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Mitochondrial fission in endothelial cells following simulated ischemia/reperfusion: Role of nitric oxide and reactive oxygen species

Randy J. Giedt^{a,b}, Changjun Yang^b, Jay L. Zweier^b, Anastasios Matzavinos^c, and B. Rita Alevriadou^{a,b,*}

^aDepartment of Biomedical Engineering, The Ohio State University, Columbus, OH 43210, USA

^bDavis Heart and Lung Research Institute, Department of Internal Medicine, Cardiovascular Medicine Division, The Ohio State University, Columbus, OH 43210, USA

^cDepartment of Mathematics, Iowa State University, Ames, IA 50011, USA

Abstract

Ischemia (I)/reperfusion (RP)-induced endothelial cell (EC) injury is thought to occur due to mitochondrial reactive oxygen species (mtROS) production. MtROS have been implicated in mitochondrial fission. We determined whether cultured EC exposure to simulated I/RP causes morphological changes in the mitochondrial network, and the mechanisms behind those changes. Since shear stress results in nitric oxide (NO)-mediated endothelial mtROS generation, we simulated I/RP as hypoxia (H) followed by oxygenated flow over the ECs (shear stress of 10 dyne/cm²). By exposing ECs to shear stress, H, H/reoxygenation (RO) or simulated I/RP and employing mitotracker staining, the differential effects of changes in mechanical forces and/or O₂ levels on the mitochondrial network were assessed. Static or sheared ECs maintained their mitochondrial network. H- or H/RO-exposed ECs underwent changes, but mitochondrial fission was significantly less compared to that in ECs exposed to I/RP. I/RP-induced fission was partially inhibited by antioxidants, a NO synthase inhibitor or an inhibitor of the fission protein dynamin-related protein 1 (Drp1), and was accompanied by Drp1 oligomerization and phosphorylation (Ser616). Hence, shear-induced NO, ROS (including mtROS), and Drp1 activation are responsible for mitochondrial fission in I/RP-exposed ECs, and excessive fission may be an underlying cause of EC dysfunction in postischemic hearts.

Keywords

Endothelial cell; Mitochondrial fission; Hypoxia/reoxygenation; Ischemia/reperfusion; Shear stress; Mitochondrial superoxide; Nitric oxide; Dynamin-related protein 1

Introduction

Endothelial cell (EC) dysfunction occurs early after cardiac reperfusion (RP) [1–2]. It is preceded by endogenous generation of superoxide radicals (O₂^{•−}) and other reactive oxygen

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*Corresponding author. Fax: (614) 247 7799., alevriadou.1@osu.edu or rita.aleviadou@osumc.edu (B.R. Alevriadou).

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species (ROS), and, in arterioles, it is followed by a decrease in EC-dependent dilation [1,3]. The greatest source of nitric oxide (NO) in the heart is the endothelial nitric oxide synthase (eNOS), and a critical physiological stimulus that activates eNOS is the shear stress exerted by flowing blood on the ECs [4]. NO is known to inhibit respiration and promote $O_2^{\bullet-}$ generation by the mitochondrial electron transport chain (ETC) [5–6]. NO reacts with $O_2^{\bullet-}$ forming peroxynitrite ($ONOO^-$) that exacerbates $O_2^{\bullet-}$ production by the ETC [7–9], and the reduction in bioavailable NO impairs EC-dependent dilation [4,10–11]. Oxidative stress is thought to induce the mitochondrial permeability transition pore (mtPTP) opening resulting in decline of the mitochondrial inner membrane potential ($\Delta\Psi_m$) and cytochrome c release/apoptosis [12–14]. The mitochondrial pathway of apoptosis is activated in ECs of coronary vessels in I/RP-exposed isolated rat hearts [15].

Mitochondria are dynamic organelles that undergo cycles of fusion and fission [16–18]. An imbalance of these two processes alters mitochondrial morphology: Disruption of fusion or enhancement of fission causes the normal, tubular mitochondrial network to initially acquire a truncated and eventually a fragmented phenotype. Conversely, disruption of fission or enhancement of fusion generates elongated tubules. The GTPase dynamin-related protein 1 (Drp1) is essential for fission, whereas the mitofusins (Mfn1, Mfn2) and optic atrophy 1 (Opa1) regulate fusion [19–20]. Use of mitochondrial toxins demonstrated that low ATP (for example, due to low $\Delta\Psi_m$) is a necessary condition, and mitochondrial ROS (mtROS) are also important, for fission initiation [21–24]. After its trafficking to mitochondria and oligomerization at fission sites [25], Drp1 interacts with Bax/Bak on the mitochondrial outer membrane resulting in outer membrane permeabilization and cytochrome c release/apoptosis [24,26–28]. Drp1 is transcriptionally controlled by p53 [29], but its activity is also regulated by different posttranslational modifications including phosphorylation at Ser616, which is associated with increased Drp1 affinity for its receptor on the mitochondrial outer membrane, Fis1 [30–33].

Although it is accepted that I/RP causes EC mitochondrial oxidative stress and mitochondrial oxidative stress is thought to drive mitochondrial fission in different cell types (transformed cells and primary neurons), little is known regarding changes in mitochondrial morphology of ECs exposed to either hypoxia (H)/reoxygenation (RO) or simulated I/RP. Cultured EC exposure to steady laminar shear stress (low arterial level at 20% O_2) is known to reproduce certain features of the I/RP-induced EC dysfunction, namely the NO/ $ONOO^-$ -mediated inhibition of respiration and resultant mitochondrial $O_2^{\bullet-}$ production [34–35]. Hence, in this study, *in vitro* I/RP was simulated as follows: During the ischemic period (I), EC monolayers were exposed to very low flow of media with no serum and glucose at 1% O_2 over their surface. During the reperfusion period (RP), EC monolayers were exposed to high flow of oxygenated (20% O_2) media with serum and glucose over their surface (corresponding to a low arterial shear stress level). By exposing cultured ECs to either static incubation, shear stress, H (same conditions were used as during ischemia), H/RO or simulated I/RP, the differential effects of changes in mechanical forces (shear stress), changes in O_2 conditions (H/RO) and changes in both mechanical forces and O_2 conditions (I/RP) on the mitochondrial network were assessed and the molecular mechanisms involved in mitochondrial fission were elucidated.

We hypothesized that H/RO and simulated I/RP would have different effects on the EC mitochondrial network morphology, and the shear-induced NO during RP, via its effect on mitochondrial respiration/mtROS production, would activate Drp1 resulting in enhanced mitochondrial fission. Using mitotracker staining, fluorescence microscopy and image analysis, we discovered that static or sheared ECs maintain their mitochondrial network morphology. Both H- and H/RO-exposed ECs underwent changes in mitochondrial morphology, but the extent of fission was significantly less compared to that in ECs exposed

to I/RP. The massive fission observed in I/RP-exposed ECs was partially inhibited by either the antioxidants *N*-acetyl-L-cysteine (NAC) and oxaloacetate (OAA) or the NOS inhibitor *N*^G-nitro-L-arginine methyl ester (L-NAME) and was accompanied by Drp1 oligomerization and phosphorylation (of both monomer and oligomer at Ser616) suggesting that fission is mainly due to shear-induced NO, ROS (and, in particular, mtROS) generation and Drp1 activation upon RP. Although Drp1 oligomerization and phosphorylation are known to regulate mitochondrial morphology of transformed cells and primary neurons exposed to different stimuli [24,30,36–37], these processes have not been documented in ECs exposed to H/RO or simulated I/RP. By characterizing the EC mitochondrial morphology changes following H/RO or simulated I/RP and shedding light on the molecular regulatory pathways, our findings establish the presence of shear stress as a determining factor for EC mitochondrial dynamics and may lead to new therapeutic strategies for protection of the coronary endothelium against the I/RP-induced injury.

Experimental Procedures

HUVEC culture

Human umbilical vein ECs (HUVECs; Cambrex, East Rutherford, NJ) were grown in complete Endothelial Cell Growth Medium (Cell Applications, San Diego, CA) in a tissue culture incubator (20% O₂). ECs (passages 3–8) were seeded onto glass slides (75×38 mm; Thermo Fisher, Waltham, MA) that were sterilized, dried and coated with a 0.5% gelatin subbing solution (Sigma-Aldrich, St. Louis, MO). ECs were used within 24 h upon confluence. Prior to treatment, EC monolayers were preincubated for 2 h in medium 199 (M199) supplemented with 0.5% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen, Carlsbad, CA) to establish a baseline and limit responses due to high levels of serum and growth factors.

EC exposure to either static, shear, H (or I), H/RO or I/RP

EC monolayers were exposed to each of the following treatments:

- a. Static incubation, in M199 (of same composition as during the preincubation period) under normoxic conditions (in a tissue culture incubator; 20% O₂) for 1 h.
- b. Shear stress, where M199 (of same composition as during the preincubation period) was flowing over the ECs at a wall shear stress of 10 dyne/cm² (low arterial range) under normoxic conditions for 1 h. Specifically, glass slides with ECs were assembled into a parallel-plate flow chamber, and the chamber was connected at both ends to a reservoir forming a flow loop [34,38–39]. ECs were exposed to a constant gravity-driven laminar shear stress, and the flow rate through the chamber was monitored by an ultrasonic sensor (Transonic Systems, Ithaca, NY). M199 was exposed to a counter-current flow of a sterile-filtered normoxic gas mixture (95% air/5% CO₂) that was humidified. Temperature was kept at 37 °C. Medium O₂ concentration was monitored real-time by an inline optical sensor (World Precision Instruments, Sarasota, FL).
- c. H (same conditions were used for simulated ischemia, I), where M199 with no glucose or serum was flowing over the ECs at a wall shear stress of 0.5 dyne/cm² (very low flow; almost equivalent to static incubation) at 1% O₂ for 1 h. During this period, M199 was equilibrated with an anoxic gas mixture (95% N₂/5% CO₂). The partial pressure of O₂ in the medium is affected by the O₂ permeability of the flow setup, but can be controlled by the flow rate. Thus, the presence of a very low flow (corresponding to 0.5 dyne/cm² wall shear stress) during H allowed for maintaining the O₂ level in the chamber at 1%.

- d. H/RO, where H, as in (c), was followed by static incubation, as in (a).
- e. I/RP, where I, as in (c), was followed by shear exposure, as in (b).

Some monolayers were treated with antioxidants, a NOS inhibitor, a NO scavenger or a Drp1 inhibitor for 30 min during the preincubation period and then subjected to static, shear, H (or I), H/RO or I/RP in M199 containing the respective compound. Specifically, some ECs were treated with either (a) the nonspecific antioxidant and glutathione precursor NAC (5 mM), (b) OAA (100 μ M), a competitive inhibitor of succinate dehydrogenase (due to its structural similarity to succinate [40]) that blocks the NO-mediated ROS production by the ETC complex II (and in part by the ETC complex III) in sheared ECs and H/RO-exposed cardiomyoblasts [41], (c) the NOS inhibitor L-NAME (500 μ M), (d) the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO; 100 μ M) or (e) the mitochondrial division inhibitor (mdivi)-1 (20 or 50 μ M), a specific pharmacological inhibitor of fission that blocks Drp1 recruitment/oligomerization on the mitochondrial outer membrane and membrane permeabilization/cytochrome c release [28] (all chemicals from Sigma-Aldrich; c-PTIO from A.G. Scientific, San Diego, CA; mdivi-1 from Enzo Life Sciences, Farmingdale, NY). The L-NAME concentration is in the range of concentrations that block the shear-induced inhibition of EC mitochondrial respiration and mitochondrial $O_2^{\bullet-}$ production [34–35]. The c-PTIO concentration was also previously shown to block the shear-induced inhibition of EC mitochondrial respiration [34]. The mdivi-1 concentrations were chosen based on the fact that mdivi-1 at 50 μ M had a maximal effect in blocking mitochondrial fission of staurosporine-treated COS cells [28] and cultured cardiomyocytes exposed to simulated I/RP (and protected the latter from cell death), whereas mdivi-1 at 10 μ M was ineffective [42]. Cell viability was determined by trypan blue exclusion (and was 90% at the end of each experimental condition).

Fluorescence detection of $\Delta\Psi_m$ and mitochondrial $O_2^{\bullet-}$ levels

For $\Delta\Psi_m$ measurement at the end of treatment, EC monolayers were incubated with tetramethylrhodamine methyl ester (TMRM; Invitrogen) at 10 nM in phosphate-buffered saline (PBS) for 20 min [43]. Images were captured (514 nm excitation/585 nm emission) from 3 optical fields using a TE2000 microscope (Nikon, Melville, NY) equipped with a CCD camera. Following background subtraction, mean fluorescence/image was calculated using Elements software (Nikon) and averaged over 3 images. For a control on mitochondrial membrane depolarization, ECs were incubated with the ETC complex III inhibitor antimycin A (10 μ M for 30 min). For mitochondrial $O_2^{\bullet-}$ measurements, ECs were incubated with mitoSOX red (Invitrogen; 3 μ M in PBS for 15 min) [44] and mean fluorescence was calculated (514 nm excitation/585 nm emission), as before.

Detection and quantification of mitochondrial network morphology

At the end of treatment, EC monolayers were incubated with mitotracker green (Invitrogen; 0.1 μ M in PBS for 20 min; according to the manufacturer, it localizes in mitochondria regardless of $\Delta\Psi_m$). Fluorescence was detected (490 nm excitation/525 nm emission) at 60 $\times$, and images were analyzed using ImageJ [45–47]. Briefly, images were contrast-enhanced at 0.5% of saturation levels, treated with a smoothing linear filter and convoluted with a 5 $\times$ 5 Laplacian kernel with non-zero diagonal terms, which defines structures based on abrupt spatial changes in fluorescence. Following convolution, images were translated into the frequency domain by ImageJ's Fast Fourier Transform algorithm and a bandpass filter was applied (to filter out structures below the optical resolution limit of 0.2 μ m). After translation into the spatial domain, images were segmented by thresholding, binarized and skeletonized according to detected thresholds. The morphology of each mitochondrial object in a final image was described by its length, aspect ratio (major/minor axis of an ellipse

equivalent to the object) and form factor ($\text{perimeter}^2/4\pi \cdot \text{area}$), and scatter plots of aspect ratio vs. form factor were generated for each image [48–50].

Western blot analysis

At the end of treatment, ECs were scraped into ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.1% SDS, 0.25% sodium deoxycholate, 1 mM NaF, 1 mM Na_3VO_4 , 1 mM PMSF, 10 $\mu\text{g}/\text{ml}$ leupeptin/aprotinin). Lysates were sonicated, centrifuged at 12,000 rpm for 15 min at 4 °C and protein concentration was determined. Samples (15 μg protein) were subjected to both non-reducing (without β -mercaptoethanol in loading buffer; $-\beta$ -ME) and reducing (with β -mercaptoethanol in loading buffer; $+\beta$ -ME) SDS-PAGE on 4–20% Tris-Glycine gels (Invitrogen). Proteins were transferred onto nitrocellulose membranes and probed with primary antibodies against Drp1 (BD Transduction Laboratories, Lexington, KY), phospho Drp1 (Ser616) (p-Drp1; Cell Signaling, Danvers, MA) and β -actin (Sigma-Aldrich) followed by appropriate secondary antibodies conjugated to horseradish peroxidase. Immunoreactive bands were visualized using enhanced chemiluminescence (Pierce, Rockford, IL).

Statistical Analysis

Data from fluorescence measurements, classification of cell populations or densitometry were expressed as mean $\pm$ SE of $n = 3$ independent experiments. Differences among treatments were determined using one-way ANOVA followed by Bonferroni's test. Regarding the analysis of mitochondrial lengths, a mixed model treated each experiment within treatment as a random variable to take the correlation of mitochondrial lengths for each experiment into account. Hypothesis testing was conducted using linear contrasts of the parameter estimates, and multiple comparisons were adjusted for using Holm's method [51]. P values <0.05 were considered significant.

Results

$\Delta\Psi_m$ and mitochondrial $\text{O}_2^{\bullet-}$ levels of ECs exposed to either shear, H (or I), H/RO or I/RP

Since low $\Delta\Psi_m$ is a necessary condition and mtROS are important for fission initiation [21–22], we used the fluorescence indicators TMRM and mitoSOX red to quantify changes in $\Delta\Psi_m$ and mitochondrial $\text{O}_2^{\bullet-}$ levels, respectively, of ECs exposed to different treatments compared to static control. As expected, incubation with antimycin A resulted in significant mitochondrial depolarization of static ECs ($\sim 50\%$ decrease of TMRM fluorescence; Fig. 1). Exposure to shear stress (10 dyne/ cm^2 at 20% O_2 for 1 h) maintained the $\Delta\Psi_m$, whereas H (same as I; very low flow at 1% O_2 for 1 h), H/RO (RO is represented by static incubation at 20% O_2 for 1 h) or I/RP (RP is represented by flow corresponding to shear stress of 10 dyne/ cm^2 at 20% O_2 for 1 h) resulted in significant mitochondrial depolarization compared to static control (equivalent to that due to static incubation with antimycin A; Fig. 1) suggesting that H (or I), H/RO and I/RP, but not shear, fulfill the necessary condition for mitochondrial fission. Regarding mitochondrial $\text{O}_2^{\bullet-}$ levels, either shear, H/RO or I/RP significantly increased mitoSOX fluorescence compared to static control; the highest signal was due to EC exposure to I/RP (Fig. 2). Both the shear and I/RP signals were significantly inhibited by either NAC, OAA or L-NAME (present both at the preincubation period and during treatments), whereas the H/RO signal was significantly inhibited by only NAC or OAA (Fig. 2), suggesting that the shear-induced NO is, at least in part, responsible for the mitochondrial $\text{O}_2^{\bullet-}$ generation during the RP period of I/RP. C-PTIO was also found to significantly inhibit the shear- and I/RP-induced increases in mitoSOX fluorescence (fold increases over static control of 2.5 ± 0.2 and 2.8 ± 0.2 , respectively; not shown), thus, further verifying the role of diffusible NO in mitochondrial $\text{O}_2^{\bullet-}$ generation.

EC mitochondrial morphology changes and the role of Drp1 activation, ROS and NO

ECs were exposed to either shear, H (or I), H/RO or I/RP; the latter in the presence or absence of either mdivi-1, NAC, OAA, L-NAME or c-PTIO, and were categorized, by blinded visual inspection of mitotracker staining, as having either intact (normal, tubular), truncated or totally fragmented mitochondria (characteristic images representing each kind of mitochondrial network morphology are shown in Fig. 3A). When ECs with truncated or fragmented mitochondria were expressed as a percentage of the total cells counted (45–100 cells were counted per experiment and data were averaged over 4 independent experiments per treatment), it was found that H (or I) and H/RO, but not shear, significantly increased the percentage of cells with truncated or fragmented mitochondria compared to static, whereas I/RP mainly resulted in cells with fragmented mitochondria. The percentage of I/RP-exposed ECs with fragmented mitochondria was significantly higher than the percentage of ECs with fragmented mitochondria following either H (or I) or H/RO (Fig. 3B). Mdivi-1 significantly and dose-dependently blocked the I/RP-induced increase in the percentage of ECs with fragmented mitochondria (Fig. 3B). NAC, OAA, L-NAME and c-PTIO also significantly inhibited ECs from acquiring fragmented mitochondria following I/RP, with NAC being the most effective (Fig. 3B; it is noteworthy that each of the inhibitors prevented the mitochondrial network from reaching the fragmented phenotype, thus keeping a larger percentage of ECs at the truncated phenotype). These data collectively suggest that Drp1 activation, ROS, especially mitochondrial $O_2^{\bullet-}$, and NO are responsible for the I/RP effect on mitochondrial network morphology.

To further assess quantitatively the mitochondrial morphological changes, characteristic images of mitotracker-stained ECs following different treatments were analyzed and corresponding scatter plots of aspect ratio vs. form factor for each mitochondrial particle per image were constructed (Fig. 4A). Both aspect ratio and form factor have a minimal value of 1 when a particle is a perfect circle, and their values increase as the shape becomes elongated (form factor, in particular, is affected by both length and branching) [48–49]. Shear resulted in a scatter plot similar to static, whereas H/RO and especially I/RP produced scatter plots with lower (closer to 1) aspect ratio and form factor values suggesting that these treatments favor mitochondrial fission over fusion. Either mdivi-1, NAC, OAA, L-NAME or c-PTIO blocked the I/RP-induced decrease in aspect ratio and form factor values (only a characteristic image and corresponding scatter plot for mdivi-1 at 50 μ M is shown; Fig. 4A). In order to present morphological changes collectively over a number of independent experiments per treatment, the length (in μ m) of each mitochondrial object per image was determined, lengths were averaged over 4–6 images per experiment and over 4 independent experiments, and box plots showing the 90th/10th and 25th/75th percentiles and medians of mitochondrial length were plotted for each treatment (Fig. 4B). Either H (or I), H/RO or I/RP were found to significantly decrease the mean mitochondrial length compared to static (shear caused no significant change), but I/RP caused the most extensive mean mitochondrial length decrease out of all the conditions studied. Either mdivi-1 (in a dose-dependent manner), NAC, OAA, L-NAME or c-PTIO significantly increased the mean mitochondrial length compared to that of I/RP-exposed ECs, with NAC being the most effective (Fig. 4B) verifying that Drp1 activation, ROS (including mtROS) and NO tip the balance towards mitochondrial fission inside I/RP-exposed ECs.

EC Drp1 activation and the role of ROS and NO

Since either oligomerization or phosphorylation of Drp1 was shown to regulate mitochondrial morphology of transformed cells and primary neurons exposed to pro-oxidant stimuli [24,30,36–37], EC lysates were analyzed using both non-reducing (– β -ME; to visualize the oligomeric/tetrameric Drp1 of ~300 kDa, besides the monomer of ~84 kDa) and reducing (+ β -ME) SDS-PAGE followed by immunoblotting with antibodies against

Drp1 and p-Drp1 (Fig. 5A). Using reducing SDS-PAGE followed by immunoblotting with the anti-Drp1 antibody, Drp1 appeared only in its monomeric form, as expected, and total Drp1 levels did not change with any treatment (Fig. 5A). Using non-reducing gels followed by immunoblotting with the anti-Drp1 antibody, shear, H (or I), H/RO and I/RP were found to significantly increase Drp1 oligomer formation compared to static (Fig. 5B). Using either non-reducing or reducing gels followed by immunoblotting with the antibody against p-Drp1, each treatment tested was found to significantly enhance the phosphorylation of Drp1 monomer compared to static (Fig. 5C). In non-reducing gels followed by immunoblotting with the same antibody, shear, H/RO and I/RP also significantly increased the phosphorylation of Drp1 oligomer compared to static (Fig. 5C).

To assess the role of ROS and NO on the I/RP-induced Drp1 activation, ECs were preincubated and exposed to I/RP in the presence of either mdivi-1 (at the highest concentration tested), NAC or L-NAME, and lysates were analyzed as before (a characteristic non-reducing gel is shown; Fig. 6A). OAA and c-PTIO were omitted, since they were the least effective among the compounds tested in maintaining the mitochondrial morphology following I/RP (Figs. 3B and 4B). Either mdivi-1, NAC or L-NAME significantly inhibited the I/RP-induced increase in Drp1 oligomerization, as well as in monomer and oligomer phosphorylation, with NAC being the most effective (Fig. 6B, C) suggesting that cellular ROS and, to a lesser extent NO, are responsible for the I/RP-induced Drp1 activation. Neither mdivi-1, NAC or L-NAME had an effect on Drp1 oligomerization and phosphorylation in static ECs (not shown).

Discussion

Our study provides the first evidence that either H, H/RO or simulated I/RP exposure results in increased mitochondrial fission in cultured vascular ECs, but simulated I/RP (where RP is represented by high flow of oxygenated media over the ECs for 1 h) causes the most drastic mitochondrial morphological changes. The enhanced mitochondrial fission following I/RP was, at least in part, mediated by Drp1 activation, endogenous NO and ROS (including mtROS) generation, as it was inhibited by the specific pharmacological inhibitor of Drp1 mdivi-1, as well as the antioxidants NAC, OAA, the NO synthase inhibitor L-NAME and the NO scavenger c-PTIO.

Each of H, H/RO and I/RP caused significant, and of similar magnitude, mitochondrial membrane depolarization compared to static control, as evidenced by TMRM fluorescence, but I/RP resulted in the highest production of mitochondrial $O_2^{\bullet-}$ that was partially dependent on endogenous NO, as detected by mitoSOX red fluorescence. The role of shear-induced NO on mtROS during RP agrees with earlier studies of ours where cultured EC exposure to steady laminar flow (low arterial level corresponding to a wall shear stress of 10 dyne/cm²) was shown to inhibit ETC complex activities, suppress O_2 consumption and increase mitochondrial $O_2^{\bullet-}$ generation, and those flow effects on EC mitochondria were, at least in part, NO/ONOO⁻-dependent [34–35]. Other groups have shown that NO generated by ECs can inhibit their own mitochondria [5], and NO from the endothelium can inhibit respiration in the myocardium [52–53]. Although EC exposure to shear stress resulted in the second highest increase in mitochondrial $O_2^{\bullet-}$ generation among all conditions tested (second to I/RP), it did not cause mitochondrial fission. This agrees with the literature where $\Delta\Psi_m$ reduction or, more correctly, ATP deprivation is essential for initiation of mitochondrial fission, and mtROS fine-tune the response under critical conditions [21–22]. Although we did not measure ATP concentrations, it is known that HUVEC exposure to H (2 h at 1.5% O_2 in culture media) results in a significant (~25%) decrease in intracellular ATP levels, and ATP is maintained mainly from glycolysis [54]. Based on that, we would expect that, in our study, EC exposure to H (1 h at 1% O_2 in media with no serum or

glucose) would inhibit ATP synthesis, thus creating favorable conditions for mitochondrial fission initiation. In contrast, shear stress in the normal range is known to maintain or even increase the ATP and $\Delta\Psi_m$ levels compared to static control [55–56]; in this study, shear (10 dyne/cm² for 1 h) maintained $\Delta\Psi_m$. Regarding the role of shear-induced endothelial mtROS, they appear to initiate signaling pathways leading to activation of transcription factors and gene expression (some of which encode for antioxidant enzymes [57–58]) and are responsible for the vasodilatory response to shear stress in patients with coronary artery disease (recently reviewed in [59]).

Morphometric analysis of the mitochondrial network was performed by blinded visual inspection of images from mitotracker-stained ECs and by mathematical/computational analysis, as previously done in studies of neurons, fibroblasts and transformed cells [48–50,60]. Either mdm1-1 or the antioxidants NAC and OAA inhibited the I/RP-induced mitochondrial fission, in agreement with studies of different cell types exposed to pro-oxidant stimuli, where fission was prevented by expression of either a dominant-negative Drp1 or the fusion protein Mfn2 [49–50] and by treatment with either general or mitochondria-targeted antioxidants [23–24]. NAC was more effective than OAA (it inhibits mtROS from ETC complex II and partially from ETC complex III [41]) in inhibiting fission. Hence, mtROS are important in mediating fission, but ROS from other sources, such as the plasma membrane-bound NAD(P)H oxidase, xanthine oxidase or uncoupled eNOS [61], via activation of the mitochondrial remodeling proteins, may also play a role in I/RP-induced fission. Use of mitochondria-targeted antioxidants and specific ROS source inhibitors could help clarify the relative contribution of each ROS source in mitochondrial fission. Regarding eNOS, if it becomes uncoupled (due to tetrahydrobiopterin oxidation) in ECs exposed to simulated I/RP, O₂^{•−} could be formed from the oxygenase site of eNOS and L-NAME would block the eNOS-derived O₂^{•−}, as well as NO, formation [62–63]. Our findings with c-PTIO verified that diffusible NO is, at least in part, responsible for the I/RP-induced mitochondrial O₂^{•−} generation and mitochondrial fission. Future experiments could use NG-monomethyl-L-arginine or asymmetric dimethylarginine, which block NO but not the O₂^{•−} generation from eNOS [64], to help clarify the role of eNOS itself as a potential source of O₂^{•−} in simulated I/RP-exposed ECs.

Drp1 is distributed mainly unassembled in the cell cytosol, but, in response to increases in cytosolic Ca²⁺ or apoptosis initiation, it undergoes self-assembly, and the recruitment of Drp1 oligomers into ring-like structures around mitochondria drives membrane constriction and fission [25,65]. Drp1 fission capacity is posttranslationally regulated by sumoylation, ubiquitination, and phosphorylation of serine residues within the GTPase effector domain [30–33]. Drp1 phosphorylation at Ser616 by cyclin-dependent kinase 1/cyclin B (Cdk1/cyclin B) results in increased Drp1 fission activity [30]. Conversely, Drp1 phosphorylation at Ser637 by protein kinase A causes Drp1 retention in the cytosol and decreased fission, whereas Drp1 dephosphorylation of Ser637 by the phosphatase calcineurin causes Drp1 translocation to the mitochondria and increased fission [33,66]. We assessed Drp1 oligomerization and phosphorylation at Ser616, as two indices of its activation. Phosphorylation at Ser616, in particular, was chosen because Bossy et al. [37] recently implicated the phosphorylation of Drp1 at Ser616 as the mechanism for mitochondrial fission induced by NO in human embryonic kidney 293 cells. Each experimental condition tested, shear, H, H/RO or I/RP, was shown to significantly increase Drp1 oligomerization and phosphorylation at Ser616 of both the Drp1 monomer and oligomer (with the exception of H in regards to phosphorylation of the Drp1 oligomer). Currently, the relative importance of Drp1 monomer vs. oligomer phosphorylation is unknown. It is also not clear if the monomers are phosphorylated and then oligomerization occurs or the oligomers get phosphorylated only after they are formed by unphosphorylated monomers. Last, studies overexpressing either phosphomimetic or non-phosphorylatable mutants are needed in order

to conclude on the involvement of the Ser616 phosphorylation site in Drp1-mediated fission inside I/RP-exposed ECs.

The I/RP-induced increases in Drp1 oligomerization and phosphorylation were inhibited by either NAC or L-NAME suggesting that oxidative/nitrosative stress drives Drp1 activation. Both the Drp1 oligomerization and phosphorylation changed in parallel to the mitochondrial $O_2^{\bullet-}$ levels (compare Figs. 2 and 5). However, Drp1 monomers are thought to be mainly in the cell cytosol and simulated I/RP may cause ROS generation by other cytosolic sources besides the mitochondrial ETC [61], implying that different ROS sources may be responsible for Drp1 monomer phosphorylation at Ser616. The fact that shear caused Drp1 activation, as evidenced by significant increases in Drp1 oligomerization and phosphorylation, without appreciable fission suggests that there are additional molecular mechanisms that regulate fission independently of Drp1 and/or that oligomerization and phosphorylation at Ser616 are not the best indicators of Drp1 activation for this particular cell type and stimulus tested. Indeed, increased fission may be the result of impaired fusion. Dissipation of $\Delta\Psi_m$ has been associated to proteolytic degradation of long Opa1 isoforms to short ones, which cannot support fusion [67–68]. Hence, maintenance of the long Opa1 isoforms in sheared ECs would support the lack of net mitochondrial morphological changes compared to static control. Furthermore, synergistic effects by phosphorylation at Ser616 and Ser637 are possible [69] suggesting that the increased fission due to phosphorylation at Ser616 may be counteracted by phosphorylation at Ser637. Last, NO may have a direct effect on fission via Drp1 S-nitrosylation at Cys644 and increase of its GTPase activity [70], although the role of S-nitrosylation on Drp1 activity was recently questioned [37].

Excessive mitochondrial fission appears to be a requisite step in intrinsic apoptosis and Drp1 function is required for apoptotic mitochondrial fission, as either expression of a dominant-negative mutant (Drp1K38A) or downregulation of Drp1 by RNAi was shown to delay mitochondrial fragmentation, cytochrome c release, caspase activation and cell death [26–28]. Understanding the role of Drp1 and the other mitochondrial-shaping proteins in mitochondrial/cell dysfunction during H/RO or *in vivo* I/RP is not straightforward, because their expression levels, posttranslational modifications and subcellular localization may all influence their activity [71]. However, Ong et al. [42] showed that inhibiting mitochondrial fission with mdivi-1 can protect the heart from I/RP-induced injury by preventing the mtPTP opening. Elongated mitochondria have enhanced mitochondrial respiration capacity and hyperpolarized $\Delta\Psi_m$ [17,72] and may be better equipped to withstand the metabolic stresses associated with I/RP. Ong et al. [42] clarified the role of mitochondrial morphological changes in cardiac cells following I/RP, but did not examine the endothelial mitochondria. eNOS-derived NO, via ONOO⁻ formation, is responsible for the inhibition of mitochondrial ETC function in postischemic cardiac tissue [53], but the role of endogenous NO in the endothelial dysfunction is not known. Using I/RP-exposed isolated hearts or *in vivo* hearts, it is difficult to assess that because: (a) Different NOS isoforms from various cell types are involved in determining the I/RP outcome, and eNOS gene knockouts in mice are counteracted by compensatory changes in other NOS isoforms [73]. (b) ROS originate from various intracellular sources and different cell types in tissue, so it is hard to isolate the effect of endothelial ROS on EC function [11,74]. (c) Postischemic EC mitochondria have been overlooked because isolation protocols yield primarily mitochondria from cardiomyocytes.

In summary, using a simulated I/RP protocol in this study, we demonstrated that oxidative/nitrosative stress results in enhanced Drp1-mediated mitochondrial fission in postischemic ECs. I/RP-induced mitochondrial fission may be an underlying mechanism for the impairment of EC mitochondrial function. However, it remains unclear, and requires conclusive experimental support to determine, whether the NO-induced mitochondrial

fragmentation upon RP causes EC death via activation of the mitochondrial pathway of apoptosis or it is a stress response aimed to eliminate damaged mitochondria via autophagy and prolong EC survival.

Highlights

- We studied mitochondrial morphology in endothelial cells exposed to simulated I/RP.
- Reperfusion was simulated as flow of oxygenated media (shear stress) over the cells.
- Mitochondria underwent extensive fission following endothelial exposure to I/RP.
- Mitochondrial fission was dependent on NO, ROS (including mtROS) and Drp1.
- We conclude that mechanical forces can play a role on mitochondrial dynamics.

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List of Abbreviations

I/RP	ischemia/reperfusion
EC	endothelial cell
mtROS	mitochondrial reactive oxygen species
NO	nitric oxide
H/RO	hypoxia/reoxygenation
Drp1	dynammin-related protein 1
O₂^{•-}	superoxide
eNOS	endothelial nitric oxide synthase
ETC	electron transport chain
ONOO⁻	peroxynitrite
mtPTP	mitochondrial permeability transition pore
$\Delta\Psi_m$	mitochondrial inner membrane potential
Mfn1, Mfn2	mitofusins 1 and 2
Opa1	optic atrophy 1
HUVECs	human umbilical vein ECs
M199	medium 199
FBS	fetal bovine serum

NAC	<i>N</i> -acetyl-L-cysteine
OAA	oxaloacetate
L-NAME	<i>N</i> ^G -nitro-L-arginine methyl ester
c-PTIO	2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide mdivi-1, mitochondrial division inhibitor-1
TMRM	tetramethylrhodamine methyl ester
PBS	phosphate-buffered saline
β-ME	β-mercaptoethanol
Cdk1	cyclin-dependent kinase 1

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Fig. 1.

Normalized TMRM fluorescence following either static EC incubation, in the presence or absence of antimycin A, or EC exposure to shear, H or I, H/RO or I/RP (each condition is defined under Experimental Procedures). Data are presented as mean $\pm$ SE of n=5 independent experiments. * P <0.05 relative to static control. † P <0.05 relative to I/RP.

Fig. 2. Normalized mitoSOX fluorescence following EC exposure to either static, shear, H or I, H/RO or I/RP in the presence or absence of either NAC, OAA or L-NAME. Data are presented as mean $\pm$ SE of n=5 independent experiments. * P <0.05 relative to static control. † P <0.05 relative to I/RP. ‡ P <0.05 relative to corresponding control.

Fig. 3.

Percentage of ECs with altered mitochondrial morphology due to different treatments. (A) Characteristic mitotracker images of ECs with intact, truncated or fragmented mitochondria (90 $\times$ magnification; scale bar is 5 μ m). Selected regions were 2-fold magnified and shown in the corresponding inserts. (B) ECs were exposed to either static, shear, H or I, H/RO or I/RP; the latter in the presence or absence of either mdivi-1 (20 or 50 μ M), NAC, OAA, L-NAME or c-PTIO. ECs having primarily truncated or fragmented mitochondria were expressed as a percentage of total cells counted (45–100 were counted per experiment). Data are presented as mean $\pm$ SE of n=4 independent experiments. * P <0.05 relative to static control. † P <0.05 relative to I/RP.

Fig. 4. Mitochondrial morphological parameters of ECs exposed to different treatments. (A) Characteristic mitotracker images of ECs exposed to static, shear, H/RO and I/RP in the presence or absence of mdivi-1 (50 μM) were processed, as described under Experimental Procedures, and corresponding scatter plots of aspect ratio vs. form factor for each mitochondrial particle per image were constructed. (B) ECs were exposed to either static, shear, H or I, H/RO or I/RP; the latter in the presence or absence of either mdivi-1 (20 or 50 μM), NAC, OAA, L-NAME or c-PTIO. Mitochondrial lengths were determined and averaged over 4–6 images per experiment and n=4 independent experiments. Box plot is showing the 90th/10th (lines) and 25/75th (filled bars) percentiles and medians (center lines) of mitochondrial length for each treatment. **P*<0.05 relative to static control. †*P*<0.05 relative to I/RP.

Fig. 5.

Drp1 activation following EC exposure to either shear, H or I, H/RO or I/RP. (A) Lysates of ECs at the end of treatment were analyzed by non-reducing ($-\beta$ -ME) and reducing ($+\beta$ -ME) SDS-PAGE followed by immunoblotting with antibodies against Drp1, p-Drp1 or β -actin. Representative blots from 3 independent experiments with similar results are shown. (B) Ratios of Drp1 oligomer/ β -actin and (C) p-Drp1/ β -actin for both monomer and oligomer are presented as mean $\pm$ SE of $n=3$ independent experiments. * $P<0.05$ relative to static control. † $P<0.05$ relative to I/RP.

Fig. 6.

Drp1 activation following EC exposure to I/RP in the presence or absence of mdivi-1, NAC or L-NAME. (A) Lysates of ECs at the end of treatment were analyzed as described in Fig. 5. Representative blots from 3 independent experiments with similar results are shown. (B) Ratios of Drp1 oligomer/β-actin and (C) p-Drp1/β-actin for both monomer and oligomer are presented as mean±SE of n=3 independent experiments. **P*<0.05 relative to static control.

†*P*<0.05 relative to I/RP.