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Fluid Mechanical Forces and Endothelial Mitochondria: A Bioengineering Perspective

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Abstract

Endothelial cell dysfunction is the hallmark of every cardiovascular disease/condition, including atherosclerosis and ischemia/reperfusion injury. Fluid shear stress acting on the vascular endothelium is known to regulate cell homeostasis. Altered hemodynamics is thought to play a causative role in endothelial dysfunction. The dysfunction is associated with/preceded by mitochondrial oxidative stress. Studies by our group and others have shown that the form and/or function of the mitochondrial network are affected when endothelial cells are exposed to shear stress in the absence or presence of additional physicochemical stimuli. The present review will summarize the current knowledge on the interconnections among intracellular Ca^{2+} - nitric oxide - mitochondrial reactive oxygen species, mitochondrial fusion/fission, autophagy/mitophagy, and cell apoptosis vs. survival. More specifically, it will list the evidence on potential regulation of the above intracellular species and processes by the fluid shear stress acting on the endothelium under either physiological flow conditions or during reperfusion (following a period of ischemia). Understanding how the local hemodynamics affects mitochondrial physiology and the cell redox state may lead to development of novel therapeutic strategies for prevention or treatment of the endothelial dysfunction and, hence, of cardiovascular disease.

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CONFLICTS OF INTEREST

The authors C.G. Scheitlin, D.M. Nair, J.A. Crestanello, J.L. Zweier and B.R. Alevriadou declare that they have no conflicts of interest.

ETHICAL STANDARDS

No human studies were carried out by the authors for this article. No animal studies were carried out by the authors for this article.

Keywords

Shear stress; reactive oxygen species; nitric oxide; oxidative stress; endothelial dysfunction; cardiovascular disease; ischemia/reperfusion; mitochondrial fission; autophagy; mitophagy

INTRODUCTION

Coronary artery disease (CAD), leading to ischemic heart disease, is among the most prevalent health problems in the world and a major cause of morbidity and mortality. The underlying cause of CAD is the development of coronary atherosclerosis.^{3,26,75,107} The vascular endothelium, due to its location at the luminal surface of every blood vessel, including those in the heart, is constantly exposed to fluid hemodynamic forces, primarily to shear stress. The local shear stress is thought to play a determinant role in endothelial cell (EC) dysfunction, the first critical step towards the development of coronary atherosclerosis.^{16,38,48,81} Following CAD treatment that results in recanalization (reperfusion; RP) of the blocked (ischemic) coronary artery, EC dysfunction occurs again; it is characterized by loss of local vasodilation, leads to adhesion of neutrophils and platelets to the endothelium, and exacerbates myocardial damage.^{5,13,40} It was recently shown that shear stress also contributes to the EC dysfunction that occurs upon RP.³⁷ Given the essential roles of ECs and one of their main products, the vasodilator nitric oxide (NO), in regulating vascular tone, inflammation and thrombosis, it is important to develop better strategies to protect the endothelium, especially the coronary endothelium.

Mitochondria are subcellular organelles that produce ATP via oxidative phosphorylation. ECs harness most of their energy from glycolysis and the endothelial mitochondria are thought to have as their primary function the production of reactive oxygen species (ROS) and mediation of redox signaling.^{42,65,102,137} It is known that mitochondria-derived ROS regulate gene expression/protein synthesis and apoptosis, but, within the last few years, it became clear that they also orchestrate the processes of mitochondrial dynamics and mitochondrial autophagy (mitophagy).^{28,53,65,66,93} Deregulation of the EC mitochondrial processes/functions can result in ROS buildup (oxidative stress), which can initiate the mitochondrial pathway of apoptosis and lead to a dysfunctional endothelium.^{42,65}

Since EC dysfunction is the first event in cardiovascular disease, local hemodynamics regulates/contributes to the dysfunction, and the dysfunction is associated with mitochondrial oxidative stress, the goal of this article is to summarize key concepts in mitochondrial free radical biology and, most importantly, to highlight the fluid shear stress effects on EC mitochondrial ROS production and redox signaling. The ultimate goal is to highlight areas where additional research is needed and to potentially identify therapies that will efficiently prevent and manage cardiovascular disease by improving the EC mitochondrial function, while taking into consideration the cell hemodynamic environment.

MITOCHONDRIAL ROS

Mammalian mitochondria consume almost all of a cell's O₂ to support oxidative phosphorylation (OXPHOS) that harnesses oxidized fuel generated in the tricarboxylic acid

cycle to synthesize ATP. The OXPHOS machinery is embedded in the inner mitochondrial membrane and consists of the electron transport chain (ETC; also called respiratory chain) and the ATP synthase. The ETC is an assembly of 4 polypeptide complexes, I, II, III and IV, and 2 electron (e) carriers, ubiquinone (Q) and cytochrome c (cyt c) (Fig. 1). Electrons from e donors, NADH and reduced flavin-adenine dinucleotide (FADH₂), enter at complexes I and II, respectively. Energy released as e travel through the ETC is used to pump protons (H⁺) out of the matrix. This creates an electrochemical gradient that results in increased mitochondrial membrane potential (Ψ_m). The potential energy stored is coupled to ATP synthesis by ATP synthase. Although the majority of O₂ is reduced to H₂O at complex IV, 1–3% of O₂ is incompletely reduced to the ROS superoxide (O₂[•]); this takes place at complexes I, II and III (Fig. 1).^{71,122} The O₂[•] production rate increases when e carriers harbor excess e, due to either OXPHOS inhibition or excessive calorie consumption. The location of O₂[•] generation is important because O₂[•] does not diffuse across membranes. Complexes I and II release O₂[•] into the matrix, whereas complex III releases O₂[•] both into the matrix and the intermembrane space (Fig. 1).⁸⁷ O₂[•] is converted to the ROS hydrogen peroxide (H₂O₂) by manganese superoxide dismutase (MnSOD) in the mitochondrial matrix and by copper zinc SOD (CuZnSOD) in the intermembrane space (and also in the cytosol).⁴⁴ At either location, H₂O₂ is decomposed, mainly by glutathione peroxidase (GPx), to H₂O. Via an Fe²⁺-catalyzed (Fenton) reaction, H₂O₂ can be converted to the potent ROS hydroxyl radical (OH[•]). H₂O₂ that escapes detoxification diffuses into the cytosol where it raises the levels of intracellular H₂O₂ and, hence, regulates redox signaling, gene expression/protein synthesis and apoptosis.^{4,12,71} The mitochondrial ETC, cytosolic xanthine oxidase, plasma membrane-bound NADPH oxidase and, in some cases, the uncoupled endothelial NO synthase (eNOS) are the major sources of intracellular ROS in vascular ECs.⁷²

SHEAR STRESS EFFECTS ON EC MITOCHONDRIAL ROS: PHYSIOLOGICAL CONDITIONS

Fluid flow is either laminar or turbulent. Laminar flow is characterized by parallel to each other streamlines and is the type of flow throughout the arterial tree (sometimes, the flow at branch points, bifurcations and curvatures is mistakenly called turbulent). There are three types of laminar flow: Steady is when fluid velocity does not vary with time; it does not occur in arteries, but it is used in *in vitro* studies. Under this flow, ECs are exposed to steady laminar shear (SS). Pulsatile and oscillatory are unsteady flows. Pulsatile is a unidirectional flow with a periodically varying magnitude yielding a positive time-average, whereas oscillatory is a bidirectional flow with a periodically varying magnitude yielding a time-average close to zero. In straight arterial segments, flow is pulsatile: ECs are exposed to pulsatile shear stress (PS) typically between 10–20 and as high as 40 dynes/cm², and these regions are spared from atherosclerotic disease. In branch points, bifurcations and curvatures, flow is oscillatory: ECs are exposed to oscillatory shear stress (OS) of about ± 4 dynes/cm² and these regions develop atherosclerosis.^{16,23,81} By exposing cultured ECs to the above flow patterns in flow chambers or cone-and-plate viscometers, it became clear that SS, PS and OS differentially modulate EC gene expression; *in vivo* studies confirmed the *in vitro* findings.^{18,24,48,97} EC exposure to SS or PS, but not OS, increased the production of

the vasodilator NO by activating eNOS and, at later times, via eNOS induction.^{52,56,67,114} SS or PS upregulated the MnSOD mRNA levels.^{1,16} OS upregulated critical NADPH oxidase subunits resulting in higher intracellular $O_2^{\bullet}$ levels compared to SS or PS.^{29,48,116} It is generally accepted that OS upregulates pro-atherogenic genes and suppresses atheroprotective ones, thereby promoting disease initiation; SS or PS does the opposite, thereby deterring disease initiation.

In NO-producing cells, such as ECs, or cells exposed to NO donors, NO modulates mitochondrial respiration as shown by O_2 consumption measurements.^{20,78} At physiological concentrations ($<1 \mu M$), NO can inhibit mitochondrial ETC complexes III and IV resulting in increased $O_2^{\bullet}$ generation.^{4,19,99} Inhibition of complex III is due to NO-induced reduction of the cytochrome *b* subunit of the complex, whereas inhibition of complex IV occurs through reversible binding of NO to the ferrocycytochrome a_3 and Cu_B^+ components. Reduced ETC activity in conditions where NO is produced (e.g. during EC exposure to hypoxia) limits the O_2 consumption by ECs, thereby increasing the availability of O_2 for the surrounding tissues. However, at higher concentrations ($>1 \mu M$), NO promotes ubiquinol oxidation with increased production of $O_2^{\bullet}$ which then reacts with NO, in a diffusion-limited reaction, to form the reactive nitrogen species (RNS) peroxynitrite (ONOO) (Fig. 1).^{99,100} ONOO has the potential to cause persistent inhibition of complex I (by oxidation/nitration), which greatly increases the $O_2^{\bullet}$ production, as well as inhibition of complexes II and IV, ATP synthase and MnSOD, and to promote cell apoptosis.^{8,9,12,14,106} Since $O_2^{\bullet}$ does not cross membranes (whereas NO diffuses freely), it is expected that ONOO will form primarily inside the matrix and the intermembrane space. Mitochondria undergo damage when ROS/RNS levels exceed their antioxidant capacity (mitochondrial oxidative stress).

Our group focused on the mitochondrial ETC and ROS production in cultured ECs subjected to SS: SS was found to inhibit each of the ETC complexes and the effect was present throughout flow exposure.⁴⁵ ETC inhibition occurred within minutes from flow onset, was accompanied by nitrotyrosine staining (a footprint of ONOO formation) and was inhibited by either the eNOS inhibitor *N*^G-nitro-L-arginine methyl ester (L-NAME) or the ONOO scavenger uric acid (UA) suggesting that intramitochondrial ONOO is, at least in part, responsible for the shear-induced inhibition of the ETC.⁴⁵ Since NO and, to a greater extent, ONOO are known to decrease mitochondrial respiration¹³² and the $O_2^{\bullet}$ formation rate by the ETC increases linearly with the partial pressure of O_2 (P_{O_2}),^{71,123} we measured O_2 consumption rates by ECs sheared at different P_{O_2} using electron paramagnetic resonance oximetry.⁵⁷ Prior to this, all published flow studies (including ours^{45,58,83,90,134,135}) had been carried out at the atmospheric O_2 concentration (21% O_2) giving rise to a P_{O_2} of 160 mmHg, which is greater than the levels in arterial blood (12–13% O_2) and much greater than the levels in venous blood (5% O_2).³¹ In agreement with our earlier findings,⁴⁵ SS was shown to inhibit respiration and the effect was more pronounced when ECs were sheared at atmospheric P_{O_2} . Either L-NAME, UA or overexpression of MnSOD abolished the shear inhibitory effect suggesting that NO/ONOO is responsible for the decrease in O_2 consumption rate by sheared ECs.⁵⁷ MitoSOX red (mitochondria-targeted $O_2^{\bullet}$ -specific probe) fluorescence verified that mitochondrial $O_2^{\bullet}$ production is enhanced in parallel with

P_{O_2} and NO/ONOO is responsible for the increased mitochondrial ROS levels in sheared ECs.⁵⁷

Shear stress induces vasodilation in conduit and resistance arteries.⁶⁸ Flow-induced dilation is mediated primarily by NO, but, in coronary arterioles from patients with CAD, it requires EC mitochondrial ROS, specifically H_2O_2 generated from $O_2^{\bullet}$ produced by the ETC.⁷⁷ Therefore, elucidating the connection between shear stress and EC mitochondrial ROS may improve our understanding of CAD. Under physiological conditions, the effect of SS or PS on EC mitochondrial $O_2^{\bullet}$ can be summarized as follows (Fig. 2, top): The onset of shear stress causes a transient increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$), due to Ca^{2+} entry from the extracellular space and/or Ca^{2+} release from the endoplasmic reticulum,^{10,86,112} which can produce mitochondrial $O_2^{\bullet}$ by stimulating the tricarboxylic acid cycle and enhancing e^- flow into the ETC.^{7,137} $[Ca^{2+}]_i$ activates eNOS, via calmodulin, resulting in enhanced NO production; NO increases mitochondrial $O_2^{\bullet}$ production via NO/ONOO-mediated ETC inhibition, but also decreases mitochondrial $O_2^{\bullet}$ levels by scavenging $O_2^{\bullet}$. We found that Ψ_m is maintained under SS.³⁷ However, it is generally expected that an increase in $[Ca^{2+}]_i$ and, hence, in mitochondrial Ca^{2+} ($[Ca^{2+}]_{mt}$) depolarizes Ψ_m and reduces mitochondrial ROS production.^{7,42,137} Adding to the complexity, EC exposure to PS was shown to hyperpolarize Ψ_m , which can also explain the shear-induced increase in mitochondrial $O_2^{\bullet}$.⁷⁴ Despite the increase in mitochondrial ROS, ECs remain healthy probably due to NO-mediated caspase inhibition, Ψ_m maintenance, mitochondrial biogenesis and upregulation of antioxidant genes.^{92,98,129} Shear-induced mitochondrial $O_2^{\bullet}$ can regulate intracellular signaling via modulation of cytosolic H_2O_2 levels. Our group showed that shear-induced mitochondrial ROS/RNS are responsible for heme oxygenase-1 upregulation.⁴⁶ When ECs were activated by chemical stimuli, EC mitochondrial ROS were found to mediate the activation of AMP kinase (AMPK) and nuclear factor- κ B.^{2,102} Overall, SS, PS and OS are expected to have differential effects on EC mitochondrial $O_2^{\bullet}$ (and, hence, on cytosolic H_2O_2 and intracellular signaling) due to their differential effects on $[Ca^{2+}]_i$ (possibly also $[Ca^{2+}]_{mt}$) and Ψ_m , eNOS activation/NO production/RNS formation and, at longer times, eNOS and antioxidant gene expression.

SHEAR STRESS EFFECTS ON EC MITOCHONDRIAL ROS: PATHOLOGICAL CONDITIONS

Although extensively studied *in vitro*, abrupt changes from static to arterial flow do not happen *in vivo*, except during RP of an ischemic vessel. A decline in EC-dependent dilation occurs soon after the burst in intracellular $O_2^{\bullet}$ by the reperfused coronary endothelium suggesting that ROS may trigger the EC injury.^{13,88} In ECs of isolated rat hearts exposed to ischemia/reperfusion (I/RP), the mitochondrial pathway of apoptosis was found to be activated suggesting that the mitochondrial ROS, in particular, may be responsible for the EC injury.^{108,109} Hence, our group focused on understanding the role of shear stress on the EC mitochondrial ROS upon the onset of RP. Since RP is reoxygenation (RO) plus reintroduction of flow, we developed an *in vitro* model of simulated I/RP (sI/RP), where severe hypoxia (1% O_2) and nutrient deprivation under static (or very low flow corresponding to <0.5 dynes/cm²) conditions for 1 h was followed by arterial-level flow of

oxygenated media (21% O₂) over the cultured ECs for 1 h.³⁷ We found that sI/RP resulted in the highest mitochondrial O₂[•] levels compared to either shear alone (without prior ischemia) or sI/RO (all under static conditions) and the signal was inhibited by L-NAME suggesting that the shear-induced NO is, at least in part, responsible for the mitochondrial O₂[•] generation during the RP period of I/RP.³⁷ Furthermore, immunocytochemistry with an antibody against nitrotyrosine verified that sI/RP leads to high ONOO generation in mitochondria of ECs compared to the relatively low ONOO formation in mitochondria of sheared ECs (with no prior exposure to ischemia).⁴⁵ Our findings agree with *in vivo* evidence where it was found that the production of both NO and O₂[•] (measured using electron paramagnetic resonance methods) is increased in the postischemic rat heart, and they react to form ONOO resulting in protein nitration and myocardial injury.¹²⁷ In *in vivo* rat and dog I/RP models and in human hearts following cardiopulmonary bypass, the concentration of nitrotyrosine is increased and blocking its formation is cardioprotective.^{47,76,133,138} It is generally accepted that small NO amounts released by eNOS are beneficial; high NO doses released following the expression of an inducible NOS in postischemic myocardium lead to enhanced ONOO formation and are harmful.^{32,60,62} However, more recently, the eNOS-derived NO upon RP following coronary ligation in mice was found responsible for inhibition of ETC complex I activity, ONOO-mediated protein tyrosine nitration and suppression of tissue O₂ consumption suggesting that the EC-derived NO leads to high intramitochondrial ONOO formation and tissue damage during early RP.¹³⁹

Hence, the role of SS or PS, as a component of RP, on postischemic EC mitochondrial O₂[•] production can be summarized as follows (Fig. 2, bottom): Following respiratory inhibition during ischemia (due to lack of O₂), RP elicits a surge in mitochondrial O₂[•] production (due to the abrupt recovery in respiration).^{7,11} [Ca²⁺]_{mt} overload occurs and is regulated by ROS, possibly via ROS-mediated inhibition of the Na⁺/Ca²⁺ exchanger that allows for [Ca²⁺]_{mt} extrusion.⁵⁹ The shear-induced burst in NO production results in formation of high intramitochondrial ONOO levels. ONOO, in conjunction with [Ca²⁺]_{mt}, inhibits the ETC, primarily at complex I, greatly increasing O₂[•] production and forming more ONOO.⁵⁵ Both high [Ca²⁺]_{mt} and ONOO are thought to lead to opening of the mitochondrial permeability transition pore (mtPTP) on the inner membrane, mitochondrial depolarization, cytochrome c release and initiation of the mitochondrial pathway of apoptosis.^{6,7,11} Release of cytochrome c effectively blocks the ETC at complex III and further enhances O₂[•] production. The importance of mitochondrial ROS/RNS in RP injury is verified by the fact that ischemic preconditioning (IPC; brief periods of I/RP prior to prolonged I/RP), the gold standard of cardioprotection, was found to attenuate the NO and mitochondrial ROS levels during RP.^{22,33,70,101} Although IPC is not clinically feasible, ischemic postconditioning (brief periods of RP/I following prolonged I and prior to prolonged RP) is, and may confer similar benefits.¹²⁶

MITOCHONDRIAL DYNAMICS - REGULATION BY ROS

In recent years, it has become clear that mitochondrial dynamics (fusion/fission and organelle motility) determines not only morphology, but also mitochondrial function.^{82,94,118} Specifically, mitochondria are known to cyclically shift between fusion

and fission (fragmentation) states (Fig. 3a). Fusion is mediated by the GTPases mitofusins (Mfns) and optic atrophy protein 1 (Opa1); fission is mediated by the GTPase dynamin-related protein 1 (Drp1) binding to mitochondrial fission factor (Mff) (with contribution by assembly proteins) on the outer membrane.^{54,117} Drp1 is distributed in the cytosol, but, once activated, it oligomerizes and gets recruited to the mitochondria, where it drives outer membrane constriction and fission.¹¹⁵ Drp1 activity is posttranslationally regulated by different mechanisms including phosphorylation and dephosphorylation of different serine residues.^{21,119} Fission produces fragments with different Ψ_m ; depolarized fragments either restore their Ψ_m and re-enter fusion or remain depolarized.^{124,125} Sustained depolarization results in phosphatase and tensin homolog-induced putative kinase 1 (PINK1) accumulation on the outer membrane and recruitment of the ubiquitin ligase parkin to PINK1.⁸⁹ Parkin ubiquitinates the Mfns and other mitochondrial proteins, and mitochondria with ubiquitinated surface proteins are targeted for elimination via mitochondrial autophagy (mitophagy).⁶⁶ The current interest in mitochondrial dynamics originates from the fact that Drp1 colocalizes with the proapoptotic B-cell lymphoma 2 (Bcl-2)-associated X protein (BAX) on the outer membrane and excessive Drp1-mediated fission contributes to apoptosis, possibly by enhancing cytochrome c release.^{34,96}

Our group focused on characterizing the relationship between bioenergetics and mitochondrial morphology in cultured ECs: We found that Ψ_m and ATP produced by glycolysis, as well as ATP produced by mitochondria, are critical for mitochondrial dynamics.³⁶ When mitochondria were depolarized or glycolysis was inhibited, there was a transient period where the fission rate increased and the fusion rate decreased before they became equal, but lower than their original values, leading to fragmentation of the network.³⁶ Although the specific role of ROS was not tested in that study, we speculate that decreased ATP levels lead to increased $[Ca^{2+}]_i$ (due to lack of ATP for the pumps that transport Ca^{2+} out of the cell and into intracellular stores), $[Ca^{2+}]_{mt}$ overload, mitochondrial depolarization, increased mitochondrial ROS and, hence, cellular ROS (due to diffusion of mitochondrial H_2O_2 into the cytosol and ROS-induced ROS release by sources, such as NADPH oxidase or xanthine oxidase). Others verified that, upon Ψ_m dissipation, the fusion machinery is inhibited (due to OPA1 proteolytic cleavage and loss of its profusion activity) and fission is enhanced (in part, due to Drp1 activation by Ca^{2+} -induced, calcineurin-mediated Drp1 dephosphorylation at Ser637).^{15,30} Although these studies have not been conducted yet, it is possible that the different flow patterns, SS, PS and OS, via differential effects on EC ATP levels and Ψ_m , may cause differential effects on the rates of mitochondrial fusion and fission. However, the biggest effect is expected to take place following Ψ_m dissipation and/or ATP depletion, such as following I/RP. Using our *in vitro* sI (1 h)/RP (1 h) model of cultured ECs, we discovered that the surge in ROS, including mitochondrial ROS, and NO upon RP plays an important role in Drp1 activation (oligomerization and phosphorylation at Ser616) and mitochondrial fission.³⁷ The sI/RP-induced fission was significantly higher compared to that following sI/RO, whereas SS alone did not alter fission (due to Ψ_m maintenance).³⁷ Overall, our findings suggested that SS (and probably PS) during RP, via modulation of the oxidative stress to which the EC is exposed, regulates the extent of fission and propensity to apoptosis. Although there is lack of *in vivo* evidence on EC mitochondrial fission due to I/RP, pretreatment of murine hearts

with an intravenous bolus of the specific Drp1 inhibitor mitochondrial division inhibitor (mdivi)-1 was shown to reduce infarct size and limit the extent of fission in cardiomyocytes suggesting that temporary inhibition of fission may be a novel strategy for cardioprotection.^{94,95}

AUTOPHAGY AND MITOPHAGY - REGULATION BY ROS

Autophagy is a cellular catabolic process in which cytoplasmic material, proteins and organelles, is transported to lysosomes for degradation; it is activated when cells are under stress (starvation, hypoxia, ischemia).^{64,66,85,105} Autophagy consists of three steps: induction, formation of autophagosomes and delivery to/fusion with lysosomes (Fig. 3b). Briefly, the mammalian target of rapamycin (mTOR) is inhibited by AMPK. Inhibition of mTOR together with activation of the phosphatidylinositol-3 kinase class III-beclin1 complex is required for induction. For autophagosome formation, the cytosolic microtubule-associated protein 1 light chain 3 (LC3) is cleaved to LC3-I and then lipidated to LC3-II. The adaptor p62 binds to both ubiquitinated mitochondrial proteins and LC3-II and tethers dysfunctional mitochondria to autophagosomes (alternative mechanisms are also available). Autophagosomes fuse with lysosomes to form autophagolysosomes where contents are degraded and amino acids/fatty acids are recycled for cell use. Autophagosome prevalence at each time point is determined by the rates of induction and processing (flux). Assessment of LC3-II levels shows “steady state” autophagy. Since LC3-II levels are also affected by the rate of autophagosomal clearance, it is important to assess both “steady state” autophagy and autophagic flux. Assessment of either LC3-II levels in the absence/presence of agents that inhibit fusion of autophagosomes with lysosomes, such as chloroquine (CQ) or ammonium chloride (NH₄Cl), or p62 levels (p62 is degraded during autophagy) is used to monitor flux.⁶³ The term “impaired autophagy” is used when either the flux is decreased (increased p62 levels) or both induction and flux are decreased. In many cell types, conditions that cause oxidative stress, including starvation, exogenous H₂O₂ or blockade of the ETC, were shown to activate autophagy.^{17,51,61,110} Besides the induction of redox-sensitive genes, other redox-regulated sites in the pathway are: (a) ROS (/RNS)-induced phosphorylation of upstream kinases AMPK (mitochondrial ROS activate AMPK; it initiates autophagy via mTOR inhibition)^{73,102,128} and c-Jun N-terminal kinase (JNK; it initiates autophagy via Bcl-2 phosphorylation that decreases Bcl-2 interaction with beclin1 leaving beclin1 available for induction),¹³¹ and (b) autophagy-related protein (Atg) 4 oxidation/inactivation leading to LC3-II accumulation and increased autophagosome formation.¹¹¹

It is possible that EC exposure to SS or PS, via activation of AMPK and JNK and changes in the cell redox status,^{41,136} may modify the levels of autophagosome formation and flux compared to static ECs. Furthermore, differences in ROS/RNS levels and kinase activation profiles¹²⁰ may lead to differential effects on autophagy in ECs under SS or PS vs. OS. *In vitro* studies need to be conducted with utmost care regarding the perfusion media composition, as autophagy is activated by serum/nutrient starvation, and appropriate controls need to be included. It was recently reported that autophagy is activated when cultured ECs or isolated rabbit carotid arteries were exposed to SS compared to static conditions.⁴³ Interestingly, pretreatment with the autophagy inducer rapamycin followed by

SS enhanced the shear-induced eNOS expression suggesting that autophagy may potentiate the beneficial effects of SS on the endothelium.⁴³ However, in the context of RP, the role of autophagy in EC survival and, in particular, the effect of shear stress on autophagy are unknown. Mitochondrial fission is a prerequisite for mitophagy¹⁰⁴ and autophagy/mitophagy are activated during cardiac ischemia (due to AMPK activation/mTOR inhibition).⁸⁴ In regards to cardiomyocytes, it was shown, *in vitro* and *in vivo*, that the flux is impaired during RO and RP, respectively, and the diminished autophagosomal clearance contributes to cardiomyocyte death.^{79,80} It is generally accepted that, in different cell types, when mitophagy becomes impaired (e.g., when the number of damaged mitochondria exceeds the cell capacity for removal), the mitophagic machinery shuts down and cells undergo apoptosis;⁶⁶ it is currently unknown if that is the case in the reperfused endothelium.

SUMMARY

There are several review papers on EC O₂[•] generation in response to chemical agonists^{35,72} and on hemodynamic regulation of ROS in ECs under different flow patterns, with emphasis on the role of OS in atherosclerosis development.^{18,25,48,91,103} There are only a limited number of review papers on EC mitochondria and their role in vascular (patho)physiology.^{65,121} This paper aimed to summarize the current knowledge on EC mitochondria, as a source of ROS, under flow conditions and, especially, under RP following ischemia. Briefly, regular flow patterns (SS or PS) produce relatively low levels of mitochondrial ROS/cytosolic ROS and high levels of NO and form relatively low levels of RNS resulting in posttranslational oxidative modifications of proteins and gene expression/protein synthesis that ensure EC health. Autophagy is expected to have a beneficial effect, but, currently, we can only speculate on the mechanisms by which autophagy interferes in the above pathways (Fig. 4, left panel). During RP, ECs are expected to produce very high levels of mitochondrial ROS/cytosolic ROS, high levels of NO, as well as high levels of RNS resulting in different profiles of posttranslational protein modifications, altered mitochondrial dynamics (fission on top of the fission that occurred during ischemia) and EC apoptosis. Autophagy and mitophagy are expected to be activated during I/RP; removal of dysfunctional mitochondria should be the EC's response to promote survival. However, with overwhelming mitochondrial damage, autophagy/mitophagy may become impaired and EC apoptosis may dominate leading to subsequent loss of myocardial function (Fig. 4, right panel). In isolated rat hearts, eNOS activity was impaired following >1 h ischemia, but recovered upon RP.³⁹ Prolonged sI (24 h) followed by RO (1 h) was recently shown to lead to EC eNOS uncoupling resulting in very high ROS (in part due to O₂[•] generation by eNOS) and diminished NO production.²⁷ With eNOS uncoupling, the profiles of oxidative/nitrosative protein modifications will be different compared to when eNOS functions properly, but the end result of increased fission and propensity to apoptosis is expected to be the same.

Only a limited number of translational studies have examined the clinical relevance of EC mitochondrial dynamics and autophagy in humans (listed in reference⁶⁵): Increased mitochondrial fission was observed in ECs obtained by biopsy from patients with diabetes, a disease characterized by ROS-mediated EC dysfunction.¹¹³ In addition, impaired autophagy

(increased p62 and decreased beclin1 levels) was discovered in ECs obtained by biopsy from elderly humans suggesting that deregulated EC autophagy/mitophagy may contribute to the increased incidence of cardiovascular disease with age.⁶⁹ It is currently unknown if altered hemodynamics, due to diabetes or aging, contributed to the impaired EC autophagy/mitophagy in these *in vivo* studies. Regarding I/RP, experiments using *ex vivo* I/RP-exposed animal hearts have established that the cardioprotection conferred by IPC, as well as by ischemic postconditioning, may be, at least in part, due to activation of autophagy/mitophagy.^{49,50,130} However, these studies mainly focused on the myocardium, not the endothelium. Furthermore, these studies did not independently examine the role of shear stress, as a component of IPC and ischemic postconditioning protocols, in EC autophagy/mitophagy. The easiest way to assess the role of shear stress would be to design *in vitro* experiments that would compare between RP (that includes flow) and RO during IPC or ischemic postconditioning; it is to be expected that shear stress, via its effects on ROS/mitochondrial ROS and NO/RNS, would play an important role in autophagy/mitophagy and the associated cardioprotection provided by either IPC or ischemic postconditioning. Overall, the *in vitro* and *ex vivo* studies presented in this review paper and the limited human *in vivo* evidence suggest that EC mitochondrial health determines the cell fate. Gaining insight into the regulation of mitochondrial oxidative stress, dynamics and autophagy by physicochemical stimuli, such as shear stress, may lead to novel therapies that will maintain mitochondrial quality control and, by preventing EC dysfunction, lead to better management of cardiovascular diseases/conditions, including CAD and I/RP injury.

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LIST OF ABBREVIATIONS

CAD	coronary artery disease
EC	endothelial cell
sI/RP	simulated ischemia/reperfusion
NO	nitric oxide
ROS	reactive oxygen species
OXPHOS	oxidative phosphorylation
ETC	electron transport chain
e	electron
Q	ubiquinone
cyt c	cytochrome c
Ψ_m	mitochondrial membrane potential

O₂[•]	superoxide radical
SOD	superoxide dismutase
GPx	glutathione peroxidase
H₂O₂	hydrogen peroxide
OH[•]	hydroxyl radical
SS	steady laminar shear stress
PS	pulsatile shear stress
OS	oscillatory shear stress
eNOS	endothelial nitric oxide synthase
RNS	reactive nitrogen species
ONOO	peroxynitrite
L-NAME	<i>N</i> ^G -nitro-L-arginine methyl ester
UA	uric acid
P_{O2}	partial pressure of O ₂
[Ca²⁺]_i	intracellular Ca ²⁺ concentration
[Ca²⁺]_{mt}	mitochondrial Ca ²⁺ concentration
AMPK	AMP kinase
RO	reoxygenation
mtPTP	mitochondrial permeability transition pore
IPC	ischemic preconditioning
Mfns	mitofusins
Opa1	optic atrophy protein 1
Drp1	dynamin-related protein 1
Mff	mitochondrial fission factor
PINK1	phosphatase and tensin homolog-induced putative kinase 1
Bcl-2	B-cell lymphoma 2
BAX	Bcl-2-associated X protein
mTOR	mammalian target of rapamycin
LC3	microtubule-associated protein 1 light chain 3
CQ	chloroquine
NH₄Cl	ammonium chloride
Atg	autophagy-related protein

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Fig. 1.

Schematic diagram of $O_2^{\bullet-}$ production by the mitochondrial ETC and its conversion to other ROS, such as H_2O_2 , and, in the presence of NO , to the RNS $ONOO^{\bullet}$. $ONOO^{\bullet}$, a potent oxidant, can cause damage to proteins, lipids and nucleic acids. H_2O_2 can also convert to $OH^{\bullet}$, another potent oxidant that can cause intramitochondrial damage.

Fig. 2.

Interrelationships among Ca^{2+} , NO/ONOO and mitochondrial $\text{O}_2^{\bullet-}$ in ECs exposed to shear stress (SS or PS) under physiological conditions (top part) or in the context of RP (following ischemia; bottom part). In the top (green) part, although the shear-induced NO increases mitochondrial $\text{O}_2^{\bullet-}$, the mitochondria maintain their function. In the bottom (red) part, both $[\text{Ca}^{2+}]_{mt}$ overload and intramitochondrial ONOO contribute to the collapse of ψ_m resulting in cytochrome c release and EC apoptosis. Mitochondrial ROS regulate, and are regulated by, $[\text{Ca}^{2+}]_{mt}$, ONOO and ψ_m . Solid lines indicate activation; broken lines indicate inhibition. (Modified from schematic in reference¹³⁷).

Fig. 3.

Mitochondrial dynamics and autophagy (for mitochondria, mitophagy). (a) Each mitochondrion cyclically shifts between fusion and fission states that are regulated by mitochondrial dynamics-related proteins: Mfns and Opa1 for fusion; Mff and Drp1 for fission. Oxidative stress is thought to activate fission and impair fusion. Mdivi-1 is a specific pharmacological inhibitor of Drp1 (and fission). Following fission, the fragment that undergoes sustained depolarization is targeted for mitophagy via PINK1 and parkin. (Modified from schematic in reference¹²⁵). (b) In autophagy, membrane structures sequester damaged organelles and proteins for delivery to and degradation by lysosomes. Key molecules involved in induction and formation of autophagosomes are listed under steps 1 and 2. Oxidative stress is thought to activate autophagy/mitophagy. Rapamycin is an agent that inhibits mTOR and induces autophagy; 3-methyladenine (3-MA) inhibits PI3K III and autophagy; CQ or NH₄Cl is used to inhibit the fusion step.

Fig. 4.

Shear stress as a determinant of EC survival vs. apoptosis. Under physiological conditions (left panel), SS or PS regulates ROS/mitochondrial ROS and RNS and, via posttranslational oxidative modifications of proteins and possibly maintenance of the autophagic machinery, ensures appropriate gene expression/protein synthesis that promotes cell health. In contrast, SS or PS during RP (right panel) results in enhanced ROS/mitochondrial ROS and RNS formation leading to posttranslational protein modifications, mitochondrial fission (further increases mitochondrial ROS) and propensity to apoptosis. Autophagy and, in particular, mitophagy play either a protective or a detrimental role. (Dashed outlined boxes indicate unknown signaling pathways).