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FORUM REVIEW ARTICLE

Regulation of Endothelial Function by Mitochondrial Reactive Oxygen Species

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Abstract

Mitochondria are well known for their central roles in ATP production, calcium homeostasis, and heme and steroid biosynthesis. However, mitochondrial reactive oxygen species (ROS), including superoxide and hydrogen peroxide, once thought to be toxic byproducts of mitochondrial physiologic activities, have recently been recognized as important cell-signaling molecules in the vascular endothelium, where their production, conversion, and destruction are highly regulated. Mitochondrial reactive oxygen species appear to regulate important vascular homeostatic functions under basal conditions in a variety of vascular beds, where, in particular, they contribute to endothelium-dependent vasodilation. On exposure to cardiovascular risk factors, endothelial mitochondria produce excessive ROS in concert with other cellular ROS sources. Mitochondrial ROS, in this setting, act as important signaling molecules activating prothrombotic and proinflammatory pathways in the vascular endothelium, a process that initially manifests itself as endothelial dysfunction and, if persistent, may lead to the development of atherosclerotic plaques. This review concentrates on emerging appreciation of the importance of mitochondrial ROS as cell-signaling molecules in the vascular endothelium under both physiologic and pathophysiologic conditions. Future potential avenues of research in this field also are discussed. *Antioxid. Redox Signal.* 15, 1517–1530.

Introduction

THE ENDOTHELIUM is well recognized as a central regulator of vascular homeostasis, and the development of endothelial dysfunction is known to precede the development of atherosclerosis and portend cardiovascular risk (168). The role of oxidative stress in the development of endothelial dysfunction and subsequent atherosclerotic disease has been the subject of extensive study over the past three decades. Initial work in this field considered reactive oxygen species (ROS) as the primarily toxic metabolic by-products with adverse effects on vascular function from direct damage to key cellular proteins and overall reduction in bioavailable endothelium-derived nitric oxide (NO). However, in recent years, a novel emerging view identifies endothelial cell ROS as prominent signaling molecules in cellular regulatory cascades, important under in both normal and pathophysiologic states (51, 77, 84, 120, 146, 150, 157, 172, 173).

As a major cellular source of ROS, mitochondria have recently garnered increased attention for their contribution to the detrimental effects of cardiovascular risk factors (7, 95, 126). Common cardiovascular risk factors, including diabetes, hypertension, and hyperlipidemia, induce pathologic alterations to the vascular phenotype through signaling pathways that require increases in mitochondrial ROS production above basal levels (113, 167, 184). These findings suggest excessive mitochondrial ROS act to encourage pathologic cell-signaling cascades under cellular conditions of overall excessive oxidative stress (125, 150, 180). However, mitochondrial-derived ROS also exhibit important physiological modulating effects on vasomotor tone in response to mechanical forces in both normal and diseased microvascular beds (90, 91). These data strongly suggest that homeostatic levels of mitochondrial ROS production act as important cell-signaling molecules, even in the absence of excessive oxidative stress.

We review the growing literature implicating mitochondrial ROS as key regulators of the vascular response to homeostatic and pathological stimuli. We discuss the regulation of the production, distribution, and destruction of mitochondrial ROS in the vascular endothelium, their role in regulating signaling cascades important in vasomotor activity, and roles in pathogenic signaling cascades in the setting of traditional cardiovascular risk factors that ultimately lead to the development of endothelial dysfunction and atherosclerosis.

Finally, we identify potential targets for favorably modulating endothelial mitochondrial ROS production for therapeutic intent.

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Mitochondrial ROS Production

General characteristics

Mitochondria reign as a major source of cellular ROS, with reports that between 0.2% and 2% of cellular mitochondrial oxygen consumption generates superoxide (24, 31, 129). Based on data from isolated mitochondrial samples, organelle concentrations of oxygen and superoxide range between 3 and 30 μM and 10 and 200 pM, respectively (4, 171). The rate of hydrogen peroxide production in isolated mitochondria varies between approximately 0.1 and 0.2 nmol/min/mg protein (136). The local concentrations of superoxide and hydrogen peroxide likely vary among tissues, based on local conditions (44). Exact tissue-specific superoxide concentrations and production rates *in vivo* remain only estimates, as our ability to make *in vivo* measurements of mitochondrial ROS remains limited.

Multiple potential mitochondrial sites of reactive oxygen species generation exist. These include Krebs cycle enzymes involved in redox reactions such as α-ketoglutarate dehydrogenase and aconitase (116), glycerol-3-phosphate dehydrogenase from the glycerol phosphate shuttle (108), and monoamine oxidase (19, 162). However, the vast majority of superoxide from mitochondria is produced by, or modulated by the complexes of the electron-transport chain (ETC) (110).

The ETC consists of four protein complexes embedded in the mitochondrial inner membrane. The spatial orientation is shown in Fig. 1. The ETC accepts electrons from reducing equivalents (NADH to complex I and $FADH_2$ produced from the glucose and fatty acid oxidation, as well as from the glycerol phosphate shuttle related to glycolysis to complex II). Donated electrons are passed down their electrochemical gradient through the complexes of the ETC, including reactive intermediaries (ubiquinone and cytochrome *c*), ultimately reducing O_2 to water. At multiple steps in this process, protons are pumped from the mitochondrial matrix into the intermembrane space, generating a proton motive force (Δp) that is a combination of the mitochondrial membrane potential created by the voltage gradient across the inner mitochondrial membrane ($\Delta\psi_m$) and the pH gradient created by proton transport during electron transfer. Δp is central to ATP production (139).

At the individual protein-complex level, the rate of production of superoxide by the mitochondrial ETC depends on several factors, including the availability of ROS sites of production, as well as oxygen tension (68, 78).

ETC sources of ROS

The major ETC sources of ROS from mitochondria are complexes I and III (24, 182). Complex II may also generate ROS (182), but this may not be independent of complex I. Local environmental conditions, including type and amount of substrate, pH, local oxygen tension, and the overall level of oxidative stress, strongly influence the site specificity and concentration of ROS produced (24).

Complex I is the initial entry point for NADH reducing equivalents and is comprised of more than 40 proteins (66, 137). Under physiologic conditions favorable to ATP production, the complex I flavin mononucleotide exposed in the mitochondrial matrix receives electrons from nicotine adenine dinucleotide (NADH) and passes these electrons through several iron-sulfur centers to the complex I ubiquinone-binding site, where electrons can reduce ubiquinone to semiquinone and subsequently to ubiquinol. In states of high $NADH/NAD^+$ ratios and minimal ATP need (*i.e.*, a fuel-replete state), electrons can be passed from the fully reduced FMN site on complex I to O_2 to produce superoxide. This mechanism is proposed as an important *in vivo* source of oxidative damage to ETC proteins (147, 164).

Alternatively, in the setting of high Δp and succinate levels and a low $NADH/NAD^+$ ratio, as may be seen when fatty acids are the primary fuel of mitochondria (87), electrons en-

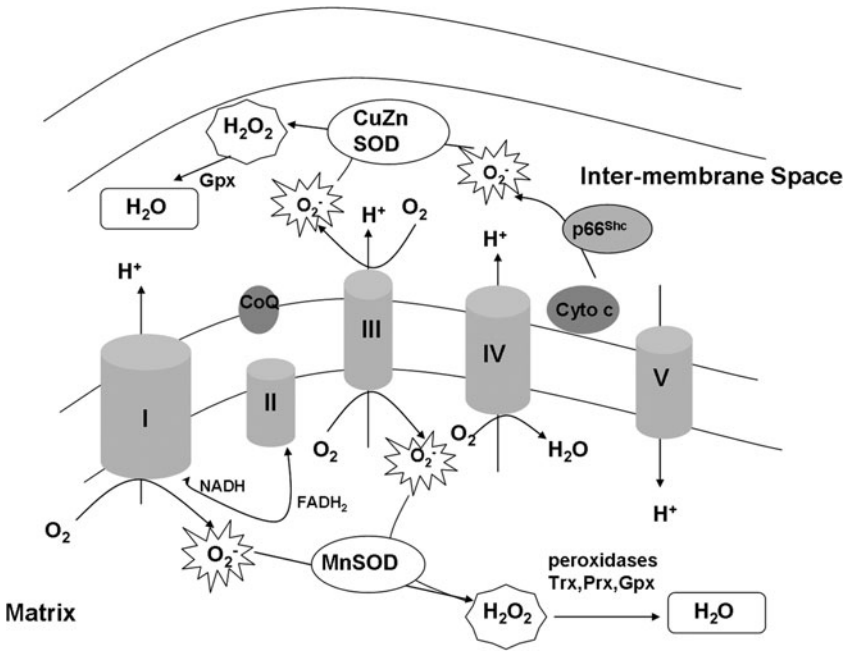

FIG. 1. Electron-transport chain and reactive oxygen species production. The process of oxidative phosphorylation receives reducing equivalents from the Krebs cycle (NADH to complex I and $FADH_2$ to complex II) and passes these electrons down the transport chain, ultimately to reduce oxygen to water. The fidelity of the process is incomplete, and the relative fidelity of the process depends on local environmental conditions. As such, oxygen can be reduced to O_2^- in at least three sites within the mitochondria: complexes, I, III, and through $p66^{Shc}$. O_2^- that does not escape the mitochondria at this point is rapidly reduced to H_2O_2 by manganese superoxide dismutase (MnSOD) and copper-zinc superoxide dismutase (CuZnSOD) in the matrix and intermembrane space, respectively. H_2O_2 either may leave the mitochondria and react with mitochondria proteins, or may be reduced to H_2O by local peroxidase enzymes. CoQ, coenzyme Q/ubiquinone; cyto C,cytochrome *c*.

tering the ETC through complex II via $FADH_2$ can backflow to complex I through reactive quinone intermediates and produce superoxide (65, 76). This reverse electron transport appears sensitive to reductions in the pH gradient between the matrix and the intermembrane space (89). The exact site of superoxide production by this mechanism is not known, but may be the ubiquinone binding site or a distal iron-sulfur cluster in complex I (87). Regardless of the mechanism, complex I–derived ROS appear to be formed primarily in the mitochondrial matrix.

Complex III also contributes to overall mitochondrial ROS production (23, 155, 182) through the two-step Q cycle. In this process, electrons are passed from ubiquinol to cytochrome *c* with reactive semiquinone intermediates that face both the matrix and the intermembrane space. Complex III can produce superoxide in either the matrix or the intermembrane space during Q-cycle inhibition, depending on whether uncoupling occurs at the matrix or the intermembrane space component of the Q cycle (59, 109). The *in vivo* physiologic importance of complex III relative to complex I ROS production remains controversial (110, 149).

A third and novel mechanism for mitochondrial ROS generation has been reported to involve $p66^{Shc}$, which can transfer electrons directly from cytochrome *c* to oxygen to form superoxide (54). This mechanism appears to be important in settings of cellular oxidative stress and particularly for apoptotic signaling (153).

Recent data suggest that ROS may be produced in bursts from mitochondria, related to brief periods of inner-membrane depolarization (165). The relation between these bursts of superoxide production and ETC complex ROS production remains to be elucidated.

Mitochondrial ROS as Cell-Signaling Molecules

Mitochondrial ROS as vascular cell-signaling molecules has significant biologic plausibility

For years, the pervasive impression has been that mitochondrial ROS are toxic by-products of energy production. However, a unique metabolic feature of endothelial cells provides the flexibility to modulate the mitochondrial respiratory rate and closely to regulate ROS production. Specifically, under most metabolic conditions, energy requirements of endothelial cells are met through anaerobic glycolysis (36) rather than through oxidative phosphorylation (37, 100, 142). Thus, endothelial mitochondrial ROS production within the ETC can be modulated by second-messenger systems without jeopardizing cellular energy requirements.

Mitochondrial ROS can escape from both the intermembrane space and the matrix into the cytosol. Superoxide in the intermembrane space may leave the mitochondria through voltage-dependent anion channels located in the outer membrane (58). However, given its electrophilic nature and short half-life, superoxide is a poor candidate molecule for mitochondrial-based cell signaling. However, superoxide is rapidly reduced hydrogen peroxide (H_2O_2) in both the intermembrane space via copper-zinc superoxide dismutase (CuZn SOD, SOD1) and the matrix, via manganese superoxide dismutase (MnSOD,SOD2) (24, 115). MnSOD is post-translationally targeted specifically to the mitochondrial matrix and appears to be centrally important in maintaining vascular homeostasis (170). ApoE-knockout mice also made deficient in MnSOD have greater impairment in endothelial function compared with ApoE-null mice (114).

An advantage of H_2O_2 as a cell-signaling molecule is its greater stability, leading to a longer *in vivo* half-life. The concentration of H_2O_2 in mitochondria is 100 times greater than that of superoxide (24). Most important, hydrogen peroxide's lack of charge allows it to pass freely through cell membranes. Emerging evidence suggests that H_2O_2 transport from mitochondria to the cytosol is enhanced by the presence of aquaporins, particularly aquaporin 8, on mitochondrial membranes (14, 15, 25). The presence of these aquaporins in the inner mitochondrial membrane appears important to maintaining cell viability through mitochondrial volume regulation (86).

Mitochondrial ROS production and removal are highly regulated

The presence of multiple pathways involved in regulating mitochondrial ROS production and destruction argues strongly for a significant role of mitochondrial ROS in cell signaling. These regulators include novel mitochondria-specific features (mitochondrial membrane potential, $\Delta\Psi_m$, dismutase and peroxidase enzymes), molecules well recognized for their importance in cell-signaling cascades (NO, calcium), and non-mitochondrial sources of cellular ROS. As described later and demonstrated in Fig. 2, these regulatory stimuli interact in coordinated fashion to modulate overall levels of mitochondrial ROS.

Mitochondrial membrane potential

As described previously, the mitochondrial membrane potential ($\Delta\Psi_m$) is the portion of the proton motive force accounted for by the transfer of electrons through the ETC. Therefore, $\Delta\Psi_m$ is sensitive to ETC substrate conditions (46, 140) pH, and the fidelity of electron transport, with higher, more polarized $\Delta\Psi_m$ generally associated with greater mitochondrial superoxide production (140). When $\Delta\Psi_m$ is maximal, protons are less avidly pumped from the matrix to the intermembrane space, reducing ETC flux and increasing the

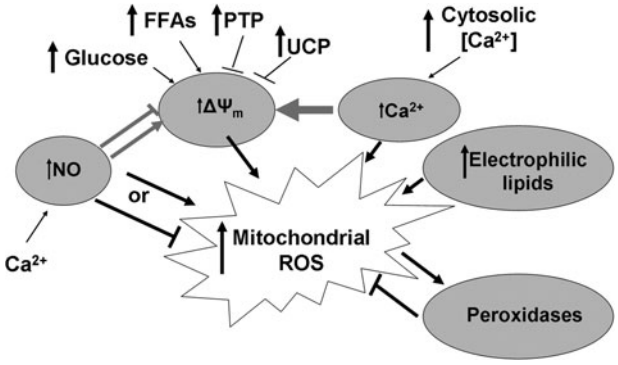

FIG. 2. Regulation of mitochondrial ROS production. Major regulators of mitochondrial ROS production include nitric oxide (NO), calcium, the mitochondrial membrane potential ($\Delta\Psi_m$), and electrophilic lipids. Interestingly, these effects are often coordinated. $\Delta\Psi_m$ is highly influenced by the cellular fuel supply, and uncoupling proteins (UCPs) and the permeability transition pore (PTP) also play significant roles in modulation of mitochondrial ROS through $\Delta\Psi_m$.

half-life of redox unstable intermediates (150). Regulatory proteins in the mitochondrial inner membrane also play a key role in modulating $\Delta\Psi_m$. Specifically, mitochondrial uncoupling proteins (UCPs) can open in the inner membrane, leading to membrane depolarization and partial uncoupling of ATP synthesis from oxidative phosphorylation by a reduction in the proton gradient. Suppression of UCP expression increases ROS production (43), whereas overexpression of UCPs may attenuate mitochondrial ROS production, dependent on cellular ATP status (46, 83). Further, UCP2 transcription is significantly upregulated in states of excessive oxidative stress and elevated $\Delta\Psi_m$, suggesting a role in feedback regulation of $\Delta\Psi_m$ (122), whereas UCP overexpression reverses excessive substrate-induced oxidative stress (113). The mitochondrial permeability transport pore (mPTP), which plays an important role in apoptotic signaling, may also play a role in ROS signal regulation (35, 165), but further data are needed, the better to elucidate its importance in ROS signaling in viable cells and tissues (69, 185).

Dismutase and peroxidase enzymes

Peroxidases are a group of compounds that enzymatically convert H_2O_2 to water. In the mitochondrial matrix, this group of compounds includes thioredoxin-2 (148), peroxiredoxin-3 (32), and glutaredoxin-2 (93). These compounds are upregulated in the setting of cellular oxidative stress likely to defend against the toxic consequences of elevated H_2O_2 levels (73, 131, 154). Coordinated regulation of peroxidase and MnSOD appears to be in part controlled by the transcriptional regulator PGC-1α (158), which in turn is upregulated by NO, another key controller of mitochondrial homeostasis in endothelial cells (18, 45). Peroxidase enzymes also are modulated by ambient calcium (104, 132).

Nitric oxide

The complex role that NO plays in regulating mitochondrial homeostasis in the endothelium has been the subject of a recent excellent review (45). NO encourages mitochondrial biogenesis through PGC-1α activation (175), and the identification of eNOS within the mitochondrial outer membrane suggests that local mitochondrial NO production may serve a mitochondrial-specific regulatory purpose (52).

NO is a known inhibitor of the respiratory chain, and the relatively high basal concentrations of NO in endothelial cells suggests that endothelial mitochondrial respiration is restricted under basal conditions (45). The relative effects of NO on respiration, $\Delta\Psi_m$, and mitochondrial ROS production are shown in Fig. 3. NO competes directly with O_2 at complex IV, reversibly inhibiting this complex and inducing ROS production (27, 128). NO also can inhibit complex I through *S*-nitrosylation (22, 28, 34, 38, 110). This is more likely to occur in settings of glutathione depletion and appears to reduce mitochondrial ROS. Interactions between NO and complex IV occur rapidly and reversibly, playing an important role in short-term regulation of respiration and ROS production, whereas complex I inhibition by nitroso compounds is longer lasting, suggesting a greater role in long-term regulation of respiration and ROS generation.

The situation becomes even further complicated in the setting of elevated systemic oxidative stress, as is seen with cardiovascular risk factors. NO reacts rapidly with excess superoxide to form peroxynitrite, which can irreversibly inhibit multiple complexes of the respiratory chain, as well as dismutase enzymes, leading initially to increased oxidative stress and increased $\Delta\Psi_m$ (53).

Calcium

The role of calcium (Ca^{2+}) in regulating mitochondrial ROS production in endothelial cells is integrally linked with other regulatory factors, including NO and $\Delta\Psi_m$. Increased calcium bioavailability from either intra- or extracellular sources increases NO production from eNOS (41). Ca^{2+} also increases flux through the Krebs cycle, leading to increased concentrations of reducing equivalents available for oxidative phosphorylation (98). Increases in cytosolic Ca^{2+} drives Ca^{2+} entry

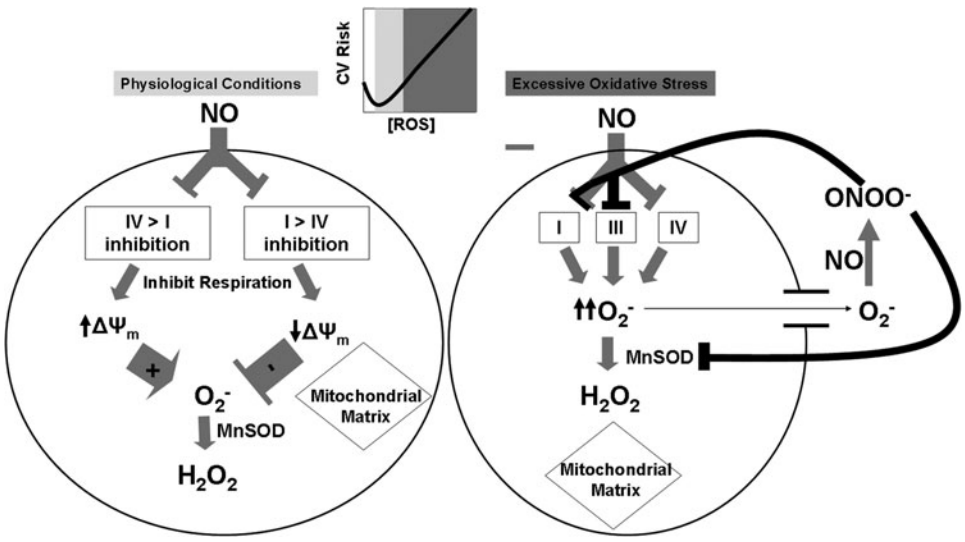

FIG. 3. Regulation of mitochondrial ROS in endothelium by NO. The overall effect of NO on mitochondrial ROS production is highly dependent on the relative inhibition of complex IV versus complex I under normal physiologic conditions. Complex IV inhibition occurs rapidly, and complex I inhibition appears to occur over a more prolonged time span. With excessive oxidative stress, as in the setting of cardiovascular risk factors (CV Risk) and atherosclerosis, superoxide reacts rapidly with NO to form peroxynitrite ($ONOO^-$), which inhibits multiple complexes of the respiratory chain as well as MnSOD. MnSOD is overwhelmed, leading to a significant increase in mitochondria-centered superoxide production. Importantly, an optimal level of ROS generation seems to exist, such that both excessive production of ROS (with reduced bioavailability of NO) and insufficient ROS (with attendant impaired physiologic signaling) can lead to a proinflammatory state and promote atherosclerosis.

into mitochondria through calcium uniporter channels and potentially mitochondria-based ryanodine receptors on the inner mitochondrial membrane (13, 39). Ca^{2+} influx into the matrix is also directly related to the magnitude of $\Delta\Psi_m$ (39). Increased calcium uptake into mitochondria also leads to an increase in mitochondrial ROS production (26). Taken together, these data suggest that a regulatory nexus of Ca^{2+}, NO, $\Delta\Psi_m$, and peroxidase enzymes coordinately modulate mitochondrial homeostasis in endothelial cells and the subsequent balance of mitochondrial ROS (Fig. 2).

Nonmitochondrial ROS sources

Emerging data suggest that nonmitochondrial-generated ROS can stimulate mitochondrial ROS production. Increased ROS production from specific, nonmitochondrial ROS-producing enzymes in the vasculature, including NADPH oxidase (42, 49, 85, 166, 174), xanthine oxidase (11), and uncoupled eNOS (30), are capable of coordinating ROS expression by mitochondria. Although this communication between nonmitochondrial ROS sources and mitochondria has been established, the optimal conditions and the mechanisms for this ROS-induced ROS release remain to be elucidated. Lipid peroxidation products with electrophilic centers may be one potential factor in this type of communication (80).

Mitochondrial ROS participate in vascular responses to mechanical stimuli

Mitochondrial ROS are tightly regulated in endothelial cells, providing the potential for a signaling role in vascular regulation, during both physiologic and pathologic stimuli. As a clinically relevant example, ROS generated from mitochondria are responsible for the vasodilator response to shear stress in patients with coronary artery disease (180).

In the microvasculature, important regulators of vascular homeostasis include not only NO and prostanoids, but also endothelium-derived hyperpolarizing factor (EDHF). Although the identity of EDHF may differ between vascular beds and in different pathologic states, H_2O_2 has been identified as an EDHF in multiple vascular beds including human coronary arterioles from patients with CAD (91, 102, 124), as well as human and mouse mesenteric arteries (96, 97).

The mechanism of flow-induced dilation in the human heart is unique. Isolated human coronary arterioles from discarded samples of the right atrial appendage ligated at the time of cardiopulmonary bypass, cannulated, pressurized, and subjected to laminar shear show an increase in superoxide and hydrogen peroxide production that is reversed by exposure to rotenone, a mitochondrial complex I inhibitor. Inhibition of complex I or complex III markedly reduces laminar shear–induced vasodilation. The mechanism of shear-induced vasodilation increases mitochondrial ROS and appears to involve an increase $\Delta\Psi_m$ that is opposed by NO-dependent ETC inhibition (60, 88). These alterations are consistent with established effects of both $\Delta\Psi_m$ and NO on mitochondrial ROS. In animal models, laminar shear upregulates both MnSOD and peroxiredoxin in the endothelium, suggesting a compensatory mechanism for tempering overall mitochondrial superoxide and H_2O_2 concentrations (1, 107).

While the mechanism for transduction of the shear signal from the cell surface to the mitochondria remains to be fully elucidated, the endothelial cytoskeleton appears to play a prominent role. Mechanical signals from the endothelial cell surface are transduced to cellular organelles through actin filaments, linking focal adhesion kinases to integrins located on the cell surface and at the abluminal basement membrane (40, 63). Cytoskeletal involvement in transducing shear-mediated dilation is observed in rat muscle arterioles, where NO and prostaglandins mediate the dilation (145), and in diseased human coronary arterioles, where hydrogen peroxide plays a key role (102). Human coronary arterioles exposed to inhibitors of actin and microtubule formation demonstrate blunted shear-induced vasodilation with a concomitant loss of superoxide and H_2O_2 production (90). The mechanism by which this signal is transmitted to the mitochondria is under investigation but may involve increases in endothelial cell calcium that are modulated by local NO production (99).

Mitochondrial ROS production in endothelial cells also appears to be important in regulating vascular responses to other mechanical stimuli. For example, both cyclic strain and stretch, important mechanical forces on the pulmonary vasculature, activate signaling cascades that stimulate production of NF-κB and endothelial cell adhesion molecules. This activation requires the production of mitochondrial ROS and an intact cytoskeleton (2, 3, 176). Redundancy exists with regard to cellular ROS sources important in shear-stress vasomotion. For example, NADPH oxidase ROS production appears to be an important mediator of pathologic cell signaling because of flow reversal and turbulent flow (55), as well as oscillatory shear (70, 71 141).

Mitochondrial ROS and pathologic vascular stressors

Excessive vascular oxidative stress not only reduces bioavailable NO through direct inactivation via production of peroxynitrite, but can also lead to eNOS uncoupling and further ROS production (81). NF-κB and protein kinase C (PKC) activation occur secondary to excessive mitochondrial ROS production in the endothelium, precipitating a range of proinflammatory and prothrombotic alterations in the endothelial phenotype (2, 9, 121, 127,156). Taken together, these data suggest that pathologic stressors common to cardiovascular risk factors can induce excessive mitochondrial ROS levels in the endothelium, leading to alterations in cell signaling and direct losses of bioavailable NO, hallmarks of endothelial dysfunction (Fig. 4).

These data support the concept that mitochondrial ROS levels serve as a barometer of overall vascular health in pathologic settings (Fig. 5) (163). As such, targeting mechanisms to reduce excessive mitochondrial ROS production may improve endothelial function and reduce cardiovascular events in those at high risk. The following sections review our current knowledge regarding the relation between cardiovascular risk factors and mitochondrial ROS, as well as the body of evidence supporting a pathogenic role for excessive mitochondrial ROS in the development of atherosclerotic disease through pathologic cell signaling.

The ability of endothelial cells to regulate mitochondrial ROS within a narrow physiologic range is important to normal vascular homeostasis. When antioxidant balancing systems are overwhelmed, as with pathologic vascular stressors, mitochondrial ROS production accelerates, leading to direct cellular oxidative damage and activation of pathologic

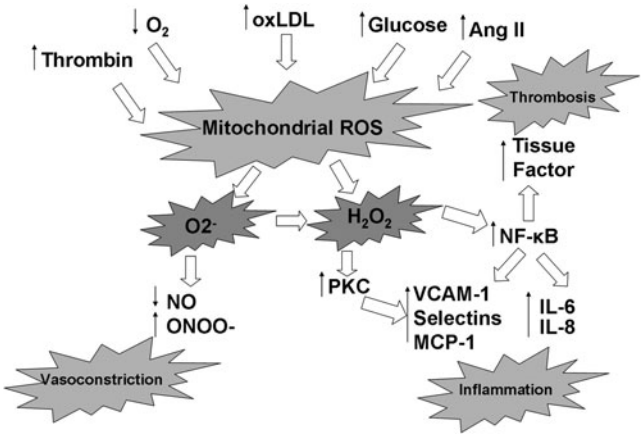

FIG. 4. Endothelial cell mitochondrial ROS response to pathologic stressors. A wide variety of pathologic stressors associated with cardiovascular risk factors are known to increase mitochondrial ROS production, including thrombin, hypoxemia, oxidized LDL, elevated angiotensin II levels, and elevated glucose levels. These have the effect of increasing both superoxide and hydrogen peroxide levels. This results in inactivation of endothelium-derived NO synthase and consumes any NO that is produced, unleashing proinflammatory signaling pathways. The consequence is increased expression of endothelial cell-adhesion molecules (VCAM-1, selectins, MCP-1), inflammatory cytokines (IL-6 and IL-8), and prothrombotic tissue factor.

cell-signaling pathways, as well as inactivation of protective mechanisms (Fig. 4). Seemingly disparate pathologic stressors, including thrombin activation of the PAR1/2 receptor (9, 33), leptin (177), hyperglycemia (113), angiotensin II (127), and hypoxemia (74, 121) all appear to induce excessive mitochondrial ROS production, leading to subsequent expression of a dysfunctional endothelial phenotype. Mechanistically, the excessive production of mitochondrial ROS reduces NO bioavailability (81) and leads to activation of NF-κB and protein kinase C (20, 156). This in turn leads to increased expression of cell-adhesion molecules, including ICAM-1, P-selectin, and E-selectin (72). Depletion of mitochondrial glutathione stores as an antioxidant defense produces a similar proinflammatory milieu with overexpression of E-selectin and VCAM-1, resulting in increased monocyte recruitment by endothelial cells (33). Mitochondrial ETC ROS production activates NF-κB resulting in increased production of the inflammatory cytokines IL-6 and IL-8 (121, 143) and prothrombotic tissue factor (9). In summary, multiple well-recognized pathophysiologic stimuli that act on the endothelium to produce a dysfunctional phenotype act at least in part through signaling pathways involving increases in mitochondrial ROS.

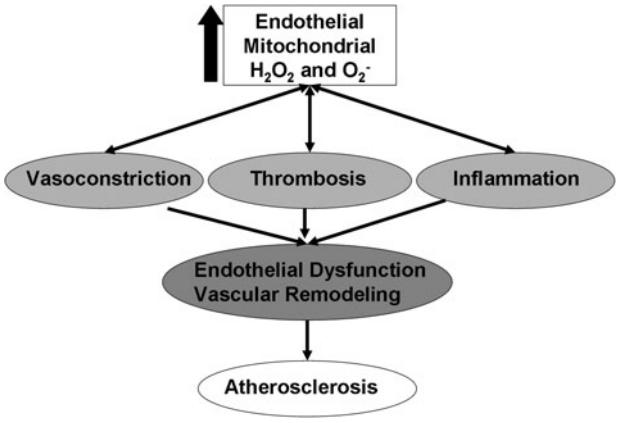

FIG. 5. Cascade of risk from excessive endothelial mitochondrial ROS production. Excessive mitochondrial ROS production induces endothelial dysfunction through multiple cell-signaling pathways. The endothelium develops this pathologic phenotype characterized by vasoconstriction, thrombosis, and inflammation. This condition is a precursor to the development of clinically relevant atherosclerosis and predicts future cardiovascular events.

Diabetes and mitochondrial ROS

A growing recognition exists of a link between mitochondrial morphology and function (133). This is best seen in the conditions of diabetes and insulin resistance, which are characterized by alterations in mitochondrial morphology, including smaller mitochondria with less-complex structure and reduced capacity for oxidative phosphorylation, elevated mitochondrial membrane potential, and reduced mitochondrial mass (106, 123). Microarray analyses of skeletal muscle from diabetic patients demonstrate reduced expression of oxidative phosphorylation–pathway elements. This can be reversed by peroxisome proliferator–activated receptor α coactivator 1α (PGC-1α) (105). PGC-1α plays an integral role in the regulation of mitochondrial biogenesis, and inhibition of PGC-1α activity leads to reductions in mitochondrial number (175). Prior *in vitro* cell culture and muscle biopsy data demonstrate decreased mitochondrial mass under hyperglycemic conditions, and this derangement also is present in the insulin-resistant offspring of type 2 diabetic patients (106).

Insulin resistance and diabetes are characterized by elevated circulating levels of glucose and free fatty acids (17, 130). In endothelial cells, hyperglycemia induces mitochondrial fission with the subsequent development of excessive mitochondrial ROS production, reduced ATP production, and blunted cell growth (179). Endothelial cell-culture data demonstrate that exposure to high glucose (30 m*M* for 7 days) increases endothelial ROS production. This phenomenon is primarily related to increased mitochondrial ROS, because blunting of this effect occurs only with pharmacologic inhibition of complex II, overexpression of UCP1, or exposure to MnSOD. Indicative of the cell-signaling capacity of mitochondrial ROS, mitochondrial ROS inhibitors also reduce NF-κB and protein kinase C activation, and blunt the production of toxic advanced glycation end products and sorbitol (113). Expression of cell-adhesion molecules on the endothelial cell surface is dependent on mitochondrial ROS in the setting of high glucose (10, 143).

Exposure of endothelial cells to high concentrations of free fatty acids (lysophosphatidylcholine and linoleic acid) increases $\Delta\Psi_m$, mitochondrial ROS, and NF-κB in human aortic endothelial cells (83). Each of these responses is inhibited by overexpression of UCP2, showing that reductions in mitochondrial ROS, linked to reductions in $\Delta\Psi_{m,}$ are responsible for the observed improvement in endothelial function. Similar

findings were noted in intact vessels in which overexpression of UCP2 in the rat aorta reversed the endothelial dysfunction induced by lysophosphatidylcholine) (83, 169).

Excessive mitochondrial ROS production in diabetes has been implicated as a "master switch" for activation of discrete pathologic signaling pathways leading to subsequent endothelial dysfunction through (a) protein kinase C activation, (b) increased age-related glycation end-product formation, and (c) increased polyol and hexosamine pathway flux (21). *In vivo* human studies have verified that hyperglycemia induces such a state of endothelial dysfunction through excessive oxidative stress. Hyperglycemia has been shown to inhibit endothelial function in the forearm microvasculature through suppression of NO bioavailability (169). This reduced dilation during acute hyperglycemia also is observed in patients with diabetes and can be improved with antioxidant therapy (12, 151, 152). The relation between human endothelial dysfunction and alterations in endothelial and inflammatory cell mitochondrial homeostasis in diabetes is relatively uncharted, although preliminary work from our laboratory suggests that altered mitochondrial homeostasis plays an important pathophysiologic role in diabetic endothelial dysfunction (unpublished results).

Dyslipidemia and mitochondrial ROS

Oxidized LDL (oxLDL) contributes fundamentally to the development of atherosclerosis (144). Mitochondrial ROS are important in the generation of oxLDL in endothelial cells (94). These oxidized lipids in turn modulate complex I activity, resulting in an increase in the expression of antioxidant genes, which attenuates the excessive oxidative stress (28, 183).

Recent data demonstrating electrophilic lipid-associated induction of mitochondrial ROS production suggest that oxidized lipids can act as messengers between nonmitochondrial sources of ROS and mitochondria (184). This concept has been buoyed by recent data demonstrating significant mitochondrial respiratory chain inhibition after exposure to extensively oxidized LDL, resulting in an increase in mitochondrial ROS production (134). The relevance of this coordinate expression of ROS to abnormalities of the vascular phenotype *in vivo* remains to be determined.

Hypertension and mitochondrial ROS

Hypertension (HTN) has a clear etiologic foundation based on enhanced oxidative stress in key target organs, including brain, kidney, and the vasculature (61). The development of HTN is associated with elevation in vascular tissue levels of superoxide, whereas infusions of superoxide scavengers can reduce blood pressure in animal models of HTN (82, 103, 111, 138). A key role for NADPH oxidase in the genesis of HTN-associated oxidative stress is well established (61, 67). However, both NADPH oxidase and mitochondria coordinately generate excessive ROS in multiple different animal models of HTN, including angiotensin II–related models (127, 167). Overexpression of mitochondrial matrix-based thioredoxin-2 in a transgenic mouse model reduces overall superoxide production and blunts the hypertensive effects of angiotensin II infusion (167). Dietary supplementation with a mitochondria-targeted antioxidant, MitoQ, attenuates the age-related hypertensive response in a spontaneous hypertensive rat model (56). These data provide feasibility for antihypertensive therapeutic interventions that target reductions in mitochondrial ROS.

Smoking and mitochondrial ROS

The adverse effects of cigarette smoke on mitochondrial homeostasis in lung tissue and myocardium have long been recognized and likely involve multiple components of tobacco smoke (57, 160). Cigarette smoking intake reduces inner mitochondrial membrane fluidity, inhibits respiration, and reduces ATP production through a switch from mitochondrial state 3 to state 4 respiration (50, 75). Recent data also demonstrate that acrolein, a reactive aldehyde in cigarette smoke, induces hyperpolarization of $\Delta\Psi_m$ with a resultant increase in ROS production (135). In addition, lipid-soluble components of cigarette smoke induce excessive mitochondrial ROS production in lung epithelial cells, contributing to toxicity in that organ (159).

The adverse effects of smoking on vascular function are well established, and both direct and second-hand exposure to cigarette smoke rapidly induces endothelial dysfunction (29, 62). Functionally, acute exposure of endothelial cells to tobacco smoke leads to a rapid reduction in $\Delta\Psi_m$ and subsequent cell death (161, 178). Further, exposure of endothelial cells to the contents of cigarette smoke leads to a short-term upregulation of proinflammatory and compensatory heat-shock and antioxidant genes (64). The inflammatory response can be reversed by the addition of the thiol antioxidant *N*-acetylcysteine, suggesting oxidative stress as a mechanism for the cigarette-smoke–related effects on the endothelium.

Similar to the alterations seen in isolated mitochondria and lung cells, cigarette smoke alters both mitochondrial morphology and function in endothelial cells, leading to mitochondrial DNA damage and cell death. Endothelial cells in the umbilical arteries of newborns of smoking mothers demonstrate an increased in mitochondrial content relative to those of nonsmoking mothers (6). In a mouse model of atherosclerosis, exposure to second-hand smoke accelerates mitochondrial DNA damage, oxidative impairment of mitochondrial enzymes, and accelerated atherogenesis (159).

Atherosclerosis and mitochondrial ROS

Multiple lines of evidence support the concept that excessive mitochondrial ROS production and its subsequent downstream signaling effects are contributory in the pathogenesis of atherosclerosis (95). Mitochondrial DNA (mtDNA) integrity, an index of mitochondrial oxidative stress, is inversely correlated with the development of atherogenesis. mtDNA is circular and contains 13 genes critical to the respiratory chain (5). The mitochondrial DNA lack of protective histones, poor DNA-repair mechanisms, and close proximity to ROS being produced in matrix and intermembrane space make it exquisitely sensitive as an indicator of oxidative damage, especially compared with genomic DNA (92). Oxidative damage to mtDNA encoding for proteins important to complex I or ATP synthase leads to further increases in mitochondrial ROS production, a process known as ROS-induced ROS release, and demonstrates a direct link between mtDNA damage and excess mitochondrial ROS (112). Increased mtDNA damage is observed histologically to a much greater degree in atherosclerotic human arterial tissue than in nonatherosclerotic regions from the same patients (8). In an

ApoE-knockout mouse model, mtDNA damage precedes the development of atherosclerotic lesions, establishing temporal plausibility for cause and effect. When a genetic deficiency of MnSOD is superimposed on this model through breeding to create mice that were also heterozygous knockouts for MnSOD, accelerated atherosclerosis is observed (8). The role of excessive mitochondrial ROS in the pathogenesis of atherosclerosis also is reinforced by ApoE-knockout mice studies, in which transgenic overexpression of thioredoxin-2 improves endothelial function and reduces the formation of atherosclerotic plaques in part by reducing oxidative stress (181), as well as human epidemiologic data suggesting that genetic polymorphisms leading to reduced MnSOD function are associated with increased atherosclerotic risk (48).

Another approach that has been used to implicate mitochondrial ROS in atherosclerosis is through modulation of mitochondrial membrane potential. As mentioned earlier, hyperpolarization leads to enhanced ROS generation. When an atherogenic mouse model undergoes bone-marrow transplant of marrow from a UCP2-knockout mouse, accelerated atherosclerosis results (16). Thus monocyte mitochondrial homeostasis can contribute to atherosclerotic plaque formation. UCP2-knockout mice fed an atherogenic diet show significantly greater aortic atheroma formation relative to control mice, suggesting that the well-known link between dietary fuel sources and atherosclerosis is critically dependent on mitochondrial homeostasis and membrane potential. This mechanistic study was recently corroborated by a genetic epidemiologic study in humans, demonstrating that a loss of function polymorphism in UCP2 is associated with elevations in cardiovascular risk in a selected population (79).

Conclusions and Future Directions

Based on the available body of evidence, mitochondrial ROS levels are tightly regulated. Regulators include mitochondria-specific enzymes, mitochondrial energetics, well-characterized second-messenger signaling molecules, and novel mechanisms for coordination of total cellular ROS production. Basal levels of mitochondrial ROS are important for maintaining vascular homeostasis in the human coronary microvasculature under normal physiological conditions. Traditional cardiovascular risk factors, which are associated with excessive vascular oxidative stress and endothelial dysfunction, exert at least a portion of their adverse effects on vascular homeostasis in cell culture and animal models through signaling pathways involving increases in mitochondrial ROS concentrations to levels significantly higher than those seen in the basal state. Finally, cell-culture and animal studies provide strong evidence that excessive mitochondrial ROS turn on mechanisms important in the pathogenesis of atherosclerosis.

A multitude of questions remain unanswered regarding the roles of mitochondrial ROS in cell signaling under both physiologic and pathophysiologic conditions. These include determining the differential expression and signaling of mitochondrial ROS under a variety of fuel-substrate states (*i.e.*, high succinate vs. high malate fuel states), the mechanisms of coordinate expression of ROS from mitochondrial and nonmitochondrial sources, and the role of mitochondria in regulating vascular endothelial function under normal physiologic conditions in conduit vessels relevant to the development of clinical atherosclerosis. Given the clear propensity for increased mitochondrial ROS production in fuel-replete states or under the influence of external regulators leading to a similar state of mitochondrial respiration, determination of mechanisms to alter mitochondrial fuel states will likely have an important impact on our understanding of the regulation of mitochondria ROS production under normal physiologic and pathophysiologic conditions. From a simplistic standpoint, exercise or limiting food intake may operate through the same mechanism for atheroprotection by reducing mitochondrial membrane potential and ROS production.

Table 1. Potential Therapeutic Targets for Reducing Excessive Mitochondrial ROS

Reduce excess mitochondrial O_2^- production	$\downarrow\Delta\Psi_m$ $\downarrow p66^{Shc}$ ↓Mitochondrial fission
Quenching of excess mitochondrial O_2^- production	↑MnSOD
Excess mitochondrial H_2O_2 destruction	↑Grx ↑Prx ↑Trx
Quench excess mitochondrial O_2^-	Mitochondrial Targeted Antioxidants
Inhibit ROS from nonmitochondrial sources	↓NADPH Oxidase ↓oxLDL

To apply the knowledge gained from the laboratory bench, translational studies are needed to determine whether mitochondrial ROS signaling pathways are relevant to the development of endothelial dysfunction and atherosclerosis in humans. Such human studies will necessitate the design and testing of novel, mitochondria-specific interventions. Potential therapeutic targets are listed in Table 1. Recent data from animal studies show that MitoQ, a mitochondria-targeted antioxidant, is an effective antihypertensive agent, demonstrating the potential promise of this approach (56). Data demonstrating an increased life span and protection from age-related endothelial dysfunction in $p66^{Shc}$-knockout animals suggest $p66^{Shc}$ as a potential target as well (47, 101). Recent data also support a role for mitochondria as generators of signaling molecules in the circulation, affecting systemic endothelial function and blood pressure (117–119). Investigations into the potential role of mitochondrial proteins as circulating hormone-like regulators of systemic vascular homeostasis (*e.g.*, coupling factor 6) may be of clinical importance.

Although numerous conceptual and methodologic challenges remain, investigations into the mechanisms and roles of mitochondrial ROS in regulating vascular homeostasis are likely to yield important, clinically relevant findings.

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Abbreviations Used

$\Delta\psi m$ = mitochondrial membrane potential
Δp = proton motive force
ApoE = apolipoprotein E
ETC = electron-transport chain
FMN = flavin mononucleotide
Grx = glutathione reductase
mPTP = mitochondrial permeability transition pore
mtDNA = mitochondrial DNA
oxLDL = oxidized LDL
Prx = peroxiredoxin
ROS = reactive oxygen species
Trx = thioredoxin
UCP = uncoupling protein