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Role of ROS signaling in differential hypoxic Ca^{2+} and contractile responses in pulmonary and systemic vascular smooth muscle cells

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Abstract

Hypoxia causes a large increase in $[Ca^{2+}]_i$ and attendant contraction in pulmonary artery smooth muscle cells (PASMCs), but not in systemic artery SMCs. The different responses meet the respective functional needs in these two distinct vascular myocytes; however, the underlying molecular mechanisms are not well known. We and other investigators have provided extensive evidence to reveal that voltage-dependent K^+ (K_V) channels, canonical transient receptor potential (TRPC) channels, ryanodine receptor Ca^{2+} release channels (RyRs), cyclic adenosine diphosphate-ribose, FK506 binding protein 12.6, protein kinase C, NADPH oxidase and reactive oxygen species (ROS) are the essential effectors and signaling intermediates in the hypoxic increase in $[Ca^{2+}]_i$ in PASMCs and HPV, but they may not primarily underlie the diverse cellular responses in pulmonary and systemic vascular myocytes. Hypoxia significantly increases mitochondrial ROS generation in PASMCs, which can induce intracellular Ca^{2+} release by opening RyRs, and may also cause extracellular Ca^{2+} influx by inhibiting K_V channels and activating TRPC channels, leading to a large increase in $[Ca^{2+}]_i$ in PASMCs and HPV. In contrast, hypoxia has no or a minor effect on mitochondrial ROS generation in systemic SMCs, thereby causing no change or a negligible increase in $[Ca^{2+}]_i$ and contraction. Further preliminary work indicates that Rieske iron–sulfur protein in the mitochondrial complex III may perhaps serve as a key initial molecular determinant for the hypoxic increase in $[Ca^{2+}]_i$ in PASMCs and HPV, suggesting its potential important role in different cellular changes to respond to hypoxic stimulation in pulmonary and systemic artery myocytes. All these findings have greatly improved our understanding of the molecular processes for the differential hypoxic Ca^{2+} and contractile responses in vascular SMCs from distinct pulmonary and systemic circulation systems.

1. Introduction

Hypoxia results in vasoconstriction in pulmonary arteries (PAs), but not in systemic arteries, to meet the respective physiological and pathological needs in these different circulation systems. Consistent with a general viewpoint that an increase in $[Ca^{2+}]_i$ is a key factor in the initiation and maintenance of contraction in vascular smooth muscle cells (SMCs), hypoxia results in a large increase in $[Ca^{2+}]_i$ in pulmonary, but not in systemic artery SMCs [see our recent review (Wang & Zheng, 2010)]. An increase in $[Ca^{2+}]_i$ in vascular SMCs results from intracellular Ca^{2+} release and extracellular Ca^{2+} influx, which are mediated by multiple ion

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channels, including ryanodine receptor Ca^{2+} release channels (RyRs), voltage-dependent K^+ (K_V) channels and store-operated Ca^{2+} (SOC) channels. Ion channels are highly sensitive to important intracellular signaling molecules reactive oxygen species (ROS). Intracellular ROS generation primarily occurs at mitochondria and NADPH oxidase (NOX) (Wang & Zheng, 2010). Thus, hypoxia may differentially affect intracellular ROS production and then regulate ion channels, leading to diverse Ca^{2+} and contractile responses in pulmonary and systemic artery myocytes.

We and other researchers have started to conduct comparative studies to determine the potential role of these important ion channels, signaling intermediates and primary molecules in the diversity of hypoxic Ca^{2+} and contractile responses in pulmonary and systemic vascular myocytes, with the ultimate objective to elucidate underlying molecular mechanisms and advance relevant clinical practices. This review summarizes the key progresses in current research from our laboratory and others.

2. Different hypoxic Ca^{2+} and contractile responses in pulmonary and systemic arteries

A detectable hypoxic response in PASMCs and PAs normally starts to occur at an O_2 tension (PO_2) of 60 mmHg or lower (Aaronson *et al.*, 2006;Wang & Zheng, 2010). Physiological hypoxic levels may vary in lungs in vivo; thus, hypoxia at different levels (10–60 mm Hg PO_2) has been often used in experiments in vitro and in vivo. Hypoxia-induced vasoconstriction in PAs (HPV) serves as an important physiological process to preserve the sufficient matching of regional alveolar ventilation and pulmonary perfusion in the lungs, but may also result in pulmonary hypertension and attendant heart failure. In contrast, hypoxia normally does not contract and may even dilate systemic (e.g., cerebral, coronary, mesenteric) arteries, leading to a fall in the arterial blood pressure to maintain a fairly constant blood flow in important organs and tissues.

An increase in $[Ca^{2+}]_i$ is a key factor in the initiation and maintenance of contraction in vascular SMCs. The increased Ca^{2+} signaling in PASMCs has been widely accepted to be a critical event for HPV (Mauban *et al.*, 2005;Moudgil *et al.*, 2005;Aaronson *et al.*, 2006;Wang & Zheng, 2010;Weir *et al.*, 2010). The distinct changes in $[Ca^{2+}]_i$ by hypoxia are closely correlated with the diversity of hypoxic contractile responses in pulmonary and systemic artery SMCs. In support of this view, we have shown that hypoxia can greatly increase $[Ca^{2+}]_i$ in pulmonary artery SMCs (PASMCs), but not in mesenteric (systemic) SMCs (MASMCs) (Wang *et al.*, 2003;Rathore *et al.*, 2006;Zheng *et al.*, 2008;Rathore *et al.*, 2008). Likewise, the hypoxic increase in $[Ca^{2+}]_i$ has been observed in PASMCs, but not in cerebral and renal artery myocytes (Vadula *et al.*, 1993;Waypa *et al.*, 2010).

3. Differential effect of hypoxia on ion channels in pulmonary and systemic artery SMCs

The hypoxic increase in $[Ca^{2+}]_i$ in PASMCs results from extracellular Ca^{2+} influx, which may occur due to the inhibition of K_V channels and activation of SOC channels (Mauban *et al.*, 2005;Moudgil *et al.*, 2005;Aaronson *et al.*, 2006;Wang & Zheng, 2010;Weir *et al.*, 2010). Extensive studies have consistently revealed that intracellular Ca^{2+} release from the sarcoplasmic reticulum (SR) through RyRs plays an important role in the hypoxic increase in $[Ca^{2+}]_i$ in PASMCs and attendant HPV. We and other investigators have started to address a fundamental question whether these ion channels may underlie the diversity of hypoxic Ca^{2+} and contractile responses in pulmonary and systemic artery SMCs. The major findings are summarized below.

(3.1) Voltage-dependent K^+ channels

Earlier collaborative research by Drs. Hume, Archer and Weir and their associates reported for the first time that hypoxia inhibits K_V channels in PASMCs (Post *et al.*, 1992). These findings were subsequently confirmed by Dr. Yuan's work (Yuan *et al.*, 1993). Inhibition of K_V channels has been thought to cause membrane depolarization and activation of voltage-dependent Ca^{2+} (Ca_V) channels, leading to extracellular Ca^{2+} influx, an increase in $[Ca^{2+}]_i$ in PASMCs, and HPV. However, scientists have argued the significance of K_V and Ca_V channels in the hypoxic responses (Turner & Kozlowski, 1997;Ward & Aaronson, 1999;Sylvester, 2001;Sham, 2002;Aaronson *et al.*, 2006;Ward & McMurtry, 2009), considering the fact that the hypoxic increase in $[Ca^{2+}]_i$ and associated HPV are preserved in the presence of K_V and Ca_V channel blockers, high extracellular K^+, and in the absence of extracellular Ca^{2+} (under conditions where Ca^{2+} influx through Ca_V channels is eliminated) (Hasunuma *et al.*, 1991;Demiryurek *et al.*, 1993;Sham *et al.*, 2000;Robertson *et al.*, 2000;Shimoda *et al.*, 2000;Dipp *et al.*, 2001;Kang *et al.*, 2002). Indeed, it is surprising to note that there has been no reports using the patch clamp technique to determine whether the hypoxic inhibition of K_V channels may, in fact, activate Ca_V channels in cultured and freshly isolated PASMCs from rats, mice and humans that have been often used in relevant studies.

Interestingly, we have recently demonstrated that membrane depolarization can cause a direct activation of Gq protein-coupled receptors (GqPCRs) to activate inositol 1,4,5-triphosphate receptor Ca^{2+} release channels (IP_3Rs) to induce Ca^{2+} release from the SR, which subsequently open neighboring RyRs, leading to further Ca^{2+} release and contraction without the involvement of Ca_V channels and associated extracellular Ca^{2+} influx in airway SMCs (Liu *et al.*, 2009b). This novel Ca^{2+} signaling mechanism may perhaps exist in PASMCs as well; as such, the hypoxic inhibition of K_V channels would cause membrane depolarization, activation of GqPCRs and then Ca^{2+} release, contributing to the hypoxic increase in $[Ca^{2+}]_i$ in PASMCs and HPV. If this hypothesis is confirmed, it may explain why the hypoxic responses are preserved when extracellular Ca^{2+} influx through Ca_V channels is eliminated.

Comparable data indicate that hypoxia inhibits K_V channels in PASMCs, but not in MASMCs (Yuan *et al.*, 1993). Further studies have reported that hypoxia for 24–72 h significantly decreases $K_V1.1$, 1.2, 1.5 and 2.1 channel α-subunit mRNA and protein expression in cultured rat PASMCs, while their expressions are unaltered in MASMCs (Wang *et al.*, 1997a;Platoshyn *et al.*, 2001). Similarly, a recent study has shown that mRNA and protein expression levels of $K_V1.1$, 1.5, 1.6, 2.1, and 4.3 channel α-subunits are decreased in PAs, but not in aorta from rats with chronic hypoxia for 3 weeks (Wang *et al.*, 2005b). On the other hand, patch clamp recordings have found that membrane depolarization produces equivalent K_V channel currents in PASMCs and MASMCs (Yuan *et al.*, 1993). Taken together, K_V channels are the hypoxic effectors in PASMCs, but not in systemic artery myocytes; as such, these ion channels may not be the specific, necessary contributors to the difference in hypoxic Ca^{2+} and contractile responses in distinct pulmonary and systemic vascular myocytes.

(3.2) Canonical transient receptor potential Ca^{2+} channels

A previous report by Robertson *et al* has shown that pretreatment with La^{3+} to inhibit SOC channels or with the SR Ca^{2+} pump inhibitor cyclopiazonic acid to deplete the SR Ca^{2+} significantly inhibits HPV in isolated rat PAs (Robertson *et al.*, 2000), indicating the involvement of SOC channels. Similar results have been observed in isolated rat lung preparations (Weigand *et al.*, 2003). Hypoxia also significantly increases Ca^{2+} entry via SOC channels in pig, rabbit and rat PASMCs (Kang *et al.*, 2003;Lin *et al.*, 2004;Ng *et al.*,

2005;Wang *et al.*, 2005a;Wang *et al.*, 2006;Ng *et al.*, 2007). SOC channels may be encoded by canonical transient receptor potential (TRPC) genes, which are known to consist of seven members designated TRPC1-7 (Nilius *et al.*, 2007;Abramowitz & Birnbaumer, 2009). Several studies have demonstrated that hypoxia for hours upregulates mRNA and protein expression levels of TRPC1, TRPC4 and/or TRPC6, and increases SOC channel currents, leading to Ca^{2+} entry in PASMCs (Fantozzi *et al.*, 2003;Lin *et al.*, 2004;Wang *et al.*, 2006;Zhang *et al.*, 2007). Therefore, Ca^{2+} entry via TRPC-encoded SOC channels may contribute to the hypoxic increase in $[Ca^{2+}]_i$ and contraction in PASMCs. Supportively, a recent report has shown that the hypoxic Ca^{2+} influx in PASMCs and HPV in lungs are reduced in TRPC6$^{-/-}$ mice (Weissmann *et al.*, 2006a). These TRPC-encoded channels are highly expressed and functional as well in systemic vascular SMCs (Inoue *et al.*, 2006); however, they cannot be activated in response to hypoxia and hence fail to cause an increase in $[Ca^{2+}]_i$ and contraction in this type of cells.

(3.3) Ryanodine receptor Ca^{2+} release channels

Considering that Ca^{2+} release from the SR via RyRs is a major component of Ca^{2+} signaling in vascular SMCs, we have sought to determine the important function of RyRs in hypoxic responses in PASMCs. Our studies using pharmacological agents and specific RyR gene deletion mice have found that RyRs are essential for the hypoxic increase in $[Ca^{2+}]_i$ in PASMCs and HPV (Wang *et al.*, 2003;Zheng *et al.*, 2004;Zheng *et al.*, 2005;Li *et al.*, 2009;Liao *et al.*, 2010). Other investigators have also provided pharmacological data to support the central role of RyRs in hypoxic cellular responses (Vadula *et al.*, 1993;Jabr *et al.*, 1997;Dipp *et al.*, 2001;Kang *et al.*, 2002;Morio & McMurtry, 2002;Ng *et al.*, 2005).

The importance of RyR-mediated Ca^{2+} release in hypoxic responses in PASMCs is reinforced by the findings that the hypoxic inhibition of K_V channels is likely to be secondary to RyR-mediated Ca^{2+} release from the SR. A previous study has shown that the hypoxic inhibition of K_V channels is mimicked by application of the RyR activator caffeine to induce RyR-dependent Ca^{2+} release, and prevented by pretreatment with caffeine to deplete RyR-expressing Ca^{2+} stores as well as the Ca^{2+} chelator 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid to buffer $[Ca^{2+}]_i$ (Post *et al.*, 1995). Moreover, inhibition of Ca^{2+} release with ryanodine, caffeine and the SR Ca^{2+} pump inhibitor thapsigargin all decrease outward K^+ currents (Vandier *et al.*, 1998). A series of well-designed studies have further provided evidence that acute hypoxia causes Ca^{2+} release from the SR by opening RyRs, which activates SOC channels, leading to Ca^{2+} influx in PASMCs (Ng *et al.*, 2005;Ng *et al.*, 2007).

Three subtypes of RyRs (RyR1, RyR2 and RyR3) are expressed in mammalian cells, and each is encoded by a distinct gene. A series of our experiments have demonstrated that all three RyR subtypes are present and functional in PASMCs (Zheng *et al.*, 2005;Zheng *et al.*, 2008;Li *et al.*, 2009;Liao *et al.*, 2010). Importantly, RyR1 gene deletion blocks the hypoxic increase $[Ca^{2+}]_i$ in PASMCs and HPV (Li *et al.*, 2009). Similarly, RyR2 or RyR3 gene deletion produces similar effects (Zheng *et al.*, 2005;Liao *et al.*, 2010). These data reveal that all three subtypes of RyRs are important for hypoxic Ca^{2+} and contractile responses in PASMCs.

With a consideration of the importance of RyRs in the hypoxic increase in $[Ca^{2+}]_i$ in PASMCs and HPV, we have started to assess their potential role in the diversity of hypoxic Ca^{2+} and contractile responses in pulmonary and systemic vascular SMCs. The results indicate the RyRs show overall high expression levels and/or functional activities in both vascular myocytes (Zheng *et al.*, 2008). In addition, we have found that RyRs are highly expressed and functional as well in other (e.g., airway) SMCs (Liu *et al.*, 2007;Liu *et al.*, 2009a;Liu *et al.*, 2009b). Collectively, RyRs are imperative for the hypoxic increase in

$[Ca^{2+}]_i$ in PASMCs and HPV, but fail to be activated by hypoxia and thus are unable to lead to Ca^{2+} and contractile responses in SMCs from systemic arteries.

(3.4) Other ion channels

Inositol triphosphate receptor Ca^{2+} release channels—Bright *et al* have shown that cyclopiazonic acid inhibits chemical hypoxia-induced rise in $[Ca^{2+}]_i$ in cultured rat PASMCs; and the inhibitory effect of cyclopiazonic acid has been attributed to the depletion of IP_3-sensitive Ca^{2+} stores (Bright *et al.*, 1995). In contrast, it has been reported that cyclopiazonic acid or thapsigargin significantly enhances HPV in freshly isolated canine pulmonary arteries (Jabr *et al.*, 1997).

In spite of these inconsistent reports, we and other investigators have provided evidence to indicate that IP_3Rs and RyRs are functionally coupled in PASMCs, by which Ca^{2+} release from the SR through IP_3Rs can activate neighboring RyRs, causing further Ca^{2+} release, a local Ca^{2+}-induced Ca^{2+} release (CICR) process, amplifying neurotransmitter-induced increase in $[Ca^{2+}]_i$ and contraction (Jabr *et al.*, 1997;Janiak *et al.*, 2001;Zheng *et al.*, 2005;Li *et al.*, 2009). We have also found that this local CICR process due to the interaction of IP_3Rs with neighboring RyRs exists as well in airway SMCs (Liu *et al.*, 2007;Liu *et al.*, 2009b). Nevertheless, further studies are necessary to directly evaluate the role of IP_3Rs in the hypoxic Ca^{2+} release in PASMCs. It should be pointed out that to date there is no studies to determine the role of IP_3Rs in the diversity of hypoxic cellular responses in pulmonary and systemic artery myocytes.

Ca^{2+}-activated Cl^- channels—We have found that hypoxia causes the opening of Ca^{2+}-activated Cl^- (Cl_{Ca}) channels in PASMCs (Wang *et al.*, 1997b;Wang *et al.*, 2003). The equilibrium potential for Cl_{Ca} channels is between −20 – −30 mV; thus, hypoxia activates Cl_{Ca} channels to produce inward (depolarizing) currents at the resting potential of about −55 mV. It is interesting to note that Cl_{Ca} currents produced by hypoxia are sustained. The sustained currents are likely to result from the removal of phosphorylation-dependent inactivation of the channels, which is mediated by Ca^{2+}/calmodulin-dependent protein kinase II (Pacaud *et al.*, 1992;Wang & Kotlikoff, 1997;Greenwood *et al.*, 2001). Moreover, mild chemical hypoxia increases the frequency and amplitude of RyR-mediated spontaneous local Ca^{2+} release (Ca^{2+} sparks) and spontaneous transient inward currents (STICs) (Wang *et al.*, 2003). Thus, hypoxia may perhaps activate RyRs and then Cl_{Ca} channels, contributing to the hypoxic extracellular Ca^{2+} influx in PASMCs and HPV.

Voltage-dependent Ca^{2+} channels—There is a report showing that hypoxia shifts the voltage-dependence of Ca_V channel opening towards the negative membrane potential in rabbit PASMCs, thereby potentiating Ca^{2+} influx (Franco-Obregon & Lopez-Barneo, 1996). Studies of oxygen sensing in carotid body cells by the same group, however, have failed to find an effect on Ca_V channel gating (Lopez-Barneo *et al.*, 1993). These results, together with previous reports that relaxation of rabbit pulmonary arteries is usually seen during hypoxic stimulation (Marriott & Marshall, 1990;MacLean *et al.*, 1993;Vadula *et al.*, 1993), suggest that such a mechanism may not play an important role in hypoxic increase in $[Ca^{2+}]_i$. As described above, a number of reports have shown that hypoxic contraction in isolated lungs, PAs and PASMCs are relatively insensitive to multiple Ca_V channel blockers (Demiryurek *et al.*, 1993;Jabr *et al.*, 1997;Robertson *et al.*, 2000;Shimoda *et al.*, 2000;Sham *et al.*, 2000;Dipp & Evans, 2001;Kang *et al.*, 2002).

Ca^{2+}-activated K^+ channels—Whether hypoxia in fact inhibits Ca^{2+}-activated K^+ (K_{Ca}) channels remains unclear, since during hypoxia this channel has been shown to be inhibited

(Park *et al.*, 1995;Peng *et al.*, 1997;Vandier *et al.*, 1998), activated (Archer *et al.*, 1996;Cornfield *et al.*, 1996;Nossaman *et al.*, 1997), and unaffected (Post *et al.*, 1995).

Taken together, the role of IP_3R, Cl_{Ca}, Ca_V and K_{Ca} channels in hypoxic cellular responses in PASMCs need further experiments for confirmation. In addition, these ion channels show similar expression levels and functional activities in pulmonary and systemic vascular SMCs, nevertheless, none of these ion channels would be able to be responded to hypoxia and accordingly can cause cellular responses in vascular SMCs from the systemic circulation system.

4. Effect of hypoxia on cyclic ADP-ribose, protein kinase C, and reactive oxygen species in pulmonary and systemic artery SMCs

Multiple intracellular molecules and proteins such as cyclic adenosine diphosphate-ribose (cADPR), protein kinase C (PKC) and ROS are known to be important intermediates to participate in Ca^{2+} signaling in vascular SMCs. Numerous studies have provided interesting information with respect to the potential important role of these intermediates in the hypoxic increase in $[Ca^{2+}]_i$ in PASMCs and HPV as well as the difference in hypoxic cellular responses in pulmonary and systemic artery myocytes, as described below.

(4.1) Cyclic adenosine diphosphate-ribose

Hypoxia has been shown to increase the activity of CD38/ADP-ribosyl cyclase and then generation of cADPR in PAs (Wilson *et al.*, 2001), and the cADPR antagonist 8-Br cADPR blocks HPV (Dipp & Evans, 2001). Thus, cADPR may perhaps serve as a mediator in the hypoxic Ca^{2+} release and contraction in PASMCs. However, cADPR has been shown to induce Ca^{2+} release from the SR by opening RyRs in variety of cell types including systemic (coronary) artery SMCs (Tang *et al.*, 2002).

We and other investigators have provided evidence to suggest that cADPR induces Ca^{2+} release from the SR by dissociating FK506 binding protein 12.6 (FKBP12.6) from RyRs in airway and coronary artery SMCs, pancreatic cells, and adrenal chromaffin cells (Noguchi *et al.*, 1997;Tang *et al.*, 2002;Wang *et al.*, 2004;Morita *et al.*, 2006). Our studies have further discovered that FKBP12.6 is involved in the hypoxic Ca^{2+} release and contraction in PASMCs (Zheng *et al.*, 2004;Liao *et al.*, 2010).

These results indicate that cADPR and FKBP12.6 function as important intermediate signaling molecules to mediate the hypoxic increase in $[Ca^{2+}]_i$ in PASMCs and HPV, but they may not be the major primary factors in determining the divergent hypoxic responses in pulmonary and systemic vascular SMCs.

(4.2) Protein kinase C

Pharmacological studies have shown that PKC may play an important role in mediating hypoxic Ca^{2+} and contractile responses in PASMCs. The PKC inhibitors prevent, whereas the PKC activators mimic and subsequently block, HPV in isolated canine and rabbit lungs, as well as isolated rat PAs (Orton *et al.*, 1990;Jin *et al.*, 1992;Barman, 1999;Weissmann *et al.*, 1999;Tsai *et al.*, 2004). It has also been reported that HPV is inhibited in isolated lungs from $PKC\varepsilon^{-/-}$ mice (Littler *et al.*, 2003).

Using multiple biochemical, pharmacological and genetic approaches, we have demonstrated that PKCε is a major isoform of the PKC family to mediate the hypoxic increase in $[Ca^{2+}]_i$ and contraction in PASMCs (Rathore *et al.*, 2006). Interestingly, the hypoxic activation of PKCε is mimicked by exogenous H_2O_2, but abolished by

pharmacological and genetic inhibition of mitochondrial ROS generation. Our studies have also found that hypoxia fails to activate PKC in MASMCs. Moreover, PKCε expression levels and responses to the PKC activator phorbol 12-myristate 13-acetate (PMA) are comparable in pulmonary and systemic artery myocytes. Apparently, hypoxia activates PKCε by increasing mitochondrial ROS generation to play a critical role in hypoxic responses in PASMCs, but this signaling molecule is not a specific, important participant in the divergent effects of hypoxia on $[Ca^{2+}]_i$ and contractility in pulmonary and systemic artery myocytes,.

(4.3) Intracellular ROS generation

Many studies have revealed that hypoxia increases intracellular ROS generation/ concentration ($[ROS]_i$) in isolated rabbit and lamb lungs, rat and dog pulmonary arteries, and cultured calf, dog and rat PASMCs (Marshall *et al.*, 1996;Killilea *et al.*, 2000;Waypa *et al.*, 2001;Paddenberg *et al.*, 2003;Brennan *et al.*, 2003;Liu *et al.*, 2003;Jernigan *et al.*, 2004;Waypa *et al.*, 2006), although hypoxia has also been reported to decrease $[ROS]_i$ in isolated rat lungs and PASMCs (Archer *et al.*, 1993;Michelakis *et al.*, 2002), as well as in microsome-enriched fractions of calf pulmonary arteries (Mohazzab & Wolin, 1994a;Mohazzab & Wolin, 1994b). Interestingly, hypoxia for minutes decreases, whereas for 48 hours increases, oxidation of dihydroethidium ($[ROS]_i$) in cultured PASMCs (Wu *et al.*, 2007).

We have also looked into the effect of hypoxia on $[ROS]_i$ in PASMCs using multiple ROS detection approaches, and found that hypoxia results in a large increase in $[ROS]_i$ (Rathore *et al.*, 2006;Wang *et al.*, 2007;Rathore *et al.*, 2008;Liao *et al.*, 2010). Recent reports using a novel, ratiometric, redox-sensitive fluorescence resonance energy transfer (FRET) probe reveal that hypoxia augments ROS signals as well in PASMCs (Waypa *et al.*, 2006;Waypa *et al.*, 2010). Using a newly developed, genetically-encoded, specific ROS biosensor HyPer (Belousov *et al.*, 2006), we have further demonstrated the hypoxic increase in $[ROS]_i$ in PASMCs (Korde & Wang, 2008).

In support of the view that ROS are critical for hypoxic Ca^{2+} and contractile responses in PASMCs, many research groups have found that exogenous H_2O_2, similar to hypoxia, induces an increase in $[Ca^{2+}]_i$ in PASMCs (Waypa *et al.*, 2002;Lin *et al.*, 2007) and vasoconstriction in PAs (Burghuber *et al.*, 1986;Seeger *et al.*, 1986;Rhoades *et al.*, 1990;Kjaeve *et al.*, 1991;Sheehan *et al.*, 1993;Yamaguchi *et al.*, 1994;Wilhelm & Herget, 1995;Jin & Rhoades, 1997;Jones *et al.*, 1997). It should be pointed out that in most of these previous studies, the concentrations of H_2O_2 used are above several hundred μM. As such, the pulmonary vasoconstriction produced is often irreversible. Despite the fact that physiological concentrations of H_2O_2 in PASMCs are unknown, our investigations have discovered that acute hypoxia (10 – 20 Torr) yields a ROS signal equivalent to that generated by exogenous H_2O_2 at 51 μM (Wang *et al.*, 2007). Using 30 μM, Pourmahram *et al* have shown that H_2O_2 evokes a reversible contraction in isolated PAs (Pourmahram *et al.*, 2008). Furthermore, pharmacological and genetic inhibition of intracellular ROS generation block the hypoxic increase in $[Ca^{2+}]_i$ in PASMCs and HPV, as summarized in our recent review (Wang & Zheng, 2010).

An earlier comparative study has shown that hypoxia decreases $[ROS]_i$ in freshly isolated rat PASMCs, but increases $[ROS]_i$ in renal (systemic) artery myocytes (Michelakis *et al.*, 2002). We have also wondered whether hypoxia differentially affects ROS signaling to be essential for the different hypoxic Ca^{2+} and contractile responses in pulmonary and systemic artery SMCs. Our data reveal that hypoxia causes a large increase in $[ROS]_i$ in freshly isolated mouse PASMCs, but not in MASMCs (Rathore *et al.*, 2006;Rathore *et al.*, 2008). In addition, hypoxia has been found to decrease $[ROS]_i$ in both cultured, passaged human

pulmonary and coronary (systemic) artery SMCs (Mehta *et al.*, 2008). More perplexedly, a recent study indicates that hypoxia produces a similar increase in ROS signals in cultured, passaged pulmonary and renal artery myocytes (Waypa *et al.*, 2010). The reason for these inconsistent data is unclear; however, different hypoxic responses may occur in freshly isolated and cultured, passaged vascular SMCs under different experimental conditions.

Nevertheless, our further investigations demonstrate that exogenous H_2O_2 mimics the hypoxic response, leading to a large increase in the activity of PKCε to contribute to the hypoxic increase in $[Ca^{2+}]_i$ and contraction in PASMCs; and exogenous H_2O_2 produces similar cellular responses in MASMCs (Rathore *et al.*, 2006;Rathore *et al.*, 2008). Evidently, a distinct increase in $[ROS]_i$ in pulmonary and mesenteric artery SMCs may principally account for the diverse hypoxic Ca^{2+} and contractile responses in these two different types of vascular myocytes.

5. Effect of hypoxia on NADPH oxidase in pulmonary and systemic artery SMCs

NOX has been thought to be a major source for intracellular ROS generation to mediate hypoxic responses in PASMCs (Weissmann *et al.*, 2006b;Wolin *et al.*, 2007). In phagocytic cells, NOX is well characterized to include the membrane-bound subunits $p22^{phox}$ and $gp91^{phox}$ (NOX2) subunits, and the cytosolic subunits $p47^{phox}$ and $p67^{phox}$. The association of these membrane-bound and cytosolic subunits is required to assemble the active NOX. Numerous studies have demonstrated that NOX inhibition by iodonium compounds attenuates the hypoxic increase in $[ROS]_i$ (Mohazzab *et al.*, 1995;Marshall *et al.*, 1996), hypoxic inhibition in K_V currents (Weir *et al.*, 1994), hypoxic increase in $[Ca^{2+}]_i$ and contraction in PASMCs (Thomas, III *et al.*, 1991;Marshall *et al.*, 1996;Zhang *et al.*, 1997), and HPV in isolated lungs and PAs (Mohazzab & Wolin, 1994a;Grimminger *et al.*, 1995;Mohazzab *et al.*, 1995;Weissmann *et al.*, 2000;Weissmann *et al.*, 2006c). The specificity of iodonium compounds as NOX inhibitors has been disputed, since these agents inhibit voltage-dependent Ca^{2+} currents in PASMCs and mitochondrial functions in heart cells (Ragan & Bloxham, 1977;Weir *et al.*, 1994). While $gp91^{phox-/-}$ mice show normal or reduced hypoxic responses (Archer *et al.*, 1999;Liu *et al.*, 2006), HPV is inhibited in $p47^{phox-/-}$ mice (Weissmann *et al.*, 2006c).

We have recently unveiled that hypoxia largely increases the activity of NOX in PASMCs; and pharmacological and genetic inhibition of NOX decrease the hypoxic increase in $[ROS]_i$, $[Ca^{2+}]_i$ and contraction (Rathore *et al.*, 2008). The hypoxic activation of NOX is mimicked by the PKC activator PMA, whereas blocked by PKCε inhibitors and gene deletion, indicating that hypoxia activates NOX by stimulating PKCε in PASMCs (Rathore *et al.*, 2008). These data, together with the previously described findings that the hypoxic activation of PKCε is secondary to mitochondrial ROS (Rathore *et al.*, 2006), suggest that hypoxia may enhances mitochondrial ROS generation, activates PKCε, and then augments NOX activity to cause further generation of intracellular ROS. This PKCε-dependent ROS-induced ROS generation plays a crucial role in the hypoxic increase in $[Ca^{2+}]_i$ in PASMCs and HPV. Supportively, exogenous H_2O_2 mimics the hypoxic effect, leading to a large increase in the activity of NOX, while pharmacological and genetic inhibition of mitochondrial ROS generation both prevent the hypoxic activation of NOX.

Our studies have also found that the major NOX subunit $gp91^{phox}$ analogues (Nox1 and Nox4, but not itself), $p22^{phox}$, $p47^{phox}$, and $p67^{phox}$ proteins are equally expressed in PASMCs and MASMCs. Hypoxia increases NOX activity in former cells, but not in the latter. In agreement with similar NOX subunit expression levels, PMA and H_2O_2 both cause an equivalent increase in the activity of NOX in PASMCs and MASMCs (Rathore *et al.*,

2008). Accordingly, NOX, like other hypoxic signaling molecules, plays a significant role in hypoxic responses in PASMCs, but not in dissimilar hypoxic responses in pulmonary and systemic systems.

6. Effect of hypoxia on mitochondrial ROS generation in pulmonary and systemic artery SMCs

As aforementioned, a series of studies from our laboratory and others have provided pharmacological and genetic evidence to indicate that mitochondria are a primary source of the hypoxic ROS generation in PASMCs (Wang & Zheng, 2010). Consistent with this view, mitochondrial inhibitors block the hypoxic increase in $[Ca^{2+}]_i$ in PASMCs, and contraction in PASMCs, pulmonary arteries and isolated lungs (Leach *et al.*, 2001;Waypa *et al.*, 2001;Waypa & Schumacker, 2002;Paddenberg *et al.*, 2003;Rathore *et al.*, 2006;Wang *et al.*, 2007;Rathore *et al.*, 2008;Liao *et al.*, 2010). It is also worth stating that pharmacological studies have suggested that the mitochondrial complex III is a main site of the hypoxic effect on ROS generation in PASMCs (Archer *et al.*, 1993;Leach *et al.*, 2001;Waypa *et al.*, 2001;Michelakis *et al.*, 2002;Waypa & Schumacker, 2002;Weissmann *et al.*, 2003;Waypa *et al.*, 2006;Rathore *et al.*, 2006;Weissmann *et al.*, 2006c;Wang *et al.*, 2007;Rathore *et al.*, 2008). Excitingly, our recent preliminary study has disclosed that siRNA-mediated gene silencing of Rieske iron–sulfur protein in the complex III blocks the hypoxic ROS generation, increase in $[Ca^{2+}]_i$ and contraction in PASMCs (Korde & Wang, 2008).

Archer and his colleagues have proposed a model that suggests different mitochondrial functions may account for the diversity of hypoxic Ca^{2+} and contractile responses in pulmonary and renal (systemic) artery SMCs (Michelakis *et al.*, 2002). In this study, they have reported that hypoxia and mitochondrial inhibitors can inhibit the tonic production of mitochondrial ROS to elicit contraction in PASMCs, but increase mitochondrial ROS generation to cause relaxation in renal artery SMCs. Likewise, we and others have also revealed that hypoxia produces a different change in $[ROS]_i$ in pulmonary and systemic artery SMCs; however, hypoxia increases $[ROS]_i$ in the former cells, and produces no effect or a minor increase in the latter cells (Rathore *et al.*, 2006;Rathore *et al.*, 2008;Waypa *et al.*, 2010).

Taking in account of all these results along with the generally agreed concept that pharmacological and genetic inhibition of mitochondrial ROS production block the hypoxic increase in $[Ca^{2+}]_i$ in PASMCs and attendant HPV, we believe that different generation of mitochondrial ROS is a primary determinant factor in the diversity of hypoxic Ca^{2+} and contractile responses in pulmonary and systemic artery SMCs.

7. Role of ROS in hypoxic effect on ion channels in pulmonary and systemic artery SMCs

Ion channels are highly regulated by the biologically important intracellular signaling molecules ROS; thus, hypoxia may differently regulate ion channels by affecting intracellular ROS generation, which leads to diverse Ca^{2+} and contractile responses in pulmonary and systemic artery myocytes. Indeed, an earlier comparative research has shown that mitochondrial inhibitors, similar to hypoxia, can cause a decrease in $[ROS]_i$ to produce K_V channel inhibition and contraction in PASMCs; on the contrary, mitochondrial inhibitors and hypoxia increase $[ROS]_i$ to lead to K_V channel augmentation and relaxation in renal artery SMCs (Michelakis *et al.*, 2002). However, it should be noted that H_2O_2-induced increase in $[Ca^{2+}]_i$ in PASMCs is unaffected by either Ca^{2+} removal in extracellular solution or the Ca_V channel blocker nifedipine (Lin *et al.*, 2007). Similarly, neither H_2O_2-evoked vasoconstriction nor the increase in $[Ca^{2+}]_i$ in PAs is affected by removal of extracellular

Ca^{2+} (Pourmahram *et al.*, 2008). Extracellular Ca^{2+} influx contributes to HPV, although the importance of K_V channels in the hypoxic extracellular Ca^{2+} influx has been argued (Turner & Kozlowski, 1997;Ward & Aaronson, 1999;Sylvester, 2001;Sham, 2002;Aaronson *et al.*, 2006;Ward & McMurtry, 2009). Collectively, the ionic mechanisms for H_2O_2-evoked pulmonary vasoconstriction are at least in part different from those for HPV. Moreover, additional experiments are needed to further evaluate the different generation of mitochondrial ROS in the hypoxic inhibition of K_V channels and associated cellular responses in pulmonary and systemic artery SMCs.

Previous reports indicate the involvement of TRPC channels in the hypoxic increase in $[Ca^{2+}]_i$ in PASMCs and HPV (Wang & Zheng, 2010); however, no study has determined the role of ROS in the hypoxic activation of these channels in PASMCs. In spite of this, SKF-96365 or La^{3+}, known to block TRPC channels, does not affect H_2O_2-induced increase in $[Ca^{2+}]_i$ in PASMCs (Lin *et al.*, 2007).

In contrast, H_2O_2-elicited increases in $[Ca^{2+}]_i$ in PASMCs and contraction in PAs are consistently shown to be inhibited or abolished by the RyR antagonist dantrolene or ryanodine (Lin *et al.*, 2007;Pourmahram *et al.*, 2008), which is comparable to their inhibitory effect on the hypoxic increase in $[Ca^{2+}]_i$ in PASMCs and HPV (Wang & Zheng, 2010). Interestingly, we have very recently provided biochemical and genetic evidence that hypoxia results in the dissociation of FKBP12.6 from RyR2 to augment the Ca^{2+} release channel activity in PASMCs (Liao *et al.*, 2010). The hypoxic dissociation of FKBP12.6 is mimicked by exogenous H_2O_2, and inhibited by blocking mitochondrial ROS generation with myxothiazol and enhancing mitochondrial ROS degradation with glutathione peroxidase-1 gene overexpression, demonstrating that the hypoxic response is secondary to an increase in mitochondrial ROS generation. Chemical and genetic removal of FKBP12.6 enhance, whereas RyR2 gene deletion blocks, the hypoxic increase in $[Ca^{2+}]_i$ in PASMCs and HPV (Zheng *et al.*, 2004;Liao *et al.*, 2010). These findings, together with the fact that FKBP12.6 and RyRs are highly expressed and functional in pulmonary and systemic vascular as well as other SMCs (Coussin *et al.*, 2000;Lohn *et al.*, 2001;Tang *et al.*, 2002;Wang *et al.*, 2004;Zheng *et al.*, 2008;Liu *et al.*, 2009a;Liu *et al.*, 2009b), imply that hypoxia largely enhances mitochondrial ROS generation to lead to the opening of RyRs, Ca^{2+} release and contraction in PASMCs, but causes no effect or an insufficient increase in mitochondrial ROS formation to produce cellular responses in systemic artery myocytes.

8. Conclusion

Hypoxia contracts pulmonary arteries, but does not contract or dilate systemic arteries. These different hypoxic contractile responses meet the unique functional needs of these two different circulation systems. Consistent with the well-established, general concept that Ca^{2+} signaling is obligatory for the initiation and maintenance of contraction in vascular SMCs, hypoxia causes a large increase in $[Ca^{2+}]_i$ in pulmonary, but not in systemic artery SMCs. The cellular and molecular mechanisms underlying the difference of the hypoxic Ca^{2+} and contractile responses in pulmonary and systemic artery SMCs are poorly understood.

As diagrammed in Figure 1, we and other scientists have provided extensive evidence to reveal that RyRs, TRPC channels, K_V channels, FKBP12.6, cADPR, PKC, ROS and NOX are the essential effectors or signaling intermediates in the hypoxic increase in $[Ca^{2+}]_i$ in PASMCs and HPV. However, these imperative hypoxic effectors and intermediates, on the whole, show high expression levels and/or intrinsic functional activities in both pulmonary and systemic vascular myocytes, and thus may not serve as primary determinants for the diverse cellular responses in vascular myocytes from these two distinct circulation systems. On the other hand, hypoxia significantly increases mitochondrial ROS generation in

pulmonary, but not in systemic artery myocytes. The increased mitochondrial ROS are not only able to induce intracellular Ca^{2+} release from the SR by opening RyRs, but may also cause extracellular Ca^{2+} influx by inhibiting K_V channels and activating TRPC channels, leading to a large increase in $[Ca^{2+}]_i$ in PASMCs and HPV; in contrast, hypoxia has no or a minor effect on mitochondrial ROS generation to influence the activity of RyR, K_V or TRPC channels, causing no or a negligible increase in $[Ca^{2+}]_i$ and contraction in systemic SMCs.

The findings from pharmacological studies suggest that the hypoxic production of mitochondrial ROS predominantly occurs at the complex III. Our recent preliminary study indicates that Rieske iron–sulfur protein in the complex III is indispensible to the hypoxic ROS generation to cause an increase in $[Ca^{2+}]_i$ in PASMCs and HPV. These data, together with the aforementioned fact that hypoxia produces a different effect on mitochondrial ROS generation to cause different Ca^{2+} and contractile responses in pulmonary and systemic artery SMCs, inspire us to conjecture that Rieske iron–sulfur protein may show different expression levels and/or functional activities to serve as a key, initial molecular determinant for the dissimilar hypoxic cellular responses in vascular SMCs from distinct pulmonary and systemic circulation systems. Clearly, further studies aimed to indentify Rieske iron–sulfur protein and/or potentially other key molecular determinants would greatly improve our understanding of the molecular mechanisms responsible for the hypoxic ROS generation, increase in $[Ca^{2+}]_i$ and contraction in PASMCs and the diversity of these hypoxic responses in pulmonary and systemic artery myocytes. New data may also help to create novel therapeutic targets for pulmonary hypertension and other related lung diseases.

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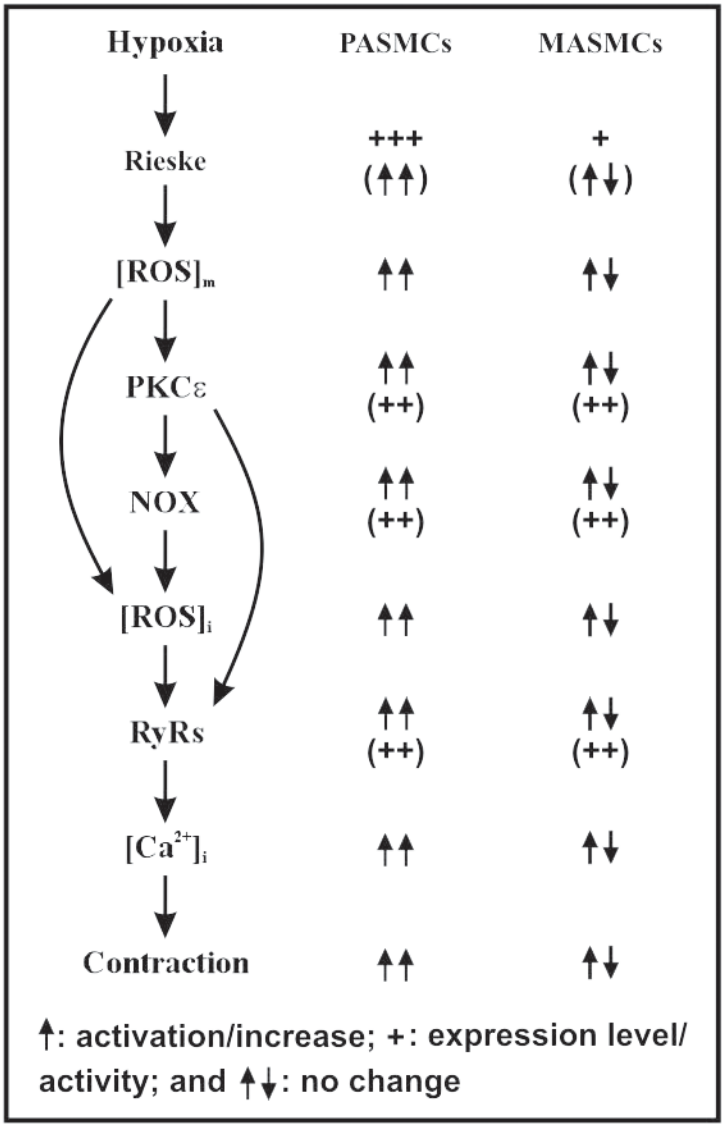

Figure 1.

A diagram depicting a potential important primary hypoxic sensing molecule (Rieske iron–sulfur protein in the mitochondrial complex III), intermediate signaling molecules (ROS, PKCε and NOX) and effectors (RyRs) in the hypoxic Ca^{2+} and contractile responses in PASMCs, and their potential roles in the diversity of hypoxic responses in PASMCs and MASMCs. ROS, reactive oxygen species; PKCε, protein kinase C-ε; NOX, NADPH oxidase; and RyRs, ryanodine receptor Ca^{2+} release channels.