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Review

Redox signaling (cross-talk) from and to mitochondria involves mitochondrial pores and reactive oxygen species

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ABSTRACT

This review highlights the important role of redox signaling between mitochondria and NADPH oxidases. Besides the definition and general importance of redox signaling, the cross-talk between mitochondrial and Nox-derived reactive oxygen species (ROS) is discussed on the basis of 4 different examples. In the first model, angiotensin-II is discussed as a trigger for NADPH oxidase activation with subsequent ROS-dependent opening of mitochondrial ATP-sensitive potassium channels leading to depolarization of mitochondrial membrane potential followed by mitochondrial ROS formation and respiratory dysfunction. This concept was supported by observations that ethidium bromide-induced mitochondrial damage suppressed angiotensin-II-dependent increase in Nox1 and oxidative stress. In another 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 oxidase via PKCε was demonstrated. The third 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. By superior molecular biological methods the authors 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 an NADPH oxidase (Nox1) in response to the fast mitochondrial ROS formation. Finally, a cross-talk between mitochondria and NADPH oxidases (Nox2) was shown in nitroglycerin-induced tolerance involving the mitochondrial permeability transition pore and ATP-sensitive potassium channels. The use of these redox signaling pathways as pharmacological targets is briefly discussed.

1. Background of redox signaling

Redox signaling pathways play an important role in many fields of biochemistry as well as medical research and provide the basis for understanding (patho)physiological mechanisms [1–13]. They allow establishing future strategies for interventions at the cellular level and therapeutic principles in animals and humans. Many aspects of the generation and regulation of redox signals by ROS derived from NADPH oxidases and mitochondria as well as NO metabolites from NO synthases are known [14]. However, many open questions remain concerning the translation of (in most part harmful) free radical reactions of superoxide, nitric oxide and their reaction products to redox signals with cellular messaging character. The developments of the last years show that mitochondria are associated in many aspects with redox reactions [15–17]. Based on estimations up to 1% of the electrons, which are involved in mitochondrial respiration, are transferred to molecular oxygen [18]. Superoxide and its product hydrogen peroxide, which are formed at the mitochondrial respiratory complexes, were demonstrated to have important signaling functions contributing to various disease states [19–21]. There is clear experimental evidence that the pores and channels in the mitochondrial membrane as well as the membrane potential are important regulators of efflux and influx of ROS/RNS from and into mitochondria [22–29]. The focus of the present review lies on the mechanisms by which mitochondrial ROS activate NADPH oxidases [30–34] and vice versa cytosolic ROS/RNS trigger mitochondrial oxidative stress and dysfunction [29,35]. Given the importance of oxidative stress in almost all cardiovascular, inflammatory and neurodegenerative diseases the process of ROS-induced ROS formation gains high importance for signaling processes in particular and pharmaceutical/clinical implications in general.

1.1. Oxidative stress and redox regulation

The discovery of superoxide dismutases (mitochondrial Mn-SOD and cytosolic/extracellular Cu,Zn-SOD) by Fridovich et al. in the 1960s [36] suggested that superoxide is formed in the organism and in living cells. Moreover, the existence of SODs implied that superoxide which is a harmful species involved in pathological processes forces the organism to express SODs for protection. Based on these observations until approximately 25 years ago, the accepted view of free radicals in

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biology was that these species destroy biological structures and only contribute to pathophysiological and harmful processes. Only for a few exceptions, a physiological role of free radicals in biological processes could be established (e.g. for monooxygenation reactions by P450s) [37]. It was in the 1970s and 80s, after decades of intensive search for the so-called "endothelium-derived relaxing factor" (EDRF) which leads to the vasodilatation of the smooth muscle, when the Noble Prize recipients Murad, Ignarro und Furchgott identified EDRF as nitric oxide ($^{\bullet}NO$) [38–40]. This discovery changed the negative view on free radicals in biology and established the understanding that these species can also confer highly important physiological processes and are involved in cellular signaling. The physiological role of $^{\bullet}NO$ in the vascular system and in the central and peripheral nervous system was extensively reviewed [41–44].

The superoxide anion ($^{\bullet}O_2^-$) can be formed from different sources such as xanthine oxidase, NAD(P)H oxidases, uncoupled NO synthases and the mitochondrial respiratory chain. In many aspects it can be regarded as a direct antagonist of $^{\bullet}NO$. A direct interaction between $^{\bullet}NO$ and superoxide could be proven by the existence of peroxynitrite ($ONOO^-$) that is formed by the diffusion-controlled reaction of $^{\bullet}NO$ with superoxide [45–48]. Peroxynitrite is a much more potent oxidant than $^{\bullet}NO$ and superoxide [49,50] and its contribution to cardiovascular and neurodegenerative diseases is meanwhile accepted [51–58]. Moreover, the reactivity of $^{\bullet}NO$ and superoxide is dramatically changed upon formation of peroxynitrite [59,60]. Therefore, the formation of peroxynitrite may be understood as the conversion of the physiological, protective properties of $^{\bullet}NO$ to those of peroxynitrite which do rather contribute to pathophysiological and harmful processes [61]. If the formation of oxidizing species such as superoxide and peroxynitrite exceeds a certain limit, the cellular redox balance is impaired and accordingly redox systems (e.g. the thiol–disulfide equilibrium) are shifted to the oxidized condition and the cellular antioxidant defense system (low molecular weight compounds such as ascorbate or tocopherol but also high molecular weight enzymatic systems such as superoxide dismutases or catalases) is overstrained [62–64]. This condition is termed oxidative stress and, in the long-run (e.g. under chronic inflammatory conditions) causes cell death (apoptosis or necrosis) and death of the whole organism.

At low concentrations oxidants/free radicals may also confer physiological functions in cellular signaling processes. A prominent example is $^{\bullet}NO$ itself, a potent regulator of vascular tone [65]. But also superoxide and peroxynitrite are involved in redox signaling by inhibiting or activating plenty of enzymatic systems by oxidative protein modifications such as thiol oxidation [49,66,67], S-nitros(yl)ation [68] and tyrosine nitration [69,70]. These oxidative protein modifications (reviewed in [71]) induce secondary signal transduction processes in the cellular network. Examples for redox-regulated proteins are the phosphatase calcineurin (oxidation/reduction at the binuclear metal center) [7], modulation of protein expression at the transcriptional level (oxidation/reduction zinc-finger motifs in transcription factors) [72,73], sulfoxidation of tyrosines phosphatases [74], modulation of the activity of cyclooxygenase (COX-1 and COX-2) and thereby the formation of prostaglandins and thromboxanes [75], modulation of mitochondrial electron transport and the respiratory chain (oxidation/reduction of iron–sulfur-cluster) [18,76] as well as the activation of protein kinase C by hydrogen peroxide [77]. Prostacyclin synthase is tyrosine-nitrated and inactivated by peroxynitrite in the low micromolar range [69,78,79] but until now, the reversibility of this inactivation by so-called denitratases lacks a biological proof [80–89]. Also the superoxide- and peroxynitrite-triggered proteasomal degradation of GTP-cyclohydrolase-1 may be regarded as a redox event and considerably influences vascular function [90,91]. More vascular and non-vascular redox events of superoxide are reviewed in [20,92–94]. Maybe these identified and the yet unidentified physiological functions of reactive oxygen and nitrogen species (ROS and RNS) are much more important than the contribution of these reactive species to pathophysiological processes as observed under oxidative stress conditions [95,96].

ROS and RNS are important mediators of inflammation that are essential for immune response and host defense. Leukocytes (such as monocytes and neutrophils) are loaded with enzymatic systems that in response to an inflammatory stimulus generate a burst of reactive oxygen and nitrogen species (from inducible NO synthase and NADPH oxidases) exceeding the normal physiological range by far [97]. Endotoxins (e.g. from bacteria) can not only induce NOS-2 in white blood cells (e.g. macrophages) but also in smooth muscle cells leading to $^{\bullet}NO$ levels in the high nanomolar range (and up to 100 μM intracellular nitrite) [98–100]. In the setting of septic shock this uncontrolled $^{\bullet}NO$ formation together with other mediators can cause uncontrolled, life-threatening hypotension (drop in blood pressure). Endotoxins also stimulate NADPH oxidases in these cells (by translocation of cytosolic regulatory subunits to the membrane-bound $gp91^{phox}$ catalytic subunit) resulting in an "oxidative burst" and huge amounts of ROS and RNS. The high concentrations of superoxide and $^{\bullet}NO$ produced in these cells lead ultimately to the formation of peroxynitrite [101,102].

1.2. Redox signaling: bioinorganic chemistry at its best

The title of this chapter was taken from a review article of Ullrich and Kissner since it reflects the elegance of the redox signaling processes and the way how nature uses harmful reactive species for the benefit of life [14]. Interactions of the radicals $^{\bullet}NO$ and superoxide form a redox system which includes nitros(yl)ations and nitrations and is part of the complex cellular signaling network. In addition, transition metal chemistry is also deeply involved because $^{\bullet}NO$, superoxide and peroxynitrite form metal complexes, which affect their reactivity. Hence, redox regulation has become an interest to bioinorganic chemists. In summary, superoxide causes vascular dysfunction not only by its direct effect on $^{\bullet}NO$ bioavailability or formation of peroxynitrite and subsequent oxidative depletion of tetrahydrobiopterin (BH_4) [103] but also by changing the prostanoid profile to a vasoconstrictive, pro-aggregatory, pro-atherosclerotic phenotype. Due to a different focus of the present review, these concepts were only briefly discussed here. The reader will find more information on these ideas in the review of Ullrich and Kissner [14] as well as in other reviews highlighting more the physiological [3] or chemical [104] aspects of this topic.

1.3. Mitochondrial pathways in redox signaling

Mitochondria are a perfect amplifier of ROS and RNS formation by several mechanisms: First, any oxidative damage at the constituents of the mitochondrial respiratory chain (e.g. by reaction with iron–sulfur-centers or critical thiols in complexes I and II but also associated proteins such as the aconitase) will lead to "uncoupling"[1] of the mitochondrial respiration with subsequent transfer of electrons to molecular oxygen increasing the mitochondrial superoxide/hydrogen peroxide formation in a positive feedback fashion [105]. Second, increased mitochondrial peroxynitrite formation will ultimately lead to nitration and inactivation of manganese superoxide dismutase (MnSOD) [70,106], which results in the impaired breakdown of mitochondrial superoxide further feeding the vicious cycle. Third, mitochondrial ROS formation itself may be stimulated by cytosolic ROS/RNS (e.g. by opening of the redox sensitive K_{ATP} channels) leading to changes in the mitochondrial membrane potential [29], an important determinant of mitochondrial ROS generation, as discussed below.

[1] It should be noted that the term "uncoupling of mitochondrial respiration" is usually used to describe distraction of mitochondrial electron flow from ATP synthesis. "uncoupling" is used here in the meaning of "autoxidation".

Another very interesting and important concept is "mitochondrial criticality" which in some situations determines between life and death [107]. These are oscillations in mitochondrial energetics, inducing a state in which the mitochondrial network of cardiomyocytes becomes very sensitive to small perturbations in ROS, resulting in the scaling of local mitochondrial uncoupling and $\Delta\Psi_m$ loss to the whole cell [108]. There is good evidence that these phenomena contribute to the genesis of potentially lethal cardiac arrhythmias in the heart during ischemia and reperfusion [109].

Since the present review will concentrate on mitochondrial redox signaling, the composition and redox sensitivity of two mitochondrial channels/pores will be discussed in detail (reviewed in [105]). Mitochondrial protein complexes that participate in critical mitochondrial/cellular processes, contain inner membrane proteins with critical vicinal thiols that can be readily oxidized by peroxynitrite [110,111]. The mitochondrial permeability transition pore (mPTP) itself is subject to redox regulation (Fig. 1) [105]. Its major constituents are the voltage-dependent anion channel (VDAC), the adenine nucleotide translocase (ANT) and the matrix-located regulatory subunit cyclophilin D (CypD). ANT is known to be affected by oxidants including peroxynitrite [111,112]. ANT can form a complex with the outer membrane protein VDAC, and oxidation of ANT vicinal thiols leads to the translocation of CypD, forming the basic unit of the pore. Oxidant-stimulated formation of VDAC–ANT–CypD complex triggers mPTP opening and promotes the release of pro-apoptotic proteins to the cytosol [110]. Peroxynitrite leads to mPTP opening [111,113,114], a process that is partially based on oxidation of critical thiols in ANT [111]. Peroxynitrite may also nitrate essential tyrosines in VDAC [115], which contributes to the formation of the active mPTP complex and/or opening of the pore. These processes also underlie the phenomenon of ischemic preconditioning, a scenario in which brief intermittent periods of ischemia provide protection against subsequent ischemic injury [116]. It is meanwhile well understood that mPTP plays a critical role in mitochondrial redox signaling, which is involved in ischemic preconditioning [117–119]. A number of studies show, that ROS formation is essential for ischemic preconditioning [26,27,120] and that protective effects of this phenomenon are lost upon addition of antioxidants [121,122]. Also the involvement of the mPTP is well defined since genetic deletion of CypD resulted in the loss of protective effects elicited by ischemic preconditioning [117,119], indicating that mPTP is essential for mitochondrial ROS (especially hydrogen peroxide) release [118,123].

In an informative editorial, R.P. Brandes has discussed the role of NADPH oxidase-derived cytosolic ROS in triggering mitochondrial ROS formation [29]. This concept is based on the generation of cytosolic ROS by NADPH oxidases and subsequent reaction of superoxide with the mitochondrial ATP-sensitive potassium channels (mtK_{ATP}) in the mitochondrial membrane (Fig. 2) [124]. Upon mtK_{ATP} opening the electrophoretic influx of potassium cations into the matrix causes depolarization of the mitochondrial membrane ($\Delta\psi_m\downarrow$) along with matrix swelling and alkalinization [125]. Matrix alkalinization in turn has been suggested to be responsible for the increase in H_2O_2 formation observed in cardiomyocytes treated with the mtK_{ATP} opener diazoxide [126–128]. In their original research paper, Kimura et al. have shown that angiotensin-II triggered activation of NADPH oxidases in the myocardium confers ischemic preconditioning, which was blocked by the NADPH oxidase inhibitor apocynin [129]. The protective effect of angiotensin-II on lipid peroxidation and p38 MAP kinase activation in the myocardium was also lost when mtK_{ATP} channels in cardiac myocytes were blocked by 5-hydroxydecanoate (5-HD). In addition, angiotensin-II dependent ROS formation from cardiac myocytes was suppressed by 5-HD although this compound had no effect on oxidative burst in isolated leukocytes. The role of ROS in this process was previously demonstrated by inhibition of pharmacological preconditioning by exogenously added ROS via 5-HD-induced blockade of the mtK_{ATP} channels [128]. These observations indicate that mitochondria are an important constituent of the positive feedback loops leading to an amplification of ROS production and ROS-dependent signaling. In the next chapters I will present some ideas from recent studies on how mitochondria are involved in redox signaling and ROS-triggered ROS.

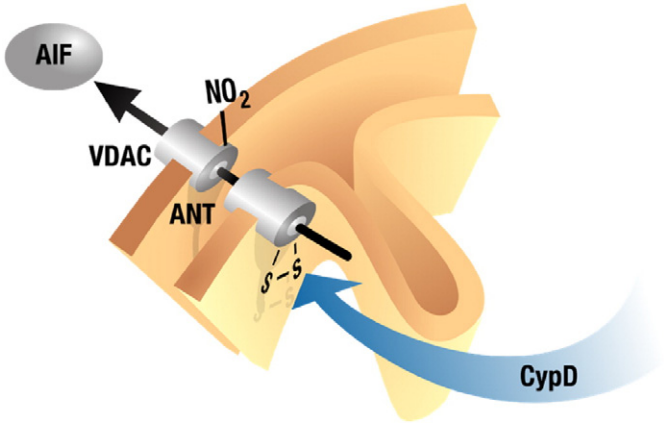

Fig. 1. Constituents and oxidative opening of the mPTP. Tyrosine nitration of the voltage-dependent anion channel (VDAC), disulfide formation in the adenine nucleotide translocase (ANT) and translocation of the matrix-located regulatory subunit cyclophilin D (CypD) increase the opening probability of the mPTP and the release of apoptosis-inducing factors (AIF). Adopted from Radi et al. [105].

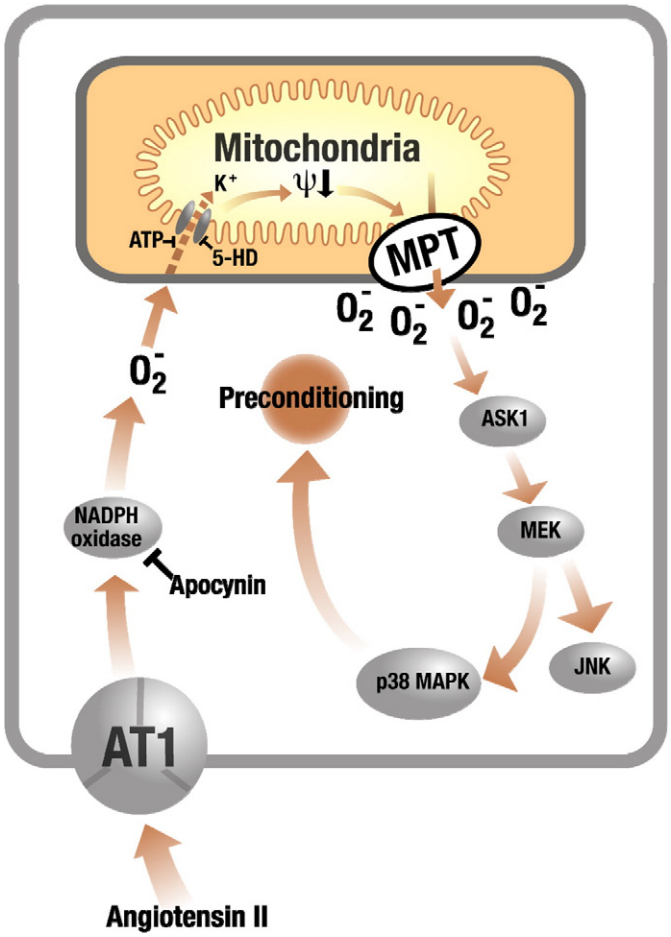

Fig. 2. Angiotensin-II triggered activation of NADPH oxidases with subsequent opening of mitochondrial K_{ATP} channels by Nox-derived ROS and mitochondrial ROS release via mPTP represent an amplification mechanism for angiotensin-II induced oxidative stress and contribute to angiotensin-II mediated preconditioning via p38 MAPK and JNK pathway. Adopted from Brandes [29].

2. Redox signaling (cross-talk) in angiotensin-II induced hypertension

2.1. Molecular mechanisms of angiotensin II-mediated mitochondrial dysfunction: linking mitochondrial oxidative damage and vascular endothelial dysfunction

In their recent publication Doughan et al. from the group of S. Dikalov proposed a new mechanism on how NADPH oxidase derived superoxide and hydrogen peroxide trigger mitochondrial dysfunction and ROS formation via the K_{ATP} channel [35]. For this purpose the authors used angiotensin-II treated endothelial cells (an experimental ex vivo model of hypertension). The authors started out with measurements of hydrogen peroxide release from isolated mitochondria from bovine aortic endothelial cells (BAECs) by EPR and amplex red/peroxidase fluorescence. All treatments were performed with intact cells, then mitochondria were isolated and subjected to analysis. Angiotensin-II increased H_2O_2 release which was blocked by the NADPH oxidase inhibitor apocynin, the PKC inhibitor chelerythrine, the radical scavenger uric acid, the K_{ATP} channel blockers 5-HD or glibenclamide as well as the NOS inhibitor L-NAME. Stimulation of mitochondrial electron flow always led to similar observations regardless whether substrates of complex I (malate/glutamate) or complex II (succinate) were used. Angiotensin-II or phorbol ester had no effect on ROS production in isolated mitochondria itself, indicating that cytosolic/membranous components are involved. The purity of mitochondrial preparations was probed by Western blotting using antibodies against GAPDH (cytosolic), cytochrome c oxidase (mitochondrial) and $p22^{phox}$ (membranous). The increase in H_2O_2 production in mitochondria from angiotensin-II treated BAECs was suppressed by the knock-down of the NADPH oxidase subunit $p22^{phox}$ by siRNA. Blockade of the mPTP by cyclosporine A decreased the H_2O_2 release from the mitochondria isolated from angiotensin-II treated BAECs. The importance of the mitochondrial membrane potential was demonstrated by the effect of the K^+/H^+ antiporter nigericin. Mitochondrial membrane potential collapsed in the mitochondria from angiotensin-II treated cells (detected by JC-1 fluorescence) and was normalized by apocynin, uric acid, ebselen (a GPx mimetic), 5-HD and glibenclamide and collapsed completely upon addition of the K_{ATP} channel opener diazoxide. Mitochondrial respiration (states 3 and 4 as well as RCR) was impaired and mitochondrial GSH levels were decreased by angiotensin-II treatment and normalized by apocynin, 5-HD and glibenclamide. Nitric oxide formation (measured by EPR-based detection of $Fe(DETC)_2$) in BAECs was decreased by angiotensin-II treatment and rescued by uric acid and 5-HD. With this study, Dikalov et al. provide a new hypothesis on the sequence of pathogenesis in the setting of hypertension and a detailed picture of the cross-talk between cytosolic and mitochondrial ROS sources (Fig. 3): Angiotensin-II activates NADPH oxidases in the cytoplasmic membrane and subsequent ROS formation will activate mitochondrial K_{ATP} channels leading to depolarization of mitochondrial membrane potential. Decreased $\Delta\psi_m$ confers increased mitochondrial ROS formation and mPTP opening. Mitochondrial ROS (most probably hydrogen peroxide) activate PKC triggering the vicious cycle of NADPH oxidase activation. By this mechanism mitochondria act as an amplifier for an oxidative stress positive feedback loop.

2.2. Cross-talk between mitochondria and NADPH oxidase: effects of mild mitochondrial dysfunction on angiotensin-II mediated increase in Nox isoform expression and activity in vascular smooth muscle cells

Recently, Wosniak et al. from the laboratory of F.R.M. Laurindo revealed a new mechanism on how mild mitochondrial dysfunction regulates angiotensin-II dependent activation of NADPH oxidases in vascular smooth muscle cells (VSMCs) [32]. For this purpose VSMCs were treated with low concentrations of ethidium bromide (EtBr,

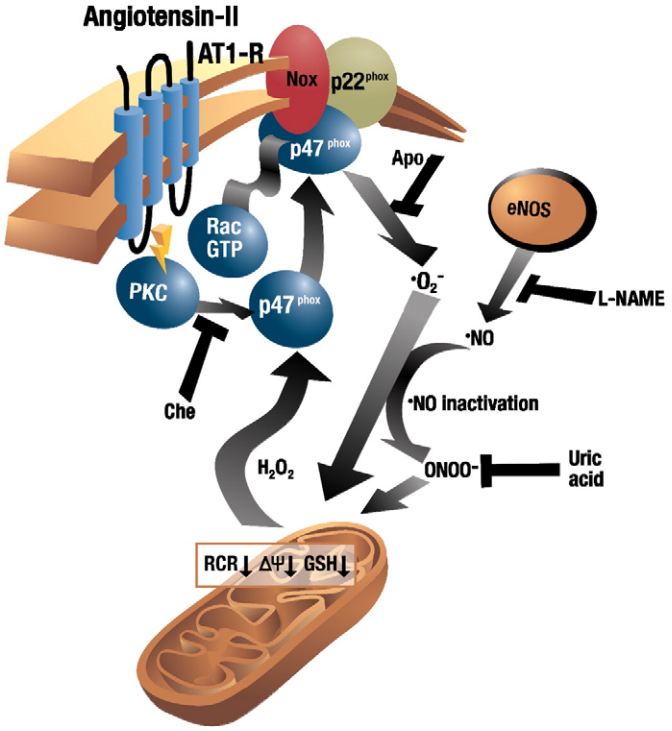

Fig. 3. Proposed model of angiotensin-II induced mitochondrial dysfunction via kindling radicals from NADPH oxidase. These Nox/NOS-derived ROS and RNS open the mtK_{ATP} channels decreasing the mitochondrial membrane potential which results in "uncoupling" of mitochondrial respiration. Mitochondria release H_2O_2, which in turn further activates NADPH oxidase via PKC, resulting in increased intracellular superoxide production, reduced $^{\bullet}NO$ bioavailability and finally endothelial/vascular dysfunction. Adopted from Doughan et al. [35].

250 ng/ml). Despite appreciable loss of mtDNA (pseudo ρ^0 cells), VSMC remained viable, had only 29% less oxygen consumption and 16% greater baseline hydrogen peroxide but unchanged glutathione levels. Most striking was the observation that angiotensin-II triggered increase in membrane NADPH oxidase activity (measured by lucigenin-derived chemiluminescence as well as dihydroethidine-derived 2-hydroxyethidium formation) was lost in EtBr treated cells along with failure to upregulate Nox1 mRNA. As a possible explanation, protein disulfide isomerase, an NADPH oxidase regulator, exhibited increased expression and inverted pattern of migration to membrane fraction after EtBr. These results unravel functionally relevant cross-talk between mitochondria and NADPH oxidase, which markedly affects redox responses to angiotensin-II at the transcriptional level.

3. Hypoxia activates NADPH oxidase to increase $[ROS]_i$ and $[Ca^{2+}]_i$ through the mitochondrial ROS-PKCε signaling axis in pulmonary artery smooth muscle cells

In their recent publication, Rathore et al. from the group of Y.-X. Wang have identified the molecular mechanisms by which mitochondrial ROS activate PKCε leading to activation of NADPH oxidases [31]. For this purpose the authors used mouse pulmonary arteries. In the first part of the study it was shown that hypoxia activates NADPH oxidase activity (most probably Nox1) in these vessels by increased translocation of $p47^{phox}$ to the plasma membrane. It should be noted that hypoxia/reoxygenation (equivalent to ischemia/reperfusion) involves many mitochondrial processes such as mitochondrial ROS formation [16,130,131]. Pharmacological (apocynin) and genetic ($p47^{phox-/-}$) inhibitions of NADPH oxidase significantly attenuated the hypoxic increase in ROS formation (DCF fluorescence) and membranous Nox activity in freshly isolated mouse pulmonary artery smooth muscle cells. Hypoxia-induced Nox activation and intracellular ROS formation were

also prevented by treatment with the unspecific PKC inhibitor chelerythrine, with a specific PKCε translocation peptide inhibitor but not with GÖ6976, an inhibitor shown to block conventional, but not novel, PKC isozymes. In addition to pharmacological inhibition, genetic deletion of PKCε (PKCε$^{-/-}$ mice) abolished the effect of hypoxia on Nox activity and intracellular ROS formation. Inhibition of mitochondrial ROS generation with rotenone or myxothiazol blocked the hypoxia-induced increase in Nox activity. Further support for an essential role of mitochondria-derived ROS in this signaling process came from experiments demonstrating that enhanced mitochondrial and cytosolic H_2O_2 decomposition by GPx-1 gene overexpression (GPx-1*tg*) prevented, whereas inhibition of mitochondrial and cytosolic H_2O_2 breakdown by GPx-1 gene deletion (GPx-1$^{-/-}$) augmented the hypoxia-induced increase in Nox activity in pulmonary arteries. Finally, it was demonstrated that exogenously added H_2O_2 also increased Nox activity in the pulmonary arteries. This study used elegant experiments based on different specific inhibitors of PKCε, Nox-subunit and GPx-1 knockout mice to demonstrate ROS (H_2O_2)-driven signaling in the setting of hypoxia (Fig. 4). It should be noted that under hypoxic conditions $^{\bullet}$NO can be easily generated from nitrite resulting in peroxynitrite formation in the presence of superoxide. Indeed, the study by Rathore et al. does not disprove a role of peroxynitrite but only demonstrates that superoxide is involved and a species that is scavenged by GPx, a well-known sink for peroxynitrite [132].

4. Link between mitochondria and NADPH oxidase 1 isozyme for the sustained production of reactive oxygen species and cell death

In a previously published study Lee et al. from the group of H.-D. Um have shown that serum withdrawal promotes the production of ROS in human 293T cells by stimulating both the mitochondria and Nox1 [133]. An analysis of their relationship revealed that the mitochondria respond to serum withdrawal within a few minutes, and the ROS (measured by flow cytometry using dichlorofluorescein fluorescence) produced by the mitochondria triggers Nox1 action by stimulating phosphoinositide 3-kinase (PI3K, by Western blotting) and Rac1. Activation of the PI3K/Rac1/Nox1 pathway was evident 4–8 h after but not earlier than serum withdrawal initiation, and this time lag was found to be required for an additional activator of the pathway, Lyn (proven by siRNA), to be expressed. Cycloheximide and actinomycin D, which inhibit protein synthesis and transcription, respectively, were used to examine the important role of gene transcription and enzyme expression in this process. Functional analysis suggested that, although the mitochondria contribute to the early (0–4 h) accumulation of ROS, the maintenance of the induced ROS levels to 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 KCN or the PI3K/Rac1/Nox1 pathway using the dominant negative mutants (PI3K-M, Rac1-M) or small interfering RNAs. This suggests that mitochondrial ROS is an essential trigger but not enough to promote cell death, which requires the sustained accumulation of ROS by the subsequent action of Nox1. Contribution of Nox2 and Nox4 was excluded by a lack of effect of siRNAs for these genes. Overall, this study shows a signaling link between the mitochondria and Nox1, which is crucial for the sustained accumulation of ROS and cell death in serum withdrawal- induced signaling (Fig. 5).

5. Cross-talk between reactive oxygen species from mitochondria and NADPH oxidases — implications for endothelial dysfunction associated with nitrate tolerance and aging

The last example of redox signaling between mitochondria and NADPH oxidases is based on a report of Wenzel et al. from the laboratory of A. Daiber (Fig. 6) [30]. Redox signaling was observed in an animal model of nitroglycerin-induced nitrate tolerance, an experimental model of vascular oxidative stress and dysfunction and of the interactions of nitric oxide, superoxide and peroxynitrite [134–137]. Organic nitrates need to undergo bioactivation to confer their vasodilatory effects via nitric oxide or an NO-related species (e.g. S-nitrosothiols and heme-NO) [138]. Due to pharmacologically undesired side-reactions (e.g. NO could block cytochrome c oxidase) there is simultaneous formation of superoxide [139]. Sources of superoxide include organic nitrate-induced "uncoupling" of the mitochondrial respiratory chain [140,141] as well as organic nitrate-triggered activation of the renin–angiotensin–aldosterone system (RAAS) and resulting PKC-dependent activation of vascular NADPH oxidases [142,143]. The simultaneous formation of $^{\bullet}$NO and superoxide leads to an increase in peroxynitrite levels inducing inhibition of the mitochondrial aldehyde dehydrogenase (ALDH-2) by thiol oxidation [8,144], desensitization of soluble guanylyl cyclase and subsequent impaired $^{\bullet}$NO down-stream signaling by thiol oxidation [145], decrease in $^{\bullet}$NO bioavailability by direct trapping of $^{\bullet}$NO by superoxide, uncoupling of NO synthase by peroxynitrite-triggered depletion of its co-factor tetrahydrobiopterin (BH_4) [146] as well as inhibition of prostacyclin synthase by peroxynitrite-dependent tyrosine nitration [147]. All of these events contribute to nitrate tolerance and cross-tolerance (endothelial dysfunction) [137] and

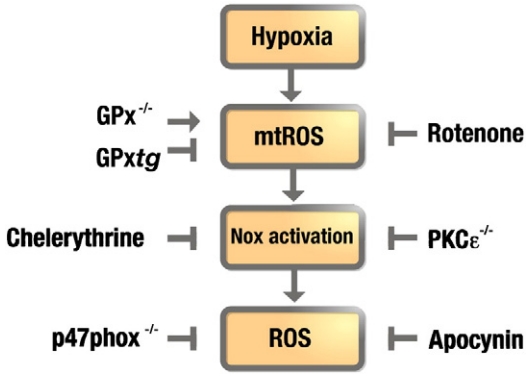

Fig. 4. Proposed model of hypoxia-induced NADPH oxidase activation by mitochondrial H_2O_2 release. The effect of hypoxia on NADPH oxidase activity was demonstrated by the Nox inhibitor apocynin and p47^{phox} knockout. The role of PKCε was demonstrated by specific inhibitors and PKCε knockout. The important contribution of mitochondrial ROS was shown by suppression of hypoxia effects by rotenone and by GPx-1 overexpression and deletion. Based on the results of Rathore et al. [31].

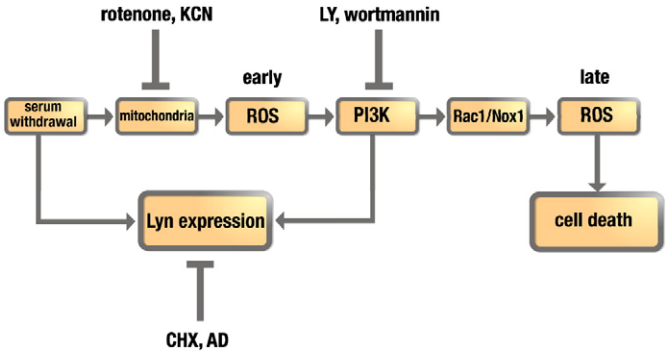

Fig. 5. Serum withdrawal induces two pathways, one leading to the mitochondrial production of ROS and the other leading to Lyn expression. Although the former event occurs immediately after serum withdrawal, the latter is evident 4 h later. PI3K is then activated in a mitochondrial ROS and Lyn-dependent manner. The activated PI3K in turn stimulates Nox1 by inducing the translocation of Rac1 to membrane fractions and the Rac1/Nox1 interaction. Consequently, the accumulation of ROS is extended into the late phase (4–8 h), and cells lose their viability. Adopted from Lee et al. [133].

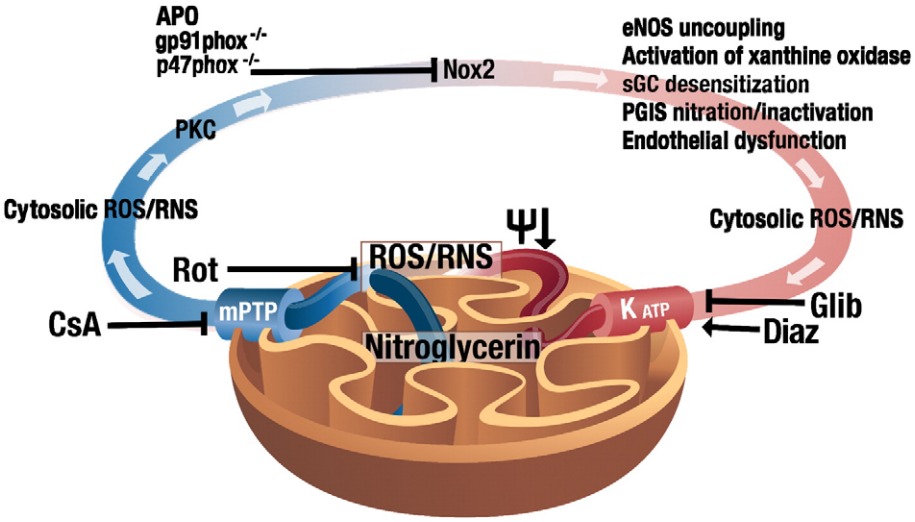

Fig. 6. Proposed hypothetical scheme of the cross-talk between mitochondrial and cytosolic (NADPH oxidase-derived) reactive oxygen and nitrogen species in the setting of nitrate tolerance. ALDH-2, mitochondrial aldehyde dehydrogenase; CsA, cyclosporin A; Rot, rotenone; mPTP, mitochondrial permeability transition pore; APO, apocynin; Glib, glibenclamide; Diaz, diazoxide; PGIS, prostacyclin synthase; sGC, soluble guanylyl cyclase; eNOS, endothelial NO synthase; PKC, protein kinase C; K_{ATP}, ATP-dependent potassium channel. Adopted from Wenzel et al. [30].

may be prevented by induction of intrinsic antioxidants as observed for the tolerance-free organic nitrate pentaerithrityl tetranitrate [121,148–150] or by co-therapy with exogenously added antioxidants such as hydralazine or lipoic acid [8,151].

The involvement of mitochondrial superoxide formation as well as hydrogen peroxide signaling in nitrate tolerance was previously demonstrated at the molecular level using $MnSOD^{+/-}$ and $GPx\text{-}1^{-/-}$ mice [135,141,152]. Both deficiencies amplified nitroglycerin-induced cross-tolerance to endothelium-dependent vasodilators (endothelial dysfunction) whereas GPx-1 deficiency only caused impaired endothelial function (decreased acetylcholine response) but showed no effect on nitroglycerin potency (nitrate tolerance). Further support for a role of mitochondrial ROS in nitrate tolerance came from Esplugues et al. using mitochondria-targeted antioxidants (Mito-Q and a GSH-ester) and cells with depleted mitochondrial proteins (so-called ρ^0 cells) [140]. These observations are in accordance with our recently published concept on how nitroglycerin–induced mitochondrial ROS formation via the permeability transition pore activates cytosolic NADPH oxidases, leading to endothelial dysfunction [30]. Surprisingly, endothelial dysfunction (sensitive to NADPH oxidases) and vascular dysfunction (sensitive to mitochondria) were dependent on the activation of distinct oxidant sources, a finding supported by the fact that $gp91^{phox-/-}$ and $p47^{phox-/-}$ mice developed tolerance but no endothelial dysfunction in response to nitroglycerin treatment. The mechanism underlying this concept is based on mtROS-driven PKC activation which in turn will activate NADPH oxidases. The NADPH oxidase-dependent cytosolic ROS and RNS formation will then uncouple eNOS, nitrate prostacyclin synthase and desensitize sGC. Previous experimental studies have shown that increased oxidative stress in cellular tissue per se is able to activate the oxidase in a positive feedback fashion [153]. This cross-talk was further substantiated by the observation that it was blocked by in vivo and ex vivo administrations of the mitochondrial permeability pore inhibitor cyclosporine A, which selectively improved endothelial dysfunction, whereas nitrate tolerance was not affected. This fully agrees with our concept that mitochondrial hydrogen peroxide or peroxynitrite is a potent trigger of NADPH oxidase activation and subsequent NOS uncoupling. Blockade of mPTP will remove this trigger. However, cyclosporine A does not decrease nitroglycerin-induced intramitochondrial oxidative stress, it rather traps ROS and RNS in the matrix, explaining its lack of effect on nitrate tolerance. In contrast, the respiratory complex I inhibitor rotenone improved endothelial dysfunction and tolerance since it removes mitochondrial oxidative stress, which largely contributes to nitrate tolerance by oxidative inhibition of ALDH-2, and removes the trigger for NADPH oxidase activation in the cytosol.

Finally, we were able to demonstrate a feedback redox signaling from the cytosol to the mitochondria: In vivo or ex vivo treatment with the K_{ATP} opener diazoxide caused a nitrate tolerance-like phenomenon in control animals, whereas the K_{ATP} inhibitor glibenclamide improved tolerance in nitroglycerin-treated animals. These observations indicate that maybe the essential step in nitroglycerin-triggered nitrate tolerance is the induction of a vicious cycle consisting of mitochondrial and Nox-derived ROS/RNS formation with each stimulating the other in a positive feedback fashion. Very similar effects of rotenone (Rot), cyclosporine A (CsA), diazoxide (Diaz) and glibenclamide (Glib) in an experimental model of angiotensin-II induced hypertension have been discussed above in detail [35]. A role of K_{ATP} channels for NADPH oxidase-driven activation of mitochondrial ROS formation via changes in the membrane potential was previously proposed and also discussed above [29]. One may also speculate that in a second pathway, nitroglycerin via its hypotensive action may cause an activation of the renin–angiotensin–aldosterone system [154], leading to increased circulating levels of angiotensin-II and aldosterone, and therefore to an activation of the NADPH oxidase. This concept is further corroborated by the demonstration that in vivo treatment with an AT_1 receptor blocker was able to prevent the development of nitroglycerin-induced endothelial dysfunction in an animal model of nitrate tolerance [155]. In addition, our present findings could explain why treatment with an AT_1 receptor blocker was not able to prevent the development of nitroglycerin-induced nitrate tolerance in human subjects [156,157].

We propose that a similar cross-talk exists in the aging vasculature and that aging-induced mtROS can activate cytosolic ROS/RNS sources leading to age-related vascular dysfunction [158]. This proposal is based on the finding that mtROS formation increases with age (and is higher in $MnSOD^{+/-}$ mice) and endothelial function is impaired with age (to a higher extend in $MnSOD^{+/-}$ mice). There was a highly significant inverse correlation between mitochondrial ROS formation and endothelial function as well as mitochondrial ROS formation and mitochondrial DNA damage. Considering the fact that most ROS sources have redox switches, a cross-talk between all known ROS sources may be possible: The interaction of mitochondria and NADPH oxidases was just discussed above, but also eNOS can be uncoupled by oxidative stress and depletion of the essential co-factor BH_4 [103]or oxidation of a critical zinc–thiolate-center [159] functions

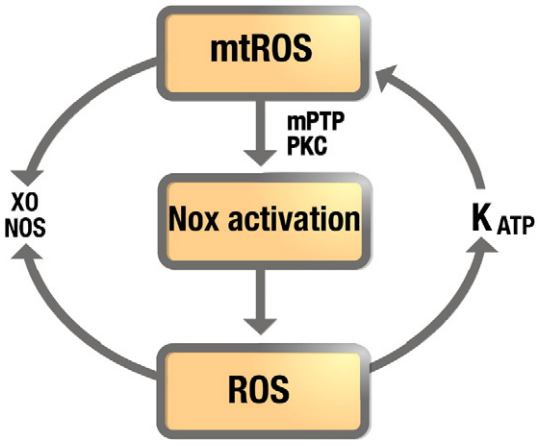

Fig. 7. Proposed concept of cross-talk of different ROS and RNS sources.

as a redox switch that determines whether eNOS generates nitric oxide, superoxide or peroxynitrite. The same applies for xanthine oxidoreductase since the dehydrogenase form requires oxidative and proteolytic modifications to become the oxidase form generating superoxide [160–163]. Accordingly, it is not surprising that in many disease states all sources of ROS are active. For example, we have observed increased NADPH oxidase activity, mitochondrial ROS formation, eNOS uncoupling and high serum xanthine oxidase activity in experimental diabetes [164–166]. The concept of ROS-triggered ROS may therefore be extended to all known sources of ROS (Fig. 7).

6. Outlook/Perspective

Hopefully, this review could demonstrate the importance of redox signaling in physiology and disease. Besides the role of NADPH oxidases and xanthine oxidase, mitochondria are of great importance since, even when not directly affected or involved in a disease state, they may become involved by the above described cross-talk pathways and confer an amplification mechanism for cellular oxidative stress. In the future, redox signaling may be an attractive target for drug development, but, due to its complexity requires careful evaluation and understanding of the pathways involved since blockade at the wrong target could not only improve disease states but also suppress physiologically important signaling pathways with detrimental consequences. The development of mitochondria-targeted antioxidants and use of these new tools may for management of disease and pathophysiological complications [140,167–172] may show the direction for future strategies to mimic the antioxidant defense system of the organism, which until now, was rather traced by quite simple and primitive attempts [173–177]. A very promising strategy may be based on the control of mitochondrial channels such as the mPTP or the mtK_{ATP} [117,125], considering that a pharmacological role of these channels beyond cardiac protection and ischemia reperfusion seems plausible.

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