Respiratory Physiology & Neurobiology 174 (2010) 212–220

Contents lists available at ScienceDirect

Respiratory Physiology & Neurobiology

journal homepage: www.elsevier.com/locate/resphysiol

Review

Reactive oxygen species signaling in pulmonary vascular smooth muscle☆

Francisco Perez-Vizcaino*, Angel Cogolludo, Laura Moreno

Department of Pharmacology, School of Medicine, University Complutense of Madrid and Ciber Enfermedades Respiratorias (Ciberes), 28040 Madrid, Spain

ARTICLE INFO

Article history:
Accepted 17 August 2010

Keywords:
Hydrogen peroxide
NADPH oxidase
Mitochondria
Hypoxic pulmonary vasconstriction

ABSTRACT

In recent years, it has become evident that reactive oxygen species (ROS) play a critical role in the regulation of several physiological and pathophysiological processes. Herein we review the main sources, targets and pathophysiological roles of ROS in pulmonary vascular smooth muscle. Mitochondria and NADPH oxidases represent the major sources of ROS in vascular cells. In addition, ROS can be produced by different pathways of arachidonic acid metabolism, endothelial NO synthase (eNOS) and xantine oxidase.

There is increasing evidence for the role of ROS, specially hydrogen peroxide, as signaling moieties to induce increase in intracellular calcium concentration ($[Ca^{2+}]_i$) and contraction in pulmonary artery smooth muscle cells (PASMC) through the modulation of a variety of targets, such as Rho kinases (ROCK), protein kinase C (PKC), voltage-gated potassium K^+ (Kv) channels and ryanodine receptors (RyR). Thus, an increase in ROS has been reported to contribute to the responses induced by different vasoconstrictor stimuli, including hypoxia. Finally, results from recent studies highlighting the involvement of ROS in the development of pulmonary hypertension are discussed in the present paper.

1. Introduction

"Reactive oxygen species" (ROS) is a common term that identifies a variety of oxygen containing substances with high reactivity with other biomolecules. ROS include both free radicals (substance containing one or more unpaired electrons) such as superoxide (O_2^-), hydroxyl ($OH^\bullet$), peroxyl ($RO_2^\bullet$), and hydroperoxyl ($HO_2^\bullet$) radicals, and non radical species such as hydrogen peroxide (H_2O_2) and other peroxides (ROOH). In parallel to ROS, the term reactive nitrogen species (RNS) is widely used to identify highly reactive substances containing nitrogen (and oxygen too) such as nitric oxide ($NO^\bullet$, a free radical) and NO derived substances such as peroxynitrite ($ONOO^-$). ROS and RNS are intimately related (Fig. 1).

ROS are constantly formed by cells in both enzymatic and non enzymatic reactions. They may interact with a huge number of cellular components including macromolecules and small molecules. Due to their high instability and the occurrence of radical chain reactions, ROS may trigger uncontrolled chemical processes in the cellular milieu. Excessive production of ROS induces irreversible alterations of proteins, lipids and DNA resulting in tissue damage. Therefore, ROS concentrations must be tightly regulated and are constantly removed by antioxidant defenses. Again, ROS may be scavenged by enzymes and by non enzymatic antioxidants. The facts that a family of specific enzymes have evolved to scavenge superoxide and peroxide and the pathological, or even lethal, consequences observed after pharmacological inhibition or gene deletion of these enzymes (Lebovitz et al., 1996) highlight the crucial importance of ROS toxicity in cells. 'Oxidative stress' is an expression that describes the consequences of an imbalance in ROS homeostasis due to an excessive formation and/or to decreased antioxidant defenses. ROS toxicity is usually mediated by hydroxyl radical while peroxynitrite is the main toxic form of RNS.

Besides their toxic effects at high concentrations, in recent years it has become evident that ROS play an essential role in the regulation of several physiological and pathophysiological processes. The enzymatic sources of ROS are regulated in response to different stimuli and the generated ROS interact specifically with several cellular targets including enzymes, ion channels and transporters, directly or by modifying other redox intermediates such as glutathione or thioredoxin. Thus, ROS can no longer be conceived merely as toxic byproducts of cell metabolism. In fact, ROS can act as intermediates in signaling pathways in much the same manner as classical second messengers. The main ROS involved in cell signaling pathways are superoxide and hydrogen peroxide. Because superoxide is electrically charged, it is highly reactive and it is rapidly dismutated into hydrogen peroxide, its concentrations are usually in the picomolar–nanomolar range and its diffusion is limited. Thus, superoxide acts as an intracellular messenger. In contrast, hydrogen peroxide is less reactive, its concentrations are in the high nanomolar–micromolar range and it diffuses better across hydrophobic membranes (Schroder and Eaton, 2008). Thus hydrogen peroxide can be regarded both as intra- as well as inter-cellular

☆ "This paper is part of a special issue entitled "Physiological Redox: Regulation in Respiratory, Vascular, and Neural Cells", guest-edited by Schumacker and Ward".

* Corresponding author. Tel.: +34 913941477; fax: +34 9139411464.
E-mail address: fperez@med.ucm.es (F. Perez-Vizcaino).

1569-9048/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.resp.2010.08.009

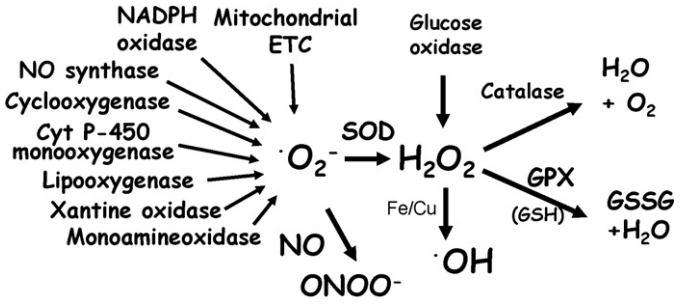

Fig. 1. Schematic diagram illustrating the main sources of ROS generation and endogenous antioxidant systems. Multiple sources can generate superoxide (O_2^-) which is rapidly dismutated to hydrogen peroxide (H_2O_2). Superoxide can also react with nitric oxide (NO) to form peroxynitrite ($ONOO^-$), resulting in NO inactivation. Hydrogen peroxide produced from superoxide dismutation or by glucose oxidase is converted into H_2O by catalase and glutathione peroxidase (GPx) but, in the presence of iron or cupper, can also produce the highly reactive hydroxyl radical ($OH^•$) by the Fenton reaction.

messenger. In this paper we review the sources, the targets and the pathophysiological role of ROS in pulmonary vascular smooth muscle cells (VSMC).

2. Sources of ROS in the pulmonary vasculature

2.1. NADPH oxidases

The structure and function of the NADPH oxidase was first characterized in neutrophils where it was reported that the activation of phagocytes determined the migration of the cytoplasmic subunits of the NADPH oxidase to the membrane, leading to the activation of the catalytic core of the enzyme (gp91^{phox} subunit) and subsequent production of superoxide. The availability of the human genome sequence led to the identification of novel homologs of the catalytic subunit gp91^{phox}, which are now termed as NOX (NADPH oxidase systems) and where gp91^{phox} is renamed NOX2. In humans, seven isoforms have been identified and classified into three groups based on evolutionary criteria: the group close to NOX2 (NOX1, NOX3, NOX4), the group of dual oxidases (DUOX1 and DUOX2) and NOX5. The different isoforms of the NOX family vary in their mechanisms of activation and distribution, which suggests they are involved in different physiological functions (Bedard and Krause, 2007). NOX-1, NOX-2, NOX-4 and NOX-5 have been detected in VSMCs, endothelium and adventitia and are probably the main source of ROS in the cardiovascular system (Briones and Touyz, 2010; Perez-Vizcaino et al., 2002).

The prototype NADPH oxidase (NOX2), found in phagocytic cells, is a multi-enzyme complex whose catalytic center is formed by the flavocytochrome b558, which is composed of two subunits, p22^{phox} and NOX-2 or its analogs. The activation of this complex requires the recruitment of several cytosolic subunits (p47^{phox}, p40^{phox}, p67^{phox} and Rac-1) to the cell membrane and its association with flavocytochrome b558 (Bedard and Krause, 2007). NOX1, the first and closest homolog of NOX2 to be described, is highly expressed in the colon epithelium but show intermediate- to low-level of expression in other cell types, including vascular smooth muscle cells and endothelial cells. NOX1 requires the presence of at least two novel cytosolic subunits, NOXO1 (NOX organizer 1 = p47^{phox} homolog) and NOXA1 (NOX activator 1 = p67^{phox} homolog). NOX-4 was originally identified as an NADPH oxidase homolog highly expressed in the kidney and suggested to be an O_2 sensor involved in controlling the production of erythropoietin. NOX4, which is strongly expressed in endothelial cells and pulmonary artery smooth muscle cells (PASMCs), does not seem to require cytosolic subunits and it might be constitutively active.

2.1.1. NOX in the pulmonary vasculature

In lung tissue, NOX1, NOX2, NOX4 and p22^{phox}, as well as the cytosolic subunits NOXA1, NOXO1, p47^{phox}, p40^{phox} and p67^{phox} are expressed with NOX2, NOX4 and p22^{phox} showing apparently the highest levels in vascular cells. In endothelial cells, NOX4 appears to be expressed at a higher level than NOX2. In PASMCs, both NOX4 and NOX1 predominate whereas NOX4 seems to be the most abundant isoform expressed in the adventitia (Brandes and Kreuzer, 2005; Mittal et al., 2007; Pendyala et al., 2009).

Vascular NOX, especially NOX4, seem to be constitutively active generating superoxide and its metabolic products such as hydrogen peroxide. Additionally, NOX can be activated by a wide range of stimuli including G-protein coupled receptor agonists (angiotensin II, thrombin, endothelin, serotonin, thromboxane A_2), cytokines (tumor necrosis factor alpha and transforming growth factor beta) or changes in oxygen levels (Briones and Touyz, 2010). The signaling pathways leading to NOX activation in vascular cells is best characterized for NOX2 and NOX1. Phosphorylation and activation of p47^{phox} and p22^{phox} by different kinases modulate the activity of these enzymes in both systemic and pulmonary arteries (Bedard and Krause, 2007; Cogolludo et al., 2006; Rathore et al., 2008). ROS generated by vascular NOX may then act as signaling molecules activating a wide range of pathways involved in the control of pulmonary vascular tone, cell proliferation and apoptosis; inflammatory responses; fibrosis and calcification, as described in Section 4. A number of drugs have been reported to be NOX inhibitors: (1) Diphenylene iodonium (DPI) is a pan-flavin enzyme irreversibly inhibitor which, in addition to NOX, inhibits other sources of ROS, including xantine oxidase or mitochondrial complex I. (2) Apocynin is a pro-drug that is oxidized by peroxidases and has long been used as an NOX inhibitor. Apocynin seems to inhibit the translocation of p47^{phox} to the membrane and its interacion with NOX2 or other isoforms. However, it has been suggested that apocynin may not actually inhibit NOX at all in vascular smooth muscle, but may act as an antioxidant (Heumuller et al., 2008). Other possible targets of this drug include Rho kinase (Schluter et al., 2008). (3) gp91ds-tat is a peptide inhibitor designed to mimic a region of gp91^{phox} that interacts with p47^{phox} and, thus, to interfere with NADPH oxidase assembly and activation. However, this peptide may lack selectivity because of the homology of this region in other NOX isoforms. Although more compounds with a NOX-inhibitory function have been identified, to date, none of the currently available "NOX inhibitors" are selective and thus precaution must be taken when interpreting the results with these drugs. Similarly, knockdown (siRNA) or knockout strategies, although specific, may activate compensatory mechanisms as those seen in human pulmonary artery endothelial cells (PAECs) for NOX2 and NOX4 (Pendyala et al., 2009).

2.2. Mitochondria

Mitochondria are also a major source of ROS production in the cardiovascular system under physiological conditions. The matrix contains enzymes involved in the metabolism of pyruvate and fatty acids to produce acetyl CoA as well as the enzymes of the citric acid cycle (except succinate dehydrogenase which is bound to the inner mitochondrial membrane (IMM) as part of complex II) which oxidizes acetyl CoA to CO_2, yielding during this process three molecules of NADH and one molecule of FADH2. NADH and FADH2 are used as electron donors by the electron transport chain (ETC) complex to generate the proton gradient in the IMM used to synthesize ATP.

Oxidation of NADH by complex I and FADH2 by complex II reduces ubiquinone to ubiquinol. Ubiquinol is then reoxidized by complex III. Oxidation of ubiquinol to ubiquinone involves the formation of ubisemiquinone and takes two cycles within complex III

with the two electrons of ubiquinol being sequentially transferred to complex IV where oxygen is reduced by cytochrome c oxidase (CcO). Under physiological conditions, approximately 2–3% of oxygen consumed by mitochondria is incompletely reduced and yields ROS. Although the specific sites where electrons are lost have not been completely elucidated, ROS seems to be generated in complex I, II and III, with complex I and III apparently being the main sites (Stowe and Camara, 2009). Superoxide can then be dismutated to hydrogen peroxide by the mitochondrial Mn superoxide dismutase (SOD) and the cytosolic CuZn SOD.

Complex I is one of the two entry sites (with complex II) for reducing equivalents and is probably the major source of mitochondrial ROS generation under physiological conditions. Several sites between the flavin complex and the quinone site have been proposed to generate superoxide within complex I. For example, rotenone inhibits electron transfer from ubiquinone binding site to complex I and blocks superoxide release by this mechanism. Ubisemiquinone is believed to be the other main source of mitochondrial ROS by undergoing auto-oxidation and generating superoxide in both Qo (intermembrane space) and Qi (matrix) sites of complex III. Antimycin A is thought to bind to the Qi site leading to accumulation of ubisemiquinone at Qo site and subsequent increase in superoxide. The increase in ROS induced by antimycin A can be blocked by either stigmatellin or myxothiazol which are thought to prevent ubisemiquinone formation within complex III (reviewed by Stowe and Camara, 2009).

2.2.1. Mitochondrial ROS generation in the pulmonary vasculature

Production of ROS by vascular mitochondria can be affected by modulation of the mitochondrial biogenesis, fussion and fission processes and mitochondrial bioenergetics (Erusalimsky and Moncada, 2007). Regulation of these processes occurs at multiple levels, including transcription, postranscriptional modifications (i.e., phosphorylation) and intracellular localization. Factors modulating mitochondria include the concentration of oxygen, the availability of electron donors, the activity of uncoupling proteins (UCP), cytokines and vasoactive mediators (NO, angiotensin-II, thromboxane A_2) (Bailey et al., 2005; Rathore et al., 2006; Waypa et al., 2010). In addition, ROS may lead to a greater production of ROS by a self amplifying mechanism (ROS-induced ROS release). Indeed, both angiotensin-II and hypoxia have been shown to increase mitochondrial ROS production and, via protein kinase C (PKC), a subsequent NOX activation in PASMCs and aortic ECs (Doughan et al., 2008; Rathore et al., 2008).

Particularly relevant to the cardiovascular system is the modulation of mitochondrial ATP and superoxide production by tissue oxygen concentration. Despite the amount of superoxide anion generated by isolated mitochondria increases when the oxygen concentration increases, ROS levels are elevated in cardiac myocytes not only during reperfusion-reoxygenation following ischemia-hypoxia but also during ischemia and hypoxia (Stowe and Camara, 2009). Similarly, increased ROS production induced by hypoxia in PASMCs has been reported by several groups (Rathore et al., 2006, 2008; Waypa et al., 2006) although the opposite has been found by others (Archer et al., 1993), as discussed in more detail in Section 4.3. In addition, exposure to prolonged periods of hypoxia also modifies the respiration/ROS generation rate by modifying the expression of enzymes involved in the supply of electron donors (i.e., pyruvate dehydrogenase or lactate dehydrogenase), ETC components (CcO subunits) or antioxidants systems (SOD, heme-oxygenase 1) following the activation of transcription factors such as hypoxia-inducible factor (HIF) and AP-1 (Semenza, 2007). Furthermore, activation of HIF represents another example of self regulation of ROS production by ROS levels. Although constitutively expressed, HIF-1alpha has a very short life under normoxic conditions because of its hydroxylation by proline hydroxylases which signals HIF-1alpha for degradation. Under hypoxic conditions, proline hydroxylases are inhibited as a consequence of substrate deficiency (low $[O_2]$) and oxidation by Complex III-generated ROS. Inhibition of proline hydroxylases allows for HIF-1alpha stabilization and the transcriptional regulation of target genes by HIF (Semenza, 2007). Finally, regulation of mitochondrial ROS production has also been observed following activation of other transcription factors with a pathophysiological role in pulmonary hypertension, such as the PPAR gamma coactivator PGC-1 alpha (Rabinovitch, 2008; Semenza, 2007).

NO and its metabolite nitrite, are other well-characterized mitochondrial regulators. Modulation of mitochondria by NO involves both cGMP-dependent mechanisms (i.e., promotion of mitochondrial biogenesis) and cGMP-independent mechanisms (i.e., binding to CcO). The interaction between NO and CcO represents the best well-known example of modulation of mitochondria by NO. NO seems to bind the CcO in two ways, by nitrosylation (addition of NO to cysteines) of the heme group or by oxidation to nitrite in the active site (Erusalimsky and Moncada, 2007). In either case, NO prevents O_2 binding, inhibits mitochondrial respiration and may increase superoxide production as consequence of the inhibition in electron flux through CcO. These effects are thought to trigger activation of AMP-activated protein kinase (AMPK) and redox-sensitive factors seen in the presence of NO in a wide number of cells (Erusalimsky and Moncada, 2007).

2.3. Arachidonic acid metabolism

The metabolism of arachidonic acid can also cause the generation of superoxide. Arachidonic acid can be metabolized by three major pathways:

- The *cyclooxygenase (COX) pathway*, also called endoperoxide H synthase (PGHS), which leads to the formation of prostaglandins (PGs: E_1, E_2, $F_{2\alpha}$, D_2, H_2 and I_2 or prostacyclin) and thromboxanes (TXs: A_2 and B_2). The PGs and TXs are synthesized from arachidonic acid by the two isoforms of COX, COX-1 and COX-2, with different characteristics in their regulation, tissue distribution and drug susceptibility (Mitchell and Warner, 1999). Both enzymes catalyze the formation of PGH_2 from arachidonic acid and can generate ROS during the second stage of the synthesis of prostanoids, due to its peroxidase activity (Kukreja et al., 1986). COX is a potential source of superoxide in the pulmonary circulation at least in certain conditions (Perez-Vizcaino et al., 2002). As described in Section 4.1, activation of COX can also stimulate other sources of ROS through the actions of its metabolites (Cogolludo et al., 2006).
- *The lipooxygenase pathway*, which produces hidroxyeicosatetranoic acids and leukotrienes, may be also mediating the rise in ROS generation seen in the pulmonary circulation under certain pathophysiological conditions.
- *The cytochrome P-450 monooxygenase pathway*, which can generate various epoxides and hydroxy acids.

2.4. Endothelial NO synthase (eNOS)

eNOS is a homodimeric complex that catalyzes the formation of NO from L-arginine by electron transfer between the cofactor NADPH, the heme prosthetic group and tetrahydrobiopterin (BH_4). Under some conditions, the limited availability of the cofactor BH_4 or the substrate L-arginine, uncoupling of the enzyme allows the electrons to be transferred to oxygen instead of L-arginine, with the subsequent production of superoxide and decreased production of NO (Belik et al., 2009b; Konduri et al., 2007). It should be

noted that although eNOS have attracted most of the interest, all three NOS isoforms have been shown to be susceptible to uncoupling.

2.5. Xantine oxidase

Xanthine oxidase (XO), present in the vascular endothelium, catalyzes the oxidation of hypoxanthine to xanthine and xantine to uric acid, generating NADH and superoxide during these reactions. The XO-hypoxanthine system is also one important pathway to generate oxidative stress *in vivo*, and XO is upregulated by hypoxia in cultured pulmonary arterial endothelial cells (Partridge et al., 1992).

2.6. Other sources

Monoamineoxidase generates superoxide during the metabolism of amines. Superoxide can be also generated by nonenzymatic reactions by auto-oxidation of several endogenous substances including cathecols. Hydrogen peroxide is also a byproduct of glucose oxidase (Fig. 1).

3. Targets of ROS

Reactive oxygen species serve as important intracellular and intercellular second messengers to regulate a variety of downstream signaling pathways through reactions with protein residues, mainly cysteine, which leads to disulfide formation, albeit protein carbonylation by ROS has recently been proposed as a novel redox signaling in PASMC (Wong et al., 2008). In this section, we focus on those ROS targets, which by modulating cytosolic calcium concentration ($[Ca^{2+}]_i$) or Ca^{2+} sensitivity, play a central role in the regulation of pulmonary vascular reactivity (Fig. 2).

3.1. Protein kinases

3.1.1. Rho kinases (ROCKs)

ROCKs are serine/threonine kinases which play an important role in a wide range of cellular functions including motility, proliferation, apoptosis and contraction. There is now considerable evidence supporting the involvement of ROCKs in the regulation of vascular tone and in the development of a variety of cardiovascular disorders (Loirand et al., 2006). Two isoforms (ROCK-1 and ROCK-2) have been identified and both are expressed in vascular smooth muscle. ROCKs are well known effectors of RhoA, a member of the Ras family of small GTP binding proteins. In its active GTP-bound form, RhoA activates ROCK which phosphorylates and inactivates myosin light chain phosphatase (MLCP). The inhibition of MLCP leads to an increase in MLC phosphorylation, which promotes contraction independently of changes in $[Ca^{2+}]_i$ concentration, also referred to as Ca^{2+} sensitization. It is now well established that the RhoA/ROCK pathway is the major regulator of the Ca^{2+} sensitization of the contractile proteins.

In systemic arteries, the RhoA/ROCK pathway is activated by ROS generated by xanthine/XO or mitochondria (Bailey et al., 2005). Alternatively, the RhoA/ROCK pathway may also modulate ROS generation. Thus, long term treatment with the ROCK inhibitor fasudil reduces NOX expression and superoxide production by the endothelium. In pulmonary arteries (PA), ROS also seem to signal through the RhoA/ROCK pathway. Thus, an increase in ROS mediates the augmented basal and ET-1-stimulated RhoA activity after chronic hypoxia (Jernigan et al., 2008). These responses were abolished by the superoxide scavenger tiron, thus suggesting a role for superoxide. Accordingly, Knock et al. (2009) have reported that the superoxide generator LY83583 induced translocation of ROCK-2 from the nucleus to the cytosol and evoked a vasoconstriction sensitive to the ROCK inhibitor Y27632 in rat pulmonary arteries. Although the activation of RhoA by superoxide seems very consistent, some discrepancies have arisen in relation with hydrogen peroxide. Thus, while after 1 h treatment with hydrogen peroxide (25 μM) the activity of RhoA is increased in cultured PASMC and tended to increase in PA endothelial cells (Chi et al., 2010), contraction induced by acute application of hydrogen peroxide (30 μM) is unaffected by ROCK inhibition (Pourmahram et al., 2008). Additionally, contraction of the ductus arteriosus induced by hydrogen peroxide (10 μM) is sensitive to Rho kinase inhibitors (Cogolludo et al., 2009b).

3.1.2. Protein kinase C

PKC includes a family of several isoforms that can be categorized into conventional or cPKC (α, β_1, β_2, and γ), novel or nPKC (δ, ε, η, θ, υ and μ), and atypical or aPKC (ζ and λ/ι) isoforms. PASMC have been found to express at least PKCα, PKC δ, PKC ε PKC λ/ι, PKCυ and PKCζ (Moreno et al., 2007; Rathore et al., 2006; Ward et al., 2004). ROS have been shown to activate different PKC isoforms. In isolated PA, hydrogen peroxide-induced Ca^{2+} sensitization and constriction was abolished by PKC inhibitors and was associated with PKCα activation (Pourmahram et al., 2008). Rathore et al. (2006) found that exogenous hydrogen peroxide, mimicking hypoxia, increases PKCε activity. More recently, these authors have shown that pharmacologic and genetic inhibition of PKCε also blocks the hypoxia-induced ROS production and proposed a model in which mitochondrial-derived ROS activate PKCε, which subsequently activates NOX-dependent ROS generation, providing a positive feedback mechanism involved in hypoxic-induced increase in ROS in PASMC (Rathore et al., 2008).

Apart from ROCK and PKCs, other kinases have been implicated in the regulation of pulmonary vascular smooth muscle tone (reviewed by Ward et al., 2004). Src-family kinase is probably the most important non-receptor tyrosine kinase in terms of tension development by regulating a number of downstream effectors, including MAP kinases (ERK1/2 and p38 subfamilies). This kinase has been implicated as an important signaling molecule responsible for ROS-induced cellular signaling cascades in vascular smooth muscle (Oeckler et al., 2005). Thus, hydrogen peroxide causes phosphorylation of extracellular signal-regulated kinases (ERK)1/2 and p38 mitogen-activated protein kinase (MAPK) and pulmonary vasoconstriction probably through a Src-dependent mechanism.

3.2. Potassium channels

Voltage-gated potassium K^+ (Kv) channels, particularly Kv1.5 and Kv2.1, play an important role in the regulation of pulmonary vascular tone and their inhibition has been shown to contribute to hypoxic pulmonary vasoconstriction (HPV; Archer et al., 1993). The redox hypothesis of HPV developed by Weir and Archer (1993) states that inhibition of mitochondrial oxidative phosphorylation by hypoxia decrease ROS production, which in turn, cause the cytosol of PASMC to become more reduced leading to the inhibition of the redox-sensitive Kv channels. In favour of this hypothesis oxidizing and reducing agents increase and decrease Kv current amplitude, respectively (Olschewski et al., 2004). Similarly, thiol oxidation by diamide causes pulmonary vasodilation attributed to K^+ channels opening (Schach et al., 2007). This idea is also supported by the fact that hydrogen peroxide or its analog tert-butyl hydroperoxide increase the activity of Kv1.5 channels expressed in heterologous systems (Schach et al., 2007). However, when tested in freshly isolated rat PASMC, tert-butyl hydroperoxide (at relatively low concentration 5 μM) rather than increase, caused a marked inhibition of the native Kv current (Cogolludo

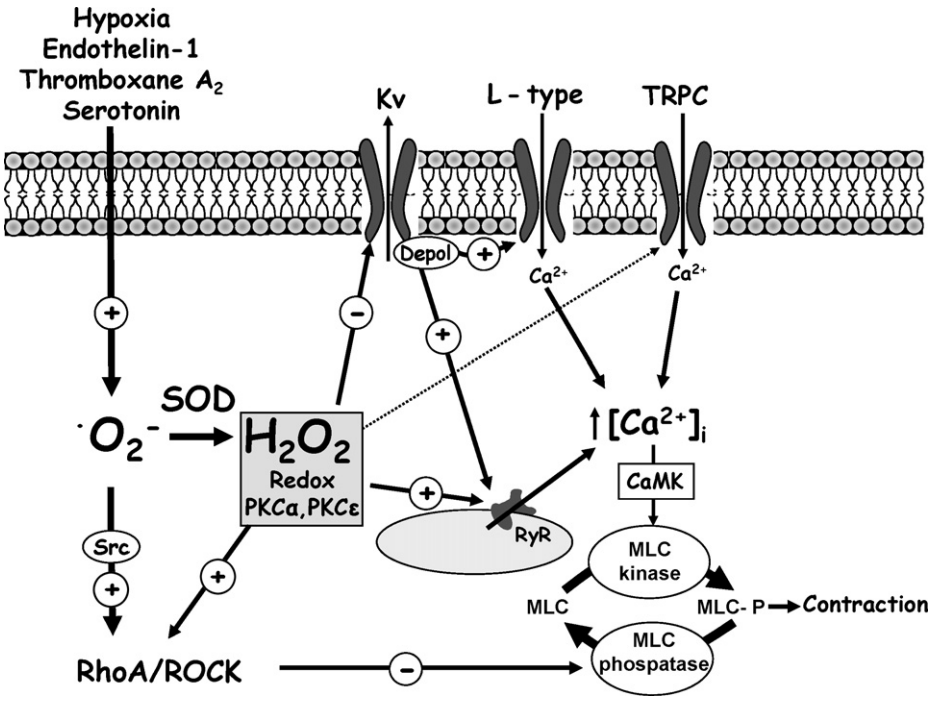

Fig. 2. Mechanisms implicated in ROS-induced pulmonary vasoconstriction. Schematic diagram showing the molecular targets and the potential signaling pathways involved in ROS-induced elevation of intracellular Ca^{2+} ($[Ca^{2+}]_i$) and contraction in pulmonary arteries. Superoxide anions are dismutated by SOD to hydrogen peroxide, which plays a prominent role as a second messenger modulating a number of molecular targets leading to an increase in $[Ca^{2+}]_i$ and contraction. Alternatively, superoxide may also directly activate the Rho/ROCK pathway through a Src-dependent mechanism. A variety of vasoconstrictor stimuli have been reported to signal through an increase in ROS production. The possible role of TRPC channels is indicated with a dotted line since, albeit suggested, has not been established. CaMK Ca^{2+}/calmodulin dependent kinase; Depol, depolarization; Kv, voltage-gated potassium channels; L-type, voltage-gated L-type Ca^{2+} channels; MLC, myosin light chain; MLC-P, myosin light chain phosphorylated; RyR, ryanodine receptors; Src, src family kinases; TRPC, canonical transient receptor potential channels.

et al., 2006). Moreover the hypoxia-induced Kv channel inhibition is prevented by catalase (Frazziano et al., 2007). These results indicate that Kv channel inhibition can also be associated with an increase in hydrogen peroxide as proposed in the ROS hypothesis for HPV. Interestingly, hydrogen peroxide also inhibits the oxygen-sensitive Kv current and causes constriction of the ductus arteriosus (Cogolludo et al., 2009b; Reeve et al., 2001).

In previous studies, our group has reported the essential role of PKCζ in the inhibition of Kv channels by U46619 (Cogolludo et al., 2003) and hypoxia (Cogolludo et al., 2009a) in rat freshly isolated PASMC. Nevertheless, this kinase is involved in hypoxic-induced ROS generation rather than in ROS-mediated Kv channel inhibition (Cogolludo et al., 2008). As mentioned above, hydrogen peroxide may activate other PKCs in pulmonary arteries such as PKCα (Pourmahram et al., 2008) and PKCε (Rathore et al., 2006), which could mediate the inhibition of Kv channels. In agreement with this idea, Rainbow et al. (2009) have recently shown that PKCα and PKCε are involved in the Kv channel inhibitory effect of endothelin-1 and angiotensin II, respectively, in mesenteric arteries.

Altogether the presented evidence indicates that the effects of ROS on K^+ channel function (and on vascular tone, see below) are complex and may reflect the modulation of the α or β subunits through different mechanisms including, among others, redox regulation and phosphorylation by protein kinases.

3.3. Ca^{2+} entry and Ca^{2+} release

The mobilization of Ca^{2+} from intracellular stores is of critical importance in the regulation of vascular smooth muscle tone and a number of publications have demonstrated the role of Ca^{2+} release from ryanodine-sensitive stores in pulmonary vasoconstriction (reviewed in Aaronson et al., 2006; Wang and Zheng, 2010; Ward and McMurtry, 2009). The three subtypes of ryanodine receptors (RyR) present in mammalian cells (RyR1, RyR2 and RyR3) are expressed in pulmonary arteries. Gene deletion of RyR1 largely blocked the increase in $[Ca^{2+}]_i$ induced by acute hypoxia in cultured PASMC (Li et al., 2009). Interestingly, these authors also showed that the increase in $[Ca^{2+}]_i$ and contraction following membrane depolarization with high K^+ was also markedly reduced in PASMC from RyR1$^{-/-}$ and RyR1$^{+/-}$ mice. These results suggest that mechanisms causing depolarization (such as inhibition of potassium channels) can be coupled through a yet unknown signal to RyR-dependent Ca^{2+} release. RyR blockade with ryanodine (50 μM) inhibits hydrogen peroxide-induced increase in $[Ca^{2+}]_i$ in cultured PASMC (Lin et al., 2007) and elevation in $[Ca^{2+}]_i$ and vasoconstriction in isolated rat PA (Pourmahram et al., 2008).

The canonical transient receptor potential (TRPC) channels constitute one of the major subfamilies of the transient receptor potential (TRP) family of ion channels. This subfamily includes seven members TRPC1-7, all of which are expressed in the pulmonary arteries. TRPC channels are considered main candidates for store operated channels (SOC) as well as receptor operated channels (ROC). Among the different TRPCs, TRPC3 and TRPC4 are redox sensitive whilst TRPC1 and TRPC6 are likely to be major determinants of cation entry in intrapulmonary arteries (Weissmann et al., 2006) which might occur through a ROS-dependent signaling. However, direct evidence of TRPC channels modulation by ROS in PA is lacking. The fact that hydrogen peroxide-induced increase in $[Ca^{2+}]_i$ and constriction of pulmonary arteries are unaffected by removal of extracellular Ca^{2+} or by TRPC channel inhibitors (Lin et al., 2007; Pourmahram et al., 2008) argues against an involvement of these channels in ROS-induced responses in pulmonary arteries. Moreover tert-butyl hydroperoxide rather than activating caused a profound inhibition of store depletion-dependent capacitative

calcium entry in PAEC (Florea and Blatter, 2008), an effect widely recognized for a variety of non vascular cells, especially platelets.

3.4. Guanylyl cyclase

It is well known that superoxide interacts rapidly with NO and attenuates its availability to stimulate soluble guanylyl cyclase (sGC), leading to a reduced capacity of endothelial cells to induce vasodilatation (i.e., endothelial dysfunction) in systemic and pulmonary (Lopez-Lopez et al., 2008) vessels. However, peroxide is a known activator of sGC and may induce relaxation by this pathway (Wolin, 2009).

3.5. Spatial localization for ROS signaling

ROS generating systems, particularly NOX and the mitochondrial ETC, are now recognized to have specific subcellular localizations. Spatial distribution allows for local increases of ROS levels close to appropriate downstream targets. This localization of the ROS signal is essential for activating rapid and specific redox signaling events (Ushio-Fukai, 2009) in a variety of processes, including cell proliferation, migration, differentiation and gene expression. Unfortunately, the available information of redox signaling compartmentalization in the regulation of vascular function is still scarce. Notably, ceramide-enriched membrane domains provide a spatial reorganization of signaling molecules upon stimulation and are now considered as important redox signaling platforms (Zhang et al., 2009). An increase in neutral sphingomyelinase (nSMase)-derived ceramide plays a key role in the rise of ROS generation (Cogolludo et al., 2008), inhibition of Kv channels and contraction induced by hypoxia in rat pulmonary arteries (Cogolludo et al., 2009a). In addition, hypoxia induced the association of $p47^{phox}$ with caveolin-1 and with Kv1.5 channels (Cogolludo et al., 2008), which is consistent with an important role of compartmentalization of signaling molecules in the HPV response. Rathore et al. (2008) proposed that elevation in mitochondrial ROS generation activates NADPH oxidase, consequently further increasing ROS levels. Since ceramide stimulates the generation of ROS and conversely nSMase is a redox-sensitive enzyme which can be activated by an increase in ROS (Jana and Pahan, 2004), it is likely that the nSMase-NADPH oxidase signal might function as a redox amplification pathway necessary for HPV.

4. Pathophysiological role of ROS

In contrast to the systemic arteries which regulate blood flow and oxygen delivery to the organs and tissue depending on their demands, PA regulate blood flow into the lung in order to achieve the highest oxygen saturation possible. Despite several regulatory pathways are common to systemic and pulmonary arteries, a number of mechanisms operate specifically in the latter, particularly those involving oxygen and ROS. Thus, PA respond to hypoxia with a vasoconstrictor response while a vasodilation is observed in systemic arteries.

4.1. Contractile and vasodilator effects of ROS in the pulmonary circulation

Both superoxide and hydrogen peroxide are modulators of vascular tone. The responses to exogenously added ROS are heterogeneous and both contractile and relaxant responses can be observed depending on the different forms of ROS used, the blood vessel studied, and the existence of a previous contractile tone. Superoxide usually contracts pulmonary arteries in a reversible manner. The contractile effect induced by superoxide generated by XO could be completely blocked by catalase, leading to the general assumption that superoxide is rapidly converted to hydrogen peroxide by mitochondrial Mn-SOD and cytosolic CuZn-SOD and that hydrogen peroxide, and not superoxide, acts as the signaling moiety that initiates the resulting vasoconstriction (Rhoades et al., 1990). However, the vasoconstriction induced by superoxide generated intracellularly using LY83583 was insensitive to catalase suggesting that it was not due to its conversion to hydrogen peroxide which is also consistent with a different signaling pathway for each ROS as mentioned above (Knock et al., 2009).

On the other hand hydrogen peroxide has both contractile and relaxant effects. Both effects may have a physiological role since hydrogen peroxide has been proposed for both endothelium-dependent contractile factor (EDCF) and for endothelium-dependent hyperpolarizing factor (EDHF) (Tang and Vanhoutte, 2009). In PA under basal tone, hydrogen peroxide induces a contractile response (Cogolludo et al., 2006; Pourmahram et al., 2008). However, in arteries stimulated with $PGF_{2\alpha}$, peroxide causes a transient contraction followed by relaxation. The differential response to peroxide can be observed even in two different portions of the same vessel under the same experimental conditions. Thus, in the chicken ductus arteriosus the segment close to the aorta and the segment close to the pulmonary artery relaxes and contracts, respectively, in response to hydrogen peroxide (Cogolludo et al., 2009b).

To our knowledge, the effects of hydroxyl radical have not been directly analyzed in the pulmonary vessels but this radical may mediate the irreversible effects of hydrogen peroxide when used at toxic concentrations (>100 μM). In systemic vessels, hydroxyl radical mediated contractions are not reversible and thus can be attributed to permanent vascular damage.

Besides the direct effects on smooth muscle, ROS can modulate vascular tone via the release of, or the interaction with, other vasoactive factors. The rapid reaction of superoxide with NO is a well known mechanism to induce endothelial dysfunction in systemic and pulmonary arteries. Exogenous addition of SOD induces an endothelium-derived NO-dependent relaxation (Villamor et al., 2003) and potentiates the effect of exogenously added NO in endothelium denuded piglet PA (Lopez-Lopez et al., 2001). These data indicate that basal superoxide levels may modulate arterial tone via scavenging NO even in the absence of imposed oxidative stress. This effect is much more evident when superoxide levels are increased by exogenous addition of superoxide generating systems, by upregulation of superoxide sources under pathological conditions or by inhibition of endogenous SOD (Lopez-Lopez et al., 2008, 2001; Villamor et al., 2003).

ROS can also influence vascular tone by the release of prostanoids via COX-dependent and independent pathways. The relationship between ROS and COX is rather complex. COX is a potential source of superoxide in the pulmonary circulation (Perez-Vizcaino et al., 2002) and, conversely, ROS stimulates COX. Moreover, COX metabolites such as TXA_2 also stimulate other sources of ROS such as NOX (Cogolludo et al., 2006). The pulmonary vasoconstrictor effect of superoxide in perfused rabbit lungs was prevented by COX inhibitors and was associated to the formation of thromboxanes, leukotrienes, and prostaglandins (Tate et al., 1984). Endothelial cells can release ROS which stimulate COX in the vascular smooth muscle with subsequent stimulation of the TP receptors by the produced prostanoids. In addition, isoprostanes are a family of prostanoid derivatives produced by nonenzymatic peroxidation of esterified arachidonic acid, through the action of ROS and RNS (Belik et al., 2009a). In fact, plasma or urinary levels of isoprostanes are widely used markers of ROS production in animals and humans. Isoprostanes exert mainly contractile responses in the pulmonary circulation via activation of TP receptors although relaxant effects can also be observed for some isoprostanes (Gonzalez-Luis et al., 2010).

4.2. Effects of ROS on proliferation and apoptosis of PASMC

The vascular remodeling process is characterized by hypertrophy and *de novo* muscularization of the vessel media. Fetal PASMCs show increased proliferation in response to hydrogen peroxide or to increasing superoxide using a Cu/Zn SOD inhibitor. This suggests that both superoxide and hydrogen peroxide stimulate proliferation (Wedgwood et al., 2001). Conversely, superoxide dismutase and catalase, superoxide dismutase/catalase mimetics and the antioxidant ascorbic acid prevented serum-induced proliferation in fetal PASMC and in the long term stimulated apoptosis (Wedgwood and Black, 2003; Wedgwood et al., 2001). As described above for the regulation of vascular tone, ROS may also potentially regulate PASMC proliferation indirectly via reducing the antiproliferative effects of NO or by releasing COX-derived prostanoids or isoprostanes which also induce PASMC proliferation.

4.3. Role in the signaling of vasoactive factors and hypoxia

In the vasculature, ROS signaling has been implicated in a large number of pathways. There is increasing evidence for the role of hydrogen peroxide as a signaling moiety to induce contraction in PASMC, therefore contributing to the response induced by different stimulus. Thus, increase in ROS has been observed for different vasoconstrictors such as TXA_2, endothelin-1, serotonin and angiotensin-II in pulmonary arteries via different signaling pathways (Briones and Touyz, 2010; Cogolludo et al., 2006; Jernigan et al., 2008). Accordingly, inhibition of ROS production or scavenging ROS partially inhibits the contractile responses to these vasoconstrictors.

HPV reflects an intrinsic property of PASMC, which allows shifting blood flow from hypoxic to normoxic lung areas, thereby coupling ventilation and perfusion. Multiple hypotheses have emerged but no convincing explanation has been given yet to explain the molecular basis of HPV (Ward and McMurtry, 2009). The mechanism is supposed to involve a putative redox-based O_2 sensor regulating the activity of effector proteins and there is a large body of evidence indicating a role of the ROS superoxide and hydrogen peroxide as signaling intermediates. However, whether ROS increase or decrease in hypoxia and the source of ROS is a matter of intense debate (Ward and McMurtry, 2009). Since the redox theory for HPV was proposed by Archer et al. (1993), data supporting opposing theories on the effects of hypoxia on ROS production (decrease vs. increase) and the molecular sources of ROS (mitochondria vs. NADPH oxidase) have emerged. The mitochondrial redox theory of HPV proposed that there is a tonic production of ROS in the mitochondrial electron transport chain in PASMCs that is inhibited by hypoxia. In contrast, multiple groups, using different approaches, sustained that hypoxia paradoxically increases ROS production in PA and that HPV can be prevented by scavengers of ROS. Recent studies indicate that these apparently contrary hypotheses may all be true. Thus, hypoxia causes a decrease in ROS generation in the mitochondrial matrix compartment, whereas it increases regulated ROS production in the mitochondrial intermembrane space, which diffuses to the cytosol (Waypa et al., 2010). Moreover, hypoxia-induced elevation of mitochondrial ROS diffusing into the cytosol can trigger the activation of NOX further increasing ROS levels (Rathore et al., 2008).

ROS also mediate the proliferative effect of several vasoactive factors in pulmonary vascular smooth muscle cells. Serotonin is taken up by PASMC via the specific serotonin transporter and stimulates MAPK activity and proliferation through the formation of superoxide anion (Maclean and Dempsie, 2010). In bovine PASMCs, serotonin induces ROS through activation of NADPH oxidase. However, in human PASMCs ROS is produced via the breakdown of serotonin by monoamine oxidase. ET-1 also stimulates PASMC proliferation via induction of ROS (Wedgwood et al., 2001). Moreover, hypoxia-induced PASMC proliferation also involves mitochondria-derived ROS (Hu et al., 2010). Thus, opening of mitochondrial K_{ATP} channels followed by a depolarization of mitochondria might play an important role in hypoxic proliferation of human PASMCs through cytochrome C accumulation or mitochondrial overproduction of hydrogen peroxide.

4.4. Pulmonary hypertension

Pulmonary hypertension (PH) is characterized by a progressive elevation of pulmonary arterial pressure due to changes in the structure and function of pulmonary arteries, ultimately inducing right ventricular failure and death (Rabinovitch, 2008). Persistent pulmonary hypertension of the newborn (PPHN) constitutes a subgroup of PH occurring in newborns which do not undergo a normal transition of the pulmonary circulation at birth and is also associated with a significant risk of death. Besides these dramatic conditions, mild elevated pulmonary pressure has been suggested to be deleterious. A recent study has correlated small increases in PA pressure, below the threshold for a diagnosis of PH, with a negative impact on survival in the general population, independently of other known risk factors (Lam et al., 2009). Elevated pulmonary pressure results from a decrease in arterial lumen through the combination of: (1) increased contractility of small PA, (2) proliferation and remodeling of endothelial and smooth muscle cells and (3) thrombosis. Several lines of evidence suggest that oxidative stress contributes to the pathogenesis of PH. The *in vitro* studies mentioned above indicate that ROS may play an important role in all three mechanisms for decreased arterial lumen. One of the early events contributing to the pathophysiology of all forms of PH is endothelial dysfunction (Rabinovitch, 2008). Increased superoxide in the vessel wall and the subsequent decrease in NO bioavailability is a typical marker of endothelial dysfunction.

Several sources of ROS are activated or upregulated in PH. Thus, NOX subunits are prominently upregulated in pulmonary arteries during chronic hypoxia-induced PH and in models of PPHN such as the fetal lambs with increased pulmonary flow (Grobe et al., 2006). Hypoxia-exposed newborn rats show increased serum and lung XO activity, vascular XO-derived superoxide production and vascular nitrotyrosine formation (Jankov et al., 2008). Additionally, uncoupled eNOS contributes to generate ROS in PH induced by monocrotaline in rats, in PH induced by high flow in fetal lambs (Konduri et al., 2007), the PH observed in caveolin-1 knockout mice (Wunderlich et al., 2008) and in endoglin (+/−) mice (Toporsian et al., 2010). Because eNOS uncoupling may result from ROS-induced oxidation of the eNOS cofactor BH_4, uncoupled eNOS may amplify the ROS signal. Microparticles have been described as biological vectors of endothelial dysfunction in several pathologies. Interestingly, plasma microparticles from hypoxic pulmonary hypertensive animals increased oxidative stress in pulmonary endothelial cells via XO and mitochondrial ETC (Tual-Chalot et al., 2010).

Hypoxia-induced pulmonary artery hyperreactivity was also associated with isoprostane release and prevented by TP receptor antagonists and catalase (Delannoy et al., 2010). Chronic hyperoxia in newborns also induces PH. This effect is associated to increased ROS and isoprostanes and prevented by ROS scavengers and TP receptor antagonists but not by COX inhibitors suggesting that these COX-independent postanoids mediate PH in this model (Belik et al., 2009a).

Due to the role of ROS in PH, several strategies designed to diminish ROS levels have been proposed as potential therapeutic treatment of PH. Scavengers of ROS have shown beneficial effects in animal models of PH. Thus, treatment with recombinant human SOD is able to enhance eNOS expression and function in

animal models of PPHN through enhancement of ROS clearance and restoration of BH_4 levels (Farrow et al., 2008). Gene transfer of extracellular SOD also ameliorated monocrotaline-induced PH in rats (Kamezaki et al., 2008). Similarly, catalase, the SOD mimetic MnTMPyP, SOD/catalase mimetics such as tempol have also shown beneficial effect in PH (Delannoy et al., 2010). An alternative strategy is to inhibit the ROS generating sources. The XO inhibitor allopurinol limited oxidative stress in the lung and attenuated hypoxia-induced vascular remodeling in neonates with PH (Jankov et al., 2008). Inhibition of eNOS with L-NAME was also able to decrease ROS and reverse PH in caveolin-1 knockout mice (Wunderlich et al., 2008) in which superoxide is produced by uncoupled eNOS. However, this later approach may not be extrapolated to other models of PH or in humans since it also removes the protective effects of NO. Remarkably, current standard treatments of PH such as PDE5 inhibitors have also been reported to lower ROS levels (Hemnes et al., 2008).

4.5. Diabetes

Diabetes mellitus is associated with systemic oxidative stress and cardiovascular disease. However, recent basic and epidemiological studies suggest also a link between diabetes and pulmonary oxidative stress and vascular dysfunction (Fouty, 2008). In rat PA, type 1 diabetes upregulates $p47^{phox}$ and induces an increase in superoxide leading to endothelial dysfunction in pulmonary arteries (Lopez-Lopez et al., 2008).

5. Conclusions

Over the last thirty years a large body of evidence has grown indicating a role for ROS in systemic and pulmonary human vascular disease. Yet, the most important proof of concept is lacking: there are no available therapeutic interventions directly targeting ROS to prevent or treat the most common forms of systemic or pulmonary vascular disease. Antioxidant vitamins (vitamin C, E or beta-carotene) have shown no benefit in the prevention of ischemic heart disease or stroke. Whether these or other antioxidants have any beneficial effect on pulmonary vascular disease is presently unknown.

Acknowledgements

The research of the authors is supported by Ministerio de Ciencia e Innovación (grants SAF2008-03948 and AGL2007-66108), European Union FP7-PEOPLE (AOR-PERG05-GA-2009-249165) and Mutua Madrileña.

References

Aaronson, P.I., Robertson, T.P., Knock, G.A., Becker, S., Lewis, T.H., Snetkov, V., Ward, J.P., 2006. Hypoxic pulmonary vasoconstriction: mechanisms and controversies. J. Physiol. 570, 53–58.

Archer, S.L., Huang, J., Henry, T., Peterson, D., Weir, E.K., 1993. A redox-based O2 sensor in rat pulmonary vasculature. Circ. Res. 73, 1100–1112.

Bailey, S.R., Mitra, S., Flavahan, S., Flavahan, N.A., 2005. Reactive oxygen species from smooth muscle mitochondria initiate cold-induced constriction of cutaneous arteries. Am. J. Physiol. Heart Circ. Physiol. 289, H243–H250.

Bedard, K., Krause, K.H., 2007. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. Physiol. Rev. 87, 245–313.

Belik, J., Gonzalez-Luis, G.E., Perez-Vizcaino, F., Villamor, E., 2009a. Isoprostanes in fetal and neonatal health and disease. Free Radic. Biol. Med..

Belik, J., Jerkic, M., McIntyre, B.A., Pan, J., Leen, J., Yu, L.X., Henkelman, R.M., Toporsian, M., Letarte, M., 2009b. Age-dependent endothelial nitric oxide synthase uncoupling in pulmonary arteries of endoglin heterozygous mice. Am. J. Physiol. Lung Cell. Mol. Physiol. 297, L1170–L1178.

Brandes, R.P., Kreuzer, J., 2005. Vascular NADPH oxidases: molecular mechanisms of activation. Cardiovasc. Res. 65, 16–27.

Briones, A.M., Touyz, R.M., 2010. Oxidative stress and hypertension: current concepts. Curr. Hypertens. Rep. 12, 135–142.

Cogolludo, Moreno, L., Cobeño, L.F.G., Castañeda, J., Gonzalez, C., Villamor, E., Perez-Vizcaino, F., 2008. Neutral sphingomyelinase and protein kinase Cζ are involved in hypoxic pulmonary vasoconstriction. Faseb J. 22, 1174–1179.

Cogolludo, A., Frazziano, G., Cobeno, L., Moreno, L., Lodi, F., Villamor, E., Tamargo, J., Perez-Vizcaino, F., 2006. Role of reactive oxygen species in Kv channel inhibition and vasoconstriction induced by TP receptor activation in rat pulmonary arteries. Ann. N Y Acad. Sci. 1091, 41–51.

Cogolludo, A., Moreno, L., Bosca, L., Tamargo, J., Perez-Vizcaino, F., 2003. Thromboxane A2-induced inhibition of voltage-gated K+ channels and pulmonary vasoconstriction: role of protein kinase Czeta. Circ. Res. 93, 656–663.

Cogolludo, A., Moreno, L., Frazziano, G., Moral-Sanz, J., Menendez, C., Castaneda, J., Gonzalez, C., Villamor, E., Perez-Vizcaino, F., 2009a. Activation of neutral sphingomyelinase is involved in acute hypoxic pulmonary vasoconstriction. Cardiovasc. Res. 82, 296–302.

Cogolludo, A.L., Moral-Sanz, J., van der Sterren, S., Frazziano, G., van Cleef, A.N., Menendez, C., Zoer, B., Moreno, E., Roman, A., Perez-Vizcaino, F., Villamor, E., 2009b. Maturation of O_2 sensing and signaling in the chicken ductus arteriosus. Am. J. Physiol. Lung Cell. Mol. Physiol. 297, L619–L630.

Chi, A.Y., Waypa, G.B., Mungai, P.T., Schumacker, P.T., 2010. Prolonged hypoxia increases ROS signaling and RhoA activation in pulmonary artery smooth muscle and endothelial cells. Antioxid. Redox Signal. 12, 603–610.

Delannoy, E., Courtois, A., Freund-Michel, V., Leblais, V., Marthan, R., Muller, B., 2010. Hypoxia-induced hyperreactivity of pulmonary arteries: role of cyclooxygenase-2, isoprostanes, and thromboxane receptors. Cardiovasc. Res. 85, 582–592.

Doughan, A.K., Harrison, D.G., Dikalov, S.I., 2008. Molecular mechanisms of angiotensin II-mediated mitochondrial dysfunction: linking mitochondrial oxidative damage and vascular endothelial dysfunction. Circ. Res. 102, 488–496.

Erusalimsky, J.D., Moncada, S., 2007. Nitric oxide and mitochondrial signaling: from physiology to pathophysiology. Arterioscler. Thromb. Vasc. Biol. 27, 2524–2531.

Farrow, K.N., Lakshminrusimha, S., Reda, W.J., Wedgwood, S., Czech, L., Gugino, S.F., Davis, J.M., Russell, J.A., Steinhorn, R.H., 2008. Superoxide dismutase restores eNOS expression and function in resistance pulmonary arteries from neonatal lambs with persistent pulmonary hypertension. Am. J. Physiol. Lung Cell. Mol. Physiol. 295, L979–L987.

Florea, S.M., Blatter, L.A., 2008. The effect of oxidative stress on Ca2+ release and capacitative Ca2+ entry in vascular endothelial cells. Cell Calcium 43, 405–415.

Fouty, B., 2008. Diabetes and the pulmonary circulation. Am. J. Physiol. Lung Cell. Mol. Physiol. 295, L725–L726.

Frazziano, G., Cogolludo, A., Moreno, L., Lodi, F., Cobeno, L., Tamargo, J., Perez-Vizcaino, F., 2007. Involvement of reactive oxygen species in Kv channels inhibition induced by hypoxia in rat pulmonary arteries. Faseb J. 21, A1171.

Gonzalez-Luis, G., Perez-Vizcaino, F., Blanco, C.E., Villamor, E., 2010. Age-related changes in isoprostane-mediated relaxation of piglet blood vessels. Front. Biosci. (Elite Ed) 2, 369–379.

Grobe, A.C., Wells, S.M., Benavidez, E., Oishi, P., Azakie, A., Fineman, J.R., Black, S.M., 2006. Increased oxidative stress in lambs with increased pulmonary blood flow and pulmonary hypertension: role of NADPH oxidase and endothelial NO synthase. Am. J. Physiol. Lung Cell. Mol. Physiol. 290, L1069–L1077.

Hemnes, A.R., Zaiman, A., Champion, H.C., 2008. PDE5A inhibition attenuates bleomycin-induced pulmonary fibrosis and pulmonary hypertension through inhibition of ROS generation and RhoA/Rho kinase activation. Am. J. Physiol. Lung Cell. Mol. Physiol. 294, L24–L33.

Heumuller, S., Wind, S., Barbosa-Sicard, E., Schmidt, H.H., Busse, R., Schroder, K., Brandes, R.P., 2008. Apocynin is not an inhibitor of vascular NADPH oxidases but an antioxidant. Hypertension 51, 211–217.

Hu, H.L., Zhang, Z.X., Chen, C.S., Cai, C., Zhao, J.P., Wang, X., 2010. Effects of mitochondrial potassium channel and membrane potential on hypoxic human pulmonary artery smooth muscle cells. Am. J. Respir. Cell Mol. Biol. 42, 661–666.

Jana, A., Pahan, K., 2004. Human immunodeficiency virus type 1 gp120 induces apoptosis in human primary neurons through redox-regulated activation of neutral sphingomyelinase. J. Neurosci. 24, 9531–9540.

Jankov, R.P., Kantores, C., Pan, J., Belik, J., 2008. Contribution of xanthine oxidase-derived superoxide to chronic hypoxic pulmonary hypertension in neonatal rats. Am. J. Physiol. Lung Cell. Mol. Physiol. 294, L233–L245.

Jernigan, N.L., Walker, B.R., Resta, T.C., 2008. Reactive oxygen species mediate RhoA/Rho kinase-induced Ca2+ sensitization in pulmonary vascular smooth muscle following chronic hypoxia. Am. J. Physiol. Lung Cell. Mol. Physiol. 295, L515–L529.

Kamezaki, F., Tasaki, H., Yamashita, K., Tsutsui, M., Koide, S., Nakata, S., Tanimoto, A., Okazaki, M., Sasaguri, Y., Adachi, T., Otsuji, Y., 2008. Gene transfer of extracellular superoxide dismutase ameliorates pulmonary hypertension in rats. Am. J. Respir. Crit. Care Med. 177, 219–226.

Knock, G.A., Snetkov, V.A., Shaifta, Y., Connolly, M., Drndarski, S., Noah, A., Pourmahram, G.E., Becker, S., Aaronson, P.I., Ward, J.P., 2009. Superoxide constricts rat pulmonary arteries via Rho-kinase-mediated Ca(2+) sensitization. Free Radic. Biol. Med. 46, 633–642.

Konduri, G.G., Bakhutashvili, I., Eis, A., Pritchard Jr., K., 2007. Oxidant stress from uncoupled nitric oxide synthase impairs vasodilation in fetal lambs with persistent pulmonary hypertension. Am. J. Physiol. Heart. Circ. Physiol. 292, H1812–H1820.

Kukreja, R.C., Kontos, H.A., Hess, M.L., Ellis, E.F., 1986. PGH synthase and lipoxygenase generate superoxide in the presence of NADH or NADPH. Circ. Res. 59, 612–619.

Lam, C.S., Borlaug, B.A., Kane, G.C., Enders, F.T., Rodeheffer, R.J., Redfield, M.M., 2009. Age-associated increases in pulmonary artery systolic pressure in the general population. Circulation 119, 2663–2670.

Lebovitz, R.M., Zhang, H., Vogel, H., Cartwright Jr., J., Dionne, L., Lu, N., Huang, S., Matzuk, M.M., 1996. Neurodegeneration, myocardial injury, and perinatal death in mitochondrial superoxide dismutase-deficient mice. Proc. Natl. Acad. Sci. U.S.A. 93, 9782–9787.

Li, X.Q., Zheng, Y.M., Rathore, R., Ma, J., Takeshima, H., Wang, Y.X., 2009. Genetic evidence for functional role of ryanodine receptor 1 in pulmonary artery smooth muscle cells. Pflugers Arch. 457, 771–783.

Lin, M.J., Yang, X.R., Cao, Y.N., Sham, J.S., 2007. Hydrogen peroxide-induced Ca2+ mobilization in pulmonary arterial smooth muscle cells. Am. J. Physiol. Lung Cell. Mol. Physiol. 292, L1598–L1608.

Loirand, G., Guerin, P., Pacaud, P., 2006. Rho kinases in cardiovascular physiology and pathophysiology. Circ. Res. 98, 322–334.

Lopez-Lopez, J.G., Moral-Sanz, J., Frazziano, G., Gomez-Villalobos, M.J., Flores-Hernandez, J., Monjaraz, E., Cogolludo, A., Perez-Vizcaino, F., 2008. Diabetes induces pulmonary artery endothelial dysfunction by NADPH oxidase induction. Am. J. Physiol. Lung Cell. Mol. Physiol. 295, L727–L732.

Lopez-Lopez, J.G., Perez-Vizcaino, F., Cogolludo, A.L., Ibarra, M., Zaragoza-Arnaez, F., Tamargo, J., 2001. Nitric oxide- and nitric oxide donors-induced relaxation and its modulation by oxidative stress in piglet pulmonary arteries. Br. J. Pharmacol. 133, 615–624.

Maclean, M.R., Dempsie, Y., 2010. The serotonin hypothesis of pulmonary hypertension revisited. Adv. Exp. Med. Biol. 661, 309–322.

Mitchell, J.A., Warner, T.D., 1999. Cyclo-oxygenase-2: pharmacology, physiology, biochemistry and relevance to NSAID therapy. Br. J. Pharmacol. 128, 1121–1132.

Mittal, M., Roth, M., Konig, P., Hofmann, S., Dony, E., Goyal, P., Selbitz, A.C., Schermuly, R.T., Ghofrani, H.A., Kwapiszewska, G., Kummer, W., Klepetko, W., Hoda, M.A., Fink, L., Hanze, J., Seeger, W., Grimminger, F., Schmidt, H.H., Weissmann, N., 2007. Hypoxia-dependent regulation of nonphagocytic NADPH oxidase subunit NOX4 in the pulmonary vasculature. Circ. Res. 101, 258–267.

Moreno, L., Frazziano, G., Cogolludo, A., Cobeno, L., Tamargo, J., Perez-Vizcaino, F., 2007. Role of protein kinase Czeta and its adaptor protein p62 in voltage-gated potassium channel modulation in pulmonary arteries. Mol. Pharmacol. 72, 1301–1309.

Oeckler, R.A., Arcuino, E., Ahmad, M., Olson, S.C., Wolin, M.S., 2005. Cytosolic NADH redox and thiol oxidation regulate pulmonary arterial force through ERK MAP kinase. Am. J. Physiol. Lung Cell. Mol. Physiol. 288, L1017–L1025.

Olschewski, A., Hong, Z., Peterson, D.A., Nelson, D.P., Porter, V.A., Weir, E.K., 2004. Opposite effects of redox status on membrane potential, cytosolic calcium, and tone in pulmonary arteries and ductus arteriosus. Am. J. Physiol. Lung Cell. Mol. Physiol. 286, L15–L22.

Partridge, C.A., Blumenstock, F.A., Malik, A.B., 1992. Pulmonary microvascular endothelial cells constitutively release xanthine oxidase. Arch. Biochem. Biophys. 294, 184–187.

Pendyala, S., Gorshkova, I.A., Usatyuk, P.V., He, D., Pennathur, A., Lambeth, J.D., Thannickal, V.J., Natarajan, V., 2009. Role of Nox4 and Nox2 in hyperoxia-induced reactive oxygen species generation and migration of human lung endothelial cells. Antioxid. Redox Signal. 11, 747–764.

Perez-Vizcaino, F., Lopez-Lopez, J.G., Santiago, R., Cogolludo, A., Zaragoza-Arnaez, F., Moreno, L., Alonso, M.J., Salaices, M., Tamargo, J., 2002. Postnatal maturation in nitric oxide-induced pulmonary artery relaxation involving cyclooxygenase-1 activity. Am. J. Physiol. Lung Cell. Mol. Physiol. 283, L839–L848.

Pourmahram, G.E., Snetkov, V.A., Shaifta, Y., Drndarski, S., Knock, G.A., Aaronson, P.I., Ward, J.P., 2008. Constriction of pulmonary artery by peroxide: role of Ca2+ release and PKC. Free Radic. Biol. Med. 45, 1468–1476.

Rabinovitch, M., 2008. Molecular pathogenesis of pulmonary arterial hypertension. J. Clin. Invest. 118, 2372–2379.

Rainbow, R.D., Norman, R.I., Everitt, D.E., Brignell, J.L., Davies, N.W., Standen, N.B., 2009. Endothelin-I and angiotensin II inhibit arterial voltage-gated K+ channels through different protein kinase C isoenzymes. Cardiovasc. Res. 83, 493–500.

Rathore, R., Zheng, Y.M., Li, X.Q., Wang, Q.S., Liu, Q.H., Ginnan, R., Singer, H.A., Ho, Y.S., Wang, Y.X., 2006. Mitochondrial ROS-PKCepsilon signaling axis is uniquely involved in hypoxic increase in [Ca2+]i in pulmonary artery smooth muscle cells. Biochem. Biophys. Res. Commun. 351, 784–790.

Rathore, R., Zheng, Y.M., Niu, C.F., Liu, Q.H., Korde, A., Ho, Y.S., Wang, Y.X., 2008. Hypoxia activates NADPH oxidase to increase [ROS]i and [Ca2+]i through the mitochondrial ROS-PKCepsilon signaling axis in pulmonary artery smooth muscle cells. Free Radic. Biol. Med. 45, 1223–1231.

Reeve, H.L., Tolarova, S., Nelson, D.P., Archer, S., Weir, E.K., 2001. Redox control of oxygen sensing in the rabbit ductus arteriosus. J. Physiol. 533, 253–261.

Rhoades, R.A., Packer, C.S., Roepke, D.A., Jin, N., Meiss, R.A., 1990. Reactive oxygen species alter contractile properties of pulmonary arterial smooth muscle. Can. J. Physiol. Pharmacol. 68, 1581–1589.

Schach, C., Xu, M., Platoshyn, O., Keller, S.H., Yuan, J.X., 2007. Thiol oxidation causes pulmonary vasodilation by activating K+ channels and inhibiting store-operated Ca2+ channels. Am. J. Physiol. Lung Cell. Mol. Physiol. 292, L685–L698.

Schluter, T., Steinbach, A.C., Steffen, A., Rettig, R., Grisk, O., 2008. Apocynin-induced vasodilation involves Rho kinase inhibition but not NADPH oxidase inhibition. Cardiovasc. Res. 80, 271–279.

Schroder, E., Eaton, P., 2008. Hydrogen peroxide as an endogenous mediator and exogenous tool in cardiovascular research: issues and considerations. Curr. Opin. Pharmacol. 8, 153–159.

Semenza, G.L., 2007. Oxygen-dependent regulation of mitochondrial respiration by hypoxia-inducible factor 1. Biochem. J. 405, 1–9.

Stowe, D.F., Camara, A.K., 2009. Mitochondrial reactive oxygen species production in excitable cells: modulators of mitochondrial and cell function. Antioxid. Redox Signal. 11, 1373–1414.

Tang, E.H., Vanhoutte, P.M., 2009. Prostanoids and reactive oxygen species: team players in endothelium-dependent contractions. Pharmacol. Ther. 122, 140–149.

Tate, R.M., Morris, H.G., Schroeder, W.R., Repine, J.E., 1984. Oxygen metabolites stimulate thromboxane production and vasoconstriction in isolated saline-perfused rabbit lungs. J. Clin. Invest. 74, 608–613.

Toporsian, M., Jerkic, M., Zhou, Y.Q., Kabir, M.G., Yu, L.X., McIntyre, B.A., Davis, A., Wang, Y.J., Stewart, D.J., Belik, J., Husain, M., Henkelman, M., Letarte, M., 2010. Spontaneous adult-onset pulmonary arterial hypertension attributable to increased endothelial oxidative stress in a murine model of hereditary hemorrhagic telangiectasia. Arterioscler. Thromb. Vasc. Biol. 30, 509–517.

Tual-Chalot, S., Guibert, C., Muller, B., Savineau, J.P., Andriantsitohaina, R., Martinez, M.C., 2010. Circulating microparticles from pulmonary hypertensive rats induce endothelial dysfunction. Am. J. Respir. Crit. Care Med..

Ushio-Fukai, M., 2009. Compartmentalization of redox signaling through NADPH oxidase-derived ROS. Antioxid. Redox Signal. 11, 1289–1299.

Villamor, E., Kessels, C.G., Fischer, M.A., Bast, A., de Mey, J.G., Blanco, C.E., 2003. Role of superoxide anion on basal and stimulated nitric oxide activity in neonatal piglet pulmonary vessels. Pediatr. Res. 54, 372–381.

Wang, Y.X., Zheng, Y.M., 2010. ROS-dependent signaling mechanisms for hypoxic Ca(2+) responses in pulmonary artery myocytes. Antioxid. Redox Signal. 12, 611–623.

Ward, J.P., Knock, G.A., Snetkov, V.A., Aaronson, P.I., 2004. Protein kinases in vascular smooth muscle tone—role in the pulmonary vasculature and hypoxic pulmonary vasoconstriction. Pharmacol. Ther. 104, 207–231.

Ward, J.P., McMurtry, I.F., 2009. Mechanisms of hypoxic pulmonary vasoconstriction and their roles in pulmonary hypertension: new findings for an old problem. Curr. Opin. Pharmacol. 9, 287–296.

Waypa, G.B., Guzy, R., Mungai, P.T., Mack, M.M., Marks, J.D., Roe, M.W., Schumacker, P.T., 2006. Increases in mitochondrial reactive oxygen species trigger hypoxia-induced calcium responses in pulmonary artery smooth muscle cells. Circ. Res. 99, 970–978.

Waypa, G.B., Marks, J.D., Guzy, R., Mungai, P.T., Schriewer, J., Dokic, D., Schumacker, P.T., 2010. Hypoxia triggers subcellular compartmental redox signaling in vascular smooth muscle cells. Circ. Res. 106, 526–535.

Wedgwood, S., Black, S.M., 2003. Induction of apoptosis in fetal pulmonary arterial smooth muscle cells by a combined superoxide dismutase/catalase mimetic. Am. J. Physiol. Lung Cell. Mol. Physiol. 285, L305–L312.

Wedgwood, S., Dettman, R.W., Black, S.M., 2001. ET-1 stimulates pulmonary arterial smooth muscle cell proliferation via induction of reactive oxygen species. Am. J. Physiol. Lung Cell. Mol. Physiol. 281, L1058–L1067.

Weissmann, N., Dietrich, A., Fuchs, B., Kalwa, H., Ay, M., Dumitrascu, R., Olschewski, A., Storch, U., Mederos y Schnitzler, M., Ghofrani, H.A., Schermuly, R.T., Pinkenburg, O., Seeger, W., Grimminger, F., Gudermann, T., 2006. Classical transient receptor potential channel 6 (TRPC6) is essential for hypoxic pulmonary vasoconstriction and alveolar gas exchange. Proc. Natl. Acad. Sci. U.S.A. 103, 19093–19098.

Wolin, M.S., 2009. Reactive oxygen species and the control of vascular function. Am. J. Physiol. Heart Circ. Physiol. 296, H539–H549.

Wong, C.M., Cheema, A.K., Zhang, L., Suzuki, Y.J., 2008. Protein carbonylation as a novel mechanism in redox signaling. Circ. Res. 102, 310–318.

Wunderlich, C., Schmeisser, A., Heerwagen, C., Ebner, B., Schober, K., Braun-Dullaeus, R.C., Schwencke, C., Kasper, M., Morawietz, H., Strasser, R.H., 2008. Chronic NOS inhibition prevents adverse lung remodeling and pulmonary arterial hypertension in caveolin-1 knockout mice. Pulm. Pharmacol. Ther. 21, 507–515.

Zhang, Y., Li, X., Becker, K.A., Gulbins, E., 2009. Ceramide-enriched membrane domains—structure and function. Biochim. Biophys. Acta 1788, 178–183.