Emerging Roles of Mitochondria ROS in Atherosclerotic Lesions: Causation or Association?

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Mitochondrial-derived reactive oxygen species (mtROS) is one of the major sources of cellular ROS, and excessive mtROS is associated with atherosclerosis progression in both human and mouse models. This review aims to summarize the most recent studies showing the existence, the causes and pathological consequences of excessive mtROS in atherosclerosis. Despite numerous association and causation studies demonstrating the importance of mtROS in atherosclerosis progression, the failure of antioxidant therapy in human randomized clinical trials demands more definitive, cell-type specific investigations. Better mechanistic understanding of mtROS in atherosclerosis may lead to more effective therapeutic strategies.


Key words: Mitochondrial ROS, Atherosclerosis, Macrophage, Inflammation, Antioxidant therapy

Introduction: Mitochondrial Derived ROS and its Regulation

Mitochondrial reactive oxygen species (mtROS) are natural by-products of the electron transportation chain (ETC), a key step in the generation of ATP through oxidative phosphorylation (OXPHOS). In this process, as the electrons are shuttled through a group of protein complexes (complex I-V), electron leakage from Complex I and III results in partial reduction of oxygen and the production of superoxide (O2•−). O2•− is then converted to an intermediate product hydrogen peroxide (H2O2) by matrix magnesium superoxide dismutase (MnSOD, SOD2) or CuZnSOD (SOD1) in the mitochondrial intermembrane space. H2O2 is further reduced to H2O through glutathione peroxidase (GPX) and circulating reduced glutathione (GSH) and oxidized glutathione (GSSG). This antioxidant system is essential for preventing the generation and accumulation of highly reactive products such as peroxynitrite (ONOO−), a product of nitric oxide (NO) with O2•−, and hydroxal radical (•HO), a product of the Fenton reaction between Fe2+ with H2O2. Consequently, this antioxidant system protects the mitochondria from mtROS-induced mtDNA damage, protein oxidation, and lipid peroxidation under normal and pathological conditions.

Convincing evidence supports the concept that mtROS plays physiologic roles through second messenger generation and signal transduction reactions to maintain cellular homeostasis in the vascular wall. For example, sheer stress-induced H2O2 formation and vasodilation are mediated by O2•− from mitochondria. However, excessive mtROS has been implicated in pathophysiologic processes in humans and animal models, including cardiovascular, neurodegenerative diseases, and aging. The balance between mtROS generation and clearance controls mitochondrial oxidative status. This balance is regulated by the mitochondria metabolic status, cross inner-membrane potential (ΔΨ), ETC complexes and uncoupling proteins (UCPs), components of mitochondrial fusion and fission machinery (DRP1, OPA1 and MFNs), and mitochondrial autophagy “mitophagy”.

Evidence of Excessive mtROS in Atherosclerotic Lesions

Due to the physical proximity of mtDNA to
ETC-derived ROS and the lack of protective histone or DNA repair mechanisms that occur in the nuclear genome, mtDNA is much more vulnerable to oxidative damage than genomic DNA. 8-OHdG (8-hydroxydeoxyguanosine) is one of the major DNA products formed upon oxidative damage of DNA in various pathological conditions. 8-OHdG accumulation has been observed in circulating leukocytes and in various cell types in atherosclerotic lesions of human and animal models. We have recently shown that non-nuclear (mitochondrial) 8-OHdG exists in macrophages in aortic root lesions of WD-fed Ldlr mice, and it is significantly associated with total atherosclerotic lesion area. In line with this finding, a 5-kb mtDNA deletion, referred to as the common mtDNA deletion, has been identified in mouse and human atherosclerotic lesions and was found to be closely associated with the extent of atherosclerosis. Furthermore, human and rabbit atherosclerotic lesions react with an antibody that recognizes an oxidized form of cardiolipin, a phospholipid exclusively expressed in mitochondria, suggesting excessive mtROS. Thus, several lines of evidence indicate that excessive mtROS-induced oxidative damage occurs in atherosclerotic lesions of both animal models and humans.

Why does Excessive mtROS Occur in Atherosclerosis?

Failure of Mitochondrial Antioxidant Functions

The antioxidant enzyme systems located in the inner membrane of mitochondria are the first line of defense against the excessive production of mtROS from ETC. Emerging evidence supports the notion that in the early stages of disease development, antioxidant enzyme levels and activities are compensatorily enhanced to maintain redox homeostasis. However, as the disease progresses, antioxidant enzyme levels declines. With regard to atherosclerosis, the aorta of Apoe−/− mice were shown to respond to atherogenic stimuli by an early increase and then subsequent decrease in the expression of antioxidant enzymes, including mitochondrial specific antioxidant GPX1 and SOD2. Similarly, MnSOD expression and parallel enzyme activities were reported to be enhanced only in viable cells, but not in apoptotic cells, in human atherosclerotic lesions. In advanced atherosclerosis or atherosclerosis accompanied with hemodialysis (HD), diabetes, and smoking, excessive mtROS is observed and is associated with a marked decrease in the activity of these antioxidant enzymes. For example, HD patients were shown to have a significantly higher carotid artery intima media thickness (CIMT) compared with healthy controls, and the levels of SOD and GPX were negatively correlated with CIMT. Another study showed that the specific activity of SOD2 in Apoe−/− mice exposed to second hand smoke was significantly decreased and mitochondria function was impaired. In another study, endothelial cells isolated from type 2 diabetes mice had a lower level of SOD2 expression compared with non-diabetic controls, which was associated with a higher amount of mtROS and impaired endothelial function. Together, these observations suggest that mitochondrial oxidative stress and damage in the advanced stages of atherosclerosis are due, at least in part, to the failure of antioxidant mechanisms. The underlying mechanisms explaining the failure of mitochondrial antioxidant function in advanced disease stages are not completely understood. Examples of hypotheses being explored in this area include peroxynitrite-mediated inactivation of MnSOD and proteasome-mediated degradation of certain antioxidant enzymes.

Experimental models have been used to test the concept that antioxidant enzyme disruption can promote atherosclerosis. For example, athero-prone regions of the aorta in heterozygous Sod2+/− Apoe−/− mice have accelerated atherosclerosis, mtDNA damage, and accumulation of 3-nitrosylated proteins, which is a protein marker of excess ROS. These changes were further amplified after exposure to cigarette smoke, which enhances the degree of oxidative stress. Moreover, Apoe−/− mice with a targeted deletion of mitochondrial-localized GPX1 had accelerated atherosclerosis compared with Gpx1+/+ control mice, but only after longterm WD feeding or under diabetic conditions. Our group has addressed this issue by quelling endogenous mtROS in macrophages. We used a macrophage-targeted transgenic catalase (mCAT) model in which the presence of catalase in the mitochondria matrix degrades H2O2. Macrophages in mCAT lesions were protected from mtDNA oxidation, and the mice had decreased atherosclerosis in the Ldlr−/− background. The general role of ROS in atherosclerosis represents a highly controversial area. One possible explanation is the differential roles of various ROS-generating systems in lesional cells. For example, mouse models of atherosclerosis with deletion of the NADPH oxidase (NOX) component P47 or with overexpression of catalase, which reduces cytosolic H2O2, had less atherosclerosis. However, no athero-protective effect was found in a model with overexpression of Cu/ZnSOD (SOD1), a cytosolic SOD isoform that reduces O2•−. In contrast to these conflicting results...
regarding cytosolic ROS, the results of mtROS studies have been consistent. Thus, it seems that while mtROS is clearly pro-atherogenic, the role of cytosolic and nuclear ROS depends on individual reactive species. The explanation may lie in divergent intracellular signaling pathways triggered by ROS from specific cellular compartments. For example, one study showed cytosolic SOD1 can suppress smooth muscle cell (SMC) proliferation through the inhibition of mitogenic ERK/P38 MAPK pathways. In contrast, mitochondrial SOD2 suppresses SMC proliferation by inhibiting JAK2/STAT signaling\(^{30}\). Another study demonstrated that angiotensin II activated the kinases MAPK, JNK and ERK5 through NOX-derived ROS, while ET-1 activated these enzymes through mtROS\(^{4}\). As another example, we demonstrated that mtROS activates NF-κB-mediated induction of the chemokine CCL2 (MCP-1) after TLR activation in macrophages, whereas cytosolic ROS has the opposite effect\(^{14}\). Therefore, in order to guide the development of antioxidant therapy, we need a more thorough understanding of the differential roles of the various ROS-generating systems in cells in the setting of specific disease settings.

**Autophagy (Mitophagy) Dysfunction**

Macroautophagy (autophagy) is a cellular process that delivers cytoplasmic contents to lysosomes for degradation and recycling. Because normal mitochondrial function is critical for cell survival, cells develop a defense mechanism against aberrant or damaged mitochondria. This machinery involves selective recognition, sequestration, and subsequent clearance of damaged mitochondria using the common machinery of macroautophagy. This process has been called mitochondrial autophagy, or mitophagy\(^{31}\). As such, mitophagy is another layer of protection against excessive mtROS, because mtROS is known to accumulate in damaged mitochondria. Interestingly, mtROS itself regulates mitophagy. Excessive mtROS can lead to mitochondrial depolarization, and the loss of ΔΨ can trigger E3 ubiquitin ligase-mediated Parkin activation. Parkin then ubiquitinates mitochondrial proteins, and these ubiquitinated proteins serve as a signal for mitophagy\(^{32, 33}\). Parkin itself can be modified by oxidative/nitrosative stress but then inactivated under excessive oxidative stress conditions\(^{34, 35}\), which can lead to the failure of mitophagy. ROS can also directly regulate autophagosomes formation. ATG4, an essential component of autophagy, is subject to oxidation and subsequent inactivation by excessive ROS, which can then lead to dysregulation of mitophagy\(^{35}\).

An interesting hypothesis is that basal mitophagy is athero-protective by disposing of damaged mitochondria and this process may go awry in advanced atherosclerosis. Defective mitophagy would then promote mitochondrial dysfunction and cell apoptosis, which is a process that can lead to the formation of necrotic cores and unstable plaques. Two recent mouse atherosclerosis studies studied the effect of deletion of the essential autophagy protein ATG5 in macrophages\(^{36, 37}\). In one study, the primary observation was larger lesion area associated with enhanced inflammasome activation. Given links between mtROS and inflammasome activation\(^{38, 39}\), the authors proposed that the potential underlying mechanism was related to a defect in mitophagy, and although mtROS was not measured, there was an increase in lesional protein oxidation and superoxide. The other study found an increase in necrotic lesions in the ATG5-deficient mice, which was associated with an increase in lesional NOX activity and ROS. Here again mtROS was not assayed, but ROS from NOX and other sources has been shown in other models to induce mtROS from mitochondria in a process called ROS-induced ROS release (RIRR)\(^{40}\). Although mtROS was not specifically assayed, we found that the mitochondria in macrophages of the ATG5-deficient lesions demonstrated loss of normal cristae morphology and were swollen (unpublished data). Thus, it is possible that production of mtROS could result from cytosolic ROS accumulation\(^{41, 42}\), and without mitophagy, mtROS-damaged mitochondria accumulate and activate mitochondrial cell death pathways\(^{40, 43}\). This hypothesis is supported by data from our group (unpublished data) and others that mtROS is higher in ATG5-deficient vs. wild-type macrophages\(^{44}\).

A recent study showed that oxLDL induced TLR9 activation through mtROS and mtDNA damage in human umbilical vein endothelial cells (HUVECs). Inhibition of autophagy increased mtROS-induced mtDNA damage and TLR9 activation after oxLDL treatment in vitro. Consistent with that, enhancing autophagy decreased mtROS and TLR9 activation. These data raise the possibility that mtROS-induced mtDNA damage that escapes mitophagy can induce a potent TLR9 activation in atherosclerosis\(^{45}\). In summary, it is possible that a failure of mitophagy in advanced atherosclerosis promotes excessive mtROS and the accumulation of damaged mitochondria, which in turn could trigger inflammatory responses and cell death. However, this hypothesis is based on models in which overall autophagy is disabled. Thus, more specific models in which mitophagy is specifically inactivated or enhanced are needed to test the role of mitophagy in atherosclerosis.
Mitochondrial Fission and Fusion

The mitochondrial dynamic processes of fission and fusion influence and integrate with multiple physiologic and pathophysiologic processes, including mitosis, mitochondria metabolism, mitochondrial quality control (mitophagy), mtROS, and cell death. Diseases such as pulmonary arterial hypertension, arterial restenosis, hypertension, Parkinson’s disease, and obesity and diabetes have been associated with abnormalities in mitochondrial dynamics. Mitochondrial fission and fusion are regulated by several different GTPases. Mitofusin 1 (MFN1) and mitofusin 2 (MFN2) regulate outer membrane fusion, whereas optic atrophy protein 1 (OPA1) mediates inner membrane fusion. Mitochondrial fission is activated by a cytosolic molecule called dynamin-related protein 1 (DRP1) and its docking molecule, such as mitochondrial fission protein 1 (FIS1) and mitochondrial fission factor-1 (MFF1). It is known that under stress conditions such as hyperglycemia, ischemia reperfusion, neurodegeneration, and aging, mitochondria usually undergo hyperfission. Hyperfission has been shown to induce excessive mtROS in vascular smooth muscle cells, cardiomyocytes, renal cells, fibroblast, and neurons. MFN2 deficiency and DRP1 activation have been linked to smooth muscle cell (SMC) hyperproliferation in pulmonary artery hypertension. In atherosclerosis, data suggest that mitochondria dynamic changes may facilitate mtROS production. For example, Mfn2 mRNA was progressively reduced in the lesions of Apoe-/- mice artery during the development of atherosclerosis. Given that mtROS increases with lesion progression, this observation suggests the theoretical possibility that a decrease in MFN2 may progressively shift the mitochondria morphology toward hyperfission, which in turn may contribute to excessive mtROS production. Consistent with this idea, a study in rabbit reported that overexpression of MFN2 was associated with reduced atherosclerosis. Interestingly, diabetic venous endothelial cells were shown to have increased mitochondrial fission and a higher level of the mitochondrial fission protein Fis1, which could contribute to mtROS overproduction and enhanced susceptibility to atherosclerosis. While these studies hint at some interesting associations between mitochondrial dynamics and atherosclerosis, more precise in vivo models and more in-depth mechanistic studies are needed to address the functional significance of mitochondrial fission and fusion in atherosclerosis.

Dysregulation of UCP2

Uncoupling protein 2 (UCP2) is a mitochondria inner membrane protein that decreases mtROS production and is the dominant form of UCP that is expressed in vascular cells. UCP2 promoter polymorphisms in humans are associated with multiple pathologic conditions, including obesity, diabetes, and atherosclerosis. Additionally, UCP2 expression is increased in the aorta of cholesterol-fed C57BL/6J mice. Under this background, knocking out Ucp2 lead to accelerated atherosclerosis. In another mouse model, Apoe-/- mice transplanted with bone marrow from Ucp2-/- mice have larger and more macrophage-rich atherosclerotic lesions compared with Ucp2+/+ transplanted mice. The increase in atherosclerosis is associated with increased mtROS production. Interestingly, the compensatory enhancement of antioxidant activity, including SOD2 and GPX1, that normally occurs in early lesions is blunted in the setting of UCP2 deficiency. These data suggest that UCP2 is up-regulated in response to an atherogenic diet and is required to maintain normal antioxidant activity in the mitochondria of vascular cells. Mechanistically, UCP2 can suppress mtROS production, maintain normal endothelial function (eNOS release), promote vascular relaxation, decrease NF-κB activation, and inhibit the expression of the pro-inflammatory adhesion molecule VCAM-1 expression in endothelial cells. Overexpression of UCP2 in THP-1 monocytes quenches steady-state ROS production, which would be expected to decrease transendothelial migration of monocytes. Taken together, these data suggest that UCP2 is a part of the protective compensatory response that maintains the adaptive activation of SOD2 and GPX1 and their activities during the early stage of atherosclerosis. While UCP2 can be activated by O2•- (66), how UCP2 is up-regulated and activated during early atherosclerosis and how it is affected by atherosclerosis progression are not known. Thus, further causation studies are required to test the hypothesis that UCP2 dysfunction contributes to the failure of antioxidant capability and leads to mtROS overproduction in advanced atherosclerosis.

Consequences of Excessive mtROS

Overproduction of mtROS may Increase Inflammation in Atherosclerosis

mtROS has been linked to the activation of inflammatory pathways involving NF-κB, TLR9, and the inflammasome. Our group reported that mtROS can activate the IKK-NF-κB pathway and thereby enhance the induction of the chemokine CCL2 in LPS-treated macrophages. In atherosclerotic lesions, suppressing mtROS in macrophages reduces inflam-
mitratory cytokine expression, including TNF-α and iNOS. Similar to bacterial DNA, mtDNA contains inflammation-inducing unmethylated CpG motifs. In ischemia-reperfusion, shock, tissue injury settings, and systemic inflammatory syndromes, damaged mtDNA is sensed by TLR9 and thereby amplifies the inflammatory response. Blocking mtROS-induced mtDNA leakage has been shown to suppress TLR9 activation in HUVECs in vitro and in Ldlr−/− lesions. Activation of TLR9 signaling correlates with an increased macrophages in lesions, suggesting a pro-inflammatory role of mtDNA in atherosclerosis. Furthermore, human and animal studies have provided evidence supporting the causative role of the NLRP3-IL-1B inflammasome activation in atherosclerosis, and mtROS and oxidative mtDNA damage has been linked to inflammasome activation under multiple pathological conditions. The exact role of a mtROS-inflammasome axis in atherosclerosis requires further investigation using tools that specifically manipulate and measure these processes.

**mtROS and mtDNA Damage in Atherosclerosis**

mtDNA contains 13 genes encoding essential proteins involved in oxidative phosphorylation (OXPHOS), 12S and 16S ribosomal RNAs, and 22 transfer RNAs. As mentioned previously in this review, mtDNA is more vulnerable to ROS-induced damage than nuclear DNA. Signs of mtDNA damage, including mtDNA mutations and deletions, and the aforementioned 5-kb mtDNA deletion have been identified in atherosclerotic lesions and are correlated with the extent of atherosclerosis. Additionally, mtDNA mutations in the MT-RNR1, MT-TL1, MT-ND2, MT-ND5 and MT-CYB genes are associated with atherosclerosis in human plaques. Diabetes is one of the major risk factors for atherosclerosis, and there is a report that diabetic atherogenesis is associated with decreased mtDNA copy number.

A recent study examined Apoe−/− mice that were haploinsufficient for the protein kinase ATM, which coordinates both nuclear and mitochondrial DNA repair. These mice developed multiple features of metabolic syndrome and accelerated atherosclerosis, with increased frequency of the 5-kb mtDNA deletion and reduced oxidative phosphorylation. These changes were abrogated by transplantation of WT bone marrow into the ATM-deficient mice, indicating the involvement of myeloid-derived cells. To further clarify the role of mtDNA in atherosclerosis, the same group targeted DNA polymerase gamma (polG), the only enzyme responsible for proof-reading activity during mtDNA proliferation, and they found that these Polg−/−Apoe−/− mice accumulated somatic point mutations in mtDNA and accelerated atherosclerosis. Mechanistically, mtDNA damage directly compromises OXPHOS, and Polg−/−Apoe−/− lesions had enhanced mtDNA damage, reduced levels of ETC complex I, II and IV, and dysfunction of mitochondrial OXPHOS. To probe the cellular mechanisms, the investigators treated HUVECs and human aortic smooth muscle cells (HASMCs) with H2O2 and ONOO− and found mtDNA deletions, decreased mtDNA-encoded mRNA and proteins, reduced ATP levels, and loss of ΔΨ. These data support the notion that somatic mtDNA mutations are sufficient to cause atherosclerosis progression.

**Lipid Peroxidation and Abnormal Protein Modification**

mtROS also leads to abnormal mitochondrial protein modification, including cysteine oxidation, cysteine nitrosylation and tyrosine nitration, and lipid peroxidation followed by degradation. O2•− interacts with NO to form OONO−, a highly reactive species that can nitrate tyrosine residues and nitrosylate cysteine residues. •HO derived from H2O2 through the Fenton reaction leads to mtDNA oxidative damage and protein oxidation. These protein modifications can result in detrimental consequences, such as alteration of protein function, activation of immune and inflammatory responses, and activation of cell death pathways. Oxidatively modified proteins and lipoproteins, including oxidized LDL, lipid peroxidation products, and nitrated tyrosines, have been identified in patients with coronary artery disease and in atherosclerotic animal models. Mitochondrial antioxidant enzymes and ETC complexes could be potential targets of such abnormal protein modifications. For example, tyrosine nitration of MnSOD2 by OONO− causes inactivation of MnSOD2, which may contribute to the overproduction of mtROS in vascular cells. Another study using rat pulmonary microvascular endothelial cells demonstrated that NO and mitochondrial derived O2•− altered mitochondrial function through tyrosine nitration of a mitochondrial protein called NDUF8, which then triggered RIP1-mediated cell necrosis. Moreover, oxidized cardio-lipin increases as atherosclerosis progresses, which may not only be a marker of mtROS but might contribute to pathophysiology. In summary, mtROS-induced modification of mitochondrial proteins and lipids could very well contribute to the pro-atherosclerotic effects of mtROS, but specific in vivo causation studies are needed to investigate this mechanism.
An increasing numbers of studies have revealed the association between mtROS and atherosclerosis, and a few of these have begun to address the critical issue of causation. Moreover, mechanistic studies have explored how a healthy level of mtROS is maintained in physiology; how it may go awry in pathophysiology; and how this imbalance can contribute to the development and progression of cardiovascular disease.

**Conclusion**

Fig. 1. Regulation of healthy level and excessive mtROS.

A. Under physiological conditions, mtROS is generated as a byproduct of electron transport and quenched by the mitochondrial antioxidant enzymes (MnSOD and GPX). Moreover, mtROS production is tempered by uncoupling protein 2 (UCP2). In the course of normal mitochondrial physiology, sporadic episodes of mitochondrial dysfunction are handled by a process involving mitochondrial fission and mitophagy. Fission is promoted by the proteins DRP1 and FIS1, and the converse process of fusion is promoted by MFN1/2 and OPA1. PINK1 and Parkin mediate the ubiquitination and recognition of damaged mitochondria by the mitophagy complex, which is depicted by the double membrane structure. B. Under pathophysiologic conditions, excess mtROS, especially the highly reactive molecules \( \cdot \text{HO} \) and \( \text{OONO}^- \), can result from (1) inactivation or degradation of MnSOD; (2) inactivation of Gpx; (3) dysfunction of UCP2; (4) dysregulation of mitochondrial fission/fusion dynamics; (5) or inactivation of mitophagy lead to mitophagy deficiency. Excessive mtROS can damage electron complex complexes and reduce ATP generation; oxidatively damage mtDNA, leading to inflammasome activation; trigger cytochrome C (CytoC) release and apoptosis; and open the mitochondria permeable transition pore (mPTP) to cause cell necrosis.
ogy, including advanced atherosclerosis; and how excessive mtROS promotes disease progression (Fig. 1). However, the failure of antioxidant therapy in human randomized clinical trials demands more definitive, cell-type specific investigations. Further understanding of these issues in the area of advanced atherosclerosis progression may provide new and more specific therapeutics. This more targeted approach may overcome some of the inconsistencies that have plagued the general field of anti-oxidant therapy for atherosclerotic vascular disease.

Conflict of Interest

None.

Acknowledgement

We thank Dr. Wei Wang for figure illustration. Dr. Ying Wang has received support from an AHA pre-doctoral training grant, and Dr. Tabas has been funded by National Institutes of Health grants R01HL075662 and R01HL106019.

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