Mitochondrial reactive oxygen species-mediated signaling in endothelial cells

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Zhang DX, Gutterman DD. Mitochondrial reactive oxygen species-mediated signaling in endothelial cells. Am J Physiol Heart Circ Physiol 292: H2023–H2031, 2006. First published January 19, 2007; doi:10.1152/ajpheart.01283.2006.—Once thought of as toxic by-products of cellular metabolism, reactive oxygen species (ROS) have been implicated in a large variety of cell-signaling processes. Several enzymatic systems contribute to ROS production in vascular endothelial cells, including NAD(P)H oxidase, xanthine oxidase, uncoupled endothelial nitric oxide synthase, and the mitochondrial electron transport chain. The respiratory chain is the major source of ROS in most mammalian cells, but the role of mitochondria-derived ROS in vascular cell signaling has received little attention. A new paradigm has evolved in recent years postulating that, in addition to producing ATP, mitochondria also play a key role in cell signaling and regulate a variety of cellular functions. This review focuses on the emerging role of mitochondrial ROS as signaling molecules in vascular endothelial cells. Specifically, we discuss some recent findings that indicate that mitochondrial ROS regulate vascular endothelial function, focusing on major sites of ROS production in endothelial mitochondria, factors modulating mitochondrial ROS production, the physiological and clinical implications of endothelial mitochondrial ROS, and methodological considerations in the study of mitochondrial contribution to vascular ROS generation.

REACTIVE OXYGEN SPECIES (ROS), such as superoxide (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$), are traditionally thought to be toxic by-products of cellular metabolism, which nonspecifically damage nucleic acids, proteins, lipids, and other cellular components. There is now a large body of evidence showing that ROS at moderate concentrations act as signaling molecules and play an important role in the regulation of various vascular cell functions (16, 32, 45, 57, 85). In vascular endothelial cells, ROS regulate vascular tone, oxygen sensing, cell growth and proliferation, apoptosis, and inflammatory responses. The heterogeneous response profile to ROS may reflect localized actions or qualitative differences due to concentration. In addition to these regulatory functions under physiological conditions, excessive or sustained ROS have been implicated in the pathogenesis of various cardiovascular diseases, such as atherosclerosis, hypertension, diabetic cardiovascular complications, and ischemia-reperfusion injury (52).

A variety of cellular enzyme systems are potential sources of ROS, including NAD(P)H oxidase, xanthine oxidase, uncoupled endothelial nitric oxide (NO) synthase (eNOS), arachidonic acid metabolizing enzymes including cytochrome P-450 enzymes, lipoxygenase and cyclooxygenase, and the mitochondrial respiratory chain (52, 59). Although the contribution of individual sources may depend on the tissues and cells involved, four enzyme systems thought to predominate in vascular endothelial ROS generation: NAD(P)H oxidase, xanthine oxidase, uncoupled eNOS, and mitochondrial electron leakage (52, 59). The former three enzymes have been extensively studied, and their importance has been demonstrated in various physiological and pathological settings. However, the mitochondrial respiratory chain is the major source of ROS in most mammalian cells. Excess production of ROS from mitochondria has been implicated in aging and a range of degenerative diseases (15, 34, 75).

Until recently, the functional significance of mitochondria-derived ROS in vascular endothelial cells has received little attention. This is partly attributable to the observations that vascular cells in general exhibit low metabolic activity (69, 70) and that mitochondria-generated ROS are less well regulated compared with other enzymes like NAD(P)H oxidase (28). However, a paradigm shift has occurred in recent years, focusing greater attention on a potential key role of mitochondrial ROS in vascular signaling (35).

Production of ROS in Mitochondria

Within mitochondria, the primary site of ROS generation is the electron transport chain. There are four protein complexes associated with the respiratory chain. Complex I, or NADH-ubiquinone oxidoreductase, accepts electrons from NADH; these electrons are carried to complex II, succinate dehydrogenase, and used to oxidize succinate to fumarate. Electrons continue to travel down their electrochemical gradient to complex III (ubiquinol-cytochrome c oxidoreductase), and subsequently to complex IV (cytochrome c oxidase), and are finally used to reduce molecular oxygen to water. Although the majority of molecular oxygen is reduced at complex IV to water, 1–4% of the oxygen is incompletely reduced to O$_2^-$, which can yield other ROS via various enzymatic or nonenzymatic reactions (Fig. 1).
The mitochondrial electron transport chain generates $O_2^-$ primarily at complexes I and III. Complex III produces $O_2^-$ by autoxidation of the ubisemiquinone radical intermediate (QH$^-$), formed during the Q cycle in the complex, with the Qo site of the complex close to the intermembrane space being the major site of $O_2^-$ production. The Qi site of complex III located close to the matrix side is less likely to react with oxygen and form $O_2^-$ since the Qi site firmly binds QH$^-$ and stabilizes it. Indeed, selective inhibitors of the Qi portion of the cycle, such as antimycin B, prolong the lifetime of ubisemiquinone at the Qo site and hence result in excess release of $O_2^-$. Conversely, inhibition of the proximal Qo site by compounds such as myxothiazol inhibits the formation of ubisemiquinone at the Qo site and thus reduces the production of $O_2^-$. Although not well understood, myxothiazol has also been reported to induce $O_2^-$ production since it allows formation but not oxidation of QH$^-$ at the distal niche of the Qo site (62). Complex III has the capacity to release $O_2^-$ to both sides of the mitochondrial inner membrane, depending on the portion of the Q cycle involved (38, 61).

In contrast, complex I-derived $O_2^-$ seems predominately released into the matrix. Although precise mechanisms of $O_2^-$ generation are largely unknown, it is suggested that complex I produces $O_2^-$ by reverse electron transfer from complex II upon succinate oxidation in the absence of NADH-linked substrates or in much lower amounts in the forward electron transfer from the NADH-linked substrates (51). Significant $O_2^-$ production from complex I was observed via reverse electron transfer, and this mechanism may account for more physiologically relevant ROS produced from mitochondria (47). It is suggested that an iron-sulfur cluster distal in the electron transfer route of the complex could be the site of electron leak and $O_2^-$ production (51).

Each mitochondrial site of superoxide generation may play a distinct role during different stimuli. Complex III has been described as the main site of ROS production in human umbilical vein endothelial cells during hypoxia-reoxygenation (81) and after stimulation with TNF-$\alpha$ (60). In contrast, complex II plays a more important role in lysophosphatidylcholine-induced ROS formation in these cells (84). It is unproved but likely that this complex II-dependent ROS production is mediated via the reverse electron transport mechanism. Complex I and/or III are responsible for much of the generated ROS that elicits dilation in response to shear stress in human coronary arteriolar endothelial cells (54).

The primary ROS produced by mitochondria is $O_2^-$, either in the matrix or the intermembrane space. As a charged species, $O_2^-$ is not readily diffusible across mitochondrial membranes. However, the mitochondrial permeability transition pore, containing the voltage-dependent mitochondrial anion channel, might serve as a conduit for intermembranous mitochondrial $O_2^-$ to pass through the outer mitochondrial membrane and into the cytosol (37). Probably a more important mechanism for transmembrane movement of reduced oxygen involves dismutation to $H_2O_2$ by superoxide dismutase (SOD). Once generated, the uncharged ROS $H_2O_2$ can easily move across the membrane.

There are three isoforms of SOD in the vessel wall: copper/zinc SOD (CuZn-SOD or SOD1), manganese SOD (Mn-SOD or SOD2), and an extracellular CuZn-SOD (EC-SOD or SOD3) (31). CuZn-SOD is located in the cytosol, nucleus, and intermembrane space of mitochondria (68). Mn-SOD is expressed solely in the mitochondrial matrix, and EC-SOD is found in the extracellular space. Each contributes to the reduction of superoxide to $H_2O_2$. The physiological importance of Mn-SOD is demonstrated by the fact that in contrast to other SOD isoforms, genetic elimination of this isoform is embryonically lethal (49, 53).

$H_2O_2$ can be further reduced by catalase and glutathione peroxidase. Glutathione peroxidase uses GSH to reduce $H_2O_2$.
and lipid peroxides to water and corresponding alcohols, respectively. This is the primary mechanism for eliminating H$_2$O$_2$ in cytosol and mitochondria. Catalase is located mainly in peroxisomes and exclusively catalyzes H$_2$O$_2$ to water. Catalase may be an important protective mechanism against a high concentration of H$_2$O$_2$ due to its higher $K_m$ for H$_2$O$_2$ compared with glutathione peroxidase (79). There are other enzymes, such as thioredoxin (91) and glutathione S-transferase, and antioxidants, such as ubiquinol and cytochrome $c$, that also help inactivate ROS generated from the mitochondria. In the presence of transition metals (e.g., copper and iron), H$_2$O$_2$ can generate the highly reactive hydroxyl radical via the Fenton reaction or the Haber-Weiss reaction. Hydroxyl radicals are short-lived, highly reactive, and contribute significantly to local organelle damage through protein modification.

**Regulation of Mitochondrial ROS Production**

A number of physiological factors regulate or modulate mitochondrial oxidant generation. Among those, three factors have been extensively studied, including mitochondrial membrane potential ($\Delta\psi$), intracellular Ca$^{2+}$, and NO (Fig. 2). The generation of mitochondrial ROS is dependent on $\Delta\psi$. High $\Delta\psi$ seems to favor the production of ROS, particularly at complex III. This is thought to be due to the slowed electron transport and the prolongation of QH occupancy in the complex. Conversely, uncouplers of oxidative phosphorylation (e.g., 2,4-dinitrophenol) and mitochondrial uncoupling proteins (UCPs), a family of mitochondrial anion carriers that induce proton leak across the inner membrane, suppress mitochondrial $\Delta\psi$, resulting in reduced formation of ROS (30, 33, 44, 50, 67). Interestingly, there is negative feedback regulation between mitochondrial ROS generation and membrane uncoupling. It has been reported that O$_2^-$ directly activates UCPs through lipid peroxidation products and reactive aldehydes (80). Ischemic preconditioning (IPC) has been reported to activate UCPs and thereby protect cells from subsequent ischemia-reperfusion injury. Although the mechanism of UCP activation in IPC remains to be determined, it is possible that ROS generated during IPC are involved in this process (64).

Mitochondria participate in intracellular Ca$^{2+}$ homeostasis via several Ca$^{2+}$ uptake and release pathways (9). In this context, mitochondria behave as a high-capacity, low-affinity transient Ca$^{2+}$ store. An increase in cytosolic Ca$^{2+}$ concentration induces Ca$^{2+}$ entry across the mitochondrial inner membrane and results in an elevation in the mitochondrial matrix Ca$^{2+}$ concentration ([Ca$^{2+}$]$_{im}$). Although this response serves primarily to buffer large, more pathophysiological changes in intracellular Ca$^{2+}$ and may not be invoked by smaller transient changes that occur with physiological signaling, recent evidence indicates that mitochondria may act as a facilitating factor in the spreading of Ca$^{2+}$ signals. These Ca$^{2+}$-regulating functions are supported by the observations of the close apposition between mitochondria and Ca$^{2+}$-release channels of endoplasmic reticulum, such as inositol 1,4,5-trisphosphate [Ins(1,4,5)P$_3$] and ryanodine receptors, as well as proximity between mitochondria and plasma membrane (17).

The effects of elevated [Ca$^{2+}$]$_{im}$ on mitochondrial ROS production are complex, and experimental findings are controversial (12, 17). Overall, it appears that Ca$^{2+}$ increases mitochondrial ROS production under conditions of partial mitochondrial membrane depolarization or in the presence of some degree of mitochondrial electron transport inhibition. Several mechanisms have been suggested: 1) Ca$^{2+}$ stimulates tricarboxylic acid (TCA) cycle and enhances electron flow into the respiratory chain; 2) Ca$^{2+}$ stimulates NO production from NO synthase, resulting in the inhibition of complex IV; 3) Ca$^{2+}$ dissociates cytochrome $c$ from the inner mitochondrial membrane and at higher concentrations induces release of cytochrome $c$ across the outer membrane (12). Cytochrome $c$ is a potent antioxidant, and its loss can result in more ROS liberation from mitochondria (92). However, under some other conditions, mitochondrial Ca$^{2+}$ uptake has no effect or slightly reduces mitochondrial ROS production because the increase in [Ca$^{2+}$]$_{im}$ serves to collapse $\Delta\psi$ (12). Interestingly, it has been
Mitochondrial ROS Signaling in Endothelial Cells

Shear stress-induced vasodilation. The endothelium plays a key role in vascular homeostasis by synthesizing and releasing several vasorelaxing factors, including NO, prostacyclin, as well as endothelium-derived hyperpolarizing factors (EDHFs). Although the identity of EDHFs vary with species, vascular bed, and vascular size, H$_2$O$_2$ has been described as an EDHF-mediating shear- or acetylcholine-induced smooth muscle relaxation in mouse and human small arteries (54–56). The enzymatic sources of H$_2$O$_2$ in many of these arterial beds remain unclear. However, in coronary arterioles from patients with coronary disease, H$_2$O$_2$ mediates flow-induced dilation (54). Liu et al. (54) demonstrated that shear stress-induced H$_2$O$_2$ formation and vasodilation are mediated by O$_2^\cdot$ originating from mitochondria. H$_2$O$_2$ is a small, uncharged, and nonradical ROS and can freely diffuse through cellular membranes, including human endothelial membranes where it can activate large-conductance Ca$^{2+}$-activated potassium channels in the underlying vascular smooth muscle to elicit dilation (54). In addition, the steady-state concentration of mitochondria-derived H$_2$O$_2$ exceeds that of O$_2^\cdot$ by a factor of 100 (15). These characteristics are consistent with intercellular signaling of mitochondria-derived ROS in the vasculature.

Consistent with other studies regarding mitochondrial O$_2^\cdot$ production, complexes III and I are the primary sites of O$_2^\cdot$ production in response to shear stress in human coronary arterioles (54). Rotenone, an inhibitor of complex I, and myxothiazol, an inhibitor of complex 3, markedly attenuated flow-induced dilation and associated O$_2^\cdot$ and H$_2$O$_2$ generation. In contrast, apocynin, a selective inhibitor of NAD(P)H oxidase, does not affect shear-induced ROS generation. It is important to note that rotenone does not nonspecifically inhibit flow-induced dilation simply by reducing cellular ATP formation since cyanide in a dose that inhibits complex IV and ATP formation has no effect on flow-induced dilation. In addition, rotenone has no effect on relaxation responses to papaverine, an endothelium-independent dilator.

It remains unknown how shear stress triggers the release of ROS from mitochondria in endothelial cells. One potential mechanism may involve cytoskeletal microfilaments that functionally connect sarcolemmal and mitochondrial membranes either directly or indirectly. It is possible that shear stress exerted on the plasma membrane induces structural changes in endothelial cytoskeleton, which is transduced to mitochondria, resulting in an alteration of mitochondrial function and a subsequent production of ROS production. Indeed, we found preliminary evidence that a disruption of cytoskeletal elements reduces a flow-induced increase in mitochondrial ROS and dilation in human coronary arterioles (unpublished observations). Cytoskeletal inhibitors have also been shown to reduce flow-mediated dilation in rabbit aorta and rat gracilis arteries where NO rather than H$_2$O$_2$ is the mediator of dilation (40, 78).

Yan et al. (89) recently reported that flow-induced dilations are decreased in mesenteric arterioles of Mn-SOD$^{-/-}$ mice relative to wild-type control mice. The authors proposed that this effect occurs because the associated decrease in activity of Mn-SOD leads to less dismutation of mitochondrial O$_2^\cdot$ and greater cytosolic trafficking of O$_2^\cdot$ via the voltage-dependent mitochondrial anion channel, resulting in the quenching and inactivation of NO (37). This cycle of events leads to reduced NO-dependent relaxations. Lower levels of Mn-SOD could also result in a reduced ability to convert O$_2^\cdot$ in mitochondria to H$_2$O$_2$, an endogenous vasodilator. Therefore, it will be of interest to determine whether reduced H$_2$O$_2$ formation in Mn-SOD$^{-/-}$ mice results in impaired relaxation responses in those vascular beds where H$_2$O$_2$ plays a more dominant role in the regulation of vascular tone.

Nonvasomotor response to mechanic stimuli. Mechanical stimulation of endothelial cells induces a large number of nonvasomotor responses, including activation of many signal pathways, changes in gene expression, and remodeling of cytoskeletal organization and cellular morphology (21, 23). An important step in this mechanotransduction cascade is the phosporylation of focal adhesion kinase (FAK). FAK is redox sensitive and can be activated by ROS, such as H$_2$O$_2$ (8, 83).
However, the source of ROS production and the relationship between the oxidant signal and the activation of FAK remain obscure. Recent studies indicate that, in pulmonary arterial endothelial cells, mitochondrial ROS serve as a missing link in stretch-induced phosphorylation of FAK (2, 3). It is proposed that mechanical stretch increases the production of ROS from mitochondria, which then signal the activation of protein kinase C (PKC). PKC then phosphorylates FAK and initiates a wide range of responses in endothelial cells.

Consistent with its potential role in shear-induced vasomotor responses, the cytoskeleton is involved in the activation of FAK by transmitting mechanical strain to ROS production in mitochondria (2). In another recent study, Ichimura et al. (41) reported that pressure elevation stimulates mitochondrial ROS production via Ca$^{2+}$-dependent signaling pathway. They found that in lung venular capillaries, pressure challenge induces cytosolic Ca$^{2+}$ oscillations followed by mitochondrial Ca$^{2+}$ oscillations, which in turn stimulate mitochondrial ROS production. It remains unknown whether cytoskeleton activation and Ca$^{2+}$ increase represent two independent pathways leading to mechanical stimulus-induced mitochondrial ROS production or whether they are part of the same signaling pathway. Mitochondria may serve to sensitize cytoplasmic Ca$^{2+}$ signaling since mitochondria-derived ROS induce Ca$^{2+}$ release from ryanodine receptors on the endoplasmic reticulum in endothelial and other vascular cells (1, 88).

**Hypoxia-reoxygenation response.** Tissue hypoxia can develop in a number of conditions, such as reduced gas exchange in the lung, decreased local blood flow, increased tissue metabolic activity, and travel to a high altitude. In endothelial cells, hypoxia initiates a number of responses that include cell growth and proliferation, increase in permeability, and changes in cell-surface adhesion molecules. Recent studies indicate that mitochondrial ROS signal downstream responses to tissue hypoxia. Mitochondria respond to cellular hypoxia by paradoxically increasing the generation of ROS; therefore, they may act as oxygen sensors in the signal cascade of hypoxic responses (19). Indeed, ROS generated from mitochondria in response to hypoxia trigger NF-κB activation and subsequent transcriptional production of IL-6, resulting in an increase in endothelial permeability (72). In another study, mitochondrial ROS were found to contribute to hypoxia-induced activation of AMP-activated protein kinase, which is thought to play a role in cellular defense responses (74).

It remains unclear how hypoxia stimulates ROS production from mitochondria (42). Hypoxic conditions might cause feedback inhibition of the electron transport chain and hence lead to increased O$_2^-$ generation, particularly in the complex III. Alternatively, low oxygen levels decrease mitochondrial proton leaks, which lead to higher Δψ and consequently increased O$_2^-$ production. It is also possible that increased O$_2^-$ production is not a response to hypoxia per se but instead mediated by NO. Under hypoxic condition, there is enhanced inhibition of complex IV by NO, and this may contribute to an increase in ROS (71, 74).

Notably, mitochondria-derived ROS have also been implicated in the hypoxic response of other vascular cells. Among those, the role of mitochondrial ROS in hypoxic pulmonary vasoconstriction has received much attention (86, 87). In pulmonary circulation, mitochondria act as an oxygen sensor, and changes in mitochondrial ROS in response to hypoxia importantly contribute to pulmonary vasoconstriction during hypoxia. However, there is a controversy as to how mitochondria-derived ROS contribute to hypoxic responses. One model proposes that hypoxia triggers an increase in mitochondrial ROS, which in turn induce Ca$^{2+}$ release from intracellular stores, opening of Ca$^{2+}$ channels in the plasma membrane, and vessel constriction (86). An opposing model proposes that a drop in oxygen tension decreases the production of mitochondrial ROS. The consequential shift of the cytosol to a more reduced state inhibits voltage-dependent potassium channels resulting in Ca$^{2+}$ influx and vasoconstriction (87). A recent study suggested that the biochemical mechanism for mitochondrial ROS changes in response to hypoxia is not intrinsic to the mitochondrial respiratory chain alone but may involve other unidentified factors that contribute to the discrepancy in mitochondrial ROS responses to hypoxia (39).

There is apparently a U-shaped curve defining the relationship between tissue oxygenation and mitochondrial ROS generation. Hyperoxia or reoxygenation after hypoxia also induces ROS formation from the electron transport chain (14, 81). It has been reported that reoxygenation after hypoxia stimulates ROS production from complex III in human umbilical vein endothelial cells. The lipid molecule ceramide plays an important role in this increase in ROS production, possibly by directly modulating the activity of complex III (81).

Multiple ROS-generating pathways coexist within vascular tissue, and cross talk between these pathways may be present in endothelial cell responses to hypoxia and/or reoxygenation. As an example, Aley et al. (1) recently reported that hypoxia triggers Ca$^{2+}$ release from the endoplasmic reticulum via two distinct pathways, which involve both mitochondria- and NAD(P)H oxidase-derived ROS. In one pathway, hypoxia modulates Ins(1,4,5)P$_3$ receptor-dependent Ca$^{2+}$ mobilization from the endoplasmic reticulum through ROS derived from NAD(P)H oxidase. In the second pathway, hypoxia induces Ca$^{2+}$ release from ryanodine receptors, an effect requiring mitochondrial ROS generation (1). There is cross talk between these two regulatory pathways, and hypoxia-induced mitochondrial ROS production augments Ca$^{2+}$ release from Ins(1,4,5)P$_3$ receptors. Interestingly, mitochondrial ROS-stimulated Ca$^{2+}$ release from ryanodine receptors has also been reported in vascular smooth muscle cells. In a recent study, Xi et al. (88) found that mitochondrial ROS activate large-conductance Ca$^{2+}$-activated potassium channels on cerebral arterial smooth muscle and cause vasorelaxation by liberating Ca$^{2+}$ from ryanodine-sensitive sarcoplasmic reticulum store (88).

**Mitochondrial ROS in Cardiovascular Disease**

Mitochondrial ROS have been implicated in the pathogenesis of cardiovascular diseases, such as atherosclerosis, hypertension, and diabetes. Although an extensive discussion is beyond the scope of this review, some important features relevant to vascular pathophysiology are summarized. The mitochondrial dysfunction theory postulates that excess release of ROS from mitochondria is responsible for the inflammatory vascular reaction that leads to cardiovascular disease (6, 73). Many cardiovascular risk factors, including hyperglycemia and insulin resistance, hypercholesterolemia and oxidation of LDL, hyperhomocysteinemia, tobacco smoke exposure, and aging,
can adversely affect the function of endothelial cell mitochondria via various mechanisms, resulting in increased ROS production. This contributes to endothelial dysfunction and ultimately to the development of cardiovascular disease. In addition, excessive mitochondrial ROS affect mitochondrial membranes, proteins and DNA, and cause further mitochondrial dysfunction through a vicious cycle. In addition to endothelial cells, increased production of mitochondrial ROS in other vascular cells (e.g., smooth muscle cells) may also contribute to the development of vascular lesions (7). It remains unclear whether a cross-link in mitochondrial ROS production exists among different vascular cell types.

The mitochondrial dysfunction theory is well illustrated in hyperglycemia-induced cellular damage in endothelial cells and other target organ cells involved in diabetic complications (13, 66). Intracellular hyperglycemia increases the mitochondrial proton gradient through excess production of electron donors (NADH and FADH2) for the TCA cycle, resulting in increased mitochondrial production of ROS. Increased ROS then activate poly(ADP-ribose) polymerase and, in turn, decrease the activity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Decreased GAPDH activity activates multiple pathways, including activation of PKC and subsequently NF-κB, resulting in reduced expression of eNOS, increased production of vasoconstrictors like endothelin-1, and activation of many proinflammatory genes. All these changes can potentially contribute to the pathogenesis of diabetic microvascular damage. Interestingly, diabetic macrovascular disease seems to involve increased free fatty-acid flux and oxidation in endothelial cells and consequent overproduction of mitochondrial ROS (29).

The specific ROS involved in cell signaling seem to vary depending on the pathophysiological setting. As an example, cytokine-induced activation of NF-κB is inversely related to Mn-SOD expression (4). Catalase and GSH peroxidase overexpression have no effect (4). These observations suggest that NF-κB activation by inflammatory cytokines is mediated by O2•− rather than H2O2.

Methods for Evaluating Vascular Mitochondrial Contribution to ROS Generation

Fluorescent dyes such as dichlorodihydrofluorescein have been widely used to detect intracellular ROS in endothelial cells. In conjunction with mitochondria-specific markers and confocal microscopy, these indicators can assay ROS generation from a mitochondrial source (48, 84). To minimize fluorescence from nonmitochondrial compartments, some indicators have been modified to target mitochondria specifically. For example, mitoSOX is a triphenylphosphonium (TPP+) -linked dihydroethidium compound. It exploits the steep electrochemical gradient across the mitochondrial inner membrane to concentrate the TPP tag more than a 100-fold within the mitochondria compared with the cytosol (77). MitoSOX has effectively been used to monitor lysophosphatidylcholine-induced mitochondrial ROS production (84).

To examine mitochondrial ROS production in vascular endothelial cells, many studies rely on inhibitors of the electron transport chain or chemical uncouplers of the mitochondrial potential. This pharmacological approach is powerful, but specificity of inhibitors is critical. For example, rotenone is a complex I inhibitor but is also a microtubule-depolymerizing agent (10, 76). Thienoyl trifluoracetone is a selective complex II inhibitor, whereas myxothiazol and stigmastatin inhibit complex III. Myxothiazol and stigmatellin at high concentrations may inhibit other components of respiratory chain, including complex I (25, 90). FCCP is a mitochondrial uncoupler with a host of resultant cellular physiological changes. In addition, pseudo-rho0 cells, depleted of mitochondrial DNA, have been used to confirm the critical role of mitochondria in ROS-mediated responses (48). A molecular approach that uses small-interfering RNA to inhibit mitochondrial function and ROS production is an important complementary approach useful in cell culture systems (36).

The use of mitochondrial antagonists is instrumental in identifying the sites of ROS production in both the isolated mitochondria preparations and in intact cells. However, results obtained from these inhibitors are not always consistent. Mitochondrial inhibitors can have divergent effects on ROS production in isolated mitochondria versus intact cells or in intact cells under different experimental conditions. When intact cells are used, rotenone has been shown to either decrease or increase mitochondrial ROS production in endothelial cells (3, 54, 84). The reasons for this discrepancy are not entirely clear but could relate to differences in mitochondrial respiratory state (17). Complex III-derived O2− seems to dominate in state 3 respiration; therefore, inhibition by rotenone of downstream electron flow into the Q cycle of complex III reduces O2− generation (82). On the other hand, complex I appears to be the main source of O2− in state 4 respiration, when electron transport rate and ATP synthesis are low and substrates are highly reduced (46). Under this situation, administration of rotenone, which blocks complex I distal to site of O2− production, increases ROS production.

Another potential problem with mitochondrial inhibitors is that the interruption of mitochondrial function may alter other aspects of cell metabolism, such as ATP synthesis, and thus complicate the interpretation of results (59). Although endothelial cells are highly glycolytic (74) and inhibition of mitochondrial respiration would have a minimal effect on cellular ATP concentrations, it is important to use some alternative approaches to confirm the findings obtained from mitochondrial inhibitors. In this context, mitochondrial-targeted antioxidants may represent a promising alternative tool in studying mitochondrial ROS. Several agents that have been chemically bonded to TPP and thus selectively scavenge mitochondrial ROS have been developed, including vitamin E and quinine derivatives mito-E (84) and mito-Q (26), as well as the nitroxide derivative Mito-carboxy proxyl (27). A molecular approach to prevent H2O2 release from mitochondria has also been reported by using adenovirus-mediated overexpression of catalase in the mitochondria (5).

Summary and Conclusion

In summary, mitochondria are not simply ATP-producing organelles but also play a key role in cell signaling. Accumulating evidence indicate mitochondrial ROS, once thought of as toxic by-products of cell respiration, function as signaling molecules in vascular endothelial cells. The mitochondrial electron transport chain represents an important source of ROS in endothelial cells, and the production of ROS from mitochondria...
dria is under tight control by a number of mechanisms. Mitochondrial ROS signaling has been implicated in the regulation of vascular tone, adaptive changes to mechanical stimuli, and vascular responses to hypoxia-reoxygenation. Excess production of mitochondrial ROS leads to a disruption of normal ROS signaling and mitochondrial dysfunction, which contributes to the pathogenesis of cardiovascular disease. Our understanding of this signaling role of mitochondrial ROS in vascular endothelial cells is at an early stage, and future research in this area remains an exciting challenge.

REFERENCES


