Endothelial cell respiration is affected by the oxygen tension during shear exposure: role of mitochondrial peroxynitrite

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Jones CI III, Han Z, Presley T, Varadaraj S, Zweier JL, Ilangovan G, Alevriadou BR. Endothelial cell respiration is affected by the oxygen tension during shear exposure: role of mitochondrial peroxynitrite. Am J Physiol Cell Physiol 295: C180–C191, 2008. First published May 14, 2008; doi:10.1152/ajpcell.00549.2007.—Cultured vascular endothelial cell (EC) exposure to steady laminar shear stress results in peroxynitrite (ONOO−) formation intramitochondrially and inactivation of the electron transport chain. We examined whether the “hyperoxic state” of 21% O2, compared with more physiological O2 tensions (PO2), increases the shear-induced nitric oxide (NO) synthesis and mitochondrial superoxide (O2•−) generation leading to ONOO− formation and suppression of respiration. Electron paramagnetic resonance oximetry was used to measure O2 consumption rates of bovine aortic ECs sheared (10 dyn/cm2, 30 min) at 5%, 10%, or 21% O2 or left static at different PO2. Flow in the presence of an endothelial NO synthase (eNOS) inhibitor or a ONOO− scavenger abolished the inhibitory effect. EC transfection with an adenovirus that expresses manganese superoxide dismutase in mitochondria, and not a control virus, blocked the inhibitory effect. Intracellular and mitochondrial O2•− production was higher in ECs sheared at 21% than at 5% O2, as determined by dihydroethidium and MitoSOX red fluorescence, respectively, and the latter was, at least in part, NO-dependent. Accumulation of NO metabolites in media of ECs sheared at 21% O2 was modestly increased compared with ECs sheared at lower PO2, suggesting that eNOS activity may be higher at 21% O2. Hence, the hyperoxia of in vitro EC flow studies, via increased NO and mitochondrial O2•− production, leads to enhanced ONOO− formation intramitochondrially and suppression of respiration.

shear stress; endothelium; mitochondria; reactive oxygen species

MITOCHONDRIA ARE THE SUBCELLULAR organelles for the production of cellular energy, but they also activate intracellular signaling pathways that modulate cell proliferation or promote cell cycle arrest and apoptosis by oxidative or nitrosative reactions. These reactions are regulated by the matrix concentration of nitric oxide (NO), which diffuses to the mitochondria from the cytosolic NO synthase (NOS) isoforms and is thought to be produced also locally by a mitochondrial NOS variant (11). In rat heart, skeletal muscle and liver mitochondria (15, 57, 58), isolated rat hearts (59), and whole animals (68), NO at physiological concentrations binds reversibly and in competition with oxygen (O2) to the reduced binuclear center CuAa3 of cytochrome-c oxidase (complex IV) of the electron transport chain (ETC) and inhibits mitochondrial O2 uptake. At slightly higher concentrations, NO oxidizes ubiquinol of ubiquinol-cytochrome c reductase (complex III) to increase unstable ubisemiquinone, which, by univalent electron transfer to O2, produces the reactive O2 species (ROS) superoxide (O2•−) (57, 58). O2•− generated from that location is released both into the matrix and intermembrane space (51), where it is dismutated to hydrogen peroxide (H2O2) by manganese superoxide dismutase (MnSOD) and copper zinc SOD (CuZnSOD), respectively (29). Hence, mitochondrial NO metabolism involves regulation of O2 consumption, H2O2 production, and the effects of the freely diffusible (to the cytosol) H2O2 on cell signaling and gene expression (11).

NO and O2•− combine at a diffusion-limited reaction rate to form peroxynitrite (ONOO−) (40), which is able to nitrate and/or oxidize amino acid side chains of mitochondrial proteins and result in irreversible inhibition of NADH-ubiquinone reductase (complex I), succinate-ubiquinone reductase (complex II), complex IV, aconitase, and ATPase (12, 13, 18, 65). In agreement with its effects on ETC components, ONOO− potentiated the inhibition of respiration in isolated cardiac muscle compared with the inhibition elicited by NO alone and made it irreversible with time (79). Under stress conditions, such as those at reperfusion (RP) following ischemia (I), where NO production is upregulated in endothelial cells (ECs) and cardiomyocytes, either because of increases in Ca2+-dependent NOS activity via changes in shear stress and intracellular Ca2+ concentration or because of expression of inducible NOS isoforms, ONOO− formation predominates over O2•− dismutation (24, 76). ONOO−-mediated inactivation of complex I increases O2•− production from that location, and O2•− generated by complex I is released exclusively into the matrix (16, 51, 63–65). Animal I/RP models showed that, upon RP, the ETC complex activities are reduced, complex I is the main source of mitochondrial ROS (2, 31, 54), and NO derived from endothelial NOS (eNOS) is the one responsible for ONOO− formation, inhibition of complex I activity, and suppression of tissue O2 consumption (84).

We recently showed that cultured human umbilical vein EC (HUVEC) exposure to steady laminar shear stress results in inactivation of the ETC complexes I, II/III, and IV, which is, at least in part, due to ONOO− formation in the mitochondria (30). Specifically, when ECs were preincubated and sheared in the presence of either the eNOS inhibitor Nω-nitro-l-arginine methyl ester (l-NAME) or the ONOO− scavenger uric acid (UA), the flow effect on complexes I and II/III was attenuated.

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and the effect on complex IV was abolished. Inhibition of complex IV is expected in NO-producing cells (17). However, shear-induced inhibition affected all complexes (including complex I), was accompanied by nitrotyrosine staining in the mitochondria, and was prevented by UA, indicating that endogenous eNOS-derived NO diffuses to the mitochondria where it reacts with $O_2^{•−}$ generated by the ETC resulting in mitochondrial ONOO$^•$ formation, and the latter is responsible for the inhibition of ETC at multiple sites (30).

All flow studies with cultured ECs, as well as the reoxygenation (RO) phase of hypoxia (H)/RO studies, including work from our group (30, 37, 48, 52, 80, 81), have been carried out at the atmospheric $O_2$ concentration (21% $O_2$), which gives rise to a partial pressure of $O_2$ ($P_{O2}$) of ~160 mmHg, above the levels in arterial blood (75–100 mmHg; 10–13% $O_2$) and well above the levels in venous blood (35 mmHg; 5% $O_2$) or the levels to which ECs are exposed in tissue capillaries (mean capillary blood $P_{O2}$ in the heart is ~20 mmHg; 2.6% $O_2$) (72).

Since it is known that 1–4% of $O_2$ reacting with the ETC is incompletely reduced to $O_2^{•−}$, and the $O_2^{•−}$ formation rate by the ETC complexes III and I increases linearly with $O_2$ concentration (42, 74), we hypothesized that, under flow, the relative “hyperoxic state” of 21% $O_2$ raises the $O_2$ concentration in EC mitochondria and enhances the formation of $O_2^{•−}$, which, via the reaction with shear-induced NO, increases the intramitochondrial ONOO$^•$ levels and potentiates the inhibition of respiration. Although it is known that (1) either hypoxia (21–100% $O_2$) or H (1–3% $O_2$)/RO (21% $O_2$) increases the mitochondrial $O_2^{•−}$ generation and inhibits respiration (9, 21, 33, 67, 71) and (2) shear at 21% $O_2$ increases the EC ONOO$^•$ formation intramitochondrially (30), the respiratory function of sheared ECs at 21% $O_2$ and at lower, but physiologic, $O_2$ levels has not been examined.

In the present study, cultured bovine aortic ECs (BAECs) were exposed to steady laminar flow (shear stress of 10 dyn/cm$^2$) for different periods of time at $O_2$ levels of either 21% (atmospheric level), 10% (close to the 12.5% $O_2$ that accurately simulates the arterial $O_2$ level for people in cities with elevation close to sea level), or 5% (venous $O_2$ level). Corresponding static controls were maintained at the same $O_2$ levels. Electron paramagnetic resonance (EPR) oximetry, a technique capable of measuring $P_{O2}$ changes vs. time from a relatively small number of cells (34, 53), was used to measure EC $O_2$ consumption rates. Since ONOO$^•$ is formed in mitochondria of ECs sheared at 21% $O_2$ (30) and ONOO$^•$ is known to greatly suppress respiration (79), we hypothesized that the $O_2$ consumption rates would be lower for sheared vs. static ECs and would be modulated by the $P_{O2}$ during flow. Since the matrix $O_2^{•−}$ concentration is controlled by MnSOD, the effect of MnSOD overexpression on EC respiration was also examined. Determination of intracellular and mitochondrial $O_2^{•−}$ production, and the dependence of the latter on NO, by ECs static or sheared at different $P_{O2}$ were based on the oxidation of the fluorophore dihydroethidium (DHE) and its mitochondria-targeted derivative MitoSOX red, respectively. A possible mechanism that would explain why shear-induced inhibition of respiration may be less at $O_2$ levels <21% is that eNOS activity may be lower under shear at <21% $O_2$, leading to reduced NO production and mitochondrial ONOO$^•$ generation. Hypoxia is known to attenuate NO production by ECs from different vascular beds (44, 70, 78), whereas hyperoxia was shown to increase eNOS expression and activity in bovine pulmonary artery ECs (44). To test whether low, but physiologic, $P_{O2}$ affects eNOS activity during shear, NO production and eNOS phosphorylation at a key regulatory site were examined in ECs sheared at different $O_2$ levels.

**EXPERIMENTAL PROCEDURES**

**EC culture.** Primary BAECs were purchased from Cambrex (East Rutherford, NJ) and cultured in Dulbecco’s modified Eagle’s medium (DMEM) with l-glutamine and NaHCO$_3$ supplemented with 10% fetal bovine serum (FBS) and antibiotics. ECs (passages 3–6) were seeded onto glass slides (75 × 38 mm; Fisher Scientific, Pittsburgh, PA) that were sterilized, air-dried, and coated with a 0.5% gelatin subbing solution that contained 0.05% potassium chromate sulfate (Sigma, St. Louis, MO). EC monolayers were used within 24 h upon confluence.

**EC exposure to shear stress.** Three glass slides with EC monolayers were assembled side-by-side into a parallel-plate flow chamber, and the chamber was connected at both ends to a reservoir forming a flow loop (26). ECs were exposed to a constant gravity-driven laminar shear stress of 10 dyn/cm$^2$ (low arterial range). Flow rate through the chamber was monitored by an ultrasonic flow sensor (Transonic Systems, Ithaca, NY). The $P_{O2}$ in the system was controlled by injecting a gas mixture of air, $N_2$, and CO$_2$ to effect the desired final medium $O_2$ concentrations of either 5%, 10%, or 21%, while maintaining 5% CO$_2$. The balance of the gases was controlled by a manual gas proportioner (Aalborg, Orangeburg, NY), so that the $O_2$ concentration, which was measured immediately downstream of the flow chamber, remained at target. Recirculating culture medium was constantly exposed to a counter-current flow of the sterile-filtered gas mixture that was warmed and humidified by bubbling through water; this permitted the use of protein-rich medium (with 10% FBS) without foaming. Medium $O_2$ concentration and temperature were monitored real-time by inline optical $O_2$ and temperature sensors (World Precision Instruments, Sarasota, FL). The temperature of the entire system was kept at 37°C. Corresponding static incubations of EC monolayers were placed in preequilibrated media in humidified incubators (Heraeus Instruments, Tewksbury, MA) preset for specified desired $O_2$ concentrations of either 5% or 21% $O_2$ for the same time periods as the sheared monolayers. Some ECs were preincubated with either 100 μM of L-NAME for 4 h or 50 μM of UA for 30 min (both from Sigma) and either subjected to shear or left static in medium containing the same concentration of the respective drug. Cell viability was determined by trypsin blue exclusion at the end of treatment, and it was ≥90%. The chosen concentration of the eNOS inhibitor is known to inhibit NO production by sheared BAECs (77). The $O_2$ concentration was chosen on the basis of the scavenging of ONOO$^•$ formed by either chemically stimulated BAECs (85) or sheared HUVECs (30).

**EC infection with adenoviral vectors.** The replication-deficient adenovirus Ad.MnSOD, which contains cDNA coding for human MnSOD including that for the mitochondrial leader sequence cloned into Ad.cytomegalovirus-link, expresses MnSOD in mitochondria and was purchased from the Vector Core Facility of the University of Iowa (86). HUVEC infection with Ad.MnSOD is known to produce an increase in expression of the 22-kDa protein and in total SOD activity, and colocalization experiments using confocal microscopy have shown that MnSOD protein is localized in the mitochondria (23). The infectious units were at 1 × 10$^{10}$ plaque-forming units (pfu)/ml and were determined by assessing pfu on 293 cells. ECs were infected for 48 h before shear exposure (~80% confluency) at a multiplicity of infection (MOI) of 100 in culture medium. MnSOD expression was tested in transfected ECs by assaying protein content using Western blot analysis. Simultaneously with treatment groups, control ECs were treated with Ad.Emptyy, which contains no foreign cDNA (also purchased from the Vector Core Facility of the University of Iowa).
Measurement of O₂ consumption. At the end of shear exposure, ECs were harvested and centrifuged, and the pellet was resuspended at a density in the order of 10⁷ cells/ml in modified Krebs-Henseleit buffer (in mM: 117.3 NaCl, 4.7 KCl, 1.3 MgSO₄, 1.2 CaCl₂, 1.2 KH₂PO₄, and 25 NaHCO₃; pH 7.4) supplemented with 20 mM glucose, in agreement with earlier respirometry studies (17). EC respiration was measured immediately using EPR oximetry with lithium phthalocyanine (LiPc) as the probe in a Bruker ER-300 X-band EPR spectrometer fitted with a TM110 microwave cavity, as described previously (34, 36, 53). This technique is based on the principle of EPR line broadening by O₂ and is capable of determining O₂ concentration with a resolution of submicromolar concentrations in small volumes. The EPR line width vs. O₂ calibration curve was constructed using known ratios of premixed O₂ and N₂ gases (36). LiPc crystals were added to EC suspensions, incubated for 10 min at 37°C, and sampled in a 50-µl glass capillary sealed at both ends. Po2 (mmHg) was computed from the EPR line width using the calibration curve. Plots of Po2 vs. time were obtained, and the slope of the linear section represented the O₂ consumption rate (VO₂, mmHg/min). Po2 and VO₂ data were used to fit the equation (27):

\[
\frac{VO₂}{VO₂max} = \frac{Po₂}{(Po₂ + P₀₀)}
\]

where VO₂ is the respiration rate at given Po₂, VO₂max is the maximum respiration rate, and P₀₀ is the Po₂ at which the respiration rate is 50% of maximum. The parameters VO₂max and P₀₀ were obtained using nonlinear least-squares fitting of the data. Knowing the solubility of O₂ in H₂O (1.22 nmol/mmHg at 37°C) and the cell density, VO₂max was expressed in nmol O₂·min⁻¹·10⁻⁶ cells (53).

Fluorescence detection of intracellular and mitochondrial O₂⁻⁻⁻. DHE and MitoSOX red (514 nm excitation/585 nm emission) were used to detect intracellular and mitochondrial O₂⁻⁻⁻ production, respectively, and 4',6-diamidino-2-phenylindole (DAPI; 359 nm excitation/461 nm emission) was used to label cell nuclei (Molecular Probes, Eugene, OR). Although DHE is widely used for the detection of intracellular O₂⁻⁻⁻ (6), it is known that the hydrothiowine moiety of DHE (and its mitochondria-targeted derivative, MitoSOX) reacts with O₂⁻⁻⁻ to specifically produce hydroxyethidium (OH-Etd⁻⁻⁻) and can react with different oxidants (such as H₂O₂ in the presence of heme proteins or peroxides) to produce ethidium (Etd⁺⁺). Both products form a complex with DNA, which greatly enhances their fluorescence when excited at 514 nm (83). While investigating the MitoSOX oxidation by O₂⁻⁻⁻, it was shown that OH-Etd⁻⁻⁻ presents an excitation maximum at 396 nm that is not present in the excitation spectrum of Etd⁺⁺ (66). Hence, in the present study, MitoSOX was excited by either the 514 or the 405 nm laser line of confocal microscopes to more accurately assess O₂⁻⁻⁻ production in mitochondria of sheared ECs, as was recently described for hyperglycemic ECs (61). Specifically, at the end of shear, EC monolayers were incubated with either DHE or MitoSOX red (10 µM) and DAPI (1 µM) for 10 min in the dark at 37°C, washed with PBS, and mounted with Fluoromount-G (Southern Biotech, Birmingham, AL), and images (20× for DHE, 62× for MitoSOX red and DAPI) were obtained by confocal microscopy (Zeiss LSM 510, Peabody, MA, for excitation at 514 or 359 nm; Olympus Fluoview 1000, Center Valley, PA, for excitation at 405 nm) and overlaid using LSM software. Digital images from three fields of view were collected per experiment and corrected for autofluorescence, and the background fluorescence was excluded from calculations by thresholding. The mean fluorescence intensity per image was calculated and averaged over the three images, using MetaMorph software (Universal Imaging, Downingtown, PA). Some EC monolayers were preincubated with either t-NNAME (100 µM for 4 h), the ETC complex III inhibitor antimycin A (10 µM for 30 min), or both and were either left static or sheared in the presence of the respective drug. Image acquisition conditions were kept constant for comparison between treatments.

Measurement of NO production. Medium samples were drawn at different time points during flow exposure (with replacement of same volume of fresh medium to maintain circulating medium volume), and the accumulation of NO metabolites [nitrite (NO₂⁻⁻⁻⁻) and nitrate (NO₃⁻⁻⁻⁻⁻; NO₃)] was measured using a chemiluminescence analyzer (Sievers 270B; Sievers Instruments, Boulder, CO), as described previously (30). For each experiment, a standard curve was constructed using different concentrations of NaNO₃ for calculation of NO₃ content per sample. The background signal in perfusion medium was subtracted from each measured value, and values were mathematically corrected for the dilution effect of medium replacement.

Western blot analysis. ECs were scraped into ice-cold lysis buffer (0.06 M Tris·HCl, 2% SDS, and 5% glycerol, pH 6.8). Cell extracts were homogenized by sonication, boiled for 5 min, and centrifuged at 10,000 rpm for 10 min at 4°C to remove the insoluble material. Protein concentration in cell lysates was determined using the bicinchoninic acid assay (Pierce, Rockford, IL). Supernatant containing 30 µg of protein was mixed with 2X sample buffer and applied in duplicate on 4–20% Tris·HCl gels (Bio-Rad Laboratories, Hercules, CA) for protein separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis at 100-V constant voltage. Proteins were electrophoretically transferred at 100-V constant voltage at room temperature for 1 h into nitrocellulose membranes (Bio-Rad). After incubation in blocking solution (10 mM Tris·HCl, 0.2% nonfat milk, 100 mM NaCl, and 0.1% Tween 20, pH 7.5), duplicate membranes were hybridized with primary antibodies against either eNOS and phospho-eNOS (Ser1177), respectively, or MnSOD and β-actin, respectively. The anti-eNOS antibody was from Transduction Laboratories (Lexington, KY), anti-p-eNOS (Ser1179; based on bovine eNOS sequence and equivalent to human Ser1177) from Upstate Biotechnology (Lake Placid, NY), anti-MnSOD from Abcam (Cambridge, MA), and anti-β-actin from Sigma. Membrane-bound primary antibodies were detected using secondary antibodies conjugated with alkaline phosphatase. Immunoblots were developed on films using the enhanced chemiluminescence method (Bio-Rad).

Statistical analysis. The VO₂max and P₀₀ values are expressed as means ± SE of n ≥ 3 independent experiments. Significant differences between treatments (static vs. shear at 21% O₂, 30 min; 5% O₂, 30 min; 21% O₂, 16 h) were determined using the SAS 9.1 software (Cary, NC) to perform one-way analysis of variance (ANOVA) followed by Bonferroni’s test for pairwise comparisons. VO₂max values of ECs that were either kept static (21% O₂) or sheared (21% O₂, 30 min) with or without t-NNAME or UA were analyzed similarly. Means ± SE of normalized (to corresponding controls) fluorescence from n ≥ 3 independent experiments were estimated for ECs labeled with either DHE (excitation at 514 nm) or MitoSOX (excitation at 514 and 405 nm) and either left static (21% O₂) or sheared (5% or 21% O₂, 30 min). Means ± SE of normalized (to corresponding controls) fluorescence from n ≥ 3 independent experiments were estimated for ECs labeled with MitoSOX (excitation at 514 nm) and either left static (21% O₂) or sheared (21% O₂, 30 min) with or without t-NNAME or UA were analyzed similarly. In the case of NO₂⁻⁻⁻ production measurements vs. time from either static or sheared ECs at different O₂ levels, NO₂⁻⁻⁻ production data from each treatment and at each time point were expressed as means ± SE of n independent experiments. Data were analyzed using a mixed-effect model with treatment (5 levels: static, 5% O₂; static 21% O₂; shear, 5% O₂; shear, 10% O₂; shear, 21% O₂), time (4 levels: 5, 15, 30 and 60 min), and their interaction as fixed effects and trial and replicate (nested within trial) as random effects. Data from time zero were not used in the analysis, since NO₂⁻⁻⁻ production was uniformly zero at that time point. Comparisons were made between treatment conditions at each time point using a Bonferroni correction for multiple comparisons. P values <0.05 were considered significant.
RESULTS

Shear stress inhibits EC respiration, and inhibition depends on \( P_{O_2} \). Since NO is known to reduce the \( V_{O_2} \), either via competitive binding to complex IV or via formation of \( \cdot ONOO^- \) that irreversibly inhibits the ETC at multiple sites (15, 57, 58, 79), and EC exposure to steady laminar flow increases the NO production resulting in \( \cdot ONOO^- \) formation in the mitochondria (30), we tested whether respiration is decreased in sheared ECs. BAECs were sheared (10 dyn/cm\(^2\)) for 30 min at either 5%, 10%, or 21% \( O_2 \), and corresponding controls were left static at either 5% or 21% \( O_2 \) for the same period. The \( P_{O_2} \) measurements vs. time (Fig. 1A) were performed on EC suspensions (2.4 \( \times 10^7 \) cells/ml) at the end of treatment by EPR oximetry and are representative ones of three experiments with similar results. The slope of the linear portion of the \( P_{O_2} \) vs. time data represents the \( V_{O_2} \). \( V_{O_2} \) was reduced in sheared ECs compared with static controls, and the inhibition of respiration was greater at the highest \( P_{O_2} \) tested (21% \( O_2 \)) (Fig. 1A). No difference in respiration was observed between ECs that were left static at different \( O_2 \) concentrations or between ECs that were sheared at 5% or 10% \( O_2 \) (Fig. 1A). The inhibition observed after 30 min shear at 21% \( O_2 \) was still present when ECs were sheared for prolonged time (16 h) (Fig. 1B). The \( P_{O_2} \) measurements vs. time (Fig. 1B) were performed on EC suspensions (1.3 \( \times 10^7 \) cells/ml) at the end of treatment and are representative of three experiments with similar results. The \( P_{O_2} \) of ECs treated identically was significantly higher in 2.4 \( \times 10^7 \) vs. 1.3 \( \times 10^7 \) cells/ml, in agreement with a previous report (35) that \( V_{O_2} \) is proportional to the cell density.

Analysis of \( O_2 \) consumption kinetics: \( V_{O_2\ max} \) of sheared ECs is lower at the highest \( P_{O_2} \) tested. Since there was no difference in respiratory function between ECs sheared at 5% vs. 10% \( O_2 \), all subsequent studies focused on ECs sheared at either 5% or 21% \( O_2 \). The \( P_{O_2} \) measurements vs. time (Fig. 2A) were performed on EC suspensions (0.8 \( \times 10^7 \) cells/ml) at the end of treatment by EPR oximetry and are representative of six experiments with similar results. Data in the low \( P_{O_2} \) range were analyzed using Eq. 1, and \( V_{O_2} \) vs. \( P_{O_2} \) lines were plotted (Fig. 2B). It is important to notice that, even under the lowest \( P_{O_2} \) tested (5% \( O_2 \); 35 mmHg), static and sheared ECs respire at their maximum rates (\( V_{O_2\ max} = V_{O_2\ max} \) at either 5% or 21% \( O_2 \)). For this characteristic run, \( V_{O_2\ max} \) of static ECs was the same at 5% and 21% \( O_2 \) (3.5 and 3.7 mmHg/min, respectively), whereas, for sheared ECs, \( V_{O_2\ max} \) at 5% \( O_2 \) was 2.7 mmHg/min and at 21% \( O_2 \) was 1.4 mmHg/min. \( P_{50} \) of static ECs was the same at 5% and 21% \( O_2 \) (4.0 and 4.2 mmHg), remained the same when ECs were sheared at 5% \( O_2 \) (3.9 mmHg), but declined when ECs were sheared at 21% \( O_2 \) (1.6 mmHg). To quantify the effect of flow at different \( O_2 \) levels on the respiratory parameters, \( V_{O_2\ max} \) (in nmol \( O_2 \cdot \text{min}^{-1} \cdot 10^6 \) cells) and \( P_{50} \) (in mmHg) values from \( n = 3–6 \) independent experiments were expressed as means \( \pm \) SE of 6 different treatments (static vs. shear at either 21% or 5% \( O_2 \) for 30 min; static vs. shear at 21% \( O_2 \) for 16 h) (Table 1). \( V_{O_2\ max} \) of sheared ECs at 21% \( O_2 \) for either 30 min or 16 h was significantly different from \( V_{O_2\ max} \) of corresponding static controls and of sheared ECs at 5% \( O_2 \) for 30 min. \( P_{50} \) of sheared ECs at 21% \( O_2 \) for either 30 min or 16 h was significantly different from \( P_{50} \) of corresponding static controls (Table 1).

\( \text{NO} \) and mitochondrial \( \cdot ONOO^- \) are responsible for the inhibitory effect of flow on \( \text{EC respiration} \). To determine the role of shear-induced \( \text{NO} \) and the resultant \( \cdot ONOO^- \) formation on the observed reduction of \( V_{O_2} \), ECs were preincubated and sheared at 21% \( O_2 \) for 30 min in the presence of either 100 \( \mu \)M of the eNOS inhibitor \( L\)-NAME or 50 \( \mu \)M of the \( \cdot ONOO^- \) scavenger UA. The \( P_{O_2} \) measurements vs. time (Fig. 3A) were performed on EC suspensions (2.4 \( \times 10^7 \) cells/ml) at the end of treatment and are representative of three experiments with similar results. Either \( L\)-NAME or UA abolished the inhibitory effect of shear at 21% \( O_2 \) on \( \text{EC respiration} \); \( V_{O_2\ max} \) (means \( \pm \) SE of \( n = 3 \) independent experiments) of sheared ECs (4.3 \( \pm \) 0.3 nmol \( O_2 \cdot \text{min}^{-1} \cdot 10^6 \) cells) was significantly different from \( V_{O_2\ max} \) of corresponding static controls (9.5 \( \pm \) 0.6 nmol \( O_2 \cdot \text{min}^{-1} \cdot 10^6 \) cells) and of sheared ECs in the presence of either \( L\)-NAME (9.3 \( \pm \) 0.7 nmol \( O_2 \cdot \text{min}^{-1} \cdot 10^6 \) cells) or UA (9.1 \( \pm \) 0.2 nmol \( O_2 \cdot \text{min}^{-1} \cdot 10^6 \) cells). There was no difference in respiration between static ECs that were preincubated...
Mitochondrial $O_2^{•−}$ production by sheared ECs increases with $P_{O_2}$ and depends on NO. Shear exposure (at either 5% or 21% $O_2$ for 30 min) caused an increase in fluorescence of either DHE (excitation at 514 nm) or MitoSOX red (excitation at 514 and 405 nm), as monitored by confocal microscopy, and the increase was higher when ECs were sheared at 21% than at 5% $O_2$ (Fig. 4A). No noticeable increase in fluorescence occurred in static controls maintained at either $P_{O_2}$ (only static control at 21% $O_2$ is shown; Fig. 4A). The MitoSOX fluorescence signals of sheared ECs were significantly different from those of their corresponding static controls at either excitation, and signals at 405 nm excitation were only slightly lower than signals at 514 nm excitation, suggesting that MitoSOX oxidation in ECs sheared at either 5% or 21% $O_2$ is predominantly $O_2^{•−}$-specific (Fig. 4B). The fluorescence signals of ECs labeled with either DHE or MitoSOX (excitation at 514 and 405 nm) and sheared at 5% $O_2$ were significantly different from the respective signals of ECs sheared at 21% $O_2$ (Fig. 4B). Since there was no appreciable difference in MitoSOX fluorescence between the two excitations, the rest of the fluorescence experiments were performed at an excitation of 514 nm. MitoSOX red is known to not get oxidized by NO or ONOO$^−$ (66). L-NAME (100 $μM$) did not affect the MitoSOX fluorescence signal in static ECs, probably because of the fact that the relatively low amount of NO produced is not sufficient to significantly inhibit complex IV and enhance $O_2^{•−}$ generation by the mitochondrial ETC (Fig. 5, A and B). Shear (21% $O_2$ for 30 min) as before, increased the MitoSOX fluorescence due to increased NO generation and intramitochondrial ONOO$^−$ formation, which are expected to increase the mitochondrial $O_2^{•−}$ generation at the level of ETC complexes III and I. Preincubation and shear at 21% $O_2$ in the presence of L-NAME (100 $μM$) significantly decreased the MitoSOX fluorescence signal compared with sheared ECs, suggesting that NO and/or its product, ONOO$^−$, is a major contributor to the mitochondrial generation of $O_2^{•−}$ (Fig. 5, A and B). To test whether MitoSOX fluorescence of sheared ECs in the presence of L-NAME is restored when a respiratory inhibitor is supplied, antimycin A (10 $μM$) was used. Antimycin A, by binding to cytochrome $b$, increases the concentration of ubiquinone, resulting in increased $O_2^{•−}$ generation from complex III (73). As expected, and left static at 21% $O_2$ for 30 min with or without L-NAME or UA (only data from static ECs without any drug are shown; Fig. 3A). The NO fraction that forms ONOO$^−$ or UA (only data from static ECs without any drug are shown; inset of Fig. 3B) were performed on EC suspensions (0.8 $×$ 10$^7$ cells/ml) as before and are representative of 6 experiments with similar results. $P_{O_2}$ measurements vs. time were performed on EC suspensions (0.8 $×$ 10$^7$ cells/ml) as before and are representative of 6 experiments with similar results. $P_{O_2}$ measurements vs. time were performed on EC suspensions (0.8 $×$ 10$^7$ cells/ml) as before and are representative of 6 experiments with similar results. $P_{O_2}$ measurements vs. time were performed on EC suspensions (0.8 $×$ 10$^7$ cells/ml) as before and are representative of 6 experiments with similar results. $P_{O_2}$ measurements vs. time were performed on EC suspensions (0.8 $×$ 10$^7$ cells/ml) as before and are representative of 6 experiments with similar results.

![Graph A](image1.png)

**Fig. 2.** Analysis of $O_2$ consumption kinetics. A: BAECs were sheared (10 dyn/cm$^2$) for 30 min at either 5% or 21% $O_2$, and corresponding controls were left static at either 5% or 21% $O_2$ for the same period. $P_{O_2}$ measurements vs. time were performed on EC suspensions (0.8 $×$ 10$^7$ cells/ml) as before and are representative of 6 experiments with similar results. B: data in A were analyzed using Eq. 1, and $O_2$ consumption rate ($V_{O_2}$) vs. $P_{O_2}$ lines were plotted.

&

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$P_{O_2}$, % $O_2$</th>
<th>Time</th>
<th>$V_{O_2\text{max}}$, nmol $O_2$-min$^{-1}\cdot10^{-6}$ cells</th>
<th>$P_{50}$, mmHg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Static</td>
<td>21</td>
<td>30 min</td>
<td>9.8 ±0.8</td>
<td>4.9 ±0.8</td>
</tr>
<tr>
<td>Shear</td>
<td>21</td>
<td>30 min</td>
<td>4.1 ±0.7*†</td>
<td>2.6 ±0.4*</td>
</tr>
<tr>
<td>Static</td>
<td>5</td>
<td>30 min</td>
<td>9.3 ±1.4</td>
<td>4.6 ±0.6</td>
</tr>
<tr>
<td>Shear</td>
<td>5</td>
<td>30 min</td>
<td>8.1 ±0.6</td>
<td>4.3 ±0.7</td>
</tr>
<tr>
<td>Static</td>
<td>21</td>
<td>16 h</td>
<td>9.6 ±0.4</td>
<td>4.7 ±0.7</td>
</tr>
<tr>
<td>Shear</td>
<td>21</td>
<td>16 h</td>
<td>4.6 ±0.7†</td>
<td>2.5 ±0.4*</td>
</tr>
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Values are means ± SE; $n = 3–6$ experiments. Plots of $P_{O_2}$ versus time were obtained from electron paramagnetic resonance (EPR) oximetry measurements on endothelial cell (EC) suspensions. Before EPR oximetry, ECs were either static or sheared at 10 dyn/cm$^2$ for either 30 min or 16 h. Maximum respiration rate ($V_{O_2\text{max}}$) and $P_{O_2}$ at which $V_{O_2}$ is 50% of maximum ($P_{50}$) values were determined, as described. Using the solubility of $O_2$ in H$_2$O at 37°C and the known cell number of each experiment, $V_{O_2\text{max}}$ was expressed in nmol $O_2$-min$^{-1}\cdot10^{-6}$ cells. *$P < 0.05$ versus corresponding static control. †$P < 0.05$ versus shear at 5% $O_2$ for 30 min.
antimycin A significantly increased the fluorescence of static ECs (Fig. 5, A and B). However, it significantly blocked the shear-induced increase in fluorescence, possibly because shear exposure inhibits complex III activity by ~70% (30) and decreased complex III activity reduces the enzyme-mediated O$_2^\cdot{^-}$ production (14). Last, when either static or sheared ECs were treated with both l-NAME and antimycin A, the signal was significantly increased compared with either static or sheared ECs treated with just l-NAME, respectively, suggesting that MitoSOX fluorescence of ECs in the absence of NO is sensitive to O$_2^\cdot{^-}$ generation by the ETC complex III (Fig. 5, A and B).

NO$_x$ production by sheared ECs increases with PO$_2$. Since less NO production by ECs sheared at either 5% or 10% O$_2$, compared with ECs sheared at 21% O$_2$, would lead to less mitochondrial ONOO$^-$ formation and partially restore respiration, NO$_x$ production in culture media of ECs exposed to static or shear conditions at different PO$_2$ was measured vs. time using a chemiluminescence analyzer. Data at each time point and treatment are means ± SE of five independent experiments (Fig. 6A). Stress at 21% O$_2$ elicited a transient burst in NO$_x$ production within the first 15 min from the onset of flow followed by a lower sustained release, in agreement with earlier studies (41, 77). When ECs were sheared at either 5% or 10% O$_2$, the initial burst occurred, but it was smaller in magnitude, so during the rest of the time...
(15–60 min), the NOx production by sheared ECs at 21% O2 was significantly higher than the production by ECs either sheared at lower PO2 or left static at 5% or 21% O2. At 5 min from the flow onset, the NOx production by sheared ECs at 21% O2 was significantly higher than that by ECs in all other conditions except from shear at 10% O2 (Fig. 6A). Since Ser1179 (based on bovine eNOS sequence and equivalent to human Ser1177) phosphorylation is considered the most important of the regulatory eNOS phosphorylation sites and diverse stimuli, including shear stress, cause early phosphorylation of this site (7, 50), we examined whether shear at different PO2 alters eNOS activity via Ser1179 phosphorylation. ECs were either sheared at 5%, 10%, or 21% O2 or left static at 5% or 21% O2. At 30 min, and cell lysates were analyzed by Western blot with antibodies specific for phospho-eNOS (Ser1179) or total eNOS (protein loading control). Shear greatly increased Ser1179 phosphorylation at each PO2 tested compared with corresponding static controls, but there was no difference in the ratio of phospho-eNOS (Ser1179) to total eNOS among ECs sheared at either 5%, 10%, or 21% O2 (Fig. 6B).

**DISCUSSION**

The present study provides the first evidence that shear-induced NO, via formation of ONOO⁻ in the mitochondria, reduces the rate of O2 consumption by cultured vascular ECs exposed to steady laminar flow (Figs. 1–3 and Table 1). The degree of inhibition of EC respiration depends on the PO2 during shear exposure; the effect is greater when flow is carried out at the atmospheric O2 concentration (Figs. 1A and 2 and Table 1). Exposure of cultured ECs to 21% O2 is mildly hyperoxic compared with the in vivo normoxia, and, hence, in the present study, BAECs were sheared at an arterial level of shear stress under either 5%, 10%, or 21% O2. Our choice of 10% O2 is within the normal O2 level for the arterial wall.
an earlier study, shear stress (21% O2) was shown to increase the relative hyperoxic 21% O2 is consistent with our hypothesis static, 5% O2; static, 21% O2) at the same time point. † ONOO

this, either the eNOS inhibitor L-NAME or the ONOO

than the suppression due to NO alone (79). In agreement with

expected to be higher at 21% vs. 5% O2.

Higher PO2 may result in increased shear-induced NO formation, in particular at 21% O2), O2

NO that forms ONOO

diffuse across cell membranes, the fraction of shear-induced

formation by the ETC is known to

A

B

ONOO

MitoSOX fluorescence (excitation at 405 nm) showed that the mitochondrial O2−−− levels are increased in parallel with the Po2 during shear exposure, and a comparison between the two excitation wavelengths showed that, even when the excitation is at 514 nm, most of the fluorescence is due to O2−−−−− specific oxidation products (Fig. 4). MitoSOX red fluorescence (excitation at 514 nm) suggested that the shear-induced NO is, at least in part, responsible for the generation of O2−−− by the ETC (Fig. 5). To accurately demonstrate mitochondrial O2−−− generation, the oxidation product OH-Etd− in mitochondrial cell fractions should be detected by HPLC, as described for whole EC lysates (83). However, MitoSOX fluorescence (excitation at 514 nm) from L-NAME-treated sheared ECs responded to mitochondrial O2−−− generation induced by antimycin A (Fig. 5).

In general, our findings using fluorescence microscopy agree with the literature that mitochondrial utilization of excess NO involves ubiquinol oxidation that increases the O2−−− production rate, and formation of ONOO− that, via inhibition of the ETC at multiple sites, amplifies the O2−−− generation (11, 57, 58).

One novelty of the present study is the technique used to measure O2 consumption rates by the relatively low number of ECs exposed to flow in a perfusion chamber: EPR oximetry with LiPc as the probe, a technique capable of yielding high-resolution PO2 vs. time data, similar to the data normally

accurate for arterial blood Po2 of people who live at high altitude), whereas 5% O2 is mildly hypoxic for arterial ECs (72). Greater inhibition of respiration for ECs sheared under the relative hyperoxic 21% O2 is consistent with our hypothesis that higher Po2 may result in increased shear-induced NO production and O2−−− generation by the ETC leading to higher ONOO− formation in the mitochondria and enhanced inhibition of respiration. Even if shear-induced NO production remained the same at 21% vs. 5% O2 (according to Fig. 6A, it is increased at 21% O2), O2−−− formation by the ETC is known to increase linearly with Po2 (42, 74) and, since O2−−− does not diffuse across cell membranes, the fraction of shear-induced NO that forms ONOO− intramitochondrially would be expected to be higher at 21% vs. 5% O2.

ONOO− is known to suppress respiration to a greater degree than the suppression due to NO alone (79). In agreement with this, either the eNOS inhibitor l-NAME or the ONOO− scavenger UA, at concentrations known to, at least in part, prevent the shear (21% O2)-induced inhibition of complex I activity (30) [complex I is inactivated by ONOO− (12)], blocked the shear (21% O2)-induced inhibition of respiration (Fig. 3A). In an earlier study, shear stress (21% O2) was shown to increase nitrotyrosine staining, a “footprint” of ONOO− generation, in HUVEC mitochondria, and the signal was inhibited by preincubation and shear exposure in the presence of UA (30).

According to a report (69), UA does not scavenge ONOO− itself, as is mostly stated in the literature, but the nitrogen dioxide radical (NO2−), a product of the reaction between ONOO− and CO2. The finding that EC transfection with Ad.MnSOD, which expresses MnSOD in the mitochondria, before flow treatment blocked the shear-induced inhibition of respiration (Fig. 3B) provides evidence that the enhanced suppression of respiration for ECs sheared at 21% O2 is due to mitochondrial ONOO− formation. O2−−− is known to react with NO at a faster rate [k1 = 1.9 × 10^10 M−1 s−1 (38)] than with MnSOD [k2 = 2.3 × 10^7 M−1 s−1 (39)]. Although there is a 10-fold difference in the rate constants and NO levels may be in the low μM range inside the mitochondria, it is the local concentration of MnSOD that determines whether NO will outcompete MnSOD and react with O2−−− to generate ONOO− or MnSOD will outcompete NO and prevent ONOO− formation. MnSOD concentration in the mitochondria is ~10 μM (61), and EC infection with Ad.MnSOD (MOI of 100) was found to cause a fivefold increase in MnSOD levels/activity (23), thus making possible the prevention of shear-induced ONOO− formation in the mitochondria of ECs infected with Ad.MnSOD.

MitoSOX fluorescence (excitation at 405 nm) showed that the mitochondrial O2−−− levels are increased in parallel with the Po2 during shear exposure, and a comparison between the two excitation wavelengths showed that, even when the excitation is at 514 nm, most of the fluorescence is due to O2−−−−− specific oxidation products (Fig. 4). MitoSOX red fluorescence (excitation at 514 nm) suggested that the shear-induced NO is, at least in part, responsible for the generation of O2−−− by the ETC (Fig. 5). To accurately demonstrate mitochondrial O2−−− generation, the oxidation product OH-Etd− in mitochondrial cell fractions should be detected by HPLC, as described for whole EC lysates (83). However, MitoSOX fluorescence (excitation at 514 nm) from L-NAME-treated sheared ECs responded to mitochondrial O2−−− generation induced by antimycin A (Fig. 5).

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One novelty of the present study is the technique used to measure O2 consumption rates by the relatively low number of ECs exposed to flow in a perfusion chamber: EPR oximetry with LiPc as the probe, a technique capable of yielding high-resolution PO2 vs. time data, similar to the data normally obtained with respirometry (27), in small volumes of cell suspensions containing as few as 104 cells for certain cell types (36). EPR oximetry has been used to measure the Vpo2 of static ECs from mouse aorta (~4 nmol O2·min−1·10−6 cells) (53), but it has never been used to measure the Vpo2 of sheared ECs from any species or vascular bed (note that Vpo2 = Vpo2max at ≥ 3% O2; Fig. 2B). Using EPR oximetry, we found that static BAECs have a Vpo2 of ~10 nmol O2·min−1·10−6 cells (sheared at 21% O2) and BAECs have ~50% of that; Table 1), which is higher but in the same order of Vpo2 values measured for static HUVECs or porcine aortic ECs (2–3 nmol O2·min−1·10−6 cells) using standard respirometry with a Clark-type O2 electrode (10, 17). The estimated Pso for static BAECs (~5 mmHg) is in the same range of Pso values reported for HUVECs (60), and sheared at 21% O2 BAECs
have ~50% of that (Table 1). P50 is analogous to the apparent Michaelis-Menten constant K_m in purified enzymatic reactions. However, since the O_2-dependence of respiration is sensitive to each component of the ETC and not only to complex IV, the mitochondrial P50 (reciprocal value of O_2 affinity) is potentially a function of each element in the ETC, in contrast to the terminological implications of a K_m constant. The lower P50 value in the case of sheared at 21% O_2 ECs shows that there is a shift in O_2 binding (toward higher affinity) in the mitochondria of sheared ECs, possibly because of oxidative/nitrosative modifications of ETC proteins. In general, P50 increases with the rate of oxidative phosphorylation, which is mediated by the ETC (27). This agrees with our findings that P50 values were decreased when ECs were sheared at 21% compared with static conditions, since shear-induced ONOO^- formation in the mitochondria leads to inhibition of the ETC (30).

In agreement with a study by Moncada’s group (17), who showed that the VO_2 of cultured ECs is dependent on Po_2 only when the cells are activated to synthesize NO, no difference in VO_2 was found between static BAECs at either 5% or 21% O_2 (Figs. 1A and 2 and Table 1). In their study, EC treatment with the neuropeptide bradykinin, which activates eNOS, induced production of NO that interacted at the level of the ETC complex IV and inhibited respiration; inhibition was greater at lower PO_2 that resembled the physiological range in tissues (17). In the present study, endogenous NO production due to shear exposure inhibited respiration, but the effect was more pronounced at 21% O_2 compared with that at lower PO_2 levels, because of ONOO^- formation in the mitochondria of ECs sheared at 21% O_2. Although both studies examined the effect of endogenous NO on EC respiration at various PO_2 levels, the difference in their findings is probably due to the difference in the EC NO response elicited by diverse stimuli, a mechanical (shear stress) and a chemical one (bradykinin): EC exposure to steady laminar flow increases NO production in a biphasic manner with an initial rapid production that is Ca^2+/-calmodulin-dependent followed (after ~15 min; Fig. 6A) by a less rapid, sustained production that is independent of further changes in Ca^2+/-calmodulin (41). In contrast, bradykinin-mediated NO production is comparable to the initial shear stress-mediated, Ca^2+/-calmodulin-dependent NO production without the sustained component (41). Sustained shear-induced NO production involves phosphorylation of eNOS at several sites and its interaction with proteins, such as caveolin and heat shock protein 90 (HSP90) (6, 8), and may be the missing link for the accumulation of high NO levels in mitochondria and the resultant ONOO^- formation. The same extent of inhibition of respiration was present even after prolonged (16 h) shear exposure (Fig. 1B), which agrees with the decreased activities of ETC complexes measured in an earlier study (30).

Since the rate of formation of ONOO^- is first order in the concentrations of both NO and O_2, our findings of ONOO^- formation intramitochondrially (Fig. 3B and Ref. 30) suggest that, at the relative hyperoxic 21% O_2, either the NO concentration increases (primarily due to eNOS activation and possibly due to changes in mitochondrial ROS activity) or the mitochondrial O_2^- concentration increases, or both (the latter seems to be the case, on the basis of Figs. 4, 5, and 6A) (62). There is evidence in the literature that hyperoxia increases both the NO synthesis and the ROS generation from the ETC: Short-term BAEC incubation at PO_2 levels in the physiological range led to a decrease in the rate of thapsigargin-induced NO_3 production, compared with that at atmospheric PO_2, suggesting that eNOS activity declines at lower PO_2 (78). At prolonged times (24 h), changes in O_2 concentrations from 95% to 3% caused a progressive decrease in eNOS mRNA and protein levels, which correlated with changes in enzyme activity, in cultured BAECs, and bovine pulmonary artery ECs (44). Sheep pulmonary microvascular EC exposure to hyperoxia (100% O_2, 30 min) increased the cellular O_2^- production, as detected by EPR spectroscopy, and blocking the ETC with rotenone decreased the O_2^- signal (67). Detection of ROS in capillary ECs by in situ imaging of 2',7'-dichlorofluorescein fluorescence increases in perfused rat lungs showed that ROS formation increases with PO_2 (21-100% O_2, 90 min) and originates from mitochondria (9).

Our NO_3 measurements in media of BAECs sheared at different PO_2 levels (Fig. 6A) suggest that shear at 21% O_2 may increase eNOS activity to a greater extent than shear at lower PO_2 levels, leading to enhanced ONOO^- formation and suppression of respiration. Since NO_3 is the degradation product of ONOO^- and its nitrating intermediates (43) and ONOO^- formation is enhanced under shear at 21% O_2 (Fig. 3 and Ref. 30), ONOO^- may contribute to the elevated NO_3 production measurement. Last, the degree of eNOS phosphorylation at Ser1179 (equivalent to human Ser1177), a critical requirement for eNOS activation (50), was found to be the same among ECs sheared at either 5%, 10%, or 21% O_2 (Fig. 6B). Increased eNOS activity under shear at 21% O_2 compared with shear at lower PO_2 levels may be due to differences in posttranslational protein modifications [nitrosylation, acylation, or phosphorylation of other sites, such as phosphorylation at Ser635 (5, 8)] and/or alterations in interactions between eNOS and its regulatory proteins calmodulin, caveolin, HSP90, and platelet EC adhesion molecule-1 (3, 22, 25, 50).

In eNOS-transfected EC lines, the dynamic regulation of respiration by endogenous NO was shown to provide protection against H_2O_2-mediated injury and death (55), probably by maintaining mitochondrial membrane potential and preventing apoptosis (4). Although the functional significance of mitochondrial-derived ROS in ECs has received little attention, it is well documented that EC mitochondrial O_2^- production from ETC complexes I and III is responsible for the shear-induced dilation in human coronary arterioles (46, 82). It is also known that, during EC exposure to cyclic strain, mitochondrial ROS mediate the activation of nuclear factor-kB and upregulation of vascular cell adhesion molecule-1 (1). During EC exposure to flow, ONOO^- was found to mediate the activation of c-Jun NH_2-terminal kinase (JNK), a mitogen-activated protein kinase that determines cell survival in response to environmental stress (28). Formation of ONOO^- in the mitochondria of sheared ECs (Fig. 4 and Ref. 30) suggests that the intramitochondrial ONOO^- is probably responsible for activation of JNK, which is a signaling molecule in the pathway leading to growth arrest by laminar shear stress (45).

Our finding that flow at hyperoxic PO_2 causes nitrosative stress to the EC mitochondria and inhibition of respiration agrees with studies in reperfused isolated heart models, where lowering the PO_2 during early RP had a beneficial effect on mitochondrial bioenergetic parameters such as O_2 consumption, ETC complex activities, mitochondrial ROS production and lipid peroxidation, and improved cardiac performance (49,
I/R-induced EC injury is believed to be self-inflicted, resulting from a burst of endogenous ONOO− upon the onset of RP, and, due to decreased bioavailable NO, it predisposes ECs to increased neutrophil adhesion (24, 76). In agreement with that, bolus injections of ONOO− into isolated hearts were shown to acutely inhibit EC-dependent coronary vasodilation (75). Furthermore, in a rat model of splanchic artery occlusion/RP, use of the ONOO− decomposition catalyst 5,10,15,20-tetraakis(2,4,6-trimethyl-3,3-disulfonylphenyl)porphinato iron reduced P-selectin staining localized mainly in vascular ECs and improved bowel survival, suggesting that its beneficial effect may be due to inhibition of neutrophil-mediated damage in the later stages of RP (19). Although minimization of intracellular ONOO− was shown to limit the postischemic EC inflammatory response, the contribution of mitochondrial ONOO− in the EC dysfunction can only be derived from in vitro studies with H/R/RO-treated cells. These studies showed that the mitochondria are a source of EC ROS production following H or anoxia (1–2 h)/RO (1 h), the ROS release site is the ETC complex III, and the neutrophil-EC adhesion at prolonged RO (10 h) is mediated by the postanoxic EC mitochondrial ROS production (32, 33, 71). RO following H (4 h) induced loss of mitochondrial membrane potential and cytochrome c release, suggesting the mitochondria as the initiation site of EC apoptosis (20). However, H/R/RO studies do not include the flow component that is essential in the RP phase of I/RP, and, as a result, do not provide information on the shear-induced NO generation upon RP and the resultant mitochondrial ROS/RNS production.

In summary, our data collectively demonstrate that endogenous NO, via formation of ONOO− in the mitochondria, significantly reduces the rate of O2 consumption by cultured vascular ECs exposed to steady laminar flow at the atmospheric Po2. When flow experiments are performed at lower, more physiological Po2 levels, the endogenous NO production is modestly reduced and the mitochondrial O2− generation (in part, due to the decline in NO) is decreased, leading to less ONOO− formation intramitochondrially and, hence, insignificant suppression of respiration. The present study points to the importance of conducting in vitro flow studies at physiological O2 concentrations, since the dynamic regulation of respiration by endogenous NO, the NO synthesis, and the generation of mitochondrial ROS are all, at least in part, regulated by Po2.

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