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REVIEW

Mitochondrial pathways for ROS formation and myocardial injury: the relevance of $p66^{Shc}$ and monoamine oxidase

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Abstract Although mitochondria are considered the most relevant site for the formation of reactive oxygen species (ROS) in cardiac myocytes, a major and unsolved issue is where ROS are generated in mitochondria. Respiratory chain is generally indicated as a main site for ROS formation. However, other mitochondrial components are likely to contribute to ROS generation. Recent reports highlight the relevance of monoamine oxidases (MAO) and $p66^{Shc}$. The importance of these systems in the irreversibility of ischemic heart injury will be discussed along with the cardioprotective effects elicited by both MAO inhibition and $p66^{Shc}$ knockout. Finally, recent evidence will be reviewed that highlight the relevance of mitochondrial ROS formation also in myocardial failure and atherosclerosis.

Keywords Oxidative stress · Mitochondria · $p66^{Shc}$ · Monoamine oxidase

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Introduction

The complete reduction of O_2 into two molecules of H_2O requires four electrons. This process occurs sequentially since O_2 accepts only one electron at a time. Consequently, O_2 reduction inevitably implies the formation of partially reduced intermediates. In particular, the addition of the first electron yields superoxide anion ($O_2\cdot^-$) that becomes hydrogen peroxide when one additional electron is added. A further single electron reduction produces the hydroxyl radical (·OH) that accepting another electron becomes H_2O. $O_2\cdot^-$ and ·OH are oxygen radicals (i.e., containing unpaired electrons) and represent quite reactive species. H_2O_2 is less reactive per se, but, according to the Fenton reaction, in the presence of Fe^{2+} or Cu^+ generates ·OH. These partially reduced forms of oxygen are commonly referred to as reactive oxygen species (ROS), which also include the highly reactive singlet oxygen ($^1O_2$). This excited form of oxygen is produced by the Haber-Weiss reaction (i.e., the non-enzymatic dismutation of $O_2\cdot^-$) or high energy irradiation (i.e., UV light).

ROS formation and toxicity are counterbalanced by a complex defense system. The most efficacious strategy is the enzymatic removal of ROS that is catalyzed by superoxide dismutases (SODs) and peroxidases [48, 49]. Through SOD reaction $O_2\cdot^-$ is transformed into H_2O_2 that is then reduced into water by peroxidases including catalase and glutathione peroxidases. Reduction of oxidized molecules can be also catalyzed by thioredoxin and peroxyredoxin [101]. The enzymatic defenses are paralleled by non-enzymatic mechanisms that rely upon antioxidants such as vitamins A, E and C, ubiquinone, urate, lipoic acid, and glutathione [50].

The imbalance between formation and removal of ROS is termed oxidative stress and plays a major role in all

cardiac diseases. In this respect, major attention has been focused on the relationship between oxidative stress and ischemia/repefusion injury [15, 22, 50, 73]. Nevertheless, an increased formation of ROS is suggested to be involved in heart failure [24, 50, 59, 89] as well as in atherosclerosis [6, 35, 40, 56, 66].

Besides altering every cell component, oxidative stress increases the occurrence of cell death. To this end, a relevant consequence of ROS accumulation is the increased susceptibility to opening of the mitochondrial permeability transition pore (PTP) [10] that is recognized as a major determinant of myocyte injury during post-ischemic reperfusion [4, 38]. PTP opening is especially sensitive to oxidative stress since it is favored by decreases in $NADPH(H^+)/NADP^+$ and -SH/-S–S ratios [32, 33]. Recent evidence suggests that PTP opening and ROS formation are linked in a vicious cycle. In fact, besides being a likely consequence of oxidative stress, PTP opening has been shown to increase mitochondrial ROS formation in cardiac myocytes [54]. In addition, the reduced susceptibility to PTP opening elicited by ischemic preconditioning appears to be associated with a decrease in mitochondrial oxidative stress [27]. On the other hand, it must be pointed out that ROS can also inhibit PTP opening. This appears to be the case with low doses of H_2O_2 [31] and singlet oxygen [90].

Although major emphasis has been put on the pathological consequences resulting from ROS-induced derangements of every macromolecule, ROS also contribute to several physiological processes [50]. The myriad of roles, at times contrasting, are likely to be related to the involvement of ROS, especially H_2O_2, in signaling pathways [52, 60]. Signaling modulation by ROS is mostly due to oxidation of cysteinyl residues resulting in covalent modifications of the affected proteins. These changes are potentially reversible due to the operation of several enzymes such as protein disulfide isomerase, glutaredoxin, and thioredoxin that catalyze the reduction of the oxidized cysteines [11, 60]. ROS modulate signaling pathways through three major modalities:

(i) activation of protein kinases. Besides the well-established modulation of extracellular signal-regulated kinase (ERK), Jun N-terminal kinase (JNK), apoptosis signal-regulating kinase 1 (ASK-1) and protein kinase C (PKC) [5, 65, 60], recent elegant evidence has been reported that ROS also modulate the myocardial activity of cyclic AMP-dependent protein kinase (PKA) [17], cyclic GMP- dependent protein kinase (PKG) [20] and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) [43];
(ii) H_2O_2 formation downstream of receptors activated by cytokines and growth factors, including insulin [12, 50, 52];
(iii) inhibition of protein phosphatases, such as protein tyrosine phosphatase 1B and PTEN [46, 93, 60].

Regarding the protective mechanisms related to ischemic preconditioning, the role of ROS, reactive nitrogen species (RNS), and their downstream cellular targets is somewhat controversial (see [44] for extensive review). However, ROS/RNS appear to play a critical role in the signal transduction pathway leading to PKC activation.

Mitochondrial ROS formation

ROS are produced at various intracellular sites, yet it is generally accepted that in cardiac myocytes the largest amount of ROS are formed within mitochondria [5, 39, 53, 72, 97]. Besides impairment of energy metabolism and ionic homeostasis, formation of ROS [21, 80, 97] represents an additional process through which mitochondrial dysfunction accelerates, or even determines, the evolution of cell injury toward necrosis or apoptosis [9, 37, 58, 62, 76].

The large majority of oxygen delivered to mitochondria is fully reduced to water at the level of Complex IV. In this terminal step of the mitochondrial respiratory chain, oxygen is reduced sequentially, yet reaction intermediates remain bound to Complex IV, so that only the final product, i.e., water, is released. Nevertheless, electrons flowing through the respiratory chain can be donated to oxygen at other sites, but in these cases the reduction is not complete, resulting in the release of partially reduced forms, especially superoxide anion. In fact, $O_2\cdot^-$ is formed at the level of Complex I and III and is then rapidly dismutated into H_2O_2 by SOD [49]. Besides the classical notion of the mitochondrial localization of MnSOD or SOD-2, also CuZnSOD (SOD-1), commonly referred to as the cytosolic isoform, is present in the intermembrane space of mitochondria [78].

The relevance of SOD as an antioxidant defense has been demonstrated by genetic approaches. Mice lacking SOD-2 display widespread organ damage associated with severe mitochondrial dysfunctions [68], and heterozygous deficiency of this enzyme impairs postischemic recovery of the heart [2]. On the other hand, SOD-2 overexpression elicits cardioprotection against the ischemia/reperfusion injury [25]. In addition, the absence of SOD-2 increases mtDNA damage and accelerates atherosclerosis in apoE knockout mice [77] providing support to the tight link between mitochondrial oxidative stress and atherogenesis [66].

The mitochondrial formation of ROS might be modulated by $NO^{\cdot}$ [86, 91] as a consequence of the inhibition of cytochrome oxidase [8, 18, 28, 57, 97]. This reversible

process can be transformed into irreversible alterations of respiratory chain when NO· formation is sustained. Indeed, NO· reacting with $O_2\cdot^-$ generates peroxynitrite, which can produce the irreversible nitration of proteins [7]. Interestingly, a proteomic study showed that one-third of the proteins nitrated during inflammatory challenge are of mitochondrial origin [3].

It must be pointed out that ROS are also produced within mitochondria at sites other than the inner mitochondrial membrane [36, 39], such as monoamine oxidase (MAO) and $p66^{Shc}$. These additional mitochondrial processes produce significant amounts of ROS. For instance, in brain mitochondria the highest rate of H_2O_2 formation from respiratory chain, as observed in the presence of the Complex III inhibitor antimycin A, is 48-fold lower than that originating from MAO activity [21]. Therefore, generation of ROS, especially H_2O_2, far from being just an unfortunate side effect of respiration, is catalyzed by specific enzymes, such as MAO and $p66^{Shc}$. It is tempting to speculate that under physiological conditions the inner mitochondrial membrane could scavenge ROS produced at other mitochondrial or cellular sites. Then, the increase in ROS formation detected under pathological conditions might result, at least in part, from the loss of scavenging properties of the inner mitochondrial membrane due to its dysfunction.

The following paragraphs will focus on the relevant contribution of MAO and $p66^{Shc}$ to both mitochondrial ROS formation and cell injury.

$p66^{Shc}$ and cardiovascular pathology

Role of $p66^{Shc}$ in mitochondrial ROS generation

$p66^{Shc}$ is a vertebrate splice variant of $p52^{Shc}$ and $p46^{Shc}$, two cytoplasmic adaptor proteins involved in the propagation of intracellular signals from activated tyrosine kinases to Ras [82]. The Shc acronym indicates proteins sharing a C-terminal SH2 domain adjacent to a collagen homology (CH) region [71]. $p66^{Shc}$ has the same modular structure as $p52^{Shc}$/$p46^{Shc}$ (SH2-CH-PTB) and contains a unique N-terminal region (CH 2); however, it is not involved in Ras regulation, but rather functions in the intracellular pathway(s) that regulates ROS metabolism and apoptosis [70]. Within its additional CH region $p66^{Shc}$ becomes phosphorylated on Ser^{36} upon UV irradiation or treatment with oxidants linking $p66^{Shc}$ with redox signaling. Indeed, $p66^{Shc}$ has been shown to play a relevant role in a wide range of pathological alterations related to oxidative stress [30, 71]. A great deal of interest was attracted to $p66^{Shc}$ by the initial report that its deletion results in a 30% prolongation of lifespan [70]. Subsequently, studies carried out in a wide range of experimental models demonstrated 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 [51, 79, 96].

A decrease in ROS levels might result from either a reduced formation and/or an increased removal. Initially, the reduced oxidative stress associated with $p66^{Shc}$ absence was attributed to a possible increase in ROS catabolism related to the Forkhead transcription factor FKHR-L1 that induces the expression of several antioxidant enzymes [75, 87]. Indeed, FKHR-L1 is phosphorylated and inactivated as a downstream target of Ser^{36}-phosphorylated $p66^{Shc}$, whereas FKHR-L1 activity is remarkably increased in cells devoid of $p66^{Shc}$ [75]. However, more recently, it became clear that ROS formation is directly enhanced by $p66^{Shc}$ that localizing to mitochondria catalyzes an alternative redox reaction [51]. In particular, electrochemical experiments demonstrated that the amino terminal portion of $p66^{Shc}$ contains a redox active sequence able to couple the reduction of molecular oxygen to H_2O_2 with the oxidation of cytochrome c [51]. This reaction is crucial for $p66^{Shc}$ apoptotic function that is abolished by mutating critical residues (E132, E133 and W134) in its redox center. Notably, these mutations impair the reaction of $p66^{Shc}$ with cytochrome c without affecting other properties (i.e., binding to and phosphorylation of the tyrosine kinase receptor and other substrates, including Grb2) [51].

More recent work suggests that PKC β phosphorylation of $p66^{Shc}$ on Ser^{36} could cause its translocation to mitochondria [84]. Therefore, the increase in mitochondrial ROS formation caused by $p66^{Shc}$ appears to amplify PKC β signaling triggered by an initial oxidative stress (Fig. 1).

Since *$p66^{Shc}$* deletion is beneficial in a wide range of pathological conditions, its expression might appear as an unfortunate oversight of Mother Nature. Although the physiological roles of this protein are far from being conclusively elucidated, besides the possible contribution to heart development, recent studies relate $p66^{Shc}$-induced ROS formation to both adipogenesis and immune response [12, 45].

$p66^{Shc}$ in cardiac and vascular pathologies

A large body of evidence supports the relevance of $p66^{Shc}$ in cardiovascular pathophysiology. In adult cardiac myocytes $p66^{Shc}$ is barely detectable. However, in dog hearts its expression was found to increase progressively during the transition toward decompensated hypertrophy induced by ventricular pacing [24]. Studies in dog heart could hardly elucidate the causative link between $p66^{Shc}$-induced oxidative stress and myocardial injury that was established by investigating the deleterious effects of angiotensin II.

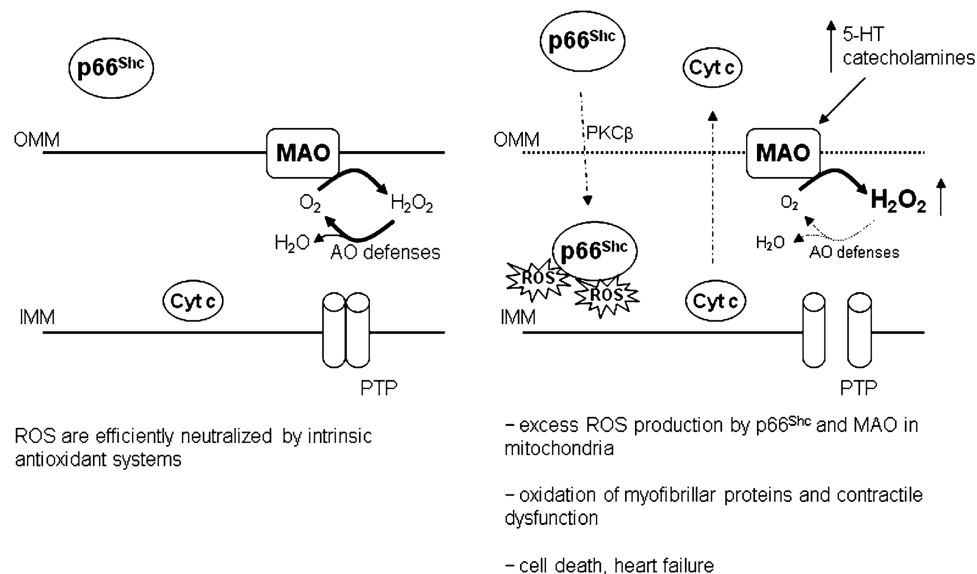


Fig. 1 Mitochondrial sources of reactive oxygen species (ROS). Under physiological conditions (*left panel*), p66^{Shc} is localized in the cytosol while ROS production by MAO is neutralized by antioxidant defenses. However, pathological stimuli (such as ischemia/reperfusion injury, *right panel*) may trigger signaling pathways that mediate p66^{Shc} translocation into the mitochondria or release of catecholamines from endogenous stores rendering them available for catabolism by MAO. Both these events result in increased ROS production that may overwhelm cellular antioxidant defenses leading to contractile dysfunction, PTP opening, cell death, and heart failure. *OMM* outer mitochondrial membrane, *IMM* inner mitochondrial membrane, cyt *c* cytochrome *c*, *MAO* monoamine oxidase, *PTP* permeability transition pore, *AO defenses* antioxidant defenses

In fact, mice devoid of p66^{Shc} displayed a significant reduction in both the occurrence of apoptosis and the degree of hypertrophy induced by a sub-pressor dose of angiotensin II [55]. While supporting the bidirectional link between oxidative stress and p66^{Shc} expression observed in other experimental models [83], these findings suggest the occurrence of a vicious cycle whereby an initial slight formation of ROS that might not affect myocardial function and viability is amplified by the increased expression of p66^{Shc} leading to a ROS overload that causes cell death and contractile impairment. Interestingly, it has been proposed that this alteration could be part of the return to a fetal phenotype observed in diseased hearts [16]. Indeed, during heart development the expression of Shc proteins is increased [47]. This might be aimed at potentiating apoptosis associated with proliferative processes required for myocardial growth. Supporting this view, p66^{Shc} knockout was found to be associated with myocardial hyperplasia, although contractile function is apparently similar to that of wild type littermates [55]. Oxidative stress and ROS formation appear to be relevant also for cells other than differentiated cardiomyocytes. In fact, the lack of p66^{Shc} was shown to protect against diabetic cardiomyopathy by preventing the senescence of cardiac progenitor cells that hampers cardiac and vascular cell turnover [89].

Besides heart failure, oxidative stress related to p66^{Shc} is likely to exacerbate ischemic injury. In this respect, the only available study was carried out in skeletal muscles using the protocol of hindlimb ischemia [100]. Upon reperfusion the extent of cell death was fivefold lower in *p66$^{Shc-/-}$* mice as compared to wild type littermates. In addition, the absence of p66^{Shc} resulted in a significant decrease of oxidative stress in both ischemic muscles and isolated endothelial cells subjected to simulated ischemia. The beneficial action of p66^{Shc} down-regulation against ischemia/reperfusion damage is also supported by the observation that protection elicited by preconditioning in neuronal SH-SY5Y cells is associated with a decreased expression of p66^{Shc} that appears to be linked to preconditioning-induced increase in NO availability [1].

The link between p66^{Shc} and vascular pathology was originally highlighted by studying the effects of hypercholesterolemia [74]. As compared to wild-type littermates hypercholesterolemic *p66$^{Shc-/-}$* mice displayed reduced levels of isoprostane and oxidized LDL, a decreased amount of foam cells, and a reduction in the extent of both apoptosis, and early atherogenic lesions. These initial findings prompted several other studies, especially in the field of diabetic vasculopathy. For instance, evidence was obtained that *p66$^{Shc-/-}$* mice are protected against glomerulopathy as shown by the maintenance of renal structure and function along with a marked reduction in oxidative stress [69]. Unfortunately, at present the translation of these experimental findings into the clinical practice is limited by the lack of drugs that might prevent the ROS forming activity of p66^{Shc}.

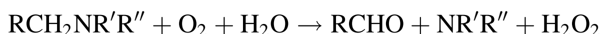
Monoamine oxidase and oxidative stress

Biochemical features

Monoamine oxidase is a flavoenzyme located within the outer mitochondrial membrane, responsible for the oxidative deamination of neurotransmitters and dietary amines. It exists in two isoforms, MAO-A and B that differ for substrate specificity and inhibitor sensitivity [42]. In both isoforms FAD is covalently bound to a cysteine residue [41]. In peripheral tissues, MAO is involved in the oxidative catabolism of amines from the blood and in preventing the entry of dietary amines into the 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 [99].

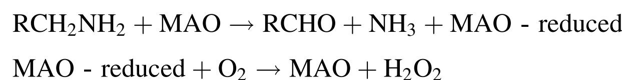
MAO-A catalyzes preferentially the oxidative deamination of norepinephrine (NE), and serotonin (5-HT) and is inhibited by low concentrations of clorgyline. In contrast, MAO-B has a higher affinity for phenylethylamine and benzylamine, and is inhibited by selegiline [99]. Both isoforms catalyze the deamination of dopamine, tyramine, octopamine, and tryptamine and are inhibited by pargyline. A wide range of MAO inhibitors is available today and these are proving to have a therapeutic value in several pathologies, including affective disorders, neurodegenerative diseases, stroke, and aging [88, 99]. 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; (iii) reversible and selective inhibitors, such as moclobemide for MAO-A and lazabemide for MAO-B.

MAO catalyze the following reaction:



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

Kinetic studies have shown that the binding of the amine group to the enzyme precedes the binding of oxygen [95]. In a first moment, the reduction of the cofactor FAD yields an aldehyde intermediate and ammonia, while in a second moment the oxidized form of the prosthetic group is restored with the concomitant production of hydrogen peroxide.



$$RCH_2NH_2 + MAO \rightarrow RCHO + NH_3 + MAO\text{ - reduced}$$
$$MAO\text{ - reduced} + O_2 \rightarrow MAO + H_2O_2$$

The aldehyde intermediates are rapidly metabolized to the corresponding acid by the action of aldehyde dehydrogenase (ALDH). A failure of this latter enzyme might increase the deleterious aspects of MAO activity generating potentially harmful aldehyde compounds that could exacerbate the damage produced by MAO-induced H_2O_2 formation. Indeed, the decrease in ALDH activity appears to be involved in both oxidative stress and nitrate tolerance [34, 98], whereas an increased activity of ALDH has been reported to result in a decreased injury of the ischemic heart [26].

Deletion of *MAO-A* and *MAO-B* genes has proven the important role of these enzymes in neurotransmitter metabolism and behavior. *MAO-A*$^{-/-}$ mice display elevated brain levels of serotonin, norepinephrine, and to a lesser extent, dopamine [23], whereas only 2-phenylethylamine is increased in MAO-B knockout mice [99]. Both MAO-A and B knockout mice show increased reactivity to stress, similar to that observed after administration of non-selective MAO inhibitors. However, these studies, and the deletion of both MAO-A and B in a rare form of human Norrie disease, indicate that MAO is not essential for survival [63]. Gene deletion has shown that MAO-A activity is important during development. A compulsive-aggressive behavior results from lack of MAO-A function in humans [19] and mice [92]. This effect, which might reflect the importance of serotonin during development, can be mimicked by the administration of MAO-A inhibitor clorgyline during the early postnatal period.

Monoamine oxidases distribution has been particularly studied in the brain, where MAO-A has been prevalently found in noradrenergic neurons whereas MAO-B has been detected in serotoninergic and histaminergic neurons and in glial cells [64]. In 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 [94].

MAO inhibition: from neurological disorders to cardiovascular diseases

The roles of MAO in terminating the actions of neurotransmitters and dietary amines in central and peripheral nervous system, as well as in extraneuronal tissues, have been extensively studied. In contrast, less attention has been given to the products of MAO activity. Monoamine catabolism results in the formation of aldehydes, ammonia and H_2O_2.

MAO is involved in numerous pathologies, in particular in neuronal and psychiatric disorders, as demonstrated by the beneficial effects elicited by MAO inhibitors. The therapeutic potential of MAO inhibition has been discovered in the early 1950s, when antituberculosis treatment with iproniazid was shown to improve the mood while reducing MAO activity [85, 88].

MAO-B appears to be involved in the loss of dopaminergic neurons that occurs in Parkinson's disease, most

likely due to the increased dopamine catabolism, resulting in elevated production of ROS responsible for the oxidative damage at the level of nigrostriatal neurons. Indeed, MAO-B inhibition has been proven to afford neuroprotection [99]. An increase in MAO-B activity in brain is also associated with diseases such as Alzheimer's or 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 [99].

Besides their implication in neurodegenerative diseases, MAO isoforms, especially MAO-A, have been shown to play a relevant role in myocardial injury caused by post-ischemic reperfusion [13], and preliminary evidence suggests that MAO-A contributes also to the maladaptive evolution of myocardial hypertrophy into failure [61] (Fig. 1). In particular, MAO-A has been demonstrated to be an important source of ROS in receptor-independent apoptotic effects of serotonin in isolated cardiomyocytes and post ischemic myocardial injury [13, 14]. In fact, MAO-dependent ROS increase appears to be relevant for serotonin-induced myocyte hypertrophy in vitro [14]. In addition, MAO-A can promote cell apoptosis through ROS-dependent sphingosine kinase inhibition with accumulation of ceramide [81]. As far as vasculature is concerned, MAO-A mediated ROS production has been shown to induce mitogenic signaling in smooth muscle cells in a process that might involve the activation of metalloproteinase MMP-2, likely contributing to vascular wall remodeling [29]. Interestingly, MAO-A activity has been reported to increase with aging [67]. It is tempting to speculate that the consequent increase in H_2O_2 formation might contribute to aging-associated pathologies, such as congestive heart failure and vascular alterations.

Considering the important role of MAO as a source of H_2O_2 that has been described both in the brain as well as in the heart following I/R injury, MAO inhibition is likely to represent an important tool for both the study and the treatment of vascular pathologies that share oxidative stress as a common denominator.

At present the relationships between $p66^{Shc}$ and MAO are still to be clarified. They might represent two independent sources of mitochondrial ROS. On the other hand, while allosteric or covalent changes do not appear to be involved in MAO activity, H_2O_2 produced by these flavoenzymes could induce conformational changes in $p66^{Shc}$, facilitating its phosphorylation and/or activation as a ROS producing enzyme. This latter possibility appears to be supported by preliminary evidence from our laboratory (unpublished data). In fact, while MAO inhibition and $p66^{Shc}$ deletion resulted in comparable degrees of protection against post-ischemic reperfusion, clorgyline did not elicit additional protection in $p66^{Shc-/-}$ mice.

In conclusion, besides opening new possibilities for therapeutical interventions against cardiovascular disorders, studies on $p66^{Shc}$ and MAO clearly indicate that mitochondrial ROS formation is not only an accidental by-product of the respiratory chain, but also that most of the intracellular oxidative stress originate in mitochondria.

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