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Novel regulators and targets of redox signaling in pulmonary vasculature

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Dysregulated redox signaling in pulmonary vasculature is central to the development of pulmonary arterial hypertension (PAH) and lung injury. Modulators of reactive oxygen species (ROS) production and downstream signaling targets are critical for mediating the physiological or pathological effects of ROS. Understanding the complex interactions between the modulators and signaling targets of ROS is essential for developing novel strategies to prevent or attenuate lung pathologies. In this review, we discuss recent studies on the modulators and targets of ROS in pulmonary endothelial and smooth muscle cells, their cellular effects, and the disease conditions associated with dysregulated redox signaling.

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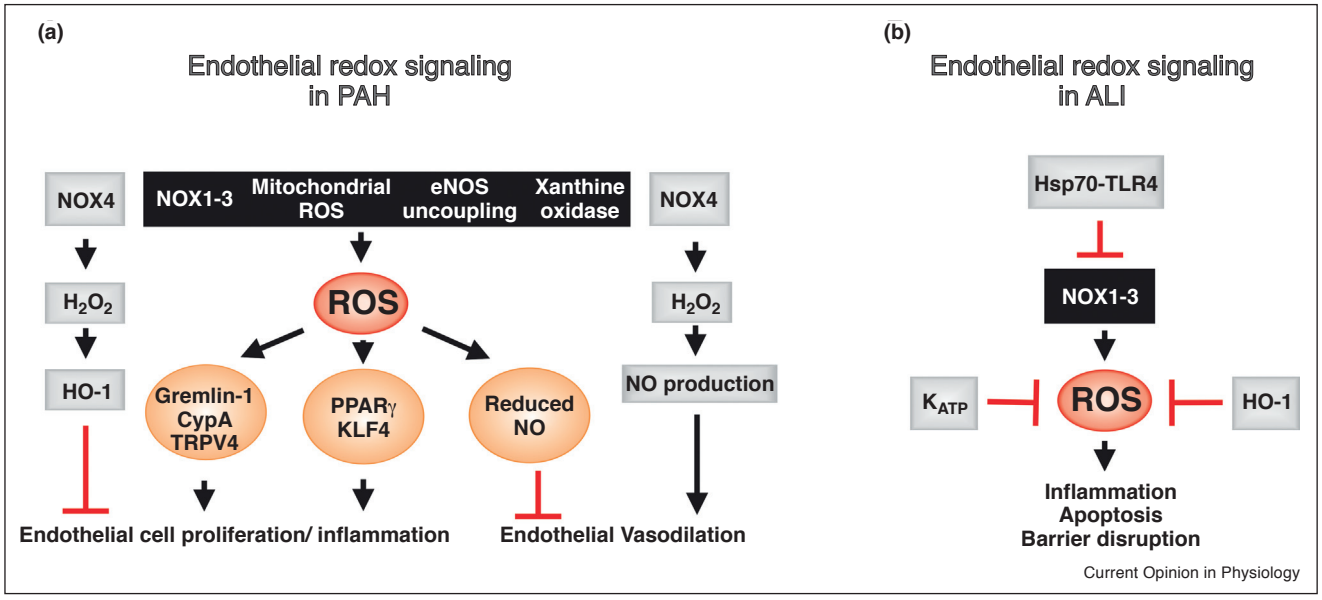
Introduction

Reactive oxygen species (ROS) comprise a set of reactive chemical intermediates of oxygen that have one or more unpaired electrons in their outer orbits. The main ROS in pulmonary circulation include superoxide radicals, hydrogen peroxide, and hydroxyl radicals. Additionally, superoxide can react with nitric oxide (NO) to form peroxynitrite, a reactive nitrogen species (RNS) that inhibits endogenous antioxidant systems via post-translational modifications [1,2]. Because of their high reactivity, ROS or RNS can oxidize proteins, DNA, lipids, or cellular membranes and alter their functions [3]. The most common sites for redox modifications of proteins are oxidant-sensitive amino acids (cysteine, selenocysteine, and methionine residues) and redox-active transition metal ion centers (heme groups, iron–sulfur centers, zinc–thiolate centers). Thus, ROS signaling plays important roles in maintaining cellular homeostasis, however, dysregulated redox signaling results in oxidative stress, which can have damaging effects on cellular functions [4].

Mitochondria and the NADPH oxidase (NOX) family of enzymes are widely regarded as the major sources of cellular ROS (Figures 1 and 2). Aerobic metabolism within the mitochondrial electron transport chain produces ROS, while NOX enzymes generate ROS (superoxide or H_2O_2) as their primary product. Four different NOX isoforms, NOX1–4, have been detected in the pulmonary vasculature [5–8]. ROS can also be generated by uncoupled nitric oxide synthase (NOS), xanthine oxidase, lipid peroxidases, and cytochrome P450 enzymes [9]. Notably, lungs express endogenous antioxidants that prevent excessive formation of ROS or limit the ROS-induced cellular damage. These include enzymes that metabolize ROS (superoxide dismutase, catalase, glutathione peroxidases, peroxiredoxins) [10], thiol reductases that reverse cysteine oxidation (thioredoxin, glutaredoxin), phase II metabolic enzymes (glutathione-*S*-transferases), small molecular weight antioxidants (glutathione and vitamins), and metal binding proteins (transferrin, lactoferrin). Several of these antioxidant systems are altered or impaired under disease conditions.

Increased NOX activity or mitochondrial dysfunction or altered endogenous antioxidant systems [10] is usually associated with higher ROS levels. In the pulmonary vasculature, an imbalance between oxidant production and antioxidant protective mechanisms is associated with deleterious effects on both smooth muscle cells (SMCs) and endothelial cells (ECs) [2,11,12•]. Oxidative stress has been associated with excessive pulmonary vasoconstriction, inflammation, and vascular remodeling, all of which contribute to the development of pulmonary arterial hypertension (PAH, Graphical Abstract). Abnormal redox signaling has also been implicated in the disruption of pulmonary endothelial barrier function and pathogenesis of acute lung injury (ALI, Graphical Abstract). Evidence for the connection between ROS and pulmonary abnormalities have been diligently summarized in previous review papers [9,13–16]. Here, we discuss the most recent discoveries on the physiological and pathological roles of ROS in the pulmonary vasculature.

Figure 1



Endothelial redox signaling mechanisms in pulmonary arterial hypertension (PAH) and acute lung injury (ALI).
(a) The major sources of reactive oxygen species (ROS) generation in pulmonary vascular endothelial cells include enzymes NOX1-3 and xanthine oxidase, mitochondria, and endothelial nitric oxide synthase (eNOS) uncoupling. In PAH, excessive ROS generation promotes endothelial cell proliferation and attenuates endothelium-dependent vasodilation. In contrast, NOX4 is thought to be protective. It releases H_2O_2, which exerts protective effects on endothelium via activation of antioxidant enzyme HO-1 and formation of NO. **(b)** Excessive ROS formation can lead to endothelial cell apoptosis and disruption of endothelial barrier, thus contributing to ALI. Hsp70-TLR4 signaling, K_{ATP} channels, and HO-1 have protective effects on ROS-induced inflammation and apoptosis in ALI.

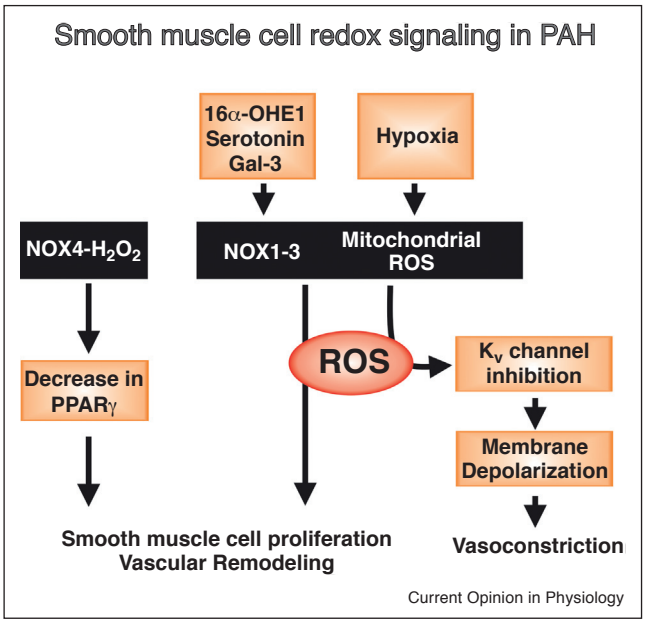
Modulators of ROS production in ECs

NOX family of enzymes

Among the NOX isoforms, NOX1–3, which primarily release superoxide, are thought to contribute to pulmonary vascular disease pathogenesis via ROS production (Figure 1). NOX4 primarily releases H_2O_2 [17] and has been suggested to have a protective effect on endothelial function [18,19]. In NOX4$^{-/-}$ mice, pulmonary endothelial tube formation and angiogenesis were attenuated [19]. Lowering the concentrations of H_2O_2 also reversed EC tube formation and angiogenesis in wild-type mice, supporting the role of NOX4–H_2O_2 signaling in EC tube formation and angiogenesis in lungs (Figure 1) [19]. Similarly, in an angiotensin II (Ang II)—induced model of oxidative stress, NOX4-deficient mice displayed endothelial dysfunction [19]. One possibility is that NOX4–H_2O_2 signaling exerts protective effects on endothelial function via increases in NO production and expression of heme oxygenase-1 (HO-1) [19]. While NO is a vasodilator and anti-inflammatory signaling molecule, HO-1 is an endogenous antioxidant enzyme [19]. Interestingly, NOX4 can localize to the endoplasmic reticulum (ER) [20,21], and this ER localization may be important for establishing the target specificity of NOX4-dependent signaling [21,22]. Additionally, endothelial NOX4–H_2O_2 signaling has also been linked with maintenance of vascular endothelial growth factor (VEGF) expression [18]. Despite numerous studies supporting an essential role for NOX4 in pulmonary vascular cells, the precise downstream mechanisms that mediate functional effects of NOX4 remain unclear.

Another enzyme of the NOX family, NOX3, was previously thought to be an insignificant player in pulmonary vasculature. However, recent studies have identified a key role for NOX3 in pulmonary vascular diseases [23,24]. For example, Yin *et al.* targeted 143 single nucleotide polymorphisms (SNPs) for 14 candidate genes and showed that the rs6557421 variant in NOX3 may be important for individual susceptibility to pulmonary hypertension (PH) [25•]. Among the SNP candidates were NOX4, NOX5, and the structural protein of caveolae, caveolin-1. However, only NOX3 SNP was associated with overall PH susceptibility. In another study, NOX3-induced ROS production led to pulmonary EC death and lung injury in mice [23]. Additionally, activation of heat shock protein 70 (Hsp70) toll-like receptor 4 (TLR-4)-Trif signaling was shown to inhibit NOX3 activity in lung ECs, attenuating ROS generation and oxidant-induced ALI (Table 1, Figure 1) [23,26]. Moreover, TLR-4-dependent activation of stanniocalcin-1 (STC-1), an endogenous antioxidant in the lungs, had a beneficial effect in ALI (Table 1) [26]. Thus, inhibition of NOX3 via a TLR-4/STC1-mediated mechanism could be a

Figure 2



Smooth muscle cell redox signaling mechanisms in PAH. In pulmonary artery smooth muscle cells, hypoxia induces mitochondrial ROS generation, which inhibits voltage-gated K^+ (K_V) channels to cause membrane depolarization and vasoconstriction. ROS generation by NOX enzyme activity is mainly responsible for smooth muscle cell proliferation and vascular remodeling in PAH. ROS generation by NOX1-3 enzymes can be activated by serotonin, 16α-hydroxyestrone (16α-OHE1), and galectin-3 (Gal-3).

potential strategy for the prevention or treatment of ALI. In this regard, Hsp70 upregulation suppressed mitochondrial ROS production and disruption of pulmonary endothelial barrier in response to bacterial toxin pneumolysin [27••], suggesting that Hsp70 upregulation may also be a therapeutic approach against pneumonia-associated lung injury (Table 1).

Another prime candidate for ROS generation, NOX1, was elevated in cultured human pulmonary artery ECs exposed to hypoxia. This was accompanied by increased ROS levels and gremlin 1 expression, which in turn stimulated EC proliferation and migration [28]. Consistent with these findings, increased levels of NOX subunit p22phox were detected in the lungs from a mouse model of hypoxia-induced PH [29••]. Importantly, mice harboring a loss of function mutation in p22phox were protected against hypoxia-induced PH, revealing an essential role of p22phox in the development of PH [29••]. p22phox was also found to promote ROS generation, vascular proliferation, migration, and angiogenesis. Thus, NOX1-ROS-gremlin 1 signaling and p22phox subunit could be targeted for potential therapy against vascular remodeling in PAH (Figure 1).

Cyclophilin A (CypA)

CypA is both a target and a modulator of cellular ROS (Figure 1). CypA is a ubiquitously expressed protein that has also been identified as a secreted oxidative stress-induced factor [30,31]. EC-derived CypA promotes inflammation through paracrine and autocrine mechanisms [32]. Importantly, oxidative stress-induced release of CypA and acetylated CypA from mouse and human MVECs, which further promoted EC oxidative stress (Table 1) [32]. A study utilizing transgenic mice that express high levels of CypA revealed a PAH phenotype in these mice by three months of age. In the same study, extracellular CypA and acetylated CypA enhanced inflammatory signals and apoptosis in MVECs [32]. This series of findings suggest that CypA or acetylated CypA release from ECs may be a potential therapeutic target for treating PAH.

K_{ATP} channels

Activation of ATP-sensitive K^+ (K_{ATP}) ion channels was recently shown to curtail ROS generation (Table 1, Figure 1) [33], thus revealing K_{ATP} channels as a novel regulator of ROS generation and a potential therapeutic target. Stimulation of endothelial K_{ATP} channels reduced lipopolysaccharide (LPS)-induced ALI by lowering ROS levels, preventing apoptosis, and suppressing endothelial inflammation. These effects were mainly attributed to the inhibition of NF-κB and MAPK signaling pathways by K_{ATP} channels [33].

Table 1

Novel modulators of ROS production in pulmonary vasculature

Modulator	Target cell	Effect	References
Hsp70-TLR4-Trif signaling	EC	Attenuates NOX3 activity, ROS production, and ALI	[23,26]
TLR4-STC1 signaling	EC	Endogenous antioxidant STC1 lowers oxidant-induced lung injury	[26,27••]
Pneumolysin	EC	Elevates ROS, damages endothelial barrier	[27••]
K_{ATP} channel	EC	Inhibits of NF-κB and MAPK signaling, lower ROS production	[33]
CypA/acetylated CypA	EC	Increases ROS levels, inflammation and apoptosis, PAH phenotype	[32]
Serotonin (5-HT)	SMC	Increases NOX1 signaling and ROS production	[47••]
Gal-3	SMC	Increases NOX expression, PA remodeling and fibrosis in PAH	[48,49]
16α-hydroxyestrone	SMC	Increased NOX1-mediated ROS production, cell proliferation	[46]

Targets of ROS in ECs

Heme oxygenase (HO-1)

HO-1 is an endogenous antioxidant and anti-inflammatory enzyme in the lungs [34]. Searching for a potential treatment for LPS-induced acute lung injury (ALI), Lin *et al.* recently investigated the role of HO-1 in ECs and other pulmonary cells [35]. Under healthy conditions, HO-1 lowered the levels of pro-inflammatory cytokines (TNF-α, IL-6 and IL-1β) and stimulated SOD activity, thereby reducing ROS levels in lung cells [35]. This observation was further supported by subsequent findings that HO-1 inhibited pro-inflammatory PI3K/Akt/NF-κB signaling and upregulated anti-inflammatory AMPK activity; effects that would be beneficial in LPS-induced ALI [34,36]. Further research is needed to determine whether the protective effect of HO-1 is derived from pulmonary ECs or other cell types.

TRPV4 channels

Recent studies on Ca^{2+} signaling mechanisms in pulmonary ECs have focused on TRP (transient receptor potential) cation channels at the EC membrane. It appears that Ca^{2+} influx through endothelial TRPV4 (TRP vanilloid 4) channels controls physiological function of pulmonary microcirculation [37] but has also been implicated in a ROS-activated mechanism for the pathogenesis of PAH (Figure 1) [37]. Marziano *et al.* recently demonstrated that a spatially restricted TRPV4-eNOS signaling mechanism in the endothelium promotes vasodilation of small, resistance-sized pulmonary arteries [37]. A subsequent study by Suresh *et al.* showed that cultured lung microvascular ECs (MVECs) from a rat model of PAH (SU5416 injection + chronic hypoxia) exhibited higher basal levels of intracellular Ca^{2+} and elevated migratory and proliferative capacity [38•] when compared to MVECs from normoxic rat. Interestingly, ROS levels were elevated in MVECs from PAH rats, and mitochondrial antioxidants lowered TRPV4 channel-dependent increases in intracellular Ca^{2+}. Similarly, TRPV4 channel inhibition also reduced intracellular Ca^{2+} levels and reversed MVEC migration and proliferation [38•], implying that abnormal migration and proliferation of MVECs in PAH may be modulated by mitochondrial ROS-induced Ca^{2+} influx via TRPV4 channels (Figure 1). The TRPV4 channel has a high Ca^{2+} conductance [39]. Therefore, it is plausible that the extent of TRPV4 channel activation determines whether the ion channel plays a beneficial or a harmful role in pulmonary vasculature.

Transcription factors

Recent studies have focused mainly on two transcription factors that can be targeted by ROS (Figure 1): peroxisome proliferator-activated receptor gamma (PPARγ) and Kruppel-like factor 4 (KLF4). The expression of PPARγ, a nuclear hormone transcription factor, was found to be reduced in ECs and SMCs during hypoxia-induced PH. Moreover, this decrease in PPARγ expression was dependent on NOX4-mediated H_2O_2 release [40–43]. In this regard, Bijli *et al.* showed that hypoxia triggers the activation of proline-rich tyrosine kinase 2 (Pyk2), which stimulates extracellular signal-regulated kinase (ERK) 1/2, promoting NF-κB-mediated NOX4 expression. The resulting H_2O_2 generation downregulates PPARγ [40]. It is not known whether PPARγ downregulation in ECs and SMCs is essential for the development of PH.

Another transcription factor, KLF4, belongs to the Kruppel-like transcription factor family and is known to be important for normal endothelial function and vascular homeostasis [44]. KLF4 is constitutively expressed in pulmonary arteries and is associated with modulation of inflammation, coagulation, and phenotypic switching [44]. In PAH, *S*-nitrosation of endothelial KFL4 was shown to contribute to endothelial dysfunction. Thus, ROS-mediated post-translational modifications of endothelial KLF4 may underlie pulmonary vascular dysfunction and development of PAH.

Modulators and targets of ROS in SMCs

Pulmonary vasoconstriction and vascular remodeling are key causative factors for increased pulmonary arterial pressures in PAH. A recent study by Jernigan *et al.* investigated the contribution of ROS in increasing pulmonary vasoconstrictor activity in a rat model of PAH (SU5416 injection + chronic hypoxia) [12•]. Hypoxia/SU5416 rats showed greater vasoconstriction of pulmonary arteries in response to endothelin-1 and this effect was dependent on ROS generation. Although ROS production increased pulmonary vasoconstriction, it did not seem to play a role in promoting vascular remodeling in this model [12•]. As discussed below, several other studies support an important role for ROS production in pulmonary vascular remodeling.

NOX and transcription factors

Among the NOX family, NOX1 and NOX4 have been consistently found to be expressed in SMCs, where they play crucial roles in cell growth [17,45]. In pulmonary artery SMCs, estrogen metabolite 16α-hydroxyestrone (16α-OHE1, Table 1, Figure 2) induced NOX1-mediated ROS production, expression of nuclear factor erythroid-related factor 2 (Nrf-2), and cell proliferation [46]. Moreover, genetic ablation of NOX1 protected the animals against development of PAH, supporting an important role for SMC NOX1 in ROS production and PAH pathogenesis.

Serotonin

Hood *et al.* recently provided evidence that serotonin (5-HT) signaling via 5-HT_{1B} receptors can stimulate NOX1-dependent ROS production in pulmonary SMCs (Table 1, Figure 2) [47••]. In human pulmonary artery SMCs, 5-HT stimulated 5-HT_{1B} receptor-Src-related

kinase-NOX1 signaling and ROS production. 5-HT-mediated ROS production also lowered the expression of transcription factor Nrf-2 and catalase activity [47••]. The fact that 5-HT, more popular for its neurotransmitter properties in mood disorders, appears to regulate redox-sensitive signaling pathways in pulmonary SMCs opens a wide range of possibilities for further exploration of neurotransmitters' role in pulmonary vascular pathologies.

Galectin-3 (Gal-3)

Gal-3 is a chimeric lectin that may regulate NOX expression and redox signaling, and is a potent driver of fibrosis [48]. In rat models of PAH, Gal-3 expression in pulmonary artery SMCs was increased, which correlated with a higher SMC migration and fibrosis. Genetic deletion of Gal-3 lowered SMC migration and fibrosis, supporting an important role for galectin-3 in pulmonary artery remodeling and fibrosis during PAH (Table 1, Figure 2) [49,50].

Concluding remarks

The physiological and pathological importance of ROS in pulmonary vasculature is firmly established. Recent studies have made significant strides into understanding novel regulators and targets of ROS (Table 1), although the specific mechanisms that can be therapeutically targeted remain elusive. Therefore, further research on targetable ROS-dependent mechanisms is needed for therapeutic benefit in pulmonary vascular diseases. It should also be noted that a significant amount of evidence for ROS regulators and their cellular effects has been derived from studies in cultured ECs or SMCs. Because of the vastly different environment in the intact blood vessels, it is plausible that native vascular cells employ different ROS signaling pathways and regulators. In this regard, studies in cell-specific knockout mice models and intact pulmonary blood vessels may provide useful insights. Identifying the source of ROS production and targeting-specific ROS involved in the pathological mechanisms will also result in better therapeutic strategies.

Conflict of interest statement

Nothing declared.

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