Free Radicals, Mitochondria, and Oxidized Lipids: The Emerging Role in Signal Transduction in Vascular Cells

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Abstract—Mitochondria have long been known to play a critical role in maintaining the bioenergetic status of cells under physiological conditions. It was also recognized early in mitochondrial research that the reduction of oxygen to generate the free radical superoxide occurs at various sites in the respiratory chain and was postulated that this could lead to mitochondrial dysfunction in a variety of disease states. Over recent years, this view has broadened substantially with the discovery that reactive oxygen, nitrogen, and lipid species can also modulate physiological cell function through a process known as redox cell signaling. These redox active second messengers are formed through regulated enzymatic pathways, including those in the mitochondrion, and result in the posttranslational modification of mitochondrial proteins and DNA. In some cases, the signaling pathways lead to cytotoxicity. Under physiological conditions, the same mediators at low concentrations activate the cytoprotective signaling pathways that increase cellular antioxidants. Thus, it is critical to understand the mechanisms by which these pathways are distinguished to develop strategies that will lead to the prevention of cardiovascular disease. In this review, we describe recent evidence that supports the hypothesis that mitochondria have an important role in cell signaling, and so contribute to both the adaptation to oxidative stress and the development of vascular diseases. (Circ Res. 2006;99:924-932.)

Key Words: apoptosis • atherosclerosis • hypertension • diabetes • environmental tobacco smoke • endothelial cells • electrophilic lipids • mitochondria • prostaglandins • redox signaling • thiols

The “free radical hypothesis” for vascular dysfunction originally postulated that reactive oxygen and nitrogen species (ROS/RNS) led to nonspecific modification of lipids, proteins, and nucleic acids, which then contributed to the etiology of the disease. However, this view has changed in recent years with the recognition that these molecules can play a role in signal transduction through specific modification of cell signaling proteins. Overall this field has come to be known as “redox cell signaling” and describes how ROS/RNS can lead to the activation of pathways that control cell differentiation and apoptosis. These mechanisms are of particular relevance to cardiovascular diseases (CVD), such as atherosclerosis and hypertension, and have been studied intensively in vascular cells. During atherosclerosis, activation of the enzymes in both infiltrating macrophages and vascular cells generate high levels of ROS/RNS, thereby changing the oxidation status of thiols on signaling proteins: the redox tone. The redox cell signaling pathways that are activated are balanced between those that protect endothelial and vascular smooth muscle cells with those that initiate cell...
ROS formation is associated with the cell signaling that species in signal transduction has emerged. Mitochondrial damage to the organelle and loss of bioenergetic function but also disruption of mitochondrial-dependent redox signaling pathways. In this review, we discuss (1) mitochondrial DNA damage in the etiology of vascular disease, (2) the mechanisms for mitochondrial ROS formation and influence on redox cell signaling events, and (3) the importance of oxidized lipids generated by enzymatic and nonenzymatic mechanisms. An increased understanding of how ROS/RNS contribute to cellular protection is now particularly important, because a number of therapeutic interventions based on decreasing these species have not demonstrated clinical benefit in vascular diseases. This is particularly striking in the case of low-molecular-weight antioxidants and inhibitors of the cyclooxygenase pathway. In both cases, we can hypothesize that these agents have limited therapeutic benefit, because ROS/RNS and oxidized lipids have potentially beneficial effects at the level of signal transduction.18

The small molecules involved in redox signaling pathways can be generated from several families of enzymes, such as the NADPH oxidases and nitric oxide synthases (NOS), in a controlled manner within the cell.19,20 These ROS/RNS are derived from the metabolism of oxygen- or nitrogen-containing compounds and their subsequent reaction products.15 Although it has been known for some time that mitochondria are a source and target for ROS/RNS, it has been only recently that a role for the formation of these species in signal transduction has emerged. Mitochondrial ROS formation is associated with the cell signaling that controls proliferation,21,22 hypertrophy, hypoxia,23 and apoptosis.24,25 The cells of the vascular system provide an interesting stage on which to examine mitochondrial signal transduction in the pathophysiology of human disease. Interestingly, among the original tenets of the free radical hypothesis is that lipid oxidation products are implicated in the progression of atherosclerosis.26 Recent studies suggest that the mitochondrion may be the site at which redox signaling mediated by lipid oxidation products is coordinated.27–29

Vascular pathologies are multifactorial, but it is clear that mitochondrial dysfunction can contribute to the pathophysiology of these diseases. This appears to not only involve damage to the organelle and loss of bioenergetic function but also disruption of mitochondrial-dependent redox signaling pathways. In this review, we discuss (1) mitochondrial DNA damage in the etiology of vascular disease, (2) the mechanisms for mitochondrial ROS formation and influence on redox cell signaling events, and (3) the importance of oxidized lipids in mediating adaptation to stress or cell death in the endothelium.

**Damage to Mitochondrial DNA and Cardiovascular Disease**

In the mitochondrion, nonspecific modification of proteins, lipids, and nucleic acids can lead to damage of these molecules and change in mitochondrial function.30 Mitochondrial DNA (mtDNA) is particularly susceptible to modification by ROS/RNS because (1) mtDNA is in close proximity to the site of ROS/RNS production; (2) mtDNA lacks histone proteins, which can protect it from oxidative damage; and (3) mitochondrial polymerases lack specificity for base excision repair and are themselves modified by ROS, which can potentially lead to changes in polymerase function and increased mutation rates in mtDNA.32 Damage to mtDNA can rapidly lead to functional changes in the cell because it encodes 13 critical protein components of the mitochondrial respiratory chain. It has been shown that mtDNA damage occurs in CVD, and this is supported by findings in human subjects, as well as studies in animal and cellular models.33–35

One of the important concepts from studies of genetic mitochondrial diseases is that there is a threshold at which damage to respiratory chain proteins results in loss of bioenergetic capacity.36 Because repair of mitochondrial proteins often requires new protein synthesis, damage to mtDNA is likely to lower this threshold (Figure 1A). Thus, the accumulation of mtDNA damage over a lifetime may increase the susceptibility to the development of pathology.34 In fact, the well known proatherogenic risk factors such as smoking, hypercholesterolemia, and obesity are all associated with increased mtDNA damage.35

For example, smokers have an increase of 6-fold in the level of mtDNA damage and a 7-fold increase in mtDNA deletions in lung tissues compared with nonsmokers.37 Exposure to either environmental tobacco smoke or smoking decreases mitochondrial respiratory chain function and enhances lipid peroxidation. The mediators involved in these effects are not clear but could involve reactive lipid aldehydes in cigarette smoke that could directly modify DNA and proteins, or carbon monoxide.38 The mechanism of carbon monoxide may involve direct inhibition of cytochrome c oxidase, resulting in decreased mitochondrial respiration, an increase in oxidative stress and lipid peroxidation, and ultimately mtDNA damage39,40 (Figure 1B).

Accumulated mtDNA damage will hinder the replacement of respiratory chain proteins damaged by ROS production. These damaged proteins are more likely to generate ROS in an uncontrolled manner, thereby accelerating bioenergetic dysfunction. In this respect, intramitochondrial antioxidants have a critical role to play because they may alter the progression of the disease by preventing damage to existing proteins. Indeed, decreased manganese superoxide dismutase (MnSOD) activity promotes atherosclerotic lesion development and increases aortic mtDNA damage in apoE−/− mice.41 Recent studies suggest that activation of the NADPH oxidases leads to crosstalk with the mitochondrion and increased ROS formation from the organelle.42 A further example of the link between accumulated mtDNA defects and susceptibility to CVD may be the impact of exposure to environmental tobacco smoke in utero. Gestational exposure to secondary tobacco smoke in mice increases the rate of development of atherosclerosis as the animals mature.43 In support of a role for mitochondria in this process, gestational exposure to cigarette smoke also increases mtDNA damage in the adult.44 The mechanisms of these effects likely involve the placental transfer of components of tobacco smoke, such as reactive lipid oxidation products,44 which may cause fetal mtDNA damage to liver,
Figure 1. Mitochondrial DNA damage and the threshold for bioenergetic dysfunction in CVD. A, When the mtDNA damage is low, the threshold for bioenergetic dysfunction is high, allowing the organelle to meet the bioenergetic needs of the cell. The dark shaded area represents “normal” mitochondrial function. As mtDNA damage progresses, the threshold at which the organelle fails to meet bioenergetic demands decreases and ROS formation increases. Factors such as environmental tobacco smoke (ETS), atherosclerosis, and inherited mtDNA mutations will result in the lowering of this threshold. With each added insult, mitochondrial ROS and mtDNA damage are increased. The overall result is a vicious cycle consisting of a decrease in protein expression and repair, which further impairs proper mitochondrial function and biogenesis. B, Cardiovascular risk factors such as hypercholesterolemia and ETS can increase mitochondrial ROS and lipid peroxidation in vascular cells. These oxidation products cause mitochondrial dysfunction through mechanisms including mtDNA damage and mitochondrial protein modification, which may further increase ROS, decrease ATP synthesis, damage vascular cell function, and affect cell survival. The resulting mitochondrial dysfunction in this scenario has been correlated with increased susceptibility to CVD.

Testing the threshold hypothesis is challenging in vivo, but the recent finding that NO may control mitochondrial biogenesis raises the possibility that synthesis of new organelles may avoid bioenergetic dysfunction by restoring a “normal” threshold.45 Because the NO-soluble guanylate cyclase pathway is impaired in early atherosclerotic lesions, then it may be possible to activate the NO signaling pathway downstream of soluble guanylate cyclase and increase mitochondrial biogenesis. Once the threshold has been restored, mtDNA damage can then be repaired. Alternatively, the reintroduction of endothelial progenitor cells to damaged areas of the vasculature may offer a source of endothelial cells containing undamaged mtDNA. However, little is known of the role of mitochondria in these progenitor cells.

Formation of ROS/RNS and Their Interaction With Mitochondria

At lower levels of ROS/RNS, damage to key targets in the mitochondrion, such as mtDNA, is prevented by the presence of intramitochondrial antioxidant defenses. The characteristics of specificity, localization, and reversibility, which are generally associated with cell signaling pathways, can now be identified in the formation of ROS at low rates from the respiratory chain. Mitochondrial superoxide is produced by the one-electron reduction of oxygen by complexes I and III of the respiratory chain and some components of the tricarboxylic acid cycle such as α-ketoglutarate dehydrogenase.46–48 Early studies using hypoxia or mitochondrial toxins characterized the formation of mitochondrial superoxide or hydrogen peroxide as an electron “leak” from the respiratory chain.46,49 It is possible that uncontrolled ROS formation also occurs pathologically when mitochondrial respiratory proteins are damaged.50 However, hyperoxia and mitochondrial toxins do not model physiological ROS production pathways and therefore do not give insights into the mechanisms of mitochondrial redox signaling.

A likely mechanism through which mitochondria may transduce and regulate cellular redox signals is through thiol switching. For example, it has been shown that the mitochondrial glutaredoxin 2 pathway modulates the redox couple that control the S-glutathionylation of proteins in the respiratory chain.51 Taken with the finding that S-glutathionylation of the 70-kDa subunit of complex I leads to superoxide formation, these data suggest there is crosstalk between thiol status and controlled formation of ROS.46,51

Mitochondrial superoxide formation can also be regulated by uncoupling proteins (UCPs), and several recent studies suggest they play a role in the etiology of CVD, although the effects appear to depend on the specific UCP isoform. Overexpression of UCP-1 in aortic smooth muscle cells increases superoxide formation, hypertension, and exacerbates atherosclerotic lesion formation.52 However, overexpression of UCP-2 in the vasculature decreases ROS generation and prevents mitochondrial overload in cardiomyocytes,53 and UCP-2 knockout is associated with increased atherosclerotic lesion formation in vivo.54 Nonetheless, these studies do suggest that changes in UCP activity (and thus superoxide formation) can contribute to vascular dysfunction.

Once formed, the mechanisms leading to superoxide signal transduction remain largely unknown but four possibilities can be proposed with some supporting evidence. The most direct of these is that superoxide itself is detected by iron–sulfur proteins such as aconitase. This “receptor” for superoxide may then release iron into the mitochondrion.55,56 This in turn could promote lipid peroxidation and the consequent formation of electrophilic lipids capable of modifying protein thiols (Figure 2). The second mechanism is the conversion of superoxide to hydrogen peroxide by the action of superoxide dismutases, which are present in both the mitochondrial matrix and intermembrane space. An important
property of hydrogen peroxide is that it readily crosses membranes and can regulate cytosolic redox-sensitive signaling pathways. Whether the hydrogen peroxide acts intramitochondrially or in the cytosol is not clear. Evidence for a role of mitochondrial hydrogen peroxide in cell signaling is supported by studies with mitochondrially targeted catalase and overexpression of MnSOD. For example, mitochondrially targeted catalase decreases hydrogen peroxide and protects cells from cytokine-mediated or hypoglycemic cell death. The third mechanism is the competitive reaction of superoxide with NO to form peroxynitrite (Figure 2). Although a role for peroxynitrite in signal transduction has been identified in a number of studies, the link to the mitochondrion remains to be fully elucidated. Lastly, an emerging concept is that the combined interaction of hydrogen peroxide and RNS with peroxidase enzymes can lead to posttranslational modification of proteins. For example, tyrosine residues can be nitrated by myeloperoxidase in the presence of both nitrite and hydrogen peroxide. This may be particularly important in cardiovascular disease because myeloperoxidase levels are an increased risk factor for CVD, and these more reduced forms of the complexes generate more ROS. In this context, reports of a mitochondrial NOS or mitochondrial targeting of cytosolic NOS isoforms are particularly interesting because of the potential for linking NO formation with control of mitochondrial ROS production. The function of the NO-cytochrome c oxidase pathway appears to be intimately related to the maintenance of oxygen gradients within tissues. This occurs at one level, through the acute modulation of actively respiring mitochondria; at a second level, through the regulation of mitochondrial biogenesis in a cGMP-dependent manner; and at a third level, through the impact of NO on the response to hypoxia. Because loss of the NO/cGMP pathway is an early event in the development of atherosclerosis, it is interesting to speculate that this loss could enhance mitochondrial dysfunction in the vessel wall through decreased mitochondrial biogenesis.

These findings have led us, and others, to propose that increased levels of NO derived from inducible NOS can substantially inhibit the respiratory chain to the detriment of adequate ATP production. This is effectively an NO-dependent hypoxia (i.e., "nitroxia") in which the generation of high levels of ROS/RNS is likely to occur. Furthermore, the sensitivity of the respiratory chain to NO inhibition changes in response to stress by mechanisms that have yet to be defined. For example, in a model of cardiac pressure overload, the concentration of NO at which cardiac function is impaired is decreased. Initially, this may be an adaptation to the hypoxic stress associated with cardiac failure. Later, the reaction of NO with superoxide increases protein nitration and may lead to mitochondrial dysfunction and progression of the disease. An interesting series of studies have also supported the concept that NO derived from endothelial NOS (eNOS) under physiological conditions is beneficial through a mechanism involving the modulation of myocardial oxygen consumption.

Although evidence implicates peroxynitrite in mitochondrial protein and DNA damage, it is also clear that it may be important in signal transduction. Although it is clear that NO is derived from NOS, the source of superoxide is not known, but may be mitochondrial. Examples of signaling proteins that can be activated by peroxynitrite include some of the mitogen activated protein kinases (MAPKs), S′-AMP-activated kinase (AMPK), and p21 ras.

**Effects of ROS/RNS on Mitochondrial Signaling**

Mitochondrially generated ROS are important for many of the signaling pathways, which contribute to cardiovascular pathologies. In some cases, the primary stimulus for activation of these pathways has been identified. For example, the
vascular agents angiotensin II, epidermal growth factor (EGF), transforming growth factor (TGF)-β, and tumor necrosis factor (TNF)-α are capable of modulating mitochondrial ROS (Figure 3). The mitochondrial contribution to growth factor signaling appears to involve the transactivation of the growth factor receptors and is associated with the protection against oxidative stress.

Studies of the mechanisms through which mitochondria integrate cell death and survival signals support the hypothesis that mitochondrial ROS/RNS play a role in these signaling pathways, particularly the MAPKs (Figure 3). Specific examples include both the extracellular signal-regulated kinases 1 and 2 (ERKs), c-jun N-terminal kinase (JNK), and p38 MAPK. The downstream effects of MAPK activation via the mitochondria are dependent on the cell type and condition. An interesting mechanism has emerged linking mitochondrial ROS formation and activation of AMPK, as previously described. Because these signaling pathways are protective, this could represent an example of the beneficial effects of peroxynitrite in signal transduction.

Investigators in the cancer field have also recognized the potential importance of mitochondrial ROS formation in the development of this disease, and these concepts may apply to CVD. In cancer, cellular proliferation can be stimulated by mitochondrial ROS, which in turn can be promoted by the accumulation of mtDNA damage. The resultant ROS can then cause the uncontrolled activation of proliferative signaling pathways. For example, oxidative stress in both cancer and CVD can enhance angiogenesis by decreasing MnSOD activity, thereby altering the regulation of MAPKs and the subsequent expression and activation of matrix metalloproteinases 2 and 9.

Lipid Oxidation in Atherosclerosis and Adaptation to Stress

The oxidative hypothesis for atherosclerosis has been critical in the development of our current understanding of the molecular mechanisms of the disease. The central concept is that oxidative modification of low-density lipoprotein (LDL) promotes a proinflammatory response, recruitment of macrophages, and the development of atherosclerotic lesions. However, it is becoming increasingly clear that vascular cells also have the capacity to adapt to oxidative stress through cell signaling mechanisms. Early studies with oxidized LDLs (oxLDLs) showed that low levels were cytoprotective, through mechanisms involving increasing levels of the intracellular antioxidant glutathione (GSH). Later studies revealed that this response was mediated by the transcriptional control of genes regulated by the electrophile response element, including heme oxygenase-1.

Although oxLDL contains a mixture of distinct lipid peroxidation products, it is likely that many of these effects are mediated by specific electrophilic lipids. In support of this hypothesis: (1) electrophilic lipids regulate GSH levels, and polymorphisms in the proteins controlling GSH synthesis are associated with increased inflammatory disease in human populations; (2) depletion of GSH or loss of heme oxygenase in animal models of cardiovascular disease enhances susceptibility to the disease process; (3) electrophilic lipids derived from both enzymatic and nonenzymatic lipid peroxidation can be detected in the vasculature in both humans with cardiovascular disease and in animal models.

One of the challenges in understanding how oxidized lipids mediate these biological effects has been the difficulty in following the fate of these lipids in the cell and monitoring the protein adducts that are formed by these molecules. We have approached this by synthesizing tagged lipids so that both protein adducts and intracellular localization can be addressed. The example shown in Figure 4 is the fluorescent
derivative of the cyclopentenone prostaglandin 15-deoxy-
Δ(12,14)-prostaglandin J₂ (15d-PGJ₂). The reticular mito-
chondrial network in endothelial cells is shown by the
staining of the mitochondrial-specific dye Mitotracker, which
colocalizes with the green fluorescence of the BODIPY-
tagged 15d-PGJ₂ (Figure 4). The mechanism for the mito-
chondrial association of the oxidized lipid is unknown but is
saturable and associated with increased stimulation of mito-
chondrial ROS formation.²⁸ We have also recently shown that
oxLDL induces mitochondrial ROS formation but the mediat-
or in this case has not been identified.²⁷ These data have led
to the hypothesis that mitochondria can detect changes in
extracellular oxidative processes and modulate cell function
through the regulation of cell signaling. It is now clear from
our own work and that of others that the reactivity of lipid
peroxidation products with cellular subproteomes, including
the electrophile-responsive proteome, leads to distinct biolog-
ical responses.¹²²–¹²⁴ Depending on the reactive lipid, these
protein modifications may contribute to protection against
vascular damage or, alternatively, to the development of
atherosclerotic responses of the endothelium. Interestingly, the
electrophilic lipids, which at high concentrations are toxic to
cells, play a critical role in mediating the adaptive response of
endothelial cells. It is now known that pharmacological
inhibition of the cyclooxygenase pathway can remove poten-
tially vasculoprotective prostaglandins, and we speculate that
the electrophilic lipid metabolites from this pathway are also
cytoprotective through the induction of antioxidant defenses.
Figure 4. Localization of cyclopentenone electrophilic lipids to the mitochondrion
in endothelial cells. Top, Structures of 15-deoxy-Δ(12,14)-prostaglandin J₂ (15d-
PGJ₂) and BODIPY-15d-PGJ₂. Asterisks denote electrophilic carbons. Bottom,
Human umbilical vein endothelial cells were incubated with BODIPY-15d-PGJ₂ (10 μmol/L)
for 30 minutes followed by Mitotracker (0.5 μmol/L) for 15 minutes
before fixing. Left, The confocal image for BODIPY-15d-PGJ₂ (green). Middle,
Mitochondria stained with Mitotracker (red). Right, The merged BODIPY and
Mitotracker images with the nuclei stained with DAPI (blue).
Future Prospects
It is now becoming clear that the mitochondrion is not only
integrated into the metabolic function of the cell but also the
signal transduction pathways that control cellular responses.
The loss of control of free radical formation from the
mitochondrion can contribute to the pathology of CVD
through a number of mechanisms including damage to
mtDNA. The challenge is now to determine the precise
molecular switches and second messengers which transduce
mitochondrial ROS to cell signals. These emerging concepts
also provide the impetus to determine how mitochondrial
dysfunction contributes to human disease. Of particular
interest are lipid oxidation products that can be generated
both nonspecifically and through the cyclooxygenase and
lipoxygenase pathways. These compounds interact with the
mitochondrion and induce the formation of ROS. Some of
these signaling pathways contribute to the adaptive anti-

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None.

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