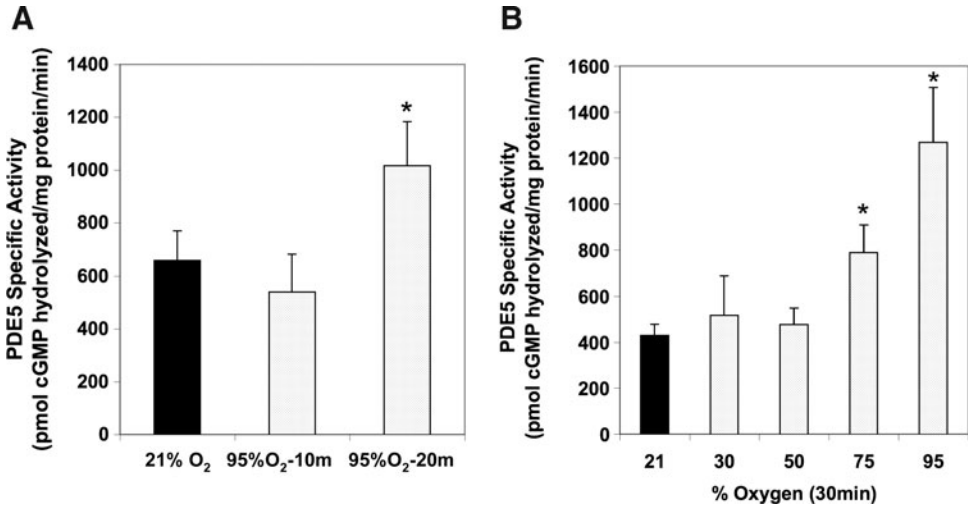
Supplementary Data

Methods

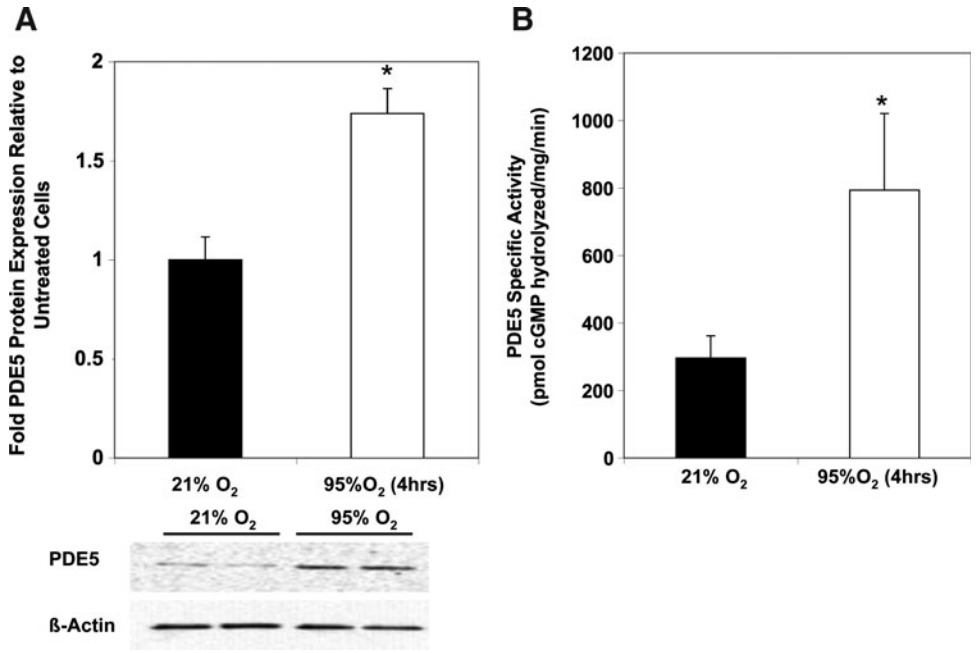
Cell Culture

Primary cultures of pulmonary artery smooth muscle cells (PASMC) from healthy fetal lambs were prepared as previously described from intrapulmonary arteries isolated from 136-day gestation fetal lambs (2). Fetal PASMC (FPASMC) identity was confirmed by immunostaining with antibodies against alpha smooth muscle actin, caldesmon, and desmin. All cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1 g/L glucose (Mediatech), 10% fetal bovine serum (FBS; Hyclone), antibiotics (Mediatech), and antimycotics (Mediatech) at 37°C in a humidified atmosphere with 5% CO_2/95% air. Cells were synchronized prior to experiments by transfer to serum-free DMEM with antibiotics and antimycotics 12 h prior to the start of the experiment. All experiments were carried out using 500,000 cells per condition between passage 2 and 6. PASMC exposed to hyperoxia were placed in a sealed humidified chamber and continuously maintained in 95% O_2/5% CO_2 (ProOx C-21, Biospherix, Lacona, NY) for 10 min, 20 min, or 4 h followed by 30 min of recovery at 21% O_2/5% CO_2. Other cells were exposed to hyperoxia of varying concentrations for 30 min: 21% O_2/5% CO_2, 30% O_2/5% CO_2, 50% O_2/5% CO_2, 75% O_2/5% CO_2, and 95% O_2/5% CO_2. Some cells were treated with DETANONOate (100 μM; Cayman Chemical, Ann Arbor, MI). Other cells were infected with either an empty adenoviral vector, Y5 (University of Iowa, Iowa City, IA), or an adenoviral construct expressing mitochondrially targeted catalase (mito-catalase, 0–1000 plaque-forming units [pfu]/cell; University of Iowa) or MnSOD (0–100 pfu/cell; ViraQuest, North Liberty, IA) to determine the appropriate dose of virus to obtain maximal antioxidant enzyme overexpression without cell toxicity. After determining the appropriate dose, the cells were infected with the appropriate adenovirus, and 48 h later, they were exposed to 21% O_2/5% CO_2 or 95% O_2/5% CO_2 for 30 min as described above followed by 30 min of recovery at 21% O_2/5% CO_2.

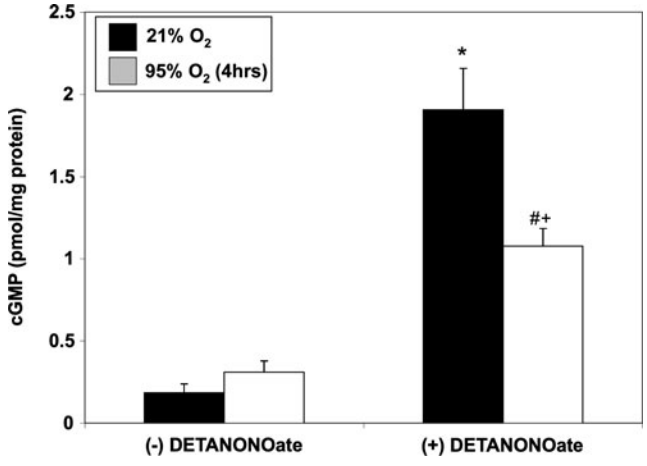
Primary cultures of adult mouse PASMC from C57Bl6 mice were prepared using a variation of the previously described technique of Marshall et al. (6, 7). Briefly, using a RV catheter, a solution of 0.5% (w/v) agarose + 0.5% iron particles in M199 media containing antibiotics (M199 serum-free media) is infused into the pulmonary vasculature. The iron particles are 0.2 μM in diameter; thus, they are excluded from the pulmonary capillary bed and lodge in the small PA. The lungs are then inflated with 1% (w/v) agarose, removed, and chilled, and the lung tissue is dissociated in cold phosphate-buffered saline (PBS). A Dynal Magnetic Particle Collector is used to pull down the iron-containing vessels. After collagenase (80 U/mL) treatment and further manual dissociation, the vessels are plated in M199 media containing 20% FBS, and antibiotics (M199 complete media) at 37°C in 21% O_2/5% CO_2 to allow cell migration from the vessels onto the culture plate. The initial plate of cells is a mixture of fibroblasts and PASMC, and the pull-down procedure is repeated on successive days to derive an enriched PASMC population and remove any residual iron particles. The enriched PASMC are maintained in M199 complete media at 37°C in 21% O_2/5% CO_2. PASMC identity is confirmed by immunostaining for smooth muscle cell markers (α-smooth muscle actin, smooth muscle myosin, caldesmon, desmin).



SUPPLEMENTARY FIG. S1. Time course and dose–response curve for hyperoxia-induced PDE5 activity. (A) Protein was harvested from FPASMC exposed to either 21% O_2/5% CO_2 or 95% O_2/5% CO_2 for 10 or 20 min. PDE5-specific cGMP hydrolytic activity was measured using a commercially available colorimetric assay ($n = 8$, read in duplicate). **(B)** Protein was harvested from FPASMC exposed to 21% O_2/5% CO_2, 30% O_2/5% CO_2, 50% O_2/5% CO_2, 75% O_2/5% CO_2, or 95% O_2/5% CO_2 for 30 min. PDE5-specific cGMP hydrolytic activity was measured using a commercially available colorimetric assay ($n = 6$, read in duplicate). Data are shown as mean ± SEM pmol cGMP hydrolyzed/mg protein/min. *$p < 0.05$ vs. FPASMC in 21% O_2/5% CO_2.



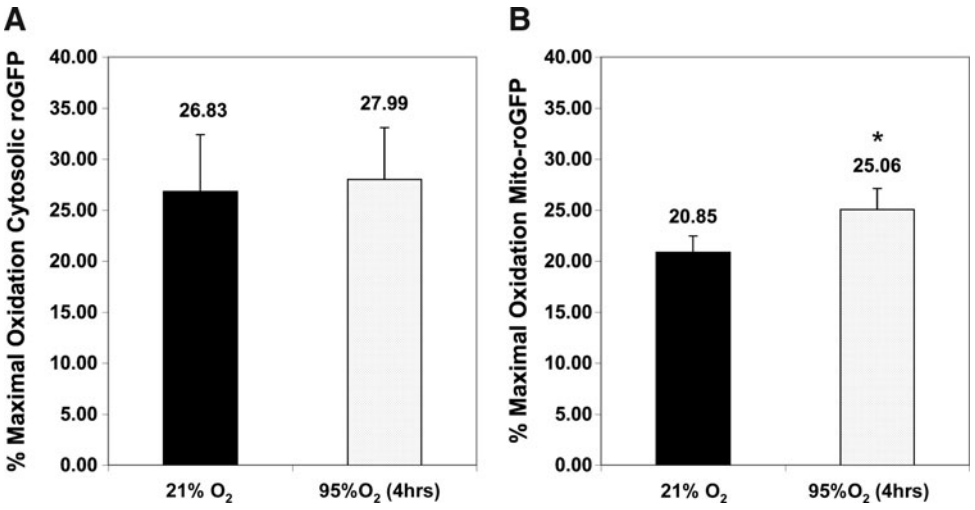
SUPPLEMENTARY FIG. S2. Four hours of 95% O_2/5% CO_2 increases PDE5 protein expression and activity. Protein was harvested from FPASMC exposed to either 21% O_2/5% CO_2 or 95% O_2/5% CO_2 for 4 h. **(A)** PDE5 protein expression analyzed by Western blot, normalized for β-actin ($n=12$). Data are shown as mean ± SEM relative to FPASMC in 21% O_2/5% CO_2. A representative Western blot is shown for PDE5 and β-actin. **(B)** PDE5-specific cGMP hydrolytic activity was measured using a commercially available colorimetric assay ($n=10$, read in duplicate). Data are shown as mean ± SEM pmol cGMP hydrolyzed/mg protein/min. *$p<0.05$ vs. FPASMC in 21% O_2/5% CO_2.



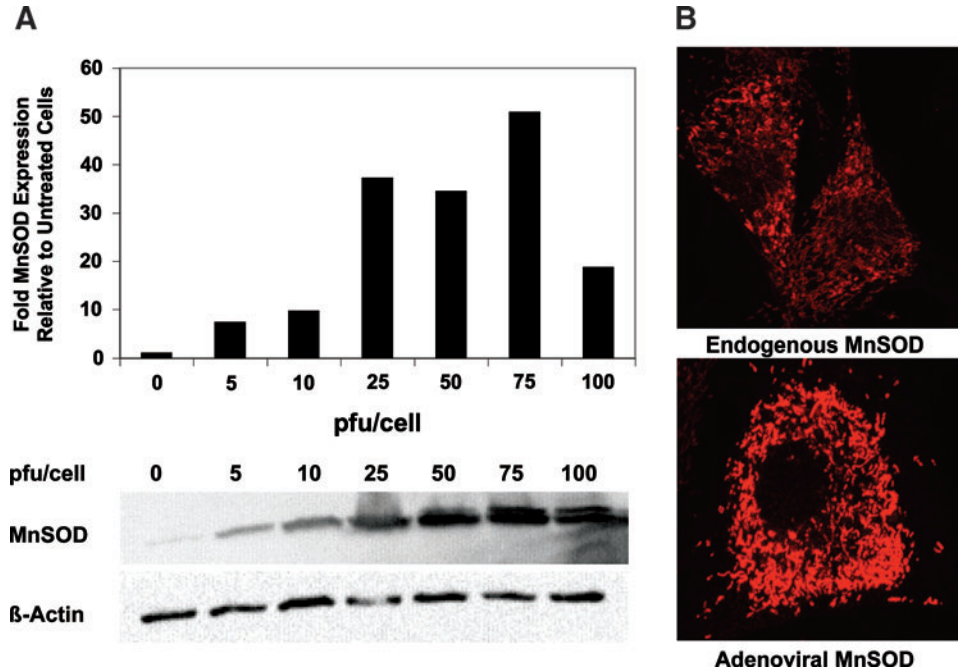
SUPPLEMENTARY FIG. S3. Four hours of 95% O_2/5% CO_2 blunts cGMP responsiveness to exogenous NO. FPASMC were exposed to 21% O_2/5% CO_2 or 95% O_2/5% CO_2 ± DETANONOate (100 μM) for 4 h. Cells were harvested and assayed for cGMP by EIA. cGMP levels were normalized for milligrams of total protein. Data are shown as mean ± SEM ($n=10$, read in duplicate). *$p<0.05$ vs. untreated FPASMC in 21% O_2/5% CO_2; $^{\#}p<0.05$ vs. untreated FPASMC in 95% O_2/5% CO_2; $^{+}p<0.05$ vs. NO-treated PASMC in 21% O_2/5% CO_2.

Hyperoxia exposure of mice and isolation of lungs and small PA

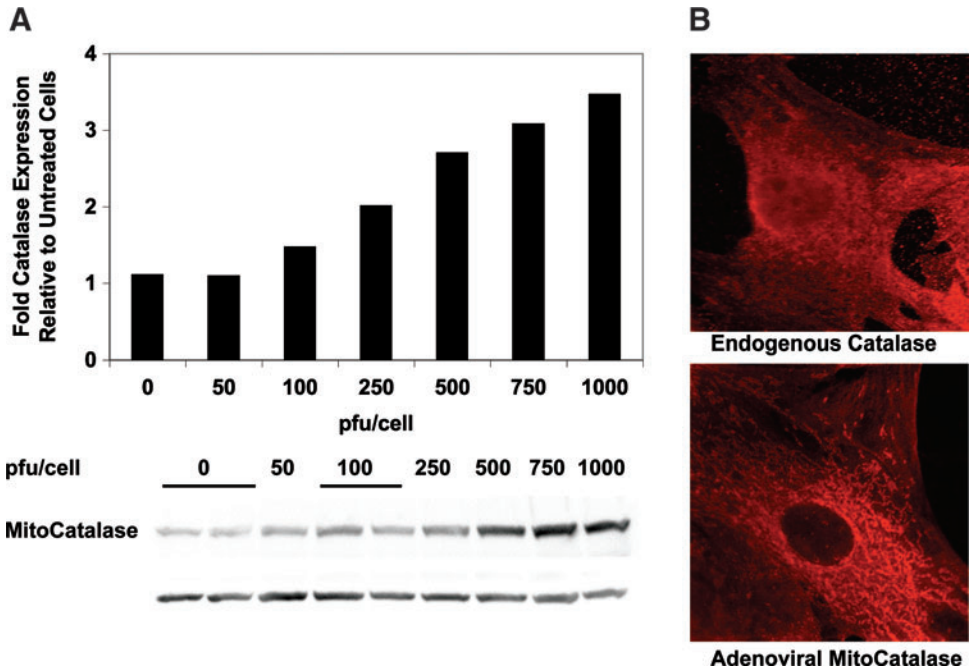
The study was approved by the Northwestern University Laboratory Animal Care Committee. Male C57Bl6 mice (Charles River, age 5–6 weeks) were placed into three experimental groups: 1) room air, nonventilated (21NV, $n=5$), 2) mechanically ventilated with 100% O_2 for 45 min (100V, $n=5$), and 3) 90% O_2 exposure for 45 min in an animal hyperoxia chamber (Biospherix; 90NV, $n=5$). Mice were ventilated with the TOPO Dual Mode Ventilator as per manufacturer instructions with peak inspiratory pressure 15 cm H_2O, PEEP 4 cm H_2O, and respiratory rate 140 breaths per minute (Kent Scientific, Torrington, CT). During ventilation, mice were anesthetized with Avertin. After exposure, animals were euthanized by isofluorane overdose followed by cervical dislocation. Lungs were perfused with PBS via the RV and PA. Lung tissue was harvested and flash-frozen for subsequent cGMP analysis. For PDE5 activity assays, small PA protein was isolated using a variation of the previously described rodent PASMC isolation technique (7). Briefly, a solution of 0.5% (w/v) agarose + 0.5% iron particles (0.2 μM) in M199 (Mediatech) was infused into the pulmonary vasculature. The iron particles are excluded from the pulmonary capillary bed and lodge in small PA. The lungs were inflated with 1% (w/v) agarose, removed, and chilled; lung tissue was dissociated in cold PBS. A Dynal Magnetic Particle was used to pull down the iron-containing vessels. After collagenase (80 U/mL) treatment and manual dissociation, the vessels were lysed and sonicated in 1 × Mg-lysis buffer (Upstate, Charlottesville, VA) supplemented with a protease inhibitor cocktail (Sigma, St. Louis, MO) and a



SUPPLEMENTARY FIG. S4. Four hours of 95% O_2/5% CO_2 induces mitochondrial, but not cytosolic, oxidation in FPASMC. Cytosolic roGFP and mito-roGFP contain surface-exposed cysteine residues that are sensitive to the local redox environment. After measuring the cellular environment using multilaser flow cytometry, the sensor is calibrated by maximally reducing it with dithiothreitol (DTT, 1 mM) and maximally oxidizing it with t-butyl hydroperoxide (TBH, 1 mM) to yield percent oxidation. FPASMC were exposed to 21% O_2/5% CO_2 or 95% O_2/5% CO_2 for 4 h, and **(A)** oxidation of cytosolic roGFP (Panel A, $n=18$) and **(B)** mito-roGFP (Panel B, $n=14$) was measured ($n=4$). Data are shown as mean ± SEM for the percent maximal oxidation of the cytosolic roGFP and mito-roGFP probes. *$p<0.05$ vs. FPASMC in 21% O_2/5% CO_2.



SUPPLEMENTARY FIG. S5. Dose–response curve for MnSOD overexpression in FPASMC. FPASMC were infected with varying doses of an adenoviral construct for MnSOD overexpression (0–100 pfu/cell). **(A)** Protein was harvested 48 h after viral infection and subjected to Western blot analysis, with β-actin normalization. Normalized expression data are shown for the representative Western blots shown for MnSOD and β-actin. **(B)** FPASMC were plated onto collagen-coated coverslips and infected with 100 pfu/cell MnSOD. After 48 h, FPASMC were fixed, permeabilized, and immunostained for MnSOD. Coverslips were imaged on a Zeiss UV LSM 510 META laser scanning confocal microscope with a 40× oil immersion lens. The top panel shows the endogenous MnSOD expression. The bottom panel shows the MnSOD expression after infection with 100 pfu/cell adenoviral MnSOD.

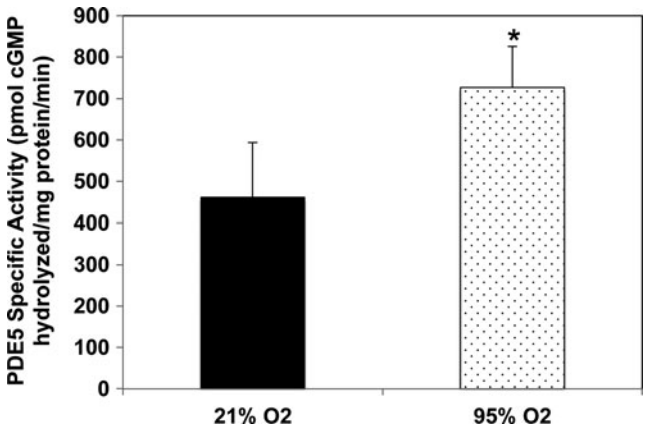


SUPPLEMENTARY FIG. S6. Dose–response curve for mito-catalase overexpression in FPASMC. FPASMC were infected with varying doses of an adenoviral construct for mito-catalase overexpression (0–1000 pfu/cell). **(A)** Protein was harvested 48 h after viral infection and subjected to Western blot analysis, with β-actin normalization. Normalized expression data are shown for the representative Western blots shown for catalase and β-actin. **(B)** FPASMC were plated onto collagen-coated coverslips and infected with 750 pfu/cell mito-catalase. After 48 h, FPASMC were fixed, permeabilized, and immunostained for catalase. Coverslips were imaged on a Zeiss UV LSM 510 META laser scanning confocal microscope with a 40× oil immersion lens. The top panel shows the endogenous catalase expression. The bottom panel shows the catalase expression after infection with 750 pfu/cell adenoviral mito-catalase.

phosphatase inhibitor cocktail (EMD Biosciences, San Diego, CA) as previously described (3), and the iron particles were removed prior to analysis of protein samples (3).

Western blot analysis

FPASMC were harvested using 1×Mg-lysis buffer (Upstate) supplemented with a protease inhibitor cocktail (Sigma) and a phosphatase inhibitor cocktail (EMD Biosciences) as previously described (2). Cell extracts were sonicated, and protein concentration was determined using the Bradford assay (1). Total protein (40 μg) was separated on a 4%–20% SDS-polyacrylamide gel (Biorad) and then transferred to a nitrocellulose membrane (Amersham, Arlington Heights, IL). Western blot was then performed as previously described (2). Briefly, membranes were blocked at room temperature with



SUPPLEMENTARY FIG. S7. Thirty minutes of 95% O_2/5% CO_2 increases PDE5 activity in adult mouse PASMC. Protein was harvested from adult mouse PASMC exposed to either 21% O_2/5% CO_2 or 95% O_2/5% CO_2 for 30 min, followed by recovery for 30 min in 21% O_2/5% CO_2. PDE5-specific cGMP hydrolytic activity was measured using a commercially available colorimetric assay ($n=6$, read in duplicate). Data are shown as mean ± SEM pmol cGMP hydrolyzed/mg protein/min. *$p=0.05$ vs. PASMC in 21% O_2/5% CO_2.

5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20 (1×TBST) and were then incubated overnight at 4°C with the primary antibody in 5% milk + 1×TBST at an appropriate dilution (1:333 for mouse anti-PDE5 [BD Transduction], 1:500 for rabbit anti-PhosphoPDE5 [Fabgennix, Frisco, TX], 1:1000 for rabbit anti-MnSOD [Assay Designs, Ann Arbor, MI], 1:1000 for rabbit anti-catalase [Abcam, Cambridge, MA] and 1:2000 for mouse β-actin [Sigma, St. Louis, MO]). The membranes were washed and incubated with the appropriate secondary antibody conjugated to horseradish peroxidase (Pierce, Rockford, IL) diluted 1:1000 in 5% milk + 1×TBST. Membranes were washed and exposed via chemiluminescence (Pierce). Bands were analyzed using a Digital Science Image Station (Kodak, Rochester, NY). PDE5, MnSOD, or Catalase expression within each Western blot was normalized to β-actin. Data are shown as fold relative to untreated control PASMC.

PDE5 activity assay

PASMC were harvested for total protein in lysis buffer supplemented with protease and phosphatase inhibitors as described above. PASMC samples were immediately placed on ice and assayed the same day as previously described (2). The protein was purified over a Centri-Spin 10 column to remove any phosphate contamination (Princeton Separations, Adelphia, NJ). Protein concentration was determined as described above. Total protein (5 μg) was assayed for cGMP hydrolytic activity using a commercially available colorimetric cyclic nucleotide phosphodiesterase assay kit (Enzo Life Sciences, Plymouth Meeting, PA). Each sample was read in four wells—two without sildenafil and two with sildenafil (100 nM)—to determine PDE5 specific cGMP-hydrolytic activity. The samples were incubated at 30°C for 30 min, and the samples were then incubated with the Biomol Green reagent with shaking at room temperature for 20 min. Results were measured using a Labsystems Multiskan EX automated plate reader at 620 nM. The difference between the picomoles of cGMP hydrolyzed per milligrams of total protein per minute without sildenafil and the picomoles of cGMP hydrolyzed per milligrams of total protein per minute with sildenafil represents the PDE5-specific cGMP-hydrolytic activity. Results are shown as the PDE5-specific picomoles of cGMP hydrolyzed per milligrams of total protein per minute for each sample.

Cyclic GMP enzyme immunoassay

PASMC were lysed with 0.1N HCl, and extracts were dried and resuspended in enzyme immunoassay (EIA) buffer (Cayman Chemical) as previously described (2). Lung cGMP from flash-frozen tissue was extracted in TCA as previously described (3). All PASMC samples were acetylated according to the manufacturer's protocol, but lung tissue cGMP was measured as nonacetylated cGMP. PASMC and lung cGMP content was measured by EIA in duplicate using a commercially available kit (Cayman Chemical). Results were measured using a Labsystems Multiskan EX automated plate reader (Thermo Electron) at 420 nm. Results are shown as picomoles of cGMP per milligrams of protein for PASMC samples and picomoles of cGMP per milligrams of tissue for lung samples.

Detection of reactive oxygen species

FPASMC were plated in a balanced salt solution and exposed to 95% O_2/5% CO_2 for 30 min as described previously (2, 8). Immediately after exposure, FPASMC were lysed in 1×Mg-lysis buffer (Upstate) supplemented with a protease inhibitor cocktail (Sigma) and a phosphatase inhibitor cocktail (EMD Biosciences) followed by sonication as previously described (2). Intracellular H_2O_2 was then measured via Amplex Red assay as per manufacturer's protocol (Invitrogen, Grand Island, NY) and previously described (9). Amplex Red fluorescence was quantified using a SpectraMax Gemini XS dual-scanning microplate spectrofluorometer (Molecular Devices). Data are shown as H_2O_2 concentration (nM) based on an H_2O_2 standard curve. Other FPASMC were loaded with MitoSOX, a fluorescent mitochondrially targeted superoxide probe, prior to hyperoxia exposure. MitoSOX fluorescence was measured by fluorescence microscopy with excitation at 518 nm and emission at 605 nm using a Nikon Eclipse TE-300 fluorescent microscope and CoolSnap digital camera. PASMC were processed and imaged in parallel, and total fluorescence is normalized to pixel area to account for cell number using Metamorph imaging software (Molecular Devices, Sunnyvale, CA).

PASMC in serum-free DMEM without phenol red and with antibiotics and antimycotics were infected in 60-mm culture dishes with either 100 pfu/cell of a cytosolic roGFP or mitochondrial matrix targeted roGFP (mito-roGFP) adenoviral construct. RoGFP is a previously characterized ratiometric fluorescent probe sensitive to cellular oxidative stress (2, 4, 5, 8). To create the cytosolic probe, surface-exposed residues in green fluorescent protein (GFP) were replaced with cysteine residues capable of forming disulfide bonds. For mito-roGFP, the mitochondrial matrix targeting sequence for cytochrome oxidase subunit IV was appended to the amino terminus, causing the expressed protein to be localized to the mitochondrial matrix compartment. Assessment of fluorescence ratios therefore provides a real-time measure of cysteine thiol redox status in that subcellular compartment (2, 4, 5, 8). For the experiments described in the online data supplement, 48 h after infection, the PASMC were exposed to 21% O_2/5% CO_2 or 95% O_2/5% CO_2 for 4 h. After exposure, PASMC were lifted from the plates and divided into three aliquots. The first aliquot was used to assess redox status while the other two were used to calibrate the probe. One aliquot was fully reduced with dithiothreitol (1 mM, Sigma), while the second was fully oxidized using t-butyl hydroperoxide (1 mM, Sigma). All aliquots were analyzed with a DakoCytomation CyAn multilaser flow cytometer in the Robert H. Lurie Comprehensive Cancer Center Flow Cytometry Facility using 405- and 488-nm excitation wavelengths, while emission was assessed at 535 nm. For each condition, the cysteine thiol redox status was calculated as percent oxidized, by comparison to the values obtained for the fully reduced and fully oxidized conditions.

Immunocytochemistry

FPASMC were plated onto collagen-treated glass coverslips and were infected with either an empty adenoviral vector, Y5 (University of Iowa, Iowa City, IA), or an adenoviral construct expressing mito-catalase (750 pfu/cell; University of Iowa) or MnSOD (100 pfu/cell; ViraQuest). Forty-

eight hours after infection, cells on coverslips were fixed with 4% formaldehyde (Fisher, Hampton, NH), permeabilized with 0.2% Triton-X (Fisher), and stained for immunocytochemistry with either anti-catalase or anti-MnSOD antibodies at a 1:50 dilution in 5% bovine serum albumin (BSA; Sigma). Utilizing a rhodamine-red labeled anti-rabbit antibody (Molecular Probes/Invitrogen) at a 1:200 dilution in 5% BSA, the intracellular expression of catalase or MnSOD at baseline and after overexpression was visualized by fluorescence microscopy with excitation at 518 nm and emission at 605 nm for rhodamine red. Fluorescent images were captured in the Northwestern University Cell Imaging Facility with a Zeiss UV LSM 510 META laser scanning confocal microscope with a 40× oil immersion lens with Zeiss LSM imaging software (Carl Zeiss MicroImaging, Thornwood, NY) using the same threshold and pinhole settings between images.

Statistical analysis

All data are expressed as the mean ± SEM. Results were analyzed by two-sided unpaired *t*-test or ANOVA with Bonferroni's *post hoc* analysis when appropriate using Prism software (GraphPad Software Inc., San Diego, CA). Statistical significance was set at $p < 0.05$.

Results

Brief hyperoxia exposure increases PDE5 activity in FPASMC

PDE5 is a key regulator of pulmonary vascular tone in the perinatal period. We have previously demonstrated that 24 h of exposure to 95%–100% O_2 increases PDE5 protein expression and activity in both isolated FPASMC and resistance pulmonary arteries from mechanically ventilated neonatal sheep (2). However, the effects of shorter time periods of O_2 exposure on PDE5 were previously unknown. In Supplementary Fig. S1A, PDE5 activity is induced with as little as 20 min of exposure to 95% O_2/5% CO_2 relative to FPASMC in 95% O_2/5% CO_2 (1016 ± 167 vs. 657 ± 112 pmol cGMP hydrolyzed/mg protein/min). Similarly, we examined a dose–response curve for oxygen exposure. Exposure to either 30% O_2/5% CO_2 or 50% O_2/5% CO_2 was not sufficient to induce PDE5 activity, but 30 min of exposure to 75% O_2/5% CO_2 was sufficient to induce PDE5 activity relative to FPASMC in 21% O_2/5% CO_2 (787 ± 120 vs. 429 ± 49 pmol cGMP hydrolyzed/mg protein/min; Supplementary Fig. S1B). It is likely that 30 min is too short of an interval to see changes in protein expression, so we also examined the effects of 4 h of exposure to 95% O_2/5% CO_2. As shown in Supplementary Fig. S2, 4 h of exposure to 95% O_2/5% CO_2 was sufficient to induce both PDE5 protein expression and activity relative to FPASMC in 21% O_2/5% CO_2 (expression: 1.74 ± 0.13-fold; activity: 793 ± 228 vs. 296 ± 66 pmol cGMP hydrolyzed/mg protein/min).

Brief hyperoxia decreases cGMP response to exogenous nitric oxide

PDE5 down-regulates nitric oxide (NO) signaling through inactivation of cGMP by hydrolysis. We sought to determine if hyperoxia-mediated induction of PDE5 activity would impact the cGMP response to an exogenous NO signal in FPASMC. After 4 h of exposure to 95% O_2/5% CO_2, the cGMP response to exogenous NO was significantly blunted vs. FPASMC in 21% O_2/5% CO_2 (1.08 ± 0.1 vs. 1.91 ± 0.25 pmol cGMP/mg protein; Online Data Supplementary Fig. S3).

Brief hyperoxia increases mitochondrial, but not cytosolic, oxidative stress

We have previously demonstrated that FPASMC exposure to 24 h of 95% O_2/5% CO_2 increases oxidative stress throughout the cells including in both the cytosol and the mitochondrial matrix (2, 4). We sought to determine if the increase in oxidative stress in one compartment preceded the other in order to begin to understand the mechanism by which hyperoxia induces cellular oxidative stress. In order to measure oxidative stress within subcellular compartments, we utilized the novel ratiometric roGFP probe, which acts to measure protein thiol oxidation within subcellular compartments. As shown in Supplementary Fig. S4, 4 h of exposure to 95% O_2/5% CO_2 did not induce any change in cytosolic oxidative stress but did increase mitochondrial matrix oxidative stress vs. the normoxic baseline (25.1 ± 2.1% vs. 20.8 ± 1.6% oxidized).

We then sought to determine if overexpression of antioxidant enzymes would be sufficient to abrogate this hyperoxia-induced mitochondrial matrix oxidative stress as described in the results section of the primary article. In order to determine the appropriate dose of adenovirally delivered antioxidant enzymes, we performed dose–response curves for each construct. Overexpression of MnSOD without evidence of toxicity to the FPASMC was seen with 25–100 pfu/cell and was easily detectable by Western blot and immunocytochemistry (Supplementary Fig. S5). Optimal overexpression of the mito-catalase required higher doses to achieve detectable overexpression (750 pfu/cell; Supplementary Fig. S6). This may at least be in part because there is no specific antibody to distinguish the mito-catalase from the endogenous catalase present throughout the cytosol. Thus, the mitochondrial overexpression must be detected against a sea of cytosolic catalase, which is highly expressed in vascular smooth muscle cells.

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