

Superoxide Generated at Mitochondrial Complex III Triggers Acute Responses to Hypoxia in the Pulmonary Circulation

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Rationale: The role of reactive oxygen species (ROS) signaling in the O₂ sensing mechanism underlying acute hypoxic pulmonary vasoconstriction (HPV) has been controversial. Although mitochondria are important sources of ROS, studies using chemical inhibitors have yielded conflicting results, whereas cellular models using genetic suppression have precluded *in vivo* confirmation. Hence, genetic animal models are required to test mechanistic hypotheses.

Objectives: We tested whether mitochondrial Complex III is required for the ROS signaling and vasoconstriction responses to acute hypoxia in pulmonary arteries (PA).

Methods: A mouse permitting Cre-mediated conditional deletion of the Rieske iron-sulfur protein (RISP) of Complex III was generated. Adenoviral Cre recombinase was used to delete RISP from isolated PA vessels or smooth muscle cells (PASMCM).

Measurements and Main Results: In PASMCM, RISP depletion abolished hypoxia-induced increases in ROS signaling in the mitochondrial intermembrane space and cytosol, and it abrogated hypoxia-induced increases in [Ca²⁺]_i. In isolated PA vessels, RISP depletion abolished hypoxia-induced ROS signaling in the cytosol. Breeding the RISP mice with transgenic mice expressing tamoxifen-activated Cre in smooth muscle permitted the depletion of RISP in PASMCM *in vivo*. Precision-cut lung slices from those mice revealed that RISP depletion abolished hypoxia-induced increases in [Ca²⁺]_i of the PA. *In vivo* RISP depletion in smooth muscle attenuated the acute hypoxia-induced increase in right ventricular systolic pressure in anesthetized mice.

Conclusions: Acute hypoxia induces superoxide release from Complex III of smooth muscle cells. These oxidant signals diffuse into the cytosol and trigger increases in [Ca²⁺]_i that cause acute hypoxic pulmonary vasoconstriction.

Keywords: oxygen sensing; Rieske iron-sulfur protein; reactive oxygen species; roGFP; hypoxic pulmonary vasoconstriction

In the lung, alveolar hypoxia triggers acute constriction of small pulmonary arteries (PA), a response termed hypoxic pulmonary vasoconstriction (HPV). This response is recapitulated in cultured PA smooth muscle cells (PASMCM), indicating that the oxygen-sensing mechanism underlying HPV is intrinsic to the

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

The molecular mechanisms of hypoxia-induced pulmonary arterial (PA) vasoconstriction remain unclear. Our previous data suggest that mitochondrial reactive oxygen species (ROS) play a central role in activating hypoxic pulmonary vascular smooth muscle constriction. Previous studies attempting to clarify the role of the mitochondrial electron transport chain (ETC) using pharmacological inhibitors have yielded conflicting results. Studies using cell culture alone are not sufficient to establish the role of specific genes for the *in vivo* response.

What This Study Adds to the Field

A mouse was developed to permit conditional deletion of the Rieske iron-sulfur protein (RISP), which is required for superoxide generation at Complex III. Depletion of the RISP gene from cultured PA smooth muscle cells (SMC) abolished hypoxia-induced ROS signaling in the mitochondrial intermembrane space and the cytosol. Abrogation of hypoxia-induced ROS signaling was also observed in systemic vascular SMC. In PASMCM, RISP depletion abolished the hypoxia-induced increases in cytosolic calcium, but responses to exogenous ROS were maintained. In precision-cut lung slices, depletion of RISP in smooth muscle abolished hypoxia-induced increases in cytosolic calcium. Deletion of the RISP gene in smooth muscle of intact mice attenuated the acute increase in right ventricular systolic pressure during hypoxia. These results demonstrate that hypoxia causes acute pulmonary vasoconstriction by stimulating the release of ROS from mitochondrial Complex III, which triggers calcium increases that cause smooth muscle contraction.

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PASMCM (1–12). Our previous work has implicated increases in reactive oxygen species (ROS) signaling during hypoxia (9, 10, 12). Previous studies using mitochondrial inhibitors and mitochondria-deficient (ρ^0) cells suggested that the electron transport chain (ETC) is required for hypoxia-induced ROS signaling in the pulmonary circulation (9, 11–18). We subsequently assessed ROS signaling in hypoxic PASMCM using roGFP, a thiol-containing, redox-sensitive reporter (19–23) targeted to compartments within mitochondria or the cytosol (10). Unlike other methods (24–26), this targeted approach permitted the differentiation of hypoxia-induced ROS changes between mitochondrial subcompartments. During hypoxia, increased oxidation was detected in the mitochondrial intermembrane space (IMS) and the cytosol, whereas oxidation in the mitochondrial matrix decreased.

Overexpression of catalase attenuated the hypoxia-induced roGFP oxidation and the associated increase in cytosolic calcium ($[Ca^{2+}]_i$), suggesting that increased mitochondrial ROS signaling is required for the hypoxia-induced increase in $[Ca^{2+}]_i$.

In tumor cell lines, we and others used RNA interference to suppress the mitochondrial Complex III Rieske iron-sulfur protein (RISP) and found that RISP suppression attenuates hypoxic ROS signaling and stabilization of hypoxia-inducible factor (HIF-1 α), suggesting that RISP function is integral to the transduction of hypoxia necessary for the regulation of HIF-1 α stability (16, 27, 28). Those findings suggested that a similar O₂ sensing mechanism might function in pulmonary vascular cells. Based on that hypothesis, Korde and colleagues (29) used siRNA to suppress RISP in PASMC and found that hypoxic ROS responses were attenuated in PASMC, mitochondria isolated from PASMC, and Complex III isolated from PASMC mitochondria. These results similarly identify mitochondria as a source of increased ROS signaling during hypoxia. However, lack of a genetic model of RISP suppression has prevented testing this hypothesis *in vivo*. We have therefore generated a mouse with *loxP* sites flanking exon 2 of the RISP gene to perform these studies.

The present study examined the role of RISP depletion in ROS responses to acute hypoxia within the cytosol and mitochondrial sub-compartments and the consequences of RISP depletion on hypoxia-induced $[Ca^{2+}]_i$ signaling. For these studies, we used cultured pulmonary and systemic arterial smooth muscle cells (SASMC) and PA segments from mice. *In vivo* depletion of RISP in the smooth muscle cells of living mice permitted studies of the importance of smooth muscle RISP in the *in vivo* pulmonary vascular response to hypoxia.

Some of the results of these studies have been previously reported in the form of abstracts (30, 31).

METHODS

Expanded methods are contained in the online supplement.

Pulmonary Microvessel SMC Isolation

The Northwestern University Institutional Animal Care and Use Committee approved all animal studies. PASMC were isolated from mouse lungs as described previously (9) using a modification of the method of Marshall and colleagues (6) (*see online supplement*).

Isolated PA Vessel Segments

Mouse lungs were perfused with an agarose-iron particle mixture, which is trapped in arteries because the particles are too large to transit pulmonary capillaries. Using a magnet, the PA vessel segments were separated from surrounding lung tissue as the connected lung tissue was digested with collagenase (type IV) (*see online supplement*).

Mouse Precision-Cut Lung Slices

Precision lung slices (200- μ m thick) were obtained from mice as previously described (32) (*see online supplement*).

Conditional RISP Knockout Mouse

A mouse carrying *loxP*-flanked exon 2 of the mouse RISP gene (RISP^{fllox/fllox}) was generated by Ozgene Pty Ltd. *See online supplement* for expansion of this method.

Conditional Smooth Muscle RISP Knockout Mouse

A smooth muscle-specific deletion of RISP was generated by crossing a tamoxifen-inducible Cre recombinase driven by the SMC myosin heavy chain promoter (SMC-MHC-Cre) (33) with an RISP^{fllox/fllox} mouse to generate an SMC-MHC-Cre/RISP^{fllox/fllox} mouse in a mixed genetic background. *See online supplement* for expansion of this method.

Statistics

Changes in roGFP oxidation, YC2.3 Förster resonance energy transfer (FRET), and Fura-2 were analyzed by using a two-way analysis of variance

with repeated measures. A Newman-Keuls multiple-range test was used to evaluate significant differences between groups and times. Changes in RSVP and protein expression were analyzed by using a two-way analysis of variance. A Newman-Keuls multiple-range test was used to evaluate significant differences between groups. To control for experimental differences in the hypoxic responses, experimental studies and control experiments were always performed on the same day. Statistical significance was set at $P < 0.05$ (34).

RESULTS

Hypoxia Increases ROS in the IMS and Cytosol and Decreases ROS in the Mitochondrial Matrix

To assess ROS signaling in subcellular compartments of cultured wild-type mouse PASMC during hypoxia, we infected cells with adenoviruses harboring DNA to express roGFP targeted to the cytosol, mitochondrial matrix, or mitochondrial intermembrane space (IMS-roGFP) as we have done previously (10). We used roGFP to assess protein thiol oxidation; to our knowledge this reporter does not participate in signal transduction. Under normoxic conditions, the cytosolic roGFP oxidation averaged $20.3 \pm 3.7\%$ (Figure 1A). With the onset of hypoxia, cytosolic roGFP

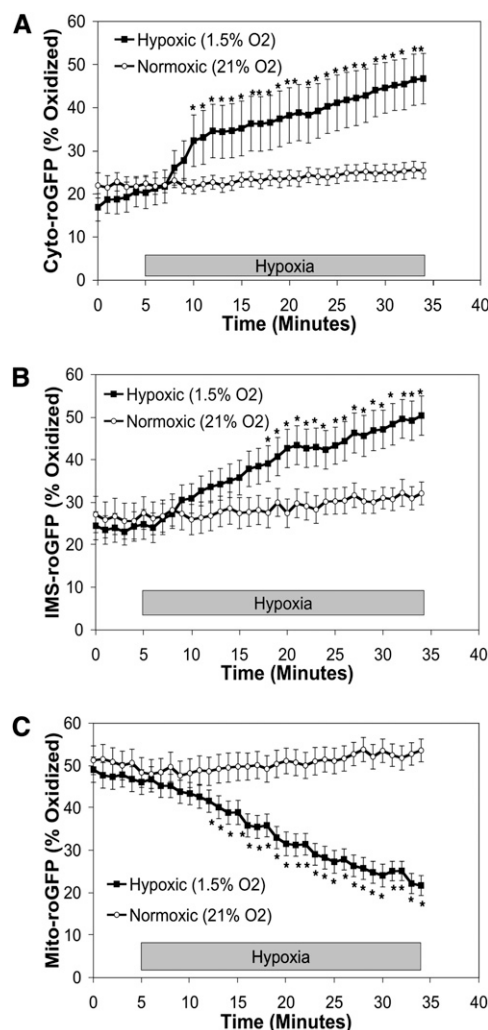


Figure 1. Effects of hypoxia on roGFP oxidation in the cytosol, intermembrane space (IMS), and mitochondrial matrix in wild-type mouse pulmonary arterial smooth muscle cells (PASMC). Averaged results from multiple experiments in wild-type mouse PASMC expressing Cyto-roGFP (A), IMS-roGFP (B), or Mito-roGFP (C) superfused with normoxic (21% O₂) or hypoxic (1.5% O₂) media. Values are means \pm SEM, $n = 6$ cover slips, 4 to 10 cells/cover slip. * $P < 0.05$ compared with normoxia.

steadily oxidized, reaching $46.7 \pm 5.8\%$ within 30 minutes (Figure 1A). Oxidation of roGFP in the IMS also increased during hypoxia, from $24.8 \pm 3.5\%$ under normoxia to $50.4 \pm 4.6\%$ by 30 minutes (Figure 1B). In contrast, oxidation in the mitochondrial matrix decreased from $46.1 \pm 2.9\%$ during normoxia to $21.7 \pm 2.3\%$ after 30 minutes of hypoxia (Figure 1C). These results in mouse PASMCM are consistent with previous results obtained in rat PASMCM (10).

Decreasing RISP Expression Attenuates Hypoxia-induced ROS Signaling in PASMCM

To determine whether intact Complex III function is required for the increase in ROS signaling during hypoxia, PASMCM from mice carrying a conditional deletion of the ubiquinol-cytochrome c reductase, *Uqcrcs1*, (*NM_025710*) (*RISP^{fllox/fllox}*) were transduced with a Cre-expressing adenovirus (200 pfu) to delete the RISP gene (*RISP-KO*). Reverse transcriptase-polymerase chain reaction demonstrated that RISP mRNA extracted from *RISP-KO* PASMCM was decreased compared with untreated PASMCM or PASMCM treated with an empty adenovirus (Figure 2A). A significant decrease in RISP protein expression in *RISP-KO* PASMCM was also confirmed by Western blot analysis (Figures 2B and 2C). Decreasing RISP expression did not affect the expression of other mitochondrial proteins, such as the cytochrome c oxidase subunit IV (COX IV) or the mitochondrial heme protein cytochrome c (see Figure E9 in the online supplement). However, it did render the mitochondrial membrane potential dependent on glycolytic ATP, which drives the ATP synthase in reverse to maintain the potential when electron transport is inhibited (Figure E4).

Cells in which RISP expression was decreased with adenoviral Cre exhibited increased baseline normoxic oxidation of Cyto-roGFP to $32.6 \pm 3.0\%$ (Figure 3A). Remarkably, however, hypoxia-induced oxidation of Cyto-roGFP was abolished. In fact, oxidation of cytosolic roGFP in *RISP-KO* PASMCM decreased to $24.7 \pm 1.6\%$ during 30 minutes of hypoxia (Figure 3A). In contrast, decreased RISP expression had no effect on the level of baseline oxidation of IMS-roGFP ($44.1 \pm 4.1\%$). However, as with Cyto-roGFP, hypoxia-induced oxidation of IMS-roGFP oxidation in *RISP-KO* cells was abrogated. Furthermore, oxidation of IMS-roGFP tended to decrease during hypoxia ($29.3 \pm 3.0\%$) (Figure 3B). Finally, in *RISP-KO* PASMCM expressing Mito-roGFP, oxidation averaged $46.6 \pm 3.2\%$ under normoxia and did not change during 30 minutes of hypoxia (Figure 3C). Thus, RISP is required for the acute increases in ROS observed in PASMCM during hypoxia.

RISP Depletion Attenuates the Hypoxia-induced Increase in $[Ca^{2+}]_i$ in PASMCM

To examine the effect of RISP depletion on the hypoxia-induced increase in $[Ca^{2+}]_i$, cytosolic calcium was assessed using a FRET sensor, YC2.3 (35), expressed in mouse PASMCM using a recombinant adenovirus (10, 12). Similar to the response observed previously in rat PASMCM (10), the YC2.3 FRET ratio increased within 2 minutes after the start of hypoxia in cells transduced with empty adenovirus, demonstrating increases in $[Ca^{2+}]_i$ ($111.0 \pm 2.9\%$ of baseline; Figure 4A). Cells in which RISP expression was decreased with adenoviral Cre exhibited alterations in both the magnitude and time course of the hypoxia-induced $[Ca^{2+}]_i$ increase. No increase in FRET ratio was observed during the initial 4 minutes, during which time hypoxia acutely increases and subsequently decreases $[Ca^{2+}]_i$ in control cells. A delayed tendency to increase (*P* value not significant) was observed well after the increase seen in control cells ($103.3 \pm$

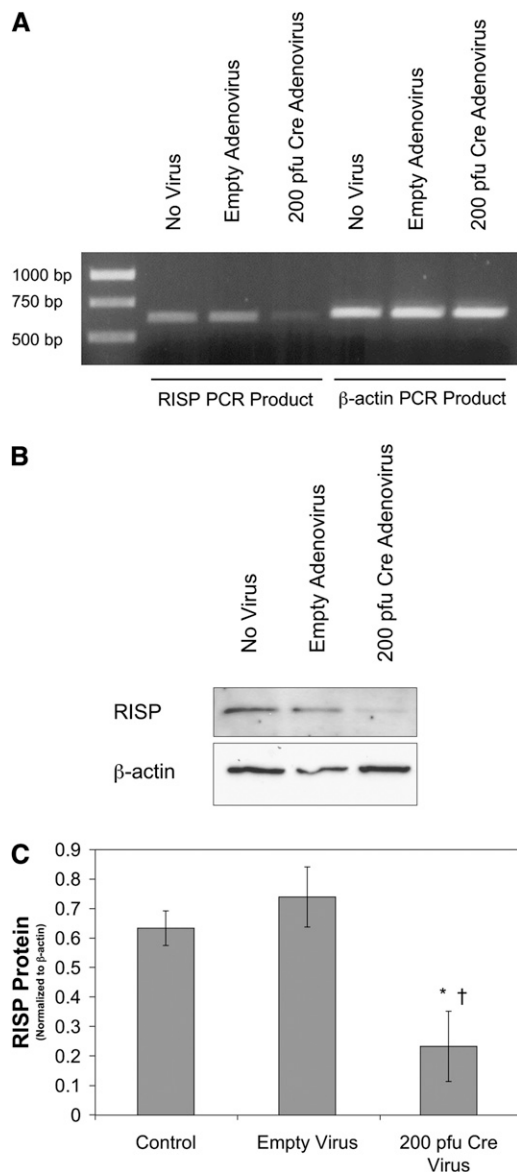


Figure 2. Effect of Cre expression on Rieske iron-sulfur protein (RISP) expression in pulmonary arterial smooth muscle cells (PASMCM) from conditional knockout mice. (A) Reverse transcriptase-polymerase chain reaction (RT-PCR) blot of mRNA extracted from uninfected (no virus), empty adenovirus-treated (200 pfu), and Cre-expressing adenovirus (200 pfu) PASMCM isolated from *RISP* conditional knockout mice. The RT-PCR product for RISP is approximately 600 bp, whereas that for β -actin (as an internal control) is approximately 540 bp. (B) Western blot of lysates from untransfected (no virus), empty adenovirus-transfected (200 pfu), and Cre-expressing adenovirus-transfected (200 pfu) PASMCM isolated from conditional knockout mice probed with antibodies against the RISP subunit or β -actin. (C) Quantified analysis of Western blots with RISP expression normalized to β -actin. Values are means \pm SEM, *n* = 3 Western blots. **P* < 0.05 compared with no virus, [†]*P* < 0.05 compared with empty adenovirus.

1.5% of baseline at the 8-min mark; Figure 4A). We confirmed that RISP depletion did not alter calcium responses to exogenous H_2O_2 application, as similar responses to H_2O_2 were observed in untreated PASMCM, in cells treated with empty adenovirus, and in cells treated with Cre adenovirus (110.2 ± 0.3 , 109.0 ± 0.7 , $107.9 \pm 0.8\%$ of baseline, respectively; Figure 4B). These results indicate that RISP is required for the acute increase in $[Ca^{2+}]_i$ signaling in response to hypoxia.

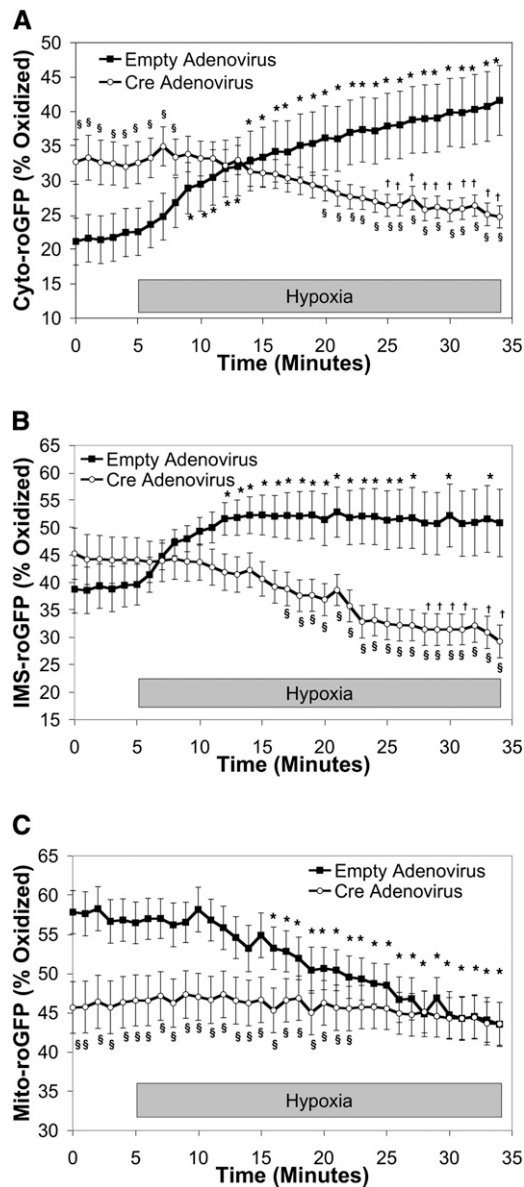


Figure 3. Effect of Rieske iron-sulfur protein (RISP) depletion on hypoxia-induced reactive oxygen species (ROS) signaling in the cytosol, intermembrane space (IMS) and mitochondrial matrix in pulmonary arterial smooth muscle cells (PASMC) from conditional knockout mice. (A–C) PASMC isolated from RISP mice and treated with empty (control) or Cre-expressing adenovirus. Average results from multiple experiments in PASMC expressing Cyto-roGFP (A), IMS-roGFP (B), or Mito-roGFP (C) superfused with normoxic (21% O₂) or hypoxic (1.5% O₂) media. Values are means ± SEM, n = 6 cover slips, 4 to 10 cells/cover slip. *P < 0.05 compared with normoxic baseline of empty virus-treated PASMC. †P < 0.05 compared with normoxic baseline of Cre-expressing PASMC. ‡P < 0.05 compared with empty virus-treated PASMC. §P < 0.05 compared with empty virus-treated PASMC.

RISP Depletion Attenuates Hypoxia-induced Increases in ROS Signaling in Systemic Arterial Smooth Muscle Cells

We previously reported that PASMC and systemic arterial smooth muscle cells (SASMC) share similar patterns of redox signaling in response to hypoxia, despite exhibiting differing downstream contractile responses (10). To determine whether the hypoxia-induced increase in cytosolic ROS signaling in SASMC also depends on Complex III, Cyto-roGFP responses were assessed in RISP-KO SASMC isolated from renal artery. In SASMC transduced with

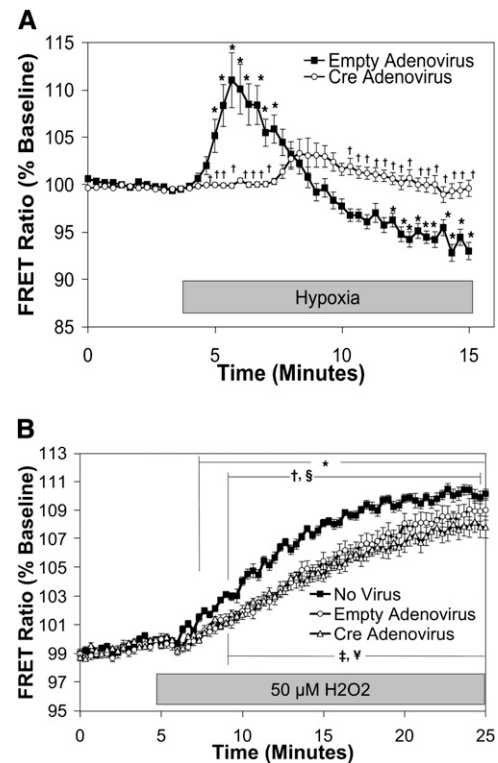


Figure 4. Effect of Rieske iron-sulfur protein (RISP) depletion on [Ca²⁺]_i in pulmonary arterial smooth muscle cells (PASMC) during hypoxia. PASMC were treated with empty (control) or Cre-expressing adenovirus. (A) Effects of hypoxia on [Ca²⁺]_i in PASMC assessed by YC2.3. Values are means ± SEM, n = 6 cover slips, 4 to 10 cells/cover slip. *P < 0.05 compared with normoxic baseline of empty virus-treated PASMC. †P < 0.05 compared with empty virus-treated PASMC. (B) Effects of exogenous H₂O₂ (50 μmol/L) on [Ca²⁺]_i in RISP-knockout mouse PASMC assessed by the calcium-sensitive, Förster resonance energy transfer (FRET) sensor YC2.3. Values are means ± SEM, n = 6 cover slips, 4 to 10 cells/cover slip. *P < 0.05 compared with baseline of untreated (no virus) PASMC. †P < 0.05 compared with baseline of empty virus-treated PASMC. ‡P < 0.05 compared with baseline of Cre-expressing PASMC. §P < 0.05 compared with empty virus-treated PASMC.

empty adenovirus, Cyto-roGFP oxidation increased during 30 minutes of hypoxia (33.3 ± 5.6 to 56.9 ± 7.0%; Figure 5A). RISP depletion increased the basal oxidation of Cyto-roGFP (47.3 ± 4.9%; Figure 5A), but oxidation decreased (37.7 ± 6.1%) during 30 minutes of hypoxia (Figure 5A). RISP depletion had no detectable effect on the minimal [Ca²⁺]_i signaling response to hypoxia, as assessed by YC2.3 FRET in SASMC (Figure 5B). These results demonstrate that Complex III is required for the hypoxia-induced ROS signaling observed in SASMC.

RISP Depletion Attenuates Hypoxia-induced Increases in ROS Signaling in Isolated PA Vessels

To determine whether hypoxia-induced ROS signaling occurs in intact vessels, ROS signaling was assessed in cells from PA vessels isolated from RISP^{fllox/fllox} mice (Figure 6A). Freshly isolated PA vessels were transduced with a Cre-expressing adenovirus, and the resulting effect of decreased RISP expression (Figure E7) on hypoxia-induced subcellular compartmental ROS signaling was assessed using Cyto- (Figure 6B) and Mito-roGFP. As seen in isolated PASMC, 30 minutes of hypoxia increased Cyto-roGFP oxidation in isolated PA from 17.2 ± 2.2% to 56.1 ± 3.8%, whereas it decreased Mito-roGFP oxidation from 52.3 ± 6.1 to

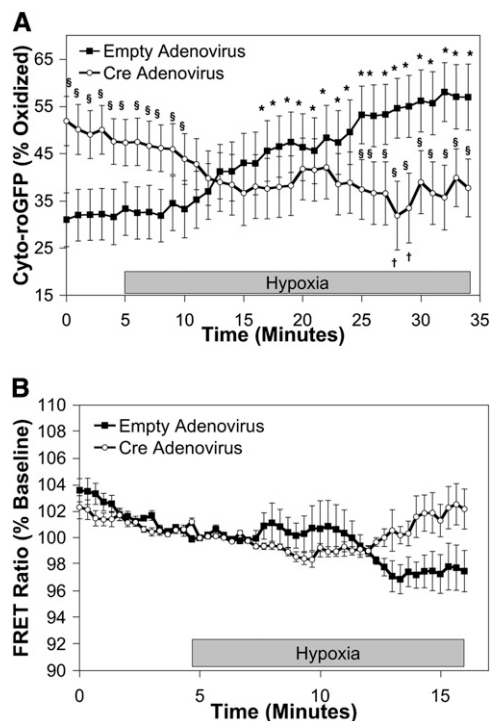


Figure 5. Effect of Rieske iron-sulfur protein (RISP) depletion on hypoxia-induced roGFP oxidation in the cytosol of mouse systemic arterial smooth muscle cells (SASMC) isolated from RISP conditional knockout mice. (A, B) SASMC isolated from RISP mice and treated with an empty (control) or Cre-expressing adenovirus. (A) Averaged results from multiple experiments in SASMC expressing Cyto-roGFP superfused with normoxic (21% O₂) or hypoxic (1.5% O₂) media. (B) Effects of hypoxia on [Ca²⁺]_i in SASMC assessed by the calcium-sensitive, Förster resonance energy transfer (FRET) sensor YC2.3. Values are means ± SEM, n = 6 cover slips, 4 to 10 cells/cover slip. *P < 0.05 compared with normoxic baseline of empty virus-treated pulmonary arterial smooth muscle cells (PASMC). †P < 0.05 compared with normoxic baseline of Cre-expressing PASMC. ‡P < 0.05 compared with empty adenovirus-infected PASMC.

35.8 ± 8.5% (Figures 6C and 6D, respectively). Decreasing RISP expression increased normoxic baseline oxidation of Cyto-roGFP to 32.6 ± 4.5% from 20.3 ± 2.4% (Figure 6C). However, hypoxia-induced oxidation of Cyto-roGFP was abolished in the cells of RISP-KO PA segments (Figure 6C). Although RISP depletion did not affect the oxidation of Mito-roGFP in RISP-KO PA vessels under normoxia, it did attenuate the progressive decrease in oxidation during acute hypoxia (Figure 6D). These results indicate that RISP is required for the changes in ROS observed in isolated PA vessels during hypoxia.

RISP Depletion Attenuates the Hypoxia-induced Increase in [Ca²⁺]_i in PASMC of Precision-Cut Lung Slices

To further assess hypoxia-induced ROS signaling *in vivo*, RISP was deleted in smooth muscle of SMC-MHC-Cre/RISP^{fllox/fllox} mice by tamoxifen injections (20 mg/kg body weight in corn oil for five consecutive days), vehicle treatment only, or untreated mice. Mice were then housed for 6 weeks to ensure complete depletion of existing protein in SMC of the tamoxifen-treated animals. At 6 weeks, a significant decrease in RISP protein in the abdominal aorta was confirmed, relative to Vehicle and Control mice (Figures 7A and 7B). The decreased SMC RISP expression had no effect on PA wall thickness, right ventricular mass as assessed by the Fulton index, or cardiac function (Figure E2). The mice remained normal in appearance and behavior despite

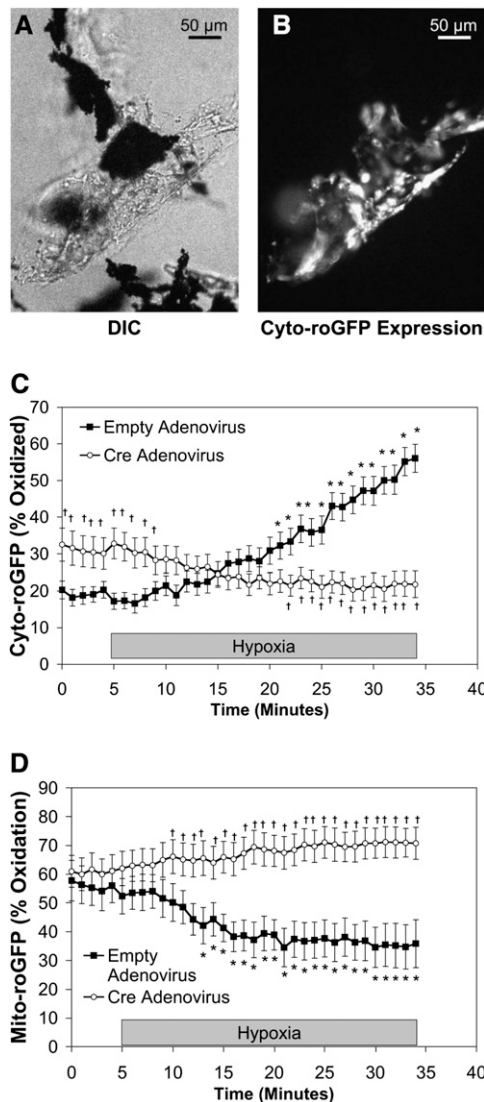


Figure 6. Effect of Rieske iron-sulfur protein (RISP) depletion on hypoxia-induced roGFP oxidation in the cytosol and mitochondrial matrix of cells in pulmonary arterial (PA) vessels isolated from RISP conditional knockout mice. As part of the isolation procedure for PA smooth muscle cells (PASMC), PA microvessel segments are generated that contain iron particles in their lumen. These iron particles allow for the microvessel segments to be constrained in the flow-through chamber using a magnet, thus allowing for their study under both normoxic and hypoxic conditions. (A) DIC image of PA vessel with iron particles (black objects) in the lumen. (B) Same PA vessel as in (A) demonstrating Cyto-roGFP fluorescence in vascular cells. Average results from multiple experiments in PA vessels expressing Cyto-roGFP (C) and Mito-roGFP (D) superfused with normoxic (21% O₂) or hypoxic (1.5% O₂) media. Values are means ± SEM, n = 6 PA vessels. *P < 0.05 compared with normoxic baseline of empty virus-treated PA vessels. †P < 0.05 compared with empty virus-treated PA vessels. DIC = differential interference contrast.

the chronic loss of Complex III function in their smooth muscle cells.

To further evaluate the role of RISP in HPV, intracellular calcium responses in the media of PA were assessed using FURA-2 in fresh precision-cut lung slices from SMC-MHC-Cre/RISP^{fllox/fllox} mice. Superfusion of lung slices from Control or Vehicle mice with hypoxic media (20 min; 1.5% O₂) caused an increase in [Ca²⁺]_i, manifested by a change in the FURA-2 ratio from baseline to 106 ± 0.9 and 105 ± 0.6% of baseline, respectively

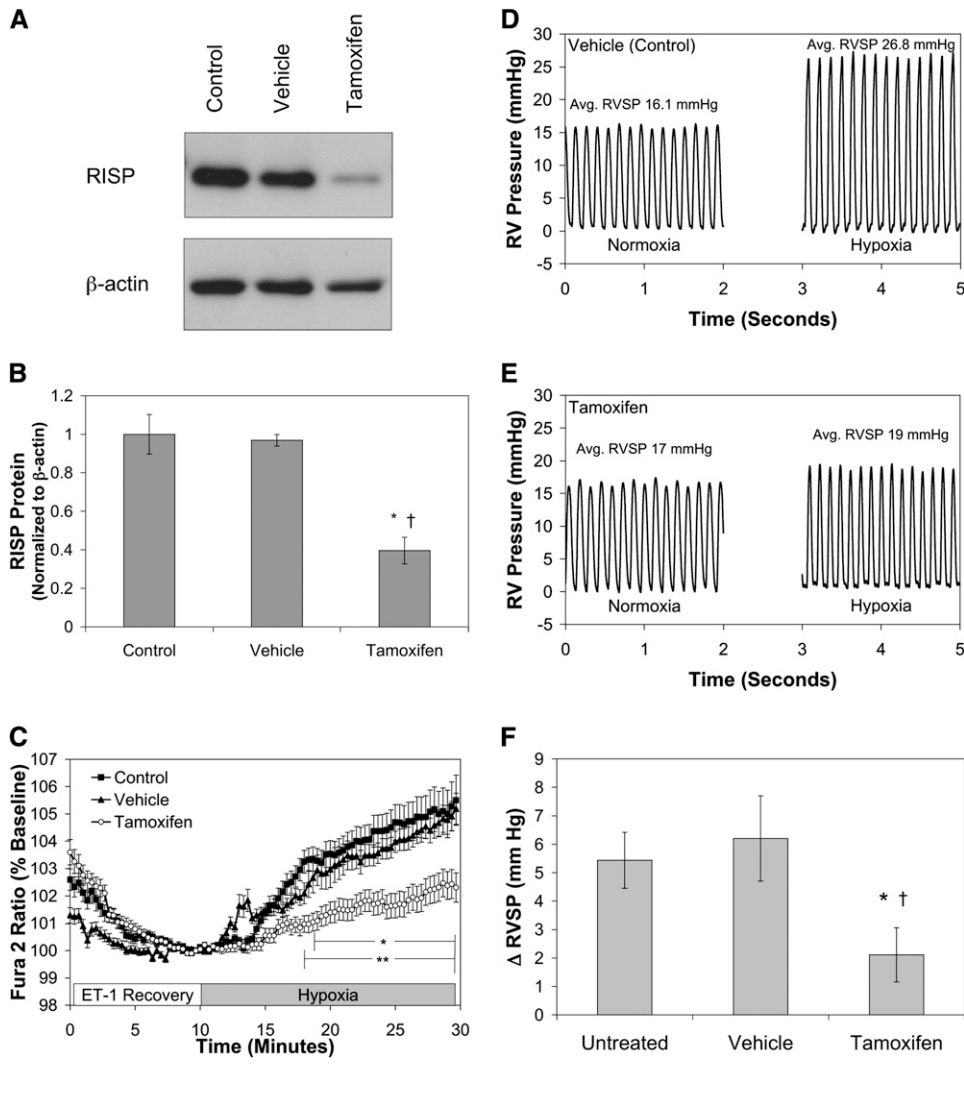


Figure 7. Effect of Riese iron-sulfur protein (RISP) depletion on hypoxia-induced increases in $[Ca^{2+}]_i$ in smooth muscle cells (SMC) of precision-cut lung slices and right ventricular systolic pressure (RVSP) of SMC- myosin heavy chain (MHC)-Cre/RISP^{fllox/fllox} mice. (A) Western blot of lysates from abdominal aorta of untreated (Control), tamoxifen vehicle (Vehicle), or tamoxifen (Tamoxifen) isolated from SMC-MHC-Cre/RISP^{fllox/fllox} mice probed with antibodies against the RISP subunit or β -actin. (B) Quantified analysis of Western blots expressed RISP expression normalized to β -actin. Values are means \pm SEM, $n = 3$ Western blots. * $P < 0.05$ compared with Control, $^\dagger P < 0.05$ compared with Vehicle. (C) Hypoxia (1.5% O_2 for 20 min)-induced changes in $[Ca^{2+}]_i$ as assessed by calcium-mediated changes in FURA-2 measured in cells within the wall of the pulmonary arteries in precision-cut lung slices. Values are means \pm SEM, $n = 6$ lung slices. * $P < 0.05$ compared with normoxic baseline for Control and Vehicle, ** $P < 0.05$ compared with Vehicle for Tamoxifen. (D) Hypoxia (5% O_2 for 1 min) increases the RVSP in the RV pressure tracing from a Vehicle (Control) mouse. (E) RISP depletion suppresses the hypoxia (5% O_2 for 1 min)-induced increase in RVSP as observed in the RV pressure tracing from a tamoxifen-treated mouse. (F) Hypoxia (5% O_2 for 1 min)-induced changes in hypoxic pulmonary vasoconstriction as denoted by changes in the RVSP of mice. Normoxic baseline RVSP for the Untreated, Vehicle, and Tamoxifen mice were 17.6 ± 1.5 , 17.3 ± 2.3 , and 17.0 ± 1.1 mm Hg, respectively. Values are means \pm SEM, $n = 7-9$ animals. * $P < 0.05$ compared with Untreated. $^\dagger P < 0.05$ compared with Vehicle.

(Figure 7C). Superfusion with hypoxia media also caused constriction of the PA (Figure E3). RISP-KO in the SMC significantly attenuated the $[Ca^{2+}]_i$ response to hypoxia in lung slice preparations ($102 \pm 0.5\%$ of baseline; Figure 7C). Thus, hypoxia-induced increases in $[Ca^{2+}]_i$ in PA of lung slices requires a functional Complex III.

RISP Depletion Attenuates Hypoxia-induced Increases in Right Ventricular Systolic Pressure of an Intact Mouse

To determine the role of RISP in the HPV response *in vivo*, hypoxia-induced changes in right ventricular systolic pressure (RVSP) were assessed in SMC-MHC-Cre/RISP^{fllox/fllox} mice ventilated under normoxic and hypoxic conditions using a Micro Tip pressure transducer catheter (Millar, Houston, TX). Changes in RVSP reflect changes in the PA pressure and thus provide an assessment of the HPV response (Figures 7D and 7E). Normoxic baseline RVSP for the Untreated, Vehicle, and Tamoxifen mice were 17.6 ± 1.5 , 17.3 ± 2.3 , and 17.0 ± 1.1 mm Hg, respectively ($P =$ not significant). When untreated or vehicle-treated mice were switched from room air to hypoxic (5% $O_2/95\%$ N_2) ventilation, RVSP increased by 5.4 ± 1.0 and 6.2 ± 1.5 mm Hg, respectively (Figure 7F). In tamoxifen-treated mice, the hypoxia-induced change

in RSVP was significantly smaller (2.1 ± 1.0 mm Hg; Figure 7F). These results demonstrate that RISP is required for the HPV response.

DISCUSSION

We developed a mouse model to permit conditional deletion of the nuclear-encoded RISP gene to assess its role in hypoxia-induced ROS and Ca^{2+} signaling in the pulmonary circulation. Using the roGFP sensor to assess subcellular oxidant signaling, we find that depletion of RISP abolishes the ROS response to hypoxia in isolated PASMC and in isolated PA segments. Depleting RISP also abolishes hypoxia-induced $[Ca^{2+}]_i$ signaling in isolated PASMC as well as in PA within freshly cut lung slices. Although the response to acute hypoxia is ablated by RISP loss, cultured PASMC retain the ability to activate $[Ca^{2+}]_i$ signaling in response to H_2O_2 , indicating that the signaling pathway downstream from ROS is not affected by RISP depletion. Depletion of RISP in smooth muscle of the intact mouse also attenuates hypoxia-induced increases in PA pressure, as assessed by changes in RVSP. Collectively these studies indicate that Complex III is required for hypoxia-induced ROS signaling that triggers the acute increase in cytosolic $[Ca^{2+}]_i$.

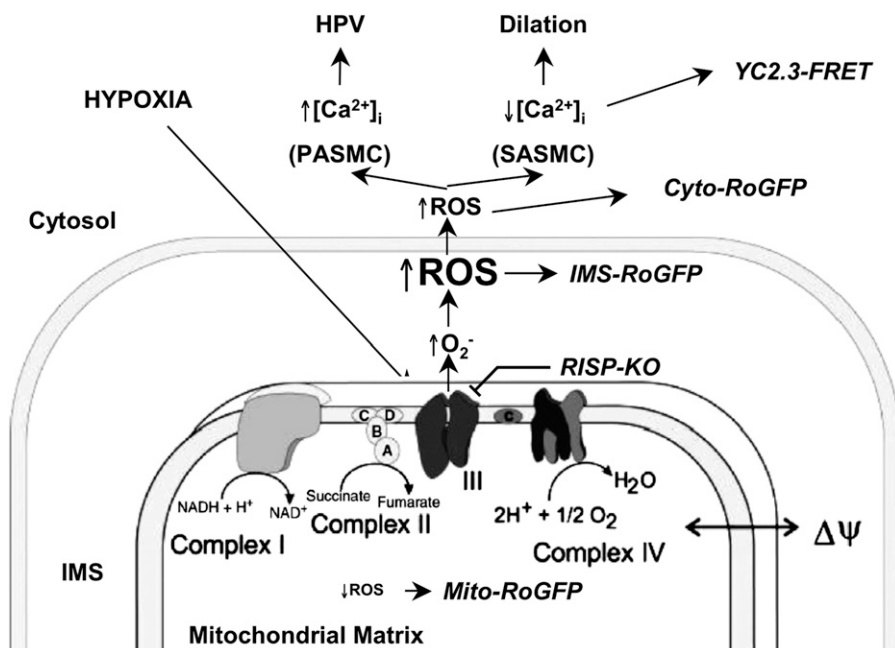


Figure 8. Model depicting mitochondria functioning as the O_2 sensor underlying the acute hypoxic pulmonary vasoconstriction (HPV) response. IMS = intermembrane space; KO = knockout; NAD^+ = nicotinamide adenine dinucleotide; $NADH$ = NAD^+ reduced; PASM = pulmonary arterial smooth muscle cells; ROS = reactive oxygen species; SASM = systemic arterial smooth muscle cells; YC2.3-FRET = the calcium-sensitive, Förster resonance energy transfer sensor.

and contraction in the pulmonary circulation (Figure 8). These findings implicate mitochondrial ROS in the oxygen-sensing response in acute HPV.

We previously showed that the cytosol and the IMS of PASM and SASM exhibit increases in oxidant signaling during hypoxia, whereas the opposite response occurs in the matrix (10). This appears to reflect an increase in the generation of superoxide at the outer surface of the inner mitochondrial membrane and a decrease in nonspecific superoxide generation in the matrix. Depletion of RISP abolished the hypoxia-induced increase in ROS in the IMS and the decrease in the matrix, which argues against the idea that ROS generation in the latter compartment is nonspecific and Po_2 dependent. Alternatively, these results could also be explained by a shift in the direction of ROS secretion from the inner mitochondrial membrane, away from the matrix compartment and toward the IMS. A hypoxia-induced shift in the conformation of Complex III would presumably be needed to explain such a change in direction of ROS release. In either case, the present study confirms and extends our previous work showing that pharmacological inhibition of Complex III with myxothiazol, which binds at the quinol oxidation (Q_o) site of the bc_1 complex, attenuated the hypoxia-induced increase in ROS and Ca^{2+} signaling in PASM as well as the HPV response in the isolated perfused lung, whereas antimycin A, which binds to the Q_i site, had no effect on the HPV response (9, 11, 12). RISP acts by transferring a single electron from ubiquinol bound at the Q_o site to cytochrome c_1 , resulting in the formation of ubisemiquinone at the Q_o site, which has the potential to transfer its electron to molecular oxygen, thus forming superoxide. By depleting RISP, the formation of ubisemiquinone at the Q_o site is prevented, thus mimicking the response to myxothiazol. Taken together, these results are also consistent with a broader body of work indicating that hypoxia increases mitochondrial ROS signaling (10, 17, 18, 27–29, 36–39).

Are mitochondria the only source of ROS during hypoxia? Studies using a genetic knockout of $p47^{phox}$ suggest that cytosolic nicotinamide adenine dinucleotide phosphate reduced oxidase systems may also contribute to the HPV response during acute hypoxia (40). The blockade of hypoxia-induced ROS responses we observe with depletion of RISP suggests that the

mitochondria may act as the initiators of ROS production, which could be amplified by engagement of nonphagocytic nicotinamide adenine dinucleotide phosphate reduced oxidase systems elsewhere in the cell. Such “ROS-induced ROS release” might permit small ROS signals generated by mitochondria to activate ROS signaling throughout the cell, thereby avoiding mitochondrial damage that might arise if the entire cellular oxidant signal originated from that organelle (15). Regardless of the source, ROS signals trigger diverse functional responses to hypoxia in PASM, including calcium signaling and AMP-regulated kinase (AMPK) activation (12, 41).

Depletion of RISP also attenuated hypoxia-induced increases in Cyto-roGFP oxidation in SASM (Figure 5A). This attenuation suggests that Complex III is required for hypoxic ROS signaling in diverse vascular cell types, and it is not consistent with the observation by Michelakis and colleagues (24) that SASM mitochondria behave reciprocally to PASM in terms of ROS generation during hypoxia. In PASM, hypoxia elicits increases in $[Ca^{2+}]_i$ to cause vasoconstriction (42–47), whereas SASM decrease $[Ca^{2+}]_i$ and undergo relaxation (24, 48, 49). Our observation that RISP depletion in SASM has no detectable effect on calcium signaling (Figure 5B) is not surprising, as SASM do not increase $[Ca^{2+}]_i$ during hypoxia. Interestingly, although hypoxia increased $[Ca^{2+}]_i$ in isolated PASM, there was a significant decrease in $[Ca^{2+}]_i$ during the second phase of the response to hypoxia (Figure 4A). This did not occur in the experiments in lung slices (Figure 7C), suggesting that other cell types may be required for a sustained increase in PASM $[Ca^{2+}]_i$ during more prolonged hypoxia.

Korde and colleagues used siRNA to decrease RISP in PASM and observed a decrease in ROS generation in isolated mitochondria under normoxia (29). However, interpretation of their data is complicated because factors regulating ROS production in the intact cell, such as the membrane potential and respiratory rate, are dramatically altered when the organelle is removed from the cell. Other studies using genetic suppression of RISP in tumor cell lines show that ROS derived from Complex III are important for hypoxic signaling (16, 27, 28). However, one cannot assume that the O_2 sensing and signaling pathways in tumor cells are identical to those in PASM. The present study

was therefore undertaken to provide a more complete assessment of its role in triggering acute responses in the pulmonary circulation.

To our knowledge, this is the first study to genetically target a subunit of the ETC to evaluate its role in the HPV response in an intact animal. Interestingly, depletion of RISP in smooth muscle of the adult mouse produced no overt phenotype (Figure E2), indicating that SMC can survive and function using glycolytic ATP, albeit with an impaired HPV response. The animals appeared healthy, and the depletion of RISP did not affect the ability of the cells to respond to exogenous H₂O₂, indicating that cells were not globally impaired. After depletion of RISP, the cultured PASMC continued to proliferate, indicating that loss of ETC function does not induce bioenergetic deficiency.

Although the present study demonstrates the importance of mitochondrial ROS for responses to acute hypoxia in the lung, chronic hypoxia in obstructive lung disease represents a major clinical problem associated with remodeling of pulmonary vessels and the development of pulmonary hypertension. It is not clear whether the O₂ sensing and ROS signaling pathways we link to the acute responses in the present report are also involved in triggering the remodeling of the pulmonary circulation during chronic hypoxia. However, given the fact that chronic depletion of RISP is well tolerated by the mice, it will be possible to test this question in future studies involving prolonged hypoxic exposure.

Author disclosures are available with the text of this article at www.atsjournals.org.

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