Am J Physiol Cell Physiol 292: C1103–C1112, 2007.
First published October 4, 2006; doi:10.1152/ajpcell.00389.2006.

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

Zhaosheng Han,[1,2] Yeong-Renn Chen,[1] Charles I. Jones III,[1,2] Guruguhan Meenakshisundaram,[1,2] Jay L. Zweier,[1] and B. Rita Alevriadou[1,2]

[1]*Davis Heart and Lung Research Institute, Department of Internal Medicine, and*
[2]*Department of Biomedical Engineering, The Ohio State University, Columbus, Ohio*

Submitted 14 July 2006; accepted in final form 30 September 2006

Han Z, Chen Y-R, Jones CI III, Meenakshisundaram G, Zweier JL, Alevriadou BR. 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 ($O_2^{\bullet-}$), 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/cm^2) 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 N^G-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 ($O_2^{\bullet-}$) 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 $O_2^{\bullet-}$ through a variety of sources, such as the enzymes of the mitochondrial electron transport chain (ETC), xanthine oxidase, cytochrome *P*-450, cyclooxygenase, lipoxygenase, uncoupled endothelial nitric oxide synthase (eNOS), and plasma membrane-bound NADPH oxidase (46, 61). Steady laminar or pulsatile shear stress cannot sustain prolonged $O_2^{\bullet-}$ 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 (H_2O_2), which originates from $O_2^{\bullet-}$ formed by the ETC (8). During state IV respiration inside the mitochondria, molecular oxygen (O_2) is incompletely reduced, resulting in $O_2^{\bullet-}$ formation predominantly at ubiquinol-cytochrome *c* reductase (QCR; complex III) and secondarily at NADH-ubiquinone reductase (NQR; complex I) (9, 44, 65). $O_2^{\bullet-}$ is released into the mitochondrial matrix, where it undergoes dismutation to H_2O_2 by manganese superoxide dismutase (MnSOD). H_2O_2 that escapes matrix glutathione peroxidase activity freely diffuses into the cytosol, where it affects the steady-state concentration of cytosolic H_2O_2 and, hence, cell redox state, signal transduction, proliferation, and apoptosis (8, 12, 44). Hyperoxia and hypoxia-reoxygenation are conditions known to increase EC ROS generation through mechanisms dependent on the ETC (38, 49, 57, 64), but the effect of shear stress on mitochondrial ROS has not been examined.

Furthermore, in cells exposed to nitric oxide (NO) donors, NO-producing cells, isolated tissues, or whole animals, NO has been shown to modulate mitochondrial respiratory functions by eliciting changes in O_2 consumption and ROS production (20, 47, 58). At physiological concentrations (10 nM-1 μM), NO is known to rapidly and reversibly inhibit cytochrome-*c* oxidase (CcO; complex IV) and complex III without promoting cytochrome *c* release (18, 50). The inhibition of complex IV occurs through reversible binding to the heme a_3-Cu_B^+ binuclear center and is competitive with O_2, whereas the inhibition of complex III leads to the autooxidation of ubisemiquinone with the subsequent generation of $O_2^{\bullet-}$ and thus H_2O_2 (50). At higher concentrations (>1 μM), NO promotes ubiquinol autooxidation with the concomitant production of $O_2^{\bullet-}$, which

Address for reprint requests and other correspondence: B. R. Alevriadou, Ohio State Univ., 610 DHLRI, 473 West 12th Ave., Columbus, OH 43210 (e-mail: rita.alevriadou@osumc.edu).

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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 $O_2^{\bullet -}$ 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 O_2 concentrations, which give rise to an extracellular O_2 concentration of ~240 μM, close to arterial levels but well above concentrations to which the cells are exposed in tissue capillaries (tissue O_2 concentration is ~30 μM) (63). This relative "hyperoxic state" probably raises the O_2 concentration in the mitochondria and may enhance the endogenous formation of mitochondrial $O_2^{\bullet -}$, 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 $NaHCO_3$ (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/cm^2 (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% CO_2) that was warmed and humidified by bubbling through water; this permits the use of protein-rich medium (with ≤10% FBS) without foaming. The temperature of the entire system was kept at 37°C. Medium O_2 concentration and temperature were monitored in real time by inline optical O_2 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/cm^2 followed by steady shear for a sustained period) and *2*) ramp flow (wall shear stress smoothly transited from 0 to 10 dyn/cm^2 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 *1*) one of the following eNOS inhibitors: 100 μM N^G-nitro-L-arginine methyl ester (L-NAME; Sigma) for 4 h, 1 mM N^G-amino-L-arginine (L-NAA; Alexis Biochemicals, San Diego, CA) for 1 h, or 1 mM N^G-methyl-L-arginine (L-NMA; Sigma) for 1 h; *2*) the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-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 (NO_2^-) and nitrate (NO_3^-); NO_x] 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 $NaNO_3$ for calculation of NO_x content per sample. The background signal in perfusion medium was subtracted from each measured value, and values were also mathematically corrected for the dilution effect of medium replacement. Use of nitro compounds, such as L-NAME, is problematic in the chemiluminescence analyzer (due to their degradation with the VCl_3/HCl used in the NO_3^- reduction); hence, only the effects of L-NAA and L-NMA on NO_x 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 (Q_1) (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 NaN_3, phospholipid (0.15 mg/ml), 0.1 mM Q_1, 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$\cdot$min$^{-1}\cdot$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

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determined by measuring the increase in absorbance at 550 nm and confirmed by inhibition with antimycin A (30 μM). Specific activity (nmol ferricytochrome *c* reduced·min^{-1}·mg $protein^{-1}$) was calculated with a molar extinction coefficient of 21 $mM^{-1}cm^{-1}$. The electron transfer activity of complex IV was assayed by measuring ferrocytochrome *c* oxidation and was further confirmed by inhibition with KCN (16, 54). Briefly, an appropriate amount of cell lysate was added to an assay mixture (1 ml) containing 50 mM phosphate buffer, pH 7.4, and 60 μM ferrocytochrome *c*. Complex IV activity was determined by measuring the decrease in absorbance at 550 nm and confirmed by inhibition with KCN (100 μM). Specific activity (nmol ferrocytochrome *c* oxidized·min^{-1}·mg $protein^{-1}$) was calculated with a molar extinction coefficient of 21 $mM^{-1}cm^{-1}$. Specific activity of each complex in sheared samples was normalized to the specific activity of the same complex in the corresponding static control samples.

Immunocytochemistry for nitrotyrosine. At the end of the flow period, glass slides were removed from the chamber, and EC monolayers were incubated with the mitochondrion-specific probe Mitotracker Deep Red 633 (500 nM; Molecular Probes, Eugene, OR) in complete growth medium for 15 min, washed in phosphate-buffered saline (PBS), fixed in ice-cold methanol for 15 min, and incubated overnight with a blocking solution of 5% bovine serum albumin in PBS. Next, EC monolayers were washed with PBS, incubated with rabbit anti-nitrotyrosine primary antibody (1:50 in blocking solution for 1.5 h; Upstate, Lake Placid, NY) followed by an Alexa 488-conjugated goat anti-rabbit secondary antibody (1:50 in blocking solution for 1 h; Molecular Probes), washed, and dried. VectaShield (Vector Laboratories, Burlingame, CA) was applied to a central region on each slide before application of a coverslip and sealant. Fluorescence images for Mitotracker Deep Red 633 and Alexa 488 were obtained by confocal fluorescence microscopy (LSM 510; Zeiss, Thornwood, NY) with a ×40 objective and overlaid with LSM Image Browser software, generating a merged image for each costained specimen.

$ONOO^-$ formation and tyrosine nitration occur in postischemic tissues (22, 69, 74). Hence, as a positive control for nitrotyrosine staining, cultured ECs were exposed to "ischemia-reperfusion" as follows. During "ischemia," EC monolayers assembled in the flow chamber were incubated in modified Krebs-Henseleit buffer (mM: 117.3 NaCl, 4.7 KCl, 25 $NaHCO_3$, 1.3 $MgSO_4$, 1.2 KH_2PO_3, and 1.23 $CaCl_2$, supplemented with 10 sodium lactate, pH 7.4) and equilibrated with 95% N_2-5% CO_2 for 2 h. To achieve complete anoxia, 1% (vol/vol) EC-Oxyrase (Oxyrase, Mansfield, OH), an oxidase mixture that reduces O_2 to H_2O, was added in the medium (45). At the end of "ischemia," monolayers were subjected to "reperfusion" by application of shear stress (10 dyn/cm^2) with the perfusion medium equilibrated with a normoxic gas mixture (95% air-5% CO_2) for 1 h and processed for nitrotyrosine staining.

Statistical analysis. In the case of specific activities of respiratory complexes in ECs exposed to different treatments or the same treatment but for different time periods, results are expressed as means ± SE of three independent experiments. Significance was determined by using Minitab software (Minitab, State College, PA) to perform one-way analysis of variance followed by Tukey's tests for pairwise comparisons. *P* values <0.05 were considered significant.

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

Complex	Culture Medium (M199)		
	+2% FBS	+2% FBS + GS	+10% FBS + GS
Complex I	15.30±0.11	21.97±2.33	33.50±0.91*†
Complex II/III	0.31±0.08	0.43±0.06	0.74±0.18
Complex IV	1.40±0.13	1.51±0.20	2.92±0.01*†

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.

RESULTS

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 $NaHCO_3$, L-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).

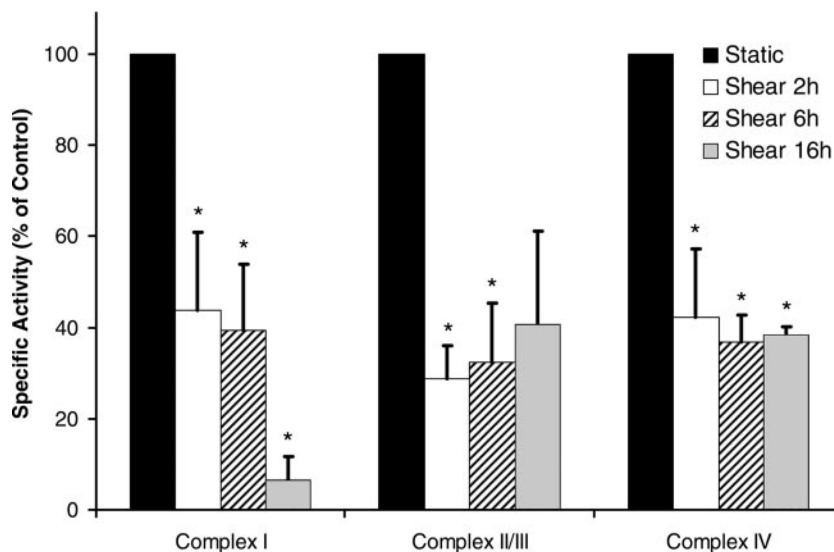

Fig. 1. Effect of prolonged human umbilical vein endothelial cell (HUVEC) exposure to steady laminar shear stress (step flow) on the electron transfer activities of mitochondrial complexes. ECs were exposed to a wall shear stress of 10 dyn/cm^2 for 2, 6, or 16 h, and, at the end of each flow period, 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.

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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), NO_x production was measured at different times after the onset of flow. Under our experimental conditions, prolonged step flow elicited a transient burst in NO_x production within the first hour, followed by a lower sustained release (Fig. 2*A*). The shear-induced increase in NO_x 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 NO_x production and mitochondrial complex activities were examined at shorter times and under two different flow profiles, step versus ramp. When NO_x 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. 2*B*). Under ramp flow, an initial burst still occurred within the first 15 min but it was much smaller in magnitude,

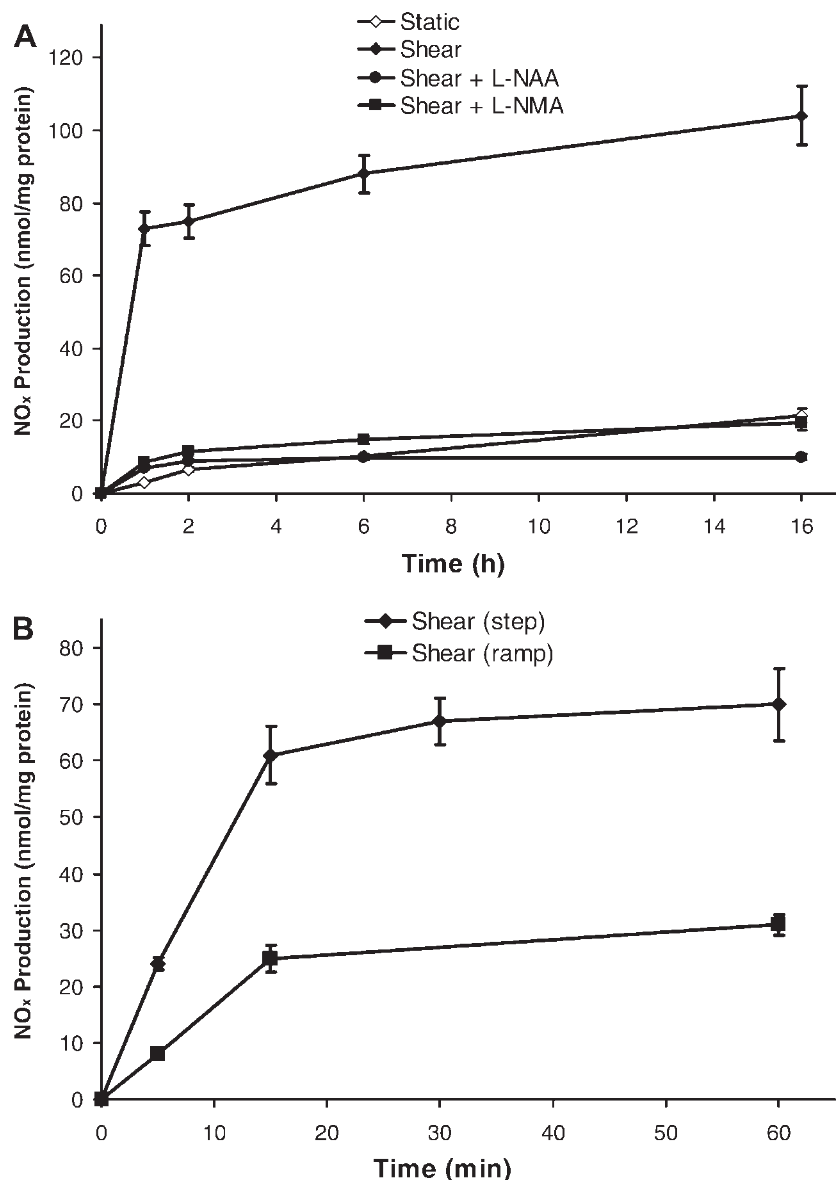

Fig. 2. NO_2^- + NO_3^- (NO_x) production during HUVEC long-term shear exposure (step flow; *A*) and short-term shear exposure (either step or ramp flow; *B*). Medium samples were drawn at different time points after the flow onset, and the accumulation of NO_2^- + NO_3^- was measured with a chemiluminescence analyzer. Some EC monolayers were preincubated and sheared in the presence of either N^G-amino-L-arginine (L-NAA) or N^G-methyl-L-arginine (L-NMA) at 1 mM. For step flow, the wall shear stress was instantaneously increased from 0 (static) to 10 dyn/cm^2, followed by steady shear for a total of 60 min or 16 h, whereas for ramp flow, the wall shear stress was linearly increased from 0 to 10 dyn/cm^2 during the first 5 min and then sustained for a total of 60 min. Data are means ± SE of 3 independent experiments.

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so at the end of 60 min NO_x production under ramp flow was less than half of the production under step flow (Fig. 2*B*). 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 μM. 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 $ONOO^-$ scavenger UA at 50 μM. 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 $ONOO^-$-mediated protein nitration, nitrotyrosine was detected in HUVECs exposed to 1 h of step flow (shear stress of 10 dyn/cm^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_2 for 1 h) resulted in a substantially more

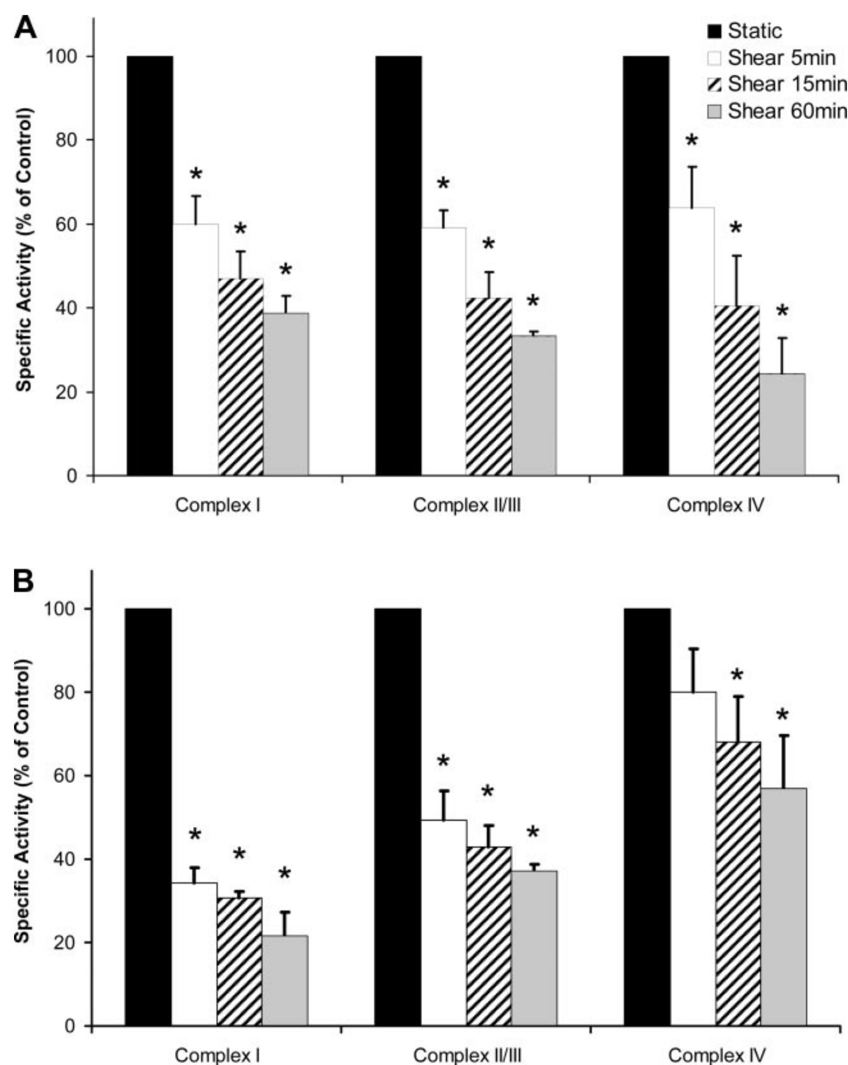

Fig. 3. Effect of short-term HUVEC exposure to either step flow (*A*) or ramp flow (*B*) on the electron transfer activities of mitochondrial complexes. ECs were exposed to either step or ramp flow (final wall shear stress of 10 dyn/cm^2) for 5, 15, or 60 min, and lysates were analyzed for specific activities 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.

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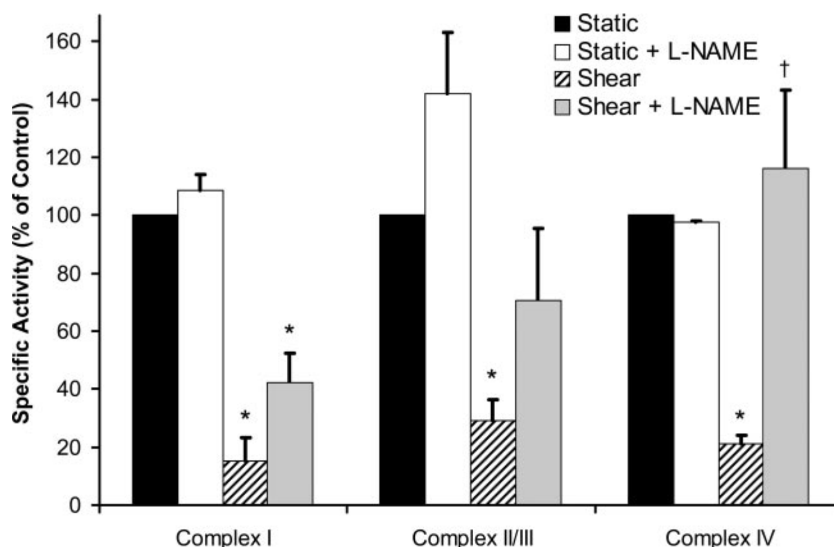

Fig. 4. Effect of an endothelial nitric oxide (NO) synthase (eNOS) inhibitor on the inhibitory effect of shear stress on mitochondrial ETC complex activities. Some EC monolayers were preincubated with 100 μM N^G-nitro-L-arginine methyl ester (L-NAME) and then either sheared (step flow) at 10 dyn/cm^2 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 ± SE; $n = 3$. $^*P < 0.05$ vs. static control; $^†P < 0.05$ vs. sheared sample.

intense nitrotyrosine staining that also colocalized with Mitotracker (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 $ONOO^-$ 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 (≥1 μM) (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 $O_2^{\bullet -}$ production by the ETC and the high matrix pH that favors the reaction

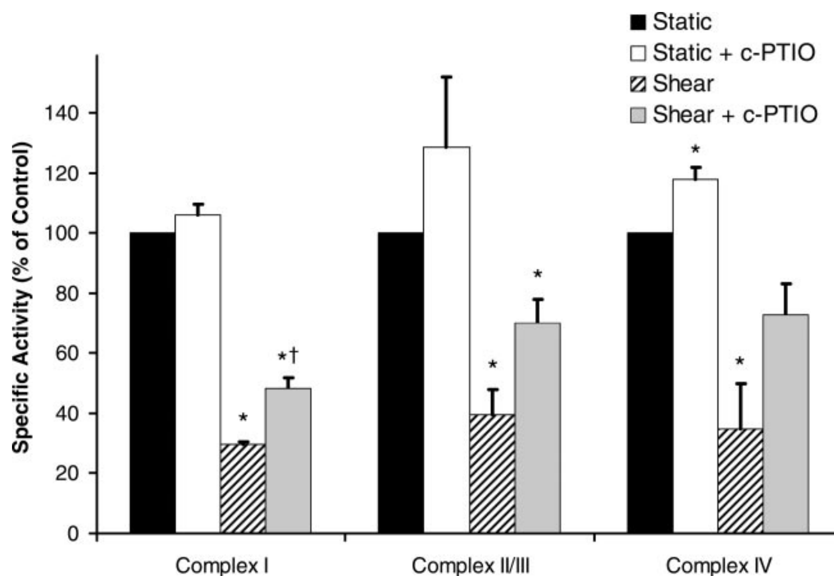

Fig. 5. Effect of a NO scavenger on the inhibitory effect of shear stress on mitochondrial ETC complex activities. Some EC monolayers were preincubated with 100 μM 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO) and then either sheared (step flow) at 10 dyn/cm^2 for 30 min 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 ± SE; $n = 3$. $^*P < 0.05$ vs. static control; $^†P < 0.05$ vs. sheared sample.

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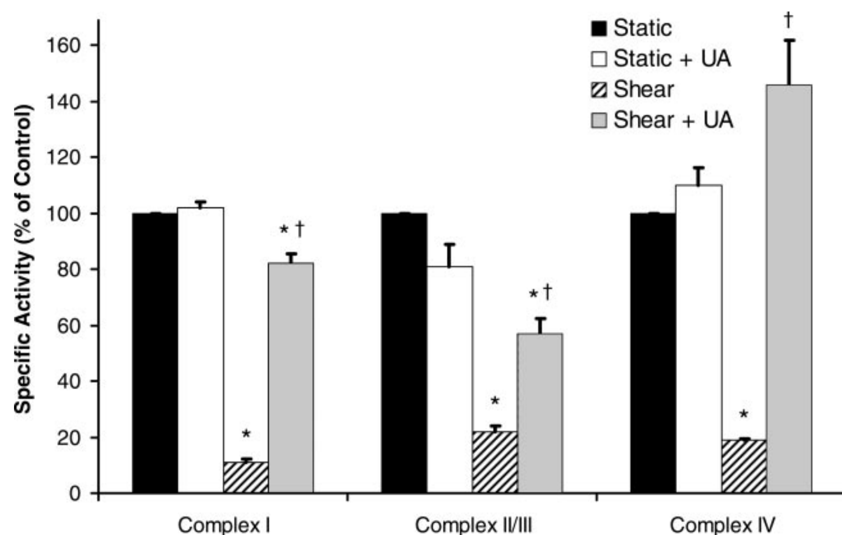

Fig. 6. Effect of a peroxynitrite ($ONOO^-$) scavenger on the inhibitory effect of shear stress on mitochondrial ETC complex activities. Some EC monolayers were preincubated with 50 μM uric acid (UA) and then either sheared (step flow) at 10 dyn/cm^2 for 30 min in the presence of UA or left in the incubator for 30 min in the presence of UA. Mitochondrial complex specific activities were measured in cell lysates, and data were processed as before. Data are means ± SE; $n = 3$. *$P < 0.05$ vs. static control; †$P < 0.05$ vs. sheared sample.

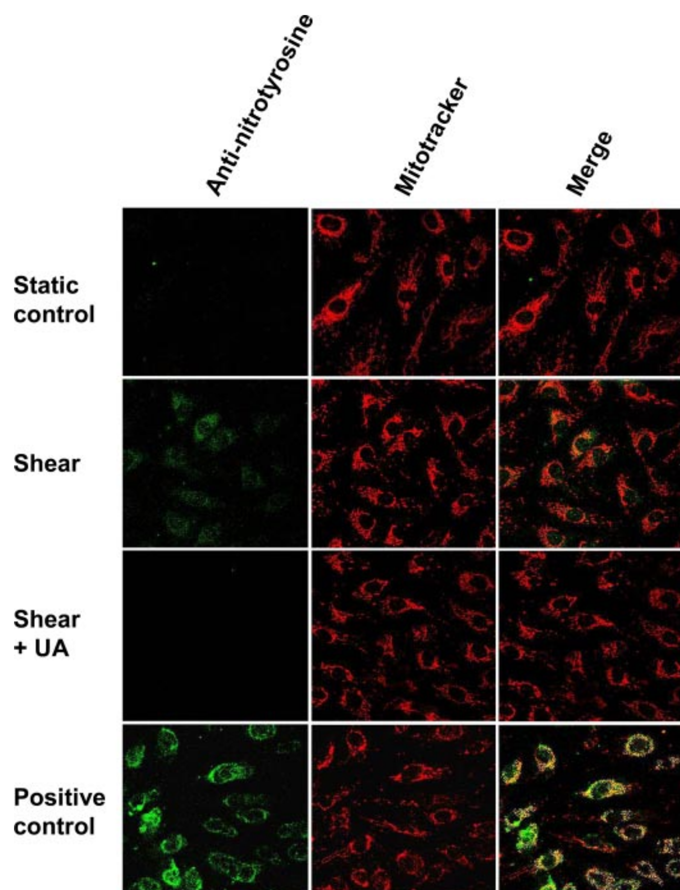

Fig. 7. Shear stress increases nitrotyrosine staining in EC mitochondria, and the signal is inhibited by UA. At the end of each treatment, static incubation in perfusion medium for 1 h, shear at 10 dyn/cm^2 for 1 h, preincubation and shear at 10 dyn/cm^2 for 1 h in the presence of 50 μM UA, or "ischemia" (static incubation under anoxia and glucose depletion for 2 h) followed by "reperfusion" (shear at 10 dyn/cm^2 for 1 h under 21% O_2), EC monolayers were loaded with Mitotracker Deep Red 633 (500 nM for 15 min), fixed, and stained for nitrotyrosine (secondary antibody was Alexa 488 conjugated). Fluorescence images were acquired with confocal microscopy (×40 magnification) and were merged in order to determine whether the increase in nitrotyrosine signal colocalized in mitochondria.

between NO and $O_2^{\bullet -}$ 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 $O_2^{\bullet -}$. Under our experimental conditions, either step or ramp steady laminar flow increased NO_x 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 ~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 $O_2^{\bullet -}$ production, there is no indication in the literature that shear increases mitochondrial ROS generation per se. Intracellular $O_2^{\bullet -}$/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 $O_2^{\bullet -}$, 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 $O_2^{\bullet -}$ and H_2O_2 production, whereas at high levels (≥1 μM), NO is thought to scavenge $O_2^{\bullet -}$ generating $ONOO^-$ (50, 51). Pathophysiolog-

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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 $O_2^{\bullet-}$ favoring the formation of $ONOO^-$. It has been demonstrated that marked hyperoxia (100% O_2) stimulates mitochondrial $O_2^{\bullet-}$ 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 O_2 are more soluble in lipid bilayers than in aqueous solution and the reaction rate between NO and O_2 is proportional to the square of the NO concentration and proportional to the O_2 concentration, the oxidation of NO by O_2 occurs more rapidly within cell membranes, including mitochondrial membranes, yielding the highly reactive nitrogen dioxide radical ($NO_2^\bullet$) (28, 59). Dinitrogen trioxide (N_2O_3) is being formed in a diffusion-controlled reaction between NO and $NO_2^\bullet$ in aqueous solution. $NO_2^\bullet$ is known to oxidize L-tyrosine, generating phenoxyl radicals, which can react further with $NO_2^\bullet$ to form 3-nitrotyrosine (68). Hence, the increased nitrotyrosine staining under flow (increased NO concentration) and atmospheric O_2 partial pressure (increased O_2 concentration) may be due to the formation of the RNS $NO_2^\bullet$ 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 $NO_2^\bullet$ and $CO_3^{\bullet-}$ that are formed from the reaction between $ONOO^-$ and CO_2 (62). If UA scavenges primarily $NO_2^\bullet$, that would explain the inhibitory effect of UA on $NO_2^\bullet$-mediated nitrotyrosine staining. Futhermore, $NO_2^\bullet$ may oxidize or nitrate a variety of molecules, while N_2O_3 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 $O_2^{\bullet-}$ generation in the ETC, and the H_2O_2 formed from the spontaneous dismutation of $O_2^{\bullet-}$ 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 H_2O_2. In the presence of UA, NO reacts with $O_2^{\bullet-}$ 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 NH_2-terminal kinase (32). Furthermore, endogenously produced NO was shown to protect ECs against H_2O_2-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.

ACKNOWLEDGMENTS

We thank Dr. A. Samouilov for technical advice and Alan Bakaletz, Manager of the Davis Heart and Lung Research Institute Microscopy Core Facility, for expert technical assistance.

GRANTS

This work was supported by National Heart, Lung, and Blood Institute Grants HL-67027 (to B. R. Alevriadou), HL-83237 (to Y.-R. Chen), HL-63744, HL-65608, and HL-38324 (to J. L. Zweier).

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