Materials and Methods:

Reagents: Antibodies against Mfn1, Drp-1 Pink-1 and Tom20 were purchased from Abcam Incorporation (Cambridge, MA) (cat. # ab57602; ab56788; ab 23707; ab56783 respectively). Antibodies against Phospho-Drp-1 (serine 616), p62, LC3B, and eNOS were obtained from Cell Signaling Technology (Danvers, MA), (cat. #3455; #5114, #3868, #9572S respectively). OPA-1 Ab was obtained BD (cat. #612606).

Goat Anti-Rabbit/Mouse IgG (H+L)-HRP Conjugate were from Bio-Rad Laboratories, Inc. (California, USA), (cat. no. #1721019 and #1706516). Growth factor-reduced Matrigel was obtained from BD Biosciences (Bedford, MA), (cat. #356230). MitoSOX Red Mitochondrial Superoxide Indicator was purchased from Thermo Fisher (cat. M36008). TEMPOL (cat. #176141) was from Sigma-Aldrich Co. LLC. Mito-TEMPOL (cat. # ALX-430-150) was obtained from Enzo Life Sciences, Inc. TMRM was purchased from Life Technologies (cat #T668). ENDO III proteins (mitochondrially targeted and mutant) were a generous gift from Dr. Glenn Wilson, Ker Ferguson and Exscien Corp. mDIVI was purchased from EMD Millipore (cat # 475856). All other reagents were from common commercial sources.

In vivo hyperoxia exposure: Treatment protocols were approved by the Institutional Animal Care and Use Committees of the Zablocki Veterans Affairs Medical Center, the Medical College of Wisconsin and Marquette University (Milwaukee, WI). For normoxia (control) rat studies, adult (68–77 days old) male Sprague-Dawley rats (Charles River; 323 ± 4 (SEM) g), were exposed to room air in chambers side by side with those exposed to hyperoxia. For hyperoxia studies, rats within the same weight (341 ± 3 g) and age range of normoxia rats were housed in a plexiglass chamber maintained at >95% O_2 for 48 hours as described [1].

Tissues from which pulmonary arteries were isolated were obtained from a local abattoir with all protocols reviewed and approved by the Medical College of Wisconsin. In brief, pulmonary arteries were carefully dissected from the lungs and washed with HEPES buffer (0.01 mol L-1, pH 7.3) to remove blood. They were then slit open along their lengths and gently scratched along the intimal surface with a surgical blade. The cells were immediately transferred to a centrifuge tube with a collagenase solution (1 mg·mL-1 in DMEM) incubated at 25°C for 8–9 min, then vortexed and centrifuged at 200× g for 10 min to obtain cell pellets. PECs were cultured in RPMI medium containing 15% FBS and 1% penicillin-streptomycin and grown to 80% confluence before use. All cells were used between *passages 2* and *6*. Control and treated cells were matched in each experiment for isolation, passage number, and time to monolayer confluency.

For siRNA studies, rat pulmonary artery endothelial cells were purchased from Procell (China cat.no. CP-R011).

Mitochondrial DNA protection [2, 3] : To test protection of mtDNA by hyperoxia, mutant inactive peptides or recombinant mitochondrial targeted Endonuclease (mt-tat-Endo III; 0.1 µg/ml as indicated in the text), was added to the culture media of PECs maintained in normoxia or hyperoxia for 48 hours. At the end of this period, **m**itochondrial **f**ragmentation **i**ndex (MFI) assays or MTT assays for cell survival were performed.

Western blots for cell and lung homogenates [4]: Cells were harvested by scraping in the presence of 0.5 ml of RIPA buffer (cat. no. 20-188; Millipore, Temecula, CA) supplemented with protease inhibitor cocktail (cat. # 539134, Millipore) [5]. Lung tissue dissected free of large airways or blood vessels were homogenized in the same buffers with inhibitors. The mixture was kept on ice for 15 min, after which lysates were centrifuged for 10 min at 20,000 *g* and the supernatants used for determining protein concentration via the Bio-Rad protein assay kit. Equal amounts of protein (10-50 µg/lane) were boiled for 5 min in Laemmli sample buffer (161-0737, Bio-Rad) supplemented with 2-mercaptoethanol, resolved on a 10% Tris-HCl SDS polyacrylamide gel (Bio-Rad), and transferred to nitrocellulose membranes. The blots were developed with specific antibodies against Mfn1 (1:1000), Drp-1 (1:1000), p62 (1:500), LC3B (1:500), and Phospho-Drp-1 (1:200), and matched secondary antibodies and visualized using ECL Plus detection reagent. Blots were first probed with a phosphospecific antibody (phospho-Drp-1), stripped with (strong antibody stripping solution, cat. no. 2504, Millipore), and reprobed with the corresponding antibody for non-phosphorylated protein (Drp-1). Images were scanned with an Alpha Image 220 Analysis System, and the relative densities were determined by ImageJ software.

Cell survival, 3-(4,5-Dimethylthiazol- 255 2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [6]: Cell viability was determined by the MTT assay, PECs with a density of 5,000 cells/well were cultured in a 96-well culture plate and then subjected to different experimental treatments as described. At the end of incubation (e.g. with hyperoxia or normoxia), the cells were incubated for 3 h in a colorless medium containing 0.5% MTT. The amount of formazan dye formed from MTT is proportional to the number of metabolically active cells. The reaction was terminated after adding 150 µl DMSO to the cells for 10 min. Absorbance at 540 nm was recorded by an ELISA plate reader.

Apoptosis assay: Detection of apoptosis by imaging/Caspase 3/7 assay [7]. Cells were seeded in a 96 well tissue culture coated plate at a density of $1x10^4$ and allowed to attach and equilibrate overnight. 12h post-attachment, media was changed, with fresh media RPMI with 5% FBS containing IncuCyte caspase 3/7 reagent at final concentration of 5 µM, per manufacturer direction (Essen Bioscience, Ann Arbor, MI, USA). Briefly, this reagent interacts with cleaved caspase 3/7, triggers the production of fluorescence in the GFP channel. Cells were then continuously exposed to either 48h of normoxic or hyperoxic conditions.

Following differential oxygen exposure, plates were transferred to the IncuCyte FLR Live Cell Imaging Device where both phase contrast and fluorescent imaging were performed at 10x magnification. IncuCyte counting algorithms were used to quantitate the number of cells expressing cleaved caspase 3/7, using a 10 µm object size filter. Following detection of apoptotic cells, a final concentration of 5 µM Vybrant DyeCycle Green stain (Thermo Fischer) was added to each well and incubated for 1.5h. After incubation, plates were again imaged using the IncuCyte FLR Live Cell Imaging Device. An apoptotic index was calculated by dividing the number of objects with detectable caspase 3/7 activity by the total number of cells present, as enumerated by Vybrant DyeCycle Green stain.

Transmission electron microscopy (TEM): Pulmonary arteries from control and hyperoxic rats exposed *in vivo* for 48 hours were dissected and processed for ultrastructural analysis by transmission electron microscopy. Dissected tissue was placed in modified Karnovski fixative containing 2% paraformaldehyde + 2.5% glutaraldehyde. Tissues were then washed with 100mM sodium cacodylate buffer and post fixed in a freshly prepared solution of 1% osmium tetroxide containing 1.25% potassium ferricyanide. Following this step, the specimens were washed with distilled water, dehydrated through graded methanol into acetonitrile followed by embedding in EMBed 812 epoxy resin. Sections (60nm) were contrasted with uranyl acetate and lead citrate and viewed in a Hitachi H600 TEM. Images were collected via Hamamatsu side mount digital camera (AMT, Woburn, MA 01801) (Hitachi-7650, Japan).

Immunocytochemistry: PECs were cultured on coverslips in 24-well culture plates. After treatments, cells were fixed with 4% paraformaldehyde at room temperature for 15 min, permeabilized with 0.5% Triton X-100 for 15 min, blocked with 3% normal bovine serum at 37°C for 30 min, then incubated with anti-Tom 20 primary antibody (mouse, 1:50) at 4°C overnight. The next day, after washing three times with PBS, the coverslips were incubated with goat anti-IgG-TR (Santa Cruz, cat. no. sc-2781) secondary antibody (1:100) for 2 h and DAPI for 15 min in the dark. The images were recorded by digital photomicrography (Nikon, Japan).

Fragmentation quantification methods: Confocal micrographs (TIFF files) of PECs stained with Tom-20, Mito-Tracker, or infected with mito-roGFP were acquired and analyzed with ImageJ software as described. Individual cells were selected using the Polygon Selection tool, copied, and transferred to new individual image files which contained only one cell each. Each individual mitochondrial image was converted to an 8-bit grayscale image. The image's threshold values were adjusted so that the mitochondria's features were distinct from the background. The image was then converted to a binary image. A custom written macro was run, which returned the number of non-contiguous mitochondrial fragments and the number of pixels in the mitochondria. The Mitochondrial Fragmentation Index (MFI) was calculated by dividing the number of non-contiguous fragments by the number of pixels and multiplying the result by 1000 [8].

In some cases, higher resolution of live PECs in real time were obtained for mitochondrial detail with MitoTracker Red (ThermoFisher cat #M7512). Cells were grown to confluency in a 4-chamber Nunc™ Lab-Tek™ II Chamber Slide™ System (ThermoFisher cat # 154461PK) in 5% FBS-RPMI media. One the day of imaging, the media was replaced with media containing10 nM MitoTracker Red for 30 minutes, then washed. The chamber slide was placed into a microscope stage incubator designed for live-cell imaging and the cells were imaged using a

laser-scanning confocal microscope at 60X magnification using an excitation wavelength of 561 nm with detection at 595/50 nm.

Mito-roGFP: A lentivirus encoding a mitochondrially targeted green fluorescent protein (mito-roGFP) at ~10^6 integrative units per ml was used to identify mitochondrial structure and the redox state of cultured PECs [9, 10]. Excitation wavelengths were 405 or 485 nm and emission at 535 nm; the ratio of the fluorescence values at 405/485 was used to assess the oxidoreductive status. Images obtained at 485 nm were also used for calculation of mitochondrial fragmentation index.

MitoSox: Cultured PECs were incubated with MitoSOX™ Red mitochondrial superoxide indicator (5 μM in PBS for 20 min) to assess for mt-ROS. Each well was rinsed three times with PBS. Images were captured (514 nm excitation/585 nm emission) from ≥3 optical fields by Nikon TE2000 microscope. Mt-ROS was quantified by comparing fold change in fluorescence intensity of treated cells versus control cells [11].

Mitochondrial membrane potential measurements. Tetramethylrhodamine, methyl ester (TMRM) is a cell-permeant dye that accumulates in active mitochondria with intact membrane potentials. In healthy cells with mitochondria that have negative mitochondrial membrane potentials, the signal is bright. With loss of mitochondrial membrane potential, TMRM signal dims or disappears. After incubation for 48 hours in normoxia or hypoxia, PECs passage 3-5 were washed with PBS, then loaded with a final concentration of 100 nM TMRM in media for 30 minutes at 37 ^{o}C, then rinsed twice with fresh PBS before imaging with a Nikon TE2000 microscope [12] with excitation and emission spectra of 514 and 585 respectively. TMRM fluorescence in cells from hyperoxic and normoxic environments were compared to assess for relative polarization.

Nitrite measurements. Nitric oxide generation was assessed by measuring the accumulation of its stable metabolite nitrite in culture supernatants of PECs using the Griess assay [13].

siRNA design and transfection [14]: To silence the expression of Drp-1 protein, rat pulmonary artery PECs (ref) were transfected with the small interfering RNA (siRNA) which was designed and synthesized by GenePharma. Non-targeted control siRNA (siNC) was used as the negative control. The sense sequence of siRNA against Drp-1 and non-targeted control sequence was listed below: Drp-1: (NM_053655.3) 5′-GCAGAACUCUAGCUGUAAUTT-3′, negative control (NC): 5′-UUCUCCGAACGUGUCCGUTT-3′. Briefly, the PECs were cultured until 50–70 % confluence. After growth arrest, siRNA were transfected with lipofectamine 2000 reagent (Invitrogen, USA) according to manufacturer's instructions. siRNA and transfection reagent were diluted in the serum-free Opti-MEM-1 medium separately, after 5 min we mixed them together. Then, we incubated the mixture (siRNA/transfection reagent) at room temperature for 20 min and added it directly onto cells. After the cells were switched to normal culture medium, cells were incubated with hyperoxia or normoxia for 48 h and used as described in the text.

Drp-1 over-expression: Plasmids for over-expression of Drp-1 were purchased from Addgene [15]. PECs at ~70% confluency were infected with plasmids for mCherry as an indicator of infection efficiency or Drp-1 (1 ug plasmid DNA) using lipofectamine 3000 (Invitrogen USA). 48 hour later, PECs were visualized with fluorescence microscopy for estimation of infection efficiency (mCherry) or harvested for western blots to assess expression of Drp-1 or phospho-Drp-1.

Transwell resistance assays [16]. Cells were plated at a density of 1x10^4 cells per well with a volume of 500μl inside the well and 750 μl outside the well in a 24 well tissue culture dish. Monolayer resistance was measured daily after 48 hours using a WPI Epithelial Volt/Ohm (TEER) Meter. When monolayer resistance reached a steady state, test agents mito-TEMPOL, ENDO-III or vehicle were added to the wells, then plates placed in normoxia or hyperoxia for 48 hours. After 48 hours, monolayer resistance was measured, and data normalized to that of concomitant vehicle controls. For studies with PECs over-expressing Drp-1, PECs in transwells were transfected and transferred to hyperoxia (or normoxia) the following day for 48 hours prior to measurements of resistance.

Scratch assay: Confluent PECs cultured in 6-well plates were wounded by 100-200 μl pipette tips, giving rise to one acellular 1-mm-wide lane per well, and the ablated cells were washed out by PBS. After that, cells were

treated with vehicle or chemicals of interest in 5% FBS DMEM. Hyperoxic PECs were maintained under 95% oxygen. The same wounded areas were photographed at time zero, 24 hrs and 48 hrs later [17].

Transwell migration assay: Migration was measured using a modified Boyden chamber with 8 µm-pore polycarbonate filter (Costar Corning, USA). Cells were trypsinized and resuspended in 1% FBS medium then seeded into transwell inserts. The experimental reagents in 10% FBS-DMEM was placed in the lower chamber. The cells were cultured for 48 h at 37 ◦C to allow migration. After that the inserts were removed. The non-migrating cells in the upper chamber were then removed with a cotton swab. The migrated cells were stained with 0.4% crystal violet in 10% ethanol for 10 min. The number of migrated cells was measured by counting the number of stained nuclei per high-power field (Nikon, Japan). Each sample was counted randomly in nine separate locations in the center of the membrane and the endothelial cell migration activity reported as number of cells migrated per field of view. Experiments for each group were performed at least three times [18].

Network formation assay: 96-well culture plates (Costar, Corning) were coated with growth factor-reduced Matrigel (BD Biosciences) in a total volume of 30 µl and allowed to solidify for 20 minutes at 37 °C. PECs were trypsinized and resuspended at 5×10^4 /mL and 200 µl of this cell suspension were added into each well. Vehicle or ROS inhibitors at the indicated concentration were added to the appropriate well. Tube formation was observed under an inverted microscope (Nikon, Japan) at 24 hours. Tube length was measured in images using Image Pro Plus [19].

Statistical analysis: Data are presented as means ± SEM. Data were first assessed for normality and equal variance. For normally distributed data with equal variance, One-way ANOVA followed by Dunnett's tests were used to compare groups. For data which failed normality testing, Kruskal-Wallis ANOVA on Ranks was performed. Data which passed normality but failed equal variance testing were compared by ANOVA on Ranks. In some cases when comparing the response of a treatment versus vehicle, unpaired t-tests were used. $P < 0.05$ was considered statistically significant. All "n"s as well as normality, variance, and statistical tests appear in the figure legends. See analysis under fragmentation index for this calculation.

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