Chapter 15

Subcellular ROS Signaling in Cardiovascular Disease

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Additional information is available at the end of the chapter

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Abstract

This review discusses recent findings that have challenged the long-held dogma in the field that reduction in reaction oxygen species (ROS) would improve clinical outcome in the patients with cardiovascular disease (CVD). Attempts will be made to shed light on the differential spatial and temporal roles of subcellular ROS in vascular endothelium in health and disease. Recent findings demonstrating that above-physiological levels of endothelial cell (EC)-specific NADPH oxidase-derived ROS *in vivo* exert beneficial effects on vascular endothelium will be discussed. The paradoxical roles of ROS in CVD suggest that subcellular sources and types of ROS may play crucial roles in the prevention, development, and progression of CVD. A better understanding of the precise mechanisms by which subcellular ROS modulate cardiovascular health and functions will certainly better prepare us with effective treatment modalities for CVD.

Keywords: reactive oxygen species, cardiovascular disease, endothelium, oxidative stress, signal transduction

1. Introduction

Cardiovascular disease (CVD) is the leading cause of death in the USA. Increased levels of reactive oxygen species (ROS) are often associated with microvascular pathology in CVD, causing endothelial dysfunction and coronary artery disease (CAD) and leading to myocardial ischemia and infarction (MI) [1–5]. However, failure of large clinical trials using antioxidants in patients with CVD [6–11], challenges the prevailing view that ROS production is damaging to the microvasculature. Indeed, findings from our laboratory and others show negative effects of ROS reduction on endothelial function and angiogenesis [12–14] and suggest that a well-regulated temporal balance of ROS production is important for normal endothelial cell (EC) function.

The paradoxical roles of ROS in CVD studied by different groups of workers also suggest that subcellular sources and types of ROS may also play crucial roles in the prevention, development, and progression of CVD. This review discusses recent findings that are challenging the long-held dogma in the field and also attempts to shed light on the differential spatial and temporal roles of subcellular ROS in vascular endothelium in CVD. A better understanding of the precise mechanisms by which subcellular ROS modulate cardiovascular health and functions will certainly better equip us with effective treatment modalities for CVD in future.

2. Cardiovascular disease (CVD)

Cardiovascular disease (CVD) is a leading cause of death and morbidity in the Western world. Recent reports suggest an increasing incidence of CVD in the developing countries too. In the USA, CVD with an annual toll of 1.2 million lives is the leading cause of deaths since 1921. At present, 85.6 million people in the country are living with CVD. In 2015, American Heart Association (AHA) reported that the prevalence of CVD among US adults is 6% with a calculated financial burden of $300 billion per year [15].

Vascular endothelium is critical for the optimal function of the heart and the vascular system, in particular due to its production of nitric oxide (NO) that regulates vascular tone and blood pressure (**Figure 1**). By regulating vascular tone including coronary vasodilation, NO plays an important role in blood supply to the myocardium (by coronary vessels) and other tissues in the body. Damage to coronary endothelium results in reduction in NO levels by reducing the level and/or activity of the enzyme, endothelial nitric oxide synthase (eNOS). This in turn contributes to the development of endothelial dysfunction (i.e. loss of vasodilation) and coronary artery disease (CAD), which may lead to ischemic insult to the heart including myocardial infarction and heart failure (HF). In myocardial ischemia, coronary vasodilation is an immediate response that may improve coronary blood flow in the heart. Another potent way myocardium employs to defend itself from ischemic insults is by preserving the existing coronary capillary vessels and/or by inducing growth of coronary vessels in the ischemic area [16–18]. Once the ischemic insult has occurred, survival of the affected cardiac tissue depends on the speed with which coronary vessels can increase blood flow. Thus, a functional coronary vascular system with endothelium-dependent NO-induced vasodilation mechanism is critical for the maintenance of coronary vascular health in the heart. Endothelial health and NO levels are also critical for the maintenance of blood pressure and prevention of hypertension that is an important risk factor for CVD.

Another major contributor to CVD is atherosclerosis, which is characterized by the accumulation of inflammatory leukocytes and lipid-laden macrophages in the vascular wall resulting in the gradual narrowing of the vascular lumens and wall thickness (**Figure 1A**). These changes, if generalized, result in arterial stiffness and can give rise to high blood pressure (hypertension); if the changes are localized, they may result in blocking of blood flow (ischemia and peripheral arterial disease), and in more severe cases, they may result in myocardial infarction, cerebrovascular disease/accident (stroke), atherosclerotic plaque rupture, and/or weakening of the vessel walls (aneurysm) (**Figure 1**).

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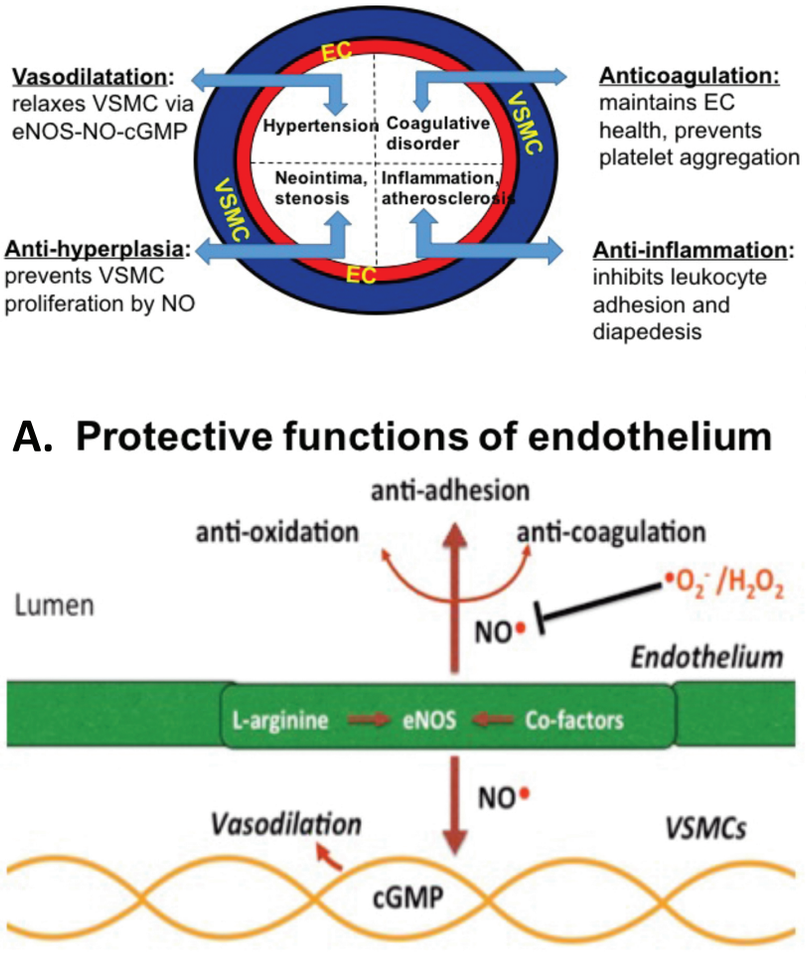

Figure 1. (A) Vascular endothelium performs critical functions in cardiovascular system, including nitric oxide (NO)-dependent vasodilation, maintenance of blood fluidity by preventing breach in the EC layer and platelet aggregation, NO-mediated inhibition of vascular smooth muscle cell (VSMC) proliferation and neointima formation, and inhibition of leukocyte adhesion to EC. Pathogenesis that may occur due to lack of specific function of EC is shown inside the circle. EC, endothelial cell; VSMC, vascular smooth muscle cell; and eNOS, endothelial nitric oxide synthase. (B) Nitric oxidase (NO)-mediated vasodilatation and other critical functions of vascular endothelium can be inhibited or blocked by excess ROS, specifically by superoxide. Endothelial nitric oxide synthase (eNOS) produces NO that acts on the luminal surface of the endothelium to prevent leukocyte adhesion and platelet aggregation/coagulation. NO diffuses to adjacent vascular smooth muscle layer (VSMC) to activate cyclic GMP (cGMP) signaling resulting in calcium ion release and relaxation of VSMC. All these NO functions can be blocked or reduced by the presence of excess ROS in the vascular wall.

Metabolic syndrome characterized by hypertension, obesity, glucose intolerance (diabetes), and hyperlipidemia is often accompanied by CVD. The endothelial dysfunction associated with metabolic syndrome has also been shown to have diminished angiogenic response and aberrant collateral vessels to chronic myocardial ischemia in large animal model [19, 20].

CVD including hypertension and heart failure (HF) are the most common cause of mortality in diabetes mellitus (DM) and usually result from DM-induced cardiomyopathy and CAD. The Framingham study showed that patients with DM are four times more likely to develop CVD. The worldwide prevalence of DM has recently been reported to be increased (total DM patients in 2015 have been projected to be over 300 millions) due to changes in lifestyle.

3. ROS and CVD cohabitation

Reactive oxygen species (ROS) has long been implicated in CVD. Increased levels of ROS are often observed in vascular tissues including coronary endothelium in CVD, and thus are believed to cause coronary endothelial dysfunction, CAD, myocardial ischemia, and infarction [1–5]. Although the mechanisms by which ROS may cause CVD has not been elucidated, increased levels of ROS, also known as oxidant stress, are believed to arise from endothelial cells (EC) resulting in loss of EC-dependent vasorelaxation and EC injury (**Figure 1B**). Oxidant-induced injury in EC in turn may result in recruitment of the inflammatory cells in the vessel wall leading to a cascade of vascular injuries. Dysfunctional EC leads to remodeling of the vascular tissues such as accelerated proliferation of the underlying vascular smooth muscle cells resulting in neointimal hyperproliferation/thickening and narrowing of the vessel lumen/stenosis (**Figure 1**). Vascular stenosis results in tissue ischemia and may also be complicated with thrombus formation. In the presence of hyperlipidemia, injury to EC may contribute to atherosclerotic changes. Remodeling of vessel wall, depending on the vascular bed affected and associated pathology/comorbidity, may result in hypertension, pulmonary hypertension, diabetic retinopathy, peripheral artery disease, myocardial ischemia, and stroke.

Interestingly, recent reports from several groups of workers demonstrated that reduction in ROS did not improve EC function and/or angiogenesis [12, 13, 21]. These findings challenged the long-held dogma that ROS are harmful and/or causative factor for developing CVD. More recently, our laboratory has shown that EC-specific increase in ROS resulted in the improvement of EC function and EC-dependent coronary vasodilation in transgenic animals [22]. All these imply that cohabitation of ROS and CVD may not be simply concluded to have deleterious effects on cardiovascular system. In addition, failure of the clinical trials (e.g. HOPE) to improve CVD using antioxidants and recent reports of beneficial effects of ROS on EC function warrant careful studies to delineate the spatial and temporal roles of ROS at the subcellular levels.

3.1. Types of ROS

ROS are reactive molecules that contain oxygen, they include molecules that have unpaired electrons, such as superoxide ($O_2^{\bullet -}$), hydroxyl anion (HO^-), and nitric oxide (NO^-) or that have the oxidizing ability but do not possess free electrons, such as hydrogen peroxide (H_2O_2), hypochlorous acid, and peroxynitrite ($ONOO^-$).

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3.1.1. Superoxide

It is produced usually as part of the metabolic processes by many intracellular enzymes such as NADPH oxidases (Nox), mitochondrial electron chain transport (ETC) system, Xanthine oxidase (XO), cytochrome P450, xanthine oxidase, lipoxygenase, myeloperoxidase, and uncoupled eNOS. $O_2^{\bullet-}$ is highly reactive and thus very unstable and has a short lifespan. It cannot cross cellular membranes and thus has a limited "area of action." Superoxide is usually converted to H_2O_2 spontaneously or can be metabolized to H_2O_2 by the antioxidant enzyme superoxide dismutases (SODs).

3.1.2. Hydrogen peroxide

As aforementioned, H_2O_2 is usually produced by dismutation of $O_2^{\bullet-}$ by SOD or by metal ions spontaneously in the Fenton reaction. Recently, Nox4 has been reported to be a source for H_2O_2; it has been reported that major ROS emanates from Nox4 enzyme is most likely H_2O_2 [23]. In comparison to superoxide, H_2O_2 is stable and can cross biological membranes, the properties that make this ROS a major player in cell signal transduction mechanisms.

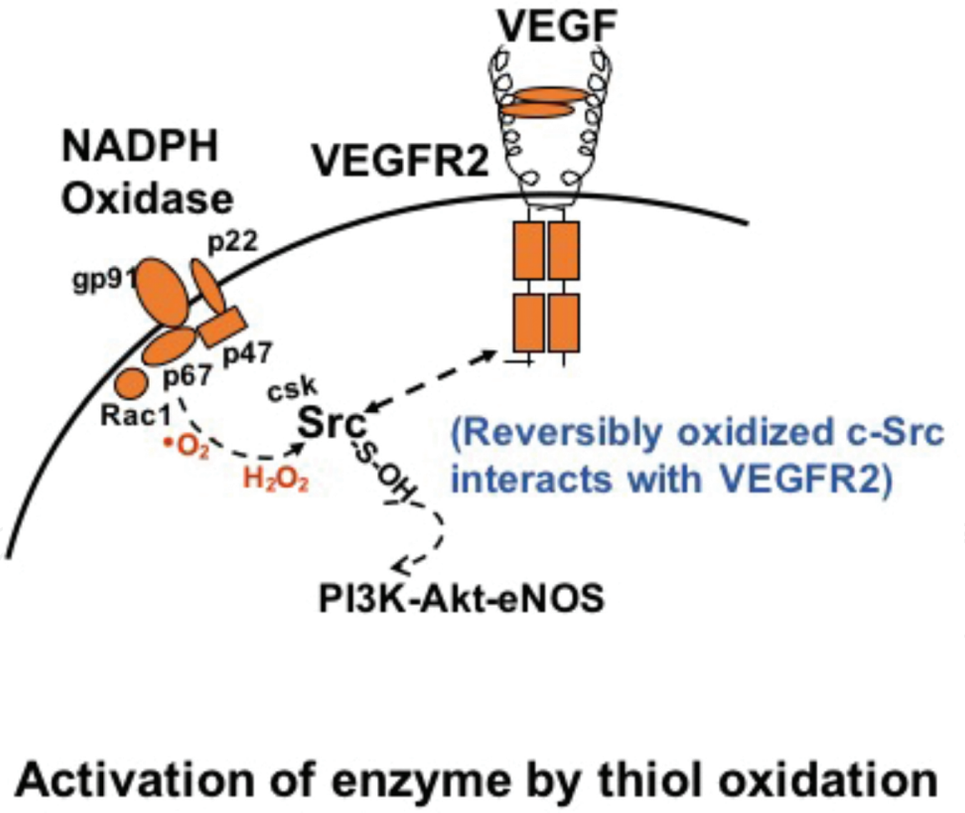

Figure 2. Reversible oxidation of thiol on cysteine residues modulate activity of the signaling molecules (kinases, phosphatases, and enzymes). NAPDH oxidase-derived ROS reversibly oxidize cysteine thiol (SH) to sulfenic acid (-SOH) on c-Src, which in turn facilitates interaction between c-Src and VEGFR2 resulting in the activation of downstream PI3K-Akt-eNOS signaling pathway in coronary endothelial cells (ECs). VEGF, vascular endothelial growth factor; VEGFR2, VEGF receptor 2; PI3K, phosphoinositol 3 kinase; Akt, protein kinase B; eNOS, endothelial nitric oxidase synthase; and Src, c-Src.

Due to its stability and membrane-crossing properties, H_2O_2 may act farther from its site of origin. H_2O_2 can react with thiol (SH) residues present on cysteine and methionine, and can catalyze the formation of the disulfide bonds (S-S) and reversible sulfenic acid (-SOH) and sulfinic acid ($-SO_2H$) moieties. All these changes can be reversed by antioxidant enzymes such as glutathione peroxidase (Gpx). However, these changes, if involves the catalytic site of the protein, can significantly modulate (activate, increase, decrease, or inhibit) the functional properties of an enzyme. It has been recently reported that c-Src oxidation by NADPH oxidase-derived ROS is crucial for c-Src and VEGF receptor 2 (VEGFR2) binding and activation of downstream c-Src-PI3K-Akt-eNOS signaling (**Figure 2**). Further oxidation of sulfinic acid to sulfonic acid ($-SO_3H$) is irreversible and does not participate in signal transduction (**Figure 2**).

3.1.3. Hydroxyl anions

HO^- is usually produced from H_2O_2 by free metals (Fenton reaction) or from the interaction between water and excited O_2. HO^- is highly reactive with a very short lifespan, is promiscuous in its interaction, and thus can cause sustained damage to DNA, amino acids, lipids, and glucose moieties mostly due to irreversibility of its interaction with biological molecules. It is thus considered a major contributor to "oxidative stress."

3.1.4. Nitric oxide

It is considered to be the "golden" molecule for cardiovascular health, which is crucial in the maintenance of the health of cardiac and vascular tissues. In EC, NO^- is produced by the enzyme eNOS and is involved in survival, growth, proliferation, and migration of vascular ECs. It is very critical for the maintenance of a continuous EC layer throughout the cardiovascular system. In fact, many of the critical functions carried out by EC are performed by NO^-, such as endothelium-dependent vasodilation (by activating cGMP pathway to decrease Ca_2^+ in vascular smooth cells, VSMC), prevention of adhesion of the anti-inflammatory cells to EC, maintenance of blood fluidity (anticoagulant, anti-thrombotic, and profibrinolytic actions), and anti-hypertrophic activity of EC through inhibition of VSMC proliferation and migration (**Figure 1**). NO, though it possess all the properties of a ROS, is often not considered as ROS by classical redox biologists. Interestingly, uncoupled eNOS may also generate superoxide.

3.1.5. Peroxynitrite

$ONOO^-$is generated by interaction between $O_2^{\bullet -}$ and NO^-. Like hydroxyl radical, peroxynitrite is also highly reactive and damaging to biological molecules including protein/enzyme due to its irreversible interaction. Thus, it is often considered as a marker for oxidative stress and/or oxidative tissue damage. In cardiovascular system, in addition to tissues damages, increase in $ONOO^-$ is considered to be an indicator of high ROS and low availability of NO^-, since $O_2^{\bullet -}$ interacts with and quenches NO^-. Decrease availability of NO^-, often marked by increase in peroxynitrite levels, is considered to be responsible for endothelial dysfunction, i.e., reduced vasorelaxation, often an initial marker for CVD.

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3.2. Subcellular sources of ROS

Biological sources of ROS are mitochondria (produced as a by-product of oxidative phosphorylation), NADPH oxidases, cytochrome P450, xanthine oxidase, lipoxygenase, myeloperoxidase, and uncoupled eNOS (**Figure 3**). While mitochondria act as the major source of ROS in most cardiovascular cells including cardiomyocytes, NADPH oxidases are the major source of intracellular ROS in vascular endothelium. EC derives most of its energy (ATP) from non-mitochondrial glycolysis, rendering it (EC) less likely to have excess ROS from mitochondrial source in physiological condition (**Figure 3**).

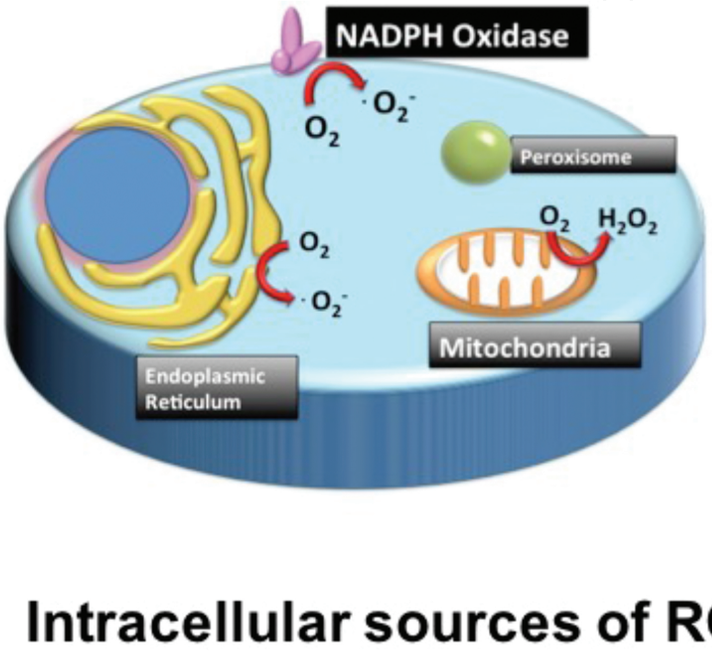

Figure 3. Subcellular sources of ROS include NADPH oxidase, mitochondria, peroxisome, lysosome, endoplasmic reticulum (ER), and cytochrome P450. NAPDH oxidases (Nox) are usually present in the cell membrane and perinuclear and ER membranes. Major species of ROS produced by NADPH oxidase is superoxide (O_2^-). Mitochondria produce ROS as a by-product of respiration/oxidative phosphorylation; electrons leaked from the electron transport chain (ETC), especially from Complexes I and III, produce superoxide in the mitochondrial matrix. Mitochondrial superoxide dismutase (MnSOD) converts superoxide to H_2O_2, which can then cross mitochondrial membrane to enter the cytosol. Nox is also found on the ER.

3.2.1. NADPH oxidase

NADPH oxidase is a multisubunit, membrane-bound protein complex that catalyzes oxidation of NADPH to $NADP^+$ and H^+, and in the process releases an electron. Molecular oxygen accepts this released electron and becomes $O_2^{\bullet -}$. There are several isoforms of NADPH oxidases, such as Rac-1-dependent NADPH oxidase (it contains Nox2/gp91^{phox}, **Figure 2**), Nox1, Nox3, Nox4, and Nox5. All isoforms have been reported to be found in the cardiovascular system except Nox3. With the exception of Nox4 (which is believed to release H_2O_2), all NADPH oxidases produce superoxide. NADPH oxidase is a major source of ROS in vascular endothelium and thus plays important roles in signal transduction in cardiovascular

system in health and disease. It has been shown that ECs possess two distinct major signaling pathways, redox-sensitive and redox-independent [24]. While PI3K-Akt-eNOS and Akt-FOXO signaling pathways were shown to be NADPH oxidase-derived ROS-dependent, PLCγ-MAPK-ERK signaling pathways were redox-independent in human coronary vascular ECs (**Figure 4**) [24]. Reduction in Nox-derived ROS resulted in inhibition of EC proliferation and migration [21, 25, 26]. Interestingly, recent findings further demonstrated that above-physiological levels (i.e. twofold increase compared to basal levels) of EC-specific Nox-derived ROS activated AMPK-eNOS signaling pathway in transgenic animals resulting in EC-dependent coronary vasorelaxation [22]. Together, these findings suggest that NADPH oxidase-derived ROS, both at the physiological and above-physiological levels, exert positive effects on EC health, growth, and function (**Figure 4**).

3.2.2. Mitochondria

Mitochondria play a major role in ROS generation in cardiovascular cells with one notable exception in ECs. Electrons leaked from ETC during oxidative phosphorylation in mitochondria produce $O_2^{\bullet -}$. Efficient mitochondrial respiration produces less ROS, however, inefficient oxidative phosphorylation gives rise to excess ROS by leaking electrons from the complexes I and III of the ETC. Mitochondrial antioxidant MnSOD (SOD2) plays a major

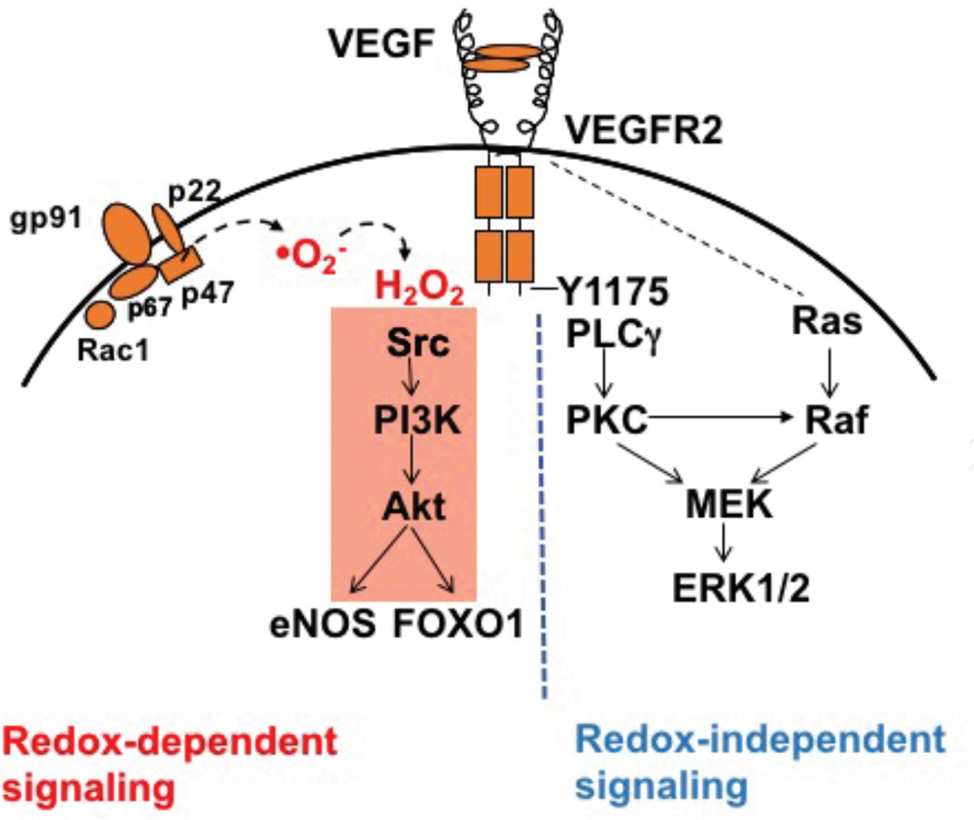

Figure 4. Coronary endothelium possesses two distinct signaling pathways: redox-sensitive PI3K-Akt-eNOS and PI3K-Akt-forkhead (FOXO) signaling (left panel) and redox-independent PLCγ-PKC-MEK-ERK1/2 signaling pathway (right panel).

role in reducing mitochondrial ROS by catalyzing superoxide to H_2O_2; Gpx further catalyzes H_2O_2 to molecular oxygen and water. Mitochondrial ROS may also increase due to increase in NO, which has been reported to inhibit Complex-I and in turn increase superoxide formation by leaking electrons into the matrix of the mitochondria.

3.2.3. Xanthine oxidase

Sulfhydryl oxidation of xanthine dehydrogenase results in xanthine oxidase formation. XO generates superoxide as a byproduct of the reaction where XO catalyzes the conversion of hypoxanthine to xanthine and xanthine to uric acid. XO is believed to produce ROS, especially H_2O_2, in ischemic conditions where oxygen tension is low. However, in ischemia-reperfusion injury, XO has been reported to be generating superoxide [27].

3.2.4. Lipoxygenase

Lipoxygenase plays an important role in hyperlipidemic state. It has been implicated in the oxidation of polyunsaturated fatty acids and in the pathology of atherosclerotic plaque formation and aortic aneurysm development.

3.2.5. Endothelial nitric oxide synthase

As aforementioned, eNOS generates NO. However, it can also produce ROS when uncoupled due to reduced availability of substrates L-arginine and/or co-factor BH4. Peroxynitrite may oxidize BH4, which in turn uncouple eNOS to form superoxide. Increased ROS is, thus, believed to activate a feed forward loop for ROS generation by uncoupling of eNOS.

3.3. Antioxidant enzymes

ROS balance is extremely critical for signal transduction and optimal functions of the cells. "Antioxidant enzymes" play an important role in redox balance. There are many cellular and extracellular enzymes that participate in oxidant metabolism, several of these are evolutionarily conserved. The term "antioxidant" is a misnomer for some of the proteins such as superoxide dismutase (SOD), which on one hand reduces the level of superoxide by converting superoxide to H_2O_2, but on the other hand increases H_2O_2, and thus results in overall increase in ROS levels. Thus, although it is called as antioxidant, SOD may well act like a pro-oxidation enzyme by converting a transient and localized ROS (O_2^-) to stable H_2O_2, which can cross membranes and thus can act farther away from its site of origin (paracrine effect).

3.3.1. Superoxide dismutases (SODs)

The very first step in regulating superoxide levels is catalyzed by this group of enzymes. Superoxide is converted to H_2O_2 by SOD. Most cells in the body including ECs have three isoforms of SOD, such as cytosolic SOD1 (Cu-Zn SOD), which catalyzes cytosolic superoxide to H_2O_2. SOD1 knockout mice have been shown to have impaired EC-dependent vasodilation [28]. In contrast, mitochondrial SOD2 (MnSOD) deletion was found to be embryonic lethal. SOD2 is a nuclear gene which upon protein translation translocated to mitochondrial matrix.

Heterozygous SOD2 mice demonstrated to have hypertension [29]. Excess ROS (e.g. ONOO) may inactivate SOD2; nitrosylation of MnSOD by ONOO has been reported to inhibit the antioxidant activity of the enzyme. Thus, increase in ONOO due to elevated ROS may result in increased mitochondrial ROS by inhibiting MnSOD activity. The "extracellular" SOD3 (Cu/ZnSOD) is a secreted protein which is localized in the outer part of the cell membrane. Deletion of SOD3 is not lethal; however, defective neovascularization has been reported in SOD3 null animals [30].

3.3.2. Glutathione peroxidase (Gpx)

Gpx catalyzes the conversion of H_2O_2 to water. Gpx is more abundantly expressed in cardiovascular cells including EC than catalase. It is also a major antioxidant protein in the mitochondria and is believed to have more critical role than catalase in regulating endothelial ROS. Gpx null animals have endothelial dysfunction [31]. It has also been reported to have severe ischemic-reperfusion injury [32] and defective angiogenesis compared to wild-type control [33].

3.3.3. Catalase

Functionally, catalase is similar to Gpx as it converts H_2O_2 to molecular oxygen and water. Structurally, catalase is a 4-heme containing enzyme. Knockout of catalase is not lethal.

3.3.4. Peroxidoredoxin (Prx)

Prx is a group of enzymes abundantly expressed in cardiovascular system. Functionally, they are similar to Gpx. Probably the most important role of Prx is to protect hemoglobin (Hb) in red blood cells (erythrocytes) where a lack of Prx has been shown to be associated with oxidation of Hb resulting in anemia.

3.3.5. Thioredoxin (Trx)

There are two isoforms of Trx present in the cardiovascular tissues, Trx-1 being present in the cytosol and Trx-2 in the mitochondria. They catalyze thiol-disulfide exchange on the cysteine residues present in the protein and thus convert the oxidized thiols of the proteins to their reduced (SH) forms. This protective action by reducing oxidized pools of proteins in the cells is crucial for redox balance. Overexpression of Trx has been shown to have protective effects against oxidative stress on cardiovascular function [34].

4. Understanding subcellular ROS is critical in CVD

It is obvious from the aforementioned discussion that endogenous ROS levels in specific subcellular compartments regulate certain signaling pathways, survival, proliferation, and pathophysiology in cardiovascular tissues. Precise understanding of the *spatial* (i.e. at different subcellular locations such as in the cytosol, mitochondria) and *temporal role of redox* (i.e. changes

in the role of ROS with time) to selectively activate downstream signaling pathways, maintain an intact and continuous EC layer throughout the cardiovascular system, maintain vasodilation in resistance arterioles, and induce a proangiogenic environment in ischemic myocardium is critical for the development of future therapeutic modalities for microvascular disease. In case of redox regulation, failure of the "all or none" approach (e.g. using global antioxidants as in the HOPE trial) also points to the end of an era that has treated any increase in cellular ROS levels as "deleterious." Instead, it is high time to consider ROS as signaling molecules, increase of which may also have "beneficial" effects during a cellular or cardiovascular crisis such as inflammation, ischemia-reperfusion, myocardial infarction, or other cardiovascular injuries including stroke. For example, an initial increase in endothelial ROS by Rac1-dependent NADPH oxidase (Nox) may have positive effects on EC survival during a time of crisis; increase in EC-specific Nox-ROS has been shown to activate a survival pathway (e.g. activation of pro-survival kinase AMPK) and improve endothelial function (coronary vasodilation) by inducing AMPK-eNOS-NO [22]. Thus, it is of utmost importance to understand the roles of modulation (increase or decrease) of subcellular (cytosolic vs. mitochondrial vs. peroxisomal/lysosomal) ROS levels in health and disease.

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