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ROS-Dependent Signaling Mechanisms for Hypoxic Ca^{2+} Responses in Pulmonary Artery Myocytes

Yong-Xiao Wang and Yun-Min Zheng

Abstract

Hypoxic exposure causes pulmonary vasoconstriction, which serves as a critical physiologic process that ensures regional alveolar ventilation and pulmonary perfusion in the lungs, but may become an essential pathologic factor leading to pulmonary hypertension. Although the molecular mechanisms underlying hypoxic pulmonary vasoconstriction and associated pulmonary hypertension are uncertain, increasing evidence indicates that hypoxia can result in a significant increase in intracellular reactive oxygen species concentration ($[ROS]_i$) through the mitochondrial electron-transport chain in pulmonary artery smooth muscle cells (PASMCs). The increased mitochondrial ROS subsequently activate protein kinase C-ε (PKCε) and NADPH oxidase (Nox), providing positive mechanisms that further increase $[ROS]_i$. ROS may directly cause extracellular Ca^{2+} influx by inhibiting voltage-dependent K^+ (K_V) channels and opening of store-operated Ca^{2+} (SOC) channels, as well as intracellular Ca^{2+} release by activating ryanodine receptors (RyRs), leading to an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and associated contraction. In concert with ROS, PKCε may also affect K_V channels, SOC channels, and RyRs, contributing to hypoxic Ca^{2+} and contractile responses in PASMCs. *Antioxid. Redox Signal.* 11, 611–623.

Introduction

It is well known that pulmonary arteries constrict in response to hypoxic exposure (<60 mm Hg Po_2). Hypoxia-induced pulmonary vasoconstriction serves as an important physiologic process that preserves the sufficient matching of regional alveolar ventilation and pulmonary perfusion in the lungs, thereby allowing sufficient oxygenation of the blood. In contrast, systemic arteries normally do not contract or even dilate in response to hypoxia to retain fairly constant blood flow to fulfill cellular metabolic demand in important organs. Despite having a unique physiologic significance, hypoxic pulmonary vasoconstriction, if sustained, may serve as a key pathologic factor leading to pulmonary hypertension and even heart failure.

The cellular and molecular mechanisms underlying the unique hypoxic pulmonary vasoconstriction and associated pulmonary hypertension remain largely elusive; however, we and many other investigators recently provided extensive evidence showing that hypoxia results in a large increase in intracellular reactive oxygen species concentration ($[ROS]_i$) in pulmonary artery smooth muscle cells (PASMCs) (10, 26, 34, 43, 47, 61, 69, 70, 97, 98, 102, 103), which is consistent with the contribution of ROS to the initiation or maintenance or both of numerous physiologic and pathologic cellular responses in virtually all types of cells. It also should be noted that the hypoxic decrease in $[ROS]_i$ has been reported (4, 49, 50, 53). Intracellular ROS can be generated by multiple resources, including the mitochondrial electron-transport chain (ETC), NADPH oxidase (Nox), xanthine oxidase, cyclooxygenase, and cytochrome P450. Among these resources, the mitochondrial ETC and Nox (4, 37, 47, 50, 61, 69, 70, 97, 102, 104, 109) have been shown to be essential for the hypoxic increase or decrease in $[ROS]_i$ in PASMCs.

A number of publications suggest that the hypoxic increase or decrease in $[ROS]_i$ can directly affect the activity of ion channels, leading to a large increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in PASMCs. For instance, hypoxia may inhibit voltage-dependent K^+ (K_V) channels by affecting $[ROS]_i$ (4, 20, 50). Presumably, hypoxic inhibition of K_V channels would result in membrane depolarization, activation of voltage-dependent Ca^{2+} (Ca_V) channels, and extracellular Ca^{2+} influx, resulting in an increase in $[Ca^{2+}]_i$. ROS also may activate ryanodine receptors/Ca^{2+}-release channels (RyRs) to induce Ca^{2+} release from the sarcoplasmic reticulum (SR), contributing to the hypoxic increase in $[Ca^{2+}]_i$ in PASMCs (40, 66). As an increase in $[Ca^{2+}]_i$ is a most important factor for cell contraction, recent studies have demonstrated that the hypoxic increase in $[Ca^{2+}]_i$ and contraction are intimately related in PASMCs (66, 72, 73). Pharmacologic and genetic interventions that inhibit or eliminate the hypoxic increase in $[Ca^{2+}]_i$ can correspondingly inhibit or eliminate

Center for Cardiovascular Sciences, Albany Medical College, Albany, New York.

the hypoxic contraction (38, 69, 70, 122). Moreover, hypoxia normally causes neither an increase in $[Ca^{2+}]_i$ nor a contraction in systemic (*e.g.*, cerebral and mesenteric) artery SMCs (69, 91, 99). In addition to the direct effect, ROS also may activate intermediate signaling molecules, such as protein kinase C-ε (PKCε), to regulate specific ion channels in concert with ROS, contributing to the hypoxic increase in $[Ca^{2+}]_i$ and associated contraction in PASMCs (69). It is interesting to note that our recent work revealed that mitochondrial ROS-dependent activation of PKCε can significantly augment Nox activity and lead to a further increase in intracellular ROS generation, which provides a positive-feedback mechanism to augment intracellular ROS generation further, contributing to the hypoxic increase in $[ROS]_i$ and $[Ca^{2+}]_i$ as well (70).

In this review, we summarize recent progress in the study of signaling mechanisms underlying the hypoxic ROS generation and attendant Ca^{2+} responses in PASMCs, particularly highlighting our own and others' latest work in the identification of specific sources, signaling cascades, and effective targets for ROS during hypoxic stimulation.

Hypoxia Causes a Significant Increase in $[ROS]_i$

ROS function as important signaling molecules mediating many physiologic and pathologic processes in virtually all types of cells. To explore the potential important role of ROS in hypoxic responses in PASMCS, Archer and his colleagues (4) examined the effect of hypoxia on intracellular ROS generation in isolated rat lungs by using lucigenin, a chemiluminescence reagent that is often used to assess superoxide anion (O_2^-) production. As shown in Table 1, they found that an acute hypoxic exposure for minutes causes a significant decrease in lucigenin-derived chemiluminescence, indicating a decrease in $[ROS]_i$. By using lucigenin chiefly for measuring O_2^- generation, dichlorodihydrofluorescein (H_2DCF) for hydrogen peroxide (H_2O_2), dihydroethidium for O_2^-, AmplexRed for H_2O_2 (or a combination of these), their associated research groups further confirmed findings that acute hypoxia for minutes decreases $[ROS]_i$ in freshly isolated rat pulmonary arteries and PASMCs (50), and hypoxia for hours decreases $[ROS]_i$ in passaged human PASMCs (49). Consistent with the hypoxic reduction in $[ROS]_i$, acute hypoxia results in a decrease in lucigenin-derived chemiluminescence in microsome-enriched fractions of calf pulmonary arteries (53). In contrast, with lucigenin, H_2DCF, dihydroethidium, and electron paramagnetic resonance, numerous research groups revealed that acute hypoxia increases, rather decreases, ROS generation in isolated rabbit and lamb lungs, rat and dog pulmonary arteries, and cultured calf, dog, and rat PASMCs (10, 26, 27, 34, 42–44, 47, 61, 97, 102, 103). Intriguingly, a study using dihydroethidium found that acute hypoxic exposure for minutes significantly and rapidly decreases, but for hours, markedly increases $[ROS]_i$ in passaged human PASMCs (114). We looked at the effect of acute hypoxia on $[ROS]_i$ in freshly isolated mouse PASMCs by using multiple approaches, including H_2DCF/DA (mainly for measuring H_2O_2), cytochrome *c* reduction assay and lucigenin (for O_2^-), and RedoxSensor Red CC-1 (for both O_2^- and H_2O_2). Our data indicate that acute hypoxia for minutes brings about a large increase in $[ROS]_i$ (69, 70, 97).

These previous controversial findings with conventional ROS-detection methods have been attributed to the use of

Table 1. Summary of Previous Reports on the Effect of Hypoxia on $[ROS]_i$ in Isolated Lungs, Pulmonary Arteries, and PASMCs

Authors	*Hypoxia*	*$[ROS]_i$*	*Preparations*	*Detection methods*	*References*
Archer *et al.*	Minutes	Decrease	Isolated rat lungs, pulmonary arteries, and PASMCs	AmplexRed, dihydroethidium, H_2DCF, Lucigenin	(4; 50)
Jernigan *et al.*	Minutes	Increase	Isolated rat pulmonary arteries	H_2DCF	(26; 27)
	Weeks	Increase			
Killilea *et al.*	Minutes	Increase	Cultured rat PASMCs	H_2DCF	(34)
Liu *et al.*	Minutes	Increase	Isolated porcine pulmonary arteries	Electron paramagnetic resonance, H_2DCF, Lucigenin	(43)
	Weeks				
Marshall *et al.*	Minutes	Increase	Cultured calf PASMCs	Lucigenin	(47)
Mehta *et al.*	Hours	Decrease	Cultured human PASMCs	Amplexred, Dihydroethidium, H_2DCF, Lucigenin	(49)
Mittal *et al.*	Weeks	Increase	Isolated mouse pulmonary arteries	Dihydroethidium	(51)
Mohazzab and Wolin	Minutes	Decrease	Microsome-enriched fractions of calf pulmonary arteries	Lucigenin	(53)
Paddenberg*et al.*	Minutes	Increase	Isolated mouse pulmonary arteries and cultured rabbit PASMCs	H_2DCF	(61)
Rathore *et al.*	Minutes	Increase	Isolated mouse PASMCs	Cytochrome *c* reduction assay, H_2DCF	(69; 70)
Wang *et al.*	Minutes	Increase	Isolated mouse PASMCs	H_2DCF, lucigenin, RedoxSensor Red CC-1	(97)
Wang *et al.*	Hours	Increase	Isolated rat pulmonary arteries	Dihydroethidium	(98)
Waypa *et al.*	Minutes	Increase	Cultured rat PASMCs	Fluorescence resonance energy transfer probe, H_2DCF	(102; 103)
Wu *et al.*	Minutes	Decrease	Cultured human PASMCs	Dihydroethidium	(114)
	Hours	Increase			

freshly isolated and cultured cells (56). Cultured cells undergo significant changes in expression levels and functional roles of hypoxia-responsible molecules in PASMCs (57, 122). Conversely, it should be noted that both the hypoxic decrease and increase in $[ROS]_i$ were observed in cultured PASMCs, as described earlier. Similar findings were made in freshly isolated PASMCs as well. Apparently, the increased generation of intracellular ROS by acute hypoxia in cultured PASMCs is not due to the cell culture *per se*; rather, they still retain indispensable parts of the hypoxia-sensing machinery. A concern also is expressed about the experimental findings in isolated lungs, because lungs are composed of a variety of cell types, which may produce distinct responses to hypoxic stimulation (78). However, this cannot well explain the observed hypoxic decrease and increase in $[ROS]_i$ in isolated lungs. Despite "everyone can be right" (100) or "ROS up, no way" (106), a recent report of using a novel, ratiometric, redox-sensitive fluorescence resonance energy-transfer probe demonstrates that acute hypoxia augments ROS signaling in isolated rat PASMCs (103). Consistent with this report, by using the newly developed, specific H_2O_2 biosensor HyPer (9), our more recent study reveals that acute hypoxia results in an increase in H_2O_2 generation in isolated mouse PASMCs (36). Nevertheless, a general agreement exists that chronic hypoxia increases $[ROS]_i$ in lungs, pulmonary arteries, and PASMCs (26, 27, 42, 51, 114). $[ROS]_i$ is most likely to be increased, playing an essential role in hypoxic responses in PASMCs.

Mitochondrial Electron-chain Transport Serves as a Primary Hypoxic Sensor That Initiates ROS Generation, Leading to an Increase in $[ROS]_i$

In vascular SMCs, one of the major resources for intracellular ROS generation is the mitochondrial ETC, wherein ROS can be generated at complex I, II, and III, with complex III appearing to be the main site. Archer and his colleagues (4, 50) reported that the complex I inhibitor rotenone and complex III postubisemiquinone site–inhibitor antimycin-A mimic and subsequently block the acute hypoxic decrease in $[ROS]_i$ in isolated rat lungs and PASMCs (4, 50). Conversely, Waypa *et al.* (102–104) showed that rotenone and the complex III preubisemiquinone-site inhibitor myxothiazol block, but do not mimic, the acute hypoxic responses in cultured rat PASMCs. These investigators also found that antimycin-A neither mimics nor inhibits the hypoxic effect. Similar observations were obtained in isolated rat pulmonary arteries (37) and rabbit lungs (109). In support, our recent studies reveal that multiple, structurally distinctive complex I inhibitor rotenone and methylphenylpyridinium iodide, complex II inhibitor nitropropionic acid and tenoyltrifluoroacetone, as well as the complex III preubisemiquinone-site inhibitor myxothiazol all do not mimic, but significantly block, the acute hypoxic increase in $[ROS]_i$ in freshly isolated mouse PASMCs (69, 70, 97). Moreover, antimycin-A and the complex IV inhibitor sodium azide neither mimic nor block the acute hypoxic response. The preventive effect of the complex I, II, and III preubisemiquinone-site inhibitors, but not the complex III postubisemiquinone-site and complex IV inhibitors, on the acute hypoxic increase in $[ROS]_i$ also were observed in vascular cells of isolated mouse lung slices (61). Collectively, the mitochondrial ETC molecules before the complex III ubisemiquinone site may act as a functional unit that serves to increase generation of ROS in PASMCs.

To complement pharmacologic studies, we and other investigators have begun to look at the effect of genetic inhibition of mitochondrial ROS generation on the hypoxic response. In mitochondria, O_2^- is rapidly converted to H_2O_2 by manganese superoxide dismutase; H_2O_2 is then degraded by glutathione peroxidase-1 (Gpx1) in mitochondria and the cytosol, as well as by catalase in the cytosol. Perceptibly, overexpression and deletion of these endogenous antioxidant molecules may specifically modify intracellular ROS levels and associated hypoxic responses in PASMCS. In agreement with this view, our recent study reveals that *Gpx1* gene overexpression to augment ROS removal attenuates the acute hypoxic increase in $[ROS]_i$ in freshly isolated mouse PASMCs, whereas *Gpx1* gene deletion to prevent ROS removal has the opposite effect (97). Similarly, adenoviral overexpression of mitochondrial catalase and Gpx1 attenuate the acute hypoxia-induced changes in the ROS signaling in cultured rat PASMCs (103). We also found that the hypoxic response is inhibited in PASMCs from mice with catalase gene overexpression (97).

Further to provide evidence for the initial role of mitochondria in the hypoxic increase in $[ROS]_i$ in PASMCs, we examined and compared the acute hypoxic increase in ROS generation in mitochondrial and nonmitochondrial areas of freshly isolated mouse PASMCs by using the specific mitochondrial marker MitoTracker and ROS-sensitive fluorescent dye H_2DCF. The results are shown in Fig. 1, indicating that the acute hypoxic increase in ROS generation occurs significantly earlier in mitochondrial areas than in nonmitochondrial areas. Additionally, the hypoxic increase in ROS generation is greater in the former areas than in the latter (97). We also recently showed that acute hypoxia results in a large increase in ROS generation in isolated mitochondria from mouse PASMCs (36). These findings further suggest that the mitochondrial ETC is an important primary hypoxic sensor that initiates ROS generation, leading to an increase in mitochondrial ROS generation ($[ROS]_m$) and then $[ROS]_i$ in PASMCs.

NADPH Oxidase Is Involved in the Hypoxic Increase in $[ROS]_i$

NADPH oxidase (Nox) is believed to be another important source for the generation of intracellular ROS in vascular SMCs. The active form of Nox is normally composed of various subunits, dependent on the cell type. In phagocytic cells, Nox is well characterized to include the membrane-bound subunits $p22^{phox}$ and $gp91^{phox}$ (Nox2) subunits, as well as the cytosolic subunits $p47^{phox}$ and $p67^{phox}$; the association of these cytosolic and membrane-bound subunits is required for the assembly of the active Nox. Previous studies with RT-PCR showed mRNA expression of $gp91^{phox}$, $p22^{phox}$, $p47^{phox}$, $p67^{phox}$, as well as the $gp91^{phox}$ analogues Nox1 and Nox4 in mouse lung tissues (51) and Nox4 in rabbit lungs (110). Immunofluorescence staining shows the presence of Nox4 protein in isolated human pulmonary arteries and cultured human PASMCs (85), as well as in human lung tissues (51). The existence of Nox 4 in human lungs has been shown by Western blotting (51). With Western blot analysis, we recently showed that the well-characterized, major phagocytic Nox membrane-bound subunit $p22^{phox}$, as well as the cytosolic subunits $p47^{phox}$ and $p67^{phox}$, are expressed in

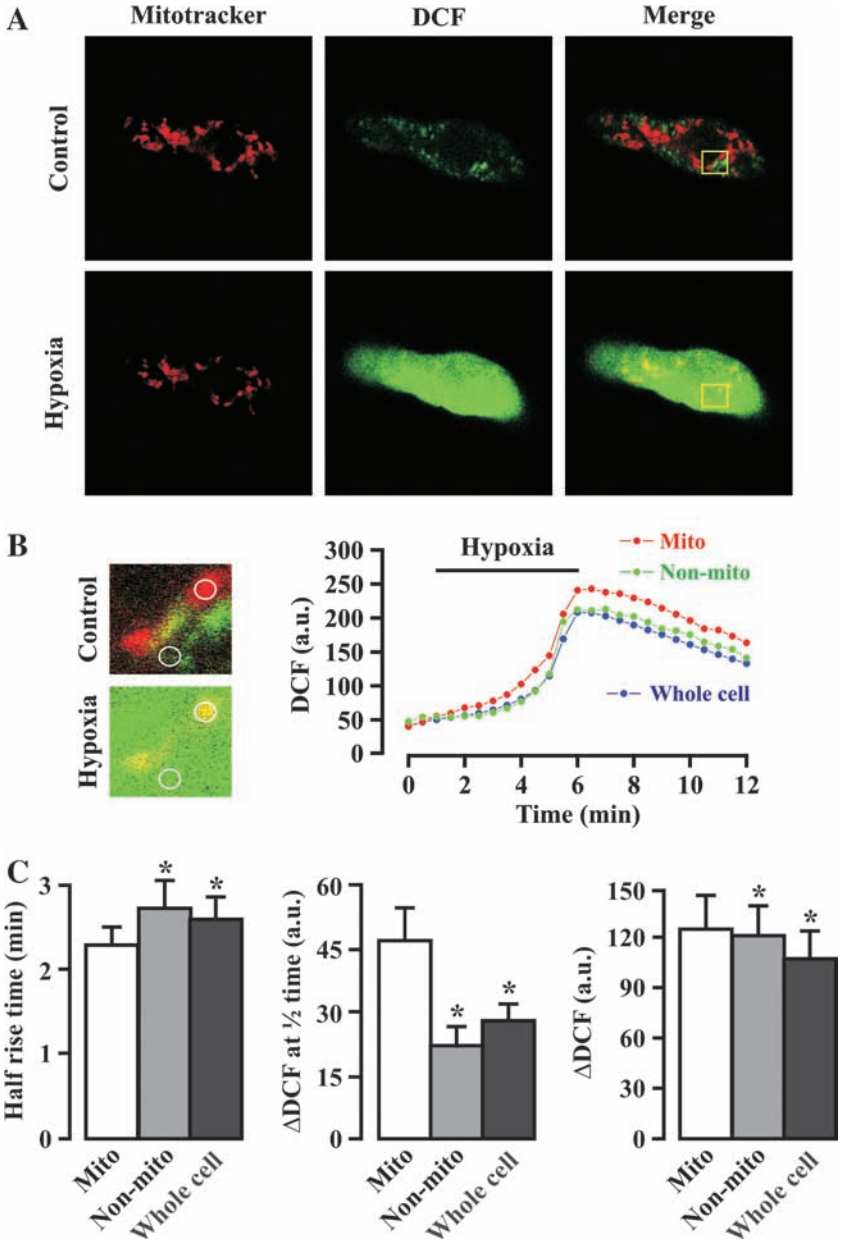

FIG. 1. Hypoxia-induced increase in ROS generation in mitochondria precedes that in nonmitochondrial areas in freshly isolated mouse pulmonary artery smooth muscle cells. (**A**) Original images show MitoTracker Deep Red 633 staining (shown as red) and DCF fluorescence (green) in a myocyte before and after hypoxia for 5 min. The superimposition of both MitoTracker staining and DCF fluorescence images produced yellow, indicating the hypoxic increase in ROS generation in mitochondria. (**B**) Extracted images were taken from the area indicated by the box in the cell shown in (**A**). The mitochondrial and nonmitochondrial area taken to measure the hypoxic increase in DCF fluorescence in the extracted images is indicated by a *circle*. Traces show the time course for the hypoxic response in mitochondrial (Mito), nonmitochondrial (Non-mito), and whole-cell areas. (**C**) Bar graph summarizes the mean half-rise time, amplitude of hypoxic increase in DCF fluorescence at the half-rise time (ΔDCF at half time), and maximal hypoxic increase in DCF fluorescence (ΔDCF) in mitochondrial, nonmitochondrial, and whole-cell areas. Data were obtained from 10 cells from five independent experiments. $*p < 0.05$ compared with mitochondrial areas. The figure is cited with permission from Wang *et al.* (97). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

endothelium-denuded mouse pulmonary arteries. Although the phagocytic Nox membrane-bound subunit $gp91^{phox}$ protein expression is not detected in pulmonary and mesenteric arteries, its analogues, Nox1 and Nox4 proteins, are observed (70).

Our study also demonstrated that acute hypoxia for minutes causes an increase in Nox activity and translocation of $p47^{phox}$, a major component of Nox, from the cytosol to the plasma membrane in endothelium-denuded mouse pulmonary arteries (70). These results suggest that Nox may contribute to the hypoxic ROS generation in PASMCs. In favor of this view, treatment with a Nox inhibitor apocynin blocks the hypoxic increase in $[ROS]_i$ in freshly isolated mouse PASMCs and Nox activity in mouse pulmonary arteries. The hypoxic

increase in $[ROS]_i$ and Nox activity are significantly prevented as well in $p47^{phox-/-}$ mouse PASMCs (Fig. 2) (70). In support of this, the Nox inhibitor diphenyleneiodonium also inhibits the hypoxic increase in $[ROS]_i$ in cultured calf PASMCs (47). Nox is another important source for the hypoxic generation of intracellular ROS in PASMCs. However, it should be noted that hypoxia may inhibit the Nox-dependent generation of intracellular ROS in a microsome-enriched fraction of calf pulmonary arteries (52).

Role of NADPH Oxidase in the Hypoxic Increase in $[ROS]_i$ Is Mediated by the Mitochondrial ROS–Protein Kinase C-ε Signaling Axis

Protein kinase C (PKC) can activate Nox to increase $[ROS]_i$, participating in a variety of cellular responses in vascular SMCs (81, 101). The PKC family consists of 12 isoforms, which can be categorized into three groups based on their structure and activation *in vitro*: the conventional PKCs (α, β_1, β_2, and γ) that are sensitive to Ca^{2+} and diacylglycerol (DAG); novel PKCs (δ, ε, η, θ, μ, and υ) that are sensitive only to DAG; and atypical PKCs (ζ and ι) that are sensitive to neither Ca^{2+} nor DAG. Damron *et al.* (17) reported that PKC isoforms (PKCα, PKCδ, PKCε, PKCζ, PKCι, and PKCυ) are expressed in cultured canine PASMCs. Our recent data reveal that PKCε protein is expressed in endothelium-denuded mouse pulmonary arteries; acute hypoxia for minutes significantly augments the total activity of PKC and PKCε (69). We more recently found that pharmacologic and genetic inhibition of PKCε blocks the hypoxia-induced increase in $[ROS]_i$ in freshly isolated mouse PASMCs (70). These findings suggest that the Nox-dependent intracellular ROS generation may be mediated by PKCε.

In support of the role of PKCε in Nox-dependent ROS generation, we showed that the conventional/novel PKC inhibitor chelerythrine and specific PKCε peptide inhibitor block the hypoxic increase in Nox activity in mouse pulmonary arteries, whereas the conventional PKC blocker Gö6796 has no effect. Our recent data further reveal that the hypoxic activation of Nox is prevented in $PKC\varepsilon^{-/-}$ mouse pulmonary arteries, and PKCε activation with PMA mimics the hypoxic response, leading to an increase of Nox activity in pulmonary arteries. This is the first report demonstrating the PKCε-dependent Nox activation as a mediator of hypoxic-induced increase in $[ROS]_i$ in PASMCs.

As the mitochondrial ETC may function as a primary oxygen sensor in the initiation of hypoxic ROS generation, we explored whether the role of PKCε is secondary to the increased generation of mitochondrial ROS and unveiled that pharmacologic inhibition of mitochondrial ROS generation with rotenone and myxothiazol both significantly prevent acute hypoxia inducing an increase in PKCε activity in mouse pulmonary arteries (69). Overexpression of Gpx1 to enhance ROS removal in mitochondria and the cytosol significantly inhibits the acute hypoxic increase in Nox activity, whereas *Gpx1* gene deletion has the opposite effect. Consistent with these results, exogenous application of H_2O_2 mimics the hypoxic response, bringing about an increase in PKCε activity. These data, together with the findings that specific inhibition of PKCε activation by pharmacologic agents and gene deletion abolishes the hypoxic activation of Nox and associated ROS generation, emphasize that the acute hypoxia-induced, Nox-dependent ROS generation is secondary to the mitochondrial ROS–PKCε signaling axis, which provides a unique positive-feedback mechanism contributing to the hypoxic increase in $[ROS]_i$ in PASMCs.

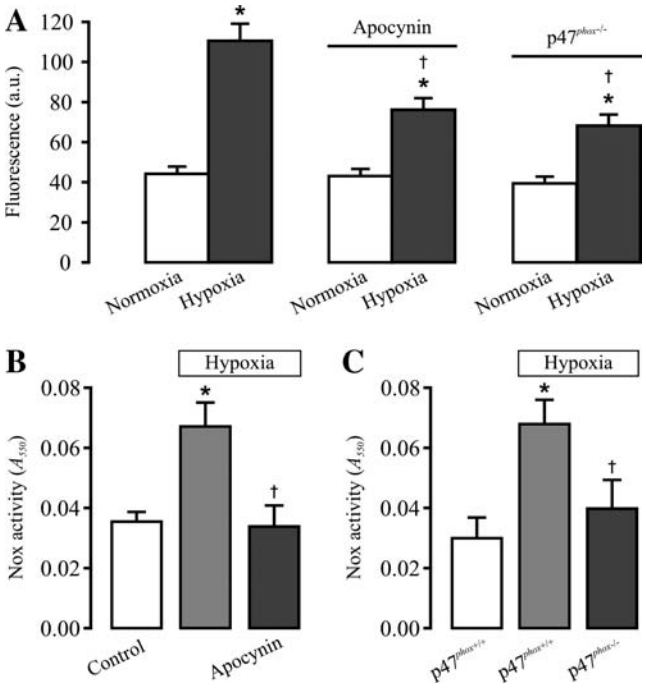

FIG. 2. Pharmacologic and genetic inhibition of NADPH oxidase significantly attenuates the hypoxic increase in $[ROS]_i$ in freshly isolated mouse pulmonary artery smooth muscle cells. (**A**) Effects of the Nox inhibitor apocynin and $p47^{phox}$ gene deletion on hypoxic increase in $[ROS]_i$ (DCF fluorescence). DCF fluorescence was recorded before (normoxia) and after hypoxia for 5 min in control ($p47^{phox+/+}$) cells, in $p47^{phox+/+}$ cells pretreated with apocynin (1 μM) for 10 min, and in $p47^{phox-/-}$ cells. Data are presented as mean ± SEM from 21 to 23 cells in four independent experiments. $^*p < 0.05$ compared with normoxia (before hypoxia); $^\dagger p < 0.05$ compared with hypoxia alone. (**B**) Effects of apocynin on the hypoxic increase in Nox activity in mouse pulmonary arteries. The activity of Nox was determined in arteries treated with normoxia, hypoxia for 5 min, and apocynin (1 μM) for 10 min plus hypoxia for 5 min. Data are presented as mean ± SEM from three independent experiments. $^*p < 0.05$ compared with control (normoxia); $^\dagger p < 0.05$ compared with hypoxia. (**C**) Effects of $p47^{phox}$ gene deletion on the hypoxic increase in Nox activity. Data are presented as mean ± SEM from three independent experiments. $^*p < 0.05$ compared with control (normoxia, $p47^{phox+/+}$); $^\dagger p < 0.05$ compared with hypoxia ($p47^{phox+/+}$). The figure is cited with permission from Rathore *et al.* (70).

An Increase in $[ROS]_i$ through the Mitochondrial ROS–PKCε–Nox Signaling Axis Is Critical for the Hypoxic Increase in $[Ca^{2+}]_i$ and Associated Contraction in PASMCs

In agreement with the concept that an increase in $[ROS]_i$ is essential for hypoxic responses in PASMCs, exogenous application of H_2O_2 for minutes, similar to acute hypoxia, induces an increase in $[Ca^{2+}]_i$ in cultured rat PASMCs (40, 104) and isolated rat pulmonary arteries (66). Exogenous O_2^- and

H_2O_2 cause pulmonary vasoconstriction in isolated rat pulmonary arteries as well (11, 30, 31, 35, 63, 66, 71, 77, 80, 113, 116). However, H_2O_2 also was found to dilate isolated calf pulmonary arteries (12).

Parallel to the effect on $[ROS]_i$, earlier studies by Archer's group (4, 50) reported that application of rotenone or antimycin-A mimics and then blocks the acute hypoxic contraction in isolated rat pulmonary arteries and lungs. Waypa *et al.* (102, 104) found that rotenone and myxothiazol, but not antimycin A, block the acute hypoxia-induced vasoconstriction in isolated rat lungs, and contraction as well as increase in $[Ca^{2+}]_i$ in cultured rat PASMCs; however, these inhibitors do not mimic the acute hypoxic responses (102, 104). Other research groups also discovered that rotenone or myxothiazol produces a similar inhibitory effect on the acute hypoxic contraction in isolated rat pulmonary arteries (37) and rabbit lungs (109). Our recent work indicates that various, structurally different mitochondrial complex I, II, and III preubisemiquinone-site inhibitors, including rotenone and methylphenylpyridinium iodide, nitroproprionic acid, tenoyltrifluoroacetone, and myxothiazol, all block, but do not reproduce the hypoxic increase in $[Ca^{2+}]_i$ and associated contraction in freshly isolated mouse PASMCs (69, 70, 97).

Interestingly, we found that pharmacologic inhibition of the complex I and II with tenoyltrifluoroacetone and tenoyltrifluoroacetone, complex I and III with methylphenylpyridinium iodide and myxothiazol, and complex II and III with tenoyltrifluoroacetone and myxothiazol do not produce an additive inhibitory effect on the acute hypoxic increase in $[Ca^{2+}]_i$ in mouse PASMCs (97). These findings further support the view that, in response to hypoxia, the mitochondrial complex molecules before the ubisemiquinone site in the complex III may operate as a functional unit to increase mitochondrial ROS generation, leading to an increase in $[ROS]_i$ and $[Ca^{2+}]_i$, as well as contraction in PASMCs.

Complementing these pharmacologic effects, a previous report showed that adenoviral overexpression of mitochondrial or cytosolic Gpx1 (or both) and catalase attenuate the acute hypoxic increase in $[Ca^{2+}]_i$ in cultured rat PASMCs (103). We also found that *Gpx1* gene overexpression to promote ROS removal inhibits the acute hypoxic increase in $[Ca^{2+}]_i$ and contraction in freshly isolated mouse PASMCs, whereas *Gpx1* gene deletion to inhibit ROS removal has the opposite effect. Catalase gene overexpression to enhance intracellular ROS clearance produces an inhibitory effect as well (97).

Our comparable study revealed that inhibition of PKCε with the conventional/novel PKC inhibitor chelerythrine or specific peptide inhibitor not only significantly diminishes the acute hypoxic increase in $[ROS]_i$, but also attenuates the hypoxic increase in $[Ca^{2+}]_i$ and contraction in mouse PASMCs; the hypoxic ROS, Ca^{2+}, and contractile responses are all blocked in PKC$\varepsilon^{-/-}$ mouse PASMCs as well (69). In support, numerous previous reports also showed that the PKC inhibitors H7, bisindolylmaleimide, calphostin C, and chelerythrine prevent, whereas the PKC activators PMA, thymelation, and farnesylthiotriazole mimic and subsequently block the acute hypoxic vasoconstriction in isolated canine and rabbit lungs, as well as isolated rat pulmonary arteries (8, 28, 60, 89, 111). Furthermore, the acute hypoxic vasoconstriction is inhibited in isolated lungs from PKC$\varepsilon^{-/-}$ mice (41). Consistent with the role of PKCε as a signaling molecule downstream of mitochondrial ROS, H_2O_2-induced pulmonary vasoconstriction in isolated rat pulmonary arteries has been found to be blocked by PKC inhibitors (29).

Similar to the inhibition of mitochondrial ETC and PKCε activity, pharmacologic inhibition of Nox by DPI has been found to block comparably the acute hypoxic increase in $[ROS]_i$ in PASMCs and vasoconstriction in isolated pulmonary arteries (47). Moreover, a number of publications show that Nox inhibition by DPI, iodonium diphenyl, and aminoethylbenzenesulfonyl fluoride all reduce the acute hypoxia-induced increase in $[Ca^{2+}]_i$ and contraction in cultured rat PASMCs (118), and vasoconstriction in isolated calf pulmonary arteries (52) and in rabbit and rat lungs (22, 88, 110). However, the specificity of iodonium compounds as Nox inhibitors has been disputed, because these agents can inhibit the mitochondrial ETC in heart cells and voltage-dependent Ca^{2+} currents in PASMCs (68, 107). Whereas gp91$^{phox-/-}$ mice show normal or reduced acute hypoxic responses (6, 42, 44), acute hypoxic vasoconstriction is inhibited in p47$^{phox-/-}$ mice (112). We recently demonstrated that apocynin, a more-specific Nox blocker, significantly reduces the acute hypoxic increase in $[Ca^{2+}]_i$ in freshly isolated mouse PASMCS, and p47^{phox} gene deletion produces a similar inhibitory effect (70). These results further support the view that the Nox-dependent increase in intracellular ROS generation contributes to the hypoxic increase in $[Ca^{2+}]_i$ and contraction in PASMCs.

ROS-dependent, Hypoxic Increases in $[Ca^{2+}]_i$ and Associated Contraction Are Mediated by Multiple Ion Channels in PASMCs

ROS-dependent, hypoxic increases in $[Ca^{2+}]_i$ and associated contraction in PASMCs are likely to be mediated by multiple ion channels, particularly K_V channels, SOC channels, and RyRs/Ca^{2+} release channels. Major recent advances in our understanding of the role of these ion channels in hypoxic Ca^{2+} and contractile responses are reviewed.

Involvement of voltage-dependent K^+ channels

K_V channels are important for control of the membrane potential and intracellular Ca^{2+} homeostasis, thereby playing a significant role in the regulation of vascular cell contraction. Extensive publications demonstrate that both acute and chronic hypoxia significantly inhibit K_V channels in PAMSCs, which may cause membrane depolarization, Ca_V channel opening, and extracellular Ca^{2+} influx, mediating the hypoxic increase in $[Ca^{2+}]_i$ and associated contraction (3, 48, 84). Rather surprisingly, no patch-clamp studies directly examine the effect of hypoxia on Ca_V channels in the cultured or freshly isolated rat, human, or mouse PASMCs. It also was noted that the hypoxic increase in $[Ca^{2+}]_i$ and associated contraction in PASMCs are preserved in the presence of K_V channel blockers, Ca_V channel blockers, and high extracellular K^+, as well as in the absence of extracellular Ca^{2+} (under conditions in which Ca^{2+} influx through Ca_V channels is eliminated) (18, 19, 23, 73, 79, 82).

K_V channels normally consist of α and β subunits. The α subunits form an actual ion-conducting pore, whereas the β subunits do not conduct ions on their own, but rather modulate the channel activity. Based on sequence homology of the hydrophobic transmembrane domains, the K_V channel α subunits can be divided into 12 classes, designated K_V1 to

12. $K_V\alpha$1.1, 1.2, 1.5, 1.6, 2.1, 4.3, and 9.3 channels have been shown to be hypoxia sensitive in functional activity or expression level or both, potentially participating in hypoxic responses in PASMCs (5, 7, 16, 24, 62, 64, 67, 93, 96). It is worth pointing out that previous studies that tried to determine the molecular identity of hypoxia-sensitive K_V channel members in PASMCs yielded conflicting results. Archer *et al.* (7) suggested that acute hypoxic inhibition of K_V currents in rat PASMCs is primarily caused by K_V2.1 block, but not K_V1.5, although they later reported that K_V1.5 plays a key role in acute hypoxic inhibition of K_V currents (5, 67). A study using anti-K_V2.1 antibody indicates that K_V2.1 channels may be a major hypoxic target in rat PASMCs (24). However, other investigators have not been able to detect K_V1.5 mRNA and K_V2.1 protein in rabbit or rat PASMCs (16, 62).

It has been reported that rotenone and antimycin-A mimic and subsequently inhibit the acute hypoxia-induced $[ROS]_i$ reduction, K_V-current inhibition, and contraction in isolated rat PASMCs (4, 50). Intriguingly, a recent study also showed that the multiple mitochondrial complex inhibitors attenuate K_V currents and shift K_V current activation to more-negative membrane voltages in rat PASMCs (20). Complementing the effect of mitochondrial inhibitors, a membrane permeable to hydrogen peroxide, *t*-butyl hydroperoxide, was found to inhibit K_V currents in rat PASMCs (13). These results suggest that K_V channels may be involved in ROS-dependent, hypoxic increase in $[Ca^{2+}]_i$ and contraction in PASMCs.

However, it was reported that neither removal of extracellular Ca^{2+} nor treatment with nifedipine to block Ca_V channels inhibits H_2O_2-induced increase in $[Ca^{2+}]_i$ in cultured rat PASMCs (40). Similarly, the use of the Ca_V channel blocker verapamil or removal of extracellular Ca^{2+} does not affect H_2O_2-evoked increase in $[Ca^{2+}]_i$ and contraction in isolated rat pulmonary arteries (66). A lack of the role of extracellular Ca^{2+} removal in H_2O_2-induced contraction in pulmonary arteries also was observed by other investigators (63, 80). Further studies are needed to resolve the reported inconsistent findings and to demonstrate further the role of K_V channel inhibition in ROS-dependent, hypoxic Ca^{2+} and contractile responses in PASMCs (78, 86, 90, 100).

Evidence also indicates that hypoxia may inhibit K_V channels by activating PKCε, contributing to the hypoxic increase in $[Ca^{2+}]_i$ and to contraction in PASMCs. A previous study showed that application of 4-aminopyridine to block K_V channels significantly augments hypoxic vasoconstriction in isolated lungs from PKC$\varepsilon^{-/-}$ mice, and K_v3.1b channel protein expression is increased in PKC$\varepsilon^{-/-}$ mouse lungs (41). These results indicate that PKCε may downregulate the expression and activity of K_V channels (such as K_v3.1b), participating in hypoxic responses in PASMCs. In support of this view, a previous report showed that PKC activation with PMA inhibits K_V currents in rat PASMCs, and this inhibition can be blocked by the selective PKC inhibitor bis-indolylmaleimide (119). Inhibition of K_V currents by endothelin-1 in cultured human PASMCs is also reversed by bis-indolylmaleimide and the general PKC inhibitor staurosporine (83). Similarly, Cogolludo *et al.* (14) reported that thromboxane A_2–induced inhibition of K_V currents in isolated rat PASMCs are attenuated by the general PKC inhibitors staurosporine, calphostin C, and Gö6983; however, the effect of thromboxane A_2 is not blocked by bis-indolylmaleimide or the conventional PKC inhibitor Gö6976 and can be prevented by the selective PKCζ pseudosubstrate inhibitor (14). These investigators further showed that Gö6976 blocks serotonin-evoked inhibition of native K_V currents in rat PASMCs and human K_V1.5 currents stably expressed in LTK cells (15), and PKCζ gene deletion prevents thromboxane A_2–induced inhibition of K_V currents in isolated mouse PASMCs (54). These data further support the concept that PKCε is involved in the hypoxic inhibition of K_V channels and also suggest that PKCε and PKCζ may differentially mediate agonist-induced responses in PASMCs.

Role of store-operated Ca^{2+} channels

ROS-dependent, hypoxic increase in $[Ca^{2+}]_i$ may result from extracellular Ca^{2+} influx due to activation of SOC channels in PASMCs. Pharmacologic studies showed that pretreatment with La^{3+} to inhibit SOC channels or cyclopiazonic acid to deplete SR Ca^{2+} significantly inhibits hypoxic vasoconstriction in isolated rat pulmonary arteries (73) and rat lungs (105). Acute hypoxia also significantly increases extracellular Ca^{2+} influx *via* SOC channels in pig, rabbit, and rat PASMCs (32, 39, 45, 58, 59, 94, 95).

The major molecular candidates for SOC channels are likely to be canonic transient receptor potential (TRPC) channels. These channels include seven members named TRPC1–7, each encoded by a different gene. All seven TRPC channels have been found to be expressed in mRNA or protein levels or both in pulmonary arteries, among which, TRPC1 and TRPC6 channels are likely to be involved in the acute and chronic hypoxic increase in $[Ca^{2+}]_i$ and contraction in PAMSCs (33, 39, 95, 108).

It is interesting to note that H_2O_2-induced increase in $[Ca^{2+}]_i$ in PASMCs does not appear to be related to TRPC channels because the general channel blockers La^{3+} and SKF-96365 fail to produce an inhibitory effect in cultured rat PASMCs. In addition, H_2O_2 does not affect Mn^{2+}-induced quenching of fura-2 fluorescence, a typical indicator of the opening of TRPC-encoded SOC channels (40). Similarly, H_2O_2-evoked vasoconstriction in isolated pulmonary arteries is not affected by SKF-96365 (66). Moreover, H_2O_2-induced increases in $[Ca^{2+}]_i$ in cultured rat PASMCs and isolated rat pulmonary arteries are not inhibited by removal of extracellular Ca^{2+} (40, 66).

Despite the lack of direct experimental evidence for the effect of PKC on TRPC channels in PASMCs, previous studies reported that SOC channels in systemic vascular (*e.g.*, coronary artery, mesenteric artery, and portal vein) SMCs are activated by the PKC activators phorbol ester phorbol 12,13-dibutyrate and 1-oleoyl-2-acetyl-*sn*-glycerol, as well as a PKC catalytic subunit, whereas they are inhibited by the PKC inhibitor chelerythrine (2, 74, 75). TRPC1 channels have been shown to be phosphorylated by PKCα, regulating store-operated Ca^{2+} entry in human endothelial cells (1). Moreover, PKCα is known to participate in the activation of SOCs in mesangial cells (46). Thus, it is interesting to determine whether a similar mechanism exits in PASMCs.

Contribution of ryanodine receptors/ Ca^{2+} release channels

The Ca^{2+} release from the SR *via* RyRs is a major component of Ca^{2+} signaling in vascular SMCs. The role of RyRs in the hypoxic Ca^{2+} release and associated contraction in

PASMCs has received extensive attention. Numerous publications have shown that the depletion of SR Ca^{2+} with caffeine (through activation of RyRs) reduces or abolishes the acute hypoxia-induced increase in $[Ca^{2+}]_i$ in cultured rat and freshly isolated canine and rat PASMCs (65, 76, 99) and vasoconstriction in isolated canine and rabbit pulmonary arteries (19, 25). Similarly, ryanodine, an agent that binds with high affinity to RyRs, largely inhibits the acute hypoxic Ca^{2+} response in cultured cat PASMCs (91) and vasoconstriction in isolated rat lungs and canine and rabbit pulmonary arteries (19, 25, 55). Other RyR antagonists, such as ruthenium red, tetracaine, and dantrolene, also significantly block the acute hypoxic increase in $[Ca^{2+}]_i$ in freshly isolated rat PASMCs and vasoconstriction in pulmonary arteries (122). These data suggest that RyRs are key targets for acute hypoxia, by which hypoxia may induce Ca^{2+} release and associated contraction in PASMCs. The importance of RyR-mediated Ca^{2+} release in hypoxic responses in PASMCs is reinforced by the findings that hypoxic inhibition of K_V channels is likely to be secondary to Ca^{2+} release from the SR (21, 65, 92). Moreover, hypoxic Ca^{2+} release through RyRs may result in the opening of SOC channels, which causes not only extracellular Ca^{2+} influx through the opening channels, but also may result in membrane depolarization, activation of Ca_V channels, and further Ca^{2+} influx, providing a positive-feedback mechanism that enhances hypoxic increase in $[Ca^{2+}]_i$ and contraction in PASMCs (58, 59).

Consistent with the potentially important role of RyRs in ROS-dependent, hypoxic Ca^{2+} and contractile responses, a previous study showed that treatment with ryanodine (50 μM) to block RyRs significantly inhibits H_2O_2-evoked, initial rapid increase in $[Ca^{2+}]_i$ in cultured rat PASMCS (40). In support, ryanodine and dantrolene abolish or greatly suppress an H_2O_2-induced increase in $[Ca^{2+}]_i$ and vasoconstriction in isolated rat pulmonary arteries (66).

Three distinct gene-encoded subtypes of RyRs (RyR1, RyR2, and RyR3) are expressed in mammalian cells. By using real-time quantitative RT-PCR, we showed that RyR1, RyR2, and RyR3 are all expressed in freshly isolated rat and mouse PASMCs (121, 122). In support of our findings, other investigators reported that all three RyR subtype mRNAs are

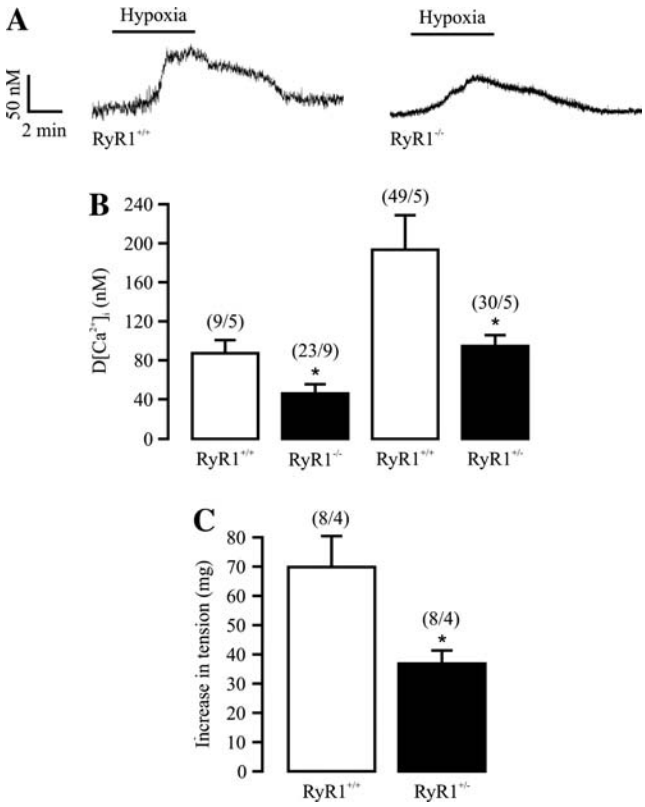

FIG. 3. RyR1 mediates the hypoxic increase in $[Ca^{2+}]_i$ and contraction in pulmonary artery smooth muscle cells. (**A**) Original recordings show that hypoxic exposure for 5 min induced an increase in $[Ca^{2+}]_i$ in an embryonic $RyR1^{+/+}$ and $RyR1^{-/-}$ mouse PASMC. (**B**) Bar graphs summarize the hypoxic increase in $[Ca^{2+}]_i$ in embryonic $RyR1^{+/+}$, embryonic $RyR1^{-/-}$, adult $RyR^{+/-}$, and adult $RyR1^{+/+}$ PASMCs. $^*p < 0.05$ compared with $RyR1^{+/+}$ cells. Numbers in parentheses indicate the numbers of cells and mice tested. (**C**) Summary of hypoxic vasoconstriction in adult $RyR1^{+/+}$ and $RyR^{+/-}$ mouse pulmonary arteries. $^*p < 0.05$ compared with $RyR1^{+/+}$ pulmonary arteries. The figure is cited with permission from Li *et al.* (38).

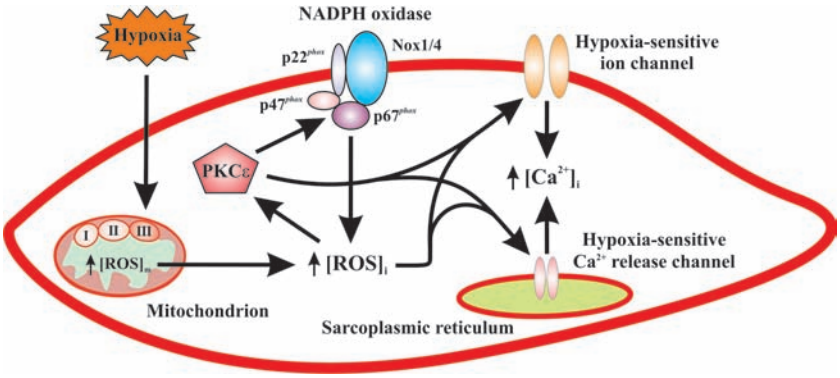

FIG. 4. A schematic diagram illustrating the signaling mechanisms for ROS-dependent, hypoxic increase in $[Ca^{2+}]_i$ and associated contraction in pulmonary artery smooth muscle cells. This includes the potential important primary hypoxic sensor mitochondrial ETC, intermediate signaling molecules ROS, PKCε, and Nox, as well as effectors hypoxia-sensitive plasmalemmal ion channels (*e.g.*, K_V channels and SOC channels) and sarcolemmal Ca^{2+} release channels (RyRs). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

present in rat intralobar pulmonary arteries (117). Expression of RyR1, RyR2, and RyR3 proteins also was observed in freshly isolated rat PASMCs by using immunofluorescence staining (122) and in isolated pulmonary arteries by using Western blot analysis (117).

We have started to explore the potential role of individual subtypes of RyRs in hypoxic responses by using genetically manipulated mice. As shown in Fig. 3, our studies revealed that acute hypoxia induces a much smaller increase in $[Ca^{2+}]_i$ in freshly isolated PASMCs from embryonic $RyR1^{-/-}$ mice at day 17, compared with wild-type mice. A decreased Ca^{2+} response also was observed in adult $RyR1^{+/-}$ mouse PASMCs. Moreover, acute hypoxic vasoconstriction is inhibited in pulmonary arteries from adult $RyR1^{+/-}$ mice (38). The acute hypoxic increase in $[Ca^{2+}]_i$ in PASMCs and vasoconstriction in pulmonary arteries are significantly diminished in adult $RyR3^{-/-}$ mice as well (122). As FK506 binding protein with a molecular mass of 12.6 kDa (FKBP12.6) is known to bind to and regulate RyR2 (115), we used $FKBP12.6^{-/-}$ mice as a unique tool in determining the potential role of RyR2 in hypoxic responses; we found that that the acute hypoxia-induced increase in $[Ca^{2+}]_i$ in PASMCs and vasoconstriction in isolated pulmonary arteries are both enhanced in $FKBP12.6^{-/-}$ mice (120). Collectively, all three RyR subtypes are involved in the hypoxic Ca^{2+} release and contraction in PASMCs.

It has been reported that addition of catalytic PKC phosphorylates RyR2 in canine cardiac microsomes. The observed extent of PKC-dependent phosphorylation of RyR2 is comparable to the level of PKC-dependent increase in the activity of RyR2 determined by [^{3}H]ryanodine-binding assay (87). By analogy, PKC is likely to regulate RyRs directly to mediate hypoxic Ca^{2+} release in PASMCs; however, this view must be demonstrated by further studies.

Conclusions

Based on our recent studies and previous publications, we present a schematic diagram, as illustrated in Fig. 4, to conclude that the mitochondrial ETC may function as a hypoxic sensor in PASMCs, by which hypoxia can significantly enhance $[ROS]_m$, leading to an initial, large increase in $[ROS]_i$. The increased $[ROS]_i$ subsequently activates the intermediate signaling molecules PKCε and Nox, providing a positive-feedback mechanism to increase further the hypoxic generation of intracellular ROS. As a consequence, ROS and PKCε synergistically result in the inhibition of plasmalemmal K_V channels (hypoxic effectors), opening of Ca_V channels, and extracellular Ca^{2+} influx, contributing to the hypoxic increase in $[Ca^{2+}]_i$ and associated contraction. In addition, ROS and PKCε may activate plasmalemmal SOC channels. As important hypoxic effectors, the opened SOC channels not only allow extracellular Ca^{2+} to enter the cell, but also cause membrane depolarization, leading to the further opening of Ca_V channels and extracellular Ca^{2+} influx. Moreover, both ROS and PKCε can in concert activate RyR1, RyR2, RyR3 or all three, inducing Ca^{2+} release from the SR, as an important process for the hypoxic Ca^{2+} and contractile responses in PASMCs. Interestingly, available evidence suggests that the hypoxic inhibition of K_V channels and activation of SOC channels are likely to be secondary to SR Ca^{2+} release. Finally, it is worth noting that, despite recent progress in the field, we are still far from fully understanding the cellular and molecular mechanisms responsible for hypoxic increases in $[Ca^{2+}]_i$ and associated contraction in PASMCs. For instance, it is unclear how the mitochondrial ETC senses hypoxia in PASMCs. To what extent each of the individual ion channels contributes to the hypoxic Ca^{2+} response remains to be determined. Thus, further studies are necessary to answer these fundamental questions and also to resolve the reported inconsistent findings.

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Address correspondence to:
Yong-Xiao Wang, M.D., Ph.D.
Center for Cardiovascular Sciences
Albany Medical College
Albany, NY 12208

E-mail: wangy@mail.amc.edu

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Abbreviations Used

$[Ca^{2+}]_i$ = intracellular Ca^{2+} concentration
Ca_V = voltage-dependent Ca^{2+}
ETC = electron-transport chain
FKBP12.6 = FK506-binding protein with a molecular mass of 12.6kDa
Gpx1 = glutathione peroxidase-1
H_2DCF = dichlorodihydrofluorescein
H_2O_2 = hydrogen peroxide
K_V = voltage-dependent K^+
Nox = NADPH oxidase
O_2^- = superoxide anion
PASMC = pulmonary artery smooth muscle cell
PKC = protein kinase C
$[ROS]_i$ = intracellular reactive oxygen species concentration
RyR = ryanodine receptor
SOC = store-operated Ca^{2+}
SR = sarcoplasmic reticulum
TRPC = canonic transient receptor potential