Analysis of Mitochondrial Morphology in Cells Experiencing a "Heart Attack"

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Abstract

Vascular endothelial cell (EC) dysfunction immediately following the recanalization of a blocked artery (known as reperfusion, RP) is attributed to the cellular generation of superoxide radicals ($O_2^{\bullet -}$) and other reactive oxygen species (ROS). Nitric oxide (NO) is known to promote ROS production from the cell mitochondria. Our lab discovered that cultured EC exposure to fluid mechanical shear stress results in endogenous NO-mediated mitochondrial ROS production. Ischemia (I)/RP-induced EC injury occurs primarily due to mitochondrial ROS production, and the decline in bioavailable NO (due to the reaction of NO with $O_2^{\bullet -}$) impairs the EC-dependent dilation in the heart coronary vessels. In general, mitochondrial ROS lead to activation of the mitochondrial apoptotic pathway. Recent literature suggests that induction of mitochondrial apoptosis correlates with mitochondrial fragmentation (fission), which may contribute to cell apoptosis/death.

Our goals are to detect and quantify changes in mitochondrial morphology due to cultured EC exposure to simulated I/RP (with or without inhibitors of ROS sources, a NO synthase inhibitor or an inhibitor of the mitochondrial fission protein Drp1), and to determine whether these changes contribute to cell apoptosis/death. Following cultured EC exposure to treatments, such as fluid shear stress, hypoxia/reoxygenation (H/RO) or ischemia/reperfusion

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(I/RP; ischemia is simulated as hypoxia; reperfusion is simulated as reoxygenation with the addition of flow), ECs are stained with mitotracker green, which is used in conjunction with fluorescence microscopy to obtain digital images of the mitochondrial network. Morphological parameters are determined for each object in an image, and these are used for quantitative comparisons of the mitochondrial network between treatments.

We found that static or sheared ECs maintain their mitochondrial network. H/RO-exposed ECs undergo changes in mitochondrial morphology, but fission is significantly less compared to that in ECs exposed to I/RP. The massive fission in I/RP-exposed ECs is inhibited by antioxidants, a NO synthase inhibitor or a Drp1 inhibitor. Hence, ROS, NO and Drp1 are all responsible for the I/RP-induced increase in mitochondrial fission. Mitochondrial morphology changes may be implicated in the induction of EC apoptosis after vessel recanalization following a heart attack suggesting that, by controlling mitochondrial morphology, we may be able to lessen the EC dysfunction and improve outcomes.

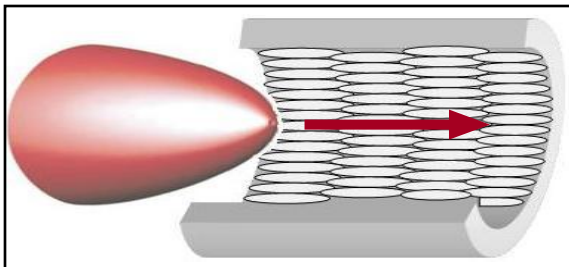

Figure 1: Display of a fully-developed laminar flow profile over endothelial cells in an artery. Endothelial cells line the interior surface of all blood vessels forming an interface between blood and the rest of the vessel wall. Shear stress is the frictional force per unit area acting on the surface of the endothelium due to the flow of viscous blood.

Introduction

Our laboratory is interested in EC mechanosignaling. "Mechanosignaling" is the understanding of signaling pathways inside cells that are exposed to defined mechanical

environments. Using a flow recirculating system, we have been submitting cultured ECs to fluid shear stresses equivalent to those that the cells encounter in their natural environment: ECs line the inside of blood vessels and, when in arteries, experience shear stresses in the order of 10-20 dyn/cm^2 (Fig. 1). We have been simulating a heart attack (myocardial infarction) and reperfusion (vessel recanalization resulting in O_2 reintroduction) by exposing cultured ECs to periods of no or very low flow (equivalent to <0.5 dyn/cm^2) under ≤3% O_2 (mimicking an ischemic event) followed by exposure to arterial flow under normoxia (mimicking a reperfusion event); an **in vitro ischemia (I)/reperfusion (RP)** protocol.

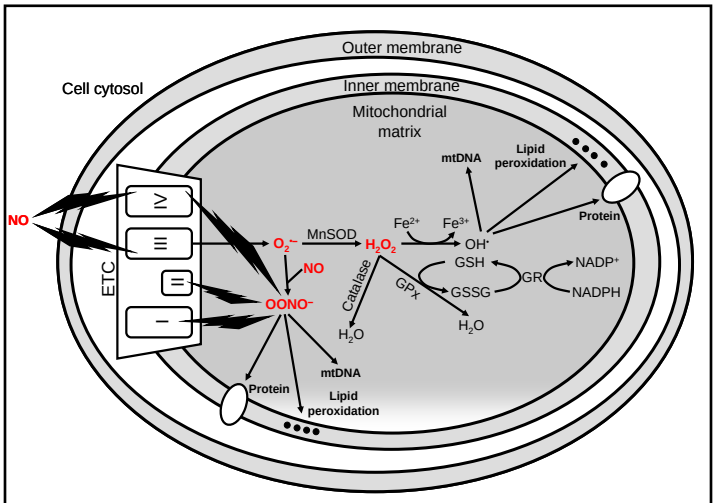

Figure 2: The role of NO in mitochondrial ROS generation. NO reversibly inhibits complexes III and IV and produces $O_2^{\bullet-}$. The product of the reaction between NO and $O_2^{\bullet-}$, $ONOO^-$, irreversibly inhibits complexes I, II and IV. H_2O_2, which originates from $O_2^{\bullet-}$ via MnSOD, is detoxified by catalase and the GSH pathway. $O_2^{\bullet-}$ liberates Fe^{2+} from ETC proteins and, in the presence of Fe^{2+}, H_2O_2 is converted to $OH^{\bullet}$. $ONOO^-$ and $OH^{\bullet}$ cause mitochondrial damage.

ECs exposed to arterial shear stress are known to increase their endogenous production rate of the vasodilator NO via activation of the enzyme endothelial NO synthase (eNOS).[1] We showed that the shear-induced NO inhibits the activities of the mitochondrial electron transport chain complexes leading to decreased mitochondrial respiration and increased production of free radicals inside the EC mitochondria (Fig. 2).[2,3] The first free radical produced is $O_2^{\bullet-}$, but this reacts inside the cell and generates other free radicals, also known as ROS (Fig. 2).[4]

It is known from studies with cultured cells, animal hearts and human hearts following cardiopulmonary bypass, that, upon I/RP, cardiac cells and especially ECs in the heart blood vessels produce high levels of ROS in their mitochondria.[5] Currently, **we are studying changes in mitochondrial network morphology in ECs exposed to *in vitro* I/RP**. In control ECs, mitochondria appear as a tubular network around the nucleus and undergo dynamic fission (fragmentation of tubules into small rods or spheres) and fusion (union of mitochondrial fragments) events (Fig. 3).[6] The protein Drp1 is known to play an important role in mitochondrial network morphology by causing fission. Drp1 activity is regulated by ROS, ATP levels, mitochondrial respiration, membrane potential, etc.[7] All of these are affected during I/RP, and may be responsible for changes in mitochondrial morphology, and changes in mitochondrial morphology may contribute to cell death in hearts of patients undergoing treatment following a heart attack.

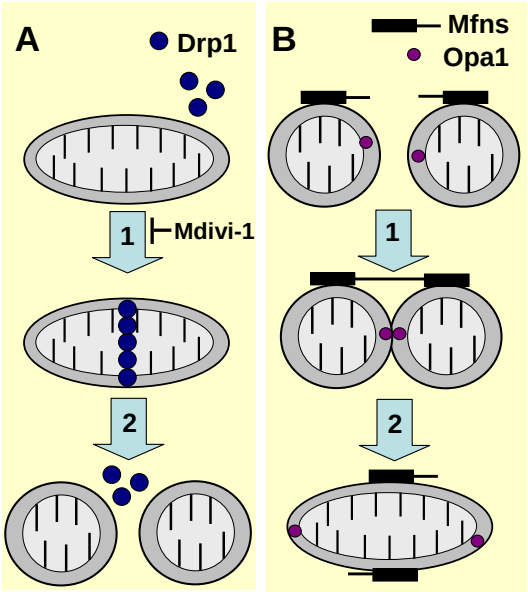

Figure 3. Schematic of mitochondrial (A) fission and (B) fusion. (A) Drp1 is recruited from the cytosol to the mitochondria at sites of scission (arrow 1; this step is inhibited by the compound mdivi-1) and couples GTP hydrolysis with mitochondrial membrane constriction and fission (arrow 2). (B) Mfns and Opa1 on the outer membrane of mitochondria interact to mediate fusion.

Hence, **the purpose of our study** is twofold; first, we want to detect and quantify changes in mitochondrial network morphology due to cell exposure to I/RP, with or without inhibitors of ROS, NO or Drp1 (use of the inhibitors may block the I/RP-induced changes in the

mitochondrial network), and second, to determine whether the changes in mitochondrial morphology contribute to the I/RP-induced increase in cell apoptosis/death.

Materials and Methods

The experimental setup consists of a parallel-plate flow chamber that connects to a flow loop (Fig. 4). This system creates gravity-controlled flow (10 dyn/cm^2; low arterial shear stress), over glass slides with cultured human ECs that are placed in the chamber. A gas proportioner controls a mixture of either 95% air/5% CO_2 for normoxic conditions or 95% N_2/5% CO_2 for ischemic or hypoxic conditions. Using this setup, we were able to create the following conditions: static cells were exposed to 0 dyn/cm^2 and 20% O_2 for 1 h in nutrient-rich media, sheared cells were exposed to 10 dyn/cm^2 and 20% O_2 for 1 h in nutrient-rich media, I/RP was created by exposing cells to 1 h of low flow (<0.5 dyn/cm^2) in 0% O_2 in starvation (no serum or glucose) media followed by 1 h of shear at 10 dyn/cm^2 in 20% O_2 in nutrient-rich media, and hypoxia/reoxygenation (H/RO) was created by exposing cells to 1 h of low flow in 0% O_2 in

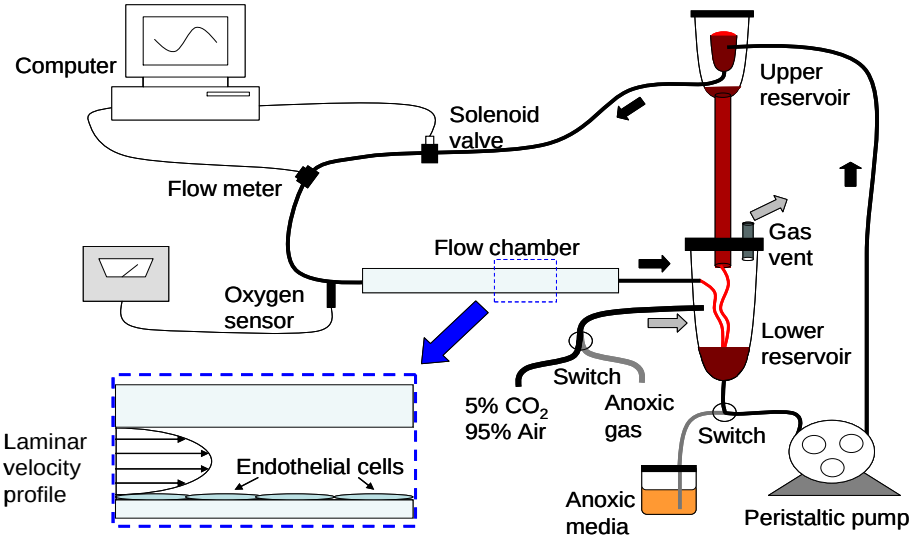

Figure 4. Flow recirculation system that exposes cultured ECs to either steady laminar shear stress or I/RP.

starvation media followed by static conditions. Hence, we can study the effects on cells of only altering mechanical forces (shear), O_2 tension (H/RO), both (I/RP), and create a control (static).

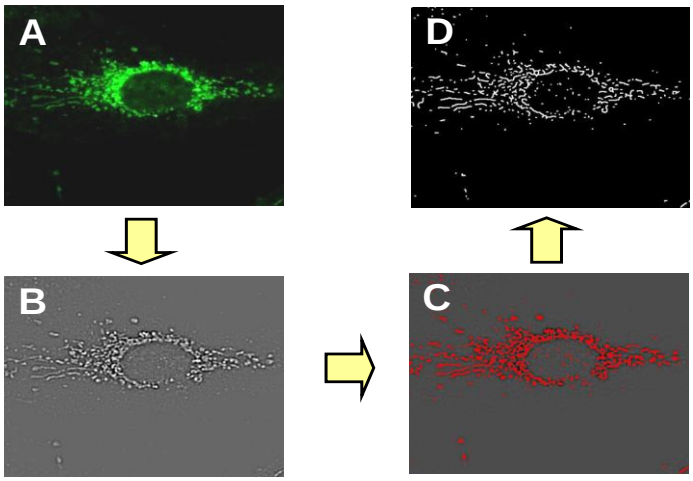

Figure 5. Digital image analysis. (A) Original image (this particular one was obtained using confocal microscopy, 100x). (B) Convolution with a kernel. (C) Segmentation by thresholding. (D) Binarization/skeletonization yield the final processed image.

After each treatment (with or without inhibitors of ROS, NO, or Drp1) cells were stained with the mitochondrial stain mitotracker green, which is used in conjunction with fluorescence microscopy to obtain digital images of mitochondrial network. The images were contrast-enhanced at 0.5% of saturation levels, treated with a smoothing linear filter, and convoluted with a 5x5 Laplacian kernel with non-zero diagonal terms, which defines structures based on abrupt spatial changes in fluorescence (Fig. 5). Images were segmented by thresholding. Various threshold detection algorithms were tested, and Otsu's was chosen. Images were binarized and skeletonized according to detected thresholds (Fig. 5). Morphological parameters, including length, aspect ratio and form factor, were determined for each mitochondrial object in the final images, and these were used for quantitative comparisons of mitochondrial morphology between conditions.

Results and Discussion

Based on the literature that has established causative relationships between mitochondrial ROS, mitochondrial fission, and cell death[8], we wish to investigate whether, and how, the mitochondrial ROS upon RP and possible changes in mitochondrial network morphology determine cell apoptosis/death. Our data showed that I/RP produces mitochondria that have low values of aspect ratio and form factor corresponding to objects that are, on average, short and more circular, implying that mitochondria in these cells have been subjected to increased fission. This is in contrast to static or sheared cells, which produced higher values of aspect ratio, form factor and length, signifying more of a healthy network morphology. H/RO-exposed cells showed an intermediate extent of fission. Cells treated with a Drp1 inhibitor, showed morphology approaching that of static cells after I/RP exposure, indicating that the changes are due to Drp1 activation. eNOS inhibitors as well as inhibitors of ROS production showed a significant improvement from I/RP-related fission. Our data provide the **first evidence that shear stress, H/RO and I/RP differentially affect the EC mitochondrial network**, and point to the injurious role of NO and mitochondrial ROS upon RP.

This project may improve our understanding of changes in mitochondrial dynamics and their repercussions on postischemic EC survival. We anticipate that the detected changes in mitochondrial morphology will be implicated in EC apoptosis/death suggesting that, **by controlling mitochondrial morphology, we may be able to lessen the I/RP-induced cellular damage and improve the clinical outcomes in heart attack patients**.

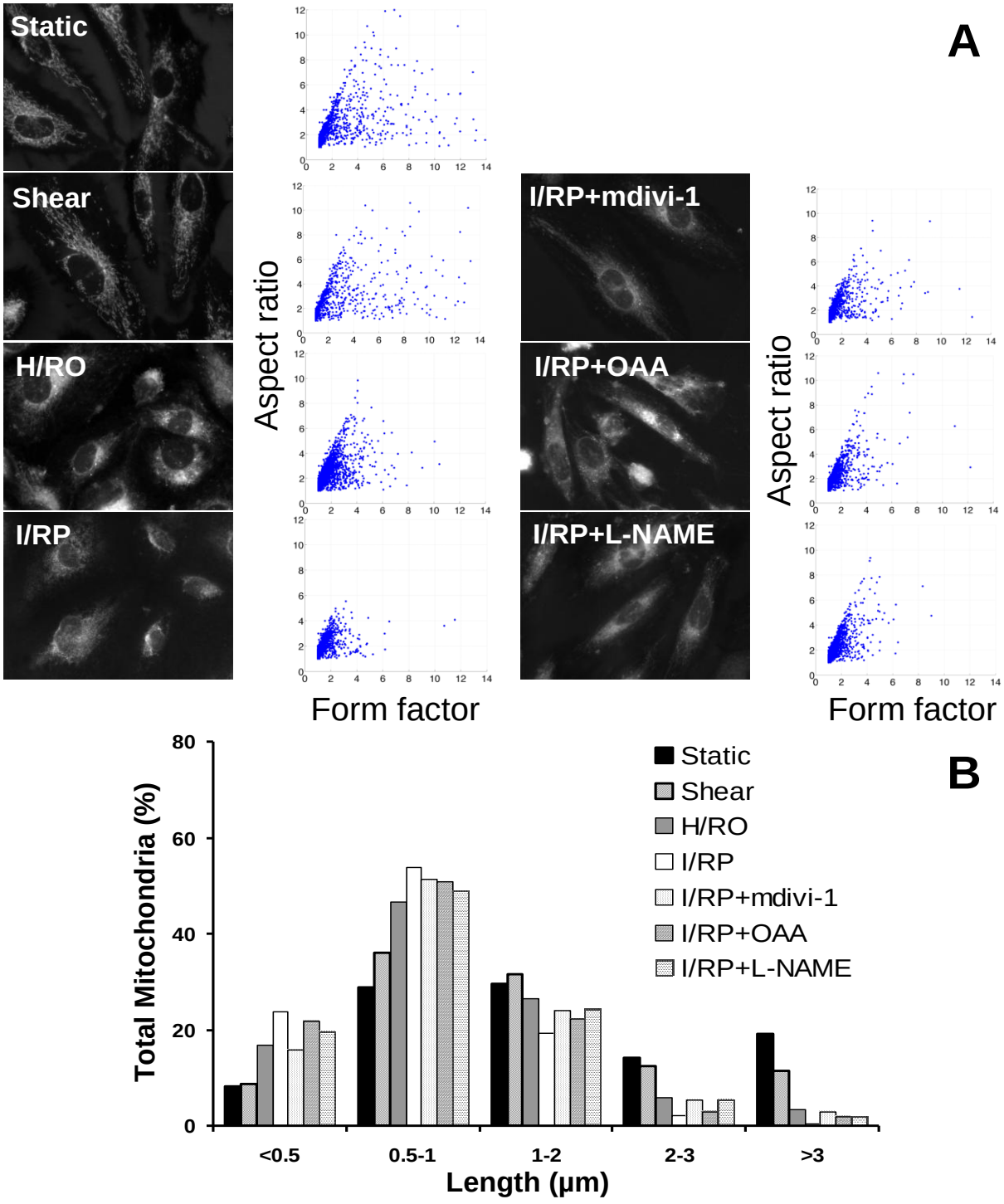

Figure 6. I/RP causes massive mitochondrial fission, and fission is, at least in part, blocked by either the Drp1 inhibitor mdivi-1, OAA or L-NAME. (A) Characteristic mitotracker images (conventional microscopy, 60x) of ECs that are either kept static or exposed to shear, H/RO, and

I/RP. Also shown are images from I/RP experiments in the presence of mdivi-1 (30 μM), OAA (inhibits mitochondrial $O_2^{\bullet-}$ generation; 100 μM) or L-NAME (inhibits NO generation from eNOS; 500 μM). Scatter plots of aspect ratio vs. form factor for each mitochondrial particle are shown to the right of each image. Notice that 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 is affected by both length and branching). (B) Length of each object in each image was determined, and data were binned into different categories from <0.5 (but >0.2) to >3 μm. Notice that I/RP had the highest % of mitochondrial objects with lengths <1 μm and the lowest % in all other categories, and this was partially prevented by either mdivi-1, OAA or L-NAME.

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