Oxygen-dependent signaling in pulmonary vascular smooth muscle

USHA RAJ1 AND LARISSA SHIMODA2
1Department of Pediatrics, Harbor-University of California at Los Angeles Research and Education Institute, University of California at Los Angeles School of Medicine, Torrance, California 90502; and 2Division of Pulmonary and Critical Care Medicine, Department of Medicine, Johns Hopkins School of Medicine, Baltimore, Maryland 21224

Raj, Usha, and Larissa Shimoda. Oxygen-dependent signaling in pulmonary vascular smooth muscle. Am J Physiol Lung Cell Mol Physiol 283: L671–L677, 2002; 10.1152/ajplung.00177.2002.—The pulmonary circulation constricts in response to acute hypoxia, which is reversible on reexposure to oxygen. On exposure to chronic hypoxia, in addition to vasoconstriction, the pulmonary vasculature undergoes remodeling, resulting in a sustained increase in pulmonary vascular resistance that is not immediately reversible. Hypoxic pulmonary vasoconstriction is physiological in the fetus, and there are many mechanisms by which the pulmonary vasculature relaxes at birth, principal among which is the acute increase in oxygen. Oxygen-induced signaling mechanisms, which result in pulmonary vascular relaxation at birth, and the mechanisms by which chronic hypoxia results in pulmonary vascular remodeling in the fetus and adult, are being investigated. Here, the roles of cGMP-dependent protein kinase in oxygen-mediated signaling in fetal pulmonary vascular smooth muscle and the effects of chronic hypoxia on ion channel activity and smooth muscle function such as contraction, growth, and gene expression were discussed.

fetal and neonatal pulmonary circulation; potassium channels; hypoxia-inducible factor 1; pulmonary vascular remodeling

IN THE FETUS, hypoxia is a physiological stimulus for the pulmonary vasculature to be constricted and the resistance to be high. However, at birth, the factors that promote vasoconstriction in utero must be downregulated, and the mechanisms that dilate the vasculature must become instantly operative. Oxygen is a powerful stimulus that sets into motion a variety of events that result in vasorelaxation. The mechanisms by which the fetal pulmonary vascular smooth muscle cell (PVSMC) responds immediately to oxygen may be unique to the fetus. Sustained exposure to hypoxia in utero and after birth leads to vasoconstriction as well as remodeling of pulmonary vessels, resulting in persistent pulmonary hypertension, which is not immediately ameliorated by oxygen. The mechanisms of vascular remodeling in the fetus and in the adult may be very different. The issues summarized in this report were considered at a featured topic session held during the Experimental Biology 2002 Meeting in New Orleans, LA.

OXYGEN-DEPENDENT SIGNALING IN FETAL PVSMC: ROLE OF cGMP-DEPENDENT PROTEIN KINASE

Control of pulmonary vasomotor tone in the perinatal period. In the transition from fetal to neonatal life, there is an immediate fall in pulmonary vascular resistance, with an almost 8- to 10-fold increase in blood flow (10, 32), which is brought about by active vasodilation of the pulmonary vasculature, induced primarily by exposure to oxygen, the mechanical effects of expansion of the lungs, stretch of the pulmonary blood vessels due to increased blood flow, and the creation of an air-liquid interface. Increased synthesis of vasodilator
agonists such as endothelium-derived nitric oxide (NO), prostacyclin, prostaglandin E₂, and bradykinin results in increased production of intracellular cGMP and cAMP in vascular smooth muscle. Intracellular accumulation of these nucleotides results in smooth muscle relaxation via a variety of mechanisms (Fig. 1). The relative importance of these two nucleotides in inducing pulmonary vasodilation in the perinatal period was investigated by Dhanakoti et al. (6). They found that ovine newborn pulmonary arteries were more sensitive to relaxation induced by cGMP than by cAMP (Fig. 2). Data from several laboratories seem to indicate that the endothelium-derived NO-guanylyl cyclase-cGMP pathway is perhaps the most important mechanism of vasodilation in the pulmonary circulation during the transition from fetal to newborn state (1, 13, 34, 37, 46). Studies in fetal and neonatal ovine pulmonary vessels indicate that cGMP-dependent protein kinase (PKG) is critically important for mediating the action of cGMP in the transitional (fetal to neonatal) pulmonary circulation (6, 12) (Fig. 3). It has also been shown that cAMP can cross activate PKG, indicating that PKG also mediates cAMP-induced relaxation in the pulmonary vasculature.

**Importance of PKG type 1 in the normal fetal to neonatal transition.** In animal models of neonatal pulmonary hypertension, the NO-cGMP pathway has been shown to be impaired (7–9, 24, 35, 38, 43). Furthermore, in some models of hypoxia-induced pulmo-

---

**Fig. 1.** Depiction of intracellular signaling pathways in pulmonary vascular smooth muscle. NO, nitric oxide; PKG, cGMP-dependent protein kinase; PKA, protein kinase A; EDNO, endothelium-derived nitric oxide; ANF, atrial natriuretic factor; AC, adenyl cyclase; GC, guanylyl cyclase; MLC, myosin light chain; MLC-p, phosphorylated myosin light chain.

---

**Fig. 2.** Relaxation responses of intact pulmonary arteries of newborn lambs to 8-bromoguanosine-3’,5’-cyclic monophosphorothioate (Rp-8-Br-PET-cGMPS) and KT-5720 on 8-Br-cGMP-induced relaxation of pulmonary arteries of newborn lambs. Experiments were performed during contraction to endothelin-1 (3 nM). The control tensions (g/mg dry wt of tissue) raised by endothelin-1 were 1.12 ± 0.13, 1.05 ± 0.11, and 1.03 ± 0.06 for untreated (control) or treated with Rp-8-Br-PET-cGMPS or KT-5720, respectively. Changes in tension are expressed as percent contraction to endothelin-1. Data are shown as means ± SE; n = 5 for all except KT-5720, n = 4 animals/group. *Significant difference (P < 0.05) from control.

---

**Fig. 3.** Effects of racemic isomer of β-phenyl-1, N²-etheno-8-bromoguanosine-3’,5’-cyclic monophosphorothioate (Rp-8-Br-PET-cGMPS) and KT-5720 on 8-Br-cGMP-induced relaxation of pulmonary arteries of newborn lambs. Experiments were performed during contraction to endothelin-1 (3 nM). The control tensions (g/mg dry wt of tissue) raised by endothelin-1 were 1.12 ± 0.13, 1.05 ± 0.11, and 1.03 ± 0.06 for untreated (control) or treated with Rp-8-Br-PET-cGMPS or KT-5720, respectively. Changes in tension are expressed as percent contraction to endothelin-1. Data are shown as means ± SE; n = 5 for all except KT-5720, n = 4 animals/group. *Significant difference (P < 0.05) from control.
nary hypertension in the neonatal (2, 42) and adult (18) animal, the vasodilator response of pulmonary vessels to cGMP is specifically impaired. Other reports in the literature indicate that hypoxia attenuates cGMP-mediated effects in vascular smooth muscle (25, 40). It is possible that the impaired pulmonary vascular responses to cGMP in these animal models of neonatal pulmonary hypertension may be explained by impaired PKG activity. Hofmann et al. (26, 33) have developed knockout mice bearing homozygous PKG type 1 null mutations (in both α and β) to eliminate PKG type 1 gene expression and abolish NO-cGMP-dependent vascular relaxation. Notably, in this knockout, there is a very high amount of fetal loss, with most dying immediately after birth. In survivors, other mechanisms of vasodilation must be in play.

Oxygen effect on cGMP-PKG-mediated pulmonary vasodilation. Because, at birth, an increase in oxygen tension is an important biological stimulus, the role of oxygen in upregulating the cGMP-PKG pathway in fetal PVSMC is being actively investigated. We have found that fetal pulmonary vessels exposed to oxygen have an augmented relaxation response to cGMP when preconstricted compared with fetal vessels that have not been exposed to oxygen (Fig. 4). This augmented relaxation response to cGMP is mostly abolished if PKG kinase activity is blocked, suggesting that the effect of oxygen is predominantly on PKG activity. Oxygen can increase PKG activity by affecting the 1) catalytic rate/amount of cGMP binding, 2) dissociation rate of bound cGMP, 3) autophosphorylation of PKG, 4) intracellular location of PKG, 5) oxidation of thiol groups in PKG protein via generation of oxygen radicals, 6) inhibition of substrate binding, 7) proteolytic rate of PKG, 8) conformational change, 9) kinetics of ATP binding, 10) activation of an inhibitor of PKG, 11) binding of other regulatory factors to allosteric site(s), and 12) complexing with other protein factors. These mechanisms need to be investigated.

Oxygen effect on pulmonary vasoactivity via reactive oxygen and nitrogen species. Biologically relevant reactive oxygen species (ROS) include superoxide anion (O₂⁻), hydrogen peroxide (H₂O₂), and hydroxy radical. Approximately 1–3% of all tissue oxygen consumption occurs through the partial reduction of oxygen to O₂⁻, with this species present at a steady-state concentration of ~10 μM, increasing to 0.01–0.1 μM in pathological states (11). Under physiological conditions, antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase, and catalase modulate ambient steady-state levels of ROS (27). In vascular smooth muscle, recent studies show that the majority of O₂⁻ produced is attributable to NAD(P)H oxidases. The cardiovascular NAD(P)H oxidases are low-output, slow-release enzymes with biochemical characteristics that differ considerably from those of neutrophil NADPH oxidase. Also, the vascular enzymes appear to have a moderate constitutive activity that is absent in phagocytes. The kinetics of activation on cellular stimulation are also unique. O₂⁻ is produced in minutes to hours in endothelial cells, vascular smooth muscle cells, and fibroblasts, in contrast to the almost instantaneous release seen in neutrophils (16). In lungs, O₂⁻ inhibits cGMP-mediated vasodilation. In contrast, H₂O₂ augments vasodilation via activation of soluble guanylyl cyclase (3).

ROS and reactive nitrogen species in PVSMC. Biologically relevant nitrogen species include NO and peroxynitrite. Endothelial and vascular smooth muscle cells produce both NO and O₂⁻ (16, 17). These two radicals can recombine to form peroxynitrite. NO reacts more rapidly with O₂⁻ than SOD and can scavenge O₂⁻ very efficiently. Although SOD concentration in cells is 1–10 μM, the rapid reaction of NO with O₂⁻ assures that peroxynitrite is always formed (11). Normally, in the cell, peroxynitrite is converted to nitrosothiols, mainly by reduced glutathione. These thiol intermediates may subsequently regenerate NO and cause vasodilation (21, 31). When cellular glutathione levels are low, as in hypoxia, peroxynitrite is likely to accumulate. Peroxynitrite is a powerful one- and two-electron oxidant that can cause DNA damage. Protein tyrosine nitration by peroxynitrite may interfere with phosphorylation/dephosphorylation signaling pathways or alter protein function (4). In pulmonary arterial smooth muscle cells, production of ROS and NO synthase may dramatically increase during hypoxia (20, 23). Recent studies identify a novel mitochondrial NO synthase (15). A drop in cellular Po₂ may stimulate NO production by this enzyme or lead to liberation of NO from heme-binding sites in cytoplasm and mitochondrial matrix (14), and this NO may readily react with O₂⁻ to form peroxynitrite. Increased production of ROS in hypoxia may decrease intracellular glutathione levels and lead to further peroxynitrite accumulation, due to reduced conversion of peroxynitrite by glutathione into thiol intermediates (5). The net effect might be that during hypoxia, there is greater accumulation of peroxynitrite than under normoxic conditions.

Previously, ROS have been viewed as toxic, leading to DNA damage and lipid oxidation. Recent data suggest that this concept may be incorrect. It is believed
that ROS are produced in a controlled fashion and have critical cellular functions as second messengers. Activation of signaling cascades and redox-sensitive transcription factors leads to induction of many genes in vascular cells (16, 19, 41). No one has studied the role of ROS and/or reactive nitrogen species in modulatingPKG activity, protein levels, and gene expression. Preliminary data were presented to show that in fetal PVSMC, increased levels of peroxynitrite are produced during hypoxia, which decrease PKG protein levels. Additionally, data were also presented to show that exogenous peroxynitrite (1 pM) decreased PKG protein levels in fetal PVSMC by ~30%, suggesting that peroxynitrite is involved in regulation of PKG protein levels during hypoxia.

**Future research directions.** The focus of future studies will be on understanding the mechanisms by which oxygen mediates intracellular signaling in PVSMC. The effects of oxygen on systemic vs. PVSMC appear to be different, an area of future investigation. Furthermore, the effects of oxygen in PVSMC in the developing fetus and newborn appear to be different from that in adult PVSMC, the mechanisms of which need to be determined.

**EFFECT OF HYPOXIA ON ION HOMEOSTASIS IN PVSMC**

Prolonged exposure to decreased oxygen tension, as occurs with many pulmonary diseases, results in pulmonary hypertension, significantly worsening prognosis. Structural remodeling, characterized by smooth muscle cell proliferation, intimal thickening, and extension of smooth muscle into previously nonmuscular arterioles (30), and active contraction of vascular smooth muscle, evidenced by acute reduction in pulmonary arterial pressure in response to vasodilatory agents (22), are commonly observed after long-term hypoxic exposure and contribute to the increase in pulmonary arterial pressure. Despite extensive characterization of the structural and functional changes that occur in pulmonary arteries in response to hypoxia, the exact cellular mechanisms underlying hypoxic pulmonary vasoconstriction, pulmonary arterial smooth muscle cell (PASMC) hypertrophy and hyperplasia, and subsequent development of pulmonary hypertension remain poorly understood.

Abnormalities in PASMC are likely to contribute to pathogenesis of hypoxic pulmonary hypertension. Because ion homeostasis regulates numerous smooth muscle cell functions, including contraction, growth, and gene expression, investigative efforts have focused on elucidating pathways involved in alterations in intracellular K\(^{+}\), Ca\(^{2+}\), and H\(^{+}\) handling in response to chronic hypoxia. To study the effects of prolonged in vivo hypoxia on PASMC function, a murine model of hypoxic pulmonary hypertension was developed (44). Adult male C57B6 mice were placed in a chamber gassed with either room air (normoxic controls) or 10% O\(_{2}\) for 3 wk. Single PASMCs were obtained by enzymatic digestion of intrapulmonary arteries. Using whole cell patch-clamp techniques, we determined that PASMCs from animals exposed to chronic hypoxia exhibit an attenuation of voltage-gated K\(^{+}\) (K\(_{V}\)) channel activity (Fig. 5), similar to results originally demonstrated by Smirnov et al. (36). The observed decrease in K\(_{V}\) current could be due to either a change in channel regulation or a change in channel expression. PCR analysis revealed a significant reduction in the expression of K\(_{V}1.2\) and K\(_{V}1.5\) channel α-subunits in pulmonary arteries from chronically hypoxic animals, suggesting that hypoxia acts to decrease K\(_{V}\) current by repressing K\(_{V}\) channel gene expression. Experiments are currently ongoing to determine the effect of chronic hypoxia on other K\(_{V}\) channel subunits and to identify the mechanisms by which a decrease in O\(_{2}\) can inhibit K\(_{V}\) channel expression.

Under normal conditions, K\(_{V}\) channels are the major regulators of resting membrane potential (E\(_{m}\)) in PASMC (45). The observed reduction in K\(_{V}\) channel activity corresponded to a depolarization of the E\(_{m}\) from ~40 to ~11 mV (Fig. 5C). These results are consistent with those first reported by Suzuki and Twarog (39) in the hypoxic rat model of pulmonary hypertension.

E\(_{m}\) plays a major role in regulating intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)). Thus inhibition of K\(_{V}\) channels and subsequent PASMC depolarization are likely to correspond to a change in cytosolic Ca\(^{2+}\). Indeed, in PASMC loaded with 5 μM fura-2 AM, a Ca\(^{2+}\)-sensitive dye, and subjected to fluorescent microscopy, resting [Ca\(^{2+}\)]\(_{i}\) was significantly elevated af-

Fig. 5. Effect of chronic hypoxia on voltage-gated K\(^{+}\) (K\(_{V}\)) currents and membrane potential (E\(_{m}\)). A: representative traces of K\(_{V}\) current, measured at test potentials from ~50 to +30 mV. Current was measured in whole cell configuration in voltage-clamp mode in the presence of 100 nM charybdotoxin to inhibit Ca\(^{2+}\)-activated K\(^{+}\) currents. B: average peak K\(_{V}\) current normalized to cell capacitance (current density) in pulmonary arterial smooth muscle cells (PASMC) from normoxic mice (n = 8) and chronically hypoxic mice (n = 5). C: bar graph represents resting E\(_{m}\) measured in PASMC from normoxic (n = 8) and chronically hypoxic mice (n = 9). E\(_{m}\) was measured in the whole cell configuration during current-clamp mode (I = 0). *Significant difference from normoxic value (P < 0.05).
Exposure to chronic hypoxia (Fig. 6A). The hypothesis is that this would be due to activation of voltage-gated Ca\(^{2+}\) channels, with hypoxia inducing a shift in \(E_m\) to a range where these channels would open and allow Ca\(^{2+}\) influx into the cell. Consistent with this hypothesis, removal of extracellular Ca\(^{2+}\) caused a rapid decrease in Ca\(^{2+}\). Surprisingly, application of nifedipine (10\(^{-6}\) M) and lanthanum (10\(^{-5}\) M), inhibitors of voltage-gated Ca\(^{2+}\) and nonselective cation channels, respectively, had no effect on resting [Ca\(^{2+}\)]\(_i\) (Fig. 6B). These results suggest that while influx of extracellular Ca\(^{2+}\) is required for maintaining the observed elevated basal [Ca\(^{2+}\)]\(_i\), the mechanism by which this occurs involves Ca\(^{2+}\) handling mechanisms other than voltage-gated Ca\(^{2+}\) and nonselective cation channels.

Elevated [Ca\(^{2+}\)]\(_i\) has been shown to contribute to cell contraction, proliferation, and changes in gene expression. In addition to [Ca\(^{2+}\)]\(_i\), intracellular pH (pHi) has been shown to play a similar modulatory role in smooth muscle cell function. The pHi was measured in PASMC from normoxic and chronically hypoxic mice using the pH-sensitive fluorescent dye 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein-AM (5 μM). Basal pHi was found to be significantly elevated in PASMC after exposure to chronic hypoxia (Fig. 7A). Although several transporters participate in pH homeostasis, studies by Quinn et al. (28) clearly demonstrated that activation of Na\(^+\)/H\(^+\) exchange was required for PASMC proliferation in response to growth factors and that inhibitors of Na\(^+\)/H\(^+\) exchange reduced pulmonary vascular remodeling in chronically hypoxic rats (29). With the use of the ammonium pulse technique, Na\(^+\)/H\(^+\) exchange activity, measured as the Na\(^+\)-dependent recovery from NHCl\(_4\)-induced acidosis, was found to be significantly increased in PASMC from chronically hypoxic mice (Fig. 7B). These data suggest that increased Na\(^+\)/H\(^+\) exchange activity in response to chronic hypoxia could account for the increase in basal pHi.

In summary, the above data indicate that prolonged exposure to alveolar hypoxia results in marked alter-

---

**Fig. 6.** Effect of chronic hypoxia on resting intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) in PASMC. A: bar graph represents resting [Ca\(^{2+}\)]\(_i\), measured with fura 2-AM in PASMC from normoxic (n = 10) and chronically hypoxic mice (n = 13). B: representative traces demonstrate the effect of removal of extracellular Ca\(^{2+}\) and application of nifedipine, a voltage-gated Ca\(^{2+}\) channel antagonist, or lanthanum, an inhibitor of nonselective cation channels, on resting [Ca\(^{2+}\)]\(_i\) in PASMC isolated from chronically hypoxic mice. *Significant difference from normoxic value (\(P < 0.05\)).

**Fig. 7.** Effect of chronic hypoxia on intracellular pH (pHi). Basal pHi, monitored in PASMC isolated from normoxic (n = 117) and chronically hypoxic mice (n = 53), was significantly higher after exposure to chronic hypoxia. B: bar graph showing the effect of chronic hypoxia on Na\(^+\)/H\(^+\) exchanger activity. Na\(^+\)/H\(^+\) exchanger activity was measured as the rate of Na\(^+\)-dependent recovery from NHCl\(_4\)-induced acidosis. PASMC from chronically hypoxic mice (n = 25) had significantly higher Na\(^+\)/H\(^+\) exchanger activity compared with PASMC isolated from normoxic mice (n = 36). In cells from both normoxic and chronically hypoxic mice, addition of ethylisopropyl amiloride (EIPA; 10\(^{-5}\) M), an inhibitor of Na\(^+\)/H\(^+\) exchanger, significantly reduced Na\(^+\)/H\(^+\) exchanger activity. All experiments were performed in the absence of bicarbonate. *Significant difference from normoxic value (\(P < 0.001\)); **significant difference from control value (\(P < 0.001\)).
ations in PASMC ion homeostasis. These changes in intracellular $K^+$, $Ca^{2+}$, and $H^+$ concentrations create conditions that promote PASMC contraction and proliferation and are likely to contribute to the pathogenesis of hypoxic pulmonary hypertension.

U. Raj was supported by National Heart, Lung, and Blood Institute (NHLBI) Grants HL-59435 and HL-47804. Investigators on the work presented by Dr. Raj were Dr. Yuansheng Gao, Dr. Sri Dhanakoti, and Fred Sander. L. Shimoda was supported by American Heart Association Scientist Development Grant AHA9930255N and by NHLBI Grant HL-67919.

**REFERENCES**


