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FORUM REVIEW ARTICLE

Redox Regulation of Ion Channels in the Pulmonary Circulation

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Abstract

Significance: The pulmonary circulation is a low-pressure, low-resistance, highly compliant vasculature. In contrast to the systemic circulation, it is not primarily regulated by a central nervous control mechanism. The regulation of resting membrane potential due to ion channels is of integral importance in the physiology and pathophysiology of the pulmonary vasculature. ***Recent Advances:*** Redox-driven ion conductance changes initiated by direct oxidation, nitration, and S-nitrosylation of the cysteine thiols and indirect phosphorylation of the threonine and serine residues directly affect pulmonary vascular tone. ***Critical Issues:*** Molecular mechanisms of changes in ion channel conductance, especially the identification of the sites of action, are still not fully elucidated. ***Future Directions:*** Further investigation of the interaction between redox status and ion channel gating, especially the physiological significance of S-glutathionylation and S-nitrosylation, could result in a better understanding of the physiological and pathophysiological importance of these mediators in general and the implications of such modifications in cellular functions and related diseases and their importance for targeted treatment strategies. *Antioxid. Redox Signal.* 22, 465–485.

Introduction

''WHY GRASS IS GREEN or why our blood is red, are mysteries which none have reach'd unto.'' John Donne (1571–1631).

The interaction between the air, the lungs, and the blood has fascinated philosophers and physiologists for centuries. In 1669, an Oxford physician, Richard Lower, reported that it was exposure to air in the lungs that caused the change from dark venous blood to bright arterial blood. However, more than another 200 years elapsed before Bradford and Dean described constriction of the pulmonary arteries (PA) caused by asphyxia (20). The recognition of the involvement of ion channels in hypoxic pulmonary vasoconstriction (HPV) has been relatively recent; of L-type calcium channels in 1976 (86) and of potassium (K^+) channels in 1992 (118). The suggestion that redox changes might play a role in the mechanism of HPV was initially made in 1986 (9) and focused on ion channels in 1993 (4). Subsequently, many laboratories have found changes in reactive oxygen species (ROS) and the redox couples GSH/GSSG and NAD(P)H/NAD(P) in the course of HPV and of chronic hypoxic pulmonary hypertension (PH).

Besides ROS, small signaling chemical species such as nitric oxide (NO), carbon monoxide (CO), and hydrogen sulfide (H_2S) molecules participate in the regulation of pulmonary vascular function. Independent of transporters, membrane receptors, or second messenger systems, they freely diffuse through cell membranes and elicit various responses. Oxidative stress is postulated to play a prominent role in the etiology of vascular and ventricular dysfunction that is associated with cardiovascular disease (53, 129, 134, 185). In the pulmonary vasculature and in the heart, changes in the ROS levels result in changes in the redox state of

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proteins, some of which can be reversed. In part, reversible protein oxidation involves the free thiol (-SH) side chain of cysteine residues that can undergo a number of redox-mediated molecular modifications which may elicit positive or negative changes in protein function (34, 185). Thus, under conditions where ROS levels are markedly elevated and/or oxidoreductase systems are impaired, there is significant alteration in the physiological function of cells, mediated by direct protein oxidation or changes in protein interaction with redox molecules. Although redox-target specification is, no doubt, dictated by the innate susceptibility of the target, additional measures are in place within the cell to prevent accumulation of ROS to toxic levels. This regulation is facilitated, in part, by the discrete subcellular compartmentalization of ROS production, the restricted availability of NADPH oxidase (Nox) activating and regulatory subunits, and the ubiquitous presence of cytosolic scavenging and neutralizing enzymes such as Cu/Zn superoxide dismutases (SODs), glutathione peroxidase (GPx), and peroxiredoxins.

The role of redox regulation in the pulmonary circulation emerged, in part, because the pulmonary vasculature constricts in response to hypoxia, while the ductus arteriosus (DA) and systemic vessels, such as the renal arteries, dilate. It is possible that redox control of ion channels might provide a mechanism which could explain these disparate responses. It is clear that the same redox signal can cause opposite changes of the gating of K^+ channels in the resistance PAs and the DA (102). For instance, the reducing agent dithiothreitol (DTT) inhibits K^+ current and causes depolarization and contraction in the smooth muscle cells (SMCs) of the PA (PASMC), while it activates K^+ current and causes hyperpolarization and relaxation of DASMCs. The oxidizing agent 5′,5′-dithiobis(2-nitrobenzoic) acid (DTNB) has the opposite effects on K^+ current, membrane polarization, and tone in PA and DA SMCs. Similar contrary actions have been described in response to changes in endogenous ROS in PA and renal artery SMCs (89). The fact that the role of redox signaling in the control of ion channels and tone in the PA is not yet agreed probably derives from the variety of ROS generated and methods of their inactivation, variation in ion channel expression in different vessels and different stages of maturation, and the use of a number of different experimental techniques. Are the relevant ROS produced in the mitochondria, by Noxs, in the plasma membrane electron transport (PMET) system, or in all of these places? Which ROS is key, superoxide anion, H_2O_2 or another radical (170)? Given that ROS may rise and fall in different places in the PASMCs during hypoxia, the location of changes is likely to be critical (168). ROS can have different physiologic and pathologic roles, and the overlap of these effects can cause confusion. In this regard, when exogenous ROS or anti-oxidant enzymes are added to cells in culture, attention should be paid as to whether the concentrations used and the sites of administration are physiological or pathological. Furthermore, since ion channels and calcium-handling proteins act as effectors, differences in the ion channels expressed in resistance and conduit vessels and in fetal and adult vessels also have to be considered. Consequently, much of the work discussed in this review is relevant to our understanding of the interaction of redox status, ion channel control, and tone in the pulmonary vasculature.

Redox Buffer Systems

Glutathione

The glutathione system includes reduced (GSH) and oxidized (GSSG) forms of glutathione, the enzymes required for its synthesis and recycling, such as gamma-glutamate cysteine ligase (γ-GCL), glutathione synthetase (GS), glutathione reductase (GR), and gamma-glutamyl transpeptidase (γ-GGT); also, the enzymes required for its use in metabolism and in mechanisms of defense against free radical-induced oxidative damage, such as glutathione s-transferases (GSTs) and GPxs. The glutathione system is one of the most important antioxidant systems for most tissues *in vivo*, and the ratio of oxidized to reduced glutathione (GSSG/GSH) is a widely used indicator of oxidative stress within different cell types. The ratio of reduced glutathione (GSH) to glutathione disulfide (GSSG; oxidized form) is >10 (43). Under normal circumstances, GSH levels within the cell are maintained *via* recycling of GSSG by the NADPH-dependent enzyme GR (Fig. 1). However, under pathological conditions, the GSH/GSSG ratio can decrease significantly as shown during ischemia/reperfusion in the right ventricle (109).

S-glutathionylation is a redox-dependent post-translational modification with growing recognition in signal transduction. Protein S-glutathionylation (P-SSG), the reversible modification of cysteine thiols by glutathione, is elevated

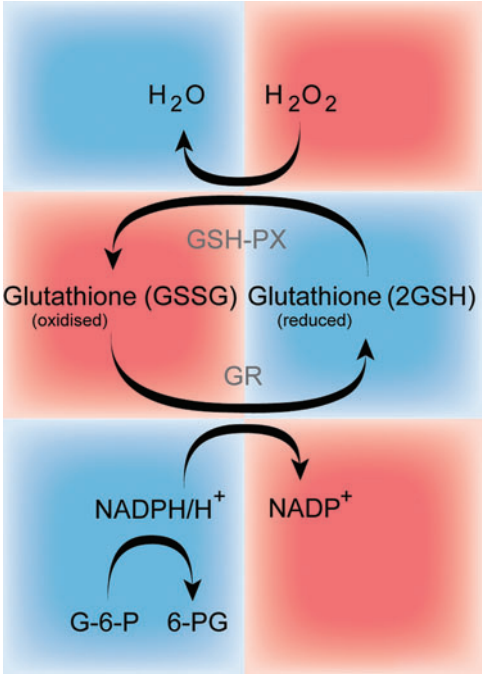

FIG. 1. The schematic of the glutathione system. GSH is synthesized from amino acids by the action of γ-glutamylcysteine synthetase and glutamyl synthase. GSH undergoes the GSH-PX coupled reaction, thereby detoxifying ROS such as H_2O_2. During this reaction, GSH is oxidized and generates GSSG, which is recycled back to GSH by the action of GR at the expense of reduced nicotinamide ($NADPH/H^+$), thus forming the redox cycle. GSH, glutathione; GSH-PX, glutathione-peroxidase; GR, glutathione reductase; H_2O_2, hydrogen peroxide; ROS, reactive oxygen species. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

in response to oxidative stress. Initially, the function of S-glutathionylation was thought to be the protection of cysteine residues against over-oxidation to sulfenic (RSOH), sulfinic (RSO_2H), or sulfonic (RSO_3H) acids, which can result in protein inactivation. Now, S-glutathionylation is considered a real regulatory event in ''redox signaling'' and it is involved in several pathways, often cross-talking with each other (180). Chen *et al.* have shown in an elegant study that oxidized glutathione (GSSG) induces dose-dependent S-glutathionylation of endothelial nitric oxide synthase (eNOS) in human endothelial cells (EC), with loss of NO and gain of superoxide ($O_2^{\bullet -}$) generation, which is associated with impaired endothelium-dependent vasodilatation in the systemic circulation (23). In the lung, bleomycin induces pulmonary fibrosis and pulmonary vascular remodeling in animal models. Day *et al.* showed, more than 10 years ago, that bleomycin up-regulates GSH and γ-glutamylcysteine synthetase after only 3 h of treatment, indicating the early involvement of the system in the development of the pulmonary vascular remodeling (26). In pulmonary arterial myocytes, the reducing agents, such as GSH, DTT, and NADH, inhibit the large conductance calcium-dependent potassium channel (BKCa) activity; whereas oxidizing agents, such as 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB) and GSSG, stimulate it (55). Although the role of ATP-sensitive K^+ channel (K_{ATP}) in the PAs is almost unknown, it is worthwhile to introduce a recent study by Yang *et al.* showing that H_2O_2 or pyridine disulfides, pharmacological tools which react with protein cysteine residues similar to S-glutathionylation, markedly inhibit K_{ATP} current carried out by the potassium inwardly rectifying channel (Kir)6.1 subunit. Simulation modeling of Kir6.1 S-glutathionylation suggested that after incorporation to residue 176, the channel remains in its closed state (181). Finally, Lock *et al.* have utilized the thiol-oxidant diamide to investigate the consequences of protein S-glutathionylation on the calcium handling protein inositol trisphosphate receptor (IP_3R) in aortic EC. The authors found that Ca^{2+}-induced Ca^{2+}-release (CICR) *via* the IP_3R is enhanced by diamide, which reacts with GSH and, therefore, promotes P-SSG formation (78).

Thioredoxin

Thioredoxin (Trx), Trx peroxidase, and thioredoxin reductase (TrxR) comprise the thioredoxin system that exists in nearly all living cells (Fig. 2). The Trx system reduces oxidized cysteine groups on proteins through an interaction with the redox-active center of TRX (Cys-Gly-Pro-Cys) and forms a disulfide bond, which, in turn, can be reduced by Trx reductase and NADPH. It functions in thiol-dependent thiol-disulfide exchange reactions and activation of transcription factors (such as NF-κB and AP-1) that are crucial to control of the reduced intracellular redox environment, cellular growth, defense against oxidative stress, and control of apoptosis. In line with these observations, both Trx and TrxR are already regarded as interesting targets for chemotherapy, and they represent novel therapeutic options for pulmonary vascular and/or right heart remodeling.

Trx is ubiquitously expressed in EC (36) and in vascular smooth muscle cells (VSMC) of normal arteries (22). While treatment with H_2O_2 increases Trx expression in EC, suggesting that Trx is an ROS-inducible protein in EC, the expression of Trx in VSMC is not regulated by ROS. It appears that Trx acts to reduce cellular oxidative stress by scavenging ROS, and might, therefore, attenuate the pathology associated with vascular disease. Trx and TrxR are present in distinct isoforms that are predominantly cytosolic (Trx1 or TrxR1) or mitochondrial (Trx2 and TrxR2). While the Trx system has been extensively investigated in lung diseases and in epithelial cells, its role in pulmonary vascular cells and in right heart diseases is largely unknown. In the pulmonary vasculature, transient-receptor potential cation (TRPC) channels are widely expressed. Xu *et al.* have detected Trx as a novel type of ion channel agonist that acts through its reduced form to break a restraining intra-subunit disulfide bridge between cysteine residues in TRPC5, thereby stimulating the channel and resulting in an increased calcium influx, either as a homomeric assembly or as a hetermultimer with TRPC1. A transduction mechanism is, therefore, revealed that can directly couple ion channel activity to extracellular reduced thioredoxin (179).

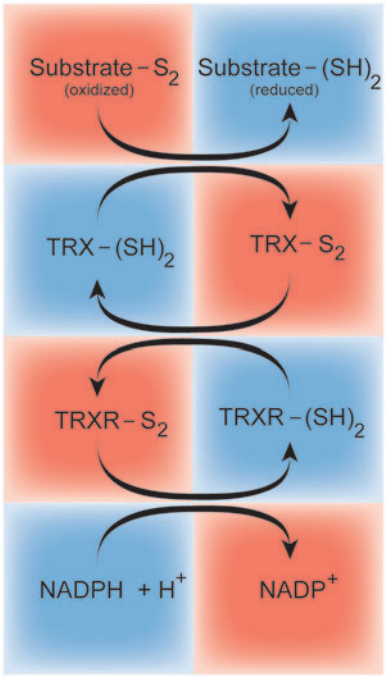

FIG. 2. The thioredoxin system. The TRX in the oxidized (TRX-S2) or reduced [TRX-$(SH)_2$] state. The oxidized thioredoxin is converted by the TRXR and NADPH to its reduced state, where TRX directly reduces disulfides in oxidized substrate proteins (Substrate-S_2). TRX, thioredoxin; TRXR, thioredoxin reductase. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

NAD(P)H/NAD(P)$^+$

The two specialized pyridine nucleotide systems (NAD^+/NADH and $NADP^+$/NADPH) are optimized for the ready exchange of electrons and for recognition by specialized nucleotide-binding domains located at the active sites of >200 different oxidoreductases (60). Moreover, these enzymes also can distinguish between reduced and oxidized states of the nucleotide and by specifically recognizing the phosphorylation state of the ribose ring, between NAD^+ and $NADP^+$. NADP(H) is recognized by enzymes that are

primarily involved in anabolic (reductive) reactions, such as lipid or cholesterol synthesis or fatty acid chain elongation; whereas NAD(H) is recognized by enzymes which catalyze catabolic reactions of glycolysis and by components of the electron transport chain (ETC) and, therefore, play an important role in (pulmonary) SMCs.

Due to the low glycolytic flux and the ability of mitochondria to oxidize cytosolic NADH by the malate-aspartate and glycerol phosphate shuttles, under normal aerobic conditions, cytosolic nicotinamide-adenine dinucleotides are mostly oxidized (*i.e.*, present as NAD^+) with an $NADH/NAD^+$ ratio <0.05 (16, 75). However, during ischemia, for example, the cytosolic $NADH/NAD^+$ ratio can increase approximately 30 times (112). The ratio of $NADH/NAD^+$ fluctuates in response to changes in metabolism. The key enzyme that regulates cytosolic $NADH/NAD^+$ levels is glyceraldehydes(s) 3-phosphate dehydrogenase (GAPDH) which is located in the sarcoplasmic reticulum (SR) membrane (178). Thus, changes in activity of this enzyme may result in more significant fluctuations of local $NADH/NAD^+$ levels in close proximity to SR-bound proteins (such as the ryanodine receptor [RyR] and sarcoplasmic/endoplasmic reticulum calcium ATPase [SERCA]) which might also explain the high compartmentalization of redox states within cytosolic subregions.

In PASMCs, changes in potassium conductance directly regulate membrane potential and thus vascular tone. Both KCa and the voltage-gated potassium channels (Kvs) are regulated by pyridine nucleotides (110, 113, 154, 157) (Fig. 3). The regulatory β-subunits of the Kv1.5 bind to pyridine nucleotides with a high affinity, enabling the Kvβ to respond to a wide range of metabolic conditions by being sensitive to changes in both NADP(H) and NAD(H) levels (77). Several observations indicate that $NAD(P)^+$ binding induces a specific conformational change that prevents Kvβ-induced inactivation, supporting K^+ conductance; whereas NADP(H) binding preserves or promotes inactivation (110, 111, 155–157, 175). In contrast, the Kv2.1 protein is not associated with a pyridine-binding subunit, such as Kvβ, and direct binding of pyridine nucleotide to Kv2.1 has not yet been demonstrated. Furthermore, recent work has shown that NAD^+ modifies the activity of the transient-receptor potential cation channel, subfamily M (TRPM), member 2 channels and, thus, regulates calcium homeostasis. Data from whole-cell patch-clamp experiments show that intracellular dialysis with NAD^+ evokes a large inward current in cells expressing TRPM2 channels. In HEK-293 cells, intracellular application of NAD^+ results in instantaneous activation of the channel, suggesting that NAD^+ directly activates the channel without the involvement of cytoplasmic or membrane components (63).

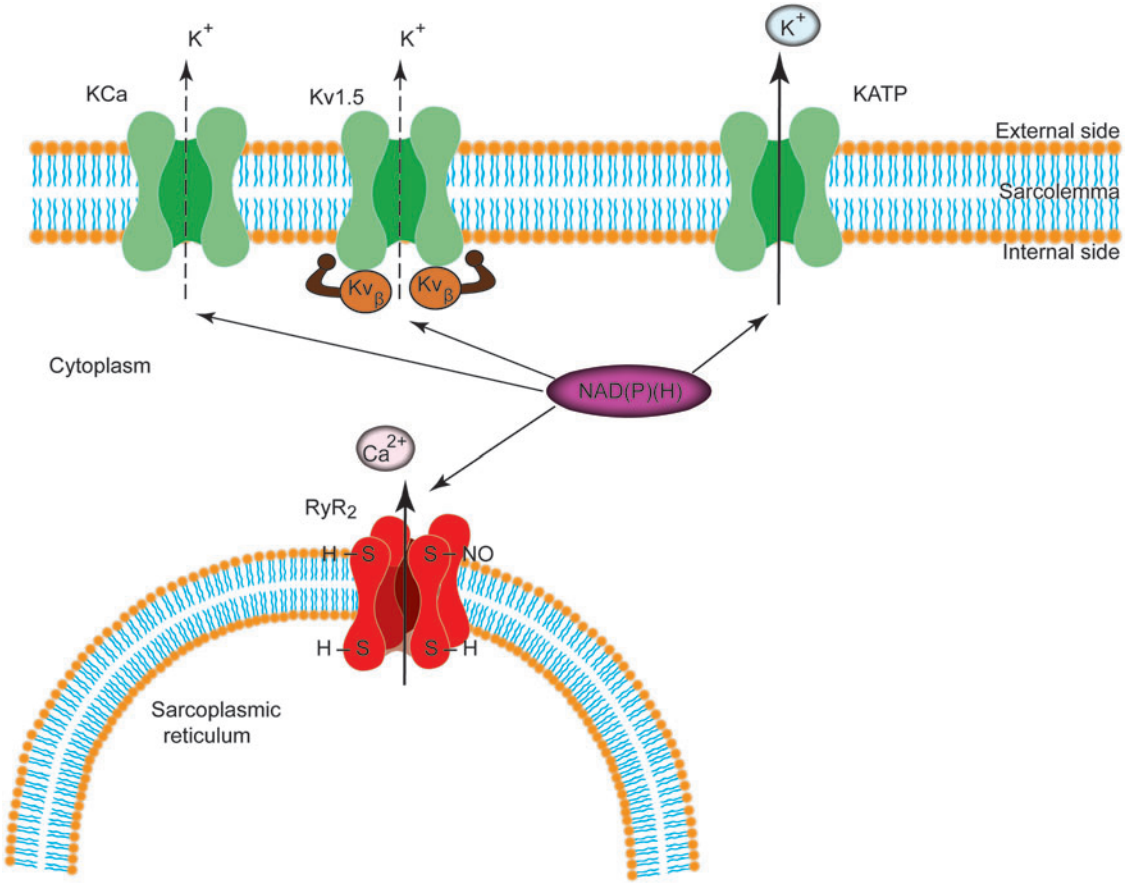

FIG. 3. Regulation of potassium channels and the RyR/Ca^{2+} release channel (RyR2) by pyridine nucleotides. Hypothetical scheme showing the regulation of plasma membrane and intracellular ion channels by NAD(P)(H). Individual cells may or may not express the entire complement of ion channels shown in the figure. RyR, ryanodine receptor. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

NO and S-nitrosylation

The radical gas NO diffuses freely from its site of production in the pulmonary EC to its target, soluble guanylate cyclase (sGC) in the smooth muscle, and is involved in a number of roles within the endothelium, regulating vascular tone, vascular growth, platelet aggregation, and modulation of inflammation (24). As such, decreased bioavailable NO results in endothelial dysfunction, which is a characteristic feature of many disease states.

NO is generated by the three isoforms of NO synthase (NOS): eNOS, neuronal (nNOS), and inducible (iNOS) isoenzyme. The classical NO signaling pathway is the activation of sGC enhancing cyclic guanosine monophosphate (cGMP) production, which, in turn, mediates vasodilatation. The alternative NO signaling pathway is S-nitrosylation (SNO; see next paragraph). NO has a modest role as a vasodilator in the pulmonary circulation under most basal physiological conditions. However, endogenous NO seems to suppress the full expression of HPV (8, 69, 70). In addition, since NO inhibits proliferation of VSMCs, it may also function to prevent the remodeling associated with the development of PH (142). Fagan *et al.* evaluated the importance of each NOS isoform in the maintenance of pulmonary vasomotor tone using isolated-perfused lungs from mice deficient in each NOS isoform. The investigators reported that under basal conditions, nNOS and iNOS have little or no role in the maintenance of low pulmonary tone in contrast to eNOS (32, 33). In addition, eNOS activity was an important determinant of pulmonary vascular responsiveness to chronic hypoxia, *in vivo* (32, 33, 143). The effect of eNOS-derived NO appeared to be most prominent under modest hypoxic stress when HPV was augmented in $eNOS^{-/-}$ mice (32, 33). The latter finding is, however, controversial (121, 144). Finally, a recent elegant study by Seimetz *et al.* highlighted the relevance of iNOS activity in bone marrow-derived cells for the development of PH *in vivo* (135). The reaction of NO with sGC results in a several 100-fold increase in production of cGMP from guanosintriphosphat (GTP). In VSMCs, the effects of cGMP are mediated through activation of its effector proteins, the cGMP-dependent protein kinase, cGMP-gated ion channels, such as calcium-activated potassium channels, and cGMP-regulated phosphodiesterases.

Recent investigations show that many effects of NO in the vasculature are mediated by S-nitrosylation, the covalent modification of a protein cysteine thiol by an NO group to generate an *S*-nitrosothiol as recently reviewed in detail (48, 74). The composition of SNO-based signaling complexes also includes NOSs; NO group donors, including *S*-nitrosoglutathione (GSNO) and other SNO-proteins that can participate in transnitrosylation reactions; and denitrosylases which curtail the signals. Ion channels and transporters participating in calcium homeostasis have been reported to be modulated by S-nitrosylation. Among Kvs, a recent report has shown that S-nitrosylation of the potassium voltage-gated channel, KQT-like subfamily, member 1 (KCNQ1) subunit facilitates the slowly activating component of the delayed rectifier K^+ current (10); whereas S-nitrosylation exerts an inhibitory influence on Kv4.3 and thus the transient outward potassium current (39). In addition, Nunez *et al.* have shown that NO inhibits hKv1.5 channels by a cGMP-dependent mechanism and by the direct S-nitrosylation of the protein (100). Sun *et al.* as well as Poteser *et al.* have reported that S-nitrosylation of the α1C subunit of the L-type calcium channel inhibits the L-type calcium current, perhaps due to eNOS-induced NO production, which is adjacent to the calcium channels in the sarcolemmal membrane caveolae (120, 147). Finally, in cardiomyocytes, the RyR/Ca^{2+} release channel (RyR2) has been shown to be activated by S-nitrosylation (40, 148) (Fig. 4). The emergence of SNOs as second messengers and of S-nitrosylation as the preeminent NO-based signal may represent a new era in pulmonary vascular physiology, as a disruption of the SNO/redox balance in myocytes, for example, in pulmonary vascular remodeling can occur. Consequently, the restoration of this equilibrium might provide a fruitful approach to restoring pulmonary vascular and/or right ventricular performance.

Reactive oxygen species

The cellular mechanisms responsible for HPV have been debated since the description of this physiological mechanism by Euler and Liljestrand (30). Since the 1970s, there has been a keen interest to identify specific ROS altered during hypoxia and their generating source(s). Thus, in the pulmonary circulation, Noxs and mitochondria have been identified as the major sources producing ROS and initiating ROS signaling. They seem to be interdependent, at least in acute HPV. However, there is still a major controversy as to whether ROS go up or down during hypoxia. Such a discrepancy is likely related to differences in preparations and detection methods (each of which has its own severe, specific limitations) as summarized in Table 1 [modified from Olschewski and Weir (104)]. Since the changes in ROS in the pulmonary circulation are not the focus of this review, we would like to highlight recent reviews dealing extensively with the topic (62, 96, 132, 150).

A number of publications suggest that the hypoxic increase or decrease in intracellular ROS concentration can affect the activity of ion channels, resulting in a large increase in intracellular Ca^{2+} concentration in PASMCs (7, 80, 81, 94, 128). Although most of these studies applied the measurement of the global intracellular calcium concentration as one of the major read-outs, unfortunately, there are a few studies specifically targeting Nox (homologues) or mitochondria under acute or chronic hypoxia (or hyperoxia) in the pulmonary circulation in order to investigate the direct interaction between the ion channels and ROS. One of the potential mechanisms by which ROS are able to regulate pulmonary vascular tone involving ion channels is *via* the control of the cellular redox potential. According to the redox theory, acute hypoxia decreases the release of a mitochondrial-derived ROS (hydrogen peroxide) and/or increases the NAD(P)H/NAD(P) ratio, resulting in contraction through the reduction of thiol groups of the channel and causing PASMC membrane depolarization as a result of closure of cell membrane potassium channels (4, 9, 95). Although *in vitro* experiments almost universally demonstrate an elevation in intracellular calcium concentration during hypoxia and similar mechanisms as for potassium channels indicated earlier might be also true for calcium channels, alternative pathways of HPV focus mainly on the source of ROS or on second messengers (cyclic adenosine diphosphate [cADP]-ribose, diacylglycerol [DAG] or calmodulin). Thus, our knowledge about Nox or mitochondria-produced ROS-driven direct regulation of calcium-handling proteins expressed in the pulmonary circulation is still very limited (136–138, 141).

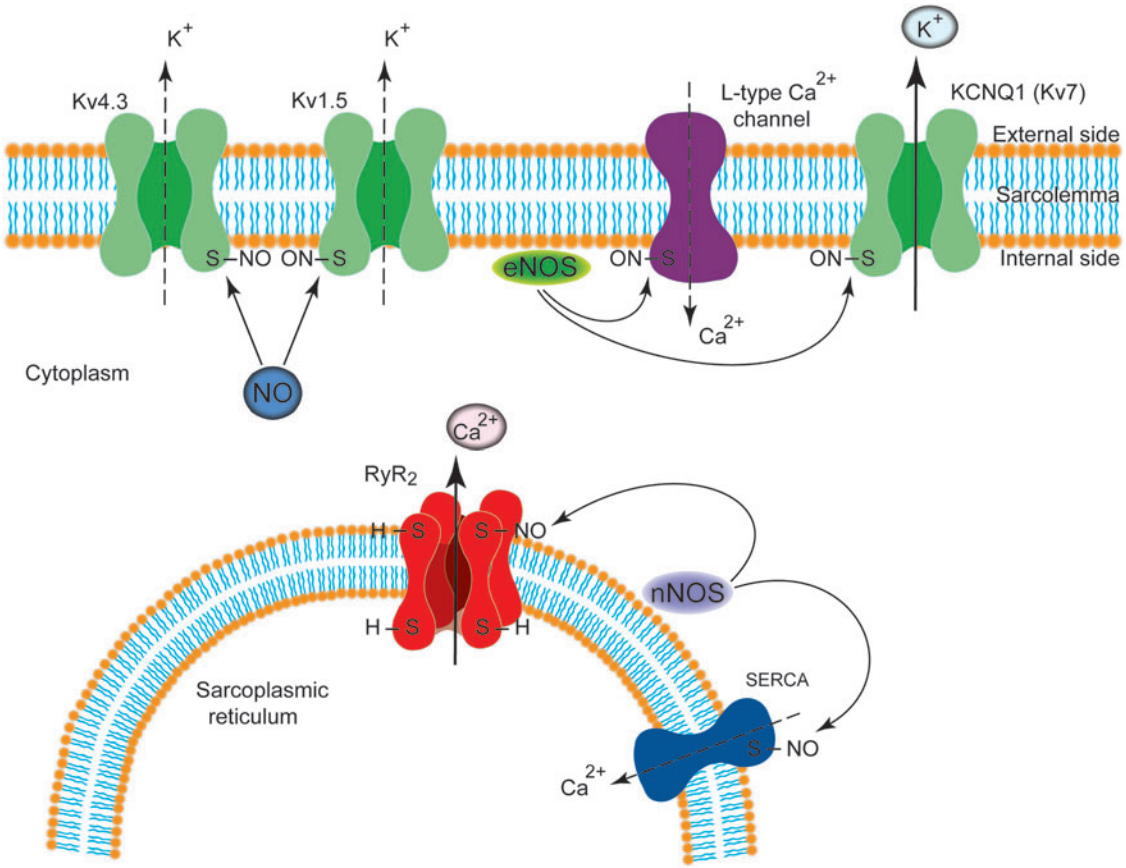

FIG. 4. S-nitrosylation of ion channels in myocytes. S-nitrosylation modulates function of different ion channels expressed either in the cell membrane (sarcolemma) or in the sarcoplasmic reticulum of myocytes. *Arrows* indicate activation, whereas *dotted line* indicates inhibition. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

NADPH oxidase. Many studies have investigated the role of the Nox in the pulmonary circulation during more than the last 20 years; however, only recently reports link their findings to Nox subtypes. The Nox family consists of seven catalytic homologues, four of which (Nox1, Nox2, Nox4, and Nox5) are found in the vasculature. These enzymes transfer electrons from NADPH to molecular oxygen, thus producing superoxide. The enzyme complex in phagocytes comprises at least five components: two cytosolic subunits p47phox and p67phox, a cell membrane-bound cytochrome b558, which consists of gp91phox (renamed Nox2) and p22phox, and a small G protein Rac. Under physiological conditions, Nox proteins (Nox1, Nox2, Nox4, and Nox5) and their products superoxide and hydrogen peroxide participate in cardiovascular homeostasis by contributing to cell differentiation, repair of damaged tissue, and regulation of vascular tone and, thus, blood pressure (68).

Acute HPV. The TWIK-related acid-sensitive potassium channel 1 (TASK-1), which is commonly expressed in oxygen-sensing cells and conveys a background K^+ current, is inactivated by hypoxia. However, TASK-1 *per se* cannot sense oxygen and may require a regulatory protein that can do so. Lee *et al.* proposed that Nox4 functions as an oxygen-sensing partner and that it modulates the oxygen sensitivity of TASK-1. In their study, the oxygen sensitivity of TASK-1 was abolished by Nox4 siRNA and Nox inhibitors, suggesting a novel function for Nox4 in the oxygen-dependent regulation of TASK-1 activity (71).

Chronic hypoxic PH. The chronic effects of ROS on ion channels are mediated *via* transcription factors. In a very recent study, Mittal *et al.* provided evidence for the regulation of the Kv1.5 channels by Nox4-derived ROS and for the development of pulmonary vascular remodeling, showing that sustained hypoxia decreases Kv channel currents by a direct effect of an Nox4-derived increase in ROS (92). Support for their findings was provided by Sturrock *et al.*, reporting the cross-talk between transforming growth factor (TGF)-β1 and Nox4, and thus implications for mechanisms of pulmonary vascular remodeling. They have suggested that TGF-β1 may facilitate proliferation by up-regulating Nox4 and ROS production, with transient oxidative inactivation of phosphatases and augmentation of growth signaling cascades (145). There is accumulating evidence that the Nox enzymes, and in particular Nox4, play an important role in chronic responses of the pulmonary vasculature to changes in oxygen tension. These exciting findings may facilitate our understanding of the pathophysiology of PH secondary to chronic hypoxia. However, elucidation of the role of redox-regulated ion channels in chronic hypoxia-driven pulmonary vascular remodeling needs further combined efforts.

Table 1. Changes in the Reactive Oxygen Species Production Under Hypoxia in Different Preparations

Tissue/preparation	*Hypoxia*	*$[ROS]_i$*	*Technique of detection*	*References*
Isolated rabbit lung	Minutes	↓	Lucigenin	Paky *et al.* (108)
Isolated rat lungs, pulmonary artery ring, PASMCs	Minutes	↓	Lucigenin, amplex red, dihydroethidium, H_2DCF	Archer *et al.* (6)
Isolated rabbit lung	Minutes	↓	Lucigenin	Weissmann *et al.* (174)
Calf pulmonary arteries	Minutes	↓	Lucigenin	Mohazzab and Wolin (93)
Rat pulmonary artery	Minutes	↓	Lucigenin, amplex red, H_2DCF	Michelakis *et al.* (89)
Rat pulmonary artery	Weeks	↓	L-012 (luminol analogue) amplex red	Bonnet *et al.* (19)
Human PASMCs	Hours	↓	Lucigenin, amplexred, dihydroethidium, H_2DCF	Mehta *et al.* (88)
Human PASMCs	Minutes	↓	Dihydroethidium	Wu *et al.* (176)
Isolated mouse lung	Minutes	↑	Electron spin resonance	Weissmann *et al.* (173)
Rat pulmonary arteries	Minutes Weeks	↑ ↑	H_2DCF	Jernigan *et al.* (56, 57)
Rat pulmonary arteries	Hours	↑	Dihydroethidium	Wang *et al.* (162)
Mouse pulmonary arteries, rabbit PASMCs	Minutes	↑	H_2DCF	Paddenberg *et al.* (107)
Porcine pulmonary arteries	Minutes Weeks	↑ ↑	Electron paramagnetic resonance, H_2DCF, lucigenin	Liu *et al.* (76)
Calf PASMCs	Minutes	↑	Lucigenin	Marshall *et al.* (85)
Rat PASMCs	Minutes	↑	H_2DCF	Killilea *et al.* (61)
Rat PASMCs	Minutes	↑	Fluorescence resonance energy transfer probe, H_2DCF	Waypa *et al.* (165, 166)
Mouse PASMCs	Minutes	↑	Cytochrome *c* reduction assay, H_2DCF	Rathore *et al.* (122, 123)
Mouse PASMCs	Minutes	↑	H_2DCF, lucigenin, Redox Sensor Red CC-1	Wang *et al.* (161)
Human PASMCs	Hours	↑	Dihydroethidium	Wu *et al.* (176)

Modified from Olschewski and Weir (104).
H_2DCF, dichlorodihydrofluorescein; PASMC, pulmonary artery smooth muscle cell; ROS, reactive oxygen species.

Mitochondria. The mitochondria play an important role in normal vascular physiology, for HPV and potentially for the development of PH, which has been extensively reviewed during recent years (2, 28, 115, 138, 140, 164, 167, 168). In the mitochondria, ROS are produced by the ETC at complexes I, II, and III. The non-charged H_2O_2 diffuses through the outer membrane to access the cytosol, superoxide reaches the cytosol through the trans-plasma-membrane electron transport protein, the voltage-dependent anion channels. During the last decade, large numbers of studies on mitochondria have been carried out, focusing on the role of the ETC as an oxygen sensor for hypoxia and HPV. Investigations providing evidence for the direct regulation of calcium-handling proteins and particularly of K^+ channels by the ETC, however, are limited. In addition, the identification of the main site for ROS production within the mitochondria is still a matter of debate.

Acute hypoxia-induced changes initiated by ROS production by mitochondria. In PASMCs and in SMCs from systemic mesenteric artery (MA), Firth *et al.* investigated differences in functional coupling between mitochondria and K^+ channels (35). They found that mitochondria are located significantly closer to the plasmalemmal membrane in PASMCs compared with MASMCs. Consistent with these findings, the effects of mitochondrial inhibitors on K^+ current were significantly more potent in PASMCs than in MASMCs, suggesting a greater structural and functional coupling between mitochondria and K^+ channels in PASMCs. In SMCs from human DA, ETC inhibitors (rotenone and antimycin) mimicked hypoxia, increasing K^+ current and reversing constriction to oxygen, as shown by Michelakis *et al.* (90). They concluded that normoxia controls human DA tone by modulating the function of the mitochondrial ETC, thereby varying mitochondrial membrane potential and the production of H_2O_2, which regulates DASMC K^+ channel activity and DA tone.

Effects of chronic hypoxia. In human PASMC, chronic hypoxia activates mitochondrial K_{ATP} channels, resulting in depolarization of mitochondrial membrane potential, increase of cytochrome C accumulation, and stimulation of mitochondrial H_2O_2 overproduction (54). However, in Fawn Hooded rats, Bonnet *et al.* have shown that mitochondrial dysfunction results in hyperpolarized mitochondria due to deficiency in components of several electron transport complexes, particularly complex I and SOD2. The net result of these abnormalities was reduced to total ROS production mimicking chronic hypoxia and causing normoxic activation of hypoxia-inducible factor (HIF)-1α, which then inhibited expression of oxygen-sensitive, voltage-gated Kv1.5, postulating a novel pathway of Kv channel regulation by mitochondrial membrane potential changes (19).

Trans-plasma membrane electron transport. The trans-plasma membrane electron transport (t-PMET) has been established with the discovery of the transmembraneous NADPH dehydrogenase (25, 42, 59). T-PMET enables

reduction of extracellular oxidants at the expense of intracellular reducing equivalents. Thus, the cell can respond to changes in the redox microenvironment, regulating a variety of biological functions, including proton pumping and cell metabolism, cell growth, or even activity of ion channels (27). The t-PMET is a balanced interplay of several components that have been identified during the last two decades. Some are ubiquitously expressed, and others are only present in certain cell types. Some utilize only a subset of electron donors or acceptors, whereas others are less specific (83). The key components of t-PMET are intracellular electron donors [*e.g.*, NAD(P)H] and either enzyme-mediated and/or shuttle-based electron transfer such as NAD(P):quinone oxidoreductase 1 (NQO1), disulfide-thiol exchanger (ENOX), members of the NOX family, and voltage-gated anionic channels also called porins or Coenzyme Q (CoQ); the last acts as a mobile electron shuttle (Fig. 5). Different strategies can be applied to identify the components that are responsible for the electron transport and to measure their activity, including measuring oxygen consumption, monitoring the disappearance of the electron donor (NAD(P)H), measuring the reduction of artificial electron acceptors, determining the amount of superoxide formation, and measuring ubiquinone reduction or hydroquinone oxidation (27).

A role for the t-PMET system and in particular the central function of Nox-driven superoxide in cardiovascular diseases, including PH and right heart failure, is very likely as discussed in detail earlier (see NADPH Oxidase section). However, most of the studies about the role of the other components have been carried out with a focus on the systemic circulation. The NQO1 in the lung has been investigated in several studies (11, 15, 16). Recently, Bongard *et al.* have shown that alterations in CoQ1 redox metabolism, either by addition of the complex I inhibitor rotenone or exposure of the animal to hyperoxia, can reveal a decrease in mitochondrial complex I activity (18). In addition, by using bovine pulmonary arterial EC, they have demonstrated that the cytoplasmic redox status, as reflected in the NADH/NAD^+ and NADPH/$NADP^+$ ratios, affects t-PMET reduction of thiazine compounds and NQO1-mediated quinone reduction (16). Ion channels in the pulmonary circulation are also affected by t-PMET. Our group described the effect of CoQ10 and duroquinone on K^+ channels in PASMCs and showed that both inhibited the whole-cell K^+ channel current, resulting in depolarization of the SMC membrane (126). Thus, CoQ10 and duroquinone, similar to hypoxia or ETC inhibition, shift the K^+ channels to the more reduced state, resulting in the closing of the channels. On balance, cellular metabolism generates excess reducing equivalents that the t-PMET system exports to oxygen. Hypothetically, hypoxia may result in a greater concentration of reducing equivalents at the cell membrane, where they can modulate the activity of the ion channels.

Redox-Sensitive Enzymes

Hydrogen peroxide and superoxide can alter the function of proteins by oxidizing aromatic residues such as histidine, tyrosine, tryptophan, and phenylalanine; sulfur-containing amino acids such as cysteine and methionine or by resulting in carbonylation of catalytic active sites containing lysine, arginine, proline, or threonine; or the racemization of lysine, arginine ,or aspartate. Thus, ROS fine tune the duration and amplification of the phosphorylation signal. The oxidation of cysteine has been the focus of the majority of redox-signaling investigations as a result of the widely acknowledged role of this redox reaction in mediating intracellular signal transduction due to forming a disulfide bond (S-S) (29, 67). From a signaling perspective, it is the reversible cysteine modification that is most relevant, because hyperoxidation tends to occur predominantly during conditions of oxidative stress, promoting enzyme inactivation and degradation [for detailed reviews, see Refs. (62, 124)].

Receptor and non-receptor TK

The level of protein tyrosine phosphorylation is the function of opposing actions of protein tyrosine kinases and protein tyrosine phosphatases (PTPs). All PTPs contain a catalytic cysteine residue in the active site, and oxidation of this residue results in the inactivation of the PTP activity (158), which is recognized as a major mechanism by which ROS regulate the level of protein tyrosine phosphorylation. However, a number

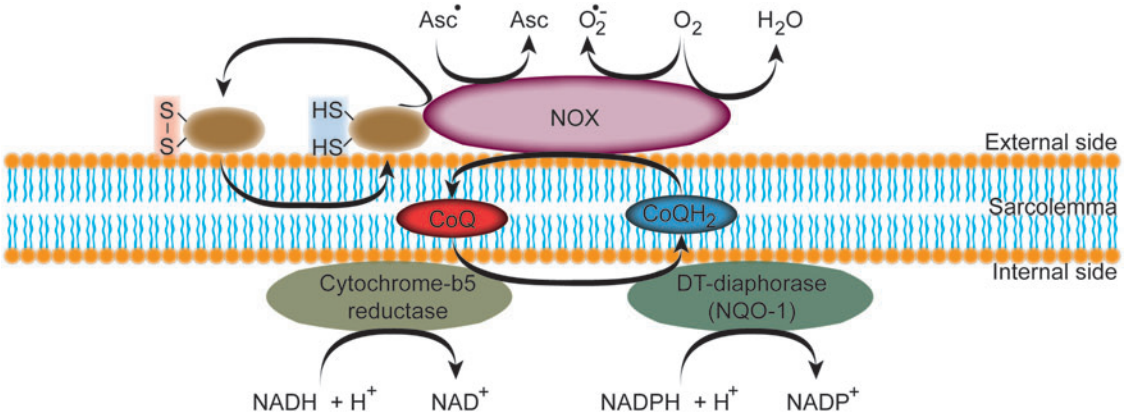

FIG. 5. Schematic representation of the t-PMET. At the cytosolic side of the plasma membrane, a quinone reductase is present that reduces CoQ to ubiquinol ($CoQH_2$). $CoQH_2$ shuttles the electrons to the NOX that is able to reduce extracellular ascorbyl radicals ($Asc^•$) produce superoxide ($O_2^{•-}$), reduce O_2 to water, and reduce protein disulfides. CoQ, Coenzyme Q; NOX, NADPH oxidase; t-PMET, trans-plasma membrane electron transport. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

of studies suggest that the proto-oncogene tyrosine-protein kinase Src (src as a short for sarcoma) also plays an important role in the cellular response to ROS, because Src specific inhibitors and dominant negative Src mutants strongly attenuate the cellular response to ROS (87).

Receptor tyrosine kinases (RTKs) such as the insulin receptor, epidermal growth factor receptor, platelet-derived growth factor (PDGF) receptor, and the protein kinase receptor c-ret have been reported to undergo direct oxidation (98). We have recently proposed that hypoxia and PDGF-BB upregulate protease-activated receptor-2 (PAR-2) expression in PASMC (66). This effect was reversed by HIF-1α depletion or the RTK inhibitor Imatinib. The PAR-2 expression observed in SMCs of pulmonary vessels of mice exposed to hypoxia was attenuated by Imatinib treatment, strengthening the link between hypoxia and RTK in PASMCs. Although there are increasing numbers of clinical studies investigating the role of RTK inhibition for the treatment of PH (38, 153), experimental evidence for the redox regulation of RTK in the pulmonary circulation is largely missing.

The non-RTK Src contains regulatory structures such as a myristoylation motif, a unique region, an Src homology (SH)3 domain, an SH2 domain, and a regulatory tail in addition to the catalytic domain. Src activity is regulated directly by reversible phosphorylation on multiple tyrosine (tyr) and serine (ser) residues, which are also targets for redox control. We recently reported that Src controls K^+ channel function in human PASMCs in response to changes in oxygen tension (Fig. 6) (97a). We showed that moderate hypoxia decreased the level of phospho-SrcTK (at tyr419) and reduced the co-localisation of the TASK-1 channel and phospho-SrcTK, resulting in closure of the channel. Our results indicate that the modulation of SrcTK is a crucial factor controlling K^+ channels, acting under normoxic conditions

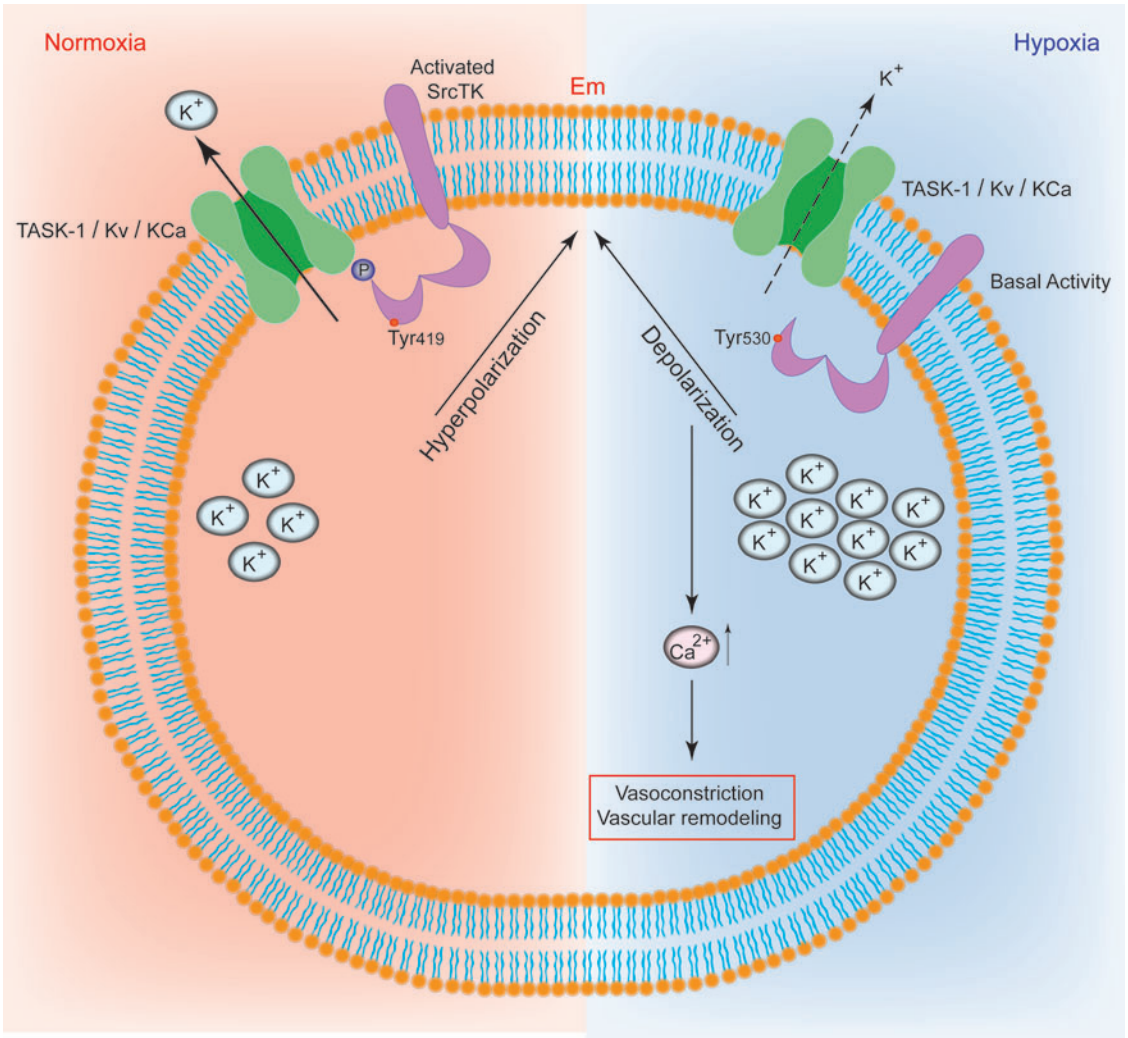

FIG. 6. Schematic representation of the t-PMET. Scheme of the proposed interplay between potassium channels and c-Src in human PASMCs. Under normoxia, the phospho-Src (active-Src, phosphorylated at Tyr419) binds to TASK-1 channels, resulting in functional TASK-1 channels. Active TASK-1 channels maintain negative resting potential in hPASMCs. In hypoxia, the phospho-Src (active-Src) is decreased. Closed potassium channels result in depolarization and increased intracellular calcium levels. (+) Indicate increase and (−) indicate decrease. Em, resting membrane potential; KCa, calcium-dependent potassium channel; Kv, voltage-gated potassium channel; PASMC, pulmonary artery smooth muscle cells; TASK-1, TWIK-related acid-sensitive potassium channel-1. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

as a cofactor to set a negative resting membrane potential in human PASMCs and low resting pulmonary vascular tone.

Redox Modulation of Ion Channels and Transporters in the Pulmonary Circulation

Redox modulation of ion channel activity seems to be an important regulatory mechanism under physiological conditions for several vasomotor functions, including not only regulation of cell membrane potential and vascular tone but also activation of transcription factors required for the expression of genes, apoptosis and necrosis, and cellular protective mechanisms against ischemic or hypoxic insults. Acute contraction of PASMCs is activated, in part, by a potassium (K^+) inhibition-induced membrane depolarization and subsequent Ca^{2+} entry through nifedipine-sensitive L-type Ca^{2+} channels (Fig. 7). The global Ca^{2+} release is the result of different localized elementary Ca^{2+} release events, termed receptor- and store-operated Ca^{2+} influx pathways (64). Extrusion of Ca^{2+} through the plasma membrane is mediated by the plasma membrane Ca^{2+}-Mg^{2+} ATPase, or Ca^{2+} pump, and the Na^+/Ca^{2+} exchanger (NCX). In addition, the inward transportation of Ca^{2+} through the reverse model of NCX is important for maintaining a high cytosolic Ca^{2+} concentration and refilling Ca^{2+} into the SR/ER *via* SERCA, when cytosolic Na^+ concentration is increased locally by increased Na^+ influx through transient-receptor potential (TRP) channels (72).

Potassium (K^+) channels

Different types of K^+ channels are sensitive to redox modulation. In the endothelial (porcine aortic endothelial cell [PAEC]) and in the PASMCs, the K^+ current is an ensemble, reflecting the activity of many different channels. At least four classes of K^+ channels have been identified in the cells of the wall of the PAs: voltage-gated K^+ channels (Kv) (31, 117, 183), calcium-dependent K^+ channels (KCa) (1, 114),

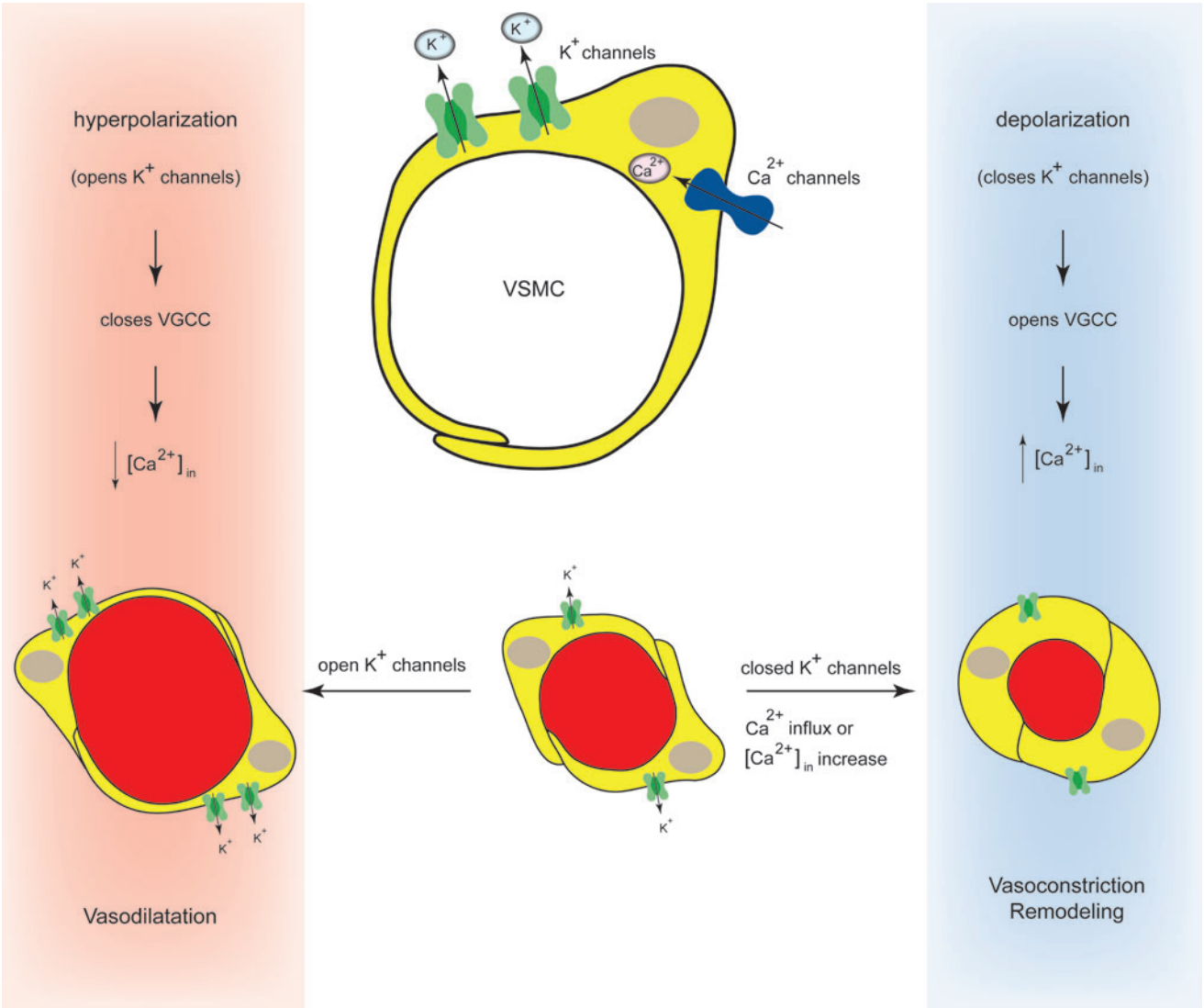

FIG. 7. Regulation of pulmonary vascular tone by potassium channels. Acute contraction of PASMCs is activated, in part, by a potassium (K^+) inhibition-induced membrane depolarization and subsequent Ca^{2+} entry through nifedipine-sensitive L-type Ca^{2+} channels. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

K_{ATP}s (99), and the more recently identified family of two-pore domain K^+ (K_{2P}) channels (103). Different types of Kv channels have been shown to be expressed in the pulmonary vasculature (5, 7, 183). At the molecular level, Kv channels are homo- or heteromultimeric tetramers that are composed of two structurally distinct subunits: the pore-forming α-subunits and the regulatory β-subunits (52). Nine families of Kv channels α-subunits are recognized from cloning studies (Kv1–9) (52, 116), each with subtypes (*e.g.*, Kv1.1–1.6). To date, 12 different Kv channel proteins have been described. We have extensively reviewed the response of the K^+ channels in PASMCs to acute and chronic hypoxia (171) and also described in our earlier work the graded response of the K^+ current to hypoxia (101).

A recent elegant report by Svoboda *et al.* shows that oxidative stress using the organic hydroperoxide *tertiary* butyl hydroperoxide (*t*BOOH) can directly result in internalization and protein degradation of Kv1.5 and that sulfenic acid modification of COOH-terminal C581 alone is sufficient to trigger these events (149). Thus, sulfenic acid modification to proteins can regulate Kv1.5 channel surface expression and stability under oxidative stress and divert channels from a recycling pathway to degradation. Redox modulation of Kv1.5 current is associated with numerous pathophysiological states that involve loss of channel protein, including PH (2). Therefore, this report might provide a novel molecular mechanism linking oxidative stress and down-regulation of channel expression observed in cardiovascular diseases. Earlier, we have reported that the same redox signal can elicit opposite effects on K^+ current, resting membrane potential, cytosolic calcium, and vascular tone in resistance PASMCs and the SMCs obtained from the DA Botalli (102). These observations support the concept that redox changes could signal the opposite effects of oxygen in the PA and DA. The thiol oxidant diamide inhibits HPV (172). Schach *et al.* have shown that this pulmonary vasodilation is markedly inhibited when the transmembrane K^+ driving force is reduced or vessels are constricted with high potassium, indicating that the thiol oxidation induces pulmonary vasodilation by opening K^+ channels (131). The role of the Nox for the ROS-induced changes in Kv current has been recently studied by Mittal *et al.* They have shown that Kv1.5 and NOX4 are colocalized in isolated PASMC. Furthermore, the Nox inhibitor and ROS scavenger apocynin as well as NOX4 siRNA reversed the hypoxia-induced decrease in Kv current density, whereas the protein levels of the channels remain unaffected by siNOX4 treatment. Determination of cysteine oxidation revealed increased NOX4-mediated Kv1.5 channel oxidation. Their observation suggests that the decreased Kv current was a direct effect of an NOX4-derived increase in ROS (92). This finding complements the report that a normoxic increase in endogenous H_2O_2 in SMCs of the DA inhibits K^+ channels and causes constriction (125). However, the similar observations in PA and DA are contrary to the opposite redox effects with diamide and other redox agents described earlier.

Oxidative modification of Kv channels has been extensively investigated by Sahoo *et al.* (130). They identified four critical cysteine residues and not only revealed the major target sites of oxidative modification in potassium voltage-gated channel subfamily H member 1 (KCNH1), also known as Kv10.1, but also provided new insights into the dynamic interplay of functional channel domains under redox modulation. The study provides solid evidence that the cysteine residues C145/C214 in the N-linker are responsible for fast modification of the ion channels, and the C532/C562 in the C-linker are responsible for slow modification of the ion channels. The authors hypothesize that in physiological levels of ROS, mild oxidative stress may act through a positive feedback by reducing Kv channel activity. Another recent experimental study has identified oxidative stress as a key contributor to Kv4 channel up-regulation due to chronic impairment of the Trx system (152). The results of Tang *et al.* suggest that expression of cardiac Kv channels is redox regulated. They have found that in myocytes from the ventricle after myocardial infarction, TrxR is markedly suppressed. This chronic impairment of the Trx system contributed to Kv4 up-regulation through sustained activation of ASK1-JNK-p38 signaling in a TrxR-dependent manner thus highlighting the Trx system as a novel therapeutic target.

Modification of Kv channels by pyridine nucleotides has been extensively reviewed recently by Kilfoil *et al.* (60). According to earlier reports, the Kv1 and Kv4 channels seem to interact with pyridine nucleotide-binding β-subunits. Binding of $NADP^+$ to Kvβ subunit removes N-type inactivation of Kv currents, whereas NADPH stabilizes channel inactivation. PAs sense and respond to environmental changes such as hypoxia. Therefore, linking K^+ transport to the metabolic state of the cell is important. In small resistance arteries, oxygen-sensitive changes in K^+ channel activity mediate HPV and decreased Kv1.5 activity may result in PH (84). It has been recently reported that *in vitro* the C-terminal peptide of Kv1.5 interacts avidly with NADPH (155). Thus, the pyridine nucleotide-binding Kvβ proteins with their ability to regulate Kv current could, in principle, impart oxygen sensitivity to Kv1.5 channels. However, the findings of the studies carried out in different species and in different cell systems are contradictory. Although the differences might be explained by differences in Kvβ expression, further work is required to determine the role of the pyridine regulation of Kv channels in HPV.

The resting potential depends on a background K^+ current comprising voltage-dependent and -independent components (46, 106) in PASMC. TASK-1 channels, described by Gurney *et al.* and also by our group (47, 103), appear to mediate the latter (46, 47). The biophysical and pharmacological properties of the voltage-dependent component were noted to bear a striking resemblance to the neuronal M-current (31). The channels responsible for the M-current are encoded by genes of the KCNQ (Kv7) family (127, 184). Along with the finding that KCNQ channel blockers are potent pulmonary vasoconstrictors (57a), the similarity of IKN to the M-current led the authors to consider the possibility that KCNQ channels mediate the voltage-dependent component of IKN (58). A triple cysteine pocket within neuronal M-channel subunits (Kv7.2–7.5) can be oxidatively modified by H_2O_2 (37, 105) and $O_2^{\bullet -}$ (75a), both of which cause augmentation of M-current. Here, they identified a reactive redox module within M-channels that is responsible for dynamic M-current modulation. Furthermore, emerging evidence demonstrates that protein S-nitrosylation is an important NO-mediated regulatory mechanism of various classes of proteins, including ion channels (146, 159). In a recent study of Asada *et al.*, the authors demonstrated that an NO donor induces S-

nitrosylation at Cys445 in the C terminus of the pore-forming α-subunit KCNQ1. They provide convincing evidence which shows that the redox motif flanking Cys445 is required for the site-specific S-nitrosylation of Cys445. S-nitrosylation at Cys445 of the KCNQ1 channel functionally regulates the KCNQ1/potassium voltage-gated channel subfamily E member 1 (KCNE1) complex channel. Since KCNQ1 contributes to the slowly activating delayed rectifier potassium channel current, this work may provide a molecular basis of NO-mediated phenomena in smooth muscle electrophysiology (10).

The large conductance Ca^{2+}-dependent potassium channel (BKCa) plays a key role in the oxygen-sensing function of the carotid body chemoreceptors. Li *et al.* have recently reported that the H_2S donor, NaHS, plays a crucial role in mediating the response of carotid body chemoreceptors to hypoxia *via* modulating the BKCa channels (73). In these cells, the BKCa currents were inhibited by hypoxia and such inhibition was mimicked by NaHS and diminished by inhibitors of cystathionine beta-synthetase (CBS), which produces H_2S from L-cysteine. Finally, mice hyperventilated in response to hypoxia, which was prevented by the CBS inhibitors amino oxyacetic acid (AOAA) and hydroxylamine. Almost 20 years ago, in PASMC and SMCs from systemic arteries, such as ear arterial smooth muscle cells (EASMC), BKCa have been reported to be redox sensitive (113). Reducing agents (DDT, GSH, and NADH) decreased PASMC, but not EASMC, KCa channel activity. However, oxidizing agents (DTNB, GSSG, and NAD) increased KCa channel activity in both PASMC and EASMC. The increased activity due to oxidizing agents was diminished by applying reducing agents. From these results, the authors suggest that the basal redox state of the PASMC KCa channel is more oxidized than that of the EASMC channel, as the response of KCa channels of the PASMC to intracellular reducing agents differs from that of the EASMC. In contrast, in isolated SMC from large conduit PAs, the KCa channel activity was unaffected by NAD and GSSG, or NADH and GSH (154). This suggests that the change in the intracellular redox state, which occurs during acute hypoxia, does not alter the activity of KCa channels in SMC from large conduit PAs. The different behavior of these channels in response to redox changes could be explained by the heterogeneity of the KCa in different tissues and may be related to the different responses of PASMC and EASMC KCa channels to hypoxia Archer *et al.* (5). Furthermore, redox-dependent modulation of activity of $Na^+ - K^+$ ATPase (65) and $Na^+ - H^+$ (177) exchange has been reported.

Calcium handling proteins

In the pulmonary circulation, the vascular tone and blood vessel diameter is determined to be due to the regulation of cytosolic/intracellular Ca^{2+}, mainly in the PASMCs of the resistance arteries. The steep ($\sim$10,000-fold) concentration gradient between the intracellular and the extracellular Ca^{2+} concentration already indicates the importance of constant and tight control of cellular Ca^{2+} homeostasis due to a complex machinery of (i) Ca^{2+}-permeable channels, enabling Ca^{2+} to flux through the membrane based on its electrochemical gradient; (ii) the Ca^{2+} pumps (*e.g.*, Ca^{2+}/Mg^{2+} ATPase or Ca^{2+} pump) transporting Ca^{2+} against its concentration gradient; and (iii) the Ca^{2+} exchangers (*e.g.*, NCX). The calcium-selective ion channels in the PASMC and EC include (i) voltage-dependent Ca^{2+} channels (VDCC) that are opened by membrane depolarization, (ii) receptor-operated Ca^{2+} channels (ROC) which are opened by DAG when membrane receptors are activated by ligands, and (iii) store-operated Ca^{2+} channels (SOC) that are opened when Ca^{2+} in the intracellular Ca^{2+} stores (*e.g.*, SR/ER) is depleted by, for example, activation of IP_3R/RyR and inhibition of SERCA on the SR/ER membrane [for a detailed review, see also Song *et al.* (141)]. In the pulmonary circulation, the investigations have focussed on measurements of ROS, pharmacological and functional studies, and mechanisms that control Ca^{2+} handling under hypoxia without determining the exact molecular site of action of the major players (138, 141) (Fig. 8).

Oxidants have been found to regulate voltage-gated Ca^{2+} channel activity on different levels, affecting their expression, trafficking, open time, and open probability. Some of these studies were able to demonstrate that the pore-forming α1-subunit, which is rich in reactive cysteines, is the molecular target for ROS. Although almost all studies have been carried out on myocytes, the mode of action of the redox modulation can be translated into VDCC in PASMCs. However, the findings are contradictory. While several reports have shown that oxidation increases the activity of voltage-gated Ca^{2+} channels, others reported inhibition of channel activity by oxidizing agents [for a more detailed review, see Hidalgo and Donoso (51)]. This might be explained either by the concentrations of ROS/RNS involved or by different effects on ion transport mechanisms in different cell types. Thus, thiol oxidizing agents (*e.g.*, thimerosal) irreversibly decreased current through the human α1C-subunit, which was reversed by DTT, expressed in HEK-293 cells (91, 133). In contrast, ROS such as H_2O_2 and $O_2^{\bullet -}$ were shown to stimulate Ca^{2+} entry through L-type voltage-gated channels in VSMC (151) and in cardiomyocytes (160). Oxidative stress also plays a major role in the pathogenesis of heart failure, and this condition involves L-type Ca^{2+} channels. Zheng et al. have very recently shown that the redox state of Ca^{2+} channels is controlled by the thioredoxin system which plays a key role in Ca^{2+} channel remodeling of the failing heart (187). Furthermore, NO also modulates VDCC by a direct redox-dependent stimulation. In ventricular myocytes, *S*-nitrosothiols (CysNO and GSNO) increased the amplitude of Ca^{2+} current as a result of S-nitrosylation of extracellular SH groups of the L-type Ca^{2+} channel, independently of the activity of kinases, phosphatases, or SR Ca^{2+} release (21, 120).

ROC and SOC provide alternative ways for Ca^{2+} influx into PAEC and/or PASMCs. Since TRP proteins are believed to underlie the molecular structure of many ROC entry channels and at least some SOC entry channels, it is logical to question whether the TRP proteins expressed in the pulmonary circulation are sensitive to regulation by redox. More than 10 TRP isoforms are expressed in vascular smooth muscle and EC, including TRPC1–7, TRPV1–4, TRPP2, TRPM2–4, -7, and -8. However, it needs to be noted that almost all investigations discussed next were carried out in heterologous expression systems or *in silico* modulations, which provides access for targeted studies of the channels. We will only briefly summarize the redox modulation of these channels, as several reviews recently discussed the existing data (138, 141, 163, 164).

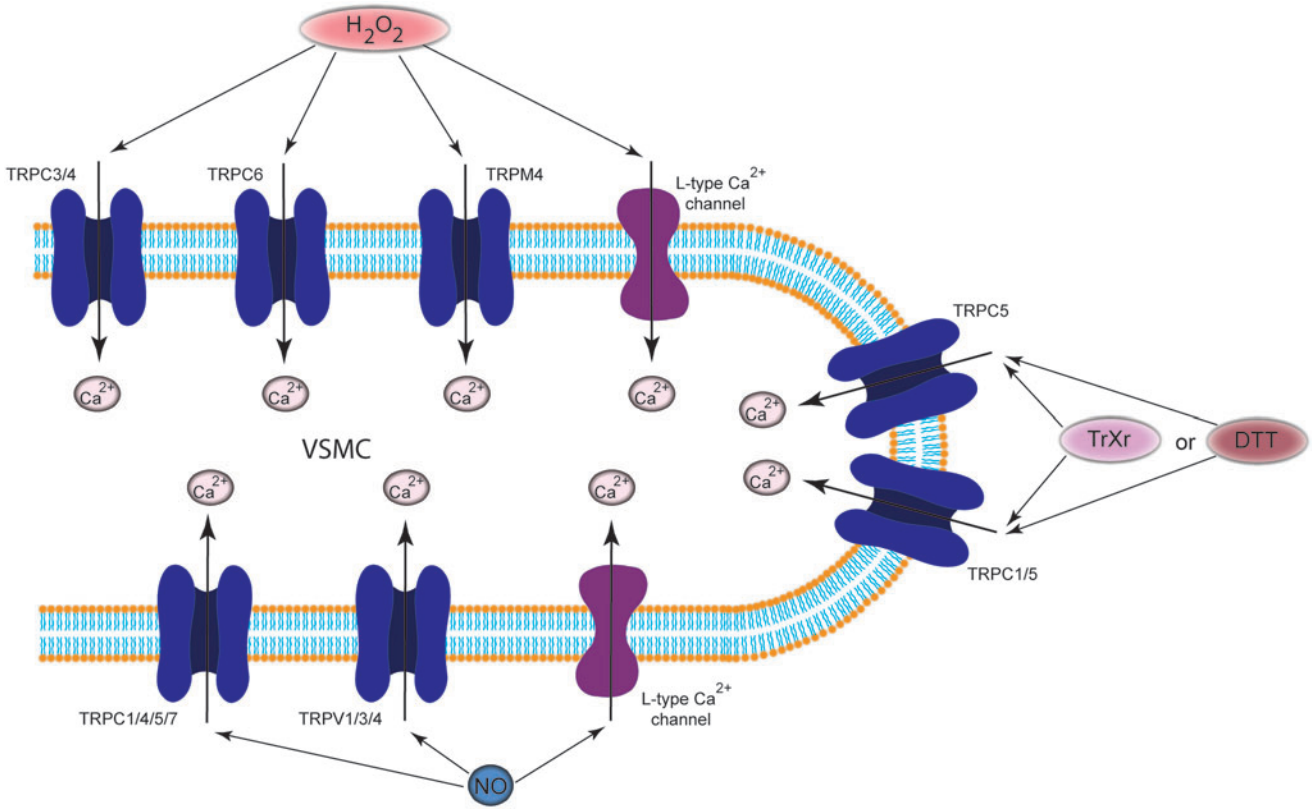

FIG. 8. Regulation of calcium selective ion channels by redox. The oxidizing agent H_2O_2 or NO directly activates calcium selective channels. In contrast, DTT or extracellular reduced Trx can increase ion current through TRPC5 homotetrameric and TRPC5/TRPC1 heterotetrameric channels. DTT, dithiothreitol; NO, nitric oxide; TRPC, transient-receptor potential cation. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

Even though there are still some uncertainties as to which compounds act directly and which act indirectly to gate the TRPM2 channel, it is clear that TRPM2 is modulated by redox changes. Using NAD^+ and ADP-ribose, Wehage *et al.* reported activation of the cationic current in TRPM2-expressing HEK cells as the consequence of redox regulation (50, 169). We have recently detected the mechano-sensitive TRPM4 in human PAs (unpublished observation). Simon *et al.* reported that H_2O_2 induces sustained activity of TRPM4, showing that oxidative modulation of the C-terminally located cysteine C1093 is crucial for the H_2O_2 effect (139). The redox regulation of TRPC3/TRPC4 channels has been extensively investigated by the Groschner group. They have shown that TRPC3 proteins are the basis of the redox-activated cation conductance of PAECs, as they are activated by H_2O_2. They identified the TRPC3/TRPC4 complex as subunits of native endothelial cation channels that are governed by the cellular redox state (12, 119). The mechanisms of the redox activation of the complex, unfortunately, remains elusive. Groschner *et al.* have described it as an ''oxidant-induced disruption of cholesterol-rich lipid rafts,'' a cross-talk between TRPC channels and signaling molecules which reside in caveolae (or lipid rafts) and further speculate that membrane cholesterol oxidation might actually be the signaling molecule that activates TRPC3 (44, 119). TRPC5 homotetrameric channels and TRPC5/TRPC1 heterotetrameric channels have been reported to be activated by DTT or extracellular reduced Trx. Both break a disulfide bond in the extracellular loop adjacent to the ion selectivity filter of TRPC5 and increase the ion conductivity of the channel (179). Similar to TRPC3 or TRPM4, TRPC6 is activated by H_2O_2 *via* modification of thiol groups of intracellular proteins. This cysteine oxidation-dependent pathway not only stimulates the TRPC6 channel by itself but also sensitizes the channels to DAG and promotes TRPC6 trafficking to the cell surface (41). TRPC5 and 7 are regulated by cysteine S-nitrosylation by NO (182). NO or the NO donor SNAP induces Ca^{2+} entry into cells, an effect that is partially reversed by additional application of the reducing agent DTT. In contrast to the other TRP channels, the mechanism is well described. The effect is specifically mediated by cysteine S-nitrosylation of the cytoplasmically accessible Cys553 and Cys558, to open the intracellular activation gate. The membrane-permeable thiol-reactive compound MTSEA mimicks this effect, but not the membrane-impermeable MTSET.

The signaling cascade underlying store-operated Ca^{2+} entry spans from receptor-mediated activation of PLC with subsequent IP_3 production to IP_3R-mediated depletion of ER Ca^{2+} stores, which, in turn, results in a conformational change, oligomerization, and translocation of stromal interaction molecule 1 (STIM1) to plasma membrane near regions of the ER, where STIM1 activates calcium release-activated calcium channel (Orai) through a direct interaction with its C-terminal region (13). The molecular identity of store-

operated Ca^{2+} influx was described first in 2006: isoforms of Orai (1, 2, and 3), as essential pore-forming units and the Ca^{2+}-sensing ER-proteins STIM1 and STIM2 (186). In addition, TRPC1 and TRPC3 have been reported to participate in the STIM-mediated Ca^{2+} entry pathway. STIM1 proteins possess several cysteine residues that could be targets for modification. Similarly, all three Orai isoforms possess predicted extracellular and intracellular cysteines. For a detailed review of the redox modulation of SOC, see Bogeski *et al.* (13). Here, we focus only on recent studies reporting modification of the newly discovered components of the SOC pathway (STIM and Orai) by redox. Recently, using electrophysiology, Ca^{2+} imaging, and site-directed mutagenesis, Bogeski *et al.* have found that Orai1 is inhibited by preincubation with H_2O_2 and that cysteines 195, 126, and 143 contribute to redox sensitivity (14). Hawkins *et al.* studied redox-mediated activation of STIM1 and found that oxidative stress results in gluthationylation of STIM1's cysteine 56, triggering STIM1 oligomerization, and, thus, they were able to activate Orai channels and induce Ca2+entry (49). In contrast, Grupe *et al.* have shown that H_2O_2 directly activates Ca^{2+} entry in an STIM-dependent manner (45). Thus, the limited number of these studies and the potential discrepancies in the recent findings clearly indicate the need for further detailed investigations of these complex signaling events. Activation of IP_3 receptors and RyRs enables Ca^{2+} release from the SR/ER to the cytosol, thus resulting in an increase in intracellular Ca^{2+} concentration and vasoconstriction in myocytes. In bovine and human aortic ECs, glutathionylation by a chemical oxidant, diamide, which selectively converts reduced glutathione (GSH) to its disulfide (GSSG), dramatically increased both the rate and magnitude of the thapsigargin-induced Ca^{2+} transient. These findings suggest that CICR *via* the IP_3R is enhanced by the formation of protein–glutathione (P-SSG) mixed disulfide, that is, glutathionylation. Similar to diamide, H_2O_2 increased the sensitivity of HAECs to both histamine and thapsigargin. The biochemical studies have shown that glutathionylation of native IP_3R1 is increased in cells challenged with H_2O_2, indicating that thiol-oxidizing agents primarily increased the sensitivity of the IP_3R to Ca^{2+} (78). Through ATP hydrolysis, SERCA and plasma membrane calcium (PMCA) pump actively clear Ca^{2+} from the cytoplasm and, thus, play a key role by maintaining cytoplasmic Ca^{2+} homeostasis. The different SERCA isoforms contain between 22 and 28 cysteine residues, and, therefore, provide a target for redox modification. According to the data available so far, only one or two of them are essential (97). SERCA inhibition *in vitro* through modification of sulfhydryl groups by either ROS or RNS has been reported in several cell types, mainly in coronary EC and in cardiomyocytes (51). Finally, the PMCA pump has also been reported to be reversibly inhibited by H_2O_2, $O_2^{\bullet -}$, $^{\bullet}OH$, and $ONOO^-$/ONOOH (51). It is very likely that residues Tyr589, Met622, and Met831 are the redox targets (82).

Conclusion

The pulmonary circulation is a target for a number of redox-active signaling factors, both circulating and locally produced. Under physiological circumstances, the concerted action of vasodilators and vasoconstrictors maintains the physiological structure and relaxed tone of the PAs. Since redox changes trigger signaling pathways to ion channels that may result in changes in ion fluxes, contraction, cell proliferation, and perhaps to vascular remodeling, to elucidate the molecular mechanisms of changes in ion channel conductance and the identification of the sites of action is essential. In the past decade, significant advancement in the understanding of protein thiol modification by pro- and antioxidants has been made. Further investigation of the interaction of redox status and ion channel gating, especially the significance of S-glutathionylation, S-nitrosylation, and protein kinase-mediated phosphorylation, will result in a better understanding of the physiological and pathophysiological importance of these mediators in general. Thus, the implications of such modifications in cellular functions and related diseases will guide our endeavour in further altering the ion channel for therapeutic purposes.

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Abbreviations Used

γ-GCL = gamma-glutamate cysteine ligase
γ-GGT = gamma-glutamyl transpeptidase
AOAA = amino oxyacetic acid
BKCa = calcium-dependent potassium channel
Ca^{2+} = calcium
cADP = cyclic adenosine diphosphate
CBS = cystathionine beta-synthetase
cGMP = cyclic guanosine monophosphate
CICR = Ca^{2+}-induced Ca^{2+}-release
CO = carbon monoxide
CoQ = Coenzyme Q
DA = ductus arteriosus
DAG = diacylglycerol
DTNB = 5,5′-dithio-bis(2-nitrobenzoic acid)
DTT = dithiothreitol
EC = endothelial cells
eNOS = endothelial nitric oxide synthase isoenzyme
ENOX = disulfide-thiol exchanger
ETC = electron transport chain
GAPDH = glyceraldehydes(s) 3-phosphate dehydrogenase
GPxs = glutathione peroxidases
GR = glutathione reductase
GS = glutathione synthetase
GSH = reduced glutathione
GSNO = *S*-nitrosoglutathione
GSSG = oxidized glutathione
GSTs = glutathione s-transferases
GTP = guanosintriphosphat
H_2DCF = dichlorodihydrofluorescein
H_2O_2 = hydrogen peroxide
HIF = hypoxia-inducible factor
HPV = hypoxic pulmonary vasoconstriction
H_2S = hydrogen sulfide
iNOS = inducible NO synthase isoenzyme
IP_3R = inositol trisphosphate receptor
K^+ = potassium
K_{ATP} = ATP-sensitive K^+ channel
KCNE1 = potassium voltage-gated channel subfamily E member 1
KCNH1 = potassium voltage-gated channel subfamily H member 1
KCNQ1 = potassium voltage-gated channel, KQT-like subfamily, member 1
Kir = potassium inwardly rectifying channel
Kv = voltage-gated potassium channel
MA = mesenteric artery
MTSEA = 2-aminoethyl methanethiosulfonate hydrobromide
MTSET = 2-(trimethylammonium)ethyl methanethiosulfonate bromide
NaHS = sodium hydrosulfide
NCX = Na^+/Ca^{2+} exchanger
nNOS = neuronal NO synthase isoenzyme
NO = nitric oxide
Nox = NADPH oxidase
NQO1 = NAD(P):quinone oxidoreductase 1
$O_2^{\bullet-}$ = superoxide
Orai = calcium release-activated calcium channel
PA = pulmonary artery
PAECs = pulmonary artery endothelial cells
PAR-2 = protease-activated receptor-2
PDGF = platelet-derived growth factor
PH = pulmonary hypertension
PMCA = plasma membrane calcium
PMET = plasma membrane electron transport
P-SSG = protein S-glutathionylation
PTP = protein tyrosine phosphatase
ROC = receptor-operated Ca^{2+} channels
ROS = reactive oxygen species
RSOH = sulfenic
RSO_2H = sulfinic
RSO_3H = sulfonic
RTK = receptor tyrosine kinase
RyR = ryanodine receptor
RyR2 = RyR/Ca^{2+} release channel
SERCA = sarcoplasmic/endoplasmic reticulum calcium ATPase
sGC = soluble guanylate cyclase
SH = Src homology
SMC = smooth muscle cell
SNO = S-nitrosylation
SOC = store-operated Ca^{2+} channels
SOD = superoxide dismutase
SR = sarcoplasmic reticulum
src = sarcoma
STIM = stromal interaction molecule
TASK-1 = TWIK-related acid-sensitive potassium channel 1
*t*BOOH = *tertiary* butyl hydroperoxide
TGF = transforming growth factor
t-PMET = trans-plasma membrane electron transport
TRP = transient-receptor potential
TRPC = transient-receptor potential cation
TRPM = transient-receptor potential cation channel, subfamily M
Trx = thioredoxin
TrxR = thioredoxin reductase
VDCC = voltage-dependent Ca^{2+} channels
VSMCs = vascular smooth muscle cells