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Role of mitochondrial reactive oxygen species in hypoxia-dependent increase in intracellular calcium in pulmonary artery myocytes

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Abstract

Previous studies examining the role of mitochondria-derived reactive oxygen species (ROS) in hypoxic responses have been mainly conducted in isolated lungs and cultured pulmonary artery smooth muscle cells (PASMCs) using mitochondrial inhibitors, and yielded largely conflicting results. Here we report that in freshly isolated mouse PASMCs, which are devoid of the mixed responses from multi-types of cells in lungs and significant changes in gene expression in cultured cells, the mitochondrial electron transport chain (ETC) complex I, II or III inhibitors blocked hypoxia-induced increases in intracellular ROS and Ca^{2+} concentration ($[\text{ROS}]_i$ and $[\text{Ca}^{2+}]_i$) without effects on their resting levels. Inhibition of the complex I plus II and/or III did not produce an additive effect. Glutathione peroxidase-1 (Gpx1) or catalase gene overexpression to enhance H_2O_2 removal remarkably reduced hypoxic increase in $[\text{ROS}]_i$ and $[\text{Ca}^{2+}]_i$, whereas Gpx1 gene deletion had the opposite effect. None of these genetic modifications changed the resting $[\text{ROS}]_i$ and $[\text{Ca}^{2+}]_i$. H_2O_2 at 51 μM caused a similar increase in DCF fluorescence ($[\text{ROS}]_i$) to that by hypoxia, but only induced 33% of hypoxic increase in $[\text{Ca}^{2+}]_i$. Moreover, H_2O_2 (5.1 μM) reversed the inhibition of the hypoxia-induced increase in $[\text{Ca}^{2+}]_i$ by rotenone. Collectively, our study using various mitochondrial inhibitors and genetic approaches demonstrate that in response to acute hypoxia, the mitochondrial ETC molecules prior to the complex III ubiquinone site act as a functional unit to increase the generation of ROS, particularly H_2O_2 , which is important for, but may not fully cause the hypoxic increase in $[\text{Ca}^{2+}]_i$ in freshly isolated PASMCs.

Keywords

hypoxia; mitochondria; reactive oxygen species; intracellular calcium; pulmonary artery smooth muscle cell

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INTRODUCTION

It is well recognized that an increase in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in pulmonary artery smooth muscle cells (PASMCs) is a primary and necessary element for hypoxic pulmonary vasoconstriction (HPV) and associated pulmonary hypertension. The hypoxia-induced increase in $[\text{Ca}^{2+}]_i$ in PASMCs results from intracellular Ca^{2+} release and extracellular Ca^{2+} influx associated with the inhibition of voltage-dependent K^+ channels and/or activation of store-operated cation channels [22]. However, the molecular processes linking hypoxia to an increase in $[\text{Ca}^{2+}]_i$ in PASMCs are not completely understood.

Recent studies have suggested that the mitochondrial electron transport chain (ETC) is involved in hypoxic contraction in isolated lungs and cultured PASMCs by affecting the generation of reactive oxygen species (ROS). However, experimental data have not only been mainly created by using mitochondrial inhibitors, but are also conflicting. It has been reported that the ETC complex I inhibitor rotenone or complex III post-ubisemiquinone site inhibitor antimycin A mimics hypoxic responses, causing a decrease in intracellular reactive oxygen species (ROS) concentration ($[\text{ROS}]_i$) and HPV in isolated rat lungs [3,23]. In contrast, Waypa *et al* have shown that rotenone and the ETC complex III pre-ubisemiquinone site inhibitor myxothiazol do not mimic, but block hypoxia-induced contraction in cultured rat PASMCs. These authors have also found that hypoxia may cause an increase, rather than a decrease in intracellular ROS generation in cultured rat PASMCs [34,35]. The lungs are apparently composed of a variety of cell types, which may produce distinct responses to hypoxic stimulation. As a result, experimental findings in lungs present the mixed responses from various types of cells, rather than defined responses observed in PASMCs [28]. Studies using cultured PASMCs have been criticized, because cultured cells undergo significant changes in expression levels and patterns of hypoxia-responsible genes [24].

In this study, we first sought to utilize freshly isolated mouse PASMCs to systemically examine whether the mitochondrial ETC complex I, II, III and IV inhibitors could mimic and then block, or block but not mimic hypoxia-induced changes in $[\text{ROS}]_i$ and $[\text{Ca}^{2+}]_i$. It is known that in mitochondria, superoxide ($\text{O}_2^{\cdot-}$) is generated through the ETC and then rapidly converted to hydrogen peroxide (H_2O_2) by manganese superoxide dismutase. H_2O_2 can be degraded by glutathione peroxidase-1 (Gpx1) in mitochondria and the cytosol, as well as by catalase in the cytosol [8,12]. Apparently, overexpression and deletion of these endogenous molecules may specifically modify intracellular ROS levels. To complement studies using mitochondrial inhibitors, thus, we next investigated the effects of genetic modifications of mitochondrial and cytosolic H_2O_2 generation by Gpx1 and/or catalase gene overexpression and deletion on hypoxia-induced change in $[\text{ROS}]_i$ and $[\text{Ca}^{2+}]_i$ as well as the resting $[\text{ROS}]_i$ and $[\text{Ca}^{2+}]_i$ (under normoxic conditions) in freshly isolated mouse PASMCs. Finally, we wanted to determine whether mitochondria-derived ROS was sufficient on its own to fully account for hypoxic responses in freshly isolated mouse PASMCs. To achieve this goal, we examined and compared hypoxia- and exogenous H_2O_2 -induced increase in $[\text{Ca}^{2+}]_i$, and then studied the effects of exogenous H_2O_2 on the inhibition of hypoxic change in $[\text{Ca}^{2+}]_i$ by mitochondrial inhibitors in freshly isolated mouse PASMCs.

MATERIAL AND METHODS

Cell preparation

Freshly isolated SMCs of mouse resistance pulmonary arteries (3rd and smaller branches) were obtained as reported previously [40]. In brief, male Swiss-Webster mice from Taconic (Germantown, NY) were euthanized by an intraperitoneal injection of sodium pentobarbital (150 mg/kg) under approved Animal Care and Use Protocol of Albany Medical College. Resistance pulmonary arteries were carefully dissected of the endothelium and connective

tissue in ice-cold, oxygenated physiological saline solution (PSS), and then cut into small pieces. The arteries were incubated in low Ca^{2+} PSS (mg/ml) containing 1.5 papain, 0.4 dithioerythritol, 1.0 bovine serum albumin (BSA), and Ca^{2+} (100 μM) for 20 min, followed by low Ca^{2+} PSS (mg/ml) containing 1.0 collagenase II, 1.0 collagenase H, 1.0 dithiothreitol and 1.0 BSA for 10 – 15 min. Finally, the tissues were washed in ice-cold low Ca^{2+} PSS for 3 times. Single cells were harvested by gentle trituration and kept on ice for daily use.

Catalase and glutathione peroxidase-1 gene (Gpx1) overexpression, as well as Gpx1 gene deletion mice were generated and maintained as described previously [10,13,39]. Freshly isolated resistance PASMCs from these transgenic and control (same background, sex and age) mice were prepared using the same procedure as described above.

Immunofluorescence staining

To determine the freshly isolated, elongate cells from mouse pulmonary arteries were smooth muscle cells, the expression of smooth muscle-specific actin and myosin were examined using immunofluorescence staining, as we described previously [41]. In brief, cells were fixed in 4 % paraformaldehyde and then incubated with 0.2 % Triton X-100. After blocked by 2.5 % BSA, cells were incubated with anti-actin (smooth muscle) or anti-myosin (smooth) antibody, followed by Alexa488-conjugated anti-mouse antibody. Immunofluorescence staining was examined using a Leica LSP2 laser scanning confocal microscope (Leica Microsystems, Mannheim, Germany).

Measurement of intercellular ROS

Intracellular ROS measurements by using chloromethyl dichlorodihydrofluorescein diacetate (CM- $\text{H}_2\text{DCF/DA}$) and RedoxSensor Red CC-1 were conducted as described previously [1, 7]. Freshly isolated cells were loaded with 5 μM CM- $\text{H}_2\text{DCF/DA}$ or Red CC-1 at room temperature for 30 min. DCF or Red CC-1 fluorescence was examined at 35 °C (controlled by Warner TC-344B temperature/heater controller), using a Zeiss LSM-510 laser scanning confocal microscope (Thornwood, NY). Fluorescent intensity was expressed in arbitrary units (a.u.) after background subtraction. In some experiments, cells were simultaneously loaded with CM-DCF-DA (5 μM) and the mitochondrial specific marker MitoTracker Deep Red 633 (75 nM) to examine the potential origin of ROS formation in mitochondria, as described previously [33].

To measure intracellular ROS by using lucigenin-derived chemiluminescence [20], lucigenin (20 μM) was added in a tube with PSS containing freshly isolated cells. Then, the tube was immediately transferred to a 20/20n Luminometer (Turner BioSystems, Sunnyvale, CA) to record the emitted chemiluminescence. The initial (maximal) values of lucigenin-derived chemiluminescence were obtained and expressed as relative light units (r.l.u.) per second per cell.

Measurement of $[\text{Ca}^{2+}]_i$

Measurements of $[\text{Ca}^{2+}]_i$ in freshly isolated mouse PASMCs were made by a dual excitation wavelength fluorescence method using an IonOptix fluorescence photometric system (Milton, MA), as described previously [40]. Cells were loaded with 5 μM fura-2/AM at room temperature for 30 min. The dye was excited at 340 and 380 nm wavelengths (Xenon 75W arc lamp). The emission fluorescence at 510 nm was detected by a photomultiplier tube at 35 °C, which was controlled by TC-344B temperature/heater controller (Warner, Hamden, CT). Background fluorescence was determined by removing the cell from the field after the experiment.

Hypoxia

Hypoxic responses were obtained by switching a perfusing solution (35 °C) equilibrated with 20% O₂, 5% CO₂ and 75% N₂ (normoxia) to a solution (35 °C) equilibrated with 1% O₂, 5% CO₂ and 94% N₂ (hypoxia), as described previously [40]. The bath pO₂ in the normoxic and hypoxic solutions were 140 and 10 – 20 Torr, as measured by using an oxygen electrode (OXEL-1, WPI). Under both conditions, pH values were the same (7.4).

Reagents

Alexa488-conjugated anti-mouse antibody, CM-H₂DCF/DA, Fura-2/AM, and MitoTracker Deep Red 633, RedoxSensor Red CC-1 were obtained from Molecular Probes (Eugene, OR); anti-actin (smooth muscle) antibody, antimycin A, anti-myosin (smooth) antibody, collagenase II, collagenase H, dithiothreitol, dithioerythritol, hydrogen peroxide (H₂O₂), 1-methyl-4-phenylpyridinium iodide (MPP), myxothiazol, 3-nitropropionic acid (NPA), papain, rotenone, sodium azide (NaN₃), and 2-tenoyltrifluoroacetone (TTFA) from Sigma (St. Louis, MO). Unless indicated otherwise, mitochondrial inhibitors were applied to the cells for 10 minutes before the start of hypoxia. To examine the effects of inhibitors, control experiments were carried out in cells from the same mice. For experiments using gene overexpression and deletion mice, cells from wildtype mice with the same background, age and sex were used for control.

Statistical analysis

Data were expressed as mean ± SE. Student's *t* test was used for determining the significance of differences between two groups, whereas one-way ANOVA employed for assaying the significance of difference between multiple comparisons. *P* < 0.05 was accepted as a level of statistical significance.

RESULTS

Effect of mitochondrial ETC complex I, II, III and IV inhibitors on hypoxia-induced change in [ROS]_i and the resting [ROS]_i in freshly isolated mouse PSMCs

We first sought to verify that the freshly isolated, elongate cells from mouse pulmonary arteries were SMCs. To reach this end, the expression of smooth muscle-specific α -actin and myosin were examined using immunofluorescence staining. As shown in Figure 1, the isolated cells were stained with both smooth muscle-specific markers, indicating that they were PSMCs.

Considering that CM-H₂DCF/DA is most frequently used to detect intracellular ROS (mainly H₂O₂) [11], we used this dye as a probe to examine the effect of hypoxia on [ROS]_i in freshly isolated mouse PSMCs. Figure 2A (*left*) shows an example of these experiments, in which hypoxia exposure for 5 min evoked a large increase in DCF fluorescence, indicating a significant increase in [ROS]_i. In a total of 54 cells, the mean DCF fluorescence was increased by 2 folds (from a resting level of 29 ± 1 a.u. to 88 ± 3 a.u.).

Next we systemically examined the effects of mitochondrial ETC complex I, II, III and IV inhibitors on hypoxia-induced increase in [ROS]_i in freshly isolated mouse PSMCs. Pretreatment with the mitochondrial ETC complex I inhibitor rotenone blocked hypoxic response in a concentration-dependent manner (0.127 – 12.7 μ M). An example of the effect of rotenone on the hypoxic increase in DCF fluorescence is presented in Figure 2A (*right*), in which pretreatment with rotenone (12.7 μ M) for 10 min significantly blocked hypoxic increase in DCF fluorescence. Compared with that in control cells (untreated with rotenone), the mean increase in DCF fluorescence following hypoxic stimulation was reduced by 89% in cells pretreated with rotenone (12.7 μ M). The concentration-dependent effect of rotenone on hypoxic increase in DCF fluorescence is summarized in Figure 2B. However, treatment of

rotenone (0.127 – 12.7 μM) for 10 min had no effect on the resting $[\text{ROS}]_i$ in freshly isolated mouse PSMCs (Figure 2C).

Similar to rotenone, the complex I inhibitor MPP (5 μM), complex II inhibitors NPA (5 mM) and TTFA (10 μM), as well as complex III pre-ubisemiquinone site inhibitor myxothiazol (10 μM) all remarkably reduced hypoxia-induced increase in DCF fluorescence (intracellular ROS generation) in freshly isolated mouse PSMCs (Figure 3A), whereas the complex III post-ubisemiquinone site inhibitor antimycin A (5.7 μM) and complex IV inhibitor NaN_3 (1 mM) did not produce any effects (Figure 3B). In addition, none of these inhibitors had an effect on the resting $[\text{ROS}]_i$.

We also examined the effect of hypoxia utilizing lucigenin-derived chemiluminescence (mainly for measuring $\text{O}_2^{\cdot -}$) and the fluorescent dye RedoxSensor Red CC-1 (for measuring both H_2O_2 and $\text{O}_2^{\cdot -}$). Hypoxic exposure for 5 min significantly increased lucigenin-derived chemiluminescence in freshly isolated mouse PSMCs. The mean value of lucigenin-derived chemiluminescence was 37 ± 4 r.l.u./s in normoxic cells and 66 ± 6 r.l.u./s in hypoxic cells ($P < 0.05$). Similarly, hypoxia for 5 min also caused a large increase in Red CC-1 fluorescence in freshly isolated mouse PSMCs. The mean increase in Red CC-1 fluorescence after hypoxic stimulation was 2.17 ± 0.34 times. These data further indicate that hypoxia may cause a significant increase in $[\text{ROS}]_i$ in freshly isolated mouse PSMCs.

We then wondered whether inhibition of mitochondrial ETC complex I, II or III could prevent hypoxic increase in lucigenin-derived chemiluminescence in freshly isolated mouse PSMCs. As shown in Figure 3C, treatment with rotenone (12.7 μM), TTFA (10 μM) and myxothiazol (10 μM) for 10 min all blocked hypoxic increase in lucigenin-derived chemiluminescence. Additionally, these inhibitors did not exert a significant effect on the resting level of lucigenin-derived chemiluminescence. Taken together, it can be stated that the inhibition of mitochondrial ETC complex I, II and III prevents the hypoxic increase in $[\text{ROS}]_i$, but does not affect the resting $[\text{ROS}]_i$ in freshly isolated mouse PSMCs, indicating that hypoxia may enhance mitochondrial ROS generation through the mitochondrial ETC complex I, II and/or III.

To further provide evidence that hypoxia may increase the generation of mitochondrial ROS, we examined and compared hypoxia-induced increases in ROS generation in mitochondrial and non-mitochondrial areas. As shown in Figure 4A, mitochondria were determined as the areas that were stained by the mitochondrial specific marker MitoTracker. The overlap of both MitoTracker staining (shown as red) and DCF fluorescence (green) images produced yellow, suggesting that hypoxia significantly increases ROS generation in mitochondria. Quantification analysis showed that hypoxia-induced increase in DCF fluorescence (ROS level) in mitochondria greatly preceded that in non-mitochondrial and whole cell areas (Figure 4B). The mean half rise time for hypoxic increase in DCF fluorescence was 2.25 ± 0.24 min in mitochondrial areas, which was shorter than that in non-mitochondrial areas (2.65 ± 0.25 min) and in whole cell areas (2.55 ± 0.24 min) ($P < 0.05$). The mean amplitude of hypoxic increase in DCF fluorescence at the half rise time and maximal hypoxic response in mitochondria were higher than those in non-mitochondrial and whole cell areas (Figure 4C). These results support the view that hypoxia enhances mitochondrial ROS generation in freshly isolated PSMCs.

Effects of genetic modification of mitochondrial and cytosolic H_2O_2 generation on hypoxia-induced change in $[\text{ROS}]_i$ and the resting $[\text{ROS}]_i$ in freshly isolated mouse PSMCs

To complement studies using mitochondrial inhibitors, we sought to determine the effect of genetic modifications of mitochondrial and cytosolic H_2O_2 generation on hypoxia-induced increase in $[\text{ROS}]_i$ in freshly isolated mouse PSMCs. As shown in Figure 5A, Gpx1 gene

overexpression to enhance H_2O_2 decomposition in mitochondria and cytosol significantly inhibited hypoxia-induced increase in DCF fluorescence. The mean increase in DCF fluorescence by hypoxia was 35 ± 2 a.u. in cells from Gpx1 gene overexpression mice and 56 ± 3 a.u. in cells from control mice, respectively ($P < 0.05$). In contrast, Gpx1 gene deletion to inhibit H_2O_2 breakdown in mitochondria and cytosol had an opposite effect. Similar to Gpx1 gene overexpression, catalase gene overexpression to increase intracellular H_2O_2 removal also remarkably inhibited hypoxic increase in DCF fluorescence. Furthermore, Gpx1 gene overexpression, Gpx1 gene deletion or catalase gene overexpression had no effect on the resting level of DCF fluorescence.

In a similar way, Gpx1 and catalase gene overexpression both prevented, whereas Gpx1 gene deletion enhanced, hypoxia-induced increase in lucigenin-derived chemiluminescence (Figure 5B) and Red CC-1 fluorescence (Figure 5C) in freshly isolated mouse PSMCs. These results complement previous studies using mitochondrial inhibitors, further indicating the hypoxic increase in intracellular ROS generation in freshly isolated PSMCs.

Effect of mitochondrial ETC complex I, II, III and IV inhibitors on hypoxia-induced change in $[\text{Ca}^{2+}]_i$ and the resting $[\text{Ca}^{2+}]_i$ in freshly isolated mouse PSMCs

Similar to our previous reports [40,41], hypoxic exposure for 5 min caused a large increase in $[\text{Ca}^{2+}]_i$ in freshly isolated mouse PSMCs. A typical example of these experiments is shown in Figure 6A (*left*). After switching hypoxia to normoxia, the hypoxia-induced increase in $[\text{Ca}^{2+}]_i$ was slowly decreased toward a resting level. The mean increase in $[\text{Ca}^{2+}]_i$ by hypoxia was 212 ± 6 nM in a total of 91 cells (from a resting level of 128 ± 5 to 340 ± 8 nM).

In parallel to inhibiting hypoxic increase in $[\text{ROS}]_i$, pretreatment with rotenone significantly blocked hypoxia-induced increase in $[\text{Ca}^{2+}]_i$ in freshly isolated mouse PSMCs. As an example shown in Figure 6A (*right*), hypoxic response was much smaller in a cell pretreated with rotenone ($12.7 \mu\text{M}$) for 10 min. The mean increase in $[\text{Ca}^{2+}]_i$ by hypoxia was 278 ± 27 nM in control cells and 75 ± 5 nM in cells pretreated with rotenone $12.7 \mu\text{M}$, respectively ($P < 0.05$). The inhibitory effect of rotenone ($0.127 - 12.7 \mu\text{M}$) was also concentration-dependent (Figure 6B). In addition, we found that application of rotenone ($0.127 - 12.7 \mu\text{M}$) for 10 min had no effect on the resting $[\text{Ca}^{2+}]_i$ (Figure 6C).

Similarly, pretreatment with MPP ($5 \mu\text{M}$), NPA (5 mM), TTFA ($10 \mu\text{M}$), and myxothiazol ($10 \mu\text{M}$) for 10 min significantly reduced hypoxic increase in $[\text{Ca}^{2+}]_i$ (Figure 7A). In contrast, antimycin A ($5.7 \mu\text{M}$) did not have a significant effect on hypoxia-induced increase in $[\text{Ca}^{2+}]_i$. The mean increase in $[\text{Ca}^{2+}]_i$ by hypoxia was 258 ± 14 nM in control cells and 272 ± 15 nM in cells pretreated with antimycin A ($P > 0.05$). At a higher concentration ($57 \mu\text{M}$), this inhibitor also failed to prevent the hypoxic response. Moreover, NaN_3 (1 mM) had no effect on hypoxic increase in $[\text{Ca}^{2+}]_i$ (Figure 7B). None of these mitochondrial inhibitors could produce an effect on the resting $[\text{Ca}^{2+}]_i$.

Since inhibition of each of the individual mitochondrial ETC complex I, II and III blocks the hypoxic response, we sought to examine whether the inhibition of both complex I and II, complex I and III, as well as complex II and III would produce an additive effect on hypoxia-induced increase in $[\text{Ca}^{2+}]_i$. As shown in Figure 7C, simultaneous treatment with rotenone ($12.7 \mu\text{M}$) and myxothiazol ($5 \mu\text{M}$) for 10 min reduced hypoxia-induced increase in $[\text{Ca}^{2+}]_i$ by 56%. This effect was not significantly different from that produced by treatment with rotenone or myxothiazol alone, in which hypoxic response was inhibited by 68% and 59%, respectively. Similarly, treatment with TTFA ($10 \mu\text{M}$) plus myxothiazol ($5 \mu\text{M}$), MPP ($5 \mu\text{M}$) plus TTFA ($10 \mu\text{M}$), and MPP ($5 \mu\text{M}$) plus myxothiazol ($10 \mu\text{M}$) did not produce an additive inhibitory effect. Taken together, our results suggest that the mitochondrial ETC molecules prior to the complex III ubiquinone site act as a functional unit to mediate hypoxia-induced

increases in $[Ca^{2+}]_i$ in freshly isolated PSMCs. In addition, simultaneous treatment with both MPP and TTFA, MPP and myxothiazol, rotenone and myxothiazol, as well as TTFA and myxothiazol had no effect on the resting $[Ca^{2+}]_i$.

Effects of genetic modification of mitochondrial and cytosolic H_2O_2 on hypoxia-induced increase in $[Ca^{2+}]_i$ and the resting $[Ca^{2+}]_i$ in freshly isolated mouse PSMCs

We also utilized the Gpx1 and catalase transgenic mice to further determine the role of mitochondrial and cytosolic H_2O_2 in hypoxic responses in freshly isolated mouse PSMCs. Figure 8A shows an example of these experiments, in which hypoxic increase in $[Ca^{2+}]_i$ was significantly inhibited in a cell from Gpx1 gene overexpression mouse. The mean increase in $[Ca^{2+}]_i$ by hypoxia was 192 ± 4 nM in control cells and 102 ± 5 nM in Gpx1 gene overexpression cells, respectively ($P < 0.05$, Figure 8B). In contrast, Gpx1 gene deletion enhanced the hypoxic response (Figure 8B). Neither Gpx1 gene overexpression nor deletion had an effect on the resting $[Ca^{2+}]_i$ (Figure 8C). Catalase gene overexpression also blocked hypoxia-induced increase in $[Ca^{2+}]_i$ (Figure 8B), but did not change the resting $[Ca^{2+}]_i$ (Figure 8C). These findings are in agreement with the effects on $[ROS]_i$, and supplement pharmacological evidence that mitochondrial ROS generation is critical for hypoxic increase in $[Ca^{2+}]_i$ and associated contraction in PSMCs.

Effect of exogenous H_2O_2 on the inhibition of hypoxia-induced increase in $[Ca^{2+}]_i$ by rotenone in freshly isolated mouse PSMCs

Here we first examined the relationship between known H_2O_2 concentrations and DCF fluorescence in freshly isolated mouse PSMCs. As shown in Figure 9A, application of H_2O_2 at 30 nM for 5 min did not induce an increase in DCF fluorescence. At 100 nM, however, this molecule evoked a slight increase in DCF fluorescence. The mean DCF fluorescence was 29 ± 3 and 36 ± 4 a.u. before and after application of H_2O_2 ($n = 8$). H_2O_2 at higher concentrations (300 nM – 3 mM) caused a large increase in DCF fluorescence in a concentration-dependent manner. The mean increases in DCF fluorescence by H_2O_2 at various concentrations are summarized in Figure 9B. There was a linear relationship between the logarithm of H_2O_2 concentrations and DCF fluorescence, yielding an equation: $y = 169.9 + 19.1 \log x$ ($r = 0.98$, $P < 0.05$). According to this equation, a mean DCF fluorescence of 88 a.u. induced by hypoxia ($n = 54$) would be equal to that yielded by H_2O_2 at 51 μ M.

In the following experiments, we examined whether H_2O_2 at 51 μ M could induce a large increase in $[Ca^{2+}]_i$ comparable to hypoxia-induced response. Application of H_2O_2 at this concentration for 5 min induced a mean increase in $[Ca^{2+}]_i$ of 86 ± 5 nM, which was only one third of the hypoxic response (259 ± 6 nM, Figure 10B). Furthermore, H_2O_2 at a lower concentration (5.1 μ M) almost failed to induce an increase in $[Ca^{2+}]_i$, but could almost fully reverse the inhibition of hypoxia-induced increase in $[Ca^{2+}]_i$ by pretreatment with the mitochondrial inhibitor rotenone (12.7 μ M) for 10 min (Figure 10A and 10B).

DISCUSSION

Archer *et al* have reported that hypoxic exposure causes a decrease in intracellular ROS generation in freshly isolated rat lungs [3,23], while other investigators have shown that hypoxia results in an increase in intracellular ROS generation in cultured rat and calf PSMCs [17,21,34]. Using CM- H_2DCF/DA as a probe to detect intracellular ROS (particularly H_2O_2), we have shown that hypoxic exposure for 5 min causes a large increase in DCF fluorescence (intracellular ROS generation) in freshly isolated mouse PSMCs, which do not show the mixed responses from multiple types of cells as in isolated lungs [28] and significant alterations in gene expression as in cultured cells [24]. Similarly, hypoxia for 5 min produces a significant increase in lucigenin-derived chemiluminescence (mainly reflecting intracellular

$O_2^{\cdot -}$) and Red CC-1 (both $O_2^{\cdot -}$ and H_2O_2) in freshly isolated mouse PASMCs, further suggesting an increase in intracellular ROS generation. The striking similarity in our findings in freshly isolated PASMCs and previous reports in cultured cells [17,21,34] indicates that the hypoxic increase in intracellular ROS generation in cultured PASMCs is not due to the cell culture per se, as it has been previously criticized to be a result of the diminished oxygen sensitivity [24], rather that cultured PASMCs may retain important parts of the hypoxic sensing machinery. In addition, it should be noted that numerous independent groups have reported the hypoxic increase in intracellular ROS generation in freshly isolated lungs and pulmonary arteries using H_2DCF , dihydroethidium, lucigenin-derived chemiluminescence, and electron paramagnetic resonance spin adduct spectra [4,15,20,26,31]. Furthermore, a recent study using a novel, ratiometric, redox-sensitive fluorescence resonance energy transfer probe elegantly demonstrates that hypoxia augments ROS signaling in cultured PASMCs [35].

The mitochondrial ETC complex I inhibitor rotenone and complex III post-ubisemiquinone site inhibitor antimycin A have been shown to mimic and subsequently to block hypoxic responses in isolated lungs [3,23], but not in cultured rat PASMCs [34]. The failure of the mitochondrial inhibitors to mimic hypoxic responses in cultured PASMCs has been thought to result from the cell culture [24]. Using freshly isolated PASMCs, here we have revealed that inhibition of the ETC complex I with rotenone or MPP, complex II with NPA or TTFA, and complex III at the pre-ubisemiquinone site with myxothiazol all significantly block hypoxia-induced increase in $[ROS]_i$, but do not affect the resting $[ROS]_i$. Inhibition of the complex III at the post-ubisemiquinone site with antimycin A or complex IV with NaN_3 has no effect on either the hypoxia-induced increase in $[ROS]_i$ or the resting $[ROS]_i$. The reason for the failure of various mitochondrial inhibitors to affect the basal level of ROS formation in freshly isolated PASMCs is unclear, but it is possible that the generation of mitochondrial ROS under the resting conditions is limited. As such, the effect of mitochondrial inhibitors, even if they have any, may not be detected by measuring the whole cell ROS level. In parallel, the basal activity of contractile machinery in PASMCs and pulmonary pressure are known to be low. Taken together, it is reasonable to believe that the inhibition of ETC complex I, II and III can block, but not mimic the hypoxic response in freshly isolated PASMCs. In agreement with this view, previous reports have shown that mitochondrial ETC complex inhibitors block, but do not reproduce hypoxic contraction in isolated rabbit lungs and rat pulmonary arteries [19,26,37]. Moreover, we have found that the hypoxic increase in ROS level occurs significantly earlier as well as is greater in mitochondrial areas than in non-mitochondrial and whole cell areas. These results further support the view that hypoxic increase in intracellular ROS generation may initially result from mitochondria in PASMCs.

To complement experimental studies using mitochondrial inhibitors, we examined the effects of genetic modification of mitochondrial and cytosolic H_2O_2 generation on hypoxic increase in $[ROS]_i$ in freshly isolated mouse PASMCs. Our data indicate that Gpx1 gene overexpression to enhance H_2O_2 removal in mitochondria and the cytosol significantly inhibits hypoxia-induced increase in $[ROS]_i$, whereas Gpx1 gene deletion has the opposite effect. Similarly, overexpression of catalase gene to enhance H_2O_2 decomposition in the cytosol also blocks hypoxia-induced increase in $[Ca^{2+}]_i$. These findings extend previous observations that adenovirus-mediated transfection of catalase inhibits hypoxia-induced increase in $[Ca^{2+}]_i$ in cultured rat PASMCs [36], and pretreatment with nitorbluetetrazolium to block H_2O_2 formation diminishes HPV in isolated rabbit lungs [38], further indicating that mitochondria-derived ROS, particularly H_2O_2 , may serve an important signaling molecule to mediate hypoxic responses in PASMCs. Additionally, we have found that Gpx1 and catalase gene overexpression as well as Gpx1 gene deletion have no effect on the resting $[ROS]_i$ in freshly isolated mouse PASMCs. These results are consistent with the findings from experiments using mitochondrial inhibitors, which do not cause a decrease in intracellular ROS generation.

CM-H₂DCF/DA is most frequently used to detect intracellular ROS (particularly H₂O₂) in living cells, since this dye becomes fluorescent after it is oxidized by ROS through an intracellular enzymatic process that requires H₂O₂, and is much less likely to leak from the cells than H₂DCF/DA [1,11,14]. However, H₂DCF/DA has received criticism for its non-specific oxidation and cytotoxicity, although loading at low concentrations (1–10 μ M) for less than 60 min in serum-free media is adequate for a variety of cell types [2,11,25]. Lucigenin-derived chemiluminescence is a useful and reliable approach to determine the generation of intracellular ROS (mainly O₂^{•-}) in vascular tissues, but it does not directly react with O₂^{•-}, which may lead to artifactual O₂^{•-} generation [11,25]. RedoxSensor Red CC-1 is a recently discovered ROS probe and has been shown as a sensitive dye to detect a broad spectrum of intracellular ROS including O₂^{•-} and H₂O₂ [7]. Because each of these probes has some weaknesses, we employed CM-H₂DCF/DA, Lucigenin and RedoxSensor Red CC-1 all for measuring intracellular ROS generation. As such, our results from the experiments using these three probes, together with mitochondrial inhibitors and genetic manipulation of ROS generation, would be fairly reliable at least in quality, which symbolizes a noteworthy strengthen of our work.

In parallel to preventing the hypoxic increase in [ROS]_i, the mitochondrial ETC complex I, II and III inhibitors rotenone, MPP, NPA, TTFA and myxothiazol all significantly block hypoxia-induced increase in [Ca²⁺]_i, but do not alter the resting [Ca²⁺]_i in freshly isolated mouse PSMCs. Consistent with these results, the augmentation of mitochondrial and/or cytosolic H₂O₂ breakdown by Gpx1 or catalase gene overexpression can block hypoxic increase in [Ca²⁺]_i without affecting the resting [Ca²⁺]_i. Additionally, the inhibition of mitochondrial and cytosolic H₂O₂ decay by Gpx1 gene deletion enhances the hypoxic increase in [Ca²⁺]_i, and has no effect on the resting [Ca²⁺]_i. Furthermore, the inhibition of the complex III at the post-ubisemiquinone site with antimycin A or complex IV with NaN₃ has no effect on either hypoxia-induced increase in [Ca²⁺]_i or the resting [Ca²⁺]_i. Overall, these data further indicate that the inhibition of the mitochondrial ETC prior to the complex III site may block, but not mimic the hypoxic response in freshly isolated mouse PSMCs.

The inhibition of any individual of the ETC complex I, II and III equally prevent hypoxic increase in [Ca²⁺]_i in freshly isolated mouse PSMCs. Moreover, the inhibition of both complex I and II with MPP and TTFA, complex I and III with rotenone (or MPP) and myxothiazol, as well as complex II and III with TTFA and myxothiazol do not produce an additive inhibitory effect on hypoxia-induced increase in [Ca²⁺]_i. These results extend previous observations that the mitochondrial ETC complex III mediates hypoxic contraction in cultured rat PSMCs [34,36] and isolated rat pulmonary arteries [19], indicating that the mitochondrial ETC complex molecules prior to the complex III ubisemiquinone site may work as a functional unit to mediate hypoxia-induced increase in [Ca²⁺]_i in PSMCs, as proposed previously [30]. In support of this view, a previous study has shown that hypoxia may switch the catalytic activity of complex II from succinate dehydrogenase to fumarate reductase, which mediates the hypoxic constriction in isolated rabbit lungs [26]. This may explain the inhibition of any individual ETC molecules prior to the complex III ubisemiquinone site can produce an inhibitory effect on hypoxia-induced increase in [Ca²⁺]_i in freshly isolated (this study) and cultured PSMCs [36], as well as hypoxic contraction in isolated lungs [34,37] and pulmonary arteries [19].

To determine whether the increased generation of mitochondrial ROS (mostly H₂O₂) is sufficient on its own to fully account for the hypoxic responses in freshly isolated mouse PSMCs, we examined and compared hypoxia- and H₂O₂-induced increase in [ROS]_i and [Ca²⁺]_i. The results indicate that H₂O₂ concentrations are positively correlated with DCF fluorescence. The concentration of H₂O₂ able to yield the DCF fluorescence equivalent to that produced by hypoxia is derived as 51 μ M. At this concentration, however, H₂O₂ only induces

33% of the hypoxia-induced increase in $[Ca^{2+}]_i$ (86 ± 5 nM for H_2O_2 response *versus* 259 ± 6 nM for hypoxic response). Consistent with our results, Waypa *et al* have reported that H_2O_2 (50 μ M) induces an increase in $[Ca^{2+}]_i$ in cultured rat PSMCs [36]. Moreover, in this study we have found that H_2O_2 at 5.1 μ M almost fails to induce an increase in $[Ca^{2+}]_i$, but completely reverses the inhibition of hypoxia-induced increase in $[Ca^{2+}]_i$ by the mitochondrial inhibitor rotenone. Therefore, the increased generation of mitochondrial ROS, particularly H_2O_2 , is necessary, but not sufficient on its own to fully induce hypoxia-induced increase in $[Ca^{2+}]_i$ in freshly isolated PSMCs. Apparently, other intracellular signaling molecules are most likely to be required for the full hypoxic response. It is known that H_2O_2 may activate protein kinase C (PKC) directly [18] or by stimulating phospholipase C (PLC) [9]. In addition, both PLC and PKC inhibitors can block H_2O_2 -induced pulmonary vasoconstriction [16,27,29]. Furthermore, there are a large lines of evidence that PKC can modify ion channels, leading to attendant enhancement of intracellular Ca^{2+} release and extracellular Ca^{2+} influx [32,42]. Taken together, mitochondria-derived ROS may not only directly cause Ca^{2+} entry, but also result in the activation of PKC, which provides a positive feedback mechanism to amplify the role of ROS in hypoxic responses in PSMCs. Further experiments, however, are needed to verify this hypothesis. Additionally, the equivalent increase in $[ROS]_i$ produced by exogenous H_2O_2 (51 μ M) and hypoxia at the whole-cell level may not denote the same authentic state of the intracellular ROS signal. Since hypoxia-generated ROS results mainly from the mitochondrial ETC, its concentration is perhaps higher within mitochondria than in non-mitochondrial and whole-cell areas. Indeed, we have found that the hypoxic increase in ROS level is greater in mitochondria than in non-mitochondrial and whole cell areas. Nevertheless, future studies by targeting ROS sensors to specific cellular compartments in animals may provide more information regarding the intracellular location of hypoxic ROS signals. Furthermore, it is worth pointing out that a recent, well-designed study has reported that lysophosphatidylcholine, a naturally occurring bioactive lipid molecule, significantly increases Ca^{2+} -dependent mitochondrial ROS generation in cultured human umbilical vein endothelial cells [33]. In agreement with this finding, Ca^{2+} -induced increase in mitochondrial ROS generation have been found in isolated mitochondria from liver, heart and neuronal cells [5, 6]. Presumably, a similar situation may occur with the hypoxic response in PSMCs, in which the increase in $[Ca^{2+}]_i$ triggered by mitochondrial ROS during hypoxia causes a further enhancement of mitochondrial ROS generation. This positive feedback system may play an important role in the development of HPV and associated pulmonary hypertension.

Summary

This study by using multiple mitochondrial inhibitors and genetically manipulated mice provides clear evidence that the mitochondrial ETC molecules prior to the complex III ubiquinone site act as a functional unit to sense acute hypoxia, thereby causing a significant increase in ROS generation in freshly isolated mouse PSMCs. The increased generation of mitochondrial ROS, particularly H_2O_2 , is essential for, but may not be sufficient on its own to fully induce the hypoxic increase in $[Ca^{2+}]_i$ and associated HPV, which possibly require the involvement of other signaling molecules (e.g., PLC and PKC).

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Figure 1.

Freshly isolated cells from pulmonary arteries express smooth muscle-specific actin and myosin. The expression of smooth muscle-specific actin and myosin were examined by immunofluorescence staining. Cells were incubated with a primary antibody for smooth muscle-specific actin (*left*) and myosin (*middle*), and then with Alexa Fluor 488-conjugated anti-mouse antibody. In negative control experiments, cells were treated identically, but not incubated with a primary antibody (*right*). Staining images were taken with a Leica LSP2 laser scanning confocal microscope.

Figure 2.

Rotenone concentration-dependently inhibits hypoxia-induced increases in $[\text{ROS}]_i$, but does not affect the resting $[\text{ROS}]_i$ in freshly isolated mouse PSMCs. (A) Recording traces show hypoxia-induced increase in DCF fluorescence (reflecting an increase in $[\text{ROS}]_i$) in a control cell (*left*) and a cell pretreated with rotenone (12.7 μM) for 10 min (*right*). The hypoxic response was much smaller in a cell pretreated with rotenone than in a control cell. (B) Bar graph summarizes the inhibitory effect of rotenone (0.127 – 12.7 μM) for 10 min on hypoxia-induced increase in DCF fluorescence (ΔDCF). * $P < 0.05$ compared with control (untreated with rotenone). (C) Summary of the effect of treatment with rotenone (0.127 – 12.7 μM) for 10 min

on the resting DCF fluorescence, indicating that rotenone did not produce any effects on the resting $[\text{ROS}]_i$.

Figure 3.

Inhibition of mitochondrial ETC molecules prior to the complex III ubisemiquinone site blocks hypoxic increases in $[\text{ROS}]_i$ in freshly isolated mouse PSMCs. (A) Graph shows that treatment with the complex I inhibitor MPP (5 μM), complex II inhibitor NPA (5 mM) or TTFA (10 μM), and complex III pre-ubisemiquinone site inhibitor myxothiazol (10 μM) for 10 min all prevented hypoxia-induced increase in DCF fluorescence (Δ DCF). * $P < 0.05$ compared with control (untreated with an inhibitor). (B) Treatment with the complex III post-ubisemiquinone site inhibitor antimycin A (5.7 μM) or IV inhibitor NaN_3 (1 mM) for 10 min had no effect on hypoxia-induced increase in DCF fluorescence (Δ DCF). (C) Treatment with rotenone (12.7 μM), TTFA (10 μM) or myxothiazol (10 μM) for 10 min blocked hypoxia-

induced increase in lucigenin-derived chemiluminescence (LDCL). * $P < 0.05$ compared with control (normoxia); $^{\dagger}P < 0.05$ compared with hypoxia alone (untreated with an inhibitor).

Figure 4.

Hypoxia-induced increase in ROS generation in mitochondria precedes that in non-mitochondrial areas in freshly isolated mouse PSMCs. (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 box in the cell shown in (A). The mitochondrial and non-mitochondrial area taken to measure hypoxic increase in DCF fluorescence in the extracted images was indicated by circle. Traces show the time course for the hypoxic response in mitochondrial (Mito), non-mitochondrial (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 $\frac{1}{2}$ time), and maximal hypoxic increase in DCF fluorescence (ΔDCF) in mitochondrial, non-mitochondrial, and whole cell areas. Data were obtained from 10 cells from 5 independent experiments. $P < 0.05$ compared with mitochondrial areas.

Figure 5.

Genetic enhancement of mitochondrial and cytosolic H_2O_2 breakdown blocks, whereas genetic inhibition of mitochondrial and cytosolic H_2O_2 breakdown augments, hypoxia-induced increases in $[\text{ROS}]_i$ in freshly isolated mouse PSMCs. (A) Gpx1 gene overexpression (Gpx1-Tg) to enhance mitochondrial and cytosolic H_2O_2 breakdown prevented, while Gpx1 gene deletion (Gpx1^{-/-}) to inhibit mitochondrial and cytosolic H_2O_2 decomposition augmented, hypoxia-induced increase in DCF fluorescence (Δ DCF). Similarly, catalase gene overexpression (CAT-Tg) to enhance cytosolic H_2O_2 decomposition also blocked hypoxic increase in DCF fluorescence. (B) Graph shows that Gpx1 and catalase gene overexpression inhibited, whereas Gpx1 gene deletion enhanced, hypoxic increase in lucigenin-derived

chemiluminescence (LDCL). (C) Summary of the inhibitory effect of Gpx1 and catalase gene overexpression as well as the enhancing effect of Gpx1 gene deletion on hypoxic increase in Red CC-1 fluorescence (Δ Red CC-1). *P < 0.05 compared with control (non-transgenic cells).

Figure 6.

Rotenone concentration-dependently inhibits hypoxia-induced increases in $[\text{Ca}^{2+}]_i$, but does not affect the resting $[\text{Ca}^{2+}]_i$ in freshly isolated mouse PSMCs. (A) Recording traces show hypoxia-induced increase in $[\text{Ca}^{2+}]_i$ in a control cell (*left*) and a cell pretreated with rotenone (12.7 μM) for 10 min (*right*). (B) Summary of the inhibitory effect of rotenone (0.127 – 12.7 μM) for 10 min on hypoxia-induced increase in $[\text{Ca}^{2+}]_i$. * $P < 0.05$ compared with control (untreated with rotenone). (C) Graph shows that treatment with rotenone (0.127 – 12.7 μM) for 10 min had no effect on the resting $[\text{Ca}^{2+}]_i$.

Figure 7.

Inhibition of mitochondrial ETC molecules prior to the complex III ubiquinone site blocks hypoxic increases in $[Ca^{2+}]_i$ in freshly isolated mouse PSMCs. (A) Graph shows that treatment with MPP (5 μ M), NPA (5 mM), TTFA (10 μ M), and myxothiazol (10 μ M) for 10 min all prevented hypoxia-induced increase in $[Ca^{2+}]_i$. * $P < 0.05$ compared with control (untreated with an inhibitor). (B) Treatment with antimycin A (5.7 μ M) or NaN₃ (1 mM) for 10 min did not affect hypoxia-induced increase in $[Ca^{2+}]_i$. (C) Graph summarizes the effect of rotenone (ROT) plus myxothiazol (MYX), TTFA plus MYX, MPP plus TTFA, and MPP plus MYX on hypoxic increase in $[Ca^{2+}]_i$. * $P < 0.05$ compared with control (untreated with an inhibitor).

Figure 8.

Genetic enhancement of mitochondrial and cytosolic H_2O_2 breakdown blocks, whereas genetic inhibition of mitochondrial and cytosolic H_2O_2 breakdown augments, hypoxia-induced increases in $[Ca^{2+}]_i$ in freshly isolated mouse PSMCs. (A) Original recordings show hypoxia-induced increase in $[Ca^{2+}]_i$ in a control cell (*left*) and a Gpx1 gene overexpression (Gpx1-Tg) cell (*right*). Please note that the hypoxic response was much smaller in a control than in a Gpx1-Tg cell. (B) Bar graph summarizes the effect of Gpx1 and catalase gene overexpression (CAT-Tg) as well as Gpx1 gene deletion (Gpx1^{-/-}) on hypoxia-induced increase in $[Ca^{2+}]_i$. * $P < 0.05$ compared with control.

Figure 9.

H_2O_2 concentration-dependently increases DCF fluorescence in freshly isolated mouse PSMCs. (A) Original recordings of the effect of H_2O_2 at various concentrations on DCF fluorescence. (B) Graph shows the relationship between H_2O_2 concentrations (30 nM – 3 mM) and DCF fluorescence. The logarithm of H_2O_2 concentrations was linearly correlated with DCF fluorescence, yielding an equation: $y = 169.9 + 19.1 \log x$ ($r = 0.98$, $P < 0.05$). Data points represent the mean values from 8 – 26 different cells. The continuous line was a line fitted to a linear equation using Origin Version-7 Software (OriginLab Corporation, USA).

Figure 10.

Exogenous application of H₂O₂ reverses the inhibition of hypoxia-induced increases in [Ca²⁺]_i by rotenone in freshly isolated mouse PSMCs. (A) Original recordings show that hypoxia and H₂O₂ (5.1 μM) induced an increase in [Ca²⁺]_i in cells pretreated with and without rotenone (12.7 μM) for 10 min. (B) Graph summarizes the effect of H₂O₂ on the inhibition of hypoxia-induced increase in [Ca²⁺]_i by rotenone. *P < 0.05 compared with control (hypoxia alone).