This Review is part of a thematic series on the **Role of Mitochondria in Cardiovascular Diseases**, which includes the following articles:

Free Radicals, Mitochondria, and Oxidized Lipids: The Emerging Role in Signal Transduction in Vascular Cells
Mitochondrial Dysfunction in Atherosclerosis
Defective Mitochondrial Biogenesis: A Hallmark of the High Cardiovascular Risk in Metabolic Syndrome?

Endothelial Mitochondria: Contributing to Vascular Function and Disease

Role of Mitochondria in Insulin Resistance

Marshall S. Runge, Guest Editor

Endothelial Mitochondria
Contributing to Vascular Function and Disease

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Abstract—Disturbances in vascular function contribute to the development of several diseases of increasing prevalence and thereby contribute significantly to human mortality and morbidity. Atherosclerosis, diabetes, heart failure, and ischemia with attendant reperfusion injury share many of the same risk factors, among the most important being oxidative stress and alterations in the blood concentrations of compounds that influence oxidative stress, such as oxidized low-density lipoprotein. In this review, we focus on endothelial cells: cells in the frontline against these disturbances. Because ATP supplies in endothelial cells are relatively independent of mitochondrial oxidative pathways, the mitochondria of endothelial cells have been somewhat neglected. However, they are emerging as agents with diverse roles in modulating the dynamics of intracellular calcium and the generation of reactive oxygen species and nitric oxide. The mitochondria may also constitute critical "targets" of oxidative stress, because survival of endothelial cells can be compromised by opening of the mitochondrial permeability transition pore or by mitochondrial pathways of apoptosis. In addition, evidence suggests that endothelial mitochondria may play a "reconnaissance" role. For example, although the exact mechanism remains obscure, endothelial mitochondria may sense levels of oxygen in the blood and relay this information to cardiac myocytes as well as modulating the vasodilatory response mediated by endothelial nitric oxide. **(*Circ Res*. 2007;100:1128-1141.)**

Key Words: mitochondria ■ endothelial cells ■ intracellular calcium ■ reactive oxygen species ■ diabetes ■ atherosclerosis

Cardiomyocytes are the major consumers of oxygen in the heart and account for approximately 75% of normal myocardial volume.[1] On the other hand, there is at least 1 capillary adjacent to every cardiomyocyte (Figure 1),[2] and cardiomyocytes are outnumbered ≈3:1 by the endothelial cells that line the microvasculature and small vessels of the heart.[1] In this review, we examine endothelial cells—cells in the "frontline" against vascular disease—and assess the role that mitochondria play in defining physiology. Data from cardiac vascular endothelial cells are used for illustration wherever possible, because the tissue of origin can affect endothelial phenotype.[3] The extent to which mitochondrial physiology varies in the endothelium of different tissues is unknown, although certainly some mitochondrial functions, such as mitochondrial DNA

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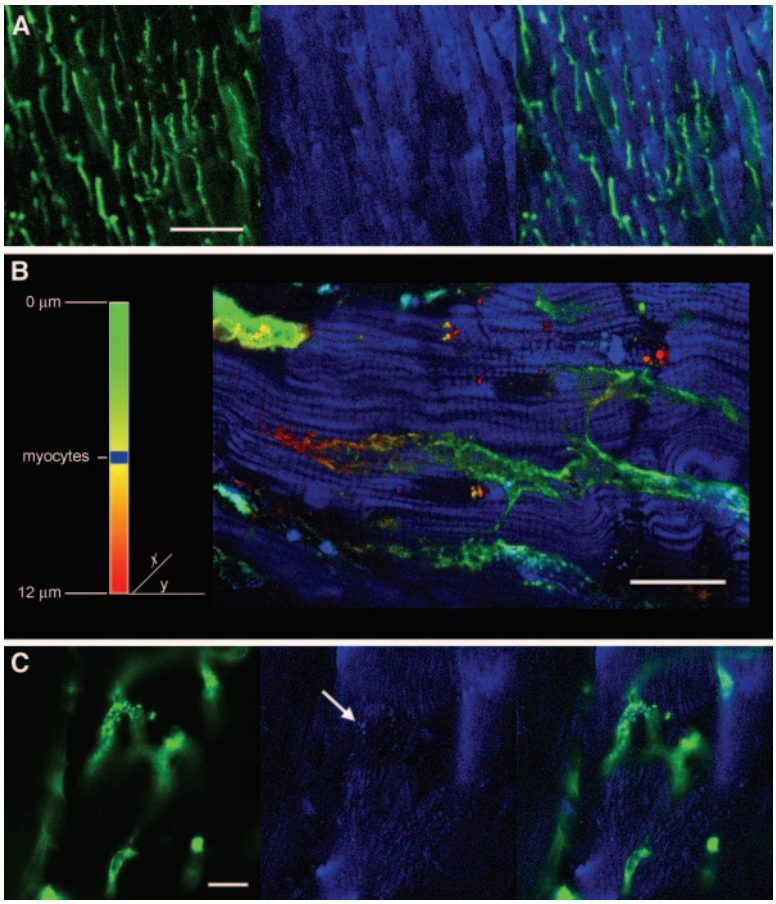

Figure 1. Endothelial cells, stained with a fluorescently labeled lectin (green), snake over and around every cardiomyocyte (blue), performing a vital "plumbing" role but also modulating the activity of mitochondria within cardiomyocytes. To obtain these images, a Zeiss LSM multiphoton microscope was used to image live cells within a 300-μm live tissue slice of rat left ventricular myocardium. Endothelium, labeled with 10 μg/mL fluorescein isothiocyanate–labeled *Bandeiraea simplicifolia* lectin, was detected by illumination at 950 nm, and emission was collected using an LP505 filter. NAD(P)H autofluorescence, which originates primarily from mitochondria within cardiomyocytes,[170] was visualized by illumination at 720 nm and emission collected between 435 to 485 nm. A, Low magnification illustrates the extent of vascularization in the myocardium. Scale bar=100 μm. B, Endothelium (colorized green to red according to depth), snakes around cardiac myocytes (blue, shown at 6-μm depth only for clarity). Scale bar=20 μm. C, At high magnification, NAD(P)H autofluorescence can be detected from endothelial mitochondria (arrow). Scale bar=10 μm.

(mtDNA) repair ability, appear to differ even between pulmonary arterial, venous, and microvascular endothelial cells.[4]

The fundamental dependence on the vasculature for the proper functioning of the heart has been evident ever since the term "cardiovascular disease" was coined. Although an essential "plumbing" role for the vasculature in facilitating the delivery of oxygen and energetic substrates for powering the high metabolic demands of the cardiomyocytes is obvious, other critical functions of the vasculature become manifest during the development of the several types of cardiovascular disease which develop when the vasculature becomes damaged. In particular, diabetes and coronary artery disease share many risk factors, and the majority of these (eg, smoking, hyperlipidemia, hyperinsulinemia, and aging) lead directly or indirectly to vascular damage.

After substrate supply, a second vital function of the endothelium is the provision of an impermeable barrier between the blood and the cardiac tissue; unlike most nonmuscular tissue, all of the arterial vasculature of the heart down to the level of the capillary bed is lined with a continuous layer of endothelial cells. The importance of the integrity of this barrier cannot be overstated, because endothelial damage can lead to leukocyte and platelet extravasation, inflammation, vascular damage, and atherosclerosis.

A third important function of the coronary vascular endothelium is the trophic support of cardiomyocytes and release of paracrine signaling peptides such as angiopoietin-1, neuregulin, platelet-derived growth factor, vascular endothelial growth factor, and adenosine, as well as signaling molecules such as reactive oxygen species (ROS), NO, and adenosine. These mediate the vital role of endothelium in regulating vascular tone. During normal physiology, endothelial cells appear able to modulate cardiac contractility, heart rate, and myocardial energetics,[5,6] and in pathological situations, this role may be extended to regulation of cardiomyocyte metabolism and survival.[6] For example, cardiac endothelial cells respond to oxidative stress by releasing neuregulin-1β, which binds to the ErbB4 receptor of neighboring cardiomyocytes and activates prosurvival signaling pathways.[7]

To understand whether endothelial mitochondria contribute to the development or progression of cardiovascular diseases, we examine, in this review, the ways in which mitochondria contribute to endothelial physiology and pathophysiology, specifically in relation to:

- The generation of NO and ROS
- Regulation of the dynamics of intracellular [Ca^{2+}] signaling
- Endothelial cells as targets of Ca^{2+} overload and oxidative damage in the vasculature, leading to activation of the mitochondrial pathway of apoptosis or induction of necrosis
- The role of endothelial cells as sensors of local oxygen concentrations

We then explore ways in which these functions may be disrupted during specific disease processes involving endothelium dysfunction.

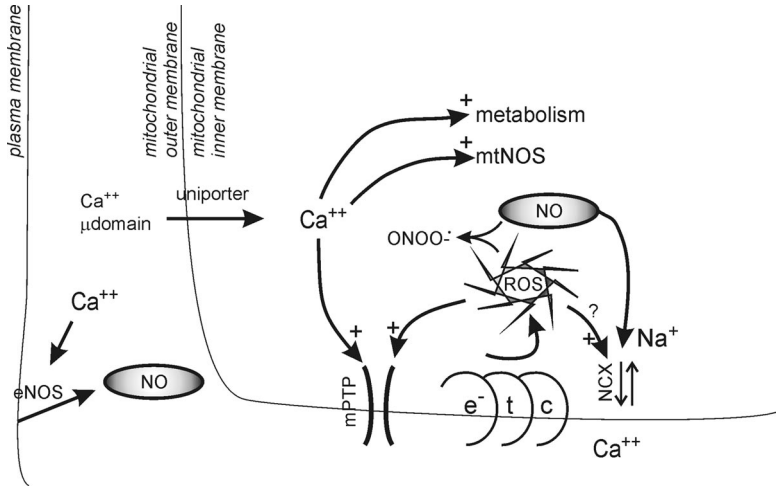

Figure 2. The mitochondria of endothelial cells are able to modulate intracellular calcium dynamics, and $[Ca^{2+}]m$ can modulate mitochondrial function. e-.t.c. indicates electron transport chain; NCX, Na^+/Ca^{2+} exchanger; $ONOO^-$, peroxynitrite. See the text for details.

The Physiology of Endothelial Mitochondria

Endothelial cells obtain a large proportion of their energy from the anaerobic glycolytic metabolism of glucose.[8–10] At least 75% of ATP synthesized by cultured pig aortic endothelial cells is provided by glycolysis.[9] It has been estimated that isolated coronary microvascular endothelial cells catabolize 99% of glucose into lactate and that their oxygen consumption is mainly attributable to the oxidation of endogenous substrates,[10,11] although it is difficult to determine how accurately these values reflect those of the cells in vivo. Because endothelial cells rely much more on glycolytic metabolism than their myocyte neighbors, the potential role of their mitochondria has been, to some degree, neglected. However, even if the endothelial mitochondrial respiratory chain were to play a limited role in energy production, it may play a physiological relevant role by virtue of its ROS-producing capacity.

Mitochondrial ROS in Endothelial Cells

ROS are probably produced continuously in all cells, through reduction of molecular oxygen by the electron transport chain to produce superoxide, which is subsequently dismutated to hydrogen peroxide.[12,13] In the normal cell, ROS form a regular part of diverse signaling pathways, but in many types of cardiovascular disease, ROS overwhelm antioxidant defenses and become damaging, a process known as oxidative stress. These ROS may originate from various sources other than mitochondria and also from other cell types in intact myocardium, making it difficult to assess in intact tissue the relative importance of mitochondrial ROS production in endothelial cells. Nevertheless, that superoxide production can occur at both complex I and complex III in mitochondria isolated from bovine aortic endothelial cells has been demonstrated in vitro.[14] It is important to keep in mind that during inflammation, such as may occur in response to atherosclerosis or ischemic injury, activated neutrophils attracted to regions of endothelial damage can generate huge quantities of ROS, which damage nearby cells.

One might speculate that intracellular pathways that use ROS as signaling molecules would be disrupted by oxidative stress, although this has yet to be specifically demonstrated in endothelial cells. Calcium-handling proteins represent a second potential target. The activity of many calcium-handling proteins of cardiomyocytes, including the ryanodine receptor and sarco-/endoplasmic reticulum calcium ATPase (SERCA), undergoes subtle regulation by redox modification of susceptible sulfhydryl groups.[15] Because ROS may influence normal Ca^{2+} handling, it follows that oxidative stress has the potential to disrupt pathways regulating intracellular Ca^{2+} homeostasis. Indeed, exposure of a permeabilized endothelial cell line to peroxide in vitro raises mitochondrial calcium concentration ($[Ca^{2+}]m$), apparently by inactivating the mitochondrial Na^+/Ca^{2+} exchanger (Figure 2).[16] This type of dysregulation may be particularly relevant in cardiovascular pathologies.

A third potential target of mitochondrial ROS is the mitochondrial electron transport chain itself. In fact, even if comparatively little superoxide were produced, it may have a significant effect via its destructive reaction with NO (Figure 2). The consequences of this reaction are complex, because it simultaneously causes the loss of molecules that may be protective (NO, ROS) or damaging (ROS) and results in the formation of peroxynitrite, which can irreversibly damage molecules including proteins of the electron transport chain (reviewed elsewhere[17]).

Endothelial NO Production

Endothelial NO is well recognized for its primary role in regulating vasoconstriction and relaxation via the constriction or relaxation of overlying smooth muscle cells.[18] Production of NO by cardiomyocytes themselves has also been irrefutably demonstrated,[17] and there is good evidence for an isoform of NO synthase (NOS) localized in cardiomyocyte mitochondria (reviewed in[17]). However, the greatest source of NO in the normal heart is undoubtedly endothelial NOS (eNOS), which is found primarily (but, despite the name, not exclusively) within endothelial cells, and is localized at the plasma membrane. NO has a half-life of seconds[19] and is probably capable of diffusing as far as the subsarcolemmal mitochondria of cardiomyocytes, which are located within 1 to 2 μm of endothelial cells.[20] NO is a reversible, competitive inhibitor at complex IV in the electron-transport chain,[21] thereby modulating the rate of oxidative metabolism, both of cardiomyocytes and of endothelial cells themselves,[22] al-

though the significance of this in intact tissue has not been irrefutably demonstrated.[23] However, it is now recognized that NO has widespread effects on the heart, modulating cardiac contraction, oxygen consumption, substrate utilization, apoptosis, and hypertrophy.[24–26] Although endothelial mitochondria may not play a major role in this aspect of normal cardiac function, it is possible that the damage that occurs to endothelial mitochondria during various pathologies may upset the delicate balance between ROS and NO levels. Mitochondrial Ca^{2+}, with its capacity to modulate both ROS and NO generation, may represent a central element in this picture.

Mitochondrial Ca^{2+} in Endothelial Cells

Although the endoplasmic reticulum (ER) is the main Ca^{2+} store in endothelial cells, ≈25% of intracellular Ca^{2+} is sequestered in the mitochondria of aortic endothelial cells.[27] The discovery that several key mitochondrial enzymes are regulated by Ca^{2+} led to the recognition that mitochondrial metabolism is responsive to physiological changes in $[Ca^{2+}]$m (Figure 2).[28,29] Thus an increase in $[Ca^{2+}]$m was shown to increase the mitochondrial ATP production in endothelial cells, as in other cell types. Increased $[Ca^{2+}]$m in response to mobilization of ER Ca^{2+} has been directly demonstrated using recombinant aequorin[30] and the fluorescent Ca^{2+} reporter pericam targeted to the mitochondrial matrix.[31] Thus, extracellular stimuli can modulate mitochondrial metabolism in endothelial cells via increasing $[Ca^{2+}]$m. Again, the significance of this in vivo is difficult to determine, especially considering that glycolytic pathways are thought to be more important for endothelial cell ATP production.

Mitochondrial calcium accumulation is mediated by the Ca^{2+} uniporter and is driven by the electrochemical potential gradient for Ca^{2+}: a function of the mitochondrial membrane potential ($\Delta\psi_m$) and the Ca^{2+} concentration gradient across the mitochondrial membrane. Therefore, situations that decrease $\Delta\psi_m$ will limit Ca^{2+} accumulation into mitochondria, which may have repercussions in terms of metabolic activity but may also protect the mitochondria from becoming overloaded with Ca^{2+}. NO has been shown to reduce $\Delta\psi_m$ in cultured calf pulmonary artery endothelial cells, thereby decreasing $[Ca^{2+}]$m.[32] Interestingly, an isoform of NOS localized to mitochondria is apparently activated by Ca^{2+}, providing a form of negative feedback on mitochondrial Ca^{2+} uptake as the NO inhibits respiration and reduces $\Delta\psi_m$.[33] This is further evidence that the mitochondria of endothelial cells are poised at the "crossroads" of Ca^{2+}, NO and ROS regulation, as proposed for the mitochondria of vascular smooth muscle cells (VSMCs).[34]

A perennial question relates to the source of the Ca^{2+} that is preferentially taken up by the mitochondria. After stimulation, net $[Ca^{2+}]$c does not appear to attain levels sufficient to promote significant uptake via the uniporter. In some cell types, mitochondria lie in very close apposition to the ER, and so are exposed to microdomains of high $[Ca^{2+}]$c following local calcium release from the ER.[35] In contrast, in an endothelial cell line derived from human umbilical vein (HUVECs), only ≈4% of mitochondria were close to (ie, within 700 nm of) the ER membrane surface,[30] although it remains possible that the intracellular architecture of an immortalized cell line growing in vitro does not exactly reflect the architecture in vivo. Despite this caveat, there is further evidence that mitochondria in endothelial cells respond to calcium microdomains that form beneath the plasma membrane, rather than close to all ER. For example, in porcine coronary artery endothelial cells, submaximal stimulation with bradykinin increased subplasmalemmal $[Ca^{2+}]$ (monitored using FFP-18), without affecting bulk calcium levels.[36] In this case, the subplasmalemmal calcium microdomains were proposed to result from vectorial Ca^{2+} release from the superficial ER toward the plasmalemma followed by extrusion of Ca^{2+} from the cell by the plasmalemmal Na^+/Ca^{2+} exchanger.[36]

In a separate model, which does not readily harmonize with that described above, it is suggested that during stimulation of endothelial cells there is a continual flux of calcium entering mitochondria through the calcium uniporter to be extruded via the Na^+/Ca^{2+} exchanger and taken up by the ER (Figure 2).[31,37] This is proposed to result in a subplasmalemmal microdomain of low calcium, which facilitates capacitative Ca^{2+} entry into the cell.[31] However, this model does not explain how calcium concentrations attain levels high enough to cause significant mitochondrial uptake. Whatever the case, it does seem apparent that one means by which endothelial mitochondria can affect vascular tone is by modulating subplasmalemmal calcium levels.

Agonists such as bradykinin and histamine, which are coupled to the production of inositol-1,4,5-triphosphate, stimulate the release of Ca^{2+} from the ER, and this mediates many of their biological effects. A role for mitochondria in the transmission of intracellular Ca^{2+} signals was first suggested by evidence that mitochondria in Ehrlich ascites tumor cells can contribute to the release of Ca^{2+} from intracellular stores in response to a small Ca^{2+} trigger signal.[38] This response, termed mitochondrial Ca^{2+}-induced Ca^{2+} release (mCICR), was subsequently demonstrated in endothelial cells.[27] The parallels between mCICR and Ca^{2+}-induced Ca^{2+} release (CICR) are limited, however. CICR involves activation of the ryanodine receptor Ca^{2+} release channel in response to increased extraluminal Ca^{2+} and terminates via as yet unknown mechanisms,[39] whereas mCICR occurs when high levels of intramitochondrial Ca^{2+} induce opening of the mitochondrial permeability transition pore (mPTP).[38,40]

The mPTP in Endothelial Cells

The mPTP is a relatively large, nonspecific pore thought to span the inner and outer mitochondrial membranes. Its core structure is controversial but is believed to consist of the adenine nucleotide translocator, the voltage-dependent anion conductance, and cyclophilin D in the matrix, which confers sensitivity of the complex to cyclosporine A, which closes the pore.[41] The survival of mice lacking cyclophilin D, voltage-dependent anion conductance, or the adenine nucleotide translocator suggests the mPTP is not necessary during normal physiology.[42–47] However, in many pathological situations, it functions as a sort of "gatekeeper": preservation of its closed conformation by treatment with cyclosporine A or

in mutants lacking cyclophilin D prevents the passage of the cells into pathways of cell death.[41–45]

In most situations in intact cells, mPTP opening appears to be definitive, because it tends to exacerbate the conditions that initiated it (ie, an increase in free Ca^{2+} and a decrease in mitochondrial membrane potential, which leads to ATP depletion, increased phosphate levels, increased acidity and oxidative stress), leading to necrosis. However, it is interesting to speculate whether there might be a "gray area" in which conditions are not quite sufficient to initiate feedback activation of mPTP opening, and the mPTP opens only transiently, possibly leading to a preconditioned state[48] or, perhaps, to activation of apoptosis rather than necrosis.[49]

Rapid changes in $\Delta\psi_m$ of individual mitochondria, or "flickering," can be revealed in cultured calf pulmonary artery endothelial cells by loading the mitochondria with the voltage-sensitive dye TMRE and exposing them to laser-induced oxidative stress,[50] similar to mitochondrial flickering first observed in cardiomyocytes.[51] This has been interpreted as episodes of transient mPTP opening,[52] but further proof is needed, because in other cell types, flickering seems to occur independently of mPTP opening.[52–55] Furthermore, even in the endothelial cells tested in the abovementioned study, the mPTP blocker cyclosporine A had no effect on flickering.[50]

Intriguingly, the mPTP in proliferating endothelial cells may be more sensitive to opening than in quiescent endothelial cells, a difference that has been capitalized on to selectively kill angiogenic endothelial cells to inhibit tumor angiogenesis in vivo.[56] The possibility that endothelial cells proliferating within atherosclerotic lesions would also be more susceptible to death pathways acting via the mPTP may merit consideration.

Endothelial Mitochondria As Sensors of the Local Environment

The positioning of the endothelial cells at the "frontline" in direct contact with the blood not only means they are particularly vulnerable to damaging molecules in the blood, but has them ideally situated for "reconnaissance" roles, ie, sensing alterations in perfusate constituents and either responding directly or transmitting reactive signals to nearby cells. An example of a direct response is the rapid increase in endothelial production of NO and other vasodilatory molecules that occurs in response to even a modest decrease in arterial partial pressure of oxygen (Pao_2), thereby causing vasodilation and increasing blood flow and O_2 supply. In various cell types, acute sensing of Po_2 appears to be performed by ROS-producing systems such as the NADPH oxidase. Mitochondria have been proposed as the central oxygen sensors in the vasculature, particularly in the specialized case of pulmonary hypoxic vasoconstriction[57] via a mechanism whereby hypoxia causes an increase in the generation of mitochondrial ROS, which then escape into the cytoplasm, although this remains controversial.[57]

Mitochondria of endothelial cells might be free to play a role as sensors of the local environment, as they are not essential for energy production. As mentioned, endothelial cells are highly glycolytic and, therefore, do not consume as much O_2 as myocytes to fuel oxidative phosphorylation. In coronary endothelial cells cultured in 5 mmol/L glucose, O_2 consumption does not decrease until Po_2 drops below 3 mm Hg, and is half maximal at 0.8 mm Hg[11] (compared with ≈1.6 mm Hg in resting cardiomyocytes[58]). ATP levels decrease in HUVECs only when Po_2 is reduced to ≈3.5 mm Hg,[8] although in cultured coronary endothelial cells, ATP levels remain constant even down to 0.1 mm Hg (either because of decreased ATP consumption or increased glycolysis).[11] In fact, inhibition of glycolysis can be tolerated by pig aortic endothelial cells for at least 3 hours, because of the fact that ATP-consuming pathways such as protein synthesis are coordinately downregulated when glycolysis is inhibited.[9,59] However, when coronary endothelial cells are cultured in palmitate and glutamine to stimulate oxidative energy production, the adenine nucleotide contents decline rapidly at <3 mm Hg oxygen.[11]

The situation is far from clear, however. Indeed, the mitochondria of cardiomyocytes themselves have previously been proposed to act as O_2 sensors.[60] In fact, despite being primarily oxidative, isolated cardiomyocytes are able to retain viability during extended periods of hypoxia (7 mm Hg for 48 hours), [61] by damping contractility until O_2 consumption matches O_2 supply. Similarly, in the myocardium, a decrease in electron transport flux (detected as an accumulation of metabolic intermediates such as NADH) does not occur until Po_2 decreases below 3 to 4 mm Hg.[62,63] Overall, the sensitivity of endothelial cells to oxygen seems fairly comparable to that of cardiomyocytes.[64]

A further issue that is not yet resolved is that, in most situations, mitochondrial ROS production is proportional to Po_2, with more ROS generated at higher O_2 tension[63]; however, ROS production certainly does appear to increase during ischemia.[65] The situation seems yet more complicated when one also considers the fact that NOS consumes O_2 in producing NO, and therefore NO production may reduce the intracellular $[O_2]$.[66] For a discussion of some of the many points of contention with regard to tissue oxygen sensing, see the report by Chadwick and Goode.[67]

An important point to keep in mind is that although the mechanism of oxygen sensing may be similar in different vascular beds, downstream signal transduction pathways must ultimately diverge, because in the pulmonary vasculature, hypoxia causes vasoconstriction, whereas coronary and skeletal muscle respond by vasodilation.

Whether or not endothelial cells are the oxygen sensors of the myocardium, they do, of course, respond to hypoxia by activating protective pathways common to many cell types. As the Po_2 is reduced to ≈3.5 mm Hg, hypoxia-inducible factor (HIF)-1α becomes stabilized in human microvascular endothelial cells.[8] Recent studies have suggested that NO produced by human endothelial cells can regulate the activity of HIF-1α and AMP-activated protein kinase (AMPK), thus affecting key response pathways to hypoxia and metabolic stress, respectively.[8] Endothelial cells also respond to hypoxia by releasing Ca^{2+} from the ER via inositol-1,4,5-triphosphate receptors (similar to the action of many agonists).[68] The mechanism involves ROS production and is suppressed when mitochondria are depolarized,[68] suggesting

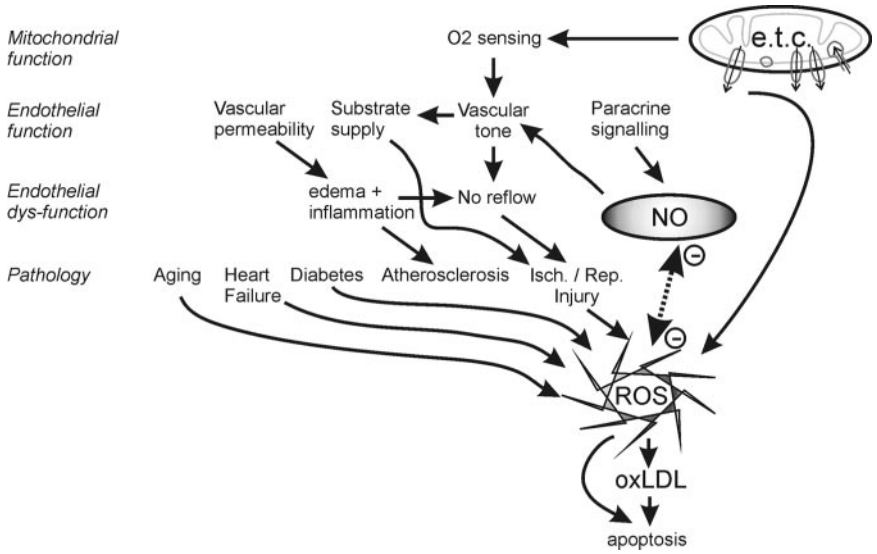

Figure 3. Mitochondrial function affects endothelial function during normal physiology and, consequently, can affect endothelial dysfunction during various cardiovascular pathologies. Many of these pathologies increase levels of oxidative stress. ROS can react with various proteins and lipids such as LDL, forming highly damaging oxLDL. By destructively reacting with NO, ROS can promote a negative-feedback pathway that further exacerbates endothelial damage and dysfunction. The central importance of ROS in this flowchart is evident, and it is possible that endothelial mitochondria contribute to ROS generation. They may also play important roles in sensing the local environment of the blood (eg, P_{O_2}). See text for details. For clarity, pathways involving calcium are not included on this figure (but see Figure 2).

that mitochondria may sense the hypoxia and transmit a signal to the ER via ROS.

Another intriguing role that has been postulated for endothelial cell mitochondria is as mechanotransducers, because they are anchored to the actin cytoskeleton. Cyclic strain was found to more than double mitochondrial ROS production in HUVECs cells.[69] Mechanotransduction required an intact actin cytoskeleton and resulted in an increase in vascular cell adhesion molecule-1 mRNA expression.[69]

Mitochondria and Endothelial Apoptosis

There are 2 main pathways of apoptosis: an extrinsic pathway, initiated by death receptor (eg, Fas, tumor necrosis factor α [TNFα] receptor) activation; and an intrinsic pathway, defined by mitochondrial cytochrome *c* release into the cytoplasm, a process modulated by proteins of the bcl2 family.[70] In some situations, mPTP opening appears to be upstream of cytochrome *c* release, and it has been suggested that bcl2 proteins exert part of their regulatory function by modulating mPTP opening.[70] However, in other cases, cytochrome *c* release proceeds independently of mPTP opening.[71] Although deletion of the mPTP component cyclophilin D greatly protects cells against necrotic death cell, no effect on either intrinsic or extrinsic pathways of apoptosis has been detected.[42,44,45] On the other hand, mCICR in myotubes was shown to be attributable to mPTP opening and results in a progressive wave of mitochondrial depolarization followed by apoptosis.[40] Recent evidence suggests that there may also be a mitochondrial apoptotic pathway distinct from that activated by proapoptotic bcl-2 family proteins, which must proceed via the mPTP because it is dependent on cyclophilin D.[72] Ultimately, whether cell death proceeds via apoptosis or necrosis after mPTP opening may depend on whether there is sufficient ATP provided by glycolytic pathways to sustain ATP-dependent apoptotic pathways[73]; if not, necrosis will result.

Although endothelial apoptosis is unlikely to occur to a significant extent in healthy vasculature, the consequences of excessive apoptosis are likely to include a deficiency in its protective function as a physical barrier.[74] Endothelial apoptosis may increase the potential for adhesion of monocytes involved in clearance of apoptotic bodies, particularly because membrane vesicles from apoptotic cells contain biologically active oxidized phospholipids.[74] This may be highly significant in the development of several pathologies, such as atherosclerosis and diabetes.

Involvement of Endothelial Mitochondria in Atherosclerosis

Properly functioning endothelium inhibits platelet aggregation, leukocyte adhesion, and VSMC proliferation by various mechanisms that include the provision of a physical barrier and the production of NO (Figure 3).[75] Consequently, damage to endothelium structure or function is considered to be among the root causes of atherosclerosis.[76] The subsequent combination of inflammation, cell death, cell proliferation, fibrosis, and eventual calcification contributes to the formation of an atheromatous plaque, which can cause clinical sequelae when it leads to stenosis or ruptures, causing a thrombus.[77] A key factor in the initial endothelial damage is the infiltration of oxidized low-density lipoprotein (oxLDL) into the arterial endothelium.[77,78] oxLDL appears to exert its toxic effects via many mechanisms[79] and has damaging effects on various aspects of endothelial function,[78] including on endothelial mitochondria.[75] Endothelial mitochondria may actually contribute to the toxic effects of oxLDL, as the mitochondria are a significant source of ROS and increase production in response to lipid oxidation products such as oxLDL.[80]

Impaired NO signaling, likely as a consequence of endothelial damage or dysfunction, has been proposed as an important mediator of atherosclerosis.[81] For example, apolipoprotein E–knockout mice are prone to developing atherosclerosis, and this propensity is further increased when they are crossed with mice lacking eNOS.[82] oxLDL (or other ROS, such as those produced by the mitochondrial electron transport chain) may enhance the degradation of NO. Despite the protective qualities of endogenous NO, excessive levels of NO can be detrimental. For example, high levels of NO are

produced by inducible NO synthase in inflammatory cells,[83] which can further damage endothelial cells. The spatial and temporal regulation of NO production and diffusion is no doubt critical to the proper regulation of endothelial function, and this is likely to be perturbed during the development of atherosclerosis.

The high turnover of endothelial cells in atherosclerosis suggests that apoptosis may contribute to the pathology.[84,85] Many proatherogenic factors, including oxLDL, angiotensin II, and oxidative stress, can induce endothelial cell apoptosis,[85,86] and oxLDL-induced apoptosis of cultured aortic endothelial cells can be prevented by a caspase inhibitor.[87] The increased production of Fas ligand and TNFα indicates that the receptor-mediated (extrinsic) pathway of apoptosis is involved in atherosclerosis,[85,88] but there is also evidence for the involvement of the mitochondrial apoptotic pathway. For example, expression of Bax and caspase 3 is increased in atherosclerotic areas.[85] Furthermore, exposure of cultured human aortic endothelial cells to oxLDL or glycated oxLDL causes a decrease in expression of antiapoptotic proteins, followed by activation of the mitochondrial apoptotic pathway.[89,90] Endothelial cell apoptosis in response to oxidative stress can be prevented by the antiapoptotic domain of Bcl-2.[91] Interestingly, cyclosporine A, an inhibitor of the mPTP, inhibits oxLDL-induced apoptosis of HUVECs,[92] although the role of mPTP in apoptosis is controversial (see above), and cyclosporine A does have many other actions. Another study using human microvascular endothelial cells found that oxLDL induced a sustained cytosolic Ca^{2+} rise that activated 2 distinct Ca^{2+}-dependent mitochondrial apoptotic pathways: the first involving the release of apoptosis inducing factor and the second proceeding via the Bid-mediated (intrinsic) mitochondrial apoptotic pathway and also requiring mPTP.[93]

Statins were developed to treat hypercholesterolemia and diabetes by inhibiting 3-hydroxy-3-methylglutaryl coenzyme A reductase and reducing cholesterol levels. However, statins have also been shown to have additional, direct protective effects on the endothelium, apparently by restoring endothelial NO production.[76,94,95] Promisingly, a recent trial observed significant regression of atherosclerotic plaques in response to "very intensive statin therapy."[96] Somewhat confusingly, however, despite apparently reducing apoptosis in human atherosclerotic plaques,[97] statins have been found to promote apoptosis in VSMCs.[98,99] Furthermore, in some cell types, statins increase the sensitivity of mPTP to opening when mitochondria are challenged with Ca^{2+}.[100,101] Although it has not yet been tested in endothelial cells, this may be attributable to a dose-dependent effect. For instance, atorvastatin exhibits a cardioprotective effect in the isolated perfused rat heart that is profoundly dose-dependent.[102]

It is worth emphasizing at this point that cardiovascular diseases involve the pathological interaction between many cell types. For example, although the loss of the functional integrity of endothelial cells appears to be fundamental in initiating the development of atherosclerosis,[74] an elegant recent study in transgenic mice provides evidence that VSMC apoptosis contributes much more significantly to the development of later aspects of vulnerable lesions.[103]

mtDNA is relatively susceptible to oxidative damage because it lacks histones and has a limited capacity for DNA repair. Thus, the extent of mtDNA damage can be used as a measure of total exposure to oxidative stress.[104] In atherosclerosis-prone apolipoprotein E–knockout mice and in human arterial specimens, the extent of atherosclerosis correlates with mtDNA damage.[105] Because mitochondrial mutations may lead to the production of greater levels of ROS, this may initiate a cycle of positive feedback. This also suggests the possibility that inherited mtDNA mutations could even initiate vascular damage and increase the risk of atherosclerosis. Over the past 10 years, mtDNA mutations have been increasingly identified as causing a wide spectrum of diseases and could conceivably be involved in the progression of atherosclerosis; however, the symptoms of mtDNA diseases tend to be much more severe in highly oxidative tissue such as muscle and brain. It may be that mtDNA mutations severe enough to cause a vascular phenotype also cause developmentally lethal defects in other tissues. However, there may be certain mutations that result, through heteroplasmic distribution of mitochondria, in compromised mitochondrial function in relatively nonoxidative cells: such an example that increases the risk of diabetes is discussed in the following section. The syndrome MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes) encompasses stroke-like episodes that, although of unknown etiology, may reflect mitochondrial dysfunction in the capillary endothelial cells in the brain.[106] In MELAS patients harboring a mtDNA mutation, abnormal and enlarged mitochondria have been detected in endothelium.[107] There is some evidence that MELAS is associated with abnormalities in blood vessels both at the level of endothelial cells and smooth muscle cells,[108,109] and flow-mediated dilatation has been found to be defective in some patients with MELAS.[110] Interestingly, MELAS-associated endothelial dysfunction was improved by prolonged L-arginine supplementation to increase eNOS activity.[110]

Involvement of Endothelial Mitochondria in Diabetes

By definition, type 2 diabetes (the more prevalent form) entails hyperinsulinemia and hyperglycemia and is also characterized by hyperlipidemia. The progression of the disease is complicated by a number of secondary problems in which the major target seems to be vascular, including a cardiomyopathy, increased occurrence of sudden death by cardiac infarction, and microvascular disease affecting the kidney and the retina.[111]The manifestation of vascular disease in the coronary arteries results in a greatly increased risk of heart failure in the diabetic population.[112,113]

There is some evidence to suggest that diabetic vascular disease results from oxidative stress. The endothelium is exposed directly to the blood and is therefore exposed directly to toxic metabolites and products such as oxLDL, which can cause damage directly by oxidative processes. In addition, the work of Brownlee, in particular, has helped to delineate several pathways by which hyperglycemia damages cells by causing increases in advanced glycation end products, increased hexosamine and polyol flux, and activation of

classical isoforms of protein kinase C, via increased levels of oxidative stress.[114] They determined that hyperglycemia inhibits eNOS activity and expression in aortic endothelial cells by increasing mitochondrial superoxide production.[115] This study used a relatively high concentration of glucose (25 mmol/L) to produce hyperglycemia, so it will be interesting to investigate whether glucose levels that would be classified as "intermediate risk" also increase mitochondrial ROS production. If so, this would strongly indicate that diabetes can lead to inhibition of endothelial NO production and derange normal vasodilation.[113]

Hyperglycemia may also affect mitochondrial Ca^{2+} dynamics. For example, exposure of cultured endothelial cells to hyperglycemic medium results in increased $[Ca^{2+}]m$ after histamine-mediated $[Ca^{2+}]c$ signaling, possibly as a consequence of altered mitochondrial morphology.[116] Hyperglycemia may also induce endothelial apoptosis and contribute to the development of atherosclerosis.[117]

The mPTP may constitute part of the pathway toward endothelial cell death in diabetes as well as other pathologies. Conditions of oxidative stress or hyperglycemia have been found to cause mPTP opening in both HUVECs and bovine aortic endothelial cells.[118] Inhibition of mPTP opening by exposure to cyclosporine A prevents this death.[118] Some antidiabetic drugs may have effects on the mPTP. One of these, metformin, appears to prevent opening of the mPTP, although this may be secondary to metabolic effects of the drug.[118]

In patients with inadequately controlled diabetes, myocardial energy utilization shifts almost exclusively to the oxidation of free fatty acids. Although the consequence of this is extensively studied in cardiomyocytes, the effect on mitochondrial metabolism in endothelial cells has been largely disregarded because they are regarded as being primarily glycolytic. Interestingly, however, recent evidence suggests that activation of the fuel-sensing enzyme AMPK in endothelial cells promotes oxidation of fatty acids (but not glucose) as a source of ATP production, whereas dependence on glycolysis is decreased.[119] This suggests that in response to the metabolic disturbance associated with diabetes, the role of mitochondria as energy sources in endothelial cells might become significant. The corollary of this observation is that processes depending on mitochondrial function in those cells could be adversely affected. For instance, isolated coronary endothelial cells can normally maintain their ATP levels down to a Po_2 of 0.1 mm Hg, but when forced to rely on oxidative pathways of ATP generation, ATP levels diminish below a Po_2 of 3 mm Hg.[11] It would be interesting to determine whether this impairs their potential ability to sense low O_2 concentrations (see under Conclusion). Interestingly, in a rat model of diabetes the hypoxia-sensing transcription factor HIF-1α is increased,[120] possibly indicating the existence of a state that has been termed "hyperglycemic pseudohypoxia."[120,121]

Involvement of Endothelial Mitochondria in Heart Failure

Heart failure occurs progressively and limits effective pump function. It is a secondary defect that occurs after damage or disorder in the heart. For example, diabetes mellitus predisposes patients to developing heart failure, partly because it causes endothelial dysfunction.[122] Endothelial dysfunction is an important component leading to heart failure. In dogs with pacing-induced heart failure, endothelium-mediated control of the coronary circulation is depressed.[123] Coronary endothelial dysfunction appears to precede heart failure in a canine coronary microembolization-induced heart failure model.[124] Although coronary endothelial flow is more difficult to measure in humans, diminished forearm vasodilatation in patients with heart failure indicates that endothelial function is also attenuated in humans.[125,126]

Levels of inflammatory markers such as TNFα are increased in patients with heart failure. TNFα is more than simply a marker of heart failure; it causes significant endothelial damage by decreasing eNOS expression in endothelium.[127] In failing human hearts, levels of eNOS protein and mRNA appear to be decreased in the endothelium.[127] In the hearts of dogs with pacing-induced failure, there is reduced NO production in coronary blood vessels[128] and diminished NO-dependent arteriolar dilation.[128] The increased level of ROS in the failing heart may react with and further deplete levels of bioavailable NO.[127] Although much of the ROS produced in response to TNFα is probably produced by nonmitochondrial enzymes, such as xanthine oxidase, NADPH oxidase, and NOS, some may originate from mitochondria, particularly when innate oxidative stress defense mechanisms become overwhelmed. Certainly, in vitro treatment of an endothelial cell line with TNFα increases mitochondrial ROS production.[129] Administration of a low-molecular-weight superoxide dismutase mimetic to dogs protects against pacing-induced heart failure and preserves coronary endothelial function.[130] Antioxidants (eg, vitamin C) seem to improve endothelial functionality and reduce the inflammatory response in patients with heart failure.[127]

As mentioned, endogenous NO may regulate myocardial mitochondrial oxidative phosphorylation by directly competing with oxygen at complex IV (see above, under section Endothelial NO Production). Thus, at a low Po_2, bradykinin decreases O_2 consumption in wild-type mice but not in mice lacking eNOS.[131] Similarly, NO derived from microvascular endothelium attenuates myocardial mitochondrial respiration in isolated myocardial muscle segments from the left ventricular free wall of canine hearts.[132] This regulatory function is lost in myocardial muscle from dogs with pacing-induced heart failure.[132] Mitochondrial inhibition by peroxynitrite, formed by reaction between NO and ROS, is irreversible, and this could contribute to cardiac dysfunction in situations such as heart failure.[133]

Involvement of Endothelial Mitochondria in Cardiac Injury From Ischemia and Reperfusion

In contrast to the chronic condition of heart failure, acute episodes of ischemia result from the occlusion of coronary arteries with atherosclerotic thrombi or other blockages. Although percutaneous coronary intervention and thrombolytic agents can relieve the ischemia and restore

blood flow, reperfusion may also precipitate a distinct set of injuries to the heart.[134]

Studies performed in the 1980s initially indicated that coronary endothelial cells in the vasculature are more resistant to ischemia than are cardiomyocytes. This was determined both by examining the ultrastructure of infarcts after up to 180 minutes of coronary occlusion of dog hearts[135] and by measuring the release of intracellular enzymes from isolated guinea pig hearts exposed to sixty minutes of anoxic perfusion followed by fifteen minutes of reperfusion.[136] The apparent innate resistance of endothelial cells seemed logical, considering that they obtain their energy primarily by glycolysis and that their energetic state can remain stable even in severe hypoxia of $pO_2 < 0.1$ mm Hg.[11] However, in recent years, injury that occurs during the reperfusion period after ischemia has been recognized as an entity distinct from that which occurs during ischemia.[137] This distinction is supported by the observation that protective modalities applied at the point of reperfusion (so-called "postconditioning") are just as effective as when applied before ischemia ("preconditioning").[138,139] When examined by electron microscopy, both apoptotic and necrotic capillary endothelial cells are detected in dog hearts that have been subjected to 45 or 90 minutes of ischemia followed by 6 hours of reperfusion.[140] Electron microscopic investigations during reperfusion after cardioplegia suggest that endothelial cells might, in fact, be even more prone to damage during reperfusion than cardiomyocytes.[141] In a recent study using isolated perfused rat hearts, apoptosis was detected initially in endothelial cells of the small coronary vessels early after reperfusion, with apoptosis spreading radially from vessels to cardiomyocytes at later time points.[142] The same group later used antibodies specific to activated (cleaved) caspases 8 and 9 to show that the intrinsic, mitochondrial pathway of apoptosis was activated predominantly in endothelial cells, whereas the extrinsic pathway was activated predominantly in cardiac myocytes.[143] Although the significance of apoptosis in ischemia and reperfusion injury is hotly debated,[144,145] some studies have shown that inhibiting caspase activity can reduce ischemia and reperfusion injury.[146,147] Whether the reduction in infarct size is direct or secondary to protection of endothelial cells is not obvious.

Perhaps of even greater significance than the question of whether endothelial survival is affected by ischemia and reperfusion is the issue of how extensively the coronary endothelium is damaged or becomes dysfunctional during ischemia and reperfusion. Damage to coronary endothelial cells certainly does occur, as evidenced by the adherence of leukocytes to "activated" endothelial cells soon after reperfusion injury of the heart in vivo (although this is also a consequence of increased production of soluble inflammatory mediators by endothelium). Inflammation, in combination with tissue edema, may result in "plugging" of capillaries and reduced capillary perfusion.[148,149] Furthermore, during reperfusion, endothelium-dependent relaxation of coronary arteries is decreased, although ischemia alone has no effect.[148,150] There is also increased extravasation of blood cells and peptides. Therefore, even if the majority of the endothelium remained viable, impairment of its trophic, tonic, and trafficking functions may drastically impair the ability of the heart to recover normal contractile function. Endothelial damage appears largely to account for the "no-reflow" phenomenon observed when regions of a deoccluded heart do not restore normal blood flow,[149] although other factors may also contribute.[151] Some studies suggest that these areas of microvascular disturbances, which expand progressively during the initial hours of reperfusion, may even promote further expansion of the region of infarct (discussed elsewhere[149]).

Endothelial dysfunction appears to be a consequence of increased ROS production at reperfusion. Mice overexpressing superoxide dismutase are resistant to capillary no-reflow after ischemia and reperfusion of the gut.[152] Activated inflammatory cells undoubtedly contribute to the ROS produced, because mice in which endothelial–lymphocyte interaction has been dampened maintain the capacity for endothelial-dependent vasodilation after ischemia and reperfusion.[153] Another source is likely to be xanthine oxidoreductase, high levels of which are found in endothelial cells after reperfusion.[154,155] Mitochondria may also contribute to the endothelial production of ROS during reperfusion.[156] In HUVECs, hypoxia and/or reoxygenation increases superoxide production, which appears to increase the permeability of endothelial cell layers via an increase in interleukin-6 secretion.[157,155] Mitochondria have been proposed as the source of the superoxide because its effects could be abrogated by inhibition of mitochondrial electron transport at either complex I or complex III, and not by inhibitors of xanthine oxidoreductase, NOS, or NADPH oxidase.[157,158] However, the story may not be so straightforward, because inhibition of complex III with antimycin is more commonly used to increase mitochondrial ROS production,[159] and diphenyleneiodonium, which inhibited superoxide production in these studies,[157] inhibits NADPH oxidase.[160]

If the endothelium is indeed an important target of reperfusion injury, then this raises the question of whether ischemic pre- or postconditioning functions primarily at the level of the myocytes or the endothelium. Preconditioning is certainly capable of increasing the survival of both cell types in vitro and in vivo.[137,150] In terms of preservation of endothelial function, both pre- and postconditioning are effective in dog models.[139,161] A novel approach for investigating endothelial dysfunction after ischemia and reperfusion in humans using a noninvasive model has been developed by MacAllister and colleagues. By inflating a blood pressure cuff to 200 mm Hg for 20 minutes and then releasing it, they are able to induce ischemia and reperfusion in the forearm of healthy human volunteers, which results in endothelial dysfunction (measured as reduced flow mediated dilation).[162] This can be prevented by a preconditioning stimulus of 3 cycles of 5 minutes of ischemia applied to the same[162] or the contralateral arm ("remote preconditioning").[163] A "postconditioning" protocol applied at the end of the period of ischemia is equally effective.[164] Importantly, experiments using a pig model indicate that remote preconditioning of the lower limb can protect against subsequent cardiac ischemia and reperfusion injury.[163] Although remote protection appears to be at least partially neuronally mediated,[165] it may also involve a humoral mediator[166] and requires the activation

of ATP-sensitive potassium (KATP) channels because it is modulated by glibenclamide or diazoxide.[167]

Evidence to date suggests that the pathways that increase endothelial cell survival during ischemia/reperfusion resemble those in cardiomyocytes and are, therefore, likely to involve the mitochondria.[150] Protection of endothelial function is likely to result from other mechanisms such as the inhibitory effects of preconditioning on expression of endothelial adhesion molecules, which results in reduced neutrophil–endothelial interactions.[150]

There is not a great deal known about mitochondrial Ca^{2+} in endothelial cells during ischemia/reperfusion. In combination with oxidative stress, calcium overload is believed to be an important component leading to mPTP opening and death in myocytes. Most assays used to measure calcium overload or mPTP opening in vivo obtain results only from the cardiomyocyte component. Similarly, mitochondria extracted from whole hearts originate mainly from cardiomyocytes, because their mitochondrial numbers greatly outnumber those in endothelial and other cell types. Hypoxia/reoxygenation stimulates intracellular calcium oscillations in human aortic endothelial cells,[168] and this may involve mitochondrial uptake of Ca^{2+} or mitochondrial ROS. However, Ca^{2+} oscillations generated in human aortic endothelial cells stimulated by histamine seem to be modulated by ROS derived from the NADPH oxidase.[169]

Angiogenesis—the formation of new vasculature—is a form of adaptation to chronic ischemia.[168] It involves the development of new coronary collateral vessels and microvascular angiogenesis and endothelial-derived NO plays a part in the complex system.[168] Intriguing recent data indicate that mitochondrial-derived ROS can modulate the angiogenic phenotype by oxidation of PTEN (phosphatase and tensin homolog),[169] although more studies are required to determine whether these results obtained using a fibrosarcoma cell line are applicable to endothelial cells or other cell types of the vasculature.

Conclusion

One point that we have tried to highlight in the discussion of the pathophysiologies here is that the same risk factors (hypercholesterolemia, hypertension, and hyperglycemia) are common to several major diseases. Each of those risk factors appears to disrupt the normal endothelial function. Endothelial mitochondria appear to modulate the intracellular dynamics of NO, ROS, and Ca^{2+}, which, in turn, control endothelial function. Damage to endothelial mitochondria may therefore represent an important step in the development of endothelial dysfunction.

Many of the syndromes described here also entail inflammation, and endothelial cells are the primary targets of circulating immune and inflammatory mediators. It will be important to establish the extent to which oxidative stress, and, in particular, excessive levels of oxLDL, can interfere with mitochondrial function in endothelium.

Another area that may merit further study is the role of the putative mitochondrial KATP (mKATP) channel in cardiac endothelial cells. Although investigation in this area has been limited by the lack of molecular information about the channels, some evidence suggests that mKATP opening protects the endothelium. It will be important to establish the specificity of the drugs used in such experiments.

In summary, it seems that the mitochondria of endothelia have been somewhat neglected, on the basis that they do not contribute significantly to energy production during normal physiology. However, with new evidence suggesting that mitochondria in various cell types are able to divide, fuse, and redistribute in response to the metabolic needs of the cell, their simple presence in endothelial cells would seem to be sufficient to indicate that they are not just there "for decoration." As the renaissance in mitochondrial research continues to redefine and extend the role of mitochondria in the cell, from modulators of calcium signaling to sources of ROS and NO to regulators of cell death, it is time to reexamine the role of mitochondria in endothelium.

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