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Crosstalk between mitochondria and NADPH oxidases

Sergey Dikalov*
Emory University School of Medicine, Atlanta, Georgia

Abstract

Reactive oxygen species (ROS) play an important role in physiological and pathological processes. In recent years, a feed-forward regulation of the ROS sources has been reported. The interaction between main cellular sources of ROS, such as mitochondria and NADPH oxidases, however, remain obscure. This work summarizes the latest findings on the role of crosstalk between mitochondria and NADPH oxidases in pathophysiological processes. Mitochondria have the highest levels of antioxidants in the cell and play an important role in the maintenance of cellular redox status, thereby acting as an ROS and redox sink and limiting NADPH oxidase activity. Mitochondria, however, are not only a target for ROS produced by NADPH oxidase but also a significant source of ROS, which under certain condition may stimulate NADPH oxidases. This crosstalk between mitochondria and NADPH oxidases, therefore, may represent a feed-forward vicious cycle of ROS production which can be pharmacologically targeted under conditions of oxidative stress. It has been demonstrated that mitochondria-targeted antioxidants break this vicious cycle, inhibiting ROS production by mitochondria and reducing NADPH oxidase activity. This may provide a novel strategy for treatment of many pathological conditions including aging, atherosclerosis, diabetes, hypertension and degenerative neurological disorders in which mitochondrial oxidative stress seems to play a role. It is conceivable that the use of mitochondria-targeted treatments would be effective in these conditions.

Introduction

Over the past several years, it has become clear that reactive oxygen species (ROS) play an important role in both physiological and pathological processes.[1, 2] Superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) have been implicated in redox regulation of cell differentiation, proliferation, migration and vasodilatation.[3-6] Under normal physiological conditions, production of ROS is highly restricted to specific subcellular sites and is down regulated by a number of negative feed-back mechanisms.[7-10] Production of ROS “in the wrong place at the wrong time” or generation of ROS in excessive amounts results in oxidative stress leading to cellular dysfunction and apoptosis which contributes to atherosclerosis,[11] heart failure,[12] hypertension,[13] ischemia/reperfusion injury,[14] cancer,[15] aging[16] and neurodegeneration.[17] While there are numerous enzyme systems that produce ROS in mammalian cells, four enzymatic systems seem to predominate. These include the NADPH oxidases,[18] xanthine oxidase,[19] uncoupled NO synthase [20] and the mitochondrial electron transport chain.[16] There is a substantial interplay between these sources, such that activation

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*To whom correspondence should be addressed: Sergey Dikalov, Ph.D., FRIMCORE Director, Division of Cardiology, Emory University School of Medicine, 1639 Pierce Drive, Atlanta, GA 30322, Tel.: 404-712-9550, Fax: 404-727-3585, dikalov@emory.edu.

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of one can lead to activation of the others (Figure 1). This can lead to feed forward processes, which further augment ROS production and oxidative stress.[21] The phenomenon of ROS-induced ROS production is very well documented: H_2O_2 activates $O_2^{\bullet -}$ production by phagocytic and non-phagocytic NADPH oxidases;[22] peroxynitrite uncouples eNOS switching from NO to $O_2^{\bullet -}$ production and increases production of mitochondrial ROS;[23, 24] H_2O_2 induces transformation of XDH into XO, a source of H_2O_2 and $O_2^{\bullet -}$.[25] The interplay between specific ROS sources, however, is not clear. Crosstalk between two major ROS sources, mitochondria and NADPH oxidases, is of particular interest.

Mitochondrial function and production of mitochondrial ROS

It is generally assumed that the major biological function of mitochondria is ATP synthesis by oxidative phosphorylation.[26] This process is based on aerobic oxidation of hydrogen and is much more efficient than anaerobic metabolism of glucose. It is based on transfer of electrons through the mitochondrial respiratory chain (Figure 2). Electrons can be supplied by either NADH at complex I or by succinate at complex II. Ubiquinone mediates electron transfer to complex III, which in turn reduces complex IV. Complex IV couples oxygen reduction to water and the proton pump, transporting protons (H^+) from the matrix to the intermembrane space. Respiring mitochondria generate the proton motive force across the inner membrane which results in a negative charge inside and produces a pH gradient.[27]

At several sites of the respiratory chain, electrons “leak” to O_2 creating $O_2^{\bullet -}$ [28, 29] (Figure 1). The main sources of mitochondrial ROS under physiological conditions are complexes I and II, which produce $O_2^{\bullet -}$ mainly on the matrix side, where it is rapidly dismutated to H_2O_2 by mitochondrial Mn-SOD (SOD2).[30, 31] Other sources of mitochondrial $O_2^{\bullet -}$ may include alpha-ketoglutarate dehydrogenase, pyruvate dehydrogenase,[32] glycerol 3-phosphate dehydrogenase, fatty acid beta-oxidation,[33] and complex III.[34, 35] H_2O_2 is a neutral molecule and will easily leave mitochondria regardless of mitochondrial energization. The amount of mitochondrial H_2O_2 is in the range of 0.1% to 2% of the electron flow.[16]

Until recently, the functional significance of mitochondria-derived ROS, particularly in vascular cells, has received little attention. This is partly due to low metabolic activity and the lack of information regarding regulation of mitochondrial ROS compared with other enzymes like NADPH oxidase.[18] However, a paradigm shift has occurred in recent years, focusing greater attention on a potential key role of mitochondrial ROS in cell signaling.[36]

A new concept is emerging that mitochondria are more than just “ATP cows”[37, 38] and ROS production by mitochondria is a part of their physiological function.[1] This process is likely to be highly regulated and we are just beginning to uncover the specific molecular mechanisms. Reverse electron transport from complex II to complex I is likely to be a major pathway for mitochondrial ROS production. It is stimulated by complex II substrate succinate and can be inhibited by proton ionophore CCCP, rotenone or the complex II inhibitors malonate or oxaloacetate (Figure 2).[39, 40] It has been recently shown that this pathway strongly depends on the pH gradient across the inner membrane (ΔpH).[41] Activation of mitochondrial ATP-sensitive potassium channels (mitoK_{ATP}) increases production of mitochondrial ROS [42, 43] and is likely to be associated with an increase of ΔpH. In this review, we are particularly interested in reverse electron transport because it can be regulated by redox-sensitive mitoK_{ATP} and mitochondrial ATP level.[44, 45]

Ischemia and apoptosis trigger $O_2^{\bullet -}$ production by complex III.[34] This may occur due to inhibition of complex IV and overreduction of the electron transport chain in cases of hypoxia or NO-mediated inhibition of complex IV which can be simulated by treatment with

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the complex III inhibitor antimycin A.[46] The contribution of complex III in production of mitochondrial $O_2^{\bullet -}$ under normal physiological conditions is, however, not clear. It is possible that $O_2^{\bullet -}$ production by complex III does not depent on mitochondrial transmembrane potential as much as reverse electron transport.[41] For example, uncoupling of mitochondria with antimycin A may inhibit production of mitochondrial ROS by reverse electron transport but stimulate $O_2^{\bullet -}$ production by complex III.[47, 48]

Mitochondrial manganese superoxide dismutase (SOD2) is a key scavenger of $O_2^{\bullet -}$ in the mitochondrial matrix. It is a nuclear-encoded protein that forms a homotetramer with each subunit binding one manganese atom. SOD2 plays critical roles in regulating redox-sensitive signaling pathways and controlling mitochondrial $O_2^{\bullet -}$.[49] By inhibiting the reaction of $O_2^{\bullet -}$ with 4Fe-4S clusters, this enzyme prevents inactivation of aconitase, complex I and complex II.[50] SOD2 is inactivated by $ONOO^-$ [51] and its activity is decreased with age [52]. Expression of SOD2 is upregulated by various cytokines and agonists in a redox-dependent manner [53]. SOD2 overexpression attenuates H_2O_2-induced apoptosis,[54] decreases lipid peroxidation and reduces the age-related decline in mitochondrial ATP.[55]

Mitochondria are not only one of the major sources of $O_2^{\bullet -}$ and H_2O_2 in vascular cells [56, 57] but are also the targets of cellular ROS [56]. Mitochondrial membranes, proteins, and mtDNA are particularly sensitive to oxidative damage.[58, 59] ROS modify mitochondrial proteins, leading to their inactivation, as in the case of SOD2 and aconitase, or alter their function, as occurs with cytochrome c [60-62]. Superoxide reacts with 4Fe-4S clusters of complex I, complex II and aconitase, resulting in the release of Fe^{3+} and altered protein function [16]. It has been shown that oxidative damage to complex I and complex II, presumably at the level of 4Fe-4S clusters, increases mitochondrial $O_2^{\bullet -}$ production. Interestingly, a decrease in complex II activity due to oxidative modification increases its $O_2^{\bullet -}$ production by 3-4 fold.[63]

Enhanced production of mitochondrial ROS is linked to mitochondrial dysfunction.[64] Mitochondrial oxidative stress not only alters the ability of the cell to generate energy but also affects cellular redox signaling [56]. ROS generated in the mitochondrial respiratory chain have been proposed as secondary messengers for activation of NFκB by TNF-α and IL-1.[65] Mitochondrial ROS, therefore, not only enhance cellular oxidative stress, but can represent an important modulator of cellular function. Growth factor receptor transactivation and its downstream signaling in response to H_2O_2 are abrogated by mitochondrial targeted antioxidants, but not by nontargeted counterparts, suggesting the involvement of mitochondrial oxidants in these events.[66]

Mitochondrial protein kinase C epsilon (PKCε) has been shown to play an important role in modulation of mitochondrial function. This enzyme is known to phosphorylate and activate the mitoK_{ATP}. Moreover PKCε is exquisitely redox sensitive and therefore is a very good candidate to transduce signals from extramitochondrial ROS leading to mitochondrial ROS production.[45] Indeed, our data implicate activation of mitoK_{ATP} in stimulation of mitochondrial ROS.[42] We, therefore, hypothesize that ROS produced by extramitochondrial NADPH oxidases stimulate PKCε, which then phosphorylates and activates mitoK_{ATP}.

Mitochondria-targeted antioxidants

Recent studies have demonstrated that decrease of mitochondrial ROS by overexpression of SOD2 protects against mitochondrial oxidative damage and myocardial dysfunction [67-69]. Low-molecular weight antioxidants, such as α-tocopherol and N-acetylcysteine, also decrease mitochondrial oxidative damage in vitro [70]. In vivo, however, these traditional

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antioxidants have limited mitochondrial accumulation.[71] A major continued challenge, therefore, is to develop mitochondria-targeted antioxidant agents that can prevent mitochondrial oxidative damage and mitochondrial dysfunction [71]. Antioxidants can be targeted to mitochondria by several methods: (i) preferential accumulation in mitochondria because of their hydrophobicity and positive charge (hydrophobic cations), (ii) binding with high affinity to an intra-mitochondrial constituent, and (iii) metabolic conversions by specific mitochondrial enzymes to reveal an active entity.[72]

The membrane potential of mitochondria within living cells is negative inside (−140 mV). As this membrane potential is far larger than in other organelles within cells, lipophilic cations such as triphenylmethylphosphonium (TPMP) selectively accumulate within mitochondria [71]. Antioxidants conjugated to TPMP, therefore, can be targeted to mitochondria. Due to their positive charge, agents such as MitoTEMPOL [73] are concentrated in the mitochondrial matrix by 1000-fold.[74] It has been reported that MitoTEMPOL is readily reduced to its hydroxylamine (MitoTEMPOL-H) by a direct reaction with ubiquinol.[73] MitoTEMPOL-H may react with $O_2^{\bullet-}$ producing MitoTEMPOL similar to the reaction of TEMPONE-H.[75] A previous report showing lack of $O_2^{\bullet-}$ scavenging by MitoTEMPOL-H was likely due to the artifact associated with direct reduction of cytochrome C by MitoTEMPOL-H.[76] Our spin trapping experiments unambiguously demonstrate scavenging of $O_2^{\bullet-}$ by hydroxylamine mitoTEMPO-H.[21]

While mitoTEMPOL acts as an SOD-mimetic converting $O_2^{\bullet-}$ molecules into H_2O_2, [21] the benefit of such agents probably exceeds that of simply scavenging superoxide, because it seems by preventing mitochondrial damage, we reduce mitochondrial electron leak and thus inhibit production of all ROS, including superoxide, hydrogen peroxide and peroxynitrite. Recently, it has been shown that pretreatment of endothelial cells with the mitochondria-targeted SOD mimetic mito-CP significantly reduced H_2O_2- and lipid peroxide-induced cellular oxidative stress [77]. Mito-CP inhibited peroxide-induced inactivation of complex I and aconitase, while restoring the mitochondrial membrane potential. In contrast, the “untargeted” carboxy proxyl (CP) did not protect the cells from peroxide-induced oxidative stress and apoptosis.

The pharmacology of mitochondria-targeted antioxidants is not well understood. For example, previously described mitoquinone ($MitoQ_{10}$) [78] may have prooxidant and proapoptotic properties due to redox cycling and generation of $O_2^{\bullet-}$ by quinone. [79, 80] Nitroxides such as TEMPO have very low toxicity making them perfect candidates for conjugation with triphenylmethylphosphonium for in vivo use, [81] but antioxidants such as Mito-CP are esters and potentially can be hydrolyzed into inactive 3-carboxyproxyl (CP) [77] and triphenylmethylphosphonium. Furthermore, micromolar concentrations of tetraphenylphosphonium inhibit oxidation of pyruvate, malate, 2-oxoglutarate and glutamate in heart mitochondria, [82] suggesting that triphenylmethylphosphonium conjugates should be used at submicromolar concentrations and tested for side effects on respiration. We have recently described in vivo applications of the mitochondria-targeted SOD mimetic mitoTEMPO, which is resistant to hydrolysis, and low doses of mitoTEMPO (25 nM in vitro and 0.7 mg/kg/day in vivo) did not reveal side effects on respiration.

The cationic, arginine-rich SS (Szeto-Schiller) tetrapeptides Dmt-D-Arg-Phe-Lys-NH2 and D-Arg-Dmt-Lys-Phe-NH employ the targeted delivery of antioxidants to the inner mitochondrial membrane [83] and have been shown to be very effective in diminishing mitochondrial ROS, inhibiting mitochondrial permeability transition, reducing cytochrome c release,[84] attenuating mitochondrial swelling, inhibiting oxidative cell death, and reperfusion injury.[72] Notably, these small peptides were concentrated 1000-fold across the

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inner mitochondrial membrane and also readily crossed the cell membrane. Preclinical studies support the use of these peptides for ischemia-reperfusion injury and neurodegenerative disorders. Although peptides have often been considered to be poor drug candidates, the few that have been studied are promising agents for the treatment of diseases.[84]

Gramicidin S-TEMPO conjugates (GS-TEMPO) preferentially accumulates in the mitochondria due to high-affinity GS binding with the inner mitochondrial membranes. The gramicidin segment was used to target the nitroxide to mitochondria because antibiotics of this type have a high affinity for bacterial membranes and because of the close relationship between bacteria and mitochondria. In a rat model of hemorrhagic shock, delayed treatment with XJB-5-131 has been shown to prolong survival time.[85]

NADPH oxidases

NADPH oxidases are a family of enzyme complexes whose primary function is to catalyze the transfer of electrons from NADPH to molecular oxygen via their “Nox” catalytic subunit, generating $O_2^{\bullet-}$ and H_2O_2. The Nox enzymes contribute to numerous biological and pathological processes including hearing and balance (Nox3), blood pressure regulation, inflammation, cell growth (Nox1/Nox2), and differentiation (Nox4).[86] The Nox proteins vary in terms of their mode of activation and localization.[87] Nox1 is expressed in smooth muscle cells, but is also present in other vascular cells. Nox2, previously known as gp91phox, is present in endothelial and phagocytic cells.[88-91] Nox3 is expressed in the brain and inner ear.[86] Nox4 is constitutively expressed and active in vascular smooth muscle and endothelial cells. [92, 93] Nox5 has been identified in human immature lymphatic tissues, in human endothelial cells, and it is activated by Ca^{2+} binding to EF-hand motifs. The Duox1/ Duox2 proteins are described as having a dual nature due to an extracellular peroxidase domain in addition to the EF-hand Ca^{2+} binding and gp91phox homology domains. Originally isolated from the thyroid, they produce the H_2O_2 that is used to oxidize iodide during thyroid hormone synthesis.[94]

It is important that Nox isoforms have not only different regulation and specific subcellular localization but also generate distinct ROS. For example, Nox4 is responsible for the basal production of H_2O_2, [95, 96] Nox1 and Nox2 generates $O_2^{\bullet-}$,[95] and Nox5 produces H_2O_2 in a Ca^{2+} dependent fashion [97] (Figure 3).

It has been recently reported that Nox4 may be expressed in the mitochondria of rat kidney cortex [98] and in the mitochondria of cardiac myocytes.[99] Ago et al. reported higher expression of Nox4 in mitochondrial fraction of cardiac myocytes compared to microsomal fraction.[100] Confocal microscopy showed significant co-localization of Nox4 with mitochondrial F1F0-ATP synthase as well as p22phox subunit of NADPH oxidases. Cysteine residues of mitochondrial proteins were more oxidized in Nox4-transgenic mice. Nox4 expression did correlate with dihydroethidium staining for superoxide and cardiac damage.[100] These studies, however, remain highly controversial since they were not able to directly demonstrate the Nox4 activity in mitochondrial preparations but showed crude NADH dependent ROS production which is known to be mediated by mitochondrial complex I. The assessment of Nox4 activity by superoxide measurements with dihydroethidium is also questionable since several groups have reported that Nox4 primarily generates H_2O_2 but not superoxide.[95, 96] Our studies did not show the presence of Nox1, Nox2, Nox4 and p22phox subunits in the mitochondria of endothelial cells.[42] It has been previously shown that Nox4 is specifically localized in focal adhesions, along stress fibers, and in the nucleus. Nox4 is co-localized with the p22phox subunit which is required for Nox4 activity.[93, 101] Unfortunately, Ago et al. did not present co-localization of p22phox

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and cardiac mitochondria. It is possible that mitochondrial localization of Nox4 reported by Block et al.[98] and Ago et al. [100] differ from previous publications [93, 101] due to distinct Nox4 antibodies used for immunostaining while many authors raised concerns regarding the specificity of some Nox4 antibodies. Although it may be intriguing to suggest the role for Nox4 in mitochondrial oxidative stress, the lack of data on mitochondrial p22phox and the absence of specific measurements of mitochondrial Nox4 activity do not support this hypothesis. It is also important that mitochondria do not require any Nox isoform to produce ROS as described above and ROS production by mitochondria can significantly surpass the amount of ROS produced by Nox4, particularly in the heart. The proposed role of Nox4 in cardiac pathology [100] may also conflict with Nox4-mediated cardiac protection against chronic load-induced stress by enhancing angiogenesis.[102] Therefore, the data on mitochondrial expression of Nox4 and its functional significance should be taken with caution and further studies of mitochondrial Nox4 are required. Meanwhile, it is conceivable that cytoplasmic Nox4 may contribute to redox sensitive upregulation of mitochondrial ROS produced in the electron transport chain via activation of PKCε, mitoK_{ATP} or modulation of thioredoxin 2 activity as described below.

Angiotensin II is the major effector hormone of the renin–angiotensin system which plays an important role in the activation of vascular NADPH oxidases by PKC and c-Src dependent pathways.[103] Initial activation of the angiotensin AT_1 receptor leads to PKC-mediated phosphorylation of $p47^{phox}$. This leads to c-Src activation and stimulation of the epidermal growth factor receptor (EGFR), which evokes phosphatidylinositol 3-kinase-dependent production of phosphatidylinositol (3,4,5)-trisphosphate and, in turn, activates Rac1 subunit of NADPH oxidase.[104] Nox4 and Nox5 do not require $p47^{phox}$ or Rac1 subunits.[105] Thus, in vascular cells, AngII primarily increases activity of Nox1 or Nox2 (Figure 3).[106] Activation of c-Src is redox sensitive and stimulated by H_2O_2,[4] which appears to represent a feed-forward mechanism whereby H_2O_2-mediated activation of c-Src amplifies NADPH oxidase activity of Nox1 and Nox2.

A correlation between endothelial Nox2 expression and hypertension has been reported.[107] Aortic Nox2 is elevated in stroke-prone SHR, in rats exposed to aldosterone plus salt and in AngII-infused mice. [108] The SOD mimetic TEMPOL inhibits redox-dependent Nox2 expression and improves pulmonary hypertension in renin transgenic rats.[109] A chimeric peptide that inhibits the association of $p47^{phox}$ with Nox2 in NADPH oxidase (gp91ds-tat) attenuates AngII-induced hypertension and decreases aortic $O_2^{\bullet-}$ production in AngII-treated rats.[110] It was found that this peptide inhibits the NADPH oxidase *in vivo* and was very effective in inhibiting Nox2 function in vitro.

Angiotensin-converting enzyme inhibitors and type I angiotensin-receptor blockers also reduce age-related mitochondrial dysfunction, attenuate hypertension-induced renal mitochondrial dysfunction, and protect against cardiac mitochondrial dysfunction in the setting of acute ischemia. [111-113] We have found that depletion of the p22phox subunit prevents mitochondrial dysfunction and the increase of mitochondrial ROS caused by AngII.[42] These findings suggest that AngII can alter mitochondrial function via activation of NADPH oxidases.[12, 42] The molecular mechanisms underlying the interplay between the NADPH oxidases and the mitochondria remain undefined.

Stimulation of mitochondrial ROS by NADPH oxidases

We have previously reported that AngII increases production of mitochondrial ROS and decreases mitochondrial membrane potential, respiratory control ratio, and low molecular weight thiols content. Activation of NADPH oxidases is an early response of endothelial cells to AngII.[114] Angiotensin II binds to the AngII type 1 receptor, leading to rapid-

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generation of ROS through PKC-dependent activation of NADPH oxidases. The deleterious effects of AngII on mitochondrial function are associated with increased cellular $O_2^{\bullet-}$ production and decreased endothelial $NO^{\bullet}$ bioavailability. Interestingly, our results indicate that AngII-mediated mitochondrial dysfunction is dependent on activation of vascular NADPH oxidases and opening of the mitoK$_{ATP}$ channels. Indeed, Paul Brookes' group showed that mitoK$_{ATP}$ channels are activated by $O_2^{\bullet-}$ and H_2O_2 but not other peroxides.[44] Our data support activation of mitoK$_{ATP}$ by ROS coming from NADPH oxidases because inhibition of NADPH oxidases and PKC by apocynin and chelerythrine completely prevented mitochondrial dysfunction induced by AngII. Apocynin is known to block the activation of NADPH oxidases, and chelerythrine selectively inhibits PKC. Both inhibitors dramatically attenuated mitochondrial ROS generation in response to AngII. Most importantly, depletion of p22phox, an essential component for NADPH oxidase function, with siRNA led to a significant decrease in ROS production in mitochondria isolated from AngII treated cells. Treatment with the mitoK$_{ATP}$ channels blocker 5-HD and glibenclamide prevented the increase in mitochondrial H_2O_2, attenuated the decrease in mitochondrial membrane potential, and preserved respiratory control ratio and low molecular weight thiols content induced by AngII. [42] Taken together, these results showed that stimulation of mitochondrial ROS by AngII requires the full enzymatic activity of NADPH oxidases and depends on activation of redox sensitive mitoK$_{ATP}$ channels (Figure 4).[42]

Our data also clearly implicate mitoK$_{ATP}$ channels in AngII–mediated mitochondrial dysfunction. As we observed, 5-HD, a specific inhibitor of mitoK$_{ATP}$ channels, and glibenclamide, a non-selective inhibitor of ATP-sensitive potassium channels, suppressed AngII-induced mitochondrial H_2O_2 production and membrane potential depolarization, prevented the decrease in mitochondrial RCR and reduced thiols content. The mechanism by which AngII regulates mitoK$_{ATP}$ channels activity is unclear. An involvement of $O_2^{\bullet-}$ and PKC in activation of mitoK$_{ATP}$ channels has been suggested in vascular smooth muscle cells and cardiac cells.[115, 116] AngII activated NADPH oxidase-derived $O_2^{\bullet-}$ is capable of stimulating the opening of the mitoK$_{ATP}$ channels via a direct action on the sulfhydryl groups of this channel.[117] Opening of these channels has been proposed to increase potassium influx causing matrix alkalinization, swelling, mild mitochondrial uncoupling and ROS production.[118] It is conceivable that mitoK$_{ATP}$ channels are both downstream and upstream of mitochondrial ROS providing feed-forward regulation.

Interestingly, acute treatment with the mitoK$_{ATP}$ channels inhibitor 5-HD after four hours incubation with AngII brought back endothelial $O_2^{\bullet-}$ production to baseline levels and restored $NO^{\bullet}$ bioavailability in endothelial cells. The decline in $O_2^{\bullet-}$ generation and recovery of $NO^{\bullet}$ production by 5-HD implies that mitochondrial ROS indeed enhances endothelial oxidative stress by a feed-forward mechanism (Figure 4).[42]

Activation of NADPH oxidases by mitochondrial ROS

It has been shown that opening of mitoK$_{ATP}$ channels with diazoxide in rat vascular smooth muscle cells depolarized the mitochondrial membrane potential and increased cellular $O_2^{\bullet-}$ detected by dihydroethidium.[115] Activation of mitoK$_{ATP}$ channels with diazoxide stimulates $O_2^{\bullet-}$ production on mitochondrial complex I[43]; however, dihydroethidium does not detect mitochondrial $O_2^{\bullet-}$.[21] The increase in dihydroethidium fluorescence indicates $O_2^{\bullet-}$ production in the cytoplasm by NADPH oxidases.[21] These data therefore suggest that stimulation of mitochondrial $O_2^{\bullet-}$ may increase the activity of NADPH oxidases leading to enhanced $O_2^{\bullet-}$ production in the cytoplasm. However, the exact mechanism of this process is not clear.

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Studies with human embryonic kidney 293T cells have shown that serum withdrawal promotes the production of mitochondrial ROS and the activation of Nox1.[119] Mitochondria respond to serum withdrawal within a few minutes, and the ROS produced by the mitochondria trigger Nox1 action by stimulating phosphoinositide 3-kinase (PI3K) and Rac1. Activation of the PI3K/Rac1/Nox1 pathway was evident 4-8 hours after serum withdrawal initiation. Functional analysis suggested that, although the mitochondria contribute to the early accumulation of ROS, the maintenance of the induced ROS levels in the later (4-8 h) phase required the action of the PI3K/Rac1/Nox1 pathway. Serum withdrawal-treated cells eventually lost their viability, which was reversed by blocking either the mitochondria-dependent induction of ROS using rotenone or the PI3K/Rac1/Nox1 pathway using the dominant negative mutants or small interfering RNAs. This suggests that mitochondrial ROS are essential but not sufficient to promote cell death, which requires the sustained accumulation of ROS by the subsequent action of Nox1.[119]

Recently, effects of mild mitochondrial dysfunction on AngII-mediated increase in Nox isoform expression and activity in vascular smooth muscle cells have been described.[120] Mild mitochondrial dysfunction due to mitochondrial DNA damage after 24 h incubation of rabbit aortic smooth muscle with ethidium bromide (EtBr) resulted in 29% less oxygen consumption and 16% greater baseline hydrogen peroxide. The normally observed increase in NADPH oxidase activity after AngII was completely abrogated after EtBr, together with failure to upregulate Nox1 mRNA expression. Similar loss in AngII redox response occurred after 24 h of antimycin A treatment. These results implicate mitochondria in regulation of expression and activity of NADPH oxidase.

The role of mitochondrial ROS was further investigated in endothelial cells with overexpression or depletion of mitochondrial superoxide dismutase (SOD2). Transfection of HAEC with an SOD2 plasmid increased mitochondrial SOD2 activity by 2.4-fold, while depletion of SOD2 with siRNA decreased SOD2 activity by 2.7-fold. In cells transfected with a GFP control plasmid, AngII stimulation doubled NADPH oxidase activity. In contrast, AngII had no effect on NADPH oxidase activity in HAEC transfected with the SOD2 plasmid. Interestingly, depletion of SOD2 increased NADPH oxidase activity in unstimulated cells. Furthermore, AngII stimulation of SOD2 depleted cells resulted in higher activity of NADPH oxidase.[21]

Analysis of intact cells showed that AngII increased the $O_2^{\bullet -}$ to a similar extent in non-transfected cells or cells treated with a GFP control plasmid. SOD2 overexpression completely prevented AngII-stimulated $O_2^{\bullet -}$ production while not affecting $O_2^{\bullet -}$ production in unstimulated cells. SOD2 depletion enhanced both basal and AngII-stimulated $O_2^{\bullet -}$ in intact cells. These data confirmed that modulation of mitochondrial $O_2^{\bullet -}$ by mitoTEMPO or changing SOD2 levels affects the production of cellular $O_2^{\bullet -}$ by NADPH oxidases. Fluorescence microscopy with mitoSOX showed that SOD2 depletion increased both basal and AngII–stimulated mitochondrial superoxide production, and that this could be inhibited by mitoTEMPO. It is important to note that mitoTEMPO treatment inhibited cellular $O_2^{\bullet -}$ and mimicked SOD2 overexpression in SOD2 depleted cells, rescuing SOD2 depleted cells.[21]

Crosstalk between mitochondria and NADPH oxidases

The data described above suggest that activation of NADPH oxidases may increase production of mitochondrial ROS and vice versa: increase of mitochondrial ROS may activate NADPH oxidases. We have suggested that this represents an ongoing feed-forward cycle. Indeed, acute treatment of AngII-stimulated cells with mitochondria-targeted SOD

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mimetic mitoTEMPO reduced mitochondrial superoxide measured by mitoSOX, completely blocked the increase in NADPH oxidase activity measured in the membrane fraction and abrogated production of cytoplasmic $O_2^{\bullet-}$ measured in intact cells using dihydroethidium and HPLC.[21] It is important that mitoTEMPO or SOD2 overexpression did not affect basal NADPH oxidase activity in unstimulated cells but only inhibited Nox activity in stimulated cells. Since AngII stimulates Nox1 and Nox2 activity, we think that these NADPH oxidase isoforms are particularly important in the crosstalk between mitochondria and NADPH oxidases (Figure 4).

An interesting example of crosstalk between mitochondrial and Nox-derived ROS has been reported in nitrate tolerance.[110] Nitrate tolerance was induced by nitroglycerin infusion in male Wistar rats. Isometric tension studies revealed that genetic deletion or inhibition of NADPH oxidases improved endothelial function, whereas nitrate tolerance was unaltered. Vice versa, nitrate tolerance was attenuated by co-treatment with the respiratory chain complex I inhibitor rotenone or the mitochondrial permeability transition pore blocker cyclosporine A. Both compounds improved endothelial function, suggesting a link between mitochondrial and Nox-derived ROS. Mitochondrial respiratory chain-derived ROS are critical for the development of nitrate tolerance, whereas Nox-derived ROS mediate nitrate tolerance-associated endothelial dysfunction.[110]

Comparison of AngII and diazoxide showed that both mitochondrial ROS and NADPH oxidase contribute to redox-sensitive mitogen-activated protein kinase activation in rat vascular smooth muscle cells in vitro and in rat aorta in vivo.[115] Similarly, AngII treatment with diazoxide depolarized the mitochondrial membrane potential and increased cytoplasmic superoxide production measured with DHE, resulting in phosphorylated MAP kinases (ERK1/2, p38, and JNK), which were suppressed by the specific inhibitor of mitoK_{ATP} channels 5-hydroxydecanoic acid. These results reveal that stimulation of mitochondrial ROS by NADPH oxidase dependent pathway or directly by diazoxide is required for maintenance of cytoplasmic superoxide production and redox-sensitive activation of MAP kinase.[115]

Overall, at least six different examples of cross-talk between mitochondrial and Nox-derived reactive oxygen species (ROS) have been reported.[121] In the first model, AngII is discussed as a trigger for NADPH oxidase activation with subsequent ROS-dependent opening of mitoK_{ATP} channels in endothelial cells, leading to depolarization of mitochondrial membrane potential followed by mitochondrial ROS formation and respiratory dysfunction.[42] In the second model, direct stimulation of mitoK_{ATP} channels with diazoxide in smooth muscle cells mimicked the effect of AngII on cytoplasmic production of superoxide by NADPH oxidases and redox-sensitive activation of MAP kinase.[115] This concept was supported by observations that ethidium bromide-induced mitochondrial damage suppressed AngII-dependent increase in Nox1 and oxidative stress.[120] In the third example, hypoxia was used as a stimulator of mitochondrial ROS formation and by using pharmacological and genetic inhibitors, a role of mitochondrial ROS for the induction of NADPH oxidases via PKCε was demonstrated.[122] The fourth model was based on cell death by serum withdrawal that promotes the production of ROS in human 293T cells by stimulating both the mitochondria and Nox1.[119] These studies showed that mitochondria were responsible for the fast onset of ROS formation followed by a slower but long-lasting oxidative stress condition based on the activation of Nox1 in response to the fast mitochondrial ROS formation. Fifth, cross-talk between mitochondria and Nox2 was shown in nitroglycerin-induced nitrate tolerance involving the mitochondrial permeability transition pore and ATP-sensitive potassium channels.[123] Finally, it has been shown that interplay between NADPH oxidase and mitochondria is mediated by the level of mitochondrial superoxide both in vitro and in vivo.[21] In this work it was shown that depletion of

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mitochondrial SOD2 increases the NADPH oxidase activity while SOD2 overexpression attenuates activation of NADPH oxidases.[21]

Taken together, these studies indicate that the interplay between mitochondrial and NADPH oxidase-derived $O_2^{\bullet-}$ constitutes a feed-forward cycle in which the NADPH oxidases increase mitochondrial ROS, which further activates the cytoplasmic NADPH oxidases and increases cellular $O_2^{\bullet-}$ production, diminishing $NO^{\bullet}$ bioavailability and uncoupling eNOS.[124] The effect of mitochondrial ROS on NADPH oxidase activity is quite likely mediated by c-Src [5] which can be stimulated by H_2O_2.[4] Indeed, activation of NADPH oxidases has been reported to be a biphasic process in which the first phase requires direct activation by AngII followed by a second phase of sustained activation that is H_2O_2 dependent.[104] This could explain why inhibition of mitochondrial H_2O_2 by mitoTEMPO results in decrease of NADPH oxidase activity.[21] Our current findings indicate that scavenging of mitochondrial $O_2^{\bullet-}$ using mitochondria-targeted antioxidants can interrupt this vicious cycle and down-regulate NADPH oxidase activity.[21] In the present study, we summarized the latest findings on the potential role of the interplay between mitochondria and NADPH oxidases in pathophysiological processes.

The interplay between mitochondrial ROS and NADPH oxidases may be rather specific and does not necessarily represent a general feed-forward mechanism due to an additive effect of ROS from Noxes and ROS from the mitochondria. Both mitochondria and NADPH oxidases are physically associated with the endoplasmic reticulum, and their redox signals can be very site specific without exposing the whole cell to elevated ROS. This crosstalk can be mediated by endoplasmic reticulum stress [125] which normally does not involve xanthine oxidase or uncoupled eNOS localized in the caveolae of the extracellular membrane. The crosstalk between mitochondrial ROS and NADPH oxidases likely plays an important role in normal physiological redox cell signaling. Under normal physiological conditions, production of ROS is highly restricted to specific subcellular sites and is down regulated by a number of negative feed-back mechanisms.[7-10] Production of ROS in excessive amounts due to overstimulation by AngII, high glucose, fat or hypoxia results in oxidative stress and transforms this feed-forward redox signaling into a vicious cycle (Figure 4) which contributes to the development of many pathological conditions.[21]

Hypertension

While ROS do not regulate blood pressure under normal conditions, they clearly contribute to the elevation in blood pressure in the setting of hypertension. ROS mediate the potent vasoconstrictor and hypertrophic effects of AngII and treatment with antioxidants decreases AngII-induced hypertension [126, 127]. PEG-SOD very effectively lowers blood pressure in AngII-treated rats, but not in normal rats [128]. Blood pressure is normal in mice lacking subunits of the NADPH oxidase, but these animals have a blunted hypertensive response to both AngII and DOCA-salt treatment [129]. Genetic overexpression of NADPH oxidase stimulates the hypertensive response to AngII [127], while overexpression of SOD attenuates the rise in blood pressure.[126]Using mice deficient in the NADPH oxidase subunit $p47^{phox}$ and mice lacking the endothelial NO synthase, it has been found that hypertension is a result of the cascade involving production of ROS from the NADPH oxidases leading to oxidation of tetrahydrobiopterin and uncoupling of endothelial NO synthase (eNOS).[20]

The role of mitochondrial oxidative stress in hypertension has been suggested in the work of Julian Wider and colleagues using mice overexpressing human thioredoxin 2 (Tg(hTrx2).[130] Systolic arterial blood pressure was not different between Tg(hTrx2) and wild-type animals under baseline conditions but the AngII-induced hypertension in Tg(hTrx2) mice was significantly attenuated. Aortic endothelium-dependent relaxation was reduced in wild-type

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mice after AngII infusion but was nearly unchanged in transgenic mice. Elevated vascular ROS and expression of NADPH oxidase subunits in response to AngII infusion were significantly attenuated in Tg(hTrx2) mice. Mitochondrial superoxide was increased after AngII infusion in wild-type mice but not in Tg(hTrx2) mice. The precise molecular mechanisms of regulation of mitochondrial ROS and NADPH oxidase by thioredoxin 2, however, remain unclear.

Co-infusion of mitochondria-targeted antioxidant mitoTEMPO and AngII attenuated hypertension decreased mitochondrial $O_2^{\bullet-}$, reduced cellular NADPH oxidase activity, inhibited vascular $O_2^{\bullet-}$ production and prevented the loss of endothelial NO.[21] Treatment of mice in vivo with mitoTEMPO decreased blood pressure by 30 mm Hg following establishment of both AngII-induced and DOCA-salt hypertension, while a similar dose of non-targeted TEMPOL was not effective. In vivo, mitoTEMPO decreased vascular $O_2^{\bullet-}$ produced by NADPH oxidases, increased vascular $NO^{\bullet}$ production and improved endothelial-dependent vasorelaxation. Interestingly, transgenic mice overexpressing mitochondrial SOD2 demonstrated attenuated AngII-induced hypertension and reduced vascular oxidative stress similar to mice treated with mitoTEMPO [21] while SOD2+/− mice were predisposed to both age-related and salt-induced hypertension.[131] These studies show that mitochondrial $O_2^{\bullet-}$ is important for the development of hypertension and that antioxidant strategies specifically targeting this organelle could have therapeutic benefit in this and possibly other diseases.[21]

Atherosclerosis

Oxidative modification of LDL and its transport into the subendothelial space of the arterial wall at the sites of endothelial damage are considered initiating events for atherosclerosis.[132] Oxidative modification of LDL results from the interaction of reactive oxygen species and reactive nitrogen species, produced from vascular wall cells and macrophages, with LDL. The resulting increased oxidative and nitrosooxidative stress induces endothelial dysfunction by impairing the bioactivity of endothelial nitric oxide and promotes leukocyte adhesion, inflammation, thrombosis, and smooth muscle cell proliferation - all processes that exacerbate atherosclerosis.[133] Studies in mice that are deficient in p47phox and gp91phox NADPH oxidase subunits show that ROS produced by these oxidases contribute to atherosclerosis.[134]

It has been recently shown that SOD2 deficiency increases endothelial dysfunction in ApoE-deficient mice.[11] Mice heterozygous for mitochondrial SOD2 (SOD2(+/−)) with apoE deficiency (apoE(−/−)) had increased formation of atherosclerotic lesions. Mitochondrial dysfunction, resulting from SOD2 deficiency, increased mtDNA damage and accelerated atherosclerosis in apoE knockout mice, consistent with the notion that increased ROS production and DNA damage in mitochondria are early events in the initiation of atherosclerosis. Mitochondrial dysfunction can result in apoptosis, favoring plaque rupture. [132]

Taken together, these data may suggest a crosstalk between NADPH oxidases and mitochondrial ROS in the development of atherosclerosis. These data also suggest that NADPH oxidases can be a pharmacological target for treatment of atherosclerosis. Indeed, recent developments in mitochondrial-targeted antioxidants that concentrate on the matrix-facing surface of the inner mitochondrial membrane in order to protect against mitochondrial oxidative damage may have therapeutic potential as a treatment for atherosclerosis.[135]

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Cancer

Oxidative stress plays an important role in malignant transformation and cancer progression.[15] The incidence of melanoma is increasing worldwide, and the prognosis for patients with high-risk or advanced metastatic melanoma remains poor despite the advances in the field.[136] As prostate cancer and aberrant changes in ROS become more common with aging, ROS signaling may play an important role in the development and progression of this malignancy. Oxidative stress is associated with several pathological conditions including inflammation and infection. Chronic increases in ROS over time are known to induce somatic mutations and neoplastic transformation.[137]

Melanoma proliferation was reduced by inhibition of NADPH oxidases.[138] Accumulating evidence suggests that ROS function as signaling molecules to mediate various growth-related responses including angiogenesis. Vascular endothelial growth factor (VEGF), one of the major angiogenesis factors, is induced in growing tumors and stimulates EC proliferation and migration. NADPH oxidases are activated by various growth factors including VEGF and angiopoietin-1 as well as hypoxia and ischemia, and ROS derived from this oxidase are involved in VEGFR2 autophosphorylation, and diverse redox signaling pathways leading to induction of transcription factors and genes involved in angiogenesis. Dietary antioxidants appear to be effective for treatment of tumor angiogenesis. Recent progress on the role of ROS derived from NADPH oxidases and redox signaling events involved in angiogenesis implicates NADPH oxidases as potential therapeutic targets for tumor angiogenesis.[139]

Recent studies suggest that mitochondria control Nox1 redox signaling and the loss of control of this signaling contributes to breast and ovarian tumorigenesis.[140] Inactivation of mitochondrial genes in rho(0) cells led to down-regulation of Nox1 while the transfer of wild type mitochondrial genes restored Nox1 expression to a level comparable to that in the parental cell line. Superoxide levels were reversed to parental levels in hybrid cells when Nox1 expression was restored by transfer of wild type mitochondria. Increasing mitochondrial superoxide levels also increased the expression of Nox1 in parental cells. Nox1 was highly expressed in breast and ovarian tumors and its expression positively correlated with expression of cytochrome C oxidase encoded by mtDNA. This study demonstrates the existence of cross talk between the mitochondria and NADPH oxidase in ovarian cancer.[140]

Among the available antioxidants, vitamin E was of the greatest interest to researchers. But collective data from all the different clinical trials, including the α-tocopherol, β-carotene prevention trial (ATBC), the heart outcome prevention evaluation-the ongoing outcomes trial (HOPE-TOO), the prostate, lung, colorectal and ovarian trial (PLCO), and the selenium and vitamin E cancer prevention trial (SELECT), were a complete disappointment due to the conclusion that the overall risks for cancer were unaffected by supplemental dietary antioxidants. Thus, treatment strategies aimed to reduce ROS production, rather than ROS neutralization, might offer an effective means against prostate cancer in particular and other malignancies in general.[137]

Cancer cells are known to have reduced levels of SOD2 expression and higher mitochondrial potential than non-malignant cells.[141] Increased expression of mitochondrial SOD2 appears to have adaptive and radioprotective effects.[142] Similarly, mitochondria-targeted antioxidants show promising therapeutic strategies to reduce detrimental effects of radiation exposure.[143] However, both SOD2 overexpression and mitochondria-targeted antioxidants may diminish efficacy of chemotherapy because they will counteract the increase of pro-apoptotic oxidative stress in cancer cells.

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We have recently shown cross talk between mitochondrial ROS and NADPH oxidases,[21] where SOD2 depletion increased NADPH oxidase activity and SOD2 overexpression attenuated activation of NADPH oxidases. Furthermore, treatment with mitochondria-targeted SOD mimetic mitoTEMPO mimics the effect of SOD2 overexpression.[21] Because cancer may be associated with both reduced SOD2 [141] and increased NADPH oxidase activity, we suggest that crosstalk between NADPH oxidases and mitochondrial ROS may play an important role in malignant transformation and cancer progression. Further studies are required to elucidate the specific role of these interactions in cancer pathophysiology.

Diabetes

Oxidative stress mediated by hyperglycemia-induced generation of ROS contributes significantly to the development and progression of diabetes and related vascular complications.[144] Michael Brownlee suggested that the mitochondrial electron transport chain plays a key role in a hyperglycemia-induced overproduction of superoxide and the development of secondary complications such as endothelial dysfunction.[145] In ρ °endothelial cells, removal of the mitochondrial electron transport chain completely inhibited hyperglycemia-induced ROS production. Interestingly, NADPH oxidases have been also implicated as a major source of ROS generation in the vasculature in response to high glucose and advanced glycation end-products.[146] On the other hand, many studies emphasize the role of mitochondrial dysfunction and mitochondrial ROS in diabetes.[147] One theory suggests that overproduction of ROS by NADPH oxidases leads to mitochondrial dysfunction.[144] Other theories emphasize that diabetes-induced defects in the electron transport chain promote ROS overproduction.[147] Interestingly, diabetes may be associated with increased opening of mitoK_{ATP} channels [148] which may be important in reduced insulin secretion and ischemic preconditioning.[149] These data potentially suggest the presence of feed-forward interaction between NADPH oxidases and mitochondria in the settings of hyperglycemia and diabetes which may be potentially mediated by activation of mitoK_{ATP} channels similar to results recently described in endothelial cells.[21] The pathophysiological role of this cross-talk in diabetes has not been fully investigated.

Neurodegeneration

Mitochondrial oxidative stress has been implicated in cognitive longevity.[150] Human cognition depends on the ability of the central nervous system to sustain high rates of energy production continuously throughout life while maintaining a healthy internal electrochemical environment. However, the central nervous system is especially susceptible to oxidative stress. The brain contains large amounts of iron, ascorbate, glutamate (a free radical-generating excitatory neurotransmitter), and highly peroxidizable unsaturated fatty acids. The brain consumes large volumes of oxygen (about 20% of whole-body O_2 consumption) and has a relatively poor antioxidant defense system. Because the rate of ROS production in human tissues is proportional to the rate of local oxygen consumption,[151] and the rate of oxygen consumption in the brain elevates with increasing demand for cognitive functions requiring planning, inductive thinking, and flexible thought,[152] the brain is a continuously operating, high-intensity oxidation/antioxidation battleground prone to oxidative imbalance during times of high demand for complex cognitive activity.[150]

Parkinson’s disease, Alzheimer’s disease and Huntington’s disease are neurodegenerative diseases with distinct clinical and morphological manifestations. However, a common feature of different neurodegenerative diseases is an impairment of mitochondrial energy metabolism in brain cells due to the critical role of mitochondria in glutamate excitotoxicity and other forms of cell death via apoptotic or necrotic pathways. The proposed mechanisms by which mitochondria induce cell death are the Ca^{2+}-dependent disruption of mitochondrial

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electrical membrane potential (ΔΨ) and opening of the pore with nonspecific permeability, known as the mitochondrial permeability transition pore (mPTP).[153] Biochemical studies have suggested the impairment of mitochondrial complex I in Parkinson's disease. Recent experimental work has modeled this abnormality using complex I inhibitor rotenone. Chronic rotenone exposure increased mitochondrial ROS, impaired mitochondrial respiration, accurately recapitulated the pathological, biochemical, and behavioral features of Parkinson's disease.[154]

Recently, NADPH oxidases have been associated with neurodegenerative disorders and related complications.[155] For example, NADPH oxidases are activated in brains from Alzheimer's disease patients and are upregulated in Parkinson's disease.[156] The NADPH oxidases may participate in ROS production in neurons and microglial cells,[157] potentially diluting their toxicity.[155] In these cell types, increased intracellular oxidative stress and AngII stimulate the expression and the activity of NADPH oxidases.[150]

It is conceivable that production of mitochondrial ROS can stimulate the expression of NADPH oxidases in the brain resulting in pro-inflammatory and pro-apoptotic vicious cycles. Interestingly, epidemiological studies in humans show both positive and negative effects of the use of antioxidant supplements on healthy cognitive aging and on the risk of developing Alzheimer disease.[158] Furthermore, it has been reported that mitochondria-targeted antioxidant SkQ1 reduced learning in the MWM task in Wistar rats but resulted in higher locomotor and exploratory activity and less anxiety.[159] Antioxidant enriched diet leads to rapid learning improvements, memory improvements after prolonged treatment and cognitive maintenance. In the brains of aged treated dogs, oxidative damage is reduced and there is some evidence of reduced Alzheimer disease-like neuropathology.[158] These data suggest that antioxidant treatments targeting ROS production by mitochondria or NADPH oxidases in the brain may be beneficial; however, further studies are required to minimize the risk of impairment by physiological redox dependent processes in the brain.

Aging

In 1972 Denham Harman suggested that free radical damage of mitochondria can be a key determinant of the aging process.[160] It has been shown that mitochondrial dysfunction and damage of mtDNA as a result of endogenous and mitochondrial ROS play an important role in the degenerative processes.[161] Although the limitations of this hypothesis has been recently criticized by David Gems and Linda Partridge,[162] there are many compelling studies demonstrating that mitochondrial $O_2^{\bullet -}$ production results in damage to macromolecules in spite of such defensive enzymes as superoxide dismutases and glutathione peroxidase, leading to the progressive dysfunction that we see as senescence.[16] Increased mitochondrial uncoupling and cell ATP depletion are evident in human muscle nearly a decade before accumulation of irreversible DNA damage that causes defects in mitochondrial electron transport chain. New evidence points to the reduction in activators of biogenesis (e.g. PGC-1alpha) and to degradation of mitochondria, allowing accumulation of molecular and membrane damage in aged mitochondria. The early dysfunction appears to be reversible based on improved mitochondrial function in vivo and elevated gene expression levels after exercise training. New molecular and in vivo findings regarding the onset and reversibility of mitochondrial dysfunction with age indicate the potential: 1) for diagnostic tools to identify patients at risk for severe irreversible defects later in life; and 2) for development of an intervention to delay the tempo of aging and improve the quality of life of the elderly.[163]

Recently, the role of NADPH oxidases in the aging process has been emphasized.[164] It is well known that the AngII mediated upregulation of NADPH oxidases contributes to age-

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related cardiovascular phathological conditions such as hypertension, heart failure and diabetes. Interestingly, blocking AngII signalling protects against degenerative processes and promotes longevity in rodents. Altogether these findings open the perspective for exploring AngII signaling in therapeutic interventions in inflammatory diseases and aging-related tissue injury.[165] Although it is widely assumed that mitochondria are the predominant source of ROS relevant for the aging process, the role of ROS generated by NADPH oxidases has been largely overlooked in aging theories. From an experimental point of view, there is now abundant evidence for the involvement of NOX enzymes in age-associated diseases.[164]

We suggest that the role of these distinct sources of ROS can be reconciled on the basis of crosstalk of NADPH oxidases and mitochondrial ROS. The role of Noxes in the aging process itself and their relative contribution as compared to mitochondria need further investigations.

Cardiac dysfunction

Emerging evidence suggests the involvement of NADPH oxidases in cardiac physiological and pathophysiological processes.[166] Definitive evidence for the involvement of NADPH oxidases in pathological hypertrophy came from experiments in Nox2−/− mice.[167] ROS affect cellular Ca^{2+} regulation at several levels, notably via redox modifications of key amino acid residues involved in the function and gating properties of intracellular and plasma membrane ion channels and transporters — e.g., L-type channels, the Na^{+}/Ca^{2+} exchanger, the sarcoplasmic reticulum ATPase and the ryanodine receptor.[168] The regulation of Ca^{2+} in cardiac myocytes is centrally important not only in excitation-contraction coupling but also in many other processes such as the regulation of gene expression and cellular energetics.

Recently, it has been shown that mitochondrial ROS regulate the cardiac sodium channel.[169] Mitochondria-targeted antioxidant mitoTEMPO and malonate reduced mitochondrial ROS and normalized the activity of cardiac sodium channels. Furthermore, mice with mitochondria-targeted expression of catalase are resistant to cardiac hypertrophy.[170] These data indicate the critical role of mitochondrial ROS in cardiac hypertrophy and failure.

Interestingly, the pathophysiological role of NADPH oxidases and mitochondrial ROS is substantially overlapped. This indicates that not only different sources of ROS contributes to cardiac dysfunctions but also suggest crosstalk between NADPH oxidases and mitochondrial ROS. Indeed, inhibition of type I angiotensin-receptor blockers reduces age-related mitochondrial dysfunction, attenuates hypertension induced renal mitochondrial dysfunction, and protects against cardiac mitochondrial dysfunction in the setting of acute ischemia.[111-113] On the other hand, the role of mitochondrial ROS in NADPH oxidase-mediated processes such as cardiomyocyte differentiation and endothelin signaling has been reported.[171, 172] These data support the crosstalk of NADPH oxidases and mitochondrial ROS in cardiac pathophysiological processes; however, further studies are necessary.

Future directions

The role of mitochondrial oxidative stress in pathological conditions is very well documented; however, the role of mitochondrial ROS in physiological processes and adaptive responses is less clear. It is conceivable that mitochondrial ROS affect cell proliferation, cell transformation, survival and differentiation via interaction with NADPH oxidases.[1] The specific molecular mechanisms of crosstalk between NADPH oxidases and mitochondria have to be further investigated.[173] Mitochondria may represent an important node in the regulation of NADPH oxidase expression [120] and activity.[21] It is therefore

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interesting to speculate that mitochondria may provide both feed-forward and feed-back regulations of NADPH oxidases. The mechanisms preventing the development of oxidative stress, however, may decline with age due to mitochondrial impairment associated with reduced mitochondrial membrane potential, diminished redox status and decreased ATP level. This will drive a feed-forward vicious cycle of ROS production by mitochondria and NADPH oxidases. Interestingly, current findings indicate that scavenging of mitochondrial $O_2^{\bullet-}$ using mitochondria-targeted antioxidants can interrupt this vicious cycle using very low therapeutical doses.[21] There are many common conditions including aging, atherosclerosis, diabetes and degenerative neurological disorders in which mitochondrial oxidative stress seems to play a role.[16, 174] It is conceivable that mitochondria-targeted interventions would be effective in these conditions.

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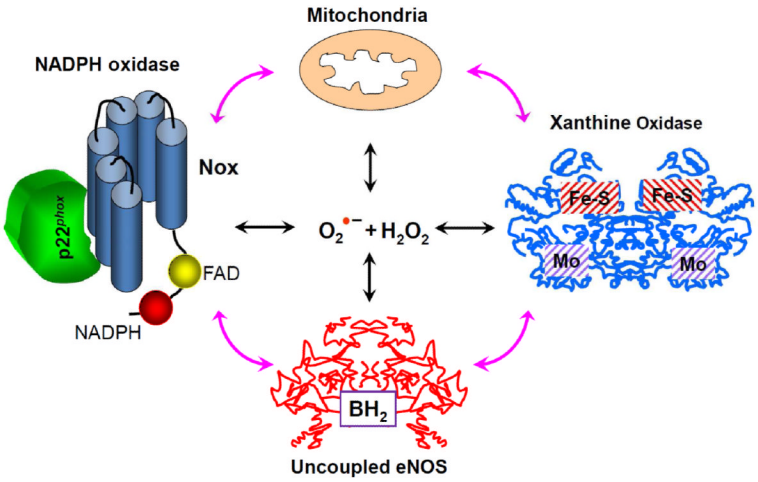

Figure 1.
Interaction of various sources of reactive oxygen species ROS. The production of ROS from any one source can lead to activation of the NADPH oxidases, conversion of xanthine dehydrogenase to xanthine oxidase, can stimulate the production of mitochondrial ROS or result in uncoupling of the endothelial nitric oxide synthase (eNOS).

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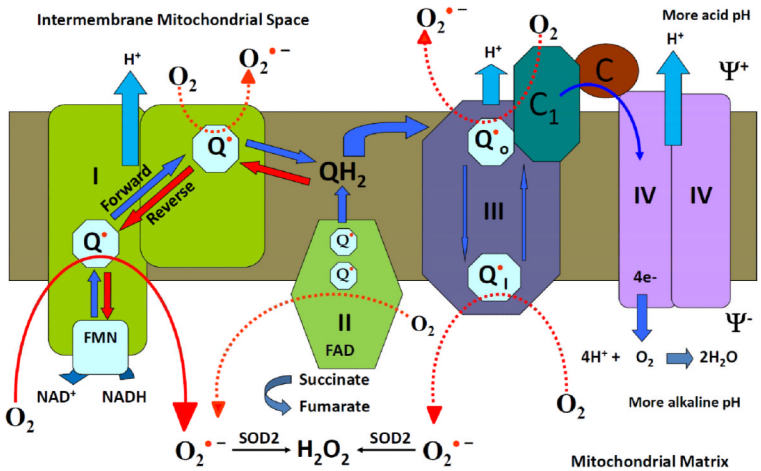

Figure 2.

Schematic presentation of the mitochondrial electron transport chain and production of mitochondrial $O_2^{\bullet -}$.

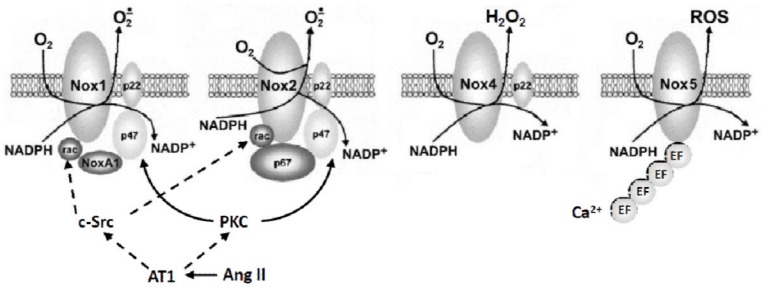

Figure 3.
Structural homology of the vascular NADPH oxidases.

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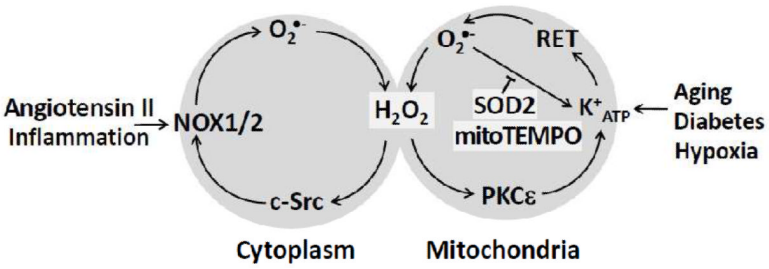

Figure 4.
Proposed crosstalk between mitochondria and NADPH oxidases.