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Peroxiredoxin-5 targeted to the mitochondrial intermembrane space attenuates hypoxia-induced reactive oxygen species signalling

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Abstract

The ability to adapt to acute and chronic hypoxia is critical for cellular survival. Two established functional responses to hypoxia include the regulation of gene transcription by HIF (hypoxia-inducible factor), and the constriction of pulmonary arteries in response to alveolar hypoxia. The mechanism of O₂ sensing in these responses is not established, but some studies implicate hypoxia-induced mitochondrial ROS (reactive oxygen species) signalling. To further test this hypothesis, we expressed PRDX5 (peroxiredoxin-5), a H₂O₂ scavenger, in the IMS (mitochondrial intermembrane space), reasoning that the scavenging of ROS in that compartment should abrogate cellular responses triggered by the release of mitochondrial oxidants to the cytosol. Using adenoviral expression of IMS-PRDX5 (IMS-targeted PRDX5) in PSMCs (pulmonary artery smooth muscle cells) we show that IMS-PRDX5 inhibits hypoxia-induced oxidant signalling in the IMS and cytosol. It also inhibits HIF-1 α stabilization and HIF activity in a dose-dependent manner without disrupting cellular oxygen consumption. IMS-PRDX5 expression also attenuates the increase in cytosolic [Ca²⁺] in PSMCs during hypoxia. These results extend previous work by demonstrating the importance of IMS-derived ROS signalling in both the HIF and lung vascular responses to hypoxia.

Keywords

hypoxia; hypoxia-inducible factor (HIF); hypoxic pulmonary vasoconstriction; peroxiredoxin-5; reactive oxygen species (ROS); redox-sensitive GFP (roGFP)

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AUTHOR CONTRIBUTION

Simran Sabharwal, Gregory Waypa and Paul Schumacker designed the experiments. Gregory Waypa isolated rat PSMCs and performed calcium experiments. Jeremy Marks performed immunogold staining of IMS-roGFP with the help of the University of Chicago electron microscopy core. Simran Sabharwal cloned the IMS constructs and performed all of the remaining experiments. Simran Sabharwal and Paul Schumacker wrote the paper with input from all of the authors. All of the authors contributed to the data interpretation.

INTRODUCTION

Adaptation to low levels of oxygen (hypoxia) is critical for survival, as all multicellular organisms require molecular oxygen (O_2) for respiratory and metabolic functions. Hypoxia triggers adaptive mechanisms at the molecular, cellular and organismal levels. At the molecular level, transcriptional responses to hypoxia are driven primarily by the hypoxia-inducible factors HIF-1 and HIF-2, which regulate the genes responsible for cellular pathways, including glycolysis, angiogenesis, proliferation, metabolism and other processes critical for mediating survival.

HIF-1 is a heterodimer consisting of α - and β -subunits. Both are ubiquitously expressed, however, stability of the α -subunit is regulated inversely with cellular O_2 levels. Under normoxic conditions, the α -subunit is hydroxylated at conserved proline residues by PHD (prolyl hydroxylase) through a reaction that consumes O_2 . The hydroxylated subunit is linked by the LIMD1 scaffolding protein to the VHL (von Hippel–Lindau) protein, which serves as the E3 ubiquitin ligase that targets it for proteasomal degradation. Under hypoxic conditions, PHD activity and HIF-1 α degradation are inhibited, allowing heterodimerization with the β -subunit and translocation to the nucleus where they interact with hypoxia-response elements in the promoter/enhancer regions of target genes. HIF-1 α activity, but not its stability, is further regulated via hydroxylation of an asparaginyl group by the protein FIH (factor inhibiting HIF). Some HIF-dependent responses occur rapidly, such as the shift from oxidative to glycolytic metabolism, whereas other effects such as angiogenesis and vasculogenesis require more prolonged hypoxic exposure [1–7].

An example of an adaptive response at the organismal level is seen in the lung, where acute hypoxic exposure elicits calcium-mediated constriction in the PSMCs (pulmonary artery smooth muscle cells). This response limits blood flow to hypoxic areas of the lung, thereby improving the efficiency of gas exchange. Termed HPV (hypoxic pulmonary vasoconstriction), this response is mediated by hypoxia-induced increases in cytosolic calcium ($[Ca^{2+}]_i$) derived from intracellular stores in the endoplasmic reticulum and from the entry of extracellular Ca^{2+} through voltage-dependent and -independent channels [8,9].

Previous work from our laboratory and others identified the importance of ROS (reactive oxygen species) signals, particularly H_2O_2 , in triggering the HIF and HPV responses [10–12]. In that regard, hypoxia elicits an increase in ROS release from the mitochondrial ETC (electron transport chain), leading to increases in oxidant signalling in the IMS (mitochondrial intermembrane space) and the cytosol [13–19]. Pharmacological inhibitors of the ETC initially suggested that complex III of the ETC is critical for hypoxia-induced ROS generation, on the basis of their ability to abolish hypoxic responses [10,11]. Subsequent studies demonstrated that suppression of electron flux through complex III, induced by suppression of the Rieske iron–sulfur protein or knockout of cytochrome *c*, abolished ROS signalling and hypoxic responses [15,16]. Those studies implicated the ubiquinone-binding site near the outer leaflet of the inner mitochondrial membrane (the Q_o site) as a likely site of ROS release. Experimental deletion of proteins critical for ETC function, or the addition of specific mitochondrial inhibitors, abrogated ROS signalling and hypoxia responses. However, they also abolished oxidative phosphorylation and O_2 consumption by the

respiratory chain. Accordingly, other investigators have challenged that model by arguing that the loss of O₂ consumption caused by inhibition of the ETC introduces an experimental artifact by increasing the oxygen tension of cells in culture, or by redistributing intracellular O₂ away from mitochondria and towards PHDs [20]. The localized increase in the availability of O₂ at PHDs, and the loss of mitochondrial ATP generation, has been argued to explain the associated loss of hypoxic responsiveness [21]. In support of the ROS signalling model, mitochondria-targeted antioxidant compounds have also been shown to abolish HIF responses to hypoxia [22]. However, skepticism has persisted because these compounds can also inhibit ETC function at higher concentrations [23]. In the present study, we reasoned that the expression of an H₂O₂ scavenger within the IMS should provide an alternative test of this hypothesis, by preventing the transmission of oxidant signals from the outer surface of the inner membrane to the cytosol. Assuming that such a system does not interfere with ETC activity, it would test the specific requirement of mitochondrial oxidant signals for hypoxic responses.

Peroxisiredoxins are thiol-dependent peroxidases that attack the O–O bond of H₂O₂, alkyl hydroperoxides and peroxynitrite using a conserved cysteine residue at the active site. This results in formation of a sulfenic acid that must be reduced by another cysteine thiol. Peroxisiredoxins are grouped depending on the location of the active cysteine residue. The typical 1-cysteine peroxiredoxins require an external thiol for reduction, whereas the typical 2-cysteine group form homodimers with one subunit reducing the sulfenic acid of the other. The atypical 2-cysteine peroxiredoxins contain the resolving thiol within the same protein and resolve the sulfenic acid to a disulfide bond which is reduced by thioredoxins, restoring the enzyme to its original form. PRDX5 (peroxiredoxin-5) is the only mammalian atypical 2-cysteine peroxiredoxin and is widely expressed among tissues and in many subcellular compartments, including the cytosol and nucleus, and in peroxisomes and mitochondria [24]. In the present study we demonstrate that expressing PRDX5 in the IMS (IMS-PRDX5) to scavenge H₂O₂ effectively attenuates both HIF stabilization and cytosolic Ca²⁺ influx under hypoxic conditions.

EXPERIMENTAL

Rat PASMC isolation

The Northwestern University Institutional Animal Care and Use Committee approved all animal studies. Rats were anaesthetized with Avertin (2,2,2-tribromoethanol) at a dose of 240 mg/kg of body weight. The level of anaesthesia was monitored by toe pinch reflex. The rats were killed prior to the removal of their lungs by exsanguination through the severing of the abdominal aorta while fully anaesthetized. PASMC isolation was based on a method developed by Waypa et al. [12] and Marshall et al. [25]. Briefly, freshly excised rat heart and lungs were rinsed with PBS containing penicillin and streptomycin (1%). The right ventricle was cannulated, and the pulmonary vasculature was flushed with PBS (30 ml). By use of the pulmonary artery cannula, growth medium 199 (M199, 30 ml) containing low-melting-point agarose (0.5%) and iron particles (Fe₃O₄; 0.5%) was flushed through the pulmonary vasculature. The iron particles were too large to pass through the capillaries; therefore, only the arteries were filled with the agarose and iron particles. Furthermore,

Fe₃O₄ is poorly soluble in the medium employed for PASMCM isolation. The airways were filled via the trachea with M199 (15 ml) containing low-melting-point agarose (1%) without iron. The lungs were placed in ice-cold PBS to cause the agarose to set. After 10 min, the lobes were dissected free and finely minced in a Petri dish. Lung fragments were resuspended and washed three times with PBS by use of a magnet to retain the iron-containing fragments. The iron-containing pieces were resuspended in M199 (25 ml) containing collagenase (type IV, 80 units/ml) and incubated at 37°C for 30–60 min. To remove extravascular tissue, fragments were drawn through an 18-gauge needle. Iron-containing fragments were washed three times with M199 containing FBS (20%, v/v) and drained. The resulting fragments were placed in a T-75 flask and resuspended in M199 containing FBS (10%, v/v). The flasks were incubated at 37°C with CO₂ (5%) in air for 4 or 5 days, during which time the SMCs (smooth muscle cells) were observed to migrate and adhere to the bottom of the flask. After 4 or 5 days, the medium and iron-containing particles were transferred to a new flask containing fresh medium. The adherent SMCs continued to propagate until the cells were 75% confluent, at which time they were trypsinized and passed by a magnet to remove any residual iron particles. Cells isolated by this method were confirmed to be PASMCMs as described previously [12]. The isolated cells were cultured for 1–2 weeks and then used for the next 1–2 weeks.

Molecular cloning

The mouse smac/Diablo IMS targeting sequence was PCR-amplified from mouse cDNA using the following primers with XhoI/HindIII restriction enzyme sites: the forward primer was 5'-GATCTCGAGATGGCGGCTCTGAGAAGT-3' and the reverse primer was 5'-GGCAAGCTTAATAGGAACCGCACA-3'. The redox-sensitive GFP roGFP, first described by Remington and co-workers [26], was PCR-amplified from the cytosolic targeted roGFP in the VQ Ad5CMV K-NpA adenoviral shuttle vector (ViraQuest) as described previously [18] using the following primers with HindIII/NotI restriction enzyme sites: forward primer, 5'-GAGAAGCTTATGGTGAGCAAGGGCGAG-3'; reverse primer, 5'-TATGCGGCCGCTTAACCTGTACAGCTCGTC-3'. Full-length PRDX5 was PCR-amplified from human cDNA using the following primers with HindIII/EcoRI restriction enzyme sites and a haemagglutinin tag at the C-terminus end of the protein: forward primer, 5'-GAGAAGCTTATGGCCCCAATCAAGGTGGGAGATGCC-3'; reverse primer, 5'-GGCGAATTCTCAAGCGTAATCTGGAACATCGTATGGGTAGAGCTGTGAGATGATATT-3'. The smac/Diablo-targeting sequence was ligated to both the roGFP and PRDX5 constructs which were then inserted into the VQ Ad5CMV K-NpA shuttle vector to create recombinant adenoviruses for use with our cells. roGFP was targeted to the matrix as described previously [18] by adding a 48 bp targeting sequence from cytochrome oxidase subunit IV to the 5' end of the coding sequence.

Immunogold staining and electron microscopy

Rat PASMCMs were transduced with an adenovirus expressing either IMS-roGFP (IMS-targeted roGFP) or matrix-targeted roGFP. Cells were trypsinized after 48 h, collected and centrifuged. The pellet was then transferred to aluminum sample holders and frozen in a Baltec HPM 010 high-pressure freezer (RMC Products). Frozen samples were freeze-substituted in uranyl acetate (0.1%) and glutaraldehyde (0.25%) in acetone at –80°C for 5

days, then warmed to -50°C over 18 h. After several acetone rinses, samples were placed into an automatic freeze substitution system (Leica EM AFS) and infiltrated with Lowicryl HM20 resin at -50°C in four graded steps (25%, 50%, 75% and 100%) over 2 days. Thereafter, resin was changed hourly three times. Samples were polymerized under UV light at -80°C for 72 h. Sections (100 nm thick) of samples embedded in Lowicryl HM20 were placed on Formvar-coated gold slot grids. The sections were blocked for 20 min with a 5% (w/v) solution of non-fat dried skimmed milk powder in TBST (TBS plus 0.1% Tween 20). Grids were incubated in anti-eGFP antibody (Abcam ab290, 1:20 dilution in 2.5% non-fat dried skimmed milk powder in TBST) at room temperature for 1 h. The sections were rinsed in a stream of TBS plus 0.5% Tween 20 and then incubated in goat anti-rabbit IgG (1:80) conjugated to 10 nm gold particles for 1 h. Control procedures omitting the primary antibody were performed. Images were obtained using a FEI Tecnai TF30 intermediate voltage electron microscope operating at 300 kV.

Immunostaining and confocal microscopy

PASMCs were plated on collagen-coated coverslips and infected with either the IMS-roGFP or IMS-PRDX5 virus. At 36–48 h post-infection the cells were fixed in 3% formaldehyde and 0.25% glutaraldehyde in PBS for 15 min and washed in PBS three times. Cells were then permeabilized in 0.1% Triton X-100 for 5 min, the aldehyde groups were reduced by exposing the cells to three 5 min washes in 0.5 mg/ml sodium borohydride, and blocked in 1% NGS (natural goat serum) in PBS for 1 h. Cells were then incubated in primary antibody in 1% NGS for 1 h followed by incubation in secondary antibody for 1 h prior to affixing the coverslips to slides for imaging. Antibodies used were: anti-MnSOD (manganese superoxide dismutase; Stressgen, #SOD-111, 1:200 dilution) and high-affinity anti-HA (Roche, #11867423001, 1:200 dilution), Alexa Fluor[®] 488 (Invitrogen, 1:400 dilution) and Alexa Fluor[®] 568 (Invitrogen, 1:400 dilution). PBS washes followed each step. Confocal images were obtained using a Zeiss LSM 510 META laser-scanning confocal microscope with a 40 $\times$ oil immersion lens and the Zeiss LSM imaging software (Carl Zeiss MicroImaging).

Oxygen consumption

Rat PASMCs were trypsinized and resuspended in full medium at a concentration of 2×10^6 cells/ml. The respiration rate of rat PASMCs was measured with a Clark-type oxygen electrode (Oxygen electrode Units DW1, Hansatech Instruments) at 37°C .

Seahorse XF-24 extracellular flux analysis

Rat PASMCs were infected with either a virus with an empty expression vector [2000 pfu (plaque-forming units)] or an equivalent dose of IMS-PRDX5 virus 36 h prior to conducting the mitochondrial stress test in accordance with the manufacturer's instructions. At 24 h post-infection, the cells were trypsinized, counted and plated at 40000 cells per well in a Seahorse XF-24 cell culture plate. Growth medium was replaced with Seahorse medium at 37°C 1 h before assessing basal oxygen consumption for approximately 1 h. Mitochondrial inhibitors were then added to assess mitochondrial proton leak (oligomycin, 1 μM), maximal respiration rate [FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone), 2 μM], and non-mitochondrial respiration (Antimycin A, 1 μM and rotenone, 1 μM). Cells from each

well were counted again after the experiment and results were normalized to the number of cells.

Hypoxia

Cells were placed in an environmental hypoxia chamber (Coy Laboratory Products) maintained at 1.5% O₂, 5% CO₂ and the balance N₂. For Western blot experiments, pre-equilibrated hypoxic medium was added to cells at the start of the experiment and tissue culture dishes were gently rocked on an oscillating platform prior to cell lysate collection. Cells for ROS and calcium experiments were placed in a static incubator inside the glove box.

Epifluorescence microscopy

Coverslips were placed into a flow-through chamber consisting of two coverslips separated by a stainless steel spacer ring. In the chamber, cells were superfused with a BSS (balanced salt solution) consisting of 117 mM NaCl, 4 mM KCl, 18 mM NaHCO₃, 0.76 mM MgSO₄, 1 mM NaH₂PO₄, 1.21 mM CaCl₂ and 5.6 mM glucose and bubbled with O₂/CO₂/N₂ gas mixtures at 37°C in a water-jacketed column. Normoxic cells were superfused with BSS equilibrated with 5% CO₂, 21% O₂ and the balance N₂. For hypoxia, BSS was bubbled with 5% CO₂, 1.5% O₂ and the balance N₂. Images were collected using a 16-bit cooled CCD (charge-coupled device) detector and were assessed using Metafluor software (Universal Imaging).

ROS measurements

Cells were plated on collagen-coated 25-mm-diameter glass coverslips and infected with virus for 24 h prior to placing them in normoxia or hypoxia for 12–30 h. For cytosolic and roGFP measurements, epifluorescence microscopy was carried out with excitation wavelengths of 400 and 485 nm and an emission wavelength of 535 nm. Regions of interest were outlined in the 485/400 ratiometric images produced by the Metafluor software and images were taken at 1 min intervals for the duration of the experiment. A stable baseline was monitored for 5 min and averaged prior to fully reducing the probe with 1 mM DTT and then fully oxidizing the probe with 1 mM TBH (t-butyl hydroperoxide). For matrix roGFP, flow cytometry was used. PSMCs exposed to normoxia or hypoxia for 18 h were trypsinized and separated into three aliquots and immediately placed on ice. One aliquot was measured as a baseline reading, one aliquot was fully reduced with DTT, and one aliquot was fully oxidized with TBH. All aliquots were analysed with a DakoCytomation CyAn multilaser flow cytometer using 405 nm and 488 nm excitation wavelengths, whereas emission was assessed at 535 nm. All results were calculated as the percentage oxidation of the respective roGFP probe, using the fully oxidized and reduced values as reference values.

Antibodies and Western blotting

Cells were lysed in a buffer consisting of 50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 40 mM 2-glycerophosphate, 1 mM PMSF, 10 mM NaF, 250 μ M sodium orthovanadate and Complete[™] protease inhibitor cocktail (Roche). Hypoxic samples were lysed inside the Coy chamber to prevent sample exposure to oxygen. Lysates

were separated on SDS/PAGE and transferred to nitrocellulose membranes that were blotted with primary antibodies. Blots were further incubated with secondary HRP (horseradish peroxidase)-conjugated antibodies (Cell Signaling Technology) and stained with ECL reagent (GE Healthcare). Chemiluminescence was detected on film and quantified using ImageJ (NIH). The primary antibodies used were: anti- β -actin (Abcam, #ab6276, 1:10000 dilution), anti-HIF-1 α (Cayman Chemical, #10006421, 1:1000 dilution), anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase; Cell Signaling Technology, #14C10, 1:1000 dilution) and anti-PDHK1 (pyruvate dehydrogenase kinase-1; Cell Signaling Technology, #C47H1, 1:1000 dilution).

Calcium measurements

Cells were plated on to 10-mm-diameter glass coverslips and infected with virus approximately 36 h prior to conducting experiments. A genetically encoded FRET-based sensor was used to measure $[Ca^{2+}]_i$. YC2.3 is a high-affinity Ca^{2+} sensor, consisting of CFP and citrine, linked by a calmodulin-M13 hinge region [27]. When bound to Ca^{2+} , FRET between CFP and citrine increases. An increase in $[Ca^{2+}]_i$ is reflected by an increase in the citrine/CFP intensity ratio (535/470). YC2.3 was packaged in a recombinant adenovirus to permit efficient expression in PSMCs. As a buffered salt solution was being used to perfuse the cells, endothelin 1 (10 nM) was added to the perfusate 15 min before hypoxia to prime the cells for hypoxia-induced PSMC contraction [28]. The 15 min was enough time to allow for the YC2.3 ratio to return to baseline after the endothelin 1-induced increase in $[Ca^{2+}]_i$. This had the added benefit of confirming that the virally treated cells retained the ability to increase cytosolic calcium via a receptor-mediated trigger.

Statistics

ANOVA was used to identify differences between multiple experimental groups. Newman–Keuls post-hoc analysis was used to determine significance between groups. YC2.3 data were analysed using a Student's *t* test. Significance was defined as $P < 0.05$.

RESULTS

Targeting protein constructs to the IMS

A 57-amino-acid leader sequence from the mouse smac/Diablo protein was used to target PRDX5 and roGFP constructs to the IMS. The first 53 amino acids of this sequence are cleaved upon entry into the IMS, leaving the unbound protein with only four additional amino acids on its N-terminus [29].

Rat PSMCs expressing the IMS-roGFP construct were immunostained for the mitochondrial protein MnSOD, showing that colocalization was restricted to the mitochondria (Figures 1A–1C). Rat PSMCs expressing IMS-PRDX5, using the same smac/Diablo targeting sequence, showed a colocalization pattern with MnSOD comparable with that of the IMS-roGFP construct (Figures 1D–1F). To ensure that the smac/Diablo-targeting sequence specifically targeted proteins only to the IMS, electron micrographs of IMS-roGFP and matrix-targeted roGFP were compared. Immunogold staining of rat PSMCs expressing the smac/Diablo-targeted IMS-roGFP shows localization of the protein

to the cristae and periphery of the mitochondria, indicating successful targeting of the construct to the IMS (Figure 1G, and Supplementary Figure S1 at <http://www.biochemj.org/bj/456/bj4560337add.htm>). This contrasted with immunogold staining of matrix-targeted roGFP, which shows labelled particles on the inside of the inner mitochondrial membrane (Figure 1H).

Mitochondrial respiration and function is not adversely affected by IMS-PRDX5 expression

As previously noted, many of the mitochondria-targeted small-molecule antioxidants have been shown to interfere with mitochondrial oxygen consumption [23]. Therefore, oxygen consumption rates were measured using a Clark-type oxygen electrode. Rat PSMCs infected with increasing doses of the IMS-PRDX5 virus demonstrated oxygen consumption levels equivalent to wild-type rat PSMCs and rat PSMCs infected with a virus containing an empty expression vector (Figure 2A). These results indicate that IMS-PRDX5 expression does not affect cellular respiration.

A recent study demonstrated that small molecule antioxidants also disrupt the mitochondrial response to inhibitors [30]. Therefore we assessed the effects of IMS-PRDX5 on mitochondrial function using a Seahorse Biosciences XF-24 extracellular flux analyser. Figure 2(B) shows that at the highest dose of the IMS-PRDX5 virus (2000 pfu), the basal oxygen consumption rates of rat PSMCs are comparable with cells infected with the empty expression vector virus. Furthermore, the IMS-PRDX5-infected cells responded similarly to control cells when exposed to various mitochondrial inhibitors, demonstrating similar levels of proton leak, maximal respiration and non-mitochondrial respiration. Collectively, these data demonstrate that IMS-PRDX5 is less disruptive to mitochondrial function than many small-molecule antioxidants.

IMS-PRDX5 expression attenuates hypoxic oxidant changes in multiple cellular compartments in a dose-dependent manner

Rat PSMCs were exposed to prolonged hypoxia (12–30 h of 1.5% O₂) or normoxia (21% O₂) and their oxidant levels were assessed using the IMS-roGFP construct. Prolonged hypoxia was used because: (i) our previous studies had already examined acute responses to hypoxia [18]; and (ii) the present study focused on HIF-1 transcriptional responses, which occur on a scale of hours, not minutes. The oxidation level of IMS-roGFP in normoxic rat PSMCs was $50.5 \pm 1.4\%$ compared with $65.3 \pm 2.5\%$ during prolonged hypoxia (Figure 3A). These results are consistent with previous results obtained during hypoxic exposures of 30 min in identical cells [18]. This relatively small increase in hypoxia-induced roGFP oxidation (~15%) is consistent with a signalling role for mitochondrial ROS, rather than a damaging stress response. Rat PSMCs infected with a virus containing an empty expression vector and exposed to prolonged hypoxia had comparable oxidant levels with wild-type cells, whereas cells infected with the IMS-PRDX5 virus showed a dose-dependent attenuation of the hypoxic increase in oxidant levels in the IMS (Figure 3A, and Supplementary Figure S2A at <http://www.biochemj.org/bj/456/bj4560337add.htm>).

If ROS released from complex III crosses the IMS and affects redox signalling in the cytosol, then expression of an antioxidant in the IMS should abrogate hypoxia-induced

oxidant stress in the cytosol as well. Using the cytosolic roGFP sensor, we found the normoxic and hypoxic oxidation levels to be $22.3 \pm 1.5\%$ and $36.4 \pm 1.5\%$ respectively (Figure 3B). Empty vector-infected cells were not different from wild-type cells, whereas IMS-PRDX5 attenuated the hypoxic increases in oxidant stress in a dose-dependent manner (Figure 3B and Supplementary Figure S2B). These results support the conclusion that hypoxia-induced ROS signalling from complex III of the electron transport chain traverses the IMS to affect redox signalling in the cytosol.

Next, we assessed oxidant levels in the mitochondrial matrix. We previously reported that hypoxia decreases oxidant stress in the matrix [18], which is consistent with our current findings (Figure 3C). Again, empty-vector-infected cells exhibited comparable decreases in oxidant stress compared with untreated cells. Interestingly, IMS-PRDX5 attenuated the hypoxia-induced decrease in matrix oxidation in a dose-dependent manner (Supplementary Figure S2C). This unexpected result suggests that oxidant release to the IMS during hypoxia might represent an O_2 -dependent shift in the directional release of membrane-generated ROS between the IMS and the matrix compartments.

IMS-PRDX5 expression attenuates HIF-1 α stabilization and activity in a dose-dependent manner

To determine whether an IMS-targeted antioxidant could attenuate HIF-1 α stabilization and activity, cells were exposed to hypoxia or normoxia for 8 h. Cell lysates were collected and Western blots were performed to measure relative HIF-1 α levels. DFO (desferrioxamine), an iron chelator that inhibits PHD function by binding free Fe^{2+} that PHD requires for HIF-1 α hydroxylation, was used as a positive control. Figure 4(A) shows that DFO stabilizes HIF-1 α in normoxia, as does hypoxia. Cells infected with a virus containing an empty expression vector showed no difference in HIF-1 α stabilization compared with hypoxic controls, but cells infected with IMS-PRDX5 virus showed a dose-dependent decrease in hypoxic HIF-1 α stabilization (Supplementary Figure S2D), with significant attenuation achieved at the highest dose used.

Having demonstrated an attenuation of hypoxia-induced HIF-1 α stabilization by IMS-PRDX5, we next sought to determine whether this abrogated HIF-1 α activity. To assess HIF-1 α function, Western blot analysis was performed for two well-known glycolytic targets under HIF-1 α regulation, GAPDH and PDHK1 [31,32]. Figures 4(B) and 4(C) show dose-dependent decreases in GAPDH and PDHK1 protein levels respectively, by IMS-PRDX5 expression (Supplementary Figures S2E–S2F). Although the 2000 pfu dose of IMS-PRDX5 was the only one to show significant effects on HIF-1 α stabilization, both the 2000 pfu and 1500 pfu doses produced significant decreases in hypoxic HIF-1 α activity as measured by protein levels of these well-described HIF-1 α target genes.

IMS-PRDX5 expression decreases cytosolic influx of Ca^{2+} during acute hypoxia

To determine whether IMS-PRDX5 attenuates hypoxia-induced increases in cytosolic calcium, we expressed the Ca^{2+} -sensitive FRET sensor, YC2.3, to assess changes in cytosolic Ca^{2+} levels. Cells infected with a virus containing an empty expression vector and exposed to acute hypoxia exhibited a maximal increase in FRET ratio that averaged $19.4 \pm$

1.3% over normoxic baseline levels (Figure 5). Cells infected with 2000 pfu/cell of IMS-PRDX5 exhibited a significantly smaller increase in $[Ca^{2+}]_i$ upon hypoxic exposure, averaging $14.3 \pm 1.6\%$. Although some responsiveness remained, the cellular transduction efficiency was incomplete, so not every analysed cell expressed IMS-PRDX5 (results not shown). Nonetheless, these results show that the acute HPV response can be mitigated by the expression of an IMS-targeted antioxidant.

DISCUSSION

In the present study we expressed a H_2O_2 scavenger, PRDX5, in the IMS of the mitochondria. This targeted antioxidant significantly attenuated hypoxia-induced increases in thiol oxidation in both the IMS and cytosol, as assessed using roGFP sensors targeted to different subcellular compartments. This decrease in oxidant signalling ablated hypoxic stabilization and transcriptional activity of HIF-1 α and significantly decreased the amplitude of Ca^{2+} signalling in response to acute hypoxia.

These results are consistent with prior reports that ROS signalling from the mitochondria is required for cellular oxygen sensing, both in terms of the acute Ca^{2+} response in PSMCs and in terms of the HIF response to hypoxia in other cell types [10–12,15–19]. We confirmed previous data that oxidant stress increases in both the IMS and cytosol during hypoxia, whereas it decreases in the mitochondrial matrix. Our previous work implicated H_2O_2 by showing that scavenging H_2O_2 in the cytosol by expressing catalase or glutathione peroxidase in that compartment was sufficient to block the cytosolic increase in hypoxic oxidant stress [17,18]. Another study also showed that cytosolic catalase expression attenuated HIF-1 α stabilization, whereas matrix catalase expression had only a minor effect on HIF-1 α stabilization [15]. Since the cytosol is the site where HIF-1 α is regulated, and is also the site of calcium-mediated activation of PSMC contraction, redox signalling in this compartment should be critical for activating multiple cellular responses to hypoxia. The present study advances this field by revealing that mitochondrial IMS redox transmission is essential for the activation of these adaptive responses to hypoxia. The PRDX5-mediated decrease in ROS signalling in the IMS was associated with a decrease in the cytosol, resulting in the attenuation of HIF-1 α stabilization, HIF-1 α transcriptional activity, and hypoxia-induced increases in cytosolic $[Ca^{2+}]_i$.

Previous studies from our laboratory and from others implicate ROS production from mitochondria as signalling molecules that act to inhibit PHD activity. Many of the studies supporting that model utilized mitochondrial inhibitors, or genetic modifications that inhibited the electron transport chain, to limit ROS production [10,11,13–16,19]. Other investigators then raised the alternative explanation that the loss of oxygen consumption itself was responsible for our results, rather than the decrease in ROS generation. For example, Hagen and co-workers [33,34] asserted that hypoxia regulates HIF-1 α stability by depriving PHD of O_2 , an essential substrate required for the hydroxylation reaction. According to their model, normal oxygen consumption by mitochondria significantly lessens the intracellular O_2 tension relative to the extracellular space. Under conditions of moderate extracellular hypoxia, this 'oxygen sink' results in more severe depletion of intracellular O_2 , which decreases PHD activity by limiting its O_2 supply. The decrease in hydroxylation

would then limit HIF-1 α protein degradation and promote its stabilization [33,34]. A corollary to this theory is that mitochondrial inhibitors, or genetic interventions that inactivate the electron transport chain, would decrease O₂ consumption, thereby increasing the intracellular O₂ tension and increasing PHD activity to promote HIF-1 α degradation.

A related explanation was proposed by Metzen and co-workers, who reported that highly confluent cells in a tissue culture dish can generate a sufficient O₂ gradient across a static layer of culture medium to produce intracellular hypoxia that is sufficient to stabilize HIF-1 α , even though the head-space gas contains >20% O₂ [20]. In that situation, addition of a mitochondrial inhibitor would abolish HIF-1 α stabilization because it would halt mitochondrial O₂ consumption, thereby restoring normoxia in the cells as the medium O₂ tension rises to equilibrate with the head-space gas. In the present study, we avoided this artefact by studying subconfluent cells and by maintaining the culture dishes on an oscillating platform during hypoxia, to enhance convective mixing to prevent the development of a gradient in the medium. Other investigators have used mitochondria-targeted chemical antioxidants to attenuate the ROS signals under hypoxia. Agents such as MitoQ do abolish HIF stabilization in hypoxia, but these compounds have also been reported to decrease oxygen consumption at higher concentrations [22,23], raising questions about whether they, too, act by increasing intracellular O₂ levels.

Other studies have used small molecules that bind to the UQCRB subunit of complex III to disrupt hypoxic ROS generation [35,36]. Complex III, located within the inner membrane, contains two sites where ROS generation can conceivably occur – the Q_o site on the IMS side and the Q_i site on the matrix side. Neither of these novel small molecules binds near the Q_o or Q_i sites, so the mechanism by which they disrupt hypoxic ROS generation remains unclear. The present study is the first to utilize a genetic intervention that does not affect the ETC to disrupt ROS signalling in the IMS. Expression of PRDX5 in the IMS allows the scavenging of ROS signals without disturbing the normal functioning of the ETC. These findings underscore the importance of mitochondrial ROS signals in the hypoxic inhibition of PHD and subsequent stabilization of HIF-1 α , as opposed to a cellular redistribution of molecular oxygen.

Some investigators have argued that PHDs function as oxygen sensors because they require O₂ as a substrate in the hydroxylation of HIF-1 α [37,38]. Certainly, PHD is fully inhibited in the absence of O₂. The K_m for O₂ of PHD has been reported to be approximately 100 μ M, which could result in a slowing of activity even at physiological levels of tissue hypoxia (10–50 μ M) [39,40]. But the role that O₂-limited activity plays in the regulation of HIF-1 α stability *in vivo* is not fully established. Importantly, *in vitro* studies of enzymatic activity by a recombinant protein exclude contributions from post-translational modifications, such as the possible role of ROS in modifying the redox status of the Fe²⁺ in PHD. Recent studies examining the sensitivities of the three HIF hydroxylation sites to various interventions report that ROS can significantly inhibit the activity of FIH, but not PHD [41,42]. This implies that ROS may play some role in modifying HIF activation and transcription, but not in HIF stabilization. Our data are inconsistent with that conclusion, because they provide evidence that mitochondrial ROS contribute to HIF stabilization. If ROS played no role, either by directly or indirectly inhibiting PHD, then scavenging ROS signalling should have

failed to affect HIF stabilization. Further insight could be gained by dissecting the effects of IMS-PRDX5 on all three HIF hydroxylation sites, but that approach would require appropriate hydroxylation-specific antibodies [41].

An unexpected finding is that IMS-PRDX5 expression increased thiol oxidation during hypoxia in the matrix compared with controls. We previously attributed the decrease in matrix oxidant stress during hypoxia to an O₂-dependent decrease in nonspecific ROS generation [18]. These new data suggest that matrix redox status may be influenced by redox conditions in the IMS. Although the mechanism for this coupling is unknown, the IMS-PRDX5 effect might reflect a shift in the relative direction of ROS release from the mitochondrial inner membrane, away from the IMS and towards the matrix. Previous studies have demonstrated that ROS release from the membrane can occur in either direction, although the factors regulating this balance are unknown [43].

Cellular responses to hypoxia are critical for survival of both cells and organisms. HIF is central to the main response to hypoxia, yet controversy still exists as to the role that ROS play in the regulation of its stabilization and activation. In the present study we provide evidence for the crucial role of mitochondrial ROS in the HIF pathway. It is critical to understand the molecular mechanisms responsible for these responses in order to find better therapies for diseases associated with tissue hypoxia or aberrant activation of these hypoxic pathways. These insights, as well as future studies, should clarify the path forward to manipulating the HIF pathway for disease management.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

BSS	balanced salt solution
DFO	desferrioxamine
ETC	electron transport chain

FIH	factor inhibiting HIF
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HIF	hypoxia-inducible factor
HPV	hypoxic pulmonary vasoconstriction
IMS	mitochondrial intermembrane space
IMS-PRDX5	IMS-targeted peroxiredoxin-5
IMS-roGFP	IMS-targeted redox-sensitive GFP
MnSOD	manganese superoxide dismutase
NGS	natural goat serum
PASMC	pulmonary arterial smooth muscle cell
PDHK1	pyruvate dehydrogenase kinase-1
pfu	plaque-forming units
PHD	prolyl hydroxylase
PRDX5	peroxiredoxin-5
roGFP	redox-sensitive GFP
ROS	reactive oxygen species
SMC	smooth muscle cell
TBH	t-butyl hydroperoxide
TBST	TBS plus 0.1 % Tween 20

REFERENCES

1. Wang GL, Semenza GL. Purification and characterization of hypoxia-inducible factor 1. *J. Biol. Chem.* 1995; 270:1230–1237. [PubMed: 7836384]
2. Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, Gaskell SJ, von Kriegsheim A, Hebestreit HF, Mukherji M, Schofield CJ, et al. Targeting of HIF- α to the von Hippel-Lindau ubiquitylation complex by O₂-regulated prolyl hydroxylation. *Science.* 2001; 292:468–472. [PubMed: 11292861]
3. Ivan M, Kondo K, Yang H, Kim W, Valiando J, Ohh M, Salic A, Asara JM, Lane WS, Kaelin WG Jr. HIF α targeted for VHL-mediated destruction by proline hydroxylation: implications for O₂ sensing. *Science.* 2001; 292:464–468. [PubMed: 11292862]
4. Maxwell PH, Wiesener MS, Chang GW, Clifford SC, Vaux EC, Cockman ME, Wykoff CC, Pugh CW, Maher ER, Ratcliffe PJ. The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. *Nature.* 1999; 399:271–275. [PubMed: 10353251]
5. Huang LE, Gu J, Schau M, Bunn HF. Regulation of hypoxia-inducible factor 1 α is mediated by an O₂-dependent degradation domain via the ubiquitin-proteasome pathway. *Proc. Natl. Acad. Sci. U.S.A.* 1998; 95:7987–7992. [PubMed: 9653127]
6. Foxler DE, Bridge KS, James V, Webb TM, Mee M, Wong SC, Feng Y, Constantin-Teodosiu D, Petursdottir TE, Bjornsson J, et al. The LIMD1 protein bridges an association between the prolyl hydroxylases and VHL to repress HIF-1 activity. *Nat. Cell Biol.* 2012; 14:201–208. [PubMed: 22286099]

7. Lando D, Peet DJ, Whelan DA, Gorman JJ, Whitelaw ML. Asparagine hydroxylation of the HIF transactivation domain a hypoxic switch. *Science*. 2002; 295:858–861. [PubMed: 11823643]
8. Waypa GB, Schumacker PT. Hypoxia-induced changes in pulmonary and systemic vascular resistance: where is the O₂ sensor? *Respir. Physiol. Neurobiol.* 2010; 174:201–211.
9. Mungai PT, Waypa GB, Jairaman A, Prakriya M, Dokic D, Ball MK, Schumacker PT. Hypoxia triggers AMPK activation through reactive oxygen species-mediated activation of calcium release-activated calcium channels. *Mol. Cell. Biol.* 2011; 31:3531–3545. [PubMed: 21670147]
10. Chandel NS, Maltepe E, Goldwasser E, Mathieu CE, Simon MC, Schumacker PT. Mitochondrial reactive oxygen species trigger hypoxia-induced transcription. *Proc. Natl. Acad. Sci. U.S.A.* 1998; 95:11715–11720. [PubMed: 9751731]
11. Chandel NS, McClintock DS, Feliciano CE, Wood TM, Melendez JA, Rodriguez AM, Schumacker PT. Reactive oxygen species generated at mitochondrial complex III stabilize hypoxia-inducible factor-1 α during hypoxia: a mechanism of O₂ sensing. *J. Biol. Chem.* 2000; 275:25130–25138. [PubMed: 10833514]
12. Waypa GB, Chandel NS, Schumacker PT. Model for hypoxic pulmonary vasoconstriction involving mitochondrial oxygen sensing. *Circ. Res.* 2001; 88:1259–1266. [PubMed: 11420302]
13. Bell EL, Klimova TA, Eisenbart J, Moraes CT, Murphy MP, Budinger GR, Chandel NS. The Q_o site of the mitochondrial complex III is required for the transduction of hypoxic signaling via reactive oxygen species production. *J. Cell Biol.* 2007; 177:1029–1036. [PubMed: 17562787]
14. Brunelle JK, Bell EL, Quesada NM, Vercauteren K, Tiranti V, Zeviani M, Scarpulla RC, Chandel NS. Oxygen sensing requires mitochondrial ROS but not oxidative phosphorylation. *Cell Metab.* 2005; 1:409–414. [PubMed: 16054090]
15. Guzy RD, Hoyos B, Robin E, Chen H, Liu L, Mansfield KD, Simon MC, Hammerling U, Schumacker PT. Mitochondrial complex III is required for hypoxia-induced ROS production and cellular oxygen sensing. *Cell Metab.* 2005; 1:401–408. [PubMed: 16054089]
16. Mansfield KD, Guzy RD, Pan Y, Young RM, Cash TP, Schumacker PT, Simon MC. Mitochondrial dysfunction resulting from loss of cytochrome *c* impairs cellular oxygen sensing and hypoxic HIF- α activation. *Cell Metab.* 2005; 1:393–399. [PubMed: 16054088]
17. Waypa GB, Guzy R, Mungai PT, Mack MM, Marks JD, Roe MW, Schumacker PT. Increases in mitochondrial reactive oxygen species trigger hypoxia-induced calcium responses in pulmonary artery smooth muscle cells. *Circ. Res.* 2006; 99:970–978. [PubMed: 17008601]
18. Waypa GB, Marks JD, Guzy R, Mungai PT, Schriewer J, Dokic D, Schumacker PT. Hypoxia triggers subcellular compartmental redox signaling in vascular smooth muscle cells. *Circ. Res.* 2010; 106:526–535. [PubMed: 20019331]
19. Waypa GB, Marks JD, Guzy RD, Mungai PT, Schriewer JM, Dokic D, Ball MK, Schumacker PT. Superoxide generated at mitochondrial complex III triggers acute responses to hypoxia in the pulmonary circulation. *Am. J. Respir. Crit. Care Med.* 2013; 187:424–432. [PubMed: 23328522]
20. Doege K, Heine S, Jensen I, Jelkmann W, Metzen E. Inhibition of mitochondrial respiration elevates oxygen concentration but leaves regulation of hypoxia-inducible factor (HIF) intact. *Blood*. 2005; 106:2311–2317. [PubMed: 15947089]
21. Zhou J, Eleni C, Spyrou G, Brune B. The mitochondrial thioredoxin system regulates nitric oxide-induced HIF-1 α protein. *Free Radical Biol. Med.* 2008; 44:91–98. [PubMed: 18045551]
22. Kelso GF, Porteous CM, Coulter CV, Hughes G, Porteous WK, Ledgerwood EC, Smith RA, Murphy MP. Selective targeting of a redox-active ubiquinone to mitochondria within cells: antioxidant and antiapoptotic properties. *J. Biol. Chem.* 2001; 276:4588–4596. [PubMed: 11092892]
23. Pan Y, Mansfield KD, Bertozzi CC, Rudenko V, Chan DA, Giaccia AJ, Simon MC. Multiple factors affecting cellular redox status and energy metabolism modulate hypoxia-inducible factor prolyl hydroxylase activity *in vivo* and *in vitro*. *Mol. Cell. Biol.* 2007; 27:912–925. [PubMed: 17101781]
24. Knoops B, Goemaere J, Van der Eecken V, Declercq JP. Peroxiredoxin 5: structure, mechanism, and function of the mammalian atypical 2-Cys peroxiredoxin. *Antioxid. Redox Signaling*. 2011; 15:817–829.

25. Marshall C, Mamary AJ, Verhoeven AJ, Marshall BE. Pulmonary artery NADPH-oxidase is activated in hypoxic pulmonary vasoconstriction. *Am. J. Respir. Cell. Mol. Biol.* 1996; 15:633–644. [PubMed: 8918370]
26. Dooley CT, Dore TM, Hanson GT, Jackson WC, Remington SJ, Tsien RY. Imaging dynamic redox changes in mammalian cells with green fluorescent protein indicators. *J. Biol. Chem.* 2004; 279:22284–22293. [PubMed: 14985369]
27. Miyawaki A, Griesbeck O, Heim R, Tsien RY. Dynamic and quantitative Ca^{2+} measurements using improved cameleons. *Proc. Natl. Acad. Sci. U.S.A.* 1999; 96:2135–2140. [PubMed: 10051607]
28. McMurtry IF. Angiotensin is not required for hypoxic constriction in salt solution-perfused rat lungs. *J. Appl. Physiol.* 1984; 56:375–380. [PubMed: 6561197]
29. Ozawa T, Natori Y, Sako Y, Kuroiwa H, Kuroiwa T, Umezawa Y. A minimal peptide sequence that targets fluorescent and functional proteins into the mitochondrial intermembrane space. *ACS Chem. Biol.* 2007; 2:176–186. [PubMed: 17348629]
30. Reily C, Mitchell T, Chacko BK, Benavides G, Murphy MP, Darley-Usmar V. Mitochondrially targeted compounds and their impact on cellular bioenergetics. *Redox Biol.* 2013; 1:86–93. [PubMed: 23667828]
31. Semenza GL. Targeting HIF-1 for cancer therapy. *Nat. Rev. Cancer.* 2003; 3:721–732. [PubMed: 13130303]
32. Kim JW, Tchernyshyov I, Semenza GL, Dang CV. HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia. *Cell Metab.* 2006; 3:177–185. [PubMed: 16517405]
33. Chua YL, Dufour E, Dassa EP, Rustin P, Jacobs HT, Taylor CT, Hagen T. Stabilization of hypoxia-inducible factor-1 α protein in hypoxia occurs independently of mitochondrial reactive oxygen species production. *J. Biol. Chem.* 2010; 285:31277–31284. [PubMed: 20675386]
34. Hagen T, Taylor CT, Lam F, Moncada S. Redistribution of intracellular oxygen in hypoxia by nitric oxide: effect on HIF1 α . *Science.* 2003; 302:1975–1978. [PubMed: 14671307]
35. Jung HJ, Shim JS, Lee J, Song YM, Park KC, Choi SH, Kim ND, Yoon JH, Mungai PT, Schumacker PT, Kwon HJ. Terpestacin inhibits tumor angiogenesis by targeting UQCRB of mitochondrial complex III and suppressing hypoxia-induced reactive oxygen species production and cellular oxygen sensing. *J. Biol. Chem.* 2010; 285:11584–11595. [PubMed: 20145250]
36. Jung HJ, Kim KH, Kim ND, Han G, Kwon HJ. Identification of a novel small molecule targeting UQCRB of mitochondrial complex III and its anti-angiogenic activity. *Bioorg. Med. Chem. Lett.* 2011; 21:1052–1056. [PubMed: 21215626]
37. Ratcliffe PJ. Oxygen sensing and hypoxia signalling pathways in animals: the implications of physiology for cancer. *J. Physiol.* 2013; 591:2027–2042. [PubMed: 23401619]
38. Kaelin WG Jr. Cancer and altered metabolism: potential importance of hypoxia-inducible factor and 2-oxoglutarate-dependent dioxygenases. *Cold Spring Harbor Symp. Quant. Biol.* 2011; 76:335–345. [PubMed: 22089927]
39. Hirsila M, Koivunen P, Gunzler V, Kivirikko KI, Myllyharju J. Characterization of the human prolyl 4-hydroxylases that modify the hypoxia-inducible factor. *J. Biol. Chem.* 2003; 278:30772–30780. [PubMed: 12788921]
40. Koivunen P, Hirsila M, Kivirikko KI, Myllyharju J. The length of peptide substrates has a marked effect on hydroxylation by the hypoxia-inducible factor prolyl 4-hydroxylases. *J. Biol. Chem.* 2006; 281:28712–28720. [PubMed: 16885164]
41. Tian YM, Yeoh KK, Lee MK, Eriksson T, Kessler BM, Kramer HB, Edelman MJ, Willam C, Pugh CW, Schofield CJ, Ratcliffe PJ. Differential sensitivity of hypoxia inducible factor hydroxylation sites to hypoxia and hydroxylase inhibitors. *J. Biol. Chem.* 2011; 286:13041–13051. [PubMed: 21335549]
42. Masson N, Singleton RS, Sekimik R, Trudgian DC, Ambrose LJ, Miranda MX, Tian YM, Kessler BM, Schofield CJ, Ratcliffe PJ. The FIH hydroxylase is a cellular peroxide sensor that modulates HIF transcriptional activity. *EMBO Rep.* 2012; 13:251–257. [PubMed: 22310300]
43. Muller FL, Liu Y, Van Remmen H. Complex III releases superoxide to both sides of the inner mitochondrial membrane. *J. Biol. Chem.* 2004; 279:49064–49073. [PubMed: 15317809]

Figure 1. A 57-amino-acid leader sequence from the mouse smac/Diablo protein targets proteins to the IMS

(A–C) Confocal images showing IMS-roGFP colocalized with the mitochondrial protein MnSOD. (D–F) Confocal images showing IMS-PRDX5 colocalized with MnSOD. Nuclei are stained blue with DAPI. (G and H) roGFP was labelled with a colloidal gold-conjugated antibody. Electron micrograph shows IMS-roGFP localized to the IMS and cristae compared with the matrix roGFP located inside the mitochondria.

Figure 2. Mitochondrial respiration and function are not adversely affected by IMS-PRDX5 expression

(A) Clarke electrode measurements of oxygen consumption by wild-type rat PASMCs, cells infected with an empty vector virus or with increasing doses of IMS-PRDX5 virus. (B) Oxygen consumption rates obtained with a Seahorse flux analyser in rat PASMCs infected with an empty vector virus or IMS-PRDX5. Results are means $\pm$ S.E.M. $n = 3$ experiments for each condition.

Figure 3. IMS-PRDX5 expression in rat PSMCs abrogates hypoxic oxidant changes in multiple cellular compartments in a dose-dependent manner

Rat PSMCs were exposed to hypoxia for 12–30 h prior to (A) IMS and (B) cytosolic roGFP studies on an epifluorescence microscope. (C) Matrix roGFP studies were performed after 18 h of hypoxic exposure and measured by flow cytometry. The results are means ± S.E.M. * $P < 0.05$ compared with normoxic wild-type. + $P < 0.05$ compared with hypoxic wild-type and 2000 pfu empty virus. ‡ $P < 0.05$ compared with 2000 pfu empty virus.

Figure 4. IMS-PRDX5 expression attenuates acute hypoxic HIF-1 α stabilization and activity in a dose-dependent manner

Rat PASMCs were exposed to hypoxia for 8 h prior to collecting cell lysates for Western blots to assess HIF-1 α stabilization and activity. β -actin was used as a loading control. DFO, an iron chelator, was used as a positive control. (A) HIF-1 α . * P 0.05 compared with normoxic wild-type. + P 0.05 compared with hypoxic wild-type and 2000 pfu empty virus. (B) GAPDH. * P 0.05 compared with hypoxic wild-type. (C) PDHK1. * P 0.05 compared with hypoxic wild-type and 2000 pfu empty virus. The results are means $\pm$ S.E.M. n = 3 for each condition.

Figure 5. IMS-PRDX5 expression attenuates acute hypoxic Ca^{2+} influx

Cytosolic Ca^{2+} levels were assessed using the FRET-based sensor YC2.3. A stable normoxic baseline was monitored for 5 min prior to adding endothelin-1 to the medium for 10 min to prime the cells. Medium was then switched to hypoxia and cells were monitored for 15 min. The maximum increase in the YC2.3 ratio under hypoxic conditions was expressed as a percentage increase over the average normoxic value. The results are means $\pm$ S.E.M. $n = 32$ coverslips per condition with approximately four to six cells per coverslip. * $P < 0.05$ compared with 2000 pfu empty virus.