Shear-induced reactive nitrogen species inhibit mitochondrial respiratory complex activities in cultured vascular endothelial cells

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Shear-induced reactive nitrogen species inhibit mitochondrial respiratory complex activities in cultured vascular endothelial cells. Am J Physiol Cell Physiol 292: C1103–C1112, 2007. First published October 4, 2006; doi:10.1152/ajpcell.00389.2006.—There is evidence that nitric oxide (NO), superoxide (O2•−), and their associated reactive nitrogen species (RNS) produced by vascular endothelial cells (ECs) in response to hemodynamic forces play a role in cell signaling. NO is known to impair mitochondrial respiration. We sought to determine whether exposure of human umbilical vein ECs (HUVECs) to steady laminar shear stress and the resultant NO production modulate electron transport chain (ETC) enzymatic activities. The activities of respiratory complexes I, II/III, and IV were dependent on the presence of serum and growth factor supplement in the medium. EC exposure to steady laminar shear stress (10 dyn/cm2) resulted in a gradual inhibition of each of the complexes starting as early as 5 min from the flow onset and lasting up to 16 h. Ramp flow resulted in inhibition of the complexes similar to that of step flow. When ECs were sheared in the presence of the NO synthase inhibitor Nω-nitro-L-arginine methyl ester (L-NAME; 100 μM), the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO; 100 μM), or the peroxynitrite (ONOO−) scavenger uric acid (UA; 50 μM), the flow-inhibitory effect on mitochondrial complexes was attenuated. In particular, L-NAME and UA abolished the flow effect on complex IV. Increased tyrosine nitration was observed in the mitochondria of sheared ECs, and UA blocked the shear-induced nitrotyrosine staining. In summary, shear stress induces mitochondrial RNS formation that inhibits the electron flux of the ETC at multiple sites. This may be a critical mechanism by which shear stress modulates EC signaling and function.

Oxidative stress; mitochondria; endothelium

Electron paramagnetic resonance spectroscopy, the most direct technique for detection of free radicals, has shown that increases in blood flow (and wall shear stress) trigger endothelium-derived free radical generation both in vivo and in isolated, perfused rabbit aortas, suggesting that this mechanism of free radical generation may contribute to vascular oxidative damage under pathological conditions (43). Similar to the in vivo and ex vivo observations, it has been shown that exposure of cultured vascular endothelial cells (ECs) to steady laminar shear stress with a perfusion system transiently increases intracellular superoxide (O2•−) levels, and the shear-induced free radicals (reactive oxygen species; ROS) act as second messengers in EC signaling, transcriptional activation, and de novo protein synthesis (17, 23, 34, 71, 72). The decrease in ROS over time is thought to result from the induction of a set of genes with antioxidant properties, and transcriptional profiling supports this hypothesis (14, 70).

ECs have the ability to generate O2•− through a variety of sources, such as the enzymes of the mitochondrial electron transport chain (ETC), xanthine oxidase, cytochrome P-450, cyclooxygenase, lipooxygenase, uncoupled endothelial nitric oxide synthase (eNOS), and plasma membrane-bound NADPH oxidase (46, 61). Steady laminar or pulsatile shear stress cannot sustain prolonged O2•− production, NADPH oxidase activity, and upregulation of expression of the different complex subunits, whereas oscillatory shear can, suggesting that flow-induced ROS production is achieved, at least in part, via activation of NADPH oxidase (23, 36, 37, 60). However, the mitochondria are recognized as the major cellular source of hydrogen peroxide (H2O2), which originates from O2− formed by the ETC (8). During state IV respiration inside the mitochondria, molecular oxygen (O2) is incompletely reduced, resulting in O2•− formation predominantly at ubiquinol-cytochrome c reductase (QCR; complex III) and secondarily at NADH-ubiquinone reductase (NQR; complex I) (9, 44, 65). O2•− is released into the mitochondrial matrix, where it undergoes dismutation to H2O2 by manganese superoxide dismutase (SOD). H2O2 that escapes matrix glutathione peroxidase (GPx) can be detoxified by the ETC (8). However, the oxygen consumption in the mitochondria has been observed to increase in the presence of free radicals (43). The inhibition of complex IV occurs through irreversible binding to the heme a3-Cu4 binuclear center and is competitive with O2, whereas the inhibition of complex III leads to the autooxidation of ubisemiquinone with the subsequent generation of O2•− and thus H2O2 (50). At higher concentrations (>1 μM), NO promotes ubiquinol autooxidation with the concomitant production of O2•−, which

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then reacts with NO, in a diffusion-limited reaction, to form the reactive nitrogen species (RNS) peroxynitrite (ONOO⁻) (51). ONOO⁻, as a strong oxidant, has the potential to cause persistent inhibition of complex I (due to nitration of the complex), followed by inhibition of succinate-ubiquinone reductase (SQR; complex II) (due to iron removal from iron-sulfur clusters), complex IV, the ATP synthase, MnSOD, and other proteins, and to promote permeability transition pore (PTP) opening, cytochrome c release, and apoptosis (11–13, 21, 56).

Since EC exposure to steady laminar shear stress produces NO by activating eNOS via several posttranslational mechanisms and at longer times via eNOS induction (35, 39, 42), and NO inhibits the activities of complexes IV and III (18, 50), we hypothesized that the endogenous shear-induced NO would differentially modulate the enzymatic activities of the ETC and either an eNOS inhibitor or a NO scavenger would reverse the inhibitory effect. The onset of flow has been shown to increase EC tyrosine nitration, a marker of RNS formation (32), suggesting that the transient shear-induced increase in O₂⁻ levels may lead to ONOO⁻ formation. However, the subcellular localization of nitrotyrosine staining has not been reported. We hypothesized that RNS/ONOO⁻ may be generated in mitochondria and, since ONOO⁻ inhibits complexes I, II, and IV (13, 21, 56), the effect of a ONOO⁻ scavenger on the activities of mitochondrial ETC complexes under shear was examined. It is worth noting that studies with cultured ECs, including the present study, are generally carried out at atmospheric O₂ concentrations, which give rise to an extracellular O₂ concentration of ~240 μM, close to arterial levels but well above concentrations to which the cells are exposed in tissue capillaries (tissue O₂ concentration is ~30 μM) (63). This relative “hypoxic state” probably raises the O₂ concentration in the mitochondria and may enhance the endogenous formation of mitochondrial O₂⁻, thereby increasing the cytotoxic potential of NO via the formation of ONOO⁻.

EXPERIMENTAL PROCEDURES

EC culture. Primary human umbilical vein ECs (HUVECs) were purchased from Vec Technologies (Rensselaer, NY) and cultured in complete growth medium MCDB 131 with 10% fetal bovine serum (FBS), phenol red, antibiotics, and 0.2 μg/ml growth factor supplement (GS) (Vec Technologies). ECs (passages 3–8) 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 chromium sulfate (Sigma, St. Louis, MO). Cell monolayers were used within 24 h on confluence. Confluent monolayers were incubated overnight with phenol red-free M199 supplemented with 2% FBS (Invitrogen, Carlsbad, CA), 1.4 mg/ml NaHCO₃ (Sigma), 0.1 mg/ml l-glutamine (Sigma), 100 U/ml penicillin (Invitrogen), 100 μg/ml streptomycin (Invitrogen), and 0.2 μg/ml GS (Vec Technologies).

EC exposure to shear stress. Three glass slides with confluent monolayers were assembled side by side in a parallel-plate flow chamber, and the chamber was connected at both ends to a reservoir forming a flow loop (29, 71, 72). ECs were exposed to a constant gravity-driven laminar shear stress of 10 dyn/cm² (low arterial range). Flow rate through the chamber was monitored by an ultrasonic flow sensor (Transonic Systems, Ithaca, NY). Recirculating medium was constantly exposed to a countercurrent flow of a sterile-filtered gas mixture (95% air-5% CO₂) that was warmed and humidified by bubbling through water; this permits the use of protein-poor medium (with ≤10% FBS) without foaming. The temperature of the entire system was kept at 37°C. Medium O₂ concentration and temperature were monitored in real time by inline optical O₂ and temperature sensors (World Precision Instruments, Sarasota, FL). ECs were exposed to one of two laminar flow profiles that were generated by using an inline proportional solenoid valve (Farmington Engineering, Madison, CT): 1) step flow (instantaneous wall shear stress increase from 0 to 10 dyn/cm² followed by steady shear for a sustained period) and 2) ramp flow (wall shear stress smoothly transited from 0 to 10 dyn/cm² over 5 min and then sustained for a desired period). Flow control programs were executed by LabVIEW (National Instruments; Austin, TX) on a PC. Some EC monolayers were preincubated with L-NNAME for 4 h, 1 mM N⁴-nitro-l-arginine methyl ester (L-NNAME; Sigma) for 4 h, 1 mM N⁴-amino-l-arginine (L-NAA; Alexis Biochemicals, San Diego, CA) for 1 h, or 1 mM N³-methyl-l-arginine (L-NMA; Sigma) for 1 h; 2) the NO scavenger 2-(4-carboxyphenoxy)-1,4,4,5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO, 100 μM; A.G. Scientific, San Diego, CA) for 30 min; or 3) the ONOO⁻ scavenger uric acid (UA, 50 μM; Sigma) for 30 min and then subjected to shear in medium containing the same concentration of the respective drug. Corresponding static controls were preincubated and maintained in the incubator for the same time periods and in the same medium as the perfusion medium. Concentrations of the eNOS inhibitors and NO scavenger were in the range of concentrations shown to inhibit the NO increase due to stimulation of cultured HUVECs by either chemical stimulation or flow exposure (4, 5, 27, 66). The UA concentration was chosen based on inhibition of ROS/RNS formation by chemically stimulated cultured bovine aortic ECs (75). Cell viability was determined by Trypan blue exclusion at the end of each treatment, and in each case it was ≥90%.

Measurement of NO production. Medium samples were drawn at different time points during flow exposure (with replacement of same volume of fresh medium in order to maintain circulating medium volume), and the accumulation of NO metabolites [nitrite (NO₃⁻) and nitrate (NO₂⁻); NO₃] was measured with a chemiluminescence analyzer (Sievers 270B; General Electric, Boulder, CO) (1). For each experiment, a standard curve was constructed with different concentrations of NaNO₃ for calculation of NOx content per sample. The background signal in perfusion medium was subtracted from each measurement, and values were also mathematically corrected for the dilution effect of medium replacement. Use of nitro compounds, such as L-NNAME, is problematic in the chemiluminescence analyzer (due to their degradation with the VCl/HCl used in the NO₃ reduction); hence, only the effects of L-NAA and L-NMA on NOx production were tested.

Assay of enzymatic activities of mitochondrial ETC. At the end of each treatment, ECs were harvested, resuspended in 3 mM HEPES buffer, pH 7.2, containing 0.25 M sucrose, 0.5 mM EGTA, and 2.5% protease inhibitor cocktail (Sigma), sonicated on ice, and centrifuged at 6,000 g for 20 min at 4°C. The supernatant was analyzed for mitochondrial complex activities on a UV/VIS spectrophotometer (model 2401; Shimadzu Scientific Instruments, Columbia, MD).

The electron transfer activity of complex I was determined in EC lysate by following the rotenone-sensitive oxidation of NADH initiated by ubiquinone-1 (Q₁) (54). Briefly, an appropriate amount of cell lysate was added to an assay mixture (0.5 ml) containing 20 mM potassium phosphate buffer, pH 8.0, 2 mM NaN₃, phospholipid (0.15 mg/ml), 0.1 mM Q₁, and 0.15 mM NADH. Complex I activity was determined by measuring the decrease in absorbance at 340 nm and confirmed by inhibition with rotenone (40 μM). Specific activity (nmol NADH oxidized·min⁻¹·mg protein⁻¹) was calculated with a molar extinction coefficient of 6.22 mM⁻¹·cm⁻¹. The electron transfer activity of succinate-cytochrome c reductase (SCR; complex II/III) in EC lysate was assayed by measuring ferricytochrome c reduction (54). Briefly, an appropriate amount of cell lysate was added to an assay mixture (0.5 ml) containing 50 mM phosphate buffer, pH 7.4, 0.3 mM EDTA, 50 μM KCN, 19.8 mM succinate, and 50 μM ferricytochrome c. Complex II/III activity was...
Culture medium composition regulates mitochondrial ETC complex activities. When just-confluent EC monolayers were switched from complete culture medium to variations of the perfusion medium, specifically M199 + 2% FBS, M199 + 2% FBS + GS, or M199 + 10% FBS + GS (in all media NaHCO₃, 1-glutamine, and penicillin-streptomycin were added), and incubated overnight, the specific activities of respiratory complexes I, II/III, and IV were found to be affected by the presence of FBS and GS in the media (Table 1).

Table 1. Levels of respiratory complex specific activities in HUVECs cultured in different media

<table>
<thead>
<tr>
<th>Complex</th>
<th>+2% FBS</th>
<th>+2% FBS + GS</th>
<th>+10% FBS + GS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complex I</td>
<td>15.30±0.11</td>
<td>21.97±2.33</td>
<td>33.50±0.91†</td>
</tr>
<tr>
<td>Complex II/III</td>
<td>0.31±0.08</td>
<td>0.43±0.06</td>
<td>0.74±0.18</td>
</tr>
<tr>
<td>Complex IV</td>
<td>1.40±0.13</td>
<td>1.51±0.20</td>
<td>2.92±0.01‡</td>
</tr>
</tbody>
</table>

Data (in nmol NADH oxidized·min⁻¹·mg protein⁻¹ for complex I, in nmol cytochrome c reduced·min⁻¹·mg protein⁻¹ for complex II/III, and in nmol cytochrome c oxidized·min⁻¹·mg protein⁻¹ for complex IV) are means ± SE specific activities of 3 independent experiments. Confluent monolayers were incubated overnight in each of the above media, and the specific activities of mitochondrial complexes were measured as described in EXPERIMENTAL PROCEDURES. FBS, fetal bovine serum; GS, growth factor supplement; HUVEC, human umbilical vein endothelial cell. *P < 0.05 vs. M199 + 2% FBS. †P < 0.05 vs. M199 + 2% FBS + GS.

Fig. 1. Effect of prolonged human umbilical vein endothelial cell (HUVEC) exposure to steady laminar shear stress (step flow) on the electron transfer activities of mitochondrial complexes. ECs were exposed to a wall shear stress of 10 dyn/cm² for 2, 6, or 16 h, and, at the end of each flow period, ECs were harvested and lysates were analyzed for activities of mitochondrial electron transport chain (ETC) complexes on a spectrophotometer, as described in EXPERIMENTAL PROCEDURES. The specific activities of complexes I, II/III, and IV (in nmol NADH oxidized·min⁻¹·mg protein⁻¹, nmol ferricytochrome c reduced·min⁻¹·mg protein⁻¹, and nmol ferrocytochrome c oxidized·min⁻¹·mg protein⁻¹, respectively) in sheared samples were normalized with respect to the specific activities in their corresponding static controls. Data are presented as means ± SE of 3 independent experiments. *P < 0.05 vs. static control.
Both FBS, in a dose-dependent manner, and GS tended to increase the specific activities, but only the specific activities of complexes I and IV in ECs cultured in M199 + 10% FBS + GS were significantly different from the activities of the respective complexes in ECs cultured in either M199 + 2% FBS or M199 + 2% FBS + GS. On the basis of these observations for all subsequent studies, the medium used for overnight incubation and perfusion was chosen to be M199 + 2% FBS + GS.

Long-term shear exposure (step flow) inhibits mitochondrial ETC complex activities. When EC monolayers were exposed to long-term step flow of either 2, 6, or 16 h, each complex specific activity was found to be inhibited by ~50% independently of the duration of shear exposure, the only exception being the complex I specific activity at 16 h, which was further decreased compared with the activity at 2 or 6 h (Fig. 1). All complex specific activities of ECs at 2, 6, or 16 h of shear exposure were significantly different from the specific activities of the respective complexes in static control ECs. Since NO and RNS are known to inhibit mitochondrial complex activities (11, 13), NOX production was measured at different times after the onset of flow. Under our experimental conditions, prolonged step flow elicited a transient burst in NOX production within the first hour, followed by a lower sustained release (Fig. 2A). The shear-induced increase in NOX production was completely blocked by preincubation and shearing in the presence of either of the eNOS inhibitors L-NAA and L-NMA at 1 mM.

Short-term exposure to either step or ramp flow inhibits mitochondrial ETC complex activities. Since most complex specific activities had reached a plateau by 2 h of shear, both NOX production and mitochondrial complex activities were examined at shorter times and under two different flow profiles, step versus ramp. When NOX production was measured at shorter times (between 5 and 60 min) under step flow, the initial burst occurred within the first 15 min from the onset of flow (Fig. 2B). Under ramp flow, an initial burst still occurred within the first 15 min but it was much smaller in magnitude,
so at the end of 60 min NO\textsubscript{x} production under ramp flow was less than half of the production under step flow (Fig. 2B). During the same time period, all mitochondrial complex activities gradually declined, but within 5 min they had already reached a significant difference compared with static controls (Fig. 3). No significant difference was found between the specific activities of each complex at any time point under step versus ramp flow (Fig. 3).

**NO is responsible for inhibitory effect of shear stress on mitochondrial ETC complex activities.** To determine the role of NO in the inhibition of enzymatic activities of mitochondrial ETC, ECs were preincubated and sheared (step flow) for 30 min in the presence of either the eNOS inhibitor L-NAME or the NO scavenger c-PTIO at 100 \(\mu\text{M}\). L-NAME partially blocked the inhibitory effect of shear on complexes I and II/III and completely abolished the shear effect on complex IV (Fig. 4). The specific activity of complex IV under shear + L-NAME was significantly different from the specific activity of complex IV under shear and not different from the specific activity of the same complex in static controls (Fig. 4). At the concentration tested, c-PTIO partially blocked the inhibitory effect of shear on each of the complexes (Fig. 5). Either L-NAME or c-PTIO tended to increase or maintain the complex specific activities of static control cells; c-PTIO caused a significant increase in the specific activity of complex IV in static control cells (Fig. 5).

**RNS are responsible for inhibitory effect of shear stress on mitochondrial ETC complex activities.** To determine the role of shear-induced RNS in the inhibition of mitochondrial complex activities, ECs were preincubated and sheared (step flow) for 30 min in the presence of the ONOO\textsuperscript{-} scavenger UA at 50 \(\mu\text{M}\). Shear + UA significantly increased the specific activities of complexes I, II/III, and IV compared with sheared samples, but only the specific activity of complex IV was restored to the same level as in the static control cells (Fig. 6). To localize the effects of ONOO\textsuperscript{-}-mediated protein nitration, nitrotyrosine was detected in HUVECs exposed to 1 h of step flow (shear stress of 10 dyn/cm\textsuperscript{2}) by immunocytochemistry and confocal fluorescence microscopy. An increase in nitrotyrosine staining was observed in sheared ECs compared with static controls, which to a major extent colocalized with the mitochondrial marker Mitotracker, and was abolished when ECs were preincubated and sheared in the presence of UA (Fig. 7). Exposing ECs to “ischemia” (static incubation under anoxia and glucose depletion for 2 h) followed by “reperfusion” (flow of perfusion medium under 21% O\textsubscript{2} for 1 h) resulted in a substantially more

![Fig. 3. Effect of short-term HUVEC exposure to either step flow (A) or ramp flow (B) on the electron transfer activities of mitochondrial complexes. ECs were exposed to either step or ramp flow (final wall shear stress of 10 dyn/cm\textsuperscript{2}) for 5, 15, or 60 min, and lysates were analyzed for specific activities of mitochondrial ETC complexes on a spectrophotometer, as described in EXPERIMENTAL PROCEDURES. Data were normalized with respect to the specific activities in corresponding static controls and are presented as means ± SE of 3 independent experiments. *\(P < 0.05\) vs. static control.](http://ajpcell.physiology.org/)
intense nitrotyrosine staining that also colocalized with Mito-tracker (Fig. 7).

DISCUSSION

The present study provides the first evidence that cultured human EC exposure to steady laminar shear stress results in inactivation of respiratory complexes I, II/III, and IV that is, at least in part, due to formation of RNS in the mitochondria. The shear-induced inhibitory effect on complexes I and II/III was decreased when ECs were sheared in the presence of either an eNOS inhibitor or a ONOO\(^{-}\)/H\(_2\)O\(_2\) scavenger, whereas, under the same conditions, the effect on complex IV was totally abolished. Shear-induced inhibition of complex IV is consistent with the fact that immediate and reversible inhibition of complex IV occurs in cells exposed to physiological levels of NO or in NO-producing cells (18, 20). Persistent inhibition of complexes I, II, and IV, due to \(S\)-nitrosylation of cysteine residues, is known to develop after several hours of cell exposure to pathophysiological levels of NO (\(\geq 1 \mu M\)) (19, 55, 73). However, under our experimental conditions, and although the reversibility of inhibition was not examined, inhibition of mitochondrial complexes occurred within 5 min from the flow onset, affected all complexes examined, was accompanied by nitrotyrosine staining, and was inhibited by UA, indicating that ONOO\(^{-}\) may play a role in the shear-induced effect on the ETC complexes. The ONOO\(^{-}\) action is thought to be irreversible, resulting from chemical reactions that involve oxidation/nitration of mitochondrial complexes I, II, and IV (11, 13, 21, 56).

Formation of RNS, most likely ONOO\(^{-}\), in cultured bovine aortic ECs shortly after the onset of steady laminar flow was reported by other investigators, but the localization of nitrotyrosine staining was not examined (32). Our finding that nitrotyrosine staining in sheared HUVECs colocalized with a mitochondrial marker agrees with the general belief that the mitochondrial matrix is the preferred suborganelle site for the formation of oxidizing NO species, due to \(O_2\) production by the ETC and the high matrix pH that favors the reaction

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**Fig. 4.** Effect of an endothelial nitric oxide (NO) synthase (eNOS) inhibitor on the inhibitory effect of shear stress on mitochondrial ETC complex activities. Some EC monolayers were preincubated with 100 \(\mu M\) \(N^\\text{G}-\text{nitro-L-arginine methyl ester (L-NAME)}\) and then either sheared (step flow) at 10 dyn/cm\(^2\) for 30 min in the presence of L-NAME or left in the incubator for 30 min in the presence of L-NAME. Mitochondrial complex specific activities were measured in cell lysates, and data were processed as before. Data are means \(\pm\) SE; \(n=3\). *\(P<0.05\) vs. static control; †\(P<0.05\) vs. sheared sample.

**Fig. 5.** Effect of a NO scavenger on the inhibitory effect of shear stress on mitochondrial ETC complex activities. Some EC monolayers were preincubated with 100 \(\mu M\) 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO) and then either sheared (step flow) at 10 dyn/cm\(^2\) for 30 min in the presence of c-PTIO or left in the incubator for 30 min in the presence of c-PTIO. Mitochondrial complex specific activities were measured in cell lysates, and data were processed as before. Data are means \(\pm\) SE; \(n=3\). *\(P<0.05\) vs. static control; †\(P<0.05\) vs. sheared sample.
between NO and O$_2^{-}$ to produce ONOO$^-$ (31, 67). The high probability of protein nitration in the matrix is reflected by the fact that a substantial number of mitochondrial proteins are nitrated in vivo (2). The physiological function of mitochondrial protein nitration is unknown, but it was found to be reversible under hypoxia, implying that it may serve signaling purposes (41). Furthermore, the subcellular localization of nitrated proteins under physiological conditions in rats was recently examined by high-resolution immunoelectron microscopy, and robust labeling was observed in EC mitochondria (33).

The increased mitochondrial formation of RNS due to shear exposure must be a consequence of increased production of NO and O$_2^{-}$, and H$_2$O$_2$. Under our experimental conditions, either step or ramp steady laminar flow increased NOx production from cultured HUVECs, but to a different extent, in agreement with earlier studies (30, 42), and eNOS-produced NO is expected to diffuse into the mitochondria. Although the actual NO concentration was not measured, an earlier study used a NO sensor and found that the peak NO concentration achieved in the perfusion chamber due to NO release by sheared bovine aortic ECs (arterial step flow) was $\approx 150$ nM, well below the pathophysiological range (40). Furthermore, a Ca$^{2+}$/calmodulin-dependent mitochondrial NOS has been reported, but questions remain regarding its precise molecular structure (10). Since shear elevates the cytosolic Ca$^{2+}$ concentration (40) leading to Ca$^{2+}$ accumulation in the mitochondria, this would also activate the mitochondrial NOS (24). Regarding mitochondrial O$_2^{-}$ production, there is no indication in the literature that shear increases mitochondrial ROS generation per se. Intracellular O$_2^{-}$/ROS production is known to increase within minutes from the onset of steady laminar shear stress (17, 23, 34, 71, 72), and this is likely due to the fact that an in vitro system is artificial (under normal circumstances in vivo, ECs do not go acutely from no flow to arterial levels of shear stress). However, the mitochondrial ETC is known to produce O$_2^{-}$, and our findings suggest that the shear-induced NO-mediated inhibition of the ETC may further enhance ROS production. At moderate NO levels, NO is known to increase O$_2^{-}$ and H$_2$O$_2$ production, whereas at high levels (\approx 1 \mu M), NO is thought to scavenge O$_2^{-}$ generating ONOO$^-$ (50, 51). Pathophysiological...
ical NO levels would require the activation of the inducible NOS, but this is not the case in ECs exposed to flow, especially since the shear-induced inhibition of ETC complexes and nitrotyrosine staining occur within a time period that would not allow for de novo protein synthesis. Since no hemoglobin is present to scavenge NO in vitro (3) and NO is more soluble in lipid bilayers, it is possible that, within cell membranes and specifically mitochondrial membranes, the shear-induced eNOS-produced NO reaches pathophysiological levels resulting in ONOO− formation. There is also the possibility that the relative “hyperoxic state” of in vitro flow studies compared with typical tissue levels (although similar to arterial levels) enhances the endogenous formation of mitochondrial O2−, favoring the formation of ONOO−. It has been demonstrated that marked hyperoxia (100% O2) stimulates mitochondrial O2− production in microvascular ECs (57).

There is one more possible scenario that may explain the nitrotyrosine staining and the NO/RNS-mediated inactivation of ETC complexes, as well as the protective effect of UA, without necessarily involving the formation of mitochondrial ONOO−; since both NO and O2 are more soluble in lipid bilayers than in aqueous solution and the reaction rate between NO and O2 is proportional to the square of the NO concentration and proportional to the O2 concentration, the oxidation of NO by O2 occurs more rapidly within cell membranes, including mitochondrial membranes, yielding the highly reactive nitrogen dioxide radical (NO2•) (28, 59). Dinitrogen trioxide (N2O3) is being formed in a diffusion-controlled reaction between NO and NO2− in aqueous solution. NO2− is known to oxidize L-tyrosine, generating phenoxyl radicals, which can react further with NO2• to form 3-nitrotyrosine (68). Hence, the increased nitrotyrosine staining under flow (increased NO concentration) and atmospheric O2 partial pressure (increased O2 concentration) may be due to the formation of the RNS NO2• resulting from the autooxidation of NO. According to a report, UA does not scavenge ONOO− itself, as is mostly stated in the literature, but the radicals NO2• and CO2•− that are formed from the reaction between ONOO− and CO2 (62). If UA scavenges primarily NO2−, that would explain the inhibitory effect of UA on NO2•-mediated nitrotyrosine staining. Furthermore, NO2• may oxidize or nitrate a variety of molecules, while N2O3 can nitrosate/nitrosylate amines or thiols (52, 53), possibly resulting in inactivation of respiratory complexes under flow.

Although both L-NAME and UA totally reversed the shear-induced effect on complex IV, UA was more effective in reversing the shear-induced effect on complex I compared with either L-NAME or c-PTIO. Since RNS are formed from NO, it would be expected that eNOS inhibitors and NO scavengers would be as effective as UA in blocking the shear-induced inhibition of mitochondrial complex activities. However, complex I is one of the two sites responsible for O2− generation in the ETC, and the H2O2 formed from the spontaneous dismutation of O2− was shown to partially inactivate complex I by damaging its iron-sulfur clusters (15). When NO is absent, because of either eNOS inhibition or NO scavenging, self-inactivation of complex I may occur by H2O2. In the presence of UA, NO reacts with O2•− to form ONOO−, which is scavenged by UA, and the self-inactivation of complex I does not occur, possibly explaining the almost complete reversal of the shear-induced response.

In any cell type exposed to NO, glycolysis is critical to cell survival because even moderate levels of NO invariably inhibit mitochondrial respiration and thus ATP production. ECs are able to maintain sufficient ATP levels via the glycolytic pathway because of NO- and shear-induced upregulation of glyceraldehyde-3-phosphate dehydrogenase (7, 25). However, at pathophysiological NO levels, NO-induced RNS can induce apoptosis via mitochondrial PTP opening, cytochrome c release, and caspase activation (11). Although our findings imply that steady laminar shear stress forms RNS in EC mitochondria (evidenced by rapid inhibition of ETC complexes I, II/III, and IV and mitochondrial nitrotyrosine staining), apoptosis is not expected to occur. Prolonged steady laminar shear stress is known to be an antiapoptotic stimulus in part due to induction of a set of genes with antioxidant properties (26, 70). It is possible that mitochondrial RNS formation is an upstream event in shear-induced signaling. In agreement with this, others have shown that shear-induced RNS mediate the activation of c-Jun NH2-terminal kinase (32). Furthermore, endogenously produced NO was shown to protect ECs against H2O2-induced death, and the protective effect of NO was lost in cells devoid of mitochondria, suggesting that NO may exert its cytoprotective effect against oxidative stress via regulation of mitochondrial respiration (48), possibly via maintenance of the mitochondrial membrane potential (6).

In summary, ECs exposed to steady laminar shear stress generate increased levels of NO leading to formation of RNS in the mitochondria and resultant inactivation of mitochondrial ETC complexes I, II/III, and IV. This shear-induced downregulation of mitochondrial electron transport could be a fundamental adaptive mechanism that regulates EC signaling, function, and survival.

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