mitochondria contain their own DNA, along with their own transcriptional machinery (similar to bacteria) reveals their extracellular origin [3, 58, 59]. The main advantage of mitochondria is the redistribution of genes in relation to bio-energetic membranes, giving eukaryotes a genomic asymmetry compared with extreme polyploidy in giant bacteria [60]. The most likely explanation for why humans have mitochondria may be that it was simply already available and through endocytosis yielded an immediate advantage over designing a novel electron transport chain [44, 58].

Eukaryotic cells with mitochondria are highly successful. First, compartmentalization localizes a metabolic pathway into a specialized compartment providing greater

efficacy for fatty acid oxidation, tricarboxylic acid (TCA) cycling, and oxidative phosphorylation. Second, the ability to conceal (as much as possible) ROS produced during cellular respiration from the cytoplasm and DNA is valuable. Third, insertion of a novel genome into the early nucleocytoplasm could provide for a new set of enzymes [3, 56, 58].

Chemiosmotic coupling

Chemiosmosis is a chemical process whereby ions move across a selectively permeable membrane down an electrochemical gradient. More specifically, with respect to ATP generation, chemiosmosis is linked to the movement of hydrogen ions across a membrane during cellular respiration or photosynthesis. When hydrogen ions move from a high concentration compartment to a site of low concentration, the resulting electrochemical gradient can be used to make ATP. This process is called chemiosmosis because it refers to the diffusion of water across a membrane (osmosis), which is used in most Bacteria, Archaea, and chloroplasts, as well as in mitochondria [55].

The importance of this ATP-generating system is now well accepted, but this agreement was not always the case. Decades of debate—commonly known as the 'ox-phos' disputes—preceded the current consensus [3]. The idea that ion gradients across a membrane functioning as the intermediary in ATP synthesis was met with initial resistance, which Leslie Orgel summarized as "the most counter-intuitive idea in biology since the presentation of Darwin's *On the Origin of Species*" [3, 61]. Mitchell [62] proposed the 'chemiosmotic coupling' hypothesis in 1961 suggesting that most ATP synthesis in respiring (oxidative phosphorylation) cells derives from the electrochemical gradient across the inner membranes of mitochondria, thereby using the energy of NADH and $FADH_2$ formed from the breakdown of energy-rich molecules such as glucose. However, this rather elaborate chemistry was also counterintuitive to the prevailing view at that time, which assumed storage of energy of electron transfer as a stable high potential intermediate.

OXIDATIVE STRESS AND COMPLEX LIFE

Geochemical evidence

Ancestral Earth's atmosphere was anoxic with an estimated O_2 concentration of less than 10^{-5} of the present atmospheric level – and perhaps much lower [24, 63]. In the absence of photosynthetic O_2, abiotic ROS production may have been among the

critical primordial sources of molecular oxygen. It has been postulated that H_2O_2 was a key player in the evolution of life on Earth, as it could have provided the driving force for the evolutionary progression from anoxygenic photosynthetic organisms to oxygenic photosynthesizers [64, 65]. This assumption has been supported by experiments with pyrite in aqueous suspensions that yield H_2O_2 in the absence of O_2 [66, 67] (equation 4):

$$2H_2O(l) + 2Fe^{(III)}\text{-py} \rightarrow H_2O_2 + 2Fe^{(II)}\text{-py} + 2H^+ \quad (4)$$

Several studies have highlighted the possibility of abiotically formed ROS as an early source of O_2. For example, there is a geochemical model for pyrite-dependent aqueous formation of ROS ($OH\cdot$ and H_2O_2) in the subsurface of Mars [68]. Further, Europa, the icy satellite of Jupiter, contains H_2O_2, which can lead to the formation of O_2 and ROS by way of radiolysis and photolysis [69, 70]. Also, abiotically formed O_2 has been discovered recently in the atmosphere of Rea, one of Saturn's icy moons [71]. The study by Slesak et al. [67] supports the hypothesis that photochemically produced O_2 and aerobic metabolisms were present on early Earth and in primordial organisms before the evolution of O_2-producing cyanobacteria [72-74].

Redox biology and aerobic evolution

Biotic responses to the earliest accumulations of O_2 in the oceans and atmosphere highlight the great evolutionary adaptability of early life forms, as some organisms found a way to use this toxic substance to their advantage. Among the biggest riddles in science is why there is no record of animals for almost two billion years after GEO until the second rise in atmospheric O_2 (~0.8 billion years ago), a time that, nevertheless, witnessed dramatic increases in eukaryotic diversity and ecosystem structure (Figure 4) [25]. It is clear that the emergence and evolution of complex life is linked to Earth's oxygen cycle; however, the cause-and-effect relationship between planetary redox cycling and evolution of animal life is still strongly debated [75, 76]. Some researchers dispute the central role of O_2 in the development of animals and emphasize instead a delay due to the necessary development of the genetic machinery [76, 77]. Still, it is possible that during this period from 1.8 to 0.8 billion years ago, termed the "Boring Billion" because of the relatively static biogeochemical, climatic, and tectonic conditions, generally low O_2 conditions suppressed the rate of evolution compared to younger time intervals in the latest Proterozoic and the Paleozoic [25, 78, 79]. In that younger window, increases in Metazoan complexity and evolution are linked with better agreement to rising O_2 levels and the ability to use O_2 to generate energy more efficiently [78, 80].

Since aerobic metabolism generates more energy, an estimate of 1000 more enzymatic reactions can occur compared anaerobic circumstances [81, 82] thus allowing for generation of new metabolites like alkaloids, steroids and isoflavanoids which are important elements for cellular membranes and homeostasis [6, 83]. However, it is important to note that the most ancient aerobic metabolisms were related to biosynthesis rather than energy generation using pyridoxial-5-phosphate synthase enzyme [84, 85], which may have extended the timeframe of evolution from initial synthesis of biomolecules to effective harnessing of energy. Moreover, all life on Earth is homochiral, meaning exclusive use of the D-enantiomer of ribose, the sugar moiety of RNA, and the L-enantiomer of the chiral amino acids [86]. The smallest enantiomer to exist is H_2O_2, leading to the hypothesis that its specific enantiomeric interactions with RNA played an important role in shaping and altering the genetic toolbox necessary for aerobic metabolism [86]. The observation that LUCA (last universal common ancestor, 3.5-3.8 billion years ago) already possessed redox biology involving O_2 and H_2O_2 suggests that the ability to detoxify oxygen species was not a trait that emerged as a response to increased O_2 levels but rather was a crucial adaptation to the early Earth's weakly oxic microenvironments [67].

Aerobic evolution thus displays 'fitness-related traits' such as reproductive lifespan and ageing, which are constrained by trade-offs among traits [87]. Trade-offs represent the cost paid when a beneficial change in one trait is linked to a harmful change in another [88]. ROS play a major role in regulating trade-offs between lifespan, metabolic rates, and fitness and the best example is the existence of NADPH oxidase (NOX) enzymes throughout aerobic development [89, 90] (Figure 4). The primary function of NOX enzymes is to regulate and compartmentalize generation of ROS. Interestingly, molecular evolution has resulted in an increase in number and diversity within the family of NOX enzymes, with increasing complexity during Metazoan evolution [91]. Remarkably, NOX enzymes are expressed in all multicellular eukaryotes but not in prokaryotes, with most mammalian species expressing seven NOX homologues (NOX 1-5 and DUOX 1-2) [6, 92]. Diversification of the NOX gene family with Metazoan evolution suggests an adaptive role for these ROS-generating enzymes in specialized organisms. Proof of relationship is suggested by the recent discovery of NOX3 enzyme expression in the inner ears of reptiles, birds, and mammals [91] thought to be associated with balance and gravitational motion. Such innovation must be beneficial for adaptation of these species to air or land [6].

Other enzymes, like di-oxygen reductases (e.g., cytochrome oxidases type A highlight the value of ROS in aerobic evolution. Recent studies show that they

preceded other cytochrome oxidases [93], suggesting that early microbiota contained low concentrations of O_2, favoring the 'respiration-early' hypothesis. This model describes a very early origin for homologous proteins of respiratory chains in both Archea and Bacteria [94]. Regardless of how likely certain reactions are to be replaced by their anaerobic equivalents [95], O_2 reduction and ROS detoxification are not exclusive to strict aerobes. As a result, it is not feasible for most O_2-dependent reactions to be substituted by anaerobic counterparts. As such, despite the much lower O_2 levels in Earth's early atmosphere, aerobic life could have been supported even before photosynthesis resulted in vast amounts of O_2 in the atmosphere [96, 97]. The fact that ROS removal systems occur in so many strict anaerobes suggests that ROS production was unavoidable even under the globally anoxic environment of early Earth [67]. For example, Kim and colleagues recently showed that O_2 needed for the most ancient reaction of aerobic metabolism, involving synthesis of pyridoxal 5'-phosphate or pyridoxal, probably came from manganese catalase (MnCAT), which appeared at the same time [96] (equation 5):

$$2H_2O_2 \rightarrow 2H_2O + O_2 \text{ (catalyzed by MnCAT)} \quad (5)$$

Therefore, ROS, such as H_2O_2, superoxide, and $OH^{\bullet}$, possibly played a major role in the development and evolution of oxygenic pathways [67, 98-100].

Reactive oxygen species and animal physiology

Enzymatic reduction of O_2 yielded a large increase in energy production capacity, allowing the evolution of multi-cellular animal life, although, with necessary adaptations to withstand the toxic properties of O_2 [101]. Thus the ability to cope with changing O_2 levels is a key factor in evolutionary selection [102] and the existence of an intricate regulator system protecting the organism from either hypoxia or hyperoxia, and subsequent oxidative stress, is mandatory. After the GOE, diversification of life remained relatively static for about a billion and a half years, possibly indicating a necessity for the development of adaptive oxygenic physiological pathways [103]. A first perquisite to maintain oxygen homeostasis is to develop a failsafe system capable of sensing O_2, and consequently CO_2, fluctuations [104, 105]. Interestingly, in all animal taxa examined up to date, conservation of O_2 homeostasis is mediated by the O_2-dependent posttranslational hydroxylation of a heterodimeric transcription factor, called hypoxia inducible factor (HIF) [104, 106]. Recent evidence suggests that core components of the HIF-system originated in ancient lineages such as corals and nematodes [107] and developed a higher degree of complexity in larger animals with respiratory and circulatory systems possibly

because O_2 requirements became more demanding [108]. Studies report prolyl hydroxylase enzymes (PHD) –which are direct sensors of cellular O_2 tension– to be the link for the crosstalk between mitochondria and the HIF pathway [106, 109]. It is therefore highly possible that this adaptation mechanism to hypoxia was a necessary development after the GOE before animal life could appear and respond to changes in oxygenation through diversification [106] (Figure 4).

A second perquisite would have been a redox-regulating mechanism that produced antioxidants, thereby keeping ROS in a physiological range. As discussed, LUCA already possessed the ability to detoxify the O_2/H_2O_2 pathway and all organisms — from the simplest bacteria to complex mammals— adapt to oxidative stress by rapidly increasing their production of antioxidants and repairing enzymes [16]. In mammals, the major defense mechanism against oxidative and xenobiotic stress is the Kelch-like ECH-associated protein 1 (Keap1)-NF-E2-related factor 2 (Nrf2) system, which is responsible for the transcription of over 200 cytoprotective genes [110]. Given the importance of this pathway, it is possible that it is preserved and modified from simple progenitors [111]. Indeed, recent data analyzing Nrf2 sequences yielded a molecular clock demonstrating that Nrf2 first appeared in fungi around 1.5 billion of years ago [101, 111]. Remarkably, Nrf2 orthologues emerged and diverged at two time points correlating with rising atmospheric oxygen levels [101] (Figure 4). A first divergence in Nrf2 occurred during the division between fungi and Metazoa [45, 101] and the second at the division between mammals and non-mammalian vertebrates during the Late Triassic [112, 113].

With the existence of an adequate oxygen sensing and protective system, the tree of life could further diverge and higher metabolic demands could be met. These changes facilitated the evolution of an exchanging-transport system in larger Metazoans, thus removing any limitation linked to diffusion of O_2 across cell layers [15, 80]. Complexity in organismal structure and function has been related to the number of differentiated cell types and overall organism size [59, 114, 115]. Estimates of the maximum number of cell types of common ancestors combined with divergence times showed an increase from two to about ten cell types between 2.5 and 2 billion years ago and from ten to 50 cell types between 1.5 -1 billion years ago [6, 15] (Figure 4).

Increased organismal complexity with an intricate cardiovascular system and cellular compartmentalization augmented O_2 demand, however, also minimized contact with atmospheric O_2. More specifically, most mammalian cells exist in vivo at O_2 concentrations of about 2-4% and thrive best (e.g. pluripotent stem cells) under these conditions (24). Only three cell types ever encounter O_2 concentrations of

about 21% (the present atmospheric level), namely cells of the upper airways and upper regions of the lungs, cornea, and skin.

It is clear that, given the central role of O_2 in evolution, many organisms are dependent on a constant flow and mediation of internal concentration of O_2. As such, complexity of mammalian DNA has been affected by aerobic metabolism, including aerobic endurance [116]. Evidence suggests that the use of O_2 as a final electron acceptor in the mitochondrial chain played a significant role in evolution of Homo sapiens [117], as endurance running allowed hominem—possibly from the time of H. erectus [118]—to hunt and secure sufficient protein for physiological development [116]. On the other hand, flying birds, which have evolved an intrinsic pulmonary system (i.e., the presence of air sacs make it possible to extract O_2 with exhalation), should have a high aerobic capacity compared to mammals. This relationship means that birds may have a superior mechanism to cope with oxidative stress. It may even be that there is an adjustable ROS threshold for different species whereby values above are detrimental, and lower ROS concentrations could be necessary as a redox signal [2, 4, 16]. The maximum potential lifespan of birds and mammals is correlated with body size. However, birds live, on average, twice as long as mammals, which is a surprising observation given their higher metabolic rates [2, 119]. When comparing a pigeon with a rat (which have roughly the same body size), the pigeon has a life span ten-times longer than that of the rat, and remarkably its mitochondrial ROS leak is nearly ten-times lower [2, 119, 120]. It is obvious in birds that the fraction of ROS that escapes dismutation (inactivation) by anti-oxidants with respect to the total O_2 consumption is so low that there is no relationship to the rate of respiration. Nevertheless, redox state in birds makes a tremendous difference in terms of longevity [59].

Could it therefore be that humans are aerobically ‘less evolved’ and are more vulnerable to OS? Is it therefore possible that OS and concurrent disease is the price we pay for evolution and diversification of O_2- and ROS-mediated pathways? Instead of focusing only on ROS production (and its deleterious effects) itself, perhaps we should be asking how much ROS can be tolerated—particularly given that levels of tolerance differ among organisms.

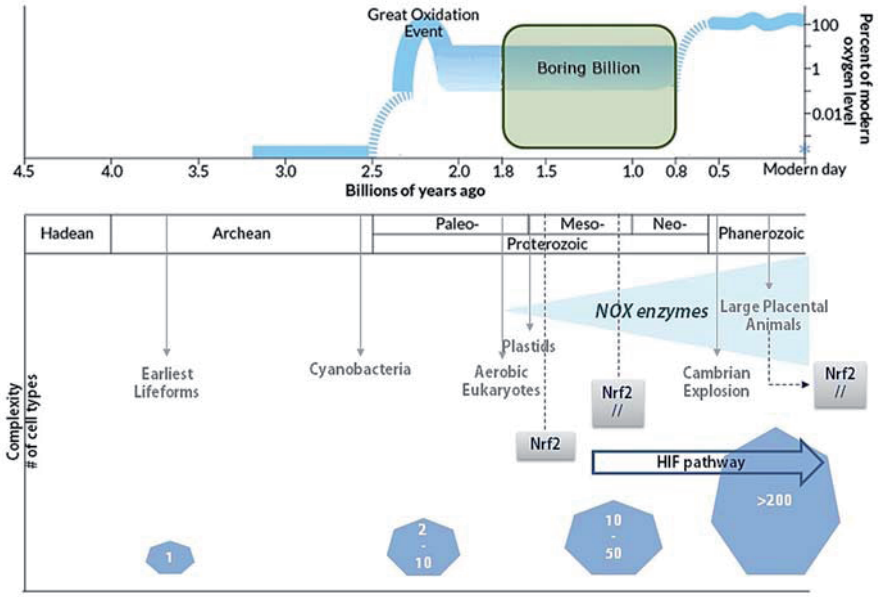


Figure 4. Diversification and complexity of life related to oxidation events over time. The first event is called the Great Oxidation Event (around 2.3 to 2.4 billion years ago), which was followed much later by the first emergence of aerobic eukaryotes and multicellularity. Another important event was the evolution of plastids (approximately 1.6 billion years ago), which provided eukaryotes with the ability to generate their own O_2 and thus seemed to have triggered a second phase in the expansion of multicellularity (10–50 cell types between 1.5 and 1.0 billion years ago ago). Also, at that time Nrf2 started to diverge (first Nrf2 //) from a precursor sequence and is first established during the division between fungi and Metazoa (approximately 1.0-1.2 billion years ago) [113]. When fungi and Metazoa diverged, core components of the hypoxia inducible factor (HIF) pathway were already in place [116]. The second major increase in atmospheric O_2 between 0.8 and 0.6 billion years ago on the O_2 time line was a precursor to the Cambrian explosion (0.54 billion years ago), which, along with the early Paleozoic intervals immediately following, was a period of most rapid and prolific speciation. A third rise in atmospheric O_2 can be seen on the graph (approximately 0.3 billion years ago ago), which has been linked to the emergence of ''gigantism'' in several arthropod groups and reptile-like animals. However, the following rapid drop in O_2 concentration (260–240 million years ago) preceded mass extinctions of these species. The rise of O_2 from about 10% to 21% over the past 205 million years is thought to have been a key factor in evolution of large placental mammals. During the Permian-Triassic boundary (approximately 252 million years ago), Nrf2 diverged yet again (second Nrf2 //) at the split between mammals and non-mammalian vertebrates [113] Specialized O_2-reducing enzymes dedicated to reactive oxygen species (ROS) generation, the NADPH oxidase (NOX) family, are primarily expressed in eukaryotes with an increasing number of NOX homologs that emerged in more complex metazoans.

CONCLUSIONS

O_2 and its derived species present us with the ultimate paradox in life and evolution—because they encompass both challenges and opportunities for life. Reactive H_2O_2 was created, even in the absence of biological O_2 production early in Earth's. This production provided opportunities to early life via the release of O_2 into the oceans as a waste product. Likely due to its favorable thermodynamic properties, O_2 was selected as terminal electron acceptor in the reduction of carbon-based reactions that created ATP, but this step also resulted in the production of reactive oxygen species (ROS) during oxidative phosphorylation. While otherwise toxic levels of ROS were tolerated through the development of anti-oxidant defense mechanisms, lower ROS levels were actually integrated into cellular signaling processes and, through evolution, have become part of normal cellular homeostasis. Nevertheless, current research still focuses on antioxidants as the 'holy grail' in combating disease and delaying ageing and ignores the physiologically beneficial roles of ROS in many cellular processes. Focus must now be placed on a more comprehensive understanding of the physiological contributions of ROS during homeostasis, which enable possible beneficial interaction between organisms and their surroundings and enhance their survival. As aerobic physiology both challenged and enhanced eukaryote diversity, O_2 toxicity led to extinction of species unable to cope—or it limited their distributions to anaerobic parts of our planet. In this sense, O_2 and ROS must be viewed as essential consequences and drivers of evolution and survival over Earth history, and their distributions and impacts through time demand further attention in future research.

ACKNOWLEDGEMENTS

We would like to thank professors **Roger Summons**, Department of Earth, Atmospheric and Planetary Sciences, Massachusetts Institute of Technology, Cambridge, USA, and **Blair Hedges**, Center for Biodiversity, Temple University, Philadelphia, USA, for their valuable feedback. **T. Lyons** acknowledges financial support from the NASA Astrobiology Institute.

REFERENCES

1. V. Trimble, Origin of the biologically important elements, Orig Life Evol Biosph 27(1-3) (1997) 3-21.
2. S.H.M. Pantopoulos K., Principles of Free Radical Biomedicine, Nova Science Publishers2011.
3. N. Lane, The Evolution of Oxidative Stress, in: S.H.M. Pantopoulos K. (Ed.), Principles of Free Radical Biomedicine, Nova Science Publishers2011.
4. Y.J. Taverne, A.J. Bogers, D.J. Duncker, D. Merkus, Reactive oxygen species and the cardiovascular system, Oxid Med Cell Longev 2013 (2013) 862423.
5. B. Halliwell, J.M. Gutteridge, Free Radicals in Biology and Medicine Oxford2015.
6. V.J. Thannickal, Oxygen in the Evolution of Complex Life and the Price We Pay, Am J Resp Cell Mol 40(5) (2009) 507-510.
7. L. Michaelis, Biological oxidations and reductions, Annu Rev Biochem 16 (1947) 1-34.
8. L. Michaelis, Fundamental principles in oxidation-reduction, Biol Bull 96(3) (1949) 293-5.
9. R. Gerschman, D.L. Gilbert, S.W. Nye, P. Dwyer, W.O. Fenn, Oxygen poisoning and x-irradiation: a mechanism in common, Science 119(3097) (1954) 623-6.
10. J.M. McCord, I. Fridovich, The utility of superoxide dismutase in studying free radical reactions. I. Radicals generated by the interaction of sulfite, dimethyl sulfoxide, and oxygen, J Biol Chem 244(22) (1969) 6056-63.
11. C.C. Winterbourn, Reconciling the chemistry and biology of reactive oxygen species, Nat Chem Biol 4(5) (2008) 278-86.
12. J.A. Lemire, J.J. Harrison, R.J. Turner, Antimicrobial activity of metals: mechanisms, molecular targets and applications, Nat Rev Microbiol 11(6) (2013) 371-384.
13. H. Sies, E. Cadenas, Oxidative stress: damage to intact cells and organs, Philos Trans R Soc Lond B Biol Sci 311(1152) (1985) 617-31.
14. T. Finkel, Oxidant signals and oxidative stress, Curr Opin Cell Biol 15(2) (2003) 247-54.
15. K.J.A. Davies, Oxidative stress, antioxidant defenses, and damage removal, repair, and replacement systems, Iubmb Life 50(4-5) (2000) 279-289.
16. B. Halliwell, Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life, Plant Physiol 141(2) (2006) 312-322.
17. N.H. Sleep, K.J. Zahnle, J.F. Kasting, H.J. Morowitz, Annihilation of Ecosystems by Large Asteroid Impacts on the Early Earth, Nature 342(6246) (1989) 139-142.
18. J.F. Kasting, EARLY EARTH Faint young Sun redux, Nature 464(7289) (2010) 687-689.

19. R.W. Carlson, E. Garnero, T.M. Harrison, J. Li, M. Manga, W.F. McDonough, S. Mukhopadhyay, B. Romanowicz, D. Rubie, Q. Williams, S.J. Zhong, How Did Early Earth Become Our Modern World?, Annu Rev Earth Pl Sc 42 (2014) 151-178.
20. S.A. Wilde, J.W. Valley, W.H. Peck, C.M. Graham, Evidence from detrital zircons for the existence of continental crust and oceans on the Earth 4.4 Gyr ago, Nature 409(6817) (2001) 175-8.
21. M.S. Dodd, D. Papineau, T. Grenne, J.F. Slack, M. Rittner, F. Pirajno, J. O'Neil, C.T.S. Little, Evidence for early life in Earth's oldest hydrothermal vent precipitates., Nature 543 (2017) 60-64.
22. J.A. Tarduno, R.D. Cottrell, W.J. Davis, F. Nimmo, R.K. Bono, A Hadean to Paleoarchean geodynamo recorded by single zircon crystals, Science 349(6247) (2015) 521-524.
23. C.I. Fassett, D.A. Minton, Impact bombardment of the terrestrial planets and the early history of the Solar System, Nat Geosci 6(7) (2013) 520-524.
24. J.F. Kasting, D. Catling, Evolution of a habitable planet, Annu Rev Astron Astr 41 (2003) 429-463.
25. T.W. Lyons, C.T. Reinhard, N.J. Planavsky, The rise of oxygen in Earth's early ocean and atmosphere, Nature 506(7488) (2014) 307-315.
26. J.J. Brocks, G.A. Logan, R. Buick, R.E. Summons, Archean molecular fossils and the early rise of eukaryotes, Science 285(5430) (1999) 1033-1036.
27. K.L. French, C. Hallmann, J.M. Hope, P.L. Schoon, J.A. Zumberge, Y. Hoshino, C.A. Peters, S.C. George, G.D. Love, J.J. Brocks, R. Buick, R.E. Summons, Reappraisal of hydrocarbon biomarkers in Archean rocks, P Natl Acad Sci USA 112(19) (2015) 5915-5920.
28. R. Buick, The Antiquity of Oxygenic Photosynthesis - Evidence from Stromatolites in Sulfate-Deficient Archean Lakes, Science 255(5040) (1992) 74-77.
29. J.L. Eigenbrode, K.H. Freeman, Late Archean rise of aerobic microbial ecosystems, P Natl Acad Sci USA 103(43) (2006) 15759-15764.
30. N.J. Planavsky, D. Asael, A. Hofmann, C.T. Reinhard, S.V. Lalonde, A. Knudsen, X.L. Wang, F.O. Ossa, E. Pecoits, A.J.B. Smith, N.J. Beukes, A. Bekker, T.M. Johnson, K.O. Konhauser, T.W. Lyons, O.J. Rouxel, Evidence for oxygenic photosynthesis half a billiion years before the Great Oxidation Event, Nat Geosci 7(4) (2014) 283-286.
31. S.L. Olson, L.R. Kump, J.F. Kasting, Quantifying the areal extent and dissolved oxygen concentrations of Archean oxygen oases, Chem Geol 362 (2013) 35-43.
32. A.D. Anbar, Y. Duan, T.W. Lyons, G.L. Arnold, B. Kendall, R.A. Creaser, A.J. Kaufman, G.W. Gordon, C. Scott, J. Garvin, R. Buick, A whiff of oxygen before the Great Oxidation Event?, Science 317(5846) (2007) 1903-1906.
33. J. Garvin, R. Buick, A.D. Anbar, G.L. Arnold, A.J. Kaufman, Isotopic Evidence for an Aerobic Nitrogen Cycle in the Latest Archean, Science 323(5917) (2009) 1045-1048.

34. A.J. Kaufman, D.T. Johnston, J. Farquhar, A.L. Masterson, T.W. Lyons, S. Bates, A.D. Anbar, G.L. Arnold, J. Garvin, R. Buick, Late Archean biospheric oxygenation and atmospheric evolution, Science 317(5846) (2007) 1900-1903.
35. R.E. Kopp, J.L. Kirschvink, H. I.A., C.Z. Nash, The Paleoproterozoic snowball Earth: A climate disaster triggered by the evolution of oxygenic photosynthesis., PNAS 102(32) (2005) 11131–11136.
36. J. Farquhar, J. Savarino, S. Airieau, M.H. Thiemens, Observation of wavelength-sensitive mass-independent sulfur isotope effects during SO2 photolysis: Implications for the early atmosphere, J Geophys Res-Planet 106(E12) (2001) 32829-32839.
37. H.D. Holland, The oxygenation of the atmosphere and oceans, Philos T R Soc B 361(1470) (2006) 903-915.
38. S. Ono, J.L. Eigenbrode, A.A. Pavlov, P. Kharecha, D. Rumble, J.F. Kasting, K.H. Freeman, New insights into Archean sulfur cycle from mass-independent sulfur isotope records from the Hamersley Basin, Australia, Earth Planet Sc Lett 213(1-2) (2003) 15-30.
39. S.H. Ono, N.J. Beukes, D. Rumble, Origin of two distinct multiple-sulfur isotope compositions of pyrite in the 2.5 Ga Klein Naute Formation, Griqualand West Basin, South Africa, Precambrian Res 169(1-4) (2009) 48-57.
40. A.L. Sessions, D.M. Doughty, P.V. Welander, R.E. Summons, D.K. Newman, The Continuing Puzzle of the Great Oxidation Event, Curr Biol 19(14) (2009) R567-R574.
41. N.J. Planavsky, C.T. Reinhard, X.L. Wang, D. Thomson, P. McGoldrick, R.H. Rainbird, T. Johnson, W.W. Fischer, T.W. Lyons, Low Mid-Proterozoic atmospheric oxygen levels and the delayed rise of animals, Science 346(6209) (2014) 635-638.
42. M. Dohrmann, G. Worheide, Dating early animal evolution using phylogenomic data, Sci Rep 7(1) (2017) 3599.
43. D.B. Mills, D.E. Canfield, Oxygen and animal evolution: did a rise of atmospheric oxygen "trigger" the origin of animals?, Bioessays 36(12) (2014) 1145-55.
44. N. Lane, Evolution. The costs of breathing, Science 334(6053) (2011) 184-5.
45. S.B. Hedges, J.E. Blair, M.L. Venturi, J.L. Shoe, A molecular timescale of eukaryote evolution and the rise of complex multicellular life, Bmc Evolutionary Biology 4 (2004).
46. T.W. Lyons, D.A. Fike, A. Zerkle, Emerging Biogeochemical Views of Earth's Ancient Microbial Worlds, Elements 11(6) (2015) 415-421.
47. W. Nitschke, M.J. Russell, Hydrothermal focusing of chemical and chemiosmotic energy, supported by delivery of catalytic Fe, Ni, Mo/W, Co, S and Se, forced life to emerge, J Mol Evol 69(5) (2009) 481-96.
48. D.E. Canfield, M.T. Rosing, C. Bjerrum, Early anaerobic metabolisms, Philos Trans R Soc Lond B Biol Sci 361(1474) (2006) 1819-34; discussion 1835-6.

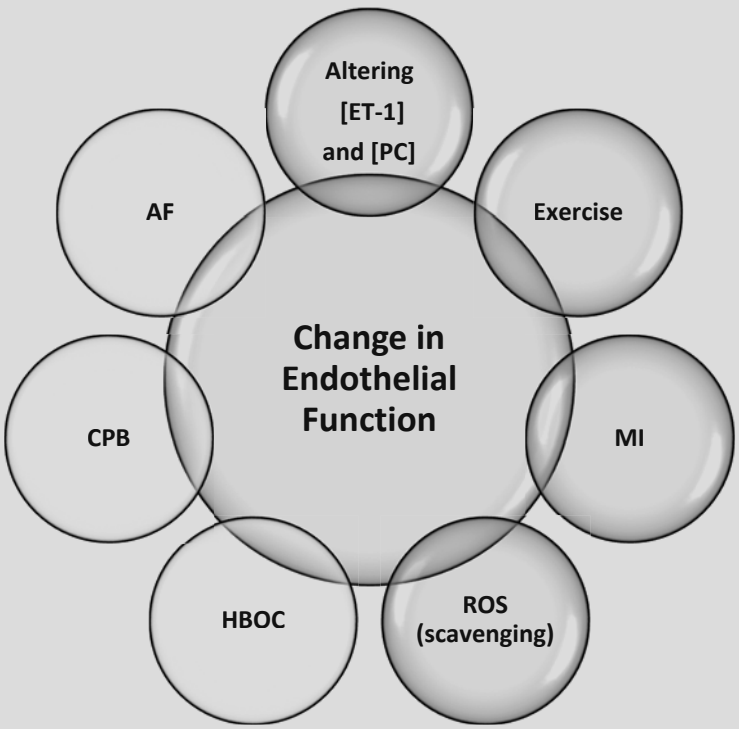
49. N. Lane, Oxygen : the molecule that made the world, Oxford University Press, Oxford, 2002.
50. G.A. Shields, B.J.W. Mills, Tectonic controls on the long-term carbon isotope mass balance, Proc Natl Acad Sci U S A 114(17) (2017) 4318-4323.
51. J. Krissansen-Totton, R. Buick, D.C. Catling, A Statistical Analysis of the Carbon Isotope Record from the Archean to Phanerozoic and Implications for the Rise of Oxygen, Am J Sci 315(4) (2015) 275-316.
52. S.B. Hedges, The origin and evolution of model organisms, Nat Rev Genet 3(11) (2002) 838-849.
53. K.O. Konhauser, E. Pecoits, S.V. Lalonde, D. Papineau, E.G. Nisbet, M.E. Barley, N.T. Arndt, K. Zahnle, B.S. Kamber, Oceanic Nickel depletion and a methanogen famine before the Great Oxidation Event, Geochim Cosmochim Ac 73(13) (2009) A678-A678.
54. T.M. Lenton, R.A. Boyle, S.W. Poulton, G.A. Shields-Zhou, N.J. Butterfield, Co-evolution of eukaryotes and ocean oxygenation in the Neoproterozoic era., Nat Geosci 7 (2014) 257–265.
55. G. Gruber, M.S.S. Manimekalai, F. Mayer, V. Muller, ATP synthases from archaea: The beauty of a molecular motor, Bba-Bioenergetics 1837(6) (2014) 940-952.
56. D.G. Searcy, Metabolic integration during the evolutionary origin of mitochondria, Cell Res 13(4) (2003) 229-38.
57. N. Lane, W. Martin, The energetics of genome complexity, Nature 467(7318) (2010) 929-34.
58. T.M. Embley, W. Martin, Eukaryotic evolution, changes and challenges, Nature 440(7084) (2006) 623-30.
59. N. Lane, Biodiversity: On the origin of bar codes, Nature 462(7271) (2009) 272-4.
60. N. Lane, W. Martin, The energetics of genome complexity, Nature 467(7318) (2010) 929-934.
61. L.E. Orgel, Are you serious, Dr Mitchell?, Nature 402(6757) (1999) 17.
62. P. Mitchell, Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism, Nature 191 (1961) 144-8.
63. J.F. Kasting, Earths Early Atmosphere, Science 259(5097) (1993) 920-926.
64. S.M. Awramik, The oldest records of photosynthesis, Photosynth Res 33(2) (1992) 75-89.
65. C.P. McKay, H. Hartman, Hydrogen peroxide and the evolution of oxygenic photosynthesis, Orig Life Evol Biosph 21 (1991) 157-63.
66. M.J. Borda, A.R. Elsetinow, M.A. Schoonen, D.R. Strongin, Pyrite-Induced Hydrogen Peroxide Formation as a Driving Force in the Evolution of Photosynthetic Organisms on an Early Earth, Astrobiology 1(3) (2001) 283-288.
67. I. Slesak, H. Slesak, J. Kruk, Oxygen and Hydrogen Peroxide in the Early Evolution of Life on Earth: In silico Comparative Analysis of Biochemical Pathways, Astrobiology 12(8) (2012) 775-784.

68. A.F. Davila, A.G. Fairen, L. Gago-Duport, C. Stoker, R. Amils, R. Bonaccorsi, J. Zavaleta, D. Lim, D. Schulze-Makuch, C.P. Mckay, Subsurface formation of oxidants on Mars and implications for the preservation of organic biosignatures, Earth Planet Sc Lett 272(1-2) (2008) 456-463.
69. R.W. Carlson, M.S. Anderson, R.E. Johnson, W.D. Smythe, A.R. Hendrix, C.A. Barth, L.A. Soderblom, G.B. Hansen, T.B. McCord, J.B. Dalton, R.N. Clark, J.H. Shirley, A.C. Ocampo, D.L. Matson, Hydrogen peroxide on the surface of Europa, Science 283(5410) (1999) 2062-4.
70. R.E. Johnson, T.I. Quickenden, P.D. Cooper, A. McKinley, C.G. Freeman, The production of oxidants in Europa's surface, Astrobiology 3(4) (2003) 823-850.
71. B.D. Teolis, G.H. Jones, P.F. Miles, R.L. Tokar, B.A. Magee, J.H. Waite, E. Roussos, D.T. Young, F.J. Crary, A.J. Coates, R.E. Johnson, W.L. Tseng, R.A. Baragiola, Cassini Finds an Oxygen-Carbon Dioxide Atmosphere at Saturn's Icy Moon Rhea, Science 330(6012) (2010) 1813-1815.
72. J. Castresana, M. Lubben, M. Saraste, D.G. Higgins, Evolution of Cytochrome-Oxidase, an Enzyme Older Than Atmospheric Oxygen, Embo J 13(11) (1994) 2516-2525.
73. M.M. Pereira, M. Santana, M. Teixeira, A novel scenario for the evolution of haem-copper oxygen reductases, Bba-Bioenergetics 1505(2-3) (2001) 185-208.
74. K.M. Towe, Aerobic Respiration in the Archean, Nature 348(6296) (1990) 54-56.
75. C.T. Reinhard, N.J. Planavsky, S.L. Olson, T.W. Lyons, D.H. Erwin, Earth's oxygen cycle and the evolution of animal life, Proc Natl Acad Sci U S A 113(32) (2016) 8933-8.
76. D.B. Mills, D.E. Canfield, Oxygen and animal evolution: Did a rise of atmospheric oxygen trigger the origin of animals?, Bioessays 36(12) (2014) 1145-1155.
77. E.A. Sperling, C.J. Wolock, A.S. Morgan, B.C. Gill, M. Kunzmann, G.P. Halverson, F.A. Macdonald, A.H. Knoll, D.T. Johnston, Statistical analysis of iron geochemical data suggests limited late Proterozoic oxygenation, Nature 523(7561) (2015) 451-4.
78. C.T. Reinhard, N.J. Planavsky, S.L. Olson, T.W. Lyons, D.H. Erwin, Earth's oxygen cycle and the evolution of animal life, P Natl Acad Sci USA 113(32) (2016) 8933-8938.
79. R. Tostevin, R.A. Wood, G.A. Shields, S.W. Poulton, R. Guilbaud, F. Bowyer, A.M. Penny, T. He, A. Curtis, K.H. Hoffmann, M.O. Clarkson, Low-oxygen waters limited habitable space for early animals, Nat Commun 7 (2016) 12818.
80. A.H. Knoll, E.A. Sperling, Oxygen and animals in Earth history, P Natl Acad Sci USA 111(11) (2014) 3907-3908.
81. J. Raymond, D. Segre, The effect of oxygen on biochemical networks and the evolution of complex life, Science 311(5768) (2006) 1764-7.

82. K. Stamati, V. Mudera, U. Cheema, Evolution of oxygen utilization in multicellular organisms and implications for cell signalling in tissue engineering, J Tissue Eng 2(1) (2011) 2041731411432365.
83. Y.Y. Jiang, D.X. Kong, T. Qin, H.Y. Zhang, How does oxygen rise drive evolution? clues from oxygen-dependent biosynthesis of nuclear receptor ligands., Biochem Bioph Res Co 391(2) (2010) 1158–1160
84. R.E. Summons, L.L. Jahnke, J.M. Hope, G.A. Logan, 2-Methylhopanoids as biomarkers for cyanobacterial oxygenic photosynthesis, Nature 400(6744) (1999) 554-7.
85. M.A. Saito, The rise of oxygen and aerobic biochemistry, Structure 20(1) (2012) 1-2.
86. R. Ball, J. Brindley, The Life Story of Hydrogen Peroxide III: Chirality and Physical Effects at the Dawn of Life, Orig Life Evol Biosph 46(1) (2016) 81-93.
87. A.K. Chippindale, A.M. Leroi, S.B. Kim, M.R. Rose, Phenotypic Plasticity and Selection in Drosophila Life-History Evolution .1. Nutrition and the Cost of Reproduction, J Evolution Biol 6(2) (1993) 171-193.
88. S. Stearns, Trade-offs in life-history evolution., Functional Ecology 3 (1989) 259-268.
89. K. Bedard, K.H. Krause, The NOX family of ROS-generating NADPH oxidases: Physiology and pathophysiology, Physiol Rev 87(1) (2007) 245-313.
90. T. Kawahara, J.D. Lambeth, Molecular evolution of Phox-related regulatory subunits for NADPH oxidase enzymes, Bmc Evolutionary Biology 7 (2007).
91. H. Sumimoto, Structure, regulation and evolution of Nox-family NADPH oxidases that produce reactive oxygen species (vol 275, pg 3249, 2008), Febs J 275(15) (2008) 3984-3984.
92. M.C. Maurel, J.L. Decout, Origins of life: Molecular foundations and new approaches, Tetrahedron 55(11) (1999) 3141-3182.
93. C. Brochier-Armanet, E. Talla, S. Gribaldo, The Multiple Evolutionary Histories of Dioxygen Reductases: Implications for the Origin and Evolution of Aerobic Respiration, Mol Biol Evol 26(2) (2009) 285-297.
94. J. Castresana, M. Saraste, Evolution of Energetic Metabolism - the Respiration-Early Hypothesis, Trends Biochem Sci 20(11) (1995) 443-448.
95. J. Raymond, R.E. Blankenship, Biosynthetic pathways, gene replacement and the antiquity of life, Geobiology 2(4) (2004) 199-203.
96. K.M. Kim, T. Qin, Y.Y. Jiang, L.L. Chen, M. Xiong, D. Caetano-Anolles, H.Y. Zhang, G. Caetano-Anolles, Protein domain structure uncovers the origin of aerobic metabolism and the rise of planetary oxygen, Structure 20(1) (2012) 67-76.
97. D.A. Stolper, N.P. Revsbech, D.E. Canfield, Aerobic growth at nanomolar oxygen concentrations, P Natl Acad Sci USA 107(44) (2010) 18755-18760.
98. J. Haqq-Misra, J.F. Kasting, S. Lee, Availability of O-2 and H2O2 on Pre-Photosynthetic Earth, Astrobiology 11(4) (2011) 293-302.

99. M.C. Liang, H. Hartman, R.E. Kopp, J.L. Kirschvink, Y.L. Yung, Production of hydrogen peroxide in the atmosphere of a Snowball Earth and the origin of oxygenic photosynthesis, P Natl Acad Sci USA 103(50) (2006) 18896-18899.
100. K.M. Towe, Early Precambrian Oxygen - Case against Photosynthesis, Nature 274(5672) (1978) 657-661.
101. R. Gacesa, W.C. Dunlap, D.J. Barlow, R.A. Laskowski, P.F. Long, Rising levels of atmospheric oxygen and evolution of Nrf2, Sci Rep 6 (2016) 27740.
102. E. Agnello, G. Malfi, A.M. Costantino, P. Massarenti, M. Pugliese, N. Fortunati, M.G. Catalano, A. Palmo, Tumour necrosis factor alpha and oxidative stress as maintaining factors in the evolution of anorexia nervosa, Eat Weight Disord-St 17(3) (2012) E194-E199.
103. P.A. Cawood, C.J. Hawkesworth, Earth's middle age, Geology 42(6) (2014) 503-506.
104. K.T. Rytkonen, J.F. Storz, Evolutionary origins of oxygen sensing in animals, Embo Rep 12(1) (2011) 3-4.
105. W. Chi, X.N. Gan, W.H. Xiao, W. Wang, S.P. He, Different evolutionary patterns of hypoxia-inducible factor alpha (HIF-alpha) isoforms in the basal branches of Actinopterygii and Sarcopterygii, Febs Open Bio 3 (2013) 479-483.
106. C. Loenarz, M.L. Coleman, A. Boleininger, B. Schierwater, P.H. Holland, P.J. Ratcliffe, C.J. Schofield, The hypoxia-inducible transcription factor pathway regulates oxygen sensing in the simplest animal, Trichoplax adhaerens, Embo Rep 12(1) (2011) 63-70.
107. R.J. Hampton-Smith, D.J. Peet, From Polyps to People A HIghly Familiar Response to Hypoxia, Ann Ny Acad Sci 1177 (2009) 19-29.
108. C.T. Taylor, J.C. McElwain, Ancient Atmospheres and the Evolution of Oxygen Sensing Via the Hypoxia-Inducible Factor in Metazoans, Physiology 25(5) (2010) 272-279.
109. S. Barth, F. Edlich, U. Berchner-Pfannschmidt, S. Gneuss, G. Jahreis, P.A. Hasgall, J. Fandrey, R.H. Wenger, G. Camenisch, Hypoxia-inducible Factor Prolyl-4-hydroxylase PHD2 Protein Abundance Depends on Integral Membrane Anchoring of FKBP38, Journal of Biological Chemistry 284(34) (2009) 23046-23058.
110. Y. Fuse, M. Kobayashi, Conservation of the Keap1-Nrf2 System: An Evolutionary Journey through Stressful Space and Time, Molecules 22(3) (2017).
111. R. Gacesa, W.C. Dunlap, P.F. Long, Bioinformatics analyses provide insight into distant homology of the Keap1-Nrf2 pathway, Free Radical Bio Med 88 (2015) 373-380.
112. D.H. Erwin, M. Laflamme, S.M. Tweedt, E.A. Sperling, D. Pisani, K.J. Peterson, The Cambrian conundrum: early divergence and later ecological success in the early history of animals, Science 334(6059) (2011) 1091-7.
113. Z.X. Luo, C.X. Yuan, Q.J. Meng, Q. Ji, A Jurassic eutherian mammal and divergence of marsupials and placentals, Nature 476(7361) (2011) 442-445.

114. D.K. Dowling, L.W. Simmons, Reactive oxygen species as universal constraints in life-history evolution, P Roy Soc B-Biol Sci 276(1663) (2009) 1737-1745.
115. N. Lane, W.F. Martin, J.A. Raven, J.F. Allen, Energy, genes and evolution: introduction to an evolutionary synthesis, Philos Trans R Soc Lond B Biol Sci 368(1622) (2013) 20120253.
116. Z. Radak, Z. Zhao, E. Koltai, H. Ohno, M. Atalay, Oxygen consumption and usage during physical exercise: the balance between oxidative stress and ROS-dependent adaptive signaling, Antioxid Redox Signal 18(10) (2013) 1208-46.
117. D.M. Bramble, D.E. Lieberman, Endurance running and the evolution of Homo, Nature 432(7015) (2004) 345-52.
118. G.D. Ruxton, D.M. Wilkinson, Thermoregulation and endurance running in extinct hominins: Wheeler's models revisited, J Hum Evol 61(2) (2011) 169-75.
119. M.K. Montgomery, A.J. Hulbert, W.A. Buttemer, The long life of birds: the rat-pigeon comparison revisited, PLoS One 6(8) (2011) e24138.
120. G. Barja, Mitochondrial oxygen consumption and reactive oxygen species production are independently modulated: implications for aging studies, Rejuvenation Res 10(2) (2007) 215-24.


Altering
[ET-1]
and [PC]
AF
Exercise
Change in
Endothelial
Function
CPB
MI
HBOC
ROS
(scavenging)

3

REACTIVE OXYGEN SPECIES AND THE CARDIOVASCULAR SYSTEM

Y.J. Taverne, A.J. Bogers, D.J. Duncker and D. Merkus

Oxidative Medicine and Cellular Longevity 2013

ABSTRACT

In the mid-1950s, free radicals were first proposed to be involved in the pathophysiology of a number of diseases [1]. However, due to their short life span and the technical difficulty of detecting them, it took till the 1980s to recognize the importance of reactive oxygen species (ROS) as important players in biological systems [2]. Nowadays, it is widely accepted that ROS play a crucial physiological role, not only in various diseases, but also in cellular homeostasis [3].

ROS are chemically reactive molecules derived from molecular oxygen and formed as a natural by-product of aerobic metabolism. During energy conversion, ROS are produced as a by-product of oxidative phosphorylation, which is presumed to be the major source of superoxide ($O_2^{\bullet -}$) production [4,5]. ROS can also be produced through a variety of enzymes including xanthine oxidase and NAD(P)H oxidase [3].

Under normal circumstances, ROS concentrations are tightly controlled by antioxidants, keeping them in the picomolar range [3]. These low concentrations of ROS enable their role as second messengers in signal transduction for vascular homeostasis and cell signaling. When excessively produced, or when antioxidants are depleted, ROS can inflict damage onto lipids, proteins, and DNA. This intracellular reduction-oxidation imbalance, called oxidative stress, can subsequently contribute to the development and/or progression of cardiovascular diseases such as atherosclerosis, ischemia-reperfusion injury, chronic ischemic heart disease, cardiomyopathy, congestive heart failure, and even ensuing arrhythmias [2,6-8].

Apparently, within cellular physiology there is a paradoxical role for ROS, which is temporally and spatially defined. In this paper we will discuss this dual role by summarizing the aspects of ROS generation and metabolization in the cardiovascular system, with focus on the role of ROS in cardiovascular cell signaling, in particular hydrogen peroxide (H_2O_2). In addition, we will discuss the role of ROS in ischemic heart disease.

MOLECULAR BASIS OF REACTIVE OXYGEN SPECIES

ROS encompass free radicals, oxygen ions, and peroxides, both organic and inorganic, but all derived from molecular oxygen. They are formed as necessary intermediates in a variety of normal biochemical reactions [3]. Only when produced in excess or not appropriately controlled, they can inflict damage within the body.

A division can be made into two groups: free radicals and other ROS. Free radicals have an extremely high chemical reactivity due to the unpaired free electron (i.e., superoxide anion $O_2^{\bullet-}$ and hydroxyl radical $OH^{\bullet}$). Other ROS like H_2O_2 and peroxynitrite ($ONOO^-$) are not considered free radicals as they lack the free unpaired electron and thus have oxidizing rather than reactive effects [3,9].

Formation of superoxide

Within living cells, $O_2^{\bullet-}$ is produced through two distinct pathways, namely, enzymatically (Figure 1) and nonenzymatically. The latter occurs when a single electron is directly transferred to oxygen by reduced coenzymes or prosthetic groups (e.g., flavins or iron sulfur clusters).

For most tissues, the primary source of $O_2^{\bullet-}$ is situated in the mitochondrial electron transport chain. It contains several redox centers that may leak approximately 1%-2% of the electrons to oxygen [5, 10]. Enzymatically, $O_2^{\bullet-}$ is produced from a variety of substrates, through different enzymes, most importantly NAD(P)H (nicotinamide adenine dinucleotide phosphate) oxidases, xanthine oxidases, and endothelial nitric oxide synthase (eNOS), as will be discussed below [11].

NADPH/NADH oxidases, located on the cell membrane of polymorphonuclear cells, macrophages, and endothelial cells, play an important role in generation of $O_2^{\bullet-}$ [3,9]. Under normal circumstances, NAD(P)H oxidases catalyze the reaction of NAD(P)H, H^+, and oxygen to form $NAD(P)^+$and H_2O_2. These oxidases are mainly present in adventitial fibroblasts but in different vascular pathologies, such as atherosclerosis and hypertension; upregulation of NAD(P)H expression has been shown in endothelial and vascular smooth muscle cells [12].

The conversion of xanthine dehydrogenase to xanthine oxidase (XO) provides another enzymatic source of $O_2^{\bullet-}$ and H_2O_2, which in turn constitutes a source of $OH^{\bullet}$. The relative amounts of $O_2^{\bullet-}$ and H_2O_2 formed by XO depend on the conditions. At physiological oxygen concentrations between 10%, and 21%, H_2O_2 constitutes

about 75% of ROS formed, whereas at lower oxygen concentrations, H_2O_2 formation from XO approaches 95% [13].

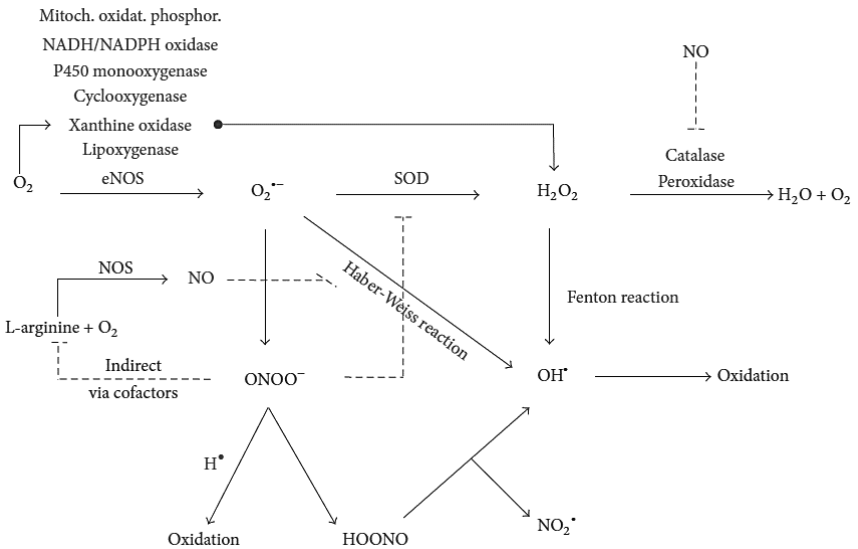


Figure 1. Summary of production and removal of various reactive oxygen species. Superoxide ($O_2^{\bullet-}$) can dismutate in several ways, either spontaneously through a reaction with superoxide dismutase (SOD), through the Haber-Weiss reaction, or in reaction with nitric oxide (NO) and its radical ($NO^\bullet$). Through SOD, hydrogen peroxide (H_2O_2) is formed and further reduced by catalase and peroxidase to form water and oxygen. Also, H_2O_2 can be formed directly from xanthine oxidase. The hydroxyl radical ($OH^\bullet$) is formed through the Haber-Weiss reaction, through the Fenton reaction, and from peroxynitrous acid (HOONO). $O_2^{\bullet-}$ can also scavenge NO to form peroxynitrite ($ONOO^-$) leading to nitroso-redox imbalance. NADH/NADPH oxidase: nicotinamide adenine dinucleotide (phosphate); (e)NOS: (endothelial) derived nitric oxide synthetase; NO: nitric oxide, NO_2: nitric dioxide; mitoch. Oxidat. phosphor, mitochondrial oxidative phosphorylation; dotted lines: inhibition.

The oxidase itself converts hypoxanthine into xanthine and xanthine into uric acid. Normally this process accounts for a small part of the ROS production but under pathological conditions, it has been proposed to mediate deleterious processes in vivo. For example, after reperfusion, large quantities of XO are released into the circulation possibly reacting with plasma purine substrates and molecular oxygen to produce ROS [14]. Chemical reactions forming molecular oxygen through NAD(P)H and xanthine oxidases are summarized in (1)–(4), respectively.

NAD(P)H oxidase reaction:

(1) $NAD(P)H + 2O_2 \rightarrow NAD(P)^+ + 2O_2^{\bullet-} + 2H^+$

Xanthine oxidase (XO) reaction:

(1) $Hypoxanthine + H_2O + 2O_2 \rightarrow Xanthine + 2O_2^{\bullet-} + 2H^+$

(2) $Xanthine + H_2O + 2O_2 \rightarrow Uric\ acid + 2O_2^{\bullet-} + 2H^+$

(3) $(Hypo)\ Xanthine + H_2O + 2O_2 \rightarrow Urate + H_2O_2$

Within vascular pathology, a potential important source of $O_2^{\bullet-}$ can be attributed to endothelial nitric oxide synthase [15]. There are three NOS isoforms, neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). In most cardiovascular tissues, nNOS and eNOS are constitutively present [3]. NOS enzymes normally catalyze the conversion of L-arginine to L-citrulline and produce nitric oxide (NO). eNOS is a heterodimer with both reductase and oxygenase domains on each monomer. In order to produce NO via eNOS, electrons must be transferred from the cofactor NAD(P)H to flavin adenine dinucleotide and flavin adenine mononucleotide to heme. The electron flow through eNOS to L-arginine, resulting in the production of NO, is dependent on the availability of its cofactors [16].

The balance between nitric oxide (NO) and $O_2^{\bullet-}$ production is regulated by the availability of tetrahydrobiopterin (BH_4). BH_4 is involved in the catalytic process of L-arginine oxidation [17]. With impaired bioavailability of BH_4, $O_2^{\bullet-}$ is released rather than NO, a condition referred to as “eNOS uncoupling” [15] (Figure 2), where electrons that normally flow from the reductase domain to the heme group, now divert towards molecular oxygen rather than L-arginine [15, 18]. In vascular disease, a major part of this catalytic enzyme is uncoupled due to BH_4 deficiency. The consequent increase in $O_2^{\bullet-}$ rapidly reacts with NO to form peroxinitrite ($ONOO^-$). $ONOO^-$ oxidizes BH_4 leading to “eNOS uncoupling,” and more production of $O_2^{\bullet-}$, thereby creating a vicious circle of ROS induced ROS production [15]. As will be discussed later on, the resulting endothelial dysfunction disturbs normal vascular responses and is associated with the development of atherosclerosis [11].

Importantly, endothelial dysfunction has been shown to be a prognostic factor for progression of atherosclerotic disease as well as cardiovascular event rate [19].

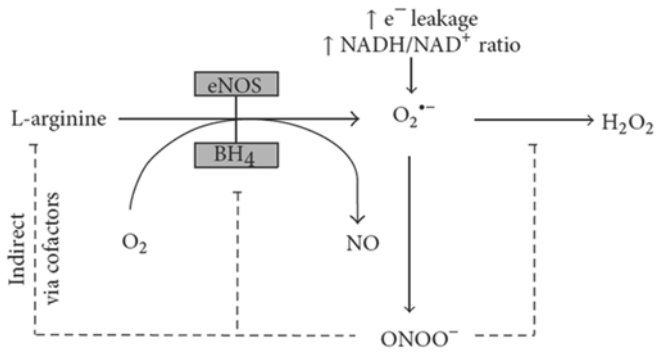


Figure 2. eNOS uncoupling. Excess superoxide ($O_2^{\bullet-}$) production, for example, after myocardial infarction, results in scavenging of nitric oxide (NO) to form peroxinitrite. The latter inhibits coupling of not only endothelial derived nitric oxide synthetase (eNOS) and tetrahydrobiopterin (BH_4), but also L-arginine and superoxide dismutase (SOD), which creates a downward spiral of enhanced $O_2^{\bullet-}$ production. Finally, eNOS gets uncoupled and produces $O_2^{\bullet-}$ rather than NO, sustaining the loop of nitroso-redox imbalance.

Reduction of superoxide

Oxidation-reduction reactions are highly similar to acid-base reactions and concern the transfer of electrons. The key in these reactions is that electrons are exchanged between reaction partners and not shared as with covalent bindings. Oxidation-reduction reactions are matched set, meaning that for every oxidation reaction there is simultaneous reduction reaction and are therefore called “half-reactions.”

Oxidation refers to loss of electrons, while reduction denotes the gain of electrons. The change of electrons between partners can be predicted by using the oxidation number, which is the algebraic difference between the number of protons and electrons in a specific ion. The produced intermediates are able to oxidize (by donating electrons) several molecules. When oxygen is scarce, cells move to a more reduced state resulting in altered function of biomolecules. This redox signaling comprises oxireductive chemical reactions that alter proteins posttranslationally, thereby creating a coupling between redox state and cell function [6].

$O_2^{\bullet-}$ can dismutate (be reduced) in several ways (Figure 1), either spontaneously through a reaction with superoxide dismutase (SOD), through the Haber-Weiss

reaction, or in reaction with NO. $O_2^{\bullet-}$ has a half-life of 10^{-9} to 10^{-11}s while in the presence of SOD, the half-life decreases to 10^{-15}s. The reaction catalyzed by SOD reduces two $O_2^{\bullet-}$ radicals to form oxygen and H_2O_2 which in turn can be fully reduced to H_2O and oxygen:

$$2O_2 + 2H^+ \rightarrow H_2O_2 + O_2 \quad (5)$$

H_2O_2 is oxidized by peroxidase and catalase. It has a half-life of 10^{-3}s in the absence of catalase and 10^{-8}s in its presence. Alternatively, H_2O_2 can react with reduced transition metals—called the Fenton reaction—to form $OH^{\bullet}$ and OH^- or $OOH^{\bullet}$ and H^+, when combined with Fe^{2+} or Fe^{3+}, respectively [3]:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^- \quad (6)$$

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + OOH^{\bullet} + H^+ \quad (7)$$

The typical range for the iron dose is 1 part of Fe per 5–25 parts of H_2O_2. The optimal pH for the Fenton reaction is between 3 and 6. When the pH is too high, iron precipitates in $Fe(OH)_3$ and will decompose H_2O_2 into oxygen. The Fenton reaction occurs predominantly at the endoplasmatic reticulum but not at mitochondria or other intracellular compartments [20]. Liu and coworkers showed that the Fenton reaction is involved in oxygen sensing, through regulation of genetic expression of hypoxia inducible factor-1 (HIF-1) [20]. An important role of HIF-1 is to establish the optimal balance between glycolytic and oxidative metabolism at any oxygen concentration to maximize ATP production without increasing ROS levels. Thus, HIF-1 induces metabolic reprogramming in cells that are oxygen deprived, thereby reducing mitochondrial respiration, minimizing $O_2^{\bullet-}$ production [21], and contributing to a fast responding oxygen-sensing system.

The reaction of $O_2^{\bullet-}$ with $NO^{\bullet}$, controlled by the rate of diffusion of both radicals, forms the very potent oxidant $ONOO^-$. $ONOO^-$ in turn is oxidized or reacts with a hydrogen radical ($H^{\bullet}$) to form the stable HOONO. The latter dismutates quickly into $OH^{\bullet}$ and free nitrogen species. Thus concentrations of $OH^{\bullet}$ increase by means of H_2O_2 and HOONO dismutation (metal independent pathway).

Alternatively, $OH^{\bullet}$ can be generated through the Haber-Weiss reaction, when superoxide radicals and H_2O_2 molecules spontaneously combine to form molecular oxygen and hydroxyl radicals:

$$O_2^{\bullet -} + H_2O_2 \rightarrow OH^{\bullet} + OH^- + O_2 \quad (8)$$

REACTIVE OXYGEN SPECIES AND CELL SIGNALING

Signal transduction pathways of cellular responses to reactive oxygen species

Most cells have been shown to generate a small burst of ROS when stimulated by, for example, cytokines, angiotensin II (Ang II), endothelin-1 (ET-1), and platelet derived growth factor (PDGF) [22], leading to the hypothesis that ROS play an important role in cellular homeostasis and communication [3] (Figure 3). ROS signaling involves alterations in the intracellular redox state and oxidative modification of regulatory and contractile proteins (Figures 4 & 5).

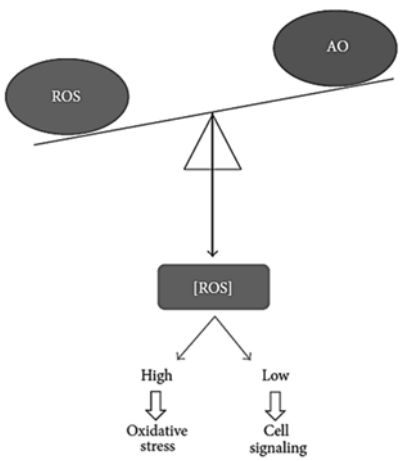


Figure 3. Redox balance. The production of reactive oxygen species (ROS) is tightly controlled by antioxidants (AOs) keeping the concentration of ROS ([ROS]) in the picomolar range. This low [ROS] is necessary for adequate cell physiology. When ROS is excessively produced or AOs are depleted, there is a high intracellular [ROS] leading to oxidative stress and resulting in cellular damage.

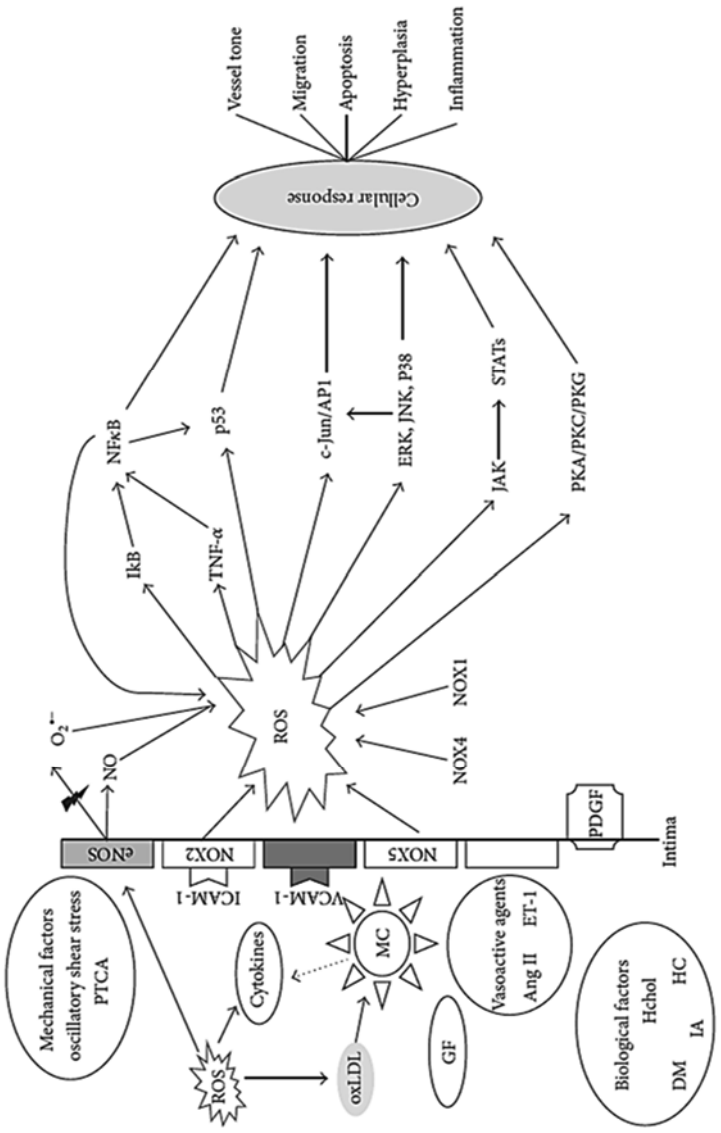


Figure 4. ROS production and vascular signaling. Several mechanical as well as circulating factors can increase ROS concentrations by acting on the tunica intima. The increased amounts of ROS activate specific second messenger systems which finally convey a cellular response. Hchol: hypercholesterolemia; DM: diabetes mellitus; IA: infectious agents; HC: homocysteine; MC: monocyte; GF: growth factors (PDGF, IGF-1, EGF, etc.); Cytokines (IL-1, TNF-α, etc.); oxLDL: oxidized low-density lipoprotein; eNOS: endothelial derived nitric oxide synthetase; PK: protein kinase A/C/G.

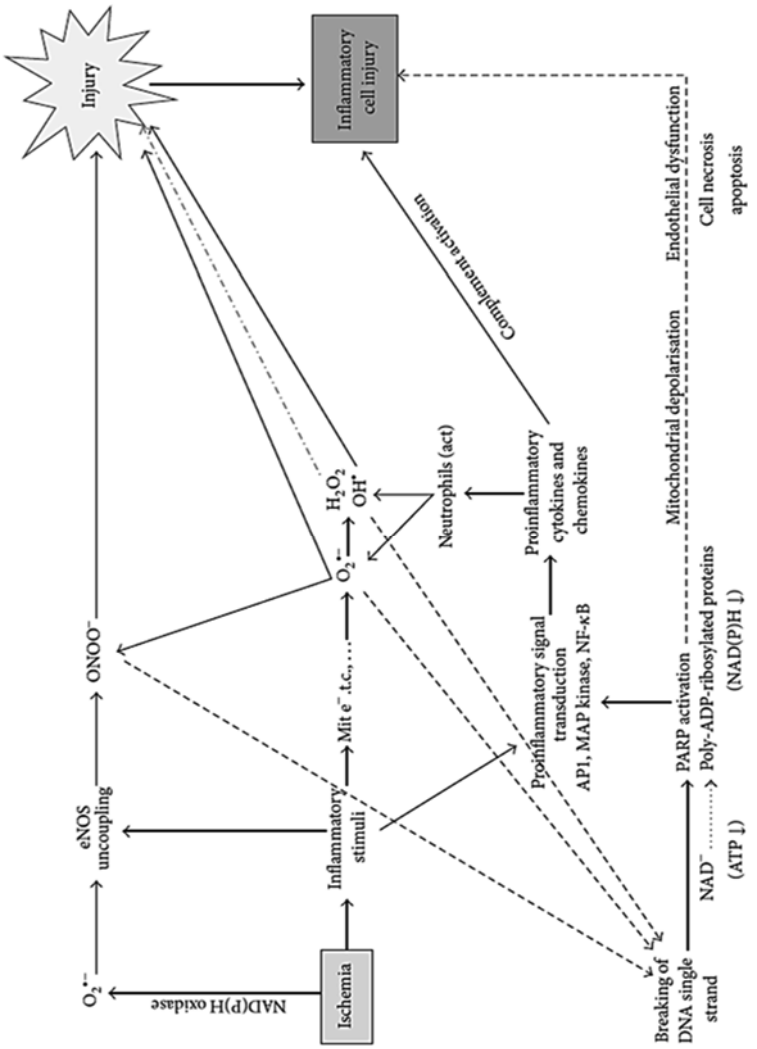


Figure 5. Different pathways leading to cell injury after ischemia. Ischemia due to atherosclerotic obstruction leads to an inflammatory process which provides the starting point of many other pathways of cellular injury via ROS production. Three main paths distinguished, being endothelial derived nitric oxide synthase (eNOS) uncoupling, mitochondrial electron transport, and proinflammatory signal molecules. Further, the produced ROS interact leading to DNA breaking and thus protein modification, with further cellular injury and dysfunction. Mit e⁻.t.c.: mitochondrial electron transport chain; ATP: adenosine triphosphate; NAD(P)H: nicotinamide adenine dinucleotide phosphate oxidase; PARP: poly-ADP-ribosylated proteins; $O_2^{•-}$: superoxide; H_2O_2: hydrogen peroxide.

The intracellular redox state within cellular homeostasis is primarily balanced by the glutathione/glutathione disulfide couple which functions as a major redox buffer, indicating/determining the redox state of the cell [23]. Glutathione (GSH) is abundantly present in the cytosol, nucleus, and mitochondria. It is synthesized in the cytosol and transported to the mitochondria and the nucleus [24]. GSH exhibits protection against ROS by (a) participating in amino acid transport through the plasma membrane, (b) scavenging $OH^{\bullet}$, H_2O_2, and lipid peroxidases via glutathione peroxidase (GPx) (catalytic reaction), (c) being a cofactor in numerous detoxifying enzymes (e.g. GPx), and (d) regeneration of the most important AOs back to their active form [25]. The latter function is linked with the redox balance of GSH with its oxidized form GSSG [26].

Glutathione exists in reduced (GSH) and oxidized (GSSG) states. When reduced, the thiol group of cysteine can donate a reducing equivalent to other unstable molecules such as ROS. By doing so, glutathione itself becomes reactive and quickly reacts with another reactive glutathione to form glutathione disulfide (GSSG). Once oxidized, glutathione can be reduced back by glutathione reductase, thereby using NAD(P)H as an electron donor. Under normal physiological conditions, more than 90% of the glutathione in the cell is in the reduced form (GSH) and less than 10% exists in the disulfide form. An increase in GSSG/GSH ratio, for example due to inactivation of glutathione reductase by $ONOO^{-}$, is considered to be indicative of oxidative stress [27].

ROS can post translationally modify proteins. Redox signaling typically involves amino acid oxidation, hydroxylation, or nitration. Targets usually are redox sensitive cysteine residues within the proteins, which have a low ionization pKa of 4-5 compared to a pKa of 8.5 of nonreactive cysteines in other proteins [28,29]. The modifications in these redox sensitive proteins alter their conformation, stability, activity and/or ability to interact with other proteins, resulting in modulation of cellular function. Redox sensitive proteins include proteins involved in calcium handling as well as contractile proteins, proteins involved in various signaling pathways and proteins involved in transcriptional activities.

Redox modulation of calcium handling proteins directly affects cardiac contraction by altering intracellular calcium. Examples of redox sensitive calcium handling proteins are calcium calmodulin kinase II (CaMKII), the ryanodine receptor on the sarcoplasmic reticulum, sarcoplasmic reticulum ATPase (SERCA), and phospholamban (for review see [29, 30]). Moreover, the contractile proteins can also be oxidatively modified by oxidation or nitrosylation [30]. Typically, oxidation of

contractile proteins is assumed to depress cardiac function, although recently some modifications have been identified that actually increase contractility. The current understanding on how oxidative stress modulates cardiac function is limited mostly because many studies have focused on isolated myofilament proteins whereas oxidative modifications of different contractile proteins occur simultaneously in vivo and act in concert. Hence, the contributions of the individual oxidative modification are difficult to establish [30].

The second group of proteins affected by direct redox modification are protein kinases and phosphatases. Since tyrosine phosphorylation is an early signaling event in many signal transduction pathways, alterations in activity through redox modification of protein kinases upstream in the signaling cascade result in indirect modulation of protein kinases more downstream in the cascade. Tyrosine phosphorylation in vascular smooth muscle cells is important in the control of vascular tone. Thus, tyrosine phosphatase inhibitors generally constrict smooth muscle, whereas tyrosine kinase inhibitors cause relaxation [28]. Oxidative modification results in inhibition of phosphotyrosine phosphatases (PTP 1A, PTP1B, and PTEN), while the protein kinase Src is activated by oxidation. Src has many targets in the cell. Interestingly, Src activates receptor tyrosine kinases such as the EGF-receptor in vascular smooth muscle cells. This activation occurs independent of EGF, and the activated receptor then acts as a signaling platform for the stimulation of phospholipase enzymes, production of lipid mediators, and activation of downstream kinases such as PI3K, Akt, ERK, and PKC [28].

PKC is directly activated by oxidation of the cysteine residues in its regulatory site, which occurs at low concentrations of oxidants. Conversly, PKC is inhibited by oxidation of cysteine residues in its catalytic domain, which occurs at higher concentrations of oxidants. Alterations in PKC activity affect many signaling cascades in the cell, including modulation of calcium sensitivity of the myofilaments and receptor tyrosine kinase signaling [28, 30]. The cAMP-dependent protein kinase A (PKA) and the cGMP-dependent protein kinase G (PKG) are also susceptible to redox modification. Both PKA and PKG are involved in regulation of vascular tone as well as cardiomyocyte contraction. When PKA oxidation occurs in its regulatory domain, it promotes dissociation of the catalytic and regulatory subunits resulting in cAMP independent PKA activation [29, 30]. However, similar to PKC, oxidation of cysteine residues in the catalytic subunit inhibits PKA activity [29]. Oxidation of PKG in its dimerization domain results in activation of the enzyme independently of the NO-cGMP pathway [29]. Oxidative modification of PKA, PKC, and PKG results in altered

phosphorylation of the myofilaments, thereby modulating cardiac as well as vascular function.

The small monomeric G-proteins ras, rac-1, and RhoA are also activated by ROS. Activation of RhoA results in its translocation to the plasma membrane and activation of Rho-kinase. Rho-kinase appears to be a key player in cardiovascular function and cardiovascular pathology. Thus, in vascular smooth muscle cells, Rho-kinase regulates calcium sensitivity of the myofilaments via inhibition of myosin light chain phosphatase [31]. Moreover, activation of Rho-kinase contributes to smooth muscle proliferation, hypertrophy and motility [32]. Rho-kinase activation is responsible for upregulation of NAD(P)H oxidases, thereby contributing to a vicious circle of ROS, leading to Rho-kinase activation resulting in more ROS production. In endothelial cells, Rho-kinase negatively regulates NO-production both by destabilizing eNOS mRNA and through impairment of eNOS activity [31, 32], thereby also contributing to augmentation of oxidative stress. In cardiomyocytes the role of Rho-kinase is less well understood although it is thought to function in a similar way to its role in vascular smooth muscle. In addition, Rho-kinase is thought to be involved in cardiomyocyte hypertrophy and apoptosis [31]. Yet, the precise role of Rho-kinase, as well as its modulation by redox regulation in cardiac myocytes, remains to be determined.

Another group of kinases that are not directly redox sensitive but very important in cardiovascular cell signaling are mitogen-activated protein kinases (MAPKs) (Figure 4). MAPKs are indirectly activated by ROS via the ROS sensitive kinases Src, PKC, ras, and the MAPK kinase kinase ASK-1 [33]. MAPKs are divided into three subgroups: extracellular signal regulated kinases (ERKs): ERK1 and ERK2; c-Jun N-terminal kinases (JNKs): JNK1, JNK2, and JNK3; and p38 kinases: p38 α, β, γ, and δ [3, 34, 35]. In addition to being indirectly activated by ROS, MAPKs are activated by environmental stresses and inflammatory cytokines, which are also known to induce oxidative stress.

The third important group of redox sensitive proteins in the cardiovascular system is involved in transcriptional activity, not only including transcription factors but also histone deacetylases (HDAC). ROS can both inhibit and stimulate cellular NF-κB signaling [36], while certain NF-κB regulated genes play a major role in regulating the amount of ROS in the cell. Also, recently ROS have been shown to directly connect the important redox sensitive transcription factors NF-κB and HIF-1, implicating a novel signaling pathway in cardiovascular pathology (Figure 5) [37].

Histone acetylation by histone acetylases promotes gene expression, while histone deacetylation by HDACs inhibits gene expression. Oxidation of HDAC4 and HDAC5 that are expressed in cardiac myocytes results in export of these HDACs from the nucleus, thereby inhibiting their activity. As these HDACs normally inhibit the transcription of prohypertrophic genese their oxidation may be involved in induction of hypertrophy [29].

The long-term consequences of ROS for cardiovascular (dys)function depend on the balance between signals promoting proliferation or growth inhibition and/or cell death. ROS can alter this balance leading to either excessive angiogenesis or loss of endothelial cells [2]. The dual role of ROS in "fine-tuning" the balance between apoptosis and excessive cell growth is illustrated by observations that, during ischemia-reperfusion injury, ROS trigger apoptosis, while ROS generated during ischemic preconditioning prevent apoptosis [38–41]. ROS generated during ischemic preconditioning are capable of up regulating expression of the Bcl-2 gene [42], which regulates the intrinsic pathway of apoptosis [43, 44]. This gene is also activated via the nuclear transcription factor NF-κB and its activation has been shown to reduce apoptosis [42, 45].

Conversely, endothelial apoptosis initiated by tumor necrosis factor-α (TNF-α) and mediated by activation of JNK can be attenuated by ROS scavenging [46]. TNF-α has been implicated in inflammatory responses of the heart and vasculature. Thus, TNF-α is one of the inflammatory cytokines that are produced in the ischemic region and surrounding myocardium following myocardial infarction. Also, vessels from subjects with diabetes are characterized by an increased TNF-α production, increased ROS production, and endothelial dysfunction [47]. Similarly, endothelial dysfunction induced by advanced glycation end products (AGEs) is mediated through elevated TNF-α expression and induction of ROS production with NF-κB functioning as the link between TNF-α and AGEs/RAGE signaling [48, 49]. TNF-α and NF-κB are interrelated in that translocation of NF-κB into the nucleus has been proposed to be pro-inflammatory and, either directly or indirectly, leading to a significant increase in TNF-α production while TNF-α activates NF-κB, which then regulates genes involved in inflammation, oxidative stress, and endothelial dysfunction [49, 50]. Interestingly, ROS produced in response to TNF-α can further activate NF-κB which again activates TNF-α creating a vicious circle [51]. Hence, ROS play a key role in the induction of vascular dysfunction in response to TNF-α [47].

The role of hydrogen peroxide in signal transduction

H_2O_2 is an interesting molecule within the ROS family. It is a waste product of mitochondrial electron transfer and, hence to be created, no additional energy is required. The chemical properties of H_2O_2, such as a short half-life, rapid metabolization by catalase, and rapid reaction with thiols, are ideal properties for H_2O_2 to act as a signaling molecule.

H_2O_2 mediates diverse physiological responses including cell differentiation, proliferation, and migration, and has been proposed to be involved in metabolic vasodilation [3, 70]. In cells stimulated with growth factors and cytokines (PDGF, EGF, insulin, TNF-α, and interleukins), the NAD(P)H oxidase gp91 Phox and homologues form the major source of H_2O_2 [34, 70–72]. However, the coupling between receptor activation to the NAD(P)H oxidase complex (Nox) is still poorly understood [73]. In order to mediate different responses, H_2O_2 modifies the activity of key signaling proteins. It catalyzes redox reactions, oxidizing primarily cysteine residues of proteins thereby altering their function. For example, the activity of tyrosine phosphatases is H_2O_2 dependent. The chemical configuration of these phosphatases contains a cysteine and arginine site resulting in a low PK_a and existing as a thiolate anion. The latter is more susceptible to H_2O_2 oxidation which abolishes its activity and is reversed by cellular thiols [74]. Not only tyrosine phosphatase but also tyrosine kinase (Src) is oxidized by H_2O_2 [75]. An overall regulation must exist, that is, both temporal and spatial, ensuring process activation (only when and where needed) and termination after exerting its effects.

In order to induce protein alterations, H_2O_2 must increase rapidly above a certain threshold, but each cell contains natural AO enzymes. Therefore H_2O_2 has to be protected from destruction. H_2O_2 is inactivated by peroxiredoxin, which is part of a family of antioxidant enzymes whose thioredoxin peroxidase activity plays an important role in protecting against oxidative stress [76]. Interestingly, H_2O_2 causes hyperoxidation, and thereby inactivation, of peroxiredoxin, prolonging H_2O_2 bioavailability. The inactive peroxiredoxin can be reactivated by the adenosine triphosphate-dependent enzyme sulfiredoxin [77].

How H_2O_2 is actually delivered to the cytosol remains incompletely understood. H_2O_2 must cross the lipid bilayer towards the target molecules in the cytosol. Although it is generally assumed that H_2O_2 crosses the membrane freely, recent research indicates some membranes to be poorly permeable to H_2O_2 [78]. A shift in membrane lipid composition or a transport through aquaporins was presented as an

alternative pathway to transfer H_2O_2 molecules to the cytosol [79]. Alternatively, H_2O_2 may also pass through gap junctions to exert its effect [80–82].

A duality is clearly present in the role for H_2O_2 in cell proliferation. On the one hand, low concentrations of H_2O_2 play an important role in regulating cell growth, although the question remains if this effect is exerted merely through second messengers (JAK/STAT) or if H_2O_2 also has a direct effect on growth. On the other hand, high concentrations of H_2O_2 are responsible for cell apoptosis while moderate doses cause the cell to arrest in the G1 phase [83]. Recently, H_2O_2-induced apoptosis was shown to be mediated through a PKC-dependent pathway, antagonized by Akt and heme oxygenase-1 [84]. Also, recent data support the hypothesis that H_2O_2 can function as a transmitter of the apoptotic signal from the region of programmed cell death to neighboring healthy cells [85]. Apoptotic cells produce H_2O_2 thereby possibly contributing to the pathogenesis of, for example, myocardial infarction and ageing. More specifically, mitochondrial produced ROS are needed for the generation of the apoptotic signal since specifically designed mitochondrial antioxidants (such as SkQs) inhibit this pathway [86].

H_2O_2 may also be involved in the response to vascular injury. Vascular smooth muscle cell (VSMC) death has been shown to occur after mechanical trauma like stenting. In response to vascular injury, potent chemotactic factors, such as bFGF, PDGF, TGF-*β*, and Ang II, are released [2]. These chemotactic factors in turn regulate VSM proliferation and migration. Two independent research groups have shown that this release of chemotactic factors is H_2O_2 dependent and thus reduced when administrating a scavenger like catalase [87, 88]. The hypothesis for ROS involvement in VSMC proliferation is further supported by a study showing that catecholamine induced VSCM proliferation can be blocked by N-acetylcysteine, tiron (superoxide scavenger), and diphenylene iodonium [89]. To allow VSMC migration and vascular remodeling, degradation of the extracellular matrix is required, which is partially accomplished by matrix metalloproteinases (MMPs). Both activity and expression of MMP-2 and MMP-9, the two MMPs thought to be most important in vascular remodeling, have been shown to be regulated by the nitroso-redox balance. Thus, H_2O_2 and $OONO^-$ increase MMP-2 activity while reduction of MMP-2 and MMP-9 can be obtained by overexpressing eNOS [90, 91]. Not only the activity of the MMP family is modulated by ROS but also their expression, thereby providing a dual mechanism for ROS to regulate vascular remodeling.

REACTIVE OXYGEN SPECIES AND PATHOPHYSIOLOGY (Figures 4 & 5)

The mitochondrial respiratory chain is one of the most prominent cellular sources of ROS. Hence, ROS production is related to oxygen consumption, making cells with high oxygen consumption more prone to oxidative stress. Oxygen consumption is particularly high in cardiac myocytes that are therefore equipped with a high number of mitochondria and a high level of respiratory chain components per milligram of mitochondrial protein. To prevent oxidative damage, these cells contain enzymatic scavengers of ROS such as SOD, glutathione peroxidase, catalase, and coenzyme Q10. Possibly together with non-enzymatic AO, they neutralize the deleterious effects of ROS. Mitochondria also possess the ability to repair themselves after oxidative damage using enzyme systems like phospholipid hydroperoxide glutathione peroxidase (PHGP). PHGP is a selenium containing enzyme directly reducing peroxidized acyl groups in phospholipids [92]. However, under pathological conditions, these protective mechanisms may fall short and make the cardiac myocytes vulnerable to oxidative damage.

With increased production of ROS, damage can be inflicted directly via oxidative modification of redox sensitive proteins [3,10]. Also, inflammation, which in turn stimulates the release of $O_2^{\cdot -}$, leads to cell injury, either directly or by depleting the natural AOs. An overview of the systems leading to cell damage via $NO^{\bullet}$, $ONOO^{-}$, $O_2^{\bullet -}$, $H_2O_2/OH^{\bullet}$, complement activation, and PARP activation is presented in Figures 3, 4 and 5.

Some examples of how oxidative stress is involved in cardiac and vascular pathologies are described in the following sections.

Hypoxia, Ischemia, and Reperfusion

Medical strategies treating acute myocardial infarction require restoration of blood flow to the ischemic region. Unfortunately, this reperfusion is associated with a burst of ROS, which may continue for hours [93], and recruitment of inflammatory cells [94, 95]. These high levels of ROS cause structural damage of the heart, capillary leak, and influence cardiomyocyte metabolism thereby impairing both systolic and diastolic function [96]. Furthermore, not only ischemic damage, but also reperfusion, can produce dysfunction of the cardiac conduction system leading to arrhythmias [7, 97]. Besides increased ROS production, hypoxia, ischemia, and

reperfusion have also been found to reduce levels of SOD, GSH, glutathione peroxidase, and ascorbate [98]. Hence, reduced scavenging further contributes to development of oxidative stress.

Reperfusion also inflicts damage on the vascular endothelium, with alterations in blood cells and micro-embolization, as well as vascular compression due to myocyte swelling, leading to changes in endothelial structure and alignment. The duration of ischemia is an important determinant of the extent of reperfusion damage. Ischemia-reperfusion damage can be reduced by ischemic preconditioning [99]. The mechanisms underlying this protection by ischemic preconditioning are incompletely understood [100]. Yet, a role for ROS as triggers for and mediators of this protective phenomenon has been consistently demonstrated. ROS can trigger preconditioning by causing activation of the mitochondrial K_{ATP} channel, which then induces generation of ROS and NO that are both required for preconditioning induced protection [101–104]. Importantly, ischemic preconditioning can be mimicked by administration of free radical donors S-morpholinosydnonimine [105] and even $ONOO^-$ [106] while preconditioning can be blocked by free radical scavengers.

Ischemic preconditioning is therefore a clear example of the so-called oxidative paradox: AOs not only reduce deleterious ROS accumulation but also molecules necessary for cardioprotection.

Atherosclerosis

The majority of cardiovascular disease is a direct consequence of atherosclerosis. The transfer of oxidized low-density lipoprotein (ox-LDL) from the vessel lumen into the tunica media is regarded as the initiator for atherosclerosis at sites with endothelial damage [107]. Mechanical factors like fluid shear stress patterns play an important role in maintenance of endothelial function and initiation of endothelial dysfunction [3]. Thus, laminar shear stress induces expression of AO genes and production of $NO^{\bullet}$, preventing apoptosis and monocyte adhesion [108]. Branched arteries exposed to oscillatory shear stress are prone to atherosclerosis. This type of flow leads to continuous NAPDH-dependent production of $O_2^{\bullet-}$ [109,110]. Increased $O_2^{\bullet-}$ can subsequently uncouple eNOS, thus creating an extra source of $O_2^{\bullet-}$ production and leading to a vicious circle of ROS-induced ROS production. Upregulation of adhesion molecules (VCAM-1, E-selectin, P-selectin, and ICAM-1) [111] at locations with disrupted flow patterns is also ROS dependent and is further

enhanced by cytokines like interleukins, TNF-α, Ang II, and vascular endothelial growth factor (VEGF) [3, 109–111]. Up regulation of adhesion molecules facilitates adherence and transmigration of leucocytes (Figures 4 and 5). Once converted to macrophages, they are capable of producing much higher amounts of ROS. ROS convert ox-LDL into highly oxidized LDL which itself is engulfed by these macrophages, forming foam cells and initiating the formation of the fatty streak.

Overall, the amount and pattern of blood flow are very important for endothelial function where aberrant flow patterns predispose to ROS production and atherosclerosis.

Clinical Evidence for Therapeutic Use of Antioxidants?

The deleterious effects of ROS can be reduced by restoring the imbalance between production and clearance of ROS [112]. Gey and colleagues found low rates of cardiovascular disease (CVD) in people consuming AO rich diets [113]. This second line of defense includes nonenzymatic antioxidant substances from dietary intake such as ascorbic acid (vitamin C), α-tocopherol (vitamin E), GSH, flavonoids, carotenoids, and others. In vitro studies indicated oxidation inhibition of low-density lipoproteins (LDL) by a number of these nonenzymatic AOs. Exogenous therapeutic administration of antioxidants has therefore been proposed as therapy for oxidative stress and cardiovascular disease. Despite some promising effects of such AO administration (Table 1), particularly in animal studies, caution should be warranted as these results could usually not be reproduced in clinical trials. Thus, conflicting results on the use of dietary supplementation of AOs, especially vitamin C, vitamin E, β-carotene, and selenium, have been presented (Table 2). AO supplementation is potentially deleterious for normal "redox homeostasis." Not only is the redox balance very delicate, but also ROS play important roles in cell signaling and are therefore essential for survival of the organism [3, 9, 114]. The most relevant AOs used in dietary supplementation are flavonoids and vitamins C and E. The beneficial cardiovascular effects of these substances may not be limited to their AO effect, as they also include anti-inflammatory, platelet inhibitory, and antithrombotic effects.

One of the most studied AO supplements in prevention of cardiovascular disease is vitamin E. Many studies suggest a protective role for vitamin E, which has led to a massive marketing of vitamin E supplements. Conversely, large randomized studies (Table 2) could not substantiate this role for vitamin E. Indeed, meta-analyses failed to find a cardio protective effect nor did it find a reduction of clinical events in high

risk patients or in patients with established disease [115–117]. Interestingly, a recent analysis even reports an increased mortality after using supplements of *β*-carotene and vitamin E [117].

When comparing Table 1 with Table 2, clinical trials fail to show a protective effect of AOs in humans. Not only are the positive outcome studies largely outnumbered by trials with no effects, they are also, in most cases, lacking the statistical power to be conclusive. The reason for failure of these AOs in clinical practice is most likely multifactorial.

First of all, it is very difficult to detect subjects with a comparable imbalance between ROS production and AO defenses. Second, biochemical aspects inherent to each substance used, posology, and intake ratio must be taken into account. The lack of knowledge of the optimal dosage and route of administration for the various AOs is a serious limitation. For example, the bioavailability of vitamin C is determined by the availability of its transporter in the small intestine, and an increase of oral administration of vitamin C can actually decrease bioavailability [118]. This example illustrates that pharmacodynamical and pharmacokinetic properties of each AO should be known to optimize their application. To be effective, it is imperative that AOs reach the specific compartments of the cell where ROS are generated. For many vascular cells, this requires uptake into the cytoplasm (or vesicles) [2]. Importantly, AOs can become oxidants in some cellular compartments.

Third, lack of clinical improvement may be attributed to the selection of the population. Most patients enrolled in the clinical trials have already established CVD and in these cases AO therapy may be too late to be effective since, in animal studies, most protocols describe administration of AOs prior to the initiation of the disease. Finally, and perhaps most important, the choice of AOs should be based on the identity and location of ROS responsible for pathology. So instead of experimenting with cocktails of AOs in human trials, basic research should focus on targeting the specific pathways of different ROS responsible for the given pathology [112, 119].

Author/ Study	Journal	Design/FU	Population*	Agents (dosage/day)	Results
Stephens et al. CHAOS [52]	Lancet	DB, PC 1.4 y (3y)	N = 2002; M and F; mean 61.8y; ischemic heart disease patients; secondary prevention	E (800mg or 400IU)	↓↓I non-fatal MI, trend ↑ CV death
Duffy et al. [53]	Lancet	DB, PC 30 d	N= 45; M and F; mean 48.5y; HT patients	C (500mg)	↓ BP in otherwise healthy HT
Boaz et al. SPACE [54]	Lancet	DB, PC 2 y	N = 196; M and F; 40-75y; haemodialysis patients	E (800 IU)	↓↓ combined endpoint of AMI = CV death + stroke
Neri et al. [55]	Clin Ther	DB, PC 15 d	N = 46; M and F; mean 40y; DM / glucose intolerance patients	NAC (600g)+C(250mg) +E(300mg)	↓ OS and inflammation
Accini et al [56]	Nutr Metab Cardiovasc Dis	DB, PC 4 m	N = 57; M and F; 23-65y; dyslipidemic patients	E (4mg); PUFAn-3 (6602mg EPA + 440 DHA); niacin (18mg); γOZ (40.2mg)	↓ OS and inflammation markers

Table 1. Major studies with possible beneficial effects of AOs on cardiovascular outcomes in humans. The CHAOS study is the largest study to report a strong decrease in nonfatal MI but, conversely, a slight increase in cardiovascular death. Other studies were performed in smaller groups. Overall, no overwhelming positive effects could be found in the studies. Population*: *N*: number of patients; M: male; F: female; y: age in years. DB: double blind; PC: placebo controlled; d: days; m: months; E: vitamin E; C: vitamin C; NAC: N-acetylcysteine; PUFAn-3: polyunsaturated fatty acids n-3; EPA: eicosapentaenoic; DHA: docosahexaenoic; *γ*OZ: *γ*-oryzanol; I: incidence; BP: blood pressure; CV: cardiovascular; MI: myocardial infarction; HT: hypertension; AMI: acute myocardial infarction; OS: oxidative stress.

Author	Journal	Design & FU	Population*	Agents (dosage /day)	Results
Hennekens et al. Physician health [57]	NEJM	DB, PC, 2x2 12 y	N = 22071; M, 40-84y; former or current smokers	βC (50mg) on alt days	No effect on CV death, AMI or all cause mortality
Rapola [58]	Lancet	DB, PC 5,3 y	N = 1862; M; 50-69y; smokers with previous MI	E (50mg)+βC (20mg)	No ↓ of MCE, ↑ risk FCHD
Virtamo [59]	Arch Intern Med	DB, PC 6.1 y	N = 27.271; M; 50-69y; smokers, no MI history; primary prevention	E (50mg)+βC (20mg)	E: ± ↓ I fatal CHD, no ↓ I non-fatal CHD; βC no effect
GISSI Prevenzione Investiga-tors [60]	Lancet	OL, PC, 2x2 3.5 y	N = 11234, M and F; startified for all age groups; AMI within 3 months; secondary prevention	E (600mg) + fish oil (10mg)	E: no effect AMI + death + stroke, fish oil: ↓ AMI + death + stroke
Yusuf et al. HOPE [61]	NEJM	DB, PC, 2x2 4.5 y	N = 9451; M and F; ≥55y; high risk CD patients; primary and secondary prevention	E (800 mg or 400IU) Ramipril	E: no effect AMI + CV death + stroke; Ramipril: ↓ AMI + CV death + stroke
De Gaetano et al. PPP [62]	Lancet	OL, PC, 2x2 3.6 y	N = 4495; M and F; mean 64.4y; high risk CD patients; primary prevention	E (300mg) Aspirin (100mg)	E: no effect Aspirin: ↓ AMI + CV death + stroke
Collins et al. HPSCG [63]	Lancet	DB, PC 5 y	N = 20.563; M and F; 40-80 y; CD, other OAD, DM patients	C (250mg) +E (600mg) +βC(20mg)	no ↓ 5y mortality

Author	Journal	Design & FU	Population*	Agents (dosage /day)	Results
Törnwall et al. [64]	Eur Heart J	DB, PC 5-8 y	N = 29.133; M; 50-69y; smokers with risk on MCE or MI history	E (50mg) or βC (20mg) or both	βC: ↑ risk non-fatal MI; E: no effect
Armitage et al. HPS [65]	BMC Med	DB, PC, 2x2 5.5 y	N = 20.53 M and F; 40-80y; high risk CD patients; primary and secondary prevention	Simvastatin (40mg) C (250mg) +E (600mg) +βC(20mg)	AO: no effect
Cook et al. WACS [66]	Arch Intern Med	DB,PC, 2x2 9.4 y	N = 8171; F; ≥ 1 CVE in history, secondary prevention	C (500mg) +E (600IU) on alt days + βC (50mg) on alt. days	No effect AMI + CV death + stroke + morbidity
Lee et al. [67]	JAMA	DB, PC, 2x2 10.1y	N = 39876; only F; >45y, healthy.	E (600IU) Asprin (100mg)	Ne benifit for major CV events. No effect on total mortality
Lonn et al. HOPE II [68]	JAMA	DB, PC 7.0 y	N=3994, >55 y with vascular disease or DM; extension of HOPE I trial.	E (400IU)	No prevention of major CV events. No prevention of cancer. Risk of HF may be ↑
Kataja-Tuomola et al. [69]	Ann Med	DB, PC, 2 × 2 6.1 y	N= 29.133, all M smokers, some with DM.	E (50mg/d) βC (20mg/d)	No protective effect on macrovascular outcomes or total mortality.

Table 2. Major studies with no beneficial effects of AOs on cardiovascular outcomes. Large multicenter studies all presented the same result that oral AOs had no beneficial effect on cardiovascular outcomes. Some studies even showed an increased risk of coronary heart disease. Population*: *N*: number of patients; M: male; F: female; y: age in years; 2 × 2: 2 × 2 factorial design comparing placebo, agent A, agent B, and combination of agent A and B; DB: double blind; OL: open label; PC: placebo controlled; E: vitamin E; C: vitamin C; βC: beta-carotene; FCHD: fatal coronary heart disease; MCE: myocardial event; CHD: coronary heart disease; AMI: acute myocardial infarction; CV: cardiovascular; OS: oxidative stress; IU: international units; HF: heart failure.

The lack of cardiovascular benefit of AOs that are presently available has initiated research on new and more effective compounds. Clinical studies show that while these novel compounds do not reduce endpoints related to atherosclerosis, they improve endothelial function by increasing local NO bioavailability and therefore endothelium-dependent vasodilatation. Promising new agents are NO-donor phenols [120] and AGI-1067 that inhibits pro-inflammatory gene expression [121]. Other potentially promising AOs act through targeting NAPDH oxidases, Nox (VAS2870), and Nox2 peptide (gp91-dstat), preventing eNOS uncoupling or inhibiting xanthine oxidase (allopurinol) [2, 72, 119]. Technological developments also allow discovery of new functions for existing AOs. For example, oxidative damage of DNA was shown to be repaired in cells by naturally occurring phenols independent of known DNA repair enzymes thereby entailing possible new approaches [122].

Moreover, targeting AO therapy at specific sites of ROS production may be more effective in treatment of cardiovascular disease than global AO therapy [123]. Mitochondrial ROS scavenging is effective in treating hypertensive rats [124] and led to the realization that mitochondria play a central role in the pathogenesis of cardiovascular disease. New pharmacological approaches enable targeting of therapeutic substances at the mitochondria [125]. In particular, AOs conjugated with triphenylphosphonium cation such as mitoquinone (MitoQ), mitovitamin E, and mitophenyltertbutyline achieve much higher concentrations in the mitochondrial membrane, than those in the cytosol, due to the negative membrane potential of the inner mitochondrial membrane [126]. A new type of compounds, named SkQs, consisting of an antioxidant moiety (plastoquinone) and a penetrating cation, has been synthetized. This group of AOs specifically prevented oxidation of mitochondrial cardiolipin, arrested H_2O_2-induced apoptosis, and blocked necrosis initiated by ROS [86, 127]. Furthermore, SkQs also appear very promising in inhibiting the development of age-related diseases [86, 128]. However, so far, no studies have been performed that target mitochondria in cardiovascular disease in humans.

CONCLUSION

ROS play a dual role in cardiovascular (patho)physiology. ROS signaling plays an important part in endothelial function, vascular tone, and cardiac function. Conversely, when excessively produced, ROS can disrupt cellular signaling and inflict cellular damage. It thus appears that concentration and location of ROS are the main determinants of their function.

Due to their very short half-life and technical difficulties of measuring ROS in vivo, little is known about the "safe margins" of ROS concentrations in cell signaling. Therefore, it is difficult to estimate which part of ROS production contributes to cellular homeostasis and normal physiological functioning and when ROS production becomes excessive and thereby detrimental.

Although the deleterious effects of ROS can potentially be reduced by restoring the imbalance between production and clearance of ROS through administration of AOs, the dosage and type of AOs should be tailored to the location and nature of oxidative stress. Continuous administration of AOs in vivo can be unfavorable for normal cell signaling which, at least partially, explains the lack of clinical evidence on the beneficial actions of AO administration.

New research should focus on matching AO therapy to oxidant stress present in the cardiovascular system. In vitro studies are extremely important to obtain knowledge on the mechanisms of oxidative damage as well as potential repair mechanisms, and when extrapolated to the in vivo setting with caution, they are likely to contribute to improve the therapeutic strategies for cardiovascular disease.

REFERENCES

1. Papa S, Skulachev VP. Reactive oxygen species, mitochondria, apoptosis and aging. Molecular and Cellular Biochemistry. 1997;174(1-2):305–319.
2. Papaharalambus CA, Griendling KK. Basic mechanisms of oxidative stress and reactive oxygen species in cardiovascular injury. Trends in Cardiovascular Medicine. 2007;17(2):48–54.
3. Dröge W. Free radicals in the physiological control of cell function. Physiological Reviews.2002;82(1):47–95.
4. Chen Q, Vazquez EJ, Moghaddas S, Hoppel CL, Lesnefsky EJ. Production of reactive oxygen species by mitochondria: central role of complex III. Journal of Biological Chemistry. 2003;278(38):36027–36031.
5. Turrens JF. Mitochondrial formation of reactive oxygen species. Journal of Physiology.2003;552(2):335–344.
6. Charles RL, Eaton P. Redox signalling in cardiovascular disease. Proteomics. 2008;2(6):823–836.
7. Lopes RD, Li L, Granger CB, et al. Atrial fibrillation and acute myocardial infarction: antithrombotic therapy and outcomes. American Journal of Medicine. 2012;125(9):897–905.
8. Vermeer F, Simoons ML, Lubsen J. Reduced frequency of ventricular fibrillation after early thrombolysis in myocardial infarction. The Lancet. 1986;1(8490):1147–1148.
9. Griendling KK, FitzGerald GA. Oxidative stress and cardiovascular injury part I: basic mechanisms and in vivo monitoring of ROS. Circulation. 2003;108(16):1912–1916.
10. Raha S, Robinson BH. Mitochondria, oxygen free radicals, disease and ageing. Trends in Biochemical Sciences. 2000;25(10):502–508.
11. Cai H, Harrison DG. Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress.Circulation Research. 2000;87(10):840–844.
12. Griendling KK, Sorescu D, Ushio-Fukai M. NAD(P)H oxidase: role in cardiovascular biology and disease. Circulation Research. 2000;86(5):494–501.
13. Kelley EE, Khoo NKH, Hundley NJ, Malik UZ, Freeman BA, Tarpey MM. Hydrogen peroxide is the major oxidant product of xanthine oxidase. Free Radical Biology and Medicine. 2010;48(4):493–498.
14. Yokoyama Y, Beckman JS, Beckman TK, et al. Circulating xanthine oxidase: potential mediator of ischemic injury. American Journal of Physiology. 1990;258(4):G564–G570.
15. Taverne YJ, de Beer VJ, Hoogteijling BA, et al. Nitroso-redox balance in control of coronary vasomotor tone. Journal of Applied Physiology. 2012;112(10):1644–1652.

16. Andrew PJ, Mayer B. Enzymatic function of nitric oxide synthases. Cardiovascular Research.1999;43(3):521–531.
17. Cosentino F, Lüscher TF. Tetrahydrobiopterin and endothelial nitric oxide synthase activity.Cardiovascular Research. 1999;43(2):274–278.
18. Kawashima S, Yokoyama M. Dysfunction of endothelial nitric oxide synthase and atherosclerosis.Arteriosclerosis, Thrombosis, and Vascular Biology. 2004;24(6):998–1005.
19. Suwaidi JA, Hamasaki S, Higano ST, Nishimura RA, Holmes DR, Lerman A. Long-term follow-up of patients with mild coronary artery disease and endothelial dysfunction. Circulation. 2000;101(9):948–954.
20. Liu Q, Berchner-Pfannschmidt U, Möller U, et al. A Fenton reaction at the endoplasmic reticulum is involved in the redox control of hypoxia-inducible gene expression. Proceedings of the National Academy of Sciences of the United States of America. 2004;101(12):4302–4307.
21. Cadenas S, Aragonés J, Landázuri MO. Mitochondrial reprogramming through cardiac oxygen sensors in ischaemic heart disease. Cardiovascular Research. 2010;88(2):219–228.
22. Thannickal VJ, Fanburg BL. Reactive oxygen species in cell signaling. American Journal of Physiology. 2000;279(6):L1005–L1028.
23. Chakravarthi S, Jessop CE, Bulleid NJ. The role of glutathione in disulphide bond formation and endoplasmic-reticulum-generated oxidative stress. EMBO Reports. 2006;7(3):271–275.
24. Shen D, Dalton TP, Nebert DW, Shertzer HG. Glutathione redox state regulates mitochondrial reactive oxygen production. Journal of Biological Chemistry. 2005;280(27):25305–25312.
25. Masella R, Di Benedetto R, Varì R, Filesi C, Giovannini C. Novel mechanisms of natural antioxidant compounds in biological systems: involvement of glutathione and glutathione-related enzymes. Journal of Nutritional Biochemistry. 2005;16(10):577–586.
26. Pastore A, Federici G, Bertini E, Piemonte F. Analysis of glutathione: implication in redox and detoxification. Clinica Chimica Acta. 2003;333(1-2):19–39.
27. Townsend DM, Tew KD, Tapiero H. The importance of glutathione in human disease. Biomedicine and Pharmacotherapy. 2003;57(3):145–155.
28. Knock GA, Ward JPT. Redox regulation of protein kinases as a modulator of vascular function.Antioxidants and Redox Signaling. 2011;15(6):1531–1547.
29. Burgoyne JR, Mongue-Din H, Eaton P, Shah AM. Redox signaling in cardiac physiology and pathology. Circulation Research. 2012;111(8):1091–1106.
30. Steinberg SF. Oxidative stress and sarcomeric proteins. Circulation Research. 2013;112(2):393–405.
31. Surma M, Wei L, Shi J. Rho kinase as a therapeutic target in cardiovascular disease. Future Cardiology. 2011;7(5):657–671.

32. Satoh K, Fukumoto Y, Shimokawa H. Rho-kinase: important new therapeutic target in cardiovascular diseases. American Journal of Physiology. 2011;301(2):H287–H296.
33. Michel MC, Li Y, Heusch G. Mitogen-activated protein kinases in the heart. Naunyn-Schmiedeberg's Archives of Pharmacology. 2001;363(3):245–266.
34. Martindale JL, Holbrook NJ. Cellular response to oxidative stress: signaling for suicide and survival.Journal of Cellular Physiology. 2002;192(1):1–15.
35. Johnson GL, Lapadat R. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. Science. 2002;298(5600):1911–1912.
36. Morgan MJ, Liu ZG. Crosstalk of reactive oxygen species and NF-κB signaling. Cell Research.2011;21(1):103–115.
37. Bonello S, Zahringer C, BelAiba RS, et al. Reactive oxygen species activate the HIF-1α promoter via a functional NFκB site. Arteriosclerosis, Thrombosis, and Vascular Biology. 2007;27(4):755–761.
38. Hattori R, Otani H, Uchiyama T, et al. Src tyrosine kinase is the trigger but not the mediator of ischemic preconditioning. American Journal of Physiology. 2001;281(3):H1066–H1074.
39. Maulik N, Sasaki H, Galang N. Differential regulation of apoptosis by ischemia-reperfusion and ischemic adaptation. Annals of the New York Academy of Sciences. 1999;874:401–411.
40. Maulik N, Yoshida T, Das DK. Regulation of cardiomyocyte apoptosis in ischemic reperfused mouse heart by glutathione peroxidase. Molecular and Cellular Biochemistry. 1999;196(1-2):13–21.
41. Becker LB. New concepts in reactive oxygen species and cardiovascular reperfusion physiology.Cardiovascular Research. 2004;61(3):461–470.
42. Maulik N, Engelman RM, Rousou JA, Flack JE, Deaton D, Das DK. Ischemic preconditioning reduces apoptosis by upregulating anti-death gene Bcl-2. Circulation. 1999;100(19):II369–II375.
43. Maulik N, Goswami S, Galang N, Das DK. Differential regulation of Bcl-2, AP-1 and NF-κB on cardiomyocyte apoptosis during myocardial ischemic stress adaptation. FEBS Letters. 1999;443(3):331–336.
44. Adams JM, Cory S. The Bcl-2 protein family: arbiters of cell survival. Science. 1998;281(5381):1322–1326.
45. Hattori R, Estrada Hernandez T, Zhu L, et al. An essential role of the antioxidant gene bcl-2 in myocardial adaptation to ischemia: an insight with antisense bcl-2 therapy. Antioxidants and Redox Signaling. 2001;3(3):403–413.
46. Xia Z, Liu M, Wu Y, et al. N-acetylcysteine attenuates TNF-α-induced human vascular endothelial cell apoptosis and restores eNOS expression. European Journal of Pharmacology. 2006;550(1–3):134–142.

47. Virdis A, Santini F, Colucci R, et al. Vascular generation of tumor necrosis factor-α reduces nitric oxide availability in small arteries from visceral fat of obese patients. Journal of the American College of Cardiology. 2011;58(3):238–247.
48. Zou W, Amcheslavsky A, Takeshita S, Drissi H, Bar-Shavit Z. TNF-α expression is transcriptionally regulated by RANK ligand. Journal of Cellular Physiology. 2005;202(2):371–378.
49. Zhang H, Park Y, Wu J, et al. Role of TNF-α in vascular dysfunction. Clinical Science.2009;116(3):219–230.
50. Gao X, Zhang H, Belmadani S, et al. Role of TNF-α-induced reactive oxygen species in endothelial dysfunction during reperfusion injury. American Journal of Physiology. 2008;295(6):H2242–H2249.
51. Guzik TJ, Mussa S, Gastaldi D, et al. Mechanisms of increased vascular superoxide production in human diabetes mellitus: role of NAD(P)H oxidase and endothelial nitric oxide synthase. Circulation.2002;105(14):1656–1662.
52. Stephens NG, Parsons A, Schofield PM, et al. Randomised controlled trial of vitamin E in patients with coronary disease: Cambridge Heart Antioxidant Study (CHAOS) The Lancet. 1996;347(9004):781–786.
53. Duffy SJ, Gokce N, Holbrook M, et al. Treatment of hypertension with ascorbic acid. The Lancet.1999;354(9195):2048–2049.
54. Boaz M, Smetana S, Weinstein T, et al. Secondary prevention with antioxidants of cardiovascular disease in endstage renal disease (SPACE): randomised placebo-controlled trial. The Lancet.2000;356(9237):1213–1218.
55. Neri S, Signorelli SS, Torrisi B, et al. Effects of antioxidant supplementation on postprandial oxidative stress and endothelial dysfunction: a single-blind, 15-day clinical trial in patients with untreated type 2 diabetes, subjects with impaired glucose tolerance, and healthy controls. Clinical Therapeutics.2005;27(11):1764–1773.
56. Accinni R, Rosina M, Bamonti F, et al. Effects of combined dietary supplementation on oxidative and inflammatory status in dyslipidemic subjects. Nutrition, Metabolism & Cardiovascular Diseases.2006;16(2):121–127.
57. Hennekens CH, Buring JE, Manson JE, et al. Lack of effect of long-term supplementation with beta carotene on the incidence of malignant neoplasms and cardiovascular disease. The New England Journal of Medicine. 1996;334(18):1145–1149.
58. Rapola JM, Virtamo J, Ripatti S, et al. Randomised trial of α-tocopherol and β-carotene supplements on incidence of major coronary events in men with previous myocardial infarction. The Lancet.1997;349(9067):1715–1720.

59. Virtamo J, Rapola JM, Ripatti S, et al. Effect of vitamin E and beta carotene on the incidence of primary nonfatal myocardial infarction and fatal coronary heart disease. Archives of Internal Medicine.1998;158(6):668–675.
60. Italiano G. Dietary supplementation with n-3 polyunsaturated fatty acids and vitamin E after myocardial infarction: results of the GISSI-Prevenzione trial. Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto miocardico. The Lancet. 1999;354(9177):447–455.
61. Yusuf S. Effects of an angiotensin-converting-enzyme inhibitor, ramipril, on cardiovascular events in high-risk patients. The New England Journal of Medicine. 2000;342(3):145–153.
62. de Gaetano G. Low-dose aspirin and vitamin E in people at cardiovascular risk: a randomised trial in general practice. Collaborative Group of the Primary Prevention Project. The Lancet. 2001;357(9250):89–95.
63. Collins R, Armitage J, Parish S, Sleight P, Peto R. MRC/BHF Heart Protection Study of antioxidant vitamin supplementation in 20 536 high-risk individuals: a randomised placebo-controlled trial. The Lancet. 2002;360(9326):23–33.
64. Törnwall ME, Virtamo J, Korhonen PA, et al. Effect of α-tocopherol and β-carotene supplementation on coronary heart disease during the 6-year post-trial follow-up in the ATBC study. European Heart Journal. 2004;25(13):1171–1178.
65. Armitage J, Collins R, Bowman L, et al. The effects of cholesterol lowering with simvastatin on cause-specific mortality and on cancer incidence in 20,536 high-risk people: a randomised placebo-controlled trial [ISRCTN48489393] BMC Medicine. 2005;3, article 6
66. Cook NR, Albert CM, Gaziano JM, et al. A randomized factorial trial of vitamins C and E and beta carotene in the secondary prevention of cardiovascular events in women: results from the women's antioxidant cardiovascular study. Archives of Internal Medicine. 2007;167(15):1610–1618.
67. Lee IM, Cook NR, Gaziano JM, et al. Vitamin E in the primary prevention of cardiovascular disease and cancer. The women's health study: a randomized controlled trial. Journal of the American Medical Association. 2005;294(1):56–65.
68. Lonn E. Effects of long-term vitamin E supplementation on cardiovascular events and cancer: a randomized controlled trial. Journal of the American Medical Association. 2005;293(11):1338–1347.
69. Kataja-Tuomola MK, Kontto JP, Männistö S, Albanes D, Virtamo JR. Effect of alpha-tocopherol and beta-carotene supplementation on macrovascular complications and total mortality from diabetes: results of the ATBC study. Annals of Medicine. 2010;42(3):178–186.

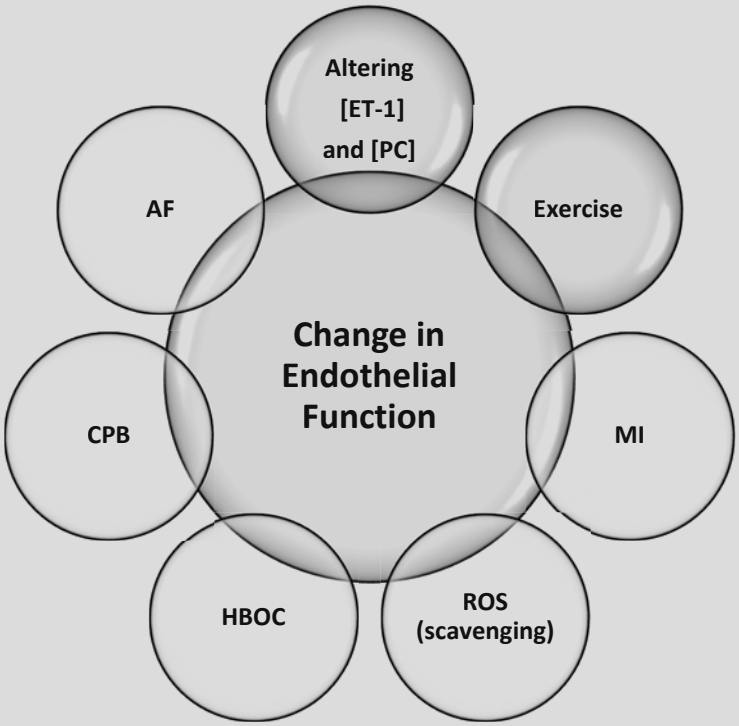
70. Ushio-Fukai M, Alexander RW. Reactive oxygen species as mediators of angiogenesis signaling. Role of NAD(P)H oxidase. Molecular and Cellular Biochemistry. 2004;264(1-2):85–97.
71. Lambeth JD. NOX enzymes and the biology of reactive oxygen. Nature Reviews Immunology.2004;4(3):181–189.
72. Sedeek M, Montezano AC, Hebert RL, et al. Oxidative stress, Nox isoforms and complications of diabetes—potential targets for novel therapies. Journal of Cardiovascular Translational Research.2012;5(4):509–518.
73. Murdoch CE, Alom-Ruiz SP, Wang M, et al. Role of endothelial Nox2 NADPH oxidase in angiotensin II-induced hypertension and vasomotor dysfunction. Basic Research in Cardiology.2011;106(4):527–538.
74. Rhee SG, Kang SW, Jeong W, Chang TS, Yang KS, Woo HA. Intracellular messenger function of hydrogen peroxide and its regulation by peroxiredoxins. Current Opinion in Cell Biology. 2005;17(2):183–189.
75. Catarzi S, Giannoni E, Favilli F, Meacci E, Iantomasi T, Vincenzini MT. Sphingosine 1-phosphate stimulation of NADPH oxidase activity: relationship with platelet-derived growth factor receptor and c-Src kinase. Biochimica et Biophysica Acta. 2007;1770(6):872–883.
76. Schröder E, Brennan JP, Eaton P. Cardiac peroxiredoxins undergo complex modifications during cardiac oxidant stress. American Journal of Physiology. 2008;295(1):H425–H433.
77. Jeong W, Bae SH, Toledano MB, Rhee SG. Role of sulfiredoxin as a regulator of peroxiredoxin function and regulation of its expression. Free Radical Biology & Medicine. 2012;53(3):447–456.
78. Malinouski M, Zhou Y, Belousov VV, Hatfield DL, Gladyshev VN. Hydrogen peroxide probes directed to different cellular compartments. PLoS ONE. 2011;6(1)e14564
79. Bienert GP, Møller ALB, Kristiansen KA, et al. Specific aquaporins facilitate the diffusion of hydrogen peroxide across membranes. Journal of Biological Chemistry. 2007;282(2):1183–1192.
80. Edwards G, Thollon C, Gardener MJ, et al. Role of gap junctions and EETs in endothelium-dependent hyperpolarization of porcine coronary artery. British Journal of Pharmacology. 2000;129(6):1145–1154.
81. Matoba T, Shimokawa H. Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in animals and humans. Journal Pharmacological Sciences. 2003;92(1):1–6.
82. Shimokawa H. Hydrogen peroxide as an endothelium-derived hyperpolarizing factor. Pflugers Archiv European Journal of Physiology. 2010;459(6):915–922.
83. Deshpande NN, Sorescu D, Seshiah P, et al. Mechanism of hydrogen peroxide-induced cell cycle arrest in vascular smooth muscle. Antioxidants and Redox Signaling. 2002;4(5):845–854.

84. Brunt KR, Fenrich KK, Kiani G, et al. Protection of human vascular smooth muscle cells from H_2O_2-induced apoptosis through functional codependence between HO-1 and AKT. Arteriosclerosis, Thrombosis, and Vascular Biology. 2006;26(9):2027–2034.
85. Pletjushkina OY, Fetisova EK, Lyamzaev KG, et al. Long-distance apoptotic killing of cells is mediated by hydrogen peroxide in a mitochondrial ROS-dependent fashion. Cell Death and Differentiation. 2005;12(11):1442–1444.
86. Skulachev VP, Anisimov VN, Antonenko YN, et al. An attempt to prevent senescence: a mitochondrial approach. Biochimica et Biophysica Acta. 2009;1787(5):437–461.
87. Sundaresan M, Yu ZX, Ferrans VJ, Irani K, Finkel T. Requirement for generation of H_2O_2 for platelet-derived growth factor signal transduction. Science. 1995;270(5234):296–299.
88. Weber DS, Taniyama Y, Rocic P, et al. Phosphoinositide-dependent kinase 1 and p21-activated protein kinase mediate reactive oxygen species-dependent regulation of platelet-derived growth factor-induced smooth muscle cell migration. Circulation Research. 2004;94(9):1219–1226.
89. Bleeke T, Zhang H, Madamanchi N, Patterson C, Faber JE. Catecholamine-induced vascular wall growth is dependent on generation of reactive oxygen species. Circulation Research. 2004;94(1):37–45.
90. Gurjar MV, Sharma RV, Bhalla RC. eNOS gene transfer inhibits smooth muscle cell migration and MMP-2 and MMP-9 activity. Arteriosclerosis, Thrombosis, and Vascular Biology. 1999;19(12):2871–2877.
91. Rajagopalan S, Meng XP, Ramasamy S, Harrison DG, Galis ZS. Reactive oxygen species produced by macrophage-derived foam cells regulate the activity of vascular matrix metalloproteinases in vitro: implications for atherosclerotic plaque stability. Journal of Clinical Investigation. 1996;98(11):2572–2579.
92. Liang H, Ran Q, Jang YC, et al. Glutathione peroxidase 4 differentially regulates the release of apoptogenic proteins from mitochondria. Free Radical Biology and Medicine. 2009;47(3):312–320.
93. Bolli R, Jeroudi MO, Patel BS, et al. Direct evidence that oxygen-derived free radicals contribute to postischemic myocardial dysfunction in the intact dog. Proceedings of the National Academy of Sciences of the United States of America. 1989;86(12):4695–4699.
94. Simoons ML, Serruys PW, Van den Brand M. Early thrombolysis in acute myocardial infarction: limitation of infarct size and improved survival. Journal of the American College of Cardiology.1986;7(4):717–728.
95. White HD, Norris RM, Brown MA, et al. Effect of intravenous streptokinase on left ventricular function and early survival after acute myocardial infarction. The New England Journal of Medicine.1987;317(14):850–855.

96. Toyokuni S. Reactive oxygen species-induced molecular damage and its application in pathology.Pathology International. 1999;49(2):91–102.
97. Majidi M, Kosinski AS, Al-Khatib SM, et al. Reperfusion ventricular arrhythmia "bursts" predict larger infarct size despite TIMI 3 flow restoration with primary angioplasty for anterior ST-elevation myocardial infarction. European Heart Journal. 2009;30(7):757–764.
98. Vaage J, Antonelli M, Bufi M, et al. Exogenous reactive oxygen species deplete the isolated rat heart of antioxidants. Free Radical Biology and Medicine. 1996;22(1-2):85–92.
99. Gori T, Di Stolfo G, Sicuro S, et al. Nitroglycerin protects the endothelium from ischaemia and reperfusion: human mechanistic insight. British Journal of Clinical Pharmacology. 2007;64(2):145–150.
100. Penna C, Mognetti B, Tullio F, et al. The platelet activating factor triggers preconditioning-like cardioprotective effect via mitochondrial K-ATP channels and redox-sensible signaling. Journal of Physiology and Pharmacology. 2008;59(1):47–54.
101. Lebuffe G, Schumacker PT, Shao ZH, Anderson T, Iwase H, Vanden Hoek TL. ROS and NO trigger early preconditioning: relationship to mitochondrial KATP channel. American Journal of Physiology. 2003;284(1):H299–H308.
102. Oldenburg O, Cohen MV, Yellon DM, Downey JM. Mitochondrial K_{ATP} channels: role in cardioprotection. Cardiovascular Research. 2002;55(3):429–437.
103. Pain T, Yang XM, Critz SD, et al. Opening of mitochondrial K(ATP) channels triggers the preconditioned state by generating free radicals. Circulation Research. 2000;87(6):460–466.
104. Heinzel FR, Luo Y, Li X, et al. Impairment of diazoxide-induced formation of reactive oxygen species and loss of cardioprotection in connexin 43 deficient mice. Circulation Research. 2005;97(6):583–586.
105. Laude K, Thuillez C, Richard V. Peroxynitrite triggers a delayed resistance of coronary endothelial cells against ischemia-reperfusion injury. American Journal of Physiology. 2002;283(4):H1418–H1423.
106. Rakhit RD, Edwards RJ, Mockridge JW, et al. Nitric oxide-induced cardioprotection in cultured rat ventricular myocytes. American Journal of Physiology. 2000;278(4):H1211–H1217.
107. Navab M, Berliner JA, Watson AD, et al. The Yin and Yang of oxidation in the development of the fatty streak: a review based on the 1994 George Lyman Duff memorial lecture. Arteriosclerosis, Thrombosis, and Vascular Biology. 1996;16(7):831–842.
108. Berk BC, Abe JI, Min W, Surapisitchat J, Yan C. Endothelial atheroprotective and anti-inflammatory mechanisms. Annals of the New York Academy of Sciences. 2001;947:93–111.

109. Godbole AS, Lu X, Guo X, Kassab GS. NADPH oxidase has a directional response to shear stress. American Journal of Physiology. 2009;296(1):H152–H158.
110. Hwang J, Saha A, Boo YC, et al. Oscillatory shear stress stimulates endothelial production of O_2^- from $p47^{phox}$-dependent NAD(P)H oxidases, leading to monocyte adhesion. Journal of Biological Chemistry. 2003;278(47):47291–47298.
111. Walpola PL, Gotlieb AI, Cybulsky MI, Langille BL. Expression of ICAM-1 and VCAM-1 and monocyte adherence in arteries exposed to altered shear stress. Arteriosclerosis, Thrombosis, and Vascular Biology. 1995;15(1):2–10.
112. Tinkel J, Hassanain H, Khouri SJ. Cardiovascular antioxidant therapy: a review of supplements, pharmacotherapies, and mechanisms. Cardiology in Review. 2012;20(2):77–83.
113. Gey KF, Puska P, Jordan P, Moser UK. Inverse correlation between plasma vitamin E and mortality from ischemic heart disease in cross-cultural epidemiology. American Journal of Clinical Nutrition.1991;53(1).
114. Fisher AB. Redox signaling across cell membranes. Antioxidants and Redox Signaling.2009;11(6):1349–1356.
115. Vivekananthan DP, Penn MS, Sapp SK, Hsu A, Topol EJ. Use of antioxidant vitamins for the prevention of cardiovascular disease: meta-analysis of randomised trials. The Lancet.2003;361(9374):2017–2023.
116. Myung SK, Ju W, Cho B, et al. Efficacy of vitamin and antioxidant supplements in prevention of cardiovascular disease: systematic review and meta-analysis of randomised controlled trials. BMJ.2013;346:p. f10.
117. Bjelakovic G, Nikolova D, Gluud LL, Simonetti RG, Gluud C. Antioxidant supplements for prevention of mortality in healthy participants and patients with various diseases. Cochrane Database of Systematic Reviews. 2008;(2)CD007176
118. McGregor GP, Biesalski HK. Rationale and impact of vitamin C in clinical nutrition. Current Opinion in Clinical Nutrition and Metabolic Care. 2006;9(6):697–703.
119. Münzel T, Gori T, Bruno RM, Taddei S. Is oxidative stress a therapeutic target in cardiovascular disease? European Heart Journal. 2010;31(22):2741–2749.
120. Boschi D, Tron GC, Lazzarato L, et al. NO-donor phenols: a new class of products endowed with antioxidant and vasodilator properties. Journal of Medicinal Chemistry. 2006;49(10):2886–2897.
121. Serebruany V, Malinin A, Scott R. The in vitro effects of a novel vascular protectant, AGI-1067, on platelet aggregation and major receptor expression in subjects with multiple risk factors for vascular disease. Journal of Cardiovascular Pharmacology and Therapeutics. 2006;11(3):191–196.

122. Zhang Q, Pan J, Zhao C, Wang Y, Jia Z, Zheng R. Non-enzymatic fast repair of DNA oxidative damage might also exist in cells. Cell Biology International. 2008;32(6):654–662.
123. O'Connor PM, Gutterman DD. Resurrecting hope for antioxidant treatment of cardiovascular disease: focus on mitochondria. Circulation Research. 2010;107(1):9–11.
124. Dikalova AE, Bikineyeva AT, Budzyn K, et al. Therapeutic targeting of mitochondrial superoxide in hypertension. Circulation Research. 2010;107(1):106–116.
125. Davidson SM. Endothelial mitochondria and heart disease. Cardiovascular Research. 2010;88(1):58–66.
126. Subramanian S, Kalyanaraman B, Migrino RQ. Mitochondrially targeted antioxidants for the treatment of cardiovascular diseases. Recent Patents on Cardiovascular Drug Discovery. 2010;5(1):54–65.
127. Skulachev MV, Antonenko YN, Anisimov VN, et al. Mitochondrial-targeted plastoquinone derivatives. Effect on senescence and acute age-related pathologies. Current Drug Targets.2011;12(6):800–826.
128. Dai DF, Johnson SC, Villarin JJ, et al. Mitochondrial oxidative stress mediates angiotensin II-induced cardiac hypertrophy and Gαq overexpression-induced heart failure. Circulation Research.2011;108(7):837–846.


Altering
[ET-1]
and [PC]
AF
Exercise
Change in
Endothelial
Function
CPB
MI
HBOC
ROS
(scavenging)

4

EXERCISE LIMITS THE PRODUCTION OF ENDOTHELIN IN THE CORONARY VASCULATURE

V. J. de Beer*, S.B. Bender*, **Y.J. Taverne**, D.J. Duncker, M.H. Laughlin and D. Merkus.
* both authors contributed equally

American Journal of Physiology: Heart and Circulatory Physiology 2011

ABSTRACT

We previously demonstrated that endothelin (ET)-mediated coronary vasoconstriction wanes with increasing exercise intensity via a nitric oxide- and prostacyclin-dependent mechanism. Therefore, we hypothesized that the waning of ET coronary vasoconstriction during exercise is the result of decreased production of ET and/or decreased ET receptor sensitivity. We investigated coronary ET receptor sensitivity using intravenous infusion of ET and coronary ET production using intravenous infusion of the ET precursor Big ET, at rest and during continuous treadmill exercise at 3 km/h in 16 chronically instrumented swine. In the systemic vasculature, Big ET and ET induced similar changes in hemodynamic parameters at rest and during continuous exercise at 3 km/h, indicating that exercise does not alter ET production or receptor sensitivity in the systemic vasculature. In the coronary vasculature, infusion of ET resulted in similar dose-dependent decreases in coronary blood flow and coronary venous oxygen tension and saturation at rest and during exercise. In contrast, administration of Big ET resulted in dose-dependent decreases in coronary blood flow, as well as coronary venous oxygen tension and saturation at rest. These effects of Big ET were significantly reduced during exercise. Altogether, our data indicate that continuous exercise at 3 km/h attenuates ET-mediated coronary vasoconstriction through reduced production of ET from Big ET rather than through reduced ET sensitivity of the coronary vasculature. The decreased ET production during exercise likely contributes to metabolic coronary vasodilation.

INTRODUCTION

Endothelin (ET)-1 is one of the most potent vasoconstrictors known. It is mainly produced in endothelial cells through cleavage from its inactive precursor, Big ET, primarily by ET converting enzyme (ECE) and, to a lesser degree, by chymase, in concert with neutral endopeptidase (NEP) [31]. Several studies have shown that ET receptor blockade results in systemic and coronary vasodilation [3, 21, 32], and hence that ET contributes to basal vascular tone in the systemic vasculature, as well as the coronary circulation. Our laboratory [21] has previously demonstrated that recruitment of coronary flow reserve during exercise is mediated, at least in part, through withdrawal of the vasoconstrictor effect of ET on the coronary vasculature. Moreover, we showed that nitric oxide (NO) and prostanoids (PGI_2) act synergistically to inhibit this vasoconstrictor influence of ET during exercise [23]. Indeed, NO and PGI_2 have been shown to reduce the production and release of ET [11, 28], while ET_A receptors can be nitrosylated by NO, thereby reducing their affinity for ET [34]. Thus, next to their direct vasodilator effects, NO and PGI_2 can induce vasodilation indirectly by limiting the production of, and/or vasoconstriction induced by, ET.

We hypothesized that the exercise-induced blunting of ET-mediated vasoconstriction in the coronary circulation results from a decrease in ET production, a decrease in ET receptor sensitivity, or a combination of these two effects. Therefore, the aim of the present study was to investigate each of these putative mechanisms by comparison of the in vivo coronary vasoconstrictor responses to Big ET and ET, in chronically instrumented swine at rest and during continuous treadmill exercise. Previous work has established that Big ET has little, if any, direct vasomotor effects, and that the vasoconstriction induced by Big ET is dependent on its conversion to ET [7, 8, 26, 27]. Hence, the magnitude of constriction to Big ET-1 approximates the extent of ET production at the vascular wall (assuming no change in ET receptor sensitivity). Comparison of the vasoconstrictor effects of Big ET-1 and ET-1 in vivo, therefore, allows examination of the ET system, with the former reflecting ET production and the latter representing ET receptor sensitivity.

METHODS

Animals

Studies were performed in accordance with the American Physiological Society's "Guiding Principles in the Care and Use of Laboratory Animals" and with approval of the Animal Care Committee of the Erasmus University Medical Center. A total of 21 Yorkshire × Landrace swine (2 to 3 mo old; 23 ± 1 kg at the time of surgery) entered the study.

Surgery

Swine were sedated (20 mg/kg ketamine and 1 mg/kg midazolam im), anesthetized (thiopental sodium 15 mg/kg iv), intubated, and ventilated with a mixture of O_2 and N_2 (1:2) to which 0.2–1.0% (vol/vol) isoflurane was added [6]. Anesthesia was maintained with midazolam (2 mg/kg iv) and fentanyl (10 $\mu g \cdot kg^{-1} \cdot h^{-1}$ iv). Swine were instrumented under sterile conditions, as previously described [21, 22]. Briefly, a thoracotomy was performed in the fourth intercostal space. Subsequently, a polyvinylchloride catheter was inserted into the aortic arch, for the measurement of blood pressure and blood sampling for the determination of the Po_2, Pco_2, pH, O_2 saturation (So_2), and hemoglobin concentration (ABL 820, Radiometer). A high-fidelity Konigsberg pressure transducer was inserted into the left ventricle (LV) via the apex for measurement of LV pressure and maximum rate of rise in LV pressure. Fluid-filled catheters were implanted in the LV, left atrium, and the pulmonary artery. Furthermore, a small angiocatheter was inserted into the anterior interventricular vein for coronary venous blood sampling [6]. Transonic flow probes were placed around the ascending aorta and proximal left anterior descending coronary artery for measurement of cardiac output and coronary blood flow (CBF), respectively [3]. Ultrasonic crystals (Sonometrics) were placed midmyocardially in the anterior wall of the LV to determine regional myocardium function. Electrical wires and catheters were tunneled subcutaneously to the back. The chest was closed, and the animals were allowed to recover.

Animals received analgesia (0.3 mg buprenorphine im) for 2 days and antibiotic prophylaxis (25 mg/kg amoxicillin and 5 mg/kg gentamycin iv) for 5 days. Studies were preformed approximately 2 wk after surgery.

Experimental Protocols

Four different protocols with infusion of ET or Big ET were performed (see below). The number of swine in each protocol, as well as the overlap between protocols, is shown in Table 1.

Protocol		ET		Big ET		
		Rest	Exercise	Rest	Exercise	Total
ET	Rest	10H/8B	6H	5H	6H	
	Exercise	3B	9H/6B	5H	5H	
Big ET	Rest	4B	5B	9H/7B	8H	
	Exercise	5B	5B	6B	11H/9B	
Total						16H/13B

Table 1. Schematic representation of the overlap of animals used in the various protocols. ET, endothelin; Big ET, big endothelin; H, hemodynamic measurements; B, coronary venous blood samples.

ET.

REST (n = 10).

With swine lying quietly on the treadmill, resting hemodynamic measurements and blood samples were obtained. An intravenous infusion of ET (Sigma, E7764, dissolved in saline) was started at 10 pmol·kg^{-1}·min^{-1}; arterial and coronary venous blood samples and hemodynamic measurements were collected at the end of the 10-min infusion period. Subsequently, the dose of ET was increased to 20 pmol·kg^{-1}·min^{-1} for 10 min, at the end of which hemodynamic measurements and blood samples were taken (22). In previous experiments, a higher dose of ET resulted in vomiting (22), which precluded its use in exercise experiments.

EXERCISE (n = 9).

Swine ran continuously for 20 min at 3 km/h without (control run) or with (ET run) infusion of ET-1 (10 and 20 pmol·kg^{-1}·min^{-1}). Blood samples and hemodynamic measurements were obtained at 10 and 20 min during each protocol. Animals were allowed to rest on the treadmill for 90 min between protocols, during which time hemodynamic values returned to baseline.

Big ET.

DOSE FINDING (n = 5).

We aimed to use doses of Big ET (Sigma, E8887, dissolved in saline) that would elicit similar increases in blood pressure as the doses of ET describe above. Studies in rats have shown that, both in vivo and ex vivo, approximately twice the dosage of Big ET is needed to reach constrictor responses similar to ET at a given dose (31, 33). Therefore, Big ET was infused intravenously at rest in dosages of 10, 20 and 40 pmol·kg^{-1}·min^{-1}. Pilot experiments revealed a further increase in blood pressure upon cessation of Big ET infusion, which stabilized after ∼5 min and likely reflects the time necessary for the conversion of the vaso-inactive Big ET into the vasoactive ET. Consequently, in subsequent experiments, we infused Big ET and measured its effects 10 min after the completion of each infusion.

REST (n = 9).

With swine lying quietly on the treadmill, resting hemodynamic measurements were obtained and blood samples were collected. Big ET was infused at 20 pmol·kg^{-1}·min^{-1} for 10 min. Approximately 10 min after completion of infusion, blood samples were taken, and hemodynamic measurements were collected. Subsequently, Big ET was infused at 40 pmol·kg^{-1}·min^{-1} for 10 min. Again, hemodynamic measurements and blood samples were taken ∼10 min after completion of the infusion. The total duration of the protocol was 40 min.

EXERCISE (n = 11).

Swine ran continuously on the treadmill at 3 km/h for 40 min without (control run) or with (Big ET run) infusion of Big ET (20 and 40 pmol·kg^{-1}·min^{-1}). Identical to the rest protocol of Big ET, the two periods of infusion were followed by a 10-min conversion period. Hence, blood samples and hemodynamic measurements were obtained at 20 and 40 min. Between protocols, animals were allowed to rest on the treadmill for 90 min, during which time hemodynamic values returned to baseline.

PHENYLEPHRINE (*n* = 5).

To investigate if the coronary vasoconstriction produced by ET or Big ET was the result of a direct vasoconstrictor effect of ET on the coronary vasculature rather than an autoregulatory response to an increase in blood pressure, we performed intravenous infusions of the α_1-agonist phenylephrine (PE; Erasmus MC pharmacy, dissolved in saline; incremental dosages of 1–5 µg·kg^{-1}·min^{-1}, 10 min each) in resting swine. Since swine lack α_1-receptors in their coronary microcirculation (30),

infusion of PE results in systemic vasoconstriction and an increase in blood pressure without a direct effect on the coronary microvasculature.

Data Analysis

Digital recording and offline analysis of hemodynamic data have been described in detail elsewhere (6, 21). Systemic vascular conductance was calculated by dividing cardiac output by blood pressure. End-diastolic length (EDL) and end-systolic length (ESL) lengths were normalized to EDL resting baseline (both at rest and during exercise). Systolic segmental shortening was calculated as [(EDL − ESL)/EDL] × 100. Arterial and venous O_2 content, as well as myocardial O_2 consumption, were calculated as previously described (6, 21).

Blood pressure, systemic vascular conductance, CBF, coronary venous Po_2 ($cvPo_2$), and coronary venous So_2 ($cvSo_2$) as well as their changes from baseline values were used as indexes for vasoconstriction in the systemic and coronary circulations at rest and during exercise. In the resting protocols, baseline values were obtained before initiation of drug infusion, whereas, in the exercise protocols, baseline values were obtained from the corresponding samples taken during the control exercise run.

Statistical Analysis

Statistical analysis of hemodynamic and blood-gas data was performed using one-way (dose or exercise), or two-way (dose and exercise) ANOVA for repeated measures. When significant effects were detected ($P < 0.05$), post hoc testing for the effects of exercise and drug treatment was performed using Scheffé's test. Changes in the relation between myocardial oxygen consumption and $cvPo_2$ were assessed using linear regression analysis with dose of ET or Big ET and animal as dummy variables. Data are presented as means ± SE.

RESULTS

Dose Finding

Increases in blood pressure at rest resulting from various doses of Big ET were matched to ET-induced increases at 10 and 20 $pmol \cdot kg^{-1} \cdot min^{-1}$. As shown in Figure 1, infusion of Big ET at 10 $pmol \cdot kg^{-1} \cdot min^{-1}$ did not increase blood pressure; however, infusion of Big ET at 20 $pmol \cdot kg^{-1} \cdot min^{-1}$ resulted in an identical increase in blood pressure as ET at 10 $pmol \cdot kg^{-1} \cdot min^{-1}$. Infusion of Big ET at a rate of 40

pmol·kg^{-1}·min^{-1} resulted in an increase in blood pressure comparable to 20 pmol·kg^{-1}·min^{-1} ET. Therefore, in the remainder of the study, Big ET doses of 20 and 40 pmol·kg^{-1}·min^{-1} were used for comparison with ET doses of 10 and 20 pmol·kg^{-1}·min^{-1}.

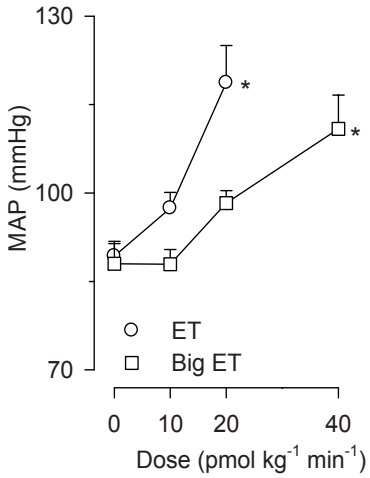


Figure 1. Dose-response study of exogenous big endothelin (Big ET) compared with ET. Shown is a dose-response curve for ET and Big ET at doses from 0 to 40 pmol·kg^{-1}·min^{-1}. MAP, mean arterial pressure. Values are means ± SE. *$P \leq 0.05$, drug effect vs. baseline (no drug).

Effects of ET and Big ET on the Systemic Circulation

ET resulted in dose-dependent systemic vasoconstriction as evidenced by a decrease in systemic vascular conductance (Figure 2) and an increase in blood pressure (Table 1 and 2). The increase in blood pressure was accompanied by decreases in heart rate and cardiac output (Table 1 and 2), likely due to activation of the baroreflex. Continuous exercise at 3 km/h (approximately 60% of estimated maximal heart rate), resulted in systemic vasodilation, reflected by the increase in systemic vascular conductance (not shown), whereas blood pressure remained essentially constant (Table 3). These exercise-induced changes in systemic hemodynamics stabilized within 3 min (not shown) and were then maintained during the entire 40 min of the longest control exercise protocol (Table 3). The responses of the systemic vasculature to infusion of either ET or Big ET were similar at rest and during exercise. Moreover, infusion of Big ET at doses of 20 and 40

pmol·kg^{-1}·min^{-1} resulted in an identical systemic vasoconstriction compared with ET at 10 and 20 pmol·kg^{-1}·min^{-1}, both at rest and during exercise (Figure 2). Together, these data show that both ET and Big ET exert a vasoconstrictor effect on the systemic vasculature, and that this effect is not influenced by exercise.

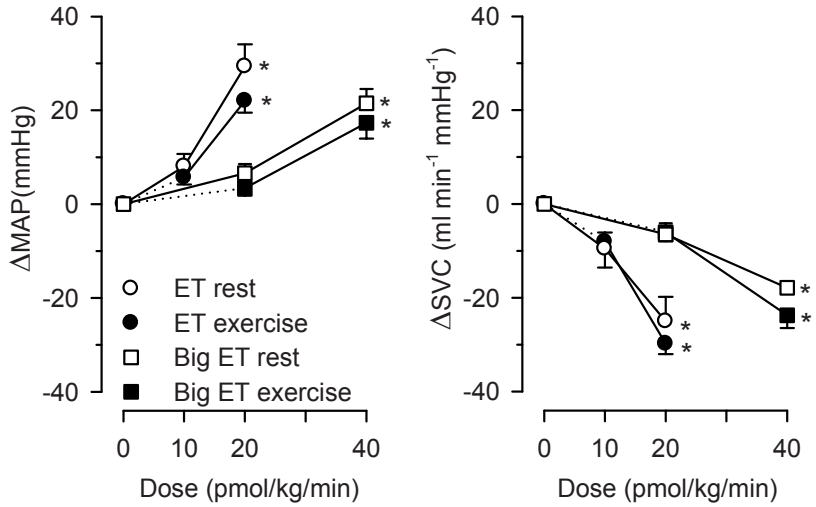


Figure 2. Systemic effects of Big ET and ET infusions at rest and during exercise. *A*: effects of ET (10 and 20 pmol·kg^{-1}·min^{-1}) and Big ET (20 and 40 pmol·kg^{-1}·min^{-1}) on MAP are not different at rest and/or during exercise. *B*: effects of ET and Big ET on systemic vascular conductance (SVC) are not different at rest and/or during exercise. Δ, Change. Values are means ± SE. *$P \leq$ 0.05, drug effect vs. control.

		Control	Dose 1	Dose 2
Systemic Hemodynamics				
Heart Rate (bpm)	ET	126 ± 6	103 ± 2*	90 ± 3*†
	Big ET	113 ± 5	102 ± 8	83 ± 7*†
Cardiac Output (L/min)	ET	5.2 ± 0.5	4.7 ± 0.4	3.8 ± 0.3*†
	Big ET	4.6 ± 0.2	4.2 ± 0.4	3.7 ± 0.3*†
Aortic Blood Pressure (mmHg)	ET	89 ± 2	97 ± 3*	119 ± 6*†
	Big ET	90 ± 3	96 ± 3*	111 ± 5*†
Left Ventricular Systolic Pressure (mmHg)	ET	110 ± 4	118 ± 4*	137 ± 10*†
	Big ET	110 ± 5	120 ± 4*	134 ± 7*†
Left Ventricular dP/dtmax (mmHg/s)	ET	2650 ± 150	2290 ± 120*	2100 ± 120*†
	Big ET	2700 ± 180	2550 ± 140	2220 ± 120*†
Left Atrial Pressure (mmHg)	ET	3 ± 1	7 ± 1*	9 ± 2*†
	Big ET	0 ± 1	4 ± 1*	6 ± 1*
Rate Pressure Product (beats min^{-1} mmHg)	ET	139 ± 9	122 ± 6*	123 ± 10*
	Big ET	123 ± 9	122 ± 10	114 ± 10
Myocardial Oxygen Balance				
Coronary Blood Flow (ml/min)	ET	41 ± 3	39 ± 3	34 ± 2*†
	Big ET	46 ± 6	43 ± 6	36 ± 5*†
Coronary Vascular Conductance (ml min^{-1} $mmHg^{-1}$)	ET	0.46 ± 0.03	0.41 ± 0.04	0.30 ± 0.03*†
	Big ET	0.45 ± 0.07	0.40 ± 0.06*	0.29 ± 0.05*†
Arterial PO_2 (mmHg)	ET	101 ± 2	96 ± 2*	99 ± 2
	Big ET	98 ± 3	97 ± 3	96 ± 3
Arterial SO_2 (%)	ET	97 ± 1	96 ± 1*	96 ± 1*
	Big ET	96 ± 1	95 ± 1	95 ± 1
Coronary Venous PO_2 (mmHg)	ET	25 ± 1	22 ± 1*	19 ± 2*†
	Big ET	23 ± 1	20 ± 1*	19 ± 1*†
Coronary Venous SO_2 (%)	ET	19 ± 2	14 ± 2*	11 ± 2*†
	Big ET	15 ± 1	11 ± 1*	8 ± 1*†
Myocardial Oxygen Consumption (µmol ml^{-1} min)	ET	171 ± 16	159 ± 14	147 ± 15*
	Big ET	209 ± 17	204 ± 15	193 ± 10

		Control	Dose 1	Dose 2
Regional Cardiac Function				
End Diastolic Length (mm)	ET	10 ± 0	10.1 ± 0.2	10.3 ± 0.2
	Big ET	10 ± 0	10.2 ± 0.2	10.4 ± 0.3
End Systolic Length (mm)	ET	8.4 ± 0.2	8.5 ± 0.3	8.9 ± 0.4
	Big ET	8.5 ± 0.2	8.5 ± 0.2	9.0 ± 0.3
Systolic Shortening (%)	ET	16.4 ± 2.4	15.8 ± 2.2	13.3 ± 2.7
	Big ET	15.5 ± 1.8	16.2 ± 2.7	14.0 ± 2.3

Table 2. Effects of Big ET and ET in swine at rest Values are means ± SE of 16 swine. Big ET Dose 1 = 20 pmol kg^{-1} min^{-1}; Dose 2 = 40 pmol kg^{-1} min^{-1}. ET Dose 1 = 10 pmol kg^{-1} min^{-1}; Dose 2 = 20 pmol kg^{-1} min^{-1}. dP/dtmax, maximum rate of rise in pressure; PO_2, oxygen tension; SO_2, oxygen saturation. * $P < 0.05$ vs rest control. † $P < 0.05$ vs dose 1.

Effects of Big ET and ET on the Coronary Circulation

Infusion of ET resulted in a dose-dependent decrease in CBF (Table 2, Figure 3). The decreased CBF resulted in a decrease in myocardial oxygen delivery that exceeded the (small) decrease in myocardial oxygen consumption. As a result, myocardial oxygen extraction increased, resulting in a decrease in $cvPo_2$ and $cvSo_2$ (Figure 3). Altogether, these data indicate a direct vasoconstrictor effect of ET on the coronary vasculature at rest. To compare ET-induced coronary vasoconstriction at rest and during exercise, changes in CBF, $cvPo_2$, and $cvSo_2$ due to exercise need to be taken into account. The CBF, $cvPo_2$, and $cvSo_2$ during exercise in the presence of ET were, therefore, compared with their respective values in a control exercise trial (Table 3). Progressive infusions of ET induced similar decreases in CBF, $cvPo_2$, and $cvSo_2$ at rest and during exercise (Figure 3), indicating that ET receptor sensitivity was unaltered during exercise. The shift in the relation between myocardial oxygen consumption and $cvPo_2$ is commonly used to assess changes in coronary resistance vessel tone. Figure 4 shows that ET caused a parallel shift in the relation between myocardial oxygen consumption and $cvPo_2$, supporting the interpretation that the vasoconstrictor effect of ET is not influenced by exercise. At the highest dose of ET, myocardial contractility tended to be reduced, as evidenced by a tendency toward a reduction in systolic shortening, which occurs red both at rest and during exercise ($P < 0.1$, Tables 2 and 3) . Altogether these findings indicate that ET receptor sensitivity in the coronary circulation is not altered by exercise.

		Control 1	Dose 1	Control 2	Dose 2
Systemic Hemodynamics					
Heart Rate (bpm)	ET	180 ± 5	165 ± 3*	183 ± 7	153 ± 4*†
	Big ET	184 ± 8	169 ± 8*	183 ± 10	159 ± 10*
Aortic Blood Pressure (mmHg)	ET	82 ± 2	88 ± 2*	81 ± 3	103 ± 3*†
	Big ET	84 ± 2	87 ± 2	84 ± 3	101 ± 4*†
Cardiac Output (L/min)	ET	7.71 ± 0.37	7.54 ± 0.32	7.80 ± 0.27	6.89 ± 0.30*†
	Big ET	8.14 ± 0.33	7.94 ± 0.32	8.42 ± 0.37	7.60 ± 0.42*
Left Ventricular Systolic Pressure (mmHg)	ET	106 ± 4	111 ± 3*	107 ± 3	122 ± 4*†
	Big ET	110 ± 3	112 ± 3	113 ± 5	124 ± 5*†
Left Ventricular dP/dt_{max} (mmHg/s)	ET	3460 ± 150	3260 ± 140	3530 ± 220	3110 ± 100
	Big ET	3290 ± 310	3280 ± 260	3410 ± 400	3250 ± 320
Left Atrial Pressure (mmHg)	ET	5 ± 1	8 ± 1*	6 ± 0	9 ± 1*
	Big ET	5 ± 1	6 ± 1	4 ± 1	9 ± 1*†
Rate Pressure Product (beats min^{-1} mmHg)	ET	190 ± 8	183 ± 6	195 ± 10	186 ± 6
	Big ET	208 ± 14	195 ± 14	213 ± 18	203 ± 18
Myocardial Oxygen Balance					
Coronary Blood Flow (ml/min)	ET	68 ± 5	66 ± 5	72 ± 6	62 ± 7
	Big ET	70 ± 6	68 ± 5	72 ± 8	68 ± 7
Coronary Vascular Conductance (ml min^{-1} mHg^{-1})	ET	0.83 ± 0.07	0.75 ± 0.06*	0.90 ± 0.09	0.60 ± 0.06*†
	Big ET	0.80 ± 0.07	0.76 ± 0.07	0.84 ± 0.09	0.67 ± 0.08*†
Arterial PO_2 (mmHg)	ET	99 ± 2	96 ± 3	94 ± 4	89 ± 4
	Big ET	101 ± 3	100 ± 4	98 ± 3†	99 ± 3
Arterial SO_2 (%)	ET	97 ± 0	96 ± 1*	96 ± 1	93 ± 1
	Big ET	97 ± 0	97 ± 0	97 ± 1	96 ± 1

		Control 1	Dose 1	Control 2	Dose 2
Coronary Venous PO_2 (mmHg)	ET	22 ± 0	20 ± 1*	21 ± 0	15 ± 1*†
	Big ET	21 ± 1	21 ± 1	20 ± 1	17 ± 1*†
Coronary Venous SO_2 (%)	ET	15 ± 2	12 ± 2*	13 ± 1†	6 ± 1*†
	Big ET	13 ± 1	12 ± 1	12 ± 1	8 ± 1*†
Myocardial Oxygen Consumption (µmol ml^{-1} min)	ET	362 ± 23	360 ± 26	344 ± 28	338 ± 28
	Big ET	344 ± 28	338 ± 28	364 ± 40	372 ± 38
Regional Cardiac Function					
End Diastolic Length (mm)	ET	10.2 ± 0.1	10.2 ± 0.1	10.2 ± 0.1	10.3 ± 0.1
	Big ET	10.7 ± 0.4	10.5 ± 0.4	10.6 ± 0.4	10.6 ± 0.4
End Systolic Length (mm)	ET	8.5 ± 0.2	8.6 ± 0.2	8.5 ± 0.2	8.7 ± 0.1*†
	Big ET	8.5 ± 0.3	8.3 ± 0.3	8.5 ± 0.2	8.5 ± 0.3
Systolic Shortening (%)	ET	16.1 ± 3.1	16.4 ± 2.4	16.5 ± 2.6	15.1 ± 2.2
	Big ET	20.2 ± 1.9	20.7 ± 1.8	20.0 ± 1.7	19.8 ± 1.5

Table 3. Effects of ET and Big ET in swine during exercise. Values are means ± SE of 16 swine. Big ET Dose 1 = 20 pmol kg^{-1} min^{-1}; Dose 2 = 40 pmol kg^{-1} min^{-1}. ET Dose 1 = 10 pmol kg^{-1} min^{-1}; Dose 2 = 20 pmol kg^{-1} min^{-1}. dP/dtmax, maximum rate in rise of pressure; PO_2, oxygen tension; SO_2, oxygen saturation. * $P < 0.05$ vs rest control. † $P < 0.05$ vs dose 1

	Control	2 μg kg^{-1} min^{-1}	5 μg kg^{-1} min^{-1}
Systemic Hemodynamics			
Heart Rate (bpm)	139 ± 8	98 ± 6*	88 ± 6*
Aortic Blood Pressure (mmHg)	93 ± 2	114 ± 5*	135 ± 7*
Rate Pressure Product (beats min^{-1} mmHg)	156 ± 10	134 ± 5	140 ± 8
Myocardial Oxygen Balance			
Coronary Blood Flow (ml/min)	62 ± 8	56 ± 10*	57 ± 7*
Coronary Vascular Conductance (ml min^{-1} $mmHg^{-1}$)	0.66 ± 0.07	0.51 ± 0.09*	0.42 ± 0.05*
Coronary Venous PO_2 (mmHg)	23 ± 1	23 ± 2	23 ± 1
Coronary Venous SO_2 (%)	20 ± 2	17 ± 2	18 ± 2
Myocardial Oxygen Consumption (mol min^{-1})	261 ± 38	227 ± 40	237 ± 29

Table 4. Effects of Phenylephrine on the systemic and coronary circulations. Values are means ± SE of 5 swine. PO_2, oxygen tension; SO_2, oxygen saturation;. * $P < 0.05$ vs corresponding control.

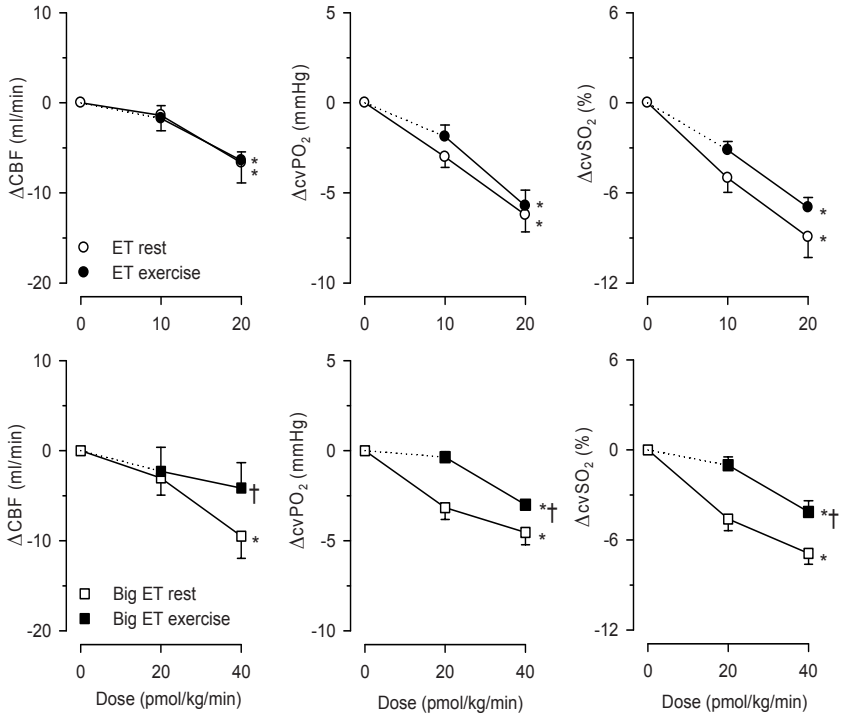


Figure 3. Coronary vasoconstrictor effects of ET (*A–C*) and Big ET (*D–F*) infusions at rest and during exercise. *A* and *D*: coronary blood flow (CBF). *B* and *E*: coronary venous oxygen tension ($cvPO_2$). *C* and *F*: coronary venous oxygen saturation ($cvSO_2$). *A–C*: effects of ET (10 and 20 pmol·kg^{-1}·min^{-1}) are similar at rest and during exercise. *D–F*: effects of Big ET (20 and 40 pmol·kg^{-1}·min^{-1}) are reduced during exercise. Values are means ± SE. **P* ≤ 0.05, drug effect vs. control; †*P* ≤ 0.05, effect of Big ET or ET altered during exercise.

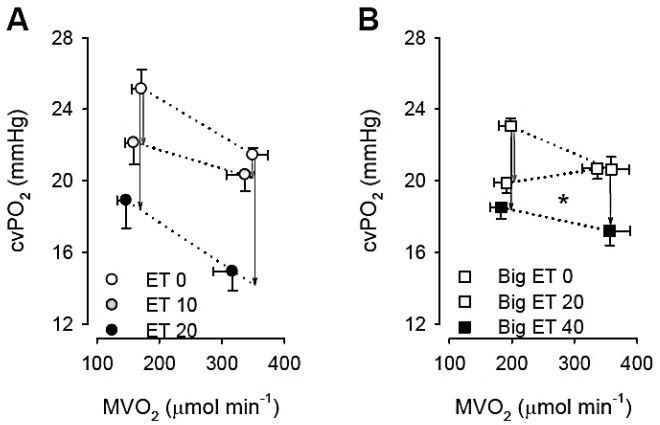


Figure 4. Effects of ET and Big ET infusions on the myocardial oxygen balance. *A*: incremental dosages of ET (0, 10, and 20 pmol·kg^{-1}·min^{-1}) resulted in a parallel shifts of the relation between myocardial oxygen consumption ($M\dot{V}O_2$) and $cvPO_2$. *P* value for rotation: 0.9. Vertical arrows indicate the change in $cvPO_2$ in response to ET at rest (*left*) and during exercise (*right*) at constant $M\dot{V}O_2$. *B*: Big ET (20 pmol·kg^{-1}·min^{-1}) resulted in a significant change in slope of the relation between $M\dot{V}O_2$ and $cvPO_2$, indicating a decreased conversion of Big ET to ET during exercise. In the presence of 40 pmol·kg^{-1}·min^{-1} Big ET, the rotation was not significant. However, when both doses were combined, a significant rotation was observed ($P = 0.049$). Vertical arrows indicate the change in $cvPO_2$ in response to Big ET at rest (*left*) and during exercise (*right*). Dotted lines were used because the measurements at rest (low $M\dot{V}O_2$) and during exercise (high $M\dot{V}O_2$) were performed in different groups of animals. *$P \leq 0.05$, rotation of the relation between $M\dot{V}O_2$ and $cvPO_2$ compared with control conditions.

Infusion of Big ET also resulted in a dose-dependent decrease in CBF at rest (Table 2). Since myocardial oxygen consumption was not influenced by infusion of Big ET, the resultant decrease in myocardial oxygen delivery necessitated an increase in myocardial oxygen extraction, and hence $cvPo_2$ and $cvSo_2$ progressively decreased (Figure 3). These data show that Big ET induces vasoconstriction of the coronary vasculature at rest. Moreover, especially at the highest dose, Big ET tended to reduce systolic shortening ($P = 0.1$, Table 2). The effects of Big ET on the coronary vasculature, as well as systolic shortening at rest, were similar to the effects induced by ET. During continuous exercise at 3 km/h, the vasoconstrictor effect of Big ET on the coronary vasculature was significantly reduced compared with resting conditions, as evidenced by blunted decreases in CBF, $cvPo_2$, and $cvSo_2$ (Figure 3). Furthermore, Big ET produced a significant increase in slope ($P = 0.049$ vs. control)

of the relation between myocardial oxygen consumption and $cvPo_2$ (Figure 4). Additionally, there was no effect of Big ET infusion on systolic shortening during exercise (Table 3).

Altogether, our data suggest that, during exercise, the conversion of Big ET to ET is decreased in the coronary vasculature compared with resting conditions. In contrast, the sensitivity of the coronary vasculature to ET seems unperturbed.

Effects of PE on the Systemic and Coronary Vasculature at Rest

PE was infused intravenously in incremental dosages up to 5 $\mu g \cdot kg^{-1} \cdot min^{-1}$, and its infusion resulted in systemic vasoconstriction, as evidenced by dose-dependent increases in blood pressure that were accompanied by decreases in heart rate (Table 4). PE infusion at a dose of 2 $\mu g \cdot kg^{-1} \cdot min^{-1}$ resulted in a similar blood pressure increase and heart rate decrease compared with the highest doses of Big ET and ET. Infusion of PE at a dose of 5 $\mu g \cdot kg^{-1} \cdot min^{-1}$ resulted in an even more pronounced increase in blood pressure and further decreases in heart rate and systemic vascular conductance. CBF also decreased upon infusion of PE, whereas $cvPo_2$ and $cvSo_2$ remained essentially unchanged (Figure 5). The discrepant response between CBF and $cvPo_2$ and $cvSo_2$ indicates that the decrease in CBF is due to a decrease in myocardial oxygen consumption rather than a direct coronary vasoconstrictor effect of PE.

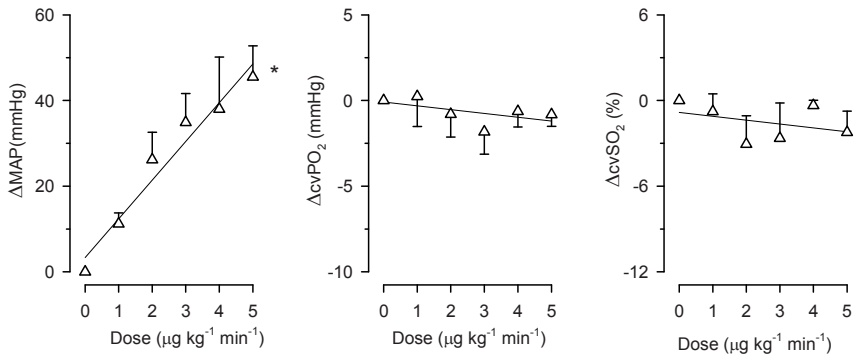


Figure 5. Systemic and coronary effects of phenylephrine (PE) infusion at rest. *A*: MAP; *B*: $cvPo_2$; *C*: $cvSo_2$. *A*: PE (1–5 $\mu g \cdot kg^{-1} \cdot min^{-1}$) causes dose-dependent systemic vasoconstriction. *B* and *C*: PE does not cause coronary vasoconstriction. Values are means ± SE. $^*P \leq 0.05$, drug effect vs. control.

DISCUSSION

The main findings of the present study were that: *1*) the systemic vasoconstrictor effects of both ET and Big ET in resting swine were similar to those produced during exercise; *2*) the coronary vasoconstrictor effects of ET were also maintained during exercise; however, *3*) the coronary vasoconstrictor effect of Big ET during exercise was attenuated compared with resting conditions. Together, these findings indicate that, during exercise, in the coronary vasculature, ET receptor sensitivity is maintained, while the conversion of Big ET to ET is blunted. The implications of these findings will be discussed below.

Exercise-induced Modulation of the ET System in the Systemic Circulation

Twofold higher dosage of Big ET compared with ET were required to obtain a similar vasoconstrictor response in both the systemic and the coronary vasculature, indicating that the conversion of Big ET to ET occurs at a similar rate in the systemic and coronary vasculature. The finding that the dose ratio of Big ET to ET is approximately two is consistent with data in anesthetized rodents [31, 33], but contrasts results in humans that demonstrate a comparable potency of Big ET vs. ET [25]. These differences are difficult to explain, but may be related to species differences in ECE activity or dosage of Big ET that was 4- to 10-fold lower in the human studies.

In the present study, we found that the systemic vasoconstriction induced by ET infusion was similar at rest and during continuous exercise, suggesting that ET receptor sensitivity in the systemic vasculature does not change during exercise. Moreover, the systemic vasoconstrictor effects of Big ET were also similar at rest and during exercise, indicating that the conversion of Big ET to ET is maintained during exercise. These data appear to be at odds with our laboratory's previous study, in which we observed that contribution of endogenous ET to systemic vascular tone decreased during exercise [21]. In that study, however, the withdrawal of ET-mediated vasoconstriction only occurred at the highest exercise intensities (4 and 5 km/h), whereas swine ran at 3 km/h in the present study, since it was not possible to maintain voluntary exercise at a higher intensity for 40 min. It is, therefore, possible that the exercise intensity used in the present study was not sufficient to demonstrate a reduced production of ET in the systemic vasculature.

After conversion of Big ET to ET, secretion occurs predominantly on the abluminal side of the vessel. Therefore, local concentrations can vary and the vasoconstriction in response to Big ET infusion may also vary between vascular beds. Withdrawal of the vasoconstrictor influence of endogenous or exogenous ET during exercise in active skeletal muscle can, therefore, be offset by an enhanced effect of ET in nonexercising muscle or visceral tissues. Indeed, it was recently observed in humans that the vasoconstrictor effect of exogenously administered ET was lower in the exercising leg compared with the resting leg [36]. Moreover, studies by Maeda et al. [13, 14, 17], in humans exercising with one leg, showed that the concentration of ET increased in the venous blood of the nonexercising leg, whereas it remained unchanged in the exercising leg. We, therefore, cannot exclude the possibility that the effects of Big ET and/or ET during exercise decreased in the vasculature of exercising muscle, whereas they may have increased in vascular beds of inactive muscle and/or visceral organs.

Exercise-Induced Modulation of the ET System in the Coronary Circulation

Under resting conditions, the coronary vasculature is maintained in a state of constriction, so-called basal tone, by a balance of vasodilator and vasoconstrictor factors released from the autonomic nervous system, the cardiac myocytes, as well as from within the vasculature itself. ET is an important factor in the maintenance of basal tone. Thus, at rest, there is continuous production of ET that is in equilibrium with its clearance, resulting in a modest vasoconstrictor effect, as illustrated by the modest vasodilator effect of ET receptor blockade with tezosentan [21]. Our data show that an increase in ET concentration through infusion of ET or Big ET results in vasoconstriction, most likely through a temporary disbalance between “production” and clearance. Studies in literature show that, not only the magnitude, but also the duration of this constriction, depends on the concentration of ET. Thus a bolus injection of a low dose of ET (4 pmol) induces a partially transient decrease in CBF in isolated rat hearts, whereas a high dose of ET (400 pmol) resulted in a sustained decrease in CBF [24]. This is also true for prolonged infusions in vivo [12]: ET infusions at 5 $ng \cdot kg^{-1} \cdot min^{-1}$ iv for 1 h in conscious dogs result in a 10-fold increase in ET plasma levels, but only in a small decrease in CBF that is almost instantaneously reversed upon cessation of the infusion. Higher dosages of 10 and 20 $ng \cdot kg^{-1} \cdot min^{-1}$, which resulted in a 40- and 80-fold increase in ET plasma levels, not only result in more severe vasoconstriction, but also in

progressively slower reversal of the constriction [12], suggesting that clearance receptors may be overwhelmed by the high circulating plasma concentrations of ET.

During exercise, the balance between vasodilators and vasoconstrictors is shifted in favor of vasodilation to maintain myocardial perfusion, and hence myocardial oxygen delivery, commensurate with the increased oxygen demand of the myocardium. Our laboratory has previously shown that the coronary vasoconstrictor effect of the endogenous ET system wanes within minutes during exercise [21], thereby contributing to the exercise-induced vasodilation. Thus it appears that the balance between production and clearance of endogenous ET can be adjusted rapidly, most likely because the concentrations of endogenous ET present in the myocardial interstitium, as well as in the circulation, are low compared with the concentrations present in response to exogenous infusion of ET. To provide evidence that this waning of the ET-induced vasoconstriction during increased metabolic demand is not simply the result of masking the vasoconstrictor influence of ET by an increasing number of vasodilator systems, we previously simulated exercise in vitro, by electrical stimulation of cardiac myocytes and adding their supernatant to isolated coronary arterioles, thereby uncoupling myocardial oxygen supply (i.e., the vasculature) and demand (i.e., the myocytes), while simultaneously preventing the influence of volatile and neurohumoral vasoactive factors [20]. Supernatant of unstimulated myocytes resulted in ET-mediated vasoconstriction that decreased with increasing stimulation rates, providing further evidence that withdrawal of ET-mediated vasoconstriction contributes to metabolic coronary vasodilation. Our laboratory subsequently showed in vivo that this effect of blunting the ET vasoconstrictor influence was mediated by NO and prostacyclin (PGI_2) [23]. NO has been shown to reduce the affinity of the ET_A receptor [34, 35], and, since both NO [1, 11] and prostacyclin [29] can limit ET production, we hypothesized that the waning of the ET vasoconstriction with increasing exercise intensity was due to a reduction in ET_A receptor sensitivity and/or ET production.

In the present study, we showed that the vasoconstriction induced by ET infusion is similar at rest and during exercise, whereas the vasoconstriction induced by infusion of Big ET was reduced during exercise. Hence, the reduced vasoconstrictor influence of ET during exercise is unlikely to be caused by decrease in receptor sensitivity. Yet the baroreceptor reflex response to the increase in blood pressure may have caused a decrease in sympathetic activity and an increase in parasympathetic activity that could potentially influence coronary resistance vessel tone and may differentially affect the responses to (Big) ET at rest and during exercise. Our laboratory has previously shown that, in swine, blocking muscarinic

receptors with atropine results in coronary vasodilation, but that this vasodilation is mediated through an increase in sympathetic activity, as it is abolished after prior β-blockade [4, 5]. Thus an increase in parasympathetic activity could influence coronary vascular tone by decreasing β-adrenergic activity, possibly resulting in vasoconstriction. Since β-adrenergic activity is already minimal under resting conditions, it is unlikely that activation of the parasympathetic nervous system contributed to the coronary vasoconstriction in response to either ET or Big ET at rest. It is, however, possible that, when sympathetic activity is high during exercise, a reduction in β-adrenergic coronary vasodilation contributed to the vasoconstriction. This would then result in an overestimation of the vasoconstriction induced by ET and Big ET during exercise, and an underestimation of the difference between rest and exercise. Following this line of reasoning, it is possible that a reduction in β-adrenergic vasodilation during exercise following ET and Big ET masked an exercise-induced decrease in coronary sensitivity to ET. As the magnitude of the increase in blood pressure and the decrease in heart rate were similar for ET and Big ET, the reduced vasoconstrictor response to Big ET compared with ET is unlikely to be caused by baroreceptor reflex-mediated activation of the parasympathetic or inhibition of the sympathetic nervous system, but rather results from a decreased production and/or release of ET.

Due to its abluminal secretion [2], ET plasma concentrations do not adequately reflect its concentrations in the interstitium, and production/release of ET cannot simply be measured by its arteriovenous concentration difference. To our knowledge, only a few studies have investigated the production of ET in the heart during exercise, although production of ET from Big ET in the heart has been demonstrated at rest [25]. Furthermore, Maeda and coworkers [15, 16] demonstrated that prolonged exercise (~45 min) increased mRNA for preproendothelin, as well as ET protein levels in the rat heart. However, since these measurements were made using the entire LV of the rat, it is unclear whether these results are indicative of elevated ET production in cardiac myocytes, vascular cells, or both, during exercise. Moreover, in these experiments, rats were anesthetized immediately following exercise, and their hearts were removed. It is, therefore, possible that the increase in ET is due to the termination of exercise rather than to exercise itself. Recent studies have shown that ET production can be reduced by increases in endothelial cGMP [11], whereas oxidative stress enhances ET production through stabilization of preproendothelin mRNA [18]. As NO alleviates oxidative stress and enhances cGMP production, this may provide a pathway by which NO can reduce ET production. In accordance with this suggestion, our

laboratory previously demonstrated that endothelial NO synthase inhibition results in an increase in ET-mediated vasoconstriction in the coronary vasculature during exercise [23].

Since our laboratory previously found that withdrawal of the vasoconstrictor effect of endogenous ET occurred within 10 min of exercise [21], it is unlikely that this is due to a change in transcription of preproendothelin mRNA. We, therefore, investigated the ET pathway downstream of preproendothelin mRNA, using infusion of Big ET. In a preliminary study, we found that the vasoconstrictor effect of Big ET progressed after cessation of the infusion and stabilized after ~5 min. This delay was most likely due to the necessary conversion of Big ET into the vasoactive ET and is in accordance with the 3-min half time of uptake of Big ET into endothelial cells [10]. The measurements of the vascular effects of Big ET were, therefore, performed 10 min after cessation of its infusion. This required a longer exercise protocol for the Big ET infusions (40 min) compared with the ET infusions (20 min). Exercise resulted in significant decreases in $cvPo_2$ and $cvSo_2$ that were slightly larger after 20 min of exercise compared with 10 min of exercise, but stable thereafter. To correct for these small changes in $cvPo_2$ and $cvSo_2$ during control exercise, we calculated the effects of Big ET and ET during exercise by subtracting the values obtained during the control run from the values during Big ET or ET infusion at the corresponding time points. CBF and other cardiovascular parameters measured were very stable over the entire 40 min of control exercise, making it unlikely that the differences between the responses to ET and Big ET were induced by the differences in duration of exercise. Moreover, the repeated-measures design of the experiments enabled the use of each animal as its own control, thereby increasing statistical power for the analyses.

The present study shows that the loss of ET-mediated vasoconstriction during exercise is due to decreased conversion of Big ET to ET in the coronary vasculature. These data are in accordance with data from a previous study that showed that cardiac myocytes can modulate the production of ET (by a phosphoramidon-sensitive enzyme) in the coronary arterioles [19]. There are two pathways by which Big ET can be converted into ET, both of which are sensitive to phosphoramidon. First, ECE can directly cleave Big ET at position 21, thereby resulting in formation of mature ET [9, 37]. Second, chymase can cleave Big ET at position 31, resulting in $ET_{1–31}$, that can be subsequently cleaved by NEP to produce ET [31]. Future studies are necessary to determine whether the decreased conversion of Big ET to ET during exercise results from inhibition of ECE, chymase, and/or NEP.

Coronary Vasoconstriction: Direct Effect or Autoregulatory Response?

Administration of Big ET and ET resulted in significant increases in blood pressure that were accompanied by significant, probably baroreceptor reflex-mediated, decreases in heart rate. The increase in blood pressure (i.e., LV afterload) and the decrease in heart rate have counterbalancing effects on myocardial oxygen consumption. Thus only the highest dose of ET at rest resulted in a significant decrease in myocardial oxygen consumption. Nevertheless, significant vasoconstriction occurred as evidenced by the decreases in CBF and $cvPo_2$ and $cvSo_2$. It could be argued that this vasoconstriction is the result of an autoregulatory response to the increase in blood pressure or a metabolic response to the decrease in myocardial oxygen consumption, rather than a direct coronary vasoconstrictor effect caused by ET. To test this hypothesis, we performed experiments with infusion of the α_1-agonist PE. Swine express α_1-adrenoceptors in the systemic, but not the coronary, resistance vessels (30). Administration of PE causes systemic vasoconstriction, thereby increasing blood pressure, without directly affecting the coronary microcirculation. Thus administration of PE can be used to produce autoregulatory coronary vasoconstriction across a range of blood pressures. Administration of PE resulted in a small decrease in myocardial oxygen consumption, resulting in a decrease in CBF. However, myocardial oxygen delivery and myocardial oxygen demand decreased to the same extent, so that $cvPo_2$ and $cvSo_2$ remained essentially unchanged. In contrast, direct vasoconstriction in the coronary resistance vessels produced by infusions of Big ET and ET resulted in decreases not only in CBF but most importantly also in $cvPo_2$ and $cvSo_2$ (Figure 6). These results underline the importance of studying myocardial oxygen balance, i.e., measuring $cvPo_2$ and $cvSo_2$, to assess vasomotor tone responses of coronary resistance vessels to drug interventions at rest and during exercise. Thus $cvPo_2$ and $cvSo_2$ reflect changes in resistance vessel tone independent of autoregulatory responses.

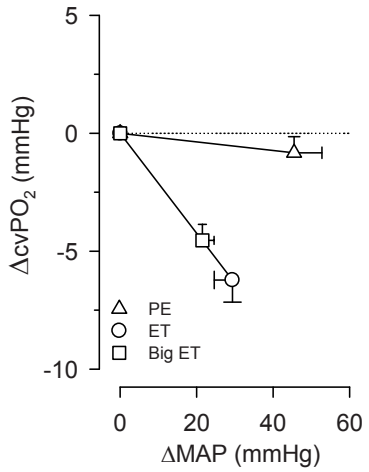


Figure 6. Effects of PE, ET, and Big ET infusions on coronary vasomotor tone and MAP. Comparison of infusions of PE (5 μg·kg^{-1}·min^{-1}), ET (20 pmol·kg^{-1}·min^{-1}), and Big ET (40 pmol·kg^{-1}·min^{-1}) at rest shows that coronary vasoconstriction induced by ET or Big ET is not due to autoregulation. Values are means ± SE.

CONCLUSION

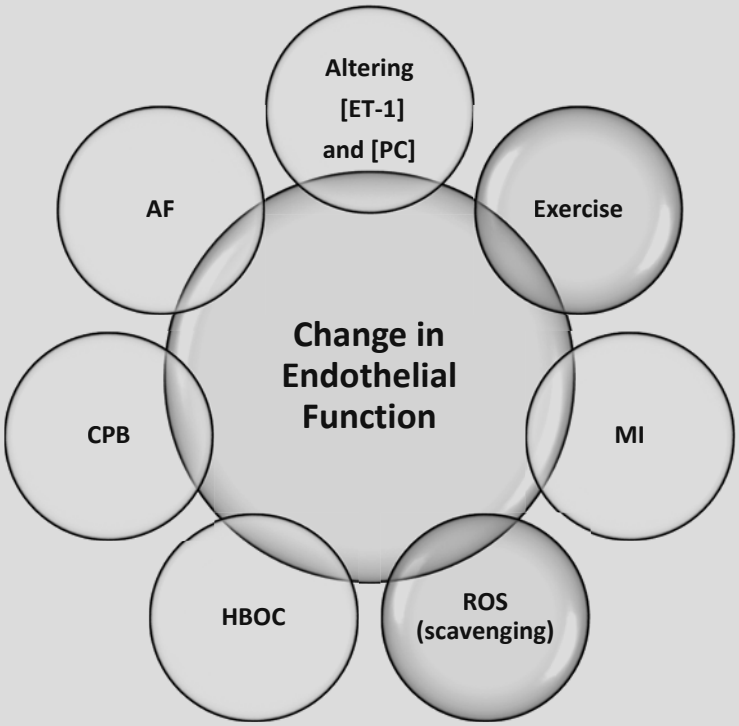
During exercise, the production of ET from its precursor Big ET is reduced in the coronary vasculature, while ET receptor sensitivity appears to be unaffected. The reduced production of ET during exercise contributes to reduction of coronary vascular tone, thereby facilitating recruitment of flow reserve during exercise-induced metabolic coronary vasodilation.

REFERENCES

1. Boulanger C, Luscher TF. Release of endothelin from the porcine aorta. Inhibition by endothelium-derived nitric oxide. J Clin Invest 85: 587–590, 1990
2. Chabrier PE. The role of endothelin in the vessel wall. Baillieres Clin Haematol 6: 577–591, 1993
3. de Beer VJ, Sorop O, Pijnappels DA, Dekkers DH, Boomsma F, Lamers JM, Duncker DJ, Merkus D. Integrative control of coronary resistance vessel tone by endothelin and angiotensin II is altered in swine with a recent myocardial infarction. Am J Physiol Heart Circ Physiol 294: H2069–H2077, 2008
4. Duncker DJ, Haitsma DB, Liem DA, Verdouw PD, Merkus D. Exercise unmasks autonomic dysfunction in swine with a recent myocardial infarction. Cardiovasc Res 65: 889–896, 2005
5. Duncker DJ, Stubenitsky R, Verdouw PD. Autonomic control of vasomotion in the porcine coronary circulation during treadmill exercise: evidence for feed-forward beta-adrenergic control. Circ Res 82: 1312–1322, 1998
6. Duncker DJ, Stubenitsky R, Verdouw PD. Role of adenosine in the regulation of coronary blood flow in swine at rest and during treadmill exercise. Am J Physiol Heart Circ Physiol 275: H1663–H1672, 1998
7. Haynes WG, Ferro CE, Webb DJ. Physiologic role of endothelin in maintenance of vascular tone in humans. J Cardiovasc Pharmacol 26, Suppl 3: S183–S185, 1995
8. Hein TW, Ren Y, Yuan Z, Xu W, Somvanshi S, Nagaoka T, Yoshida A, Kuo L. Functional and molecular characterization of the endothelin system in retinal arterioles. Invest Ophthalmol Vis Sci 50: 3329–3336, 2009
9. Hisaki K, Matsumura Y, Fujita K, Maekawa H, Takaoka M, Morimoto S. Differences in potency of big endothelin-1-induced pressor action in rat isolated perfused mesenteric artery, hindquarter and lung. Life Sci 54: 275–280, 1994
10. Johnstrom P, Fryer TD, Richards HK, Maguire JJ, Clark JC, Pickard JD, Davenport AP. Positron emission tomography of [18F]-big endothelin-1 reveals renal excretion but tissue-specific conversion to [18F]-endothelin-1 in lung and liver. Br J Pharmacol 159: 812–819, 2010
11. Kelly LK, Wedgwood S, Steinhorn RH, Black SM. Nitric oxide decreases endothelin-1 secretion through the activation of soluble guanylate cyclase. Am J Physiol Lung Cell Mol Physiol 286: L984–L991, 2004
12. Leadley RJ, Jr, Zhu JL, Goetz KL. Effects of endothelin-1 and sarafotoxin S6b on regional hemodynamics in the conscious dog. Am J Physiol Regul Integr Comp Physiol 260: R1210–R1217, 1991

13. Maeda S, Miyauchi T, Goto K, Matsuda M. Alteration of plasma endothelin-1 by exercise at intensities lower and higher than ventilatory threshold. J Appl Physiol 77: 1399–1402, 1994
14. Maeda S, Miyauchi T, Goto K, Matsuda M. Differences in the change in the time course of plasma endothelin-1 and endothelin-3 levels after exercise in humans. The response to exercise of endothelin-3 is more rapid than that of endothelin-1. Life Sci 61: 419–425, 1997
15. Maeda S, Miyauchi T, Sakai S, Kobayashi T, Goto K, Sugishita Y, Matsuda M. Endothelin-1 in the heart during exercise. J Cardiovasc Pharmacol 31, Suppl 1: S392–S394, 1998
16. Maeda S, Miyauchi T, Sakai S, Kobayashi T, Iemitsu M, Goto K, Sugishita Y, Matsuda M. Prolonged exercise causes an increase in endothelin-1 production in the heart in rats. Am J Physiol Heart Circ Physiol 275: H2105–H2112, 1998
17. Maeda S, Miyauchi T, Sakane M, Saito M, Maki S, Goto K, Matsuda M. Does endothelin-1 participate in the exercise-induced changes of blood flow distribution of muscles in humans? J Appl Physiol 82: 1107–1111, 1997
18. Mawji IA, Robb GB, Tai SC, Marsden PA. Role of the 3′-untranslated region of human endothelin-1 in vascular endothelial cells. Contribution to transcript lability and the cellular heat shock response. J Biol Chem 279: 8655–8667, 2004
19. Merkus D, Brzezinska AK, Zhang C, Saito S, Chilian WM. Cardiac myocytes control release of endothelin-1 in coronary vasculature. Am J Physiol Heart Circ Physiol 288: H2088–H2092, 2005
20. Merkus D, Duncker DJ, Chilian WM. Metabolic regulation of coronary vascular tone: role of endothelin-1. Am J Physiol Heart Circ Physiol 283: H1915–H1921, 2002
21. Merkus D, Houweling B, Mirza A, Boomsma F, van den Meiracker AH, Duncker DJ. Contribution of endothelin and its receptors to the regulation of vascular tone during exercise is different in the systemic, coronary and pulmonary circulation. Cardiovasc Res 59: 745–754, 2003
22. Merkus D, Houweling B, van den Meiracker AH, Boomsma F, Duncker DJ. Contribution of endothelin to coronary vasomotor tone is abolished after myocardial infarction. Am J Physiol Heart Circ Physiol 288: H871–H880, 2005
23. Merkus D, Sorop O, Houweling B, Boomsma F, van den Meiracker AH, Duncker DJ. NO and prostanoids blunt endothelin-mediated coronary vasoconstrictor influence in exercising swine. Am J Physiol Heart Circ Physiol 291: H2075–H2081, 2006
24. Neubauer S, Ertl G, Haas U, Pulzer F, Kochsiek K. Effects of endothelin-1 in isolated perfused rat heart. J Cardiovasc Pharmacol 16: 1–8, 1990

25. Pernow J, Kaijser L, Lundberg JM, Ahlborg G. Comparable potent coronary constrictor effects of endothelin-1 and big endothelin-1 in humans. Circulation 94: 2077–2082, 1996
26. Plumpton C, Haynes WG, Webb DJ, Davenport AP. Phosphoramidon inhibition of the in vivo conversion of big endothelin-1 to endothelin-1 in the human forearm. Br J Pharmacol 116: 1821–1828, 1995
27. Pollock DM, Opgenorth TJ. ETA receptor-mediated responses to endothelin-1 and big endothelin-1 in the rat kidney. Br J Pharmacol 111: 729–732, 1994
28. Prins BA, Hu RM, Nazario B, Pedram A, Frank HJ, Weber MA, Levin ER. Prostaglandin E2 and prostacyclin inhibit the production and secretion of endothelin from cultured endothelial cells. J Biol Chem 269: 11938–11944, 1994
29. Razandi M, Pedram A, Rubin T, Levin ER. PGE2 and PGI2 inhibit ET-1 secretion from endothelial cells by stimulating particulate guanylate cyclase. Am J Physiol Heart Circ Physiol 270: H1342–H1349, 1996
30. Schulz R, Oudiz RJ, Guth BD, Heusch G. Minimal alpha 1- and alpha 2-adrenoceptor-mediated coronary vasoconstriction in the anaesthetized swine. Naunyn Schmiedebergs Arch Pharmacol 342: 422–428, 1990
31. Simard E, Jin D, Takai S, Miyazaki M, Brochu I, D'Orleans-Juste P. Chymase-dependent conversion of Big endothelin-1 in the mouse in vivo. J Pharmacol Exp Ther 328: 540–548, 2009
32. Takamura M, Parent R, Cernacek P, Lavallee M. Influence of dual ET_A/ET_B-receptor blockade on coronary responses to treadmill exercise in dogs. J Appl Physiol 89: 2041–2048, 2000
33. Verbeuren TJ, Mennecier P, Merceron D, Simonet S, de Nanteuil G, Vincent M, Laubie M. Phosphoramidon inhibits the conversion of big ET-1 into ET-1 in the pithed rat and in isolated perfused rat kidneys. J Cardiovasc Pharmacol 22, Suppl 8: S81–S84, 1993
34. Wiley KE, Davenport AP. Nitric oxide-mediated modulation of the endothelin-1 signalling pathway in the human cardiovascular system. Br J Pharmacol 132: 213–220, 2001
35. Wiley KE, Davenport AP. Physiological antagonism of endothelin-1 in human conductance and resistance coronary artery. Br J Pharmacol 133: 568–574, 2001
36. Wray DW, Nishiyama SK, Donato AJ, Sander M, Wagner PD, Richardson RS. Endothelin-1-mediated vasoconstriction at rest and during dynamic exercise in healthy humans. Am J Physiol Heart Circ Physiol 293: H2550–H2556, 2007
37. Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, Yazaki Y, Goto K, Masaki T. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature 332: 411–415, 1988


Altering
[ET-1]
and [PC]
AF
Exercise
Change in
Endothelial
Function
CPB
MI
HBOC
ROS
(scavenging)

5

REACTIVE OXYGEN SPECIES ARE NOT CRITICAL METABOLIC REGULATORS IN EXERCISE HYPEREMIA IN HEALTHY SWINE

Y.J. Taverne, A.Verzijl, D.J. Duncker, D. Merkus.

In preparation 2017

ABSTRACT

Coupling blood flow to organ metabolism is required to ensure adequate distribution of oxygen to metabolically active tissue and identification of those mechanisms responsible for metabolic hyperemia during exercise has proven to be challenging. Reactive oxygen species (ROS), and more specific hydrogen peroxide (H_2O_2), have been suggested to act as regulatory mechanism in exercise hyperemia. Therefore, we first examined the overall effects of ROS on systemic, pulmonary and coronary vascular tone during exercise hyperemia by scavenging all ROS. Subsequently we investigated whether increasing production of H_2O_2 and/or decreasing breakdown of H_2O_2 affected exercise-induced vasodilation in the systemic, pulmonary and coronary vasculature. The main findings of this study were that during exercise, scavenging ROS did not alter vasoreactivity possibly due to an intact antioxidant system and presumably low production of free radicals. Furthermore, contrasting previous research, pharmacological endogenous H_2O_2 up-regulation did not exert vasodilatory properties in the systemic, pulmonary or coronary vasculature possibly due to the fact that superoxide ($O_2^{\bullet -}$) production is not upregulated. The lack of effect of pharmacologically induced alterations to a possible vasodilator substance on the extent of exercise hyperemia may reflect a compensation by other vasodilatory substances This shows great redundancy of the system in healthy subjects rather than excluding the involvement of ROS. However, it is clear that, only when endothelial dysfunction becomes apparent, a ROS mediated contribution to exercise hyperemia ensues. Further research should focus on these mechanisms of H_2O_2 derived vasodilation in health and disease.

INTRODUCTION

Coupling blood flow to organ metabolism is required to ensure adequate distribution of oxygen to metabolically active tissue, while avoiding excessive increases in cardiac output. Metabolic dilation, or metabolic hyperemia, is therefore critical to maintain sufficient oxygen delivery for aerobic metabolism and organ function [1, 2]. The identification of the mechanism(s) responsible for metabolic hyperemia during exercise has proven to be challenging [3-5], and the current consensus is that multiple pathways and factors, derived from endothelium (i.e. nitric oxide, prostanoids, endothelin and endothelium derived hyperpolarizing factor), sympathetic nervous system (i.e. beta feedforward vasodilation), and tissue metabolites, (H^+, adenosine) act in concert [6-8]. As regulation of blood flow during exercise requires a coordinated and integrated approach to adequately deliver oxygen and nutrients throughout the body, an optimal regulatory mechanism would directly couple oxygen consumption to the production of a vasodilator.

Reactive oxygen species (ROS) are chemically reactive molecules derived from molecular oxygen. ROS are produced during many biological processes and can act as signaling molecules and have been suggested to play an intricate role in regulation of vasoreactivity [9-11]. During energy conversion in the mitochondria, superoxide ($O_2^{\bullet-}$) is produced as a by-product of oxidative phosphorylation [10, 12, 13]. Thus, any increase in mitochondrial activity in tissue to generate ATP required for the exercising muscle, results in increased generation of $O_2^{\bullet-}$. $O_2^{\bullet-}$ can modulate vascular tone by inhibiting the opening of voltage dependent K (Kv)-channels in vascular smooth muscle, but its action-range is limited by its high reactivity and inability to pass cellular membranes. Breakdown of $O_2^{\bullet-}$, or dismutation, is catalyzed by superoxide dismutase (SOD), thereby forming hydrogen peroxide (H_2O_2). As Chilian and colleagues showed that production of H_2O_2 by cardiomyocytes is increased with increasing metabolism, and that H_2O_2 is capable to induce coronary vasodilation [2], they proposed that H_2O_2 couples coronary blood flow to myocardial metabolism. Indeed, H_2O_2 has all the properties to be a metabolic dilator, such as a short half-life, due to its breakdown by catalase, and the fact that it is freely permeable (9, 14). Similarly, recent data from the group of Kang et al. provided evidence for enhanced mitochondrial function and metabolic dilation in SOD overexpressed mice [15].

To provide further evidence for a role of H_2O_2 in metabolic hyperemia, it needs to be shown that augmenting the production of H_2O_2 or inhibiting its breakdown impacts metabolic vasodilation. Therefore, we first examined the overall effects of

ROS on systemic, pulmonary and coronary vascular tone during exercise hyperemia scavenging all ROS by a cocktail of N-acetylcysteine, an antioxidant that has been shown to optimize cellular thiol redox state by scavenging $O_2^{\bullet-}$ [16, 17], mercaptopropionylglycine, a potent cell-permeable scavenger that predominantly impacts hydroxyl radicals and peroxynitrite [18, 19], and the SOD mimetic 4 hydroxy-TEMPO (Tempol). Subsequently, we investigated whether increasing production of H_2O_2 by administration of the SOD mimetic, and/or decreasing breakdown of H_2O_2 by inhibiting catalase with 3-amino-1,2,4-triazole [19] affected exercise-induced vasodilation in the systemic, pulmonary and coronary vasculature.

METHODS

Animals

Studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH Publication No. 85–23, Revised 1996), and with prior approval of the Animal Care Committee of the Erasmus Medical Center. Nineteen, two- to three-month-old Yorkshire × Landrace pigs of either sex were entered into the study.

Surgery

Surgical protocol was described elsewhere [20-22]. In short, swine were sedated (20 mg/kg ketamine and 1 mg/kg midazolam im), anesthetized (thiopental sodium 15 mg/kg iv), intubated, and ventilated with a mixture of O_2 and N_2 (1:2) to which 2-3% (vol/vol) isoflurane was added to maintain anesthesia [20, 21]. Under sterile conditions, the chest was opened via the fourth left intercostal space, and a fluid-filled polyvinylchloride catheter was inserted into the aortic arch for aortic blood pressure measurement (Combitrans pressure transducers, Braun) and blood sampling. A Transit-time flow probe (Transonic Systems) was positioned around the ascending aorta for measurement of cardiac output. A microtipped pressure transducer ($P_{4.5}$, Konigsberg Instruments) was inserted into the left ventricle via the apex. Polyvinylchloride catheters were inserted into the left ventricle (LV) to calibrate the Konigsberg transducer LV pressure signal, into the left atrium to measure pressure, and into the pulmonary artery to administer drugs. Finally, a transit-time flow probe (Transonic Systems) was placed around the left anterior descending coronary artery [20]. Catheters were tunneled to the back, and animals were allowed to recover, receiving analgesia (0.3 mg buprenorphine im and a slow

release fentanyl patch) and antibiotic prophylaxis (25 mg/kg amoxicillin and 5 mg/kg gentamicin iv) for 5 days [20, 21].

Experimental Protocols

Studies were performed 1–3 wk after surgery with animals resting and exercising on a motor-driven treadmill up to 80-86% of maximal heart rate . Four protocols (as described below) were performed on different days and in random order. All chemicals were obtained from Sigma.

With swine lying quietly on the treadmill, resting hemodynamic measurements consisting of heart rate (HR), LV pressure (LVP), mean pulmonary pressure (MPAP), mean aortic pressure (MAP), left atrial pressure (LAP), aortic and coronary blood flow (CBF) were obtained. Hemodynamic measurements were repeated and rectal temperature was measured with animals standing on the treadmill. Subsequently, swine were subjected to a four-stage exercise protocol (1–4 km/h) while hemodynamic variables were continuously recorded and blood samples collected during the last 60 s of each 3 min exercise stage at a time when hemodynamics had reached a steady state. After the exercise protocol was completed, animals were allowed to rest on the treadmill for 90 min after which partially overlapping subgroups of animals underwent a second exercise protocol, in the presence of different modulators of ROS as outlined below. If different protocols were performed in the same animal, they were performed on different days and in random order. We have previously shown excellent reproducibility of the hemodynamic response in consecutive bouts of exercise [21, 23].

Effects of scavenging all ROS

The first subgroup of swine (N=7) received a mixture of antioxidants in order to scavenge as many ROS as possible. This mixture consisted of 4-hydroxy-TEMPO (Tempol) 45 mg.kg^{-1} [24, 25], 150 mg.kg^{-1} *N*-acetyl-L-cysteine (NAC) [16, 26, 27] and 2-mercapto-propionyl-glycine (MPG) which was continuously infused during exercise at 1 mg·kg^{-1}·min^{-1} [17, 28-30]. Tempol is a superoxide dismutase mimetic thereby stimulating the formation of H_2O_2 from $O_2^{\bullet-}$ [31], NAC an acetylated cysteine residue which has been shown to optimize cellular thiol redox state [16, 17] and MPG a potent cell-permeable scavenger of hydroxyl radicals and peroxynitrite [18, 19].

Effects of increasing endogenous of H_2O_2

The second subgroup of swine (N=9) received a dosage of 45 mg.kg^{-1} Tempol alone to increase the conversion of $O_2^{\bullet-}$ to H_2O_2 and the exercise protocol was repeated.

The third subgroup (N=9) received 3-amino-1,2,4-triazole (ATZ) at 50 mg.kg^{-1} [32] Catalase catalyzes the decomposition of hydrogen peroxide to water and oxygen and can be inhibited by ATZ, thereby increasing the amount of H_2O_2.

The last subgroup of four swine received the combination of ATZ 50 mg.kg^{-1} and Tempol 45 mg.kg^{-1} thereby aiming to enhance the formation of H_2O_2 while simultaneously inhibiting the reduction of H_2O_2 to water and oxygen.

Statistical Analysis

Statistical analysis of hemodynamic data was performed with SPSS 22 (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp). Unpaired t-test was used to compare single data points with respect to resting state, and to compare to controls. The effects of drug treatment, time and exercise were compared using a two-way analysis of variance (ANOVA) for repeated measures. When significant effects were detected, Bonferroni correction was applied. Statistical significance was accepted when $P \leq 0.05$. Data are presented as mean ± SEM.

RESULTS

Effects of exercise in control animals

Exercise up to 4 km/h in control conditions resulted in a doubling of CO ($P < 0.05$) which was principally due to increase in HR (Table 1) since stroke volume (SV) was minimally affected (not shown). Systemic vasodilation occurred, as evidenced by a decrease in systemic vascular resistance, while MAP was maintained more or less constant throughout incremental levels of exercise (Figure 1 and 2). MPAP doubled during exercise ($P < 0.05$) (Figure 1 and 3), which was the result of the exercise-induced increase of left atrial pressure (not shown) and CO (Table 1), as pulmonary vascular resistance (PVR) did essentially not change. CBF significantly increased with incremental levels of exercise to meet the higher metabolic demand of the myocardium due to a significant increase in coronary vascular conductance (Figure 1 and 4).

		Exercise level (km/h)				
	Treatment	Lying	1	2	3	4
HR (bpm)						
	Control	117±9	154±9†	162±8†	177±9*†	217±12*†
	ROS scavenger	142±11	174±11†	190±10*†‡	215±13*†‡	246±12*†
	Control	148±4	185±10†	190±7†	198±4†	205±15†
	Tempol	143±11	176±8†	188±9*†	205,1±9*†	222±5*†
	Control	150±5	173±12	179±16	199±19*†	193±17
	ATZ	141±7	157±11	178±11*†	189±9*†	209±6*†
	Control	132±10	177±16†	184±15†	191±9†	197±23
	Tempol-ATZ	141±18	179±15†	193±9†	202±4†	231±15†
CO (ml/min)						
	Control	3,8±0,3	5,4±0,6†	5,7±0,7†	6,2±0,7*†	7,3±0,6*†
	ROS scavenger	4,7±0,6	5,0±0,4	5,7±0,4*	6,5±0,4*	7,0±0,4*†
	Control	4,4±0,8	6,4±1,2†	6,3±1,2†	6,9±1,3*†	7,1±1,4†
	Tempol	4,9±1,1	5,9±1,1†	6,5±1,2*†	6,9±1,3*†	7,6±1,4*†
	Control	4,1±0,7	6,0±1,0†	6,3±0,8†	7,3±1,1*†	7,2±0,9†
	ATZ	4,7±1,1	5,5±0,7	6,1±1,0†‡	6,5±0,9*†	7,4±1,3†
	Control	3,6±0,8	5,0±1,3	4,9±1,0†	5,1±1,0†	5,2±0,6†
	Tempol-ATZ	3,1±0,3	5,0±1,1	5,3±0,9†	5,6±1,0*†‡	6,0±1,0*†
LvP max mmHg)						
	Control	101±7	104±6	108±5	107±5*	116±6
	ROS scavenger	81±10	92±7	95±6‡	91±14†	110±6
	Control	106±5	111±4	112±4	117±5	124±6
	Tempol	117±10	118±7	115±5	125±11	134±12
	Control	93±14	110±13†	108±11†	112±11†	111±11†
	ATZ	101±13	111±13	112±13†	115±12†	115±13†
	Control	93±14	110±13†	108±11†	112±11†	111±11†
	Tempol-ATZ	101±13‡	111±13	112±13	115±12	115±13*

Table 1. Systemic hemodynamics. * P ≤ 0.05 compared to previous, † P ≤ 0.05 compared to rest, ‡ P < 0.05 control versus treatment. HR, heart rate; CO, cardiac output; LvP max, maximum left ventricular pressure.

Scavenging ROS

Administration of a combination of ROS scavengers significantly (ANOVA P = 0.04) decreased MAP by 12 ± 5 mmHg at rest, and this effect decreased slightly during exercise (Figure 1). CO was unchanged, although a decrease in stroke volume was compensated by an increase in HR (Table 1). ROS scavenging resulted in systemic vasodilation as evidenced by a decrease in systemic vascular resistance (SVR) at rest, but this vasodilator effect waned during exercise. ROS scavenging had no effect on the pulmonary vasculature, as PVR did not change either at rest or during exercise, which together with the unaltered CO, resulted in similar MPAP before and after ROS scavenging (Figure 1, Table 1). Similarly, ROS scavenging did not influence coronary vascular resistance (CVR) or coronary blood flow (CBF) either at rest or during exercise.

Increasing hydrogen peroxide

Administration of the superoxide dismutase mimetic Tempol to enhance the conversion from $O_2^{\bullet-}$ to H_2O_2 had no effect on either MAP or SVR. Surprisingly, inhibition of catalase by ATZ, increased MAP at rest, resulting in a, probably baroreceptor reflex mediated, decrease in HR ($P < 0.05$). During exercise however, ATZ had no effect on either MAP or SVR. Combination of Tempol and ATZ to simultaneously enhance production and inhibit breakdown of H_2O_2 resulted in a small non-significant decrease in MAP and SVR particularly during exercise.

In the pulmonary vasculature, administration of Tempol or ATZ resulted in a slight increase in PVR, and a concomitant increase in MPAP. Combined administration of Tempol and ATZ had no significant effect on PVR or MPAP (Figure 3).

Coronary vascular conductance seemed to be improved (non-significant) after Tempol, an effect which was more attributable to Tempol alone compared to ATZ or Tempol + ATZ, however without alteration of coronary blood flow (Figure 4).

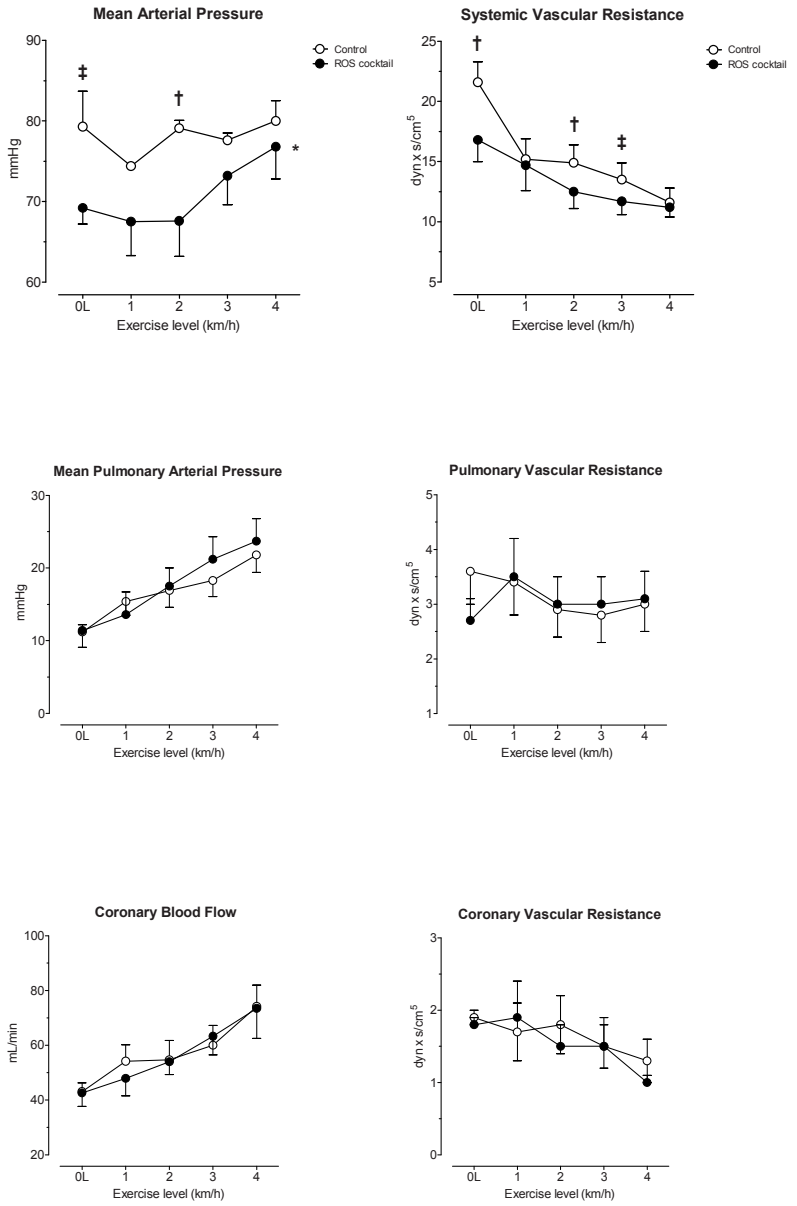


Figure 1. Effects of ROS scavengers on systemic, pulmonary and coronary circulation in exercising swine. † $P \leq 0.05$ and ‡ $P < 0.1$ comparing ROS scavenger cocktail to controls. * $P \leq 0.05$ overall effects over time between ROS scavenger cocktail and controls; N = 7.

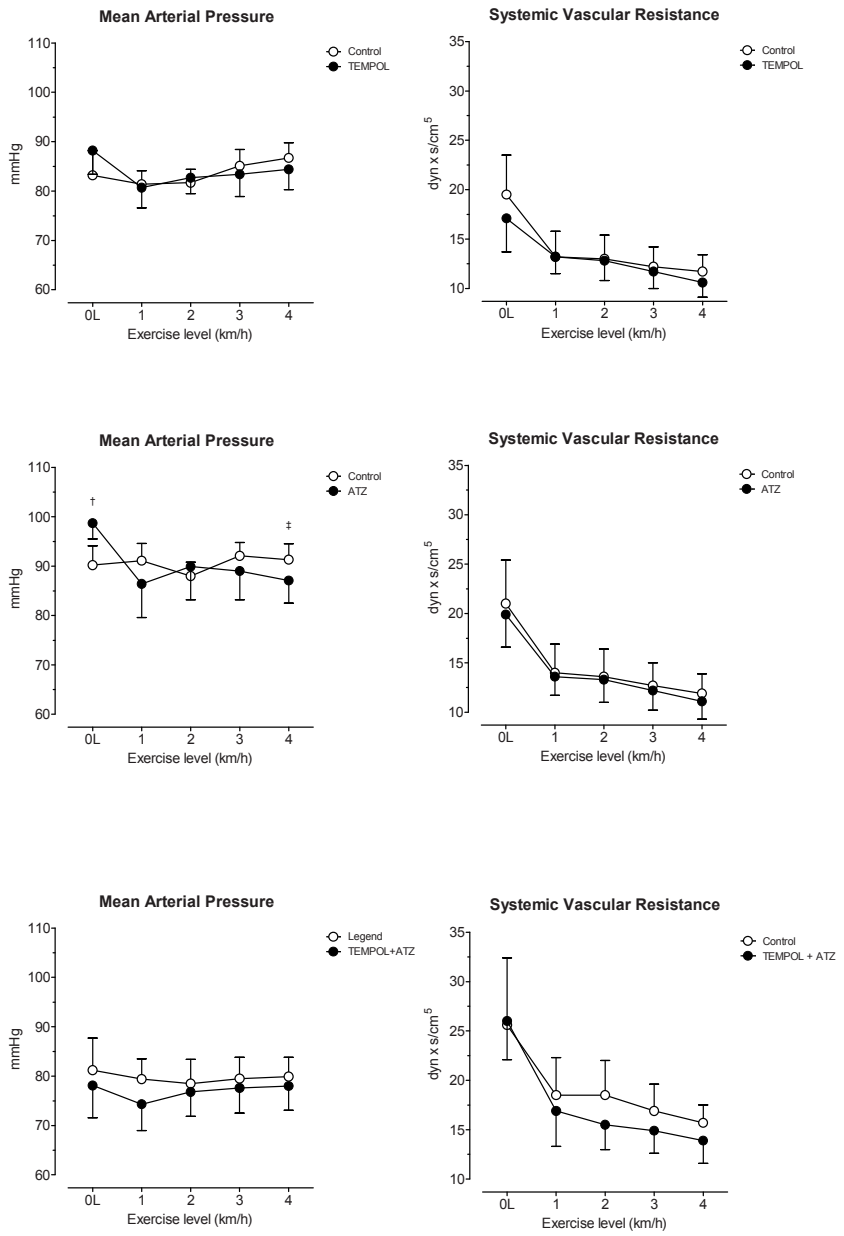


Figure 2. Effects of upregulating H_2O_2 on the systemic circulation in exercising swine. † $P \leq 0.05$ and ‡ $P < 0.1$ comparing compound to controls; Tempol N=9, ATZ N=9 and Tempol + ATZ N= 4.

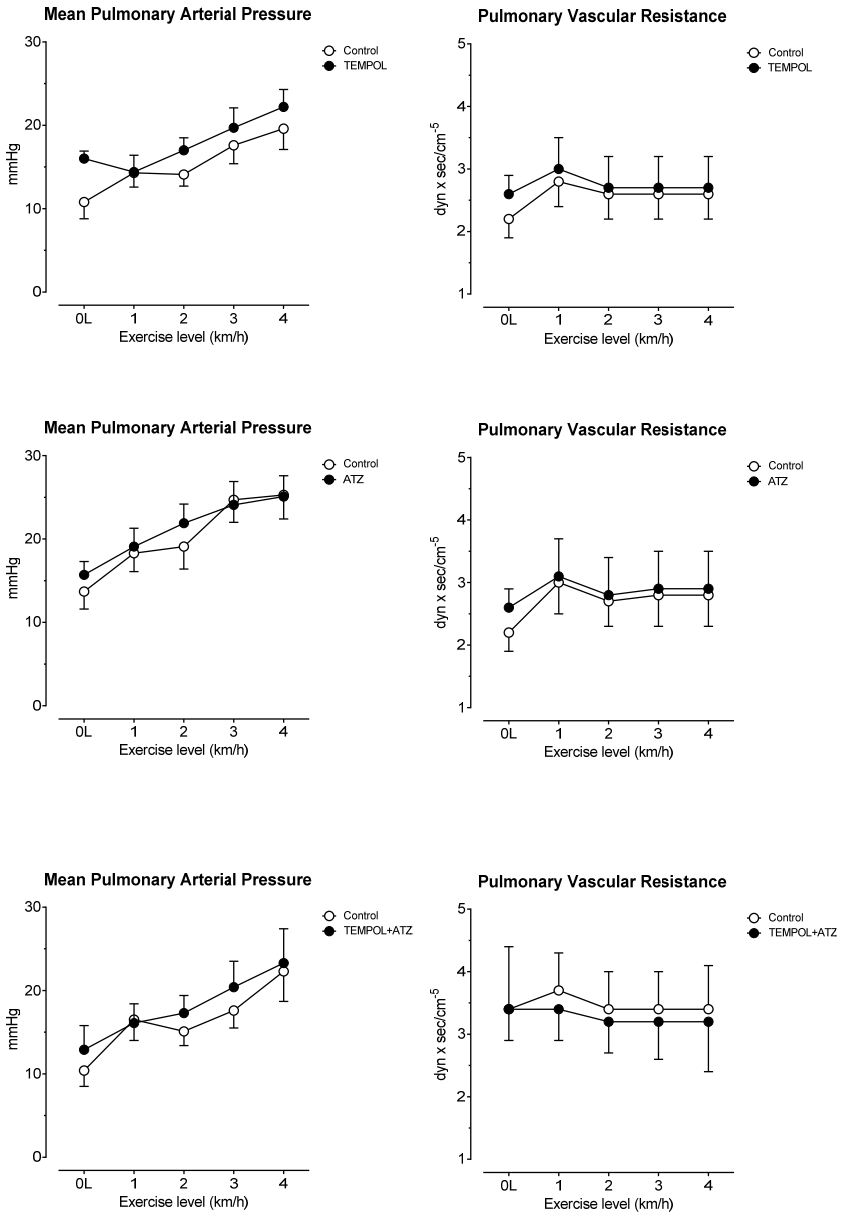


Figure 3. Effects of upregulating H_2O_2 on the pulmonary circulation in exercising swine. † P ≤ 0.05 and ‡ P < 0.1 comparing compound to controls; Tempol N=9, ATZ N=9 and Tempol + ATZ N= 4.

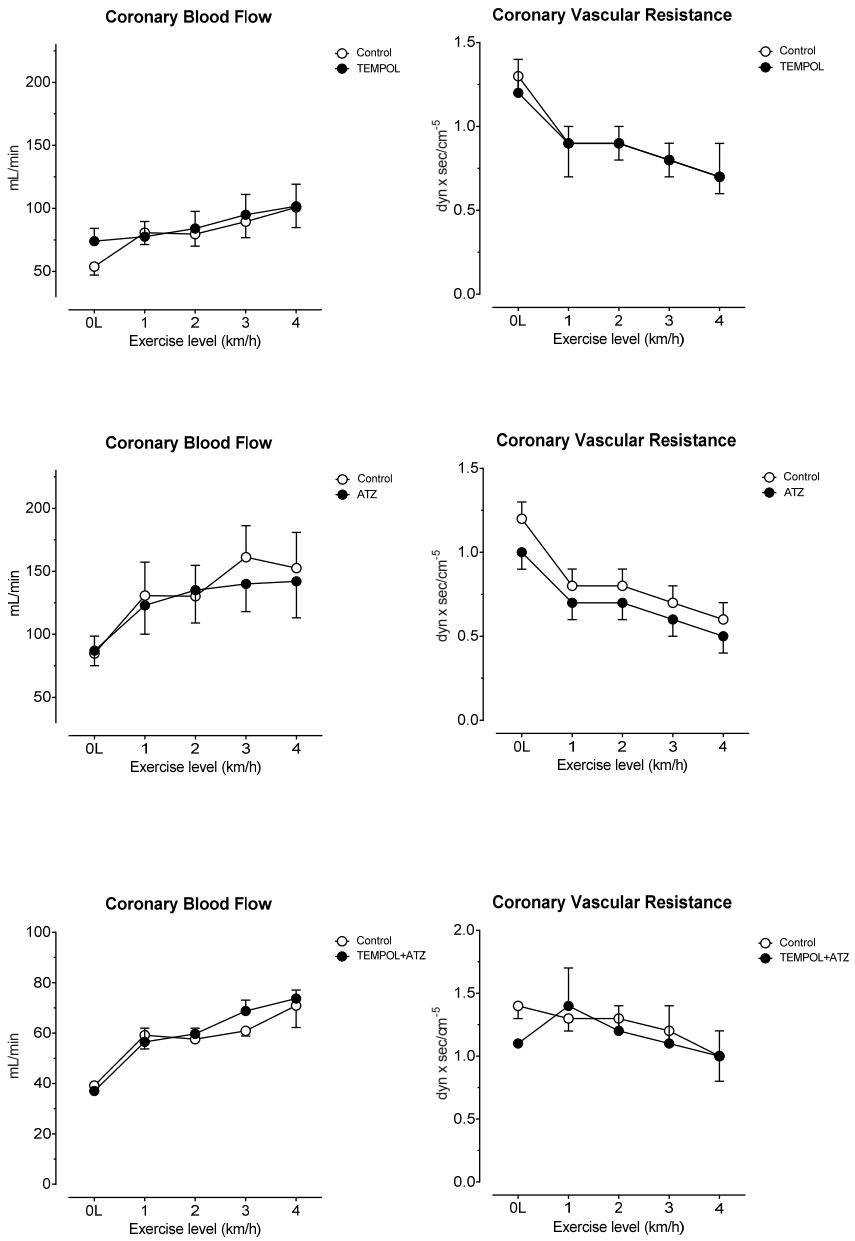


Figure 4. Effects of upregulating H_2O_2 on the coronary circulation in exercising swine. † $P \leq 0.05$ and ‡ $P < 0.1$ comparing compound to controls; Tempol N=9, ATZ N=9 and Tempol + ATZ N= 4.

DISCUSSION

To our knowledge, this is the first study to determine the effects of ROS, and more specific H_2O_2, in exercise hyperemia in exercising healthy swine. The main findings of this study were that during exercise, scavenging ROS did not alter vasoreactivity possibly due to an intact antioxidant system and presumably low production of free radicals. Furthermore, contrasting previous research, pharmacological endogenous H_2O_2 up-regulation did not exert vasodilatory properties in the systemic, pulmonary or coronary vasculature possibly due to the fact that superoxide ($O_2^{\bullet-}$) production is not upregulated.

Free radicals and exercise hyperemia

It is clear that with transient ischemia, changes in muscular O_2 levels, with concomitant $O_2^{\bullet-}$ production created by compression of vasculature during contraction, can act as an important trigger for vasodilation [33]. Under normal physiological conditions $O_2^{\bullet-}$ is enzymatically produced by a variety of oxidases, including xanthine oxidase, NADPH oxidase [10, 34]. However, the major source of $O_2^{\bullet-}$ is its production as a by-product of oxidative phosphorylation in the mitochondria. This is even more true during exercise, when the increased work load of skeletal muscle obviously requires an enormous increase in ATP production. Similarly, hemodynamic adjustments necessary to augment cardiac output during exercise cause an increase in each of the major determinants of myocardial oxygen demand e.g. heart rate, contractility and ventricular work [35]. Both in skeletal and cardiac muscle, O_2 flux through the mitochondrial transport chain is therefore augmented (up to 100-fold higher than resting values in contracting muscles), which is accompanied by excessive ROS production. The reactive nature of particularly $O_2^{\bullet-}$ requires activation of protective pathways, i.e. antioxidant systems that mainly convert $O_2^{\bullet-}$ to H_2O_2, in order to maintain cellular homeostasis [11, 36-38].

In our study, scavenging of free radicals did reduce vascular resistance in the systemic vasculature, but not in pulmonary and coronary vascular beds. However, the systemic vasodilator effect was not enhanced during exercise, suggesting that ROS neither contribute to exercise hyperemia in skeletal muscle nor in the myocardium. Opposing results exist on whether scavenging of free radicals alters vascular resistance, whether $O_2^{\bullet-}$ or H_2O_2 are mediators of exercise hyperemia, and/or whether exercise could tilt the balance towards oxidative stress. Data from the current study concur with previous results from our laboratory [30] and together suggest that either the amount of free radicals produced is very low, or that enough

SOD is present and activated during exercise to reduce $O_2^{\bullet-}$, and that the vasoconstrictor influence of $O_2^{\bullet-}$ is balanced by a similar vasodilator influence of H_2O_2 (see below). These data are seemingly in contrast with studies that do show a role for $O_2^{\bullet-}$ in regulation of vascular tone in skeletal muscle. However, although initial studies reported that ROS generation during exercise was directly related to the elevated oxygen consumption occurring with increased mitochondrial activity, and calculated a 50- or 100-fold increase in $O_2^{\bullet-}$ generation by skeletal muscles during aerobic contractions [39-41], a more recent paper reassessed the rate of production of ROS by mitochondria [42]. This group concluded that the upper estimate of the total fraction of oxygen utilized that forms superoxide was ~0.15% [42]; a value that is several orders of magnitude lower than the original estimate of 2–5% [43]. Moreover, during physiological exercise, transient increased ROS levels form an important stimulus for muscle cells to adapt to chronic exercise [38], very similar to the adaptive ischemic preconditioning response [44, 45]. Thus, acute bouts of exercise independently increase SOD activity [46-48]. In the post-exercise period, cytoplasmic SOD enzyme activity gradually returns to baseline, while its mitochondrial counterpart continues to increase [49]. Interestingly, glutathione peroxidase activity after exercise is dependent on the muscle type [49] and catalase activity appears not to be affected by acute exercise [50]. Although upregulation of antioxidants is muscle and/or fiber type-specific [45], the weight of evidence suggests that the antioxidant response is upregulated during acute exercise and the role of $O_2^{\bullet-}$ may be very limited.

Hydrogen peroxide and vasoreactivity

In healthy subjects, $O_2^{\bullet-}$ levels are tightly controlled by superoxide dismutase (SOD) [51, 52] keeping them in the picomolar range. SOD catalyzes the reaction of $O_2^{\bullet-}$ into H_2O_2 and O_2. The abundance of SOD in healthy blood vessels limits the amount of peroxynitrate ($ONOO^-$) [53] formed under normal conditions, particularly when keeping in mind that the reaction between NO and $O_2^{\bullet-}$ to form $ONOO^-$ is 3–4 times faster than the reduction of $O_2^{\bullet-}$ by SOD [53]. The direct relation between metabolism and $O_2^{\bullet-}$, the rapid conversion from $O_2^{\bullet-}$ to H_2O_2, the stability and diffusion ability of H_2O_2, as well as its capability to act as EDHF to cause vasodilation [54-56], make H_2O_2 an ideal candidate to act a signaling molecule. However, in the present study, neither stimulating reduction of $O_2^{\bullet-}$ to H_2O_2, nor blocking reduction of H_2O_2, nor a combination of these interventions affected vascular tone in either the coronary or the systemic vasculature in exercising swine. Our data contrast findings in isolated rat myocytes and in the canine heart in vivo, that show H_2O_2 to

be increased with increasing metabolism and that the increase in H_2O_2 is linearly related to the increase in coronary blood flow [2] as well as findings in SOD2 overexpressing mice that show enhanced coronary metabolic vasodilation in response to stress [15]. It is unlikely that these divergent effects are due to species differences in the response to H_2O_2, as H_2O_2 has been shown to cause vasodilation in vitro in a variety of species and vessels from different vascular beds, i.e. in bovine pulmonary arteries [57], bovine coronary arteries [58], cat cerebral arteries [59], porcine coronary arteries [60-62] and mice mesenteric arteries [56]. The lack of effect of pharmacologically induced alterations to a possible vasodilator substance on the extent of exercise hyperemia may reflect a compensation by other vasodilatory substances [63]. This shows great redundancy of the system in healthy subjects rather than excluding the involvement of ROS. Alternatively, it is possible that rats and mice, that have high metabolic rates, produce more $O_2^{\bullet-}$ and more H_2O_2 than healthy awake swine. The same may be true for the anesthetized dogs, in which ventilation with room air supplemented with oxygen [2, 55] may have caused supra-normal oxygen levels and oxidative stress in the myocardium.

This explanation is consistent with the observation that, upon the onset of cardiovascular disease (CVD), and more specific coronary artery disease, which is accompanied by an increase in oxidative stress, there is a switch in vasoactive substances produced by the endothelium from NO and prostanoids to a mitochondrial-derived H_2O_2 [64-66]. Conversely, under conditions of endothelial dysfunction, some studies propose a synergistic effect of H_2O_2 on the production of NO [64, 67]. Indeed, the group of Thenchaisri and coworkers reported restoration of coronary vasodilation during exercise in Yucatan miniature swine models with coronary occlusion which was associated with the activation of nitric oxide synthase and where H_2O_2 plays an important role [68]. They reported a concentration of 0.3-1M H_2O_2 to restore vasodilation [68] under pathological conditions. Also, the group of Miura hypothesized that H_2O_2 could act as a compensatory mechanism for reduced bioavailability of NO [69] in such cases. It should be noted however, that switching to a ROS-dependent pathway brings along a physiological cost as H_2O_2 has, opposed to NO [70], pro-inflammatory and proliferating traits in the vasculature possibly worsening cardiovascular disease [70-72].

Thus, it is conceivable to assume that ROS and H_2O_2 more specific are players in cellular homeostasis and exercise hyperemia in subjects with cardiovascular disease. However, in healthy subjects, enough antioxidant pathways exist keeping redox states balanced and exercise does not potentiate an oxidative stress reaction strong enough to be detected or attenuated in normal vascular physiology.

CONCLUSION

Vascular response to exercise and H_2O_2 in particular is an interesting and unexplored topic in the area of exercise physiology. In our study, modulating H_2O_2 concentrations by stimulating its production and/or inhibiting its degradation did not change exercise hyperemia, implying that the amount of $O_2^{\bullet-}$ produced is kept in the picomolar range by natural antioxidants thereby balancing redox reactions and a myriad of compensatory systems exist to compensate for loss of H_2O_2. It is clear that, only when endothelial dysfunction becomes apparent, a ROS mediated contribution to exercise hyperemia ensues. Further research should focus on these mechanisms of H_2O_2 derived vasodilation in health and disease.

REFERENCES

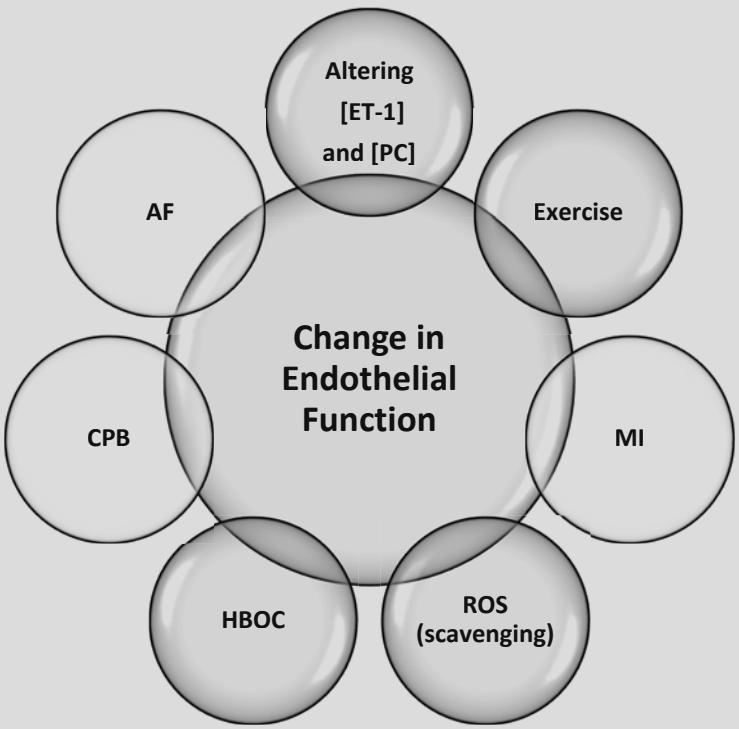
1. Feigl EO. Coronary physiology. Physiol Rev. 1983;63(1):1-205.
2. Saitoh S, Zhang C, Tune JD, Potter B, Kiyooka T, Rogers PA, et al. Hydrogen peroxide: a feed-forward dilator that couples myocardial metabolism to coronary blood flow. Arterioscler Thromb Vasc Biol. 2006;26(12):2614-21.
3. Fisslthaler B, Popp R, Kiss L, Potente M, Harder DR, Fleming I, et al. Cytochrome P450 2C is an EDHF synthase in coronary arteries. Nature. 1999;401(6752):493-7.
4. Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature. 1980;288(5789):373-6.
5. Trinity JD, Broxterman RM, Richardson RS. Regulation of exercise blood flow: Role of free radicals. Free Radic Biol Med. 2016.
6. Mortensen SP, Gonzalez-Alonso J, Damsgaard R, Saltin B, Hellsten Y. Inhibition of nitric oxide and prostaglandins, but not endothelial-derived hyperpolarizing factors, reduces blood flow and aerobic energy turnover in the exercising human leg. J Physiol. 2007;581(Pt 2):853-61.
7. Schrage WG, Dietz NM, Eisenach JH, Joyner MJ. Agonist-dependent variablity of contributions of nitric oxide and prostaglandins in human skeletal muscle. J Appl Physiol (1985). 2005;98(4):1251-7.
8. Schrage WG, Joyner MJ, Dinenno FA. Local inhibition of nitric oxide and prostaglandins independently reduces forearm exercise hyperaemia in humans. J Physiol. 2004;557(Pt 2):599-611.
9. Beny JL, von der Weid PY. Hydrogen peroxide: an endogenous smooth muscle cell hyperpolarizing factor. Biochem Biophys Res Commun. 1991;176(1):378-84.
10. Droge W. Free radicals in the physiological control of cell function. Physiol Rev. 2002;82(1):47-95.
11. Taverne YJ, Bogers AJ, Duncker DJ, Merkus D. Reactive oxygen species and the cardiovascular system. Oxid Med Cell Longev. 2013;2013:862423.
12. Chen Q, Vazquez EJ, Moghaddas S, Hoppel CL, Lesnefsky EJ. Production of reactive oxygen species by mitochondria: central role of complex III. J Biol Chem. 2003;278(38):36027-31.
13. Halliwell B. Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life. Plant Physiol. 2006;141(2):312-22.
14. Matoba T, Shimokawa H, Kubota H, Morikawa K, Fujiki T, Kunihiro I, et al. Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in human mesenteric arteries. Biochem Biophys Res Commun. 2002;290(3):909-13.
15. Kang PT, Chen CL, Ohanyan V, Luther DJ, Meszaros JG, Chilian WM, et al. Overexpressing superoxide dismutase 2 induces a supernormal cardiac

function by enhancing redox-dependent mitochondrial function and metabolic dilation. J Mol Cell Cardiol. 2015;88:14-28.
16. Supinski GS, Stofan D, Ciufo R, DiMarco A. N-acetylcysteine administration alters the response to inspiratory loading in oxygen-supplemented rats. J Appl Physiol (1985). 1997;82(4):1119-25.
17. Mitsos SE, Askew TE, Fantone JC, Kunkel SL, Abrams GD, Schork A, et al. Protective effects of N-2-mercaptopropionyl glycine against myocardial reperfusion injury after neutrophil depletion in the dog: evidence for the role of intracellular-derived free radicals. Circulation. 1986;73(5):1077-86.
18. Bolli R, Jeroudi MO, Patel BS, Aruoma OI, Halliwell B, Lai EK, et al. Marked reduction of free radical generation and contractile dysfunction by antioxidant therapy begun at the time of reperfusion. Evidence that myocardial "stunning" is a manifestation of reperfusion injury. Circ Res. 1989;65(3):607-22.
19. Akita Y, Otani H, Matsuhisa S, Kyoi S, Enoki C, Hattori R, et al. Exercise-induced activation of cardiac sympathetic nerve triggers cardioprotection via redox-sensitive activation of eNOS and upregulation of iNOS. Am J Physiol Heart Circ Physiol. 2007;292(5):H2051-9.
20. Merkus D, Houweling B, Mirza A, Boomsma F, van den Meiracker AH, Duncker DJ. Contribution of endothelin and its receptors to the regulation of vascular tone during exercise is different in the systemic, coronary and pulmonary circulation. Cardiovasc Res. 2003;59(3):745-54.
21. Duncker DJ, Stubenitsky R, Verdouw PD. Autonomic control of vasomotion in the porcine coronary circulation during treadmill exercise: evidence for feed-forward beta-adrenergic control. Circ Res. 1998;82(12):1312-22.
22. De Wijs-Meijler DP, Stam K, van Duin RW, Verzijl A, Reiss IK, Duncker DJ, et al. Surgical Placement of Catheters for Long-term Cardiovascular Exercise Testing in Swine. J Vis Exp. 2016(108):e53772.
23. Duncker DJ, Stubenitsky R, Tonino PA, Verdouw PD. Nitric oxide contributes to the regulation of vasomotor tone but does not modulate O(2)-consumption in exercising swine. Cardiovasc Res. 2000;47(4):738-48.
24. Chatterjee PK, Cuzzocrea S, Brown PA, Zacharowski K, Stewart KN, Mota-Filipe H, et al. Tempol, a membrane-permeable radical scavenger, reduces oxidant stress-mediated renal dysfunction and injury in the rat. Kidney Int. 2000;58(2):658-73.
25. McDonald MC, Zacharowski K, Bowes J, Cuzzocrea S, Thiemermann C. Tempol reduces infarct size in rodent models of regional myocardial ischemia and reperfusion. Free Radic Biol Med. 1999;27(5-6):493-503.
26. Matuszczak Y, Farid M, Jones J, Lansdowne S, Smith MA, Taylor AA, et al. Effects of N-acetylcysteine on glutathione oxidation and fatigue during handgrip exercise. Muscle Nerve. 2005;32(5):633-8.
27. Reid MB, Stokic DS, Koch SM, Khawli FA, Leis AA. N-acetylcysteine inhibits muscle fatigue in humans. J Clin Invest. 1994;94(6):2468-74.

28. Jarrar D, Wang P, Cioffi WG, Bland KI, Chaudry IH. Critical role of oxygen radicals in the initiation of hepatic depression after trauma hemorrhage. J Trauma. 2000;49(5):879-85.
29. Myers ML, Bolli R, Lekich RF, Hartley CJ, Roberts R. N-2-mercaptopropionylglycine improves recovery of myocardial function after reversible regional ischemia. J Am Coll Cardiol. 1986;8(5):1161-8.
30. Taverne YJ, de Beer VJ, Hoogteijling BA, Juni RP, Moens AL, Duncker DJ, et al. Nitroso-redox balance in control of coronary vasomotor tone. J Appl Physiol (1985). 2012;112(10):1644-52.
31. Wilcox CS. Effects of tempol and redox-cycling nitroxides in models of oxidative stress. Pharmacol Ther. 2010;126(2):119-45.
32. Brown JM, Grosso MA, Terada LS, Whitman GJ, Banerjee A, White CW, et al. Endotoxin pretreatment increases endogenous myocardial catalase activity and decreases ischemia-reperfusion injury of isolated rat hearts. Proc Natl Acad Sci U S A. 1989;86(7):2516-20.
33. Manevich Y, Al-Mehdi A, Muzykantov V, Fisher AB. Oxidative burst and NO generation as initial response to ischemia in flow-adapted endothelial cells. Am J Physiol Heart Circ Physiol. 2001;280(5):H2126-35.
34. Turrens JF. Mitochondrial formation of reactive oxygen species. J Physiol. 2003;552(Pt 2):335-44.
35. Duncker DJ, Bache RJ. Regulation of coronary blood flow during exercise. Physiol Rev. 2008;88(3):1009-86.
36. Ji LL. Antioxidants and oxidative stress in exercise. Proc Soc Exp Biol Med. 1999;222(3):283-92.
37. Powers SK, Ji LL, Leeuwenburgh C. Exercise training-induced alterations in skeletal muscle antioxidant capacity: a brief review. Med Sci Sports Exerc. 1999;31(7):987-97.
38. Radak Z, Zhao Z, Koltai E, Ohno H, Atalay M. Oxygen consumption and usage during physical exercise: the balance between oxidative stress and ROS-dependent adaptive signaling. Antioxid Redox Signal. 2013;18(10):1208-46.
39. Urso ML, Clarkson PM. Oxidative stress, exercise, and antioxidant supplementation. Toxicology. 2003;189(1-2):41-54.
40. Kanter MM. Free radicals, exercise, and antioxidant supplementation. Int J Sport Nutr. 1994;4(3):205-20.
41. Powers SK, Jackson MJ. Exercise-induced oxidative stress: cellular mechanisms and impact on muscle force production. Physiol Rev. 2008;88(4):1243-76.
42. St-Pierre J, Buckingham JA, Roebuck SJ, Brand MD. Topology of superoxide production from different sites in the mitochondrial electron transport chain. J Biol Chem. 2002;277(47):44784-90.
43. Boveris A, Chance B. The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. Biochem J. 1973;134(3):707-16.

44. Alleman RJ, Katunga LA, Nelson MA, Brown DA, Anderson EJ. The "Goldilocks Zone" from a redox perspective-Adaptive vs. deleterious responses to oxidative stress in striated muscle. Front Physiol. 2014;5:358.
45. Steinbacher P, Eckl P. Impact of oxidative stress on exercising skeletal muscle. Biomolecules. 2015;5(2):356-77.
46. Hitomi Y, Watanabe S, Kizaki T, Sakurai T, Takemasa T, Haga S, et al. Acute exercise increases expression of extracellular superoxide dismutase in skeletal muscle and the aorta. Redox Rep. 2008;13(5):213-6.
47. Hollander J, Fiebig R, Gore M, Ookawara T, Ohno H, Ji LL. Superoxide dismutase gene expression is activated by a single bout of exercise in rat skeletal muscle. Pflugers Arch. 2001;442(3):426-34.
48. Lawler JM, Powers SK, Visser T, Van Dijk H, Kordus MJ, Ji LL. Acute exercise and skeletal muscle antioxidant and metabolic enzymes: effects of fiber type and age. Am J Physiol. 1993;265(6 Pt 2):R1344-50.
49. Radak Z, Asano K, Inoue M, Kizaki T, Oh-Ishi S, Suzuki K, et al. Superoxide dismutase derivative reduces oxidative damage in skeletal muscle of rats during exhaustive exercise. J Appl Physiol (1985). 1995;79(1):129-35.
50. Ji LL. Exercise and oxidative stress: role of the cellular antioxidant systems. Exerc Sport Sci Rev. 1995;23:135-66.
51. Karlsson K, Marklund SL. Extracellular superoxide dismutase in the vascular system of mammals. Biochem J. 1988;255(1):223-8.
52. Landmesser U, Merten R, Spiekermann S, Buttner K, Drexler H, Hornig B. Vascular extracellular superoxide dismutase activity in patients with coronary artery disease: relation to endothelium-dependent vasodilation. Circulation. 2000;101(19):2264-70.
53. Beckman JS, Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. Am J Physiol. 1996;271(5 Pt 1):C1424-37.
54. Rogers PA, Dick GM, Knudson JD, Focardi M, Bratz IN, Swafford AN, Jr., et al. H2O2-induced redox-sensitive coronary vasodilation is mediated by 4-aminopyridine-sensitive K+ channels. Am J Physiol Heart Circ Physiol. 2006;291(5):H2473-82.
55. Saitoh S, Kiyooka T, Rocic P, Rogers PA, Zhang C, Swafford A, et al. Redox-dependent coronary metabolic dilation. Am J Physiol Heart Circ Physiol. 2007;293(6):H3720-5.
56. Matoba T, Shimokawa H, Nakashima M, Hirakawa Y, Mukai Y, Hirano K, et al. Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in mice. J Clin Invest. 2000;106(12):1521-30.
57. Cherry PD, Omar HA, Farrell KA, Stuart JS, Wolin MS. Superoxide anion inhibits cGMP-associated bovine pulmonary arterial relaxation. Am J Physiol. 1990;259(4 Pt 2):H1056-62.
58. Iesaki T, Gupte SA, Kaminski PM, Wolin MS. Inhibition of guanylate cyclase stimulation by NO and bovine arterial relaxation to peroxynitrite and H2O2. Am J Physiol. 1999;277(3 Pt 2):H978-85.

59. Fraile ML, Conde MV, Sanz L, Moreno MJ, Marco EJ, Lopez de Pablo AL. Different influence of superoxide anions and hydrogen peroxide on endothelial function of isolated cat cerebral and pulmonary arteries. Gen Pharmacol. 1994;25(6):1197-205.
60. Barlow RS, White RE. Hydrogen peroxide relaxes porcine coronary arteries by stimulating BKCa channel activity. Am J Physiol. 1998;275(4 Pt 2):H1283-9.
61. Hayabuchi Y, Nakaya Y, Matsuoka S, Kuroda Y. Hydrogen peroxide-induced vascular relaxation in porcine coronary arteries is mediated by Ca2+-activated K+ channels. Heart Vessels. 1998;13(1):9-17.
62. Matoba T, Shimokawa H, Morikawa K, Kubota H, Kunihiro I, Urakami-Harasawa L, et al. Electron spin resonance detection of hydrogen peroxide as an endothelium-derived hyperpolarizing factor in porcine coronary microvessels. Arterioscler Thromb Vasc Biol. 2003;23(7):1224-30.
63. Clifford PS, Hellsten Y. Vasodilatory mechanisms in contracting skeletal muscle. J Appl Physiol (1985). 2004;97(1):393-403.
64. Sato A, Sakuma I, Gutterman DD. Mechanism of dilation to reactive oxygen species in human coronary arterioles. Am J Physiol Heart Circ Physiol. 2003;285(6):H2345-54.
65. Liu Y, Zhao H, Li H, Kalyanaraman B, Nicolosi AC, Gutterman DD. Mitochondrial sources of H2O2 generation play a key role in flow-mediated dilation in human coronary resistance arteries. Circ Res. 2003;93(6):573-80.
66. Phillips SA, Hatoum OA, Gutterman DD. The mechanism of flow-induced dilation in human adipose arterioles involves hydrogen peroxide during CAD. Am J Physiol Heart Circ Physiol. 2007;292(1):H93-100.
67. D.J. S, W.Y. S, H.Y. R, H.S. A, K.S. C. Induction of Vasodilation by Hydrogen Peroxide and its Applications in Exercise Science. Biol Sport. 2012;29:87-92.
68. Thengchaisri N, Shipley R, Ren Y, Parker J, Kuo L. Exercise training restores coronary arteriolar dilation to NOS activation distal to coronary artery occlusion: role of hydrogen peroxide. Arterioscler Thromb Vasc Biol. 2007;27(4):791-8.
69. Miura H, Bosnjak JJ, Ning G, Saito T, Miura M, Gutterman DD. Role for hydrogen peroxide in flow-induced dilation of human coronary arterioles. Circ Res. 2003;92(2):e31-40.
70. Forstermann U. Nitric oxide and oxidative stress in vascular disease. Pflugers Arch. 2010;459(6):923-39.
71. Durand MJ, Dharmashankar K, Bian JT, Das E, Vidovich M, Gutterman DD, et al. Acute exertion elicits a H2O2-dependent vasodilator mechanism in the microvasculature of exercise-trained but not sedentary adults. Hypertension. 2015;65(1):140-5.
72. Cai H. Hydrogen peroxide regulation of endothelial function: origins, mechanisms, and consequences. Cardiovasc Res. 2005;68(1):26-36.


Altering
[ET-1]
and [PC]
AF
Exercise
Change in
Endothelial
Function
CPB
MI
HBOC
ROS
(scavenging)

6

NORMALIZATION OF HEMOGLOBIN-BASED OXYGEN CARRIER-201 INDUCED VASOCONSTRICTION

Targeting nitric oxide and endothelin

Y.J. Taverne, D. de Wijs-Meijler, M. Te Lintel Hekkert, P.F. Moon-Massat, G.P. Dubée, D.J. Duncker, D. Merkus

Journal of Applied Physiology 2017

ABSTRACT

Hemoglobin oxygen carrier (HBOC)-201 is a cell-free modified hemoglobin solution potentially facilitating oxygen uptake and delivery in cardiovascular disorders and hemorrhagic shock. Clinical use has been hampered by vasoconstriction in the systemic and pulmonary beds. Therefore, we aimed to *1*) determine the possibility of counteracting HBOC-201-induced pressor effects with either adenosine (ADO) or nitroglycerin (NTG); *2*) assess the potential roles of nitric oxide (NO) scavenging, reactive oxygen species (ROS), and endothelin (ET) in mediating the observed vasoconstriction; and *3*) compare these effects in resting and exercising swine. Chronically instrumented swine were studied at rest and during exercise after administration of HBOC-201 alone or in combination with ADO. The role of NO was assessed by supplementation with NTG or administration of the eNOS inhibitor N^{ω}-nitro-L-arginine. Alternative vasoactive pathways were investigated via intravenous administration of the ETA/ETB receptor blocker tezosentan or a mixture of ROS scavengers. The systemic and to a lesser extent the pulmonary pressor effects of HBOC-201 could be counteracted by ADO; however, dosage titration was very important to avoid systemic hypotension. Similarly, supplementation of NO with NTG negated the pressor effects but also required titration of the dose. The pressor response to HBOC-201 was reduced after eNOS inhibition and abolished by simultaneous ETA/ETB receptor blockade, while ROS scavenging had no effect. In conclusion, the pressor response to HBOC-201 is mediated by vasoconstriction due to NO scavenging and production of ET. Further research should explore the effect of longer-acting ET receptor blockers to counteract the side effect of hemoglobin-based oxygen carriers.

INTRODUCTION

Hemoglobin-based oxygen carrier (HBOC)-201 is a cell-and endotoxin-free, glutaraldehyde-polymerized hemoglobin solution produced by chemical modification of hemoglobin extracted from isolated bovine red blood cells [25]. HBOCs may be used in the treatment of cardiovascular disorders, and hemorrhagic shock, in particular; however, side effects include systemic and pulmonary blood pressure elevations, plasma volume expansion, lower cardiac output and reduction in heart rate [24, 39, 41, 53]. Despite these potentially unfavorable effects, studies in human subjects with documented coronary disease showed that HBOC-201 had no effect on left ventricular (LV) stroke work index or any of the measured coronary function parameters [53].

The most important HBOC-201 side effect is systemic and pulmonary vasoconstriction [24, 49]. Consequently, this study first aimed to determine the possibility of reversing the HBOC- 201 pressor effects via simultaneous administration of adeno- sine (ADO), a nitric oxide (NO)-independent vasodilator, or the NO-donor nitroglycerin (NTG). The pressor effect of HBOC-201 has been ascribed to scavenging of NO, an important endogenous vascular relaxing factor [5, 6, 18, 44, 45, 60]. Free hemoglobin (Hb) undergoes rapid ($\sim 10^7$ $M^{-1} \cdot s^{-1}$) [19, 23] and irreversible reaction with NO to form metHb, where Hb kinetically behaves as a dioxygenase enzyme [1, 12]. In the following slower processes, iron-NO complexes are formed that may further deplete NO concentrations [1]. However, although disruption of the NO-mediated cascade may be an important contributor to transient systemic and pulmonary hypertension, it is not the only possible pathway.

Oversupplying oxygen (O_2) can stimulate vasoconstriction, to protect against the oxygen burst, but can also stimulate reactive oxygen species (ROS) formation that may result in further scavenging of NO [33, 61]. By scavenging NO, conversion from pro-endothelin to endothelin (ET) is no longer inhibited, thereby increasing the release of this vasoconstrictor [3, 28, 52]. Also, free radicals generated by the auto-oxidation of hemoglobin may contribute to the enhanced release of ET [59]. Therefore, the second aim of this study was to address the potential roles of NO scavenging, ROS, and/or endothelin in the HBOC-201 systemic and pulmonary pressor effects NO has been shown to contribute to exercise-induced vasodilation in skeletal muscle, the heart, as well as the pulmonary vasculature in many [9, 22, 51] but not all studies [13]. A state of decreased NO and increased ROS and ET production resembles some aspects of endothelial dysfunction, a phenomenon that

may have exaggerated effects during exercise. Hence, the third aim of this study compared the effects of HBOC-201 in resting and exercising swine.

METHODS

Animals

Studies were performed in accordance with the Council of Europe Convention (ETS123)/Directive (86/609/EEC) for the protection of vertebrate animals used for experimental and other scientific purposes and with approval of the Animal Care Committee at Erasmus MC, University Medical Center Rotterdam. A total of 16 Yorkshire X Landrace swine (2–3 mo old, 22 ± 1 kg at the time of surgery) of either sex (11 female and 5 male) entered the study. After completing all experimental protocols, animals were euthanized by an intravenous overdose of pentobarbital sodium.

Surgical procedures

Detailed surgical procedures have been previously described [8, 37]. In brief, under deep anesthesia, a thoracotomy was performed in the fourth left intercostal space. Fluid-filled catheters were placed in the aorta, pulmonary artery, left atrium, and LV for measurement of pressure, infusion of drugs and blood sampling. In addition, a flow probe (Transonic Systems) was placed around the ascending aorta for measurement of cardiac output. All catheters were exteriorized at the back of the animal and filled with heparinized saline. The thorax was closed in layers, and the animal was allowed to recover for at least one week. Antibiotic prophylaxis (amoxicillin, 25 mg/kg iv) was provided for 5–7 days starting immediately before surgery. Immediate postoperative analgesia was provided by buprenorphine (0.015 mg/kg im), while a slow-release fentanyl patch (12 µg/h) maintained postoperative analgesia for 72 h. Studies were performed 1–3 wk after surgery, with animals resting and exercising on a motor-driven treadmill up to 85–90% of maximal heart rate. Three main protocols (as described below) were performed on different days and in random order. All chemicals were obtained from Sigma, and HBOC-201 (13 g/dl) was obtained from OPK Biotech.

Experimental Protocols

Hemodynamic effects and reproducibility of HBOC-201 infusion

With swine lying on the treadmill, resting hemodynamic measurements consisting of heart rate (HR), LV pressure, first derivative of LV pressure (dP/d*t*), mean aortic pressure (MAP), pulmonary artery pressure (PAP), left atrial pressure, and cardiac output were obtained. Subsequently, swine were subjected to a five-stage exercise protocol (1–5 km/h) while hemodynamic variables were continuously recorded, and blood samples were collected during the last 60 s of each 3-min exercise stage at a time when hemodynamics had reached a steady state. Blood samples were used for determination of Hb, oxygen content, and lactate using an automated blood gas analyzer (ABL800; Radiometer). After the exercise protocol was completed, animals were allowed to rest on the treadmill for 90 min, after which HBOC-201 (10 ml/kg iv) was infused over a period of 30 min. At the end of infusion, the exercise protocol was repeated. We have previously shown excellent reproducibility of the hemodynamic response in consecutive bouts of exercise [9].

Hemodynamic effects and reproducibility of HBOC-201 infusion

With swine lying quietly on the treadmill, resting hemodynamic measurements consisting of heart rate (HR), LV pressure, first derivative of LV pressure (dP/d*t*), mean aortic pressure (MAP), left atrial pressure, aortic and coronary blood flow (CBF) were obtained. Hemodynamic measurements were repeated and rectal temperature was measured with animals standing on the treadmill. Subsequently, swine were subjected to a five-stage progressive exercise protocol (1–5 km/h) while hemodynamic variables were continuously recorded and blood samples collected during the last 60 s of each 3 min exercise stage at a time when hemodynamics had reached a steady state. After the exercise protocol was completed, animals were allowed to rest on the treadmill for 90 min, after which HBOC-201 (10 ml/kg iv) was infused over a period of 30 min. At the end of infusion, the exercise protocol was repeated. We have previously shown excellent reproducibility of the hemodynamic response in consecutive bouts of exercise [16,17].

Also, in three pigs, we assessed the reproducibility of the hemodynamic responses to HBOC-201 infusion by administration of three separate doses of HBOC-201 (10 ml/kg iv), separated by 5 ± 1 days.

Reversal of pressor effect of HBOC-201 by adenosine and NO.

After performing a control run, six animals received HBOC-201 combined with the vasodilator ADO. Administration of ADO was started 10 min after the start of HBOC-201 infusion and continued till the end of the second run. The infusion rate of ADO (25 mg/ml) was titrated to obtain a stable MAP similar to that before HBOC administration.
To determine the involvement of NO in HBOC-201- induced hypertension, in six swine, the NO donor nitroglycerin (NTG) was infused starting 10 min after the start of HBOC-201 infusion. To prevent a direct interaction between the NO donor and HBOC-201, HBOC-201 and NTG were infused through separate catheters. The infusion rate of NTG (1 mg/ml) was titrated to obtain a stable MAP similar to that before HBOC administration.

To further investigate the role of endogenous NO, NO production was inhibited using the NO synthase inhibitor N^{W}-nitro-L-arginine (L-NNA, 20 mg/kg iv) in five swine [9]. After administration of L-NNA, swine underwent an L-NNA exercise trial. Ninety minutes later, HBOC-201 (10 ml/kg iv) was given to the animals, and they underwent a second exercise trial. As previously shown [57], L-NNA has a long-lasting effect so no additional L-NNA was administered before the second exercise protocol.

Other Vasoactive pathways

Loss of NO reduces ROS scavenging and may increase the production of the potent vasoconstrictor ET. To determine the involvement of ET and ROS in HBOC-201-induced hypertension, HBOC-201 was infused after prior administration of an ET-receptor antagonist or a cocktail of ROS scavengers.

ET receptor blockade

After completing a control exercise proto- col, animals were allowed to rest on the treadmill for 90 min. Then, the mixed ET_A and ET_B receptor (ET_A/ET_B) antagonist tezosentan was intravenously administered over 10 min in a dose of 3 mg/kg iv (slow bolus), followed by a continuous infusion of 6 $mg \cdot kg^{-1} \cdot h^{-1}$ iv in four swine (38). HBOC-201 (10 ml/kg iv) was started upon completion of the tezosentan slow bolus. When HBOC-201 infusion was completed, the exercise protocol was repeated.

ROS scavengers

We used a mixture of different substances to scavenge all ROS during HBOC administration. The mixture consisted of *N*-acetylcysteine (NAC; 150 mg/kg iv), 4-hydroxy-2,2,6,6- tetramethylpiperidin-1-oxyl (Tempol; 30 mg/kg iv), and mercaptopropionyl glycine (MPG; 1 mg/kg iv) [26]. NAC is an aminothiol and

synthetic precursor of intracellular cysteine and glutathione and is thus considered an important antioxidant (6). It is generally assumed that the antioxidant and free radical scavenging activities of NAC are attributable to in- creasing intracellular glutathione levels; however, NAC also possesses a reducing property through its thiol-disulfide exchange activity [2, 26]. Tempol is a stable piperidine nitroxide and scavenges superoxide anions in vitro and may act as an SOD mimetic [26]. Tempol also reduces the formation of hydroxyl radicals either by scavenging superoxide anions or hydroxyl radicals (via the Fenton or Haber-Weiss reactions) (26). *N*-2-mercap- topropionylglycine (MPG) is a synthetic thiol compound that is not highly radical specific and scavenges different types of ROS, including $O_2^{\bullet-}$, $ONOO^-$ and $^{\bullet}OH$ [2, 26, 57]. After completing a control exercise protocol, the animals were allowed to rest on the treadmill for 90 min. Then, the scavenger mixture was administered in five swine, starting 10 min before the HBOC-201 infusion. The administration of NAC and Tempol was completed before administration of HBOC-201, while MPG infusion continued throughout HBOC-201 administration and the subsequent exercise protocol.

Data and statistical analysis

Digital recording and offline analysis of hemodynamic variables have been described in detail elsewhere (10, 11, 38). Systemic vascular resistance (SVR) was computed as mean aortic blood pressure divided by cardiac output. Pulmonary vascular resistance (PVR) was computed as mean pulmonary arterial pressure minus mean left atrial pressure divided by cardiac output. Body lactate production/consumption was calculated as the product of cardiac output and arterio-mixed venous lactate difference. Statistical analysis of hemodynamic data was performed with SPSS 22 [IBM (Armonk, NY) released 2013. IBM SPSS Statistics for Windows, Version 22.0]. Since no differences between male and female swine were found in the response to HBOC-201 administration alone, data from both sexes were pooled. The effects of drug treatment and exercise were compared using a two-way ANOVA for repeated measures. When significant effects were detected, post hoc testing was performed using paired or unpaired *t*-test, with Bonferroni correction. Statistical significance was accepted when $P < 0.05$. Data are presented as means ± SE.

RESULTS

Hemodynamic Effects and Reproducibility of HBOC-201 administration

Administration of HBOC resulted in significant pressor effects in the systemic and pulmonary circulations with an increase in MAP (27 ± 3 mmHg) and PAP (14 ± 1 mmHg). These pressor responses were accompanied by a probable baroreflex-mediated decrease in HR, which together with a slight decrease in stroke volume, resulted in a decrease in cardiac output (Table 1). These pressor effects were the result of significant systemic and pulmonary vasoconstriction, as evidenced by significant increases in SVR and PVR. There was no sign of anaerobic metabolism, as arterial and mixed venous lactate levels (Table 2) and body lactate consumption (not shown) were maintained. The hemodynamic responses to HBOC-201 administration occurred during the first 10 min of HBOC-201 administration after which they stabilized. More- over, a second and third HBOC administration with 5–7 days washout in between yielded hemodynamic responses similar to the first administration (Figure 1).

The pressor response to HBOC-201 was maintained during exercise (Figure 1). In the systemic circulation, both MAP and SVR remained elevated throughout the exercise protocol as compared with control, although the elevation of SVR tended to wane with incremental levels of exercise. Similarly, in the pulmonary circulation both PAP and PVR remained elevated at all exercise intensities.

Reversal of pressor effect of HBOC-201 by adenosine and NO in the systemic and pulmonary vasculature

Co-infusion of ADO was carefully titrated to maintain MAP at a level similar to MAP before HBOC-201 infusion (Figure 1). Dosages required to stabilize MAP fluctuated throughout the experiment, but they were on average 0.17 ± 0.01 $mg \cdot kg^{-1} \cdot min^{-1}$ (range between 0.08 and 0.38 $mg \cdot kg^{-1} \cdot min^{-1}$). Although the HBOC-201-induced changes in SVR and PVR were abolished by ADO (Figure 1), PAP tended to remain slightly higher ($P = 0.08$) due to a slight increase in left atrial pressure (not shown). MAP increased by ~15 mmHg upon cessation of ADO infusion (not shown).

	Treatment	Exercise level (km/h)					
		$Rest_{Lying}$	1	2	3	4	5
Systemic hemodynamics							
HR (bpm)	Control	124 ± 5	170 ±9*	177 ± 9*	188 ± 8*	218± 10*	244 ±10*
	HBOC-201	100 ± 3†	138 ± 5*†	148 ± 6*†	161 ± 5*†	192 ± 7*	221 ± 8*
MAP (mmHg)	Control	89 ± 3	83 ± 3	83 ± 3	83 ± 2	84 ± 2	87 ± 3
	HBOC-201	113 ± 3†	105 ± 3†	105 ± 2*†	103 ± 2*†	104 ± 2*†	104 ± 2*†
SV (ml/beat)	Control	38 ± 2	43 ± 2*	43 ± 2	43 ± 2	40 ± 2	39 ± 2
	HBOC-201	42 ± 2	46 ± 2	44 ± 2	44 ± 2	40 ± 2	40 ± 2
CO (l/min)	Control	4.7 ± 0.2	7.4 ± 0.3*	7.6 ± 0.3*	8.1 ±0.2*	8.8 ± 0.3*	9.8 ± 0.3*
	HBOC-201	4.3 ± 0.2	6.4 ± 0.2*†	6.7±0.2*†	7.2±0.2*†	8.2 ± 0.2*	9.0 ± 0.3*
SVR ($mmHg.l^{-1}.min$)	Control	19.3±0.08	11.3 ± 0.3*	11.0 ± 0.4*	10.3±0.4*	9.7 ± 0.4*	9.0 ± 0.4*
	HBOC-201	26.6±1.3†	16.7±0.6*†	16.1±0.6*†	14.6±0.6*†	12.8±0.5*†	11.6±0.5*†

	Treatment	Exercise level (km/h)					
		$Rest_{Lying}$	1	2	3	4	5
Pulmonary hemodynamics							
MPAP (mmHg)	Control	14 ± 1	21 ± 2*	21 ± 1*	23 ± 1*	28 ± 1*	31 ± 1*
	HBOC-201	26 ± 2†	32 ± 2†	34 ± 3*†	35 ± 2*†	39 ± 2*†	42 ± 2*†
LAP (mmHg)	Control	1.5 ± 1.0	2.4 ± 1.0	3.3 ± 0.6	4.9±0.6*	7.7 ± 1.0*	8.3 ± 1.0*
	HBOC-201	9.8±0.7†	7.7 ± 1.1†	7.0 ± 1.0†	8.1±1.1†	9.0 ± 0.7	9.6 ± 1.0
PVR ($mmHg.l^{-1}.min$)	Control	2.7 ± 0.2	2.5 ± 0.2	2.4 ± 0.2	2.3 ± 0.2	2.3 ± 0.2	2.4 ± 0.2
	HBOC-201	3.8±0.4†	4.1 ± 0.4†	4.3 ± 0.5†	4.1 ± 0.4†	4.0 ± 0.4†	3.8 ± 0.4†

Table 1. Hemodynamic effects of HBOC-201 at rest and during exercise. Data are means ± SE. HR, heart rate; MAP, mean arterial pressure; SV, stroke volume; CO, cardiac output; SVR, systemic vascular resistance; MPAP, mean pulmonary artery pressure; LAP, left atrial pressure; PVR, pulmonary vascular resistance; HBOC-201, hemoglobin-based oxygen carrier 201. *$P < 0.05$ vs. $Rest_{Lying}$. †$P < 0.05$ vs. control.

The exogenous administration of NO, by co-infusion of the NO-donor NTG, was also titrated to counteract systemic pressor responses to HBOC-201. The dose of NTG required to stabilize MAP increased from 0.11 ± 0.01 $mg \cdot kg^{-1} \cdot min^{-1}$ at 20 min of HBOC-201 infusion to 0.22 ± 0.06 $mg \cdot kg^{-1} \cdot min^{-1}$ upon completion of HBOC-201 infusion (P = 0.05) and remained essentially unchanged during the exercise protocol, being 0.16 $mg \cdot kg^{-1} \cdot min^{-1}$ at maximal exercise (range from 0.06 to 0.49 $mg \cdot kg^{-1} \cdot min^{-1}$). This dose of NTG negated the HBOC-201-induced increase in SVR as well as PVR and, thereby, the elevated pressures in these vascular beds (Figure 2). Similar to ADO, MAP increased upon cessation of the NTG-infusion (not shown).

Endothelial NOS (eNOS) blockade with L-NNA resulted in peripheral vasoconstriction, as evidenced by a significant increase in SVR and an increase in MAP. The increase in MAP was accompanied by increases in LV systolic pressure, as well as left atrial pressure, and probably, by a baroreflex-mediated decrease in HR and cardiac output, as stroke volume was not altered (Figure 2). However, subsequent infusion of HBOC-201 did not result in a further increase in MAP or SVR. In contrast to the findings in the systemic circulation, HBOC-201 induced an increase in mean PAP and PVR even in the presence of L-NNA (Figure 2). Thus HBOC-201 induced further pulmonary vasoconstriction following the vasoconstriction produced by L-NNA, both at rest and during exercise, suggesting that, in addition to scavenging of NO, HBOC-201 exerts its vasoconstrictor effect through another pathway in the pulmonary circulation. Of note, when HBOC-201 and L-NNA were co-infused, systemic pressor responses appeared to be increased, as compared with the effect of HBOC-201 alone (Figure 2), indicating that not all NO is scavenged by HBOC-201.

The exogenous administration of NO, by co-infusion of the NO-donor NTG, was also titrated to counteract systemic pressor responses to HBOC-201. The dose of NTG required to stabilize MAP increased from 0.11 ± 0.01 $mg \cdot kg^{-1}$ min^{-1} at 20 min of HBOC-201 infusion to 0.22 ± 0.06 $mg \cdot kg^{-1}$ min^{-1} upon completion of HBOC-201 infusion ($P \leq 0.05$) and remained essentially unchanged during the exercise protocol, being 0.16 $mg \cdot kg^{-1} \cdot min^{-1}$ at maximal exercise (range from 0.06 to 0.49 $mg \cdot kg^{-1} \cdot min^{-1}$). This dose of NTG negated the HBOC-201-induced increase in SVR as well as PVR and, thereby, the elevated pressures in these vascular beds (Figure 2). Similar to ADO, MAP increased upon cessation of the NTG-infusion (not shown).

	Treatment	Exercise level (km/h)					
		$Rest_{Lying}$	1	2	3	4	5
Arterial							
Hemoglobin	Control	8.4 ± 0.2	8.7 ± 0.2*	8.8 ± 0.2	8.9 ± 0.2	9.0 ± 0.2	9.3 ±0.2*
(g%)	HBOC-201	9.0 ± 0.2	9.3 ± 0.1†	9.5 ± 0.2†*	9.6 ± 0.2*†	10.1 ± 0.2†*	10.4 ± 0.2†*
Met-hemoglobin	Control	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
(%)	HBOC-201	1.1 ± 0.1†	1.2 ± 0.1†	1.2 ± 0.1†	1.2 ± 0.1†	1.2 ± 0.1†	1.1 ± 0.1†
Plasma hemoglobin	Control	0.002 ± 0.01	0.001± 0.01	0.002 ± 0.01	0.002 ± 0.01	0.002± 0.01	0.002±0.01
(g%)	HBOC-201	2.06±0.05†	2.08±0.04†	2.04±0.05†	2.03±0.05†	2.03±0.05†	2.05±0.05†
Sa_{O2}	Control	97 ± 1	95 ± 1	98 ± 1	98 ± 1	96 ± 1	94 ± 1*
(%)	HBOC-201	91 ± 1	90 ± 1†	90 ± 1†	90 ± 1†	90 ± 1†	90 ± 1†
O_2 Hb	Control	96 ± 1	94 ± 1	95 ± 1	95 ± 1	95 ± 1	94 ± 1
(%)	HBOC-201	89 ± 1†	88 ± 1†	89 ± 1†	89 ± 1†	89 ± 1†	88 ± 1†
PO_2	Control	102 ± 2	94 ± 3*	97 ± 2	96 ± 3	94 ± 2	90 ± 3*
(mmHg)	HBOC-201	102 ± 2	94 ± 3*	98 ± 3	94 ± 3	92 ± 3*	90 ± 3*
PCO_2	Control	42 ± 1	41 ± 1	40 ± 1	40 ± 1	39 ± 1	38 ± 1*
(mmHg)	HBOC-201	43 ± 1	41 ± 1	41 ± 1	43 ± 1	39 ± 1*	38 ± 1*

	Treatment	Exercise level (km/h)					
		$Rest_{Lying}$	1	2	3	4	5
pH	Control	7.44 ± 0.01	7.46 ± 0.01	7.47 ± 0.01*	7.47 ± 0.01*	7.48 ± 0.01*	7.48 ± 0.01*
(mmHg)	HBOC-201	7.45 ± 0.01	7.46 ± 0.01	7.46 ± 0.01	7.46 ± 0.01	7.47 ± 0.01*	7.47 ± 0.01
Lactate	Control	1.1 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	1.3 ± 0.1	1.9 ± 0.2*
(mmol/l)	HBOC-201	1.0 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	0.9 ± 0.1	1.3 ± 0.1	2.2 ± 0.4*
Mixed venous							
SaO_2	Control	50 ± 1	39 ± 1*	37 ± 1*	37 ± 1*	33 ± 1*	26 ± 2*
(%)	HBOC-201	39 ± 2†	30 ± 2*†	29 ± 2*†	29 ± 2*†	25 ± 2*†	22 ± 2
PO_2	Control	42 ± 1	37 ± 1	36 ± 1*	36 ± 1*	33 ± 0.5	31 ± 1*
(mmHg)	HBOC-201	38 ± 1†	33 ± 1†	33 ± 1*†	33 ± 1*†	30 ± 1†	28 ± 1
PCO_2	Control	51 ± 1	50 ± 2	52 ± 1	51 ± 1	51 ± 1	51 ± 1
(mmHg)	HBOC-201	53 ± 1	54 ± 1	54 ± 1	52 ± 1	53 ± 2	52 ± 2
pH	Control	7.36 ± 0.01	7.36 ± 0.01	7.38 ± 0.01	7.37 ± 0.01	7.38 ± 0.01	7.32 ± 0.01
(mmHg)	HBOC-201	7.35 ± 0.01	7.35 ± 0.01	7.35 ± 0.01	7.35 ± 0.01	7.35 ± 0.01	7.34 ± 0.01
Lactate	Control	1.0 ± 0.1	0.8 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	1.1 ± 0.1	1.6 ± 0.2*
(mmol/l)	HBOC-201	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	1.1 ± 0.1	1.9 ± 0.4*

Table 2. Effects of HBOC-201 on blood gas values at rest and during exercise. Data are means ± SE; *n* = 11. SaO2, oxygen saturation; O_2 Hb, fraction of oxyhemoglobin in total hemoglobin; PO_2, O_2 tension; PCO_2, CO_2 tension. *$P \leq 0.05$ vs. Rest. †$P \leq 0.05$ vs. control.

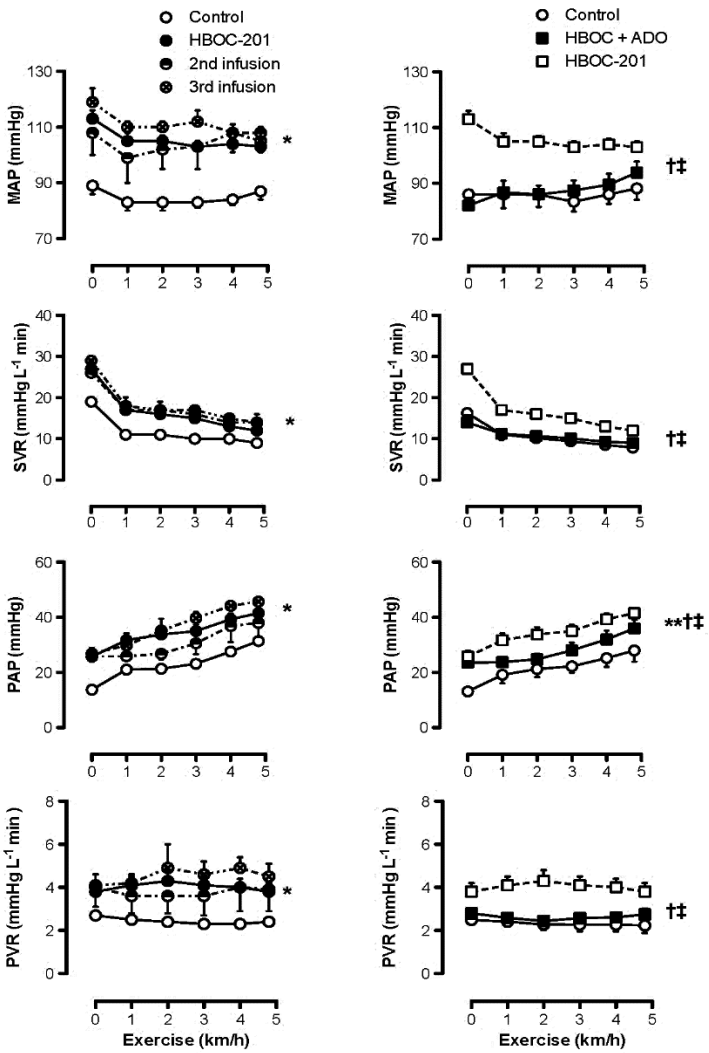


Figure. 1. Systemic and pulmonary hemodynamics at rest and during exercise following administration of hemoglobin-based oxygen carrier (HBOC)-201 alone (*n* = 3 pigs, *left*), demonstrating reproducibility of 3 separate administrations of HBOC-201, and in combination with infusion of adenosine (ADO) (*n* = 6 pigs in full crossover study design, *right*). MAP, mean arterial pressure; SVR, systemic vascular resistance; PAP, pulmonary artery pressure; PVR, pulmonary vascular resistance. **P* ≤ 0.05, ***P* ≤ 0.10, compared with control; †*P* ≤ 0.05, compared with HBOC-201 alone; ‡*P* ≤ 0.05, effect of HBOC Ado different from HBOC-201 alone.

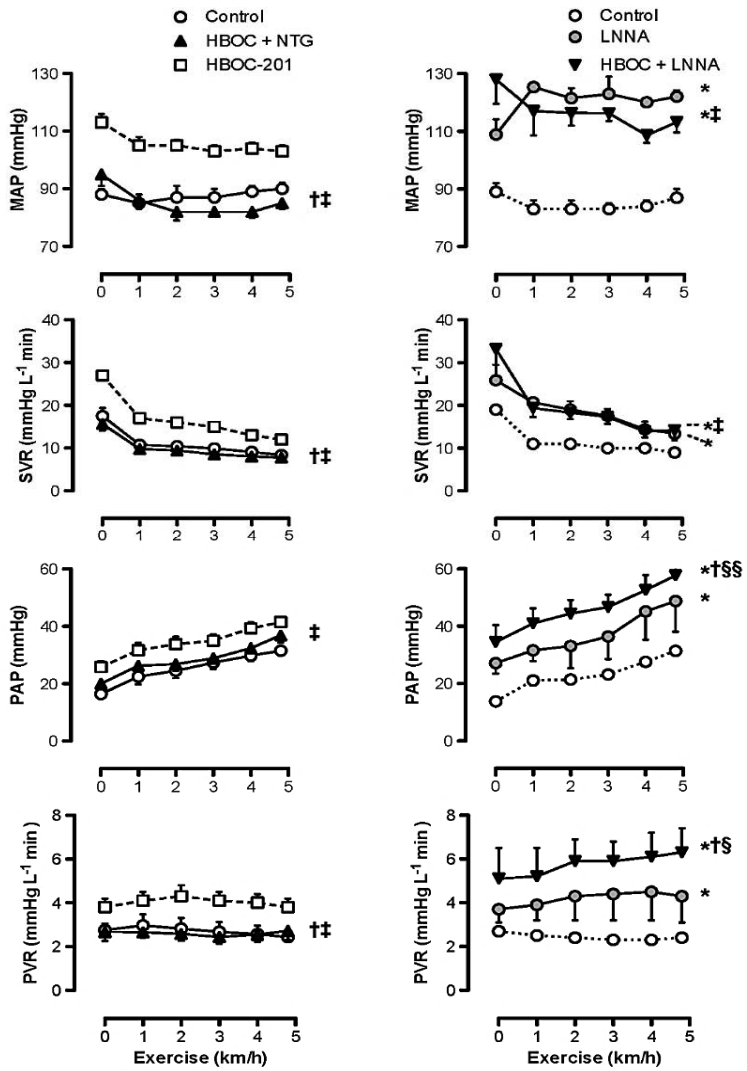


Figure 2. Systemic and pulmonary hemodynamics at rest and during exercise following administration of the nitric oxide (NO)-donor nitroglycerin (NTG; *left*) or the eNOS inhibitor *N*_-nitro-L-arginine (L-NNA; *right*) in combination with HBOC-201. **P* ≤ 0.05, compared with control; †*P* ≤ 0.05, compared with HBOC-201 alone; ‡*P* ≤ 0.05, effect of HBOC NTG different from HBOC-201 alone; §*P* ≤ 0.05, §§ *P* ≤ 0.1 compared with L-NNA alone; *n* = 6 pigs (NTG) or 5 pigs (L-NNA) in a full crossover study design. For the sake of clarity, statistics comparing HBOC-201 with control are not shown, but they are identical to Figure 1.

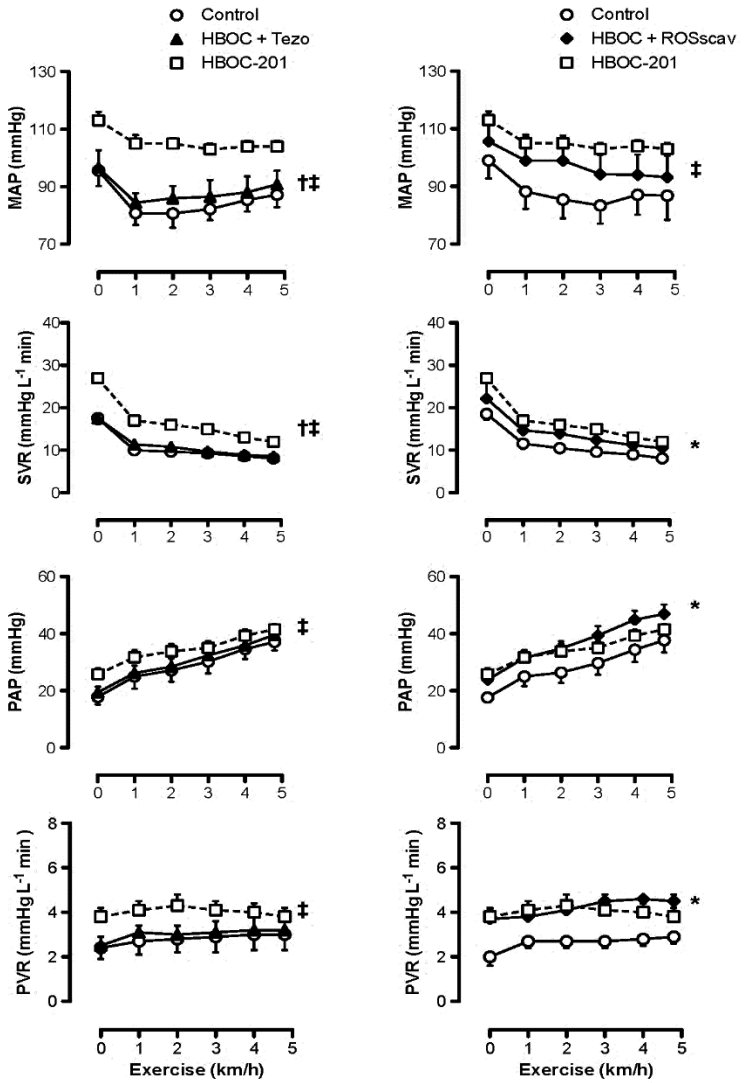


Figure 3. Systemic and pulmonary hemodynamics at rest and during exercise following administration of the endothelin ET_A/ET_B-receptor blocker Tezosentan (*n* = 4 pigs in full crossover study design; *left*) **or a reactive oxygen species (ROS) scavenger cocktail** comprised of *N*-acetylcysteine (NAC), Tempol, and mercaptopropionyl glycine (MPG; *n* = 5 pigs in full crossover study design; *right*) in combination with HBOC-201. **P* ≤ 0.05, compared with control; †*P* ≤ 0.05, compared with HBOC-201 alone; ‡*P* ≤ 0.05, effect of HBOC Tezo or ROS different from HBOC-201 alone. For the sake of clarity, statistics comparing HBOC-201 with control are not shown, but are identical to Figure 1.

Other vasoactive pathways

Administration of the mixed ETA and ETB receptor antagonist tezosentan reduced MAP by 10 ± 4 mmHg ($P \leq 0.05$), and negated the systemic hypertension caused by subsequent HBOC-201 by preventing the increase in SVR (Figure 3). Also, in the pulmonary circulation, HBOC-201 had no effect on either pulmonary pressure or PVR, in the presence of tezosentan (Figure 3). These data suggest that activation of the endothelin system is an important contributor to the vasoconstrictor response to HBOC-201.

ROS scavenging in itself had no significant effect on MAP (Δ MAP 12 ± 8 mmHg, $P=0.21$). Co-infusion of ROS scavengers with HBOC-201 slightly reduced the effect of HBOC-201 on mean arterial pressure but did not significantly affect SVR (Figure 3). Similarly, in the pulmonary vascular bed, no reduction of pressor effects could be detected at rest, and the effects of HBOC-201 tended to be exacerbated during exercise following administration of the ROS scavenger cocktail (Figure 3).

DISCUSSION

In the present study we report, in accordance with previous publications [40, 46, 60, 63–65], that intravenous administration of HBOC-201 resulted in systemic and pulmonary hypertension as a result of vasoconstriction, which was maintained during exercise. Pressor responses could be prevented by co-infusion of NTG or ADO both at rest and during exercise; however, this required careful titration of the dosage of these vasodilators. eNOS inhibition prevented HBOC-201-induced increase in systemic vasoconstriction, and it reduced but did not abolish HBOC-201-induced pulmonary vasoconstriction. ETA/ETB blockade with tezosentan prevented the HBOC-201-induced pressor responses in the systemic and pulmonary vasculature, while ROS-scavenging tended to blunt the pressor response in the systemic but not pulmonary vasculature.

Abolishing pressor effects

The main hemodynamic effects of HBOC-201 occurred during the first 10 min of its administration and were maintained throughout the entire infusion and after infusion. Repeated administration of HBOC-201, following complete washout, induced virtually identical effects, corroborating results from ECMO priming with HBOC-201 in piglets [21, 62] and indicating that no immune reaction occurred in response to the protein and that repeated administration is safe. Also, plasma

clearance of HBOC-201, which has been shown to follow first-order pharmacokinetics with an elimination half time of 20 h, for either single or multiple dosage regimens [25], was comparable with previous studies [25, 34]. As anticipated, HBOC-201 produced an increase in arterial Hb, met-Hb, and plasma metHb (Table 2); however, the level of metHb in this study was well below toxic levels [32], and co-infusion of NTG with HBOC did not elevate metHb levels further (not shown).

The pressor effect of HBOC has been ascribed to the scavenging of NO, primarily by plasma ferrous heme, thereby lowering NO concentration [63]. Our results support previous findings that NTG is capable of negating HBOC-201-induced vasoconstriction and the accompanying increases in systemic and pulmonary blood pressures [27]. NTG reduces vascular resistance in small and large vessels through endothelium independent, but NO-mediated, vasodilation [66]. However, earlier studies were skeptical using NTG co-administration as a therapeutic option to negate the pressor effect of HBOC due to its short half-life, requiring continuous infusion. Moreover, profound systemic vasodilation and hypotension might occur in response to NTG, potentially jeopardizing resuscitation from hemorrhagic shock [27, 31]. In the present study, we avoided NTG-induced hypotension through careful NTG titration to maintain MAP within physiological limits. Importantly, the NTG dosage required to normalize systemic pressures was also capable of normalizing pulmonary pressures. NTG was compared with ADO, a purine nucleoside and principal NO-independent vasodilator [14, 37]. At ADO infusion rates that eliminated HBOC-induced systemic hypertension, the pulmonary pressor effect was not fully eliminated, despite restoration of normal PVR. The persistence of the pulmonary pressor effect was likely due to elevated left atrial pressure, secondary to adenosine-induced negative cardiac inotropy [20].

Indirect oxidation of hemoglobin involves a process of co-oxidation in which the methemoglobin-forming agent is co-oxidized with heme iron by hemoglobin-bound oxygen (HbO_2) [4]. $O_2^{\bullet -}$ and H_2O_2 are produced when HbO2 accepts electrons from ferrous heme and the methemoglobin-forming agent. However, ROS scavenging only marginally influenced the pressor response to HBOC-201 in the systemic vasculature, while the pulmonary pressor response was unaffected, suggesting that ROS do not play a major role in the pressor effect of HBOC-201. Alternatively, it is possible that oxidative stress indirectly modulates vascular tone. Indeed, it has been shown that oxidative stress enhances ET production through stabilization of prepro-endothelin mRNA [15, 35, 56]. In the present study, the ET-receptor blocker

tezosentan was capable of negating the pressor responses of HBOC-201 in both the systemic and pulmonary vasculature. Therefore, it is plausible that pressor effects of HBOC-201 may result from disinhibition of endothelin synthesis and release [17, 47]. ET is a potent and long-lasting vasoconstrictor and ET-receptor blockade could, therefore, potentially provide another strategy to oppose HBOC-201-induced vasoconstriction. Although tezosentan is a relatively short-acting receptor blocker with a half-life of 3 h [7], longer acting ET-receptor blockers are available. ET-receptor blockade would require less patient monitoring and have a lower risk of inducing hypotension.

Effects of HBOC-201 during exercise

To our knowledge, this is the first study to analyze the effect of a cell-free oxygen carrier on exercise hyperemia. In the normal healthy vasculature, exercise-induced vasodilation is regulated via an intricate interplay of vasoactive molecules, including NO, ROS, and ET [9, 22, 30]. As outlined above, HBOC-201 could potentially scavenge NO and enhance production of ROS and ET and could, therefore, interfere with exercise-induced vasodilation. However, although the pressor responses of HBOC-201 were essentially unaffected by exercise, SVR did decrease during exercise following administration of HBOC-201, indicating that exercise-induced vasodilation is essentially intact. To assess the role of NO in HBOC-201 pressor effects at rest and during exercise, HBOC administration was repeated following eNOS inhibition. If indeed, scavenging of NO is the main contributor to the pressor effect of HBOC-201, eNOS inhibition would be expected to block a further pressor effect by HBOC-201. Indeed, in accordance with previous studies [50, 64], following inhibition of eNOS and the consequent vasoconstriction, no additional vasoconstriction was induced by HBOC-201 infusion in the systemic vasculature. In contrast, HBOC-201 did result in a further increase in PVR. It is not clear why the pulmonary and systemic vasculature responded differently to the combination of L-NNA and HBOC-201. However, it is possible that L-NNA did not completely inhibit eNOS in the pulmonary circulation, although this is unlikely given the high dose (20 mg/kg iv) of L-NNA administered.

An alternative explanation for the divergent effects in the systemic and pulmonary vasculature is that it has been shown that the nature of the chemical interaction between NO and Hb is dependent on the amount of oxygen present. Formation of FeIINOHb occurs principally when Hb is deoxygenated (T-state) in peripheral tissue. NO bound to the heme-group can be transferred to a specific cysteine residue (β93Cys) upon re-oxygenation of Hb in the lung, resulting in formation of SNO-Hb [54]. This SNO-Hb formation can also occur directly but only when Hb is oxygenated

(R-state). From SNO-Hb, NO can be either released or transferred to another thiol group, thereby preserving part of NO signaling [36, 55]. Thus *S*-nitrosylation of Hb is governed, in part, by the state of the Hb molecule undergoing an allosteric shift from R to T shift during passage in the circulatory system [29]. These varying degrees of Hb S-nitrosylation at different molecular states (R and T) may explain, at least in part, the different hemodynamic responses to HBOC-201 in the systemic and pulmonary vasculature following NOS inhibition by L-NNA. In peripheral micro vessels, because of low oxygen tension, SNO-Hb levels are low and NO released from SNO-Hb may be consumed by biological targets and catabolic reactions, such as those mediated by GSNO reductase [55]. Consequently, the availability of bioactive NO may be closely coupled to de novo synthesis by NOS that is inhibitable by L-NNA, leaving little residual NO for scavenging by HBOC-201. By contrast, bioactive NO in the form of SNO-Hb is abundant in lungs and well protected inside erythrocytes but, upon release, is susceptible to scavenging by free Hb, manifesting as a further increase in PVR following eNOS inhibition. A third explanation may be that the vasoconstrictor effect of HBOC-201 is not solely mediated through scavenging of NO. A ROS scavenging cocktail failed to appreciably alter hemodynamic responses to HBOC-201 and, unlike either NTG or ADO, failed to restore SVR or PVR to control levels. As glutaraldehyde-polymerized HBOC in itself was shown to exhibit catalase-like properties, it is possible that the increase in free radicals induced by administration of this HBOC was negated by HBOC itself [58]. Indeed, lipid peroxidation as measured by thiobarbituric acid reactive substances was not significantly elevated by glutaraldehyde-polymerized HBOC.Similar to our study, these observations suggest that HBOC-201 fails to significantly stimulate ROS formation or that any HBOC-induced increase in ROS is adequately scavenged by endogenous antioxidants. However, it is possible that in certain disease states generally characterized by elevated oxidative stress [57], such as reperfusion following myocardial infarction or in the presence of severe endothelial dysfunction, HBOC may exacerbate oxidative stress, either directly or through scavenging NO. In vitro studies have suggested that HBOCs may amplify ROS formation that could, in turn, react with NO to generate nitroxide radicals [33] and/or uncouple NO synthase secondary to insufficient cofactors tetrahydrobiopterin (BH4) and NADPH required to convert L-arginine to L-citrulline and NO [43].

The pattern of SVR and PVR during exercise with HBOC-201 very much resembles the pattern found with ET antagonism, as we previously showed that the vasodilator effect of ET-receptor blockade waned with increasing exercise intensity in the systemic circulation while it increased in the pulmonary vasculature [38]. Moreover,

reduced bioavailability of NO and/or oxidative stress could contribute to overexpression of ET [3]. Although we did not measure plasma ET-levels in the present study, an increase in ET-mediated vasoconstriction as a cause of the pressor effects of HBOC-201 is consistent with the ability of tezosentan to negate these pressor effects both at rest and during exercise. Importantly, dosing of the ET-receptor blocker tezosentan did not need to be altered during exercise, and endothelin-antagonists by themselves have only very modest effects on hemodynamics, making endothelin antagonists clinically attractive antagonists of the pressor effect of HBOC-201.

Finally, several clinical trials have shown that other HBOC products with other compositions than HBOC-201 may increase the risk of myocardial infarction and death [42]. However, clinical studies conducted with HBOC-201 in patients with documented cardiovascular disease showed both intravenous and intracoronary infusion of HBOC-201 to be safe and well tolerated [53].

Methodological considerations

Although plasma ET-1 measurements could possibly strengthen the conclusion that HBOC-201-induced vasoconstriction is ET mediated, a number of both physiological and methodological issues complicate interpretation of such measurements. First, ET is released for more than 80% into the abluminal side, while less than 20% is secreted into the lumen side. Hence circulating ET-1 does not reflect the local concentration of ET-1 in the vessel wall. Second, an ET-mediated pressor effect may be caused by changes in ET-receptor sensitivity through altered nitrosylation of the ET-receptors. Finally, we have previously found that L-NNA and indomethacin yield a false-positive result in plasma ET-1 measurements [22]. Similarly, an interaction with HBOC-201 cannot be excluded, making it difficult to interpret the results. Therefore, blocking ET receptors with tezosentan is the best way to assess the interaction of HBOC-201 with the ET system.

CONCLUSION AND FUTURE DIRECTIONS

HBOC-201 can disrupt hemodynamic homeostasis, mimicking some aspects of endothelial dysfunction, resulting in elevated systemic and pulmonary blood pressures. HBOC-201 induced vasoconstriction is mediated by scavenging NO and likely by upregulating ET production. Pressor effects can be restored by NO donors or direct vasodilators, such as nitroglycerin or ADO, but dosages must be carefully monitored to avoid hypotension. However, hemodynamic normalization was more easily achieved via administration of an ET receptor blocker. Future studies should

focus on co-administration of long-acting ETA receptor antagonists (e.g., ambrisentan or sitaxentan) and, although oxygen derived free radicals do not appear to play a significant role in HBOC-201-induced pressor responses of healthy subjects, the possible role of ROS in HBOC-induced vasoconstriction in subjects with documented preexisting endothelial dysfunction would be of interest.

ACKNOWLEDGMENTS

Experiments were performed at the Division of Experimental Cardiology, Department of Cardiology, Thorax center, Erasmus University Medical Center Rotterdam, The Netherlands. The views expressed in this article are those of the authors and do not necessarily reflect the official policy or position of the Department of the Navy, Department of Defense, nor the U.S. Government. P. F. Moon-Massat is a military service member (or an employee or contractor of the U.S. Government). This work was prepared as part of his official duties. Title 17 U.S.C. §105 provides that "Copyright protection under this title is not available for any work of the United States Government." Title 17 U.S.C. § defines a U.S. Government work as work prepared by a military service member or employee of the U.S. Government as part of that person's official duties.

REFERENCES

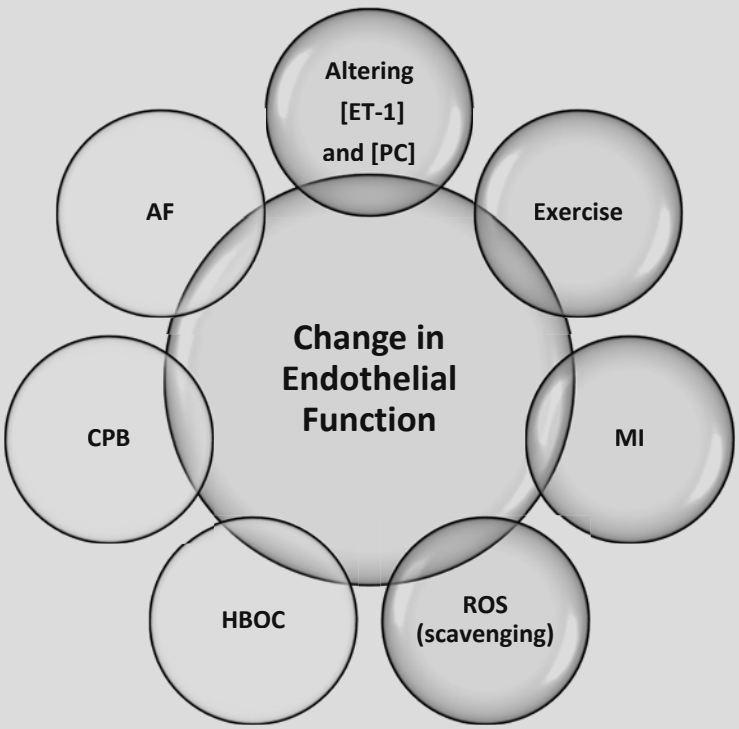
1. Alayash AI. Blood substitutes: why haven't we been more successful? Trends Biotechnol 32: 177–185, 2014.
2. Atmaca G. Antioxidant effects of sulfur-containing amino acids. Yonsei Med J 45: 776–788, 2004. \
3. Biro GP. Adverse HBOC-endothelial dysfunction synergism: a possible contributor to adverse clinical outcomes? Curr Drug Discov Technol 9:194–203, 2012.
4. Bradberry SM, Aw TC, Williams NR, Vale JA. Occupational methaemoglobinaemia. Occup Environ Med 58: 611–615, 2001.
5. Buehler PW, Alayash AI. Toxicities of hemoglobin solutions: in search of in-vitro and in-vivo model systems. Transfusion 44: 1516–1530, 2004.
6. Cabrales P, Friedman JM. HBOC vasoactivity: interplay between nitric oxide scavenging and capacity to generate bioactive nitric oxide species. Antioxid Redox Signal 18: 2284–2297, 2013.
7. Dingemanse J, Clozel M, van Giersbergen PL. Pharmacokinetics and pharmacodynamics of tezosentan, an intravenous dual endothelin receptor antagonist, following chronic infusion in healthy subjects. Br J Clin Pharmacol 53: 355–362, 2002.
8. Duncker DJ, Oei HH, Hu F, Stubenitsky R, Verdouw PD. Role of KATP channels in regulation of systemic, pulmonary, and coronary vasomotor tone in exercising swine. Am J Physiol Heart Circ Physiol 280: H22–H33, 2001.
9. Duncker DJ, Stubenitsky R, Tonino PA, Verdouw PD. Nitric oxide contributes to the regulation of vasomotor tone but does not modulate O2-consumption in exercising swine. Cardiovasc Res 47: 738–748, 2000.
10. Duncker DJ, Stubenitsky R, Verdouw PD. Autonomic control of vasomotion in the porcine coronary circulation during treadmill exercise: evidence for feed-forward beta-adrenergic control. Circ Res 82: 1312–1322, 1998.
11. Duncker DJ, Stubenitsky R, Verdouw PD. Role of adenosine in the regulation of coronary blood flow in swine at rest and during treadmill exercise. Am J Physiol Heart Circ Physiol 275: H1663–H1672, 1998.
12. Eich RF, Li T, Lemon DD, Doherty DH, Curry SR, Aitken JF, Mathews AJ, Johnson KA, Smith RD, Phillips GN Jr, Olson JS. Mechanism of NO-induced oxidation of myoglobin and hemoglobin. Biochemistry 35: 6976–6983, 1996.
13. Frandsenn U, Bangsbo J, Sander M, Höffner L, Betak A, Saltin B, Hellsten Y. Exercise-induced hyperaemia and leg oxygen uptake are not altered during effective inhibition of nitric oxide synthase with N(G)-nitro-L-arginine methyl ester in humans. J Physiol 531: 257–264, 2001.

14. Fuller RW, Maxwell DL, Conradson TB, Dixon CM, Barnes PJ. Circulatory and respiratory effects of infused adenosine in conscious man. Br J Clin Pharmacol 24: 309–317, 1987.
15. Ganesh D, Kumarathasan P, Thomson EM, St-Germain C, Blais E, Crapo J, Vincent R. Impact of superoxide dismutase mimetic AEOL 10150 on the endothelin system of Fischer 344 rats. PLoS One 11:e0151810, 2016.
16. Gow AJ, Stamler JS. Reactions between nitric oxide and haemoglobin under physiological conditions. Nature 391: 169–173, 1998.
17. Gulati A, Sen AP, Sharma AC, Singh G. Role of ET and NO in resuscitative effect of diaspirin cross-linked hemoglobin after hemorrhage in rat. Am J Physiol Heart Circ Physiol 273: H827–H836, 1997.
18. Hai CM. Systems biology of HBOC-induced vasoconstriction. Curr Drug Discov Technol 9: 204–211, 2012.
19. Han TH, Hyduke DR, Vaughn MW, Fukuto JM, Liao JC. Nitric oxide reaction with red blood cells and hemoglobin under heterogeneous conditions. Proc Natl Acad Sci USA 99: 7763–7768, 2002.
20. Headrick JP, Peart JN, Reichelt ME, Haseler LJ. Adenosine and its receptors in the heart: regulation, retaliation and adaptation. Biochim Biophys Acta 1808: 1413–1428, 2011.
21. Henderson CLAC, Anderson CM, Sorrells DL, Wilson BJ, Dick EJ, DiGeronimo RJ. The use of a hemoglobin-based oxygen-carrying solution (HBOC-201) for extracorporeal membrane oxygenation in a porcine model with acute respiratory distress syndrome. Pediatr Crit Care Med 5: 384–390, 2004.
22. Houweling B, Merkus D, Dekker MM, Duncker DJ. Nitric oxide blunts the endothelin-mediated pulmonary vasoconstriction in exercising swine. J Physiol 568: 629–638, 2005.
23. Huang KT, Huang Z, Kim-Shapiro DB. Nitric oxide red blood cell membrane permeability at high and low oxygen tension. Nitric Oxide 16: 209–216, 2007.
24. Irwin DC, Foreman B, Morris K, White M, Sullivan T, Jacobs R, Monnet E, Hackett T, TissotvanPatot MC, Hamilton KL, Gotshall RW. Polymerized bovine hemoglobin decreases oxygen delivery during normoxia and acute hypoxia in the rat. Am J Physiol Heart Circ Physiol 295: H1090–H1099, 2008.
25. Jahr JS, Moallempour M, Lim JC. HBOC-201, hemoglobin glutamer-250 (bovine), Hemopure (Biopure Corporation). Expert Opin Biol Ther 8:1425–1433, 2008.
26. Kappers MH, de Beer VJ, Zhou Z, Danser AH, Sleijfer S, Duncker DJ, van den Meiracker AH, Merkus D. Sunitinib-induced systemic vasoconstriction in swine is endothelin mediated and does not involve nitric oxide or oxidative stress. Hypertension 59: 151–7, 2012.
27. Katz LM, Manning JE, McCurdy S, Sproule C, McGwin G Jr, Moon-Massat P, Cairns CB, Freilich D. Nitroglycerin attenuates vasoconstriction of HBOC-

201 during hemorrhagic shock resuscitation. Resuscitation 81: 481–487, 2010.
28. Kelly LK, Wedgwood S, Steinhorn RH, Black SM. Nitric oxide decreases endothelin-1 secretion through the activation of soluble guanylate cyclase. Am J Physiol Lung Cell Mol Physiol 286: L984–L991, 2004.
29. Kulandavelu S, Balkan W, Hare JM. Regulation of oxygen delivery to the body via hypoxic vasodilation. Proc Natl Acad Sci USA 112: 6254–6255, 2015.
30. Laughlin MH, Davis MJ, Secher NH, van Lieshout JJ, Arce-Esquivel AA, Simmons GH, Bender SB, Padilla J, Bache RJ, Merkus D, Duncker DJ. Peripheral circulation. Compr Physiol 2: 321–447, 2012.
31. Levien LJ, Hodgson RE, James MF. Hemoglobin-based blood substitutes and risk of myocardial infarction and death. JAMA 300: 1295, 2008.
32. Linberg R, Conover CD, Shum KL, Shorr RG. Hemoglobin based oxygen carriers: how much methemoglobin is too much? Artif Cells Blood Substit Immobil Biotechnol 26: 133–148, 1998.
33. Liu L, Hausladen A, Zeng M, Que L, Heitman J, Stamler JS. A metabolic enzyme for S-nitrosothiol conserved from bacteria to humans. Nature 410: 490–494, 2001.
34. Lowe KC. Genomics and blood substitutes for 21st century Europe ("EuroBloodSubstitutes"). Artif Cells Blood Substit Immobil Biotechnol 34: 589–599, 2006.
35. Mawji IA, Robb GB, Tai SC, Marsden PA. Role of the 3'-untranslated region of human endothelin-1 in vascular endothelial cells. Contribution to transcript lability and the cellular heat shock response. J Biol Chem 279: 8655–8667, 2004.
36. McMahon TJ, Moon RE, Luschinger BP, Carraway MS, Stone AE, Stolp BW, Gow AJ, Pawloski JR, Watke P, Singel DJ, Piantadosi CA, Stamler JS. Nitric oxide in the human respiratory cycle. Nat Med 8: 711–717, 2002.
37. Merkus D, Haitsma DB, Fung TY, Assen YJ, Verdouw PD, Duncker DJ. Coronary blood flow regulation in exercising swine involves parallel rather than redundant vasodilator pathways. Am J Physiol Heart Circ Physiol 285: H424–H433, 2003.
38. Merkus D, Houweling B, Mirza A, Boomsma F, van den Meiracker AH, Duncker DJ. Contribution of endothelin and its receptors to the regulation of vascular tone during exercise is different in the systemic, coronary and pulmonary circulation. Cardiovasc Res 59: 745–754, 2003.
39. Mongan PD, Moon-Massat PF, Rentko V, Mihok S, Dragovich A, Sharma P. Regional blood flow after serial normovolemic exchange transfusion with HBOC-201 (Hemopure) in anesthetized swine. J Trauma 67: 51–60, 2009.
40. Moon-Massat P, Scultetus A, Arnaud F, Brown A, Haque A, Saha B, Kim B, Sagini E, McGwin G Jr, Auker C, McCarron R, Freilich D. The effect HBOC-201 and sodium nitrite resuscitation after uncontrolled haemorrhagic shock in swine. Injury 43: 638–647, 2012.

41. Muir WW, Ilangovan G, Zweier JL, Moon-Massat PF, Rentko VT. Vital organ tissue oxygenation after serial normovolemic exchange transfusion with HBOC-201 in anesthetized swine. Shock 35: 597–603, 2011.
42. Natanson C, Kern SJ, Lurie P, Banks SM, Wolfe SM. Cell-free hemoglobin-based blood substitutes and risk of myocardial infarction and death: a meta-analysis. JAMA 299: 2304–2312, 2008.
43. Pacher P, Beckman JS, Liaudet L. Nitric oxide and peroxynitrite in health and disease. Physiol Rev 87: 315–424, 2007.
44. Patel RP. Biochemical aspects of the reaction of hemoglobin and NO: implications for Hb-based blood substitutes. Free Radic Biol Med 28: 1518–1525, 2000.
45. Reiter CD, Wang X, Tanus-Santos JE, Hogg N, Cannon RO 3rd, Schechter AN, Gladwin MT. Cell-free hemoglobin limits nitric oxide bioavailability in sickle-cell disease. Nat Med 8: 1383–1389, 2002.
46. Rodriguez C. Vitturi DA, He J, Vandromme M, Brandon A, Hutchings A, Rue LW 3rd, Kerby JD, Patel RP. Sodium nitrite therapy attenuates hypertensive effects of HBOC-201 via nitrite reduction. Biochem J 422: 423–432, 2010.
47. Rohlfs RJ, Bruner E, Chiu A, Gonzales A, Gonzales ML, Magde D, Magde MD Jr, Vandegriff KD, Winslow RM. Arterial blood pressure responses to cell-free hemoglobin solutions and the reaction with nitric oxide. J Biol Chem 273: 12128–12134, 1998.
48. Rueckschloss U, Quinn MT, Holtz J, Morawietz H. Dose-dependent regulation of NAD(P)H oxidase expression by angiotensin II in human endothelial cells: protective effect of angiotensin II type 1 receptor blockade in patients with coronary artery disease. Arterioscler Thromb Vasc Biol 22: 1845–1851, 2002.
49. Sakai H, Hara H, Yuasa M, Tsai AG, Takeoka S, Tsuchida E, Intaglietta M. Molecular dimensions of Hb-based O2 carriers determine constriction of resistance arteries and hypertension. Am J Physiol Heart Circ Physiol 279: H908–H915, 2000.
50. Sampei K, Ulatowski JA, Asano Y, Kwansa H, Bucci E, Koehler RC. Role of nitric oxide scavenging in vascular response to cell-free hemoglobin transfusion. Am J Physiol Heart Circ Physiol 289: H1191–H1201, 2005.
51. Schrage WG, Joyner MJ, Dinenno FA. Local inhibition of nitric oxide and prostaglandins independently reduces forearm exercise hyperaemia in humans. J Physiol 557: 599–611, 2004.
52. Schultz SC, Grady B, Cole F, Hamilton I, Burhop K, Malcolm DS. A role for endothelin and nitric oxide in the pressor response to diaspirin cross-linked hemoglobin. J Lab Clin Med 122: 301–308, 1993.
53. Serruys PW, Vranckx P, Slagboom T, Regar E, Meliga E, de Winter RJ, Heyndrickx G, Schuler G, van Remortel EA, Dubé GP, Symons J. Haemodynamic effects, safety, and tolerability of haemoglobin-based oxygen carrier-201 in patients undergoing PCI for CAD. EuroIntervention 3: 600–609, 2008.

54. Singel DJ, Stamler JS. Chemical physiology of blood flow regulation by red blood cells: the role of nitric oxide and S-nitrosohemoglobin. Annu Rev Physiol 67: 99–145, 2005.
55. Stamler JS, Jia L, Eu JP, McMahon TJ, Demchenko IT, Bonaventura J, Gernert K, Piantadosi CA. Blood flow regulation by S-nitrosohemoglobin in the physiological oxygen gradient. Science 276: 2034–2037, 1997.
56. Stow LR, Jacobs ME, Wingo CS, Cain BD. Endothelin-1 gene regulation. FASEB J 25: 16–28, 2011.
57. Taverne YJ, de Beer VJ, Hoogteijling BA, Juni RP, Moens AL, Duncker DJ, Merkus D. Nitroso-redox balance in control of coronary vasomotor tone. J Appl Physiol (1985) 112: 1644–1652, 2012.
58. Toma VA, Farcas_ AD, Roman I, Sevastre B, Hathazi D, Scurtu F, Damian G, Silaghi-Dumitrescu R. Comparative in vivo effects of hemoglobin-based oxygen carriers (HBOC) with varying prooxidant and physiological reactivity. PLoS One 11: e0153909, 2016.
59. Touyz RM, Schiffrin EL. Reactive oxygen species in vascular biology: implications in hypertension. Histochem Cell Biol 122: 339–352, 2004.
60. Tsai AG, Cabrales P, Manjula BN, Acharya SA, Winslow RM, Intaglietta M. Dissociation of local nitric oxide concentration and vasoconstriction in the presence of cell-free hemoglobin oxygen carriers. Blood 108: 3603–3610, 2006.
61. Winslow RM. Oxygen: the poison is in the dose. Transfusion 53: 424–437, 2013.
62. York GB, DiGeronimo RJ, Wilson BJ, Cofer BR, Breuer CK, Josephs JD, Smith DL, Sorrells DL. Extracorporeal membrane oxygenation in piglets using a polymerized bovine hemoglobin-based oxygen-carrying solution (HBOC-201). J Pediatr Surg 37: 1387–1392, 2002.
63. Yu B, Raher MJ, Volpato GP, Bloch KD, Ichinose F, Zapol WM. Inhaled nitric oxide enables artificial blood transfusion without hypertension. Circulation 117: 1982–1990, 2008.
64. Yu B, Shahid M, Egorina EM, Sovershaev MA, Raher MJ, Lei C, Wu MX, Bloch KD, Zapol WM. Endothelial dysfunction enhances vasoconstriction due to scavenging of nitric oxide by a hemoglobinbased oxygen carrier. Anesthesiology 112: 586 –594, 2010.
65. Yu B, Volpato GP, Chang K, Bloch KD, Zapol WM. Prevention of the pulmonary vasoconstrictor effects of HBOC-201 in awake lambs by continuously breathing nitric oxide. Anesthesiology 110: 113–122, 2009.
66. Zhang J, Somers M, Cobb FR. Heterogeneous effects of nitroglycerin on the conductance and resistance coronary arterial vasculature. Am J Physiol 264: H1960–H1968, 1993.


Altering
[ET-1]
and [PC]
AF
Exercise
Change in
Endothelial
Function
CPB
MI
HBOC
ROS
(scavenging)

7

PROSTANOIDS SUPPRESS THE CORONARY VASOCONSTRICTOR INFLUENCE OF ENDOTHELIN AFTER MYOCARDIAL INFARCTION

V. J. de Beer, **Y.J. Taverne**, D.W. Kuster, A. Najafi, D.J. Duncker and D. Merkus

American Journal of Physiology: Heart and Circulatory Physiology 2011

ABSTRACT

Myocardial infarction (MI) is associated with endothelial dysfunction resulting in an imbalance in endothelium-derived vasodilators and vasoconstrictors. We have previously shown that despite increased endothelin (ET) plasma levels, the coronary vasoconstrictor effect of endogenous ET is abolished after MI. In normal swine, nitric oxide (NO) and prostanoids modulate the vasoconstrictor effect of ET. In light of the interaction among NO, prostanoids, and ET combined with endothelial dysfunction present after MI, we investigated this interaction in control of coronary vasomotor tone in the remote noninfarcted myocardium after MI. Studies were performed in chronically instrumented swine (18 normal swine; 13 swine with MI) at rest and during treadmill exercise. Furthermore, endothelial nitric oxide synthase (eNOS) and cyclooxygenase protein levels were measured in the anterior (noninfarcted) wall of six normal and six swine with MI. eNOS inhibition with N^{ω}-nitro-L-arginine (L-NNA) and cyclooxygenase inhibition with indomethacin each resulted in coronary vasoconstriction at rest and during exercise, as evidenced by a decrease in coronary venous oxygen levels. The effect of L-NNA was slightly decreased in swine with MI, although eNOS expression was not altered. Conversely, in accordance with the unaltered expression of cyclooxygenase-1 after MI, the effect of indomethacin was similar in normal and MI swine. L-NNA enhanced the vasodilator effect of the $ET_{A/B}$ receptor blocker tezosentan but exclusively during exercise in both normal and MI swine. Interestingly, this effect of L-NNA was blunted in MI compared with normal swine. In contrast, whereas indomethacin increased the vasodilator effect of tezosentan only during exercise in normal swine, indomethacin unmasked a coronary vasodilator effect of tezosentan in MI swine both at rest and during exercise. In conclusion, the present study shows that endothelial control of the coronary vasculature is altered in post-MI remodeled myocardium. Thus the overall vasodilator influences of NO as well as its inhibition of the vasoconstrictor influence of ET on the coronary resistance vessels were reduced after MI. In contrast, while the overall prostanoid vasodilator influence was maintained, its inhibition of ET vasoconstrictor influences was enhanced in post-MI remote myocardium.

INTRODUCTION

Congestive heart failure is the only major cardiovascular disorder of which the incidence has increased over the past decade, which is principally due to a reduction in mortality associated with acute myocardial infarction (MI). Consequently, MI has become an increasingly important risk factor for the development of congestive heart failure [5]. The loss of viable myocardial tissue and the consequent left ventricular (LV) dysfunction result in neurohumoral activation, which, in conjunction with altered mechanical loading conditions of the LV, initiates the process of LV remodeling (consisting of LV hypertrophy and dilation). Although these adaptations are aimed at restoring cardiac pump performance, LV remodeling has been shown to be an independent risk factor for later development of congestive heart failure [5]. The mechanisms that contribute to the progression from mild LV dysfunction to overt congestive heart failure are still incompletely understood but could involve an impaired supply of O_2 to the hypertrophied myocardium. This concept is supported by observations in humans and animals with LV dysfunction after MI, indicating a reduction in coronary flow reserve of up to 35% in the surviving postinfarct LV myocardium (see reference 12 for review). Furthermore, hemodynamic and neurohumoral abnormalities associated with LV dysfunction are exacerbated during exercise [24, 32], which is accompanied by small perturbations in the myocardial O_2 balance as O_2 supply fails to match the increased O_2 demand [9, 34].

A distinct feature of swine with a recent MI is an elevation of circulating endothelin (ET) levels at rest and during treadmill exercise [24, 34]. We [34] therefore previously tested the hypothesis that an increased vasoconstrictor influence of ET contributed to the perturbations in myocardial oxygen balance but found a paradoxical loss of coronary vasoconstrictor influence by ET. The mechanism for the loss of ET constrictor influence was not addressed in that study. However, in normal swine, we observed that nitric oxide (NO) and prostacyclin act in concert to blunt the ET-induced coronary vasoconstriction particularly during exercise [40]. These findings suggest that NO and prostacyclin may also be responsible for negating the vasoconstrictor influence of ET in remodeled myocardium after MI.

In light of these considerations, the present study was undertaken to test the hypothesis that NO and prostacyclin are responsible for the abolished ET vasoconstrictor influence in swine with a recent MI. In view of previous observations that NO-mediated vasodilation is maintained or slightly decreased in remote myocardium after MI [25], whereas expression of cyclooxygenase (COX)-1 and COX-2 is increased in the human heart after MI [50], we further hypothesized that

inhibition of ET would be particularly prostanoid and to a lesser extent NO mediated. For the purpose of testing these hypotheses, swine were chronically instrumented, subjected to permanent ligation of the left circumflex coronary artery or a sham procedure, and studied ~2 wk later while running on a treadmill. To investigate whether alterations in myocardial endothelial nitric oxide synthase (eNOS), COX-1, and/or COX-2 contributed to altered influences of either NO or prostanoids on coronary vasomotor tone, protein expression of eNOS, COX-1, and COX-2 was measured in normal myocardium as well as in the remote myocardium after MI.

METHODS

Animals

Studies were performed in accordance with the American Physiological Society's Guiding Principles in the Care and Use of Laboratory Animals and with approval of the Animal Care Committee of the Erasmus Medical Center . A total of 35 crossbred Yorkshire × Landrace swine of either sex (2 to 3 mo old; 22 ± 1 kg at the time of surgery) were entered into the study.

Surgical Procedures

Swine were sedated (20 mg/kg ketamine im + 1 mg/kg midazolam im), anesthetized (thiopental sodium: 15 mg/kg iv), intubated, and ventilated with a mixture of O_2 and N_2 (1:2) [17]. Anesthesia was maintained with midazolam (2 mg/kg + 1 $mg \cdot kg^{-1} \cdot h^{-1}$ iv) and fentanyl (10 $\mu g \cdot kg^{-1} \cdot h^{-1}$ iv). Swine were instrumented under sterile conditions as previously described [34]. Briefly, a thoracotomy was performed in the fourth intercostal space. Subsequently, a polyvinylchloride catheter was inserted into the aortic arch for the measurement of aortic pressure and blood sampling for the determination of PO_2, PCO_2, pH (ABL 505; Radiometer), O_2 saturation, and hemoglobin concentration (OSM3; Radiometer). A high-fidelity Konigsberg pressure transducer was inserted into the LV via the apex for measurement of LV pressure and maximum rate of rise in LV pressure ($LVdP/dt_{max}$). Fluid-filled catheters were implanted for measurement of blood pressure and/or infusion of drugs in the LV, left atrium, and pulmonary artery. Furthermore, a small angiocatheter was inserted into the anterior interventricular vein for coronary venous blood sampling. Flow probes were placed around the proximal left anterior descending coronary artery (2.5–3 mm; Transonic Systems) for measurement of coronary blood flow and the aorta (14–16 mm; Skalar) for measurement of both cardiac output and stroke

volume [33]. Finally, in all animals the proximal part of the left coronary circumflex artery (LCx) was exposed, but only in 17 swine that were designated to the MI group the LCx was permanently occluded with a silk suture [7]. Three MI swine died during surgery, due to recurrent fibrillation. Electrical wires and catheters were tunneled subcutaneously to the back. The chest was closed, and the animals were allowed to recover.

Animals received analgesia (0.3 mg buprenorphine im) for 2 days and antibiotic prophylaxis (25 mg/kg amoxicillin and 5 mg/kg gentamycin iv) for 5 days. One MI swine died overnight following surgery.

Exercise Protocols

Studies were performed 1–3 wk after surgery with swine [13 MI and 18 normal (N) animals] exercising on a motor driven treadmill. Excellent reproducibility of consecutive exercise trials has been reported previously [10, 14, 16, 44]. Four exercise protocols were performed on four different days and in random order.

ET receptor blockade.

With swine (8 MI; 12 N) lying quietly on the treadmill, resting hemodynamic measurements were obtained and blood samples collected. Hemodynamic measurements were repeated and rectal temperature was measured with animals standing on the treadmill. Subsequently, a four-stage (1–4 km/h) treadmill exercise protocol was started for MI swine, whereas a five-stage exercise protocol (1–5 km/h) was started for normal swine; each exercise stage lasted 2–3 min. Hemodynamic variables were continuously recorded and blood samples were collected during the last 60 s of each stage. After completing the control exercise protocol, animals were allowed to rest on the treadmill for 90 min. Then, the mixed ET_A and ET_B receptor (ET_A/ET_B) antagonist tezosentan (a gift from Dr Clozel, Actelion Pharmaceuticals) was intravenously administered over 10 min in a dose of 3 mg/kg, followed by a continuous infusion of 6 $mg \cdot kg^{-1} \cdot h^{-1}$ iv [31], and the exercise protocol was repeated.

eNOS inhibition and ET receptor blockade.

Ninety minutes after nine MI and nine normal swine had undergone a control exercise trial, animals received the NO synthase inhibitor N^{ω}-nitro-L-arginine [L-NNA; Sigma (Zwijndrecht, The Netherlands); 20 mg/kg iv; Ref. 10] and underwent a second exercise trial. Ninety minutes later, a subset of animals (6 MI; 8 N) animals

received tezosentan (3 mg/kg iv + 6 mg·kg^{-1}·h^{-1} iv) and underwent a third exercise trial.

COX inhibition and ET receptor blockade.

Ninety minutes after 11 MI and 10 normal swine had undergone a control exercise trial (as described above), animals received the COX inhibitor indomethacin (Sigma) in a dose of 10 mg/kg iv infused over 10 min [36] and 5 min later underwent a second exercise trial. Another 90 min later, a subset of animals (7 MI; 8 N) animals received indomethacin in a dose of 5 mg/kg iv, which resulted in hemodynamic conditions that were identical to those following administration of 10 mg/kg before the second exercise trial [37]. Subsequently, animals received tezosentan (3 mg/kg iv + 6 mg·kg^{-1}·h^{-1} iv) and underwent a third exercise trial.

Expression of eNOS and COX.

A separate group of swine (*n* = 12) was used for determination of eNOS, COX-1, and COX-2 in the anterior LV wall, corresponding to the LV region in which coronary blood flow studies were performed. Initial surgery was performed as described above, but no catheters were implanted [11]. The LCx coronary artery was dissected free in all 12 swine and ligated to induce MI in 6 swine. These swine were killed 3 wk after induction of MI or sham operation. Tissue homogenates were prepared from frozen LV subendocardial tissue from the anterior wall, which represents remote noninfarcted area in MI animals. Protein levels were studied by immunoblotting against COX-1 (1:1,000; sc-7950; Santa Cruz Biotechnology, Santa Cruz, CA), COX-2 (1:500; 160112; Cayman Chemical, Ann Arbor, MI), and eNOS (1:1,000; 610297; BD Transduction Laboratories, San Jose, CA) and normalized to α-actin (1:1,000; A1804; Sigma) used as a loading control.

Data Acquisition and Analysis

Digital recording and off-line analysis of hemodynamic data and computation of myocardial O_2 consumption ($M\dot{V}O_2$) and myocardial O_2 extraction (MEO_2) have been described in detail elsewhere [16, 17, 33]. Statistical analysis of hemodynamic data was performed using three-way (MI, drug treatment, and exercise) or two-way (MI and exercise) ANOVA for repeated measures, as appropriate. When significant effects were detected post hoc testing for the effects of exercise, drug treatment and MI were performed using Scheffé's test. To test for the effects of MI and drug treatment on the relation between $M\dot{V}O_2$ and either coronary venous O_2 tension (Pcv_{O2}), saturation (Scv_{O2}), or MEO_2, regression analysis was performed with each animal as a dummy variable and with MI, drug treatment, and $M\dot{V}O_2$ as independent

variables. Band densities of the Western blots were compared using an unpaired *t*-test. Statistical significance was accepted at $P \leq 0.05$ (two-tailed). Data are presented as means ± SE. Since statistical analyses revealed no differences between male and female swine, data from both sexes were pooled. Data on part of the normal swine have been previously reported [37].

RESULTS

Effects of MI on Hemodynamics and Myocardial Oxygen Balance

Ligation of the LCx results in an infarction of 20–25% of the LV [42]. The surviving myocardium hypertrophies as evidenced by a significant increase in LV weight to body weight ratio from 3.1 ± 0.2 g/kg in normal swine to 3.7 ± 0.2 g/kg in swine with MI, despite obvious thinning of the scar tissue compared with normal myocardium.

Swine with a MI displayed significant LV dysfunction as evidenced by decreases in $LVdP/dt_{max}$, stroke volume, and LV systolic pressure and an increase in LV filling pressure at rest, but particularly during exercise, compared with normal swine (Figure 1).

In normal swine, the exercise-induced increase in myocardial O_2 delivery matched the increase in myocardial O_2 demand; allowing MEO_2 to be maintained constant, so that Pcv_{O2} and Scv_{O2} remained unaltered during exercise. In the remote myocardium of MI swine, MEO_2 was slightly higher at rest and particularly during exercise, reflecting a subtle mismatch between O_2 delivery and demand, although this was not accompanied by significant decreases in Scv_{O2} (Figure. 1).

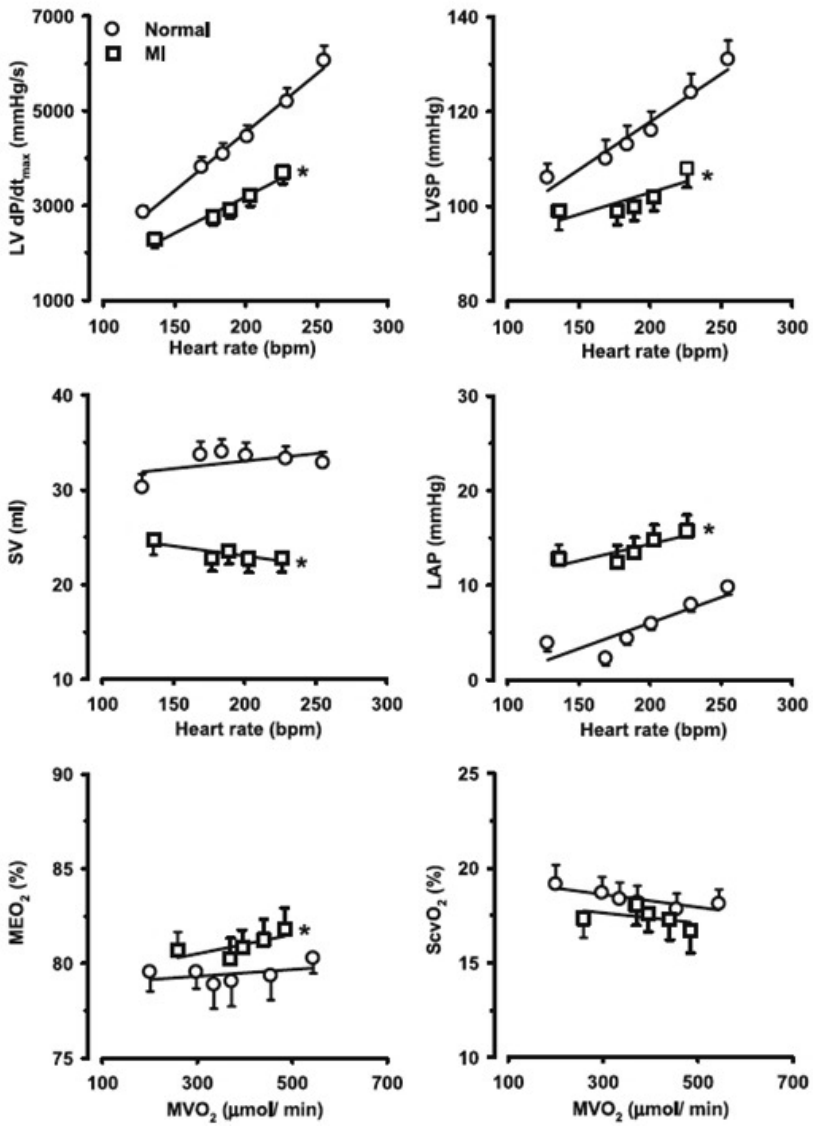


Figure 1. Effects of a myocardial infarction (MI) on systemic hemodynamic variables and coronary blood gas values. LVdP/dt_{max}, maximum rate of rise in left ventricular (LV) pressure; LVSP, LV systolic pressure; SV, stroke volume; LAP, left atrial pressure; MEO_2, myocardial oxygen extraction; Scv_{O2}, coronary venous oxygen saturation; bpm, beats/min. MI: n = 13; normal: n = 18. Values are means ± SE. *P≤0.05 vs. normal swine.

	Treatment	MI		Normal		
		Rest	4km/h	Rest	4km/h	5km/h
HR	Control for L-NNA	137 ± 3	230 ± 5*	132 ± 5	225 ± 6*	249 ± 6*
(bpm)	L-NNA	103 ± 4†	205 ± 5*†	103 ± 6†	191 ± 6*†	226 ± 7*†
	Control for Indo	135 ± 4	233 ± 9*	121 ± 4	228 ± 8*	252 ± 6*
	Indo	98 ± 3†	177 ± 5*†	86 ± 6†	173 ± 5*†	202 ± 7*†
MAP	Control for L-NNA	86 ± 3	78 ± 1*§	90 ± 2	87 ± 3	89 ± 3
(mmHg)	L-NNA	120 ± 5†	111 ± 4*†	122 ± 2†	123 ± 2†	121 ± 3†
	Control for Indo	91 ± 2	84 ± 3*	91 ± 2	88 ±3*	90 ± 3
	Indo	122 ± 5†	92 ± 4*†	123 ± 6†	101 ± 5*†	99 ± 4*†
LVSP	Control for L-NNA	97 ± 4§	108 ± 4*	108 ± 2	123 ± 6*	130 ± 6*
(mmHg)	L-NNA	125 ± 5†	127 ± 4†§	132 ± 4†	142 ± 4†	151 ± 4*†
	Control for Indo	104 ± 3	114 ± 4*	101 ± 2	116 ± 5*	124 ± 5*
	Indo	129 ± 5†	122 ± 5†	133 ± 7†	123 ± 8	128 ± 7
$LVdP/dt_{max}$	Control for L-NNA	2410 ±160§	3900 ± 220*§	3360 ± 180	4880 ± 320*	5430 ± 350*
(mmHg/s)	L-NNA	2180 ± 160	4540 ± 220*†	2380 ± 110†	5260 ± 290*	6270 ± 330*†
	Control for Indo	2520 ± 130	4120 ± 410*	2550 ± 170	4630 ± 430*	5270 ± 350*
	Indo	2550 ± 120	3570 ± 300*†	2210 ± 110†	3890 ± 260*†	4510 ± 420*†
LAP	Control for L-NNA	13 ± 2§	18 ± 1§	3 ± 2	11 ± 1*	13 ± 1*
(mmHg)	L-NNA	15 ± 2§	17 ± 1§	10 ± 1†	10 ± 1	11 ± 1
	Control for Indo	10 ± 2§	16 ± 2*§	3 ± 1	8 ± 1*	9 ± 1*
	Indo	17 ± 3†	19 ± 3†§	10 ± 2†	7 ± 1	8 ± 1

Table 1. Hemodynamic data in swine with and without MI at rest and during maximum exercise in the presence and absence of eNOS and/or COX blockade. Data are means ± SE. MI, myocardial infarction; eNOS, endothelial nitric oxide synthase; COX, cyclooxygenase; HR, heart rate; Indo, indomethacin; MAP, mean arterial pressure; LVSP, left ventricular systolic pressure; LVdP/dt_{max}, maximum rate of rise in left ventricular pressure; LAP, left atrial pressure; L-NNA, N^{ω}-nitro-L-arginine (MI: n = 9; normal: n = 9); Indo, indomethacin (MI: n =11; normal: n =10). * $P \leq 0.05$ vs. rest; † $P \leq 0.05$ vs. control; § $P \leq 0.05$ vs. normal swine.

Role of NO and Prostanoids in the Regulation of Coronary Resistance Vessel Tone after MI

The effects of L-NNA and indomethacin on hemodynamics and blood gases at rest and during exercise are shown in Tables 1 and 2. In both swine with MI and in normal swine, inhibition of the production of NO or prostanoid production resulted in systemic vasoconstriction as evidenced by the increase in blood pressure, which resulted in a, probably baroreceptor reflex mediated, decrease in heart rate (Table 1). The increase in blood pressure and the decrease in heart rate had opposing effects on myocardial oxygen consumption, which therefore remained unaltered in the presence of either L-NNA or indomethacin (Table 2).

	Treatment	MI		Normal		
		Rest	4km/h	Rest	4km/h	5km/h
Art Hb	Control for L-NNA	7.2 ±0.2	8.0 ± 0.3*	7.7 ± 0.2	8.6 ± 0.2*	8.8 ± 0.2*
(g%)	L-NNA	7.3 ± 0.2	8.6 ± 0.2*	7.3 ± 0.3	8.7 ± 0.2*	9.3 ± 0.2*†
	Control for Indo	7.8 ± 0.2	8.5 ± 0.2*	7.7 ± 0.3	8.7 ± 0.3*	8.9 ± 0.3*
	Indo	8.6 ± 0.3†	8.8 ± 0.2	7.9 ± 0.4	8.4 ± 0.2	8.7 ± 0.3*
Part SO_2	Control for L-NNA	92 ± 1	93 ± 1	93 ± 1	93 ± 1	92 ± 1
(%)	L-NNA	93 ± 1	92 ± 1	93 ± 1	93 ± 1	92 ± 1
	Control for Indo	93 ± 1	92 ± 1	92 ± 1	91 ± 1	91 ± 1
	Indo	94 ± 1†	93 ± 1	93 ± 1†	94 ± 1†	93 ± 1†
Art PO_2	Control for L-NNA	101 ± 3	94 ± 4	99 ± 2	95 ± 5	88 ± 3*
(mmHg)	L-NNA	96 ± 2	91 ± 4*	100 ± 3	95 ± 5	97 ± 7
	Control for Indo	101 ± 2	96 ± 3	101 ± 3	96 ± 4	91 ± 4
	Indo	120 ± 4†	103 ± 4*	115 ± 4†	109 ± 2†	98 ± 3*†
Art PCO_2	Control for L-NNA	44 ± 1	40 ± 0*	43 ± 1	40 ± 1*	39 ± 1*
(mmHg)	L-NNA	43 ± 1	38 ± 1*	41 2±	36 ± 2*	34 ± 2*
	Control for Indo	44 ± 1	41 ± 1*	42 ± 1	39 ± 1*	39 ± 1*
	Indo	34 ± 1†	36 ± 1†	36 ± 2†	35 ± 0†	35 ± 1†
PCV_{CO2}	Control for L-NNA	54 ± 1	54 ± 1	56 ± 1	55 ± 1	52 ±1
(mmHg)	L-NNA	55 ± 1	50 ± 2*	56 ± 1	52 ± 2*	52 ± 2*
	Control for Indo	52 ± 2	51 ± 1§	54 ± 3	56 ± 2	54 ± 2
	Indo	44±1†	47 ± 1*†	51 ± 4	54 ± 4	53 ± 3

	Treatment	MI		Normal		
		Rest	4km/h	Rest	4km/h	5km/h
CBF	**Control for L-NNA**	70 ± 4	126 ± 9*	66 ± 6	128 ± 11*	147 ± 12*
(ml/min)	**L-NNA**	62 ± 5†	114 ± 10*	58 ± 5†	116 ± 12*†	134 ± 12*†
	Control for Indo	72 ± 6§	129 ± 11*	52 ± 4	104 ± 9*	127 ± 9*
	Indo	53 ± 5†	105 ± 9*†	43 ± 5	84 ± 7*†	99 ± 6*†
MVO_2	**Control for L-NNA**	232 ± 11	472 ± 42*	241 ± 23	529 ± 41*	620 ± 47*
(μmol/min)	**L-NNA**	221 ± 20	482 ± 47*	222 ± 26	525 ± 54*	630 ± 54*
	Control for Indo	251 ± 26	515 ± 57*	192 ± 14	406 ± 23*	544 ± 39*
	Indo	240 ± 28	476 ± 49*	181 ± 22	388 ± 37*	460 ± 35*†
MEO_2	**Control for L-NNA**	79 ± 2	81 ± 1	81 ± 1	82 ± 1	82 ± 1
(%)	**L-NNA**	82 ± 1	83 ± 1	85 ± 1†	87 ± 1*†	86 ± 1†
	Control for Indo	80 ± 1	81 ± 1	81 ± 1	82 ± 1	83 ± 1
	Indo	87 ± 2†	88 ± 1†	88 ± 1†	90 ± 1†	89 ± 1†

Table 2. Blood gas values in swine with and without MI at rest and during maximum exercise in the presence and absence of eNOS and/or COX blockade. Data are means ± SE. For L-NNA, MI: *n* = 9; normal: *n* = 9. For Indo: MI *n* = 11; normal: *n* = 10. Art, arterial; Hb, hemoglobin; SO2, oxygen saturation; PO2, oxygen tension; PCO2, carbon dioxide tension; PcvCO2, coronary venous O2 tension; CBF, coronary blood flow; $M\dot{V}O_2$, myocardial oxygen consumption; MEO2, myocardial oxygen extraction. *$P \leq 0.05$ vs. rest; †$P \leq 0.05$ vs. control; §$P \leq 0.05$ vs. normal swine.

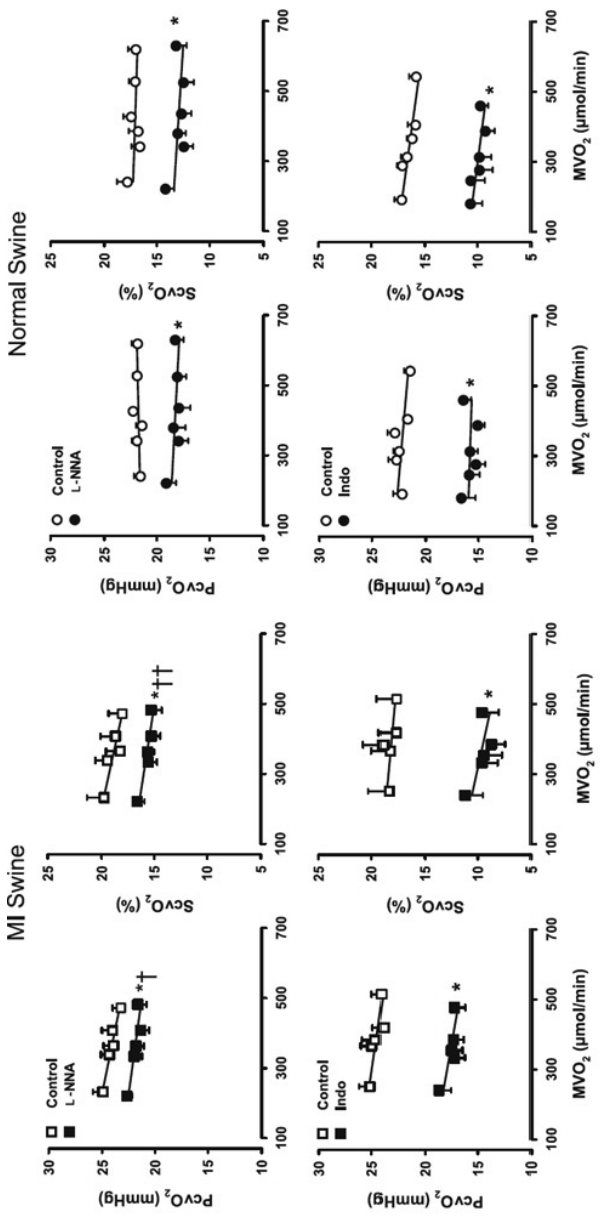


Figure 2. Effects of endothelial nitric oxide synthase (eNOS) inhibition with N^{ω}-nitro-L-arginine [L-NNA; MI: $n = 9$; normal $n = 9$] and cyclooxygenase inhibition with Indomethacin (Indo; MI: $n = 11$; normal: $n = 10$) on the coronary venous oxygen tension (Pcv_{O2}) and saturation (Scv_{O2}) in swine with and without an MI. Values are means ± SE. *$P \leq 0.05$ drug vs. control; $^{\dagger}P \leq 0.05$, $^{\dagger\dagger}P \leq 0.06$, drug effect different after MI.

Role of NO and Prostanoids in the Loss of ET Coronary Vasoconstrictor Influence after MI

The effects of tezosentan in the absence and presence of L-NNA or indomethacin on hemodynamics and blood gases at rest and during exercise are shown in Tables 3 and 4. Irrespective of the presence of L-NNA or indomethacin, administration of tezosentan resulted of systemic vasodilation as evidenced by a decrease in blood pressure, resulting in an increase in heart rate (Table 3). The decrease in blood pressure and the increase in heart rate had opposing effects on myocardial oxygen consumption, which therefore was not altered by administration of tezosentan either in the presence or absence of L-NNA or indomethacin in MI swine. In normal swine, however, administration of tezosentan resulted in a slight increase of myocardial oxygen consumption under resting conditions both in the absence and presence of L-NNA and indomethacin (Table 4).

In accordance with previous studies from our laboratory [7, 34], tezosentan did not affect Pcv_{O2} or Scv_{O2} in MI swine, indicating a lack of coronary vasodilation in response to ET_A/ET_B receptor blockade and hence a lack of coronary vasoconstrictor influence by endogenous ET (Figure 3). In contrast, tezosentan produced vasodilation in normal swine, which gradually waned with increasing exercise intensities ($P < 0.05$ for both Pcv_{O2} and Scv_{O2}).

	Treatment	MI		Normal		
		Rest	4km/h	Rest	4km/h	5km/h
HR (beats per min)	Control	142 ± 4	235 ± 9*	131 ± 4	233 ± 6*	264 ± 5*
	Tezo	150 ± 6	239 ± 6*	150 ± 4†	244 ± 7*†	269 ± 5*
	L-NNA	107 ± 4	203 ± 8*	103 ± 6	191 ± 6*	226 ± 7*
	L-NNA+Tezo	124 ± 4†	212 ± 8*	118 ± 5†	204 ± 6†	236 ± 8
	Indo	98 ± 3	177 ± 5*	86 ± 7	174 ± 5*	204 ± 7*
	Indo+Tezo	111 ± 6	175 ± 6*	97 ± 7†	178 ± 8*	212 ± 9*
MAP (mmHg)	Control	88 ± 2	81 ± 3*§	95 ± 2	90 ± 2*	92 ± 2
	Tezo	79 ± 2†§	76 ± 2†§	89 ± 2†	82 ± 1*†	84 ± 2*†
	L-NNA	119 ± 6	107 ± 4*§	122 ± 2	123 ± 2	121 ± 3
	L-NNA+Tezo	111 ± 5†	91 ± 4*	111 ± 4†	101 ± 2†	100 ± 2†
	Indo	122 ± 5	92 ± 4*	124 ± 7	104 ± 5	101 ± 4
	Indo+Tezo	100 ± 4†	83 ± 4*†	111 ± 3	88 ± 3*†	89 ± 3*†
LVSP (mmHg)	Control	100 ± 4	113 ± 4*	107 ± 3	124 ± 4*	131 ± 4*
	Tezo	97 ± 3	110 ± 4*	110 ± 3	123 ± 4*	128 ± 5*
	L-NNA	128 ± 7	129 ± 4	132 ± 4	142 ± 4	151 ± 4*
	L-NNA+Tezo	120 ± 6†	117 ± 6	117 ± 8	130 ± 4†	137 ± 4*†
	Indo	129 ± 5	122 ± 5*	135 ± 7	128 ± 7*	131 ± 7*
	Indo+Tezo	118 ± 3	119 ± 6	126 ± 3	118 ± 3	126 ± 4

	Treatment	MI		Normal		
		Rest	4km/h	Rest	4km/h	5km/h
LVdP/dt_{max}	Control	2430 ± 170	4090 ± 350	2720 ± 110	5100 ± 370*	5690 ± 360*
(mmHg/s)	Tezo	2450 ± 90	3900 ± 330	3400 ± 210†	5070 ± 360*	5520 ± 390*
	L-NNA	2140 ±170	4350 ± 230	2380 ±110	5260 ± 290*	6270 ± 330*
	L-NNA+Tezo	2470 ± 180†	3890 ± 210	3040 ± 130†	5120 ± 230*	5610 ± 230*
	Indo	2430 ± 160	3450 ± 260	2410 ± 110	4210 ± 190*	5030 ± 330*
	Indo+Tezo	2540 ± 180	3220 ± 220†§	2850 ± 210†	4160 ± 230*	4940 ± 290*
LAP	Control	13 ± 2§	18 ± 2*§	4 ± 1	8 ± 1*	11 ± 1*
(mmHg)	Tezo	12 ± 2§	18 ± 2*§	4 ± 1	8 ± 1*	10 ± 1*
	L-NNA	17 ± 2§	17 ± 2§	10 ± 1	10 ± 1*	11 ± 1*
	L-NNA+Tezo	14 ± 2§	16 ± 3	2 ± 2†	10 ± 1	12 ± 1
	Indo	17 ± 3	19 ± 3*§	10 ± 2	7 ± 1*	8 ± 2*
	Indo+Tezo	12 ± 2†	16 ± 3†§	8 ± 1	7 ± 1	8 ± 1

Table 3. Hemodynamic variables in swine with and without MI at rest and at maximum exercise: effects of tezosentan. Data are means ± SE. For L-NNA, MI: *n* = 6; normal: *n* = 8. For Indo, MI: *n* = 7; normal: *n* = 8. For tezosentan (Tezo), MI *n* = 8; normal: *n* = 12. HR, heart rate; MAP, mean arterial pressure; LVSP, left ventricular systolic pressure; LVdP/dtmax, maximum rate of rise in left ventricular pressure; LAP, left atrial pressure. **P* ≤ 0.05 vs. rest; †*P* ≤ 0.05 effect of Tezo; §*P* ≤ 0.05 vs. normal swine.

	Treatment	MI		Normal		
		Rest	4km/h	Rest	4km/h	5km/h
Art Hb	Control	7.6 ± 0.2	8.3 ± 0.4*	7.9 ± 0.3	8.6 ± 0.2*	8.9 ± 0.2*
(g%)	Tezo	7.9 ± 0.3	8.2 ± 0.4*	8.6 ± 0.3†	8.6 ± 0.2	8.8 ± 0.2
	L-NNA	7.2 ± 0.3	8.6 ± 0.2*	7.2 ± 0.3	8.7 ± 0.2*	9.3 ± 0.2*
	L-NNA-Tezo	8.0 ± 0.2	8.6 ± 0.1*	8.0 ± 0.3†	8.7 ± 0.2*	8.9 ± 0.2*
	Indo	8.8 ± 0.4	9.0 ± 0.3*	8.0 ± 0.4	8.4 ± 0.2	8.7 ± 0.2
	Indo+Tezo	7.8 ± 0.3†	8.1 ± 0.3†	8.4 ± 0.3	8.3 ± 0.2	8.5 ± 0.2
Art SO_2	Control	92 ± 1	90 ± 1	92 ± 1	93 ± 1	92 ± 1
(%)	Tezo	91 ± 1	89 ± 1§	92 ± 1	93 ± 1	92 ± 1
	L-NNA	93 ± 1	92 ± 1	93 ± 1	93 ± 1	92 ± 1
	L-NNA-Tezo	92 ± 1	91 ± 2	93 ± 1	93 ± 1	92 ± 1
	Indo	94 ± 1	92 ± 2	93 ± 1	93 ± 1	92 ± 1
	Indo+Tezo	93 ± 1	94 ± 1	93 ± 1	93 ± 1†	92 ± 1
Art PO_2	Control	98 ± 4	85 ± 4*	97 ± 3	92 ± 3	88 ± 3*
(mmHg)	Tezo	90 ± 2†§	83 ± 4§	99 ± 3	94 ± 3	91 ± 3
	L-NNA	95 ± 2	86 ± 3	98 ± 2	93 ± 5	94 ± 7
	L-NNA-Tezo	90 ± 4	80 ± 3*	95 ± 3	88 ± 2	94 ± 7
	Indo	122 ± 6	101 ± 5	115 ± 5	107 ± 5	97 ± 4*
	Indo+Tezo	107 ± 4†	100 ± 3*	115 ± 4	100 ± 5*	95 ± 4*
Art PCO_2	Control	45 ± 1	41 ± 1*	44 ± 1	40 ± 1*	39 ± 1*
(mmHg)	Tezo	46 ± 1	42 ± 1*	45 ± 1	40 ± 1*	39 ± 1*
	L-NNA	43 ± 1	38 ± 1*	43 ± 1	37 ± 1*	36 ± 1*
	L-NNA-Tezo	46 ± 2	45 ± 4*	44 ± 1	38 ± 1*	37 ± 1*
	Indo	34 ± 1	37 ± 2*	39 ± 4	38 ± 4	39 ± 4
	Indo+Tezo	40 ± 1†§	37 ± 1	37 ± 1	35 ± 1	34 ± 1*

	Treatment	MI		Normal		
		Rest	4km/h	Rest	4km/h	5km/h
PCV_{CO2}	Control	55 ± 1	54 ± 2	56 ± 1	52 ± 1	54 ± 2
(mmHg)	Tezo	56 ± 1	53 ± 2	58 ± 1	52 ± 2*	52 ± 2*
	L-NNA	54 ± 2	48 ± 2	56 ± 1	51 ± 2*	50 ± 1*
	L-NNA-Tezo	59 ± 7	56 ± 9*	57 ± 3	51 ± 2	48 ± 1*†
	Indo	43 ± 1	47 ± 1	47 ± 2	51 ± 2	50 ± 2
	Indo+Tezo	52 ± 2†§	48 ± 1*	48 ± 1	49 ± 2	49 ± 275
CBF	Control	75 ± 6	126 ± 12*	58 ± 6	125 ± 11*	147 ± 12*
(ml/min)	Tezo	75 ± 7	131 ± 12*	64 ± 7†	127 ± 12*†	145 ± 12*
	L-NNA	63 ± 6	115 ± 12*	58 ± 6	116 ± 13*	134 ± 13*
	L-NNA-Tezo	65 ± 6	114 ± 12*	68 ± 8†	126 ± 13*†	143 ± 14*†
	Indo	51 ± 7	94 ± 10*	39 ± 6	85 ± 9*	98 ± 8*
	Indo+Tezo	62 ± 8†	109 ± 14*	44 ± 5†	85 ± 8*	106 ± 9*
MVO_2	Control	269 ± 20	488 ± 47*	213 ± 18	498 ± 38*	601 ± 47*
(µmol/min)	Tezo	277 ± 22	503 ± 46*	247 ± 22†	500 ± 40*	557 ± 44*
	L-NNA	222 ± 26	488 ± 68*	215 ± 28	523 ± 61*	628 ± 61*
	L-NNA-Tezo	239 ± 25	443 ± 58*	261 ± 31†	519 ± 60	610 ± 74*
	Indo	244 ± 42	423 ± 58*	169 ± 26	395 ± 46*	466 ± 41*
	Indo+Tezo	242 ± 46	450 ± 90*	194 ± 24†	373 ± 40*	464 ± 41*
MEO_2	Control	81 ± 1§	83 ± 1§	81 ± 1	80 ± 1	80 ± 1*
(%)	Tezo	81 ± 1§	84 ± 1§	78 ± 1†	79 ± 1†	79 ± 1
	L-NNA	83 ± 1	83 ± 1	85 ± 1	86 ± 1*	85 ± 1
	L-NNA-Tezo	82 ± 1	80 ± 2†	82 ± 2	82 ± 1	82 ± 1
	Indo	88 ± 2	90 ± 2	89 ± 1	90 ± 1	90 ± 1
	Indo+Tezo	85 ± 2	86 ± 2	87 ± 2	87 ± 1†	87 ± 1†

.

Table 4. Blood gas values in swine with and without MI at rest and at maximum exercise: effects of tezosentan. Data are means ± SE. For Tezo, MI: *n* = 8; normal: *n* _ 12. For L-NNA, MI: *n* _ 6; normal: *n* = 8. For Indo, MI: *n* = 7; normal *n* = 8. **P* ≤ 0.05 vs. rest; †*P* ≤ 0.05, effect of Tezo; §*P* ≤ 0.05 vs. normal swine

Pretreatment with L-NNA unmasked a small vasodilator response to tezosentan in MI swine, which was principally confined to exercise. These responses qualitatively resembled those in normal swine in which L-NNA also selectively enhanced tezosentan-induced vasodilation during exercise. Quantitatively, however, the vasodilator response during exercise was less in MI compared with normal swine. These findings suggest that not only the overall vasodilator influence of NO (Figure 2), but also the vasodilator influence through inhibition of ET, was reduced in swine with MI (Figure 3).

Pretreatment with indomethacin unmasked a marked vasodilator response to tezosentan in MI swine, both at rest and during exercise. In contrast, in normal swine indomethacin unmasked a vasodilator response to tezosentan only during exercise, while leaving the vasodilator response to tezosentan under resting conditions unaffected. These findings suggest that in MI swine prostanoids have increased importance in limiting the vasoconstrictor influence of ET and that the vasodilator effect of prostanoids in MI swine, particularly under resting conditions, is partly mediated through inhibition of the ET system.

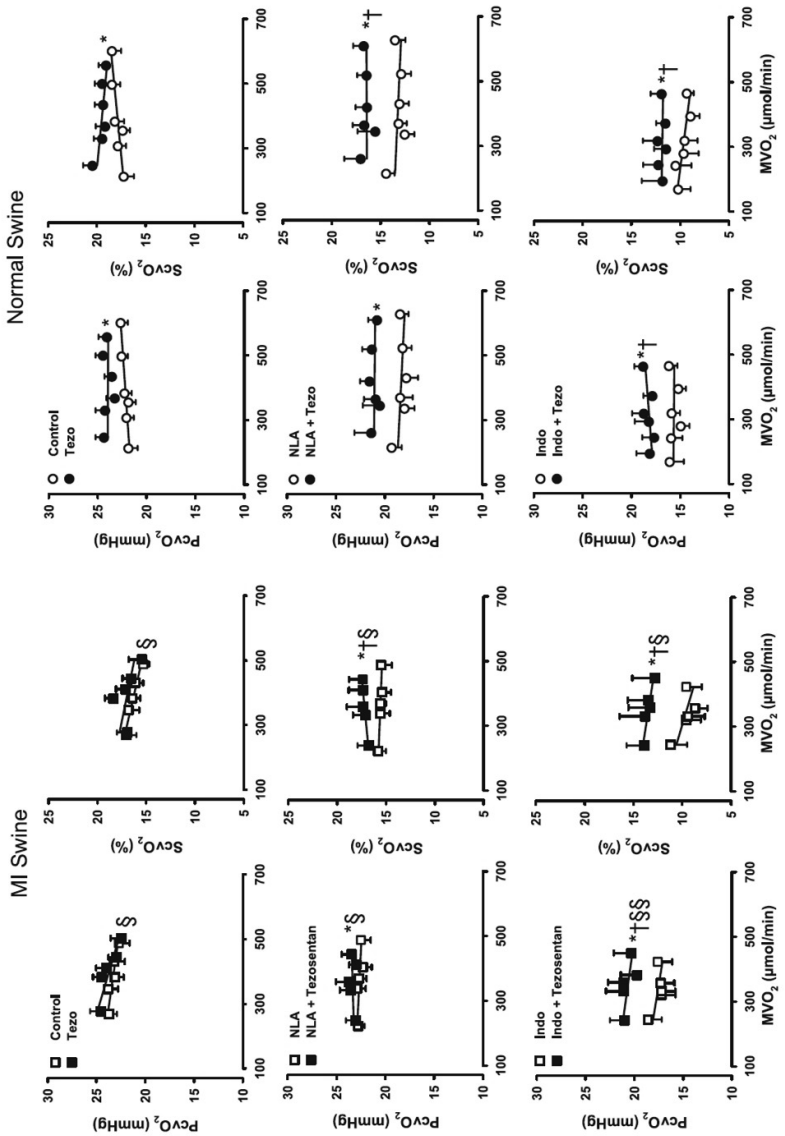


Figure. 3. Effects of ETA/B receptor blockade with tezosentan (Tezo; MI: *n* _ 8; normal: *n* _ 12) **in the presence and absence of eNOS inhibition with L-NNA** (MI: *n* = 6; normal: *n* = 8) **and cyclooxygenase inhibition with Indo** (MI: *n* = 7; normal: *n* = 8) **on coronary venous oxygen tension (PcvO2) and saturation (ScvO2) in swine with and without MI.** Values are means ± SE. **P* ≤ 0.05, effect of Tezo; †*P* ≤ 0.05, effect of Tezo different after prior L-NNA or Indo; §*P* ≤ 0.05, §§*P* ≤ 0.07, drug effect different after MI.

DISCUSSION

The main findings of the present study were that in remote remodeled myocardium of swine with LV dysfunction due to a recent MI the vasoconstrictor response to L-NNA was blunted; furthermore, although pretreatment with L-NNA enhanced the vasodilator response to tezosentan after MI, the inhibitory effects of L-NNA on the endogenous ET system were attenuated compared with normal swine. In contrast to the effects of L-NNA, the vasoconstrictor responses to indomethacin were maintained in swine with MI. Moreover, pretreatment with indomethacin enhanced vasodilator responses to tezosentan, compared with normal swine. However, despite the altered coronary vasomotor influences of NO and/or prostanoids, the protein expression of eNOS, COX-1, and COX-2 was unaltered in the remote myocardium of swine with MI compared with normal swine. The implications of these findings will be discussed in detail below.

Methodological Considerations

Intravenous administration of blockers of vasoactive substances not only results in changes in coronary resistance vessel tone but also in marked changes in systemic hemodynamics. Thus both COX and eNOS inhibition result in systemic vasoconstriction, thereby increasing arterial pressure and, probably via activation of the baroreceptor reflex, resulting in a decrease in heart rate. Conversely, ET_A/ET_B receptor blockade causes mild systemic vasodilation, thereby decreasing arterial pressure and resulting in an increase in heart rate. These alterations in blood pressure are likely to cause autoregulatory adjustments in coronary vascular tone and thereby influence coronary vascular resistance independent of the direct effects of prostanoids, NO, and ET. In addition, the alterations in systemic hemodynamics may influence myocardial work and thereby myocardial O_2 consumption, which will result in adjustments of myocardial oxygen supply and hence coronary blood flow will change. Therefore, changes in coronary blood flow and/or coronary vascular resistance in response to inhibition of eNOS, COX, and blockade of ET_A/ET_B receptors do not adequately reflect regulatory changes in coronary resistance vessel tone.

To study the regulation of coronary resistance vessel tone in vivo, and corrected for changes in myocardial metabolism, the relation between coronary venous O_2 levels and myocardial O_2 consumption is commonly used [13, 45]. For example, a decrease

in resistance vessel tone increases myocardial O_2 supply at a given level of myocardial O_2 consumption, allowing a decrease in myocardial O_2 extraction and leading to an increased coronary venous O_2 content and hence an upward shift of the relation between myocardial O_2 consumption and coronary venous O_2 levels. The coronary venous oxygen content thus represents an index of myocardial tissue oxygenation (i.e., the balance between oxygen delivery and oxygen consumption) that is determined by coronary resistance vessel tone [13, 45]. In the present study, we observed that, under resting conditions, Pcv_{O2} was slightly elevated while Scv_{O2} was unaltered in swine with MI compared with normal swine. These findings suggest a change in the oxygen dissociation curve. The oxygen dissociation curve can be influenced by pH, PCO_2, and 2,3-diphosphoglycerate (2,3-DPG; Ref. 27). A decrease in pH and an increase in PCO_2, as occur in exercising muscle, facilitate the dissociation of O_2, thereby promoting the diffusion of O_2 to the exercising tissue. As can be appreciated from Table 2, neither pH nor PCO_2 changed after MI, suggesting that alterations in 2,3-DPG may be responsible for this shift in the oxygen dissociation curve. In support of this notion, Kedziora et al. [28] showed that 2,3-DPG in erythrocytes was significantly increased in patients 3 wk after MI. The increase in 2,3-DPG decreases the affinity of hemoglobin for O_2 and could have resulted in an increase in Pcv_{O2} while Scv_{O2} remained unaltered. Importantly, however, the effects of acute administration of indomethacin, L-NNA, and tezosentan did not appear to be influenced by the oxygen dissociation curve, as changes in Pcv_{O2} and Scv_{O2} produced by each of these pharmacological agents were similar.

Integrated Endothelial Control of Coronary Resistance Vessel Tone after MI

In accordance with previous studies from our laboratory, we showed in the present study that myocardial oxygen demand of the remote, noninfarcted myocardium, is slightly increased after MI, both at rest and during exercise. The increase in myocardial oxygen demand is most likely due to the LV hypertrophy as the magnitude of the increase in myocardial oxygen demand matches the increase in the LV-to-body weight ratio after MI. Any increase in myocardial oxygen demand necessitates an elevation of coronary blood flow and hence recruitment of coronary flow reserve. We have previously shown that withdrawal of ET-mediated vasoconstriction contributes to the recruitment of flow reserve both in the healthy myocardium during exercise [33] as well as in the remodeled myocardium after MI [34; present study]. The withdrawal of ET-mediated coronary vasoconstriction after MI was not due to a loss of ET receptors, as coronary arterioles isolated from

remote myocardium after MI displayed a more pronounced vasoconstriction in response to ET [34]. The withdrawal of ET-mediated vasoconstriction during exercise in the normal heart was shown to be mediated by NO and prostanoids [37]; these data indicate that recruitment of coronary flow reserve during exercise depends critically on a healthy well-functioning endothelium. Since endothelial dysfunction has been shown to be present after MI [4, 7, 47, 49], we investigated in the present study if the contributions of the three main endothelial vasoactive systems (NO, prostanoids, and ET) to control of coronary resistance vessel tone in remodeled myocardium were altered at rest and/or during exercise.

Endothelial dysfunction results in a loss of NO bioavailability [21], which could exacerbate LV dysfunction by increasing LV afterload, while simultaneously inducing coronary vasoconstriction and hampering myocardial O_2 supply [15, 29, 41]. Loss of NO may therefore have contributed to the slight increase in myocardial oxygen extraction at rest and during exercise as observed in the present study. In a previous study from our laboratory [25], we showed that exercise-induced NO-mediated coronary vasodilation was not significantly blunted after MI, although receptor-mediated NO production was reduced. The current study (in a different group of animals) showed a small, but statistically significant, reduction in NO-mediated coronary vasodilator influence after MI. This reduced vasodilator influence of NO was present both at rest and during exercise. Although eNOS expression was not altered in the remote myocardium after MI compared with normal myocardium, it is still possible that eNOS activity may have been lower due to loss of eNOS phosphorylation [38] or increased plasma levels of the endogenous eNOS inhibitor asymmetric dimethylarginine [20, 23]. The reduced NO-mediated vasodilation may have also been due to increased NO scavenging by reactive oxygen species. Indeed, studies in rats [1, 2] as well as a recent study in swine from our own laboratory [3] suggest that oxidative stress is increased in the remote myocardium after MI. Importantly, we [37] have previously shown that NO does not only have a direct vasodilator effect but also reduces ET-mediated vasoconstriction since the withdrawal of ET necessary for recruitment of flow reserve during exercise in normal swine is mediated in part through NO. However, the present study shows that NO-mediated inhibition of the ET vasoconstrictor influence was significantly reduced after MI. The latter observation, in conjunction with the observation that the overall ET coronary vasoconstrictor influence is abolished in MI swine, implies that vasoactive substances other than NO (possibly prostanoids) must have been responsible for the complete inhibition of ET influence on the coronary resistance vessels after MI.

In the normal heart, COX-1 is expressed constitutively [46, 50, present study]. In accordance with these data, we [36] have previously shown that endogenous prostanoids contribute to regulation of coronary vascular tone at rest and during exercise in healthy swine. In contrast, inhibition of prostanoid production has very little effect in healthy humans [19] and dogs [6] either at rest or during exercise. As the effects of endogenous prostanoids decrease with age in both pigs [48] and humans [52], the discrepancy between our studies in juvenile pigs and the human/canine (adult) studies may reflect a difference in age-dependent contribution of prostanoids to regulation of coronary vascular tone.

Coronary artery disease and MI trigger an inflammatory response both in the LV and the coronary vasculature. Although inflammation is a natural repair response to tissue damage, it may eventually contribute to myocardial damage, LV remodeling, and ultimately the development of heart failure [26, 51]. A significant part of the inflammatory response is mediated by prostanoids that are produced by COX-1 and COX-2. While COX-1 is already present in the healthy coronary vasculature [46, 50], COX-2 is mainly induced by shear stress and at sites of inflammation and could therefore become more important after MI. Indeed, studies in humans [50] and rats [43] found an increase in COX-2 in the infarcted myocardium and border zone, and the contribution of prostanoids to regulation of coronary vascular tone in humans increases with the progression of coronary artery disease. Distal to angiographically minimally diseased coronary arteries, inhibition of prostanoid production induces mild vasoconstriction at rest that increases during exercise [8], whereas vasoconstriction is most pronounced in patients with coronary artery disease at rest [18, 22, 39] and during exercise [18, 39]. In contrast to the increased expression of COX-2 in the infarcted myocardium, neither COX-1 nor COX-2-expression changes in the remote myocardium after MI [43; present study], which is consistent with the unaltered overall coronary vasodilator influence of endogenous prostanoids in this area. Yet, the indirect effects of prostanoids on coronary vasomotor tone, via inhibition of the ET system, were increased. COX-1 and COX-2 end products influence the ET system on various levels; for instance, thromboxane A_2 stimulates the production of pre-pro-ET [30] the precursor of the vasoactive ET, while both PGE_2 and PGI_2 inhibit ET production as well as secretion [40]. The present study shows that after MI an increased inhibition of the ET system by prostanoids occurs in the coronary vasculature, which contributes to the recruitment of flow reserve to fulfill the increased oxygen demand of the myocardium. However, further experiments specifically designed to discern alterations in the role of the individual

COX end products after MI are required to further investigate their role in the regulation of coronary vascular tone in health and disease.

PERSPECTIVE AND CONCLUSIONS

Coronary vasomotor tone is tightly regulated by a multitude of vasodilator and vasoconstrictor influences, which, in addition to any direct effects on the vascular smooth muscle, can also impact each other's expression, secretion, and receptor activation [7, 9, 37]. After MI, endothelial and neurohumoral control of coronary vasomotor tone is altered [12]. To maintain perfusion of the hypertrophied remote myocardium after MI in the presence of endothelial dysfunction (reduced NO vasodilation), coronary flow reserve is recruited not only by increasing metabolic vasodilation via K_{ATP} channel activation [35] but surprisingly also by suppression of endogenous vasoconstrictor influences of ET [7, 34] and angiotensin II [7, 32]. The present study shows that the suppression of the vasoconstrictor influences of ET on the coronary resistance vessels in the remote remodeled myocardium is not due to NO, of which the overall vasodilator influence and the inhibition of ET influence were blunted. In contrast, despite an unchanged overall vasodilator influence, prostanoids acted to negate the coronary vasoconstrictor influences of ET. These observations underscore the highly complex interactions between vasodilator and vasoconstrictor influences exerted by the endothelium on the coronary resistance vessels.

REFERENCES

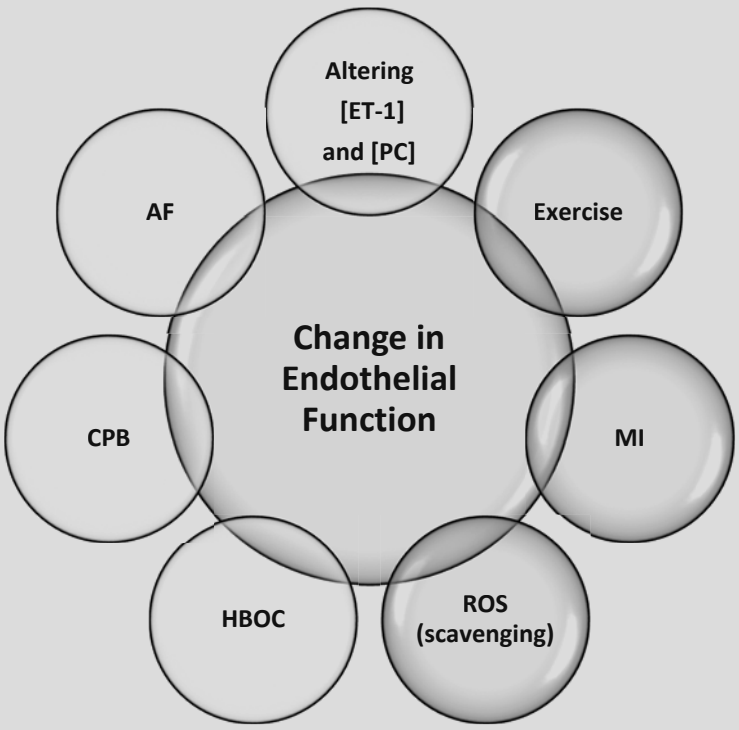
1. Berges A, Van Nassauw L, Timmermans JP, Vrints C. Role of nitric oxide during coronary endothelial dysfunction after myocardial infarction. Eur J Pharmacol 516: 60–70, 2005.
2. Berges A, Van Nassauw L, Timmermans JP, Vrints C. Time-dependent expression pattern of nitric oxide and superoxide after myocardial infarction in rats. Pharmacol Res 55: 72–79, 2007.
3. Boontje NM, Merkus D, Zaremba R, Versteilen A, de Waard MC, Mearini G, de Beer VJ, Carrier L, Walker LA, Niessen HW, Dobrev D, Stienen GJ, Duncker DJ, van der Velden J. Enhanced myofilament responsiveness upon beta-adrenergic stimulation in post-infarct remodeled myocardium. J Mol Cell Cardiol 50: 487–499, 2011.
4. Chen SM, Tsai TH, Hang CL, Yip HK, Fang CY, Wu CJ, Guo GB. Endothelial dysfunction in young patients with acute ST-elevation myocardial infarction. Heart Vessels 26: 2–9, 2011.
5. Colucci WS, Braunwald E. Pathophysiology of heart failure. In: Heart Disease–A Textbook of Cardiovascular Medicine (6 ed.), edited by Braunwald E, Zipes DP, Libby P: W.B. Saunders, 2001, p. 503–534.
6. Dai XZ, Bache RJ. Effect of indomethacin on coronary blood flow during graded treadmill exercise in the dog. Am J Physiol Heart Circ Physiol 247:H452–H458, 1984.
7. de Beer VJ, Sorop O, Pijnappels DA, Dekkers DH, Boomsma F, Lamers JM, Duncker DJ, Merkus D. Integrative control of coronary resistance vessel tone by endothelin and angiotensin II is altered in swine with a recent myocardial infarction. Am J Physiol Heart Circ Physiol 294: H2069–H2077, 2008.
8. Duffy SJ, Castle SF, Harper RW, Meredith IT. Contribution of vasodilator prostanoids and nitric oxide to resting flow, metabolic vasodilation, and flow-mediated dilation in human coronary circulation. Circulation 100: 1951–1957, 1999.
9. Duncker DJ, Bache RJ. Regulation of coronary blood flow during exercise. Physiol Rev 88: 1009–1086, 2008.
10. Duncker DJ, Bache RJ. Regulation of coronary vasomotor tone under normal conditions and during acute myocardial hypoperfusion. Pharmacol Ther 86: 87–110, 2000.
11. Duncker DJ, Boontje NM, Merkus D, Versteilen A, Krysiak J, Mearini G, El-Armouche A, de Beer VJ, Lamers JM, Carrier L, Walker LA, Linke WA, Stienen GJ, van der Velden J. Prevention of myofilament dysfunction by beta-blocker therapy in postinfarct remodeling. Circ Heart Fail 2: 233–242, 2009.

12. Duncker DJ, de Beer VJ, Merkus D. Alterations in vasomotor control of coronary resistance vessels in remodelled myocardium of swine with a recent myocardial infarction. Med Biol Eng Comput 46: 485–497, 2008.
13. Duncker DJ, Merkus D. Exercise hyperaemia in the heart: the search for the dilator mechanism. J Physiol 583: 847–854, 2007.
14. Duncker DJ, Oei HH, Hu F, Stubenitsky R, Verdouw PD. Role of KATP - channels in regulation of systemic, pulmonary, and coronary vasomotor tone in exercising swine. Am J Physiol Heart Circ Physiol 280: H22–H33, 2001.
15. Duncker DJ, Stubenitsky R, Tonino PA, Verdouw PD. Nitric oxide contributes to the regulation of vasomotor tone but does not modulate O(2)-consumption in exercising swine. Cardiovasc Res 47: 738–748, 2000.
16. Duncker DJ, Stubenitsky R, Verdouw PD. Autonomic control of vasomotion in the porcine coronary circulation during treadmill exercise: evidence for feed-forward beta-adrenergic control. Circ Res 82: 1312–1322, 1998.
17. Duncker DJ, Stubenitsky R, Verdouw PD. Role of adenosine in the regulation of coronary blood flow in swine at rest and during treadmill exercise. Am J Physiol Heart Circ Physiol 275: H1663–H1672, 1998.
18. Edlund A, Berglund B, van Dorne D, Kaijser L, Nowak J, Patrono C, Sollevi A, Wennmalm A. Coronary flow regulation in patients with ischemic heart disease: release of purines and prostacyclin and the effect of inhibitors of prostaglandin formation. Circulation 71: 1113–1120, 1985.
19. Edlund A, Sollevi A, Wennmalm A. The role of adenosine and prostacyclin in coronary flow regulation in healthy man. Acta Physiol Scand 135: 39–46, 1989.
20. Feng Q, Lu X, Fortin AJ, Pettersson A, Hedner T, Kline RL, Arnold JM. Elevation of an endogenous inhibitor of nitric oxide synthesis in experimental congestive heart failure. Cardiovasc Res 37: 667–675, 1998.
21. Fraccarollo D, Widder JD, Galuppo P, Thum T, Tsikas D, Hoffmann M, Ruetten H, Ertl G, Bauersachs J. Improvement in left ventricular remodeling by the endothelial nitric oxide synthase enhancer AVE9488 after experimental myocardial infarction. Circulation 118: 818–827, 2008.
22. Friedman PL, Brown EJ Jr, Gunther S, Alexander RW, Barry WH, Mudge GH Jr, Grossman W. Coronary vasoconstrictor effect of indomethacin in patients with coronary-artery disease. N Engl J Med 305: 1171–1175, 1981.
23. Gray GA, Patrizio M, Sherry L, Miller AA, Malaki M, Wallace AF, Leiper JM, Vallance P. Immunolocalisation and activity of DDAH I and II in the heart and modification post-myocardial infarction. Acta Histochem 112: 413–423, 2010.
24. Haitsma DB, Bac D, Raja N, Boomsma F, Verdouw PD, Duncker DJ. Minimal impairment of myocardial blood flow responses to exercise in the remodeled left ventricle early after myocardial infarction, despite significant hemodynamic and neurohumoral alterations. Cardiovasc Res 52: 417–428, 2001.

25. Haitsma DB, Merkus D, Vermeulen J, Verdouw PD, Duncker DJ. Nitric oxide production is maintained in exercising swine with chronic left ventricular dysfunction. Am J Physiol Heart Circ Physiol 282: H2198–H2209, 2002.
26. Hansen PR. Role of neutrophils in myocardial ischemia and reperfusion. Circulation 91: 1872–1885, 1995.
27. Hlastala MP, Woodson RD. Saturation dependency of the Bohr effect: interactions among H_, CO2, and DPG. J Appl Physiol 38: 1126–1131, 1975.
28. Kedziora H, Lao A, Golinski A, Cieslinska D, Kedziora J, Tkaczewski W. Myocardial infarction Changes in the concentrations of high-energy compounds and free amino acids in erythrocytes. Atherosclerosis 40: 359–364, 1981.
29. Kiowski W, Sutsch G, Schalcher C, Brunner HP, Oechslin E. Endothelial control of vascular tone in chronic heart failure. J Cardiovasc Pharmacol 32, Suppl 3: S67–73, 1998.
30. Lubrano V, Baldi S, Ferrannini E, L'Abbate A, Natali A. Role of thromboxane A(2) receptor on the effects of oxidized LDL on microvascular endothelium nitric oxide, endothelin-1, and IL-6 production. Microcirculation 15: 543–553, 2008.
31. Merkus D, Haitsma DB, Fung TY, Assen YJ, Verdouw PD, Duncker DJ. Coronary blood flow regulation in exercising swine involves parallel rather than redundant vasodilator pathways. Am J Physiol Heart Circ Physiol 285: H424–H433, 2003.
32. Merkus D, Haitsma DB, Sorop O, Boomsma F, de Beer VJ, Lamers JM, Verdouw PD, Duncker DJ. Coronary vasoconstrictor influence of angiotensin II is reduced in remodeled myocardium after myocardial infarction. Am J Physiol Heart Circ Physiol 291: H2082–H2089, 2006.
33. Merkus D, Houweling B, Mirza A, Boomsma F, van den Meiracker AH, Duncker DJ. Contribution of endothelin and its receptors to the regulation of vascular tone during exercise is different in the systemic, coronary and pulmonary circulation. Cardiovasc Res 59: 745–754, 2003.
34. Merkus D, Houweling B, van den Meiracker AH, Boomsma F, Duncker DJ. Contribution of endothelin to coronary vasomotor tone is abolished after myocardial infarction. Am J Physiol Heart Circ Physiol 288: H871–H880, 2005.
35. Merkus D, Houweling B, van Vliet M, Duncker DJ. Contribution of KATP - channels to coronary vasomotor tone regulation is enhanced in exercising swine with a recent myocardial infarction. Am J Physiol Heart Circ Physiol 288: H1306–H1313, 2005.
36. Merkus D, Houweling B, Zarbanoui A, Duncker DJ. Interaction between prostanoids and nitric oxide in regulation of systemic, pulmonary, and coronary vascular tone in exercising swine. Am J Physiol Heart Circ Physiol 286: H1114–H1123, 2004.
37. Merkus D, Sorop O, Houweling B, Boomsma F, van den Meiracker AH, Duncker DJ. NO and prostanoids blunt endothelin-mediated coronary

vasoconstrictor influence in exercising swine. Am J Physiol Heart Circ Physiol 291: H2075–H2081, 2006.
38. Mount PF, Kemp BE, Power DA. Regulation of endothelial and myocardial NO synthesis by multi-site eNOS phosphorylation. J Mol Cell Cardiol 42: 271–279, 2007.
39. Pacold I, Hwang MH, Lawless CE, Diamond P, Scanlon PJ, Loeb HS. Effects of indomethacin on coronary hemodynamics, myocardial metabolism and anginal threshold in coronary artery disease. Am J Cardiol 57: 912–915, 1986.
40. Prins B, Hu R, Nazario B, Pedram A, Frank H, Weber M, Levin E. Prostaglandin E2 and prostacyclin inhibit the production and secretion of endothelin from cultured endothelial cells. J Biol Chem 269: 11938–11944, 1994.
41. Recchia FA, McConnell PI, Bernstein RD, Vogel TR, Xu X, Hintze TH. Reduced nitric oxide production and altered myocardial metabolism during the decompensation of pacing-induced heart failure in the conscious dog. Circ Res 83: 969–979, 1998.
42. Schaper W, Remijsen P, Xhonneux R. The size of myocardial infarction after experimental coronary artery ligation. Z Kreislaufforsch 58: 904–909, 1969.
43. Scheuren N, Jacobs M, Ertl G, Schorb W. Cyclooxygenase-2 in myocardium stimulation by angiotensin-II in cultured cardiac fibroblasts and role at acute myocardial infarction. J Mol Cell Cardiol 34: 29–37, 2002.
44. Stubenitsky R, Verdouw PD, Duncker DJ. Autonomic control of cardiovascular performance and whole body O2 delivery and utilization in swine during treadmill exercise. Cardiovasc Res 39: 459–474, 1998.
45. Tune JD, Gorman MW, Feigl EO. Matching coronary blood flow to myocardial oxygen consumption. J Appl Physiol 97: 404–415, 2004.
46. Vane JR, Bakhle YS, Botting RM. Cyclooxygenases 1 and 2. Annu Rev Pharmacol Toxicol 38: 97–120, 1998.
47. Wang Y, Wang S, Wier WG, Zhang Q, Jiang H, Li Q, Chen S, Tian Z, Li Y, Yu X, Zhao M, Liu J, Yang J, Zhang J, Zang W. Exercise improves the dilatation function of mesenteric arteries in post-myocardial infarction rats via a PI3K/Akt/eNOS pathway-mediated mechanism. Am J Physiol Heart Circ Physiol 299: H2097–H2106, 2010.
48. Willis AP, Leffler CW. NO and prostanoids: age dependence of hypercapnia and histamine-induced dilations of pig pial arterioles. Am J Physiol Heart Circ Physiol 277: H299–H307, 1999.
49. Zhang HL, Zhao M, He X, Jiang HK, Yu XJ, Ma X, Zang WJ. [Alterations in aortic vasomotor function in rats with chronic heart failure and its mechanism]. Sheng Li Xue Bao 62: 317–324, 2010.
50. Zidar N, Dolenc-Strazar Z, Jeruc J, Jerse M, Balazic J, Gartner U, Jermol U, Zupanc T, Stajer D. Expression of cyclooxygenase-1 and cyclooxygenase-2 in the normal human heart and in myocardial infarction. Cardiovasc Pathol 16: 300–304, 2007.

51. Zidar N, Jeruc J, Balazic J, Stajer D. Neutrophils in human myocardial infarction with rupture of the free wall. Cardiovasc Pathol 14: 247–250, 2005.
52. Zinkevich N, Wittenburg A, Gutterman D. Role of cyclooxygenase in flow-induced dilation of human coronary arterioles depends upon age. Circulation 122: A15764, 2010.


Altering
[ET-1]
and [PC]
AF
Exercise
Change in
Endothelial
Function
CPB
MI
HBOC
ROS
(scavenging)

8

NITROSO-REDOX BALANCE IN CONTROL OF CORONARY VASOMOTOR TONE

YJ Taverne, VJ de Beer, B Hoogteijling, RP Juni, AL Moens, DJ Duncker, D Merkus

Journal of Applied Physiology 2012

ABSTRACT

Reactive oxygen species (ROS) are essential in vascular homeostasis but may contribute to vascular dysfunction when excessively produced. Superoxide anion ($O_2^{\bullet-}$) can directly affect vascular tone by reacting with K^+ channels and indirectly by reacting with nitric oxide (NO), thereby scavenging NO and causing nitroso-redox imbalance. After myocardial infarction (MI), oxidative stress increases, favoring the imbalance and resulting in coronary vasoconstriction. Consequently, we hypothesized that ROS scavenging results in coronary vasodilation, particularly after MI, and is enhanced after inhibition of NO production. Chronically instrumented swine were studied at rest and during exercise before and after scavenging of ROS with *N*-(2-mercaptoproprionyl)-glycine (MPG, 20 mg/kg iv) in the presence or absence of prior inhibition of endothelial NO synthase (eNOS) with N^{ω}-nitro-L-arginine (L-NNA, 20 mg/kg iv). In normal swine, MPG resulted in coronary vasodilation as evidenced by an increased coronary venous O_2 tension, and trends toward increased coronary venous O_2 saturation and decreased myocardial O_2 extraction. These effects were not altered by prior inhibition of eNOS. In MI swine, MPG showed a significant vasodilator effect, which surprisingly was abolished by prior inhibition of eNOS. Moreover, eNOS dimer/monomer ratio was decreased after MI, reflecting eNOS uncoupling. In conclusion, ROS exert a small coronary vasoconstrictor influence in normal swine, which does not involve scavenging of NO. This vasoconstrictor influence of ROS is slightly enhanced after MI. Since inhibition of eNOS abolished rather than augmented the vasoconstrictor influence of ROS in swine with MI, while eNOS dimer/monomer ratio was decreased, our data imply that uncoupled eNOS may be a significant source of $O_2^{\bullet-}$ after MI.

INTRODUCTION

Nitric oxide (NO) and superoxide ($O_2^{\bullet-}$) are key players in cellular nitroso-redox balance and are required for normal vascular homeostasis. The importance of the nitroso-redox balance in the cardiovascular system is underlined by studies that show that oxidative stress, i.e., a disturbance of the nitroso-redox balance, contributes to the pathogenesis of diabetes, hypertension, and atherosclerosis [1, 16, 38, 49, 50, 66]. Although several studies have shown that oxidative stress is increased after a myocardial infarction (MI), even in the remote myocardium [7, 9], and that the increased oxidative stress contributes to endothelial dysfunction in isolated large coronary arteries [6], the influence of the increased oxidative stress on the coronary microvasculature in the remote myocardium after MI in vivo has not been investigated to date.

Under normal physiological conditions $O_2^{\bullet-}$ is enzymatically produced by a variety of oxidases, including xanthine oxidase and NADPH oxidase and as a by-product of oxidative phosphorylation in the mitochondria, which is presumed to be the major source of $O_2^{\bullet-}$ production [18, 67]. $O_2^{\bullet-}$ can affect vascular function either directly, by reducing the opening probability of K^+ channels, or indirectly, by quenching NO and forming $ONOO^-$, both leading to vasoconstriction [60]. To prevent the deleterious actions of high concentrations of $O_2^{\bullet-}$, its concentration is tightly controlled and kept in the picomolar range by superoxide dismutase (SOD) thereby creating H_2O_2, a membrane-permeable vasodilator [37, 41] that has been suggested to be the factor that couples myocardial metabolism to coronary vasomotor tone [56].

After MI, $O_2^{\bullet-}$ is excessively produced, resulting in oxidative stress even in the remote, noninfarcted myocardium [7, 9], which may result in enhanced coronary vasoconstriction. Given the role of the mitochondrial respiratory chain as a major source of $O_2^{\bullet-}$, oxidative stress is likely to increase during exercise. An increased $O_2^{\bullet-}$-mediated vasoconstriction may therefore directly contribute to the relative hypoperfusion of the remote noninfarcted myocardium that is particularly observed during exercise [29]. Thus we hypothesize that the vasoconstrictor effect of $O_2^{\bullet-}$ in the remote, noninfarcted myocardium is enhanced after MI, especially during exercise. To test this hypothesis, we investigated the effects of *N*-(2-mercaptoproprionyl)-glycine (MPG), a synthetic aminothiol antioxidant that acts primarily, although perhaps not exclusively [2, 59], through scavenging of $O_2^{\bullet-}$ [16], in chronically instrumented swine with and without a recent MI.

In addition to its direct coronary vasoconstrictor effect, oxidative stress may result in a shift in the nitroso-redox balance toward the formation of peroxynitrite ($ONOO^-$), thereby reducing NO bioavailability, and counteracting the physiological NO-mediated quiescent and dilated state of blood vessels [64]. Moreover, $O_2^{\bullet-}$ can lead to uncoupling of endothelial NO synthase (eNOS), through oxidation of its cofactor tetrahydrobiopterin (BH_4), thereby causing a shift from NO to $O_2^{\bullet-}$ production [14, 31] and further aggravating microvascular dysfunction. We therefore also investigated whether eNOS uncoupling occurred in the remote myocardium after MI, and if the contribution of eNOS-dependent $O_2^{\bullet-}$ was altered after MI.

METHODS

Animals

Studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH Publication No. 85–23, Revised 1996), and with prior approval of the Animal Care Committee of the Erasmus Medical Center. Twenty-six two- to three-month-old Yorkshire × Landrace pigs (21.4 ± 0.4 kg for normal swine and 20.9 ± 0.2 kg for MI swine at the time of surgery) of either sex were entered into the study.

Surgery

Twenty-six swine were sedated (20 mg/kg ketamine and 1 mg/kg midazolam im), anesthetized (thiopental sodium 15 mg/kg iv), intubated, and ventilated with a mixture of O_2 and N_2 (1:2) to which, if necessary, 0.2–1.0% (vol/vol) isoflurane was added [21, 42]. Anesthesia was maintained with midazolam (2 mg/kg + 1 $mg \cdot kg^{-1} \cdot h^{-1}$ iv) and fentanyl (10 $\mu g \cdot kg^{-1} \cdot h^{-1}$ iv). Under sterile conditions, the chest was opened via the fourth left intercostal space, and a fluid-filled polyvinylchloride catheter was inserted into the aortic arch for aortic blood pressure measurement (Combitrans pressure transducers, Braun) and blood sampling. A Transit-time flow probe (Transonic Systems) was positioned around the ascending aorta for measurement of cardiac output. A microtipped pressure transducer ($P_{4.5}$, Konigsberg Instruments) was inserted into the left ventricle via the apex. Polyvinylchloride catheters were inserted into the left ventricle (LV) to calibrate the Konigsberg transducer LV pressure signal, into the left atrium to measure pressure, and into the pulmonary artery to administer drugs. A small angiocatheter was inserted into the anterior interventricular vein for coronary venous blood sampling. Finally, a transit-time flow probe (Transonic Systems) was placed around the left anterior descending coronary artery [42]. In all swine the proximal part of the left coronary circumflex artery (LCx)

was exposed, but only in 14 animals was the LCx permanently occluded with a silk suture to produce a MI [29]. Catheters were tunneled to the back, and animals were allowed to recover, receiving analgesia (0.3 mg buprenorphine im) for 2 days and antibiotic prophylaxis (25 mg/kg amoxicillin and 5 mg/kg gentamicin iv) for 5 days [21, 42]. Two MI swine died overnight, most likely due to ventricular fibrillation.

Experimental Protocols

Studies were performed 1–3 wk after surgery with animals resting and exercising on a motor-driven treadmill up to 85–90% of maximal heart rate. Two protocols (as described below) were performed on different days and in random order. All chemicals were obtained from Sigma.

Effect of scavenging of ROS.

With swine lying quietly on the treadmill, resting hemodynamic measurements consisting of heart rate, LV pressure, first derivative of LV pressure (dP/d*t*), mean aortic pressure, left atrial pressure, aortic and coronary blood flow (CBF) were obtained and arterial and coronary venous blood samples collected in 8 normal and 11 MI swine. Hemodynamic measurements were repeated and rectal temperature was measured with animals standing on the treadmill. Subsequently, swine were subjected to a four-stage exercise protocol (1–4 km/h) while hemodynamic variables were continuously recorded and blood samples collected during the last 60 s of each 3 min exercise stage at a time when hemodynamics had reached a steady state. After the exercise protocol was completed, animals were allowed to rest on the treadmill for 90 min after which animals received the ROS scavenger MPG [1 $mg \cdot kg^{-1} \cdot min^{-1}$, continuous infusion [34, 44, 45, 48, 62] and the exercise protocol was repeated.

We have previously shown excellent reproducibility of the hemodynamic response in consecutive bouts of exercise [20, 21].

Effect of ROS scavenging after NO blockade.

Ninety minutes after 11 normal and 8 MI swine had undergone a control exercise trial, animals received NO synthase inhibitor N^{ω}-nitro-L-arginine (L-NNA, 20 mg/kg iv) [20] and underwent a second exercise trial. Ninety minutes later, MPG (1 $mg \cdot kg^{-1} \cdot min^{-1}$, continuous infusion) was given to the animals and they underwent a third exercise trial. Because L-NNA has a long-lasting effect (mean arterial pressure, 123 ± 4 mmHg after administration of L-NNA before the second exercise trial and

121 ± 4 mmHg before administration of MPG prior to the third exercise trial), no additional L-NNA was administered before the third exercise protocol.

Blood gas measurements.

Blood samples were kept in iced syringes until the conclusion of each exercise trial. Measurements of PO_2 (mmHg), PCO_2 (mmHg), and pH were then immediately performed with a blood-gas analyzer (model 600, Acid-Base Laboratory, Radiometer, Copenhagen, Denmark). Hemoglobin (Hb; g/100 ml) and Hb O_2 saturation (SO_2) were measured with a hemoximeter (OSM3, Radiometer). Myocardial O_2 delivery (MDO_2 = CBF × arterial O_2 content), myocardial O_2 consumption [MVO_2 = CBF × (arterial O_2 content − coronary venous O_2 content)], and myocardial O_2 extraction ($MEO_2 = MVO_2/MDO_2$) were computed using the blood-gas values and CBF [21].

It is noteworthy that pigs have a negligible native collateral circulation, so that an acute occlusion of the LCx (without reperfusion) results in a transmural MI of the lateral wall encompassing 20–25% of the LV [29, 57]. Even though 1–3 wk after LCx occlusion some "collateral" flow to the fibrotic infarct zone is present [~20% of flow to the normal myocardium [68]], it is important to note that the anterior interventricular vein selectively drains the LAD perfusion territory [8], so that changes in O_2 balance in the infarcted area will not be reflected in the coronary venous O_2 content in the anterior interventricular vein.

eNOS Uncoupling

A separate group of swine (*n* = 12) was used for determination of eNOS uncoupling. Initial surgery was performed as described above, but no catheters were implanted. The LCx coronary artery was dissected free in all 12 swine, and ligated in 6 swine to induce MI. All 12 swine were euthanized 3 wk after induction of MI or sham operation. One MI pig was excluded from analysis due to a very small infarct size (<10% of LV).

Subendocardial tissue from the remote, noninfarcted anterior free wall was used. Myocardium was homogenized for 10 s in lysis buffer of the following composition: 50 mM Tris·HCl, pH 7.5; 150 mM NaCl; 0.1% SDS; 0.5% deoxycholate; 1% NP-40; protease inhibitors; 1 mM PMSF. Samples (60 μg of protein) were separated on a gel containing 0.375 M Tris, pH 8.8. Low-temperature SDS-PAGE was performed for detection of eNOS monomer and dimer [46]. The protein samples were subjected to SDS-PAGE with 7% self-made SDS-Tris gels run overnight. Gels and buffers were equilibrated at 4°C before electrophoresis, and the buffer tank was placed in an ice

bath during electrophoresis to maintain the low temperature. Subsequent to SDS-PAGE, the proteins were transferred for 3 h to nitrocellulose membranes. The blots were then probed as routine Western blot with primary NOS3 antibody (1:5,000, Santa Cruz Biotechnology, Heidelberg, Germany) and secondary rabbit anti-mouse IgG antibody conjugated with horseradish peroxidase (HRP; 1:1,000, Santa Cruz Biotechnology, Heidelberg, Germany), and detected by enhanced chemiluminescence substrate (Perkin Elmer) with LAS 3000 CCD camera (Fujifilm). The images were then analyzed with ImageJ (NIH). Data are presented as arbitrary units, which are a combination of band size and intensity.

Data Analysis

Hemodynamic data were digitally recorded and analyzed off-line. Effects of free radical scavenging (MPG) and eNOS inhibition (L-NNA) and MI on systemic hemodynamics were assessed with ANOVA for repeated measures followed by post hoc tests (Scheffe) where appropriate. Statistical comparison of individual data points in the table between normal and MI swine was performed using an unpaired *t*-test. To test for the effects of MI and drug treatment (MPG and/or L-NNA) on the relation between MVO_2 and coronary venous O_2 tension ($cvPO_2$), coronary venous O_2 saturation ($cvSO_2$), or MEO_2, regression analysis was performed using MI, drug treatment, and MVO_2 as well as their interaction as independent variables and assigning a dummy variable to each animal. Band densities of the Western blots were compared between normal and MI swine using an unpaired *t*-test. Statistical significance was accepted at $P \leq 0.05$ (2 tailed). Data are presented as means ± SE.

RESULTS

Systemic Hemodynamics and LV Function

Despite the loss of viable myocardial tissue, encompassing 20–25% of the LV [29, 57], the LV weight-to-body weight ratio in MI swine (3.5 ± 0.1 g/kg) tended to be higher than in normal swine (3.3 ± 0.2 g/kg), reflecting hypertrophy of surviving myocardium. MI resulted in LV dysfunction, as evidenced by a lower stroke volume, LV systolic pressure, and LV dP/dt_{max}, and a twofold higher mean left atrial pressure (all $P < 0.05$ by ANOVA). Exercise resulted in blunted increments of stroke volume and LV dP/dt_{max} in MI compared with normal swine, while the increase in heart rate was maintained (Figure 1, Table 1).

Scavenging of ROS through administration of MPG had little effect on LV function in either normal or MI swine as LV systolic pressure (Figure 1), left atrial pressure

(Table 1), and the relation between heart rate and dP/dt_{max} were unaffected by administration of MPG (Figure 1). Mean arterial pressure decreased slightly in both normal and MI swine. The decrease in mean arterial pressure was accompanied by an increase in heart rate in normal swine, while stroke volume was slightly reduced (Table 1), resulting in an unaltered cardiac output. In swine with MI, both heart rate and stroke volume remained unaffected by administration of MPG. Moreover, radical scavenging had no significant effect on peripheral vascular tone as systemic vascular conductance (SVC) did not change significantly after administration of MPG (Figure 1).

eNOS blockade with L-NNA resulted in peripheral vasoconstriction as evidenced by a decrease in SVC and an increase in mean arterial pressure (Figure 1). The increase in mean arterial pressure was accompanied by increases in LV systolic pressure as well as left atrial pressure, and was accompanied by a probably baroreflex-mediated decrease in heart rate and cardiac output, as stroke volume was not altered (Table 1). The effects of eNOS inhibition were similar in normal swine and swine with MI.

In the presence of L-NNA, ROS scavenging with MPG resulted in a small but significant increase in SVC and a decrease in mean arterial pressure, reflecting mild systemic vasodilation. The decrease in mean arterial pressure resulted in a probably baroreceptor reflex-mediated increase in heart rate and cardiac output. The decreases in LV systolic pressure and left atrial pressure following MPG administration in the presence of L-NNA most likely occurred in parallel to the decrease in mean arterial blood pressure, as neither stroke volume (Table 1) nor the relation between heart rate and LV dP/dt_{max} changed (Figure 1). The systemic hemodynamic effects of MPG after eNOS inhibition were similar in normal swine and swine with MI.

Table 1. Effect of MPG on hemodynamics in the presence and absence of NO synthase inhibition in healthy and MI swine. Data are means ± SE. Con, control; MPG, *N*-(2-mercaptoproprionyl)-glycine; HR, heart rate; SV, stroke volume; CO, cardiac output; LAP, left atrial pressure, CBF, coronary blood flow, Hbart, arterial hemoglobin; MI, myocardial infarction. * $P < 0.05$ vs. rest; † $P < 0.05$, effect of MPG; ‡ $P < 0.05$, effect of L-NNA; § $P < 0.05$ vs. normal swine.

	Treatment	MI	Exercise Level (km/h)				
			$Rest_{Lying}$	1	2	3	4
HR	Control	-	137 ± 8	179 ± 10*	192 ± 10*	198 ± 8*	224 ± 8*
(beats/min)	MPG	-	155 ± 9†	191 ± 9*	198 ± 10*	214 ± 12*	229 ± 11*
	L-NNA	-	98 ± 4‡	139 ± 6*‡	154 ± 7	172 ± 7*‡	201 ± 9*‡
	L-NNA+MPG	-	115 ± 7†‡	154 ± 7*	168 ± 7*	187 ± 8*	213 ± 8*
	Control	+	136 ± 9	176 ± 7*	187 ± 8*	198 ± 8*	213 ± 7*
	MPG	+	132 ± 8§	182 ± 8*	190 ± 5*	200 ± 7*	218 ± 10*
	L-NNA	+	130 ± 12	173 ± 11*‡	178 ± 7*	192 ± 6	203 ± 11
	L-NNA+MPG	+	134 ± 12	167 ± 8*	181 ± 8*	186 ± 7*	204 ± 7*
SV	Control	-	32 ± 3	37 ± 3*	38 ± 3*	38 ± 3*	37 ± 4*
(ml)	MPG	-	31 ± 3†	34 ± 3*†	35 ± 3*†	36 ± 4*†	36 ± 3*
	L-NNA	-	33 ± 2	38 ± 2*‡	38 ± 2*‡	37 ± 2*	36 ± 2*
	L-NNA+MPG	-	33 ± 2	34 ± 2	34 ± 2	35 ± 2	34 ± 2
	Control	+	26 ± 3	29 ± 3	28 ± 3§	29 ± 3	29 ± 3§
	MPG	+	27 ± 3	26 ± 2§	28 ± 3	28 ± 3	30 ± 3
	L-NNA	+	23 ± 3‡	24 ± 3‡	24 ± 3‡	25 ± 3	27 ± 4‡
	L-NNA+MPG	+	25 ± 4‡	25 ± 3‡	26 ± 4‡	26 ± 4	25 ± 3‡
CO	Control	-	4.3 ± 0.4	6.4 ± 0.6*	7.1 ± 0.7*	7.4 ± 0.6*	8.1 ± 0.6*
(l/min)	MPG	-	4.5 ± 0.3	6.2 ± 0.5*	6.6 ± 0.5*	7.2 ± 0.6*	7.9 ± 0.6
	L-NNA	-	3.5 ± 0.3	5.5 ± 0.4*	6.0 ± 0.4*	6.7 ± 0.4*	7.5 ± 0.4*
	L-NNA+MPG	-	4.0 ± 0.3†	5.6 ± 0.4*	6.1 ± 0.4*	6.8 ± 0.4*	7.6 ± 0.5*
	Control	+	3.3 ± 0.3§	5.1 ± 0.5*§	5.0 ± 0.3*§	5.6 ± 0.4*§	6.0 ± 0.5*§
	MPG	+	3.3 ± 0.3§	4.7 ± 0.3*§	5.3 ± 0.5*§	5.5 ± 0.4*§	6.2 ± 0.6*§
	L-NNA	+	2.8 ± 0.3	4.0 ± 0.4*	4.2 ± 0.4*	4,8 ± 0.4*	5.3 ± 0.6*
	L-NNA+MPG	+	2.9 ± 0.4	4.0 ± 0.4*	4.5 ± 0.6*	4.8 ± 0.6*	5.2 ± 0.8*

	Treatment	MI	Exercise Level (km/h)				
			$Rest_{Lying}$	1	2	3	4
LAP	Control	-	2.2 ± 1.9	2.4 ± 1.6	3.2 ± 1.5	4.0 ± 1.4	4.8 ± 1.4
(mmHg)	MPG	-	2.0 ± 1.3	2.7 ± 1.6	3.6 ± 1.3	5.7 ± 1.4	5.8 ± 1.3
	L-NNA	-	8.4 ± 1.3‡	7.5 ± 1.1‡	8.0 ± 1.3‡	8.2 ± 1.2	8.0 ± 1.4
	L-NNA+MPG	-	5.2 ± 1.5†	1.9 ± 1.2*†‡	3.8 ± 1.3†	4.5 ± 1.5†	5.4 ± 1.8
	Control	+	12.1 ± 2.6§	12.8 ± 2.7§	14.1 ± 2.4§	15.5 ± 2.4§	16.9 ± 2.5§
	MPG	+	7.6 ± 2.8	8.9 ± 3.0	10.6 ± 2.7	11.3 ± 3.0	13.7 ± 3.1
	L-NNA	+	8.4 ± 1.3‡	7.5 ± 1.1‡	8.0 ± 1.3‡	8.2 ± 1.2	8.0 ± 1.4
	L-NNA+MPG	+	5.2 ± 1.5†	1.9 ± 1.2*†‡	3.8 ± 1.3†	4.5 ± 1.5†	5.4 ± 1.8
CBF	Control	-	53 ± 4	78 ± 8*	84 ± 8*	87 ± 7*	105 ± 9*
(ml/min)	MPG	-	52 ± 4	76 ± 6*	80 ± 7*	89 ± 7*	103 ± 9*
	L-NNA	-	53 ± 4	79 ± 6*	86 ± 6*	94 ± 6*	113 ± 7*
	L-NNA+MPG	-	61 ± 4‡	80 ± 5*	86 ± 6*	97 ± 7*	112 ± 7*
	Control	+	46 ± 4	59 ± 3*§	63 ± 4*§	70 ± 4*	76 ± 4*§
	MPG	+	44 ± 4	60 ± 5*§	66 ± 6*	70 ± 5*§	74 ± 4*§
	L-NNA	+	55 ± 5	67 ± 7*	68 ± 7	69 ± 4*§	75 ± 5*§
	L-NNA+MPG	+	51 ± 6	64 ± 9	68 ± 10	60 ± 4‡§	68 ± 4§
Hb art	Control	-	9.0 ± 0.3	9.3 ± 0.3	9.5 ± 0.2*	9.4 ± 0.2	9.5 ± 0.2*
(g%)	MPG	-	9.5 ± 0.4	9.6 ± 0.2	9.6 ± 0.2	9.5 ± 0.2	9.6 ± 0.2
	L-NNA	-	8.1 ± 0.2	8.8 ± 0.2*	8.8 ± 0.3	9.2 ± 0.2*‡	9.5 ± 0.2*‡
	L-NNA+MPG	-	8.6 ± 0.2†	9.1 ± 0.1*‡	9.2 ± 0.2*	9.4 ± 0.2*‡	9.6 ± 0.2*‡
	Control	+	8.1 ± 0.4	8.4 ± 0.4	8.4 ± 0.5	8.4 ± 0.3§	8.8 ± 0.4*
	MPG	+	8/3 ± 0.4§	8.8 ± 0.4*†	8.7 ± 0.4*	8.7 ± 0.4*	8.8 ± 0.3*
	L-NNA	+	7.4 ± 0.4	8.1 ± 0.4*	8.2 ± 0.4*	8.7 ± 0.2*	8.9 ± 0.3*
	L-NNA+MPG	+	7.6 ± 0.4§	8.3 ± 0.5*	8.3 ± 0.5*	9.0 ± 0.2*	9.4 ± 0.3*

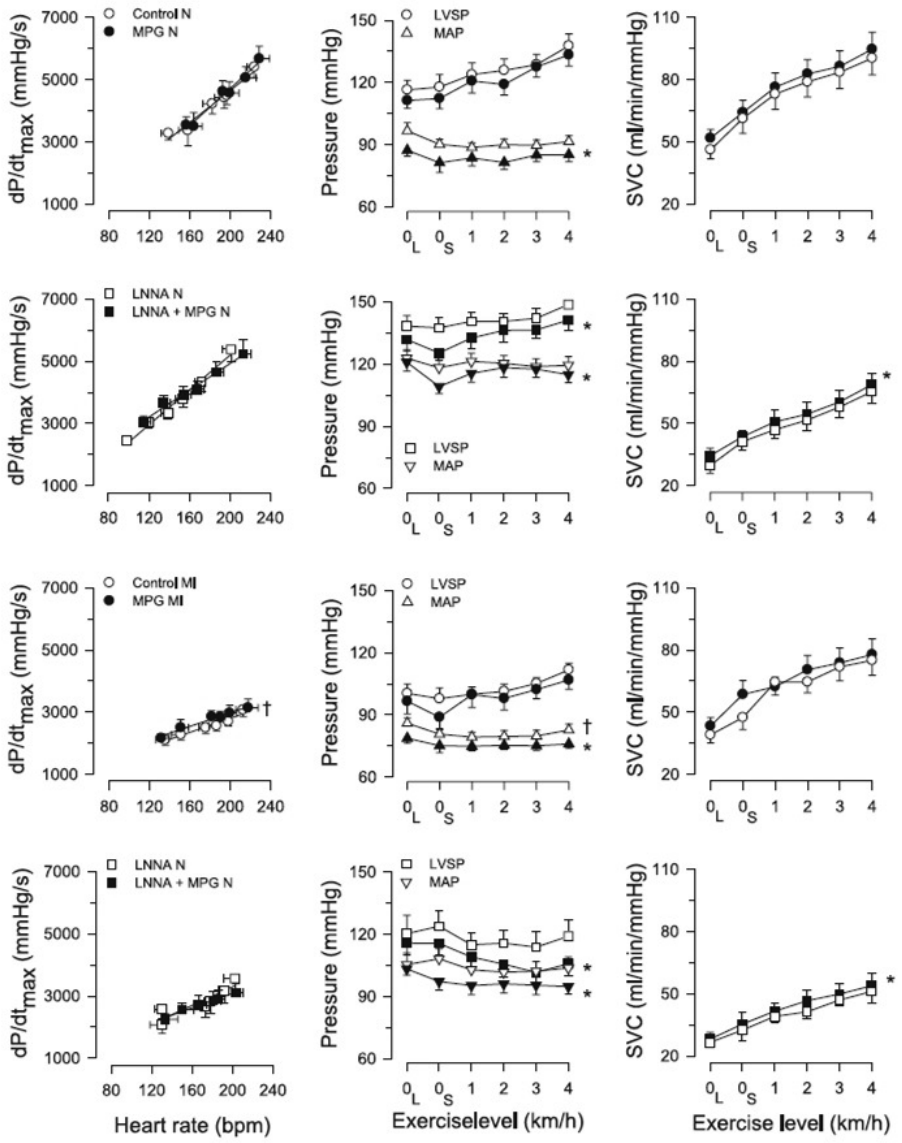


Figure 1. Changes in cardiac function and systemic hemodynamics in response to *N*-(2-mercaptoproprionyl)-glycine (MPG) in normal swine (N) and swine with myocardial infarction (MI) in the absence and presence of endothelial nitric oxide synthase (eNOS) inhibition with *N*_-nitro-L-arginine (L-NNA). dP/dtmax, maximum of first derivative of left ventricular pressure as an index of contractility; LVSP, left ventricular systolic pressure; MAP, mean arterial pressure; SVC, systemic vascular conductance; 0L is rest, lying; 0S is rest, standing. Data are means ± SE. **P* ≤ 0.05, effect of MPG. †*P* ≤ 0.05, control relation different in normal swine vs. swine with MI.

Coronary Circulation

In accordance with previous studies from our laboratory [20, 30, 43], coronary blood flow and myocardial O_2 delivery increased commensurate with the exercise-induced increase in myocardial O_2 consumption, so that $cvSO_2$ and $cvPO_2$ remained constant (Figure 2). After MI, the increase in coronary blood flow to the remote, noninfarcted myocardium was slightly less than the increase in myocardial O_2 consumption, forcing the heart to increase its O_2 extraction as reflected by a slight decrease in particularly $cvSO_2$ with increasing exercise intensity, while $cvPO_2$ was less affected (Figure 2).

In normal swine, MPG resulted in coronary vasodilation as evidenced by a significant increase in $cvPO_2$ and a tendency toward an increase in $cvSO_2$ (P = 0.13) and decreased MEO_2 (P = 0.15). The effects of MPG were similar at rest and during exercise (Figure 2). In the remote coronary vasculature of swine with MI, the vasodilator effect of MPG was significantly increased, as the shifts in $cvSO_2$ were significantly larger compared with normal swine (Figure 2), indicating increased oxidative stress in the coronary vasculature.

In accordance with previous studies from our laboratory [20, 30, 43], inhibition of eNOS with L-NNA resulted in coronary vasoconstriction in both normal swine and swine with MI, as evidenced by a decrease in $cvSO_2$ and $cvPO_2$ and an increase in MEO_2 (Figure 2). Subsequent administration of MPG resulted in small but significant vasodilation in normal swine (Figure 2). However, the effect of MPG after L-NNA was not different from its effect under control conditions, suggesting that ROS exert a direct effect on the coronary vasculature that is not mediated through scavenging of NO. Surprisingly, in swine with MI, MPG had no effect on $cvSO_2$ or $cvPO_2$ in the presence of L-NNA (Figure 2). Thus the vasodilator effect of MPG was abolished by prior eNOS inhibition, suggesting that, in the remote myocardium of swine with MI, eNOS contributes to formation of ROS, most likely $O_2^{\bullet-}$, which is in accordance with our recent observation that DHE staining was increased in the remote myocardium after MI [9].

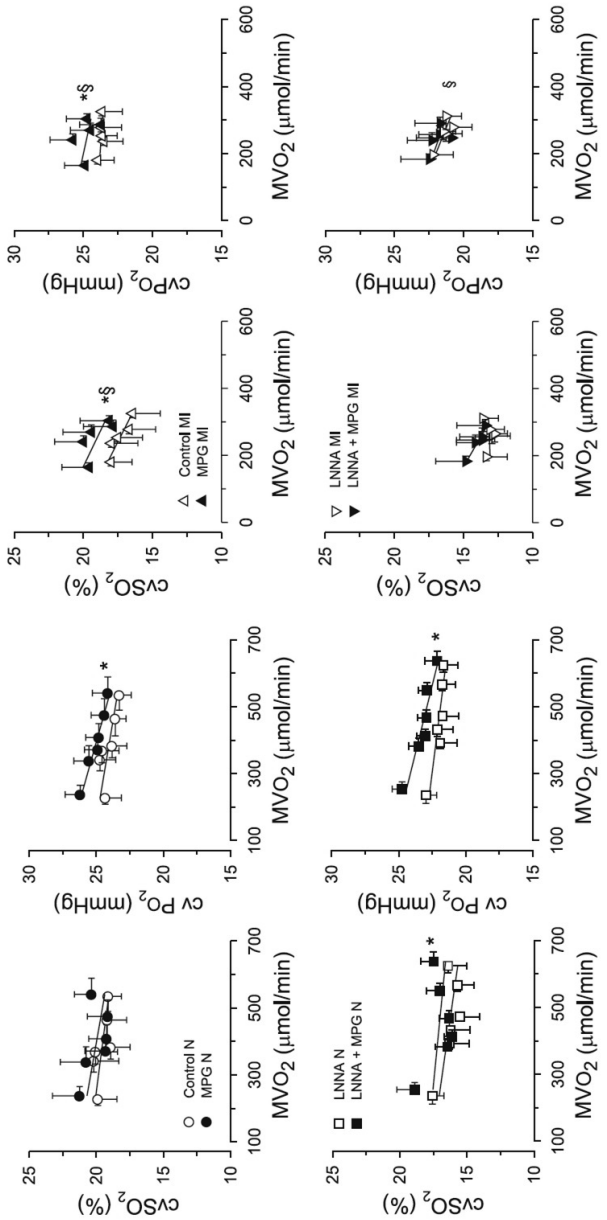


Figure 2. Changes in myocardial oxygen balance in response to MPG in normal swine (left) vs. swine with MI (right) in the absence (top) and presence of eNOS inhibition with L-NNA (bottom). MVO_2, myocardial O_2 consumption; $cvPO_2$, coronary venous O_2 tension; $cvSO_2$, coronary venous O_2 saturation. Data are means ± SE. **P* < 0.05, effect of MPG. §*P* < 0.05, effect of MPG different in normal swine vs. swine with MI.

eNOS Uncoupling

Total eNOS as well as the eNOS dimer-monomer ratio were decreased in tissue from swine with MI compared with normal swine (Fig. 3), indicating that eNOS expression was decreased whereas eNOS uncoupling was increased in swine with MI.

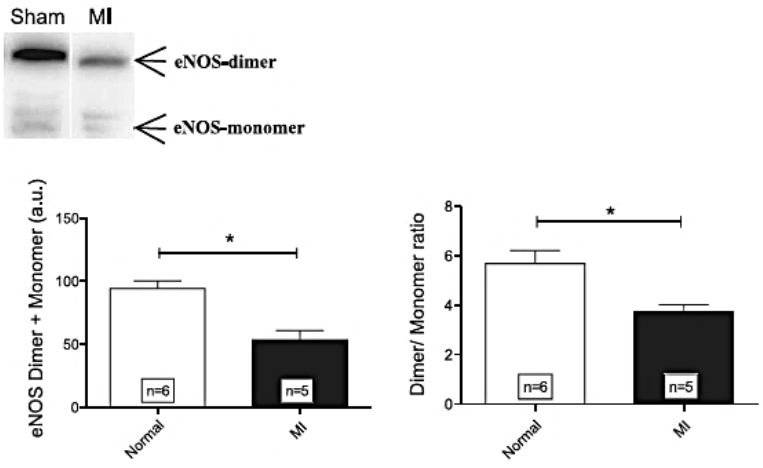


Figure 3. Changes in eNOS expression (dimer + monomer) and eNOS uncoupling (dimer/monomer ratio) in normal swine and swine with MI. Data are means ± SE. *P < 0.05, effect of MI.

DISCUSSION

The major findings of the present study are that *1*) ROS cause coronary vasoconstriction in normal swine that is not mediated through altered bioavailability of NO; *2*) the vasoconstrictor influence of ROS is enhanced in the remote myocardium after MI; *3*) inhibition of eNOS reduced rather than enhanced the vasoconstrictor influence of ROS after MI, and *4*) eNOS dimer-monomer ratio was decreased in myocardial tissue from swine with MI, suggesting that (uncoupled) eNOS is a significant source of $O_2^{\bullet-}$. Implications of these findings will be discussed below.

Methodological Considerations

Choice of MPG.

We chose to use MPG as a ROS scavenger as MPG is not highly radical specific and scavenges different types of ROS, including $O_2^{\bullet -}$, $ONOO^-$ and $OH^\bullet$ [2, 59]. Although radiolabeling showed that MPG primarily accumulates in the mitochondria [13], it is highly diffusible and has been shown to scavenge radicals both in the intracellular and extracellular compartment [10]. Thus MPG is able to access most sources of free radical production [35, 52], which allowed us to assess the effects of changes in the cellular nitroso-redox balance on coronary vascular tone independent of the source of free radicals. Our observation that administration of MPG resulted in vasodilation suggests that MPG acts through scavenging of a vasoconstrictor ROS, most likely $O_2^{\bullet -}$. In the remainder of the discussion we will, therefore, focus on the role of $O_2^{\bullet -}$, although a contribution of other vasoconstrictor ROS like $ONOO^-$ and $OH^\bullet$ cannot be excluded.

Myocardial O_2 balance as an index of coronary vascular tone.

Intravenous administration of blockers of vasoactive systems results in changes in systemic hemodynamics that not only affect myocardial oxygen demand but also affect coronary perfusion pressure and perfusion time, and may therefore result in autoregulatory and metabolic changes in resistance vessel tone [29, 65]. Changes in CBF or coronary vascular resistance following blockade of a vasoactive system therefore reflect not just the response to the blocker that was administered. Under basal resting conditions, the heart is characterized by a high level (80%) of O_2 extraction [21, 22]. Accordingly, the ability of the coronary resistance vessels to dilate in response to increments in myocardial O_2 demand is extremely important to maintain an adequate O_2 supply to the myocardium. A sensitive way to study alterations in coronary vascular tone in relation to myocardial metabolism, independent of changes in systemic hemodynamics, is the relationship between coronary venous O_2 levels and myocardial O_2 consumption [29, 65]. Thus an increased coronary resistance vessel tone will reduce coronary blood flow, and hence myocardial O_2 delivery, at a given level of myocardial O_2 consumption, forcing the myocardium to increase its O_2 extraction in order to meet myocardial O_2 demand, thus resulting in a lower coronary venous O_2 level. Conversely, a decrease in resistance vessel tone increases myocardial O_2 delivery at a given level of myocardial O_2 consumption, resulting in an increased coronary venous O_2 level.

Coronary venous O_2 levels thus represent an index of myocardial tissue oxygenation (i.e., the balance between O_2 supply and O_2 demand) that is determined by coronary resistance vessel tone. Accordingly, the relations between MVO_2 and $cvSO_2$, and $cvPO_2$ were used for interpretation of changes in coronary resistance vessel tone.

Effects of Nitroso-Redox Balance on Cardiac Function

Oxidative modification of calcium transporters [75] as well as contractile proteins [19] has been shown to contribute to cardiac dysfunction. In the present study, we found a slight decrease in stroke volume, but no further evidence for altered cardiac function, after scavenging free radicals with MPG in normal swine, suggesting that free radicals produced in the normal heart are effectively scavenged by normal antioxidant mechanisms, and therefore do not affect cardiac function.

After MI, cardiac function is hampered due to loss of functional myocardial cells, leading to impaired ventricular contraction, as evidenced by decreases in LV systolic pressure, LV dP/dt_{max}, and stroke volume, which results in elevated LV filling pressures in an attempt to maintain function. A recent study from our laboratory [9] showed that DHE staining of the remote myocardium of swine with MI is increased compared with normal animals, indicating that $O_2^{\bullet-}$ generation is increased. Thus oxidative modification of myofilaments may contribute to the observed LV dysfunction after myocardial infarction. Indeed, cardiac myocytes, isolated from the remote myocardium, displayed both systolic (reduced maximal force) and diastolic (increased calcium sensitivity) dysfunction [69]. Nevertheless, administration of MPG did not alter global cardiac function in swine with MI, indicating that an acute reduction in oxidative stress with MPG did not immediately improve LV function.

Effects of Nitroso-Redox Balance on the Vasculature

Healthy swine.

The effect of ROS is determined by their rate and location of production as well as by their rate and route of degradation. $O_2^{\bullet-}$ generation occurs in all layers of the vascular wall [64]. Because it is highly reactive, non- membrane permeable and because antioxidant defense mechanisms are abundantly present, the actions of $O_2^{\bullet-}$ will be restricted to the subcellular compartment in the proximity of its source

of generation [24, 51, 55, 63]. There are several ways in which $O_2^{\bullet-}$ may alter vascular tone. Thus $O_2^{\bullet-}$ can react with NO to form $ONOO^-$, thereby limiting NO bioavailability and decreasing NO-mediated vasodilation [23]. Moreover, $O_2^{\bullet-}$ can oxidatively alter various K^+ channels in vascular smooth muscle cells [28, 40], thereby depolarizing the membrane potential and resulting in vasoconstriction. In addition, $O_2^{\bullet-}$ can react with the sarco(endo)plasmic reticulum Ca^{2+} (SERCA) pump in pig coronary artery smooth muscle cells, while plasma membrane Ca^{2+} pumps are relatively resistant to oxidative modification [25, 26]. However, SERCA inhibition by $ONOO^-$ depletes the SR from Ca^{2+} and is therefore more likely to induce vasodilation [27, 71].

In healthy subjects, $O_2^{\bullet-}$ levels are tightly controlled by superoxide dismutase (SOD) [32, 29, 61]. SOD catalyzes the reaction of $O_2^{\bullet-}$ into H_2O_2 and O_2. The abundance of SOD in healthy blood vessels limits the amount of $ONOO^-$ formed under normal conditions although the reaction between NO and $O_2^{\bullet-}$ to form $ONOO^-$ is 3–4 times faster than the reduction of $O_2^{\bullet-}$ by SOD [5]. H_2O_2, a potent vasodilator [37, 41, 54, 56] that is formed by SOD, is more stable and diffusible than $O_2^{\bullet-}$ and is, therefore, under normal physiological conditions likely to affect vascular tone. Consequently, the net effect of ROS scavenging with MPG on vascular tone depends on the relative quantities of $O_2^{\bullet-}$ and H_2O_2.

ROS scavenging with MPG had no significant effect on vascular tone in the systemic circulation, suggesting that either the amount of free radicals produced is very low or that the vasoconstrictor influence of $O_2^{\bullet-}$ is balanced by a similar vasodilator influence of H_2O_2. After inhibition of eNOS, however, administration of MPG did result in slight but statistically significant systemic vasodilation. Hence, our data indicate that minimal scavenging of $O_2^{\bullet-}$ by NO does occur, which limits the direct effect of $O_2^{\bullet-}$ on the systemic vasculature.

In the coronary circulation, however, free radical scavenging with MPG did have a small but significant vasodilator effect both at rest and during exercise, indicating that the vasoconstrictor effect of $O_2^{\bullet-}$ exceeds the vasodilator effect of H_2O_2, resulting in a net vasoconstrictor effect of ROS on the coronary vasculature. We did not investigate the source of the free radicals, but the observation that inhibition of NADPH-oxidase did not affect vascular tone in awake dogs [74] suggests that NADPH-oxidase is not the source of $O_2^{\bullet-}$ in the coronary vasculature of healthy animals. Recent studies in rats imply that the respiratory chain of the mitochondria in the cardiac myocytes is the main source of $O_2^{\bullet-}$ [56]. $O_2^{\bullet-}$ derived from the

mitochondria is converted by SOD to the vasodilator H_2O_2, which is membrane permeable, diffuses to the microvasculature, and is responsible for the coupling of increased myocardial metabolism to vasodilation of the coronary microvessels [56, 73]. During exercise, myocardial O_2 consumption doubles and even moderate exercise may increase mitochondrial free radical production, possibly beyond endogenous antioxidant defenses, resulting in oxidative stress [17, 53, 58]. However, administration of a SOD mimetic had no effect on coronary vasomotor tone in awake dogs either at rest or during exercise [12], indicating that endogenous SOD is capable of converting the majority of $O_2^{\bullet-}$. These findings are in accordance with the findings in the present study that the effect of MPG was not increased during exercise. The apparent discrepancy between studies implying a prominent vasodilator role for endogenous $O_2^{\bullet-}$-derived H_2O_2 and the present study that shows that scavenging $O_2^{\bullet-}$ results in coronary vasodilation (and consequently that $O_2^{\bullet-}$ exerts a vasoconstrictor influence) may be due to different locations where MPG and SOD act with respect to the possible sources of $O_2^{\bullet-}$ in the vasculature.

Interestingly, eNOS inhibition appears to have very little effect on subsequent ROS scavenging with MPG (present study) or on the vasoconstrictor effect of catalase on canine coronary arterioles in vivo [73], suggesting that $O_2^{\bullet-}$ exerts a direct vasoconstrictor effect on the coronary vasculature rather than an effect that is mediated through altered bioavailability of NO.

Altogether, the data in the present study suggest that $O_2^{\bullet-}$ has a small direct vasoconstrictor effect on the coronary vasculature that is not mediated through altering bioavailability of NO and that is not altered during exercise.

Enhanced contribution of ROS to vascular tone after MI.

Several studies have indicated that MI may result in oxidative stress in the systemic vasculature [3, 32, 33]. The increased systemic oxidative stress usually occurs within days to weeks after MI [3, 33] and may be secondary to neurohumoral activation and subsequent activation of NADPH oxidase [4]. In the present study, the effects of MPG in the systemic vasculature were similar in normal and MI swine, indicating that MI did not result in a generalized increase in oxidative stress.

In contrast to our findings in the systemic vasculature, the coronary vasodilator effects of MPG (present study), as well as the DHE staining of remote myocardium

[9], were increased in swine with MI compared with normal swine, indicating an increased ROS production even in the microvasculature supplying the remote, noninfarcted myocardium. These data are in accordance with findings that $O_2^{\bullet-}$ production is increased in the remote coronary arteries of rats with MI [6] as well as in monocytes/macrophages within the intima, media, and adventitia in vessels with coronary artery disease [11]. NADPH oxidase has been suggested as a potential source of $O_2^{\bullet-}$ in the remote myocardium after MI [7]. Moreover, in a dog model of LV failure as a result of chronic rapid pacing [74], inhibition of NADPH oxidase with apocynin resulted in significant coronary vasodilation, suggesting that NADPH oxidase was the source of $O_2^{\bullet-}$ in these animals with LV dysfunction.

An alternative cause of the increased $O_2^{\bullet-}$ in the coronary vasculature may be its decreased scavenging by NO. eNOS expression was decreased in remote myocardium of MI swine in the present study, although such decrease was not found in a recent study from our laboratory in a different group of MI swine [15]. An explanation for these discordant results is not readily found, but the decrease in eNOS seemed to be related to infarct size in the present study as in one swine, with a very small MI (<10% of the left ventricle), that was excluded from the analysis, eNOS expression was found to be similar as in normal swine. Moreover, since we did not use a loading control in the present study, we cannot fully exclude that subtle differences in protein loading may have influenced our results. Importantly, eNOS uncoupling, as measured by the eNOS dimer/monomer ratio (being independent of protein loading), was increased in swine with MI. eNOS uncoupling by oxidation of its cofactor BH_4 and/or substrate depletion results in generation of $O_2^{\bullet-}$ rather than NO by eNOS [47, 70, 72]. Consistent with a role of eNOS in generation of $O_2^{\bullet-}$, the vasodilator effect of MPG in swine with MI disappeared after L-NNA. Nevertheless, L-NNA resulted in significant coronary vasoconstriction at rest and during exercise, indicating that despite the decreased eNOS expression and the increased eNOS uncoupling, eNOS-mediated NO production still exerted a net coronary vasodilator effect. In accordance with these findings, agonists were still capable of inducing NO-dependent coronary vasodilation, albeit that this vasodilation was impaired in the remote coronary vasculature after MI [30], which is consistent with the reduced eNOS expression in the present study.

Altogether, the data in the present study suggest that uncoupled eNOS is the most important source of $O_2^{\bullet-}$ in the remote coronary vasculature after MI. It is however possible that $O_2^{\bullet-}$ production by NAPDH oxidase provides the initial $O_2^{\bullet-}$, which acts to uncouple eNOS, thereby initiating a process that results in more $O_2^{\bullet-}$ production.

CONCLUSIONS

Our results suggest that ROS, and most likely $O_2^{\bullet-}$, contribute to coronary vasoconstriction in normal swine that is not mediated through altered bioavailability of NO. The contribution of ROS to vascular tone is enhanced after MI. The observation in MI swine that eNOS inhibition reduced rather than augmented the coronary vasoconstrictor influence of ROS, while the eNOS dimer/monomer ratio was decreased, suggests that after MI, uncoupled eNOS acts as a significant source of $O_2^{\bullet-}$.

REFERENCES

1. Arimura K, Egashira K, Nakamura R, Ide T, Tsutsui H, Shimokawa H, Takeshita A. Increased inactivation of nitric oxide is involved in coronary endothelial dysfunction in heart failure. Am J Physiol Heart Circ Physiol 280: H68–H75, 2001.
2. Atmaca G. Antioxidant effects of sulfur-containing amino acids. Yonsei Med J 45: 776–788, 2004.
3. Bauersachs J, Bouloumie A, Fraccarollo D, Hu K, Busse R, Ertl G. Endothelial dysfunction in chronic myocardial infarction despite increased vascular endothelial nitric oxide synthase and soluble guanylate cyclase expression: role of enhanced vascular superoxide production. Circulation 100: 292–298, 1999.
4. Bauersachs J, Widder JD. Endothelial dysfunction in heart failure. Pharmacol Rep 60: 119–126, 2008.
5. Beckman JS, Koppenol WH. Nitric oxide, superoxide, peroxynitrite: the good, the bad, ugly. Am J Physiol Cell Physiol 271: C1424–C1437, 1996.
6. Berges A, Van Nassauw L, Timmermans JP, Vrints C. Role of nitric oxide during coronary endothelial dysfunction after myocardial infarction. Eur J Pharmacol 516: 60–70, 2005.
7. Berges A, Van Nassauw L, Timmermans JP, Vrints C. Time-dependent expression pattern of nitric oxide and superoxide after myocardial infarction in rats. Pharmacol Res 55: 72–79, 2007.
8. Bier J, Sharaf B, Gewirtz H. Origin of anterior interventricular vein blood in domestic swine. Am J Physiol Heart Circ Physiol 260: H1732–H1736, 1991.
9. Boontje NM, Merkus D, Zaremba R, Versteilen A, de Waard MC, Mearini G, de Beer VJ, Carrier L, Walker LA, Niessen HW, Dobrev D, Stienen GJ, Duncker DJ, van der Velden J. Enhanced myofilament responsiveness upon beta-adrenergic stimulation in post-infarct remodeled myocardium. J Mol Cell Cardiol 50: 487–499, 2011.
10. Cai M, Ogawa R. Effects of free radical scavengers, methylprednisolone, and ulinastatin on acute xanthine and xanthine oxidase-induced lung injury in rats. Circ Shock 43: 71–78, 1994.
11. Cathcart MK. Regulation of superoxide anion production by NADPH oxidase in monocytes/macrophages: contributions to atherosclerosis. Arterioscler Thromb Vasc Biol 24: 23–28, 2004.
12. Chen Y, Hou M, Li Y, Traverse JH, Zhang P, Salvemini D, Fukai T, Bache RJ. Increased superoxide production causes coronary endothelial dysfunction and depressed oxygen consumption in the failing heart. Am J Physiol Heart Circ Physiol 288: H133–H141, 2005.

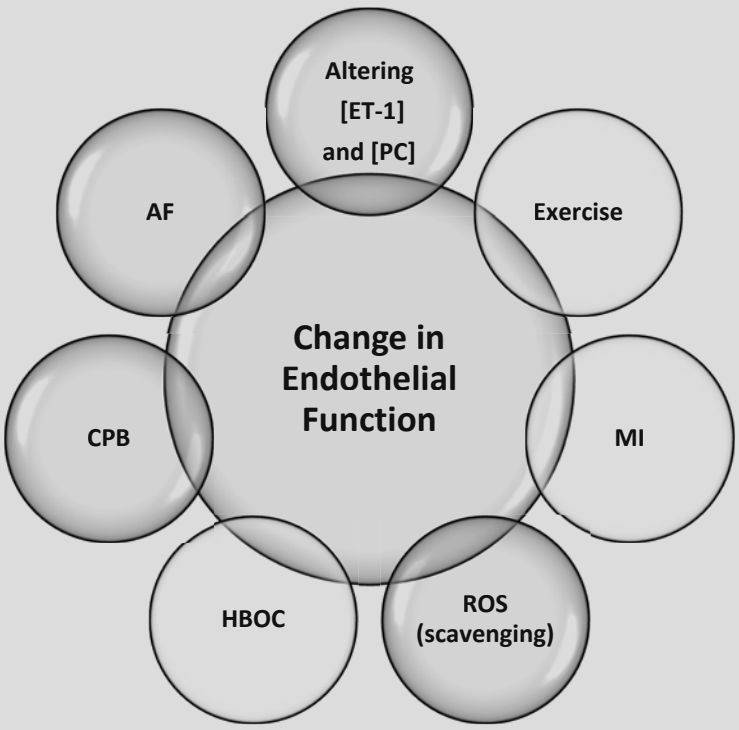
13. Chiba T, Kitoo H, Toshioka N. [Studies on thiol and disulfide compounds. I. Absorption distribution metabolism and excretion of 35S-2-mercaptopropionylglycine]. Yakugaku Zasshi 93: 112–118, 1973.
14. Cosentino F, Luscher TF. Tetrahydrobiopterin and endothelial nitric oxide synthase activity. Cardiovasc Res 43: 274–278, 1999.
15. de Beer VJ, Taverne YJ, Kuster DW, Najafi A, Duncker DJ, Merkus D. Prostanoids suppress the coronary vasoconstrictor influence of endothelin after myocardial infarction. Am J Physiol Heart Circ Physiol 301: H1080–H1089, 2011.
16. Deneke SM. Thiol-based antioxidants. Curr Top Cell Regul 36: 151–180, 2000.
17. Di Meo S, Venditti P. Mitochondria in exercise-induced oxidative stress. Biol Signals Recept 10: 125–140, 2001.
18. Droge W. Free radicals in the physiological control of cell function. Physiol Rev 82: 47–95, 2002.
19. Duncan JG, Ravi R, Stull LB, Murphy AM. Chronic xanthine oxidase inhibition prevents myofibrillar protein oxidation and preserves cardiac function in a transgenic mouse model of cardiomyopathy. Am J Physiol Heart Circ Physiol 289: H1512–H1518, 2005.
20. Duncker DJ, Stubenitsky R, Tonino PA, Verdouw PD. Nitric oxide contributes to the regulation of vasomotor tone but does not modulate O2-consumption in exercising swine. Cardiovasc Res 47: 738–748, 2000.
21. Duncker DJ, Stubenitsky R, Verdouw PD. Autonomic control of vasomotion in the porcine coronary circulation during treadmill exercise: evidence for feed-forward beta-adrenergic control. Circ Res 82: 1312–1322, 1998.
22. Feigl EO. Coronary physiology. Physiol Rev 63: 1–205, 1983.
23. Forstermann U. Nitric oxide and oxidative stress in vascular disease. Pflügers Arch 459: 923–939, 2010.
24. Griendling KK, Minieri CA, Ollerenshaw JD, Alexander RW. Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. Circ Res 74: 1141–1148, 1994.
25. Grover AK, Samson SE. Effect of superoxide radical on Ca_2-pumps of coronary artery. Am J Physiol Cell Physiol 255: C297–C303, 1988.
26. Grover AK, Samson SE, Fomin VP. Peroxide inactivates calcium pumps in pig coronary artery. Am J Physiol Heart Circ Physiol 263: H537–H543, 1992.
27. Grover AK, Samson SE, Fomin VP, Werstiuk ES. Effects of peroxide and superoxide on coronary artery: ANG II response and sarcoplasmic reticulum Ca2_ pump. Am J Physiol Cell Physiol 269: C546–C553, 1995.
28. Gutterman DD, Miura H, Liu Y. Redox modulation of vascular tone: focus of potassium channel mechanisms of dilation. Arterioscler Thromb Vasc Biol 25: 671–678, 2005.
29. Haitsma DB, Bac D, Raja N, Boomsma F, Verdouw PD, Duncker DJ. Minimal impairment of myocardial blood flow responses to exercise in the remodeled left ventricle early after myocardial infarction, despite

significant hemodynamic and neurohumoral alterations. Cardiovasc Res 52: 417–428, 2001.
30. Haitsma DB, Merkus D, Vermeulen J, Verdouw PD, Duncker DJ. Nitric oxide production is maintained in exercising swine with chronic left ventricular dysfunction. Am J Physiol Heart Circ Physiol 282: H2198–H2209, 2002.
31. Hare JM. Nitric oxide and excitation-contraction coupling. J Mol Cell Cardiol 35: 719–729, 2003.
32. Indik JH, Goldman S, Gaballa MA. Oxidative stress contributes to vascular endothelial dysfunction in heart failure. Am J Physiol Heart Circ Physiol 281: H1767–H1770, 2001.
33. Inoue T, Ide T, Yamato M, Yoshida M, Tsutsumi T, Andou M, Utsumi H, Tsutsui H, Sunagawa K. Time-dependent changes of myocardial and systemic oxidative stress are dissociated after myocardial infarction. Free Radic Res 43: 37–46, 2009.
34. Jarrar D, Wang P, Cioffi WG, Bland KI, Chaudry IH. Critical role of oxygen radicals in the initiation of hepatic depression after trauma hemorrhage. J Trauma 49: 879–885, 2000.
35. Jolly SR, Kane WJ, Bailie MB, Abrams GD, Lucchesi BR. Canine myocardial reperfusion injury. Its reduction by the combined administration of superoxide dismutase and catalase. Circ Res 54: 277–285, 1984.
36. Karlsson K, Marklund SL. Extracellular superoxide dismutase in the vascular system of mammals. Biochem J 255: 223–228, 1988.
37. Kobayashi T, Kamata K. Modulation by hydrogen peroxide of noradrenaline-induced contraction in aorta from streptozotocin-induced diabetic rat. Eur J Pharmacol 441: 83–89, 2002.
38. Lancel S, Tissier S, Mordon S, Marechal X, Depontieu F, Scherpereel A, Chopin C, Neviere R. Peroxynitrite decomposition catalysts prevent myocardial dysfunction and inflammation in endotoxemic rats. J Am Coll Cardiol 43: 2348–2358, 2004.
39. Landmesser U, Merten R, Spiekermann S, Buttner K, Drexler H, Hornig B. Vascular extracellular superoxide dismutase activity in patients with coronary artery disease: relation to endothelium-dependent vasodilation. Circulation 101: 2264–2270, 2000.
40. Liu Y, Gutterman DD. Oxidative stress and potassium channel function. Clin Exp Pharmacol Physiol 29: 305–311, 2002.
41. Matoba T, Shimokawa H, Kubota H, Morikawa K, Fujiki T, Kunihiro I, Mukai Y, Hirakawa Y, Takeshita A. Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in human mesenteric arteries. Biochem Biophys Res Commun 290: 909–913, 2002.
42. Merkus D, Houweling B, Mirza A, Boomsma F, van den Meiracker AH, Duncker DJ. Contribution of endothelin and its receptors to the regulation of vascular tone during exercise is different in the systemic, coronary and pulmonary circulation. Cardiovasc Res 59: 745–754, 2003.

43. Merkus D, Houweling B, Zarbanoui A, Duncker DJ. Interaction between prostanoids and nitric oxide in regulation of systemic, pulmonary, and coronary vascular tone in exercising swine. Am J Physiol Heart Circ Physiol 286: H1114–H1123, 2004.
44. Mitsos SE, Askew TE, Fantone JC, Kunkel SL, Abrams GD, Schork A, Lucchesi BR. Protective effects of N-2-mercaptopropionyl glycine against myocardial reperfusion injury after neutrophil depletion in the dog: evidence for the role of intracellular-derived free radicals. Circulation 73: 1077–1086, 1986.
45. Mitsos SE, Fantone JC, Gallagher KP, Walden KM, Simpson PJ, Abrams GD, Schork MA, Lucchesi BR. Canine myocardial reperfusion injury: protection by a free radical scavenger, N-2-mercaptopropionyl glycine. J Cardiovasc Pharmacol 8: 978–988, 1986.
46. Moens AL, Takimoto E, Tocchetti CG, Chakir K, Bedja D, Cormaci G, Ketner EA, Majmudar M, Gabrielson K, Halushka MK, Mitchell JB, Biswal S, Channon KM, Wolin MS, Alp NJ, Paolocci N, Champion HC, Kass DA. Reversal of cardiac hypertrophy and fibrosis from pressure overload by tetrahydrobiopterin: efficacy of recoupling nitric oxide synthase as a therapeutic strategy. Circulation 117: 2626–2636, 2008.
47. Munzel T, Daiber A, Ullrich V, Mulsch A. Vascular consequences of endothelial nitric oxide synthase uncoupling for the activity and expression of the soluble guanylyl cyclase and the cGMP-dependent protein kinase. Arterioscler Thromb Vasc Biol 25: 1551–1557, 2005.
48. Myers ML, Bolli R, Lekich RF, Hartley CJ, Roberts R. N-2-mercaptopropionylglycine improves recovery of myocardial function after reversible regional ischemia. J Am Coll Cardiol 8: 1161–1168, 1986.
49. Pacher P, Obrosova IG, Mabley JG, Szabo C. Role of nitrosative stress and peroxynitrite in the pathogenesis of diabetic complications. Emerging new therapeutical strategies. Curr Med Chem 12: 267–275, 2005.
50. Pacher P, Szabo C. Role of the peroxynitrite-poly(ADP-ribose) polymerase pathway in human disease. Am J Pathol 173: 2–13, 2008.
51. Pagano PJ, Clark JK, Cifuentes-Pagano ME, Clark SM, Callis GM, Quinn MT. Localization of a constitutively active, phagocyte-like NADPH oxidase in rabbit aortic adventitia: enhancement by angiotensin II. Proc Natl Acad Sci USA 94: 14483–14488, 1997.
52. Persad S, Takeda S, Panagia V, Dhalla NS. Beta-adrenoceptor-linked signal transduction in ischemic-reperfused heart and scavenging of oxyradicals. J Mol Cell Cardiol 29: 545–558, 1997.
53. Powers SK, Jackson MJ. Exercise-induced oxidative stress: cellular mechanisms and impact on muscle force production. Physiol Rev 88: 1243–1276, 2008.
54. Rogers PA, Dick GM, Knudson JD, Focardi M, Bratz IN, Swafford AN Jr, Saitoh S, Tune JD, Chilian WM. H2O2-induced redox-sensitive coronary vasodilation is mediated by 4-aminopyridine-sensitive K_ channels. Am J Physiol Heart Circ Physiol 291: H2473–H2482, 2006.

55. Rueckschloss U, Quinn MT, Holtz J, Morawietz H. Dose-dependent regulation of NAD(P)H oxidase expression by angiotensin II in human endothelial cells: protective effect of angiotensin II type 1 receptor blockade in patients with coronary artery disease. Arterioscler Thromb Vasc Biol 22: 1845–1851, 2002.
56. Saitoh S, Zhang C, Tune JD, Potter B, Kiyooka T, Rogers PA, Knudson JD, Dick GM, Swafford A, Chilian WM. Hydrogen peroxide: a feed-forward dilator that couples myocardial metabolism to coronary blood flow. Arterioscler Thromb Vasc Biol 26: 2614–2621, 2006.
57. Schaper W, Remijsen P, Xhonneux R. The size of myocardial infarction after experimental coronary artery ligation. Z Kreislaufforsch 58: 904–909, 1969.
58. Sen CK, Atalay M, Hanninen O. Exercise-induced oxidative stress: glutathione supplementation and deficiency. J Appl Physiol 77: 2177–2187, 1994.
59. Sochman J. N-acetylcysteine in acute cardiology: 10 years later: what do we know and what would we like to know?! J Am Coll Cardiol 39: 1422–1428, 2002.
60. Stamler JS, Lamas S, Fang FC. Nitrosylation: the prototypic redoxbased signaling mechanism. Cell 106: 675–683, 2001.
61. Stralin P, Karlsson K, Johansson BO, Marklund SL. The interstitium of the human arterial wall contains very large amounts of extracellular superoxide dismutase. Arterioscler Thromb Vasc Biol 15: 2032–2036, 1995.
62. Tanaka M, Fujiwara H, Yamasaki K, Sasayama S. Superoxide dismutase and N-2-mercaptopropionyl glycine attenuate infarct size limitation effect of ischaemic preconditioning in the rabbit. Cardiovasc Res 28: 980–986, 1994.
63. Touyz RM, Schiffrin EL. Ang II-stimulated superoxide production is mediated via phospholipase D in human vascular smooth muscle cells. Hypertension 34: 976–982, 1999.
64. Touyz RM, Schiffrin EL. Reactive oxygen species in vascular biology: implications in hypertension. Histochem Cell Biol 122: 339–352, 2004.
65. Tune JD, Richmond KN, Gorman MW, Feigl EO. Control of coronary blood flow during exercise. Exp Biol Med (Maywood) 227: 238–250, 2002.
66. Turko IV, Murad F. Protein nitration in cardiovascular diseases. Pharmacol Rev 54: 619–634, 2002.
67. Turrens JF. Mitochondrial formation of reactive oxygen species. J Physiol 552: 335–344, 2003.
68. van der Giessen WJ, van Woerkens LJ, Saxena PR, Roelandt JR, Verdouw PD. Nisoldipine improves blood flow to skeletal muscles in conscious pigs with chronic heart failure. Eur Heart J 11: 552–559, 1990.
69. van der Velden J, Merkus D, Klarenbeek BR, James AT, Boontje NM, Dekkers DH, Stienen GJ, Lamers JM, Duncker DJ. Alterations in myofilament function contribute to left ventricular dysfunction in pigs early after myocardial infarction. Circ Res 95: e85–e95, 2004.

70. Vasquez-Vivar J, Kalyanaraman B, Martasek P, Hogg N, Masters BS, Karoui H, Tordo P, Pritchard KA Jr. Superoxide generation by endothelial nitric oxide synthase: the influence of cofactors. Proc Natl Acad Sci USA 95: 9220–9225, 1998.
71. Walia M, Samson SE, Schmidt T, Best K, Kwan CY, Grover AK. Effects of peroxynitrite on pig coronary artery smooth muscle. Cell Calcium 34: 69–74, 2003.
72. Xia Y, Tsai AL, Berka V, Zweier JL. Superoxide generation from endothelial nitric-oxide synthase. A Ca2_/calmodulin-dependent and tetrahydrobiopterin regulatory process. J Biol Chem 273: 25804–25808, 1998.
73. Yada T, Shimokawa H, Hiramatsu O, Shinozaki Y, Mori H, Goto M, Ogasawara Y, Kajiya F. Important role of endogenous hydrogen peroxide in pacing-induced metabolic coronary vasodilation in dogs in vivo. J Am Coll Cardiol 50: 1272–1278, 2007.
74. Zhang P, Hou M, Li Y, Xu X, Barsoum M, Chen Y, Bache RJ. NADPH oxidase contributes to coronary endothelial dysfunction in the failing heart. Am J Physiol Heart Circ Physiol 296: H840–H846, 2009.
75. Zima AV, Blatter LA. Redox regulation of cardiac calcium channels and transporters. Cardiovasc Res 71: 310–321, 2006.


Altering
[ET-1]
and [PC]
AF
Exercise
Change in
Endothelial
Function
CPB
MI
HBOC
ROS
(scavenging)

9

ATRIAL NADPH STIMULATED SUPEROXIDE PRODUCTION AND ALTERED REDOX STATE IN THE DEVELOPMENT OF ATRIAL FIBRILLATION AFTER CARDIAC SURGERY

Y. J. Taverne, D.J. Thuijs, C.P. Teuwen, L.A. Blonden, P. Knops, D.J. Duncker, N.M. de Groot, A.J. Bogers, D. Merkus

Submitted 2017

ABSTRACT

Atrial fibrillation after cardiac surgery (POAF) increases patient morbidity and prolongs hospitalization. Recent studies indicate that inflammation and oxidative stress associated with cardiac surgery play a significant role in the pathogenesis of POAF, however, the mechanism remains unclear. We investigated the relationship between preoperative plasma markers of oxidative stress and inflammation, with a particular focus on myocardial nicotinamide adenine dinucleotide phosphate (NADPH) oxidase expression and activity in patients developing POAF.

40 Consecutive cardiac surgery patients were included where age and poor left ventricular function were strong independent predictors for the occurrence of POAF (48%). Fasting blood samples and right atrial appendage were collected before and after cardiopulmonary bypass (CBP). Plasma markers of lipid peroxidation tended to be higher (P=0.09) in the POAF group after CBP. POAF patients had higher preoperative circulating levels of IL-6 (P=0.06), IL-10 (P=0.01) and TNF-α (P=0.07) that correlated with plasma ET-1 (r=0.34, P=0.04) and right atrial appendage NOX2 expression (r=0.48, P=0.03). Logistic regression identified TBARS (odds ratio 1.19; 95% CI 0.99-1.44, P=0.05) and pre-operative NADPH-oxidase activity (odds ratio 1.11; 95% CI 1.03-1.19, P=0.004) as strong independent predictors of POAF.

Preoperative redox imbalance is prevalent in patients with POAF and high levels of pro-inflammatory cytokines stimulate NADPH oxidase to release superoxide. Future studies should focus on the value of (other) plasma markers of oxidative stress, preventing the activation of specific oxidases and tailor ROS alteration to tissue specific production rather that systemic ROS eradication.

INTRODUCTION

Atrial fibrillation after cardiac surgery (POAF) occurs within the first 2 to 5 postoperative days in 35%-50% of patients [1, 2]. AF increases morbidity by predisposing to hemodynamic decline and increased risk of stroke, with concomitant prolonged hospitalization and significant additional health care costs [1].

Despite extensive research and administration of antiarrhythmic therapy and cardiac protection during surgery, the incidence of POAF remains undiminished [3]. Although specific risk factors for POAF such as age, diabetes, length of ischemic period and mechanical damage have been identified, these have failed to be useful predictors of POAF in the clinical setting [1, 3]. Moreover, lack of knowledge concerning the underlying mechanism has hampered effective prevention of POAF [3-5].

Recent studies indicate that inflammation and oxidative stress, associated with cardiac surgery (and cardiopulmonary bypass (CBP) in particular), play a significant role in the development of POAF [6-9]. Ischemia–reperfusion injury during cardiac surgery leads to the formation of reactive oxygen species (ROS), causing oxidative stress and a systemic inflammatory response, contributing to endothelial and myocardial dysfunction [9-13]. Endothelial dysfunction results in an imbalance between production of vasodilators (i.e nitric oxide (NO)) and vasoconstrictors (i.e endothelin-1 (ET-1) and superoxide($O_2^{\bullet -}$)), as well as activation of NADPH-oxidase and 'uncoupling' of endothelial nitric oxide synthase (eNOS). Recent data suggest that both NADPH-oxidase and 'uncoupling' of eNOS contribute significantly to ROS production and may therefore be key factors in the development of POAF [8, 13, 14].

Importantly, an increasing body of evidence suggests that the presence of (pro)inflammatory factors and production of ROS and reactive nitrogen species resulting in oxidative and nitrosative stress during cardiac surgery contribute to a shortening of the atrial effective refractory period predisposing to the development of POAF [15, 16]. It is, however, unclear whether the redox dysbalance predisposing to POAF already exists preoperatively and is aggravated by cardiac surgery or whether it is solely due to cardiac surgery. Moreover, the origin of ROS as well as their role in the ontogenesis of POAF, remains to be elucidated.

In the present pilot study, we hypothesized that preoperative endothelial dysfunction induces a pro-inflammatory state, resulting in a redox imbalance that predisposes to development of AF after an inflammatory insult, i.e. cardiac surgery. We therefore prospectively compared cardiac surgery patients developing POAF to

those remaining in sinus rhythm (SR) and aimed to investigate: (a) to what extent preoperative endothelial dysfunction, as evidenced by a decrease in nitrate/nitrite and an increase in endothelin-1 (ET-1), are associated with the development of POAF; (b) if a preoperative increased inflammatory state and/or redox imbalance predisposes to the development of POAF; and (c) whether NADPH-oxidase expression and activity are increased in the atria of patients with POAF and may contribute to increased production of ROS in the atria.

METHODS

Patient enrollment

This single-institution, prospective cohort study, as approved by the Erasmus Medical and Ethical Review Committee, enrolled forty consecutive patients scheduled for elective primary coronary bypass grafting, valvular surgery (mitral or aortic; equal in both groups), or a combination of the two and using cardiopulmonary bypass (CPB). All patients studied provided informed consent.

Exclusion criteria included a preceding history of AF or other atrial arrhythmias and the consequential usage of anti-arrhythmic drug other than beta-blockers. Further, patients with significant comorbidities such as advanced hepatic disease (cirrhosis) and chronic renal failure were excluded. Standard anesthetic and surgical protocols were performed in all patients. Patient baseline characteristics are presented in Table 1.

Postoperatively, patient's heart rhythm was continuously monitored for a minimum of 92h in SR or longer when AF developed. POAF was defined, according to the guidelines [17], as a sustained period of AF for at least 30s as examined by electrocardiogram requiring electrical or chemical cardioversion. Subjects who developed POAF were compared to patients remaining in SR (Table 1).

N= 40	SR 21 (52%)	AF 19 (48%)	P value
Male gender	16 (76)	11 (57)	NS
Age	65.5 ± 1.4	71.2 ± 1.5	0.04
LVEF (%)	57.1 ± 1.0	52.3 ± 1.4	0.03
Beta-blocker use	17 (81)	12 (63)	NS
Statin use	19 (91)	13 (68)	NS
Previous smokers	12 (57)	14 (74)	NS
Diabetes mellitus	6 (29)	2 (11)	NS
Hypertension	10 (48)	8 (42)	NS

Table 1. Baseline patient characteristics. LVEF; left ventricular ejection fraction. P values non-significant (NS) when P > 0.05. Where appropriate, data are presented ± SEM.

Tissue harvesting and blood sampling

Fasting blood samples were collected in addition to standard blood sampling (2 EDTA tubes) according to our local protocol at T0A (preoperative), T0B (6h postoperative), T1 (24h postoperative), T2 (48h postoperative) and T4 (96h postoperative). After blood collection, blood tubes were placed on ice, immediately centrifuged at 4° Celsius at 3500rpm and stored at -80° Celsius until analysis. Whenever blood samples were not placed immediately on ice, centrifuged and stored at -80° Celsius (for logistic reasons), samples were excluded from the study.

Right atrial appendage samples were harvested as described previously [18], with cold sharp dissection and handled in a non-traumatic fashion. Briefly, two 2-0 braided polyesters sutures (Ti-Cron, Covidien) were placed in the right atrial appendage. Before venous cannulation, the first atrial sample was collected, the venous cannula placed and the proximal purse-string tightened to secure the venous cannula. The distal suture remained loose to allow perfusion with blood and cardioplegia, but also reperfusion after removal of the cross-clamp. The second tissue sample was taken upon removal of the cannula before tightening of the distal purse-string. Both tissue samples were immediately snap frozen in liquid nitrogen until analysis and when enough tissue was available both lucigenin and Q-PCR analyses (see below) were performed.

Tissue and blood analysis

Tissue samples were snap frozen in liquid nitrogen and stored at -80^{o}C until analysis. Plasma samples were aliquoted and stored at -80^{o}C until analysis. All samples were prepared and analyzed following guidelines provided in the manufacturers

protocols. All measurements were carried out by a single investigator (LB) blinded to the patients' post-operative outcome.

Plasma assays

The TBARS assay kit and TNF-α (human) EIA kit and nitrate/nitrite colorimetric assay kit were obtained from Cayman Chemical. Human IL-6 and IL-10 Elisa kits were obtained from Thermo Fisher Scientific and the Endothelin-1 Elisa kit from Enzo Life Sciences. EIA and colorimetric measurements were performed on a Thermo Electron Corporation Multiskan EX plate reader.

Tissue analyses - ROS detection

Protein was extracted from the tissue in ice-cold Krebs-Hepes buffer pH 7.4 (KHB, containing 99 mM NaCl, 4.7 mM KCl, 1.0 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.9 mM $CaCl_2$, 20 mM Hepes, 25 mM $NaHCO_3$, 11.1 mM Glucose) supplemented with complete protease inhibitor cocktail using a PRO2000 homogenizer set at maximum speed with 3 bursts of 10 seconds.

100 µg Protein (Pierce BCA Protein Assay Kit, Thermo Electron Corporation, Multiskan EX plate reader) was assessed for ROS content by lucigenin enhanced chemiluminescence (5 µM, Sigma Aldrich, M80-10) in the absence or presence of 100 µM NADPH (Sigma Aldrich, N750) using a Thermo Electron Corporation, Luminoskan Ascent microplate luminometer. Light emission data (obtained as the sum from 75 measurements, 1000 msec integration time) were normalized to protein content and expressed as Relative Light Units per gram protein (RLU/g).

Tissue analyses - gene expression

Tissue samples were prepared for RNA extraction using a PRO2000 homogenizer set at maximum speed with 3 bursts of 10 seconds. Total RNA was extracted from the homogenates using the protocol for fibrous tissue with the Qiagen RNeasy mini kit, Cat. No. 74104. cDNA was generated from 500 ng RNA (Nanodrop spectrophotometer, Isogen Life Science) using the Bioline SensiFAST cDNA synthese kit, Cat. No. Bio-65054 on a Biometra T-professional thermocycler.

Quantitative PCR was performed using the Bioline SensiMix SYBR & Fluorescein kit, Cat. No. QT615-05 on either a BIO-RAD myiQ iCycler or BIO-RAD CFX, C1000 Touch Thermal Cycler. Relative mRNA gene expression levels were determined using Biogazelle's qBase$^+$ qPCR data analysis package.

The target gene and primer sets are shown in Table 2.

	Target gene
eNOS	*F: GAAGGCGACAATCCTGTATGGC, R: TGTTCGAGGGACACCACGTCAT*
ECE1	*F: GCTCATCTACCACAAAGTGACGG, R: GTCATAGACCACAATAGGCTCGG*
ET_A	*F: CACTTTTCGTGGCACAGAGC, R: CCCACCATTCCCACGATGAA*
ET_B	*F: CAGAAAGCCTCCGTGGGAATCA, R: ACAGCCAGAACCACAGAGACCA*
NOX2	*F: CTCTGAACTTGGAGACAGGCAAA, R: CACAGCGTGATGACAACTCCAG*
NOX4	*F: ACCAGATGTTGGGGCTAGGA, R: CTCCTGGTTCTCCTGCTTGG*
PPET-1	*CTACTTCTGCCACCTGGACATC, R: TCACGGTCTGTTGCCTTTGTGG*
βActin	*F: TCCCTGGAGAAGAGCTACGA, R: AGCACTGTGTTGGCGTACAG*
GAPDH	*F: TGCCAAATATGATGACATCAAGAA, R: GGAGTGGGTGTCGCTGTTG*

Table 2. Target gene set. eNOS, endothelial nitric oxide synthase; ECE, endothelin converting enzyme; ET_A, endothelin receptor type A; ET_B ,endothelin receptor type B; NOX2 and NOX4, nicotinamide adenine dinucleotide phosphate-oxidase isoforms; PPET-1, pre-pro-endothelin-1; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase.

Statistical analysis

Continuous variables were compared using the Student's t-test. Plasma values were assessed with ANOVA for repeated measures followed by post hoc testing (with Bonferroni correction) when appropriate. To identify variables as indices for POAF, a Pearson correlation test was performed. Afterwards, variables were entered in a multiple logistic regression model using block entry of variables. Criterion for entry in the analysis was P≤0.05. Variables that were previously shown to affect the occurrence of POAF (e.g. use of beta-blockers [1] and statins [19]) and affect the NADPH oxidase status (e.g. diabetes mellitus [20]) were also included in the model. No interactions were fitted in the model. Statistical analysis of data was performed with SPSS 22 (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp). Statistical significance was accepted when $P \leq 0.05$. Data are presented as mean ± SEM.

RESULTS

Nineteen patients (48%) developed AF after cardiac surgery (Table 1) with the highest incidence, as previously reported [1, 8, 17], on the second and third post-operative day. No significant differences were detected between patients with POAF and SR with respect to sex, DM, smoking, hypertension and use of beta blockers and

statins. However, patients who developed POAF were slightly older (P = 0.04) and had a worse preoperative left ventricular ejection fraction (LVEF) (P = 0.03) (Table 1). No differences were detected in terms of cross-clamp time and atrial distention between groups (not shown). Age (r = 0.3, P = 0.018) and preoperative LVEF (r = -0.31, P = 0.015) correlated with the incidence of POAF. Multivariate analysis of baseline characteristics and surgical procedure showed age (odds ratio = 1.07, CI: 0.97-1.15; P = 0.05) and EF (odds ratio = 0.9, CI: 0.84-0.99; P = 0.03) as strong independent predictive factors for POAF.

Inflammation and endothelial function

POAF patients tended to have higher pre-surgery plasma levels of the pro-inflammatory cytokines IL-6 (P = 0.06) and TNF- α (P = 0.07) and significantly higher plasma concentration of the anti-inflammatory cytokine IL-10 (P = 0.01) than patients remaining in SR (Figure 1 and 2). IL-6 and IL-10 were strongly correlated both preoperatively (r = 0.60; P < 0.01), and at post-operative day 1 (r = 0.57, P < 0.001). TNF-α was measurable in 5 out of 19 patients that developed AF and in 2 out of 21 patients remaining in SR (P = 0.08). Endothelial dysfunction as indicated by higher levels of circulating ET-1 was more pronounced in the POAF group as compared to SR (Figure 2). However, pre-operative NO, CRP and lactate were similar between groups (Figure 1 and 2).

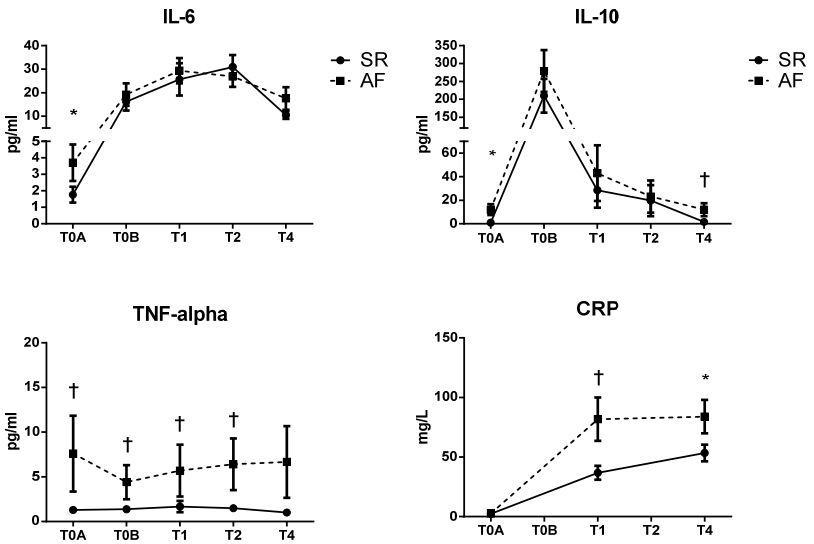


Figure 1. Inflammatory status as measured in blood plasma. * P ≤ 0.05; † P ≤ 0.1, AF versus SR at corresponding time points.

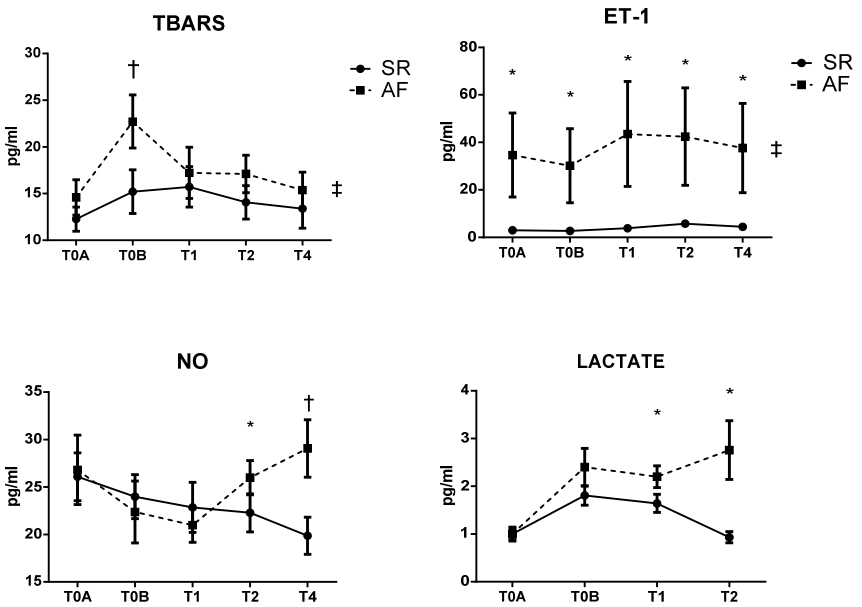


Figure 2. Blood plasma analyses of lipid peroxidation (TBARS) and vasoactive substances. Differences between SR and POAF group at corresponding time points: * P ≤ 0.05; † P ≤ 0.1. ‡ overall difference over time between SR and POAF group with P ≤ 0.05.

CPB activated both pro- and anti-inflammatory pathways in both groups as indicated by the increase in IL-6, CRP and IL-10 after surgery in both groups (Figure 1 and 2). The increase in pro-inflammatory factors was accompanied by in an increase in OS, as indicated by an increase in lipid peroxidation products (TBARS) and a decrease in NO after surgery. The increase in CRP was more pronounced in patients who developed POAF (P = 0.02), moreover, CRP tended to be higher already before onset of POAF at day 1 postoperatively (P = 0.08) (Figure 2).

In the POAF group, overall lipid peroxidation (TBARS) was significantly elevated compared to the SR group (MANOVA P=0.013) (Figure 2). Furthermore, the CPB-induced peak in TBARS tended to be higher in the POAF group after CPB (P = 0.09) however the rest of the individual time-points failed to reach statistical significance between groups. Although both TNF-α and ET-1 were higher in the POAF group, CPB did not significantly affect their levels. Surprisingly, plasma nitrate/nitrate, as an index of NO was higher in the patients with POAF at 48 and 96 hours post-CPB

(Figure 2). This increase in nitrite/nitrate occurred after initiation of the POAF-treatment with amiodaron.

At the end of the hospitalization period, similar to the observations prior to surgery, the levels of IL-6, Il-10, TNF-α and ET-1 all tended to be higher in patients with POAF as compared to patients in SR. In addition, CRP and lactate were more elevated in the patients with POAF. Leukocyte counts did not differ between the two groups (not shown).

All blood plasma variables were fitted into a stepwise regression model where preoperative TBARS concentration was the most important independent predictor of development of postoperative AF, with an odds ratio 1.19; 95% CI 0.99-1.44, P = 0.05.

Oxidative stress and ET in right atrial tissue

NOX-2, but not NOX-4 expression was significantly higher in the atrial tissue of patients that developed POAF (P=0.02) already at baseline (Figure 3). In accordance with the increased NOX-2 expression, the increase (presented as delta) in lucigenin-fluorescence, an index for $O_2^{\bullet-}$ production, upon stimulation with NADPH was significantly larger in right atrial tissue obtained from patients in the POAF group at baseline (P = 0.02) (Figure 4). Moreover, NADPH-stimulated $O_2^{\bullet-}$ production showed significant correlations with higher TNF-α levels (r = 0.48, P = 0.03), CRP (r = 0.83, P = 0.004) and age increments (r = 0.6, P = 0.034). Although there was an inverse correlation between NOX-2 and eNOS (r = -0.39; P = 0.008), neither baseline eNOS expression nor mRNA expression of different components of the ET-system (ECE, ppET, ET_A, ET_B) were different between groups.

Following CPB, the NADPH-induced $O_2^{\cdot-}$ production did not change in patients that would develop POAF, whereas it increased in the patients that would remain in SR (Figure 3). Hence, the difference in NADPH-induced superoxide-production that was present in atrial tissue upon initiation of surgery, disappeared towards the end of CBP. Moreover, ECE, ET_A and NOX-2 expression declined significantly in the POAF group (P = 0.02, P = 0.01 and P = 0.02 respectively). Lower ECE and ET_A expression correlated with NOX-2 (r = 0.31; P = 0.05) and NOX-4 (r = 0.42; P = 0.007), whereas an inverse correlation was seen between eNOS expression and ECE-1 (r = -0.40; P = 0.01) as well as NOX-2 (r = -0.34; P = 0.03). Interestingly, the higher eNOS expression after CPB in the POAF group showed a strong correlation with TBARS concentration (r = 0.83; P < 0.001).

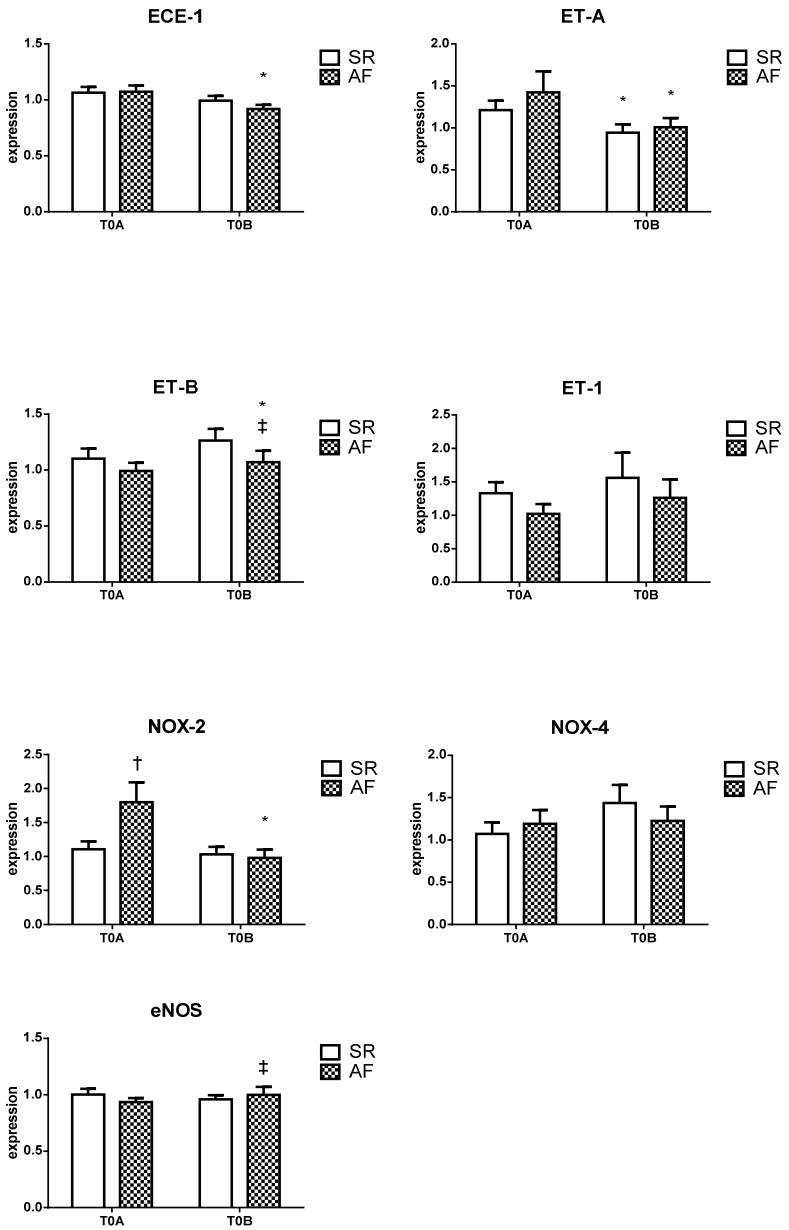


Figure 3. Q-PCR right atrial tissue. Significance levels $P \leq 0.05$; † difference SR and POAF before CPB (T0A), ‡ difference SR and POAF after CPB (T0B), * difference between T0A and T0B within SR or POAF.

Logistic regression identified NADPH-stimulated $O_2^{\bullet-}$ release at baseline (odds ratio 1.11; 95% CI 1.03-1.19, P = 0.004) and NOX-2 expression at baseline (odds ratio 2.36, 95% CI 1.01-5.52, P = 0.04) as the most important independent predictors of POAF.

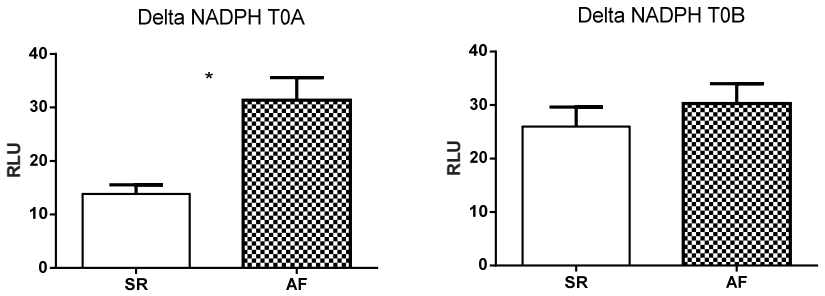


Figure 4. Lucigenin analyses right atrial tissue before (T0A) and after (T0B) cardiopulmonary bypass. Amounts of $O_2^{\bullet-}$ produced from NADPH oxidase before and after CPB. * P ≤ 0.05; difference between SR and AF. $O_2^{\bullet-}$ production after CPB increased significantly in patients remaining in SR (P = 0.02).

DISCUSSION

To our knowledge, this is the first study to analyze the potential link between inflammatory status, oxidative and nitrosative stress and the production of vasoactive substances such as ET-1 and NO, in the development of AF after cardiac surgery. The main findings of our study are that patients developing POAF tend to have higher pre-operative plasma levels of IL-6, IL-10, TNF-α, and ET-1, which are accompanied by increased tissue expression of NOX-2 and higher NADPH-induced $O_2^{\bullet-}$ production as compared to patients remaining in SR. The higher plasma levels of TBARS, TNF-α and ET-1 persist throughout the hospitalization period, whereas CRP and lactate only increase in POAF patients post-surgery. Overall, TBARS, NOX-2 expression and NADPH-stimulated $O_2^{\bullet-}$ release are the most is the most important independent predictors of POAF.

Risk Factors and Atrial Fibrillation

Many studies have tried to identify patient and intraoperative factors that predict the occurrence of AF after cardiac surgery. Only increasing age has been identified as a consistent independent predictor for POAF between different studies [1, 5, 17,

21]. Other factors including male gender [22], diabetes [23, 24], hypertension [1, 22, 25] LVEF [1, 26], preoperative use of beta blockage and statins [27], type of surgery [1, 3] and cross clamp time [1, 25] have been suggested as strong predictors of POAF. However, those risk factors show a large variability between different studies, and therefore fail to consistently and independently predict POAF [5], most likely because multiple factors play a role in the pathogenesis of POAF. Similarly, in our study, albeit small powered, only higher age and worse preoperative LVEF were associated with a higher incidence of POAF whereas the other presumed risk factors did not correlate with the occurrence of POAF. It is important to realize that most of these variables are traits of underlying (patho)physiological processes, and that predictive power may increase for factors that are related to the pathophysiology of AF. Indeed, ageing has been associated with atrial structural electrophysiological changes which may explain its strong predictive power [28, 29]. Moreover, in our study, worse LVEF correlated significantly with higher expression of ECE ($r = -0.38$, $P = 0.016$) and tended to correlate with expression NOX-2 ($r = -0.28$, $P = 0.07$).

Inflammation and atrial fibrillation

Recent studies imply a prominent role of systemic inflammatory response in the pathogenesis of POAF. Thus, steroids [30] and statins [31] that are known for their anti-inflammatory effects, reduce the incidence of POAF. In the present study, we investigated the link between blood inflammatory markers and the stimulation of $O_2^{\bullet-}$ production through NADPH-oxidase in atrial tissue. Our study is the first to show a prominent systemic inflammatory response with a strong association between IL-6 level, TNF-α level, the activation of the complement-CRP pathway, increased expression of NOX-2 and enhanced NADPH-stimulated superoxide production, with development of POAF. Interestingly, the inflammatory response associated with CPB, did not further stimulate NADPH-oxidase to increase superoxide production in the POAF group, although a significant increase was seen in the SR group. These results suggest a worse preoperative inflammatory state that may be linked with oxidative tissue injury through NADPH-oxidase in the development of POAF.

Oxidative stress and atrial fibrillation

Many known global risk factors for POAF can been linked to OS. Moreover, recent studies showed urine biomarkers of OS to be higher post-surgery in patients that developed AF [32], while gene signatures of pathways involved in OS were different in atrial tissue of patients that developed POAF [33]. Oxidative damage to the myocardium has been shown to be involved in electrophysiological remodeling with higher incidence of AF [4, 6, 13]. However, it is still unclear whether OS is in fact the

initiating factor of POAF and what the main source of OS is. NADPH-oxidases have emerged as a potential enzymatic source for ROS in the pathogenesis of atrial remodeling and the prevalence of AF. The NADPH-oxidase family consist of multi-subunit enzymatic complexes where NOX-2 and NOX4 isoforms are primarily expressed in the heart [34, 35]. The present study identifies NOX-2, and not NOX-4, to be upregulated in atrial tissue of patients that developed POAF. Recent controlled clinical trials showing that preventing activation of NADPH oxidases effectively lowers POAF incidence in patients after cardiac surgery [30, 31]. In accordance with the study of Kim et al [13], our data show that NADPH-stimulated superoxide production in the right atrial appendage is the most important independent risk factor for the occurrence of POAF [8, 13]. Hence, together with previous studies [4, 8, 13, 36, 37], our data are consistent with the concept that $O_2^{\bullet-}$ production by NADPH oxidase, and more specific NOX-2, is at the center of POAF pathophysiology.

AF is associated with myocardial OS leading to electrophysiological changes, such as shortening of the effective refractory period which facilitates POAF [8, 27]. In addition, OS may contribute to the endocardial and endothelial dysfunction observed in these patients, which may facilitate thrombus formation in the atrium [12]. Both endocardial and endothelial dysfunction during AF may further contribute to increased production of $O_2^{\bullet-}$ [38]. Excess ROS production results in scavenging of NO, thereby forming peroxynitrite ($ONOO^-$), which may subsequently uncouple eNOS, resulting in production of superoxide instead of NO [39]. However, it remains unclear whether the pathophysiological consequences of increased ROS production are due to myocardial reduction of NO production or due to direct protein oxidation [8]. When ROS production exceeds AO defenses and NO bioavailability is depressed, ET-1 levels increase possibly contributing to further endothelial dysfunction [40, 41]. ET-1 stimulates ROS production in human endothelial cells [42, 43], and cardiac myocytes [44] and both ET_A [42] and ET_B [43] receptors have been reported to be involved in $O_2^{\bullet-}$ production from NADPH stimulation as well as from the mitochondrial transport chain [44]. In our study, ET-1 levels were significantly higher in the POAF group. Since ET-1 is secreted predominantly on the abluminal side and circulating ET-1 measurements not only reflect endothelial and/or endocardial dysfunction in the atrium [45], we measured ECE, ET_A, ET_B and preproET-1 RNA expression in the atrial myocardium. No significant differences between patients who developed POAF and patients in SR were found at the start of CPB, whereas the vasodilator ET_B receptor tended to be decreased at the end of CPD in patients with POAF. These data suggest that activation of the ET-pathway is not the prime stimulus for NADPH-driven ROS production in the atrium of POAF patients.

Both overall systemic OS, measured by the amount of lipid peroxidation (TBARS), and the peak in TBARS following CPB were higher in the POAF group, reflecting the redox imbalance with lower antioxidant capacity in patients developing POAF [4, 6, 9, 13]. Global risk factors like LVEF and age, correlated strongly with the amount of TBARS. A significant increase in oxidative stress, with a concomitant decrease in circulating nitrate/nitrite following CPB was observed in both patients with POAF and patients remaining in SR. Intriguingly, eNOS mRNA correlates with the amount of $O_2^{\bullet-}$ present at the start of CPB, suggesting that eNOS uncoupling may contribute to $O_2^{\bullet-}$ production in the right atrium. Following treatment for AF with amiodarone, plasma levels of NO rose, which is in accordance with previous studies [46] and suggests recoupling of eNOS as a direct effect of amiodarone administration.

The involvement of OS in the multifactorial etiogenesis of POAF is underlined by studies administrating antioxidants, like N-acetylcysteine or statins, showing a decrease in serum levels of molecular markers of oxidative stress in patients undergoing cardiac surgery, that are accompanied by a decrease in the incidence of POAF [27, 47, 48]. Similarly, controlled clinical trials preventing activation of NADPH oxidases by vasoactive substances (f.e. statins [31, 37, 49] and steroids [30]) were effective in preventing POAF in cardiac surgery patients. It should however be noted that reducing oxidative stress in a wide range of cardiovascular diseases has failed to show beneficial effects in large clinical trials [10, 50]. It is therefore important to realize that a decrease in plasma markers of lipid or protein oxidation, may not necessarily reflect a reduction in oxidative injury in the atrial appendages [8]. Thus, a specific focus should be to target the local chain of free radical production instead of trying to eradicate systemic ROS in patients with cardiovascular disease [1, 5, 8, 13, 50].

CONCLUSION AND FUTURE PERSPECTIVES

In this small pilot study, we show that a preoperative redox imbalance is present in patients developing POAF and high levels of pro-inflammatory cytokines stimulate NADPH oxidase to release $O_2^{\bullet-}$ (summarized in figure 5).

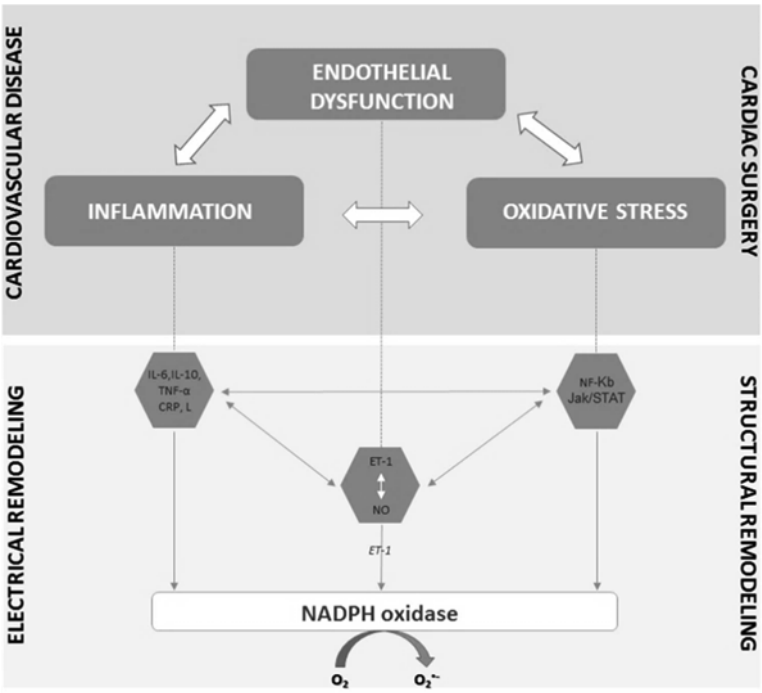


Figure 5. Summary of the suggested different pathways leading to POAF.

The inflammatory stimulus of CPB promotes production of $O_2^{\cdot-}$ from NADPH oxidase in patients remaining in SR, but not in patients developing POAF, possibly indicating a more balanced redox system and functional antioxidant system. Our data suggests that the most important $O_2^{\cdot-}$ producing oxidase is NOX-2 and can be seen as a strong independent predictor of the development of AF after cardiac surgery, independent from type of surgery. Plasma measurement of TBARS seems to be a valuable attribute in the prediction of POAF, and indicates that the altered redox state continues during hospitalization (where eNOS uncoupling could possibly play a role) providing a downward spiral with electrophysiological changes leading to POAF. Given the current data, future studies should focus on the value of other plasma markers of oxidative stress, preventing the activation of specific oxidases (f.e. with apocynin) and tailor ROS alteration to tissue specific production rather that systemic ROS eradication.

REFERENCES

1. Mathew JP, Fontes ML, Tudor IC, Ramsay J, Duke P, Mazer CD, Barash PG, Hsu PH, Mangano DT, Investigators of the Ischemia R, Education F and Multicenter Study of Perioperative Ischemia Research G. A multicenter risk index for atrial fibrillation after cardiac surgery. JAMA. 2004;291:1720-9.
2. Villareal RP, Hariharan R, Liu BC, Kar B, Lee VV, Elayda M, Lopez JA, Rasekh A, Wilson JM and Massumi A. Postoperative atrial fibrillation and mortality after coronary artery bypass surgery. J Am Coll Cardiol. 2004;43:742-8.
3. Auer J, Weber T, Berent R, Ng CK, Lamm G and Eber B. Risk factors of postoperative atrial fibrillation after cardiac surgery. J Card Surg. 2005;20:425-31.
4. Antoniades C, Demosthenous M, Reilly S, Margaritis M, Zhang MH, Antonopoulos A, Marinou K, Nahar K, Jayaram R, Tousoulis D, Bakogiannis C, Sayeed R, Triantafyllou C, Koumallos N, Psarros C, Miliou A, Stefanadis C, Channon KM and Casadei B. Myocardial redox state predicts in-hospital clinical outcome after cardiac surgery effects of short-term pre-operative statin treatment. J Am Coll Cardiol. 2012;59:60-70.
5. Kaireviciute D, Aidietis A and Lip GY. Atrial fibrillation following cardiac surgery: clinical features and preventative strategies. Eur Heart J. 2009;30:410-25.
6. Clermont G, Vergely C, Jazayeri S, Lahet JJ, Goudeau JJ, Lecour S, David M, Rochette L and Girard C. Systemic free radical activation is a major event involved in myocardial oxidative stress related to cardiopulmonary bypass. Anesthesiology. 2002;96:80-7.
7. Levy JH and Tanaka KA. Inflammatory response to cardiopulmonary bypass. Ann Thorac Surg. 2003;75:S715-20.
8. Kim YM, Kattach H, Ratnatunga C, Pillai R, Channon KM and Casadei B. Association of atrial nicotinamide adenine dinucleotide phosphate oxidase activity with the development of atrial fibrillation after cardiac surgery. J Am Coll Cardiol. 2008;51:68-74.
9. Ramlawi B, Otu H, Mieno S, Boodhwani M, Sodha NR, Clements RT, Bianchi C and Sellke FW. Oxidative stress and atrial fibrillation after cardiac surgery: a case-control study. Ann Thorac Surg. 2007;84:1166-72; discussion 1172-3.
10. Taverne YJ, Bogers AJ, Duncker DJ and Merkus D. Reactive oxygen species and the cardiovascular system. Oxid Med Cell Longev. 2013;2013:862423.
11. Lopes RD, Li L, Granger CB, Wang TY, Foody JM, Funk M, Peterson ED and Alexander KP. Atrial fibrillation and acute myocardial infarction: antithrombotic therapy and outcomes. Am J Med. 2012;125:897-905.
12. Cai H, Li Z, Goette A, Mera F, Honeycutt C, Feterik K, Wilcox JN, Dudley SC, Jr., Harrison DG and Langberg JJ. Downregulation of endocardial nitric oxide synthase expression and nitric oxide production in atrial fibrillation:

potential mechanisms for atrial thrombosis and stroke. Circulation. 2002;106:2854-8.

13. Kim YM, Guzik TJ, Zhang YH, Zhang MH, Kattach H, Ratnatunga C, Pillai R, Channon KM and Casadei B. A myocardial Nox2 containing NAD(P)H oxidase contributes to oxidative stress in human atrial fibrillation. Circ Res. 2005;97:629-36.
14. Violi F, Pastori D, Pignatelli P and Loffredo L. Antioxidants for prevention of atrial fibrillation: a potentially useful future therapeutic approach? A review of the literature and meta-analysis. Europace. 2014;16:1107-16.
15. Wijesurendra R and Casadei B. Atrial supply-demand mismatch in atrial fibrillation: The missing link between rapid rate and atrial remodeling? Heart Rhythm. 2015;12:1001-2.
16. Van Wagoner DR. Electrophysiological remodeling in human atrial fibrillation. Pacing Clin Electrophysiol. 2003;26:1572-5.
17. Kirchhof P, Benussi S, Kotecha D, Ahlsson A, Atar D, Casadei B, Castella M, Diener HC, Heidbuchel H, Hendriks J, Hindricks G, Manolis AS, Oldgren J, Popescu BA, Schotten U, Van Putte B, Vardas P, Agewall S, Camm J, Baron Esquivias G, Budts W, Carerj S, Casselman F, Coca A, De Caterina R, Deftereos S, Dobrev D, Ferro JM, Filippatos G, Fitzsimons D, Gorenek B, Guenoun M, Hohnloser SH, Kolh P, Lip GY, Manolis A, McMurray J, Ponikowski P, Rosenhek R, Ruschitzka F, Savelieva I, Sharma S, Suwalski P, Tamargo JL, Taylor CJ, Van Gelder IC, Voors AA, Windecker S, Zamorano JL and Zeppenfeld K. 2016 ESC Guidelines for the management of atrial fibrillation developed in collaboration with EACTS. Eur J Cardiothorac Surg. 2016;50:e1-e88.
18. Ramlawi B, Feng J, Mieno S, Szabo C, Zsengeller Z, Clements R, Sodha N, Boodhwani M, Bianchi C and Sellke FW. Indices of apoptosis activation after blood cardioplegia and cardiopulmonary bypass. Circulation. 2006;114:I257-63.
19. Maack C, Kartes T, Kilter H, Schafers HJ, Nickenig G, Bohm M and Laufs U. Oxygen free radical release in human failing myocardium is associated with increased activity of rac1-GTPase and represents a target for statin treatment. Circulation. 2003;108:1567-74.
20. Guzik TJ, Mussa S, Gastaldi D, Sadowski J, Ratnatunga C, Pillai R and Channon KM. Mechanisms of increased vascular superoxide production in human diabetes mellitus: role of NAD(P)H oxidase and endothelial nitric oxide synthase. Circulation. 2002;105:1656-62.
21. Schnabel RB, Yin X, Gona P, Larson MG, Beiser AS, McManus DD, Newton-Cheh C, Lubitz SA, Magnani JW, Ellinor PT, Seshadri S, Wolf PA, Vasan RS, Benjamin EJ and Levy D. 50 year trends in atrial fibrillation prevalence, incidence, risk factors, and mortality in the Framingham Heart Study: a cohort study. Lancet. 2015;386:154-62.
22. Aranki SF, Shaw DP, Adams DH, Rizzo RJ, Couper GS, VanderVliet M, Collins JJ, Jr., Cohn LH and Burstin HR. Predictors of atrial fibrillation after coronary

artery surgery. Current trends and impact on hospital resources. Circulation. 1996;94:390-7.
23. Echahidi N, Mohty D, Pibarot P, Despres JP, O'Hara G, Champagne J, Philippon F, Daleau P, Voisine P and Mathieu P. Obesity and metabolic syndrome are independent risk factors for atrial fibrillation after coronary artery bypass graft surgery. Circulation. 2007;116:I213-9.
24. Zacharias A, Schwann TA, Riordan CJ, Durham SJ, Shah AS and Habib RH. Obesity and risk of new-onset atrial fibrillation after cardiac surgery. Circulation. 2005;112:3247-55.
25. Almassi GH, Schowalter T, Nicolosi AC, Aggarwal A, Moritz TE, Henderson WG, Tarazi R, Shroyer AL, Sethi GK, Grover FL and Hammermeister KE. Atrial fibrillation after cardiac surgery: a major morbid event? Ann Surg. 1997;226:501-11; discussion 511-3.
26. Choi YS, Shim JK, Hong SW, Kim DH, Kim JC and Kwak YL. Risk factors of atrial fibrillation following off-pump coronary artery bypass graft surgery: predictive value of C-reactive protein and transfusion requirement. Eur J Cardiothorac Surg. 2009;36:838-43.
27. Carnes CA, Chung MK, Nakayama T, Nakayama H, Baliga RS, Piao S, Kanderian A, Pavia S, Hamlin RL, McCarthy PM, Bauer JA and Van Wagoner DR. Ascorbate attenuates atrial pacing-induced peroxynitrite formation and electrical remodeling and decreases the incidence of postoperative atrial fibrillation. Circ Res. 2001;89:E32-8.
28. Kojodjojo P, Kanagaratnam P, Markides V, Davies DW and Peters N. Age-related changes in human left and right atrial conduction. J Cardiovasc Electrophysiol. 2006;17:120-7.
29. Mariscalco G, Engstrom KG, Ferrarese S, Cozzi G, Bruno VD, Sessa F and Sala A. Relationship between atrial histopathology and atrial fibrillation after coronary bypass surgery. J Thorac Cardiovasc Surg. 2006;131:1364-72.
30. Halonen J, Halonen P, Jarvinen O, Taskinen P, Auvinen T, Tarkka M, Hippelainen M, Juvonen T, Hartikainen J and Hakala T. Corticosteroids for the prevention of atrial fibrillation after cardiac surgery: a randomized controlled trial. JAMA. 2007;297:1562-7.
31. Patti G, Chello M, Candura D, Pasceri V, D'Ambrosio A, Covino E and Di Sciascio G. Randomized trial of atorvastatin for reduction of postoperative atrial fibrillation in patients undergoing cardiac surgery: results of the ARMYDA-3 (Atorvastatin for Reduction of MYocardial Dysrhythmia After cardiac surgery) study. Circulation. 2006;114:1455-61.
32. Wu JH, Marchioli R, Silletta MG, Masson S, Sellke FW, Libby P, Milne GL, Brown NJ, Lombardi F, Damiano RJ, Jr., Marsala J, Rinaldi M, Domenech A, Simon C, Tavazzi L and Mozaffarian D. Oxidative Stress Biomarkers and Incidence of Postoperative Atrial Fibrillation in the Omega-3 Fatty Acids for Prevention of Postoperative Atrial Fibrillation (OPERA) Trial. J Am Heart Assoc. 2015;4.

33. Kertai MD, Qi W, Li YJ, Lombard FW, Liu Y, Smith MP, Stafford-Smith M, Newman MF, Milano CA, Mathew JP, Podgoreanu MV, Duke Perioperative G and Safety Outcomes Investigative T. Gene signatures of postoperative atrial fibrillation in atrial tissue after coronary artery bypass grafting surgery in patients receiving beta-blockers. J Mol Cell Cardiol. 2016;92:109-15.
34. Maejima Y, Kuroda J, Matsushima S, Ago T and Sadoshima J. Regulation of myocardial growth and death by NADPH oxidase. J Mol Cell Cardiol. 2011;50:408-16.
35. Youn JY, Zhang J, Zhang Y, Chen H, Liu D, Ping P, Weiss JN and Cai H. Oxidative stress in atrial fibrillation: an emerging role of NADPH oxidase. J Mol Cell Cardiol. 2013;62:72-9.
36. Adam O, Frost G, Custodis F, Sussman MA, Schafers HJ, Bohm M and Laufs U. Role of Rac1 GTPase activation in atrial fibrillation. J Am Coll Cardiol. 2007;50:359-67.
37. Reilly SN, Jayaram R, Nahar K, Antoniades C, Verheule S, Channon KM, Alp NJ, Schotten U and Casadei B. Atrial sources of reactive oxygen species vary with the duration and substrate of atrial fibrillation: implications for the antiarrhythmic effect of statins. Circulation. 2011;124:1107-17.
38. Dudley SC, Jr., Hoch NE, McCann LA, Honeycutt C, Diamandopoulos L, Fukai T, Harrison DG, Dikalov SI and Langberg J. Atrial fibrillation increases production of superoxide by the left atrium and left atrial appendage: role of the NADPH and xanthine oxidases. Circulation. 2005;112:1266-73.
39. Taverne YJ, de Beer VJ, Hoogteijling BA, Juni RP, Moens AL, Duncker DJ and Merkus D. Nitroso-redox balance in control of coronary vasomotor tone. J Appl Physiol (1985). 2012;112:1644-52.
40. Lerman A, Holmes DR, Jr., Bell MR, Garratt KN, Nishimura RA and Burnett JC, Jr. Endothelin in coronary endothelial dysfunction and early atherosclerosis in humans. Circulation. 1995;92:2426-31.
41. Bohm F and Pernow J. The importance of endothelin-1 for vascular dysfunction in cardiovascular disease. Cardiovasc Res. 2007;76:8-18.
42. Dong F, Zhang X, Wold LE, Ren Q, Zhang Z and Ren J. Endothelin-1 enhances oxidative stress, cell proliferation and reduces apoptosis in human umbilical vein endothelial cells: role of ETB receptor, NADPH oxidase and caveolin-1. Br J Pharmacol. 2005;145:323-33.
43. Duerrschmidt N, Wippich N, Goettsch W, Broemme HJ and Morawietz H. Endothelin-1 induces NAD(P)H oxidase in human endothelial cells. Biochem Biophys Res Commun. 2000;269:713-7.
44. Deng W, Baki L and Baumgarten CM. Endothelin signalling regulates volume-sensitive Cl- current via NADPH oxidase and mitochondrial reactive oxygen species. Cardiovasc Res. 2010;88:93-100.
45. Taverne YJ, de Wijs-Meijler DP, Te Lintel Hekkert M, Moon-Massat PF, Dube GP, Duncker DJ and Merkus D. Normalization of Hemoglobin-based Oxygen Carrier-201 Induced Vasoconstriction: Roles of nitric oxide scavenging and endothelin. J Appl Physiol (1985). 2017:jap 00677 2016.

46. Uysal A, Azak S, Colak MC, Burma O, Ozguler IM, Ustundag B and Bayar MK. Perioperative High-Dose Amiodarone Elevates Nitric Oxide Levels in Patients Undergoing Coronary Artery Bypass Surgery. Biomedical Research. 2013;24:486-492.
47. Ozaydin M, Peker O, Erdogan D, Kapan S, Turker Y, Varol E, Ozguner F, Dogan A and Ibrisim E. N-acetylcysteine for the prevention of postoperative atrial fibrillation: a prospective, randomized, placebo-controlled pilot study. Eur Heart J. 2008;29:625-31.
48. Tossios P, Bloch W, Huebner A, Raji MR, Dodos F, Klass O, Suedkamp M, Kasper SM, Hellmich M and Mehlhorn U. N-acetylcysteine prevents reactive oxygen species-mediated myocardial stress in patients undergoing cardiac surgery: results of a randomized, double-blind, placebo-controlled clinical trial. J Thorac Cardiovasc Surg. 2003;126:1513-20.
49. Wassmann S, Laufs U, Muller K, Konkol C, Ahlbory K, Baumer AT, Linz W, Bohm M and Nickenig G. Cellular antioxidant effects of atorvastatin in vitro and in vivo. Arterioscler Thromb Vasc Biol. 2002;22:300-5.
50. Griendling KK and FitzGerald GA. Oxidative stress and cardiovascular injury: Part II: animal and human studies. Circulation. 2003;108:2034-40.

10

DISCUSSION AND FUTURE PERSPECTIVES

GENERAL DISCUSSION

The aim of this thesis was to study the intricate interplay between nitric oxide (NO), endothelin-1 (ET-1) and reactive oxygen species (ROS), which together form an important regulatory system for vascular function. Disruption of this balance is considered to play a major role in development and progression of vascular dysfunction and, consequently, in many forms of cardiovascular disease. Despite great progress in the field of free radical biology and advances in cardiovascular medicine, we still do not have a complete understanding of the underlying mechanisms of cardiovascular disease and consequences of pathophysiological elevated ROS in cardiovascular tissue. Therefore, the interrelationship of these pathways, and more specifically NO-ET-ROS, was investigated under different conditions of endothelial (dys)function in animal models and patients. This final chapter discusses these results in a broader context and presents directives for future research implementing these findings and possibly providing new insights in physiology and pathophysiology of radical biology in cardiovascular medicine. The first part of the discussion involves the idiosyncrasy in aerobe evolution that encompasses the switch to oxidative phosphorylation and consequential production of free radicals. Using oxygen as a terminal electron acceptor evolved over millions of years and lies at the source of for example ischemic heart disease. Studying the origins of our oxidative metabolism helps to gain more insight in these ancient messenger systems. We then proceed implementing this knowledge into redox biology and the development of cardiovascular disease. Second, a series of studies shed light on second messenger pathways under conditions of healthy endothelium, however, challenged by physical exercise and pharmacological interference, thereby stressing endothelial functionality. Finally, we examined the diseased endothelium with altered vascular control with effects on the remote myocardium.

EVOLUTION, REDOX BIOLOGY AND CARDIOVASCULAR DISEASE

A hallmark of vertebrate evolution is the development of endothelial lining of the circulatory system, which is defined by a layer of epithelial cells expressing baso-apical polarity. This intricate part of the cardiovascular system evolved in an ancestral vertebrate some 540-510 million years ago to optimize flow dynamics, barrier function and localize immune and coagulation functions [1]. The endothelium serves to maintain vascular homeostasis through multiple complex interactions with cells in the vessel wall, lumen and surrounding tissue [1]. In a quiescent state, endothelial cells balance the release of various vasodilating factors (like NO, prostanoids and presumably hydrogen peroxide (H_2O_2)) and vasoconstricting factors (for example ET, superoxide ($O_2^{\bullet -}$) and peroxynitrate ($ONOO^-$)) to couple blood flow to metabolic demand and thus maintain tissue homeostasis [2,3]. Within this complex interplay of endothelial derived factors lies an evolutionary trade-off, since the potential of growth of an organism is limited by the potential of oxygen and nutrient delivery to all cells. Thus, at some point, cells will have reached their maximum size above which their metabolic demands can no longer be met by diffusion, mandating a circulatory system [4].

Given the necessity of a closed and interactive endothelialized system within concomitant development of endothermia, another major evolutionary trade-off for early life had to develop. Higher energy demands mandated the usage of O_2 to produce ATP and thus allowing for a more energy-efficient metabolic pathway. However, O_2 is also a highly toxic agent, and evolution has generated an armamentarium of coping mechanisms, including anti-oxidants, to curtail its toxic effects. This necessary adaptation could possibly explain why evolution of complex life stalled for about one billion years (called the “Boring Billion” between 1.8 and 0.8 billion of years ago in Earth’s history). Interestingly, this period is compacted between the two great oxidation events and, although some important events occurred -such as development of eukaryotic cells and evolution of multicellularity- evolution of life during this period was relatively slow (as compared to the Cambrian explosion). Evidence accumulates demonstrating that early antioxidant systems, such as the nuclear factor erythroid 2-related factor 2 (Nrf2) evolved to overcome the metabolic toxicity of oxygen [5] possibly explaining the “delay” in evolution.
Moreover, just as the thermo-dynamical properties of O_2 were harnessed for aerobic metabolism during evolution, so have the chemical properties of ROS been adopted for the purpose of cell signalling and regulation, hence forming an intricate part of the evolution of animal life. In order to fully understand the overall

importance of free radicals in human physiology, evolution of ROS and their generation must be explored; even throughout early Earth's history. Therefore, Chapter 2 provides an overview of O_2 derived free radicals, their discovery, chemistry, and physiological role in the evolution of complex life. O_2 and its radicals present the ultimate paradox to life and evolution—as they encompass both challenges and opportunities for life. Even in the absence of biological O_2 production early in Earth's history, a reactive species, H_2O_2, was created providing an opportunity for early life through the release of O_2 into the atmosphere as a waste product. Likely due to its favourable thermodynamic properties, O_2 was selected as terminal electron acceptor in the reduction of carbon-based reactions that created ATP, but this step also resulted in the production of ROS during oxidative phosphorylation. While toxic levels of ROS detoxified through the development of antioxidant defence mechanisms, lower ROS levels were actually integrated into cellular signalling processes and, through evolution, have become part of normal cellular homeostasis. However, current research still focuses on antioxidants as the 'holy grail' in combatting disease and delaying ageing thereby ignoring the physiologically beneficial roles of ROS in many cellular processes. Consequently, focus must now be placed on a more thorough understanding of the physiological contributions of ROS during homeostasis, which enables possible beneficial interactions of organisms with their surroundings and enhance their survival. As aerobic physiology both challenged and enhanced eukaryote diversity, O_2 toxicity led to extinction of species unable to cope or limited their distribution to anaerobic realms of our planet. Thus, O_2 and ROS truly can be understood as essential consequences and drivers of evolution and survival throughout Earth's history.

It is increasingly recognized that oxidative phosphorylation is tightly coupled to O_2 concentration. As presented in Chapter 2, O_2 bursts in Early Earth led to the extinction of species unable to cope with the atmospheric rise in oxygen. The introduction of O_2 highlights the great evolutionary adaptability of early life forms, as some organisms found a way to use this toxic substance to their advantage. Enzymatic reduction of O_2 yielded a large increase in energy production capacity, allowing the evolution of multi-cellular animal life. However, notwithstanding the benefit of using O_2, it is still a very toxic agent that requires antioxidants to curtail its toxicity. Invariably, all organisms—from the simplest bacteria to complex mammals—adapt to oxidative stress by rapidly increasing their production of antioxidants and repair enzymes [6, 7]. Thus, in mammals, a set of ~40 genes is promptly activated in a highly coordinated response to oxidative stress [6-8]. The use of O_2 and ROS in normal physiology clearly presents a conundrum for understanding fitness and survival of mammalian species [9, 10]. A possible

explanation for this paradox could be antagonistic pleiotropy [11]. This concept is based on that idea that genes are selected because of their beneficial effects during early life, which can mediate deleterious effects in later life [11]. As such, complexity of mammalian DNA has been affected by aerobic metabolism, including aerobic endurance [12]. Evidence suggests that the use of O_2 as a final electron acceptor in the mitochondrial chain played a significant role in evolution of Homo sapiens [13] as endurance running allowed hominem—possibly from the time of H. erectus [14]—to hunt and thus providing enough proteins for physiological development [10].

On the other hand, building an ATP-yielding mechanism through oxidative phosphorylation dictates the necessity of a constant flow of O_2 to metabolic active tissue. When blood flow is hampered, due to for example endothelial dysfunction leading to hypoxia and ischemia, a state of intracellular reduction-oxidation imbalance (called oxidative stress) occurs. In Chapter 3, the apparent paradoxical role of ROS within cellular physiology was analysed and described. Under normal circumstances, ROS concentrations are tightly controlled by antioxidants, keeping them in the picomolar range [15]. These low concentrations of ROS enable their role as second messengers in signal transduction for vascular homeostasis and cell signaling. When excessively produced, or when antioxidants are depleted, ROS can inflict damage onto lipids, proteins, and DNA. This intracellular reduction-oxidation imbalance can subsequently contribute to the development and/or progression of cardiovascular diseases such as atherosclerosis, ischemia-reperfusion injury, chronic ischemic heart disease, cardiomyopathy, congestive heart failure, and arrhythmias [15].

Due to their very short half-life and the technical difficulties of measuring ROS in vivo, little is known about the “safe margins” of ROS concentrations in cell signaling. Therefore, it is difficult to estimate which part of ROS production contributes to cellular homeostasis and normal physiological functioning and at what time ROS production becomes excessive and thereby detrimental. Although deleterious effects of ROS can potentially be reduced by restoring the imbalance between production and clearance of ROS through administration of antioxidants, the dosage and type of antioxidants should be tailored to the location and nature of oxidative stress. Continuous administration of antioxidants in vivo can be unfavorable for normal cell signaling which, at least partially, explains the lack of clinical evidence on

beneficial actions of antioxidant administration. Further research should focus on matching antioxidant therapy to oxidant stress present in the cardiovascular system, while in vitro studies are extremely important to obtain knowledge on the mechanisms of oxidative damage as well as potential repair mechanisms. However, when extrapolating those results to the in vivo setting, caution should be warranted. Nevertheless, fundamental research focusing on second messenger pathways in health and disease, is likely to contribute to the improvement of therapeutic strategies for cardiovascular disease. Chapter 2 & 3 are summarized in Figure 1.

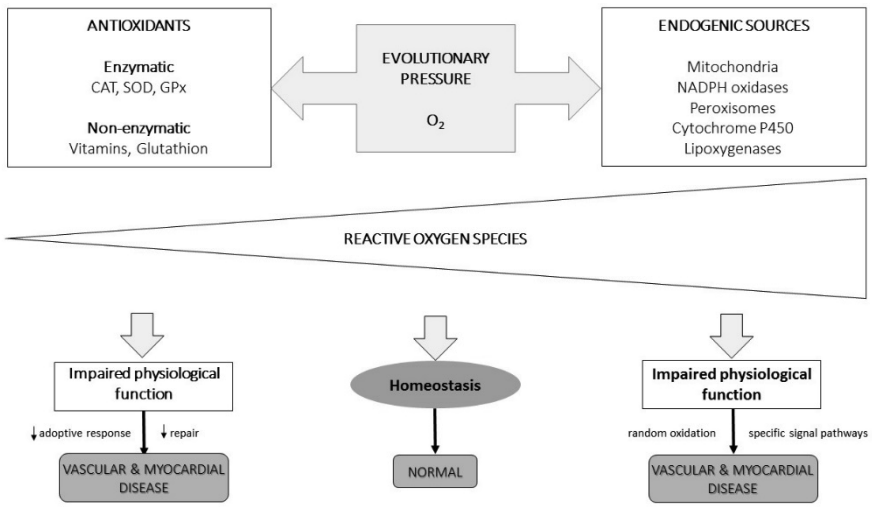


Figure 1. Evolutionary pressure leading to the delicate redox balancing system. Intricate pathways that evolved during evolution of multicellular life maintaining free radical concentrations in the picomolar range in order to preserve cellular homeostasis. Cardiovascular disease is apparent when ROS production exceeds antioxidant capacity or antioxidant production is hampered. CAT, catalase; SOD, superoxide dismutase; CPx, glutathione peroxidase; NADPH, nicotinamide adenine dinucleotide phosphate.

ENDOTHELIAL CONTROL OF VASOREACTIVITY IN HEALTH

Not all free radicals are considered to be deleterious and as stated, concentration and temporal aspects are of importance. One key player in redox reactions but also vasoreactivity is NO, which causes vasodilation. Its major opponent, ET-1, forms an integrated pathway with NO creating a diametrical opposite. In addition to being a potent vasoconstrictor, ET-1 has also been described as a potent stimulator of

oxidative stress [16, 17], thereby initiating a possible silencing mechanism for the NO dependent inhibition of ET-1. ET-1 is one of the most potent vasoconstrictors known and several studies have shown that ET receptor blockade results in systemic and coronary vasodilation [18-20] and that ET contributes to basal vascular tone in the systemic vasculature, as well as the coronary circulation. Our laboratory previously demonstrated [18] that recruitment of coronary flow reserve during exercise is mediated, at least in part, through withdrawal of the vasoconstrictor effect of ET on the coronary vasculature. Therefore, in **Chapter 4**, we investigated the hypothesis that exercise-induced blunting of ET-mediated vasoconstriction in the coronary circulation results from a decrease in ET production, a decrease in ET receptor sensitivity, or a combination of these two effects. Previous work already established that its precursor (Big ET), has little, if any, direct vasomotor effects, and that the vasoconstriction induced by Big ET is dependent on its conversion to ET [21-24]. Hence, the magnitude of constriction to Big ET-1 approximates the extent of ET production at the vascular wall (assuming no change in ET receptor sensitivity). Comparison of the vasoconstrictor effects of Big ET-1 and ET-1 in vivo, therefore, allows examination of the ET system, with the former reflecting ET production and the latter representing ET receptor sensitivity. Thus by comparison of these two substances, we could delineate whether waning from ET-induced coronary vasoconstriction during exercise resulted from a decreased production and / or decreased receptor sensitivity. The main findings were that, while the coronary vasoconstrictor effect of ET was maintained, the coronary vasoconstrictor effect of Big ET was attenuated during exercise. These differences were not the result of systemic influences, as systemic vasoconstrictor effects of ET and Big ET were comparable and rest and during exercise. Together, these findings indicate that, during exercise, in the coronary vasculature, ET receptor sensitivity is maintained, while the conversion of Big ET to ET is blunted.

Coupling blood flow to metabolism requires vasomotor regulation for adequate delivery of O_2 to metabolically active organ systems. In **Chapter 4**, we already discussed the adaptation in the ET-1 pathway allowing for metabolic hyperemia, however, current consensus states that a multitude of pathways act in concert. Recent years, one pathway gained much attention possibly coupling oxygen consumption to the production of an endothelial derived relaxing factor [25]. H_2O_2 production by cardiomyocytes has been shown to be augmented with increased metabolism and is capable to induce coronary vasodilation [26, 27]. Indeed, H_2O_2, created from the reduction of $O_2^{\bullet -}$, has all the properties to be a metabolic dilator, such as a short half-life (due to its brake down by catalase) and the fact that it is

freely permeable [28, 29]. However, in order to fulfill this role, it needs to be shown that augmenting the production of H_2O_2 or inhibiting its breakdown impact metabolic vasodilation. Thus in **Chapter 5**, we first examined the overall effects of ROS on systemic, pulmonary and coronary vascular tone during exercise hyperemia by scavenging all ROS. Subsequently we investigated whether increasing the production and / or decreasing breakdown of H_2O_2 would indeed affect exercise-induced vasodilation. Despite extensive research showing that exercise increases ROS production [30-32], more specific mitochondrial $O_2^{\bullet-}$ production, our study showed no alteration in vasoreactivity leading to exercise hyperemia concurring with our results presented in **Chapter 8**. However, caution is warranted and a number of aspects need to be addressed. First, it is likely that adoptive antioxidant systems are well established and fully functional keeping ROS in the picomolar range [14, 33, 34]. Second, it is possible that, despite the O_2 flux generated during exercise (as high as 100x higher than resting values in contracting muscles), concomitant ROS production (especially $O_2^{\bullet-}$), is very low. Mitochondria have long been the potential source with a 50- or 100-fold increase in $O_2^{\bullet-}$ generation by skeletal muscles during aerobic contractions [35-37]. However, Brand and colleagues [38] have recently reassessed the rate of production of ROS by mitochondria and have concluded that the upper estimate of the total fraction of oxygen utilized that forms $O_2^{\bullet-}$ was ~0.15%; a value that is several orders of magnitude lower than the original estimate of 2–5% [39]. Overall, $O_2^{\bullet-}$ appears not to be the prime coupler of increased oxygen demand to increased blood flow during exercise in healthy swine, nor does it seem to be produced in such amounts that adding antioxidants, above a fully functioning redox system, would result in further vasodilation and increased blood flow. Nonetheless, these findings do not exclude ROS involvement in exercise hyperemic responses. Contrasting, it is well documented that upon the onset of cardiovascular disease, and more specific coronary artery disease, there is a switch from the NO/prostanoids pathway to a mitochondrial derived H_2O_2 dependent mechanism [40, 41]. Indeed, the group of Thengchaisri and coworkers reported restoration of coronary vasodilation during exercise training in Yucatan miniature swine models with coronary occlusion, which was associated with the activation of nitric oxide synthase and where H_2O_2 plays an important role [42]. Thus, it is conceivable to assume that ROS, and H_2O_2 more specific, are important players in cellular homeostasis and exercise hyperemia, however, in healthy subjects, enough antioxidant pathways exist keeping redox states balanced. Clearly, exercise does not potentiate an oxidative stress reaction strong enough to be detected or attenuated in normal vascular physiology. However, the lack of effect of pharmacologically modulating a particular vasodilator mechanism of exercise hyperemia may also be

due to compensation by other vasodilator substances [43], rather than excluding the involvement of ROS. Thus, it is possible that only when endothelium function becomes impaired, vasodilatory signaling pathways switch to a ROS mediated mechanism in exercise hyperemia. The results of **Chapter 4 & 5** are summarized in Figure 2.

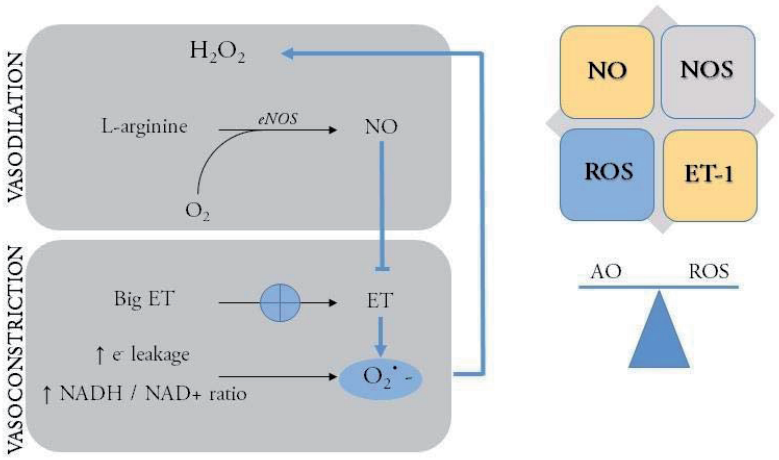


Figure 2. Effects of exercise on coronary and systemic circulation. Nitric oxide (NO) inhibits endothelin-1 (ET-1) production while ET-1 itself stimulates production of superoxide ($O_2^{\bullet-}$) (blue arrow) from NADPH oxidase. $O_2^{\bullet-}$ produced (from NADPH oxidase or due to mitochondrial leak) is quickly catalyzed into hydrogen peroxide (H_2O_2) (blue line). Conversion of Big ET to ET is blunted during exercise in the coronary circulation (blue circle), while systemic vasoconstrictor effects of ET and Big ET are maintained and comparable during rest and exercise. During exercise hyperemia, cellular homeostasis is preserved due to a fully functional antioxidant system with minimal production of reactive oxygen species. Also, the amount of $O_2^{\bullet-}$ produced is quickly converted into H_2O_2 which is presumed to be a vasodilatory substance. Main players in exercise hyperemia are NO and ET-1 forming a diametrical opposite (yellow boxes). Free radical pathway is important during exercise, however, the amounts of ROS produced are kept within physiological range (blue box) with an intact redox balance. Nitric oxide synthase (NOS), and endothelial derived NOS (eNOS), remains coupled and therefore functional.

When vasomotor function is impaired, for example by reduced generation of NO, the presence of oxidative stress or upregulation of ET; vasoreactivity shifts toward a reduced vasodilation, a pro-inflammatory state and prothrombotic properties. This phenomenon, termed endothelial dysfunction, can result from and / or contribute to several disease processes, such as hypertension, coronary artery disease, chronic

heart failure, peripheral artery disease and diabetes (**Chapter 3)**. It is important to notice that every injury to the arterial endothelium represents a primary event in atherogenesis. Conversely, it is possible for the endothelium to undergo transient states of dysfunction without morphological damage. As presented in **Chapter 5**, during exercise, endothelial function is challenged, however, metabolic demands can be met by a fully functional second messenger and antioxidant system [14]. Also, states of endothelial dysfunction can be the inadvertent consequence of therapeutic interventions and pharmacological interference. One example of such a substance is hemoglobin-based oxygen carrier (HBOC)-201, studied in **Chapter 6**. It is a cell- and endotoxin free, glutaraldehyde-polymerized hemoglobin solution produced by chemical modification of hemoglobin extracted from isolated bovine red blood cells [44]. HBOC's may be used in the treatment of cardiovascular disorders and for the priming of heart-lung machines. However, the most important side effect, impeding widespread clinical usage, is systemic and pulmonary vasoconstriction [45, 46]. Pressor effects of HBOC-201 have been ascribed to scavenging NO and ROS formation due to the oxidative burst by oversupplying oxygen [47-52]. ROS can further scavenge NO (see also **Chapter 3 & 8**) and this scavenging interferes with the NO-mediated inhibition of the conversion from pre-endothelin to endothelin (**Chapter4**), thereby increasing vasoconstrictor potential [53-55]. In **Chapter 6**, different pathways leading to transient endothelial dysfunction with systemic and pulmonary pressor effects after HBOC-201 administration, were studied where we determined the possibility for pressure effects to be reversed by administrating an NO-independent dilator and addressed the potential roles of NO scavenging, ROS and / or ET in exercising swine. Our results show that HBOC-201 can disrupt hemodynamic homeostasis, mimicking some aspects of endothelial dysfunction, resulting in systemic and pulmonary hypertension as a result of vasoconstriction which was not altered by exercise. Pressure responses are mediated by scavenging of NO and possibly by up-regulation of ET production. Pressor effects can be restored by NO donors (NTG) or direct vasodilators (adenosine), but dosages must be carefully monitored to avoid hypotension. On the other hand, ET receptor blockade with tezosentan prevented the HBOC-201-induced pressor responses in the systemic and pulmonary vasculature, while ROS scavenging tended to reduce the pressure responses only in the systemic but not in the pulmonary vasculature. As hemodynamic normalization was more easily achieved via administration of an ET receptor blocker, future studies should focus on long-acting ET_A receptor agonists (e.g. ambrisentan or sitaxentan). Furthermore, despite the fact that free radicals do not appear to play a significant role in HBOC-201 induced pressure responses in healthy subjects, it is

clear that they are embedded in the mechanics of these responses. Therefore, further research on ROS and HBOC-201 induced vasoconstriction in subjects with documented pre-existing endothelial dysfunction (e.g. atherosclerosis) would be of interest. Figure 3 summarizes different vasoactive pathways during transient endothelial dysfunction based on pharmacological interference as discussed in Chapter 6.

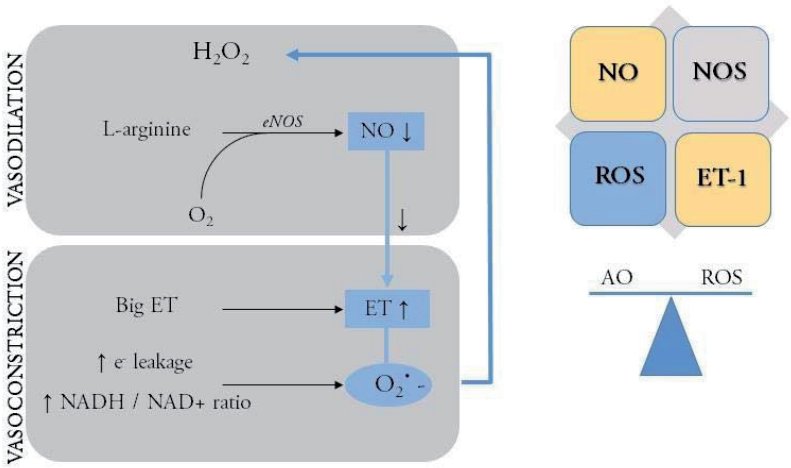


Figure 3. Pharmacologically (HBOC) induced endothelial dysfunction. Pressor effects were mediated by scavenging of NO, thus inhibiting the regulatory function on ET-1, and possibly by up-regulation of ET production. Free radicals did not appear to play a significant role in HBOC induced pressure responses in healthy subjects due to a fully functional antioxidant system preventing the downward spiral seen in cardiovascular pathology. Main players in HBOC induced pressor effects are NO and ET-1 (yellow boxes), while oxidative stress (blue box) is prevented due to a fully functional redox balance.

ENDOTHELIAL CONTROL OF VASOREACTIVITY IN CARDIOVASCULAR DISEASE

A distinct feature (in swine and humans) of a recent myocardial infarction (MI) is an elevation of circulating ET levels at rest and during treadmill exercise [56, 57]. A paradoxically decreased vasoconstrictor influence of ET protects against the perturbations in myocardial oxygen balance, through a loss of coronary vasoconstrictor influence by ET [56]. As in healthy subjects, NO and prostacyclin act in concert to blunt the ET-induced coronary vasoconstriction particularly during exercise [58], NO and prostacyclin may also be responsible for negating the vasoconstrictor influences of ET in remodeled myocardium after MI. Production of NO (and prostanoids) increases with shear stress (e.g. as the result of exercise) [59, 60]; and because NO and prostanoids have been shown to be able to modify the release of ET [61], we investigated whether the withdrawal of ET-mediated coronary vasoconstriction during exercise is mediated through NO and/or prostanoids in swine with a recent MI. In Chapter 7, in accordance with our previous observations [18], we demonstrated that inhibition of NO and prostanoids, alone or in concert, enhance the ET induced vasoconstriction of the coronary resistance vessels during exercise. Furthermore, the vasodilators NO and prostanoids did not affect the ET-induced vasoconstriction at rest. Current results suggest that both NO and prostanoids induce vasodilation via their respective pathways, as well as influencing the ET system particularly during exercise, thus accommodating exercise hyperaemia (see Chapter 4 & 5). Both NO and prostanoids can interact with the ET system at various levels. These vasodilators can limit the production and/or release of ET [54, 62, 63] and NO has been reported to decrease ET_A receptor sensitivity [64]. Taken together, these results show that the suppression of the vasoconstrictor influences of ET on the coronary resistance vessels in the remote remodeled myocardium is not due to NO, of which the overall vasodilator influence and the inhibition of ET influence were blunted. In contrast, despite an unchanged overall vasodilator influence, prostanoids acted to negate the coronary vasoconstrictor influences of ET. These observations underscore the highly complex interactions between vasodilator and vasoconstrictor influences exerted by the endothelium on the coronary resistance vessels. Correspondingly, an additional analysis on a subgroup of swine presented in Chapter 7 showed a small but significant reduction in NO-mediated coronary vasodilator influence after MI which was present at rest and during exercise. It could be speculated that this reduced vasodilation resulted from an increase in NO by scavenging of ROS or a decrease in NO production, due to either a decrease in eNOS expression or eNOS phosphorylation [65, 66]. In

accordance with increased NO scavenging studies in rats [67-69] and from our own laboratory [70], data suggest that oxidative stress is increased in the remote myocardium after MI. Importantly, we have previously shown that NO does not only have a direct vasomotor effect, but also reduces ET-mediated vasoconstriction since the withdrawal of ET necessary for the recruitment of flow reserve during exercise in normal swine is mediated in part through NO [61].

It becomes apparent that NO and $O_2^{\bullet-}$ are key players required for normal vascular homeostasis and form a chemical entity defined as the nitroso-redox balance. The importance of the nitroso-redox balance in the cardiovascular system (see **Chapter** 3) is underlined by studies showing that oxidative stress, i.e., a disturbance of the nitroso-redox balance, contributes to the pathogenesis of diabetes, hypertension, and atherosclerosis [71-76]. As presented in **Chapter 7**, oxidative stress is increased after MI and studies show that oxidative imbalance even resides the remote myocardium [68, 70] contributing to endothelial dysfunction in isolated large coronary arteries [67]. However, the influence of increased oxidative stress on the coronary microvasculature in the remote myocardium after MI in vivo was not yet investigated leading to **Chapter 8** examining the effects of ROS in the remote myocardium after MI. As presented in **Chapter 3**, under normal physiological conditions, $O_2^{\bullet-}$ is enzymatically produced by a variety of oxidases, including xanthine oxidase and NADPH oxidase and as a by-product of oxidative phosphorylation in the mitochondria [15,77]. $O_2^{\bullet-}$ can affect vascular function either directly, by reducing the opening probability of K^+ channels, or indirectly, by quenching NO and forming $ONOO^-$, both leading to vasoconstriction [78]. To prevent deleterious actions of high concentrations of $O_2^{\bullet-}$, its concentration is tightly controlled and kept in the picomolar range by superoxide dismutase (SOD) thereby creating H_2O_2, a membrane-permeable vasodilator [29, 79] that has been suggested to be the factor that couples myocardial metabolism to coronary vasomotor tone [25]. After MI, $O_2^{\bullet-}$ is excessively produced, resulting in oxidative stress even in the remote, non-infarcted myocardium [68, 70], which may result in enhanced coronary vasoconstriction. Given the role of the mitochondrial respiratory chain as a major source of $O_2^{\bullet-}$, oxidative stress is likely to increase during exercise. An increased $O_2^{\bullet-}$ mediated vasoconstriction may therefore directly contribute to the relative hypoperfusion of the remote non-infarcted myocardium that is particularly observed during exercise [80]. In this study, our results suggest that ROS, and most likely $O_2^{\bullet-}$, contribute to coronary vasoconstriction in normal swine that is not mediated through altered bioavailability of NO. This vasoconstrictor influence of ROS is enhanced in the remote myocardium after MI. Further, inhibition of eNOS

reduced rather than enhanced the coronary vasoconstrictor influence of ROS after MI while the eNOS dimer-monomer ratio was decreased in myocardial tissue from swine with MI, suggesting that (uncoupled) eNOS is a significant source of $O_2^{\bullet-}$. Vasoactive alterations after myocardial infarction are summarized in Figure 4.

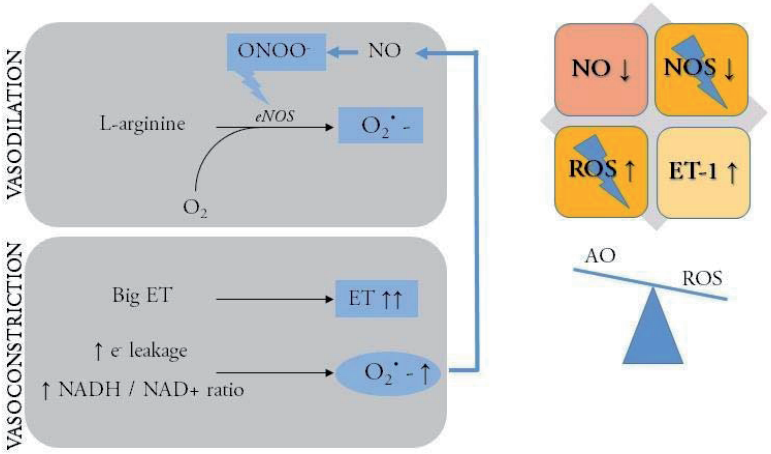


Figure 4. Nitros-redox imbalance after myocardial infarction. After myocardial infarction, ET levels increase (yellow box) and oxidative stress is increased in the remote myocardium with reduced NO levels diminishing the inhibitory effect of NO on ET. Furthermore, superoxide ($O_2^{\bullet-}$) scavenges NO (red box & blue line) thereby forming peroxynitrite ($ONOO^-$). $ONOO^-$ itself uncouples endothelial nitric oxide synthase (eNOS, orange box) with consequential production of ROS, more specific $O_2^{\bullet-}$ (orange boxes). Under pathological conditions with endothelial dysfunction, all players are involved creating a downwards spiral. A redox imbalance is present with increased production of $O_2^{\bullet-}$ and a dysfunctional antioxidant system aggravating endothelial dysfunction diminishing vasodilatory capacity.

It is clear that endothelial dysfunction increases oxidative stress and thus also pro-inflammatory agents. Further, augmented $O_2^{\bullet-}$ production scavenges circulating NO leading to nitrosative stress, thereby influencing the NO-ET balance. Up till now, all studies were performed in swine whilst creating either transient or permanent endothelial dysfunction and looking at different outcomes. Recent studies indicated that inflammation and oxidative stress associated with cardiac surgery – and cardiopulmonary bypass (CBP) in particular- play a significant role in the development of postoperative atrial fibrillation (POAF) [81-83]. Extending results from our previous work to the clinical setting led to the hypothesis that the outcome atrial fibrillation could possibly be the result of a worse preoperative oxidative and

inflammatory state (**Chapter 9**). POAF occurs within the first 2 to 5 postoperative days in 35%-50% of patients [81-83]. AF increases morbidity by predisposing to hemodynamic decline and increased risk of stroke, with concomitant prolonged hospitalization and significant additional health care costs. Despite extensive research and administration of antiarrhythmic therapy and cardiac protection during surgery, POAF incidence remains practically unchanged and may in fact be increasing [84, 85]. Although specific risk factors for POAF such as age, diabetes, length of ischemic period, mechanical damage have been identified; all have failed to be useful as predictors of POAF in the clinical setting [81, 82, 84, 86, 87]. Moreover, lack of knowledge concerning the underlying mechanism leading to POAF delays the progression of AF prevention [84, 88, 89]. Recent studies indicated that inflammation and oxidative stress associated with cardiac surgery – and CBP in particular- play a significant role in the development of POAF [90-95]. In this study, we compared patients after cardiac surgery remaining in sinus rhythm to those with AF. Our pilot study clearly shows a prominent role of the systemic inflammatory response with a strong association between the IL-6 level, TNF-α, the activation of the complement-CRP pathway and NADPH oxidase stimulated $O_2^{\bullet-}$ production with consequential development of POAF. Interestingly, the inflammatory response associated with CPB, did not further stimulate NADPH oxidase to increase $O_2^{\bullet-}$ production in the POAF group, however, a significant increase was seen in the SR group. These results indicate that a worse preoperative inflammatory state can be linked with oxidative tissue injury through NADPH oxidase in the development of POAF. Indeed, recent controlled clinical trials preventing activation of NADPH oxidases, effectively lower POAF incidence in patients after cardiac surgery [96, 97]. Our results agree with previous studies [88, 93, 98-101] placing NADPH oxidase, and more specific NOX-2, at the center of POAF pathophysiology. More specific, our data show that, in accordance with the study of Kim et al [99], NADPH-stimulated superoxide production in the right atrial appendage is the most important independent risk factor for the occurrence of POAF [93, 99]. We also found that a worse preoperative redox imbalance is present in patients developing POAF and high levels of pro-inflammatory cytokines stimulate NADPH oxidase to release $O_2^{\bullet-}$. The inflammatory stimulus of cardiopulmonary bypass promotes production of $O_2^{\bullet-}$ from NADPH oxidase in patients remaining in SR, but not in patients developing POAF, possibly indicating a more balanced redox system and functional antioxidant system. Plasma measurement of lipid peroxidation (TBARS) seems to be a valuable attribute in the prediction of POAF, and indicates that the altered redox state continues during hospitalization and possibly leads to eNOS uncoupling providing the downward spiral with electrophysiological changes leading to POAF. Given the

current data, future studies should focus on preventing activation specific oxidases (f.e. with apocynin) and tailor ROS alteration to tissue specific production rather that systemic ROS eradication. Pathophysiological pathways of POAF are summarized in Figure 5.

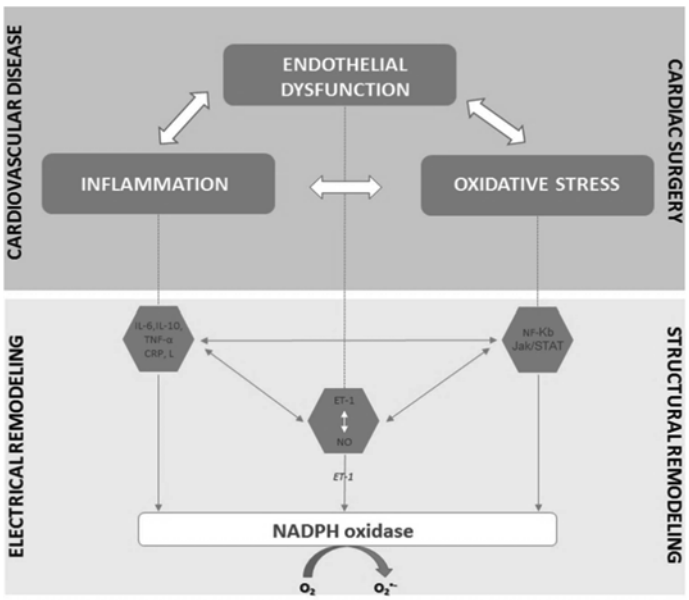


Figure 5. Pathophysiological pathways of POAF coupling endothelial dysfunction to oxidative stress and inflammation.

CONCLUSION AND FUTURE PERSPECTIVES

Extensive research over the last decades has placed the endothelium at the center of cardiovascular disease. Our and other studies clearly show an intricate control system of vasoreactivity that is the result of millions of years of evolution. This delicate system balances autocrine, paracrine and endocrine derivatives, some in the picomolar range, to maintain cardiovascular homeostasis. A trait of oxidative phosphorylation is the production of ROS and evolution has created a pallet of antioxidants throughout time to withstand oxidative and nitrosative damage. During times of endothelial damage or dysfunction, alternative pathways are activated to compensate for the altered vasoreactivity. Only when deleterious adaptations of the endothelium overcome regulatory mechanisms will the imbalance between substances with vasodilating and anti-prothrombogenic features (such as NO, and possibly H_2O_2), and substances with vasoconstricting (such as $O_2^{\bullet -}$ and ET-1) and pro-thrombotic features (such as ET-1) progress. This state, called endothelial dysfunction, will eventually lead to a myriad of cardiovascular diseases. Knowledge of its hallmark position in cardiovascular disease and assessment of its functionality, and thus placing endothelial dysfunction at the source of cardiovascular disease, already showed promising results in preventing cardiovascular disease. New knowledge now shifts the paradigm of oxidative stress from a presumed beneficial believe of total ROS eradication towards a concept of ROS adaptation and modification in cardiovascular (patho)physiology. Further, new tools are now being examined to assess endothelial function in the clinical setting and possibly use this new parameter to further prevent cardiovascular disease before it becomes overt. Detection of endothelial dysfunction, more specific the severity of endothelial dysfunction, could be a useful tool to implement in risk stratifications and possibly change treatment strategies in international guidelines.

REFERENCES

1. Widlansky ME, Gokce N, Keaney JF, Jr., Vita JA. The clinical implications of endothelial dysfunction. J Am Coll Cardiol. 2003;42(7):1149-60.
2. Sarelius I, Pohl U. Control of muscle blood flow during exercise: local factors and integrative mechanisms. Acta Physiol (Oxf). 2010;199(4):349-65.
3. Duncker DJ, Merkus D. Exercise hyperaemia in the heart: the search for the dilator mechanism. J Physiol. 2007;583(Pt 3):847-54.
4. Monahan-Earley R, Dvorak AM, Aird WC. Evolutionary origins of the blood vascular system and endothelium. J Thromb Haemost. 2013;11 Suppl 1:46-66.
5. Gacesa R, Dunlap WC, Barlow DJ, Laskowski RA, Long PF. Rising levels of atmospheric oxygen and evolution of Nrf2. Sci Rep-Uk. 2016;6.
6. Halliwell B. Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life. Plant Physiol. 2006;141(2):312-22.
7. Davies KJ. An overview of oxidative stress. IUBMB Life. 2000;50(4-5):241-4.
8. Sitte N, Merker K, Von Zglinicki T, Grune T, Davies KJ. Protein oxidation and degradation during cellular senescence of human BJ fibroblasts: part I--effects of proliferative senescence. FASEB J. 2000;14(15):2495-502.
9. Bramble DM, Lieberman DE. Endurance running and the evolution of Homo. Nature. 2004;432(7015):345-52.
10. Lambeth JD. Nox enzymes, ROS, and chronic disease: An example of antagonistic pleiotropy. Free Radical Bio Med. 2007;43(3):332-47.
11. Davies KJ. The broad spectrum of responses to oxidants in proliferating cells: a new paradigm for oxidative stress. IUBMB Life. 1999;48(1):41-7.
12. Lambeth JD. Nox enzymes, ROS, and chronic disease: an example of antagonistic pleiotropy. Free Radic Biol Med. 2007;43(3):332-47.
13. Kirkwood TB, Rose MR. Evolution of senescence: late survival sacrificed for reproduction. Philos Trans R Soc Lond B Biol Sci. 1991;332(1262):15-24.
14. Radak Z, Zhao Z, Koltai E, Ohno H, Atalay M. Oxygen consumption and usage during physical exercise: the balance between oxidative stress and ROS-dependent adaptive signaling. Antioxid Redox Signal. 2013;18(10):1208-46.
15. Droge W. Free radicals in the physiological control of cell function. Physiol Rev. 2002;82(1):47-95.
16. Callera GE, Tostes RC, Yogi A, Montezano AC, Touyz RM. Endothelin-1-induced oxidative stress in DOCA-salt hypertension involves NADPH-oxidase-independent mechanisms. Clin Sci (Lond). 2006;110(2):243-53.
17. Li L, Fink GD, Watts SW, Northcott CA, Galligan JJ, Pagano PJ, et al. Endothelin-1 increases vascular superoxide via endothelin(A)-NADPH oxidase pathway in low-renin hypertension. Circulation. 2003;107(7):1053-8.

18. Merkus D, Houweling B, Mirza A, Boomsma F, van den Meiracker AH, Duncker DJ. Contribution of endothelin and its receptors to the regulation of vascular tone during exercise is different in the systemic, coronary and pulmonary circulation. Cardiovasc Res. 2003;59(3):745-54.
19. Takamura M, Parent R, Cernacek P, Lavallee M. Influence of dual ET(A)/ET(B)-receptor blockade on coronary responses to treadmill exercise in dogs. J Appl Physiol (1985). 2000;89(5):2041-8.
20. de Beer VJ, Sorop O, Pijnappels DA, Dekkers DH, Boomsma F, Lamers JM, et al. Integrative control of coronary resistance vessel tone by endothelin and angiotensin II is altered in swine with a recent myocardial infarction. Am J Physiol Heart Circ Physiol. 2008;294(5):H2069-77.
21. Haynes WG, Ferro CE, Webb DJ. Physiologic role of endothelin in maintenance of vascular tone in humans. J Cardiovasc Pharmacol. 1995;26 Suppl 3:S183-5.
22. Hein TW, Ren Y, Yuan Z, Xu W, Somvanshi S, Nagaoka T, et al. Functional and molecular characterization of the endothelin system in retinal arterioles. Invest Ophthalmol Vis Sci. 2009;50(7):3329-36.
23. Plumpton C, Haynes WG, Webb DJ, Davenport AP. Phosphoramidon inhibition of the in vivo conversion of big endothelin-1 to endothelin-1 in the human forearm. Br J Pharmacol. 1995;116(2):1821-8.
24. Pollock DM, Opgenorth TJ. ETA receptor-mediated responses to endothelin-1 and big endothelin-1 in the rat kidney. Br J Pharmacol. 1994;111(3):729-32.
25. Saitoh S, Zhang C, Tune JD, Potter B, Kiyooka T, Rogers PA, et al. Hydrogen peroxide: a feed-forward dilator that couples myocardial metabolism to coronary blood flow. Arterioscler Thromb Vasc Biol. 2006;26(12):2614-21.
26. Breton-Romero R, Lamas S. Hydrogen peroxide signaling in vascular endothelial cells. Redox Biol. 2014;2:529-34.
27. Lucchesi PA, Belmadani S, Matrougui K. Hydrogen peroxide acts as both vasodilator and vasoconstrictor in the control of perfused mouse mesenteric resistance arteries. J Hypertens. 2005;23(3):571-9.
28. Beny JL, von der Weid PY. Hydrogen peroxide: an endogenous smooth muscle cell hyperpolarizing factor. Biochem Biophys Res Commun. 1991;176(1):378-84.
29. Matoba T, Shimokawa H, Kubota H, Morikawa K, Fujiki T, Kunihiro I, et al. Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in human mesenteric arteries. Biochem Biophys Res Commun. 2002;290(3):909-13.
30. Saitoh S, Kiyooka T, Rocic P, Rogers PA, Zhang C, Swafford A, et al. Redox-dependent coronary metabolic dilation. Am J Physiol Heart Circ Physiol. 2007;293(6):H3720-5.
31. Samora JB, Frisbee JC, Boegehold MA. Hydrogen peroxide emerges as a regulator of tone in skeletal muscle arterioles during juvenile growth. Microcirculation. 2008;15(2):151-61.

32. Sindler AL, Delp MD, Reyes R, Wu G, Muller-Delp JM. Effects of ageing and exercise training on eNOS uncoupling in skeletal muscle resistance arterioles. J Physiol. 2009;587(Pt 15):3885-97.
33. Ji LL. Antioxidants and oxidative stress in exercise. Proc Soc Exp Biol Med. 1999;222(3):283-92.
34. Powers SK, Ji LL, Leeuwenburgh C. Exercise training-induced alterations in skeletal muscle antioxidant capacity: a brief review. Med Sci Sports Exerc. 1999;31(7):987-97.
35. Urso ML, Clarkson PM. Oxidative stress, exercise, and antioxidant supplementation. Toxicology. 2003;189(1-2):41-54.
36. Kanter MM. Free radicals, exercise, and antioxidant supplementation. Int J Sport Nutr. 1994;4(3):205-20.
37. Powers SK, Jackson MJ. Exercise-induced oxidative stress: cellular mechanisms and impact on muscle force production. Physiol Rev. 2008;88(4):1243-76.
38. Lambert AJ, Brand MD. Reactive oxygen species production by mitochondria. Methods Mol Biol. 2009;554:165-81.
39. Boveris A, Chance B. The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. Biochem J. 1973;134(3):707-16.
40. Sato A, Sakuma I, Gutterman DD. Mechanism of dilation to reactive oxygen species in human coronary arterioles. Am J Physiol Heart Circ Physiol. 2003;285(6):H2345-54.
41. Phillips SA, Hatoum OA, Gutterman DD. The mechanism of flow-induced dilation in human adipose arterioles involves hydrogen peroxide during CAD. Am J Physiol Heart Circ Physiol. 2007;292(1):H93-100.
42. Thengchaisri N, Shipley R, Ren Y, Parker J, Kuo L. Exercise training restores coronary arteriolar dilation to NOS activation distal to coronary artery occlusion: role of hydrogen peroxide. Arterioscler Thromb Vasc Biol. 2007;27(4):791-8.
43. Clifford PS, Hellsten Y. Vasodilatory mechanisms in contracting skeletal muscle. J Appl Physiol (1985). 2004;97(1):393-403.
44. Jahr JS, Moallempour M, Lim JC. HBOC-201, hemoglobin glutamer-250 (bovine), Hemopure (Biopure Corporation). Expert Opin Biol Ther. 2008;8(9):1425-33.
45. Serruys PW, Vranckx P, Slagboom T, Regar E, Meliga E, de Winter RJ, et al. Haemodynamic effects, safety, and tolerability of haemoglobin-based oxygen carrier-201 in patients undergoing PCI for CAD. EuroIntervention. 2008;3(5):600-9.
46. Irwin DC, Foreman B, Morris K, White M, Sullivan T, Jacobs R, et al. Polymerized bovine hemoglobin decreases oxygen delivery during normoxia and acute hypoxia in the rat. Am J Physiol Heart Circ Physiol. 2008;295(3):H1090-H9.

47. Buehler PW, Alayash AI. Toxicities of hemoglobin solutions: in search of in-vitro and in-vivo model systems. Transfusion. 2004;44(10):1516-30.
48. Cabrales P, Friedman JM. HBOC vasoactivity: interplay between nitric oxide scavenging and capacity to generate bioactive nitric oxide species. Antioxid Redox Signal. 2013;18(17):2284-97.
49. Hai CM. Systems biology of HBOC-induced vasoconstriction. Curr Drug Discov Technol. 2012;9(3):204-11.
50. Patel RP. Biochemical aspects of the reaction of hemoglobin and NO: implications for Hb-based blood substitutes. Free Radic Biol Med. 2000;28(10):1518-25.
51. Stamler JS, Jia L, Eu JP, McMahon TJ, Demchenko IT, Bonaventura J, et al. Blood flow regulation by S-nitrosohemoglobin in the physiological oxygen gradient. Science. 1997;276(5321):2034-7.
52. Tsai AG, Cabrales P, Manjula BN, Acharya SA, Winslow RM, Intaglietta M. Dissociation of local nitric oxide concentration and vasoconstriction in the presence of cell-free hemoglobin oxygen carriers. Blood. 2006;108(10):3603-10.
53. Biro GP. Adverse HBOC-endothelial dysfunction synergism: a possible contributor to adverse clinical outcomes? Curr Drug Discov Technol. 2012;9(3):194-203.
54. Kelly LK, Wedgwood S, Steinhorn RH, Black SM. Nitric oxide decreases endothelin-1 secretion through the activation of soluble guanylate cyclase. Am J Physiol Lung Cell Mol Physiol. 2004;286(5):L984-91.
55. Schultz SC, Grady B, Cole F, Hamilton I, Burhop K, Malcolm DS. A role for endothelin and nitric oxide in the pressor response to diaspirin cross-linked hemoglobin. J Lab Clin Med. 1993;122(3):301-8.
56. Merkus D, Houweling B, van den Meiracker AH, Boomsma F, Duncker DJ. Contribution of endothelin to coronary vasomotor tone is abolished after myocardial infarction. Am J Physiol Heart Circ Physiol. 2005;288(2):H871-80.
57. Duncker DJ, Haitsma DB, Liem DA, Verdouw PD, Merkus D. Exercise unmasks autonomic dysfunction in swine with a recent myocardial infarction. Cardiovasc Res. 2005;65(4):889-96.
58. Prins BA, Hu RM, Nazario B, Pedram A, Frank HJ, Weber MA, et al. Prostaglandin E2 and prostacyclin inhibit the production and secretion of endothelin from cultured endothelial cells. J Biol Chem. 1994;269(16):11938-44.
59. Koller A, Sun D, Huang A, Kaley G. Corelease of nitric oxide and prostaglandins mediates flow-dependent dilation of rat gracilis muscle arterioles. Am J Physiol. 1994;267(1 Pt 2):H326-32.
60. Osanai T, Fujita N, Fujiwara N, Nakano T, Takahashi K, Guan W, et al. Cross talk of shear-induced production of prostacyclin and nitric oxide in endothelial cells. Am J Physiol Heart Circ Physiol. 2000;278(1):H233-8.

61. Merkus D, Sorop O, Houweling B, Boomsma F, van den Meiracker AH, Duncker DJ. NO and prostanoids blunt endothelin-mediated coronary vasoconstrictor influence in exercising swine. Am J Physiol Heart Circ Physiol. 2006;291(5):H2075-81.
62. McMurray JJ, Ray SG, Abdullah I, Dargie HJ, Morton JJ. Plasma endothelin in chronic heart failure. Circulation. 1992;85(4):1374-9.
63. Boulanger C, Luscher TF. Release of endothelin from the porcine aorta. Inhibition by endothelium-derived nitric oxide. J Clin Invest. 1990;85(2):587-90.
64. Wiley KE, Davenport AP. Nitric oxide-mediated modulation of the endothelin-1 signalling pathway in the human cardiovascular system. Br J Pharmacol. 2001;132(1):213-20.
65. Shi Y, Vanhoutte PM. Reactive oxygen-derived free radicals are key to the endothelial dysfunction of diabetes. J Diabetes. 2009;1(3):151-62.
66. Vanhoutte PM, Shimokawa H, Tang EH, Feletou M. Endothelial dysfunction and vascular disease. Acta Physiol (Oxf). 2009;196(2):193-222.
67. Berges A, Van Nassauw L, Timmermans JP, Vrints C. Role of nitric oxide during coronary endothelial dysfunction after myocardial infarction. Eur J Pharmacol. 2005;516(1):60-70.
68. Berges A, Van Nassauw L, Timmermans JP, Vrints C. Time-dependent expression pattern of nitric oxide and superoxide after myocardial infarction in rats. Pharmacol Res. 2007;55(1):72-9.
69. Krijnen PA, Nijmeijer R, Meijer CJ, Visser CA, Hack CE, Niessen HW. Apoptosis in myocardial ischaemia and infarction. J Clin Pathol. 2002;55(11):801-11.
70. Boontje NM, Merkus D, Zaremba R, Versteilen A, de Waard MC, Mearini G, et al. Enhanced myofilament responsiveness upon beta-adrenergic stimulation in post-infarct remodeled myocardium. J Mol Cell Cardiol. 2011;50(3):487-99.
71. Arimura K, Egashira K, Nakamura R, Ide T, Tsutsui H, Shimokawa H, et al. Increased inactivation of nitric oxide is involved in coronary endothelial dysfunction in heart failure. Am J Physiol Heart Circ Physiol. 2001;280(1):H68-75.
72. Deneke SM. Thiol-based antioxidants. Curr Top Cell Regul. 2000;36:151-80.
73. Lancel S, Tissier S, Mordon S, Marechal X, Depontieu F, Scherpereel A, et al. Peroxynitrite decomposition catalysts prevent myocardial dysfunction and inflammation in endotoxemic rats. J Am Coll Cardiol. 2004;43(12):2348-58.
74. Pacher P, Obrosova IG, Mabley JG, Szabo C. Role of nitrosative stress and peroxynitrite in the pathogenesis of diabetic complications. Emerging new therapeutical strategies. Curr Med Chem. 2005;12(3):267-75.
75. Pacher P, Szabo C. Role of poly(ADP-ribose) polymerase-1 activation in the pathogenesis of diabetic complications: endothelial dysfunction, as a common underlying theme. Antioxid Redox Signal. 2005;7(11-12):1568-80.

76. Turko IV, Murad F. Protein nitration in cardiovascular diseases. Pharmacol Rev. 2002;54(4):619-34.
77. Turrens JF. Mitochondrial formation of reactive oxygen species. J Physiol. 2003;552(Pt 2):335-44.
78. Stamler JS, Lamas S, Fang FC. Nitrosylation. the prototypic redox-based signaling mechanism. Cell. 2001;106(6):675-83.
79. Kobayashi T, Kamata K. Modulation by hydrogen peroxide of noradrenaline-induced contraction in aorta from streptozotocin-induced diabetic rat. Eur J Pharmacol. 2002;441(1-2):83-9.
80. Haitsma DB, Bac D, Raja N, Boomsma F, Verdouw PD, Duncker DJ. Minimal impairment of myocardial blood flow responses to exercise in the remodeled left ventricle early after myocardial infarction, despite significant hemodynamic and neurohumoral alterations. Cardiovasc Res. 2001;52(3):417-28.
81. Aranki SF, Shaw DP, Adams DH, Rizzo RJ, Couper GS, VanderVliet M, et al. Predictors of atrial fibrillation after coronary artery surgery. Current trends and impact on hospital resources. Circulation. 1996;94(3):390-7.
82. Mathew JP, Fontes ML, Tudor IC, Ramsay J, Duke P, Mazer CD, et al. A multicenter risk index for atrial fibrillation after cardiac surgery. JAMA. 2004;291(14):1720-9.
83. Villareal RP, Hariharan R, Liu BC, Kar B, Lee VV, Elayda M, et al. Postoperative atrial fibrillation and mortality after coronary artery bypass surgery. J Am Coll Cardiol. 2004;43(5):742-8.
84. Auer J, Weber T, Berent R, Ng CK, Lamm G, Eber B. Risk factors of postoperative atrial fibrillation after cardiac surgery. J Card Surg. 2005;20(5):425-31.
85. Creswell LL, Schuessler RB, Rosenbloom M, Cox JL. Hazards of postoperative atrial arrhythmias. Ann Thorac Surg. 1993;56(3):539-49.
86. Carnes CA, Chung MK, Nakayama T, Nakayama H, Baliga RS, Piao S, et al. Ascorbate attenuates atrial pacing-induced peroxynitrite formation and electrical remodeling and decreases the incidence of postoperative atrial fibrillation. Circ Res. 2001;89(6):E32-8.
87. Echahidi N, Mohty D, Pibarot P, Despres JP, O'Hara G, Champagne J, et al. Obesity and metabolic syndrome are independent risk factors for atrial fibrillation after coronary artery bypass graft surgery. Circulation. 2007;116(11 Suppl):I213-9.
88. Antoniades C, Demosthenous M, Reilly S, Margaritis M, Zhang MH, Antonopoulos A, et al. Myocardial redox state predicts in-hospital clinical outcome after cardiac surgery effects of short-term pre-operative statin treatment. J Am Coll Cardiol. 2012;59(1):60-70.
89. Kaireviciute D, Aidietis A, Lip GY. Atrial fibrillation following cardiac surgery: clinical features and preventative strategies. Eur Heart J. 2009;30(4):410-25.

90. Clermont G, Vergely C, Jazayeri S, Lahet JJ, Goudeau JJ, Lecour S, et al. Systemic free radical activation is a major event involved in myocardial oxidative stress related to cardiopulmonary bypass. Anesthesiology. 2002;96(1):80-7.
91. Levy JH, Tanaka KA. Inflammatory response to cardiopulmonary bypass. Ann Thorac Surg. 2003;75(2):S715-20.
92. Gaudino M, Andreotti F, Zamparelli R, Di Castelnuovo A, Nasso G, Burzotta F, et al. The -174G/C interleukin-6 polymorphism influences postoperative interleukin-6 levels and postoperative atrial fibrillation. Is atrial fibrillation an inflammatory complication? Circulation. 2003;108 Suppl 1:II195-9.
93. Kim YM, Kattach H, Ratnatunga C, Pillai R, Channon KM, Casadei B. Association of atrial nicotinamide adenine dinucleotide phosphate oxidase activity with the development of atrial fibrillation after cardiac surgery. J Am Coll Cardiol. 2008;51(1):68-74.
94. Ramlawi B, Otu H, Mieno S, Boodhwani M, Sodha NR, Clements RT, et al. Oxidative stress and atrial fibrillation after cardiac surgery: a case-control study. Ann Thorac Surg. 2007;84(4):1166-72; discussion 72-3.
95. Ramlawi B, Feng J, Mieno S, Szabo C, Zsengeller Z, Clements R, et al. Indices of apoptosis activation after blood cardioplegia and cardiopulmonary bypass. Circulation. 2006;114(1 Suppl):I257-63.
96. Halonen J, Halonen P, Jarvinen O, Taskinen P, Auvinen T, Tarkka M, et al. Corticosteroids for the prevention of atrial fibrillation after cardiac surgery: a randomized controlled trial. JAMA. 2007;297(14):1562-7.
97. Patti G, Chello M, Candura D, Pasceri V, D'Ambrosio A, Covino E, et al. Randomized trial of atorvastatin for reduction of postoperative atrial fibrillation in patients undergoing cardiac surgery: results of the ARMYDA-3 (Atorvastatin for Reduction of MYocardial Dysrhythmia After cardiac surgery) study. Circulation. 2006;114(14):1455-61.
98. Chang JP, Chen MC, Liu WH, Yang CH, Chen CJ, Chen YL, et al. Atrial myocardial nox2 containing NADPH oxidase activity contribution to oxidative stress in mitral regurgitation: potential mechanism for atrial remodeling. Cardiovasc Pathol. 2011;20(2):99-106.
99. Kim YM, Guzik TJ, Zhang YH, Zhang MH, Kattach H, Ratnatunga C, et al. A myocardial Nox2 containing NAD(P)H oxidase contributes to oxidative stress in human atrial fibrillation. Circ Res. 2005;97(7):629-36.
100. Adam O, Frost G, Custodis F, Sussman MA, Schafers HJ, Bohm M, et al. Role of Rac1 GTPase activation in atrial fibrillation. J Am Coll Cardiol. 2007;50(4):359-67.
101. Reilly SN, Jayaram R, Nahar K, Antoniades C, Verheule S, Channon KM, et al. Atrial sources of reactive oxygen species vary with the duration and substrate of atrial fibrillation: implications for the antiarrhythmic effect of statins. Circulation. 2011;124(10):1107-17.

11

SUMMARY

SUMMARY

In this thesis we studied the intricate interplay between nitric oxide (NO), endothelin-1 (ET-1) and reactive oxygen / nitrogen species (ROS/RNS) on vascular function in health and cardiovascular disease.

Chapter 1 is a general introduction to this thesis. This chapter forms the background against which the research hypotheses are explained and the aims of this thesis are postulated.

Chapter 2 provides an overview of oxygen (O_2)-derived free radicals, their discovery, chemistry, and physiological role in the evolution of complex life. More specific, we created a molecular timeframe explaining the necessary adaptations in redox biology preceding the Cambrian explosion. Enzymatic reduction of O_2 yielded a large increase in energy production capacity, allowing the evolution of multi-cellular animal life. However, notwithstanding the benefit of using O_2, it is still a very toxic agent that requires antioxidants to curtail its toxicity. O_2 and its derived species thus present the ultimate paradox to life and evolution—as they encompass both challenges and opportunities for life.

In **Chapter 3**, we analysed and described the apparent paradoxical role of ROS within cellular physiology. Under normal circumstances, reactive oxygen species (ROS) concentrations are tightly controlled by antioxidants, keeping them in the picomolar range. Only when produced in excess will they evoke oxidative damage. Although the deleterious effects of ROS can potentially be reduced by restoring the imbalance between production and clearance of ROS through administration of antioxidants; we demonstrate that many studies fail to show beneficial effects. Hence, the dosage and type of antioxidants should be tailored to the location and nature of oxidative stress and current awareness on redox physiology should shift from ROS eradication to ROS adaptation.

One key player in redox reactions but also vasoreactivity is nitric oxide (NO) causing vasodilation. Its major opponent, endothelin (ET) -1, forms an intrinsic pathway with NO creating a diametrical opposite. In addition to being a potent vasoconstrictor,

ET-1 has also been described as a potent stimulator of oxidative stress thereby initiating a possible silencing mechanism for the NO dependent inhibition of ET-1. Therefore, in **Chapter 4**, we investigated the hypothesis that exercise-induced blunting of ET-mediated vasoconstriction in the coronary circulation results from a decrease in ET production, a decrease in ET receptor sensitivity, or a combination of these two effects. We found that during exercise, in the coronary vasculature, ET receptor sensitivity is maintained, while the conversion of Big ET to ET is blunted.

Coupling blood flow to metabolism requires vasomotor regulation for adequate delivery of O_2 to metabolically active organ systems. In Chapter 4 we already discussed the adaptation in the ET-1 pathway allowing for metabolic hyperemia, however, the current consensus states that a multitude of pathways act in concert. Recent years, one pathway gained much attention possibly coupling oxygen consumption to the production of an endothelial derived relaxing factor. Thus in **Chapter 5**, we first examined the overall effects of ROS on systemic, pulmonary and coronary vascular tone during exercise hyperemia by scavenging all ROS. Subsequently we investigated whether increasing the production and / or decreasing brake down of hydrogen peroxide (H_2O_2) would indeed affect exercise-induced vasodilation. Despite extensive research showing that exercise increases ROS production more specific mitochondrial superoxide production, in our study scavenging of free radicals did not alter vascular resistance in systemic, pulmonary and coronary vascular beds. These results imply that either enough superoxide dismutase is present and most likely upregulated to reduce superoxide ($O_2^{\bullet -}$), the amount of free radicals produced is very low, or that the vasoconstrictor influence of $O_2^{\bullet -}$ is balanced by a similar vasodilator influence of H_2O_2.

During exercise, endothelial function is challenged; nevertheless, metabolic demands can be met by a fully functional second messenger and antioxidant system. However, states of endothelial dysfunction can also be assigned to pharmacological interference. In **Chapter 6**, different pathways leading to transient endothelial dysfunction with systemic and pulmonary pressor effects after HBOC-201 administration were studied where we determined the possibility for pressure effects to be reversed by administrating an NO-independent dilator and addressed the potential roles of NO scavenging, ROS and / or ET in exercising swine. Our results show that HBOC-201 can disrupt hemodynamic homeostasis, mimicking some aspects of endothelial dysfunction, resulting in systemic and pulmonary

hypertension as a result of vasoconstriction. These pressure responses were mediated by scavenging of NO and possibly by up-regulation of ET production. ROS scavenging did not appear to play a significant role in HBOC-201 induced pressure responses in healthy subjects. It is therefore clear that they are embedded in the mechanics of these responses.

A distinct feature (in swine and human subjects) of a recent myocardial infarction (MI) is an elevation of circulating ET levels at rest and during treadmill exercise. Increased vasoconstrictor influence of ET contributes to the perturbations in myocardial oxygen balance, however, with a paradoxical loss of coronary vasoconstrictor influence by ET. In **Chapter** 7, we investigated whether NO and prostacyclin may be responsible for negating the vasoconstrictor influences of ET in remodeled myocardium after MI. Here we showed that inhibition of NO and prostanoids, enhance the ET induced vasoconstriction of the coronary resistance vessels during exercise. Furthermore, the vasodilators NO and prostanoids did not affect the ET-induced vasoconstriction at rest. Current results suggest that both NO and prostanoids induce vasodilation via their respective pathways, as well as influencing the ET system particularly during exercise, thus accommodating exercise hyperaemia. These results imply that NO and prostanoids decreased ET production and/or ET receptor sensitivity during exercise resulting in the observed exercise hyperaemia. Correspondingly, an additional analysis on a subgroup of swine showed a small but significant reduction in NO-mediated coronary vasodilator influence after MI which was present at rest and during exercise. This reduced vasodilation possibly resulted from an increased in NO scavenging (by $O_2^{\bullet-}$) or a decrease in NO production, due to either a decrease in eNOS expression or eNOS phosphorylation.

In **Chapter 8**, following the results presented in **Chapter 7**, we consequently investigated the effects of ROS in the remote myocardium after MI. It becomes apparent that NO and $O_2^{\bullet-}$ are key players required for normal vascular homeostasis and form a chemical entity defined as nitroso-redox balance. We show that, after MI, $O_2^{\bullet-}$ is excessively produced, resulting in OS even in the remote, non-infarcted myocardium which may result in enhanced coronary vasoconstriction. Further, inhibition of eNOS reduced rather than enhanced the coronary vasoconstrictor influence of ROS after MI while the eNOS dimer-monomer ratio was decreased in

myocardial tissue from swine with MI, suggesting that (uncoupled) eNOS is a significant source of $O_2^{\bullet-}$.

It is clear that endothelial dysfunction increases oxidative stress and thus also pro-inflammatory agents. Further, augmented $O_2^{\bullet-}$ production scavenges circulating NO leading to nitrosative stress thus influencing the NO-ET balance (**Chapter 8**). Recent studies indicated that inflammation and oxidative stress (OS) associated with cardiac surgery – and cardiopulmonary bypass (CBP) in particular- play a significant role in the development of postoperative atrial fibrillation (POAF). In **Chapter 9,** taken previous results into account, we compared patients remaining in sinus rhythm to patients developing POAF. The inflammatory stimulus of CPB promotes production of $O_2^{\bullet-}$ from NADPH oxidase in patients remaining in SR, but not in patients developing POAF, possibly indicating a more balanced redox system and functional antioxidant system. Our data suggests that the most important $O_2^{\bullet-}$ producing oxidase is NOX-2 and can be seen as a strong independent predictor of the development of AF after cardiac surgery, independent from type of surgery. Plasma measurement of TBARS seems to be a valuable attribute in the prediction of POAF, and indicates that the altered redox state continues during hospitalization (where eNOS uncoupling could possibly play a role) providing a downward spiral with electrophysiological changes leading to POAF.

Chapter 10 discusses the results of this thesis in a broader context and presents directives for future research implementing these findings and possibly providing new insights in physiology and pathophysiology of radical biology in cardiovascular medicine.

12

NEDERLANDSE SAMENVATTING

NEDERLANDSE SAMENVATTING

In dit proefschrift werd de interactie onderzocht tussen stikstof oxide (NO), endotheline-1 (ET-1), vrije zuurstof /stikstof radicalen (VZR/VSR) en hun effect op de vaatfunctie bij gezonde dieren, dieren met met een myocard infarct, en patiënten met cardiovasculair lijden.

Hoofdstuk 1 behelst de algemene introductie van dit proefschrift en vormt de achtergrond voor het verklaren van de verschillende hypotheses en het postuleren van de doelstellingen binnen dit proefschrift.

In **Hoofdstuk 2** wordt een overzicht gegeven over de ontdekking, chemie en fysiologie van vrije zuurstofradicalen (VZR); in het bijzonder over hun rol in de evolutie van complex cellulair leven. Meer specifiek werd een moleculaire tijdschaal gecreëerd, welke de noodzakelijke adaptaties in redox biologie duidelijk maakt, leidend tot de Cambrische explosie. Enzymatische reductie van zuurstof (O_2) zorgde voor een enorme toename in energie productie wat de diversificatie in multi-cellulariteit mogelijk maakte. Echter, O_2 is heel erg toxisch en het gebruik ervan vergt antioxidanten om de toxische werking tegen te gaan. O_2 en afgeleide radicalen omvatten dus zowel mogelijkheden als uitdagingen voor het leven op aarde en kunnen gezien worden als de ultieme paradox van evolutie.

In **Hoofdstuk 3** werd een analyse gemaakt van deze paradoxale rol van VZR en werd de werking binnen de cel fysiologie beschreven. Bij gezonde individuen worden de concentraties VZR in de pico molaire concentratie gehouden door antioxidanten. Echter, bij excessieve productie van VZR, treedt er oxidatieve schade op. Hoewel de schadelijke effecten van VZR potentieel kunnen worden tegen gegaan door de redox disbalans tussen aanmaak en wegvangen van VZR te herstellen, wordt er aangetoond dat veel studies er niet in slagen om dit effect te bewijzen. Het is duidelijk dat het toedienen van antioxidanten dosis en locatie afhankelijk is en specifiek moet worden afgestemd op de aard van oxidatieve stress. Zodoende, dient binnen de redox fysiologie de huidige gedachte van VZR eradicatie naar VZR adaptatie te verschuiven.

Een belangrijke component binnen vasoreactiviteit en redox reacties is stikstofoxide (NO), welke vasodilatatie veroorzaakt. NO is de sterkste tegenpool van endotheline-1 (ET-1) en beiden vormen ze een intrinsiek fysiologisch systeem. ET-1 is niet alleen een potente vasoconstrictor, maar wordt ook beschreven als belangrijke stimulator van oxidatieve stress, waardoor een potentiele interdictie van het inhibitie effect van NO op ET-1 bestaat. Zodoende werd in **Hoofdstuk 4** de hypothese onderzocht dat inspanning geïnduceerde vervlakking van ET gemedieerde coronair vasoconstrictie het gevolg is van een verlaagde ET productie, een verlaagde ET receptor sensitiviteit, of een combinatie van beide effecten. Deze studie wees uit dat er gedurende inspanning in de coronair circulatie een nivellatie optreedt van de omzetting van de voorloper van ET (Big ET) naar ET, echter met behoud van de ET receptor sensitiviteit.

Vasoreactiviteit staat in voor het koppelen van de doorbloeding aan metabolisme en zodoende voor adequate O_2 voorziening aan actieve orgaan systemen. In **Hoofdstuk 4** werd de adaptatie van het ET-1 systeem in inspanningshyperemie al besproken, echter huidige inzichten beschrijven aan veelvoud aan mechanismen verantwoordelijk voor de optredende vasodilatatie. Recent kreeg één mechanisme veel meer aandacht alwaar zuurstofconsumptie wordt gekoppeld aan een endothelium afgeleide relaxatie factor (EDRF). Zodoende werd in **Hoofdstuk 5** nader onderzoek verricht naar dit mechanisme. Verschillende antioxidanten werden toegediend waardoor VZR werden weggevangen en de algemene effecten op de systeem, pulmonaal- en coronair circulatie konden worden onderzocht. Meer specifiek werd de omzetting van superoxide ($O_2^{\bullet-}$) naar waterstofperoxide (H_2O_2) versterkt ter verificatie van de mogelijke vasodilatatie effecten van H_2O_2. Ondanks veel studies die beschrijven dat inspanning zorgt voor een toename aan VZR, en meer specifiek $O_2^{\bullet-}$ productie vanuit de mitochondriën, werd er in deze studie, na toedienen van antioxidanten, geen effect gezien in vaatweerstand van de systeem, pulmonaal- en coronair circulatie. Deze resultaten tonen aan dat er of voldoende $O_2^{\bullet-}$ dismutase aanwezig is, alwaar de productie vermoedelijk is versterkt, om $O_2^{\bullet-}$ af te breken; de hoeveelheid geproduceerde VZR heel laag is; of dat het vasoconstrictie effect van $O_2^{\bullet-}$ wordt tegengegaan door de vasodilatatie invloed van H_2O_2.

Gedurende inspanning wordt de endotheel functie geprovoceerd, echter kan het hoofd worden geboden aan de metabolische vraag gezien er sprake is van een goed functionerend secundair boodschapper en antioxidant systeem. Een staat van transiente endotheel dysfunctie kan verkregen worden door farmacologische

invloeden, zoals beschreven in Hoofdstuk 6. Hier worden diverse systemen onderzocht die leiden tot de endotheel dysfunctie en dientengevolge systemische en pulmonaal vasoconstrictie effecten toegeschreven aan HBOC-201. De mogelijkheid tot reversibiliteit van deze effecten inclusief de potentiele rol van NO inhibitie, VZR en / of ET-1 werden tijdens inspanning onderzocht in gezonde varkens. De resultaten tonen aan dat HBOC-201 de hemodynamische homeostase kan verstoren en zodoende bepaalde aspecten van endotheliaal dysfunctie imiteert. Dit uit zich in een gegeneraliseerde vasoconstrictie met consecutieve systemische en pulmonaal hypertensie ten gevolge van wegvangen van NO en mogelijke op-regulatie van ET-1. Het wegvangen van VZR leek weinig effect te hebben op de HBOC-201 geïnduceerde hypertensieve respons in gezonde varkens. Het is dus duidelijk dat VZR geïncorporeerd zijn in de functionaliteit van dergelijk respons.

Een uitgesproken kenmerk van een recent myocard infarct (in varkens en mensen) is de toename van circulerend ET tijdens rust en inspanning. De toegenomen vasoconstrictie invloed van ET draagt bij aan de verstoringen van de myocardiale zuurstofbalans, met een paradoxaal verlies aan ET invloed op coronair vasoconstrictie. In Hoofdstuk 7 werd onderzocht of NO en prostacycline verantwoordelijk zouden zijn voor de tegenstrijdige effecten van ET in het geherstructureerde myocardium na een myocard infarct. Deze studie maakt duidelijk dat door inhibitie van NO en prostanoïden, de ET geïnduceerde vasoconstrictie van de coronair weerstandsvaten toeneemt gedurende inspanning. Voorts hadden de vasodilatatoren NO en prostanoïden geen effect op de ET-geïnduceerde vasoconstrictie tijdens rust. Dit betekent dat beide NO en prostanoïden hun vasodilatator effect uitoefenen via hun respectievelijke routes, alsook de beïnvloeding van het ET systeem, meer specifiek tijdens inspanning, en derhalve de accommodatie van inspanningshyperemie. NO en prostanoïden verlaagden derhalve de ET productie dan wel ET receptor sensitiviteit gedurende inspanning welke resulteerde in inspanningshyperemie. Een additionele analyse op een subgroep varkens toonde een kleine significante afname in NO gemedieerde coronair vasodilatatie na myocard infarct en dit gedurende rust en inspanning. Deze reductie was hoogstwaarschijnlijk het gevolg van een verhoogd wegvangen van NO door $O_2^{\bullet -}$ of een verlaagde NO productie door verlaagde eNOS expressie of fosforylatie van eNOS.

Volgend op de resultaten gepresenteerd in Hoofdstuk 7, werden de effecten van VZR op het myocardium nader geanalyseerd in Hoofdstuk 8. Het moge duidelijk zijn dat NO en $O_2^{\bullet-}$ hoofdspelers zijn in normale vasculaire homeostase en vormen een chemische entiteit gedefinieerd als nitroso-redox balans. In dit hoofdstuk wordt aangetoond dat, na myocard infarct, $O_2^{\bullet-}$ excessief wordt geproduceerd resulterend in oxidatieve stress, zo ook in het afgelegen, niet geïnfarceerde myocardium resulterend in toegenomen coronair vasoconstrictie. Verder verlaagde inhibitie van eNOS de vasoconstrictie invloed van VZR na myocard infarct, terwijl de eNOS dimeer-monomeer ratio was gereduceerd in cardiaal weefsel van varkens na myocard infarct. Dit toont aan dat "uncoupled" eNOS een significante bron voor $O_2^{\cdot-}$ productie is na myocard infarct.

Endotheel dysfunctie verhoogt oxidatieve stress en dusdanig ook pro-inflammatoire aspecten. Daarnaast vangt een verhoogde $O_2^{\bullet-}$ concentratie het circulerende NO weg leidend tot nitrosatieve stress, met beïnvloeding van de NO-ET balans. Recente studies tonen aan dat inflammatie en oxidatieve stress geassocieerd met hartchirurgie, en meer specifiek cardiopulmonale bypass, een belangrijke rol spelen in het ontstaan van postoperatief atrium fibrilleren (POAF). In Hoofdstuk 9 wordt er verder gegaan op de resultaten van Hoofdstuk 8 en werden patiënten die na hartoperatie in sinus ritme bleven vergeleken met diegene die postoperatief atrium fibrilleren ontwikkelden. In deze kleine studie werd aangetoond dat er sprake is van een preoperatieve redox disbalans in patiënten met POAF en dat verhoogde preoperatieve concentraties aan inflammatoire cytokines NADPH oxidase stimuleren tot het produceren van $O_2^{\bullet-}$ bij patiënten die in sinusritme blijven indicatief voor een intact redox systeem en functioneel antioxidant systeem. Voorts is de belangrijkste bron van $O_2^{\bullet-}$ NOX-2 en kan de activatie gezien worden als een significante onafhankelijke voorspeller van POAF, onafhankelijk van het type chirurgie. Bloed plasma TBARS metingen zijn waardevolle voorspellers van POAF en betuigen een veranderde redox status welke zich verder zet gedurende hospitalisatie (alwaar eNOS "uncoupling" een rol kan spelen). Zodoende wordt een neerwaartse spiraal gecreëerd met elektrofysiologische aanpassing resulterend in POAF.

Hoofdstuk 10 bespreekt de resultaten van dit proefschrift binnen een breder kader en geeft instructies voor verder onderzoek met implementatie van de gepresenteerde data. Zodoende kunnen nieuwe inzichten in de fysiologie en pathofysiologie van radicaal biologie worden gecreëerd binnen het veld van cardiovasculaire geneeskunde.

13

ADDENDUM

PhD portfolio

Curriculum vitae

Dankwoord

PHD PORTFOLIO

Name PhD student:	Yannick J.H.J. Taverne
Erasmus MC department:	Experimental Cardiology
	Cardiothoracic Surgery
Research School:	Cardiovascular Research School (COEUR)
PhD period:	2012-2017
Title thesis:	Integrated Control of Endothelial Vasoreactivity
Promotors:	Prof.dr. D.J.G.M. Duncker
	Prof.dr. A.J.J.C. Bogers
Date of defense thesis:	01-12-2017

Academic training

2013 – Present:	**Resident in training Cardiothoracic Surgery**, Thorax Center Erasmus MC Rotterdam, The Netherlands.
2012	**Resident not in training Cardiothoracic Surgery,** Thorax Center Erasmus MC Rotterdam, The Netherlands.
2007 – 2010:	**Master of Science in Clinical Research,** NIHES, Rotterdam, the Netherlands.
2005 – 2012:	**Medical School,** Erasmus University Rotterdam, the Netherlands.
2003 – 2009:	**Specialization in Functional and Applied (cardiac) Anatomy and Evolutionary Biology**. University of Ghent and Catholic University of Leuven, Belgium / Erasmus MC Rotterdam, the Netherlands.
1998 – 2003:	**Master in Physical Therapy and Rehabilitation Sciences.** KHBO Bruges in association with the Catholic University of Leuven, Belgium.

PhD training

GENERAL	Year	ECTS
Biomedical English Writing and Communication	2008	0.15
English medical writing	2009	0.85
RESEARCH SKILLS		
Statistics		
Introduction to Data Analysis	2008	0.7
Regression Analysis	2008	1.9
Survival Analysis	2008	1.9
Modern Statistical Methods	2008	4.3
Principles of Epidemiologic Data Analysis	2010	0.9
Advanced Analysis of Prognostic Studies	2010	0.9
Methodology		
Principles of Research in medicine and Epidemiology	2007	0.7
Introduction to Methods for Decision Making in Medicine	2007	0.7
Methods of Clinical Research	2007	0.7
Case-control Studies	2007	0.7
Clinical Trials	2007	0.7
Pharmaco-epidemiology	2007	4.3
Study design	2007	0.9
Introduction to Clinical Research	2007	0.9
Intervention Research and Clinical Trials	2008	0.9
Diagnostic Research	2008	0.9
Advanced Topics in Decision-making in Medicine	2008	0.9
Prognostic Research	2008	0.7
Topics in Meta-analysis	2009	0.9
Pharmaco-epidemiology and Drug Safety	2010	1.9
Advanced Topics in Clinical Trials	2010	0.9
In depth courses		
Management and Health Care Organization, Harvard, Boston, USA	2009	0.9
Ethical Bases in Health Care, Harvard, Boston, USA	2009	0.9
COEUR Research Seminars and Lectures	2009-2013	4.5
Congenital EACTS Windsor, UK	2016	1
Cardiac 2 EACTS Windsor, UK	2015	1
Cardiac 1 EACTS Windsor, UK	2016	1
COEUR couses (6)	2007-2014	9

TEACHING		
ERCATHAN in depth functional anatomy and physiology courses	2007-2017	10
Supervising research students medical curriculum	2012-2017	5
PRESENTATIONS / INTERNATIONAL SYMPOSIA		
EACTS Vienna, Austria	2017	1
Aortic Valve, Paris, France	2016	1
EACTS Amsterdam, The Netherlands	2016	1
Aortic Valve, Paris, France	2015	1
Heart Evolution and Surgical anatomy Pittsburg, USA	2015	1
EACTS Vienna, Austria	2014	1
Young Investigator Cardiac Physiology, Papendal, The Netherlands	2012	0.9
Erasmus lectures	2012-2017	3.6
TOTAL ECTS		**68.1**

CURRICULUM VITAE

Yannick Taverne was born on the 20th of June 1979 in Roeselare, Belgium. He finished high school in 1997 and started his master in physical therapy and rehabilitation sciences at KHBO, in association with the Catholic University Leuven, Belgium. In 2003 he graduated cum laude and continued his training in cardiopulmonary rehabilitation and research. After graduating, while working as a physical therapist, he continued his specialization in functional and applied anatomy, more specific on evolutionary biology of the cardiopulmonary system. In 2005, he started medical school at the Erasmus University in Rotterdam, the Netherlands. During his medical training he continued working and expanded his anatomy specialization while starting the scientific forum ERCATHAN (European CardioThoracic *functional* Anatomy), which provides highly specialized clinical anatomy master classes and research. Within this format, courses are given throughout Europe on surgical and functional anatomy of the heart and lungs.

In 2006, Yannick was selected to participate in a Research Master for medical students, offered by the Erasmus MC Graduate School. This program enabled him to combine a Master of Science in Clinical Research with his medical degree. Part of this program was spent at the Harvard School of Public Health, Harvard University, Boston, MA, USA. As part of this research master, he performed one year of research at the division of Experimental Cardiology, Thorax Center, Erasmus MC, under the supervision of prof. dr. D.J.G.M. Duncker and prof. dr. A.J.J.C. Bogers. After finishing medical school in 2012, he started working as a resident at the department of Cardiothoracic Surgery were in 2013, he entered the training program for cardiothoracic surgery under supervision of prof. dr. A.P. Kappetein. During his training he completed his thesis.

DANKWOORD

Bij een proefschrift hoort een dankwoord. Bijna iedereen die een proefschrift in handen krijgt, bladert direct door naar het dankwoord. De kans is groot dat u dit nu ook hebt gedaan. Het is ook een belangrijk onderdeel alwaar de ruimte bestaat om mensen te vermelden die dit werk mede mogelijk hebben gemaakt. Hier schuilt natuurlijk ook het gevaar dat sommigen per abuis kunnen worden vergeten. Daarom wil ik alvast starten met iedereen, die me de laatste jaren heeft geholpen, te bedanken voor hun steun en toeverlaat. Zonder hen had ik dit niet alleen voor elkaar kunnen krijgen. Een aantal mensen wil ik hier in het bijzonder benoemen.

Allereerst, wil ik beginnen met het bedanken van mijn promotoren.

Prof. dr. Duncker, beste Dirk, ik kan me nog goed herinneren toen ik in het eerste jaar bij u langskwam met de vraag of ik onderzoek kon doen. Gelukkig vond u het een goed plan en kon ik bij u op het lab aan de slag. Ik ken weinig mensen die zo gedreven en hartstochtelijk kunnen vertellen over de ins-en-outs van het menselijk functioneren. Even een “zijspoor” naar de vergelijkende fysiologie, de relatie met de (specifieke vorm van de) aorta druk curve en de implicatie van de fysiologie in de bredere vraagstukken des levens, zorgden altijd voor mooie discussies. Deze enorme stroom aan kennis resulteerde dan ook in de nodige “rood-markeringen” op de verschillende papers; uiteindelijk leidend tot dit proefschrift. Kortom, Dirk, bedankt voor de kans en het vertrouwen!

Beste prof. Bogers, al vroeg in de opleiding geneeskunde kwam ik op uw deur aankloppen met de vraag wat er van iemand werd verwacht om thoraxchirurg te worden. U heeft me toen heel nuttige tips gegeven met een plek als oudste co, ANIOS en uiteindelijk een opleidingsplek binnen de thoraxchirurgie van het Erasmus MC. Binnen mijn onderzoek, hoewel heel basaal, was u altijd bereid mee te denken om nieuwe ideeën uit te werken en vind ik uw kritische blik bewonderenswaardig. Prof. Bogers, ook hier veel dank voor de begeleiding en kansen binnen de thoraxchirurgie die het schrijven van dit proefschrift mede mogelijk hebben gemaakt.

Mijn copromotor dr. Merkus, beste Daphne, waar moet ik beginnen om je te bedanken. Ondanks de drukte en de vele verplichtingen slaagde je er toch in om de tijd te maken voor de vele studenten. Even een kort bericht sturen en meestal kon ik snel terecht om wat door te spreken. Ik bewonder je drive en vindingrijkheid, zeker tijdens de operaties! Ik denk dat het wel duidelijk is dat jij de ‘McGuyver’ van de

23ste verdieping bent. Ik heb enorm veel gehad aan de correcties en tips bij het schrijven van dit proefschrift. Daphne, dank je voor de leuke tijd samen op het lab en ik hoop dat we in de toekomst nog lang mogen samen werken! PS. De Belgische speciaal bieren volgen nog; zoals beloofd.

Wat betreft de vrienden, mijn maten (ook wel: min moatn); ik had me voor genomen om het kort te houden. Echter, kan ik het toch niet laten even stil te staan bij deze bijzondere groep mensen.

Mijn paranimfen, Tim en Ralph; mannen van het eerste uur! Ooit hebben we een pact gesloten (toen we nog jong en onbezonnen waren,… en ik nog haar had) dat we elkaar zouden steunen bij onze promoties. En hier staan we dan! Two down, one to go! Jongens, bedankt voor alles, het was geen makkelijk jaar, maar jullie waren er wel! Wat een avondje stappen in België, een race parcourtje over de pechstrook en meerdere nachtjes snijzaal (met equivalent aantal uren in de Witte Aap) niet allemaal kunnen opleveren!

Jean, Hilco, Juanito. De soms harde aanpak en 'no nonsens' mentaliteit is iets wat ik altijd enorm heb geapprecieerd. Het "niet lullen, maar poetsen" heeft ons allen gebracht waar we nu zijn, uiteraard met de nodige ontspanningen en verbruik van gerstenat! Of het nu gaat om een harde "knal" in Noord-Holland, een toiletbezoek in Coo, wat Franse kaas in de haardos of een veel te kleine sauna die eigenlijk evengoed een badkuip zou kunnen zijn; het maakt allemaal niet uit! Gewoon jezelf zijn!

Hann, Hannus, Hannibal; we go way back. Als ouwe rot van het lab weet je maar al te goed wat voor effect dit soort basaal onderzoek op de vorming van een maagzweer kan hebben! Menig frustratie kon echter altijd goed worden aangepakt op de squashbaan waar je nooit verlegen zat om deze beste fors te laten rennen. Gelukkig werden dergelijke uitputtingsslagen ruim gecompenseerd met de immer groeiende collectie van het betere graan destillaat. Daarnaast heb ik kunnen ondervinden dat je leuze "mi casa, su casa" wel heel letterlijk is; echt super bedankt voor die tijd!

Kortom, jongens, zoals West-Vlaanderens 'beste zanger' het ooit stelde: *"min moatn, ik zie uder gerne, wa zo kik doen zondr gidre"*!

De leden van de commissie: prof. dr. Zijlstra, prof. dr. Takkenberg, prof. dr. Kleinrensink, dr. Mik en dr. Verheule wil ik graag bedanken voor het zetelen in deze promotie.

Meer specifiek wil ik hier prof. dr. Kleinrensink bedanken. Beste Gert-Jan, de vrijheid en geboden mogelijkheden op de snijzaal zijn in de meeste centra ondenkbaar. Toch had je er vertrouwen in mij (en ons bijzondere groepje maten) aan de slag te laten gaan. Immer enthousiast begeleidde je me in de exploitaties; van EARP, ERCATHAN, Belgische bieravonden tot het in een vaag verleden gebeurde en volledig onderdrukte (ergens weggestopt in de diepste krochten van ons bewustzijn) uitstap naar Renesse. GJ, vele malen dank voor de kansen, de leuke gesprekken en steun doorheen de jaren.

Graag wil ik ook alle coauteurs en de mensen op de 23ste verdieping bedanken voor hun bijdrage aan de publicaties, het prettig samenwerken en de leuke sfeer. Meer specifiek wil ik Annemarie bedanken. Echt een super tijd met je gehad op het lab, nooit verlegen om een grapje en altijd bereid extra data te helpen analyseren. Daarbij komt nog dat je, ondanks groot protest, echter na enige stimulatie, toch bereid bent om de draadjes te voeren.

Veel dank gaat uit naar de staf cardiothoracale chirurgie voor de begeleiding en sturing tijdens mijn opleiding. De ruimte en vrijheid die ik van de opleidingsgroep verkreeg hebben er mede voor gezorgd dat dit proefschrift kon worden afgerond. Meer specifiek wil ik hier ook mijn opleiders vermelden. Prof. dr. Kappetein, beste AP, tijdens de eerste 5 jaar van mijn opleiding stond uw deur altijd open voor overleg, maar ook om te spuien wanneer het even tegen zat. Uw toewijding aan onderzoek is een grote inspiratiebron en de updates over de laatste publicaties, meer specifiek onder begeleiding van wat gastronomie, werden altijd meer dan geapprecieerd. AP, het ga u goed in uw verdere carrière en bedankt voor de afgelopen jaren! Dr. Bekkers, beste Jos, recent bent u de nieuwe opleider geworden. Er waait een nieuwe wind door het opleidingsklimaat en dit wordt door ons allen uiterst gewaardeerd. Uw passie tot opleiden werkt als een stimulans voor mijn laatste jaar en het doet me deugd te merken dat u alles in het werk stelt om dit zo goed mogelijk in te vullen!

Special thanks to prof. dr. Timothy Lyons from the department of Earth Sciences, University of California. I am very grateful that you embraced our early contacts with enthusiasm leading to a paper combining different areas of expertise. It was a very nice experience for me to get involved in a field that normally would never be incorporated into medical research. I learned a lot and, who knows, maybe in the future another quest awaits.

Marga, Jan, Maarten-Jan; het is wat om er zo'n Belg bij te krijgen. Hebben jullie ook niet om gevraagd en plots staat hij aan je deur. Heel fijn dat ik zo hartelijk werd opgenomen in de familie! Enorm veel steun aan jullie gehad in een toch wel heel bewogen jaar. Nu is de tijd aangebroken (en me dunkt is er geen weg meer terug) om die mooie fles Amarone maar eens aan te vallen!

Mutty, hoezeer je hier niet bij had willen zijn. Menig keer hebben we het er over gehad hoe je deze dag voor je zag en hoe trots je was! De hardste strijd heb jij geleverd, hard heb je gevochten, maar het mocht niet baten. Dit wil ik daarom graag aan je opdragen! Padre, aan jouw de taak om voor jullie beiden waar te nemen. Het geeft me enigszins rust dat ze sowieso wist dat alles wel goed zou komen met ons. Lieve ouders, bedankt voor alles!

Sanne, mon amour; last but not least. Teveel ogen lezen dit mee en daarom zal ik het hier kort houden. Mijn rots in de branding, ik hou van je!

Graag wil ik volgende sponsors bedanken voor hun financiële bijdrage aan dit proefschrift.

Getinge Group

Chipsoft B.V.

Vascutek Nederland

QP&S N.V.

Krijnen Medical

ABN AMRO Groep

Thoraxcentrum Research B.V., Erasmus Medisch Centrum

Experimentele Cardiologie, Erasmus Medisch Centrum

Cardialysis

Hans Huysmans Foundation