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Reactive oxygen species and vascular remodeling in cardiovascular diseases

Title in Spanish: *Especies reactivas de oxígeno y remodelado vascular en enfermedades cardiovasculares*

Andrea Aguado[1], M. Soledad Avendaño[1], Ana M. Briones[1], Mercedes Salaices[1]*

[1]Departamento de Farmacología, Facultad de Medicina, Universidad Autónoma de Madrid, Instituto de Investigación Hospital Universitario La Paz (IdiPAZ), 28029, Madrid, Spain

ABSTRACT: Reactive oxygen species (ROS) are reactive derivatives of O_2 metabolism produced by all types of vascular cells. ROS play an important role in both physiological and pathological situations by acting as intracellular signaling molecules which regulate vascular function and structure. Accordingly, oxidative stress is implicated among other processes in inflammation, hypertrophy, migration, growth/apoptosis and extracellular matrix protein turnover which are important processes involved in vascular remodeling in cardiovascular diseases. In the cardiovascular system, the major source of ROS is the NADPH oxidase family of enzymes composed by seven members where NOX-1 and NOX-4 are the main isoforms in vascular smooth muscle cells. This review highlights the importance of NOX-derived ROS in vascular biology and focuses on the potential role of oxidative stress in vascular remodeling.

RESUMEN: Las especies reactivas de oxígeno son derivados reactivos del metabolismo del O_2 producido por todos los tipos celulares a nivel vascular. Las especies reactivas de oxígeno juegan un papel importante en situaciones tanto fisiológicas como patológicas mediante su actuación como moléculas de señalización intracelular que regulan la función y estructura vascular. De esta manera, el estrés oxidativo está implicado, entre otros procesos, en la inflamación, hipertrofia, migración, proliferación/apoptosis y reciclaje de proteínas de matriz extracelular, los cuales son procesos importantes implicados en el remodelado vascular durante enfermedades cardiovasculares. En el sistema cardiovascular, la mayor fuente de especies reactivas de oxígeno es la familia de enzimas NADPH oxidase formadas por siete miembros donde NOX-1 y NOX-4 son las principales isoformas en células musculares lisas vasculares. Esta revisión destaca la importancia de las especies reactivas de oxígeno derivadas de NOX en la biología vascular y se centra en el papel potencial del estrés oxidativo en el remodelado vascular.

*Corresponding Author: mercedes.salaices@uam.es
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1. INTRODUCTION

Cardiovascular diseases are the first cause of death globally, accounting for more than 30% of deaths which means that 17.5 million people die annually from cardiovascular disease. In Spain, the number of deaths due to cardiovascular diseases in 2014 was over 129,000 people and this number will increase in 2020 reaching 142.000 deaths. According to the "Centro de Estudios Económicos y Empresariales" the direct costs of cardiovascular diseases have been estimated over 5.900 million of euros in 2014 and the indirect costs due to morbidity associated with cardiovascular disease as well as absence from work have been estimated over 60 million euros.

Reactive oxygen species (ROS) are essential mediators of cell physiology. They can modulate the activity of many signaling molecules including kinases, phosphatases, transcription factors and cytoskeleton proteins and thus, they regulate different cellular processes. ROS play an important physiological role in controlling vascular tone and structure and they can also contribute to pathological mechanisms related to endothelial dysfunction, vascular reactivity, arterial remodeling and vascular inflammation. The NADPH oxidases are the main source of ROS in the cardiovascular system. Seven members have been characterized and depending on the isoform, they are expressed in different cardiovascular cell types and cellular compartments regulating diverse functions such as proliferation, migration, differentiation, apoptosis, senescence and inflammatory responses (1-3). For several decades, the role of NADPH oxidase in chronic granulomatous disease has been known due to the key role of NADPH oxidase in neutrophils. This concept has been now extended to cardiovascular diseases since strong evidences have demonstrated that NADPH oxidase-

derived ROS released from phagocytic and vascular cells are involved in many processed associated to cardiovascular disease. Thus, many studies performed mainly in animal models have demonstrated that increased oxidative stress state is necessary for the initiation and progression of vascular disease that may ultimately lead to heart attack and strokes. Some members of the NADPH oxidases are constitutively expressed in the vasculature. However, different hormones, inflammatory mediators or hemodynamic stimuli important in cardiovascular diseases, increase the activity or the expression of NADPH oxidase isoforms leading to a deleterious oxidative stress status in the cardiovascular system. These changes trigger the production of growth factors, proteases and cellular adhesion molecules by different vessel cell types leading to structural changes in the wall of the vessel in a process known as vascular remodeling.

In this review, we discuss in detail the composition and regulation of the main NOX enzymes expressed in the media layer (NOX-1 and 4) and their roles in vascular remodeling associated with cardiovascular diseases.

2. VASCULAR REMODELING

2.1. Artery structure

Arteries are divided in three concentrical layers from the inside out: intima, media and adventitia which are organized in cellular components and extracellular matrix (ECM) (Figure 1).

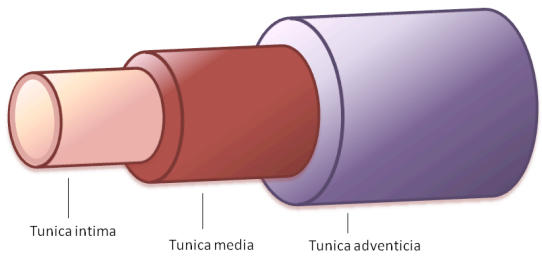

Figure 1. Artery structure. Wall section showing all layers of an artery wall.

- Intima: it is in the inner part of the vessel and comprises a monolayer of endothelial cells which lay in the basement membrane. This layer of endothelial cells is separated from the media layer by the internal elastic lamina which is a fenestrated lamina of elastic fibers. The intima layer is important in the control of vascular function and structure because endothelial cells are an important source of vasoconstrictor/vasodilator and proliferative/antiproliferative factors.

- Media: this layer includes circumferentially arranged vascular smooth muscle cells (VSMCs) and variable amounts of ECM. The tunica media is separated from the tunica adventitia by a second layer of elastic fibers, the external elastic lamina. In response to different vasoactive factors and hemodynamic forces, VSMCs can release a variety of substances which affect vascular tone and structure.

- Adventitia: it is mainly formed by fibroblasts but it also contains macrophages and mast cells and different components of the ECM. In the last years, it has become evident that the adventitia is not only a mechanical support for the vessel but also an active player of the regulation of vascular tone and structure by releasing different factors.

The ECM is a gel-like form which functions as a scaffolding structure for the vascular cells and determines the elasticity and mechanical properties of the vessels. Their components are synthetized by different cell types of the vascular wall. The two main ECM proteins are collagen and elastin; while elastin confers the elastic properties to vessels, collagen provides the strength (4). There are other ECM proteins that are in less quantity such as glycoproteins, proteoglycans and integrins that are involved in several cellular processes (4). Among them, tenascin-C (TN-C), which is an inducible glycoprotein, expressed predominantly in embryonic, remodeled adult tissues and in pathological conditions, is particularly interesting. Competitive binding of TN-C to ECM proteins and their counterpart cell-surface receptors mediates its ability to modulate cell-ECM interactions. The capacity of TN-C to interact with a wide range of ECM molecules may also enable it to contribute to the structural organization of the ECM. In addition, TN-C can promote migration and proliferation by direct activation of cell-surface growth factor receptors and cellular differentiation by up-regulating androgen receptor and endothelin type 1 receptor expression (5). Thus, TN-C relevance relies on its implication in vascular cell differentiation, proliferation and migration (5).

2.2. Types of vascular remodeling

It is now accepted that the vascular wall can change its structure in order to maintain the appropriate lumen size to permit normal blood flow. This process is termed vascular remodeling (6). This ability of the arteries to adapt its structure in response to physiological and pathological conditions is essential in situations such as pregnancy or aging but also in many arterial diseases. Thus, the inability of the vessels to remodel appropriately is considered a form of "vascular failure" that can lead to pathologic states such as hypertension, atherosclerosis or restenosis (7). This process is active and involves structural changes including cell growth, death, migration and the synthesis or degradation of the ECM (7).

Vascular remodeling can occur with or without growth of the vessel wall (i.e. hypertrophic, eutrophic or hypotrophic) and with smaller, greater or similar lumen size (inward, outward or compensated) (8). Vascular remodeling often differs depending on the vessel type or the cardiovascular disease model (Figure 2).

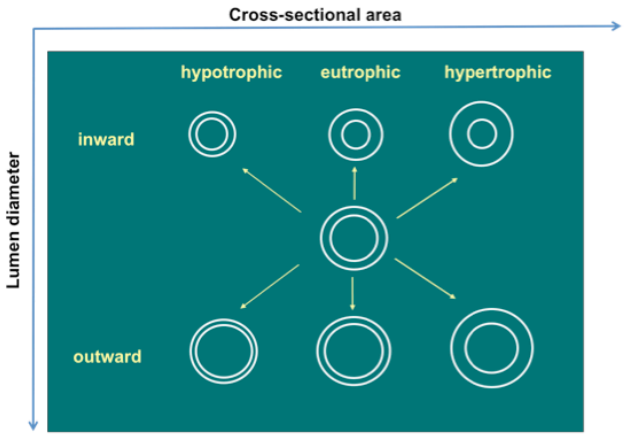

Figure 2. Types of vascular remodeling. Classification refers to changes on the lumen diameter (inward: upper row or outward lower row) and vessel cross-sectional area (hypotrophic: left column; eutrophic: center column and hypertrophic: right column). Adapted from Mulvany (8).

- **Hypertrophic remodeling** is characterized by an increase in the media thickness, media/lumen and vascular cross sectional area associated with a more evident contribution of cell growth (8). This type of remodeling is characteristic of large arteries in ageing or in pathologies like hypertension (9) or restenosis which is associated with proliferation and migration of different cells types (7).

- **Hypotrophic remodeling** is associated with a decrease in the amount of material (i.e. diminished cross sectional area) around the vessel wall (8). This type of remodeling may be related to apoptosis processes and/or to rearrangement of the material in the vessel wall (10). Hypotrophic remodeling has been shown in renal afferent arterioles from spontaneously hypertensive rats (SHR) (11) and in mesenteric resistance arteries from ouabain-induced hypertensive animals (12). Moreover, patients with autosomal dominant hyperimmunoglobulin E syndrome were found to have a high prevalence of hypotrophic remodeling in carotid arteries with an increased circumferential stress and enhanced susceptibility to dilation and aneurysm formation associated to angiotensin II (AngII) and apolipoprotein E (13).

- **Eutrophic remodeling** is characterized by a decrease in the outer and lumen diameters and an increase in the media thickness and the media/lumen ratio with no change in the wall cross sectional area (8). It has been suggested that this type of remodeling is due to rearrangement of the same amount of wall material around a smaller vessel lumen (14, 15). The mechanisms leading to this type of remodeling are poorly known but some authors suggest that a combination of inward growth and peripheral apoptosis or prolonged vasoconstriction of vascular cells embedded in an expanded ECM can lead to eutrophic remodeling (9, 16).

The importance of the vascular structural abnormalities in cardiovascular diseases, such as hypertension, lies on the fact that in patients it has been demonstrated that the media to lumen ratio parameter has a prognostic value of cardiovascular events in a high-risk population (17, 18). Thus, the presence of structural alterations in the microcirculation may be considered an important link between hypertension and ischemic heart disease, heart failure, cerebral ischemic attacks, and renal failure (15).

Vascular tone and structure are regulated by the equilibrium between vasodilator- antiproliferative-antifibrotic factors and vasoconstrictor- proliferative-profibrotic factors, which are released in large part, by the ECs and VSMCs in response to mechanical or chemical stimuli. The imbalance between these substances leads to the endothelial dysfunction and/or the vascular remodeling observed in cardiovascular diseases. Vascular remodeling can be induced by dynamic interactions between local growth factors, vasoactive substances and hemodynamic stimuli being all important mediators in the vascular adaptation process. The number of mediators involved in altered vascular structure is continuously growing; however, to date it is well admitted that AngII, cytokines, prostanoids and ROS have a key role (7).

2.3. Cell proliferation and migration

As mentioned, wall thickening is one of the main features of many cardiovascular diseases. Depending on the specific vascular bed and pathology, the cellular and non-cellular events leading to altered vascular structure might be different. Thus, hypertension causes arterial media thickening with or without cell growth, and ECM deposition in both humans and animal experimental models; however, atherosclerosis and reaction to injury such as endothelial denudation or restenosis cause intimal thickening associated to variable degrees of alterations in the surrounding ECM (19, 20) (Figure 3). Although it is known that during vascular remodeling VSMC proliferation and migration are processes that take place, their regulation is not exactly known.

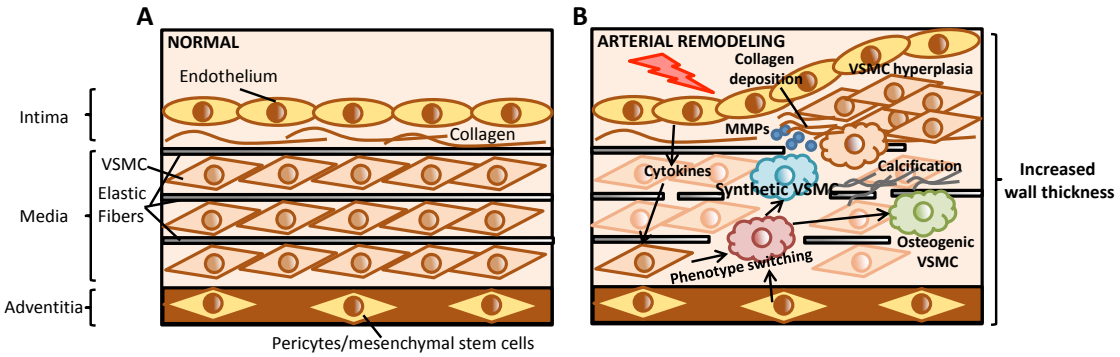

Figure 3. Pathophysiological mechanisms of arterial remodeling. Cross sectional schematic view of the arterial wall in **(A)** normal situation or **(B)** during arterial remodeling. Thickening of the wall is the main feature of arterial remodeling. Elastic fiber degradation, extracellular matrix calcification, collagen deposition and vascular smooth muscle cell migration and phenotype switching lead to adaptation of the vascular wall. Matrix metalloproteinase (MMP). Modified from van Varik et al. (20).

Intimal thickening can occur in blood vessels as a consequence of physiological process as occurs in ageing, in response to increased intraluminal pressure, or after vascular injury as observed in balloon dilatation, stent implantation or atherosclerosis processes (21). Because of its importance, many *in vivo* models of VSMC growth and proliferation such as the carotid ligation mouse model have been developed. In this model, an intima lesion characterized by enrichment of VSMCs occurs in response to luminal narrowing leading to the formation of the neointima (19, 21). Neointima is part of the reparative response to injury and its formation involves an important inflammatory component with infiltration of inflammatory cells and release of cytokines and chemokines, thrombosis, increase in the number of VSMCs and matrix production leading to a reduction in vessel diameter (19, 22, 23). The increased number of VSMCs is mainly originated by migration from the underlying media and proliferation, although there are other processes involved such as transdifferentiation of endothelial cells or differentiation from circulating precursors (7, 20, 21) (Figure 3).

The involvement of cell proliferation and/or migration in hypertensive vascular remodeling mainly depends of the vascular bed and the experimental model studied. Thus, coronary but not mesenteric vessels from SHR show increased VSMC number (24). In addition, administration of AngII, the main effector peptide of the renin-angiotensin-aldosterone system (RAAS) lead to a progressive increase in blood pressure and media thickening through migration, proliferation and hypertrophy of the VSMCs, being this effect mediated through the AngII type 1 receptor (AT_1R) (7, 25-28) (Figure 4). Besides hemodynamic and humoral factors, in the last years it has become evident that vascular infiltration of immune inflammatory cells and pro-inflammatory mediators such as ROS are key contributors to the vascular remodeling observed in this pathology (29-31).

Cell proliferation and migration begin with stimulation of cell surface receptors that transduce the external signal to a series of coordinated responses inside the cell. Diverse signal transduction systems such as nuclear factor-kappa B (NF-kB), the activator protein-1 (AP-1), the mitogen activated protein kinases (MAPKs) or the phosphatidylinositol-3-kinase (PI3K)/Akt pathways have been proposed to translate the stimulus within VSMCs (32). However, despite of the growing information regarding the mechanisms controlling VSMC migration and proliferation in response to stimuli such as AngII (28), the regulation in response to other stimuli is less known.

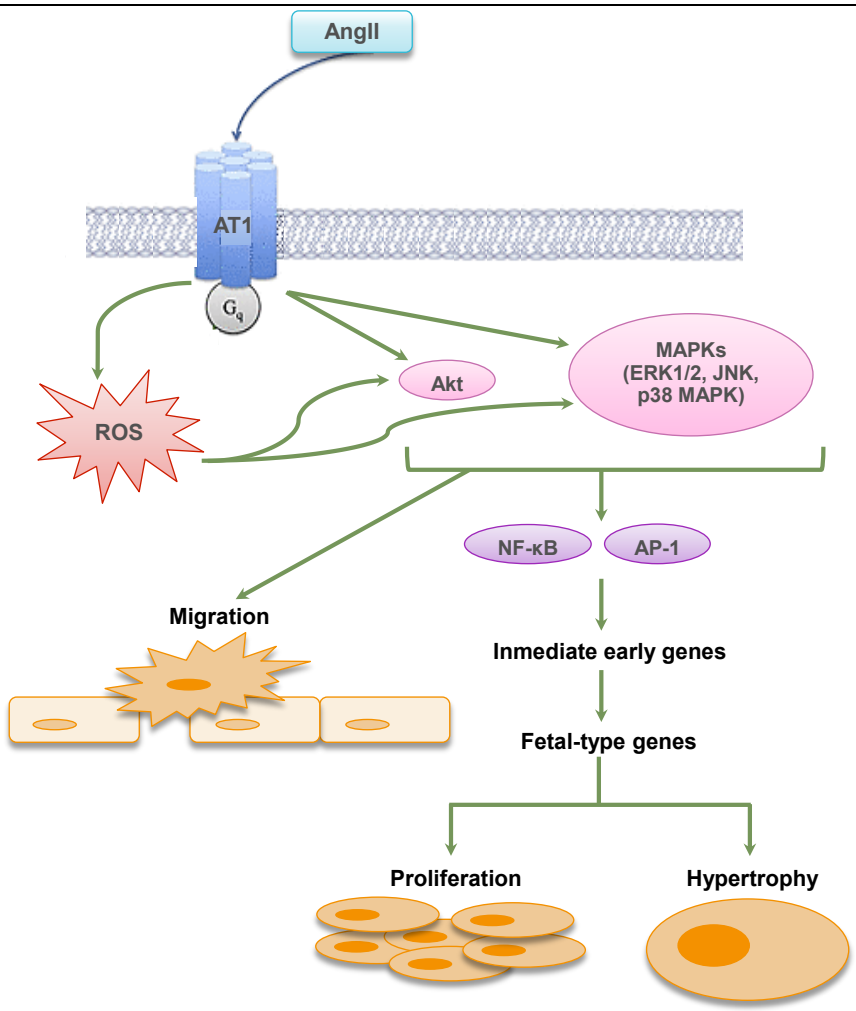

Figure 4. Role of ROS in AngII induces proliferation, migration and/or hypertrophy of VSMCs. Arrows indicate the main biological end points preceding cell proliferation, migration and hypertrophy in response to AngII. Adapted from Chiou et al. (28).

3. REACTIVE OXYGEN SPECIES

ROS are reactive derivatives of the oxygen metabolism with superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$) being of major importance. There is an apparent paradox between the roles of ROS as essential biomolecules in the regulation of many cellular functions and as toxic by-products of metabolism that may be related at least in part, to differences in the concentrations of ROS produced. Thus, at low intracellular concentrations, ROS have a key role in the physiological regulation of vascular tone, cell growth, adhesion, differentiation, senescence and apoptosis. However, excessive ROS levels may be associated with the development of several cardiovascular diseases (33, 34).

$O_2^{\bullet-}$, H_2O_2 and $ONOO^-$ are produced by almost all cell types including vascular cells. Besides NADPH oxidase, other sources of ROS in the vascular wall include mitochondria, xanthine oxidase (XO), uncoupled endothelial nitric oxide synthase (eNOS), endoplasmic reticulum, cyclooxygenase (COX), cytochrome P450 and lipoxygenase (35, 36). Mitochondria are a major cellular source of ROS. There are several sites in the electron-transport chain where oxygen can be reduced to $O_2^{\bullet-}$, with complexes I and III being the sites with the greatest capacity (37). XO catalyzes the sequential oxidation of hypoxanthine to xanthine and xanthine to urate and can generate $O_2^{\bullet-}$ and H_2O_2 (38). XO is mainly expressed in the endothelium and both its protein expression and $O_2^{\bullet-}$ production can be activated by AngII (39). eNOS uses L-arginine as substrate and tetrahydrobiopterin (BH_4) as cofactor to generate NO. However, under pathological conditions, L-arginine or BH_4 deficiency induces eNOS uncoupling resulting in $O_2^{\bullet-}$ production (40).

$O_2^{\bullet-}$ is highly reactive, has a short half-life and is unable to diffuse across biological membranes except possibly via ion channels (33). $O_2^{\bullet-}$ can dismute to H_2O_2, both spontaneously and enzymatically via any of the three isoforms of the superoxide dismutase (SOD): cytosolic Cu/Zn-SOD or SOD1, mitochondrial Mn-SOD or SOD2 and extracellular EC-SOD or SOD3 (Figure 5). As mentioned, H_2O_2 can also be formed directly by some types of NOX such as NOX-4, DUOX-1 and -2 (1). H_2O_2 is more stable than $O_2^{\bullet-}$ and crosses membranes through some members of the aquaporin family (41). H_2O_2 is

rapidly metabolized to water and oxygen by several enzymatic systems such as glutathione peroxidase, catalase and the thioredoxin system (35, 42, 43) (Figure 5). In the presence of transition metals (such as Fe^{2+}) H_2O_2 can be converted to hydroxyl radicals ($HO^{\bullet}$), which are highly reactive and can cause damage to lipids, proteins and DNA. In addition, NO which has a very short half-life, can react with $O_2^{\bullet-}$ to form $ONOO^-$ that is capable of modifying the structure and function of proteins. Thus, ROS regulation is important to maintain redox environment of the cell. When there is an imbalance between oxidants and antioxidant systems increased ROS steady-state levels start multiple pathologies including inflammation and cardiovascular disease (1, 35). At low intracellular concentrations, ROS have a key role in the physiological regulation of vascular tone, cell growth, adhesion, differentiation, senescence and apoptosis (1, 44). However, an increase in the amount of ROS leads to pathological processes such as endothelial dysfunction, inflammation and proliferation or migration of VSMCs leading to vascular remodeling.

The mechanisms responsible of ROS-associated pathological effects are multiple and include quenching of vasodilator NO by $O_2^{\bullet-}$, generation of vasoconstrictor lipid peroxidation products, depletion of BH_4, and induction of fibrosis through activation of matrix metalloproteinases (45). At intracellular level, ROS induce different processes such as increased intracellular calcium, activation of growth and inflammatory transcription factors and activation of different signaling pathways such as mitogen activated protein kinases (MAPK), protein tyrosine phosphatases, tyrosine kinase, PI3K, and RhoA/ROCK (34).

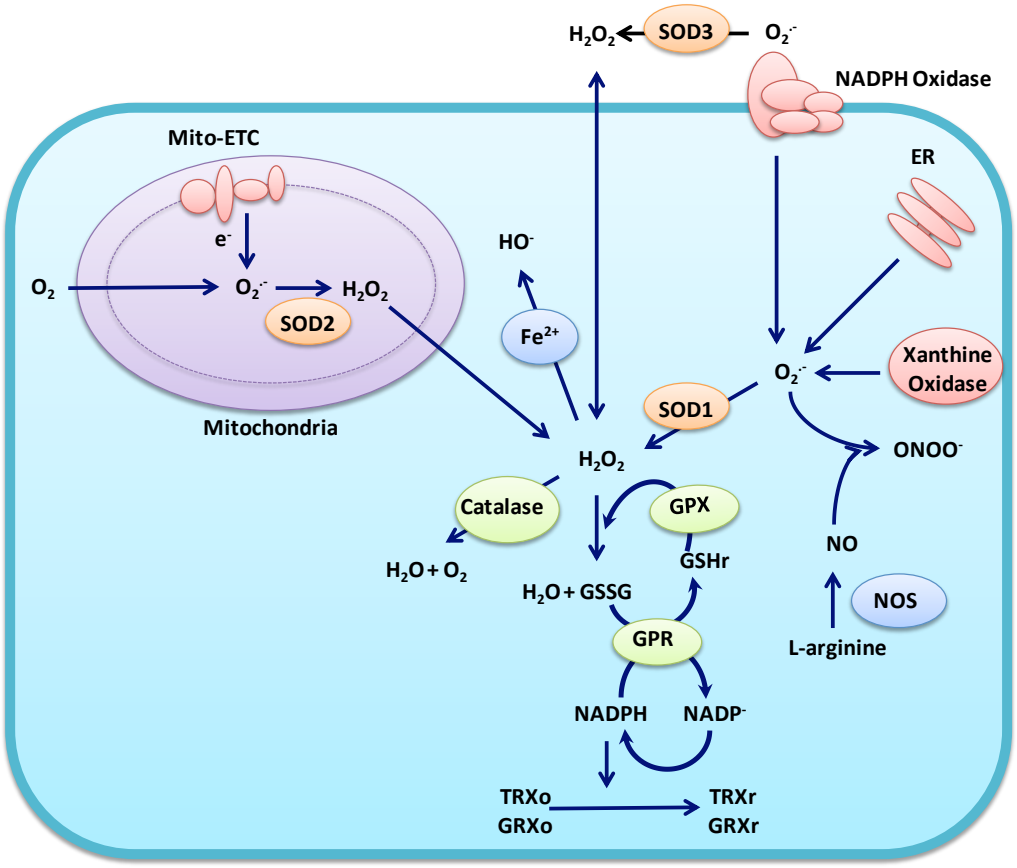

Figure 5. Reactive oxygen species formation and metabolism. Major sources of ROS generation include the mitochondrial electron transport chain (Mito-ETC), endoplasmic reticulum (ER) system, NADPH oxidase and xanthine oxidase. Superoxide anion ($O_2^{\bullet-}$) is the main initial free radical specie which can be converted to other reactive species. In the mitochondria, $O_2^{\bullet-}$ is generated by the capture of electrons escaping from the Mito-ETC by molecular oxygen (O_2). $O_2^{\bullet-}$ can be rapidly converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD), which is converted to H_2O by catalase, glutathione peroxidase (GPX) or the thioredoxin (TRX) systems. In the presence of transition metals (such as Fe^{2+}), H_2O_2 can be converted to hydroxyl radicals ($HO^{\bullet}$) NO has a very short half-life and can react with superoxide to form $ONOO^-$. Glutathione reductase (GPR); glutaredoxin oxidized (GRXo); glutaredoxin reduced (GRXr); glutathione reduced (GSHr); glutathione oxidized (GSSG); thioredoxin oxidized (TRXo); thioredoxin reduced (TRXr). Adapted from Trachootham et al. (43).

ROS can act as second messengers activating different intracellular signaling pathways. Particularly, H_2O_2 induces post-translational oxidative modifications on sulfur containing amino acid of proteins. Although methionine and cysteine residues can be targets, the most important is the cysteine thiol group. ROS react with the sulfur atom of cysteine side chains leading to the formation of sulfenic acids (-SOH) that can affect proteins

implicated in cell migration. Depending on the environmental oxidative state these reactions are reversible or irreversible. Thus, in a reducing environment in the cell (normal status) this process is quickly reverted. Conversely, in a strongly oxidative environment, the sulfenic form is unstable and can undergo further oxidation via disproportionation (a type of redox reaction where a species is simultaneously reduced and oxidized to form two different products) to sulfinic ($-SOH_2$) species. Under greater oxidative stress, the sulphonic ($-SO_3H$) species can be created. Other possibilities for post-translational cysteine modifications include glutathionylation (-SSG) or the formation of an inter- or intramolecular disulfide bond (-SS-), thus causing protein oxidative damage (45-47).

It is also well established, that redox-dependent signaling pathways in VSMCs include modifications in the activity of protein tyrosine kinases such as Src, Ras, JAK2, Pyk2, PI3K and EGFR, as well as MAPK, particularly p38 MAPK, ERK1/2 and ERK5 which as mentioned, have a key role in cell migration and proliferation and hence in pathological vascular remodeling (45). These processes probably occur through oxidation/reduction of protein tyrosine phosphatases (PTP), which are susceptible to oxidation and inactivation by ROS. Increased intracellular ROS also induces an increase in intracellular free calcium concentration ([Ca2+]i) and an increase in intracellular pH (pH_i) that also contribute to altered contraction and remodeling observed in pathological situations where ROS have a prominent role (45). Rho GTPases and actin are also sensitive to these modifications leading to actin cytoskeleton reorganization (45, 47, 48). Thus, ROS are able to induce VSMC proliferation and migration by a number of different intracellular signaling pathways (Figure 6).

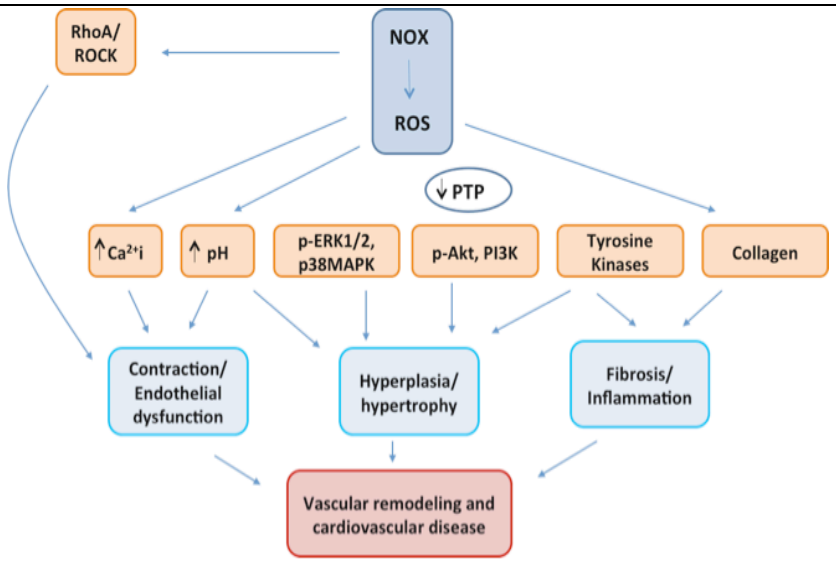

Figure 6. Intracellular mechanisms activated by ROS that participate in cardiovascular disease. NOX-derived ROS activate different signaling pathways as well as increase in pH and Ca^{2+}. These processes lead to different cellular responses that will end in cardiovascular disease. Adapted from Briones and Touyz (45).

To date, a number of studies have demonstrated that stimuli important for cardiovascular diseases induce VSMC migration and/or proliferation *via* ROS (49). For example, AngII regulates FAT atypical cadherin 1 (Fat1) expression and activity and induces Fat1-dependent VSMC migration via activation of AT_1R, ERK1/2, and NOX-1-derived ROS (50). Similarly, PDGF-induced VSMC migration is ROS dependent and the Src/PDK1/PAK1 signaling pathway is important as a ROS-sensitive mediator of migration (51). Moreover, in VSMCs H_2O_2 induces cell migration by inducing the expression of a cytoskeleton protein, ARPC2, through a p38 MAPK-dependent mechanism (41)

3.1. NADPH oxidases

As mentioned, NADPH oxidases are the major source of ROS in the vascular wall in physiological and pathological conditions (1, 34, 52-54). The main catalytic function of NADPH oxidases is the generation of ROS, thus differing from the rest of the ROS-producing enzymes which produce ROS as a by-product of their activity. NADPH oxidase reduces oxygen to superoxide anion ($O_2^{\bullet-}$), being NADPH the electron donor; thus, there is an electron transfer from the cytosol across biological membranes. There are seven NADPH oxidases isoforms in mammals and all of them have a catalytic subunit called NOX (NOX-1-5) or DUOX (DUOX-1-2 also called NOX-6-7) and up to seven regulatory subunits (Figure 7).

NOX-1, NOX-2, NOX-4 and NOX-5 are expressed in the cardiovascular system. NOX-2 is the classical NOX that was primarily characterized in leukocytes. NOX-1, NOX-2 and NOX-3 activities are regulated by cytosolic adaptor proteins or "NOX organizers" (p47phox or NOXO1 and p40phox) and "NOX activators" (p67phox or NOXA1) that bind GTP-Rac and affect the flow of electrons (Figure 7). The p22phox component forms a stable heterodimeric complex with NOX core components (NOX-1-4), required for post-translation processing or

maturation into active oxidases. In NOX-1/NOX-3 systems, p22phox also promotes plasma membrane targeting of the oxidases and provides a docking site for NOX organizers. However, NOX-4 only depends on p22phox in order to be active, is constitutively activated, and ROS production is regulated by Poldip2 (Figure 7). NOX-5 and DUOX are Ca^{2+}-responsive oxidases that contain Ca^{2+}-binding domain (Figure 7). NOX-1, NOX-2, NOX-3 and NOX-5 produce $O_2^{\bullet -}$ while NOX-4, DUOX-1 and DUOX-2 produce H_2O_2 (34, 55).

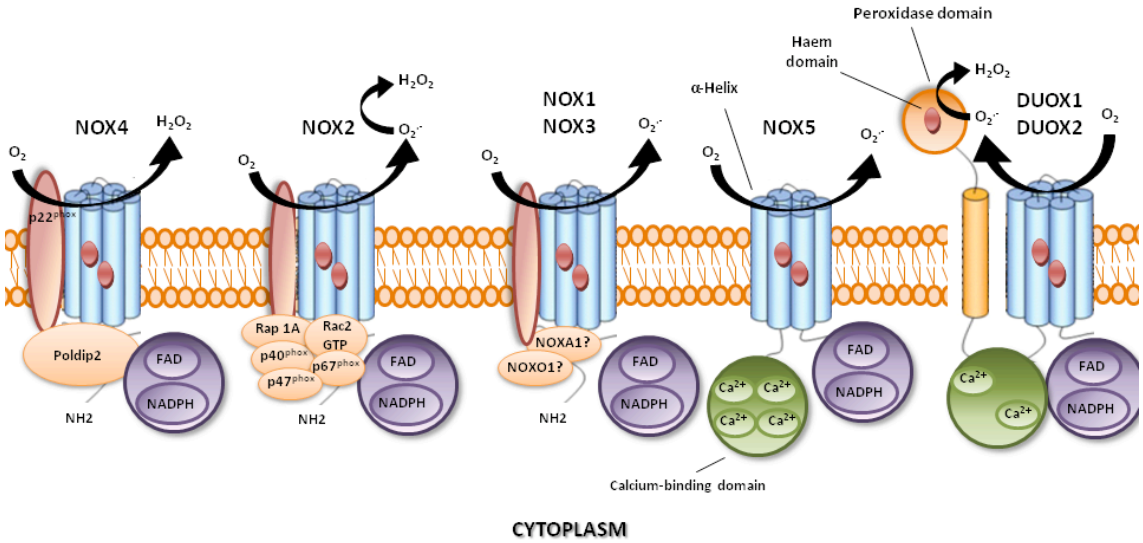

Figure 7. Subunit composition of the seven mammalian NADPH oxidase isoforms. The catalytic subunits of NADPH oxidase (NOX) 1-5, dual oxidase (DUOX) 1 and 2 are shown in blue. The stabilization subunit p22phox is shown in red. Cytosolic organizers: p40phox, NOX organizer 1(NOXO1) and p47phox; cytosolic activators: p67phox and NOX activator 1 (NOXA1); and small GTPases (RAC1 and RAC2), are shown in grey. Polymerase δ-interacting protein 2 (POLDIP2) and calcium-binding domains motifs are shown in orange or green respectively. Adapted from Montezano and Touyz (34) and Guichard et al. (55).

Within the vascular wall, NOX isoforms locations vary depending on the cell type and the cellular compartments. Thus, endothelial cells express NOX-1, NOX-2, NOX-4 and NOX-5; VSMCs mainly express NOX-1, NOX-4 and NOX-5; and adventitial fibroblasts mainly express NOX-2 and NOX-4 (1). It is noteworthy that NOX-5 is only expressed in human cells (1). NOX distribution in subcellular compartments also varies within the cell. In VSMCs, NOX-1 is localized to the plasma membrane, caveolae and endosomes while NOX-4 seems to be in focal adhesions, endoplamic reticulum and nucleus (1, 56). Additionally, NOX-4 seems to be present in the mitochondria of cardiomyocytes (56).

Because of their preferential expression in VSMCs and their importance in vascular remodeling, in the next part of the Review we will focus on specific aspects of NOX-1 and NOX-4 including available information on regulation, function and their role in vascular remodeling.

3.1.a. NOX-1

NOX-1 is expressed in colon epithelium and also in other tissues including the vascular wall where it seems to be up-regulated in pathological conditions or after exposure to different agonists important in cardiovascular disease (54). Thus, in VSMCs NOX-1 is up-regulated by AngII (57, 58), $PGF_{2\alpha}$ and PDGF (59), IFN-γ (60) or IL-1β (61). In addition, vascular NOX-1 expression is elevated in several *in vivo* animal models of hypertension such as two-kidney two-clip renovascular hypertensive rats, DOCA salt hypertensive rats and AngII-infused mice (62-64). Moreover, NOX-1 expression is elevated during restenosis following balloon angioplasty (65). However, the role of NOX-1 in atherogenesis remains controversial with NOX-1 being undetected in atherosclerotic rabbit (66) or human lesions (67, 68) and overexpressed in aorta from $ApoE^{-/-}$ mice (69).

NOX-1 promoter has different binding sites for transcription factors including a member of CREB/ATF

family (59), AP-1 (70), NF-κB (71) or Janus kinase/Signal transducers and activators of transcription (JAK/STAT) (60) (Figure 8).

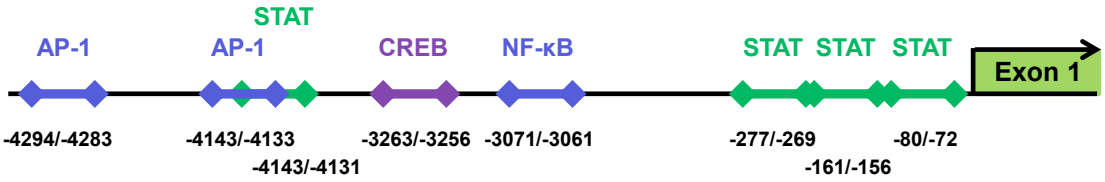

Figure 8. Structure of human NOX-1 gene promoter. Binding sites for transcription factors involved in NOX-1 expression.

Most of these studies have evaluated transcriptional regulation of NOX-1. However, to our knowledge, no studies have demonstrated post-transcriptional regulation of NOX-1. In fact, regulation of NOX-1 mRNA through its 3'UTR is conceivable because of the presence of AREs which are implicated in mammalian mRNA degradation. Accordingly, our group has described in VSMCs a new mechanism whereby in the presence of AngII plus IL-1β, NOX-1 expression is potentiated through HuR-dependent NOX-1 mRNA stabilization. Moreover, exacerbated NOX-1 expression is responsible for an increased NADPH oxidase activity, ROS production and cell migration (72).

3.1.b. NOX-4

NOX-4 is very abundant in kidney and it seems ubiquitously expressed mainly in differentiated cells. NOX-4 is mostly found in focal adhesions and in the endoplasmic reticulum (73-75). As mentioned, its structure differs from NOX-1 and enables the protein to directly produce H_2O_2 (76, 77). It has been suggested that the predominant factor controlling NOX-4-dependent ROS formation is the expression level of the enzyme (44); therefore, the knowledge of the mechanisms responsible of its expression is very important.

It seems now accepted that NOX-4 is constitutively active (56). However, less clear is whether NOX-4 expression can be modulated and variable data regarding NOX-4 induction are found in the literature. Thus, hypoxia induces NOX-4 expression in pulmonary artery SMC (78, 79) and TGF-β induces NOX-4 in cardiomyocytes and vascular cells (80-82). However, thrombin, PDGF and peroxisome proliferator-activated receptor-γ (PPAR-γ) ligands reduce NOX-4 expression in VSMCs and endothelial cells, (57, 83, 84). Moreover, other stimuli including AngII and IL-1β have demonstrated to up-regulate, decrease or no affect NOX-4 expression in vascular cells (57, 58, 76, 83, 85). Our group has proposed that IL-1β decreases NOX-4 expression in VSMCs and consequently H_2O_2 production involved in cell migration (72). Reasons for these differences remain elusive but different locations in different cell types or presence of different NOX-4 isoforms might contribute to the observed findings (54).

In vivo studies have tried to shed light on the role of NOX-4 in cardiovascular disease; however, findings are still far from being conclusive. Depending on the pathology or the blood vessel studied, increased, decreased or unchanged NOX-4 expression can be found (56). Thus, in SHR, NOX-4 levels have been reported to be unchanged in aged aorta (86). In contrast, NOX-4 mRNA expression seems to be higher in basilar arteries (87) or aorta (64) from SHR compared to normotensive Wistar-Kyoto rats. Similarly, increased NOX-4 expression has been observed in the renal cortex of aldosterone-salt rats and in aorta of AngII-infused mice (88, 89). In human atherosclerosis, NOX-4 expression is increased in intimal lesions of coronary arteries (67); however, in experimental atherosclerosis, NOX-4 expression is unchanged in the aorta of $ApoE^{-/-}$ mice or in primate models (90, 91).

NOX-4 regulation seems to be mostly transcriptional (Figure 9). NOX-4 has been proposed to be a housekeeping gene because its promoter region contains many GC bases (92). E2F1 transcription factor is involved in the basal NOX-4 expression in rodent VSMCs (93). Sp3 and three GC-boxes containing putative Sp/Klf binding sites are also essential for the basal expression of the *NOX-4* gene (94). Furthermore, in human endothelial cells, NOX-4 basal transcription is dependent of the deacetylation of transcription factor(s) and polymerase(s) (95). Regarding the inducible expression of NOX-4, JAK/STAT and NF-κB seem to be involved in NOX-4 expression in response to IFN-γ or TNF-α (60, 71). In addition, hypoxia induces NOX-4 through a hypoxia-inducible factor-1α (HIF-1α) dependent mechanism contributing to maintain ROS levels in smooth muscle cells from pulmonary artery (79). However, the mechanisms whereby NOX-4 is down-regulated are poorly understood. JunD, a member of the AP-1 family of transcription factors, is emerging as a major gatekeeper against oxidative stress. Interestingly, JunD knockout mice show an increased vascular expression of NOX-4 (96). However, additional mechanisms might contribute to NOX-4 down-regulation in response to different stimuli. Our group has suggested that a repressor of new synthesis is necessary for IL-1β-mediated NOX-4 transcriptional down-regulation which binds to NOX-4 proximal promoter (72).

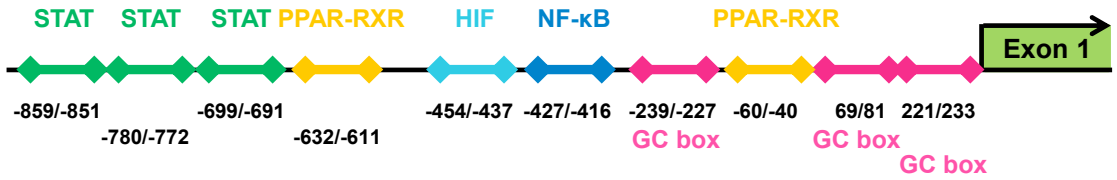

Figure 9. Structure of human NOX-4 gene promoter. Binding sites for transcription factors involved in NOX-4 expression. Retinoid X receptor (RXR).

3.2. Role of NOX-derived ROS in vascular remodeling

NOXs are important in physiological processes including host defense, aging, and cellular homeostasis. However, the up-regulation of different NOXs, including NOX-1 and NOX-4, has been implicated in several cardiovascular diseases such as atherosclerosis, hypertension, diabetes, isquemia/reperfusion, restenosis or abdominal aortic aneurisms. Thus, NOX-derived ROS contribute to the oxidative stress, vascular inflammation, endothelial dysfunction and vascular remodeling observed in these cardiovascular pathologies (1, 34, 52-54, 99). The mechanisms whereby NOX-derived ROS contribute to altered vessel structure include modulation of cell growth, apoptosis, migration, inflammation and ECM production (1, 25). This is based on both *in vitro* and *in vivo* studies using genetically modified animals and experimental models of hypertension, atherosclerosis, aneurysms and others. However, although a causal relationship has clearly been demonstrated in many animal studies, an effective ROS-modulating therapy still remains to be established by clinical studies. In addition, despite of the amount of literature available on this subject, the regulation of specific NOXs in vascular cells is not completely understood.

As mentioned, many *in vitro* studies have demonstrated the role of oxidative stress as facilitator of different processes leading to vascular remodeling (26, 41, 50). In addition, several studies in different animal models, have demonstrated the key role of ROS from different origins in vascular remodeling in cardiovascular diseases such as hypertension. Thus, in stroke-prone SHR, tempol, a SOD analogue, decreased vascular $O_2^{\bullet-}$concentration, increased antioxidant status and reduced vascular remodeling observed in this hypertensive model (100). Accordingly, in the AngII-infused mouse and deoxycorticosterone acetate-salt-induced hypertensive rat models, apocynin, a non-specific NADPH oxidase inhibitor, prevented structural alterations and collagen deposition (64, 101, 102). Finally, mito-TEMPO, a mitochondria-targeted SOD mimetic, also reduced structural alterations induced by AngII infusion (64). On the other hand, exercise training induces beneficial effects in the structure and/or mechanics of resistance arteries in hypertension probably through effects on oxidative stress (24). In addition, different drugs (angiotensin-converting-enzyme inhibitors and AngII and mineralocorticoid receptor blockers) with demonstrated beneficial effects on vascular remodeling are able to reduce ROS generation in experimental models and in humans with cardiometabolic pathologies (9, 103). Besides having a role on hypertensive vascular remodeling, ROS are also involved in vascular remodeling in the context of other cardiovascular diseases such as abdominal aortic aneurisms or atherosclerosis and the reader is referred to excellent reviews on this subject (104, 105).

Regarding the specific NOX isoform involved in vascular remodeling, genetic manipulation *in vitro* or *in vivo* using transgenic knockout or overexpressing mice have yielded additional although not conclusive results. It seems that NOX-1-derived ROS are implicated in migration of different cell types, such as in VSMCs stimulated with thrombin, PDGF or bFGF (106, 107). NOX-1 also plays a role in proliferation since targeting NOX-1 with antisense or siRNA or genetic deletion in VSMCs inhibits proliferation induced by different stimuli (107-109). Similarly, in the wire injury-induced neointima formation model, both proliferation and apoptosis were reduced in NOX-1 knockout mice (NOX-$1^{y/-}$) but there was little difference in mice overexpressing NOX-1 compared with wild type mice (107). Accordingly, proliferation and migration were reduced in response to PDGF in cultured NOX-$1^{y/-}$ VSMCs and increased along with ECM production in cells overexpressing NOX-1 compared with wild type VSMCs (107), suggesting that NOX-1 is required for the neointima formation. Several studies have evaluated the role of NOX-1 in vascular remodeling in response to AngII. AngII induces VSMC proliferation and migration as well as carotid artery hyperplasia in rats via AT_1R interaction with NOX-1 (26). Interestingly, in response to AngII, NOX-$1^{y/-}$ mice showed a marked reduction in aortic media hypertrophy (110, 111), but this reduction was due to a marked decrease in ECM accumulation and not in the number of VSMCs since AngII-induced VSMC proliferation was conserved (110). Conversely, (112) demonstrated that AngII did elicit similar hypertrophic response in the thoracic aorta of NOX-$1^{y/-}$ and NOX-$1^{y/+}$ mice although superoxide production was blunted in NOX-$1^{y/-}$. According to these findings, our group has also demonstrated that AngII plus IL-1β induced NOX-1-dependent VSMC migration (72). Finally, transgenic mice overexpressing NOX-1 in VSMCs showed markedly greater superoxide production, systolic blood pressure and aortic hypertrophy in response to AngII than their littermate controls, which were partially reversed by tempol treatment (52). Altogether, these findings suggest that cell specific location of NOX-1 might be the key to modulate hypertrophic vascular remodeling being NOX-1 from VSMCs of fundamental importance.

Regarding mechanisms activated by NOX-1-derived ROS, it has been demonstrated that in the presence of some stimuli, NOX-1 activates different proteins involved in cell adhesion and migration such as paxilin, Rac, RhoA and cofilin (44). Moreover, recently NOX-1 has been shown to be involved in matrix metalloproteinase-9 expression, a metalloproteinase essential in cell migration since NOX-1 siRNA reduced matrix metalloproteinase-9 expression (44).

The functional role of NOX-4 in vascular cells is under debate (54, 56). NOX-4 depletion leads to a loss of differentiation markers gene expression in adult VSMCs, while in mouse embryonic stem cells, NOX-4 overexpression increased VSMC differentiation markers (113, 114). These results suggest that NOX-4 contributes to the maintenance of a differentiated state of the cell preventing cell activation or proliferation (44, 54, 113, 115), suggesting a protective effect of NOX-4. However, transgenic mice with cardiac specific overexpression of NOX-4 showed decreased left ventricular function with enhanced $O_2^{\bullet-}$, production in the heart, which was accompanied by increased apoptosis and fibrosis, suggesting a deleterious role for NOX-4 (116). Interestingly, NOX-$4^{-/-}$ mice developed exaggerated contractile dysfunction, hypertrophy and cardiac dilatation during exposure to chronic overload, whereas mice with cardiomyocyte-targeted overexpression of NOX-4 were protected (117). The different functions of NOX-4 might also depend on the disease model or stimulus to be studied (56). In the AngII-infused mouse model, aortas from NOX-4-deficient animals developed increased inflammation, media hypertrophy and endothelial dysfunction compared to their wild type littermates (111) suggesting that NOX-4 might act as a protective enzyme. Besides acting on differentiation, proliferation and migration, NOX-4 has a role in other processes involved in vascular remodeling such as apoptosis, senescence and cell cycle (54). Indeed, 7-ketocholesterol-induced apoptotic events were abolished silencing NOX-4 expression, while NOX-4 down-regulation inhibited TGF-β1-dependent cell proliferation in VSMCs and PASMCs respectively by regulating ROS production and signaling cascades (81, 118). Thus, it has been suggested that NOX-4 might regulate fundamental cellular processes that contribute to each of these responses (54).

Reasons for so different roles for NOX-1 and NOX-4 in vascular biology are far from being clarified. As mentioned, NOX-4 is a special NOX because it has a high constitutive activity, is highly expressed in some cells such as endothelial cells and its subcellular location is different to other NOXs (56). Moreover, different from NOX-1 and NOX-2, NOX-4 releases predominantly H_2O_2. Although not extensively studied, H_2O_2 in the media and endothelial layers may have different functions. Thus, smooth muscle-specific catalase overexpression blocks the H_2O_2-mediated AngII-induced vascular hypertrophy (119) whereas endothelial-specific catalase overexpression prevents exercise-stimulated induction of eNOS (120). Future studies with improved tools will reveal the true nature of the role of NOX-4 in both health and disease (56).

4. CONCLUSSIONS AND PERSPECTIVES

ROS production in the vasculature by vascular and non-vascular cells is a highly regulated process. ROS act as signaling molecules, mainly through oxidative modification of proteins and subsequent activation or inhibition of different proteins involved in different processes including cell signaling or gene transcription. In cardiovascular diseases, ROS contribute to vascular injury by promoting among other processes vascular cell growth, migration, ECM protein deposition, activation of matrix metalloproteinases or inflammation, which in turn will favor vascular remodeling. The NADPH oxidase family, is an important source of ROS in the arterial wall during cardiovascular diseases and modulate vascular remodeling. As for the specific NOX isoform NOX-1 and NOX-4 seem to be particularly important, however, it is well known that activation of other NOXs (NOX-2 and NOX-5) also contribute to O2•− production in rodent and/or human VSMCs (54). The above findings suggest that strategies to reduce ROS may have therapeutic potential in cardiovascular alterations in patients. However, results in humans on this aspect have been not clarifying (34). It has been proposed that prevention of ROS generation using specific inhibitors of ROS producing enzymes such as those of the NADPH oxidase family may be better to reduce oxidative stress than attempting to scavenge ROS once they have generated (1). However, to date no selective inhibitors of NOX that can be used in clinics have been developed. Long-term awaited studies are necessary to know if such strategies would be useful in vascular remodeling associated to cardiovascular diseases.

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