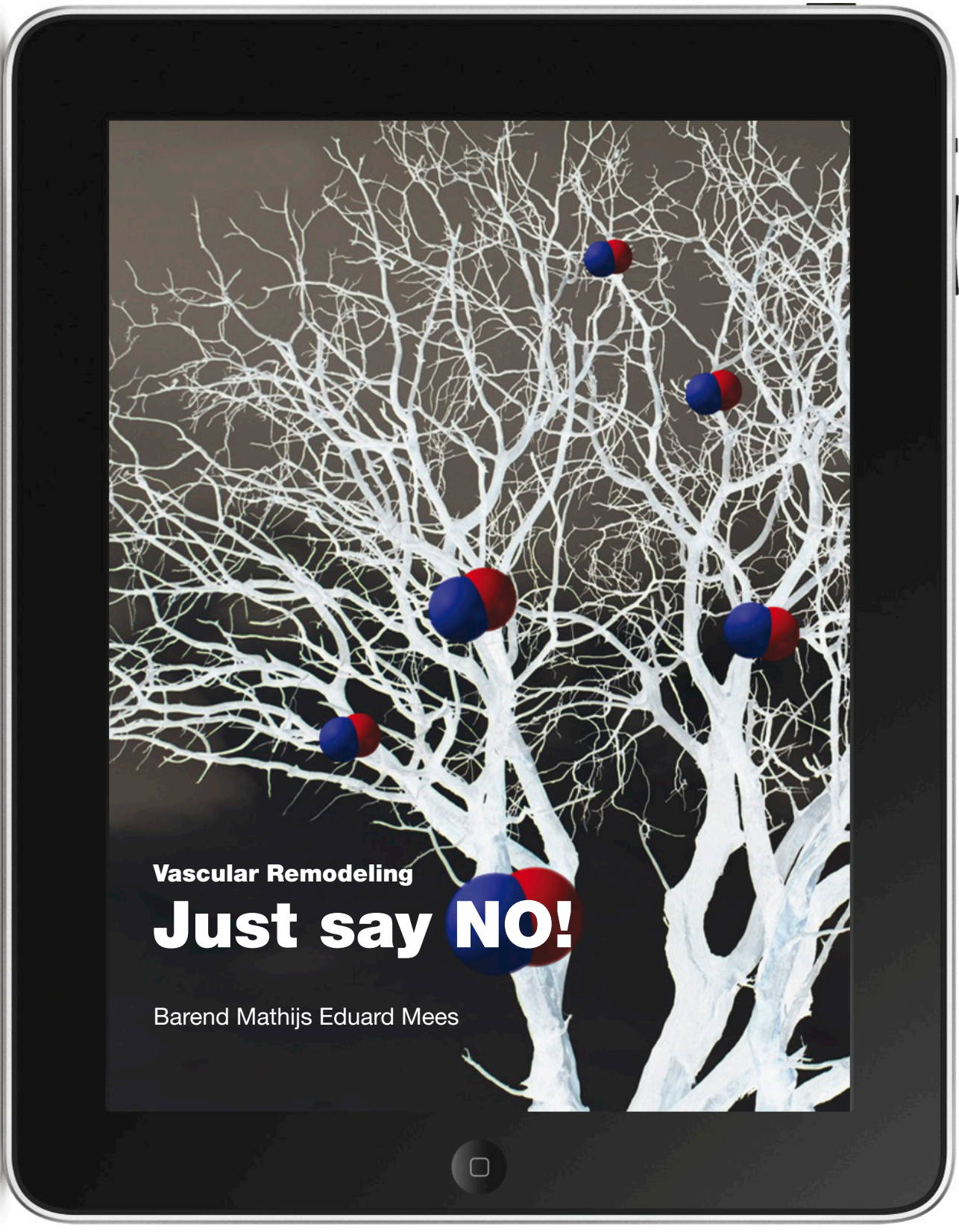
Vascular Remodeling
Just say NO!
Barend Mathijs Eduard Mees

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Vascular Remodeling: Just say NO!

Vasculaire remodellering: Zeg maar NO!

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op gezag van de rector magnificus

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Contents

Chapter 1

Introduction

Barend Mees

Partly adapted from:
A Different Outlook on the Role of Bone Marrow Stem Cells in Vascular Growth: Bone Marrow Delivers Software, not Hardware

Matthias Heil, Tibor Ziegelhoeffer, Barend Mees and Wolfgang Schaper

Circ. Res. 2004;94;573-574

From the Max-Planck-Institute for Physiological and Clinical Research (M.H., T.Z., W.S.) and Kerckhoff-Clinic (T.Z.), Bad Nauheim, Germany; and Departments of Vascular Surgery and Cell Biology and Genetics (B.M.), Erasmus Medical Center, Rotterdam, the Netherlands.

Introduction

"You are only as old as your endothelium", as often stated by Paul M Vanhoutte, when opening his famous "Mechanisms of Vasodilation" meetings from 1980 onwards, highlights the importance of endothelium and the continuous process of vascular remodeling in many physiological and pathophysiological situations as embryogenesis, wound healing, tumor growth and ischemic disease. In chronic and acute cardiovascular occlusive disease, different types of vascular remodeling contribute to tissue repair and vascular growth. Firstly, collateral growth represents the expansive growth of preexisting vessels, forming collateral bridges between arterial networks. Secondly, neovascularization refers to vascular growth from a combination of three different mechanisms: vasculogenesis is the formation of blood vessels by endothelial progenitors while angiogenesis refers to capillary sprouting or intussusceptive growth and arteriogenesis to the subsequent stabilization of these new vascular structures by mural cells[1]. Distinct, but partially overlapping, cellular and molecular pathways drive collateral growth and neovascularization (Figure 1)[2]. Hypoxia is known to stimulate neovascularization in the setting of ischemia, whereas fluid shear stress (FSS) might be the most important trigger for initiation of collateral growth. Besides these specific initial triggers, all types of vascular remodeling share growth factors, chemokines, proteases, and inflammatory cells, which play different roles in promoting and refining these processes.

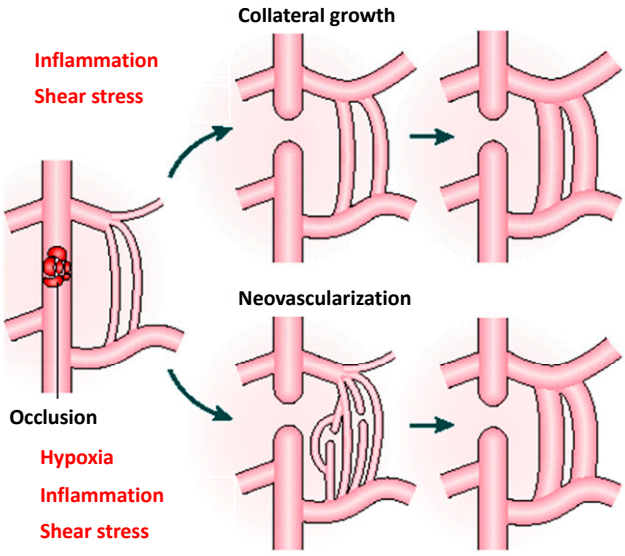

Figure 1. During postocclusive vascular remodeling, increased shear stress in preexisting arterioles is the most important stimulus for collateral growth, while hypoxia in the more distant vascular bed is the major trigger for neovascularization. See text for further description of differences in vascular remodeling pathways.

Therefore, in case of occlusive arterial disease, the different types of vascular remodeling are considered as complementary parts of the process of blood flow and ischemic tissue perfusion recovery.

Collateral Growth

Following an arterial occlusion outward remodeling of preexistent interconnecting arterioles occurs by proliferation of vascular smooth muscle and endothelial cells. This is initiated by deformation of the endothelial cells through increased pulsatile fluid shear stress (FSS) caused by the steep pressure gradient between the high preocclusive and the very low postocclusive pressure regions that are interconnected by collateral vessels.

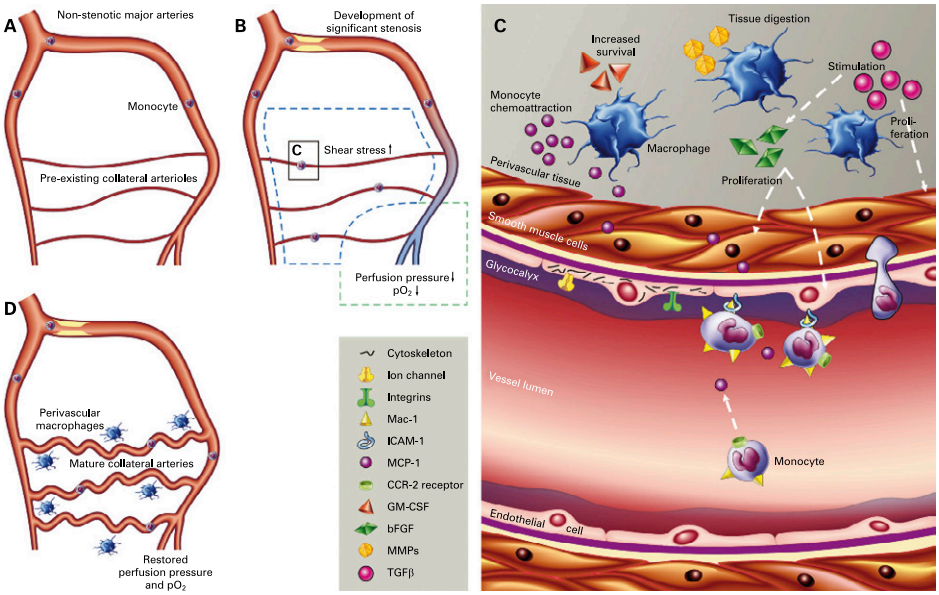

Figure 2. Mechanisms of collateral growth; increased shear stress in preexisting collateral arteries activates the endothelium, leading to an inflammatory-like reaction with invading monocytes and finally endothelial and smooth muscle cell proliferation. (Figure taken from Schirmer et al[5] with permission)

The primary physiological response to FSS is an activation of endothelial cells via transduction of the mechanical stimulus, and a large number of genes are controlled by shear stress responsive elements in their promoters[3,4]. Increased FSS results in increased production of endothelial adhesion molecules and chemokines and its receptors (Figure 2). The most extensively studied chemokine contributing to collat-

eral growth is monocyte chemoattractant protein-1 (MCP-1). MCP-1 and its receptor chemokine receptor 2 (CCR2) are significantly upregulated in collateral arteries and in absence of MCP-1 or CCR2 collateral growth is severely impaired. A second important pathway activated by FSS is fractalkine/CX3C receptor 1, which is involved in adhesion and migration of leukocytes. Furthermore, increased FSS may also enhance synthesis of proinflammatory cytokines as tumor necrosis factor α (TNFα) and interleukin-6 (IL-6). Thus, production of the above cytokines, chemokines and adhesion molecules lead to attraction and activation of proinflammatory cells (monocytes, NK cells and T-cells) into the adventitial space or attachment of these cells to the endothelium. Inflammatory cells then produce proteases and growth factors to digest the extra-cellular scaffold and allow motility and provide space for the new cells. The bulk of new tissue production is carried by the smooth muscle cells of the media, which transform their phenotype from a contractile into a synthetic and proliferative one[6,7].

Vasculogenesis

For many years, the prevailing dogma stated that vessels in the embryo developed from endothelial progenitors, whereas sprouting of vessels in the adult resulted only from division of differentiated endothelial cells. However, evidence from the last decades indicates that endothelial progenitors contribute to vessel growth both in the embryo and in specific conditions in the adult, including ischemia, inflammation and malignancy. Endothelial cells differentiate in the embryo from angioblasts[8] and in the adult from endothelial progenitor cells (EPC) that are derived not only from bone marrow, but also from other tissues like adipose tissue, liver, spleen and even vessel wall[9-12]. In the condition of postischemic vascular remodeling, progenitor cells are mobilized and attracted towards the ischemic tissue via homing signals as a response to hypoxia. This recruitment of stem cells to the neovascularization sites strongly resembles an inflammatory response. Once in the vicinity of ischemic tissue, progenitor cells interact with the endothelial monolayer in a similar way as leucocytes interact with activated endothelial cells[13]. The activated endothelium produces chemoattractant molecules as stromal-cell derived factor-1 (SDF-1) and adhesive molecules as selectin and intercellular adhesion molecule-1 (ICAM-1) and binds progenitor cells to their equivalent receptors chemokine receptor 4 (CXCR4), placenta growth factor-1 (PlGF-1) and β2 integrin. Subsequently the endothelial lineage allows progenitor cells to incorporate in the endothelium or infiltrate the vessel wall through increased vasopermeability. Progenitor cells will then differentiate into an endothelial cell phenotype or produce different factors involved in tissue remodeling exerting an important paracrine effect.

Angiogenesis and Arteriogenesis

The nascent vascular bed expands by sprouting and matures into a system of stable vessels. Hypoxia is the main stimulus for expansion of the vascular bed and occurs when tissue oxygen demand is beyond the limit of oxygen diffusion. Hypoxia triggers endothelial cell sprouting by signaling through hypoxia-inducible transcription factors (HIFs)[14]. HIF-1 and HIF-2 upregulate many angiogenic genes, but the induction of vascular endothelial growth factor-A (VEGF-A) is the most remarkable. VEGF-A is a key regulator of physiological and pathological angiogenesis. Two receptor tyrosine kinases, VEGFR-1 and VEGFR-2, mediate its biological effects[15]. Furthermore, hypoxia also controls the inflammatory reaction. Activation of HIF-1α is essential for myeloid cell infiltration and activation and it modulates adhesion capacity of inflammatory cells, through a mechanism independent of VEGF. As endothelial cells migrate during vessel sprouting the extracellular matrix is proteolytically broken down and its composition altered. This proteolytic remodeling of the extracellular matrix is an extremely balanced process[16]. Establishment of a functional vascular network further requires that nascent vessels mature into durable vessels. The association of pericytes and smooth muscle cells with newly formed vessels regulates endothelial cell proliferation, survival, migration, differentiation, vascular branching, blood flow and vascular permeability. At least two important molecular pathways are involved in regulating this process: the platelet-derived growth factor B (PDGFB) pathway and the transforming growth factor β (TGFβ) pathway[17]. Finally, flow has an essential role in determining whether neovessels regress or persist. By affecting several factors (including growth factors, integrins and matrix metalloproteinases) flow stimulates hyperplasia of endothelial and smooth muscle cells and induces the reorganization of endothelial junctions and the deposition of extracellular matrix, all of which contribute to vessel maturation[1].

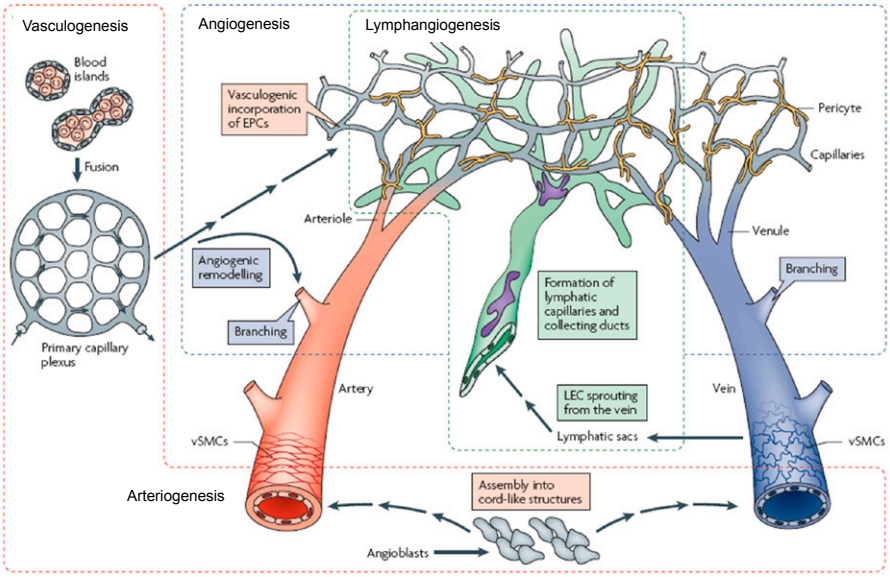

Figure 3. Neovascularization is the combination of vasculogenesis, angiogenesis and arteriogenesis. (Figure taken from Adams & Alitalo[18] with permission)

Discovery of Nitric Oxide

There are few discoveries with the magnitude of the impact that nitric oxide (NO) has had on biology during the 25 years since its discovery as an important molecule in the cardiovascular system. NO was discovered in 1772 by Joseph Priestly as a clear, colorless gas with 6 - 10 seconds lifetime *in vivo*. In 1980, Robert Furchgott discovered that endothelial cells produce an endothelium-derived relaxing factor (EDRF) in response to stimulation by acetylcholine[19]. In 1987, Salvador Moncada and Louis Ignarro independently proved that EDRF is NO[20,21]. A decade earlier, Ferid Murad had shown that nitrovasodilators (e.g., exogeneous NO, nitroglycerin, sodium nitroprusside) are able to relax vascular tissues[22]. All this work contributed to the recognition of NO being a major signaling molecule in the cardiovascular system. In 1992, the cover of the journal Science proclaimed NO as the molecule of the year and awarding the Nobel Prize in Physiology and Medicine to Furchgott, Ignarro and Murad six years later underlined the importance of their work. In 1999, Moncada was the most highly cited U.K. scientist of the decade, reflecting his seminal work on NO. Since then, NO has been found to be involved in almost every area of biomedicine, including cardiovascular function, neurotransmission, pain, diabetes, wound healing and tissue repair, skin renewal, cancer, immune function, infection, respiratory function and eye disease[23].

Nitric Oxide Regulation

NO is a gaseous signaling molecule that is synthesized by the nitric oxide synthase (NOS) enzyme in almost all tissues and organs. There are three isoforms of NOS that are encoded by different genes. Their names are derived from the tissue from which they were initially isolated[24]. Neuronal NOS (nNOS/NOS1) was isolated from the brain and was the first NOS enzyme to be identified[25]. Inducible NOS (iNOS/NOS2) was isolated from macrophages[26]. The third NOS enzyme, endothelial NOS (eNOS/NOS3) was isolated from bovine aortic endothelial cells[27]. NO is synthesized by all three NOS isoforms by the conversion of L-arginine to L-citrulline. eNOS and nNOS are calcium-dependent whereas iNOS is calcium-independent. However, all isoforms require calmodulin binding to achieve full catalytic activity. The availability of L-arginine and the cofactor for the reaction tetrahydrobiopterin (BH4), affect the production of NO. In addition, guanosine triphosphate (GTP) cyclohydrolase 1, which catalyzes GTP to dihydrobiopterin triphosphate, appears to be the rate-limiting enzyme. Reduced substrate and cofactor availability, conditions that may pertain in disease states, results in NOS uncoupling where superoxide anion (O_2-) is produced preferentially over NO.

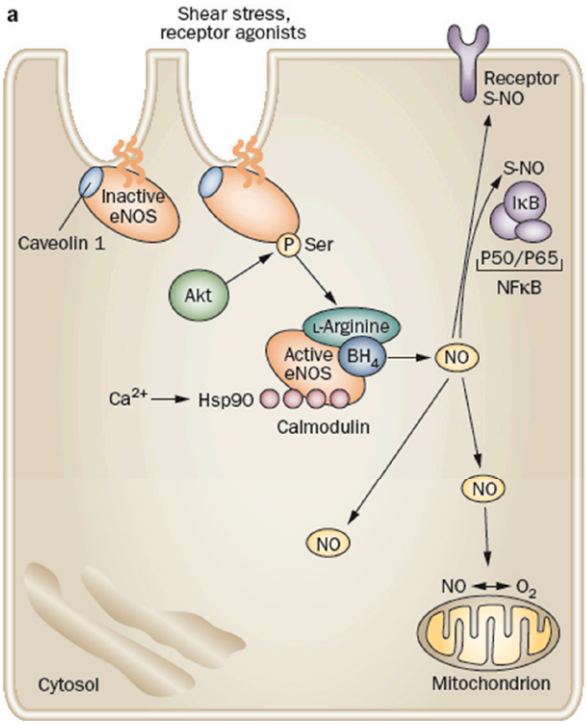

Figure 4. NO is produced in the endothelial cell upon exposure to shear stress or receptor antagonists (eg., acetylcholine, bradykinin, VEGF). (Figure taken from Rabelink et al[30] with permission)

iNOS produces more NO than eNOS but it also has a greater tendency to uncouple and produce free radical oxygen species (ROS) as superoxide anion. At low concentrations ROS acts physiologically, however at higher concentrations superoxide anion alone can be damaging and moreover also react with NO to produce peroxynitrite, thereby causing further damage.

The NOS enzymes are also regulated by phosphorylation. Phosphorylation of eNOS at specific residues (as serine 1777), mainly through the Akt/phosphatidylinositol 3-kinase (PI3K)-mediated pathway, causes the enzyme to produce higher levels of NO[24]. Finally, the activity of eNOS is also regulated by its cellular localization and by its interaction with a number of different proteins. Localization of eNOS to the Golgi region and the plasmalemmal caveolae is required for optimal NO production[28]. Regulation via protein–protein interactions both positively and negatively affect NOS activity[29].

Nitric Oxide in the Cardiovascular System

In the cardiovascular system, NO has an astonishingly wide range of physiological and pathophysiological activities. NO influences vascular homeostasis in many ways beyond modulation of vasomotion, such as inhibition of smooth muscle cell proliferation, platelet aggregation, platelet and monocyte adhesion to the endothelium, low-density lipoprotein (LDL) oxidation, expression of adhesion molecules and endothelin production. Defects in these functions usually result from decreased NO bioavailability and dysfunction in the NOS enzymes and are responsible for diseases such as atherosclerosis, coronary artery disease, diabetes, hypercholesterolemia, hypertension, immune reactions (transplant rejections), inflammation, migraine, meningitis, peripheral vascular disease, vascular restenosis, septic shock, sickle cell disease and stroke[31].

Nitric Oxide-Based Therapies

The involvement of NO in such a wide variety of disease states has made it an attractive target for both pharmacological and gene therapies. In 1998 Pfizer introduced Viagra®, a drug with a mechanism of action (phosphodiesterase inhibition) based on the NO-cGMP system, which revolutionized the management of erectile dysfunction. In the following years many experimental studies were performed to develop a therapeutic increase in NO bioavailability using pharmacological agents, NO-releasing donor compounds or NOS gene therapy. However, the complex regulation of NOS in the cardiovascular system needs to be carefully considered in the context of gene therapy

with substrate availability, enzyme uncoupling and superoxide generation potentially having deleterious effects, in particular in different vascular disease states[32].

Nitric Oxide in Vascular Remodeling

Vasodilation

Nitric oxide is clearly the major endothelium-dependent vasodilator produced by large blood vessels. Endothelial cells produce NO via eNOS and NO activates the cyclic guanosine monophosphate (cGMP) pathway through soluble guanylate cyclase (sGC) leading to vascular smooth muscle cell relaxation and vasodilation. In the aorta or carotid arteries of mice deficient in eNOS (eNOS KO mice), endothelium-dependent vasodilation is eliminated[33]. However, the vasodilatory roles for NO in arterioles and venules appear to be more complex and the importance of NO as a vasodilatory agent (deciphered by the sensitivity of the response to blockage of NOS) depends on the stimulus (flow versus other agonists such as acetylcholine, VEGF, histamine), the vascular bed studied (coronary, cremaster, gracillus, mesentery), the species (human, rat, mouse, hamster) and the number of endothelial-smooth muscle interconnections (i.e. myo-endothelial gaps)[33]. For example, in eNOS KO mice, the local vasodilatory actions of acetylcholine or flow induced changes in blood flow are not diminished, due to compensation by upregulation of nNOS, endothelium-derived hyperpolarizing factor (EDHF) and vasodilatory prostaglandins[34,35]. However, the local vasodilatory action of histamine was absent from arterioles in eNOS KO mice[36].

Another theory imparting NO as an important factor in microvascular blood flow control is the concept that NO bound to hemoglobin in red blood cells can serve as a stable NO-adduct for delivery at sites of resistance[37]. Although this principle can be demonstrated in model systems, the physiological role of NO via its release from hemoglobin in regulating blood flow in the microcirculation remains controversial.

Finally, studies on postocclusion vascular remodeling demonstrated that apart from endothelial cells producing NO also bone marrow-derived cells homing towards ischemic tissue were able to produce NO and thus induce vasodilation[38].

Collateral growth

Collateral growth is characterized as the response of adult arteries with structural changes to changes in blood flow, during which the lumen is controlled by an immediate physiological adjustment in vascular tone induced by the change in flow, and by a delayed anatomical change that occurs when the change in flow persists[39]. During this early adaptive response to increased fluid shear stress a strong vasodilation results in a transient, temporary increase in flow through the collaterals. The persistent increase

in shear stress subsequently triggers endothelial production of a host of molecules (growth regulatory, inflammatory and adhesive) leading to the complex inflammatory-based mechanism of collateral enlargement with endothelial and vascular smooth muscle cell proliferation. While ample evidence exists for the function and critical role of endothelial NO in vasomotion and angiogenesis, the role of eNOS in collateral growth is less clear, and is even known as the NO-paradox[6].

On the one hand, increased shear stress upregulates the expression of eNOS augmenting endothelial NO production, probably resulting in the rapid vasodilatory response. In contrast, NO also acts as a potent inhibitor of the expression of adhesion molecules and smooth muscle cell proliferation, which are both indispensable for collateral growth. NO has emerged as a crucial endogenous antiinflammatory mediator in a number of pathophysiological states including hypercholesterolemia and ischemia-reperfusion injury. More specifically, NO can act by downregulating cytokines, resulting in the downregulation of endothelial cell adhesion molecules (ECAMs). The mechanism of leukocytes recruitment is mediated by these ECAMs such as the selectin family (P- and E-selectin), which are important modulators of leukocyte-endothelium interaction via leukocytes rolling along the endothelium and adhesion to the endothelium. Once the cells begin to roll, they can then firmly attach to the endothelium via integrin interaction with endothelial intercellular adhesion molecules (ICAMs) to promote leukocyte adhesion[33]. Pharmacological studies have shown that NOS inhibitors could increase leukocyte adhesion, an effect that can be reversed by large amounts of exogenous L-arginine. Similar studies using eNOS KO mice confirmed that upon activation of an inflammatory response, eNOS derived NO is critical for the reduction of leukocytes adhesion and the extent of tissue injury. Using another inflammatory model, namely the myocardial ischemia–reperfusion injury, NO was also shown to provide a protective role in the injury cascade leading to inflammation[33].

In addition to the role of NO as an inhibitor of leucocyte adhesion, NO in the microcirculation can also prevent platelets from adhering to the endothelium and assist with the dis-aggregation of activated platelets to the endothelium or underlying basement membrane. This concept was unequivocally demonstrated in mice deficient for eNOS, in which platelet adhesion was increased and consequently bleeding times were decreased[40,41].

Finally, while eNOS upregulation in the vessel wall was demonstrated in immunohistochemical studies of collateral arteries, eNOS-deficient mice display decreased vascular remodeling in response to femoral artery ligation and suffer from severe peripheral ischemic complications. Recent studies have attributed this effect in eNOS KO mice to impaired collateral growth, angiogenesis, vasculogenesis, or a combination of these[42-44]. However, in these studies the different competing or complementary mechanisms of vascular growth could not be isolated because of the use of a severe ischemia model,

which causes substantial damage to the lower limb. Thus, the role of eNOS in the different types of vascular remodeling could not be elucidated. Therefore, in absence of an *in vivo* model specific for collateral growth, the role of eNOS in collateral growth remains paradoxical.

Vasculogenesis

The first step in vasculogenesis is mobilization of progenitor cells in their microenvironment, sometimes described as stem cell niche. A variety of cytokines (granulate-colony-stimulating factor (G-CSF), SDF-1, VEGF, PlGF and erythropoietin) induce the mobilization of progenitor cells via activation of proteases (cathepsins, MMPs), which interfere with receptor ligand-interactions that maintain progenitor cells in their environment[45]. This process is dependent on eNOS-mediated release of NO. In eNOS KO mice MMP-9 induction via VEGF signaling is significantly decreased, thus mobilization from bone marrow progenitor cells is impaired and the neovascularization response to ischemia hampered[42]. Transplantation of wild-type eNOS-expressing bone marrow cells was not able to rescue the neovascularization response, indicating that NO provided by the local (ischemic) environment also regulates progenitor cell functions.

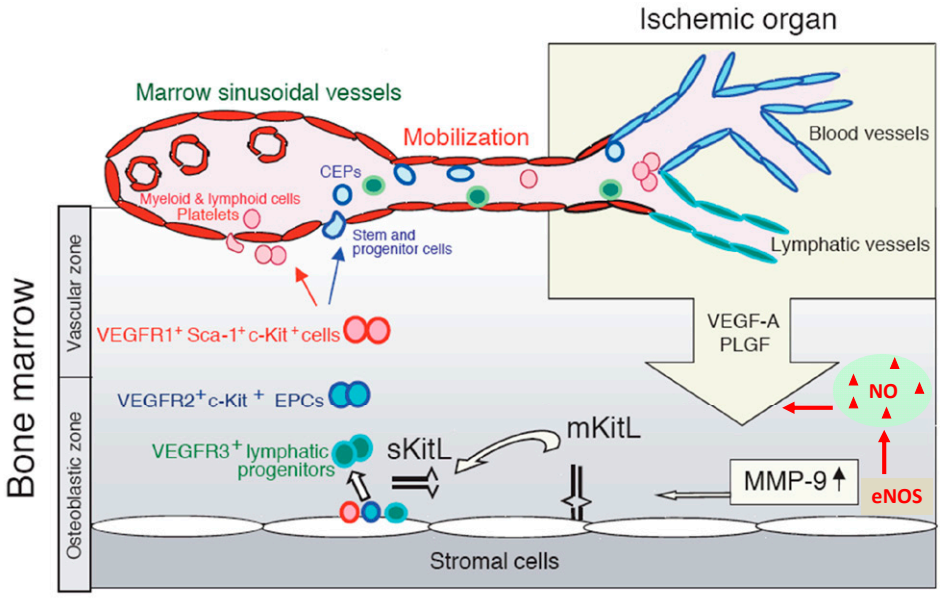

Figure 5. eNOS is a mediator of progenitor cell mobilization via VEGF-A/MMP-9 pathway. (Figure modified from Rafii & Lyden[46] with permission)

Furthermore, progenitor cells are able to produce growth factors involved in vascular remodeling. At arrival at the target ischemic tissue, progenitor cells interact with the activated endothelium through SDF-1 and CXCR4 signaling and release NO via an eNOS-dependent pathway. Production of NO subsequently induces a marked vasodilation and disruption of vascular endothelial-cadherin/β-catenin complexes, leading to increased vascular permeability. Progenitor cells from eNOS-deficient mice were not able to induce this vasodilation and vasopermeability demonstrating that the ability of progenitor cells to produce NO is critical for their proangiogenic function[38].

Angiogenesis and arteriogenesis

The mechanisms that regulate angiogenesis in hypoxia or hypoxic microenvironment are modulated by various pro- and antiangiogenic factors. Hypoxia-inducible factors (HIFs) have been established as the basic and major inducers of angiogenesis, up-regulating expression of these angiogenic proteins. A complex interplay with NO exists upstream from HIF activation, as NO drives HIF-1α stabilization under normoxia while NO favors the degradation of HIF-1α under hypoxia[47]. Thus, via HIF-1α, NO is able to mediate the activation of several angiogenic proteins.

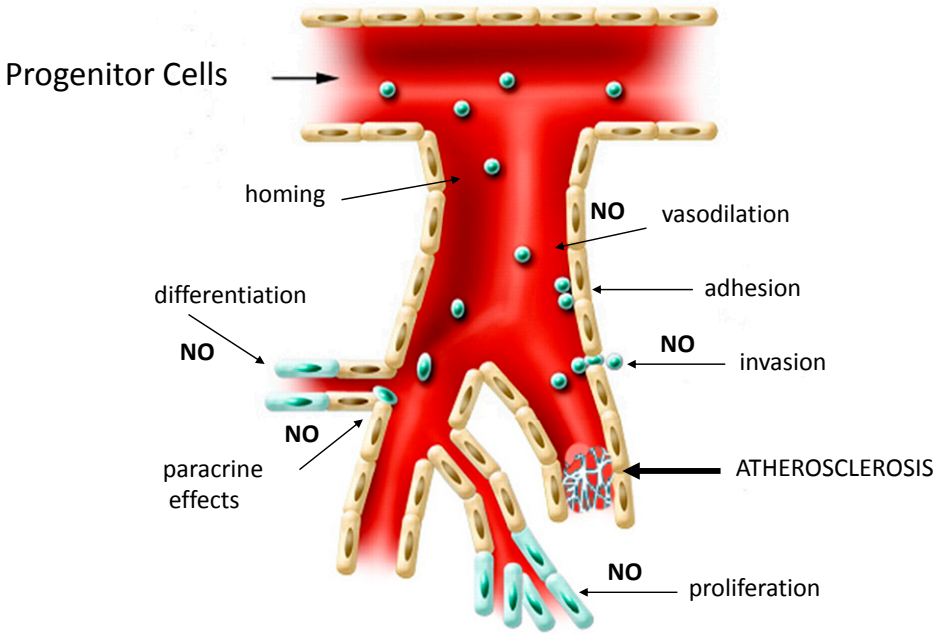

Figure 6. Consecutive proangiogenic effects of NO release in neovascularization. (Figure modified from Dimmeler et al[48] with permission)

When activated by HIFs, angiogenic growth factors as VEGF, PDGF, angiopoietin-1 (Ang-1) or sphingosine 1-phosphate (S1-P) signal through their receptors on endothelial cells and activate endothelial NO synthase. This process is mediated through increased calcium concentration, by activating Akt via the phosphatidylinositol 3-kinase (PI3K)-mediated pathway and the recruitment of heat-shock protein 90 (Hsp90). Vice versa, NO is able to stimulate the expression of VEGF-A and its receptors. Finally, NO release induces angiogenesis by stimulating endothelial cell migration, proliferation and survival. Although NO is an inhibitor of the TGFβ pathway inhibiting vascular smooth muscle cell proliferation in various models (pulmonary hypertension, arterial calcification) there is no evidence of hampered vessel stabilization by NO production.

Therapeutic Vascular Remodeling

To prevent or treat ischemic cardiovascular disease, the concept of therapeutic vascular remodeling, eg. the stimulation of tissue vascularization after ischemia, has been developed and recently made the step from bench to bedside. Therapeutic vascular remodeling strategies fall into two categories: single gene/protein- or cell-based.
Angiogenic growth factor (e.g., VEGF, FGFs, and hepatocyte growth factor) therapy has been tested clinically for more than 5 years. Overall, benefit for peripheral arterial occlusive disease (PAOD) patients has been disappointing[49,50]. While repeated administration of the angiogenic gene may increase the chances of success as demonstrated by a recent study treating PAOD patients with FGF-1[51], the current thought is that use of a combination of growth factors, or "master switches" (e.g., hypoxia-inducible factor (HIF)-1α), may be better strategies for successful revascularization due to the molecular complexity of collateral growth and neovascularization[52]. Moreover, recently, the concept of combining angiogenesis (determining oxygen delivery) with induction of metabolic changes (regulating oxygen consumption) by (ischemic) muscle was introduced as a promising new strategy to treat patients with ischemia[53,54].
In addition, the disappointing results may be related to a combination of endothelial dysfunction and the reduced amount or the lack of responsiveness of endogenous progenitor (vascular) cells in patients with cardiovascular disease, as the success of administration of growth factors depends on the ability of progenitor cells to be recruited and to expand. In this context, the concept of (stem) cell-based revascularization emerged in 1997, when Isner and coworkers described circulating cells in adults –called 'endothelial progenitor cells' (EPC)– with the capacity to differentiate into endothelial cells (EC) and incorporate into new vessels in ischemic tissue[12]. Since then, the number of studies reporting on (stem) cell-related revascularization has exponentially increased and recent clinical studies have demonstrated the safety, feasibility and efficacy of

intracoronary and intramuscular infusion of adult bone marrow-derived mononuclear cells (BMMNC) in patients with PAOD, acute myocardial infarction, and ischemic cardiomyopathy[50,55].

However, despite this excitement regarding the possible clinical use of BMMNC, in atherosclerosis, diabetes and other risk factors for cardiovascular diseases the availability of bone marrow cells is reduced. In addition their function is impaired to varying degrees hampering proneovascularization effects of autologous cell-based therapies[50,55]. Moreover, the safety of BMMNC treatment has been questioned by studies finding an increase in atherosclerotic plaque size after BMMNC treatment[56,57]. This potentially hazardous dual effect of therapeutic neovascularization on atherogenesis is explained by the many common pathways of both mechanisms and has been named the "Janus Phenomenon"[58].

Therefore, although pioneering clinical experience with stem cell-related therapy seems promising, many questions remain unanswered and it seems too early for general clinical use of this technique.

Aim of the thesis

Decreased bioavailability of nitric oxide is a hallmark in cardiovascular disease, resulting in or due to endothelial dysfunction. The aim of the present thesis was to unravel the role of NO in vascular remodeling. To this aim, we developed in our laboratory two different lines of transgenic mouse models with increased human eNOS expression, leading to elevated bioavailability of NO in physiological and pathophysiological circumstances. Using these eNOS transgenic (eNOS TG) mice we studied the complex regulation of eNOS in collateral growth, cell adhesion and neovascularization and the effects of elevated NO production on these processes of vascular remodeling. Furthermore, in order to increase the clinical relevance, we evaluated the effects of eNOS overexpression under pathophysiological conditions as diabetes and atherosclerotic disease. Finally, we developed a new concept of eNOS transgenic progenitor cell transplantation for therapeutic vascular remodeling in ischemic disease.

References

1. Carmeliet, P. Angiogenesis in health and disease. *Nat Med* 9, 653-660 (2003).
2. Silvestre, J.S., Mallat, Z., Tedgui, A. & Levy, B.I. Postischaemic neovascularization and inflammation. *Cardiovasc Res* 78, 242-249 (2008).
3. Shyy, J.Y., Li, Y.S., Lin, M.C., Chen, W., Yuan, S., Usami, S. & Chien, S. Multiple cis-elements mediate shear stress-induced gene expression. *J Biomech* 28, 1451-1457 (1995).
4. Lehoux, S., Castier, Y. & Tedgui, A. Molecular mechanisms of the vascular responses to haemodynamic forces. *J Intern Med* 259, 381-392 (2006).
5. Schirmer, S.H., van Nooijen, F.C., Piek, J.J. & van Royen, N. Stimulation of collateral artery growth: travelling further down the road to clinical application. *Heart* 95, 191-197 (2009).
6. Schaper, W. Collateral circulation: past and present. *Basic Res Cardiol* 104, 5-21 (2009).
7. Heil, M., Ziegelhoeffer, T., Mees, B. & Schaper, W. A different outlook on the role of bone marrow stem cells in vascular growth: bone marrow delivers software not hardware. *Circ Res* 94, 573-574 (2004).
8. Mikkola, H.K. & Orkin, S.H. The search for the hemangioblast. *J Hematother Stem Cell Res* 11, 9-17 (2002).
9. Zengin, E., Chalajour, F., Gehling, U.M., Ito, W.D., Treede, H., Lauke, H., Weil, J., Reichenspurner, H., Kilic, N. & Ergun, S. Vascular wall resident progenitor cells: a source for postnatal vasculogenesis. *Development* 133, 1543-1551 (2006).
10. Aicher, A., Rentsch, M., Sasaki, K., Ellwart, J.W., Fandrich, F., Siebert, R., Cooke, J.P., Dimmeler, S. & Heeschen, C. Nonbone marrow-derived circulating progenitor cells contribute to postnatal neovascularization following tissue ischemia. *Circ Res* 100, 581-589 (2007).
11. Miranville, A., Heeschen, C., Sengenes, C., Curat, C.A., Busse, R. & Bouloumie, A. Improvement of postnatal neovascularization by human adipose tissue-derived stem cells. *Circulation* 110, 349-355 (2004).
12. Asahara, T., Murohara, T., Sullivan, A., Silver, M., van der Zee, R., Li, T., Witzenbichler, B., Schatteman, G. & Isner, J.M. Isolation of putative progenitor endothelial cells for angiogenesis. *Science* 275, 964-967 (1997).
13. Zampetaki, A., Kirton, J.P. & Xu, Q. Vascular repair by endothelial progenitor cells. *Cardiovasc Res* 78, 413-421 (2008).
14. Pugh, C.W. & Ratcliffe, P.J. Regulation of angiogenesis by hypoxia: role of the HIF system. *Nat Med* 9, 677-684 (2003).
15. Ferrara, N., Gerber, H.P. & LeCouter, J. The biology of VEGF and its receptors. *Nat Med* 9, 669-676 (2003).
16. Luttun, A., Dewerchin, M., Collen, D. & Carmeliet, P. The role of proteinases in angiogenesis, heart development, restenosis, atherosclerosis, myocardial ischemia, and stroke: insights from genetic studies. *Curr Atheroscler Rep* 2, 407-416 (2000).
17. Jain, R.K. Molecular regulation of vessel maturation. *Nat Med* 9, 685-693 (2003).
18. Adams, R.H. & Alitalo, K. Molecular regulation of angiogenesis and lymphangiogenesis. *Nat Rev Mol Cell Biol* 8, 464-478 (2007).
19. Furchgott, R.F. & Zawadzki, J.V. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* 288, 373-376 (1980).
20. Ignarro, L.J., Buga, G.M., Wood, K.S., Byrns, R.E. & Chaudhuri, G. Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc Natl Acad Sci U S A* 84, 9265-9269 (1987).

21. Palmer, R.M., Ferrige, A.G. & Moncada, S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* 327, 524-526 (1987).
22. Katsuki, S., Arnold, W., Mittal, C. & Murad, F. Stimulation of guanylate cyclase by sodium nitroprusside, nitroglycerin and nitric oxide in various tissue preparations and comparison to the effects of sodium azide and hydroxylamine. *J Cyclic Nucleotide Res* 3, 23-35 (1977).
23. Chatterjee, A., Black, S.M. & Catravas, J.D. Endothelial nitric oxide (NO) and its pathophysiologic regulation. *Vascul Pharmacol* 49, 134-140 (2008).
24. Michel, T. & Feron, O. Nitric oxide synthases: which, where, how, and why? *J Clin Invest* 100, 2146-2152 (1997).
25. Bredt, D.S. & Snyder, S.H. Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. *Proc Natl Acad Sci U S A* 87, 682-685 (1990).
26. Hevel, J.M., White, K.A. & Marletta, M.A. Purification of the inducible murine macrophage nitric oxide synthase. Identification as a flavoprotein. *J Biol Chem* 266, 22789-22791 (1991).
27. Pollock, J.S., Forstermann, U., Mitchell, J.A., Warner, T.D., Schmidt, H.H., Nakane, M. & Murad, F. Purification and characterization of particulate endothelium-derived relaxing factor synthase from cultured and native bovine aortic endothelial cells. *Proc Natl Acad Sci U S A* 88, 10480-10484 (1991).
28. Fulton, D., Fontana, J., Sowa, G., Gratton, J.P., Lin, M., Li, K.X., Michell, B., Kemp, B.E., Rodman, D. & Sessa, W.C. Localization of endothelial nitric-oxide synthase phosphorylated on serine 1179 and nitric oxide in Golgi and plasma membrane defines the existence of two pools of active enzyme. *J Biol Chem* 277, 4277-4284 (2002).
29. Sessa, W.C. eNOS at a glance. *J Cell Sci* 117, 2427-2429 (2004).
30. Rabelink, T.J., de Boer, H.C. & van Zonneveld, A.J. Endothelial activation and circulating markers of endothelial activation in kidney disease. *Nat Rev Nephrol* 6, 404-414 (2010).
31. Yetik-Anacak, G. & Catravas, J.D. Nitric oxide and the endothelium: history and impact on cardiovascular disease. *Vascul Pharmacol* 45, 268-276 (2006).
32. O'Connor, D.M. & O'Brien, T. Nitric oxide synthase gene therapy: progress and prospects. *Expert Opin Biol Ther* 9, 867-878 (2009).
33. Sessa, W.C. Molecular control of blood flow and angiogenesis: role of nitric oxide. *J Thromb Haemost* 7 Suppl 1, 35-37 (2009).
34. Meng, W., Ayata, C., Waeber, C., Huang, P.L. & Moskowitz, M.A. Neuronal NOS-cGMP-dependent ACh-induced relaxation in pial arterioles of endothelial NOS knockout mice. *Am J Physiol* 274, H411-415 (1998).
35. Sun, D., Huang, A., Smith, C.J., Stackpole, C.J., Connetta, J.A., Shesely, E.G., Koller, A. & Kaley, G. Enhanced release of prostaglandins contributes to flow-induced arteriolar dilation in eNOS knockout mice. *Circ Res* 85, 288-293 (1999).
36. Payne, G.W., Madri, J.A., Sessa, W.C. & Segal, S.S. Abolition of arteriolar dilation but not constriction to histamine in cremaster muscle of eNOS-/- mice. *Am J Physiol Heart Circ Physiol* 285, H493-498 (2003).
37. McMahon, T.J., Moon, R.E., Luschinger, B.P., Carraway, M.S., Stone, A.E., Stolp, B.W., Gow, A.J., Pawloski, J.R., Watke, P., Singel, D.J., Piantadosi, C.A. & Stamler, J.S. Nitric oxide in the human respiratory cycle. *Nat Med* 8, 711-717 (2002).
38. You, D., Waeckel, L., Ebrahimian, T.G., Blanc-Brude, O., Foubert, P., Barateau, V., Duriez, M., Lericousse-Roussanne, S., Vilar, J., Dejana, E., Tobelem, G., Levy, B.I. & Silvestre, J.S. Increase in vascular permeability and vasodilation are critical for proangiogenic effects of stem cell therapy. *Circulation* 114, 328-338 (2006).

39. Eitenmuller, I., Volger, O., Kluge, A., Troidl, K., Barancik, M., Cai, W.J., Heil, M., Pipp, F., Fischer, S., Horrevoets, A.J., Schmitz-Rixen, T. & Schaper, W. The range of adaptation by collateral vessels after femoral artery occlusion. *Circ Res* 99, 656-662 (2006).
40. Cerwinka, W.H., Cooper, D., Krieglstein, C.F., Feelisch, M. & Granger, D.N. Nitric oxide modulates endotoxin-induced platelet-endothelial cell adhesion in intestinal venules. *Am J Physiol Heart Circ Physiol* 282, H1111-1117 (2002).
41. Freedman, J.E., Sauter, R., Battinelli, E.M., Ault, K., Knowles, C., Huang, P.L. & Loscalzo, J. Deficient platelet-derived nitric oxide and enhanced hemostasis in mice lacking the NOSIII gene. *Circ Res* 84, 1416-1421 (1999).
42. Aicher, A., Heeschen, C., Mildner-Rihm, C., Urbich, C., Ihling, C., Technau-Ihling, K., Zeiher, A.M. & Dimmeler, S. Essential role of endothelial nitric oxide synthase for mobilization of stem and progenitor cells. *Nat Med* 9, 1370-1376 (2003).
43. Yu, J., deMuinck, E.D., Zhuang, Z., Drinane, M., Kauser, K., Rubanyi, G.M., Qian, H.S., Murata, T., Escalante, B. & Sessa, W.C. Endothelial nitric oxide synthase is critical for ischemic remodeling, mural cell recruitment, and blood flow reserve. *Proc Natl Acad Sci U S A* 102, 10999-11004 (2005).
44. Murohara, T., Asahara, T., Silver, M., Bauters, C., Masuda, H., Kalka, C., Kearney, M., Chen, D., Symes, J.F., Fishman, M.C., Huang, P.L. & Isner, J.M. Nitric oxide synthase modulates angiogenesis in response to tissue ischemia. *J Clin Invest* 101, 2567-2578 (1998).
45. Dimmeler, S. Regulation of bone marrow-derived vascular progenitor cell mobilization and maintenance. *Arterioscler Thromb Vasc Biol* 30, 1088-1093 (2010).
46. Rafii, S. & Lyden, D. Therapeutic stem and progenitor cell transplantation for organ vascularization and regeneration. *Nat Med* 9, 702-712 (2003).
47. Balligand, J.L., Feron, O. & Dessy, C. eNOS activation by physical forces: from short-term regulation of contraction to chronic remodeling of cardiovascular tissues. *Physiol Rev* 89, 481-534 (2009).
48. Dimmeler, S., Zeiher, A.M. & Schneider, M.D. Unchain my heart: the scientific foundations of cardiac repair. *J Clin Invest* 115, 572-583 (2005).
49. Collinson, D.J. & Donnelly, R. Therapeutic angiogenesis in peripheral arterial disease: can biotechnology produce an effective collateral circulation? *Eur J Vasc Endovasc Surg* 28, 9-23 (2004).
50. Aranguren, X.L., Verfaillie, C.M. & Luttun, A. Emerging hurdles in stem cell therapy for peripheral vascular disease. *J Mol Med* 87, 3-16 (2009).
51. Nikol, S., Baumgartner, I., Van Belle, E., Diehm, C., Visona, A., Capogrossi, M.C., Ferreira-Maldent, N., Gallino, A., Wyatt, M.G., Wijesinghe, L.D., Fusari, M., Stephan, D., Emmerich, J., Pompilio, G., Vermassen, F., Pham, E., Grek, V., Coleman, M. & Meyer, F. Therapeutic angiogenesis with intramuscular NV1FGF improves amputation-free survival in patients with critical limb ischemia. *Mol Ther* 16, 972-978 (2008).
52. Vincent, K.A., Feron, O. & Kelly, R.A. Harnessing the response to tissue hypoxia: HIF-1 alpha and therapeutic angiogenesis. *Trends Cardiovasc Med* 12, 362-367 (2002).
53. Arany, Z., Foo, S.Y., Ma, Y., Ruas, J.L., Bommi-Reddy, A., Girnun, G., Cooper, M., Laznik, D., Chinsomboon, J., Rangwala, S.M., Baek, K.H., Rosenzweig, A. & Spiegelman, B.M. HIF-independent regulation of VEGF and angiogenesis by the transcriptional coactivator PGC-1alpha. *Nature* 451, 1008-1012 (2008).
54. Carmeliet, P. & Baes, M. Metabolism and therapeutic angiogenesis. *N Engl J Med* 358, 2511-2512 (2008).
55. Segers, V.F. & Lee, R.T. Stem-cell therapy for cardiac disease. *Nature* 451, 937-942 (2008).
56. Silvestre, J.S., Gojova, A., Brun, V., Potteaux, S., Esposito, B., Duriez, M., Clergue, M., Le Ricousse-Roussanne, S., Barateau, V., Merval, R., Groux, H., Tobelem, G., Levy, B., Tedgui, A. & Mallat, Z. Trans-

plantation of bone marrow-derived mononuclear cells in ischemic apolipoprotein E-knockout mice accelerates atherosclerosis without altering plaque composition. *Circulation* 108, 2839-2842 (2003).

57. George, J., Afek, A., Abashidze, A., Shmilovich, H., Deutsch, V., Kopolovich, J., Miller, H. & Keren, G. Transfer of endothelial progenitor and bone marrow cells influences atherosclerotic plaque size and composition in apolipoprotein E knockout mice. *Arterioscler Thromb Vasc Biol* 25, 2636-2641 (2005).
58. Epstein, S.E., Stabile, E., Kinnaird, T., Lee, C.W., Clavijo, L. & Burnett, M.S. Janus phenomenon: the interrelated tradeoffs inherent in therapies designed to enhance collateral formation and those designed to inhibit atherogenesis. *Circulation* 109, 2826-2831 (2004).

Chapter 2

The eNOS Transgenic Mouse

Reduction of Blood Pressure, Plasma Cholesterol and Atherosclerosis by Elevated Endothelial Nitric Oxide

Rien van Haperen[1], Monique de Waard[2], Elza van Deel[2], Barend Mees[1], Michael Kutryk[1,2], Thijs van Aken[3], Jaap Hamming[1,4], Frank Grosveld[1], Dirk J. Duncker[2], Rini de Crom[1,4]

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From the Departments of Cell Biology & Genetics[1], Experimental Cardiology, Thoraxcenter[2], Erasmus Laboratory Animal Science Center (EDC)[3], and Vascular Surgery[4], Erasmus Medical Center, Rotterdam, The Netherlands

Abstract

In the vascular system, nitric oxide is generated by endothelial NO synthase (eNOS). NO has pleiotropic effects, most of which are believed to be atheroprotective. Therefore, it has been argued that patients suffering from cardiovascular disease could benefit from an increase in eNOS activity. However, increased NO production can cause oxidative damage, cell toxicity and apoptosis, and hence could be atherogenic rather than beneficial. To study the *in vivo* effects of increased eNOS activity, we created transgenic mice overexpressing human eNOS. Aortic blood pressure was ~20 mm Hg lower in the transgenic mice compared to control mice because of lower systemic vascular resistance. The effects of eNOS overexpression on diet-induced atherosclerosis were studied in apolipoprotein E-deficient mice. Elevation of eNOS activity decreased blood pressure (~20 mm Hg) and plasma levels of cholesterol (~17%), resulting in a reduction in atherosclerotic lesions by 40%. We conclude that an increase in eNOS activity is beneficial and provides protection against atherosclerosis.

Introduction

Endothelial nitric oxide synthase (eNOS) plays an important role in the regulation of vascular tone, vascular biology and haemostasis. For example, NO produced by eNOS causes vasodilation. Thus, eNOS knock out mice are hypertensive[1], while eNOS transgenic mice have hypotension[2]. In addition, NO reduces the activation and aggregation of platelets[3,4], attenuates adhesion of leukocytes to the endothelium[5-7], reduces the permeability of the endothelium and inhibits proliferation and migration of vascular smooth muscle cells[8]. Impaired activity of eNOS is associated with endothelial cell dysfunction[9]. For these reasons eNOS has been proposed to modulate atherosclerotic disease[10-12]. Indeed, impairment or deficiency of eNOS gives rise to accelerated atherosclerosis in animal models[10,12-14], indicating that physiological levels of eNOS are antiatherogenic. This suggests that patients at increased risk of atherosclerotic vascular disease could possibly benefit from an increase of eNOS activity by pharmacological means or (local) gene therapy. However, eNOS derived NO also has detrimental effects[15], like the generation of superoxides[16], making it difficult to predict whether increased eNOS activity is beneficial or harmful[17]. To determine whether increased eNOS activity may be beneficial, we created transgenic mice that express the human eNOS gene. These mice were crossbred to apolipoprotein E-deficient (apoE0) mice in order to evaluate the effects of a constitutive increase in eNOS activity on the development of atherosclerosis.

Experimental Procedures

Mice

A DNA fragment containing the human eNOS gene was isolated from a home made human genomic cosmid library[18] using eNOS cDNA (kindly donated by Dr. S. Janssens, Leuven, Belgium[19]) as a probe. In addition, the DNA fragment contained ~6kb of 5' natural flanking sequence, including the native eNOS promoter, and ~3kb of 3' sequence to the gene. Vector sequences were removed by restriction endonucleases. A solution of 1-2 µg/ml of DNA was used for microinjection of fertilized oocytes from FVB donor mice and transplanted into the oviducts of pseudopregnant B10xCBA mice. Founder mice and offspring were genotyped by PCR on DNA isolated from tail biopsies. Primers used were: 5'-GTC CTG CAG ACC GTG CAG C-3' (sense) and 5'-GGC TGT TGG TGT CTG AGC CG-3' (antisense). Mice were backcrossed to C57Bl6 for at least 5 generations (>96% C57Bl6). All eNOS transgenic mice were hemizygous. ApoE0 mice were obtained from the The Jackson Laboratory, Bar Harbor, ME. Male mice were used in all experiments. All animal experiments were performed in compliance with institutional (Erasmus MC,

Rotterdam, The Netherlands) and national (Ministry of Health, Welfare and Sport, The Hague, The Netherlands) guidelines.

Western blotting and immunohistochemistry

Aortas were collected and homogenized in 50 mM Tris-HCl, pH7.4 containing 1 mM EDTA, 0.25 M sucrose and 20 mM CHAPS. Western blotting was performed as described previously[20]. 25 µg of protein (BCA protein assay kit; Pierce Chemical Company, Rockford, IL) was applied to each lane. Anti-eNOS was obtained from Santa Cruz Biotechnology Inc., Santa Cruz, CA. This antibody was also used for immunohistochemistry experiments, which were performed according to Bakker et al[21].

Hemodynamic measurements

Baseline blood pressure measurements

Mice were weighed, anesthetized with ketamine (100 mg/kg ip) and xylazine (20 mg/kg ip), intubated and ventilated with a mixture of O_2 and N_2 (1/2 vol/vol) with a pressure controlled ventilator (Servo ventilator 900C, Siemens-Elema, Sweden). The ventilation rate was set at 100 strokes/min with a peak inspiration pressure of 18 cmH_2O and a positive end expiration pressure of 6 cmH_2O. After intubation the mice were placed on a heating pad to maintain body temperature at 37°C and a polyethylene catheter (PE 10) was inserted into the right carotid artery and advanced into the aortic arch, for the measurement of aortic pressure. In the first part of the study we used 12 eNOS-Tg2 and 5 eNOS-Tg3 mice for screening of eNOS expression, and compared them to 33 wild-type mice. Ten minutes after a second intraperitoneal bolus of anesthetics (100 mg/kg ketamine and 20 mg/kg xylazine) baseline blood pressure recordings were obtained.

Effect of L-NAME on systemic vascular resistance

Subsequently, we chose the eNOS-Tg2 line to determine whether the lower aortic blood pressure was the result of an NO-mediated decrease in systemic vascular resistance. For this purpose, in 17 eNOS-Tg2 mice and 17 wild-type mice a polyethylene catheter (PE 10) was inserted into the right carotid artery and advanced into the aortic arch, for the measurement of aortic pressure, while another PE 10 catheter was introduced into the right external jugular vein and advanced into the superior caval vein for infusion of L-NAME. After thoracotomy through the second right intercostal space, the ascending aorta was exposed and a transit-time flow probe (ID 1.5 mm; Transonics systems Inc. T206) was placed around the aorta for measuring aorta flow. Ten minutes after a second intraperitoneal bolus of 100 mg/kg ketamine and 20 mg/kg xylazine, baseline recordings were obtained. A continuous 10 minutes intravenous infusion of L-NAME (100 mg/kg) was started: 10 minutes after completion of the infusion, measurements were repeated.

Effects of dietary suppletion of L-arginine on baseline hemodynamics

In the eNOS-Tg2 line we studied whether L-arginine deficiency contributed to the modest effects of eNOS overexpression on systemic vascular resistance. For this purpose, in six eNOS-Tg2 male mice and seven wild-type male mice, L-arginine was supplemented in their drinking water (2.5% w/v). One week later, animals were instrumented as described above and hemodynamic measurements were performed under baseline conditions.

Data analysis

Hemodynamic data were recorded and digitized using an on-line 4 channel data acquisition program (ATCODAS, Dataq Instruments, Akron, OH), for later analysis with a program written in MatLab (Mathworks Inc, Natick, MA). Fifteen consecutive beats were selected for determination of heart rate, aortic pressure, and aorta blood flow.

eNOS activity assay

Aortas were collected and homogenized in 50 mM Tris-HCl, pH7.4 containing 1 mM EDTA, 0.25 M sucrose and 20 mM CHAPS. eNOS activity assays were performed by measuring L-arginine to L-citrulline conversion using a nitric oxide synthase assay kit (Calbiochem, La Jolla, CA; cat.no. 482700) according to the manufacturer's instructions. Protein content was measured by the BCA protein assay kit (Pierce Chemical Company, Rockford, IL).

Lipid measurements

Blood was collected via orbital puncture after an overnight fasting period. Plasma was frozen freshly or subjected to ultracentrifugation in a Beckman 42.2 Ti rotor (42000 rpm, 3 h, 12°C) at $d = 1.063$ g/ml. Tubes were sliced and two fractions were collected: very low-density lipoprotein (VLDL) + low-density lipoprotein (LDL): $d < 1.063$ g/ml, and high-density lipoprotein (HDL): $d > 1.063$ g/ml. Cholesterol was measured with the F-chol kit (Roche Molecular Biochemicals) after hydrolysis of cholesteryl esters with cholesterol esterase from *Candida cylindracea* (Roche Molecular Biochemicals).

Atherosclerosis

Atherosclerosis experiments were performed in age- and sex-matched mice. Male mice of 8 weeks were fed a Western type diet, containing 15% (w/w) cacoa butter and 0.25% (w/w) cholesterol (diet W, Hope Farms, Woerden, The Netherlands) for 6 weeks, which leads to appreciable atherosclerosis[22-24]. Animals were anesthesized using isoflurane, and in situ fixation was performed via the left ventricle of the heart using phosphate buffered formalin (4%, v/v). A Sony digital camera was used to obtain images of sections of the aortic root. These were analyzed by Scion Image processing and analyzing

software (available from www.scioncorp.com). Atherosclerosis was quantified in five sections per mouse with 80 µm intervals using the method of Paigen et al[25].

Data analysis

Analysis of data was performed using two-way or one-way analysis of variance followed by Scheffé's test, as appropriate. Statistical significance was accepted when $P<0.05$ (two-tailed). Data are presented as mean ± S.E.

Results and Discussion

For the generation of eNOS transgenic mice, we used a DNA fragment that comprised the complete human eNOS genomic sequence, including all exons and introns as well its natural flanking sequences. Therefore, our mice are different from those described by Ohashi et al which overexpressed bovine eNOS cDNA, driven by the murine pre-proendothelin-1 promoter[2]. Our approach was chosen in order to preserve the natural regulation of the gene and to prevent ectopic expression. For example, the endothelium enhancer element that is located 4.9 kilobases upstream from the transcription start site of the eNOS gene[26] is included in this construct. By using this construct we mimic the human situation in terms of regulation and tissue distribution of eNOS as much as possible.

Two independent lines with appreciable overexpression of the transgene, as measured by RT-PCR (not shown), were selected and arbitrarily designated eNOStg2 and eNOStg3. Production of human eNOS protein was demonstrated by Western blotting of aorta homogenates (Fig. 1A). Subsequently, eNOS activity in aortas from control (wild-type) and eNOS-overexpressing mice was measured, using the L-arginine to L-citrulline conversion assay[27]. In aorta, the level of active eNOS enzyme was 10-fold increased in eNOStg2 mice and 7.5-fold in eNOStg3 mice, respectively, when compared to wild-type animals (Fig 1B). Expression of human eNOS was also investigated by immunohistochemistry. There was no human eNOS staining of aortas of wild-type mice, whereas the endothelial layer of the aorta was clearly stained in both human eNOS transgenic lines (Fig 1C).

The expression pattern of the the human eNOS transgene was investigated by immunohistochemistry in heart (Fig. 2A), liver (Fig. 2B), kidney (Fig. 2C), adrenal (Fig. 2D) and testis (Fig. 2E). Sections from wild-type controls show virtually no immunostaining (not shown). The lining of the larger vessels is clearly stained (Fig. 2A, 2D and 2E) . Staining of capillaries is visible in the heart, between the cardiomyocytes. Immunoreactivity is observed in the sinusoids in the liver, and, in the kidney, in the peritubular capillaries as wells as in the capillaries from the glomeruli. In the adrenal, the cortical capillaries as well as medullary capillary sinusoids and veins are stained.

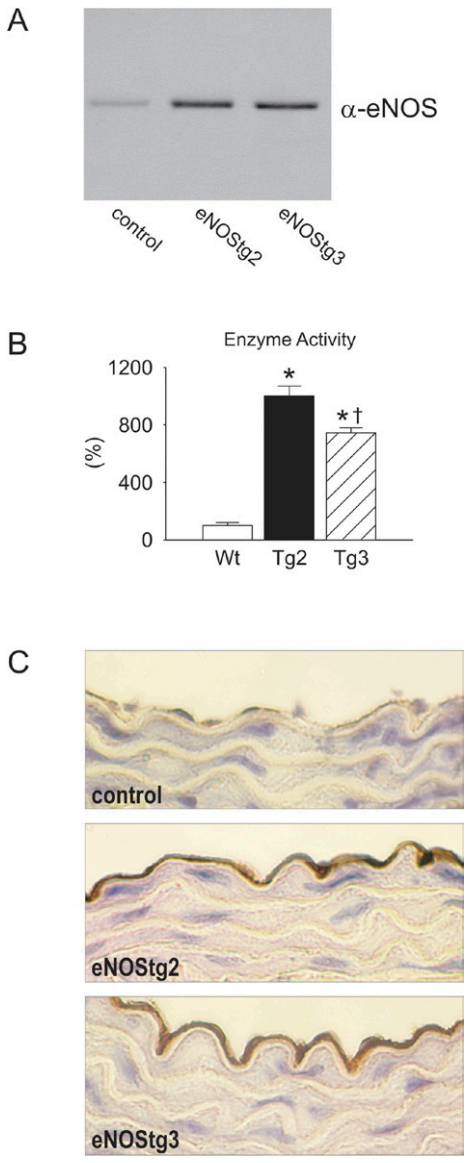

Fig. 1. Expression of eNOS in transgenic mice. (A) Western blot analysis of aortas from control (wild-type littermates), eNOStg2 or eNOStg3 mice. 25 µg of homogenate was applied per lane. The blot was probed with anti human eNOS antibody. A single protein band of the expected molecular size (~130 kDa) was detected. (B) eNOS activity was measured in aortas from control (Wt), eNOStg2 (Tg2) or eNOStg3 (Tg3) mice by the L-arginine to L-citrulline conversion assay using a nitric oxide synthase assay kit (Calbiochem). Activity is expressed as percentage of the activity in controls, which was 1.56 ± 0.31 pmol/µg/min. Each value represents the mean ± S.E. of three animals. One of three experiments is shown. *P<0.05 vs controls, †P<0.05 vs eNOStg2 mice (ANOVA followed by Scheffé's test). (C) Immunohistochemistry on aortas from control, eNOStg2 or eNOStg3 mice. Aortas were collected after *in situ* fixation. Paraffin sections were incubated with antihuman eNOS antibody and a peroxidase-conjugated secondary antibody. Original magnification, x630.

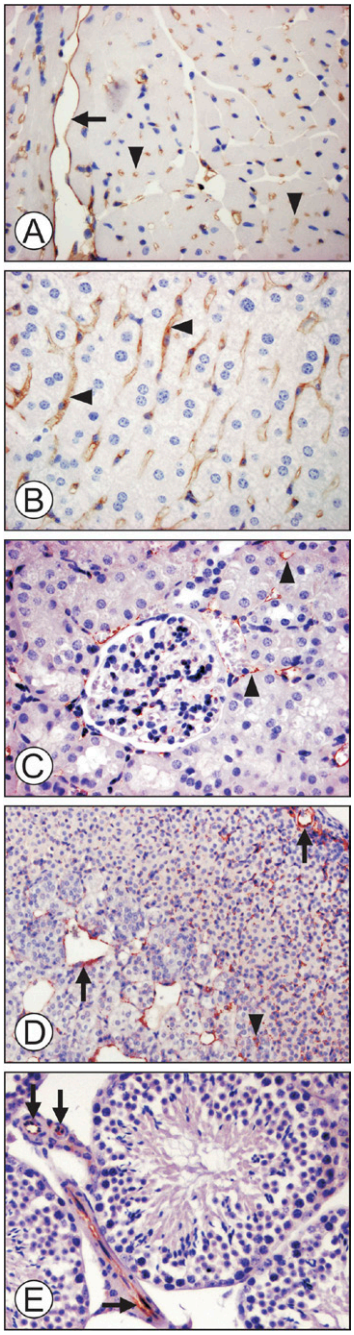

Fig. 2. Expression pattern of human eNOS in transgenic mice. Organs from eNOStg2 mice were collected after *in situ* fixation. Paraffin sections were incubated with antihuman eNOS antibody and a peroxidase-conjugated secondary antibody. A: heart, B: liver, C: kidney, D: adrenal, E: testis tissue. *Arrowheads* indicate representative immunoreactive capillaries; *arrows* indicate larger blood vessels. Original magnification, x400.

In the testis, only blood vessels between the seminiferous tubules are stained. The parenchyma cells of none of these organs show appreciable immunoreactivity. Similar results were found with sections from eNOStg3 mice (not shown).

Our results show that the genomic sequences included in the DNA fragment we used are sufficient for expression in endothelial cells. Although eNOS activity is tightly regulated at the posttranslational level[28,29], there is also extensive regulation at the transcriptional level[30,31]. The present study shows that our construct results in high level expression of human eNOS which is not prevented by a feedback mechanism.

To study the effect of increased eNOS activity on blood pressure and vascular tone, we performed hemodynamic studies[32]. Heart rates were similar for wild-type controls and eNOS transgenic lines, whereas both eNOS transgenic lines each exhibited a 20-25 mmHg lower mean aortic blood pressure compared with littermate controls (Fig. 3A). Subsequent hemodynamic studies were performed in eNOStg2, the transgenic mouse line with the highest expression. These experiments showed that the lower aortic blood pressure was the result of a 30% lower systemic vascular resistance, because mean aortic blood flow and heart rate were similar in both groups (Fig. 3B). Subsequent infusion of the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) increased systemic vascular resistance and abolished the difference between control and eNOStg2 mice. We therefore conclude that the lower basal systemic vascular resistance in eNOStg2 mice is the result of increased NO production (Fig. 3B). This is corroborated by the significantly larger increase in blood pressure in response to L-NAME in the transgenic mice. Suppletion of L-arginine had no effect on the already lower mean aortic blood pressure and heart rate in the transgenic mice (Fig. 3C). We therefore also conclude that the blood pressure lowering effect of eNOS overexpression was not limited by a shortage of substrate.

The present study shows that the lower blood pressure associated with eNOS overexpression (as reported before[2]) is the result of a lower systemic vascular resistance. Thus eNOS activity was not only increased in the larger blood vessels but also in the microcirculation. Although eNOStg2 and eNOStg3 mice showed a slight variation in eNOS activity (Fig. 1B), the degree to which blood pressure was lowered was not different. This suggests that another rate-limiting factor, or one or more compensatory mechanisms prevented a further decrease in blood pressure in the eNOStg2 mice.

To study whether the increased expression of eNOS protects the mice against the development of diet-induced atherosclerosis, eNOS transgenic mice were cross-bred to apoE0 mice, which represent a well-known mouse model for studying atherosclerosis[33,34]. The animals were fed a Western type diet for 6 weeks. As shown in Fig. 4A, overexpression of eNOS also caused a decrease in systemic blood pressure under these conditions, although heart rates were similar.

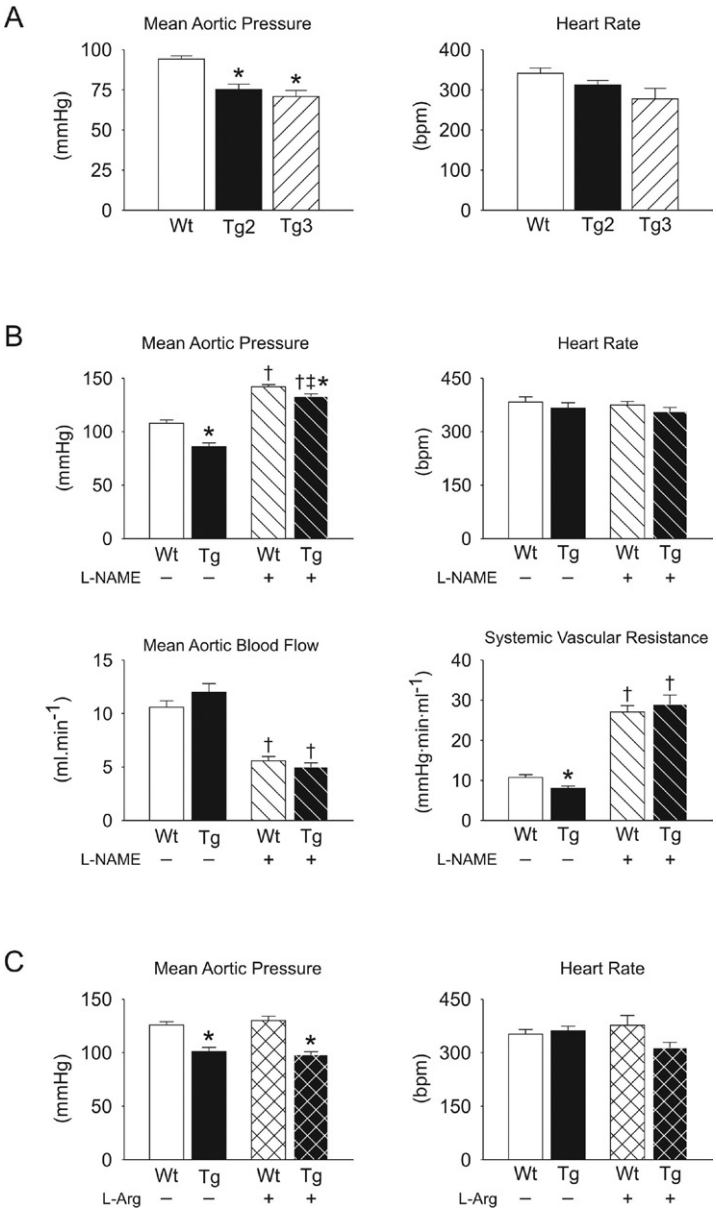

Fig. 3. Hemodynamic measurements. (A) Mean aortic pressure and heart rate were measured in anesthetized control (Wt, wild-type littermates), eNOStg2 (Tg2) or eNOStg3 (Tg3) mice. (B) Mean aortic pressure, heart rate, mean aortic blood flow and systemic vascular resistance were analyzed in control (Wt, wild-type littermates) or eNOStg2 (Tg) mice before and following infusion of L-NAME. (C) Mean aortic pressure and heart rate were measured in anesthetized control (Wt, wild-type littermates), or eNOStg2 (Tg) mice following one week of drinking water with or without L-arginine supplementation (L-Arg, 2.5% w/v). Each value represents the mean ± S.E. of ≥ five animals. *P<0.05 vs controls (Wt), †P<0.05 vs baseline (before infusion of L-NAME), ‡P<0.05 vs controls (Wt) after infusion of L-NAME (ANOVA followed by Scheffé's test).

Table 1. Plasma lipid and lipoprotein analysis

Total cholesterol concentrations in plasma from apoE-deficient control (apoE0), apoE0/eNOStg2 or apoE0/eNOStg3 mice after feeding a normal chow diet or an atherogenic diet. Cholesterol concentrations were determined by enzymatic methods. VLDL + LDL and HDL fractions were isolated by ultracentrifugation. TC: total cholesterol (mM); VLDL + LDL: cholesterol in VLDL and LDL (mM); HDL: cholesterol in HDL (mM).

		Chow	Atherogenic diet		
	n	TC	TC	VLDL+LDL	HDL
apoE0	32	10.9 ± 0.3	28.4 ± 0.9	27.3 ± 0.8	0.4 ± 0.02
apoE0/eNOStg2	22	9.2 ± 0.4**	23.3 ± 1.1**	21.9 ± 1.1***	0.4 ± 0.02
apoE0/eNOStg3	19	9.0 ± 0.2***	24.4 ± 1.1*	24.3 ± 1.0*	0.4 ± 0.02

* P<0.05
** P<0.01
*** P<0.001

Plasma cholesterol levels were measured before the start of the atherogenic diet (i.e. on normal chow diet) and at the end of the experiment (Table 1). Both eNOS transgenic lines showed a decrease in plasma cholesterol of ~15% when compared to apoE0 mice when fed a normal chow diet.

As expected, the Western diet resulted in a dramatic increase (~3-fold) in plasma cholesterol levels, while the total cholesterol concentration remained lower in the eNOS transgenic animals when compared to apoE0 controls (~ –16%). This difference was due to variations in the VLDL and LDL, which contain the bulk of the plasma cholesterol under these conditions: HDL-cholesterol concentration in plasma was 0.4 mM and did not differ between the groups (Table 1). These findings indicate that elevated eNOS activity results in a slightly more favorable (i.e. less atherogenic) lipoprotein profile, because VLDL and LDL contain the atherogenic portion of plasma cholesterol[35], whereas HDL is protective against the development of atherosclerosis[36,37].

To study the effect of eNOS overexpression on atherosclerosis, the atherosclerotic lesion areas in the aortic roots were measured. Fig. 4B shows representative examples of histological sections. Compared with apoE0 mice, we observed a decrease in atherosclerosis in both lines of eNOS transgenic mice studied (Fig. 4C).

Several studies have described a relation between plasma cholesterol and eNOS activity[38-40]. However, these investigations exclusively focused on the effects of changes in plasma cholesterol levels on eNOS activity. Hypercholesterolemia is associated with decreased eNOS activity, probably via an interaction of oxidized LDL with caveolae, the plasma membrane domains in which eNOS resides[41]. Cholesterol synthesis inhibitors (statins) have been reported to increase eNOS activity in addition to their cholesterol lowering effects, but these actions appear to be independent[38].

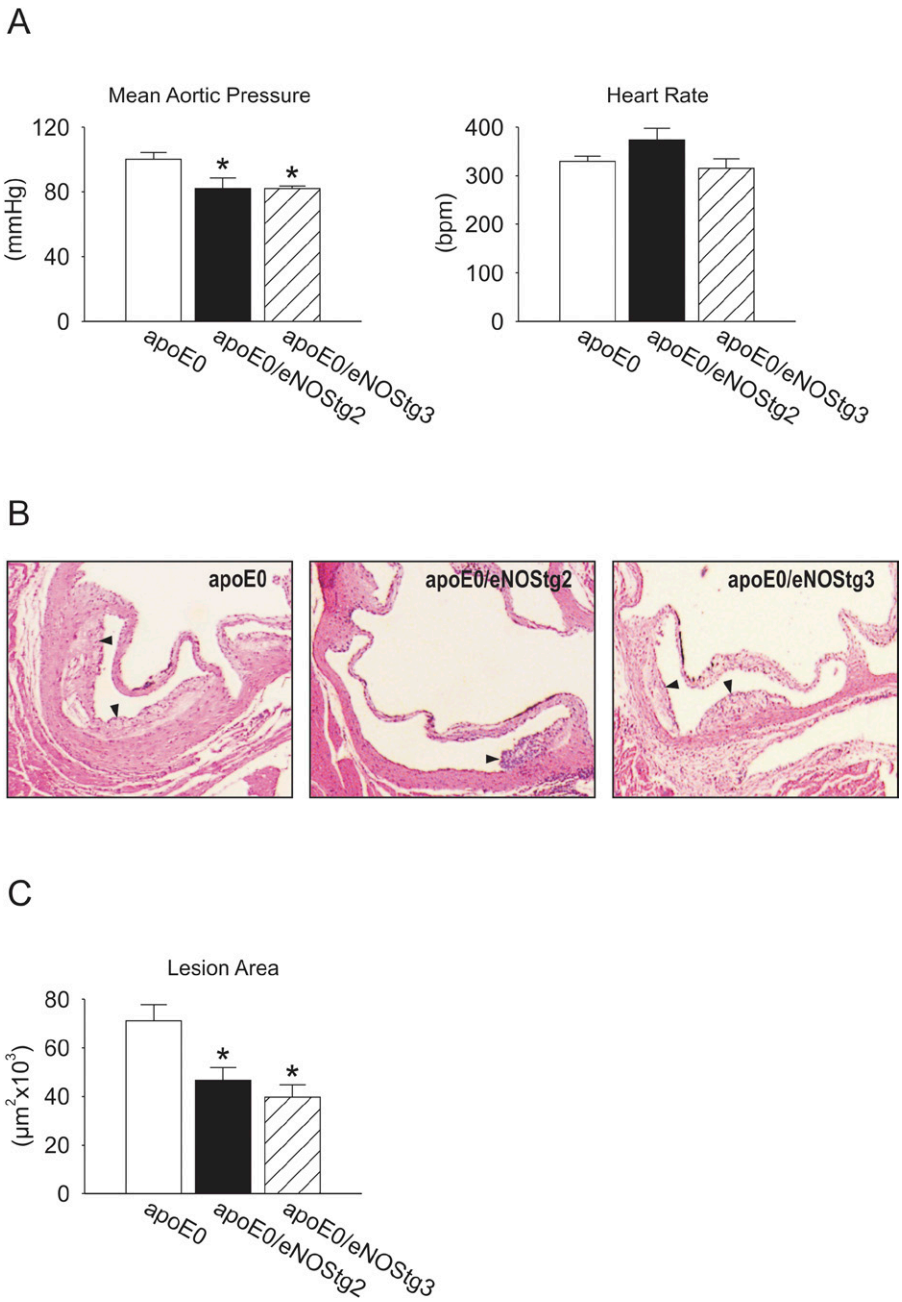

Fig. 4. Analysis of eNOS transgenic/apoE-deficient mice. (A) Mean aortic pressure and heart rate were measured in anesthetized apoE-deficient control (apoE0; n=15), apoE0/eNOStg2 (n=8) or apoE0/eNOStg3 (n=8) mice. (B) Representative photomicrographs of H&E stained paraffin sections with lesion areas in the aortic valves (*arrowheads*). Original magnification, x25. (C) Lesion area in the aortic root of apoE-deficient control (apoE0; n=32), apoE0/eNOStg2 (n=22) or apoE0/eNOStg3 (n=19) mice. Areas were measured in five sections with 80-μm intervals and expressed as μm^2 per section per animal.

In the present study we observed that the level of eNOS-expression affects the level of plasma cholesterol: plasma levels of cholesterol were about 15% lower in eNOStg/apoE0 mice as compared with apoE0 controls.

A similar difference in plasma cholesterol levels was found after feeding the mice a Western type diet for 6 weeks, indicating that eNOS overexpression alleviates diet-induced hypercholesterolemia. This effect is not caused by ectopic expression in organs involved in lipid metabolism, e.g. the liver, as the expression pattern is restricted to endothelial cells in all organs that were examined (Fig. 2). Recently it was shown that hypercholesterolemia in mice results in CD36-mediated cholesterol depletion of caveolae, followed by translocation of eNOS from caveolae and subsequent inactivation of the enzyme[42]. Thus, eNOS activity is directly related to the cholesterol content of caveolae. Possibly, the moderate decrease in plasma cholesterol that we observed in our eNOS transgenic mice is caused by a recruitment of plasma cholesterol by the endothelial cells in order to handle the increased level of eNOS protein. This small decrease in plasma cholesterol likely contributed, at least in part, to the observed lower susceptibility to diet-induced atherosclerosis.

Although it has been proposed that elevation of eNOS activity would attenuate atherosclerosis[11], serious doubts have also been expressed as to whether an increase in eNOS activity *in vivo* would have beneficial effects, because high levels of NO (e.g. as produced by inducible NO synthase) have been implicated in cell toxicity and apoptosis[15,43]. During the preparation of our manuscript, Ozaki et al[44] reported the unexpected observation that relatively modest overexpression of eNOS resulted in *increased* atherosclerosis. Based on our findings, we conclude that this conclusion cannot be generalized, taking into account the following considerations. First, because enzyme activity is ± 1.5 fold increased, the level of eNOS overexpression in the mice used by Ozaki et al is relatively low, when compared with the much higher NO production levels by activated iNOS (inducible NO synthase). A 1.5 fold increase is also rather low in terms of possible drug or gene therapy applications. Second, the observed increase in atherosclerosis is explained by measurements indicating that the overexpressed eNOS enzyme is dysfunctional in the mouse model used by Ozaki et al This finding is not unexpected, given the moderate level of overexpression in their mice, as it has been previously reported that endogenous eNOS is indeed dysfunctional in terms of NO production in apoE0 mice fed a Western type diet[45]. The results from the study by Ozaki et al are probably (at least in part) explained by the construct used by the authors, which consists of cDNA (often leading to low expression levels) and a heterologous promoter. In contrast, our results demonstrate that overexpression of human eNOS in endothelial cells indeed results in decreased atherosclerosis, most likely via lowering blood pressure and plasma cholesterol. Our study therefore suggests that elevation of eNOS activity could be beneficial for patients at risk of developing atherosclerotic disease.

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References

1. Huang, P.L., Huang, Z., Mashimo, H., Bloch, K.D., Moskowitz, M.A., Bevan, J.A. and Fishman, M.C. Hypertension in mice lacking the gene for endothelial nitric oxide synthase. *Nature* 377, 239-242 (1995).
2. Ohashi, Y., Kawashima, S., Hirata, K., Yamashita, T., Ishida, T., Inoue, N., Sakoda, T., Kurihara, H., Yazaki, Y., and Yokoyama, M. Hypotension and reduced nitric oxide-elicited vasorelaxation in transgenic mice overexpressing endothelial nitric oxide synthase. *J. Clin. Invest.* 102, 2061-2071 (1998).
3. Riddell, D.R. and Owen, J.S. Nitric oxide and platelet aggregation. *Vitam. Horm.* 57, 25-48 (1999).
4. Loscalzo, J. Nitric oxide insufficiency, platelet activation, and arterial thrombosis. *Circ. Res.* 88, 756-762 (2001).
5. Lefer, A.M. Nitric oxide: nature's naturally occurring leukocyte inhibitor. *Circulation* 95, 553-554 (1997).
6. Qian, H., Neplioueva, V., Shetty, G.A., Channon, K.M. and George, S.E. Nitric oxide synthase gene therapy rapidly reduces adhesion molecule expression and inflammatory cell infiltration in carotid arteries of cholesterol-fed rabbits. *Circulation* 99, 2979-2982 (1999).
7. Niebauer, J., Dulak, J., Chan, J.R., Tsao, P.S. and Cooke, J.P. Gene transfer of nitric oxide synthase: effects on endothelial biology. *J. Am. Coll. Cardiol.* 34, 1201-1207 (1999).
8. Li, H. and Forstermann, U. Nitric oxide in the pathogenesis of vascular disease. *J. Pathol.* 190, 244-254 (2000).
9. Harrison, D.G. Cellular and molecular mechanisms of endothelial cell dysfunction. *J. Clin. Invest.* 100, 2153-2157 (1997).
10. Barton, M., Haudenschild, C.C., d'Uscio, L.V., Shaw, S., Munter, K. and Luscher, T.F. Endothelin ETA receptor blockade restores NO-mediated endothelial function and inhibits atherosclerosis in apolipoprotein E-deficient mice. *Proc. Natl. Acad. Sci. U. S. A.* 95, 14367-14372 (1998).
11. Drummond, G.R. and Harrison, D.G. eNOS-overexpressing mice: too much NO makes the blood pressure low. *J. Clin. Invest.* 102, 2033-2034 (1998).
12. Kano, H., Hayashi, T., Sumi, D., Esaki, T., Asai, Y., Thakur, N.K., Jayachandran, M. and Iguchi, A. A HMG-CoA reductase inhibitor improved regression of atherosclerosis in the rabbit aorta without affecting serum lipid levels: possible relevance of up-regulation of endothelial NO synthase mRNA. *Biochem. Biophys. Res. Commun.* 259, 414-419 (1999).
13. Knowles, J.W., Reddick, R.L., Jennette, J.C., Shesely, E.G., Smithies, O. and Maeda, N. Enhanced atherosclerosis and kidney dysfunction in eNOS(-/-)Apoe(-/-) mice are ameliorated by enalapril treatment. *J. Clin. Invest.* 105, 451-458 (2000).
14. Kuhlencordt, P.J., Gyurko, R., Han, F., Scherrer-Crosbie, M., Aretz, T.H., Hajjar, R., Picard, M.H. and Huang, P.L. Accelerated atherosclerosis, aortic aneurysm formation, and ischemic heart disease in apolipoprotein E/endothelial nitric oxide synthase double-knockout mice. *Circulation* 104, 448-454 (2001).
15. Wever, R.M., Luscher, T.F., Cosentino, F. and Rabelink, T.J. Atherosclerosis and the two faces of endothelial nitric oxide synthase. *Circulation* 97, 108-112 (1998).
16. Xia, Y., Tsai, A.L., Berka, V. and Zweier, J.L. Superoxide generation from endothelial nitric-oxide synthase. A Ca2+/calmodulin-dependent and tetrahydrobiopterin regulatory process. *J. Biol. Chem.* 273, 25804-25808 (1998).
17. O'Donnell, V.B. and Freeman, B.A. Interactions between nitric oxide and lipid oxidation pathways: implications for vascular disease. *Circ. Res.* 88, 12-21 (2001).

18. van Haperen, R., van Tol, A., Vermeulen, P., Jauhiainen, M., van Gent, T., van den Berg, P., Ehnholm, S., Grosveld, F., van der Kamp, A. and de Crom, R. Human plasma phospholipid transfer protein increases the antiatherogenic potential of high-density lipoproteins in transgenic mice. *Arterioscler. Thromb. Vasc. Biol.* 20, 1082-1088 (2000).
19. Janssens, S.P., Shimouchi, A., Quertermous, T., Bloch, D.B. and Bloch, K.D. Cloning and expression of a cDNA encoding human endothelium-derived relaxing factor/nitric oxide synthase. *J. Biol. Chem.* 267, 14519-14522 (1992).
20. de Crom, R., van Haperen, R., Janssens, R., Visser, P., Willemsen, R., Grosveld, F. and van der Kamp, A. Gp96/GRP94 is a putative high-density lipoprotein-binding protein in liver. *Biochim. Biophys. Acta* 1437, 378-392 (1999).
21. Bakker, C.E., de Diego Otero, Y., Bontekoe, C., Raghoe, P., Luteijn, T., Hoogeveen, A. T., Oostra, B.A. and Willemsen, R. Immunocytochemical and biochemical characterization of FMRP, FXR1P, and FXR2P in the mouse. *Exp. Cell Res.* 258, 162-170 (2000).
22. Nakashima, Y., Plump, A.S., Raines, E.W., Breslow, J.L. and Ross, R. ApoE-deficient mice develop lesions of all phases of atherosclerosis throughout the arterial tree. *Arterioscler. Thromb.* 14, 133-140 (1994).
23. Murayama, T., Yokode, M., Kataoka, H., Imabayashi, T., Yoshida, H., Sano, H., Nishikawa, S. and Kita, T. Intraperitoneal administration of anti-c-fms monoclonal antibody prevents initial events of atherogenesis but does not reduce the size of advanced lesions in apolipoprotein E-deficient mice. *Circulation* 99, 1740-1746 (1999).
24. Sjoland, H., Eitzman, D.T., Gordon, D., Westrick, R., Nabel, E.G. and Ginsburg, D. Atherosclerosis progression in LDL receptor-deficient and apolipoprotein E-deficient mice is independent of genetic alterations in plasminogen activator inhibitor-1. *Arterioscler. Thromb. Vasc. Biol.* 20, 846-852 (2000).
25. Paigen, B., Morrow, A., Holmes, P.A., Mitchell, D. and Williams, R.A. Quantitative assessment of atherosclerotic lesions in mice. *Atherosclerosis* 68, 231-240 (1987).
26. Laumonnier, Y., Nadaud, S., Agrapart, M. and Soubrier, F. Characterization of an upstream enhancer region in the promoter of the human endothelial nitric-oxide synthase gene. *J. Biol. Chem.* 275, 40732-40741 (2000).
27. Garcia-Cardena, G., Fan, R., Stern, D.F., Liu, J. and Sessa, W.C. Endothelial nitric oxide synthase is regulated by tyrosine phosphorylation and interacts with caveolin-1. *J. Biol. Chem.* 271, 27237-27240 (1996).
28. Marletta, M.A. Another activation switch for endothelial nitric oxide synthase: why does it have to be so complicated? *Trends Biochem. Sci.* 26, 519-521 (2001).
29. Fulton, D., Gratton, J.P. and Sessa, W.C. Posttranslational control of endothelial nitric oxide synthase: why isn't calcium/calmodulin enough? *J. Pharmacol. Exp. Ther.* 299, 818-824 (2001).
30. Forstermann, U., Boissel, J.P. and Kleinert, H. Expressional control of the 'constitutive' isoforms of nitric oxide synthase (NOS I and NOS III). *Faseb J.* 12, 773-790 (1998).
31. Govers, R. and Rabelink, T.J. Cellular regulation of endothelial nitric oxide synthase. *Am. J. Physiol. Renal Physiol.* 280, F193-206 (2001).
32. Kamphoven, J.H., Stubenitsky, R., Reuser, A.J., van der Ploeg, A.T., Verdouw, P.D. and Duncker, D.J. Cardiac remodeling and contractile function in acid alpha-glucosidase knockout mice. *Physiol. Genomics* 5, 171-179 (2001).
33. Breslow, J.L. Mouse models of atherosclerosis. *Science* 272, 685-688 (1996).
34. Daugherty, A. Mouse models of atherosclerosis. *Am J. Med. Sci.* 323, 3-10 (2002).
35. Brown, M.S. and Goldstein, J.L. A receptor-mediated pathway for cholesterol homeostasis. *Science* 232, 34-47 (1986).

36. Gordon, D.J. and Rifkind, B.M. High-density lipoprotein--the clinical implications of recent studies. *N. Engl. J. Med.* 321, 1311-1316 (1989).
37. Libby, P. Managing the risk of atherosclerosis: the role of high-density lipoprotein. *Am. J. Cardiol.* 88, 3N-8N (2001).
38. Sessa, W.C. Can modulation of endothelial nitric oxide synthase explain the vasculoprotective actions of statins? *Trends Mol. Med.* 7, 189-191 (2001).
39. Feron, O., Dessy, C., Moniotte, S., Desager, J.P. and Balligand, J.L. Hypercholesterolemia decreases nitric oxide production by promoting the interaction of caveolin and endothelial nitric oxide synthase. *J. Clin. Invest.* 103, 897-905 (1999).
40. Feron, O., Dessy, C., Desager, J.P. and Balligand, J.L. Hydroxy-methylglutaryl-coenzyme A reductase inhibition promotes endothelial nitric oxide synthase activation through a decrease in caveolin abundance. *Circulation* 103, 113-118 (2001).
41. Everson, W.V. and Smart, E.J. Influence of caveolin, cholesterol, and lipoproteins on nitric oxide synthase: implications for vascular disease. *Trends Cardiovasc. Med.* 11, 246-250 (2001).
42. Kincer, J.F., Uittenbogaard, A., Dressman, J., Guerin, T.M., Febbraio, M., Guo, L. and Smart, E.J. Hypercholesterolemia promotes a CD36-dependent and endothelial nitric-oxide synthase-mediated vascular dysfunction. *J. Biol. Chem.* 277, 23525-23533 (2002).
43. Hoit, B.D. Two faces of nitric oxide: lessons learned from the NOS2 knockout. *Circ. Res.* 89, 289-291 (2001).
44. Ozaki, M., Kawashima, S., Yamashita, T., Hirase, T., Namiki, M., Inoue, N., Hirata, K., Yasui, H., Sakurai, H., Yoshida, Y., Masada, M. and Yokoyama, M. Overexpression of endothelial nitric oxide synthase accelerates atherosclerotic lesion formation in apoE-deficient mice. *J. Clin. Invest.* 110, 331-340 (2002).
45. d'Uscio, L.V., Baker, T.A., Mantilla, C.B., Smith, L., Weiler, D., Sieck, G.C. and Katusic, Z.S. Mechanism of endothelial dysfunction in apolipoprotein E-deficient mice. *Arterioscler. Thromb. Vasc. Biol.* 21, 1017-1022 (2001).

Chapter 3

The eNOS-GFP Transgenic Mouse

Functional Expression of Endothelial Nitric Oxide Synthase Fused to Green Fluorescent Protein in Transgenic Mice

Rien van Haperen[1], Caroline Cheng[2], Barend Mees[1], Elza van Deel[2], Monique de Waard[2], Luc C.A. van Damme[2], Teus van Gent[1], Thijs van Aken[3], Rob Krams[2], Dirk J. Duncker[2], Rini de Crom[1,4]

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From the Departments of Cell Biology & Genetics[1], Experimental Cardiology, Thoraxcenter[2], Erasmus Laboratory Animal Science Center (EDC)[3], and Vascular Surgery[4], Erasmus Medical Center, Rotterdam, the Netherlands

Abstract

The activity of endothelial nitric oxide synthase (eNOS) is subject to complex transcriptional and posttranslational regulation including the association with several proteins and variations in subcellular distribution. In the present study we describe a transgenic mouse model expressing eNOS fused to green fluorescent protein (GFP), which allows the study of localization and regulation of eNOS expression. We tested the functionality of eNOS in the eNOS–GFP mice. Expression of eNOS was restricted to the endothelial lining of blood vessels in various tissues tested, without appreciable expression in non-endothelial cells. Activity of the enzyme was confirmed by essaying the conversion of L-arginine to L-citrulline. NO production in isolated vessels was increased in transgenic mice when compared to non-transgenic control animals 4.88± 0.59 and 2.48± 0.47 μM NO, respectively, $P<0.005$). Both the mean aortic pressure and the pulmonary artery pressure were reduced in eNOS–GFP mice (both ~30%, $P<0.05$). Plasma cholesterol levels were also slightly reduced (~20%, $P<0.05$). In conclusion, eNOS–GFP mice express functional eNOS and provide a unique model to study regulation of eNOS activity or eNOS mediated vascular events, including response to ischemia, response to differences in shear stress, angiogenesis and vasculogenesis, and to study the subcellular distribution in relation with functional responses to these events.

Introduction

In endothelial cells, nitric oxide (NO) is generated by the enzyme endothelial nitric oxide synthase (eNOS) via the conversion of L-arginine to L-citrulline. NO produced by eNOS affects a number of biological processes in the vessel wall. It is important for the regulation of blood pressure and plays an important role in the aggregation of blood platelets, adhesion of leukocytes to the vessel wall and migration of vascular smooth muscle cells[1]. A decrease in NO availability is one of the hallmarks of endothelial dysfunction, which can occur in a number of cardiovascular disorders, including hypertension, heart failure, diabetes and atherosclerosis[2]. It has been shown in animal models that a decrease in eNOS activity results in accelerated atherosclerosis[3,4]. Conversely, stimulation of eNOS activity by statin treatment has been implicated in the protective actions of these drugs[5-7].

For these reasons, the regulation of eNOS activity is considered to be of major physiological and pathophysiological importance. Although eNOS was originally termed endothelial *constitutive* NOS (ecNOS)[8-10], several laboratories have found that its protein expression and enzymatic activity is under tight regulation[10-13]. In addition to regulation at the transcriptional level, protein activity is controlled in several ways. Regulatory processes include *N*-myristoylation and cysteine palmitoylation, serine/threonine phosphorylation, and protein-protein interactions. These events affect the intracellular localization of eNOS, which might influence its enzymatic activity. Normally, eNOS is localized in two subcellular compartments: the Golgi complex and the plasma membrane[14]. Although it has been suggested that translocation between these two compartments is important in the regulation of the enzymatic activity, it has been demonstrated that eNOS in both pools can be phosphorylated and activated[14]. *In vitro* studies of cells transfected with eNOS cDNA fused to DNA encoding green fluorescent protein (GFP) have provided valuable information of eNOS localization and regulation. However, to date such studies have not been performed *in vivo*. Consequently, we developed a transgenic mouse model in which an eNOS–GFP fusion protein is expressed. The results indicate that the eNOS fusion protein is functionally intact. These eNOS–GFP-tg mice can be used to study vascular reactions in which eNOS is involved, including angiogenesis or response to variations in shear stress.

Materials and Methods

Generation of eNOS–GFP transgenic mice

A genomic DNA fragment was isolated from a homemade human cosmid library. It included 6 kb of 5′ sequence, the complete eNOS gene, and 3 kb of 3′ sequence. At

the STOP codon of the eNOS gene, a linker was introduced that allowed the in frame insertion of a BamHI-NotI DNA fragment encoding eGFP which was derived from the pEGFP-N1 plasmid (BD-Sciences Clontech, Palo Alto CA). Fertilized oocytes from FVB mice were microinjected with a solution of 1-2 µg/ml in 8 mM Tris-HCl, 0.1 mM EDTA and transplanted into the oviducts of pseudopregnant B10xCBA mice. Founder mice and offspring were genotyped by PCR on DNA isolated from tail biopsies. Primers used were: 5'-GTCCTGCAGACCGTGCAGC-3' (sense) and 5'-GGCTGTTGGTGTCTGAGCCG-3' (antisense). Mice were backcrossed to C57Bl6 for at least 5 generations (>96% C57Bl6). Transgenic mice used in the present study were hemizygous. All animal experiments were performed in compliance with institutional and national guidelines.

Immunohistochemistry, fluorescence microscopy, confocal microscopy

Immunohistochemistry experiments were performed according to Bakker et al[15] on 7 µm paraffin sections. The antibodies used were raised against the carboxy terminus of eNOS (Santa Cruz Biotechnology Inc., Santa Cruz, CA, cat.nr. sc-654). For confocal microscopy, mice were sacrificed using an overdose of isoflurane (1-chloro-2,2,2-trifluoroethyl-difluoromethyl-ether). Subsequently, in situ perfusion fixation was performed by flushing 20 ml PBS through a cardiac puncture followed by 20 ml 4% (v/v) paraformaldehyde in PBS. The common carotid arteries were then harvested, fixed in 4% paraformaldehyde in PBS at 4°C, and mounted in Vectashield® (Vector Laboratories, Inc, Burlingame, CA) between glass slides. Samples were examined under a Zeiss LSM inverted laser scanning confocal fluorescence microscope. Images of GFP fluorescence were acquired after excitation with a 488-nm argon laser and after filtering the fluorescence with a 500-550 nm bandpass barrier filter. The thickness of the optical slice images is 0.6 µm.

Western blotting

Western blotting experiments were performed as described previously[16]. The antibodies used were directed against human eNOS or human eNOS phosphorylated at Ser-1177, both obtained from Santa Cruz Biotechnology Inc., Santa Cruz, CA (cat.nr. sc-654 and sc-12972, respectively).

eNOS enzyme activity and nitric oxide measurements

eNOS activity was measured in the L-arginine to L-citrulline conversion assay using a nitric oxide synthase assay kit (Calbiochem, La Jolla, CA; cat.no. 482700) as described previously[16]. For NO measurements, mice were anaesthetised with 0.2 ml i.p. sodium pentobarbital (Apharmo BV, Arnhem, The Netherlands) and intracardially perfused with 10 ml Krebs Henseleit buffer solution (118 mM NaCl, 4.7 mM KCl, 3.0 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 0.5 mM EDTA, 10 mM D-glucose and 0.1

mM L-arginine, equilibrated with 95% air and 5% CO_2 at room temperature resulting in a pH of 7.4). The aorta was dissected and in situ cannulated with two metal cannulas, proximal at the level of aortic arch and distal at the level of the diaphragm. The cannulas were connected to a homemade device keeping the vessel at its original length. Next, the vessel was transported to the set-up, where the cannulas were fixed to two holders. The two holders were placed in a chamber filled with Krebs buffer. The cannula at the proximal end of the vessel was connected to Tygon® tubing coming out of a reservoir with Krebs buffer. Between the reservoir and the cannula a flow pump (Watson Marlow, UK) and an infusion pump (Perfusor VI, B.Braun, Germany) were positioned. The settings of the pump were adjusted to perfuse the aorta with a constant flow of 10 ml/min, measured by an EMF flow sensor. This value was based on measurements of the cardiac output of our mice with a clinical Doppler device (Prosound 4000, Aloka, Japan) or by a transit-time flow probe (see below). Next, the aorta vessel was equilibrated by perfusing it for 30 minutes. During that period the experimental set up was switched to its calibration state and the NO-sensor was calibrated by infusing known NO concentrations through a tube mounted in parallel to the vessel (0.1 to 30 µM). Then, L-arginine (0.1 M) in Krebs buffer was added to the perfusate via the infusion pump. The NO-sensitive electrode was positioned at the distal end of the aorta, in order to measure the NO-production by the vessel. The electrode was connected to an NO measurement system (Iso-NO mark II, World Precision Instruments, USA). All data, including temperature, pressure drop, flow and NO measurements were stored in a PC equipped with a AD converter and analysis software (Acodas, Dataq Instruments, Natick, MA).

Hemodynamics and heart weights

Hemodynamic measurements were performed as described[16]. Shortly, blood pressure measurements were performed in mice anesthetized with ketamine (100 mg/kg ip) and xylazine (20 mg/kg ip), intubated and ventilated with a mixture of O_2 and N_2 (1/2 vol/vol). A flame stretched PE 50 polyethylene catheter was inserted into the right carotid artery and advanced into the aortic arch for measurement of aortic pressure and connected to a pressure transducer (Braun). For infusion of L-NAME, a second catheter was introduced into the right external jugular vein and advanced into the superior caval vein. After thoracotomy through the second right intercostal space, the ascending aorta was exposed and a transit-time flow probe (ID 1.5 mm; Transonics systems T206) was placed around the aorta for measuring aorta flow. Ten minutes after a second intraperitoneal bolus of 100 mg/kg ketamine and 20 mg/kg xylazine, baseline recordings were obtained. Then, a continuous 10 minutes intravenous infusion of L-NAME (100 mg/kg) was started and measurements were continued until 10 minutes after completion of the infusion. For the measurements of pulmonary artery pressure, the second

left intercostal space was opened and a 30G needle connected to a PE 10 polyethylene catheter was directly inserted into the pulmonary artery. Hemodynamic data were recorded and digitized using an on-line 4 channel data acquisition program (ATCODAS, Dataq Instruments, Akron, OH). Ten consecutive beats were selected for determination of heart rate, aortic and pulmonary artery pressures, and aortic blood flow. For determination of ventricular weights the heart was removed and the ventricles were separated from the atria, the aorta and the pulmonary artery. The right ventricle was carefully separated from the left ventricle (incl. septum) and the right and left ventricle were weighed on a microbalance (Sartorius AG Göttingen). For the measurements of dry weights, tissue fluids were removed by lyophilization.

Cholesterol and lipoprotein analysis

Blood was collected by orbital puncture after an overnight fast. Plasma from 6-10 mice was pooled and subjected to gel filtration analysis on two HR10/30 FPLC columns in tandem (Superdex 200 and Superose 6, both prepgrade, Pharmacia Biotech., Uppsala, Sweden)[17]. Cholesterol concentrations were measured with the free cholesterol C kit (WAKO, Neuss, Germany) after hydrolysis of cholesterol esters with cholesterol esterase from *Candida cylindracea*.

Data analysis

Analysis of data was performed using two-way or one-way analysis of variance followed by Scheffé's test, as appropriate. Statistical significance was accepted when $P<0.05$ (two-tailed). Data are presented as mean ± SEM.

Results

eNOS–GFP transgenic mice

We aimed to create mice transgenic for human eNOS, in which the expression resembled that of the endogenous gene as much as possible, both with respect to the expression pattern as well as the regulation. Therefore, we used a DNA fragment to generate transgenic mice that comprised the complete human eNOS genomic sequence, including the natural promoter. This fragment was isolated from a human cosmid library. In addition to the eNOS gene, the DNA fragment contained ~6 kb of 5′ sequence, including the endothelial enhancer sequence identified by Laumonnier et al[18], and ~3 kb of 3′ sequence. At the STOP codon of the eNOS gene, a DNA fragment encoding enhanced GFP was inserted in frame with the eNOS gene, in order to obtain a DNA construct encoding an eNOS–GFP fusion protein. This fragment (Fig. 1a) was used for the generation of eNOS–GFP transgenic (eNOS–GFPtg) mice.

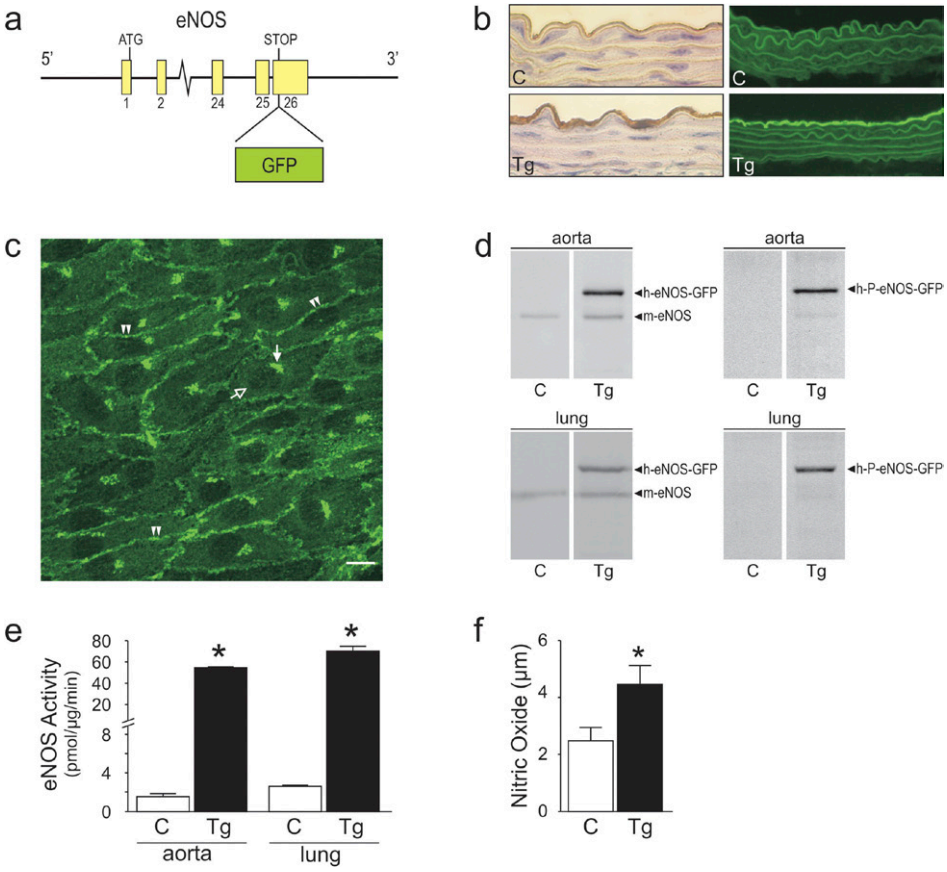

Figure 1. Transgenic mice expressing eNOS–GFP. a. The DNA construct used for the generation of eNOS–GFPtg mice consisted of the complete eNOS gene in which an eGFP encoding cassette was cloned in frame at the position of the eNOS STOP codon. The natural flanking sequences of the eNOS gene were left intact. b. Sections of aorta from control mice or eNOS–GFP transgenic mice, inspected with immunohistochemistry using an antibody directed against human eNOS (*left panels*) or by fluorescent microscopy (*right panels*). Original magnifications: 630x. c. Green fluorescence in endothelial cells from an intact carotid artery inspected with confocal microscopy. *Double arrowheads* point at fluorescence at the position of the plasma membrane, the white arrow at fluorescence at the position of the Golgi complex. The *non-filled arrow* points at the cell nucleus. Scale bar: 10 µm. d. Immunoblotting of aorta (*upper panels*) or lung (*lower panels*) homogenates with antibodies directed against human eNOS (*left panels*), which show cross-reactivity with endogenous mouse eNOS, or antibodies directed against phosphorylated Ser-1177 from human eNOS (*right panels*). e. eNOS activity measured in aorta or lung homogenates by the L-arginine to L-citrulline conversion assay. n=9. *P<0.001. f. NO production in isolated aortas measured with an NO-sensitive electrode. n=11 (C), n=8 (Tg). *P<0.005. b, d-f: C: control mice, Tg: eNOS–GFP transgenic mice.

Fig. 1b shows sections from mouse aorta, taken from eNOS–GFPtg mice or wild-type littermates (controls). Immunohistochemistry studies on these sections using an antibody directed against human eNOS revealed a strong staining of the endothelial layer of the aorta in the eNOS–GFPtg mice, while there was no staining in the media of the aorta (Fig. 1b, lower left panel). Sections from non-transgenic controls showed virtually no staining (Fig. 1b, upper left panel). Inspection with fluorescence microscopy showed a strong fluorescent signal in the endothelial layer of the aorta from eNOS–GFPtg mice (Fig. 1b, lower right panel), which is much higher than the autofluorescence from the elastic lamellae that is present in both eNOS–GFPtg mice and in wild-type controls (Fig. 1b, upper right panel). The subcellular localization was studied by confocal microscopy in the carotid artery (Fig. 1c). The expression pattern exactly matches the sites where eNOS is known to be primarily located: in the Golgi complex and at the plasma membrane. We performed Western blotting experiments with aorta and lung tissue from eNOS–GFPtg mice and non-transgenic controls (Fig. 1d). Using an antibody directed against human eNOS, both the endogenous murine eNOS is detected, caused by cross-reactivity of the antibody, as well as the eNOS–GFP fusion protein, which has a slightly higher molecular mass caused by the GFP moiety (approximately 130 kDa and 155 kDa, respectively; Fig. 1d, left panels). In addition, we used an antibody that recognizes eNOS that is phosphorylated on serine 1177 (P-eNOS), which is the activated form of the enzyme. P-eNOS–GFP is present in both aorta and lung tissue from eNOS–GFPtg mice (Fig. 1d, right panels). In order to determine whether the expressed eNOS–GFP fusion protein is catalytically active, enzymatic activity was measured in aorta and lung tissue from eNOS–GFPtg mice and non-transgenic controls. As shown in Fig. 1e, the transgenic mice show an increase in eNOS activity of approximately 35-fold in aorta and 27-fold in lung tissue. In addition, NO production was measured in isolated vessels *ex vivo*, using an NO sensor. Aortas from eNOS–GFP transgenic mice showed an increased NO production compared with aortas from control mice (Fig 1f). The data could be described by a first order kinetic reaction, which was confirmed by a Lineweaver-Burke transformation (data not shown).

Localization of eNOS–GFP

In order to investigate the cell specificity of the expression of the transgene, sections from organs of eNOS–GFPtg mice were inspected by immunohistochemistry using an antibody directed against human eNOS (Fig. 2). eNOS immunoreactivity was detected in small blood vessels in the heart (Fig. 2a), in the sinusoids in the liver (Fig. 2b), in the peritubular capillaries and in the endothelial cells in the glomeruli in the kidney (Fig. 2c) and in the capillary sinusoids in the adrenal (Fig. 2d).

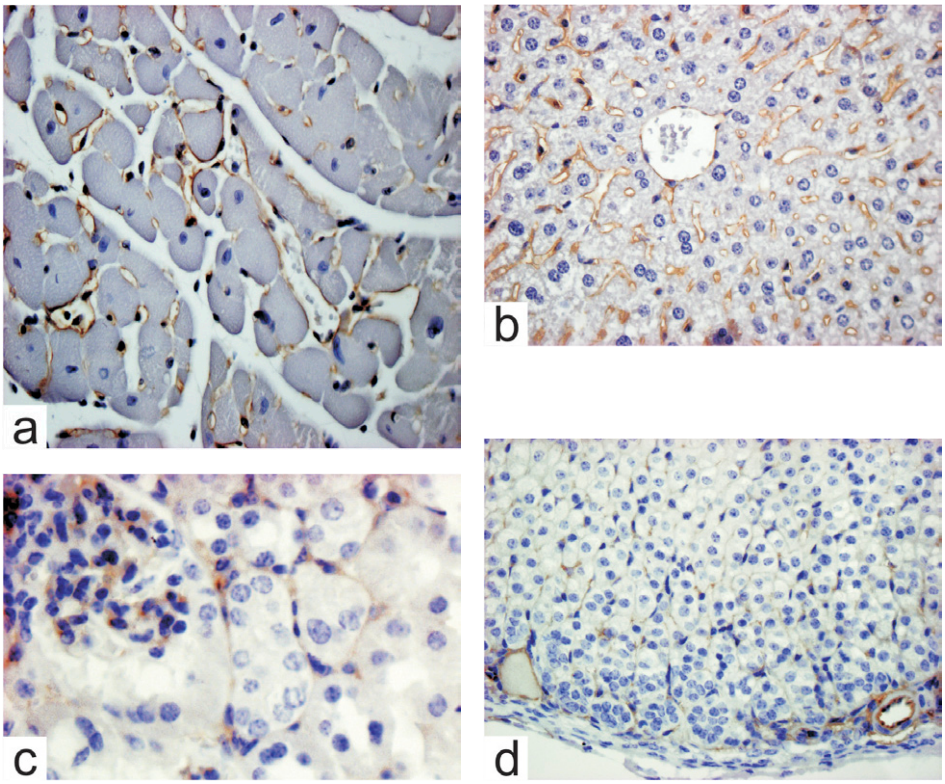

Figure 2. Localization of eNOS–GFP in various organs from eNOS–GFPtg mice visualized by immunohistochemistry using an antibody directed against human eNOS. a. heart, b. liver, c. kidney, d. adrenal. Original magnifications: 400x.

eNOS protein was not found in the parenchymal cells of these organs. Sections from non-transgenic controls showed no immunoreactivity (not shown). This expression pattern was confirmed by fluorescence microscopy. Fig. 3 shows micrographs from heart (Fig. 3a,b), liver (Fig. 3c,d), kidney (Fig. 3e,f) and adrenal gland (Fig. 3g,h). The lining of the larger vessels was clearly fluorescent (Fig. 3c). A dense capillary network was fluorescent in the heart, surrounding the cardiomyocytes, and in the adrenal. The sinusoids in the liver, as well as the peritubular capillaries in the kidney showed a positive signal, just like the capillaries of the glomeruli in the kidney. The parenchymal cells of none of these organs showed appreciable expression. In sections from non-transgenic controls, no fluorescent signal was detected (not shown).

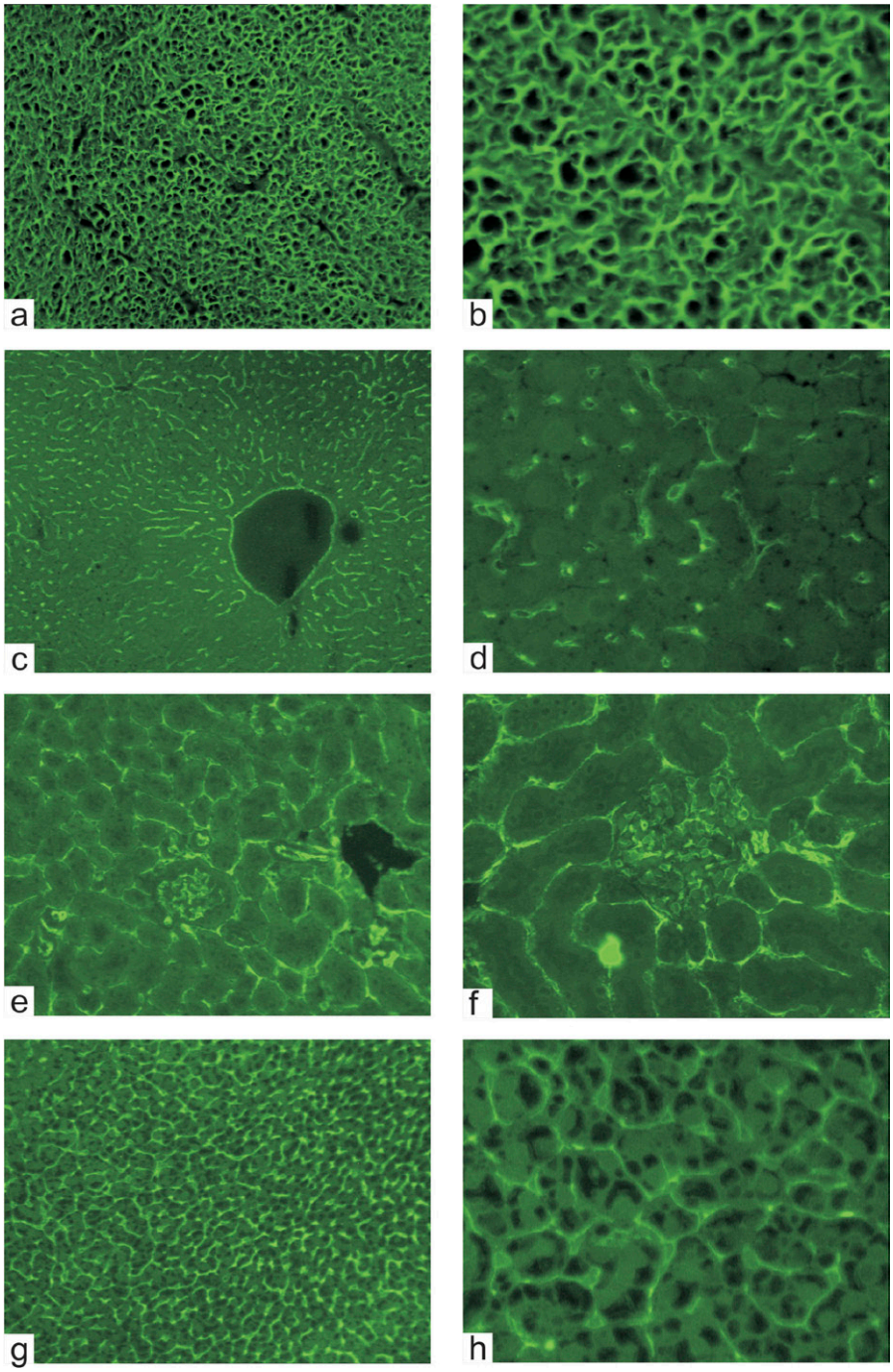

Figure 3. Localization of eNOS–GFP in various organs from eNOS–GFPtg mice visualized by fluorescence microscopy. a.,b.: heart, c.,d. liver, e.,f. kidney, g.,h. adrenal. Original magnifications: 100x (a.,c.,e.,g.), 400x (b.,d.,f.,h.).

Hemodynamic properties of eNOS–GFPtg mice

We performed hemodynamic measurements in eNOS–GFPtg mice compared to non-transgenic control mice. This was done in order to establish the functional activity of the eNOS–GFP fusion protein, as eNOS derived NO is an important modulator of vascular tone. As shown in figure 4a, the mean aortic pressure (MAP) was decreased in eNOS–GFP expressing mice compared to wild-type littermates by approximately 29%. Treatment with the NO synthase inhibitor N^G nitro-L-arginine methyl ester (L-NAME) resulted in an increase of the MAP and abolished the difference between transgenic mice and wild-type littermates. The increase in the MAP (DMAP) caused by L-NAME treatment was greater in transgenic mice than in non-transgenic controls. There were no differences found in heart rate (Fig. 4b) or mean aortic blood flow (Fig. 4c) between transgenic or control mice, with or without treatment with L-NAME. Consequently, the difference in MAP can be attributed to a difference in systemic vascular resistance (Fig. 4d). In addition to a lower pressure in the systemic bed, we also observed a lower mean pulmonary artery pressure in eNOS–GFPtg mice of 30% (Fig. 4e). We also analyzed the heart weights of the transgenic mice in comparison with controls. No differences were found (Fig. 4f).

Plasma cholesterol levels in eNOS–GFPtg mice

Finally, we measured levels of plasma cholesterol in eNOS–GFPtg mice with different genetic backgrounds as models for variations in plasma cholesterol levels. We compared wild-type background, heterozygous low-density lipoprotein receptor-deficient background, which results in a condition of slight hypercholesterolemia, and apolipoprotein E-deficient background, which results in a condition of more severe hypercholesterolemia. We found a slight decrease in cholesterol levels in all backgrounds when comparing eNOS–GFP transgenic animals with animals without this transgene (Fig. 5a). Next, we analyzed the lipoprotein profile in two of these backgrounds. In wild-type background, plasma cholesterol is predominantly found in high-density lipoproteins (HDL)[19]. Thus, the cholesterol lowering observed in eNOS–GFP transgenic animals is found in HDL (Fig. 5b). In apolipoprotein E-deficient background however, plasma cholesterol is present in the atherogenic lipoproteins, very low-density lipoproteins (VLDL) and low-density lipoproteins (LDL)[16]. In this background, expression of eNOS–GFP results in a decrease in both VLDL+LDL and HDL (Fig. 5c).

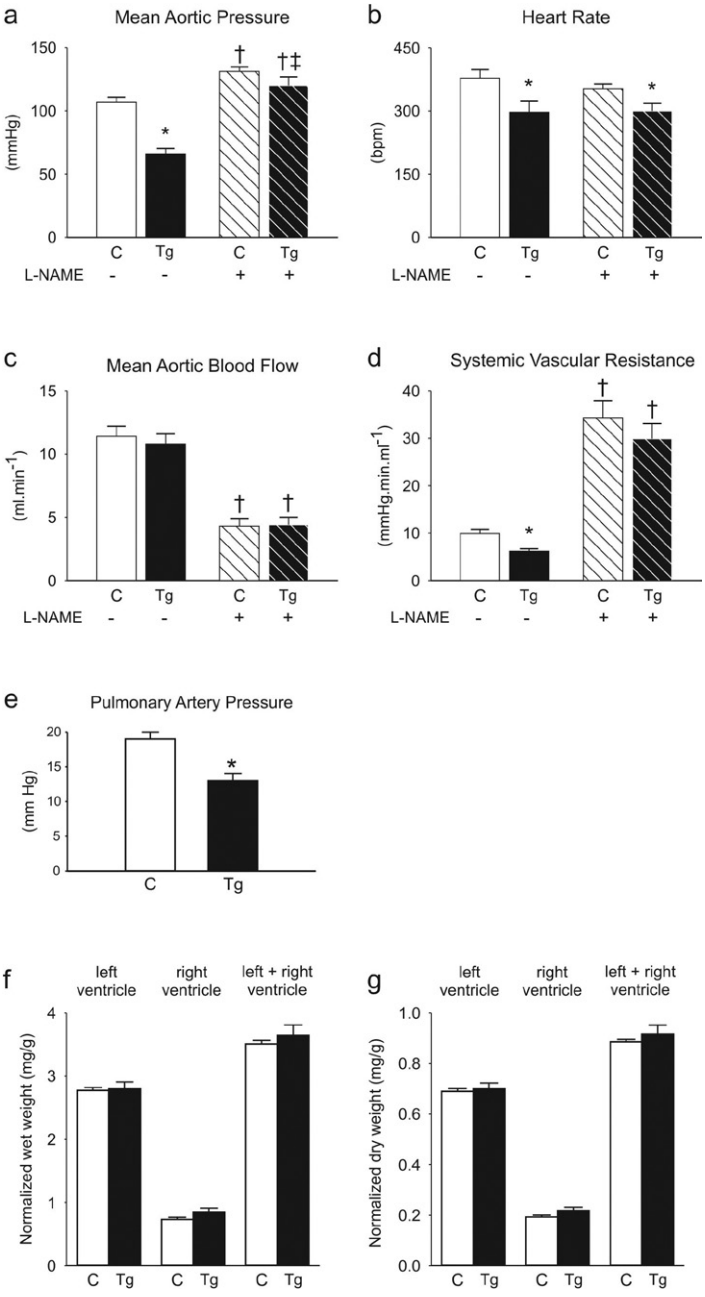

Figure 4. Hemodynamics and ventricular weights were measured in control mice (C) or eNOS–GFPtg mice (Tg) before and after addition of L-NAME. a. Mean aortic pressure; b. heart rate; c. mean aortic blood flow; d. systemic vascular resistance; e. pulmonary artery pressure; f. ventricular wet weights; g. ventricular dry weights. a-d: n=10 (C), n=9 (Tg), e: n=8 (C), n=6 (Tg), f-g: n=20 (C), n=12 (Tg). *: P<0.05 vs controls; †: P<0.05 vs controls before addition of L-NAME; ‡: P<0.05 D in C vs D in Tg (before and after addition of L-NAME).

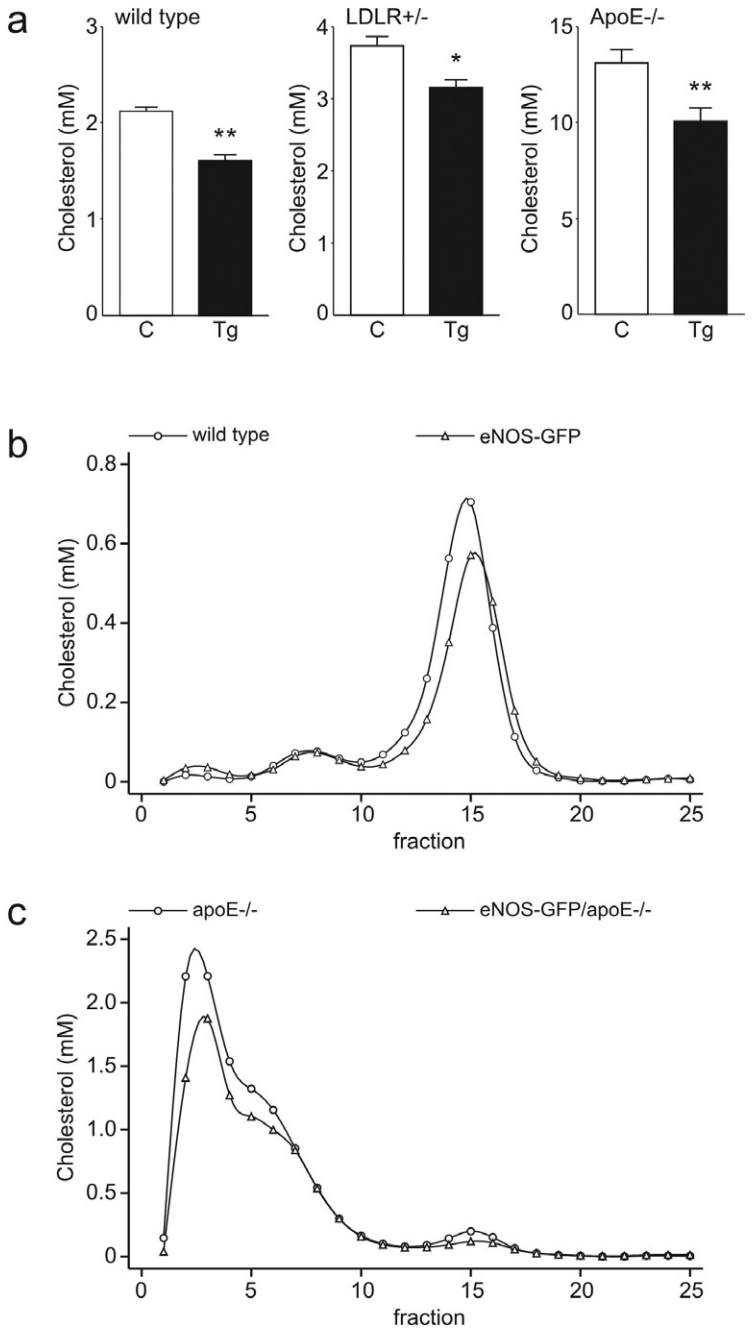

Figure 5. Cholesterol measurements. a. Plasma cholesterol concentrations of wild-type, LDLR heterozygous knock out (LDLR+/-) and apolipoprotein E homozygous knock out (ApoE-/-) mice with (Tg) or without (C) the eNOS–GFP transgene. *: P<0.01 vs controls; . **: P<0.001 vs controls. b.,c. Fractionation of lipoproteins by gel filtration of plasma from mice with wild-type background (b) or apolipoprotein E knock out background (c). Fraction 1-5 contain VLDL, 6-11 contain LDL and 12-20 contain HDL.

Discussion

We generated transgenic mice that express human eNOS in fusion with GFP. In order to preserve transcriptional regulation, we used a genomic construct which contains the intronic sequences as well as the natural flanking sequences, including the previously identified endothelial enhancer located 4.9 kilobases upstream of the transcription initiation site[18]. The expression pattern was visualized either indirectly, via immunohistochemistry using antibodies directed against eNOS, or directly via fluorescence microscopy. Both techniques showed virtually the same distribution of the eNOS–GFP transgene in a variety of organs: endothelial cells of smaller and larger vessels in various organs are positive, while the parenchymal cells are not. These results indicate that the DNA construct used for microinjections resulted in expression of the transgene predominantly, if not exclusively, in endothelial cells.

The functionality of the eNOS–GFP fusion protein was demonstrated in two different ways. Using the L-arginine to L-citrulline conversion assay, it was shown that eNOS enzyme activity is ~30 fold higher in eNOS–GFP transgenic animals compared with non-transgenic controls. In *ex vivo* measurements with an NO sensor, NO production by endothelial cells in isolated blood vessels was shown to be 1.8 fold higher when vessels from eNOS–GFP transgenic mice were used.

The apparent discrepancy between these measurements can be explained by the difference between the methods that were used. In the L-arginine to L-citrulline conversion assay, a homogenate of aortic tissue was used. Cofactors and reagents are added to the assay in order to optimize the conditions for the enzymatic reaction. Therefore, the actual measurement represents the maximal eNOS activity, thereby reflecting the total amount of enzyme present. In contrast, the *ex vivo* measurements with the NO sensor were performed in intact blood vessels. As a consequence, the cellular regulation of the activity of the enzyme is still intact. In this system, the actual NO production is measured. This is much lower than the maximal enzymatic activity, probably because posttranslational control of eNOS activity is intact.

In addition, blood pressure measurements showed that the mean aortic pressure was lower in eNOS–GFP transgenic animals compared with non-transgenic controls. This is in agreement with findings in other transgenic models of eNOS overexpression[16,20]. In the latter models, pulmonary pressures have not been measured. However, these are expected to be decreased as well, as the pulmonary vasodilator response following NO inhalation in patients is well known[21,22]. Indeed, we found lower pulmonary pressure in eNOS–GFP transgenic animals compared with non-transgenic controls.

GFP expression has been used in various transgenic mouse models and in numerous *in vitro* studies using cultured cells. It is generally assumed that the GFP protein itself does not interfere with any biological function of other proteins studied. However,

specific expression in cardiomyocytes has been reported to result in hypertrophy[23]. Although the transgene in our mouse model is expressed in endothelial cells and not in cardiomyocytes, we measured the ventricular weights of transgenic and control mice. No differences were found, demonstrating that hypertrophy probably does not occur in eNOS–GFPtg mice.

Previously, we generated and studied transgenic mice overexpressing eNOS without GFP[16]. In addition to a lower blood pressure, these animals displayed a decrease in plasma levels of cholesterol. Thus, we measured plasma cholesterol in eNOS–GFP transgenic mice and found a decrease of ~20% compared with non-transgenic mice (Fig. 5a). Plasma cholesterol in mice is for the larger part in HDL, which is generally believed to be atheroprotective[19]. Therefore, atherosclerosis in mouse models is usually studied in a genetic background with increased cholesterol in atherogenic very low-density lipoproteins (VLDL) and low-density lipoproteins (LDL). We crossbred eNOS–GFPtg mice with two of these models. In the first place, we crossbred them to low-density lipoprotein receptor-deficient mice, resulting in a background of heterozygous deficiency. This genetic background causes mild hypercholesterolemia[24]. In addition, we crossbred the mice in a apolipoprotein E-deficient background, which causes more severe hypercholesterolemia[16]. In both of these backgrounds, we found that expression of eNOS–GFP results in lower levels of plasma cholesterol (Fig. 5a). While in wild-type background a reduction in HDL-cholesterol was found (Fig. 5b), the effect in apolipoprotein E-deficient mice was primarily in VLDL+LDL cholesterol (Fig. 5c). This is in agreement with our previous work, in which it was tentatively concluded that this effect contributed to the antiatherogenic properties of elevated eNOS expression[16].

The mouse model described in the present study can be used to study the unusually complex regulation of eNOS[11,13]. Regulation takes place at the transcriptional level[25-27], and is responsive to variations in shear stress[28,29]. In addition, extensive regulation of eNOS activity at the posttranslational level exists. These pathways are probably also active in the eNOS–GFPtg mice, as these are most likely responsible for the remarkable discrepancy between the level of protein overexpression and the physiological level of NO production (compare Fig. 1d,e with Fig. 1f). The posttranslational regulatory mechanisms include protein–protein interactions, some of which affect the subcellular localization of the protein. eNOS is primarily located in the Golgi complex and in the caveolae in the plasma membrane, which were shown to be two pools of active enzyme[14], although others have suggested that active eNOS is largely restricted to caveolae[30-32]. A number of proteins have been shown to interact with eNOS and affect its activity. Caveolin, the structural protein of the caveolae, forms an inhibitory complex with eNOS[32], for which further evidence was provided by studies in caveolin-deficient mice[33]. Upon activation by calcium, calmodulin can activate eNOS by displacing it from caveolin[34,35]. Another protein that binds to eNOS and subsequently activates it is heat

shock protein 90[36]. The latter protein appears to play a role in the caveolin–calmodulin regulatory complex[37]. Recently identified proteins that interact with eNOS have been termed NOSIP and NOSTRIN[38,39]. The functions of these proteins have not been established yet. Still another way of posttranslational regulation of eNOS activity is protein modification, like palmitoylation and myristoylation[40,41], or phosphorylation, especially of the serine at position 1177 (human eNOS)[42,43].

The eNOS–GFPtg mice described in the present study are particularly suited to study various aspects of the complex regulation of eNOS activity. Subcellular localization under various conditions or dynamic changes in this pattern upon various stimuli can be studied, as well as interactions with other proteins or with the cytoskeleton[44]. Valuable information has been achieved by studying expression of eNOS–GFP cDNA in transfected cells[45,46]. Our model also allows *ex vivo* and *in vivo* studies, including real time imaging by intravital microscopy. These types of studies will be useful to further assess the impact of eNOS activity in processes like reactivity to variations in shear stress or angiogenesis under conditions of ischemia or in tumor growth.

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References

1. Li, H. & Forstermann, U. Nitric oxide in the pathogenesis of vascular disease. *J Pathol* 190, 244-254. (2000).
2. Harrison, D.G. Cellular and molecular mechanisms of endothelial cell dysfunction. *J Clin Invest* 100, 2153-2157. (1997).
3. Knowles, J.W., Reddick, R.L., Jennette, J.C., Shesely, E.G., Smithies, O. & Maeda, N. Enhanced atherosclerosis and kidney dysfunction in eNOS(-/-)Apoe(-/-) mice are ameliorated by enalapril treatment. *J Clin Invest* 105, 451-458. (2000).
4. Kuhlencordt, P.J., Gyurko, R., Han, F., Scherrer-Crosbie, M., Aretz, T.H., Hajjar, R., Picard, M.H. & Huang, P.L. Accelerated atherosclerosis, aortic aneurysm formation, and ischemic heart disease in apolipoprotein E/endothelial nitric oxide synthase double-knockout mice. *Circulation* 104, 448-454. (2001).
5. Laufs, U., La Fata, V., Plutzky, J. & Liao, J.K. Upregulation of endothelial nitric oxide synthase by HMG CoA reductase inhibitors. *Circulation* 97, 1129-1135 (1998).
6. Kano, H., Hayashi, T., Sumi, D., Esaki, T., Asai, Y., Thakur, N.K., Jayachandran, M. & Iguchi, A. A HMG-CoA reductase inhibitor improved regression of atherosclerosis in the rabbit aorta without affecting serum lipid levels: possible relevance of up-regulation of endothelial NO synthase mRNA. *Biochem Biophys Res Commun* 259, 414-419. (1999).
7. Sessa, W.C. Can modulation of endothelial nitric oxide synthase explain the vasculoprotective actions of statins? *Trends Mol Med* 7, 189-191. (2001).
8. Nishida, K., Harrison, D.G., Navas, J.P., Fisher, A.A., Dockery, S.P., Uematsu, M., Nerem, R.M., Alexander, R.W. & Murphy, T.J. Molecular cloning and characterization of the constitutive bovine aortic endothelial cell nitric oxide synthase. *J Clin Invest* 90, 2092-2096 (1992).
9. Marsden, P.A., Heng, H.H., Scherer, S.W., Stewart, R.J., Hall, A.V., Shi, X.M., Tsui, L.C. & Schappert, K.T. Structure and chromosomal localization of the human constitutive endothelial nitric oxide synthase gene. *J Biol Chem* 268, 17478-17488 (1993).
10. Forstermann, U., Boissel, J.P. & Kleinert, H. Expressional control of the 'constitutive' isoforms of nitric oxide synthase (NOS I and NOS III). *Faseb J* 12, 773-790. (1998).
11. Fulton, D., Gratton, J.P. & Sessa, W.C. Post-translational control of endothelial nitric oxide synthase: why isn't calcium/calmodulin enough? *J Pharmacol Exp Ther* 299, 818-824. (2001).
12. Govers, R. & Rabelink, T.J. Cellular regulation of endothelial nitric oxide synthase. *Am J Physiol Renal Physiol* 280, F193-206. (2001).
13. Marletta, M.A. Another activation switch for endothelial nitric oxide synthase: why does it have to be so complicated? *Trends Biochem Sci* 26, 519-521. (2001).
14. Fulton, D., Fontana, J., Sowa, G., Gratton, J.P., Lin, M., Li, K.X., Michell, B., Kemp, B.E., Rodman, D. & Sessa, W.C. Localization of endothelial nitric-oxide synthase phosphorylated on serine 1179 and nitric oxide in Golgi and plasma membrane defines the existence of two pools of active enzyme. *J Biol Chem* 277, 4277-4284 (2002).
15. Bakker, C.E., de Diego Otero, Y., Bontekoe, C., Raghoe, P., Luteijn, T., Hoogeveen, A.T., Oostra, B.A. & Willemsen, R. Immunocytochemical and biochemical characterization of FMRP, FXR1P, and FXR2P in the mouse. *Exp Cell Res* 258, 162-170 (2000).
16. Van Haperen, R., De Waard, M., Van Deel, E., Mees, B., Kutryk, M., Van Aken, T., Hamming, J., Grosveld, F., Duncker, D.J. & De Crom, R. Reduction of blood pressure, plasma cholesterol, and atherosclerosis by elevated endothelial nitric oxide. *J Biol Chem* 277, 48803-48807 (2002).

17. Lie, J., de Crom, R., van Gent, T., van Haperen, R., Scheek, L., Lankhuizen, I. & van Tol, A. Elevation of plasma phospholipid transfer protein in transgenic mice increases VLDL secretion. *J Lipid Res* 43, 1875-1880 (2002).
18. Laumonnier, Y., Nadaud, S., Agrapart, M. & Soubrier, F. Characterization of an upstream enhancer region in the promoter of the human endothelial nitric-oxide synthase gene. *J Biol Chem* 275, 40732-40741. (2000).
19. van Haperen, R., van Tol, A., Vermeulen, P., Jauhiainen, M., van Gent, T., van den Berg, P., Ehnholm, S., Grosveld, F., van der Kamp, A. & de Crom, R. Human plasma phospholipid transfer protein increases the antiatherogenic potential of high density lipoproteins in transgenic mice. *Arterioscler Thromb Vasc Biol* 20, 1082-1088. (2000).
20. Ohashi, Y., Kawashima, S., Hirata, K., Yamashita, T., Ishida, T., Inoue, N., Sakoda, T., Kurihara, H., Yazaki, Y. & Yokoyama, M. Hypotension and reduced nitric oxide-elicited vasorelaxation in transgenic mice overexpressing endothelial nitric oxide synthase. *J Clin Invest* 102, 2061-2071. (1998).
21. Hurford, W.E. Inhaled nitric oxide. *Respir Care Clin N Am* 8, 261-279 (2002).
22. Sitbon, O., Humbert, M. & Simonneau, G. Primary pulmonary hypertension: Current therapy. *Prog Cardiovasc Dis* 45, 115-128 (2002).
23. Huang, W.Y., Aramburu, J., Douglas, P.S. & Izumo, S. Transgenic expression of green fluorescence protein can cause dilated cardiomyopathy. *Nat Med* 6, 482-483 (2000).
24. van Haperen, R., van Tol, A., van Gent, T., Scheek, L., Visser, P., van der Kamp, A., Grosveld, F. & de Crom, R. Increased risk of atherosclerosis by elevated plasma levels of phospholipid transfer protein. *J Biol Chem* 277, 48938-48943 (2002).
25. Zhang, R., Min, W. & Sessa, W.C. Functional analysis of the human endothelial nitric oxide synthase promoter. Sp1 and GATA factors are necessary for basal transcription in endothelial cells. *J Biol Chem* 270, 15320-15326 (1995).
26. Karantzoulis-Fegaras, F., Antoniou, H., Lai, S.L., Kulkarni, G., D'Abreo, C., Wong, G.K., Miller, T.L., Chan, Y., Atkins, J., Wang, Y. & Marsden, P.A. Characterization of the human endothelial nitric-oxide synthase promoter. *J Biol Chem* 274, 3076-3093 (1999).
27. Wu, K.K. Regulation of endothelial nitric oxide synthase activity and gene expression. *Ann N Y Acad Sci* 962, 122-130 (2002).
28. Topper, J.N., Cai, J., Falb, D. & Gimbrone, M.A., Jr. Identification of vascular endothelial genes differentially responsive to fluid mechanical stimuli: cyclooxygenase-2, manganese superoxide dismutase, and endothelial cell nitric oxide synthase are selectively up-regulated by steady laminar shear stress. *Proc Natl Acad Sci U S A* 93, 10417-10422 (1996).
29. Go, Y.M., Boo, Y.C., Park, H., Maland, M.C., Patel, R., Pritchard, K.A., Jr., Fujio, Y., Walsh, K., Darley-Usmar, V. & Jo, H. Protein kinase B/Akt activates c-Jun NH(2)-terminal kinase by increasing NO production in response to shear stress. *J Appl Physiol* 91, 1574-1581 (2001).
30. Blair, A., Shaul, P.W., Yuhanna, I.S., Conrad, P.A. & Smart, E.J. Oxidized low density lipoprotein displaces endothelial nitric-oxide synthase (eNOS) from plasmalemmal caveolae and impairs eNOS activation. *J Biol Chem* 274, 32512-32519 (1999).
31. Uittenbogaard, A., Shaul, P.W., Yuhanna, I.S., Blair, A. & Smart, E.J. High density lipoprotein prevents oxidized low density lipoprotein-induced inhibition of endothelial nitric-oxide synthase localization and activation in caveolae. *J Biol Chem* 275, 11278-11283 (2000).
32. Everson, W.V. & Smart, E.J. Influence of caveolin, cholesterol, and lipoproteins on nitric oxide synthase: implications for vascular disease. *Trends Cardiovasc Med* 11, 246-250 (2001).
33. Schubert, W., Frank, P.G., Woodman, S.E., Hyogo, H., Cohen, D.E., Chow, C.W. & Lisanti, M.P. Microvascular hyperpermeability in caveolin-1 (-/-) knock-out mice. Treatment with a specific nitric-oxide

synthase inhibitor, L-name, restores normal microvascular permeability in Cav-1 null mice. *J Biol Chem* 277, 40091-40098 (2002).

34. Michel, J.B., Feron, O., Sase, K., Prabhakar, P. & Michel, T. Caveolin versus calmodulin. Counterbalancing allosteric modulators of endothelial nitric oxide synthase. *J Biol Chem* 272, 25907-25912 (1997).
35. Michel, T. & Feron, O. Nitric oxide synthases: which, where, how, and why? *J Clin Invest* 100, 2146-2152 (1997).
36. Garcia-Cardena, G., Fan, R., Shah, V., Sorrentino, R., Cirino, G., Papapetropoulos, A. & Sessa, W.C. Dynamic activation of endothelial nitric oxide synthase by Hsp90. *Nature* 392, 821-824 (1998).
37. Gratton, J.P., Fontana, J., O'Connor, D.S., Garcia-Cardena, G., McCabe, T.J. & Sessa, W.C. Reconstitution of an endothelial nitric-oxide synthase (eNOS), hsp90, and caveolin-1 complex in vitro. Evidence that hsp90 facilitates calmodulin stimulated displacement of eNOS from caveolin-1. *J Biol Chem* 275, 22268-22272 (2000).
38. Dedio, J., Konig, P., Wohlfart, P., Schroeder, C., Kummer, W. & Muller-Esterl, W. NOSIP, a novel modulator of endothelial nitric oxide synthase activity. *Faseb J* 15, 79-89 (2001).
39. Zimmermann, K., Opitz, N., Dedio, J., Renne, C., Muller-Esterl, W. & Oess, S. NOSTRIN: a protein modulating nitric oxide release and subcellular distribution of endothelial nitric oxide synthase. *Proc Natl Acad Sci U S A* 99, 17167-17172 (2002).
40. Robinson, L.J. & Michel, T. Mutagenesis of palmitoylation sites in endothelial nitric oxide synthase identifies a novel motif for dual acylation and subcellular targeting. *Proc Natl Acad Sci U S A* 92, 11776-11780 (1995).
41. Sakoda, T., Hirata, K., Kuroda, R., Miki, N., Suematsu, M., Kawashima, S. & Yokoyama, M. Myristoylation of endothelial cell nitric oxide synthase is important for extracellular release of nitric oxide. *Mol Cell Biochem* 152, 143-148 (1995).
42. Fulton, D., Gratton, J.P., McCabe, T.J., Fontana, J., Fujio, Y., Walsh, K., Franke, T.F., Papapetropoulos, A. & Sessa, W.C. Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature* 399, 597-601. (1999).
43. Dimmeler, S., Fleming, I., Fisslthaler, B., Hermann, C., Busse, R. & Zeiher, A.M. Activation of nitric oxide synthase in endothelial cells by Akt- dependent phosphorylation. *Nature* 399, 601-605. (1999).
44. Venema, V.J., Marrero, M.B. & Venema, R.C. Bradykinin-stimulated protein tyrosine phosphorylation promotes endothelial nitric oxide synthase translocation to the cytoskeleton. *Biochem Biophys Res Commun* 226, 703-710 (1996).
45. Liu, J., Hughes, T.E. & Sessa, W.C. The first 35 amino acids and fatty acylation sites determine the molecular targeting of endothelial nitric oxide synthase into the Golgi region of cells: a green fluorescent protein study. *J Cell Biol* 137, 1525-1535 (1997).
46. Sowa, G., Liu, J., Papapetropoulos, A., Rex-Haffner, M., Hughes, T.E. & Sessa, W.C. Trafficking of endothelial nitric-oxide synthase in living cells. Quantitative evidence supporting the role of palmitoylation as a kinetic trapping mechanism limiting membrane diffusion. *J Biol Chem* 274, 22524-22531 (1999).

Chapter 4

eNOS and Collaterals

Endothelial Nitric Oxide Synthase Activity is Essential for Vasodilation during Blood Flow Recovery, but not for Arteriogenesis

Barend Mees[2,3], Shawn Wagner[1], Elena Ninci[1], Silvia Tribulova[1], Sandra Martin[1], Rien van Haperen[2], Sawa Kostin[1], Matthias Heil[1], Rini de Crom[2,3], Wolfgang Schaper[1]

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Department of [1]Experimental Cardiology, Max-Planck-Institute for Heart & Lung Research, Bad Nauheim, Germany; Departments of [2]Cell Biology & Genetics and [3]Vascular Surgery, Erasmus University MC, Rotterdam, The Netherlands

Abstract

Arteriogenesis is the major mechanism of vascular growth, which is able to compensate for blood flow deficiency after arterial occlusion. Endothelial nitric oxide synthase (eNOS) activity is essential for neovascularization, however its specific role in arteriogenesis remains unclear. We studied the role of eNOS in arteriogenesis using three mouse strains with different eNOS expression.

Distal femoral artery ligation was performed in eNOS-overexpressing mice (eNOStg), eNOS-deficient (eNOS-/-) mice and wild-type (WT) controls. Tissue perfusion and collateral-dependent blood flow were significantly increased in eNOStg mice compared to WT only immediately following ligation. In eNOS-/- mice, although tissue perfusion remained significantly decreased, collateral-dependent blood flow was only decreased until day 7, suggesting normal, perhaps delayed collateral growth. Histology confirmed no differences in collateral arteries of eNOStg, eNOS-/- and WT mice at one and three weeks. Administration of an NO donor induced vasodilation in collateral arteries of eNOS-/- mice, but not in WT, identifying the inability to vasodilate collateral arteries as main cause of impaired blood flow recovery in eNOS-/- mice.

This study demonstrates that eNOS activity is crucial for NO-mediated vasodilation of peripheral collateral vessels following arterial occlusion but not for collateral artery growth.

Introduction

The stimulation of vascular growth has become an important therapeutic goal for prevention and treatment of tissue ischemia in cardiovascular disease and is referred to as therapeutic neovascularization. Three distinct processes of vascular growth can contribute to the recovery of blood flow and preservation of tissue: arteriogenesis, i.e. collateral artery growth, angiogenesis, i.e. sprouting of capillaries from preexisting blood vessels, and vasculogenesis, i.e. formation of blood vessels from endothelial progenitors[1]. From a therapeutic point of view, it is essential to isolate the distinct mechanisms of vascular growth, because these occur in different types of tissue and vessels and are regulated by separate stimuli. For example, in the experimental ischemic hind limb model, arteriogenesis is initiated and stimulated in collateral vessels in the upper part of the hind limb by an increase in fluid shear stress[2]. In contrast, in the lower part of the limb, both angiogenesis and vasculogenesis are mainly driven by tissue ischemia[3,4]. Clinical trials based on stimulation of therapeutic VEGF- and FGF-mediated angiogenesis have not shown convincing results[5,6]. Therefore, in this study we focus on arteriogenesis, as this is the most upstream mechanism and the most efficient one to provide bulk flow to the ischemic area after occlusion or stenosis of a major artery[7].
A potential mechanism for inducing therapeutic neovascularization is to increase the production of endothelial NO. eNOS activity has been shown essential for neovascularization. eNOS-/- mice display a decreased neovascularization in response to severe ischemia. Recent studies have attributed this to impaired arteriogenesis, angiogenesis, vasculogenesis, or a combination of these[8-10]. However, in these studies the different competing or complementary mechanisms of vascular growth could not be isolated, because the use of a severe murine ischemia *in vivo* model, which causes substantial damage to the lower limb. Thus, the role of eNOS in each specific mechanism could not be elucidated. In addition, the role of eNOS in arteriogenesis has been paradoxical. Increased shear stress is known to upregulate the expression of eNOS augmenting endothelial NO production[11-13]. In contrast, NO has also been shown to inhibit expression of adhesion molecules and smooth muscle cell proliferation, which are both indispensable for arteriogenesis[11,14,15]. Besides, given the importance of eNOS in regulating vascular tonus and blood flow, other mechanisms could be involved. In this study, we investigated the role of eNOS in arteriogenesis in both eNOS-/- and eNOStg mice using a hind limb model, which caused only minimal ischemia in the lower limb, to specifically analyze arteriogenesis. Using this murine arteriogenesis-specific model several research groups have recently described different molecular and cellular mechanisms of arteriogenesis[16-18]. Two lines of eNOStg mice have been previously generated in our laboratory and overexpress the human eNOS gene[19,20]. We have recently reported that in the eNOStg mice eNOS expression is functional and restricted to the endothelial lining in all blood

vessels. In eNOStg mice eNOS protein and eNOS activity (20-fold greater) levels in the vasculature as well as NO-production (1.8-fold increased) are significantly enhanced, causing a lower blood pressure, lower plasma cholesterol levels and less atherosclerosis. In the present study, we first compared eNOStg and WT mice and only found beneficial effects of eNOS overexpression on blood flow recovery immediately following ligation and no further favorable effects on collateral artery growth. Subsequently, we analyzed arteriogenesis in eNOS-/- and WT control mice and confirmed previously published data showing impaired blood flow recovery and increased ischemic tissue damage in eNOS-/- mice. However, we found no differences in collateral growth between eNOS-/- and WT mice. Interestingly, we discovered that in eNOS-/- mice blood flow recovery and clinical outcome after distal femoral ligation were impaired by the inability to sufficiently vasodilate collateral peripheral vessels, and not because of impaired arteriogenesis.

Materials and Methods

Animal experiments

Transgenic mice overexpressing eNOS-GFP (eNOStg) were generated as previously described[20]. Briefly, an eNOS-GFP fusion gene was made by inserting a DNA fragment encoding the enhanced green fluorescent protein (GFP) in frame at the stop codon of the complete human eNOS gene and used to perform microinjections of fertilized mouse oocytes. Hemizygous eNOStg mice were used that expressed the human eNOS gene fused to GFP under the regulation of the autologous human eNOS promoter, and that were backcrossed to C57BL/6J background for >10 generations. For further details, see supplemental methods, available online at htttp://atvb.ahajournals.org. eNOS-deficient (eNOS-/-) and littermate wild-type C57Bl/6 mice (WT) were originally purchased from Jackson Laboratories (Bar Harbor, Maine). All mice used were age- (10 to 12 weeks) and sex-matched. The experimental protocol was approved by the Animal Experiments Committee under the national Experiments on Animals Act and adhered to the rules laid down in this national law that serves the implementation of "Guidelines on the protection of experimental animals " by the Council of Europe (1986), Directive 86/609/EC.

Mouse model of femoral ligation

The surgical procedure was performed as previously described[7]. Briefly, mice were anesthetized with a mixture of ketamin (20 mg/kg) and xylazin (110 mg/kg). After minimal incision in the right mid-thigh, the right superficial femoral artery was dissected and ligated just distally to the origin of the deep femoral artery.

Limb function and muscle atrophy

For a clinical evaluation of the function of the ischemic hind limb the active movement of the right foot was scored (1=no use; 2=standing; 3=normal use without spreading toes; 4=normal use), as previously described[21]. For evaluating the extent of atrophy the weights of the excised left and right m. gastrocnemius were determined.

Tissue perfusion

Relative hemoglobin oxygen saturation measurements were performed using an AbTisSpec spectrometer (LEA, Medizintecknik) placing the probe alternately on the left and right foot. Laser Doppler perfusion imaging (LDI; Moor Instruments Ltd.) was used for recording serial relative blood flow measurements. The whole region of the foot was analyzed on either side. All measurements were performed in a preheated chamber (37°C) after five minutes of warming and under the influence of a mixture of ketamin (15 mg/kg) and xylazin (82 mg/kg). Measurements are expressed as right-to-left ratios.

Collateral-dependent blood flow

Blood flow in the three main arteries of both m. gastrocnemius was analyzed by Magnetic Resonance Imaging (MRI), as previously described[22,23]. For further details, see supplemental methods. A subset of mice from WT and eNOS-/- groups was studied after intraperitoneal injection of 5mg/kg of SNAP (*S*-nitroso-*N*-acetyl-penicillamine; Sigma).

Histology

For evaluating capillary density, cryosections (7 µm) were cut from the m. gastrocnemius and stained with an antibody against lectin (BS-1, Sigma). Capillaries and muscle fibers were counted and data expressed as capillary-to-muscle fiber ratio. We used two different techniques for collateral artery morphometry. In one set of mice, for ultrastructure, collateral vessels in the m. adductor were isolated, dissected and embedded as previously described[7]. Ultra-thin sections (1 µm) were cut and stained with toluidine blue. Collateral arteries (ligated side) and preexisting arterioles (non-ligated side) were then measured with NIH software, and subsequently diameters, wall areas and wall thicknesses were calculated. In another set of mice, the complete m. adductor was dissected and embedded. Cryosections (7 µm) were cut and immunostained with an antibody against α-SM-actin (Sigma). In both ligated and non-ligated muscles arterioles were identified and measured as above. For further details, see online data supplement.

Statistics

Statistical analysis of all data was performed using one-way ANOVA followed by a multiple comparison test. Data are reported as means ± standard error of the mean (SEM). Statistical significance was accepted when $P<0.05$ (two-tailed).

Results

eNOS-overexpressing mice

Tissue perfusion

Hemoglobin oxygen saturation measurements revealed that oxygen saturation, a marker for tissue perfusion, was only significantly increased in eNOStg mice immediately after ligation, compared with WT (R/L postligation: 0.56±0.06 versus 0.24±0.05 in eNOStg and WT respectively, P<0.001, Figure 1A). Equally, LDI measurements of the feet only showed a significant increase in relative blood flow in eNOStg mice immediately after ligation, compared with WT (R/L postligation: 0.33±0.04 versus 0.13±0.02, P<0.01, Figure 1B)

Collateral-dependent blood flow

The inflow of blood in the calf arteries is directly dependent on collateral flow in the upper part of the leg and was measured by MRI. In eNOStg mice collateral-dependent blood flow was significantly increased only immediately following ligation, matching tissue perfusion findings in the foot (R/L postligation: 0.12±0.01 versus 0.05±0.01 in eNOStg and WT, respectively; P<0.001, Figure 1C). The above measurements, showing differences in blood flow recovery between eNOStg and WT mice only immediately after the femoral ligation, suggested an increased acute maximal vasodialation in eNOStg mice but no beneficial effects of eNOS overexpression on arteriogenesis.

eNOS-deficient mice

Limb function and muscle atrophy

Limb function, assessed by a foot movement score, recovered completely to normal within two weeks in WT mice, whereas limb function of eNOS-/- mice did not even reach normal levels during complete follow-up (Figure 2A). Similarly, toe necrosis and auto-amputation were commonly seen in the eNOS-/- group, in contrary to the mice in the other groups (data not shown). Also, significant more m. gastrocnemius atrophy was seen in the eNOS-/- mice compared with WT mice (R/L muscle weight: 0.94±0.02 versus 0.78±0.04 in WT and eNOS-/-, respectively; P<0.01, Figure 2B).

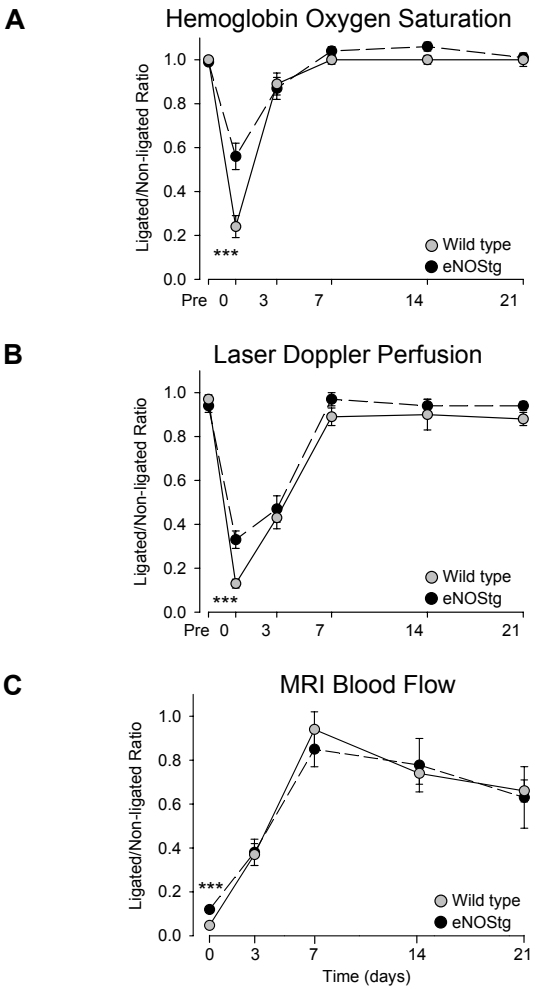

Figure 1. A. Time course of oxygen saturation in the feet (n≥10). B. Time course of tissue perfusion in the feet measured by Laser Doppler Imaging (n≥10). C. Time course of collateral-dependent blood flow in the calf muscle measured by MRI (n≥10). All measurements are expressed as ligated/non-ligated ratios, ***P<0.001 vs WT.

Tissue perfusion

Oxygen saturation remained significantly impaired in eNOS-/- mice during complete follow-up (Figure 2C). As expected, relative blood flow in the feet, assessed by LDI, remained equally impaired until three weeks after ligation (Figure 2D)

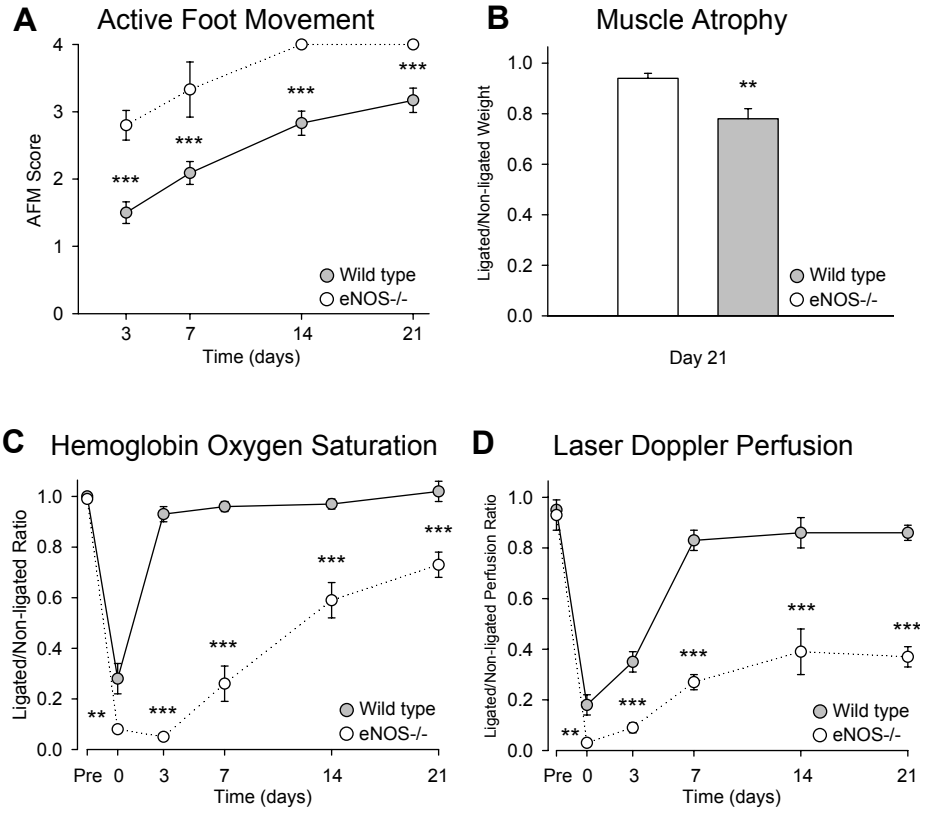

Figure 2. A. Limb function evaluated by active foot movement score (n≥6). B. M. gastrocnemius atrophy (n=8) C. Time course of oxygen saturation in the feet (n≥6). D. Time course of tissue perfusion in the feet measured by Laser Doppler Imaging (n≥6). All measurements are expressed as ligated/non-ligated ratios, ***P*<0.01 and ****P*<0.001 vs WT

Collateral-dependent blood flow

In contrast with tissue perfusion findings, a decrease in collateral-dependent blood flow was found only up to seven days after surgery in eNOS-/- mice, when compared with WT, suggesting normal or possibly somewhat delayed collateral artery growth in eNOS-/- mice (R/L blood flow at day 7: 1.02±0.07 versus 0.69±0.08 in WT and eNOS-/-, respectively; P<0.01; at day 14: 0.81±0.04 versus 0.83±0.08 in WT and eNOS-/-; Figure 3A). In eNOS-/- mice the blood flow even continued to increase and was significantly higher than in WT mice at three weeks. To circumvent the effects of vasoconstriction in eNOS-/- mice, we systemically administered at day 7 the NO-donor SNAP to both WT and eNOS-/- mice and measured collateral-dependent blood flow. In WT mice, SNAP induced vasodilation and thus an increase of blood flow in the unligated leg, whereas in the ligated leg no increase in blood flow could be induced, as vasodilation

was already at its maximal (or submaximal) level (Figure 3B). In eNOS-/- mice, however, SNAP induced vasodilation and increase of blood flow to the same extent in both legs. This suggested that normal functional collateral arteries had grown in the eNOS-/- mice, but that these were unable to vasodilate sufficiently because impaired NO production.

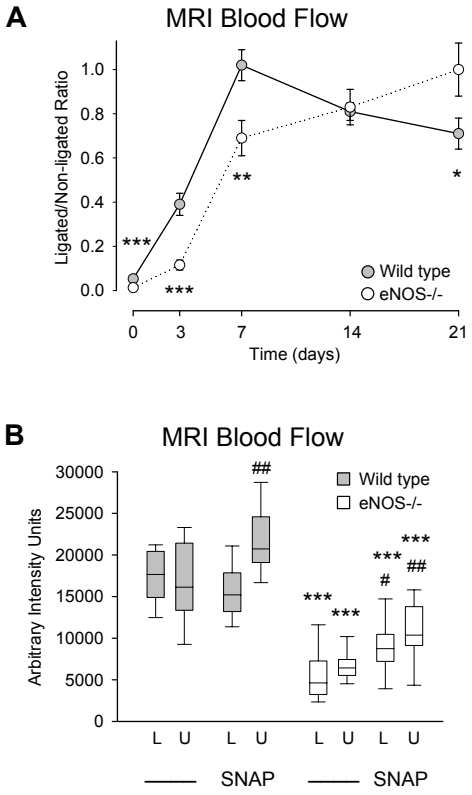

Figure 3. A. Time course of collateral-dependent blood flow in the calf muscle measured by MRI (n≥10). Measurements are expressed as ligated/non-ligated ratio, **P*<0.05, ***P*<0.01 and ****P*<0.001 vs WT. B. Collateral-dependent blood flow before and after administration of NO-donor SNAP seven days after femoral artery ligation (n≥13) Measurements are expressed as arbitrary units, $^{\#}P$<0.05 and $^{\#\#}P$<0.01 vs before SNAP, ****P*<0.001 vs WT.

Collateral morphometry

Finally, to obtain anatomic data of collateral arteries we studied collateral artery growth in the adductor muscle of the three groups of mice using two different histological analyses.

Cryosections

In complete cross-sections of the adductor muscle conducting arterioles were identified and analyzed (Figure 4A). Diameters of preexisting arterioles in the non-ligated adductor muscle did not differ between eNOStg, WT and eNOS-/- mice (in μm: 37±2 versus 33±3 versus 34±2 in eNOStg, WT and eNOS-/-, respectively; Figure 4B). Collateral diameters significantly and continuously increased after ligation, as compared with preexisting arterioles, and no differences between collateral diameters from eNOStg, WT and eNOS-/- mice were found at one and three weeks (in μm: 62±11 versus 76±11 versus 67±15 in eNOStg, WT and eNOS-/-, respectively). Wall areas of preexisting arterioles of eNOStg mice were significantly smaller than in WT mice (in μm^2: 557±51 versus 958±107 in eNOStg and WT, respectively; $P<0.01$, Figure 4C). In all three groups wall areas significantly and continuously enlarged after ligation. At one and three weeks after ligation no differences were found between the wall areas of collateral arteries from eNOStg, WT and eNOS-/- mice (in μm^2: 1098±195 versus 1610±240 versus 1598±321 in eNOStg, WT and eNOS-/-, respectively, at three weeks after ligation). Analysis of the wall thickness of collateral arteries revealed the same significant and continuous enlarging after ligation in all three groups of mice (Figure 4D). The wall thickness of collateral arteries in eNOStg mice was smaller than in WT mice, both before and after ligation. However, no differences in wall thickness were observed between eNOS-/- and WT mice, neither before nor after ligation (in μm: 8.4±0.5 versus 7.8±0.5 in WT and eNOS-/-, respectively, before ligation and 11.8±1.3 versus 10.7±0.8, in WT and eNOS-/-, at three weeks after ligation).

Ultra-thin sections

In ultra-thin sections, cut from the predilection area for collateral artery growth in the adductor muscle, preexisting arterioles or collateral arteries were identified and analyzed (Figure 5A). We did not find any significant differences in diameters and wall area between preexisting arterioles in eNOStg, WT and eNOS-/- mice (Figure 5B,C). Three weeks after ligation diameters and wall areas from collateral arteries were significantly enlarged in all three groups of mice, as compared to preexisting arterioles. However, as in cryosections, between groups no differences were found in collateral artery size. These findings confirmed the above blood flow data and indicate that collateral artery growth was normal in conditions of either deficient or increased eNOS activity.

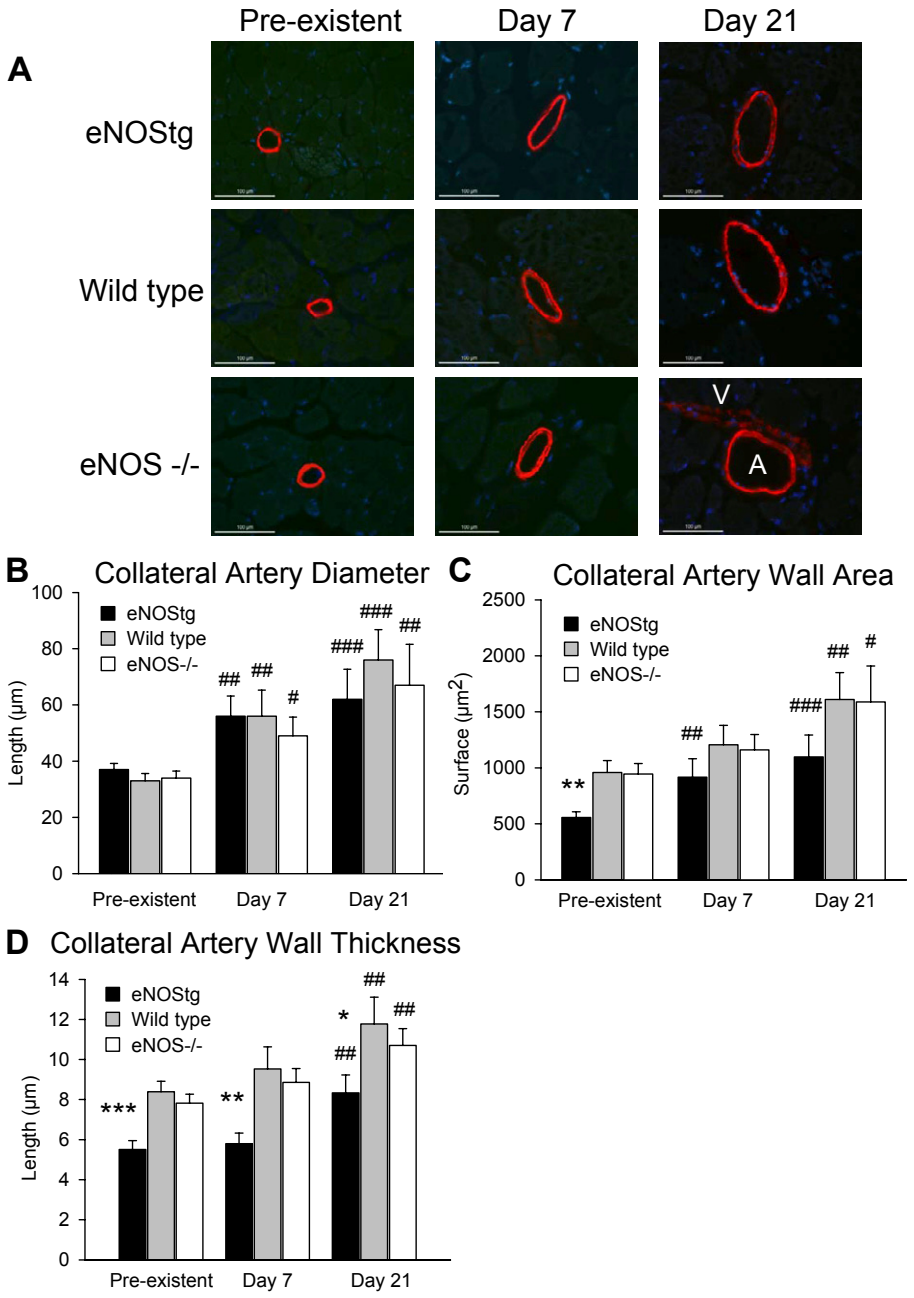

Figure 4. A. Representative photographs of cryosections with a collateral artery (A) in the adductor muscle of eNOStg, wild-type and eNOS-/- mice at different time points. Note the accompanying vein (V). Red=artery wall stained with anti-α-SMC actin, blue=nuclei stained with Dapi (magnification x400). B., C., and D. Morphometric measurements of diameter, wall area and wall thickness of collateral arteries and preexisting arterioles (n≥5). $^{\#}P<0.05$, $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$ vs preexistent, $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ vs WT.

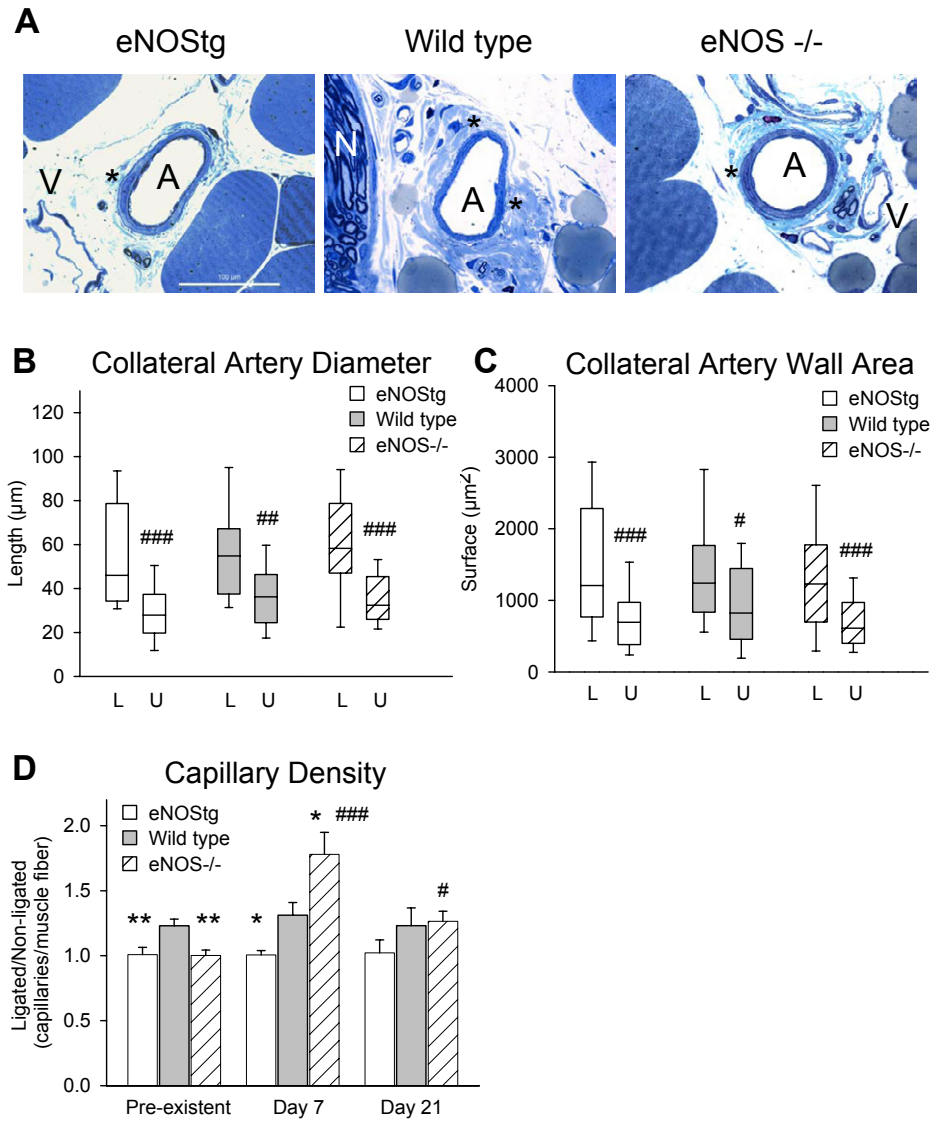

Figure 5. A. Representative photographs of ultra-thin sections with a collateral artery (A) in the adductor muscle of eNOStg, wild-type and eNOS-/- mice, 21 days after femoral artery ligation (magnification x630). Note the accompanying vein (V) and/or nerve (N). In all three collaterals at least one smooth-muscle cell nucleus (*) can be identified. B. and C. Morphometric measurements of diameter wall area of collateral arteries and preexisting arterioles (n≥5). $^{\#}P<0.05$, $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$ vs preexistent. D. Capillary density measurements in the calf muscle (n≥5). Measurements are expressed as capillary/fiber ratio, $^{\#}P<0.05$ and $^{\#\#\#}P<0.001$ vs preexistent, $^{*}P<0.05$ and $^{**}P<0.01$ vs WT.

Capillary density

Capillary density measurements in the calf muscle confirmed the arteriogenesis-specificity of our hind limb model. In unligated calf muscles of eNOStg and eNOS-/-

mice capillary density was decreased compared with WT mice (in capillary/fiber ratio: 1.01±0.06 versus 1.23±0.05 versus 1.00± 0.04, for eNOStg, WT and eNOS-/-, respectively; P<0.01; Figure 5D). After ligation the capillary/fiber ratio in calf muscles in both eNOStg and WT mice did not change, suggesting the absence of an ischemic stimulus for angiogenesis or vasculogenesis in these mice. In eNOS-/- mice, however, a significant 1.8-fold increase in capillaries was seen in the ligated leg, implying the presence of angiogenesis and tissue ischemia.

Discussion

In the present study we evaluated the role of eNOS in a hind limb model, specific for arteriogenesis. Firstly, to evaluate the possible benefits of elevated eNOS activity on collateral growth we compared transgenic mice overexpressing human eNOS with WT control mice. We only found a beneficial effect of overexpression of eNOS in the acute phase of blood flow recovery after femoral occlusion. At all later time points during follow up blood flow recovery was equal in eNOStg and WT mice. Histological analysis revealed no differences in collateral artery growth between the two groups of mice. In a previously published study performed with a different transgenic mouse overexpressing (bovine) eNOS beneficial effects of eNOS overexpression on blood flow recovery after arterial occlusion were found during complete follow-up[24]. In another study adenoviral eNOS-gene transfer in the ischemic hind limb of rats resulted in improved blood flow recovery and increase in capillary density[25]. However, in both studies a much more severe hind limb ischemia model was used, causing large ischemia and tissue damage in the lower limb and inducing both arteriogenesis in the upper limb, and angiogenesis and vasculogenesis in the lower limb. Therefore, beneficial effects of eNOS overexpression could not be extrapolated to one single type of vascular growth. Our findings suggest that the beneficial effects of eNOS overexpression on blood flow recovery in a severe hind limb ischemia model are not the result of increased arteriogenesis, but rather increased angiogenesis or vasculogenesis. Besides, the beneficial effect of eNOS overexpression in our study immediately after femoral occlusion suggests an important role for NO-mediated vasodilation in the initial phase of blood flow recovery, when vascular growth cannot have reached a substantial level yet.

Previous studies suggesting a positive role for eNOS in arteriogenesis have used different models, either the severe murine hind limb ischemia model[8] or an exercise based-model in rats[26]. However, in these studies arteriogenesis and angiogenesis were both present and not analyzed separately. The distal femoral artery ligation model used in our study is an *in vivo* model specific for collateral artery growth. In earlier studies of our group minimal hypoxia and ischemia where found in the lower leg[7,27]. Capillary density

data from our study also suggest the absence of an ischemic stimulus in the lower leg, because of no increase of capillary growth was seen after femoral occlusion in control animals. Therefore, our present study is the only study on eNOS and collateral artery growth using an *in vivo* model specific for this type of vascular growth based on acute increase of shear stress after arterial occlusion.

As we did not find beneficial effects of eNOS overexpression on arteriogenesis, we were interested to evaluate the effect of absence of eNOS activity on arteriogenesis and thus compared eNOS-/- and WT mice using the same hind limb model and measurements. In earlier studies using eNOS-/- mice and the severe hind limb ischemia model it was already demonstrated that eNOS-/- mice had a very poor blood flow recovery, suggested to be caused by impaired angiogenesis or vasculogenesis[9,10]. Equally, in our study distal femoral artery ligation caused a significantly reduced blood flow recovery as well as tissue damage in eNOS-/- mice, despite our observations of an increase in angiogenesis and normal arteriogenesis in eNOS-/- mice compared with WT. We demonstrated that the impaired blood flow recovery was not caused by impaired arteriogenesis, but by insufficient vasodilation in the early recovery phase, before collateral arteries were completely formed.

Using MRI, a relatively new technique which measures absolute (and largely deep muscular) blood flow and is less influenced by changes in skin blood flow as LDI, we found in eNOS-/- mice a decreased collateral-dependent blood flow only in the first week after ligation. In eNOS-/- mice, collateral-dependent blood flow continued to increase during three weeks of follow up, while in WT mice blood flow remained the same or was slightly decreased after two weeks. We suspect that after two weeks vasodilation is reduced in WT mice as growing collaterals adequately provide bulk flow to the distal leg, resulting in equal (or slightly decreased) net collateral blood flow after three weeks. In the eNOS-/- mice however, in the absence of vasodilation collateral blood flow continues to increase as collaterals continue to enlarge. Consequently, administration of the NO-donor SNAP confirmed the insufficient vasodilation of collateral arteries in eNOS-/- mice due to the absence of eNOS activity.

To obtain conclusive anatomic data of collateral arteries we extensively studied collateral artery growth in the adductor muscle after maximal vasodilation using two different histological analyses. Cryosections were immunohistochemically stained and used to study collateral artery growth in the general overview of the whole adductor muscle. For ultra-thin sections only the predilection area for collateral artery growth was isolated. This isolation procedure and the thinness of these sections permitted us to analyze a smaller number of vessels in more detail. Both histological analyses revealed normal collateral artery growth in eNOS-/- mice after one and three weeks, suggesting a crucial role for vascular tonus in the early phase after femoral ligation. Finally, the tissue damage in the lower limbs of eNOS-/- mice appeared irreversible,

because at three weeks of follow up muscle weights were decreased, tissue perfusion of the feet remained impaired, and collateral-dependent blood flow was still increasing. This irreversibility stresses the essential role of NO-mediated vasodilation.

Our findings confirm that eNOS activity is essential for an effective restoration of blood flow after femoral artery occlusion, however not by stimulation of arteriogenesis but by inducing NO-dependent vasodilation of the collateral-dependent peripheral vessels. Because arteriogenesis itself is not an immediate process and thus cannot protect tissue from ischemic injury in the acute phase, the combination of both adequate vasodilation (of peripheral collateral vessels) in the acute phase and vascular (ie, collateral artery) growth for continuation is necessary for successful blood flow recovery and tissue salvage.

Clinical trials aiming for therapeutic neovascularization by stimulating vascular growth using different growth factors have shown disappointing results[5,6]. These disappointing results might be due to the preexistent endothelial dysfunction in patients with atherosclerosis or diabetes. Endothelial dysfunction causes decreased vasoreactivity as a result of a reduced bioavailability of NO. Therefore, there might be (partial) analogies between these patients and eNOS-/- mice, in which arteriogenesis is not impaired but the inability to sufficiently vasodilate resulted in severe ischemic damage. However, similar to the approach to stimulate vascular growth only, stimulation of vasodilation only might not be sufficient to adequately restore blood flow distally from the occlusion in a clinical situation of chronic ischemia in the lower limb. A combination of stimulation of vascular growth and vasodilation could be essential for successful therapeutic neovascularization, specifically in conditions where endothelial dysfunction is present (ie, hypercholesterolemia and diabetes). We are currently investigating this hypothesis using both diabetic and hypercholesterolemic mice.

In summary, eNOS overexpression did not have beneficial effects for collateral artery growth. eNOS deficiency caused severe impaired blood flow recovery after arterial occlusion caused by insufficient vasodilation of collateral-dependent vessels, whereas collateral artery growth was intact. Therefore, eNOS activity is essential for an effective restoration of blood flow after femoral artery occlusion, however not by stimulation of arteriogenesis but by inducing NO-dependent vasodilation of the collateral-dependent peripheral vessels.

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References

1. Carmeliet, P. Angiogenesis in health and disease. *Nat Med* 9, 653-660 (2003).
2. Heil, M. & Schaper, W. Influence of mechanical, cellular, and molecular factors on collateral artery growth (arteriogenesis). *Circ Res* 95, 449-458 (2004).
3. Ito, W.D., Arras, M., Scholz, D., Winkler, B., Htun, P. & Schaper, W. Angiogenesis but not collateral growth is associated with ischemia after femoral artery occlusion. *Am J Physiol* 273, H1255-1265 (1997).
4. Urbich, C. & Dimmeler, S. Endothelial progenitor cells: characterization and role in vascular biology. *Circ Res* 95, 343-353 (2004).
5. Henry, T.D., Annex, B.H., McKendall, G.R., Azrin, M.A., Lopez, J.J., Giordano, F.J., Shah, P.K., Willerson, J.T., Benza, R.L., Berman, D.S., Gibson, C.M., Bajamonde, A., Rundle, A.C., Fine, J. & McCluskey, E.R. The VIVA trial: Vascular endothelial growth factor in Ischemia for Vascular Angiogenesis. *Circulation* 107, 1359-1365 (2003).
6. Simons, M., Annex, B.H., Laham, R.J., Kleiman, N., Henry, T., Dauerman, H., Udelson, J.E., Gervino, E.V., Pike, M., Whitehouse, M.J., Moon, T. & Chronos, N.A. Pharmacological treatment of coronary artery disease with recombinant fibroblast growth factor-2: double-blind, randomized, controlled clinical trial. *Circulation* 105, 788-793 (2002).
7. Scholz, D., Ziegelhoeffer, T., Helisch, A., Wagner, S., Friedrich, C., Podzuweit, T. & Schaper, W. Contribution of arteriogenesis and angiogenesis to postocclusive hindlimb perfusion in mice. *J Mol Cell Cardiol* 34, 775-787 (2002).
8. Yu, J., Demuinck, E.D., Zhuang, Z., Drinane, M., Kauser, K., Rubanyi, G.M., Qian, H.S., Murata, T., Escalante, B. & Sessa, W.C. Endothelial nitric oxide synthase is critical for ischemic remodeling, mural cell recruitment, and blood flow reserve. *Proc Natl Acad Sci U S A* (2005).
9. Murohara, T., Asahara, T., Silver, M., Bauters, C., Masuda, H., Kalka, C., Kearney, M., Chen, D., Symes, J.F., Fishman, M.C., Huang, P.L. & Isner, J.M. Nitric oxide synthase modulates angiogenesis in response to tissue ischemia. *J Clin Invest* 101, 2567-2578 (1998).
10. Aicher, A., Heeschen, C., Mildner-Rihm, C., Urbich, C., Ihling, C., Technau-Ihling, K., Zeiher, A.M. & Dimmeler, S. Essential role of endothelial nitric oxide synthase for mobilization of stem and progenitor cells. *Nat Med* 9, 1370-1376 (2003).
11. Ignarro, L.J., Napoli, C. & Loscalzo, J. Nitric oxide donors and cardiovascular agents modulating the bioactivity of nitric oxide: an overview. *Circ Res* 90, 21-28 (2002).
12. Topper, J.N., Cai, J., Falb, D. & Gimbrone, M.A., Jr. Identification of vascular endothelial genes differentially responsive to fluid mechanical stimuli: cyclooxygenase-2, manganese superoxide dismutase, and endothelial cell nitric oxide synthase are selectively up-regulated by steady laminar shear stress. *Proc Natl Acad Sci U S A* 93, 10417-10422 (1996).
13. Fleming, I., Fisslthaler, B., Dixit, M. & Busse, R. Role of PECAM-1 in the shear-stress-induced activation of Akt and the endothelial nitric oxide synthase (eNOS) in endothelial cells. *J Cell Sci* 118, 4103-4111 (2005).
14. Schaper, J., Konig, R., Franz, D. & Schaper, W. The endothelial surface of growing coronary collateral arteries. Intimal margination and diapedesis of monocytes. A combined SEM and TEM study. *Virchows Arch A Pathol Anat Histol* 370, 193-205 (1976).
15. Schmidt, H.H. & Walter, U. NO at work. *Cell* 78, 919-925 (1994).
16. Heil, M., Ziegelhoeffer, T., Pipp, F., Kostin, S., Martin, S., Clauss, M. & Schaper, W. Blood monocyte concentration is critical for enhancement of collateral artery growth. *Am J Physiol Heart Circ Physiol* 283, H2411-2419 (2002).

17. Hoefer, I.E., van Royen, N., Rectenwald, J.E., Bray, E.J., Abouhamze, Z., Moldawer, L.L., Voskuil, M., Piek, J.J., Buschmann, I.R. & Ozaki, C.K. Direct evidence for tumor necrosis factor-alpha signaling in arteriogenesis. *Circulation* 105, 1639-1641 (2002).
18. Kinnaird, T., Stabile, E., Burnett, M.S., Lee, C.W., Barr, S., Fuchs, S. & Epstein, S.E. Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote in vitro and in vivo arteriogenesis through paracrine mechanisms. *Circ Res* 94, 678-685 (2004).
19. van Haperen, R., de Waard, M., van Deel, E., Mees, B., Kutryk, M., van Aken, T., Hamming, J., Grosveld, F., Duncker, D.J. & de Crom, R. Reduction of blood pressure, plasma cholesterol, and atherosclerosis by elevated endothelial nitric oxide. *J Biol Chem* 277, 48803-48807 (2002).
20. van Haperen, R., Cheng, C., Mees, B.M., van Deel, E., de Waard, M., van Damme, L.C., van Gent, T., van Aken, T., Krams, R., Duncker, D.J. & de Crom, R. Functional expression of endothelial nitric oxide synthase fused to green fluorescent protein in transgenic mice. *Am J Pathol* 163, 1677-1686 (2003).
21. Heil, M., Ziegelhoeffer, T., Wagner, S., Fernandez, B., Helisch, A., Martin, S., Tribulova, S., Kuziel, W.A., Bachmann, G. & Schaper, W. Collateral artery growth (arteriogenesis) after experimental arterial occlusion is impaired in mice lacking CC-chemokine receptor-2. *Circ Res* 94, 671-677 (2004).
22. Wagner, S., Helisch, A., Ziegelhoeffer, T., Bachmann, G. & Schaper, W. Magnetic resonance angiography of collateral vessels in a murine femoral artery ligation model. *NMR Biomed* 17, 21-27 (2004).
23. Wagner, S., Helisch, A., Bachmann, G. & Schaper, W. Time-of-flight quantitative measurements of blood flow in mouse hindlimbs. *J Magn Reson Imaging* 19, 468-474 (2004).
24. Amano, K., Matsubara, H., Iba, O., Okigaki, M., Fujiyama, S., Imada, T., Kojima, H., Nozawa, Y., Kawashima, S., Yokoyama, M. & Iwasaka, T. Enhancement of ischemia-induced angiogenesis by eNOS overexpression. *Hypertension* 41, 156-162 (2003).
25. Smith, R.S., Jr., Lin, K.F., Agata, J., Chao, L. & Chao, J. Human endothelial nitric oxide synthase gene delivery promotes angiogenesis in a rat model of hindlimb ischemia. *Arterioscler Thromb Vasc Biol* 22, 1279-1285 (2002).
26. Lloyd, P.G., Yang, H.T. & Terjung, R.L. Arteriogenesis and angiogenesis in rat ischemic hindlimb: role of nitric oxide. *Am J Physiol Heart Circ Physiol* 281, H2528-2538 (2001).
27. Helisch, A., Wagner, S., Khan, N., Drinane, M., Wolfram, S., Heil, M., Ziegelhoeffer, T., Brandt, U., Pearlman, J.D., Swartz, H.M. & Schaper, W. Impact of mouse strain differences in innate hindlimb collateral vasculature. *Arterioscler Thromb Vasc Biol* 26, 520-526 (2006).

Chapter 5

eNOS and Adhesion

High Pressure Promotes Monocyte Adhesion to the Vascular Wall

Stéphanie Riou[1], Barend Mees[2,3], Bruno Esposito[1], Régine Merval[1], Jose Vilar[1], Dominique Stengel[4], Ewa Ninio[4], Rien van Haperen[3], Rini de Crom[2,3], Alain Tedgui[1], Stéphanie Lehoux[1]

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[1]INSERM U689; Centre de Recherche Cardiovasculaire Inserm Lariboisière; Paris, France
[2]Department of Vascular Surgery, and [3]Cell Biology & Genetics, Erasmus University Medical Center, Rotterdam, The Netherlands
[4]INSERM U525; Université Pierre et Marie Curie (UPMC-Paris 6); Faculté de Médecine Pierre et Marie Curie; Paris, France

Abstract

Hypertension is a known risk factor for the development of atherosclerosis. To assess how mechanical factors contribute to this process, mouse carotid arteries were maintained in organ culture at normal (80 mmHg) or high (150 mmHg) intraluminal pressure during 1, 6, 12 or 24h. Thereafter, fluorescent human monocytic cells (U937) were injected intraluminally and allowed to adhere for 30 minutes before washout. U937 adhesion was increased in vessels kept at 150 mmHg 12 hours (23.5±5.7 versus 9.9±2.2 cells/mm at 80 mmHg; $p<0.05$) or 24 hours (26.7±5.7 versus 8.8±1.5 cells/mm; $p<0.05$). At 24 hours, high pressure was associated with increased mRNA expression of monocyte chemoattractant protein-1, interleukin-6, keratinocyte-derived chemokine, and vascular cell adhesion molecule-1 (6.9±2.1, 4.4±0.1, 9.8±2.8 and 2.4±0.1-fold respectively; $p<0.05$), as assessed by quantitative RT-PCR and corroborated by immunohistochemistry, which also revealed an increase in intracellular adhesion molecule-1 expression. Nuclear factor κB inhibition using SN50 peptide abolished the overexpression of chemokines and adhesion molecules and reduced U937 adhesion in vessels at 150 mmHg. Moreover, treatment of vessels and cells with specific neutralizing antibodies established that monocyte chemoattractant protein-1, interleukin-6 and keratinocyte-derived chemokine released from vessels at 150 mmHg primed the monocytes, increasing their adhesion to vascular cell adhesion molecule-1 but not intracellular adhesion molecule-1 via $\alpha_4\beta_1$ integrins. The additive effect of chemokines on the adhesion of U937 cells to vascular cell adhesion molecule-1 was confirmed by *in vitro* assay. Finally, pressure-dependent U937 adhesion was blunted in arteries from mice overexpressing endothelial NO synthase. Hence, high intraluminal pressure induces cytokine and adhesion molecule expression via Nuclear factor κB, leading to monocytic cell adhesion. These results indicate that hypertension may directly contribute to the development of atherosclerosis through Nuclear factor κB induction.

Introduction

Atherosclerosis is an inflammatory disease characterized by an accumulation of leukocytes, lipids and fibrous tissue in the intima of arteries. In the early phases of atherosclerotic plaque development, activated endothelial cells express elevated amounts of adhesion molecules such as selectins (P-selectin and E-selectin) and intracellular (ICAM-1), vascular (VCAM-1) and platelet endothelial cell adhesion molecules (PECAM-1) at their surface. Cytokines and chemokines are also secreted in excess by activated vascular cells. These conditions favor the recruitment and the accumulation of monocytes and lymphocytes in the intima of vessels[1].

It is well known that the zones of the vascular tree where blood flow is disturbed or oscillatory are predisposed to the formation of atherosclerotic lesions, whereas vessels exposed to laminar shear stress remain relatively plaque-free[2], mostly credited to local release of nitric oxide (NO)[3]. However, blood pressure also influences plaque formation, arterial hypertension being an independent risk factor for atherosclerosis[4]. In hypertensive patients, high concentrations of circulating ICAM-1, VCAM-1 and E-selectin[5], as well as monocyte chemoattractive protein (MCP-1)[6] have been reported. Experimental animal models have also linked hypertension with cytokine and adhesion molecule expression, as well as the propensity for atherosclerotic plaque development. The expression of MCP-1 is more important in the aorta of hypertensive SHR rats than that of control Wistar rats[7]. Moreover, chronic hypertension due to endothelial NO synthase (eNOS) deficiency[8] or induced by clamping renal arteries[9] or by aortic constriction[10] exacerbates atherosclerosis in ApoE$^{-/-}$ mice. However, the direct role of arterial pressure in the development of atherosclerotic plaques has not yet been clearly demonstrated.

A recent study carried out in our laboratory showed that stretch of the arterial wall caused by an increase in the intraluminal pressure induces the activation and nuclear translocation of the transcriptional factor nuclear factor κB (NF-κB)[11]. This factor intervenes in the transcription of a large number of inflammatory genes coding for cytokines, chemokines and adhesion molecules[12]. Consequently, we hypothesized that high arterial pressure could contribute to the development of atherosclerotic lesions directly by inducing monocyte adhesion via NF-κB. Using an *in vitro* organ culture model of whole vessel, we assessed monocyte adhesion to the vascular endothelium of arteries kept at normal or high pressure. We also evaluated which adhesion molecules and chemokines might be implicated in this process, and verified their induction by NF-κB.

Material and methods

Organ culture

Mouse left and right carotid arteries were isolated, cannulated at both extremities, and immersed in an organ culture bath filled with Dulbecco's modified Eagle's medium (DMEM, GIBCO BRL) supplemented with 5% fetal calf serum as described previously[11]. Each arterial segment was connected to a closed perfusion circuit consisting of a 3-port reservoir, a peristaltic pump (Alitea), and a pressure chamber allowing for the application of a controlled intraluminal hydrostatic pressure. Organ culture of carotid segments was carried out under sterile conditions in an incubator containing 5% CO_2 at 37°C. The flow was set at 1.38 mL/min, allowing for renewal of the medium within the intraluminal space while creating minimal shear forces (0.5 dyne/cm^2). Likewise, to avoid the potentially confounding effect of cyclic stretch vessels were exposed to steady, continuous stretch, although we have previously shown that NF-κB is not induced in pulsatile vessels[13]. Arterial segments were kept at an intraluminal pressure of 80 mmHg for 1 hour stabilization after surgery. Thereafter, vessels were exposed to a pressure of 80 mmHg or 150 mmHg for 1, 6, 12 or 24 hours. High pressure imposed a stretch corresponding to a 20±3% increase in diameter. More details appear in the online data supplement, at http://circres.ahajournals.org.

Most experiments were undertaken using vessels from C57BL/6 mice. However, arteries from mice overexpressing (eNOS-tg) and underexpressing (eNOS$^{-/-}$) eNOS described previously[14], or their wild-type littermates were also used where indicated. In one set of experiments, vessels maintained for 24 hours at 80 mmHg were treated with lipopolysaccharide (LPS) (10 µg/mL). Some arteries kept at 80 or 150 mmHg for 24 hours were incubated with the NF-κB inhibitor peptide SN50 (AAVALLPAVLLALLAP-VQRKRQKLMP, 50 µg/mL; Upstate Biotechnology), with the pharmacological inhibitor of NF-κB ammonium pyrrolidine dithiocarbamate (PDTC, 10 µM; Sigma), or with the eNOS inhibitor N-nitro-L-arginine methyl ester (L-NAME, 10 µM, Sigma) added to the culture medium at the onset of the equilibration period.

Fluorescent cell preparation

Cells of the human monocytic cell line U937 were cultivated in RPMI medium 1640 (GIBCO BRL) containing penicillin (100 UI/L) and supplemented with 5% fetal calf serum (Boehringer-Mannheim). U937 cells were labeled with 0.5 µM fluorescent dye (CellTracker Orange CMTMR; Molecular Probes). Briefly, the cells were incubated with the fluorescent dye for 30 minutes and then resuspended in culture medium.

In order to circumvent a potential direct effect of pressure on U937 adhesion, the intraluminal pressure of all vessels was reset to 80 mmHg 30 minutes before intraluminal cell injection. The fluorescent U937 cells were injected in the lumen of cultured

vessels by the distal end and allowed to interact 30 minutes ($5x10^6$ cells/mL). After a 10-minutes washout at low-flow shear stress (0.5 dyne/cm^2), vessels were fixed in 4% paraformaldehyde for 15 minutes. Adherent cells were counted under a fluorescence microscope. In some experiments, blocking antibodies targeting VCAM-1 (25µg/mL; AF643), ICAM-1 (5µg/mL; AF796), MCP-1 (100µg/mL; AB479), IL-6 (1µg/mL; AF406) or KC (10µg/mL; AF453) (R&D Systems) were added to the intraluminal compartment 30 minutes before U937 injection. Alternatively, U937 cells were incubated with a blocking anti-α_4 (5µg/mL, BBA37 R&D Systems) or anti-β_1 integrin antibody (5µg/mL, 553715 BD PharMingen), or with a non-blocking anti-α_4 antibody (5µg/mL, 9C10 Research Diagnostics), before being injected in vessels.

Immunohistochemical analysis and quantitative RT-PCR

Details regarding immunohistochemical analysis and quantitative RT-PCR appear in the online supplement, at http://circres.ahajournals.org. Primary goat polyclonal antibodies used for immunohistochemistry and targeting VCAM-1, ICAM-1, E-selectin, MCP-1, IL-6, and KC, were obtained from Santa Cruz Biotechnology. All primers were designed using Primer Express 2 Software and are reported in Table 1.

Table 1. Forward (F) and reverse (R) primers used for the quantitative analysis of adhesion molecule and chemokine expression.

mouse VCAM-1-F	AAC CAG AAA AGT TCT GCT TGA CAA GT
mouse VCAM-1-R	ATT AAG TTA CAA CAG TCA GTC CAA GCA A
mouse E selectin-F	CAA GGC CAG CCC TCT ACC A
mouse E selectin-R	AAA GCA ATA AAA CGC TGT TTC TGT T
mouse ICAM-1-F	CCC CCC GAC ACA GGA AAG
mouse ICAM-1-R	TCC CCA GAC TCT CAC AGC ATC T
mouse MCP-1-F	GCA GCA GGT GTC CCA AAGAA
mouse MCP-1-R	ATT TAC GGG TCA ACT TCA CAT TCA A
mouse IL6-F	ATT ACA CAT GTT CTC TGG GAA ATC GT
mouse IL6-R	TAT ATC CAG TTT GGT AGC ATC CAT CA
mouse KC-F	CCA CCC GCT CGC TTC TCT GTG
mouse KC-R	CCG TTA CTT GGG GAC ACC TTT TAG CA

Cell adhesion assay

Cell culture plates were coated with recombinant mouse VCAM-1/Fc chimera (2µg/mL R&D Systems) at 4°C overnight. After washout, saturation with a 2% bovine serum albumin was performed for 1 hour. Fluorescent U937 cells (10^3/well) were made to adhere untreated or in presence of recombinant mouse IL-6 (0.06 ng/mL), MCP-1 (10 ng/mL) and/or KC (5 ng/mL R&D Systems) for 1 to 30 minutes. In another set of experiments, fluorescent U937 were made to adhere for 30 minutes in the presence

of a blocking anti-α_4 (5µg/mL, R&D Systems) or anti-β_1 integrin antibody (5 µg/mL, PharMingen). After washout, adherent U937 cells were counted under a fluorescence microscope.

Statistics

Data are presented as mean±SEM. Data were analyzed by ANOVA, and when results were found to be significant comparisons were performed by Bonferroni test or Student's paired t-test (to compare arteries from the same animal). Statistical significance was accepted for $P<0.05$.

Results

Pressure induces monocytic cell adhesion via NF-κB

The effects of high intraluminal pressure on monocytic cell adhesion were first assessed in carotid arteries maintained in culture for 1, 6, 12 or 24 hours at 80 or 150 mmHg (figure 1A). The number of adherent U937 cells did not vary significantly between vessels maintained at 80 or 150 mmHg for 1 (2.15±1.32-fold increase in cell adhesion at 150 mmHg) or 6 hours (2.19±0.66-fold). However, high intraluminal pressure led to a significant increase in U937 adhesion when maintained for either 12 hours (5.90±2.34-fold $P<0.05$) or 24 hours (6.01±2.15-fold; $P<0.05$). High intraluminal pressure likewise enhanced primary mouse mononuclear cell adherence at 24 hours (online figure 1). Thus, maintenance of vessels at high intraluminal pressure for at least 12 hours favors monocytic cell adhesion.

We have previously shown that high pressure induces NF-κB in cultured arteries[11,15]. To evaluate the role of NF-κB in pressure-dependent U937 binding to the vascular wall, some carotid arteries were treated for 24 hours with SN50, an inhibitor that binds the nuclear localization sequence on NF-κB and prevents its translocation to the nucleus. We confirmed that phosphorylation of the p65 subunit of NF-κB and its nuclear translocation was significantly more important in vessels kept at 150 mmHg for 24 hours than in arteries at 80 mmHg, and that SN50 prevented these effects. Baseline monocytic cell adhesion was not significantly affected by the NF-κB inhibitor treatment in vessels kept at 80 mmHg. However, in vessels at high pressure SN50 treatment prevented the increase U937 adhesion (4.4±1.5 cells/mm at 80 mmHg versus 8.2±3.2 cells/mm at 150 mmHg) (figure 1A). Similar results were obtained with the NF-κB inhibitor PDTC (data not shown). Therefore, we concluded that high pressure induces monocytic cell adhesion via activation of the NF-κB pathway.

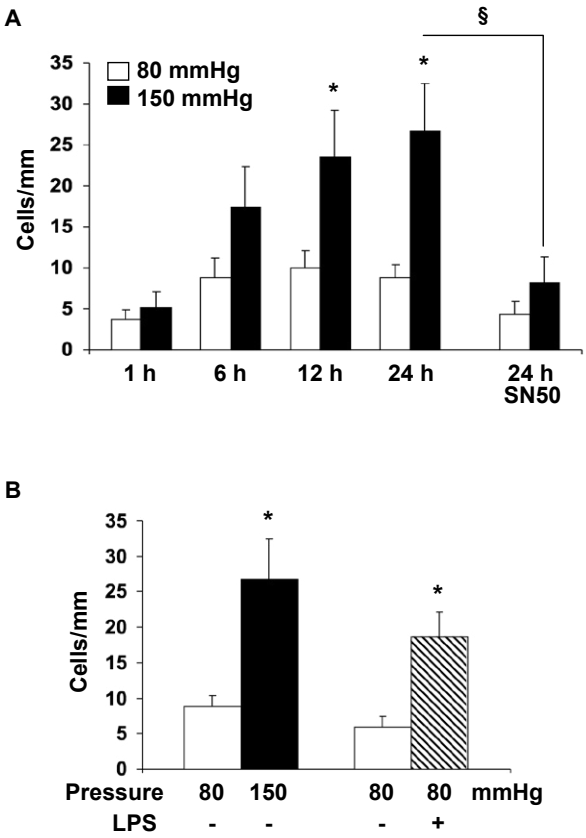

Figure 1. High intraluminal pressure leads to an increase in monocytic cell adhesion via NF-κB. A: Vessels were incubated at 80 or 150 mmHg for 1, 6, 12 or 24 hours. Fluorescent U937 cells were then injected in the intraluminal space for 30 minutes, and adherent cells were counted after washout. Monocytic cell adhesion was enhanced in vessels exposed to high pressure for 12 and 24 hours. However, in vessels cultured with a peptide inhibitor of NF-κB, SN50 (50 µg/mL), high pressure failed to induce monocyte adhesion. B: Monocytic cell adhesion in arteries incubated for 24 hours at high pressure is equivalent to that attributable to LPS (10 µg/mL). Data are mean±SEM of n=6 to 10 experiments. *P<0.05 vs 80 mmHg at the same time point, untreated. §P<0.05 vs 150 mmHg untreated at 24h.

Pressure-dependent monocytic cell adhesion is equivalent to LPS stimulation

To compare the effects of the stretch stimulus to a more traditional inflammatory mediator, vessels were cultured for 24 hours at 80 mmHg and treated or not with LPS, a known activator of endothelial cells[16,17]. We found that the number of adherent U937 cells in arteries incubated with LPS (18.7±3.5 cells/mm) was almost three-fold more important than that in arteries cultured without LPS (5.9±1.6 cells/mm, P<0.05, figure 1B), equivalent to that induced by high pressure.

Pressure-dependent expression of adhesion molecules and chemokines

We then studied the expression of adhesion molecules and chemokines potentially involved in regulating monocytic cell adhesion to the vascular wall. Quantitative RT-PCR of genes encoding VCAM-1, ICAM-1, E-selectin, MCP-1, IL-6 and KC was undertaken in vessels maintained for 24 hours at normal or high intraluminal pressure (figure 2). On the one hand, the expression of ICAM-1 and E-selectin was not different in vessels maintained at 80 or 150 mmHg. On the other hand, the expression of VCAM-1, MCP-1, KC and IL-6 was respectively 2.0±0.1, 8.1±2.0, 9.8±2.8 and 5.8±0.1 fold greater at 150 mmHg than at 80 mmHg (P<0.05).

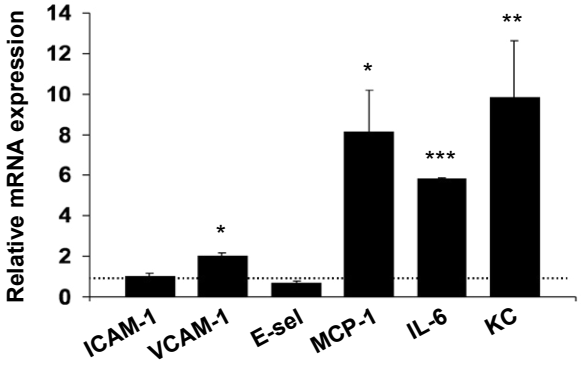

Figure 2. Quantitative RT-PCR carried out on vessels incubated during 24 hours reveals that the expression of VCAM-1, MCP-1, IL-6, and KC is upregulated in vessels incubated at 150 mmHg. The dotted line represents the expression of adhesion molecules and chemokines at 80 mmHg (100%). Data are mean±SEM of n=4 experiments evaluated in duplicate. *P<0.05, **P<0.01, ***P<0.001 vs 80 mmHg.

To verify whether pressure-dependent changes in mRNA expression corresponded to modified protein content, immunohistological staining for cytokines and adhesion molecules was evaluated. High pressure did not alter the expression of E-selectin in vessels (figure 3A), whereas staining for ICAM-1 and VCAM-1 was significantly enhanced in vessels incubated for 24 hours at 150 mmHg compared with arteries kept at 80 mmHg (figure 3A). Quantification of relative vessel wall surface staining revealed greater expression of ICAM-1 in the endothelium (6.9±0.9% versus 1.8±0.5%, P<0.01) and more VCAM-1 in the endothelium and the media (25.8±3.5% versus 9.8±1.6%, P<0.001) of arteries at 150 mmHg compared with 80 mmHg (figure 3B). The expression of MCP-1, IL-6, and KC was also increased in the endothelium and the media of vessels maintained for 24 hours at 150 mmHg versus 80 mmHg (17.4±1.1% versus 10.8±0.9%, P<0.001; 18.3±1.9% versus 6.8±1.5%, P<0.05; and 15.9±1.6% versus 8.4±3.3%, P<0.05 respectively, figure 3A and B).

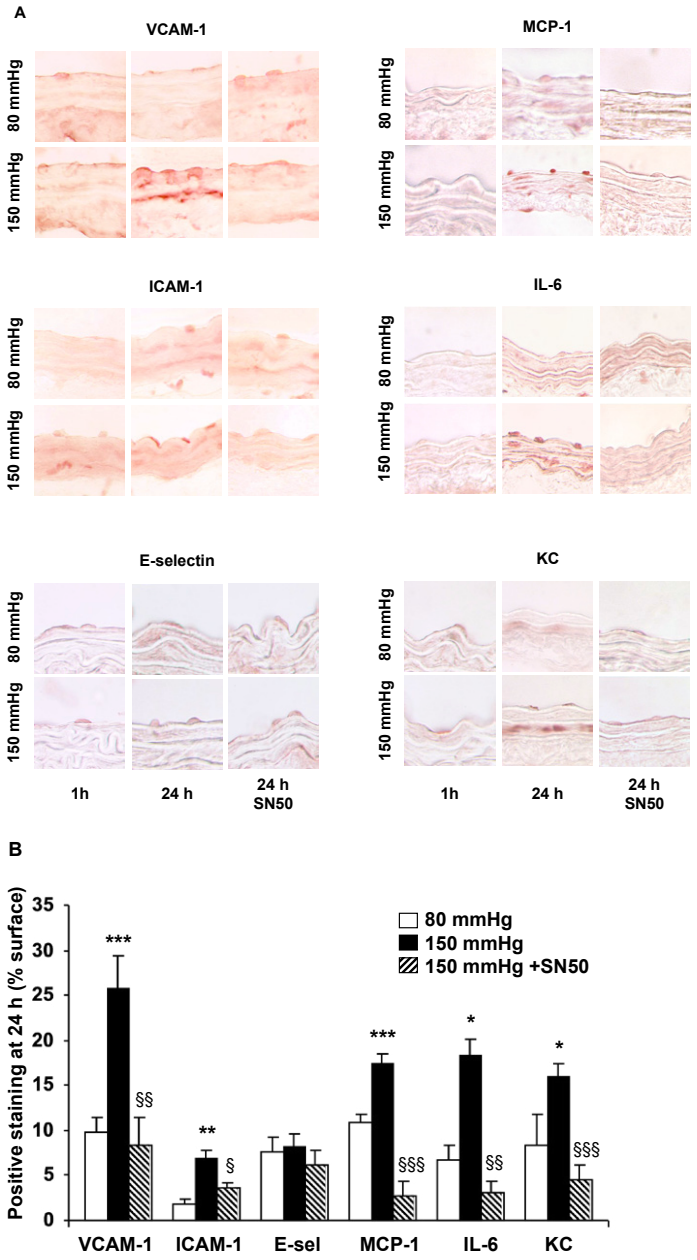

Figure 3. A: IHC studies demonstrate that high intraluminal pressure leads to an increase in the expression of VCAM-1, MCP-1 and IL-6 and KC throughout the vascular wall at 24 hours, whereas ICAM-1 expression is enhanced only in the endothelium and E-selectin levels remain unchanged. The NF-κB inhibitor SN50 prevents pressure-dependent overexpression of these proteins. Representative of 4 separate experiments. Surface staining for these molecules at 24 hours is quantified in B. Data are mean±SEM of n=4. *P<0.05, **P<0.01, ***P<0.001 vs 80 mmHg, §P<0.05, §§P<0.01, §§§P<0.001 vs 150 mmHg untreated at 24 hours.

In all cases, incubation of vessels with the inhibitor of NF-κB reduced the intensity of immunostains such that protein expression of the cytokines and adhesion molecules no longer differed between 80 and 150 mmHg (figure 3A and B). These immunohistologic studies indicated that high intraluminal pressure induces an increase in the vascular expression of adhesion molecules and chemokines via the NF-κB pathway.

Adhesion molecules and chemokines contribute to pressure-dependent monocytic cell adhesion

To determine the relative importance of adhesion molecules and cytokines in pressure-dependent U937 adhesion, vessels maintained for 24 hours at 80 or 150 mmHg were treated with blocking antibodies directed against these proteins for 1 hour before the intraluminal monocytic cell injection. The anti-ICAM-1 antibody did not affect U937 adhesion at all in vessels cultured at 80 mmHg (10.9±2.5 cells/mm) or 150 mmHg (24±3.8 cells/mm), such that the difference between the two remained significant ($P<0.01$, figure 4). In comparison, there was complete inhibition of pressure-dependent U937 adhesion in vessels treated with antibodies directed against VCAM-1 or MCP-1 (7.0±0.4 cells/mm and 6.8±1.3 cells/mm at 150 mmHg, respectively; $P<0.001$ versus 150 mmHg untreated). Blocking IL-6 and KC strongly reduced the number of adherent monocytic cells associated with high pressure ($P<0.001$), although a small increment in U937 adhesion was still distinguishable in arteries kept at 150 mmHg compared with 80 mmHg ($P<0.05$).

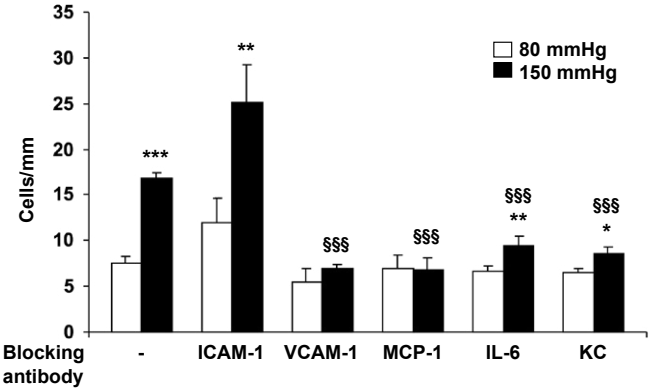

Figure 4. Quantification of monocytic cell adhesion in cultured vessels maintained at 80 or 150 mmHg for 24 hours, then treated for 1 hour with blocking antibodies targeting ICAM-1, VCAM-1, MCP-1, IL-6 or KC before intraluminal monocytic cell injection. Blocking ICAM-1 fails to prevent the increase in U937 cell adhesion associated with high pressure, but all other treatments prevent stretch-induced adhesion, either completely (VCAM-1, MCP-1) or partially (IL-6, KC). Data are mean±SEM of n=4 to 6 experiments. *P<0.05, **P<0.01, ***P<0.001 vs 80 mmHg, §§§P<0.001 vs 150 mmHg untreated.

The direct effect of chemokines on monocytic cell adhesiveness was verified an *in vitro* cell adhesion assay, which was performed using fluorescent U937 cells made to adhere to VCAM-1-coated plates. U937 adhesion was bolstered by cytokine treatment (MCP-1, IL-6 and KC), reaching 223.0±9.8 cells/well at 30 minutes compared with 102.5±18.5 cells/well for untreated monocytes (figure 5A). Cell adhesion on plastic was negligible. The independent effect of MCP-1, IL-6 and KC on monocytic cell adhesion was evaluated at 30 minutes (figure 5B). All three cytokines induced a significant increase in U937 adhesion to VCAM-1 compared with control (119.8±16.7 cells/well), rising to 169.8±17.4 (MCP-1), 162.8±15.0 (IL-6) and 167.0±15.7 cells/well (KC; $P<0.05$), and they exerted an additive effect on U937 adhesion to VCAM-1 (244.7±26.0 cells/well; $P<0.01$).

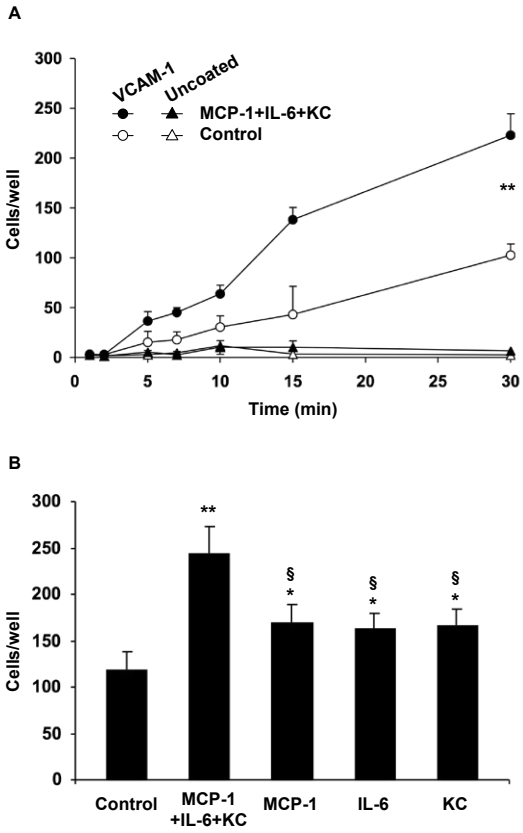

Figure 5. Quantification of U937 cell adhesion *in vitro*. A: Untreated or chemokine-stimulated monocytic cells were left to adhere for 1 to 30 minutes on VCAM-1-coated or uncoated plates. Results show that combined treatment with MCP-1, IL-6 and KC enhanced U937 cell adhesion on VCAM-1 compared with controls, whereas no cells adhered on uncoated plates. B: Treatment of monocytic cells with MCP-1, IL-6 or KC enhances their adhesion to VCAM-1-coated plates at 30 minutes, and treatment with all three chemokines at once reveals an additive effect. Data are mean±SEM of n=3 to 6 experiments. *$P<0.05$, **$P<0.01$ vs untreated control, §$P<0.05$ vs MCP-1+IL-6+KC cotreatment.

Role of α_4 and β_1 integrins in pressure-dependent monocytic cell adhesion

To establish that monocytic $\alpha_4\beta_1$, known to interact with endothelial VCAM-1, was implicated in pressure-dependent monocyte adhesion, U937 cells were treated with blocking antibodies directed against α_4 or β_1 integrin for 1 hour before intraluminal injection in vessels. The blockade of either α_4 or β_1 strongly decreased U937 adhesion in vessels maintained at 150 mmHg for 24 hours compared with untreated monocytic cells (4.9±0.6 and 7.2±1.7 respectively, versus 16.9±0.5; P<0.001; figure 6A). Similarly, pretreatment of U937 cells with the blocking anti-α_4 or anti-β_1 integrin antibodies significantly blunted their adhesion on VCAM-1-coated plates 30 minutes after combined MCP-1, IL-6, and KC stimulation (164.5±21.9 versus 93.2±4.9 with anti-α_4 and 104.1±6.7 with anti-β_1; P<0.01; figure 6B). In comparison, non-blocking anti-α_4 antibodies did not significantly interfere with monocytic cell binding to the vessel wall or to VCAM-1-coated plates (data not shown). These results strongly suggest that MCP-1, IL-6 and KC released from vessels at 150 mmHg primes monocytic cells, increasing their adhesion to VCAM-1 via $\alpha_4\beta_1$ integrins.

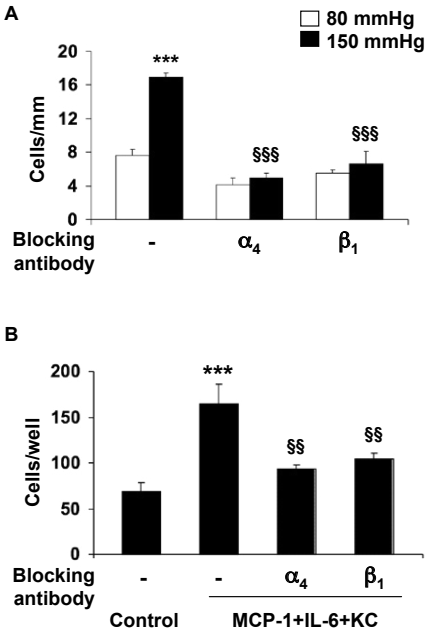

Figure 6. Key role of α_4 and β_1 integrins in monocytic cell adhesion. A: Increased U937 adhesion in vessels maintained for 24h at 150 mmHg was blunted by incubating the cells with blocking anti-α_4 or anti-β_1 antibodies 1 hour before their intraluminal injection. Data are mean±SEM of n=6 experiments. ***P<0.001 vs 80 mmHg, §§§P<0.001 vs 150 mmHg untreated. B: Similarly, the blocking anti-α_4 or anti-β_1 antibodies reduced the adhesion of chemokine-stimulated monocytic cells on VCAM-1-coated plates at 30 minutes. Data are mean±SEM of n=4. ***P<0.001 vs untreated control, §§P<0.01 vs MCP-1+IL-6+KC treated monocytes without antibody incubation.

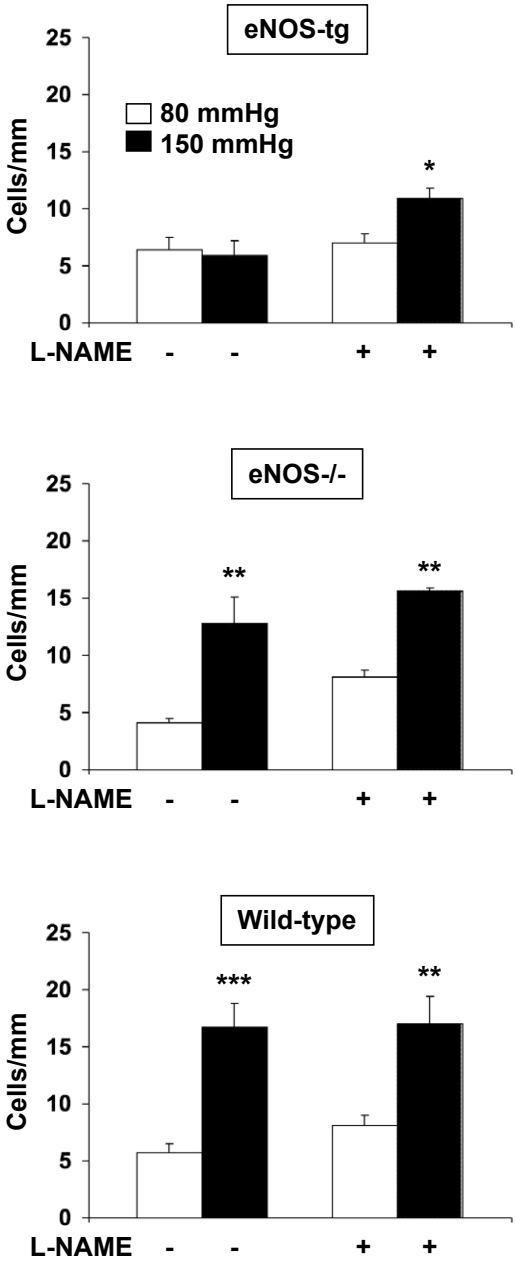

Figure 7. Overexpression of eNOS counters enhanced monocytic cell adhesion associated with high intraluminal pressure. Carotid arteries from eNOS-overexpressing (eNOS-tg), eNOS knockout and wild-type littermates were maintained for 24 hours at 80 of 150 mmHg, with or without L-NAME treatment, before U937 cell injection. Monocytic cell adhesion was similarly increased in wild-type and eNOS-/- carotids exposed to high intraluminal pressure. This response was blunted in arteries of eNOS-tg mice, but restored by treatment with L-NAME. Data are mean±SEM of n=6 to 8 experiments. *P<0.05, **P<0.01, ***P<0.001 vs 80 mmHg.

Enhanced NO production prevents pressure-induced monocyte adhesion

The protective effect of shear stress is mostly attributable to the local synthesis and release of NO via activation of eNOS. To verify whether the protective effects of NO may counterbalance the proatherosclerotic effects of high pressure, we verified stretch-induced U937 adhesion in vessels obtained from mice overexpressing (eNOS-tg) or underexpressing ($eNOS^{-/-}$) endothelial NO synthase, or from wild-type littermates. As demonstrated in figure 7, lack of NO synthase in $eNOS^{-/-}$ vessels did not affect high-pressure-induced monocyte adhesion, but overexpression of eNOS was accompanied by a marked reduction of U937 binding in vessels maintained at 150 mmHg, such that cell adhesion levels no longer varied between arteries exposed to normal or high pressure. Treatment of vessels with L-NAME restored the stretch-dependent increment in monocyte adhesion in eNOS-tg vessels but did not affect U937 binding in vessels from $eNOS^{-/-}$ mice or wild-type littermates.

Discussion

The present study reveals that exposing arteries to high intraluminal pressure induces the expression of adhesion molecules and cytokines by endothelial and smooth muscle cells, leading to increased monocyte adhesion to the vascular wall. Moreover, we show that NF-κB plays a central role in this mechanosensitive process. To the best of our knowledge, this is the first demonstration of a direct proatherogenic effect of pressure alone, independent of hormonal conditions associated with hypertension.

Many studies in human subjects and animal models have described an association between hypertension and increased atherosclerotic plaque formation[6-10,18]. However, it was recently demonstrated in a rat model of aortic coarctation that enhanced expression of adhesion molecules occurred only in aortic segments exposed to a high pressure[19]. Similarly, in a rabbit model of experimental atherosclerosis with aortic stenosis, monocyte adhesion and expression of VCAM-1 were more important in the proximal aorta where pressure is elevated than in the normotensive distal aorta[20]. These reports showed that high pressure is necessary to stimulate monocyte adhesion. Nevertheless, increased levels of angiotensin II, which characterize these models, may very well have acted synergistically with the applied stretch to produce proatherosclerotic effects. Indeed, angiotensin-converting enzyme inhibitors and angiotensin II type 1 receptor blockers prevent adhesion molecule overexpression as well as monocyte infiltration in hypertensive transgenic rats[21,22] and are associated with reduced plaque size in hypertensive $ApoE^{-/-}$, $eNOS^{-/-}$ mice[8]. However, our study clearly demonstrates that the hypertensive mechanical environment is sufficient to elicit chemokine and adhesion molecule expression leading to monocyte adhesion to the vascular wall, in the absence

of confounding hormonal factors. Moreover, even adding angiotensin II to the culture medium did not enhance U937 adhesion to the vessel wall, either at normal or high pressure, indicating that stretch is a more potent stimulus of monocytic cell adhesion than angiotensin II in whole arteries, at least in the *ex vivo* setting.

Arteries are exposed to a complex mechanical environment, including both cyclic strain and shear stress. Some studies have shown that exposing smooth muscle cells to cyclic stretch stimulates the expression of IL-6 and IL-8 via the activation of c-Jun N-terminal kinase and NF-κB pathways[23,24], whereas cyclic stretching of endothelial cells was associated with increased expression of ICAM-1[25] and E-selectin, along with greater monocyte adhesion[26]. Reproducing the tonic component of vessel stretch, continuous stretch also stimulated the expression of IL-6 via NF-κB in cultured endothelial cells[27]. Nevertheless, the phenotype of vascular cells is deeply altered *in vitro*, such that the responses to mechanical stimuli differ significantly from *in vivo* conditions where cells are exposed to a complex, tensile and 3D matricial environment. Indeed, unlike what is reported in vascular cells *in vitro*, cyclic stretch did not activate NF-κB in whole vessels[13]. Moreover, exposing arteries to a normotensive degree of stretch (80 mmHg), sufficient to maintain smooth muscle cell phenotype[28], did not activate NF-κB or induce monocyte adhesion in the present study. High intraluminal pressure alone, reminiscent of the hypertensive state, stimulated NF-κB-dependent expression of chemokines and adhesion molecules, allowing for monocytic cell adhesion. On the other hand, overexpression of eNOS, associated with enhanced NO release[14], blunted these proatherosclerotic effects of high pressure in vessels from eNOS-tg mice. Hence, hypertensive mechanical conditions facilitate atherosclerotic plaque formation in vascular regions where blood flow is low or oscillatory, whereas the protective effect of NO release prevails in vessels exposed to laminar shear stress.

It has been shown that monocytes preincubated with MCP-1 and IL-8 adhere on endothelial cells[29]. Likewise KC, the murine homologue of IL-8[30], but not MCP-1, triggered monocyte arrest on early atherosclerotic endothelium in a reconstituted flow chamber system, and blockade of $\alpha_4\beta_1$ integrins or VCAM-1, but not ICAM-1, inhibited this process[31]. Moreover, VCAM-1 and its ligand $\alpha_4\beta_1$ are critical for monocyte rolling and adhesion in early atherosclerotic lesions[32], and formation of lesions is markedly reduced in atherosclerosis-prone mice after peptide perfusion to block $\alpha_4\beta_1$, compared with unperfused mice[33]. In the present study, we found that high pressure increases the endothelial expression of ICAM-1 and induces a strong overexpression of VCAM-1, MCP-1, IL-6 and KC in all vascular cells, through induction of NF-κB. In agreement with the previous studies cited above, we demonstrated that monocyte adhesion occurs via the interaction of $\alpha_4\beta_1$ integrins with VCAM-1 rather than ICAM-1. However, blockade of MCP-1, IL-6 or KC led to a strong decrease in monocyte adhesion to the vascular wall, and our *in vitro* studies confirmed that all three cytokines are necessary to prime

monocyte adhesion. This contrasts with a previous report establishing that KC plays a predominant role in mediating monocyte adhesion in vessels from ApoE$^{-/-}$ mice fed a Western-type diet[31]; disparities between that study and our present findings indicate that the nature of the proatherosclerotic stimulus may influence which chemokines participate in monocyte recruitment. Regardless, beyond the initial endothelial cell adhesion step, the enhanced VCAM-1 and chemokine expression observed in smooth muscle cells of arteries at high intraluminal pressure could facilitate inflammatory cell infiltration in the vascular wall. Finally, the fact that all of the outcomes of high pressure described here could be reversed by blocking NF-κB highlights the key role of this transcription factor in the deleterious, proatherosclerotic effects associated with hypertensive conditions.

In summary, our results demonstrate that high intraluminal pressure alone, in the absence of external hormonal factors, is sufficient to induce chemokine and adhesion molecule expression and so trigger monocyte adhesion to vascular wall. More importantly, our work indicates that high blood pressure may directly contribute to the development of atherosclerosis though induction of NF-κB, suggesting that this pathway may provide an interesting therapeutic target to counter adverse effects of hypertension.

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References

1. Hansson, G.K. Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med* 352, 1685-1695 (2005).
2. Davies, P.F., Polacek, D.C., Handen, J.S., Helmke, B.P. & DePaola, N. A spatial approach to transcriptional profiling: mechanotransduction and the focal origin of atherosclerosis. *Trends Biotechnol* 17, 347-351 (1999).
3. Harrison, D.G., Widder, J., Grumbach, I., Chen, W., Weber, M. & Searles, C. Endothelial mechanotransduction, nitric oxide and vascular inflammation. *J Intern Med* 259, 351-363 (2006).
4. Stamler, J. Blood pressure and high blood pressure - aspects of risk. *Hypertension* 18, 95-107 (1991).
5. Peter, K., Nawroth, P., Conradt, C., Nordt, T., Weiss, T., Boehme, M., Wunsch, A., Allenberg, J., Kubler, W. & Bode, C. Circulating vascular cell adhesion molecule-1 correlates with the extent of human atherosclerosis in contrast to circulating intercellular adhesion molecule-1, E-selectin, P-selectin, and thrombomodulin. *Arteriosclerosis Thrombosis and Vascular Biology* 17, 505-512 (1997).
6. Buemi, M., Allegra, A., Aloisi, C., Corica, F., Alonci, A., Ruello, A., Montalto, G. & Frisina, N. Cold pressor test raises serum concentrations of ICAM-1, VCAM-1, and E-selectin in normotensive and hypertensive patients. *Hypertension* 30, 845-847 (1997).
7. Capers, Q.t., Alexander, R.W., Lou, P., De Leon, H., Wilcox, J.N., Ishizaka, N., Howard, A.B. & Taylor, W.R. Monocyte chemoattractant protein-1 expression in aortic tissues of hypertensive rats. *Hypertension* 30, 1397-1402 (1997).
8. Knowles, J.W., Reddick, R.L., Jennette, J.C., Shesely, E.G., Smithies, O. & Maeda, N. Enhanced atherosclerosis and kidney dysfunction in eNOS(-/-)Apoe(-/-) mice are ameliorated by enalapril treatment. *J Clin Invest* 105, 451-458 (2000).
9. Mazzolai, L., Duchosal, M.A., Korber, M., Bouzourene, K., Aubert, J.F., Hao, H., Vallet, V., Brunner, H.R., Nussberger, J., Gabbiani, G. & Hayoz, D. Endogenous angiotensin II induces atherosclerotic plaque vulnerability and elicits a Th1 response in ApoE-/- mice. *Hypertension* 44, 277-282 (2004).
10. Wu, J.H., Hagaman, J., Kim, S., Reddick, R.L. & Maeda, N. Aortic constriction exacerbates atherosclerosis and induces cardiac dysfunction in mice lacking apolipoprotein E. *Arterioscler Thromb Vasc Biol* 22, 469-475 (2002).
11. Lemarie, C.A., Esposito, B., Tedgui, A. & Lehoux, S. Pressure-induced vascular activation of nuclear factor-kappaB: role in cell survival. *Circ Res* 93, 207-212 (2003).
12. De Martin, R., Hoeth, M., Hofer-Warbinek, R. & Schmid, J.A. The transcription factor NF-kappa B and the regulation of vascular cell function. *Arterioscler Thromb Vasc Biol* 20, E83-88 (2000).
13. Lehoux, S., Esposito, B., Merval, R. & Tedgui, A. Differential regulation of vascular focal adhesion kinase by steady stretch and pulsatility. *Circulation* 111, 643-649 (2005).
14. van Haperen, R., Cheng, C., Mees, B.M., van Deel, E., de Waard, M., van Damme, L.C., van Gent, T., van Aken, T., Krams, R., Duncker, D.J. & de Crom, R. Functional expression of endothelial nitric oxide synthase fused to green fluorescent protein in transgenic mice. *Am J Pathol* 163, 1677-1686 (2003).
15. Lemarie, C.A., Tharaux, P.L., Esposito, B., Tedgui, A. & Lehoux, S. Transforming growth factor-alpha mediates nuclear factor kappaB activation in strained arteries. *Circ Res* 99, 434-441 (2006).
16. Schneeberger, P.M., van Langevelde, P., van Kessel, K.P., Vandenbroucke-Grauls, C.M. & Verhoef, J. Lipopolysaccharide induces hyperadhesion of endothelial cells for neutrophils leading to damage. *Shock* 2, 296-300 (1994).
17. Morrison, D.C., Lei, M.G., Kirikae, T. & Chen, T.Y. Endotoxin receptors on mammalian cells. *Immunobiology* 187, 212-226 (1993).

18. Franklin, S.S., Larson, M.G., Khan, S.A., Wong, N.D., Leip, E.P., Kannel, W.B. & Levy, D. Does the relation of blood pressure to coronary heart disease risk change with aging? The Framingham Heart Study. *Circulation* 103, 1245-1249 (2001).
19. Wang, H., Nawata, J., Kakudo, N., Sugimura, K., Suzuki, J., Sakuma, M., Ikeda, J. & Shirato, K. The upregulation of ICAM-1 and P-selectin requires high blood pressure but not circulating renin-angiotensin system in vivo. *J Hypertens* 22, 1323-1332 (2004).
20. Tropea, B.I., Huie, P., Cooke, J.P., Tsao, P.S., Sibley, R.K. & Zarins, C.K. Hypertension-enhanced monocyte adhesion in experimental atherosclerosis. *J Vasc Surg* 23, 596-605 (1996).
21. Luft, F.C., Mervaala, E., Muller, D.N., Gross, V., Schmidt, F., Park, J.K., Schmitz, C., Lippoldt, A., Breu, V., Dechend, R., Dragun, D., Schneider, W., Ganten, D. & Haller, H. Hypertension-induced end-organ damage : A new transgenic approach to an old problem. *Hypertension* 33, 212-218. (1999).
22. Strawn, W.B., Gallagher, P.E., Tallant, E.A., Ganten, D. & Ferrario, C.M. Angiotensin II AT1-receptor blockade inhibits monocyte activation and adherence in transgenic (mRen2)27 rats. *J Cardiovasc Pharmacol* 33, 341-351 (1999).
23. Li, L.F., Ouyang, B., Choukroun, G., Matyal, R., Mascarenhas, M., Jafari, B., Bonventre, J.V., Force, T. & Quinn, D.A. Stretch-induced IL-8 depends on c-Jun NH2-terminal and nuclear factor-kappaB-inducing kinases. *Am J Physiol Lung Cell Mol Physiol* 285, L464-475 (2003).
24. Zampetaki, A., Zhang, Z., Hu, Y. & Xu, Q. Biomechanical stress induces IL-6 expression in smooth muscle cells via Ras/Rac1-p38 MAPK-NF-kappaB signaling pathways. *Am J Physiol Heart Circ Physiol* 288, H2946-2954 (2005).
25. Cheng, J.J., Wung, B.S., Chao, Y.J. & Wang, D.L. Cyclic strain enhances adhesion of monocytes to endothelial cells by increasing intercellular adhesion molecule-1 expression. *Hypertension* 28, 386-391 (1996).
26. Yun, J.K., Anderson, J.M. & Ziats, N.P. Cyclic-strain-induced endothelial cell expression of adhesion molecules and their roles in monocyte-endothelial interaction. *J Biomed Mater Res* 44, 87-97 (1999).
27. Kobayashi, S., Nagino, M., Komatsu, S., Naruse, K., Nimura, Y., Nakanishi, M. & Sokabe, M. Stretch-induced IL-6 secretion from endothelial cells requires NF-kappaB activation. *Biochem Biophys Res Commun* 308, 306-312 (2003).
28. Birukov, K.G., Bardy, N., Lehoux, S., Merval, R., Shirinsky, V.P. & Tedgui, A. Intraluminal pressure is essential for the maintenance of smooth muscle caldesmon and filamin content in aortic organ culture. *Arterioscler Thromb Vasc Biol* 18, 922-927 (1998).
29. Gerszten, R.E., Garcia-Zepeda, E.A., Lim, Y.C., Yoshida, M., Ding, H.A., Gimbrone, M.A., Jr., Luster, A.D., Luscinskas, F.W. & Rosenzweig, A. MCP-1 and IL-8 trigger firm adhesion of monocytes to vascular endothelium under flow conditions. *Nature* 398, 718-723 (1999).
30. Bozic, C.R., Gerard, N.P., von Uexkull-Guldenband, C., Kolakowski, L.F., Jr., Conklyn, M.J., Breslow, R., Showell, H.J. & Gerard, C. The murine interleukin 8 type B receptor homologue and its ligands. Expression and biological characterization. *J Biol Chem* 269, 29355-29358 (1994).
31. Huo, Y., Weber, C., Forlow, S.B., Sperandio, M., Thatte, J., Mack, M., Jung, S., Littman, D.R. & Ley, K. The chemokine KC, but not monocyte chemoattractant protein-1, triggers monocyte arrest on early atherosclerotic endothelium. *J Clin Invest* 108, 1307-1314 (2001).
32. Huo, Y., Hafezi-Moghadam, A. & Ley, K. Role of vascular cell adhesion molecule-1 and fibronectin connecting segment-1 in monocyte rolling and adhesion on early atherosclerotic lesions. *Circ Res* 87, 153-159 (2000).
33. Shih, P.T., Brennan, M.L., Vora, D.K., Territo, M.C., Strahl, D., Elices, M.J., Lusis, A.J. & Berliner, J.A. Blocking very late antigen-4 integrin decreases leukocyte entry and fatty streak formation in mice fed an atherogenic diet. *Circ Res* 84, 345-351 (1999).

Chapter 6

eNOS and Neovascularization

Endothelial Nitric Oxide Synthase Overexpression Restores the Efficiency of Bone Marrow Mononuclear Cell-based Therapy

Barend Mees[2,3], Alice Récalde[1], Céline Loinard[1], Dennie Tempel[2,3], Marcia Godinho[2,3], José Vilar[1], Rien van Haperen[3], Bernard Lévy[1], Rini de Crom[2,3], Jean-Sébastien Silvestre[1]

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[1] INSERM, U970, Paris Cardiovascular Research Center – PARCC, Université Paris Descartes, UMR-S970, Paris, France.
[2] Departments of Vascular Surgery and [3]Cell Biology & Genetics, Erasmus University Medical Center, Rotterdam, the Netherlands.

Abstract

Bone marrow–derived mononuclear cells (BMMNC) enhance postischemic neovascularization, and their therapeutic use is currently under clinical investigation. However, cardiovascular risk factors, including diabetes and hypercholesterolemia lead to the abrogation of BMMNC proangiogenic potential. Nitric Oxide (NO) has shown to be critical for the proangiogenic function of BMMNC and increased endothelial NO Synthase (eNOS) activity promotes vessel growth in ischemic conditions. We therefore hypothesized that eNOS overexpression could restore both the impaired neovascularization response and decreased proangiogenic function of BMMNC in clinically relevant models of diabetes and hypercholesterolemia. Transgenic eNOS overexpression in diabetic, atherosclerotic and wild-type mice induced a 1.5 to 2.3-fold increase in postischemic neovascularization compared to control. eNOS overexpression in diabetic or atherosclerotic BMMNC restored their reduced proangiogenic potential in ischemic hind limb. This effect was associated with an increase in BMMNC ability to differentiate into cells with endothelial phenotype *in vitro* and *vivo* and an increase in BMMNC paracrine function including VEGF-A release and NO-dependent vasodilation. Moreover, while WT BMMNC treatment resulted in significant progression of atherosclerotic plaque in ischemic mice, eNOS transgenic atherosclerotic BMMNC treatment even had antiatherogenic effects.
Cell-based eNOS gene therapy has both proangiogenic and antiatherogenic effects and should be further investigated for the development of efficient therapeutic neovascularization designed to treat ischemic cardiovascular disease.

Introduction

To prevent or treat ischemic diseases, therapeutic neovascularization, the stimulation of tissue vascularization after ischemia, has recently progressed from bench to bedside. Strategies include transplantation of angiogenic bone marrow-derived mononuclear cells (BMMNC) or gene transfer for systemic or local up-regulation of proangiogenic proteins. Clinical studies have demonstrated the safety, feasibility and efficacy of intracoronary and intramuscular infusion of adult BMMNC in patients with peripheral arterial disease, acute myocardial infarction, and ischemic cardiomyopathy[1,2].
However, despite the excitement surrounding the possible clinical use of BMMNC, in atherosclerosis, diabetes and other risk factors for cardiovascular diseases the availability of bone marrow and progenitor cells is reduced and their function impaired to varying degrees[1,2]. Moreover, the safety of BMMNC treatment has been questioned by studies that found an increase in atherosclerotic plaque size after BMMNC treatment[3]. This potentially hazardous dual effect of therapeutic neovascularization on atherogenesis is explained by the many common pathways of both mechanisms and has been named the "Janus Phenomenon"[4].
Impaired bioavailability of Nitric Oxide (NO) is a hallmark in patients with cardiovascular disease. Moreover, the enzyme endothelial NO synthase (eNOS) has also been shown to be essential for neovascularization. It has a key regulatory function in endothelial cell growth[5], vascular remodeling[6], angiogenesis[7] and vasodilation[8] and plays a crucial role in the functional activity of BMMNC[9,10]. Thus, impaired bioavailability of NO may significantly contribute to the impaired neovascularization response to ischemia in atherosclerosis or diabetes. Therefore, using homebred transgenic mice overexpressing human eNOS[11], the purposes of the present study were to evaluate whether eNOS gene therapy would be able to improve the postischemic neovascularization response in diabetes and atherosclerosis and to restore the impaired proangiogenic potential of BMMNC without causing simultaneous detrimental proatherogenic effects, overcoming the "Janus Phenomenon".

Materials and methods

Mice

The experimental protocol was approved by the Animal Experiments Committee under the national Experiments on Animals Act and adhered to the rules laid down in this national law that serves the implementation of " Guidelines on the protection of experimental animals " by the Council of Europe (1986), (Directive 86/609/EC). C57BL/6 and apolipoprotein E-deficient (ApoE KO) transgenic mice overexpressing the human

eNOS gene under regulation of the human eNOS promoter were obtained, as previously described[11]. Mice were backcrossed to C57Bl6 for at least ten generations (>96% C57Bl6). To induce diabetes, 8-week-old mice were injected intraperitoneally with 40 mg/kg of streptozotocin (STZ; Sigma) in 0.05 mol/L Sodium Citrate, pH 4.5, daily for five days[12]. Mice were treated with or without NO synthase inhibitor N(G)-nitro-L-arginine methyl ester (L-NAME, 10 mg/kg/day in the drinking water, Sigma).

Hind limb ischemia model and quantification of neovascularization

Mice underwent surgery to induce unilateral hind limb ischemia, as previously described[13]. A total of $1x10^6$ freshly isolated bone marrow-derived mononuclear cells (BMMNC) were intravenously injected 24 hours after femoral artery ligation. Two weeks after ligation, postischemic neovascularization was evaluated by Laser Doppler Imaging and microangiography, as previously described[13].

Atherosclerosis

Plasma cholesterol levels were measured and atherosclerotic plaque lesion size and composition in the aortic root was evaluated by immunohistochemistry, as previously described[3].

NO and ROS production

NO production in BMMNC was assessed by measuring intracellular nitrosation of NO-sensitive fluorochrome 4,5-Diaminofluorescein diacetate (DAF-2/DA; Alexis Biochemicals). Briefly, BMMNC were incubated with 10 µM DAF-2/DA for 180 minutes (37°C). Exposure to light was avoided as far as possible throughout experimentation. At 180 minutes supernatants were removed and cells were washed in fresh DAF-2/DA-free buffer followed by immediate FACS analysis. A Becton Dickinson FACSCalibur analyzer was used to quantify fluorescence (excitation wavelength: 488 nm and emission wavelength: 530 nm) at the single-cell level, and data were analyzed using Cellquest version 3.3 (Becton Dickinson) software. Cellular reactive oxygen species (ROS) levels, reflecting a balance between oxidant production and removal by endogenous antioxidants, were also quantified using L-012 as described recently[12]. BMMNC were lysed in 50mmol/L Tris buffer (pH 7.5) containing protease inhibitors (Boerhinger) and centrifuged at 10 000 x g for 15 minutes at 4°C. Supernatants were then incubated with 100 mmol/L L-012 (Wako). Luminescence was counted (Perkin Elmer Topcount NXT) during 20 seconds after a 10 minutes interval allowing for the plates to become adapted to the dark.

Growth factor assay

After isolation of adherent cells in cell cultures, culture medium was collected and analyzed for vascular endothelial growth factor A (VEGF-A) by enzyme-linked immunosorbent assay (R&D Systems).

Measurement of arterial diameter in isolated femoral arteries

After 24 hours of ischemia, ischemic femoral arteries from control animals were isolated and cannulated at both extremities in a video monitored perfusion system (arteriograph, LSI). A total of $0.5x10^6$ BMMNC from WT, eNOStg control and diabetic or hypercholesterolemic mice were then perfused, as earlier described[9].

Isolation, and incorporation of bone marrow mononuclear cells

Bone marrow cells were obtained by flushing tibiae and femora of donor mice. Low-density BMMNC were then isolated by density gradient centrifugation with Ficoll. BMMNC were plated at a density of $1x10^6$ cells per cm^2 on 24-well plates (Nunc) coated with 10 µg/ml fibronectin (Sigma) and cultured up to 7 days in M199 medium supplemented with 20% FBS (Invitrogen), 0.05 mg/ml Bovine Pituitary Extract (Invitrogen), antibiotics, and 10 units/ml heparin (Leo Pharma BV). Nonadherent cells were then removed and adherent cells were analyzed by immunochemical assay with DiI-labeled acetylated low-density lipoprotein (DiI-LDL; Molecular Probes), fluorescein isothiocyanate-labeled *Bandeiraea Simplicifolia* lectin (BS-1 lectin; Sigma), goat anti-eNOS (Santa-cruz) and subsequently rabbit antigoat Alexa fluor 594. Endothelial cell phenotype was revealed by double positive staining for both DiI-LDL and BS-1 lectin and both eNOS and BS-1 lectin. These double-positive cells were negative for the monocytic marker CD45 (data not shown). Cell numbers were counted and expressed in cells per field. Three fields from each culture were counted.

To demonstrate incorporation of BMMNC-derived endothelial cells into ischemic muscles, green Alexafluor 546-labeled BMMNC ($1x10^6$ cells/100µl PBS) were intravenously administered 24 hours after induction of hind limb ischemia. The gastrocnemius muscles were harvested four days after injection of BMMNC. Incorporated BMMNC were detected by immunostaining with a biotinylated isolectin B4 (Sigma) followed by incubation with rhodamin red streptavidin (Jackson). The number of infiltrating cells was also evaluated using green Alexafluor 546-labeled BMMNC isolated from WT or eNOStg mice. Two days after BMMNC injection, the ischemic gastrocnemius muscles were harvested, weighed, minced and digested in 450 U/ml collagenase I, 125 U/ml collagenase XI, 60 U/ml DNAseI and 60 U/ml hyaluronidase (Sigma Aldrich) for 1 hour at 37°C. Cell suspensions were layered on histopaque 1083 (Sigma Aldrich) for gradient density centrifugation. In the mononuclear cells fraction, the number of cells being green Alexafluor 546/$DAPI^+$ was then evaluated on a LSRII Flow Cytometer (Becton Dickinson) with the FACSDiva software (Becton Dickinson).

Statistical analysis

Statistical analysis was performed using Student's t-test or one-way analysis of variance, followed by post-hoc analysis, as appropriate. Data are reported as mean ± standard error of the mean (SEM). Statistical significance was accepted when $P<0.05$.

Results

eNOS overexpression is proangiogenic

Postischemic hind limb neovascularization was impaired in diabetic mice and ApoE KO mice compared with WT, demonstrated by a 1.3-fold decreased foot perfusion and vessel density ($P<0.05$, Figure 1A). eNOS overexpression in the endothelium of diabetic mice and ApoE KO mice resulted in an increase in foot perfusion and vessel density ($P<0.001$), reaching above WT levels. Endothelial eNOS overexpression in WT mice resulted in the strongest neovascularization response (1.5 to 1.7-fold increase compared with WT controls; $P<0.001$). Of interest, treatment with the NOS inhibitor N(G)-nitro-L-arginine methyl esther (L-NAME) abrogated postischemic vessel growth in mice overexpressing eNOS, underscoring the role of NO in the observed protective effects.

The role of eNOS overexpression on cell-based therapeutic neovascularization was evaluated by intravenous transplantation of different BMMNC after induction of ischemia in WT, diabetic and ApoE KO recipient mice. In WT recipient mice, transplantation of WT BMMNC and eNOStg BMMNC resulted in a comparable 1.3 to 1.9-fold increase in foot perfusion and vessel density compared with saline ($P<0.01$, Figure 1B). In diabetic mice, diabetic BMMNC transplantation did not improve neovascularization response. Conversely, administration of WT BMMNC increased vessel growth in diabetic ischemic area but to a lesser extent than that of eNOStg BMMNC. Interestingly, eNOStg diabetic BMMNC transplantation resulted in a significant 1.3 to 1.5-fold increase in postischemic neovascularization and reached WT BMMNC levels compared with saline and diabetic BMMNC ($P<0.01$). Similarly, eNOS overexpression restored the therapeutic potential of ApoE KO BMMNC.

Differentiation of eNOS-overexpressing BMMNC

Western blot analysis of BMMNC confirmed an increased presence of eNOS protein in eNOS transgenic BMMNC ($P<0.05$, Figure 2A). Concordantly, NO production by eNOS transgenic BMMNC was significantly elevated compared with control BMMNC ($P<0.05$, Figure 2B). We also showed that BMMNC-derived ROS were up-regulated in diabetic and ApoEKO BMMNC, as previously described[12]. However, eNOS overexpression did not affect ROS levels (Figure 2B).

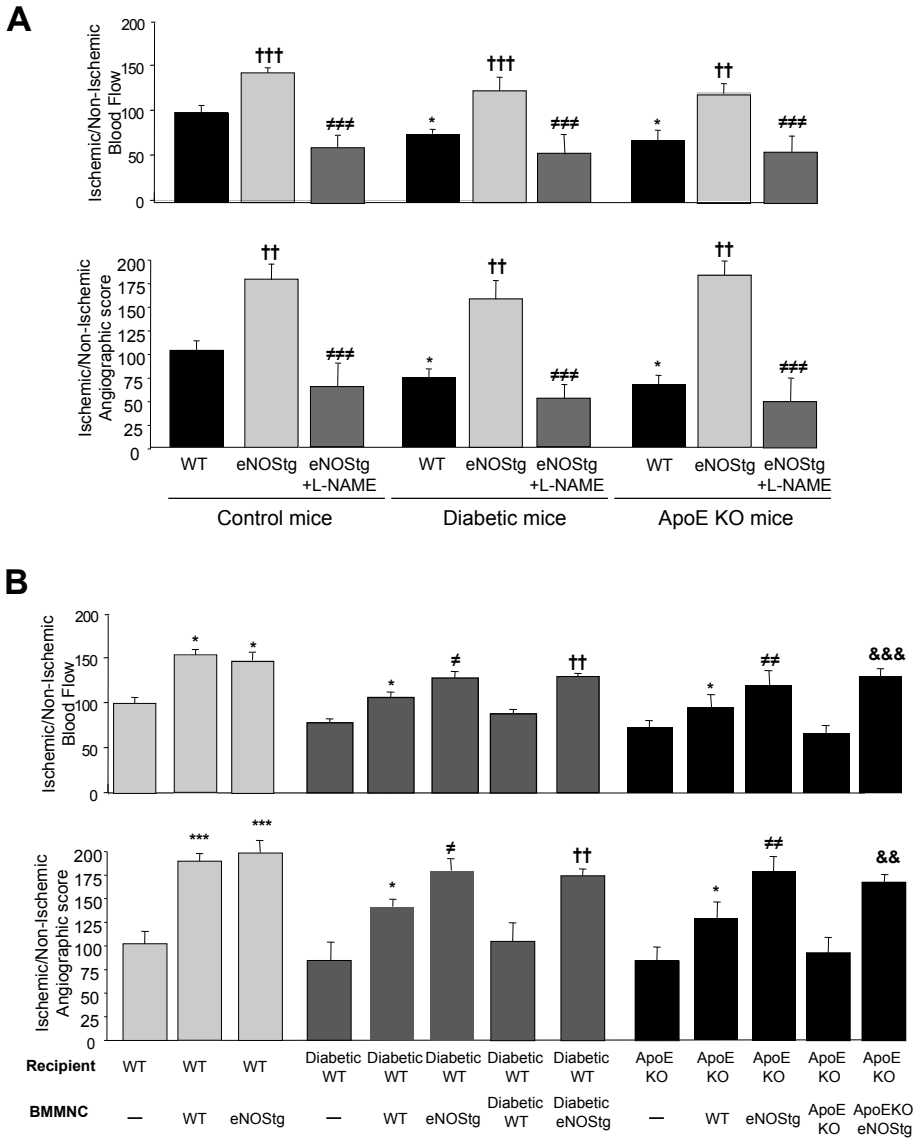

Figure 1. A) eNOS overexpression increases postischemic neovascularization. Quantification of foot perfusion (upper) measurements by Laser Doppler Imaging and vessel density measurements by microangiography (lower). Values are mean ± SEM, n=10 and *P<0.05, vs WT control mice, ††P<0.01, †††P<0.001 vs WT mice, ≠≠≠P<0.001 vs eNOS transgenic mice. B) Bone marrow eNOS overexpression restores the proangiogenic effect on postischemic neovascularization. Quantitative evaluation (upper) of foot perfusion by Laser Doppler Imaging and vessel density measurements by micro-angiography (lower). Values are ± SEM, n=10 and *P<0.05,***P<0.001 vs PBS-treated mice, ≠P<0.05, ≠≠P<0.01 vs mice receiving WT BMMNC, ††P<0.01 vs diabetic mice receiving diabetic BMMNC, &&P<0.01, &&P<0.001 vs ApoEKO mice receiving ApoEKO BMMNC.

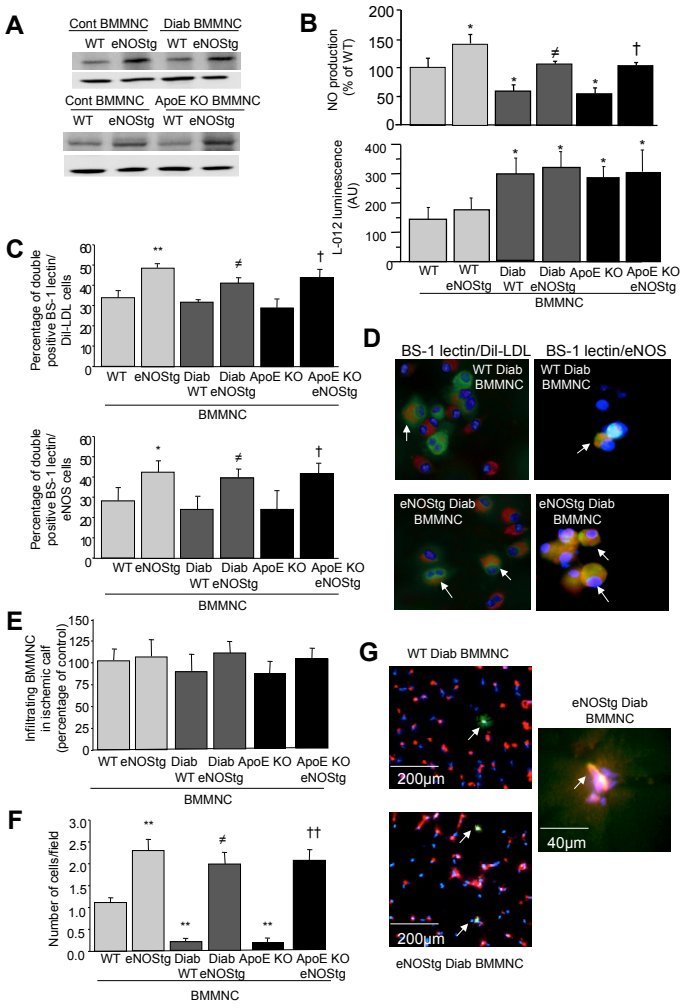

Figure 2. eNOS overexpression increases differentiation of BMMNC. Representative quantatitive Western blot (A) and NO and ROS production (B) in BMMNC isolated from WT or eNOS transgenic control, diabetic and ApoE KO mice. C) Quantification of percentage of BMMNC that double positively stained for BS-1 Lectin and DiI-LDL (upper) or BS-1 lectin and eNOS (lower), regarded as cells with endothelial phenotype. D) Representative images of BMMNC cultures. *Arrows* indicate double positive cells with endothelial phenotype respectively for DiI-LDL (red) and BS-1 lectin (green) or eNOS (red) and BS-1 lectin. Nuclei were stained with DAPI (blue). E) Quantitative evaluation of the number of infiltrating BMMNC in the ischemic area. BMMNC were isolated from WT or eNOS transgenic control, diabetic and ApoE KO mice. Two days after BMMNC injection in WT mice with hind limb ischemia, the gastrocnemius muscles were digested and the number of cells being green Alexafluor 546 ^{hi}/DAPI^{+} was then evaluated in the mononuclear cells fraction. Quantitative analysis (F) and representative photomicrographs (G) of incorporated BMMNC-derived endothelial cells in histological sections from ischemic skeletal muscles. BMMNC were stained using Alexa fluor 546 cell tracker (green). Mouse vasculature was identified by isolectin B4 staining (red). Nuclei were stained with DAPI (blue). *Arrows* indicate incorporated BMMNC-derived endothelial cells. Values are mean ± SEM, n=4. *P<0.05, **P<0.01 vs WT BMMNC, ≠P<0.05 vs WT Diab BMMNC, †P<0.05 and ††P<0.01 vs ApoE KO BMMNC.

Immunohistochemical analysis of BMMNC cultured in endothelial-specific medium demonstrated a significant increase in BMMNC differentiation into cells with endothelial phenotype of eNOS transgenic BMMNC compared with BMMNC, revealed by a greater percentage of BS-1 lectin/Dil-LDL and BS-1 lectin/eNOS double positive cells ($P<0.01$, Figure 2C-D). Similarly, eNOS overexpression increased the number of incorporated BMMNC-derived endothelial cells into capillaries of ischemic muscle (Figure 2 F-G). Conversely, administration of WT or eNOStg BMMNC did not affect the number of infiltrating BMMNC in ischemic area (Figure 2E).

Paracrine effects of eNOS-overexpressing BMMNC

BMMNC are also capable of stimulating neovascularization by secretion of proangiogenic factors. Culture medium was collected from BMMNC differentiation cell cultures. Using ELISA assay, quantitative measurements of VEGF-A demonstrated an increase in VEGF-A secretion in culture medium from eNOS transgenic BMMNC compared to WT BMMNC (Figure 3A). Finally, because proangiogenic effects of BMMNC also rely on the cells' ability to modulate vascular function, we assessed BMMNC effects on vascular diameter in isolated perfused mouse femoral arteries (238±5 μm, internal diameter). Intraluminal administration of BMMNC induced a rapid vasodilation (internal diameter increased by 27±2 μm for $5x10^5$ cells/ml). eNOS transgenic BMMNC increased vasodilation by 1.7-fold over WT BMMNC ($p<0.01$). Interestingly, WT diabetic BMMNC and ApoE KO BMMNC only slightly affected vessel diameter whereas eNOS overexpression fully restored the vasodilatory potential of diabetic and ApoE KO BMMNC (Figure 3B).

eNOS overexpression is antiatherogenic

Atherosclerotic lesions were analyzed in ApoE KO mice after induction of hind limb ischemia and subsequent BMMNC treatment. Plasma cholesterol levels were similar in all groups. Treatment with ApoE KO BMMNC did not affect plaque size or composition in contrast with treatment with WT BMMNC (60% increase in lesion size, without differences in composition, $P<0.001$, Table 1). Remarkably, treatment with eNOStg ApoE KO BMMNC had an inhibitory effect on plaque progression (44% decrease in lesion size, $P<0.05$). Furthermore, plaque composition changed due to eNOS transgenic ApoE KO BMMNC treatment into a more stable phenotype (40% decrease in macrophages, $P<0.05$).

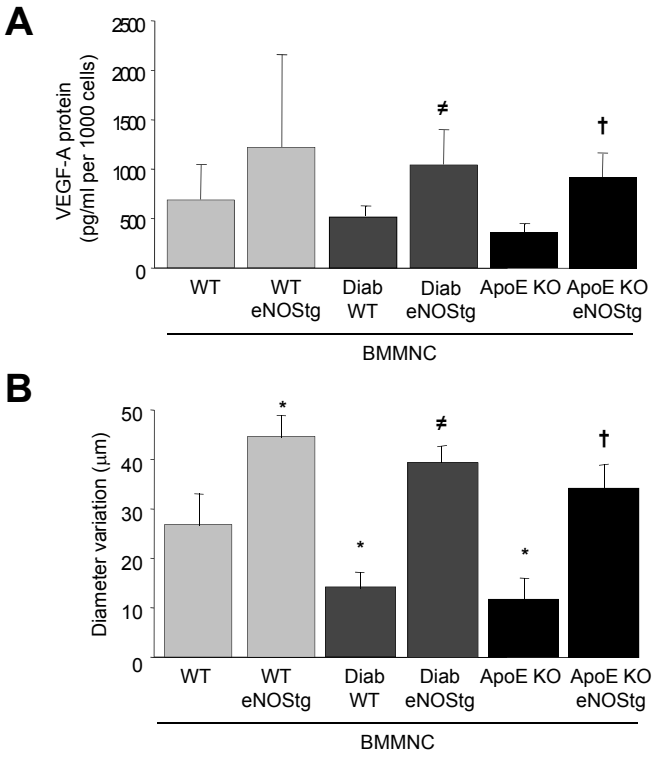

Figure 3. eNOS overexpression increases the paracrine potential of BMMNC. A) Quantification by ELISA assays of secretion of VEGF-A in cell culture medium. Values are mean ± SEM, n=4 and **P<0.01 vs WT BMMNC, ≠P<0.05 vs WT Diab BMMNC and †P<0.01 vs ApoE KO BMMNC. ND indicates not detected. B) Quantitative evaluation of ischemic femoral artery diameter isolated from control mice after intraluminal injection of BMMNC isolated from WT, eNOS transgenic control, diabetic or ApoE KO mice. Values are mean ± SEM, n=10 and *P<0.05 vs WT and ≠P<0.05 vs WT Diab BMMNC and †P<0.05 vs ApoE KO BMMNC.

Table 1. Bone marrow eNOS overexpression has inhibitory effects on atherosclerotic lesion size progression in the aortic sinus of ApoE KO mice. Values are mean ± SEM, n=10. *P<0.05 vs PBS and †P<0.05 vs ApoE KO BMMNC.

	PBS	WT BMMNC	ApoE KO BMMNC	eNOS transgenic ApoE KO BMMNC
Total cholesterol (mM)	13.2±1.6	12.8±1.2	11.9±2.4	11.4±3.5
Lesion size (μm^2)	108838±14257	178257±16687*	110654±13458	75125±9458*†
% of MOMA2 positive staining	28.3±2.4	34.2±3.1	30.4±2.7	20.1±1.9*†
% of α-actin positive staining	9.4±0.3	10.6±1.1	11.1±2.1	10.2±1.5
% of Sirius red positive staining	23.1±2.6	20.2±1.9	24.3±2.7	25.4±2.5

Discussion

In the present study, we showed that endothelial eNOS overexpression improved the postischemic neovascularization response in healthy, diabetic and atherosclerotic mice. Subsequently, eNOS overexpression in bone marrow repaired the decreased pro-angiogenic functional activity of diabetic and atherosclerotic BMMNC. Finally, the results of the present study demonstrated a unique combined proangiogenic and antiatherogenic effect of cell-based eNOS gene therapy in atherosclerotic mice.

The critical role of eNOS in postischemic neovascularization has been well established. In eNOS-deficient mice neovascularization is decreased resulting in severe limb loss[14] and in parallel, up-regulation of eNOS activity by eNOS gene delivery[15] or bovine eNOS overexpression[16] enhances postischemic blood flow recovery and limb function. In our study we confirmed these findings in healthy mice and extended them to a more clinically relevant model, the diabetic and atherosclerotic mouse. eNOS overexpression in diabetic and atherosclerotic mice induced a strong postischemic neovascularization response comparable with WT mice. Mechanisms of the positive neovascularization effects of eNOS up-regulation included increase in vasodilation[8], increase in vessel density as shown by microangiographic measurements and increase in vasculogenesis as a result of restoration of pathological bone marrow proangiogenic function. Indeed, the enhanced postischemic neovascularization response after treatment of ischemic mice with eNOS transgenic diabetic or eNOS transgenic ApoE KO BMMNC revealed that up-regulation of eNOS repaired the reduced proangiogenic function of pathological BMMNC. This was further explained by an increased differentiation into cells with endothelial phenotype, and an increase in their paracrine potential including VEGF-A release and NO-dependent vessel dilation[9]. It is noteworthy that, although no changes in the amount of total eNOS protein were observed, NO release was decreased in diabetic BMMNC compared with nondiabetic control BMMNC. Our findings are consistent with prior reports showing that hyperglycemia and diabetes are associated with impaired eNOS functions, at least in part, through inhibition of eNOS phosphorylation[17,18].

The term "Janus phenomenon" has been invented for the dual effect of protein (FGF, MCP-1)-, gene (VEGF, TNF)- or cell (BMMNC, EPC)-based therapeutic angiogenesis on progression and destabilization of atherogenesis[4]. NO appears a possible exception to the "Janus Phenomenon" having established proneovascularization and antiatherogenic effects. However, these effects have never been investigated at the same time in the same model. Our study is the first study using eNOS cell-based gene therapy for the stimulation of postischemic neovascularization and simultaneously focusing on prevention of supplemental proatherogenic effects. Interestingly, WT BMMNC treatment led to increased plaque size in ischemic atherosclerotic mice, whereas ApoE KO BMMNC treatment had no effect on lesion size, as previously described[3]. However

at the same time, we could indeed demonstrate an antiatherogenic effect of eNOStg atherosclerotic BMMNC, thus blunting the "Janus Phenomenon". In our studies, only occasional donor BMMNC were identified in the atherosclerotic lesions, making a physical or local contribution of transplanted BMMNC to plaque growth less likely. A possible other explanation could be the production of chemokines by transplanted BMMNC, however we could not detect any differences in MCP-1 and VEGF blood levels in the different BMMNC-treated mice (data not shown). It is likely that the potent antiinflammatory effect of eNOS overexpression is the main responsible factor in the antiatherogenic effect of eNOS transgenic ApoE KO BMMNC treatment, as described previously[11]. Definitely, further studies will be necessary to investigate in more detail, not only our interesting observation of the combined proangiogenic and antiatherogenic potential of eNOS cell-based therapy, but also the complete concept of BMMNC treatment-induced atherogenesis. Nevertheless, these results imply that eNOS up-regulation is a promising target for local or stem cell-based therapeutic neovascularization in patients with ischemic (cardio)vascular disease.

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References

1. Segers, V.F. & Lee, R.T. Stem-cell therapy for cardiac disease. *Nature* 451, 937-942 (2008).
2. Aranguren, X.L., Verfaillie, C.M. & Luttun, A. Emerging hurdles in stem cell therapy for peripheral vascular disease. *J Mol Med* 87, 3-16 (2009).
3. Silvestre, J.S., Gojova, A., Brun, V., Potteaux, S., Esposito, B., Duriez, M., Clergue, M., Le Ricousse-Roussanne, S., Barateau, V., Merval, R., Groux, H., Tobelem, G., Levy, B., Tedgui, A. & Mallat, Z. Transplantation of bone marrow-derived mononuclear cells in ischemic apolipoprotein E-knockout mice accelerates atherosclerosis without altering plaque composition. *Circulation* 108, 2839-2842 (2003).
4. Epstein, S.E., Stabile, E., Kinnaird, T., Lee, C.W., Clavijo, L. & Burnett, M.S. Janus phenomenon: the interrelated tradeoffs inherent in therapies designed to enhance collateral formation and those designed to inhibit atherogenesis. *Circulation* 109, 2826-2831 (2004).
5. Ziche, M., Morbidelli, L., Masini, E., Amerini, S., Granger, H.J., Maggi, C.A., Geppetti, P. & Ledda, F. Nitric oxide mediates angiogenesis *in vivo* and endothelial cell growth and migration *in vitro* promoted by substance P. *J Clin Invest* 94, 2036-2044 (1994).
6. Rudic, R.D., Shesely, E.G., Maeda, N., Smithies, O., Segal, S.S. & Sessa, W.C. Direct evidence for the importance of endothelium-derived nitric oxide in vascular remodeling. *J Clin Invest* 101, 731-736 (1998).
7. Murohara, T., Witzenbichler, B., Spyridopoulos, I., Asahara, T., Ding, B., Sullivan, A., Losordo, D.W. & Isner, J.M. Role of endothelial nitric oxide synthase in endothelial cell migration. *Arterioscler Thromb Vasc Biol* 19, 1156-1161 (1999).
8. Mees, B., Wagner, S., Ninci, E., Tribulova, S., Martin, S., van Haperen, R., Kostin, S., Heil, M., de Crom, R. & Schaper, W. Endothelial nitric oxide synthase activity is essential for vasodilation during blood flow recovery but not for arteriogenesis. *Arterioscler Thromb Vasc Biol* 27, 1926-1933 (2007).
9. You, D., Waeckel, L., Ebrahimian, T.G., Blanc-Brude, O., Foubert, P., Barateau, V., Duriez, M., Leri-cousse-Roussanne, S., Vilar, J., Dejana, E., Tobelem, G., Levy, B.I. & Silvestre, J.S. Increase in vascular permeability and vasodilation are critical for proangiogenic effects of stem cell therapy. *Circulation* 114, 328-338 (2006).
10. Landmesser, U., Engberding, N., Bahlmann, F.H., Schaefer, A., Wiencke, A., Heineke, A., Spiekermann, S., Hilfiker-Kleiner, D., Templin, C., Kotlarz, D., Mueller, M., Fuchs, M., Hornig, B., Haller, H. & Drexler, H. Statin-induced improvement of endothelial progenitor cell mobilization, myocardial neovascularization, left ventricular function, and survival after experimental myocardial infarction requires endothelial nitric oxide synthase. *Circulation* 110, 1933-1939 (2004).
11. van Haperen, R., de Waard, M., van Deel, E., Mees, B., Kutryk, M., van Aken, T., Hamming, J., Grosveld, F., Duncker, D.J. & de Crom, R. Reduction of blood pressure, plasma cholesterol, and atherosclerosis by elevated endothelial nitric oxide. *J Biol Chem* 277, 48803-48807 (2002).
12. Ebrahimian, T.G., Heymes, C., You, D., Blanc-Brude, O., Mees, B., Waeckel, L., Duriez, M., Vilar, J., Brandes, R.P., Levy, B.I., Shah, A.M. & Silvestre, J.S. NADPH oxidase-derived overproduction of reactive oxygen species impairs postischemic neovascularization in mice with type 1 diabetes. *Am J Pathol* 169, 719-728 (2006).
13. Silvestre, J.S., Thery, C., Hamard, G., Boddaert, J., Aguilar, B., Delcayre, A., Houbron, C., Tamarat, R., Blanc-Brude, O., Heeneman, S., Clergue, M., Duriez, M., Merval, R., Levy, B., Tedgui, A., Amigorena, S. & Mallat, Z. Lactadherin promotes VEGF-dependent neovascularization. *Nat Med* 11, 499-506 (2005).

14. Murohara, T., Asahara, T., Silver, M., Bauters, C., Masuda, H., Kalka, C., Kearney, M., Chen, D., Symes, J.F., Fishman, M.C., Huang, P.L. & Isner, J.M. Nitric oxide synthase modulates angiogenesis in response to tissue ischemia. *J Clin Invest* 101, 2567-2578 (1998).
15. Smith, R.S., Jr., Lin, K.F., Agata, J., Chao, L. & Chao, J. Human endothelial nitric oxide synthase gene delivery promotes angiogenesis in a rat model of hindlimb ischemia. *Arterioscler Thromb Vasc Biol* 22, 1279-1285 (2002).
16. Amano, K., Matsubara, H., Iba, O., Okigaki, M., Fujiyama, S., Imada, T., Kojima, H., Nozawa, Y., Kawashima, S., Yokoyama, M. & Iwasaka, T. Enhancement of ischemia-induced angiogenesis by eNOS overexpression. *Hypertension* 41, 156-162 (2003).
17. Du, X.L., Edelstein, D., Dimmeler, S., Ju, Q., Sui, C. & Brownlee, M. Hyperglycemia inhibits endothelial nitric oxide synthase activity by posttranslational modification at the Akt site. *J Clin Invest* 108, 1341-1348 (2001).
18. Gallagher, K.A., Liu, Z.J., Xiao, M., Chen, H., Goldstein, L.J., Buerk, D.G., Nedeau, A., Thom, S.R. & Velazquez, O.C. Diabetic impairments in NO-mediated endothelial progenitor cell mobilization and homing are reversed by hyperoxia and SDF-1 alpha. *J Clin Invest* 117, 1249-1259 (2007).

Chapter 7

Summary and Perspectives

Barend Mees

Introduction

Cardiovascular disease is currently still responsible for the largest part of morbidity and mortality in the Western world. Endothelial dysfunction due to or resulting in impaired bioavailability of Nitric Oxide (NO) is a hallmark in patients with cardiovascular disease, leading to atherosclerosis, occlusive disease and subsequent postischemic complications. Vascular remodeling is the native adaptive response to arterial occlusion, consisting of collateral growth, arteriogenesis, angiogenesis and vasculogenesis[1]. NO is thought to be essential in vascular remodeling, as decreased endothelial NO synthase (eNOS) activity inhibits vessel growth in ischemic conditions[2] and NO has shown to be critical for the proangiogenic function of bone marrow–derived mononuclear cells (BMMNC)[3,4]. In addition, cardiovascular risk factors including diabetes and hypercholesterolemia lead to a decreased quantity and proangiogenic potential of BMMNC, resulting in impaired vascular remodeling[5-7].
The aim of the present thesis was to further unravel the role of NO in vascular remodeling and to evaluate positive effects on vascular remodeling by elevated eNOS activity in physiological and cardiovascular pathophysiological conditions.

eNOS transgenic mice

In chapter 2 and 3 we describe the generation and characterization of two different eNOS transgenic (eNOS TG) mouse models[8,9]. For the generation of eNOS TG mice, we used a DNA fragment that comprised the complete human eNOS genomic sequence, including all exons and introns as well as its natural flanking sequences. By using this construct with the autologous eNOS promotor we were able to simulate the human situation in terms of regulation and tissue distribution of eNOS as much as possible. In chapter 2 we describe for the first time in literature a human eNOS TG mouse model. In chapter 3 we describe a second transgenic mouse overexpressing human eNOS fused with a green fluorescent protein (GFP), allowing the study of localization and regulation of eNOS. Localization studies revealed in both mouse models that expression of the eNOS transgene was restricted to the endothelial lining of blood vessels in various tissues tested, without appreciable expression in non-endothelial cells. In eNOS-GFP TG mice GFP was expressed along the same pattern as eNOS expression in all endothelial cells, confirming the presence of the eNOS-GFP fusion protein. Using GFP expression and confocal microscopy we could demonstrate the localization of human eNOS-GFP in the plasma cell membrane and Golgi complex of the cell, where eNOS is normally localized[10]. eNOS activity was 10-fold elevated in the eNOS TG mice. Subsequently, NO production in isolated vessels was significantly increased in transgenic mice when

compared with non-transgenic control animals. Aortic blood pressure was 20 mm Hg lower in the transgenic mice compared with control mice because of lower systemic vascular resistance. The effects of eNOS overexpression on diet-induced atherosclerosis were studied in a crossbred with apolipoprotein E-deficient (ApoE KO) mice. Elevation of eNOS activity in these eNOS TG*ApoE KO mice decreased blood pressure (-20 mm Hg) and plasma levels of cholesterol (-17%), resulting in a reduction in atherosclerotic lesions by 40%. These results seem in contradiction with earlier reports from another (bovine) eNOS transgenic mouse model, in which modest overexpression of eNOS resulted in increased atherosclerosis[11]. A probable explanation for these contradictory results lies in the fact that the promotor used for the DNA construct in the bovine eNOS mouse model was derived from the endothelin-1 gene[12], which is differentially and often even inversely regulated when compared with the activity of the eNOS promotor[13]. Thus, endothelin-1 and eNOS have opposite effects on vascular tone and the development of atherosclerosis[13]. Moreover, the marked decrease in atherosclerosis in our mouse model is in concordance with earlier studies showing increased atherosclerotic lesion size in eNOS KO mice and therefore our results confirm the atheroprotective role of eNOS[14].
In conclusion, we generated two eNOS transgenic mouse models with physiological regulation of the overexpressing human eNOS gene and a characteristic phenotype because of increased NO bioavailability, making these models a very useful tool for further research to unravel the role of eNOS in various physiological and pathophysiological conditions.

eNOS in collateral growth

In chapter 4 we studied the role of eNOS in collateral growth using three mouse strains with different levels of eNOS expression[15]. Distal femoral artery ligation was performed in transgenic eNOS-overexpressing mice (eNOS TG), eNOS-deficient (eNOS KO) mice, and wild-type (WT) controls. In the distal femoral artery ligation model there is no limb ischemia in WT mice. Therefore, it is regarded as a model specific for collateral artery growth[16]. Firstly, to evaluate the possible benefits of elevated eNOS activity on collateral growth, we compared eNOS TG with WT mice. We could demonstrate a beneficial effect of overexpression of eNOS, but only in the acute phase of blood flow recovery immediately after femoral occlusion. During three weeks of follow-up, blood flow recovery was equal in eNOS TG and WT mice. In agreement with these findings, histological analysis showed that there were no differences in collateral artery growth. Therefore, we concluded that the increase of NO bioavailability enhanced the early vasodilatory response induced by increased fluid shear stress (FSS), but did not further enhance the outward vascular remodeling of collateral arteries.

In the second part of this study we evaluated the effect of absence of eNOS activity on collateral growth and thus compared eNOS KO and WT mice using the same hind limb model and measurements. Distal femoral artery ligation caused significantly reduced blood flow recovery as well as tissue damage in eNOS KO mice, despite our observations of an increase in angiogenesis and normal collateral growth compared to WT. Histological analysis revealed growing collateral artery size to the same extent in eNOS KO and WT mice. *In vivo* the administration of an NO donor induced vasodilation in collateral arteries of eNOS KO mice, but not in WT, as they were already fully vasodilated. Therefore, we concluded that in eNOS KO mice impaired blood flow recovery leading to severe tissue damage, was the result of insufficient vasodilation in the early recovery phase, before collateral artery remodeling was complete.

In a follow-up study performed in the same laboratory, the role of NO in collateral growth was further unraveled. Distal femoral artery ligation in both eNOS KO and iNOS KO mice confirmed our results on normal collateral growth in eNOS KO mice but demonstrated a partially inhibited collateral growth in iNOS KO mice. Pharmacological unspecific NOS inhibition with L-NAME (NG-mono-methyl-L-arginine ester) markedly blocked collateral growth. Furthermore, the combination of eNOS KO mice, treated with the specific iNOS inhibitor L-NIL (L-N6-(1-iminoethyl)-L-lysine) completely abolished collateral growth. Both eNOS and iNOS appeared upregulated in FSS-stimulated collateral vessels. Finally, the NO donor diethylenetriamine (DETA) NONOate strongly stimulated collateral artery growth, activated perivascular monocytes, and increased proliferation markers[17].

More recently, it was suggested that NO also has an important role in maintaining native collateral density during natural growth to adulthood and in collateral remodeling in obstructive disease, the latter through regulation of cell proliferation. In particular, based on results from array profiling and network analysis, eNOS/NO may coordinately regulate expression of a network of genes controlling cell cycle checkpoints necessary for cell proliferation during collateral remodeling[18].

In summary, irrespective of source, NO is an essential link in the pathways leading to arteriogenesis, via a reciprocal counterbalance between NOS isoforms. NO released by shear stress-activated endothelium stimulates adhering monocytes to produce another NO cascade (and growth factors), resulting in proliferation of endothelial cells and subsequently smooth muscle cells of the media (Figure 1). NO may originate also from the monocytes/macrophages in the perivascular space where they are activated in an autocrine way and stimulated to produce mitogens. However, the paradox of NO influence and interplay on activation, regulation and mostly proliferation on both (endothelial and smooth muscle) sides of the elastic lamina still remains not completely resolved.

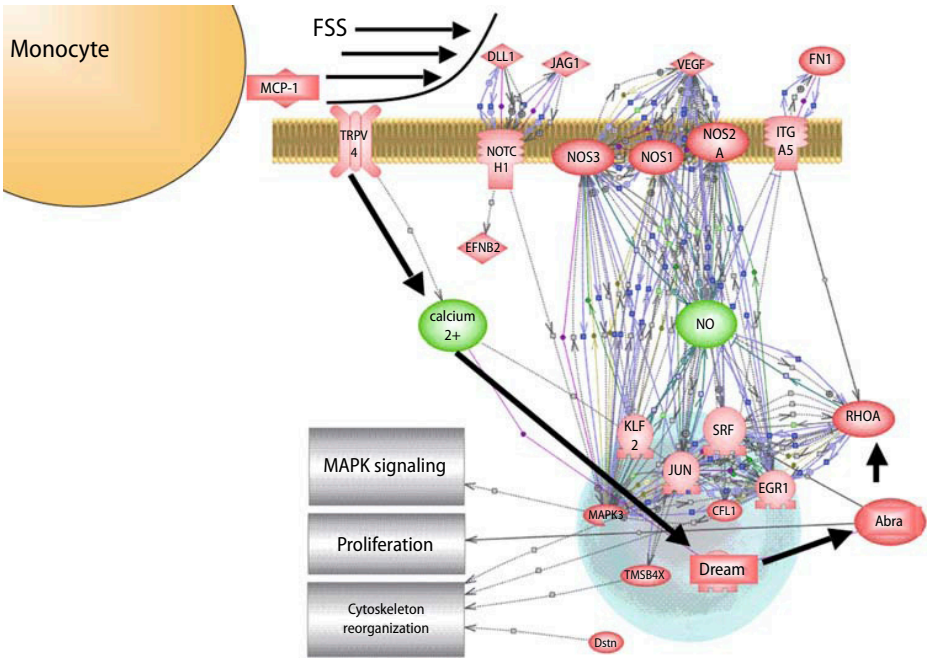


Figure 1. Role of NO in the endothelial cell during collateral growth.
Different ligands and receptors act in concert leading to an activation of a variety of transcription factors and signaling pathways via the central players calcium and NO, resulting in endothelial cell proliferation. (Figure taken from Schaper[19] with permission)

eNOS in cellular adhesion

Cellular adhesion is an indispensable mechanism in vascular remodeling, both in growth of collateral arteries as in angiogenesis during neovascularization. Furthermore, it is an important step in the development of atherosclerotic plaques. In chapter 5 we studied the pressure-induced mechanism of cellular adhesion[20]. Arteries are continuously exposed to a complex mechanical environment, including both shear stress and cyclic strain by intraluminal pressure, causing stretch of the arterial wall. Stretch of the arterial wall induces an endothelial activation leading to cellular adhesion. There has been extensive research on shear stress as a potent activator of endothelial cells, inducing adhesion of monocytes and inflammatory cells, resulting in an inflammatory response. These effects of increased shear stress are mostly attributable to the local synthesis and release of NO[21]. Hence, NO can act by downregulating cytokines, resulting in the downregulation of endothelial cell adhesion molecules (ECAMs), which are important modulators of leukocyte-endothelium interaction via leukocyte rolling along the endothelium and adhesion to the endothelium. Once the cells begin to roll, they can then

firmly attach to the endothelium via integrin interaction with endothelial intercellular adhesion molecules (ICAMs) to promote leukocyte adhesion.
An earlier study performed in our laboratory showed that stretch of the arterial wall caused by an increase in the intraluminal pressure also induces an adhesion-inflammatory pathway via the activation and nuclear translocation of the transcriptional factor nuclear factor-kappa B (NF-κB).[22] This factor regulates the transcription of a large number of inflammatory genes coding for cytokines, chemokines, and adhesion molecules[23].
To unravel the role of NO in pressure-induced cellular adhesion, we verified stretch-induced human monocyte line U937 adhesion in vessels obtained from eNOS TG mice, eNOS KO mice, or WT littermates. The lack of NO synthase in eNOS KO vessels did not affect high pressure-induced monocyte adhesion, but overexpression of eNOS was accompanied by a marked reduction of U937 monocyte binding in vessels maintained at high pressure, such that cell adhesion levels no longer varied between arteries exposed to normal or high pressure. Treatment of vessels with L-NAME restored the stretch-dependent increment in monocyte adhesion in eNOS TG vessels.
In conclusion, we suggest that hypertensive mechanical conditions facilitate cellular adhesion in vascular regions where blood flow is low or oscillatory via the transcription factor NF-κB, leading to the development of atherosclerotic lesions. However, in hypertensive vessels exposed to laminar shear stress, shear-induced NO release inhibits cellular adhesion and atherosclerotic plaque formation is hampered.

eNOS in neovascularization

The critical role of eNOS in postischemic neovascularization has been well established. In eNOS-deficient mice neovascularization is decreased resulting in severe limb loss and in parallel, up-regulation of eNOS activity by eNOS gene delivery or bovine eNOS overexpression enhances postischemic blood flow recovery and limb function[2,24,25]. In the study described in chapter 6 we confirmed these findings in healthy mice and extended them to a clinically more relevant model, the diabetic and atherosclerotic mouse[26]. eNOS overexpression in diabetic and atherosclerotic mice induced a strong postischemic neovascularization response when compared with WT mice. Mechanisms of the positive effects on neovascularization of eNOS up-regulation in ischemic tissue included increase in vasodilation[15], increase in vessel density and increase in vasculogenesis as a result of restoration of pathological endothelial and bone marrow (proangiogenic) function.
Secondly, the enhanced postischemic neovascularization response after treatment of ischemic mice with eNOS TG diabetic or eNOS TG atherosclerotic bone marrow-derived

mononuclear cells (BMMNC) revealed that up-regulation of eNOS repaired the reduced proangiogenic function of pathological BMMNC. This was explained by an increased differentiation into cells with endothelial phenotype, and an increase in their paracrine potential including VEGF release and NO-dependent vessel dilation[4]. The term "Janus phenomenon" has been suggested for the dual effect of protein (FGF, MCP-1)-, gene (VEGF, TNF)- or cell (BMMNC, EPC)-based therapeutic neovascularization on progression and destabilization of atherogenesis[27]. NO appears a possible exception to the "Janus Phenomenon" having established proneovascularization and antiatherogenic effects. However, these effects have never been investigated at the same time in the same model. Our study is the first study using eNOS cell-based gene therapy for the stimulation of postischemic neovascularization and simultaneously focusing on prevention of supplemental proatherogenic effects. We showed that WT BMMNC treatment led to increased plaque size in ischemic atherosclerotic mice, while atherosclerotic BMMNC treatment had no effect on lesion size, as previously described[28]. Moreover, we could demonstrate an antiatherogenic effect of eNOS TG atherosclerotic BMMNC, thus blunting the "Janus Phenomenon". In our studies, only occasional donor BMMNC were identified in the atherosclerotic lesions, making a physical or local contribution of transplanted BMMNC to plaque growth less likely. Another possible explanation could be paracrine effects of transplanted BMMNC through production of chemokines. It is likely that the potent antiinflammatory effect of eNOS overexpression is the main responsible factor in the antiatherogenic effect of eNOS TG atherosclerotic BMMNC treatment, as described previously[8].

In conclusion, we demonstrated that local and progenitor cell-based eNOS gene therapy has both proangiogenic and antiatherogenic effects and should be further investigated for the development of efficient therapeutic neovascularization designed to treat ischemic cardiovascular disease.

NO is a master switch in vascular remodeling

The combination of recent insights from literature and the present thesis lead to the concept of NO as one of the master switches in vascular remodeling (Figure 2). However, several key questions about the role of NO in neovascularization remain unanswered. Elucidating the role of NO in vessel specification and maturation would be of significant interest for tissue engineering and drug delivery[29]. The intracellular concentration of cGMP, a second messenger of vasodilation induced by NO, is tightly regulated by phosphodiesterases, already used clinically. Hence, it is particularly important to identify the roles in neovascularization of the cGMP-dependent and independent effects of NO[30].

Recently, an additional mechanism in tissue neovascularization, the expansion of isolated vessels by biomechanical forces, named looping angiogenesis, was discovered (Figure 3)[31]. This seems the most important mechanism of vascularization during wound healing, but has never been studied in the context of postischemic neovascularization. Future work will need to establish to which extent looping angiogenesis participates in vascular remodeling and whether there is crosstalk between the pathways for the translocation of vessels by biomechanical forces and the classical (NO-regulated) mechanisms of neovascularization.

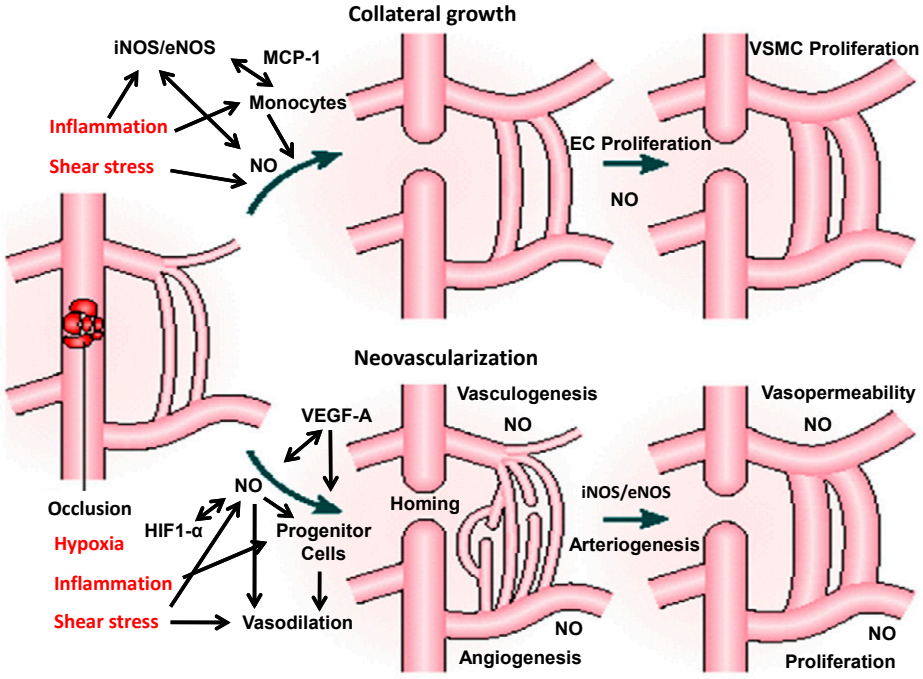


Figure 2. Both mechanisms of vascular remodeling, e.g. collateral growth and neovascularization, are modulated by NO through a complex regulation at different molecular levels. Modified from "Introduction" chapter. For more details see text.

Finally, lymphatic vessel function is as important as blood vessel function for the maintenance of tissue homeostasis, and disruption of the lymphatic system is encountered in many pathologies. Compared with our knowledge of blood vessels, our knowledge of NO function in lymphatic vessels still remains in its infancy[33].

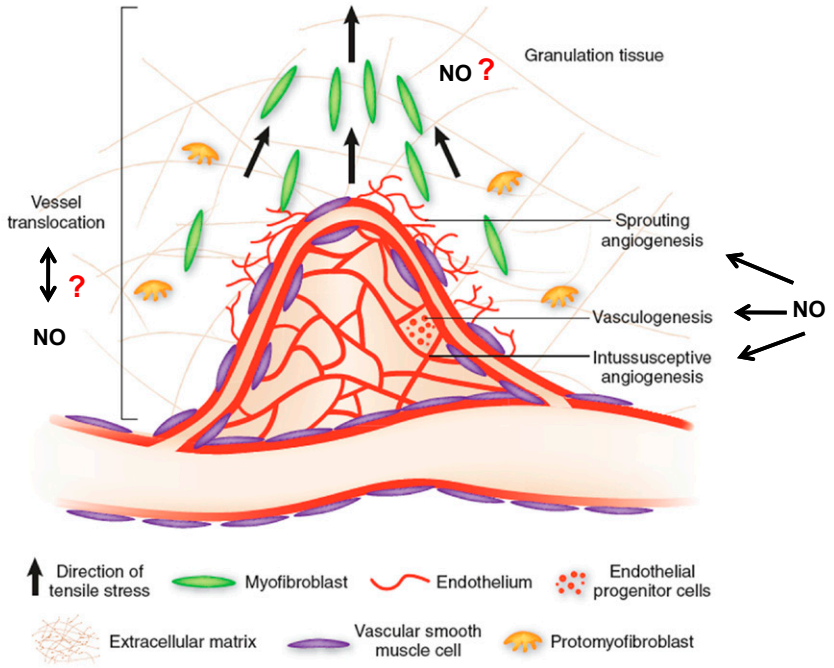


Figure 3. Looping angiogenesis, a fourth mechanism of angiogenesis for tissue vascularization, is mediated by biomechanical forces through myofibroblastic contraction pulling on existing vessel loops. (Modified from Benest & Augustin[32] with permission)

NO-based therapeutic vascular remodeling

With the current advances, especially the suggestion of the concept of NO as a master switch, the modulation of eNOS should be considered as a potent new strategy to stimulate vascular remodeling in diseases with inadequate vessel formation. Regulation of NO and NO-mediated effects can occur through multiple mechanisms, including targeting of eNOS, availability of factors that regulate NOS activity or through modulation of protein-protein interactions of NOS with its partners. For this purpose several pharmacological, gene- and stem cell therapies are under present preclinical and clinical investigations.

Modulation of eNOS activity

There are a number of factors which affect the production of NO and the ability of NO to reach or diffuse to its cellular targets. An important aspect of NOS function is the availability of substrates (L-arginine) and cofactors and the presence of NO scavenging by free radicals[34].

Under physiological conditions, the intracellular L-arginine concentration is in excess of the *K*m for eNOS, owing to L-arginine uptake via cationic amino acid transporters[35]. Under pathological conditions, NO-dependent endothelial dysfunction has been linked with reduced L-arginine transport, i.e., eNOS might become substrate limited[36]. This may be due to competition with NOS for L-arginine as substrate by other enzymes coexpressed in the same cell type, including arginase, an enzyme converting L-arginine to urea and L-ornithine, and arginine decarboxylase, a mitochondrial enzyme that produces carbon dioxide and agmatine[37]. The administration of L-arginine has been explored in conditions such as hypercholesterolemia, diabetes, and coronary artery disease. For example, L-arginine supplementation partially reversed the impairment of endothelium-dependent vasodilation in response to acetylcholine in hypercholesterolemic animals[38]. Whereas in acute studies an increase in NO bioavailability was shown, chronic L-arginine administration in patients did not have any beneficial effects[39-41]. An explanation could be that beneficial effects of chronic L-arginine administration are offset by simultaneous increments in *S*-adenosyl-L-homocysteine as a side product reducing NO bioavailability at the same time[42].

Tetrahydrobiopterin (BH4) is an essential cofactor for NOS and has profound effects on NOS function, including stabilizing its dimeric structure and facilitating and enhancing binding of L-arginine[43,44]. Reduced bioavailability of BH4 is associated with models of diabetes, atherosclerosis and hypertension[45-47]. Oral administration of BH4 shows promise for the treatment of oxidative stress-induced disorders, such as the metabolic syndrome in rats, and it slows the progression of atherosclerosis in mice and improves endothelial dysfunction in patients with coronary artery disease[48-50]. In 2008, a first study on chronic BH4 supplementation demonstrated an improvement on endothelial dysfunction in hypercholesterolemic patients[51]. Thus, BH4 represents a therapeutic relevant tool to modulate NOS function in different diseases that are characterized by reduced NOS activity or NO synthesis.

NO levels can also be raised by NO-releasing donor compounds. Delivery of NO-donors to vessel walls has resulted in decreased vessel damage in response to injury but optimization of the delivery compound is still required[52,53]. Furthermore, rather than trying to grossly substitute the effect of physiologically produced NO with exogenous sources, in many instances, the real added value would be a strategy that restores endogenous NO production from eNOS in situ.

Side effects of eNOS elevation

Upon deficiency of substrate or reduced cofactors, NOS no longer produces NO but instead transfers electrons to oxygen, thereby producing free oxygen radicals, such as superoxide (O_2-), if BH4 is lacking, or H_2O_2, if L-arginine is lacking[54,55]. These free oxygen radicals, in turn, can lead to further oxidation of BH4 (as well as other cofactors),

thereby catalyzing the whole process. If levels of superoxide increase significantly, NO outcompetes superoxide dismutase (SOD), the physiological inactivator of superoxide[56]. This reaction has the triple effect of scavenging NO, reducing its bioavailability and producing a potent oxidant, peroxynitrite (ONOO-). This condition is referred to as the so-called "uncoupled" state of eNOS. The uncoupled state of eNOS, as well as increased superoxide formation, has been associated with many vascular diseases and risk factors for atherosclerosis and consequently has been regarded as an abnormality of eNOS function[34]. Therefore, eNOS elevation, especially in pathological conditions, resulting in a rapid shortage of substrate or cofactors, may play a dual role, causing detrimental opposite effects, namely further endothelial dysfunction and progression of atherosclerotic disease.

Secondly, a disadvantage of any proangiogenic therapy is that it might also promote angiogenesis in a tumor. If NOS upregulatory therapy was applied in a patient who had undiagnosed metastatic cancer it would assist in further spread of the disease. Thus patients being enrolled into NOS proangiogenic trials would need a search for occult malignancy[57].

eNOS gene targeting

The focus of gene therapy has been to deliver the NOS gene directly to the site of injury resulting in a local increase of NO generation. The latter approach avoids the problem of systemic NO toxicity. NOS gene therapy delivery strategies include the use of either plasmid DNA, liposomes or adenoviruses as vectors with the large majority of *in vivo* studies using adenoviral vectors. From the current research reviewed, eNOS appears to be the most attractive NOS isoform for gene therapy. Administration of eNOS resulted in the best therapeutic outcome following vascular injury. Moreover, in a rat hind limb ischemia model both an adenoviral and a non-viral eNOS-gene delivery therapy induced increase in postischemic neovascularization mediated via VEGF[24,58]. In research using a mouse model of hind limb ischemia a phosphomimetic form of eNOS DNA was injected intramuscularly with electroporation to efficiently deliver the transgene. The transgene-treated (eNOS KO) animals had improved blood flow compared with untreated animals and also avoided limb loss and had healthier muscle fibers[59]. Earlier, the use of a phosphomimetic eNOS suggested an improvement over wild-type eNOS with increased NO generation and a lower tendency to uncouple[60]. Therefore, the use of this form of eNOS in cardiovascular disease models should be further evaluated.

Furthermore, adenoviruses as vectors are on the one hand efficient as a method of gene delivery but elicit an immune response. This alone can abolish any positive effect achieved by gene therapy. New vectors, both viral and non-viral, need to be developed and tested to overcome this obstacle. One strong candidate is the third-generation or helper-dependent adenoviral vector. This vector has had the coding sequence for the

viral genome deleted. Viral proteins that initiate an immune response can therefore not be synthesized[61]. This has the added effect of prolonging transgene expression, which could be advantageous in the cardiovascular setting[62].

Stem cell therapy

Stem cell-based (gene) therapy may appear as the current most promising for the stimulation of vascular remodeling in cardiovascular disease. The multifactorial mechanism of action of adult stem cells leads to positive effects on all types of vascular remodeling, including positive side effects on for example myogenesis and scar remodeling, and may therefore be the most comprehensive therapeutic option (Figure 4).

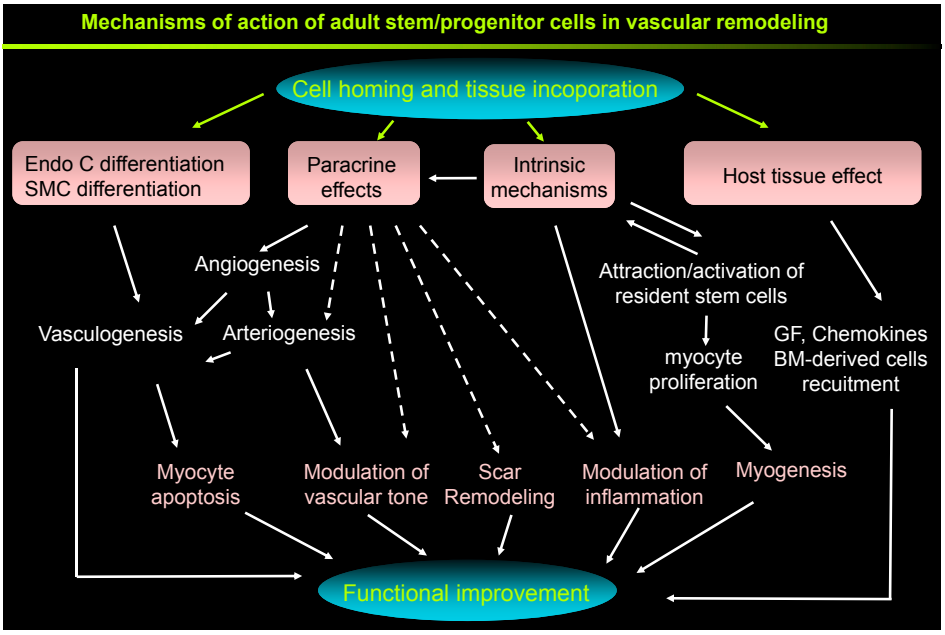


Figure 4. Multifactorial mechanisms of action of stem cells in vascular remodeling suggest stem cell-based therapy as the most promising and comprehensive for the stimulation of vascular remodeling.

However, optimizing a (stem) cell-based therapy is a long and multifactorial procedure, in which several steps need to be taken to go from the bench to bedside. Initial clinical experience may reveal new riddles that lead us back to the bench for further scrutiny before going to another clinical phase. Thus, rather than being a linear 'one-way street', therapy design can be more adequately represented as a cyclic track (Figure 5).

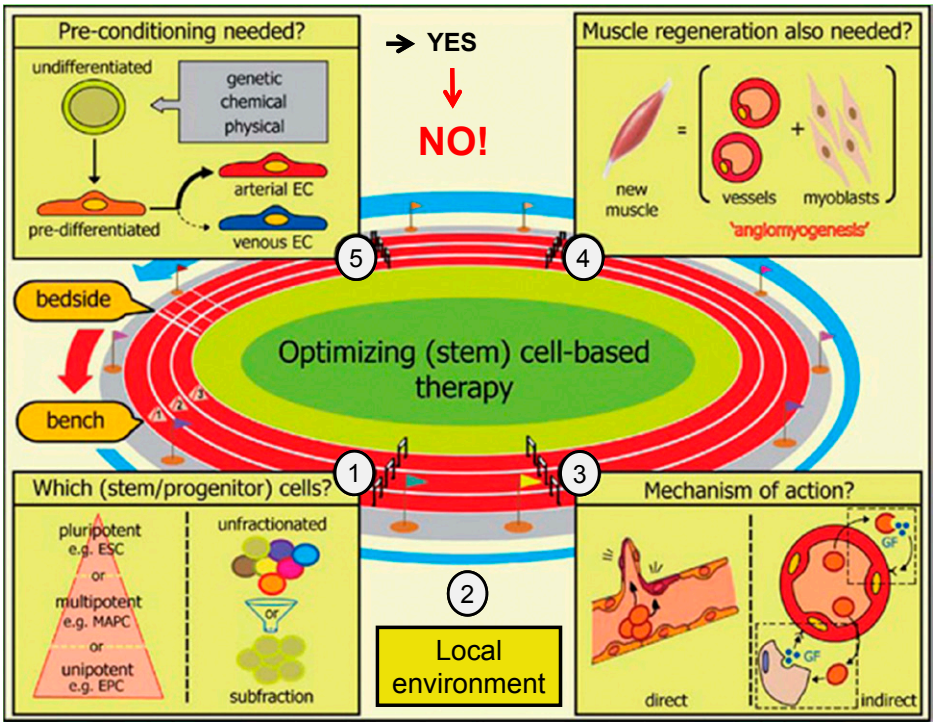


Figure 5. New hurdles in stem cell therapy for vascular remodeling. Optimizing a (stem) cell-based therapy is a long and multifactorial procedure, in which several steps ('hurdles' 1–5) need to be taken to go from the bench to the bedside. (Modified from Aranguren et al[63] with permission)

While questions about safety, dose, and administration route/timing/frequency were the hurdles to be taken in the first round, other issues may prevail in the next. The choice of the better cell source/subset is a first hurdle to be taken, which requires comparative studies between different cell types from the stem cell 'hierarchy' or between several subfractions of a certain source (hurdle 1). In our studies, we used autologous bone marrow-derived mononuclear cells, overcoming the problem of host immune response and exerting a strategy of multifactioral mechanisms of action on vascular remodeling, both direct and indirect, and on additional types of remodeling, both described in following hurdles. Secondly, the local environment should be optimized for stem cell-based neovascularization (hurdle 2). Locally elevated eNOS expression in the endothelium was shown to attract an increased amount of stem cells towards ischemic tissue. Thirdly, determining the mechanism of action is not merely an academic question but is of significant clinical relevance. Cells can directly contribute by being incorporated into nascent vessels. Alternatively, cells can deliver growth factors that instruct endogenous vascular cells (hurdle 3). We have demonstrated that eNOS-(over)expressing bone marrow cells also increase vasodilation and vasopermeability in ischemic vessels as a first step in the neovascularization response. A new mechanism

is that transplanted cells instruct host cells to secrete angiogenic growth factors. Combining revascularization with muscular regeneration ('angiomyogenesis') could be an important added value for long-term therapeutic benefit (hurdle 4). Finally, exploiting the full potential of (stem) cell therapy in ischemic cardiovascular disease may require decisions about the appropriate differentiation state, the need for arterial prespecification and other forms of genetic, chemical, or physical preconditioning (hurdle 5). In the context of this hurdle, our research on eNOS overexpression shows eNOS modification as a promising preconditioning tool for optimizing (stem) cell-based therapy. Therefore, we believe that in the near future the combination of stem cell therapy and eNOS gene targeting can lead towards a successful therapy for vascular remodeling in patients with cardiovascular disease.

And thus the final conclusion is: "Vascular Remodeling: Just say NO!"

References

1. Carmeliet, P. Angiogenesis in health and disease. *Nat Med* 9, 653-660 (2003).
2. Murohara, T., Asahara, T., Silver, M., Bauters, C., Masuda, H., Kalka, C., Kearney, M., Chen, D., Symes, J.F., Fishman, M.C., Huang, P.L. & Isner, J.M. Nitric oxide synthase modulates angiogenesis in response to tissue ischemia. *J Clin Invest* 101, 2567-2578 (1998).
3. Aicher, A., Heeschen, C., Mildner-Rihm, C., Urbich, C., Ihling, C., Technau-Ihling, K., Zeiher, A.M. & Dimmeler, S. Essential role of endothelial nitric oxide synthase for mobilization of stem and progenitor cells. *Nat Med* 9, 1370-1376 (2003).
4. You, D., Waeckel, L., Ebrahimian, T.G., Blanc-Brude, O., Foubert, P., Barateau, V., Duriez, M., Lericousse-Roussanne, S., Vilar, J., Dejana, E., Tobelem, G., Levy, B.I. & Silvestre, J.S. Increase in vascular permeability and vasodilation are critical for proangiogenic effects of stem cell therapy. *Circulation* 114, 328-338 (2006).
5. Tamarat, R., Silvestre, J.S., Le Ricousse-Roussanne, S., Barateau, V., Lecomte-Raclet, L., Clergue, M., Duriez, M., Tobelem, G. & Levy, B.I. Impairment in ischemia-induced neovascularization in diabetes: bone marrow mononuclear cell dysfunction and therapeutic potential of placenta growth factor treatment. *Am J Pathol* 164, 457-466 (2004).
6. Hill, J.M., Zalos, G., Halcox, J.P., Schenke, W.H., Waclawiw, M.A., Quyyumi, A.A. & Finkel, T. Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. *N Engl J Med* 348, 593-600 (2003).
7. Vasa, M., Fichtlscherer, S., Aicher, A., Adler, K., Urbich, C., Martin, H., Zeiher, A.M. & Dimmeler, S. Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. *Circ Res* 89, E1-7 (2001).
8. van Haperen, R., de Waard, M., van Deel, E., Mees, B., Kutryk, M., van Aken, T., Hamming, J., Grosveld, F., Duncker, D.J. & de Crom, R. Reduction of blood pressure, plasma cholesterol, and atherosclerosis by elevated endothelial nitric oxide. *J Biol Chem* 277, 48803-48807 (2002).
9. van Haperen, R., Cheng, C., Mees, B.M., van Deel, E., de Waard, M., van Damme, L.C., van Gent, T., van Aken, T., Krams, R., Duncker, D.J. & de Crom, R. Functional expression of endothelial nitric oxide synthase fused to green fluorescent protein in transgenic mice. *Am J Pathol* 163, 1677-1686 (2003).
10. Fulton, D., Fontana, J., Sowa, G., Gratton, J.P., Lin, M., Li, K.X., Michell, B., Kemp, B.E., Rodman, D. & Sessa, W.C. Localization of endothelial nitric-oxide synthase phosphorylated on serine 1179 and nitric oxide in Golgi and plasma membrane defines the existence of two pools of active enzyme. *J Biol Chem* 277, 4277-4284 (2002).
11. Ozaki, M., Kawashima, S., Yamashita, T., Hirase, T., Namiki, M., Inoue, N., Hirata, K., Yasui, H., Sakurai, H., Yoshida, Y., Masada, M. & Yokoyama, M. Overexpression of endothelial nitric oxide synthase accelerates atherosclerotic lesion formation in apoE-deficient mice. *J Clin Invest* 110, 331-340 (2002).
12. Ohashi, Y., Kawashima, S., Hirata, K., Yamashita, T., Ishida, T., Inoue, N., Sakoda, T., Kurihara, H., Yazaki, Y. & Yokoyama, M. Hypotension and reduced nitric oxide-elicited vasorelaxation in transgenic mice overexpressing endothelial nitric oxide synthase. *J Clin Invest* 102, 2061-2071 (1998).
13. Thorin, E. & Webb, D.J. Endothelium-derived endothelin-1. *Pflugers Arch* 459, 951-958 (2010).
14. Kuhlencordt, P.J., Gyurko, R., Han, F., Scherrer-Crosbie, M., Aretz, T.H., Hajjar, R., Picard, M.H. & Huang, P.L. Accelerated atherosclerosis, aortic aneurysm formation, and ischemic heart disease in apolipoprotein E/endothelial nitric oxide synthase double-knockout mice. *Circulation* 104, 448-454 (2001).

15. Mees, B., Wagner, S., Ninci, E., Tribulova, S., Martin, S., van Haperen, R., Kostin, S., Heil, M., de Crom, R. & Schaper, W. Endothelial nitric oxide synthase activity is essential for vasodilation during blood flow recovery but not for arteriogenesis. *Arterioscler Thromb Vasc Biol* 27, 1926-1933 (2007).
16. Scholz, D., Ziegelhoeffer, T., Helisch, A., Wagner, S., Friedrich, C., Podzuweit, T. & Schaper, W. Contribution of arteriogenesis and angiogenesis to postocclusive hindlimb perfusion in mice. *J Mol Cell Cardiol* 34, 775-787 (2002).
17. Troidl, K., Tribulova, S., Cai, W.J., Ruding, I., Apfelbeck, H., Schierling, W., Troidl, C., Schmitz-Rixen, T. & Schaper, W. Effects of endogenous nitric oxide and of DETA NONOate in arteriogenesis. *J Cardiovasc Pharmacol* 55, 153-160 (2010).
18. Dai, X. & Faber, J.E. Endothelial nitric oxide synthase deficiency causes collateral vessel rarefaction and impairs activation of a cell cycle gene network during arteriogenesis. *Circ Res* 106, 1870-1881 (2010).
19. Schaper, W. Collateral circulation: past and present. *Basic Res Cardiol* 104, 5-21 (2009).
20. Riou, S., Mees, B., Esposito, B., Merval, R., Vilar, J., Stengel, D., Ninio, E., van Haperen, R., de Crom, R., Tedgui, A. & Lehoux, S. High pressure promotes monocyte adhesion to the vascular wall. *Circ Res* 100, 1226-1233 (2007).
21. Cheng, C., van Haperen, R., de Waard, M., van Damme, L.C., Tempel, D., Hanemaaijer, L., van Cappellen, G.W., Bos, J., Slager, C.J., Duncker, D.J., van der Steen, A.F., de Crom, R. & Krams, R. Shear stress affects the intracellular distribution of eNOS: direct demonstration by a novel *in vivo* technique. *Blood* 106, 3691-3698 (2005).
22. Lemarie, C.A., Esposito, B., Tedgui, A. & Lehoux, S. Pressure-induced vascular activation of nuclear factor-kappaB: role in cell survival. *Circ Res* 93, 207-212 (2003).
23. De Martin, R., Hoeth, M., Hofer-Warbinek, R. & Schmid, J.A. The transcription factor NF-kappa B and the regulation of vascular cell function. *Arterioscler Thromb Vasc Biol* 20, E83-88 (2000).
24. Smith, R.S., Jr., Lin, K.F., Agata, J., Chao, L. & Chao, J. Human endothelial nitric oxide synthase gene delivery promotes angiogenesis in a rat model of hindlimb ischemia. *Arterioscler Thromb Vasc Biol* 22, 1279-1285 (2002).
25. Amano, K., Matsubara, H., Iba, O., Okigaki, M., Fujiyama, S., Imada, T., Kojima, H., Nozawa, Y., Kawashima, S., Yokoyama, M. & Iwasaka, T. Enhancement of ischemia-induced angiogenesis by eNOS overexpression. *Hypertension* 41, 156-162 (2003).
26. Mees, B., Recalde, A., Loinard, C., Tempel, D., Godinho, M., Vilar, J., van Haperen, R., Levy, B., de Crom, R. & Silvestre, J.S. Endothelial nitric oxide synthase overexpression restores the efficiency of bone marrow mononuclear cell-based therapy. *Am J Pathol* 178, 55-60 (2011).
27. Epstein, S.E., Stabile, E., Kinnaird, T., Lee, C.W., Clavijo, L. & Burnett, M.S. Janus phenomenon: the interrelated tradeoffs inherent in therapies designed to enhance collateral formation and those designed to inhibit atherogenesis. *Circulation* 109, 2826-2831 (2004).
28. Silvestre, J.S., Gojova, A., Brun, V., Potteaux, S., Esposito, B., Duriez, M., Clergue, M., Le Ricousse-Roussanne, S., Barateau, V., Merval, R., Groux, H., Tobelem, G., Levy, B., Tedgui, A. & Mallat, Z. Transplantation of bone marrow-derived mononuclear cells in ischemic apolipoprotein E-knockout mice accelerates atherosclerosis without altering plaque composition. *Circulation* 108, 2839-2842 (2003).
29. Jain, R.K. Molecular regulation of vessel maturation. *Nat Med* 9, 685-693 (2003).
30. Gu, Z., Kaul, M., Yan, B., Kridel, S.J., Cui, J., Strongin, A., Smith, J.W., Liddington, R.C. & Lipton, S.A. S-nitrosylation of matrix metalloproteinases: signaling pathway to neuronal cell death. *Science* 297, 1186-1190 (2002).

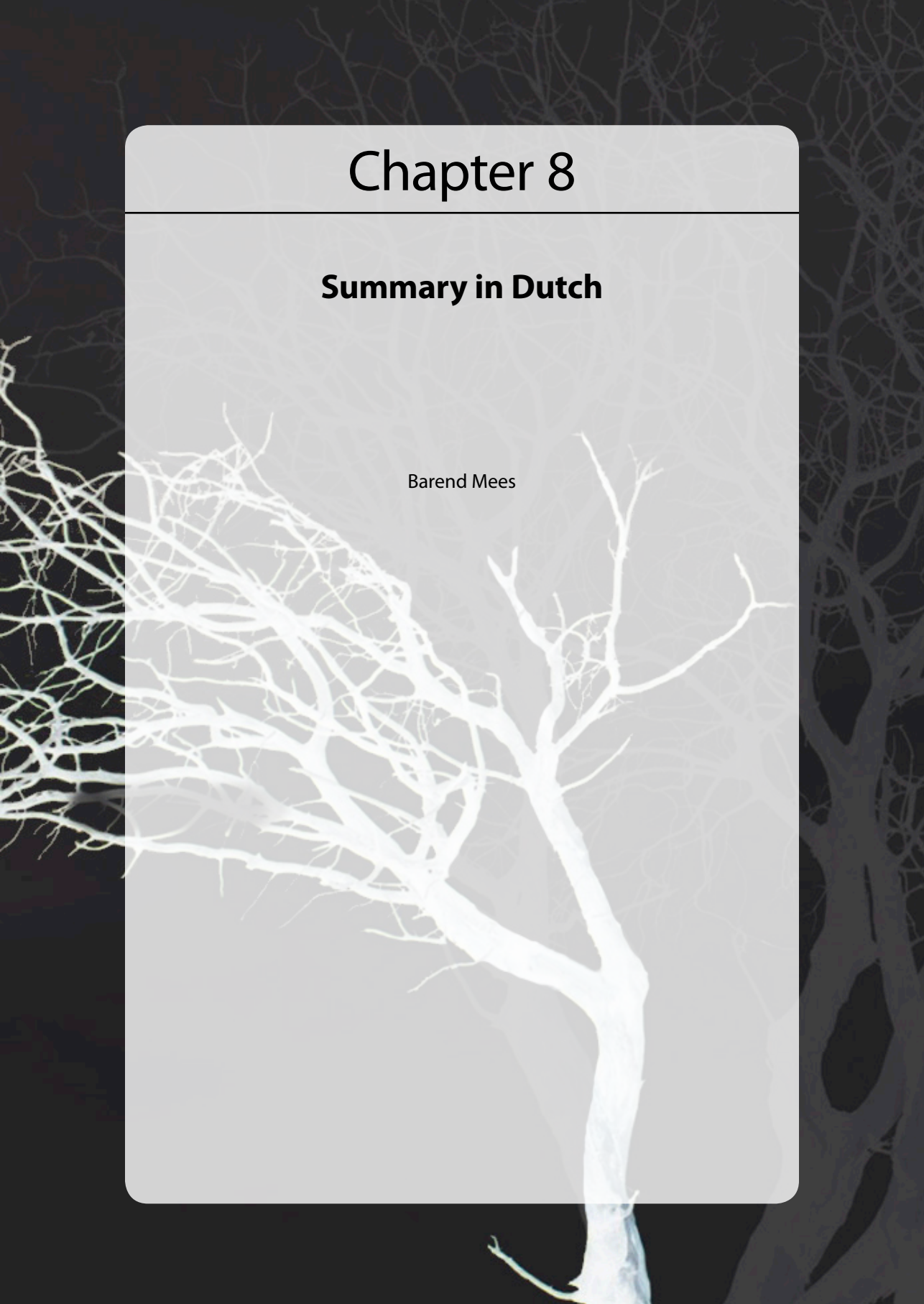
31. Kilarski, W.W., Samolov, B., Petersson, L., Kvanta, A. & Gerwins, P. Biomechanical regulation of blood vessel growth during tissue vascularization. *Nat Med* 15, 657-664 (2009).
32. Benest, A.V. & Augustin, H.G. Tension in the vasculature. *Nat Med* 15, 608-610 (2009).
33. Duda, D.G., Fukumura, D. & Jain, R.K. Role of eNOS in neovascularization: NO for endothelial progenitor cells. *Trends Mol Med* 10, 143-145 (2004).
34. Chatterjee, A., Black, S.M. & Catravas, J.D. Endothelial nitric oxide (NO) and its pathophysiologic regulation. *Vascul Pharmacol* 49, 134-140 (2008).
35. Suschek, C.V., Schnorr, O., Hemmrich, K., Aust, O., Klotz, L.O., Sies, H. & Kolb-Bachofen, V. Critical role of L-arginine in endothelial cell survival during oxidative stress. *Circulation* 107, 2607-2614 (2003).
36. Kamada, Y., Nagaretani, H., Tamura, S., Ohama, T., Maruyama, T., Hiraoka, H., Yamashita, S., Yamada, A., Kiso, S., Inui, Y., Ito, N., Kayanoki, Y., Kawata, S. & Matsuzawa, Y. Vascular endothelial dysfunction resulting from L-arginine deficiency in a patient with lysinuric protein intolerance. *J Clin Invest* 108, 717-724 (2001).
37. Hein, T.W., Zhang, C., Wang, W., Chang, C.I., Thengchaisri, N. & Kuo, L. Ischemia-reperfusion selectively impairs nitric oxide-mediated dilation in coronary arterioles: counteracting role of arginase. *FASEB J* 17, 2328-2330 (2003).
38. Cooke, J.P., Singer, A.H., Tsao, P., Zera, P., Rowan, R.A. & Billingham, M.E. Antiatherogenic effects of L-arginine in the hypercholesterolemic rabbit. *J Clin Invest* 90, 1168-1172 (1992).
39. Creager, M.A., Gallagher, S.J., Girerd, X.J., Coleman, S.M., Dzau, V.J. & Cooke, J.P. L-arginine improves endothelium-dependent vasodilation in hypercholesterolemic humans. *J Clin Invest* 90, 1248-1253 (1992).
40. Stroes, E.S., Koomans, H.A., de Bruin, T.W. & Rabelink, T.J. Vascular function in the forearm of hypercholesterolaemic patients off and on lipid-lowering medication. *Lancet* 346, 467-471 (1995).
41. Blum, A., Hathaway, L., Mincemoyer, R., Schenke, W.H., Kirby, M., Csako, G., Waclawiw, M.A., Panza, J.A. & Cannon, R.O., 3rd. Oral L-arginine in patients with coronary artery disease on medical management. *Circulation* 101, 2160-2164 (2000).
42. Loscalzo, J. Adverse effects of supplemental L-arginine in atherosclerosis: consequences of methylation stress in a complex catabolism? *Arterioscler Thromb Vasc Biol* 23, 3-5 (2003).
43. Cosentino, F. & Luscher, T.F. Tetrahydrobiopterin and endothelial nitric oxide synthase activity. *Cardiovasc Res* 43, 274-278 (1999).
44. Cosentino, F., Patton, S., d'Uscio, L.V., Werner, E.R., Werner-Felmayer, G., Moreau, P., Malinski, T. & Luscher, T.F. Tetrahydrobiopterin alters superoxide and nitric oxide release in prehypertensive rats. *J Clin Invest* 101, 1530-1537 (1998).
45. d'Uscio, L.V., Milstien, S., Richardson, D., Smith, L. & Katusic, Z.S. Long-term vitamin C treatment increases vascular tetrahydrobiopterin levels and nitric oxide synthase activity. *Circ Res* 92, 88-95 (2003).
46. Meininger, C.J., Cai, S., Parker, J.L., Channon, K.M., Kelly, K.A., Becker, E.J., Wood, M.K., Wade, L.A. & Wu, G. GTP cyclohydrolase I gene transfer reverses tetrahydrobiopterin deficiency and increases nitric oxide synthesis in endothelial cells and isolated vessels from diabetic rats. *FASEB J* 18, 1900-1902 (2004).
47. Stroes, E., Kastelein, J., Cosentino, F., Erkelens, W., Wever, R., Koomans, H., Luscher, T. & Rabelink, T. Tetrahydrobiopterin restores endothelial function in hypercholesterolemia. *J Clin Invest* 99, 41-46 (1997).
48. Wang, X., Hattori, Y., Satoh, H., Iwata, C., Banba, N., Monden, T., Uchida, K., Kamikawa, Y. & Kasai, K. Tetrahydrobiopterin prevents endothelial dysfunction and restores adiponectin levels in rats. *Eur J Pharmacol* 555, 48-53 (2007).

49. Hattori, Y., Hattori, S., Wang, X., Satoh, H., Nakanishi, N. & Kasai, K. Oral administration of tetrahydrobiopterin slows the progression of atherosclerosis in apolipoprotein E-knockout mice. *Arterioscler Thromb Vasc Biol* 27, 865-870 (2007).
50. Maier, W., Cosentino, F., Lutolf, R.B., Fleisch, M., Seiler, C., Hess, O.M., Meier, B. & Luscher, T.F. Tetrahydrobiopterin improves endothelial function in patients with coronary artery disease. *J Cardiovasc Pharmacol* 35, 173-178 (2000).
51. Cosentino, F., Hurlimann, D., Delli Gatti, C., Chenevard, R., Blau, N., Alp, N.J., Channon, K.M., Eto, M., Lerch, P., Enseleit, F., Ruschitzka, F., Volpe, M., Luscher, T.F. & Noll, G. Chronic treatment with tetrahydrobiopterin reverses endothelial dysfunction and oxidative stress in hypercholesterolaemia. *Heart* 94, 487-492 (2008).
52. Chaux, A., Ruan, X.M., Fishbein, M.C., Ouyang, Y., Kaul, S., Pass, J.A. & Matloff, J.M. Perivascular delivery of a nitric oxide donor inhibits neointimal hyperplasia in vein grafts implanted in the arterial circulation. *J Thorac Cardiovasc Surg* 115, 604-612; discussion 612-604 (1998).
53. Pearce, C.G., Najjar, S.F., Kapadia, M.R., Murar, J., Eng, J., Lyle, B., Aalami, O.O., Jiang, Q., Hrabie, J.A., Saavedra, J.E., Keefer, L.K. & Kibbe, M.R. Beneficial effect of a short-acting NO donor for the prevention of neointimal hyperplasia. *Free Radic Biol Med* 44, 73-81 (2008).
54. Kawashima, S. & Yokoyama, M. Dysfunction of endothelial nitric oxide synthase and atherosclerosis. *Arterioscler Thromb Vasc Biol* 24, 998-1005 (2004).
55. Fleming, I. & Busse, R. Molecular mechanisms involved in the regulation of the endothelial nitric oxide synthase. *Am J Physiol Regul Integr Comp Physiol* 284, R1-12 (2003).
56. Beckman, J.S. & Koppenol, W.H. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *Am J Physiol* 271, C1424-1437 (1996).
57. O'Connor, D.M. & O'Brien, T. Nitric oxide synthase gene therapy: progress and prospects. *Expert Opin Biol Ther* 9, 867-878 (2009).
58. Namba, T., Koike, H., Murakami, K., Aoki, M., Makino, H., Hashiya, N., Ogihara, T., Kaneda, Y., Kohno, M. & Morishita, R. Angiogenesis induced by endothelial nitric oxide synthase gene through vascular endothelial growth factor expression in a rat hindlimb ischemia model. *Circulation* 108, 2250-2257 (2003).
59. Qian, H.S., Liu, P., Huw, L.Y., Orme, A., Halks-Miller, M., Hill, S.M., Jin, F., Kretschmer, P., Blasko, E., Cashion, L., Szymanski, P., Vergona, R., Harkins, R., Yu, J., Sessa, W.C., Dole, W.P., Rubanyi, G.M. & Kauser, K. Effective treatment of vascular endothelial growth factor refractory hindlimb ischemia by a mutant endothelial nitric oxide synthase gene. *Gene Ther* 13, 1342-1350 (2006).
60. Fulton, D., Gratton, J.P., McCabe, T.J., Fontana, J., Fujio, Y., Walsh, K., Franke, T.F., Papapetropoulos, A. & Sessa, W.C. Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature* 399, 597-601 (1999).
61. Palmer, D.J. & Ng, P. Helper-dependent adenoviral vectors for gene therapy. *Hum Gene Ther* 16, 1-16 (2005).
62. Wen, S., Graf, S., Massey, P.G. & Dichek, D.A. Improved vascular gene transfer with a helper-dependent adenoviral vector. *Circulation* 110, 1484-1491 (2004).
63. Aranguren, X.L., Verfaillie, C.M. & Luttun, A. Emerging hurdles in stem cell therapy for peripheral vascular disease. *J Mol Med* 87, 3-16 (2009).

Chapter 8

Summary in Dutch

Barend Mees



"Een mens is net zo oud als zijn endotheel…"

Hart- en vaatziekten en kanker zijn verantwoordelijk voor het grootste gedeelte van ziekte en sterfte in de Westerse wereld in een vergelijkbare verhouding. In patiënten met hart- en vaatziekte functioneert de binnenbekleding van de bloedvaten, het endotheel, minder goed. Endotheel is de initiator en modulator van de continue aanpassing van bloedvaten tijdens vele gezonde of ziekteprocessen in ons hart- en vaatstelsel, zoals embryonale ontwikkeling, wondgenezing, hoge bloeddruk, tumorgroei en aderverkalking (atherosclerose). Wanneer bloedvaten vernauwd of afgesloten geraken, passen zij zich aan door middel van vaatverwijding of verschillende vormen van vaatgroei. Dit proces wordt vaatremodellering genoemd. Een normale vaatremodellering is essentieel voor een gezond hart- en vaatstelsel. Omgekeerd is bij hart- en vaatpatiënten dit aanpassingsvermogen gestoord als gevolg van een verminderde endotheelfunctie. Zo ontstaat atherosclerose met als gevolg vaatvernauwingen en -afsluitingen en tenslotte complicaties door zuurstoftekort, zoals een hartinfarct of weefselversterf van het been.

Zeg YES tegen vaatremodellering…

Een adequate vaatremodellering behoedt het lichaam voor complicaties van een bloedvatvernauwing. Vaatremodellering wordt gekenmerkt door een acute vaatverwijding gevolgd door vaatgroei. Er wordt onderscheid gemaakt tussen verschillende vormen van vaatgroei. Collateraalvorming beschrijft de expansieve groei van reeds aanwezige bloedvaten tot nieuwe netwerken die de bloedstroom om de afsluiting leiden. Neovascularizatie bestaat uit drie mechanismen van vaatgroei: vasculogenese beschrijft de vorming van bloedvaten door endotheliale voorloper-stamcellen, angiogenese is het ontstaan van nieuwe haarvaten en arteriogenese refereert aan de stabilisatie van deze nieuwe vasculaire structuren. De initiële trigger voor collateraalvorming is de verandering van bloedstroom door collaterale bloedvaten, terwijl de stimulans voor neovascularisatie het zuurstoftekort in het weefsel stroomafwaarts van de vaatafsluiting is. In het verdere proces hierna reguleren echter dezelfde groeifactoren en ontstekingscellen de verschillende vormen van vaatremodellering. Hierdoor worden, in het geval van vernauwend vaatlijden, de verschillende vormen van vaatremodellering beschouwd als complementaire onderdelen van het proces van bloedstroom- en weefselperfusie herstel.

Zeg NO in het hart- en vaatstelsel…

Er zijn maar weinig ontdekkingen van het kaliber van de impact die NO (stikstofmonoxide) heeft gehad op de biologie sinds 25 jaar geleden ontdekt werd dat het gas NO een belangrijk molecuul bleek in het hart- en vaatstelsel. In 1992 werd NO door het blad Science uitgeroepen tot molecuul van het jaar en in 1998 kregen de ontdekkers van de rol van NO in het cardiovasculaire systeem hiervoor de Nobelprijs. In het hart- en vaatstelsel speelt de productie van NO een essentiële rol door regulering van vaatverwijding, vaatgroei, vaatverkalking en vaatontsteking. NO wordt in vrijwel het gehele lichaam geproduceerd door het enzym NOS (*Nitric Oxide Synthase*). Voor het hart- en vaatstelsel is de productie van NO door het endotheel via eNOS (endotheliaal NOS) het belangrijkst. In patiënten met hart- en vaatziekte is als gevolg van verminderde endotheelfunctie de activiteit van eNOS gestoord en daardoor de productie van NO verminderd. Deze verminderde beschikbaarheid van NO bij alle hart- en vaatpatiënten wordt grotendeels verantwoordelijk gehouden voor het ontstaan van ziektes als atherosclerose, suikerziekte en hoge bloeddruk.

Doel van het proefschrift

Het doel van dit proefschrift was ten eerste het verder ophelderen van de precieze rol die NO speelt in vaatremodellering. Vervolgens werden de effecten van "therapeutische" verhoging van eNOS op vaatremodellering onderzocht, zowel in gezonde als zieke hart- en vaatstelsels bij muizen.

De eNOS transgene muizen

In hoofdstuk 2 en 3 worden het genereren en de karakterisering van twee verschillende genetisch gemodificeerde muizen besproken. De eNOS transgene (TG) muis heeft in zijn eigen DNA het menselijk eNOS gen (DNA fragment) ingebouwd gekregen. De eNOS-GFP TG muis heeft eveneens een menselijk eNOS gen ingebouwd gekregen, waarbij tevoren dit gen gekoppeld is aan een gen voor een fluorescerend eiwit (GFP; *Green Fluorescent Protein*) , waardoor het eNOS eiwit door het gehele lichaam gevisualiseerd kan worden door de fluorescentie van het GFP. Lokalisatiestudies laten in beide muizen zien dat het humane eNOS in alle endotheelcellen aanwezig is. In beide typen eNOS TG muizen is zowel de eNOS activiteit als de NO productie tien keer verhoogd vergeleken met niet genetisch gemodificeerde controle muizen. Hierdoor hebben de eNOS TG muizen een duidelijk lagere bloeddruk, een lager cholesterolniveau in het bloed en

significant minder atherosclerose. Concluderend, zijn deze eNOS TG muizen de enige muizen in de wereld met een verhoogd humaan eNOS, leidend tot een karakteristiek gunstig hart- en vaatrisicoprofiel en daardoor zeer bruikbaar om de rol van NO in het gezonde en zieke cardiovasculaire systeem verder te onderzoeken.

Zonder eNOS wel, maar zonder NO geen collateralen…

In hoofdstuk 4 wordt de rol van eNOS in collateraalvorming onderzocht met behulp van drie muismodellen met een verschillende hoeveelheid eNOS. In de hierboven beschreven eNOS TG muizen is de eNOS activiteit vele malen verhoogd vergeleken met een normale gezonde muis. In eNOS-deficiënte ("*knockout*"; eNOS KO) muizen staat het eNOS gen uitgeschakeld en is eNOS activiteit dus geheel afwezig. Dit diermodel is jaren geleden in een ander laboratorium ontwikkeld en inmiddels commercieel verkrijgbaar via gespecialiseerde proefdierbedrijven. Als controle muizen worden normale gezonde muizen gebruikt. Door de liesslagader af te binden halverwege het bovenbeen ontstaat een situatie specifiek voor collateraalvorming waarbij zuurstoftekort geen prominente rol speelt, maar juist de verandering van bloedstroom. Verhoging van eNOS activiteit bleek in dit model alleen een positief effect op het herstel van de bloedstroom te hebben direct na de operatie, maar uiteindelijk geen verschil te maken in collateraalvorming. Dit effect wordt veroorzaakt door een krachtige acute vaatverwijding. In eNOS KO muizen daarentegen veroorzaakte het afbinden van de liesslagader een sterk vertraagd herstel van bloedstroom en aanzienlijk weefselversterf als gevolg van de afwezigheid van eNOS. Microscopisch onderzoek liet echter evenveel en even grote collateralen zien in eNOS KO muizen als in gezonde muizen. De veroorzaakte weefselschade in de eNOS KO muizen blijkt veroorzaakt te worden door het feit dat de collateralen in de eNOS KO muizen niet kunnen vaatverwijden. De conclusie van dit hoofdstuk luidt dan ook dat bij het herstel van de bloedstroom in geval van een vaatafsluiting eNOS geen onmisbare rol speelt bij het vormen van collateralen, maar wel bij het (acute) proces van vaatverwijding. In een latere vervolgstudie is door onze onderzoeksgroep aangetoond dat NO zelf wel een onmisbare rol speelt in collateraalvorming omdat in afwezigheid van eNOS een andere vorm van NOS (inducible NOS) de noodzakelijke productie van NO overneemt. Dit gebeurt via een complex nog niet volledig ontrafeld mechanisme van balans en compensatie tussen de verschillende NOS isoformen.

eNOS beschermt tegen atherosclerose in vaten met hoge bloeddruk…

Bij het ontstaan van atherosclerotische laesies (kalkafzettingen in de vaatwand, ook wel plaques genoemd) is de belangrijke eerste stap de hechting aan het endotheel en vervolgens binnendringen in de vaatwand van ontstekingscellen uit de bloedbaan. In gedeelten van bloedvaten waar hoge bloeddruk of hoge oscillerende druk door turbulentie heerst, is het endotheel gevoeliger voor het aanhechten en binnendringen in de vaatwand van deze cellen en daarom ontstaan op deze plekken vaker plaques. In hoofdstuk 5 wordt de rol van eNOS en NO beschreven in dit mechanisme. Door een geïsoleerde muizen halsslagader, opgehangen in een proefopstelling met variabel bloeddruksysteem, werden ontstekingscellen geperfundeerd. Aan het endotheel van bloedvaten onder hoge bloeddruk hechtten drie keer zo veel ontstekingscellen als aan endotheel van bloedvaten onder normale bloeddruk. In muizen zonder eNOS (eNOS KO) was dit verschijnsel identiek. In de eNOS TG muizen daarentegen was de celaanhechting onder normale bloeddruk gelijk aan die bij gezonde muizen, maar steeg deze niet in condities van hoge bloeddruk. Concluderend, beschermt een verhoogde eNOS activiteit en dus een verhoogde NO beschikbaarheid tegen hechting van ontstekingscellen aan de vaatwand van bloedvaten onder hoge bloeddruk. Hiermee kan één van de mechanismen waardoor NO beschermt tegen het ontwikkelen van atherosclerose verklaard worden.

eNOS voor meer nieuwe vaten…

In eerdere studies is de cruciale rol van eNOS in neovascularizatie al aangetoond. In eNOS muizen blijkt de neovascularizatierespons op een doorbloedingsstoornis sterk verminderd te zijn, wat resulteert in ernstig weefselversterf. Daarentegen wordt de neovascularizatierespons juist versterkt bij proefdieren waarbij de eNOS activiteit verhoogd is, bijvoorbeeld door middel van gentherapie. In hoofdstuk 6 wordt het bovenstaande bevestigd in een gezonde situatie en vervolgens onderzocht in een hart- en vaatziekte achtergrond. Het model, dat hiervoor gebruikt wordt, is vergelijkbaar met het model uit hoofdstuk 4, omdat eveneens de liesslagader van een muis wordt afgebonden. Het gebruikte model in hoofdstuk 6 is echter een model waarbij de slagader hoger (dichter bij de buik) in de lies wordt afgebonden om niet alleen collateraalvorming door verandering van bloedstroom, maar voornamelijk neovascularizatie door het ontstane zuurstoftekort te stimuleren. In dit model hadden onze eNOS TG muizen een versterkte neovascularizatierespons na het afbinden van de liesslagader vergeleken met normale muizen, met als gevolg betere doorbloeding en meer nieuwe bloedvaatjes in het onderbeen. Muizen met een hart- en vaatziekte, zoals atherosclerose of diabetes, hadden

in hetzelfde afbindmodel echter een sterk verminderde neovascularizatierespons. Als deze muizen gekruist werden met een eNOS TG muis bleken daarentegen de eNOS TG atherosclerotische of eNOS TG diabetische muizen net zo'n adequate neovascularizatierespons te hebben als gezonde normale muizen. Verhoging van eNOS activiteit in het lokale weefsel herstelt dus de gestoorde neovascularizatierespons van muizen met een hart- en vaatziekte. Het mechanisme hierachter bestaat uit een krachtigere vaatverwijding en een toename van vaatgroei, zowel de uitgroei van bestaande als het ontstaan van nieuwe bloedvaatjes, door verhoogde NO productie door eNOS.

eNOS voor betere stamcellen en (dus) nog meer nieuwe vaten…

In het tweede deel van hoofdstuk 6 wordt de rol van stamcellen om neovascularizatie te stimuleren onderzocht. Vanuit eerdere wetenschappelijke literatuur is bekend dat stamcellen van gezonde muizen de neovascularizatierespons na afbinden van een liesslagader krachtig kunnen stimuleren en dat stamcellen van diabetische of atherosclerotische muizen dit niet of minder goed kunnen. In onze studie werden een dag na het afbinden van de liesslagader bij dezelfde muizen 1 miljoen vers geoogste stamcellen in de bloedbaan geïnjecteerd. Stamcellen van normale muizen bleken net zo goed neovascularizatie te stimuleren als stamcellen van de eNOS TG muizen. Stamcellen van atherosclerotische en diabetische muizen bleken een vergelijkbaar (namelijk geen positief) effect op de neovascularisatierespons te hebben als een injectie met placebo. Echter, stamcellen van eNOS TG atherosclerotische en eNOS TG diabetische muizen hadden hetzelfde stimulerende effect op neovascularizatie als de stamcellen van de gezonde muizen. Het verhogen van de eNOS activiteit in de stamcellen van muizen met hart- en vaatziekte is dus een manier om de verminderde neovascularizatie-stimulerende functie van deze stamcellen op te heffen. Dit gunstige effect wordt verklaard door een toegenomen NO productie van deze cellen, een toegenomen differentiatie van deze stamcellen in de richting van vaatcellen en een toename in de productie van vaat-groeifactoren door deze stamcellen.

Is NO een oplossing voor het "Janus fenomeen"…?

Janus was een mythologische Romeinse god met één hoofd en twee gezichten kijkend in tegenovergestelde richtingen. De term het "Janus fenomeen" is gebruikt voor het duale effect van therapeutische neovascularizatie op de groei van atherosclerotische plaques. In eerdere studies is aangetoond dat zowel eiwit- als stamceltherapie in een atherosclerotische muis met een afgebonden liesslagader niet alleen vaatgroei

stimuleert, maar ook een significante groei van de atherosclerotische laesies. Dit kan verklaard worden doordat de aangrijpingsmechanismen voor het stimuleren van verschillende vormen van vaatgroei, zoals arteriogenese en collateraalvorming, vaak dezelfde cellulaire mechanismen volgen als de groei van atherosclerose. NO lijkt hierop één mogelijke uitzondering, omdat van NO zowel pro-neovascularizatie als anti-atherogene effecten zijn aangetoond, hoewel altijd los van elkaar. Als gunstig neveneffect in onze studie uit hoofdstuk 6 bleek echter dat de injectie van eNOS TG atherosclerotische stamcellen in atherosclerotische muizen zelfs een remmend effect had op de groei van de atherosclerotische laesies. Dit effect zou verklaard kunnen worden door een anti-ontstekingseffect van verhoogde eNOS activiteit. Dit is de eerste keer dat in eenzelfde studie zowel pro-neovascularizatie als anti-atherogene effecten van NO zijn gevonden.

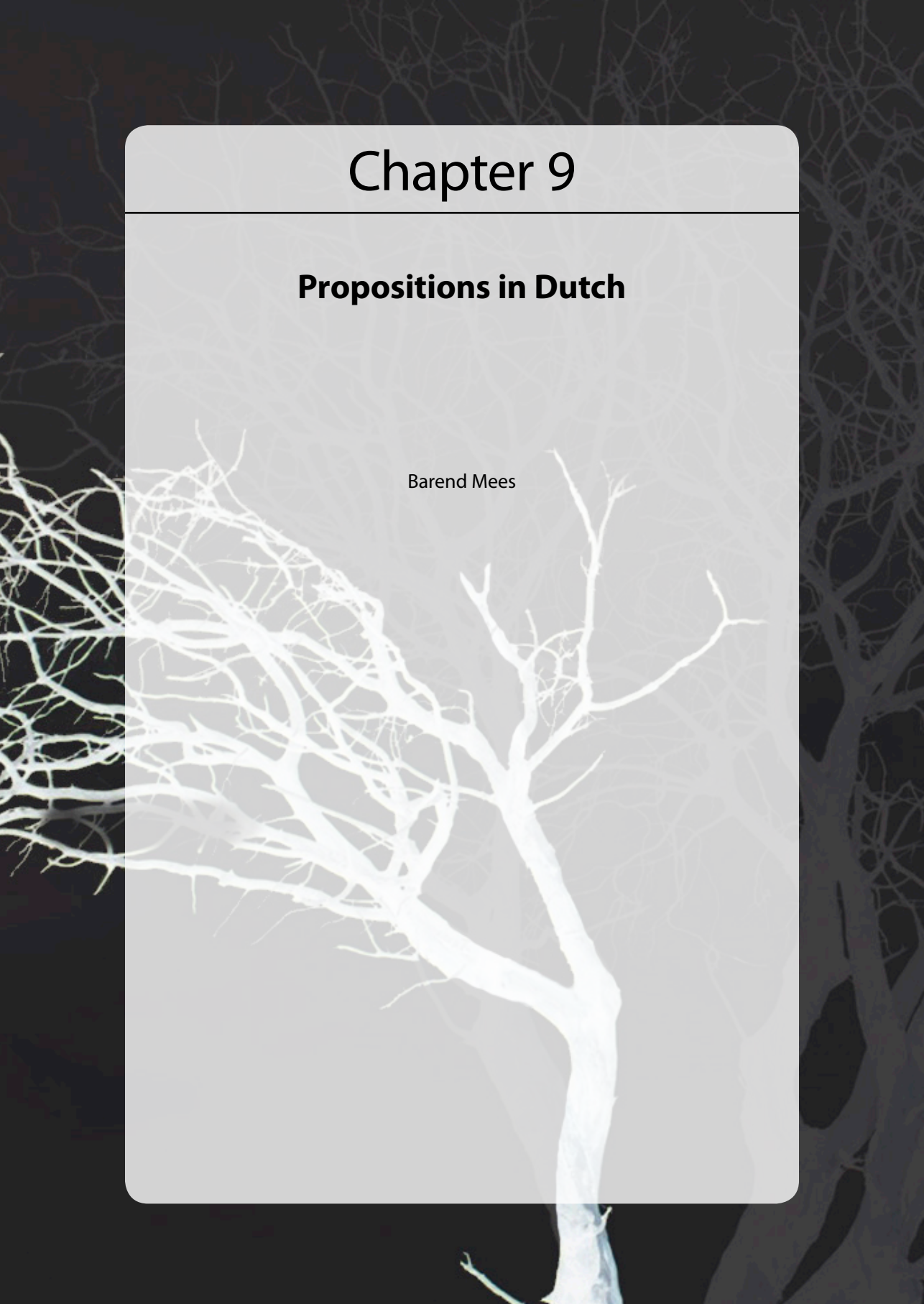
Vasculaire remodellering: Zeg maar NO!

De combinatie van inzichten in de recente literatuur en bovenstaande studies maken aannemelijk dat NO één van de belangrijkste schakels is in vasculaire remodellering. Dit maakt de stimulering van vasculaire remodellering door het verhogen van de beschikbaarheid van NO een veelbelovende kandidaat voor de ontwikkeling van een nieuwe therapie voor hart- en vaatziekten. Stimuleren van NO of NO-gemedieerde effecten kan plaatsvinden via multipele mechanismen, zoals het genetisch opreguleren van eNOS, het verhogen van substraten voor de productie van NO en het bevorderen van eiwit-eiwit interacties met eNOS. Hiervoor worden momenteel verschillende farmacologische, gen- en stamceltherapieën in preklinische of klinische situaties onderzocht. Ons inziens vormt de combinatie van stamceltherapie en eNOS gen-modulatie één van de meest kansrijke strategieën voor een succesvolle therapie voor vasculaire remodellering in patiënten met hart- en vaatziekte.

Chapter 9

Propositions in Dutch

Barend Mees



Stellingen

behorende bij het proefschrift
Vascular Remodeling: Just say NO!

Barend Mees, Juni 2011

1. Vasodilatatie via een NO-werkingsmechanisme speelt een fundamentele rol bij het herstel van de bloedstroom na een arteriele occlusie (dit proefschrift).
2. Zonder NO geen collateralen, maar zonder eNOS wel (dit proefschrift).
3. Het feit dat eNOS overexpressie zowel in diabetische muizen als in atherosclerotische muizen de gestoorde neovascularizatie-respons herstelt is een nieuwe aanwijzing dat NO fungeert als een "hoofdschakelaar" op het moederbord van vasculaire remodellering (dit proefschrift).
4. Het verhogen van eNOS activiteit is anno 2011 de meest veelbelovende strategie voor een effectieve therapeutische vasculaire remodellering zonder nadelige neveneffecten op de progressie van atherosclerose ("Janus Fenomeen"; dit proefschrift).
5. Het phenotype van de eNOS transgene muis is de droom voor iedere vaatpatiënt (dit proefschrift).
6. Therapeutische vasculaire remodellering is net als wijn maken: het geheim ligt vaak in de "terroir".
7. Ook al is de ontwikkeling van het elektronisch patiënten dossier door de politiek tot stilstand gebracht, dit proefschrift demonstreert dat de digitale revolutie in de geneeskunde niet te stoppen is.
8. Een amputatie bij een vaatpatiënt is geen operatie voor een (ongesuperviseerde) assistent.
9. Het ultieme wetenschappelijke onderzoek van de chirurg dient erop gericht te zijn om het vak van chirurg overbodig te maken.
10 Het leren zien van schoonheid is even belangrijk in de wetenschap als in het leven. (geïnspireerd door Schoonheid in de Wetenschap van Hans Galjaard)
11. "The way you cut your meat reflects the way you live" (Confucius).

Chapter 10

Thanks

Barend Mees

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Chapter 11

List of Publications

Book chapters

"Endothelial nitric oxide synthase in cardiovascular homeostasis and disease". Observations in endothelial NO synthase transgenic mice.
Duncker D, van Haperen R, van Deel E, de Waard M, Mees B, de Crom.
In: Ince C. (Ed): "The Physiological Genomics of the Critically Ill Mouse". Kluwer Academic Publishers, Amsterdam 2004.

Hoofdstuk vaatchirurgie
Barend Mees & Hero van Urk.
In: Leidraad Chirurgie, Bohn Stafleu van Loghum, Houten 2005.

Peer-reviewed articles

Go to: http://www.ncbi.nlm.nih.gov/pubmed?term=Mees%20B%20NOT%20NEJM

Chapter 12

PhD Portfolio

Erasmus MC
Universitair Medisch Centrum Rotterdam

PhD Portfolio Summary

Summary of PhD training and teaching activities

Name PhD student: Barend Mees	PhD period: 2003 – 2011
Erasmus MC Department: Cell Biology & Vascular Surgery	Promotors: Prof. H. van Urk & Prof. J. Hamming
Research School: MGC & COEUR	Supervisor: Dr. M. de Crom

1. PhD training

	Year	Workload (ECTS)
General academic skills		
- Experimental animal course (art. 9)	2003	3
- How to write an article (NTtvG)	2007	0.5
- Advanced radiation course (Boerhaave)	2010	1
Research skills		
- Basics of epidemiology (Boerhaave)	2009	1
In-depth courses (e.g. Research school, Medical Training)		
- Master in Cardiovascular Research (COEUR)	2003 – 2006	7.5
- Master of Molecular Medicine (MGC)	2003 – 2006	7.5
Presentations		
- National conferences (o.a. Chirurgendagen, Endotheeldag, SEOHS)	2003 – 2011	5
- International conferences (o.a. AHA, ACC, ESVS, ESSR, IVBM, EVGN)	2004 – 2011	5
Seminars and workshops		
- Journal club	2003 – 2006	0.5
- MGC symposia & workshops	2003 – 2005	1
- EVGN seminar (Paris)	2006	0.5

2. Teaching activities

	Year	Workload (ECTS)
Lecturing		
- Teaching OR nurses in training	2003 – 2004	0.5
- Teaching Medical students during internship	2006 – 2011	5
Supervising practicals and excursions		
- Supervising first aid examinations (Medical students)	2003 – 2004	0.5

Chapter 13

Curriculum Vitae

Personal details

Name	Mees, Barend Mathijs Eduard
Date of birth	October 10, 1975
E-mail	bmemees@hotmail.com

Profile

An ambitious team player, taking the lead towards improving performance with positive and creative energy. Always eager to innovate. Humor is an important asset. Responsible and reliable. Caring for patients, with honest interest and respect. Family man.

For more


Barend Mees


http://nl.linkedin.com/in/barendmees

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Chapter 14

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