Endothelial Cell Reactive Oxygen Species and Ca²⁺ Signaling in Pulmonary Hypertension

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1 Introduction

Within the lung vasculature, reactive oxygen species (ROS) are critical signaling intermediates in both health and disease. ROS are thought to play a particularly important role in the pathobiology of pulmonary hypertension (PH) [1, 2]. Alterations in ROS homeostasis in the pulmonary vasculature, specifically in pulmonary smooth muscle (SMC) and endothelial (EC) cells, lead to a variety of signaling events that promote enhanced vasoreactivity, increased cellular migration and proliferation, and vascular remodeling of the pulmonary arteries. For instance increased ROS activate multiple kinase pathways and, of particular interest to this review, has been shown to increase intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in various vascular beds [3]. It is now appreciated that pulmonary vascular ECs exhibit considerable phenotypic heterogeneity based on whether they reside in the large, conduit vessels, i.e., the pulmonary artery, or the smaller vessels in the microvasculature [4]. Although a significant amount is known regarding the role of ROS in abnormal EC function in the systemic circulation, it is not clear which of these pathways may also play a role in promoting changes in EC phenotype in different types of lung ECs. While more recent studies have begun utilizing ECs isolated from specific sites within the lung (i.e., conduit lung vessels versus microvasculature) in order to better understand the specific pathways underlying lung EC dysfunction in PH [5–7], significant gaps in our understanding of the specific molecular mechanisms governing lung EC function remain. In this chapter, we will review the role played by elevations in ROS, and in particular ROS-induced changes $[Ca^{2+}]_i$, in promoting abnormal vasoreactivity and EC function in PH.

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2 Pulmonary Hypertension

PH refers to a heterogeneous condition defined as increased (25 mmHg at rest) mean pulmonary arterial pressure (mPAP), leading to progressive right ventricular overload and, in some cases, failure [8]. PH is diagnosed based on clinical examination and right heart catheterization, and is classified into five main groups based on hemodynamic and pathologic differences (Table 1). Group 1 PH, also called pulmonary arterial hypertension (PAH), is characterized by very high right-sided pressures and development of occlusive lesions in the pulmonary vasculature. The etiology of PH in Group 1 includes inherited and idiopathic causes as well as PAH due to drugs and toxins, connective tissue disease, HIV, and chronic liver disease (portal hypertension). Group 2 refers to PH that occurs as a consequence of left heart dysfunction, while Group 3 is defined as PH occurring in the context of chronic hypoxia, often in the setting of parenchymal lung disease. Group 4 and Group 5 refer to elevated P_{PA} due to chronic thromboembolic and systemic diseases, respectively.

While each group in the PH classification arises from varied etiologies, in each case the underlying pulmonary vascular abnormalities fall into three main categories: (1) increased vascular tone due to sustained contraction of the arterial tree, (2) vascular wall thickening due to uncontrolled EC, SMC, and fibroblast migration and proliferation, and in the case of PAH, (3) development of occlusive lesions in the distal arterioles that obstruct blood flow. These occlusive lesions of PAH comprise multiple cell types, including ECs that are monoclonal, apoptosis-resistant and hyperproliferative. The fundamental cell signaling abnormalities that lead to this aberrant EC phenotype are still not clear, with many potential pathways remaining under investigation. Over the past decade, there has been continued interest in the role of ROS in regulating both abnormal pulmonary vascular tone and promoting migration and proliferation.

3 ROS in Pulmonary Hypertension

Accumulating evidence supports a role for increased ROS in the development and progression of PH [1, 9]. Urinary and plasma levels of oxidative stress markers have been detected in PAH patients [10, 11] while histological examination of lung sections from patients displayed increased nitrotyrosine and 8-hydroxyguanosine residues, which are by-products of oxidative stress [12]. Experimentally decreasing ROS levels with antioxidants augmented pulmonary arterial responsiveness to the vasodilator, nitric oxide (NO), in rat pulmonary arterial rings [13, 14]. Similarly, inhibition of xanthine oxidase or treatment with the ROS scavenger TEMPOL, attenuated development of chronic hypoxic PH. These data, collectively suggest that elevated ROS are involved in the pathobiology of PH, particularly by influencing Ca^{2+}-dependent pulmonary vasoreactivity and increased migration and proliferation of ECs.

4 Sources of ROS in PH

ROS can arise from multiple extracellular (i.e., infiltrating inflammatory cells) and intracellular sources [1]. Generation of intracellular ROS in the lung vasculature occurs via

mechanisms similar to those described in other cell types. For example, membrane bound NOX produce ROS in response to external agonists, while non membrane-bound enzymes, such as xanthine oxidase, aid in generation of additional cytosolic ROS [2]. Perhaps the most common source of ROS generation is mitochondria [15].

NAPDH Oxidase.

NOX is a complex enzyme comprising several protein subunits [16]. It catalyzes the transfer of two electrons from NADPH to two successive oxygen molecules, generating superoxide anions. In a variety of cell types, including ECs, NOX generates superoxide that is quickly converted to hydrogen peroxide (H_2O_2), allowing the enzyme to exert significant signaling effects on ROS-sensitive targets. In phagocytic cells, NOX-derived ROS forms part of the defense mechanism against pathogens whereas in non-phagocytic cells NOX is thought to serve primarily in a signaling capacity. The catalytic subunit has several isoforms (NOX1–5, DUOX1–2) and associates with multiple binding partners that enhance enzymatic activity (reviewed in [2, 16, 17]). Some of these binding partners are isoform specific; for instance, NOX2 associates with several "phox" proteins, including p47 and p67, and Rac1 while NOX4 associates with Poldip2 and p22 [18]. NOX1–4 are expressed in ECs, whereas both NOX2 and NOX4 have both been shown to contribute to EC proliferation [19]. NOX4 may be particularly important to EC function, as specific deletion of NOX4 inhibits microvascular and umbilical vein EC migration and proliferation. Conversely, NOX4 overexpression is sufficient to increase migration and proliferation [20].

Evidence suggests that NOX expression and activity is in fact increased in PH, which leads to pulmonary vascular hyperreactivity. For example, studies performed on homogenates of endothelium-intact pulmonary arteries isolated from a model of fetal/newborn PH demonstrated that increased NOX expression and uncoupling of endothelial nitric oxide synthase (eNOS; see Fig. 1) leads to elevated ROS production [21, 22]. Moreover, lung tissue expression of NOX subunits was increased in a murine model of chronic intermittent hypoxia (modeling PH due to obstructive sleep apnea) [23]. PH induced by either 3 or 10 days of hypoxia also increased NOX1 expression in pulmonary resistance arteries of newborn piglets [24]. On the other hand, mice with deficiency of the gp91phox (i.e., NOX2) subunit failed to develop PH with chronic sustained [25] or intermittent hypoxia [23]. Loss of gp91phox also attenuated hypoxia-induced, NO-dependent relaxation in isolated pulmonary arteries [26]. While all of these studies suggest a role for increased NOX expression/activity in PH, a caveat with the use of whole tissue homogenates or global knockouts is that the exact contribution of ECs (vs. SMCs) cannot be fully ascertained.

NOX is activated by ligation of VEGF receptor-2 (VEGFR2) via Rac1 [27]. In addition, NOX can be activated by a myriad other injurious stimuli including angiotensin II [27], tumor necrosis factor-alpha (TNF-α) [28] and shear stress [29]. The functional consequences of increased NOX-derived ROS in ECs are incompletely understood. Based on work performed in systemic ECs, basal ROS generation by NOX likely contributes to EC proliferation and angiogenesis [19, 20], although these effects are mild. The growth response is enhanced when NOX activity is stimulated by agonists known to be involved in PH, including hypoxia [30] and vascular endothelial growth factor (VEGF) [31]. Regarding the

latter, NOX-generated ROS may serve as a signal for VEGF-induced changes in EC growth [31, 32].

Once generated, NOX-derived ROS activate a variety of targets, including signaling molecules (e.g., Src, Akt, and MAP kinases) and transcription factors (e.g., NF-kB and AP-1) [28]. ROS serve as an activation signal by increasing leukocyte recruitment to the endothelium [33, 34] and initiating and propagating EC migration [35, 36]. In response to VEGF, localized production of ROS by NOX mediates interactions with TRAF4 and PAK1, two cytoskeletal proteins involved in cell motility [37, 38]. NOX-derived ROS are also critical for EC proliferation [39] via activation of Ras/ERK and JNK pathways in response to the viral protein, Tat, in a HIV-induced model of EC migration and proliferation. These data suggest that ROS play a critical role in EC movement and division, especially in response to injurious stimuli.

Mitochondrial ROS (mtROS).

Mitochondria are major sources of ROS in all cell types, and have recently come under increased scrutiny as drivers of ROS-induced EC dysfunction in PH. Complexes I and III of the electron transport chain are known mitochondrial sites of ROS generation [15, 40, 41]. Superoxide produced by complex I is released into the mitochondrial matrix, while superoxide produced by complex III can be released into either the intermembrane space or the mitochondrial matrix. Upon release, superoxide released into the intermembrane space or matrix is converted to H_2O_2 by superoxide dismutase (SOD)-1 or SOD-2, respectively. The site-specific superoxide release in mitochondria can modulate different signaling pathways [40]. Since mitochondria are thought to function as cellular oxygen sensors, mtROS may link changes in oxygen tension and hypoxia-induced alterations in cell signaling and function. In part due to the limitations surrounding ROS-measurement methods, difficulty of measuring mitochondrial O_2 in vivo and the differential effects of superoxide due to compartmentalization, the exact relationship between hypoxia and mtROS remains an area of active investigation, with lines of evidence pointing towards both decreased [42–44] and increased ROS [45, 46] in the setting of acute hypoxia (also reviewed in [1, 45, 47]).

With respect to the role of mtROS in prolonged hypoxia, which is more relevant to PH, most studies have been performed in pulmonary arterial SMCs, where one of the main consequences of altered mtROS following hypoxia is stabilization of hypoxia-inducible factor-1α (HIF-1α), the oxygen-sensitive subunit of the HIF-1 transcription factor (Fig. 2). HIF-1, a master regulator of adaptive cell responses to hypoxia [48], is constitutively ubiquitinated and degraded due to hydroxylation by proline hydroxylase domain (PHD) proteins. Since this reaction requires O_2, hypoxia leads to loss of hydroxylation secondary to decreased substrate availability and stabilization of HIF-1α [49]. HIF-1α/HIF-1β heterodimers transcriptionally regulate a variety of cellular responses to hypoxia [50]. Although the mechanisms are not yet fully clear, regulation of HIF-1α by mtROS likely involves inhibition of enzymes (i.e., PHDs) that normally aid in HIF-1α degradation [51]. For instance, quenching to H_2O_2 in the intermembrane space of the mitochondrion before it is able to reach the cytosol attenuates hypoxia induced HIF-1α stabilization [52]. Less is

known regarding the relationship between hypoxia and mtROS in pulmonary ECs. However, Al-Mehdi et al. [53] reported that, in PA ECs, acute hypoxia increased mtROS and mobilized mitochondria to the perinuclear space, leading to diffusion of mtROS into the nucleus. Taken together with reports describing induction of HIF-1α [54] expression in response to hypoxia in PA ECs, it is likely that signaling pathways similar to those described in SMCs, involving mtROS generation and HIF-1α stabilization, exist in PA ECs as well.

Main transcriptional targets of HIF-1 include metabolic enzymes that control cellular shifts from oxidative phosphorylation towards glycolysis [44]. This shift, called the Warburg effect, was initially described in cancer cells and, while less efficient in terms of ATP production, allows for increased production of other macromolecules required for cellular growth and proliferation. Under conditions of tissue hypoxia, glycolysis is a normal response to cellular stress (i.e., the Pasteur effect). However, as increasingly seen in the context of PAH, abnormal HIF stabilization during normoxic conditions may induce a metabolic shift and consequent changes in cell function. Histologic sections of lungs from patients with PAH that stain positive for markers of oxidative stress also exhibit increased HIF-1α expression [55], while pulmonary ECs from patients with PAH display glycolytic shift [56]. These data indicate that HIF is abnormally stabilized in PAH and ROS, most likely mitochondrial in origin, are probably involved. It is likely that mtROS, maladaptive HIF stabilization and glycolytic shift are related processes that participate in a feed-forward mechanism such that changes in mtROS may enhance HIF stabilization, which in turn promotes glycolytic shift, altering mitochondrial redox state. However, much remains unknown with regards to the specifics of mtROS in PAH, particularly in ECs.

While little is known about mtROS levels in lung endothelium in the context of changes in mitochondrial respiration, data collected in systemic ECs may provide important clues [41]. In general, vascular ECs do not use oxidative phosphorylation as primary source of ATP [57]. Agonists, such as glucose, Ang II, and VEGF, increase EC mtROS production [41] and also activate NOX, arguing for the possibility that EC ROS production in PH may occur at multiple sites and may actually be linked, as significant cross talk likely exists between these pathways. As an example, mitochondrial biogenesis [58] and ROS production induced by VEGF, may in turn increase NOX expression/activity, further promoting oxidative stress [59].

Cyclic stretch provides one model where the interplay between mtROS and HIF-1α stabilization has been recently investigated. Unlike shear stress, which results from flow of fluid along (or parallel to) the endothelium in a single direction, stretch is primarily determined by the intraluminal vascular pressure [60], and exerts force perpendicular to the endothelial surface. Most often used in the context of ventilator-associated lung injury, in vitro models of stretch may mimic pressure-related changes in the PA that occur during PH. While the effect of increased strain has not been evaluated in pulmonary ECs, PASMCs undergoing cyclic stretch exhibit increased mtROS and HIF-1α activity; interestingly, these findings mirror those found in SMCs isolated from newborn lambs in a pediatric PH model induced by in utero ligation of the ductus arteriosus [61, 62]. Finally, HUVECs undergoing cyclic strain generated ROS, a response that was absent in cells depleted of mitochondria [63].

Although much remains unknown regarding the role of mtROS in promoting functional changes in the pulmonary vasculature in PH, a recent report noted that induction of mtROS promoted pulmonary arterial EC migration and proliferation in a process that involved p38 MAP kinases [64]. In the systemic circulation, the role of mtROS in EC-mediated vascular dysfunction has been studied more in more detail, with mtROS promoting EC migration and proliferation directly [40, 65]. Moreover, H_2O_2 generated by mitochondria was required for EC-mediated flow-induced coronary resistance artery dilation [66]. Taken together, these data suggest that EC mtROS are likely to play a role in ROS-induced EC dysfunction during PH.

5 EC [Ca^{2+}]$_i$ in Pulmonary Hypertension

In non-excitable cells, Ca^{2+} homeostasis is maintained via several mechanisms. Ca^{2+} can enter cells from the extracellular space through plasma membrane channels or be pumped from the cell by Ca^{2+}-ATPases. Ca^{2+} is also sequestered within, and released from, organelles. In the endoplasmic reticulum (ER) , Ca^{2+} is tightly regulated by release channels and Ca^{2+} transporters on the ER membrane, while similar channels control mitochondrial Ca^{2+} influx and efflux (Fig. 2). [Ca^{2+}]$_i$ homeostasis between the cytosol and ER can be pharmacologically disrupted using agents such as thapsigargin (Tg), an agent that inhibits Ca^{2+} uptake into the ER and depletes ER stores. A full listing of endothelial Ca^{2+} channels/ transporters is beyond the scope of this chapter; however, in general, plasmalemmal Ca^{2+} channels can be receptor-operated (e.g., transient receptor potential [TRP]), voltage-gated (e.g., T-type) or activated by depletion of ER Ca^{2+} stores (store-operated) [67–69], while ER release channels belong to inositol triphosphate or ryanodine receptor families.

At baseline, lung EC cytosolic Ca^{2+} levels are tightly controlled, typically averaging 100 nM [70]. Stimuli can increase [Ca^{2+}]$_i$ by directly binding receptor-operated channels on the cell membrane, activating metabolites that subsequently agonize channels, or stimulating Ca^{2+} efflux from the ER. Limited information is available regarding changes in, and the specific role of, basal EC [Ca^{2+}]$_i$ in PH. Globally, whole genome profiling approaches showed that genes encoding Ca^{2+} signaling pathways were significantly dysregulated in ECs in a model of PH induced by the diet drug, fenfluramine [71]. Exposure of pulmonary ECs to either acute [72] or chronic hypoxia [73] increased [Ca^{2+}]$_i$, with the latter mediated via upregulation of store-operated TRP channels. Together, these data suggest a role for increased [Ca^{2+}]$_i$ in PAH EC pathobiology, but as detailed below, the specific source of [Ca^{2+}]$_i$ and the channel involved remain under investigation.

One Ca^{2+} channel that has recently come into focus in PH studies is a member of the transient receptor potential (canonical) family, TRPC4. A store-operated Ca^{2+} channel, TRPC4 plays a key role in increasing endothelial permeability in the large vessels of the lung [74], leading to the formation of perivascular cuffing [75–77]. Interestingly, rats subjected to the SU5416 plus hypoxia (SuHx) protocol, which creates a severe experimental model of PAH, displayed increased EC Ca^{2+} transients through TRPC4 [78] and perivascular edema in response to Tg compared to normoxic controls, suggesting a higher sensitivity to induction of EC hyperpermeability through activation of SOCE [79]. These responses were attenuated in *TRPC4$^{-/-}$* rats even though PA pressures were similar to wild-

type animals [79]. Loss of TRPC4 also conferred a survival benefit in SuHx rats [80], although the exact cell type (EC or SMC) responsible for this benefit remains to be determined. These data suggest that changes in SOCE mediated changes in EC permeability may contribute to mortality in PAH, though it is unclear whether the same calcium channels also contribute to other aspects of PAH pathogenesis such as formation of vaso-occlusive lesions.

6   Links Between ROS and $[Ca^{2+}]_i$ in the Lung Endothelium

ROS-induced Ca^{2+} entry.

Elevations in ROS have been shown to increase $[Ca^{2+}]_i$ and, conversely, increased $[Ca^{2+}]_i$ participates in ROS generation [3]. With respect to the former, ROS can activate Ca^{2+} channels by two mechanisms [81–83]: via direct activation through redox sensitive cysteine residues on the channels (i.e., TRPA1, TRPV4, TRPM4) or via activation by metabolites generated by elevations in ROS (i.e., TRPM2) [81, 84–86, 87]. In addition to modulating the activity of plasma membrane channels, ROS can also induce release from internal stores, presumably through an effect on ER Ca^{2+} transporters [88–91]. The proteins responsible for replenishing ER Ca^{2+} stores following depletion (i.e., STIM and Orai) are also redox sensitive [92]. It should be noted, however, that the specific radical generated and/or compartmentalization may result in different Ca^{2+} responses [93]. Further complicating ROS-mediated Ca^{2+} responses is the fact that the increase in $[Ca^{2+}]_i$ likely represents a summation of multiple, temporally distinct Ca^{2+} release phenomena, with a mix of release and influx events.

mtROS and $[Ca^{2+}]$:

Mitochondria also possess distinct Ca^{2+} channels that regulate Ca^{2+} uptake and release [94]. The mitochondrial Ca^{2+} uniporter (MCU) is responsible for Ca^{2+} transport into the mitochondria while various other pumps are responsible for Ca^{2+} efflux. In addition to buffering cytosolic Ca^{2+} levels, proximity of mitochondria to the ER allows for Ca^{2+} ion transfer between these organelles [95]. Several enzymatic reactions that occur in the mitochondria, including the portions of the Krebs cycle, require Ca^{2+} and large increases in mitochondrial Ca^{2+} can also act as a signal for impending cell failure and activate cell death [94]. Increased mitochondrial Ca^{2+} levels and mtROS are often observed under similar conditions, and mice deficient for UCP2, a mitochondrial Ca^{2+} transport protein, developed PH, suggesting a common pathway between mtROS and $[Ca^{2+}]_i$. Furthermore, pulmonary SMCs isolated from UCP2 mice exhibit stabilization of HIF under normoxic conditions. While these changes mimic those observed with hypoxia [96] or in ECs from PAH patients [97] and animal models [98], it is not clear if similar HIF stabilization was also present in ECs of UCP2-deficient mice. Thus, the role for EC UCP2 in modulating maladaptive mitochondrial function in PH remains unknown.

ROS and $[Ca^{2+}]_i$ in EC migration and proliferation.

Migration and proliferation are dependent on both ROS and $[Ca^{2+}]_i$ in a variety of tissue types [3]. In PH, increased migration and proliferation occur in both SMCs and ECs, and the anatomic site for these functional changes is at the level of the resistance arteries [68].

Unlike SMCs, where significantly more is known regarding migration and proliferation following ROS-generating stimuli, considerably less is known about the interplay of ROS and Ca^{2+} in EC, particularly lung EC. Data from systemic ECs demonstrated that exposure to VEGF increased NADPH-derived ROS, leading to posttranslational modification of SERCA2b (S-glutathiolation), increased $[Ca^{2+}]_i$, and migration. Notably, removal of a reactive cysteine on SERCA2b attenuated increases in migration by either VEGF or exogenous H_2O_2 [91, 99]. Interplay between ECs and SMCs exists as well; for example, endothelial store-operated $[Ca^{2+}]_i$ entry is important for transcriptional activation of various inflammatory factors that in turn drive pulmonary smooth muscle proliferation and vascular remodeling [73].

ROS and $[Ca^{2+}]_i$ in EC shear stress responses.

A phenomenon of particular interest in PH is shear stress. Recent studies using magnetic resonance imaging elegantly demonstrated the presence of complex flow patterns in the pulmonary arteries of patients with PAH [100, 101]. ECs are uniquely positioned to serve as sensors of changes in adjacent blood flow, with EC dysfunction occurring as a consequence of both changes in laminar flow and formation of non-laminar flow patterns. Changes in shear stress cause a wide variety of responses in ECs, from alterations in gene expression [102] to cytoskeletal reorganization [103]. With respect to ROS, both increased and abrupt cessation of blood flow triggered radical production [104–106] and increased $[Ca^{2+}]_i$ [107–109], suggesting the possibility of cross talk between these signaling pathways. Supporting this notion, flow-adapted microvascular ECs subjected to sudden disruption in flow exhibited augmented baseline expression of T-type Ca^{2+} channels and NOX-dependent ROS generation [110, 111] that was followed sequentially by an increase $[Ca^{2+}]_i$ [112, 113] that was inhibited by depletion of ER stores. However, the effect of changes in shear stress due to the complex flow patterns observed in PH has not been fully elucidated. Interestingly, high shear stress increased $[Ca^{2+}]_i$ to a greater extent in SMC from PAH patients compared to normal controls [114] through a mechanism involving increased expression of TRPM7 and TRPV4 channels. Further studies will be required to determine whether a similar response occurs through the same mechanisms in ECs.

ROS and Ca^{2+} in pulmonary vasoreactivity.

Interactions between ROS and Ca^{2+} have been implicated in several stimulus-specific mechanisms of increased vasoreactivity including: (1) hypoxic pulmonary vasoconstriction (HPV), a well-described pulmonary vascular response to acute hypoxia, (2) nitric oxide production, a key step in endothelium-mediated pulmonary vascular vasodilation, and (3) pulmonary arterial constriction in chronic hypoxia, a known mediator of PH, particularly in Class III disease.

(a)   Hypoxic Vasoconstriction: It is well documented that decreased oxygen tension causes HPV in an effort to shunt blood away from poorly oxygenated alveoli. HPV may also play some role in the development of PH, although the exact cellular mechanisms underlying contraction in response to acute and chronic hypoxia may differ depending on the length of hypoxic exposure. Nonetheless, both ROS and increased $[Ca^{2+}]_i$ are involved in HPV [115–117]. ROS are sufficient to induce pulmonary vasoconstriction; in studies

utilizing endothelium-intact pulmonary arterial rings, contraction induced by provisioning exogenous ROS was not attenuated by removal of extracellular Ca^{2+} or blocking of membrane channels, arguing for store release as the mechanism of ROS-induced Ca^{2+} influx mediating PA contraction [118–120] (Fig. 3). However, an important caveat to these studies is that it is not possible to delineate which effects were mediated by the endothelium and which might have occurred in SMCs.

(b)   Nitric Oxide Production: Nitric oxide (NO) production by ECs is a key defense mechanism against vascular dysfunction across multiple vascular beds, including the pulmonary circulation. In ECs, NO production occurs primarily via endothelial nitric oxide synthase (eNOS). Dimeric units of eNOS catalyze electron transfer from NADPH to various electron carrying intermediates to eventually produce NO from L-arginine in a process that requires molecular O_2, L-arginine and ($6R$-)5,6,7,8-tetrahydrobiopterin (BH_4). This process occurs in two steps. First, electrons are transferred from NAPDH to a heme group within eNOS where they are stabilized with the aid of O_2. Next, the electrons are used to catalyze NO production from L-arginine in a process that requires BH_4. eNOS activity is regulated by a variety of posttranslational modifications, including phosphorylation and oxidative modification of Cys residues. NO production is also determined by the availability of the critical cofactors such that when BH_4 is unavailable, eNOS cannot successfully complete electron transfer from NADPH to L-arginine, resulting in formation of superoxide radicals. Under these conditions, BH_4 deficiency uncouples the first half of the reaction (electron transfer from NADPH to the heme group) from the second half (electron transfer from the heme group to L-arginine).

Increases in cytosolic ROS decrease the ability of eNOS to efficiently produce NO primarily by decreasing BH_4 availability [121]. NOX-generated superoxide can react with NO to produce peroxinitrites ($ONOO^\bullet$) which can oxidize BH_4 to a product (BH_3) that cannot serve as a eNOS cofactor [122]. NOX-generated ROS can also oxidize cysteine residues on eNOS to decrease BH_4 binding [121] and/or oxidize a moiety that is critical for NO generation (i.e., the Zn-thiolate cluster) [123]. Through these mechanisms, increased cytosolic ROS facilitate uncoupling of eNOS, leading to a feed-forward mechanism of ROS-induced ROS generation in ECs.

While increases in ROS disrupt eNOS function via oxidative modification of critical motifs and cofactors, increases in $[Ca^{2+}]_i$ modulate eNOS function via calmodulin (CaM). As reviewed in detail elsewhere [124, 125], the sensitivity of NOS to changes in $[Ca^{2+}]_i$ is isoform-dependent. For the purposes of this review, we will limit our discussion to the calcium-sensitive, constitutively expressed eNOS; however, inducible NOS (iNOS), which is not Ca^{2+} dependent, has also been implicated in oxidative stress. Although controversy remains regarding the specifics of eNOS subcellular localization and the role of various posttranslational modifications on eNOS activity, it is generally accepted that increased $[Ca^{2+}]_i$ leads to CaM binding which in turn facilitates electron transfer towards the heme moiety where NO synthesis occurs. Thus, while increased ROS leads to uncoupling, increases in $[Ca^{2+}]_i$ activate NO production. The net result of increases in both ROS and $[Ca^{2+}]_i$ on NO production in pulmonary ECs may vary with source of ROS, and compartmentalization; studies conducted in ECs from PAH patients show both oxidant stress

[12] and increased $[Ca^{2+}]_i$ [73], with decreased NO production [126], suggesting that in PH the net effect shifts towards uncoupling/decreased NO production. Consistent with these findings, Ghosh et al. [127] recently showed that abnormal phosphorylation of eNOS contributed to decreased NO production in PAH ECs and may contribute to eNOS uncoupling [128], but further work will be needed to elucidate whether this mechanism is related to ROS and/or Ca^{2+} levels.

Once produced, NO acts within the vessel lumen, with anti-inflammatory effects on leukocytes and platelets to decrease adherence of inflammatory cells to the endothelium and intracellularly, to induce production of cyclic guanosine monophosphate (cGMP). In SMCs, cGMP promotes vasodilation. Indeed, targeting cGMP availability, by either inhibiting degradation or stimulating production, are two treatment avenues currently utilized in the treatment of PAH.

(c) Chronic Hypoxia: Increased $[Ca^{2+}]_i$ is responsible for enhanced pulmonary vasoreactivity and development of abnormal EC function that occur with chronic hypoxia [129, 130]. For instance, chronic hypoxia increased agonist-induced vasoconstriction via both ROS generation and enhancement of Ca^{2+}-sensitivity of the contractile apparatus due to ROS-induced activation of RhoA/ROCK [131]. However, most of these studies were performed either in SMCs or preparations that included ECs and SMC; thus, the EC-specific roles of ROS and Ca^{2+} in regulating vasoreactivity during chronic hypoxia remain unclear.

In summary, it is evident that inhibition of either ROS or Ca^{2+} entry invokes largely similar responses with regards to vasoreactivity in the pulmonary vasculature. However, significant gaps still exist in our understanding as to whether ROS directly activates Ca^{2+} entry in pulmonary ECs, and if so, the exact channels that are involved in this response.

7 Conclusions

Based on available evidence, elevations in ROS and $[Ca^{2+}]_i$ play key roles in the pathobiology of PH, mainly because increased ROS generation elevates EC $[Ca^{2+}]_i$. While it seems clear that elevations in ROS from NOX and mitochondria are important for migration, proliferation, and vaso-reactive responses, the role played by ROS-induced Ca^{2+} influx on these pathways, particularly in ECs, remains to be determined. Continued investigation is necessary to better understand the role of both ROS and $[Ca^{2+}]_i$ in promoting vascular reactivity, migration, and proliferation in pulmonary ECs. The hope is that this will lead to targeted therapies aimed at reversing or slowing progression of this devastating disease.

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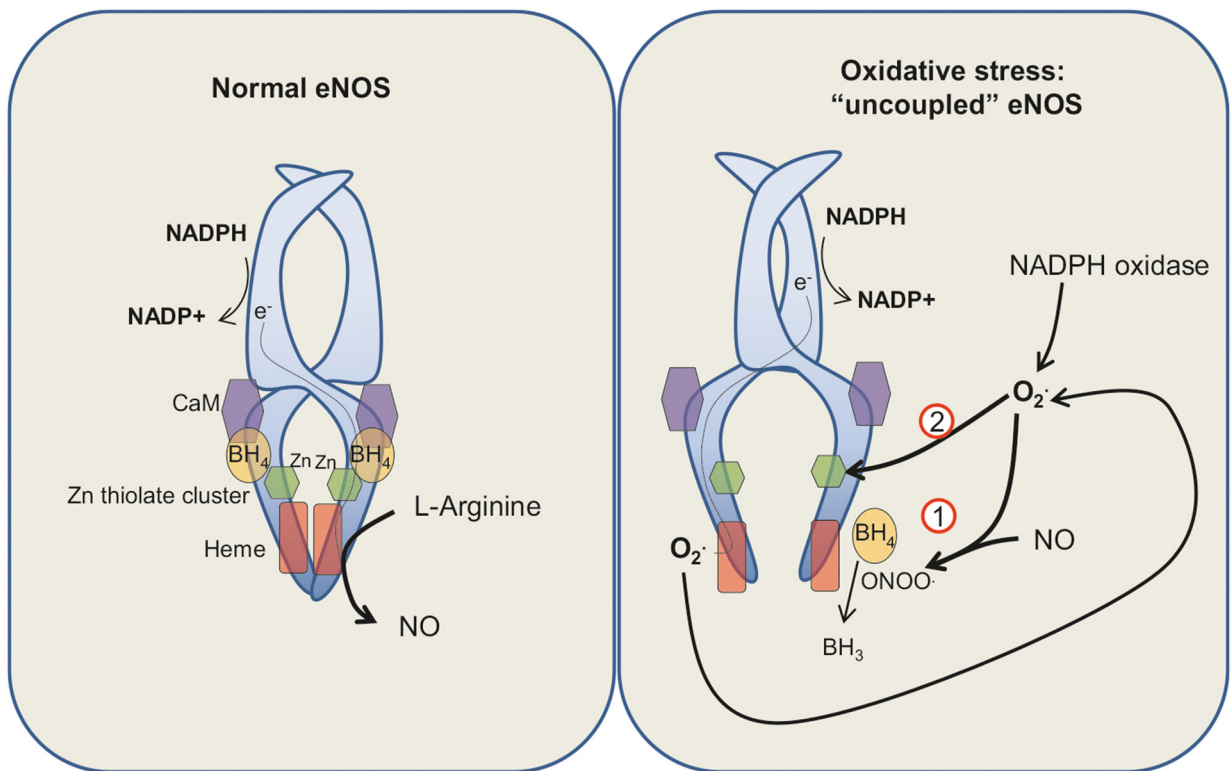
Fig. 1.

Reactive oxygen species (ROS) and endothelial nitric oxide synthase (eNOS) uncoupling. Under normal circumstances (*left panel*), eNOS utilizes various cofactors including L-arginine, molecular O_2 and ($6R$-)5,6,7,8-tetrahydrobiopterin (BH_4) to catalyze nitric oxide (NO) formation (at the heme site). The eNOS enzyme includes various regulatory subunits including a calmodulin (CaM) binding site. In situations of oxidative stress (*right panel*), local increases in superoxide ($O_2^{\bullet}$) lead to: (1) formation of peroxinitrites ($ONOO^-$) due to reactions with NO, causing oxidation of BH_4 (to BH_3) and inactivation of this critical cofactor and (2) oxidative modification of the Zn-thiolate cluster. Together, these events lead to decreased catalytic efficiency of eNOS and generation of $O_2^{\bullet}$ instead of NO. Increased $O_2^{\bullet}$ in turn can augment further uncoupling of eNOS via a feed-forward mechanism
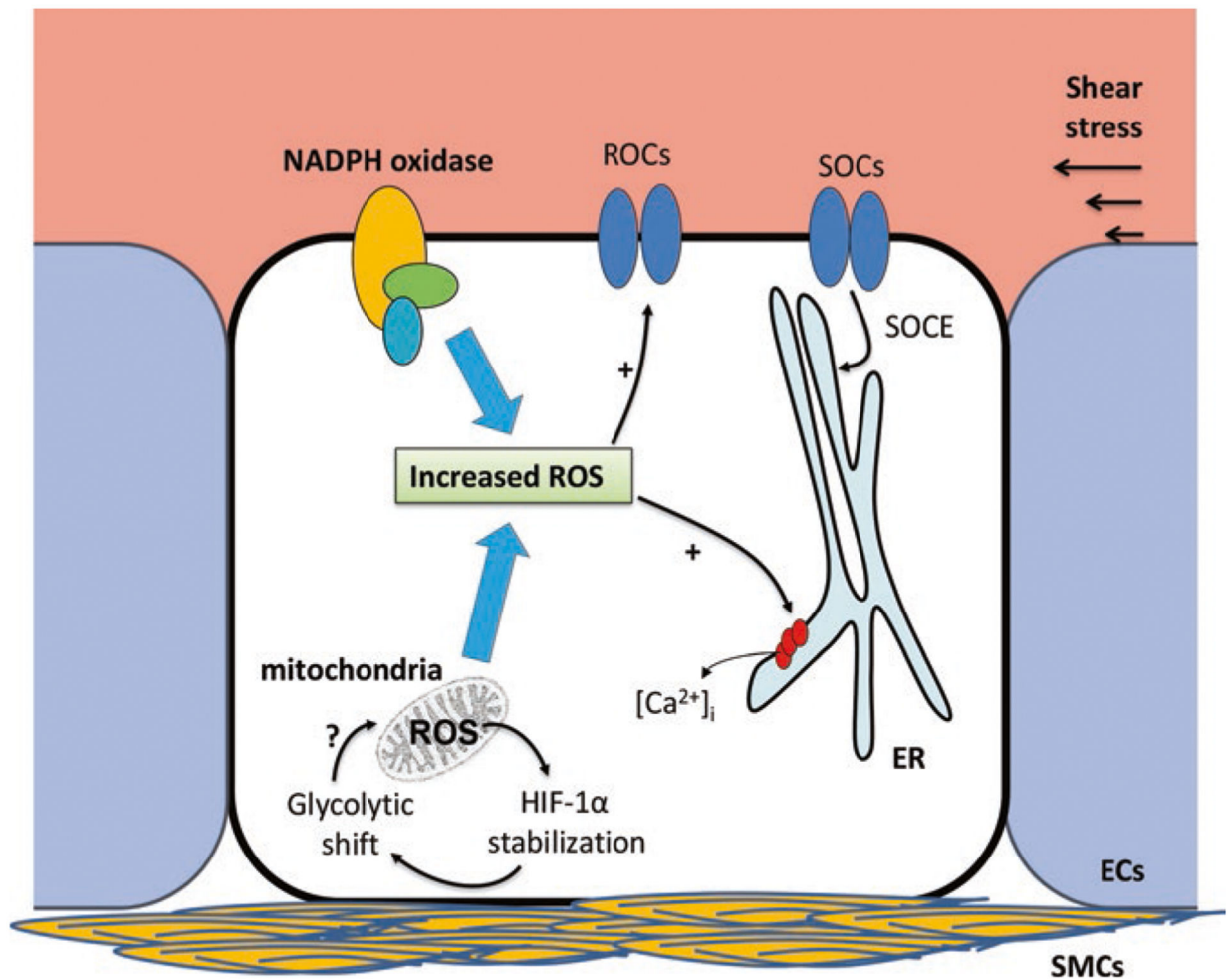
Fig. 2.
Mechanisms of reactive oxygen species (ROS)-induced Ca^{2+} entry in endothelial cells (ECs) in pulmonary arterial hypertension (PAH). Increases in ROS from cytosolic (primarily NADPH oxidase) and mitochondrial sources can lead to both release of Ca^{2+} from internal endoplasmic reticulum (ER) stores as well as influx through receptor-operated (ROC) and store-operated (SOC) channels on the cell membrane. Release of Ca^{2+} from the ER can activate SOCs in order to replete ER Ca^{2+} stores via store-operated Ca^{2+} entry (SOCE). The sources of increased ROS in ECs include: NADPH oxidase, mitochondria and physical stimuli like shear stress. Based on data from both ECs and smooth muscle cells (SMCs) in PAH, mitochondrial ROS generation may be linked to abnormal HIF-1α stabilization and glycolytic shift
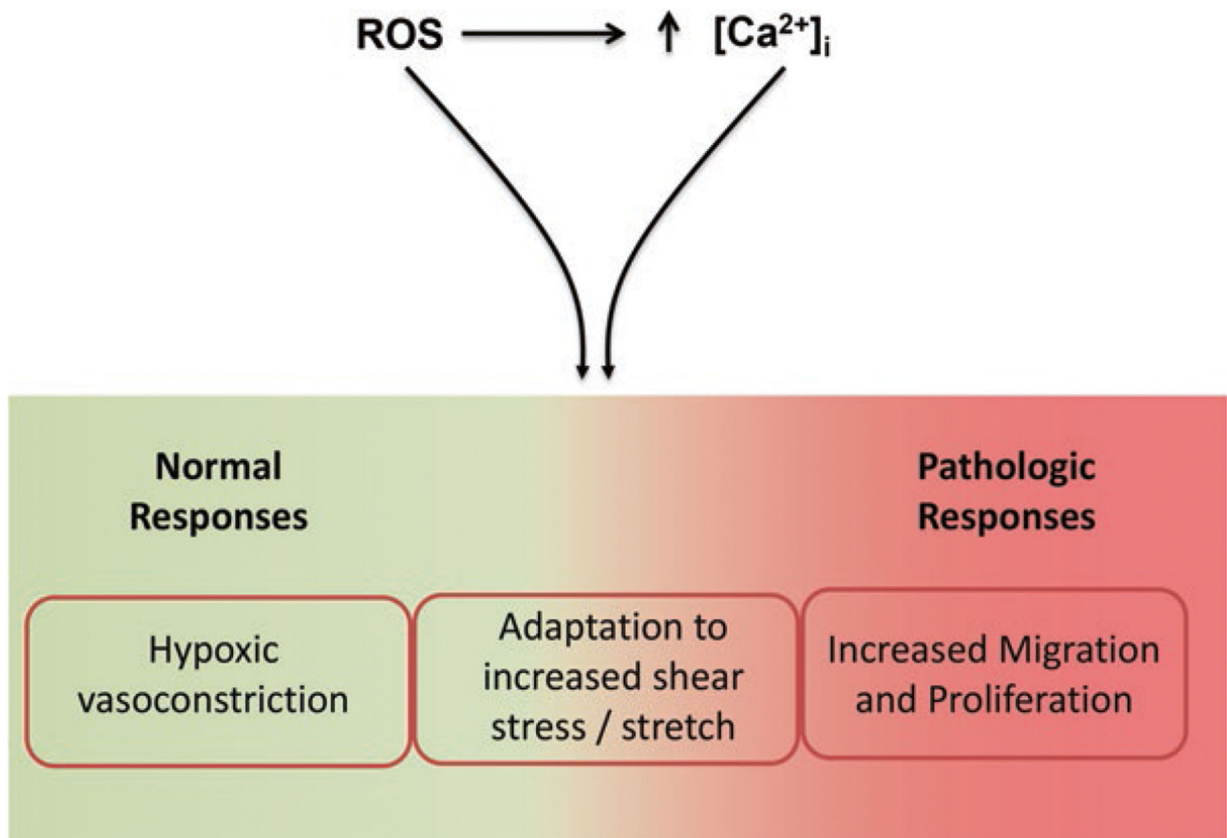
Fig. 3.
Functional consequences of elevated reactive oxygen species (ROS) and intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in pulmonary endothelial cells (ECs). Elevations of both ROS and $[Ca^{2+}]_i$ promote normal physiologic responses, such as hypoxic vasoconstriction, maladaptive responses such as EC adaptation to shear stress and stretch and lastly, pathologic responses such as increased migration and proliferation

Table 1

Classification of PH (adapted from Galie et al., *Eur Heart J* 2015)

Classification of PH
Group 1: Pulmonary Arterial Hypertension (PAH)
Idiopathic PAH
Heritable PAH due to known (e.g., BMPR2) or other mutations
Drug and toxin induced
PAH associated with systemic disease
Connective tissue disease
HIV
Portal hypertension (liver disease)
Congenital heart disease
Schistosomiasis
Group 1′: Pulmonary veno-occlusive disease Pulmonary capillary hemangiomatosis
Idiopathic
Heritable (EIF2AK4 or other mutations)
Drugs, toxins and radiation-induced
Associated with connective tissue disease or HIV infection
Group 1′′: Persistent PH of the newborn
Group 2: PH due to left heart disease
Left ventricular systolic or diastolic dysfunction
Valvular disease
Congenital/acquired left heart inflow/outflow tract obstruction and congenital cardiomyopathies
Congenital/acquired pulmonary vein stenosis
Group 3: PH due to lung diseases and/or hypoxia
PH due to chronic hypoxia (COPD, interstitial lung disease)
PH due to sleep-disordered breathing
Alveolar hypoventilation disorders
Chronic high altitude exposure
Developmental lung disease
Group 4: PH due to chronic thromboembolic disease and other pulmonary artery obstructions
Chronic thromboembolic pulmonary hypertension
Other pulmonary artery obstructions (e.g., Angiosarcoma, arteritis, parasites)
Group 5: PH due to unclear mechanism
Hematologic disorders (e.g., chronic hemolytic anemia)
Systemic disorders (e.g., sarcoidosis, LAM)
Metabolic disorders (e.g., glycogen storage disease)
Other (e.g., tumoral thrombotic microangiopathy, fibrosing mediastinitis. Chronic renal failure, segmental PH)