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Review

Mitochondria and vascular pathology

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Abstract:
Functional and structural changes in mitochondria are caused by the opening of the mitochondrial permeability transition pore (PTP) and by the mitochondrial generation of reactive oxygen species (ROS). These two processes are linked in a vicious cycle that has been extensively documented in ischemia/reperfusion injuries of the heart, and the same processes likely contribute to vascular pathology. For instance, the opening of the PTP causes cell death in isolated endothelial and vascular smooth muscle cells. Indeed, atherosclerosis is exacerbated when mitochondrial antioxidant defenses are hampered, but a decrease in mitochondrial ROS formation reduces atherogenesis.
Determining the exact location of ROS generation in mitochondria is a relevant and still unanswered question. The respiratory chain is generally believed to be a main site of ROS formation. However, several other mitochondrial components likely contribute to ROS generation. Recent reports highlight the relevance of monoamine oxidases (MAO) and $p66^{Shc}$. For example, the absence of $p66^{Shc}$ in hypercholesterolemic mice has been reported to reduce the occurrence of foam cells and early atherogenic lesions. On the other hand, MAO inhibition has been shown to reduce oxidative stress in many cell types eliciting significant protection from myocardial ischemia. In conclusion, evidence will be presented to demonstrate that (i) mitochondria are major sites of ROS formation; (ii) an increase in mitochondrial ROS formation and/or a decrease in mitochondrial antioxidant defenses exacerbate atherosclerosis; and (iii) mitochondrial dysfunction is likely a relevant mechanism underlying several risk factors (i.e., diabetes, hyperlipidemia, hypertension) associated with atherosclerosis.

Key words: oxidative stress, mitochondria, $p66^{Shc}$, monoamine oxidase

Abbreviations: apoE – apolipoprotein E, MAO – monoamine oxidase, PTP – mitochondrial permeability transition pore, ROS – reactive oxygen species, SOD – superoxide dismutase

Mitochondrial mechanisms of cell injury

The energy-linked processes and metabolic activities occurring in mitochondria are required to maintain cell viability. This uncomplicated conception is paradoxically counterbalanced by the well-established notion that mitochondria play a significant role in cell death [8, 45, 58]. In addition to the profound imbalance between ATP synthesis and utilization that occurs as a consequence of mitochondrial dysfunction [31, 71], the impairment of ionic homeostasis [12, 34, 43, 61, 69] and the formation of reactive oxygen species (ROS) [15, 63, 77] represent two additional processes through which mitochondria accelerate, or

even determine, the evolution of cell injury toward necrosis or apoptosis [8, 32, 45, 47, 58].

A large body of evidence supports the concept that ROS are formed within mitochondria under physiological and pathological conditions [3, 33, 40, 56, 77]. The superoxide anion ($O_2^{\cdot -}$), formed by Complex I and III, is rapidly transformed into hydrogen peroxide (H_2O_2) by a family of metalloenzymes, the superoxide dismutases (SOD) [38]. Particularly relevant in this process is the mitochondrial form of SOD (MnSOD or SOD-2). Widespread organ damage associated with severe mitochondrial dysfunction has been observed in mice lacking SOD-2 [52]. Recent work has demonstrated that CuZnSOD (SOD-1), commonly referred to as the cytosolic isoform, is also present in the mitochondrial intermembrane space [60].

The major link between mitochondria and vascular derangements is oxidative stress [4, 26, 35, 42, 50]. In particular, the development of atherosclerosis appears to depend on the mitochondrial metabolism of ROS. In fact, when mitochondrial antioxidant defenses are hampered, atherosclerosis is exacerbated, whereas a decrease in mitochondrial ROS formation reduces atherogenesis [50]. For instance, the absence of SOD-2 increases mtDNA damage and accelerates atherosclerosis in apoE knockout mice [59]. These findings are consistent with results obtained in ischemia/reperfusion experiments, which show that SOD-2 overexpression elicits cardioprotection [19], whereas a heterozygous deficiency of this enzyme impairs postischemic recovery of the heart [1].

In addition to the damage to all of cell's components, oxidative stress increases the occurrence of cell death, especially by apoptosis, which greatly contributes to the progress of atherosclerotic lesions. In this respect, an important consequence of ROS accumulation is increased susceptibility to opening of the mitochondrial permeability transition pore (PTP) [7]. PTP opening is especially sensitive to oxidative stress, since it is favored by decreases in $NADPH(H^+)/NADP^+$ and -SH/-S-S ratios [23, 24]. Recent evidence suggests that PTP opening and ROS formation are linked in a vicious cycle. In addition to being a likely consequence of oxidative stress, PTP opening has been shown to increase mitochondrial ROS formation in cardiac myocytes [41].

Despite the large body of evidence that relates PTP opening to cell death, especially in the case of an ischemia/reperfusion injury to the heart [29], definite proof of PTP involvement in atherogenesis is lacking. While data obtained *in vitro* indicate that PTP opening causes cell death in isolated endothelial and vascular smooth muscle cells [26, 28], no information on the effect of pharmacological or genetic inhibition of the PTP on atherogenesis *in vivo* is available.

The mitochondrial formation of ROS may be modulated by NO· [67, 72] as a consequence of the inhibition of cytochrome oxidase [6, 13, 20, 44, 77]. This reversible process can be transformed into irreversible damage to the respiratory chain when NO· formation is sustained. Indeed, the reaction of NO· with $O_2^{\cdot -}$ generates peroxynitrite, which can cause the irreversible nitration of proteins [5]. Interestingly, a proteomic study showed that one-third of the proteins nitrated during an inflammatory challenge were mitochondrial in origin [2].

It must be noted that ROS are also produced within mitochondria at sites other than the inner mitochondrial membrane [30, 33], by proteins such as monoamine oxidase (MAO) and $p66^{Shc}$. These additional mitochondrial processes produce significant amounts of ROS. For instance, in brain mitochondria MAO activity results in steady state concentrations of H_2O_2 that are 48-fold higher than those originating from the respiratory chain in the presence of antimycin A [15]. Therefore, the generation of ROS, especially H_2O_2, by mitochondria is not just an unfortunate side effect of respiration, but can also be catalyzed by specific enzymes, such as MAO and $p66^{Shc}$. It is tempting to speculate that, under physiological conditions, the inner mitochondrial membrane scavenges ROS produced at other mitochondrial or cellular sites. In such a scenario, the increase in ROS formation detected under pathological conditions might result, at least in part, from a dysfunction of the inner mitochondrial membrane's scavenging abilities.

This review will focus on the relevant contributions of MAO and $p66^{Shc}$ to both mitochondrial ROS formation and cell injury, as summarized in Figure 1.

Oxidative stress, vascular pathology and $p66^{Shc}$

The Shc family of proteins gets its name from the abbreviation of the Src homology 2 domain and a collagen-homology region [55]. Two members of this adaptor protein family, $p52^{Shc}$ and $p46^{Shc}$, are phos-

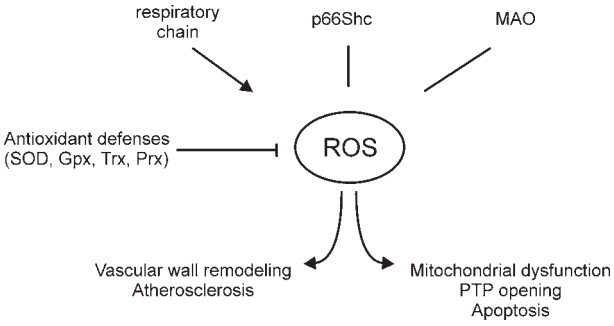

Fig. 1. Mitochondrial sources of reactive oxygen species (ROS). If not efficiently neutralized by intrinsic antioxidant systems, ROS produced by the respiratory chain, $p66^{Shc}$ and MAO in mitochondria can trigger secondary signaling pathways that lead to vascular wall remodeling and atherosclerosis. Alternatively, excess ROS production at these sites can also induce mitochondrial dysfunction resulting in PTP opening and apoptosis. Gpx – glutathione peroxidase, MAO – monoamine oxidase, Prx – peroxiedoxin, PTP – mitochondrial permeability transition pore, SOD – superoxide dismutase, Trx – thioredoxin

phorylated in response to mitotic signals and result in Ras activation. A third member of the family, $p66^{Shc}$, which is not involved in Ras activation, is composed of the entire $p52^{Shc}$/$p46^{Shc}$ sequence and an additional amino-terminal proline-rich region, named CH2, which contains a serine phosphorylation site implicated in oxidative stress signaling. Indeed, $p66^{Shc}$ has been shown to play a significant role in a wide range of pathological conditions related to oxidative stress [22, 55]. $p66^{Shc}$ garnered a great deal of attention when it was demonstrated that its deletion resulted in a 30% increase in lifespan [54]. Subsequently, studies carried out in a wide range of experimental models established quite clearly that ROS formation is reduced in cells lacking $p66^{Shc}$, and that systemic and intracellular markers of oxidative stress are diminished in $p66^{Shc-/-}$ mice [39, 62, 76]. The relationship of $p66^{Shc}$ to ROS formation was elucidated by showing that it partially localizes within mitochondria, where it catalyzes electron transfer from cytochrome *c* to oxygen [39]. More recent work suggests that PKC β phosphorylation of $p66^{Shc}$ on Ser^{36} could cause its translocation to mitochondria [65]. It seems that the increase in mitochondrial ROS formation caused by $p66^{Shc}$ amplifies the PKC β signaling triggered by an initial oxidative stress.

The link between $p66^{Shc}$ and vascular pathology was originally highlighted by studying the effects of hypercholesterolemia [57]. When compared to wild type littermates, hypercholesterolemic $p66^{Shc-/-}$ mice displayed reduced levels of isoprostane and oxidized LDL, a decreased number of foam cells, and a reduction in the extent of both apoptosis and early atherogenic lesions. These initial findings prompted several additional studies, especially in the field of diabetic vasculopathy. In studies of streptozotocin-induced diabetes, $p66^{Shc-/-}$ mice showed glycemic levels similar to wild type mice, but were protected against glomerulopathy as shown by the preservation of renal structure and function, and by a marked reduction in oxidative stress [53]. Using the same experimental model, $p66^{Shc-/-}$ mice were found to have unimpaired acetylcholine-induced vasorelaxation, due to the unchanged availability of NO [16]. In addition, the lack of $p66^{Shc}$ was shown to protect against diabetic cardiomyopathy by preventing the senescence of cardiac progenitor cells, which hampers cardiac and vascular cell turnover [70].

The findings obtained in mice lacking $p66^{Shc}$ prove beyond any doubt that mitochondrial ROS formation is not just an accidental by-product of the respiratory chain, and that most intracellular oxidative stress originates in mitochondria. However, at present the translation of these concepts into clinical practice is limited by the lack of drugs that prevent ROS formation by $p66^{Shc}$.

Monoamine oxidase

Structural features and biochemical role

Monoamine oxidase, a flavoenzyme located within the outer mitochondrial membrane, is responsible for the oxidative deamination of neurotransmitters and dietary amines. It exists in two isoforms, MAO-A and B, which differ in substrate specificity and inhibitor sensitivity [37]. In both isoforms, FAD is covalently bound to a cysteine residue [36]. MAO-A and B are anchored to the outer membrane of mitochondria through a C-terminal α-helix segment that protrudes from the basal face of the structure. The analysis of crystal diffraction data indicates that MAO-B crystallizes as a dimer; with each monomer containing a C-terminal membrane bound domain, a FAD binding domain and a substrate-binding domain [11]. In contrast to human MAO-B, human MAO-A crystallizes as a monomer [27]. Analysis of residue side chains in both active sites shows substrates to have less free-

dom of rotation in the MAO-B site than in MAO-A. The structural basis for this difference can be partially attributed to the conformational differences in the region from residue 200 to 215 that constitutes the "cavity shaping loop" in both isoforms. This loop has a more extended conformation in MAO-A and a more compact conformation in MAO-B.

In peripheral tissues, the MAO isoforms are involved in the oxidative catabolism of amines from the blood, and in preventing dietary amines from entering circulation. In the central and peripheral nervous system, intraneuronal MAO-A and -B protect neurons from exogenous amines, terminate the actions of amine neurotransmitters and regulate the contents of intracellular amine stores [79].

MAO-A, which preferentially catalyzes the oxidative deamination of norepinephrine (NE) and serotonin (5-HT), is inhibited by low concentrations of clorgyline. In contrast, MAO-B, which has a higher affinity for phenylethylamine and benzylamine, is inhibited by selegiline [79]. Both isoforms catalyze the deamination of dopamine, tyramine, octopamine and tryptamine, and are inhibited by pargyline. MAO catalyzes the following reaction:

$$RCH_2NR'R'' + O_2 + H_2O \rightarrow RCHO + NHR'R' + H_2O_2$$

Kinetic studies have shown that amine binding to the enzyme precedes oxygen binding [75]. In the first step, the reduction of the FAD cofactor yields an aldehyde intermediate and an ammonium, while, in a second step, the prosthetic group is oxidixed with the concomitant production of hydrogen peroxide.

$$RCH_2NH_2 + MAO \rightarrow RCHO + NH_4^+ + MAO\text{-reduced}$$

$$MAO\text{-reduced} + O_2 \rightarrow MAO + H_2O_2$$

The aldehyde intermediates are rapidly metabolized to the corresponding carboxylic acids by the action of aldehyde dehydrogenase (ALDH). A failure of this latter enzyme might increase the deleterious aspects of MAO activity by generating potentially harmful aldehyde compounds, and magnifying the damage done by MAO-induced H_2O_2 formation. Indeed, a decrease in ALDH activity appears to be involved in both oxidative stress and nitrate tolerance [25, 78], whereas increased ALDH activity has been reported to result in decreased injury to ischemic hearts [18].

The main physiological role of MAO is the degradation of endogenous monoamine neurotransmitters and dietary amines, such as tyramine, which may cause hypertensive crises if not properly catabolized [79]. Similarly, MAO-B in microvessels and at the blood-brain barrier has a protective function acting as a metabolic barrier, and preventing the entrance of xenobiotic and potentially toxic neurotransmitters.

The deletion of the MAO-A and MAO-B genes has proven their important role in neurotransmitter metabolism and behavior. MAO-A knockout mice have elevated brain levels of serotonin, norepinephrine and, to a lesser extent, dopamine [17], whereas only 2-phenylethylamine levels are increased in MAO-B knockout mice [79]. Both MAO-A and -B knockout mice show an increased response to stress, similar to that observed after the administration of non-selective MAO inhibitors. However, these studies, and the deletion of both MAO-A and MAO-B, in a rare form of human Norrie disease, indicate that MAO is not essential for survival [48]. Gene deletion has shown that MAO-A activity is important during development. A compulsive aggressive behavior results from a lack of MAO-A function in humans [14] and mice [73]. This effect, which might reflect the importance of serotonin during development, can be mimicked by administering the MAO-A inhibitor clorgyline during the early postnatal period.

The distribution of MAO in the brain has been well studied. MAO-A is prevalently found in noradrenergic neurons, whereas MAO-B has been detected in serotoninergic and histaminergic neurons and in glial cells [49]. With respect to peripheral tissues, MAO-A has been found in placenta, liver, intestine and thyroid gland, while platelets, liver and kidney contain mainly MAO-B. Human cardiomyocytes contain both enzymes, although MAO-A is the predominant isoform [74].

The therapeutic potential of MAO inhibitors

MAO's roles, in terminating the action of neurotransmitters in the central and peripheral nervous system and in the oxidation of dietary amines in extraneuronal tissues, have been extensively studied. However, less attention has been paid to the products of MAO activity. Monoamine catabolism results in the formation of aldehydes, ammonium cations and H_2O_2.

MAOs are involved in numerous pathologies. The important role of MAOs in neuronal and psychiatric disorders is demonstrated by the beneficial effects elicited by MAO inhibitors. The therapeutic potential of MAO inhibition was realized in the early 1950s, when iproniazid, an antituberculosis treatment, was

shown to improve mood while reducing MAO activity [66, 68]. A wide spectrum of MAO inhibitors are available today and these are proving to have therapeutic value in several pathologies, including affective disorders, neurodegenerative diseases, stroke and aging [68, 79]. MAO inhibitors are distinguished on the basis of their specificity for each isoform and the nature of their binding to the enzyme [68, 79]. They can be classified into three groups: (i) irreversible and non-selective inhibitors, such as phenelzine and tranylcypromine; (ii) irreversible and selective inhibitors, such as selegiline for MAO-B and clorgyline for MAO-A; and (iii) reversible and selective inhibitors, such as moclobemide for MAO-A and lazabemide for MAO-B.

MAO-B seems to be involved in the loss of dopaminergic neurons that occurs in Parkinson's disease, most likely due to increased dopamine catabolism, which results in an elevated production of the reactive oxygen species responsible for the oxidative damage in nigrostriatal neurons. Indeed, MAO-B inhibition has been shown to afford neuroprotection [79]. Furthermore, an increase in brain MAO-B activity is also associated with diseases such as Alzheimer's and Huntington's disease. Depression, panic attacks and personality disorders are also associated with changes in dopaminergic, noradrenergic and serotoninergic neurotransmission, which are regulated by both isoforms of MAO [79].

In addition to being implicated in neurodegenerative diseases, the MAO isoforms, especially MAO-A, have been shown to play a prominent role in myocardial injury caused by post-ischemic reperfusion [9]. Preliminary evidence also suggests that MAO-A contributes to the maladaptive evolution from myocardial hypertrophy to failure [46]. In particular, MAO-A has been demonstrated to be an important source of ROS in the receptor-independent apoptotic effects of serotonin in isolated cardiomyocytes and in post ischemic myocardial injury [9, 10]. In fact, MAO-dependent increases in ROS production appear to be relevant in serotonin-induced myocyte hypertrophy *in vitro* [10]. In addition, MAO-A can promote apoptosis through ROS-dependent sphingosine kinase inhibition that results in the accumulation of ceramide [64]. With respect to vasculature, MAO-A-mediated ROS production has been shown to induce mitogenic signaling in smooth muscle cells by a process that may involve the activation of the metalloproteinase MMP-2, which likely contributes to vascular wall remodeling [21]. Interestingly, MAO-A activity has been reported to increase with aging [51]. It is tempting to speculate that the resulting increase in H_2O_2 formation might contribute to aging-associated pathologies, such as congestive heart failure and vascular pathologies.

Considering MAO's important role as a source of H_2O_2 in both the brain and in the heart following post-I/R cardiac injury, MAO inhibition will likely represent an important tool for both the study and the treatment of vascular pathologies that share oxidative stress as a common denominator.

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