Hypoxia divergently regulates production of reactive oxygen species in human pulmonary and coronary artery smooth muscle cells

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Wu W, Platoshyn O, Firth AL, Yuan JX. Hypoxia divergently regulates production of reactive oxygen species in human pulmonary and coronary artery smooth muscle cells. Am J Physiol Lung Cell Mol Physiol 293: L952–L959, 2007. First published August 10, 2007; doi:10.1152/ajplung.00203.2007.—Acute hypoxia causes pulmonary vasoconstriction and coronary vasodilation. The divergent effects of hypoxia on pulmonary and coronary vascular smooth muscle cells suggest that the mechanisms involved in oxygen sensing and downstream effectors are different in these two types of cells. Since production of reactive oxygen species (ROS) is regulated by oxygen tension, ROS have been hypothesized to be a signaling mechanism in hypoxia-induced pulmonary vasoconstriction and vascular remodeling. Furthermore, an increased ROS production is also implicated in arteriosclerosis. In this study, we determined and compared the effects of hypoxia on ROS levels in human pulmonary arterial smooth muscle cells (PASMC) and coronary arterial smooth muscle cells (CASMC). Our results indicated that acute exposure to hypoxia (P\textsubscript{O2} = 25–30 mmHg for 5–10 min) significantly and rapidly decreased ROS levels in both PASMC and CASMC. However, chronic exposure to hypoxia (P\textsubscript{O2} = 30 mmHg for 48 h) markedly increased ROS levels in PASMC, but decreased ROS production in CASMC. Furthermore, chronic treatment with endothelin-1, a potent vasoconstrictor and mitogen, caused a significant increase in ROS production in both PASMC and CASMC. The inhibitory effect of acute hypoxia on ROS production in PASMC was also accelerated in cells chronically treated with endothelin-1. While the decreased ROS in PASMC and CASMC after acute exposure to hypoxia may reflect the lower level of oxygen substrate available for ROS production, the increased ROS production in PASMC during chronic hypoxia may reflect a pathophysiological response unique to the pulmonary vasculature that contributes to the development of pulmonary vascular remodeling in patients with hypoxia-associated pulmonary hypertension.

INTRACELLULAR PRODUCTION of reactive oxygen species (ROS), such as superoxide (O\textsuperscript{2-}) and hydrogen superoxide (HO\textsuperscript{2-}), formed from sequential transfer of electrons from molecular oxygen, in vascular smooth muscle cells plays an important role in the physiological regulation of vascular tone and vascular remodeling (20, 22). These reactive intermediates act as progenitors for other forms of ROS such as hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}), peroxynitrite (ONOO\textsuperscript{-}), and the hydroxyl radical. There are currently debates as to the nature of the dysregulation of ROS production that may contribute to pathophysiological conditions, such as pulmonary hypertension and atherosclerosis, and the disturbance of normal vascular function. Not surprisingly, the mechanisms by which hypoxia causes pulmonary vessels to constrict and coronary vessels to relax are therefore an active area of research.

It is now known that hypoxic pulmonary vasoconstriction (HPV) is an intrinsic property of the pulmonary vasculature and specifically resides in pulmonary arterial smooth muscle cells (PASMC) (31, 34). It has been reported that hypoxia inhibits voltage-gated K\textsuperscript{+} (K\textsubscript{v}) currents in PASMC (39, 58), and the hypoxia-induced closure of K\textsubscript{v} channels appears to result from a change in cytoplasmic ROS or redox status in PASMC (4). Channels comprising the K\textsubscript{v}1.5 subunit are implicated in such mechanisms (6, 26, 38). Despite extensive research into the mechanisms of HPV, there is currently no single mechanism that can entirely explain the phenomenon (41, 53). How O\textsubscript{2} levels are detected in PASMC and the subsequent signaling pathways contributing to pulmonary arterial constriction remain debated. Since their production is dependent on cellular oxygen concentration and redox status, changes in ROS have been hypothesized to be an important signaling mechanism in HPV. Potentially, changes in cellular ROS levels can sequentially modulate ion channel function and lead to pulmonary vasoconstriction.

It has been indicated that reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is an important source of increased O\textsubsuperscript{2+} in response to hypoxia (32); however, an increase in mitochondrially derived ROS is also widely supported (50). This opposes the original hypothesis that favors a decrease in ROS predominantly due to a reduction of O\textsubscript{2+} production resultant of the decreased O\textsubscript{2} supply to the mitochondria (5, 33), and, furthermore, other groups still dispute that ROS changes at all in response to hypoxia (11). NADPH oxidases have materialized as a prominent source of ROS in the vasculature and cardiovascular system (2, 9, 16). Vascular smooth muscle cells from resistance arteries are known to contain NOX2, a nonphagocytic NADPH oxidase protein that consists of a membrane-bound cytochrome b\textsubscript{558} including the catalytic gp91\textsuperscript{phox} and the p22\textsuperscript{phox} subunits, with cytosolic components including p47\textsuperscript{phox}, p67\textsuperscript{phox}, and the small Rho guanosine triphosphatase (GTPase) Rac (25). The specific subcellular localization of NADPH oxidase may be an important determinant of the fate of NADPH-derived ROS production and ROS-dependent signaling pathways. Indeed, recently NADPH has been identified as being bound to the β-subunit of K\textsubscript{v} channels (18, 19, 36); the oxidation of NADPH to NADPH\textsuperscript{+} confers a structural change to the channel complex conferring acceleration of the channel inactivation kinetics. Such data consolidate a link between ROS and K\textsuperscript{+} channels and thus the excitability of cells.
Notably, endothelin (ET)-1, which has been shown to be upregulated during hypoxia (29), has been demonstrated to mediate pulmonary vasoconstriction via NADPH oxidase-derived superoxide and is an important mediator contributing to the pathogenesis of chronic hypoxia (CH)-induced pulmonary hypertension (30, 51). ET-1 is additionally implicated in the elevation of ROS in cardiac myocytes (57) via ETA receptor-mediated regulation of NADPH oxidase activity and in NADPH oxidase-dependent smooth muscle cell proliferation (51). Another established activator of NADPH oxidases is angiotensin II (ANG II), the incongruous effects of which are attributed to the excessive production of ROS (15, 40).

The objective of this study was therefore to investigate whether there is a putative role for hypoxia-induced elevation of ROS in the mechanisms of HPV by 1) determining whether and how ROS levels change during hypoxia in PASMC and 2) comparing how hypoxia affects ROS levels in pulmonary vs. coronary vasculature, which vasoconstrict and vasodilate in response to hypoxia, respectively.

MATERIALS AND METHODS

Cell preparation and culture. Cell lines of normal human PASMC and coronary arterial smooth muscle cells (CASMC) were purchased from Clonetics (now Lonza Walkersville, Walkersville, MD). Cells were cultured in 75-cm² flasks with smooth muscle growth medium (SmGM, Cambrex) supplemented with 10% fetal bovine serum (FBS), 0.5 ng/ml human epidermal growth factor, 2 ng/ml human fibroblast growth factor, 5 µg/ml insulin, and 50 µg/ml gentamycin sulfate. Cells were maintained in an incubator under a humidified atmosphere of 5% CO²-95% air at 37°C and used between passages 4 and 8. The medium was changed after 24 h and every 48 h thereafter. Cells were subcultured onto 25-cm² coverslips with trypsin-EDTA buffer (Cambrex) at 60% confluence and allowed to adhere for a minimum of 12 h. Cells were then fed with fresh medium and grown for 24 h to allow recovery from trypsinization before experiments commenced.

ROS detection. Intracellular ROS was detected via the dye dihydroethidium (DHE; Molecular Probes). Unreacted DHE permeates live cells to accumulate as blue fluorescence in the cytoplasm. DHE can be oxidized by superoxide to a specific fluorescent product (ethidium/oxyethidium) that intercalates with DNA, accumulating as red fluorescence in the nucleus. Oxyethidium has an additional oxygen atom in its molecular structure compared with ethidium (59).

Cells grown on coverslips were mounted on a Nikon Eclipse TE-200 fluorescence microscope and superfused with normoxic physiological salt solution (PSS) during the initial 30 s of imaging. PSS included (in mM) 141 NaCl, 4.7 KCl, 1.8 CaCl₂, 1.2 MgCl₂, 10 HEPES, and 10 glucose (pH 7.4). Images were captured every 2 s to minimize photobleaching via a Photometrix digital camera, and the average fluorescence intensities for individual cells were quantified with NIH ImageJ software. The fluorescence intensity of cells loaded with DHE is expressed as arbitrary units (a.u.), and changes in the fluorescence are expressed as changes in arbitrary units.

Hypoxic exposure. Acute hypoxia was achieved as described previously (58). Briefly, cells were incubated with 80 µM DHE in the medium at 37°C for 15 min before hypoxic treatment. Cells were perfused with normoxic PSS during the initial 30 s of imaging. Hypoxic conditions were established by bubbling the superfusion solution with N₂ (100%) for 15 min to achieve oxygen tension (PO₂) ranging from 25 to 30 mmHg at 24°C. PO₂ was quantified with an oxygen electrode (Microelectrodes, Londonderry, NH) positioned in the cell chamber on the microscope stage to continuously monitor PO₂ as previously shown (58). For the CH experiments, cells cultured in serum-free smooth muscle basal medium (SmBM, Cambrex) were incubated in a chamber for 48 h at 37°C in normoxia (PO₂ = 140 mmHg) or hypoxia (PO₂ = 30 mmHg). DHE (80 µM) was added to the medium and also incubated for 15 min before imaging. In the positive control experiments, PASMC and CASMC were treated with 200 nM ANG II for 8 h to induce superoxide production (15). Polyethylene glycol-superoxide dismutase (SOD) (100 U/ml) was used to catalyze the conversion of superoxide to H₂O₂, verifying the fluorescence signal as superoxide (13). In experiments using 1 µM ET-1, cells were incubated for 48 h.

Statistical analysis. The combined data are expressed as means ± SE. Statistical analysis was performed with the unpaired Student’s t-test. Statistical differences were deemed to be significant at P < 0.05.

RESULTS

To validate the protocol for the detection of intracellular changes in the oxidative state in PASMC with DHE, positive controls were established. Over a period of 15 min, oxidation of DHE was observed by the change in the predominantly cytosolic blue DHE fluorescence to red ethidium/oxyethidium fluorescence concentrated in the nucleus, representative of the basal ROS concentration in the cell (Fig. 1A). The dye was therefore left to equilibrate for 15 min preceding the commencement of any experiment. Changes in fluorescence intensity in a single PASMC after treatment were used in this study to demonstrate an increase or decrease of ROS production in response to different stimuli. Using this method, we found that extracellular application of ANG II significantly increased ROS production in CASMC, while SOD partially inhibited the ANG II-mediated increase in ROS level (Fig. 1B).

Acute hypoxia significantly reduced superoxide production in human PASMC and CASMC. Acute hypoxia, established by superfusing with N₂-equilibrated PSS (PO₂ = 30 mmHg), rapidly reduced ROS production in both PASMC (n = 14) and CASMC (n = 30) (Fig. 2, A and B). The decreased intensity over a 14-min period is expressed as raw whole cell fluorescence (Fig. 2, A and B, left) and normalized to the basal fluorescence at time 0 min (Fig. 2, A and B, right) for PASMC (Fig. 2A) and CASMC (Fig. 2B) to facilitate comparison between the cell types. The average kinetic profiles of the acute hypoxia-induced decreases in fluorescence intensity were comparable in PASMC and CASMC (Fig. 2C).

ET-1, an endothelium-derived contracting factor proposed to be an important trigger in the development of HPV and hypoxia-associated pulmonary hypertension (30), is also known to stimulate intracellular superoxide production (51). A significant increase in PASMC ROS was stimulated by chronic treatment (48 h) with ET-1 [from 28.9 ± 2.6 a.u. (n = 14) in control to 38.3 ± 1.7 a.u. (n = 31) after chronic ET-1 treatment; P < 0.01] (Fig. 3, A and B). Subsequent exposure to acute hypoxia caused a significant (P < 0.001) time-dependent decrease in ROS levels in ET-1-treated PASMC (in which superoxide concentration was higher in both nuclei and cytosol) (Fig. 3, A and C–F). This decrease occurred at a significantly enhanced rate compared with control (Fig. 3D).

These results confirm that acute hypoxia reduces the level of ROS in PASMC, either under basal or physiological conditions or under pathophysiological conditions when superoxide production is increased by ET-1. On the other hand, ET-1 had no effect on ROS production in CASMC, with only a small, insignificant decrease in whole cell fluorescence observed (data not shown).
Chronic hypoxia significantly increases ROS levels in human PASMC but decreases ROS levels in human CASMC. Exposure of PASMC to CH (48 h), however, significantly increased superoxide production \[27.6 \pm 1.2 \text{ a.u.} (n = 28)\] and after CH treatment \[21.3 \pm 1.0 \text{ a.u.} (n = 100)\] (\(P < 0.01\) for both compared with normoxic control).

In contrast, incubation in CH decreased ROS production in CASMC from \[39.1 \pm 2.9 \text{ a.u.} (n = 57)\] in normoxia to \[27.7 \pm 1.3 \text{ a.u.} (n = 77)\] in CH (\(P < 0.01\)) (Fig. 5, A and B). Moreover, ET-1 had no significant effect on superoxide production in either normoxic or hypoxic conditions in CASMC (Fig. 5C), in contrast to PASMC, in which ET-1 significantly increased superoxide (Fig. 4). ANG II is a known activator of superoxide production in CASMC (15); incubation with ANG II instead of ET-1 did cause a significant increase in mean whole cell fluorescence \[18.6 \pm 1.2\] and \[34.9 \pm 2.8 \text{ a.u.} in absence and presence of ANG II, respectively; \(P < 0.001\)) and this increase included a substantial SOD-dependent component reflecting the catalysis of reactive intermediates such as superoxide to more stable \(\text{H}_2\text{O}_2\) (also see Fig. 1C) (59). Interestingly, SOD did significantly decrease whole cell fluorescence in both normoxic and hypoxic conditions compared with control fluorescence \(P < 0.01\), indicative of a decrease in superoxide from basal/control levels.

In contrast, incubation in CH decreased ROS production in CASMC from \[39.1 \pm 2.9 \text{ a.u.} (n = 57)\] in normoxia to \[27.7 \pm 1.3 \text{ a.u.} (n = 77)\] in CH (\(P < 0.01\)) (Fig. 5, A and B). Moreover, ET-1 had no significant effect on superoxide production in either normoxic or hypoxic conditions in CASMC (Fig. 5C), in contrast to PASMC, in which ET-1 significantly increased superoxide (Fig. 4).

**Fig. 2.** Acute hypoxia decreases ROS in both PASMC and CASMC. Averaged time-dependent decrease in whole cell fluorescence (left) and decrease normalized to basal fluorescence \((F/F_0)\) in normoxia for comparison (right) in PASMC \((A; n = 14)\) and CASMC \((B; n = 30)\). C: comparison of the normalized average kinetics of the acute hypoxia-mediated decrease in ROS over 15 min in PASMC (red) and CASMC (blue).

**Fig. 1.** Verification of the use of dihydroethidium (DHE) to measure changes in intracellular reactive oxygen species (ROS) production or ROS levels in pulmonary arterial smooth muscle cells (PASMC) and coronary arterial smooth muscle cells (CASMC). A: representative PASMC showing cytosolic blue DHE fluorescence; after 15 min, the DHE has been oxidized by ROS to be visible as red fluorescence predominantly localized in the nucleus where the oxidized ethidium/oxyethidium intercalated with DNA. The level of oxidized ethidium/oxyethidium fluorescence after 15 min was considered to be the basal ROS level and corresponds to time point 0 min in all experiments. Overlay of left and right panels shows colocalization of the respective fluorescence. B: summarized data (means \(\pm SE\) show mean whole cell fluorescence in control CASMC (open bar; \(n = 28\)) and CASMC treated with angiotensin II (A-II, filled bar; \(n = 27\)) and A-II + superoxide dismutase (SOD, gray bar; \(n = 21\)). ***\(P < 0.001\) vs. control.
DISCUSSION

A putative involvement of increased ROS in CH-induced pulmonary hypertension is consolidated in this study, where the data indicate that 1) CH induced opposite effects on superoxide production in PASMC and CASMC and 2) agonist ET-1 increased superoxide levels in PASMC but not in CASMC. The differences in superoxide production in response to hypoxia (and ET-1 treatment) between the pulmonary and coronary circulation may have significant physiological implications in terms of the effect of acute hypoxia and CH on the contractility of different vascular beds. Furthermore, the data also provide evidence for a differential regulation of superoxide production during acute hypoxia and CH in PASMC: acute hypoxia decreased basal and agonist (ET-1)-induced superoxide production, whereas CH increased superoxide production and additionally blocked ET-1-mediated increases in superoxide.

Acute hypoxia reduces superoxide in both PASMC and CASMC. The data from this study indicate that a decrease in superoxide production is involved in acute hypoxia-induced pulmonary vasoconstriction. The change in fluorescence in our experiments can be largely attributed to altered superoxide production converting DHE to oxyethidium, although ethidium is also produced, reflecting the more general cellular redox state (59). The production of oxyethidium and subsequent change in fluorescence is known to be inhibited by SOD and is not sensitive to changes in H$_2$O$_2$ concentration; therefore, the SOD-dependent component of DHE fluorescence can be attributed to a single electron reduction of molecular oxygen to superoxide (13). In our experiments, the change in ROS detected by DHE oxidation should therefore have a SOD-sensitive component more selective for superoxide and a SOD-insensitive component likely to be H$_2$O$_2$ dependent (12).

While the debate continues over a decrease (52) or paradoxical increase (24, 48) in ROS during hypoxia, it is possible that ROS are not involved in acute HPV. In our experiments acute hypoxia decreased superoxide in both PASMC and CASMC, while it is known to induce pulmonary vasoconstriction but coronary vasodilation. This notion is strengthened by the fact that, as previously reported, increase in ROS takes place over several minutes while the onset of HPV occurs within 7 s (27). Although more evidence is needed, it is possible that decreased superoxide triggers a mechanism causing PASMC contraction [e.g., via inhibition of K$_v$ channels and/or activation of transient receptor potential (TRP) cation channels] but causes CASMC relaxation [e.g., via increased cGMP and nitric oxide (42), Ca$^{2+}$ desensitization (49), and/or a lowering of cytosolic NADPH levels (21)].

Previous work in our laboratory, supported by others, has demonstrated a decrease in K$_v$1.5 currents in response to acute hypoxia specific to the pulmonary circulation or PASMC; no response was observed in mesenteric arterial SMC (26, 37, 58),
which is also difficult to attribute to the similar decreased superoxide production in PASMC and CASMC observed in this paper. However, more recently, a Kv channel β-subunit that resembles a functional NADPH-dependent enzyme to catalyze redox reactions has been identified (8, 36, 55). It is possible that the presence of this β-subunit in PASMC associated with the “hypoxic-sensitive” Kv channel α-subunits may account for the selectivity of the effect of hypoxia in pulmonary but not systemic (or coronary) arteries. On the other hand, it is also possible, and perhaps more likely, that the acute hypoxic changes in superoxide are not directly linked to the contractility of the pulmonary vessels. Acute hypoxia is also known to increase cytosolic free Ca²⁺ concentration ([Ca²⁺]cyt) in PASMC (but not in aortic SMC) via capacitative Ca²⁺ entry through TRP cation channels, a mechanism that may or may not be dependent on cellular redox state (35, 45). The decrease in superoxide in response to hypoxia most likely reflects the lower level of oxygen available as a substrate for oxygen radical production, leading to a reduced cellular redox

Fig. 4. Chronic hypoxia increases ROS production in PASMC. A: representative image of PASMC on a coverslip under basal conditions (control; top left), after chronic hypoxia (top right), in normoxia after chronic ET-1 treatment (bottom left), and in normoxia after ET-1 and SOD treatment (bottom right). B: histograms reflecting the spread of individual cell fluorescence in normoxia (top; n = 111) and after chronic hypoxia (bottom; n = 49). C: comparison of the effect of ET-1 and SOD on ROS production in normoxic (left; n = 111, 105, and 120 for control, ET-1, and ET-1 + SOD, respectively) and chronic hypoxic (right; n = 49, 110, and 100 for control, ET-1, and ET-1 + SOD, respectively) conditions. Dashed line indicates basal fluorescence at the start of the experiments. Significant changes compared with control (basal) levels: **P < 0.01 vs. normoxic control.

Fig. 5. Chronic hypoxia decreases ROS production in CASMC. A: representative image of CASMC on a coverslip under basal conditions (control), after chronic hypoxia, in normoxia after chronic ET-1 treatment (clockwise from top left). B: histograms showing the spread of individual cell fluorescence in normoxia (top; n = 57) and after chronic hypoxia (bottom; n = 82). C: comparison of the effect of ET-1 and SOD on ROS production in normoxic (left; n = 57, 72, and 38 for control, ET-1, and ET-1 + SOD, respectively) and chronic hypoxic (right; n = 82, 77, and 64 for control, ET-1, and ET-1 + SOD, respectively) conditions. Dashed line indicates basal fluorescence at the start of the experiments. Significant changes compared with control (basal) levels: **P < 0.01 vs. normoxic control.
state. This is consistent with the expected chemical effects of lower \( \text{PO}_2 \) on ROS levels. Clarification of these mechanisms specific to HPV requires further experimentation.

**Chronic hypoxia divergently affects ROS production in PASMC and CASMC.** The increased superoxide in PASMC during CH may, however, reflect a pathophysiological response unique to the pulmonary circulation that contributes to sustained pulmonary vasoconstriction and excessive pulmonary vascular remodeling in patients with hypoxia-associated pulmonary hypertension, because such increased levels were not observed in CASMC (Figs. 4 and 5).

There is a large body of evidence supporting an increase in PASMC ROS during CH (32, 50). In support of our data, a fluorescence resonance energy transfer (FRET)-based intracellular sensor has recently shown a hypoxia-induced increase in ROS (23). It is proposed that this increase in ROS is unlikely to act directly on \( \text{K}_v \) channels but may influence their activity or expression indirectly by regulating ET-1 signaling (47). ET-1, which is shown to be upregulated during hypoxia (29), has been demonstrated to mediate pulmonary vasoconstriction via NADPH oxidase-derived superoxide and is importantly proposed to contribute to HPV and the pathogenesis of CH-induced pulmonary hypertension (30, 51). ET-1 also stimulates PASMC proliferation, an effect likely to be caused by its augmentation of ROS production (51), which may contribute to the vascular remodeling observed in pulmonary hypertension. Furthermore, in ovine fetal PASMC (17), inhibition of NADPH oxidase induces apoptosis and prevents ET-1-mediated SMC proliferation. In our experiments, ET-1 did increase superoxide concentration, mimicking the effects observed with CH in PASMC. The opposite effect was observed in CASMC, where ET-1 did not increase supr O2 concentration, suggesting that the regulation of ROS production may differ between the two cell types (Fig. 5D). Therefore, ET-1 increased by hypoxia has very different effects on ROS in coronary and pulmonary vascular smooth muscle cells, and this, in part, may account for the opposite responses (coronary vasodilation vs. pulmonary vasoconstriction) to CH. Moreover, differences between systemic and pulmonary artery ROS/superoxide production in response to sustained hypoxia may be due to differential regulation of NADPH oxidases (or there may be differences in SOD activity). In pulmonary artery, both NOX1 and NOX4 are present (10) and superoxide production by these NOX isoforms is dependent on direct interaction with \( \text{p22}^{\text{phox}} \) (3), \( \text{p22}^{\text{phox}} \)-based NADH/NADPH oxidase is also implicated in the pathogenesis of atherosclerotic coronary artery disease (7, 56). However, it is possible that similar interaction is differentially regulated by hypoxia in the coronary and pulmonary circulation (or CASMC and PASMC). Both coronary and pulmonary arteries have been shown to express NOX isoforms at similar levels; however, increased superoxide production may be due to higher basal cytosolic NADPH in pulmonary arteries (21), or the subcellular localization of NOX isoforms may be crucial to the activation of downstream signaling in response to increased ROS. Such pathways remain to be fully defined.

Other possibilities exist for the divergent effects of hypoxia on ROS and vascular function. For example, TRP expression and function are also known to be upregulated in PASMC during CH. Effects may, in part, be attributed to the increased superoxide production that we and others have observed. First, ROS are known to activate adenylyl cyclase that can increase cyclic ADP-ribose (cADPR), activate ryanodine receptors, and deplete intracellular \( \text{Ca}^{2+} \) stores (e.g., sarcoplasmic/endoplasmic reticulum); sustained store depletion then activates store-operated TRP channels (notably TRPC1, 4, and 6) and causes increase in \( \text{Ca}^{2+} \)-influx (44). Furthermore, increased TRP channel expression induced by hypoxia can be regulated by hypoxia-inducible factor 1, an hypoxia-induced transcription factor that is known to be upregulated by increased superoxide (1, 46), and may therefore have profound effects on hypoxia-associated pulmonary hypertension. In addition, CH-mediated pulmonary vascular remodeling may at least partly result from (or relate to) the observed increase in production of superoxide; this may subsequently activate the MAPK pathway and cause cell proliferation, as reported in many cell types such as human, rat, and mouse pulmonary, but not systemic, fibroblasts (54); alveolar epithelial cells (43); and cancerous cells (14). Additionally, p38 MAPK plays an important role in mediating hypoxia-induced contraction of the rat pulmonary artery (28).

**Concluding remarks.** Amid an active debate on the “ups and downs” of ROS during hypoxia, the data presented in this paper provide a possible explanation for the regulation and involvement of ROS in hypoxia. During acute hypoxic exposure, ROS, particularly superoxide, decrease in both coronary and pulmonary vascular smooth muscle cells and are thus unlikely to be highly influential over the vessel contractility in both circulations that are known to dilate and constrict to hypoxia, respectively. However, superoxide regulation during more sustained, chronic periods of hypoxia is differentially regulated, increasing in PASMC and decreasing in CASMC. The increased ROS production in PASMC during CH may indeed reflect a pathophysiological response unique to pulmonary circulation that contributes to hypoxia-induced pulmonary vasoconstriction and vascular remodeling observed in patients with CH-mediated pulmonary hypertension.

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