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Themed Section: Redox Biology and Oxidative Stress in Health and Disease

REVIEW ARTICLE

Crosstalk of mitochondria with NADPH oxidase via reactive oxygen and nitrogen species signalling and its role for vascular function

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Cardiovascular diseases are associated with and/or caused by oxidative stress. This concept has been proven by using the approach of genetic deletion of reactive species producing (pro-oxidant) enzymes as well as by the overexpression of reactive species detoxifying (antioxidant) enzymes leading to a marked reduction of reactive oxygen and nitrogen species (RONS) and in parallel to an amelioration of the severity of diseases. Likewise, the development and progression of cardiovascular diseases is aggravated by overexpression of RONS producing enzymes as well as deletion of antioxidant RONS detoxifying enzymes. Thus, the consequences of the interaction (redox crosstalk) of superoxide/hydrogen peroxide produced by mitochondria with other ROS producing enzymes such as NADPH oxidases (Nox) are of outstanding importance and will be discussed including the consequences for endothelial nitric oxide synthase (eNOS) uncoupling as well as the redox regulation of the vascular function/tone in general (soluble guanylyl cyclase, endothelin-1, prostanoid synthesis). Pathways and potential mechanisms leading to this crosstalk will be analysed in detail and highlighted by selected examples from the current literature including hypoxia, angiotensin II-induced hypertension, nitrate tolerance, aging and others. The general concept of redox-based activation of RONS sources via "kindling radicals" and enzyme-specific "redox switches" will be discussed providing evidence that mitochondria represent key players and amplifiers of the burden of oxidative stress.

LINKED ARTICLES

This article is part of a themed section on Redox Biology and Oxidative Stress in Health and Disease. To view the other articles in this section visit http://onlinelibrary.wiley.com/doi/10.1111/bph.v174.12/issuetoc

Abbreviations

$\Delta\Psi_m$, mitochondrial membrane potential; 5-HD, 5-hydroxydecanoic acid; ADMA, asymmetric dimethylarginine; AT-II, angiotensin-II; BH_4, tetrahydrobiopterin; cGMP, cyclic guanosine monophosphate; COX, cyclooxygenase; eNOS, endothelial nitric oxide synthase; ET-1, endothelin-1; GCH-1, GTP cyclohydrolase-1; K_{ATP},, ATP-sensitive potassium channel; MAO, monoamine oxidase (isoforms A and B); MAPK, mitogen-activated protein kinases; MitoSOX, triphenylphosphonium dihydroethidium (mitochondria-targeted superoxide probe); MitoQ, triphenylphosphonium quinone (mitochondria-targeted antioxidant); MnSOD, manganese superoxide dismutase; mPTP, mitochondrial permeability transition pore; mtK_{ATP},, mitochondrial K_{ATP}; mtROS, mitochondrial reactive oxygen species; nNOS, neuronal nitric oxide synthase; Nox, NADPH oxidase catalytic subunit (isoforms 1, 2 and 4); PGIS, prostacyclin; PKC, protein kinase C; RNS, reactive nitrogen species (accounts in the present review mostly for peroxynitrite and nitrogen dioxide); ROS, reactive oxygen species (accounts in the present review mostly for superoxide and hydrogen peroxide); sGC, soluble guanylyl cyclase; SOD, superoxide dismutase; Src (or cSrc), tyrosine kinase; XDH, xanthine dehydrogenase; XO, xanthine oxidase

Tables of Links

TARGETS
Enzymes[a]
ALDH2, aldehyde dehydrogenase 2
eNOS, endothelial NOS
sGC, soluble guanylyl cyclase
MAO-B, monoamine oxidase B
PGIS, prostacyclin synthase (CYP8A1)
Src kinase
Voltage-gated ion channels[b]
K_{ATP} channels ($K_{ir}6.x$)

LIGANDS
AT-II, angiotensin II
Asymmetric dimethylarginine
Diazoxide
ET-1, endothelin 1
Glibenclamide
Hydrogen peroxide
NO
Pinacidil

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2006) and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 ([a b]Alexander *et al.*, 2014, 2015).

Introduction

Redox biology and impact of oxidative stress on disease progression

Since the discovery of the biological significance of reactive oxygen (ROS) and nitrogen species (RNS) (Sies, 1991), especially by identification of antioxidant enzymes such as superoxide dismutases (SODs) (McCord *et al.*, 1971) or catalases (Stadie *et al.*, 1945), much effort was made by the scientific community to elucidate the role of these reactive species in physiological and pathophysiological processes. The discovery and characterization of NADPH oxidases as superoxide or hydrogen peroxide producing enzymes provided another important milestone in the redox biology field (Griendling *et al.*, 1994; Rajagopalan *et al.*, 1996; Li and Shah, 2001; Takac *et al.*, 2011) and further supported the concept that ROS and RNS can also fulfil physiological functions, by their participation in cellular redox signalling (Stamler, 1994; Ullrich and Kissner, 2006). Similar physiological functions have been demonstrated for mitochondria-derived ROS (Sena and Chandel, 2012). There is growing body of evidence that ROS and RNS play a crucial role in cardiovascular (Cai and Harrison, 2000; Griendling and FitzGerald, 2003a, 2003b), neurodegenerative (Giasson *et al.*, 2000; Ischiropoulos and Beckman, 2003) and inflammatory diseases (Szabo, 1996; Kooy *et al.*, 1997). The underlying pathogenesis, however, and especially the physiological properties of ROS and RNS by conferring redox regulation are not fully understood.

Several clinical studies demonstrated beneficial effects of acute high-dose vitamin C infusion into the forearm on flow-mediated or acetylcholine-induced vasodilation (Heitzer *et al.*, 1996; Heitzer *et al.*, 2001b; Heitzer *et al.*, 2001a) clearly providing indirect evidence for increased oxidative stress in vessels from patients with hypercholesterolemia, diabetes type I and II or chronic smokers. Importantly, Heitzer *et al.* demonstrated that the degree of vitamin C effects on endothelial dysfunction assessed by forearm plethysmography in patients with coronary artery disease has a prognostic meaning (Heitzer *et al.*, 2001a). Patients with a strong vitamin C (above the median) response had a worse prognosis and more cardiovascular events within the next 3 years including MI, death due to myocardial infarction, stroke or revascularization procedures as compared to patients having a vitamin C response below the median (Figure 1). The main conclusion of this study was that patients with increased oxidative stress in forearm vessels may also have increased oxidative stress in coronary arteries leading to increased cardiovascular events. Indeed, studies by Kathy Griendling's group, demonstrated in specimens from coronary artery disease (CAD) patients increased oxidative stress, especially in the shoulder of the plaque. This might explain why these patients are more prone to plaque ruptures and subsequent cardiovascular events (Sorescu *et al.*, 2002).

Despite these encouraging data from acute, parenteral therapy with vitamin C or other antioxidants, almost all huge clinical trials on oral antioxidant therapy were negative in respect of hard endpoints and clinical outcome (for review see (Gori and Munzel, 2011; Chen *et al.*, 2012; Schmidt *et al.*, 2015)). There are numerous reasons for this failure, discussed in the mentioned reviews, but most reasons might compromise off-target effects of the antioxidants (e.g. interference with intrinsic redox-triggered protective pathways) and the fact that ROS from the same source might have different effects due to concentration and timing.

Molecular proof of the involvement of oxidative stress in cardiovascular disease and concept of redox-crosstalk of different sources of oxidants

A molecular proof of the involvement of oxidative stress in cardiovascular diseases is mainly based on animal experimental models with appreciable effects of genetic deletion or overexpression of NADPH oxidase subunits or of antioxidant enzymes on the disease phenotype (summarized in (Daiber *et al.*, 2009; Chen *et al.*, 2012; Karbach *et al.*, 2013)). We summarized experimental data on deletion or overexpression as well as pharmacological inhibition of different ROS sources

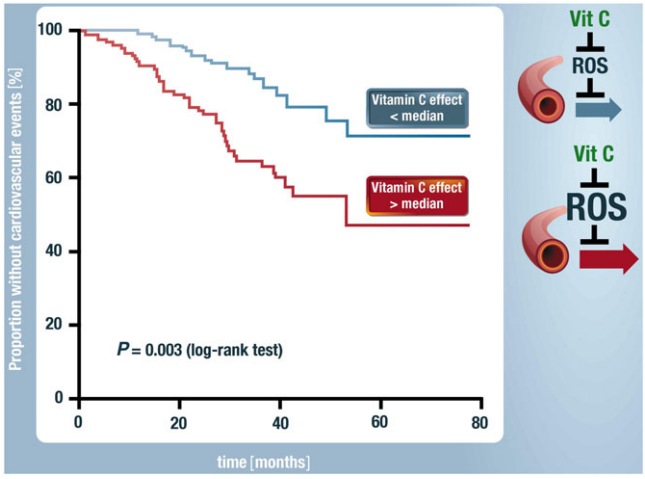

Figure 1

Indirect proof for the prognostic importance of oxidative stress. In patients with high oxidative stress in forearm vessels, compatible with a vitamin C response on endothelial function above the median, had within the next 5 years more cardiovascular events such as myocardial infarction, stroke, coronary and peripheral revascularization procedures as compared to patients with a vitamin C response below the median. Endothelial function was assessed by venous plethysmography and intraarterial infusion of the endothelium-dependent vasodilator acetylcholine. Modified from Heitzer *et al.* and Münzel, *Circulation* 2001 (Heitzer *et al.*, 2001a). With permission of Wolters Kluwer Health. Copyright 2001.

and their impact on progression and severity of hypertension (Table 1) or myocardial infarction (Table 2). However, it would be easy to extend such summary to almost all major diseases (e.g. neurodegenerative disease, inflammatory disease, metabolic syndrome/diabetes). The important role of MnSOD activity for carcinogenesis (Konzack and Kietzmann, 2014) and the impact of mitochondrial ROS on pathogenesis in brown adipose tissue in hyperlipidemia and hyperinsulinemia was recently reviewed in full detail (Markelic *et al.*, 2013).

Here, we would like to put forward the concept that oxidative stress and redox signalling is based on a complex network of various ROS and RNS sources that may interact with and stimulate each other in a positive feedback fashion. These interactions make it very difficult to pharmacologically target oxidative stress as a cause of disease development and progression (Chen *et al.*, 2012). The redox crosstalk of the major sources of ROS and RNS, mitochondria, NADPH oxidases, xanthine oxidase and eNOS, was previously reviewed in detail (Daiber, 2010; Dikalov, 2011; Schulz *et al.*, 2014) (see Table 3 for more references).

We will therefore focus on the most recent advances in this field with special emphasis on the cardiovascular system and discuss in particular the effects of the crosstalk between mitochondria and NADPH oxidases on vascular function by dysregulation/uncoupling of eNOS (Karbach *et al.*, 2013). The network of these redox interactions is even more complex since recent data suggests that low-grade inflammation (e.g. in conditions of psoriasis or rheumatoid arthritis) represents a risk factor for the development of arterial hypertension and cardiovascular disease (Harrison *et al.*, 2011; Karbach *et al.*, 2013). Also mental stress causes activation of the immune system with adverse effects on the cardiovascular system (Marvar and Harrison, 2012). Finally, inflammatory pathways themselves are redox regulated (Brune *et al.*, 2013; Janko *et al.*, 2014; Hwang *et al.*, 2013). In the following paragraphs we will highlight the interaction of different sources of ROS and RNS and how this redox crosstalk affects the vascular system.

Table 1

Important (causal) ROS sources in different hypertension models

Model and ROS source	Ref.
Critical role of mitochondrial ROS for AT-II induced hypertension.	(Doughan *et al.*, 2008; Dikalova *et al.*, 2010)
Critical role of the NADPH oxidase isoform Nox1 for AT-II induced hypertension.	(Dikalova *et al.*, 2005; Matsuno *et al.*, 2005)
Critical role of the NADPH oxidase isoform Nox2 ($gp91^{phox}$) for AT-II induced hypertension.	(Bendall *et al.*, 2007; Murdoch *et al.*, 2011; Chrissobolis *et al.*, 2012)
Critical role of the NADPH oxidase isoform Nox4 for AT-II induced hypertension.	(Wingler *et al.*, 2001; Lee *et al.*, 2013)
Critical role of the NADPH oxidase subunit $p47^{phox}$ for AT-II induced hypertension.	(Landmesser *et al.*, 2002)
Critical role of the NADPH oxidase subunit $p47^{phox}$ for DOCA-salt induced hypertension.	(Landmesser *et al.*, 2003)
Critical role of xanthine oxidase for AT-II induced hypertension.	(Landmesser *et al.*, 2007)

Redox regulation of vascular function by oxidative degradation of •NO, dysregulation/uncoupling of eNOS, desensitization of sGC, dysregulation of prostanoid synthesis and activation of the endothelin system

The first example of a redox regulation of the vascular tone was provided by Gryglewski and coworkers (Gryglewski *et al.*, 1986). They demonstrated that the vasodilator capacity of the endothelium-derived relaxing factor (EDRF, later being

Table 2

Important (causal) ROS sources in different myocardial infarction models.

Model and ROS source	Ref.
Critical role of the NADPH oxidase subunit $p47^{phox}$ for MI damage.	(Doerries *et al.*, 2007)
Critical role of the NADPH oxidase isoform Nox4 for MI damage.	(Infanger *et al.*, 2010)
Critical role of the NADPH oxidase isoform Nox2 for MI damage.	(Looi *et al.*, 2008; Somasuntharam *et al.*, 2013)
Critical role of mitochondrial ROS for MI damage.	(Piot *et al.*, 2008; Kloner *et al.*, 2012; Somasuntharam *et al.*, 2013)
Critical role of xanthine oxidase for MI damage.	(Akizuki *et al.*, 1985; Werns *et al.*, 1986; Engberding *et al.*, 2004; Doerries *et al.*, 2007)

identified as nitric oxide) in isolated denuded aortic ring segments was decreased with increasing the diffusion distance of the perfusate from the cell culture to the endothelium-devoid aortic ring segments and that upon addition of SOD vasodilation was strikingly improved. With these experiments, the authors demonstrated that superoxide is a direct antagonist of EDRF, even before its identity was widely accepted to be nitric oxide, and thereby provided the basis for a simple concept of how oxidative stress (increased superoxide formation) reduces the bioavailability of nitric oxide thereby leading to vascular dysfunction. Superoxide reacts with nitric oxide in a diffusion-controlled reaction to produce peroxynitrite (Beckman *et al.*, 1990; Beckman and Koppenol, 1996). Peroxynitrite in turn leads to endothelial(vascular) dysfunction by nitration of prostacyclin synthase (Zou and Ullrich, 1996; Zou *et al.*, 1997) by inhibition of soluble guanylyl cyclase (Weber *et al.*, 2001) and by causing eNOS uncoupling due to oxidation of the eNOS cofactor tetrahydrobiopterin (Kuzkaya *et al.*, 2003; Schulz *et al.*, 2008).

We have recently provided the concept of different "redox switches" in eNOS in order to explain its high sensitivity to oxidative stress conditions (Figure 2) (Karbach *et al.*, 2013; Schulz *et al.*, 2014). However, it should be noted that any of these switches described in Figure 2 can be activated in a certain disease condition, but usually they are simultaneously activated and one finds eNOS uncoupling, sGC desensitization and PGIS nitration along with increased COX activity at the same time (for review see (Bachschmid *et al.*, 2005; Munzel *et al.*, 2005)). Also up-regulated ET-1 activity is accompanied with oxidative stress conditions and by activation of other redox switches, which was observed in nitroglycerin-induced nitrate tolerance (Munzel *et al.*, 1995; Hink *et al.*, 2003; Sayed *et al.*, 2008; Knorr *et al.*, 2011).

Redox switches, eNOS uncoupling and impaired •NO signalling

The mechanisms underlying eNOS uncoupling including S-glutathionylation of the eNOS reductase domain, eNOS phosphorylation, oxidation of the eNOS cofactor tetrahydrobiopterin, increased intracellular ADMA levels or oxidative disruption of the zinc-sulphur complex ($ZnCys_4$) have recently been reviewed (Forstermann and Munzel, 2006; Schulz *et al.*, 2014). Therapeutic targeting of uncoupled eNOS was recently reviewed in detail (Risbano and Gladwin, 2013) (see also this virtual BJP issue Daiber *et al.*). Of note, also other NOS isoforms may uncouple and produce superoxide instead of •NO as shown for nNOS in models of metabolic syndrome and angiotensin-II induced hypertension (Zhang *et al.*, 2009; Wu *et al.*, 2014). Moreover, a protective role of nNOS in preventing eNOS uncoupling was reported (Idigo *et al.*, 2012). Increased oxidative stress also leads to an impaired •NO/cGMP-signalling by oxidative inhibition of soluble guanylyl cyclase (sGC) via oxidation of Cys122 (Brune *et al.*, 1990; Weber *et al.*, 2001; Sayed *et al.*, 2007), making the enzyme less sensitive to •NO (Figure 2). Under nitrosative stress conditions sGC may also undergo S-nitrosation at Cys243 and Cys122 (Sayed *et al.*, 2007). The discovery of compounds that are able to activate the enzyme in a •NO-independent manner, such as heme-dependent sGC stimulators and heme-independent sGC activators has led to the development of several drugs for different clinical indications (for review see (Stasch *et al.*, 2011) and this virtual BJP issue Daiber *et al.*).

Oxidative stress and the endothelin-system

The vasoconstrictor endothelin-1 (ET-1) induces expression of NADPH oxidase (gp91phox or Nox2 dependent isoform) in endothelial cells (Duerrschmidt *et al.*, 2000) and stimulates superoxide formation ex. vivo in isolated human arteries via a ET-1 receptor dependent mechanism (Cerrato *et al.*, 2012). ET-1 also increases vascular superoxide formation via activation of the NADPH oxidase in mild hypertension models (Li *et al.*, 2003a, 2003c). Vice versa, it was also shown that NADPH oxidase derived superoxide formation potentiates the vasoconstriction induced by ET-1 (Li *et al.*, 2003b). These findings provide evidence for a crosstalk comparable to that described above for the mitochondrial superoxide/hydrogen peroxide-Nox2 axis (Figure 2). The demonstration that oxidative stress per se increases the expression of ET-1 via induction of its promoter further substantiates the concept of a crosstalk involving mitochondrial superoxide/hydrogen peroxide, Nox2 and ET-1 as important constituents (Kahler *et al.*, 2000; Kahler *et al.*, 2001). In addition, ET-1 as well as AT-II can directly trigger mitochondrial calcium uptake by mitochondrial calcium microdomains via IP3-receptor (type 3) that co-localises with mitochondria and subsequent activation of the mitochondrial ryanodine receptor (mRyR1) (Pacher *et al.*, 2008). This leads to mPTP opening and release of mitochondrial superoxide/hydrogen peroxide (Seidlmayer *et al.*, 2014). Likewise, mitochondrial superoxide/hydrogen peroxide formation was associated with increased ET-1 secretion of pulmonary artery endothelial cells (Ouyang *et al.*, 2012).

Oxidative stress and prostanoid synthesis

Prostanoid synthesis is also redox regulated by nitration of the prostacyclin synthase (Zou and Ullrich, 1996; Zou *et al.*, 1997;

Table 3

Evidence for a redox crosstalk between different sources of ROS. Examples for mitochondria and NADPH oxidases.

Model and mito/Nox signaling	Ref.
Mitochondrial ROS formation triggers the expression of Nox1 gene and protein expression in tumorigenesis.	(Desouki *et al.*, 2005)
Cellular starvation activates NADPH oxidase and induces cell death through the mitochondrial ROS-PI3K signaling axis in human 293 T cells.	(Lee *et al.*, 2006)
First Evidence for a Crosstalk Between Mitochondrial and NADPH Oxidase-Derived Reactive Oxygen Species in Nitroglycerin-Triggered Vascular Dysfunction.	(Wenzel *et al.*, 2008b)
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.	(Rathore *et al.*, 2008)
Molecular Mechanisms of Angiotensin II–Mediated Mitochondrial Dysfunction. Linking Mitochondrial Oxidative Damage and Vascular Endothelial Dysfunction.	(Doughan *et al.*, 2008)
Effects of mild mitochondrial dysfunction on angiotensin II – mediated increase in Nox isoform expression and activity in SMC.	(Wosniak *et al.*, 2009)
RAGE-Induced Cytosolic ROS Promote Mitochondrial Superoxide Generation in Diabetes.	(Coughlan *et al.*, 2009)
Therapeutic Targeting of Mitochondrial Superoxide in Hypertension.	(Dikalova *et al.*, 2010)
ROS-induced ROS release in vascular biology.	(Zinkevich and Gutterman, 2011)
Nox2-generated H_2O_2 regulates mitochondria-derived superoxide production and cGMP nitration in response to lipopoly saccharide.	(Ahmed *et al.*, 2012)
Interaction of mitochondria and phagocytic NADPH oxidase via ROS and calcium signaling in cultured lymphoblasts.	(Dikalov *et al.*, 2012)
Interplay of Nox4 and mtROS in angiotensin-II induced uncoupling of eNOS.	(Lee *et al.*, 2013)
Nox2-induced production of mitochondrial superoxide in angiotensin II - mediated endothelial oxidative stress and hypertension.	(Dikalov and Nazarewicz, 2013)
Nox2 as a potential target of mitochondrial superoxide and its role in endothelial oxidative stress.	(Nazarewicz *et al.*, 2013)
Crosstalk of mitochondria and NADPH oxidases via ROS signaling in white blood cells and its relevance in the aging process, angiotensin II induced hypertension – aggravation by MnSOD deficiency and amelioration by cyclophilin D deficiency.	(Kroller-Schon *et al.*, 2014)
Interaction of mtROS with Nox2 but not Nox1, Nox4 or Nox5 in the angiotensin II-induced hypertension model via hydrogen peroxide, sSrc, PKCε and mtK_{ATP} channels.	(Dikalov *et al.*, 2014)
A role for mitochondrial oxidants in angiotensin II-induced premature senescence of human vascular smooth muscle cells by NADPH oxidases.	(Mistry *et al.*, 2013)
Mitochondrial regulation of NADPH oxidase in hindlimb unweighting rat cerebral arteries.	(Zhang *et al.*, 2014)

Bachschmid *et al.*, 2003) or by activation of cyclooxygenases in the presence of peroxides ("peroxide tone") as well as inactivation of these enzymes by nitration of critical tyrosine residues (Figure 2) (Schildknecht *et al.*, 2005; Schildknecht *et al.*, 2006; Schildknecht *et al.*, 2008; Schildknecht *et al.*, 2012). Prostanoid synthesis and ROS generation can even interact with each other in a redox crosstalk fashion (Hernanz *et al.*, 2014).

What are the biologically relevant sources of ROS and RNS in cardiovascular disease?

The failure of antioxidants to cure cardiovascular diseases was, as reported previously, likely due to their low reaction constants with the reactive species and their ineffectiveness in targeting a certain ROS/RNS source as well as their organ-specific or even subcellular accumulation (Chen *et al.*, 2012; Schmidt *et al.*, 2015). Due to this spatial and target non-specificity they may interfere on the other hand with important physiological cellular redox signalling pathways, important stress-response systems or pathogen defence mechanisms of the organism. For instance, it has been shown that cell differentiation, proliferation and migration are redox-regulated in various cell types and physiological or pathophysiological settings (Griendling and Ushio-Fukai, 1998; Cappelletti *et al.*, 2003; Schroder *et al.*, 2007; Mofarrahi *et al.*, 2008; Schroder *et al.*, 2009; Hurd *et al.*, 2012). Also pathogen defence by the immune system relies on the generation of large amounts of ROS and RNS in order to kill the pathogen/parasite and to ensure host survival (Miller and Britigan, 1997; Stolf *et al.*, 2011), as was shown for *Leishmania*

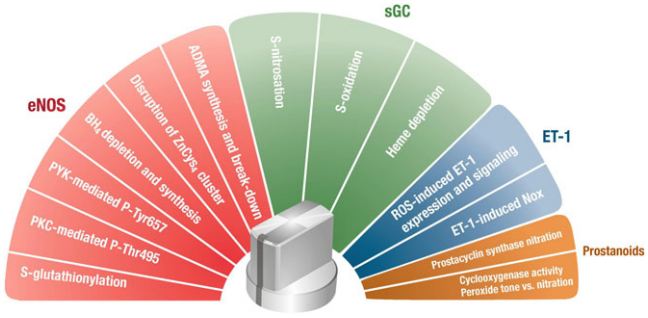

Figure 2

Redox regulation of the vascular tone. Potential „redox switches"in endothelial NO synthase (eNOS) are based on S-glutathionylation of cysteines in the reductase domain, H_2O_2/$ONOO^-$ mediated activation of PKC and protein tyrosine kinase-2 (PYK-2) dependent eNOS phosphorylation, direct oxidative depletion of the eNOS cofactor BH_4, oxidative disruption of the zinc-sulphur-complex in the dimer binding interface and regulation of ADMA synthesis/break-down by oxidants. Potential „redox switches"in soluble guanylyl cyclase (sGC) are based on S-nitrosation by species such as N_2O_3 or S-oxidation of regulatory cysteines as well as oxidation (depletion) of the heme moiety. An additional "redox switch" comprises the endothelin-1 (ET-1) and NADPH oxidase (Nox) system since oxidants can lead to increased expression of ET-1 and potentiate its vasoconstrictory properties, which can be further self-amplified by ET-1 dependent activation of NADPH oxidase. Finally, prostanoid synthesis (Cox and PGIS) represents a "redox switch" since the key enzyme of prostanoid synthesis, cyclooxygenase, is activated at low peroxide levels and nitrated/inactivated at high peroxynitrite or nitrite/H_2O_2 concentrations, whereas PGIS is nitrated/inactivated by peroxynitrite and both oxidative alterations may significantly shift the prostanoid profile from vasodilatory to vasoconstrictory conditions. Activation of all of these redox switches by ROS and RNS (most likely hydrogen peroxide, peroxynitrite or superoxide) alters endothelial and vascular function, which will favour vasoconstriction and/or the development of arterial hypertension. Any of these switches can be activated in a certain disease condition but usually they are simultaneously activated and one finds eNOS uncoupling, sGC desensitization and PGIS nitration along with increased COX activity or up-regulated ET-1 signalling at the same time.

amazonensis infection (Giorgio *et al.*, 1998; Linares *et al.*, 2001) or *Trypanosoma cruzi* invasion (Denicola *et al.*, 1993; Irigoin *et al.*, 2008). The importance of the phagocytic NADPH oxidase for the immune system is also demonstrated by the high mortality of individuals with chronic granulomatous disease (genetic $p22^{phox}$, $p40^{phox}$, $p47^{phox}$, $p67^{phox}$ or $gp91^{phox}$ mutation) (Kuhns *et al.*, 2010). Finally, highly protective stress response mechanisms such as ischemic pre- or post-conditioning rely on a tightly controlled and timely restricted formation of ROS and RNS (Das and Maulik, 2003; Dost *et al.*, 2008; Di Lisa *et al.*, 2011; Andreadou *et al.*, 2015), which can be mimicked by certain drugs (e.g. organic nitrates) (Gori and Forconi, 2005; Gori and Parker, 2008) and inhibited by antioxidants (Thomas *et al.*, 2007; Pechanova *et al.*, 2015). Considering these highly beneficial actions of oxidants in the organism this may explain at least in part the failure of classical or non-specific antioxidants so far but also stress the importance of discriminating between harmful and protective ROS and RNS sources (Figure 3A).

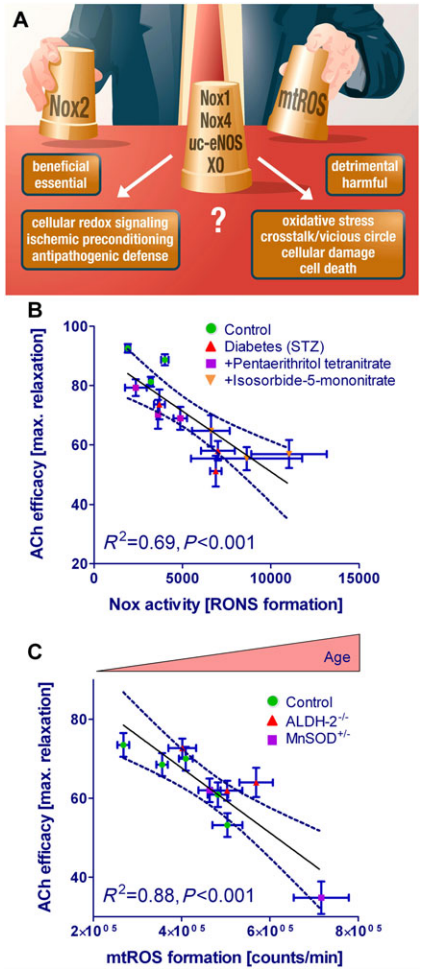

Figure 3

(A) Who's the bad guy – or which biological source of ROS formation is the most detrimental one? Likely candidates are mitochondrial ROS formation (mitochondrial superoxide/hydrogen peroxide), NADPH oxidases (Nox1, Nox2, Nox4), uncoupled eNOS (uc-eNOS) or xanthine oxidase (XO). The most challenging task for the future is the discrimination between beneficial and detrimental effects of ROS formation and signalling. (A) Direct correlation between vascular ROS formation and endothelial function. Endothelial function (measured by isometric tension recording, efficacy in %) inversely correlates with NADPH oxidase activity (measured by lucigenin-derived chemiluminescence [superoxide formation] in membrane fractions; expressed as RLU/min) in experimental type 1 diabetes mellitus and therapy with organic nitrates pentaerithrityl tetranitrate (PETN, beneficial effects) vs. isosorbide-5-mononitrate (ISMN, adverse effects). Type 1 diabetes mellitus is associated with activation of all ROS sources and organic nitrates such as isosorbide-5-mononitrate further aggravate these complications. Retraced from original data of Schuhmacher *et al.* and Daiber, *Diabetes* 2011 (Schuhmacher *et al.*, 2011). (B) Endothelial function (measured by isometric tension recording, efficacy in %) inversely correlates with mitochondrial ROS formation (measured by L-012-derived chemiluminescence [peroxynitrite, superoxide or hydrogen peroxide formation] in isolated mitochondria; expressed as RLU/min) in aging mice with antioxidant enzyme deficiencies (mitochondrial aldehyde dehydrogenase $[ALDH\text{-}2]^{-/-}$ and $MnSOD^{+/-}$). ALDH-2 deficiency leads to increased carbonyl stress since the enzyme is a major sink of toxic aldehydes. MnSOD deficiency increases mitochondrial superoxide levels and leads to increased cardiac fibrosis and impaired vascular function under oxidative stress conditions. RLU, relative light units. Retraced from original data of Wenzel *et al.* and Daiber, *Cardiovasc. Res.* 2008 (Wenzel *et al.*, 2008c).

Disease-relevant sources of oxidative stress

The identification of harmful ROS and RNS sources is further complicated by the fact that for almost any disease condition various enzymatic sources have been accused to cause the disease (Tables 1, 2). For example we have demonstrated an inverse correlation between mitochondrial ROS formation and endothelial dysfunction with increasing age (Wenzel *et al.*, 2008c), whereas in type 1 diabetes loss of endothelial function inversely correlated with the activity of NADPH oxidase (Schuhmacher *et al.*, 2011) (Figure 3B and C). Type 1 diabetes is a condition with activation of all cellular ROS sources, increased protein tyrosine nitration, eNOS uncoupling as well as low-grade inflammation and impaired organic nitrate potency (nitrate resistance) (Wendt *et al.*, 2005; Oelze *et al.*, 2010). Aging per se causes increased mitochondrial oxidative stress that may be associated with increased Nox activity and low-grade inflammation (Wenzel *et al.*, 2008a; Oelze *et al.*, 2014). ALDH-2 and MnSOD are considered important antioxidant proteins for cardioprotection (Strassburger *et al.*, 2005; Chen *et al.*, 2008). In the setting of arterial hypertension an important pathophysiological role of the NADPH oxidase subunits $p47^{phox}$, Nox1, Nox2 and Nox4 as well as mitochondrial ROS (mitochondrial superoxide/hydrogen peroxide) or xanthine oxidase was demonstrated (Table 1). For myocardial infarction it was shown that NADPH oxidase subunits such as $p47^{phox}$, Nox2, Nox4, mitochondrial superoxide/hydrogen peroxide or xanthine oxidase contribute to the size and extension of MI (Table 2). Similar observations are made for metabolic (e.g. diabetes), neurodegenerative (e.g. Alzheimer's disease) and inflammatory disease (e.g. sepsis or liver steatosis) as well as the aging process itself (Ischiropoulos and Beckman, 2003; Loguercio and Federico, 2003; Griendling and FitzGerald, 2003b; Zou *et al.*, 2004; Ceriello, 2006; Munzel *et al.*, 2010; Chen *et al.*, 2012; Kroller-Schon *et al.*, 2012; El Assar *et al.*, 2013).

A logic explanation for the identification of various sources of ROS for specific diseases such as hypertension or myocardial infarction may be the interaction with each other in a crosstalk fashion. Thus, the elimination of only one constituent of this crosstalk network normalizes the pathophysiological condition since the overall oxidative stress is reduced below a certain threshold that can be managed by the cellular antioxidant defence system. In the following sections we will discuss the proposed crosstalk concept, however, without clear differentiation between different tissues and cells. While this might be no serious problem for most of the cytosolic proteins discussed, there might be substantial differences among mitochondria isolated form different cell types in different organs, especially at the level of subpopulations. We also have to stress that a major part of the studies on the nature of the crosstalk between different ROS sources and induction of oxidative stress in general was conducted in vitro due to lack of specific tools to address ROS signalling in vivo (for review see (Schmidt *et al.*, 2015)).

Redox crosstalk of different sources of ROS and RNS

Redox crosstalk and vascular function – impact of NADPH oxidases and mitochondria

Vascular function is inversely correlated with activity of the membranous (particulate) NADPH oxidase activity in experimental type 1 diabetes mellitus (Figure 3B) (Schuhmacher *et al.*, 2011). Because of the close spatial localization of eNOS and vascular NADPH oxidases or even phagocytic NADPH oxidase from infiltrated immune cells, this ROS source may easily trigger uncoupling of eNOS, may desensitize the NO target sGC and may activate other redox-sensitive vasoactive systems such as the ET-1 synthesis and receptor signalling pathways (Figure 2). The inverse correlation of vascular function and mitochondrial superoxide/hydrogen peroxide appears to be more complex (Figure 3C) (Wenzel *et al.*, 2008c) since the release of superoxide produced in the matrix by complex I and II or more likely its dismutation product hydrogen peroxide is required for eliciting their detrimental effects on other cellular structures, such as induction of endothelial dysfunction or activation of other ROS sources. According to the concept of "kindling radicals" (or also "bonfire" hypothesis), initial formation of ROS (e.g., from NADPH oxidases or mitochondria) triggers the activation of secondary ROS sources (e.g. xanthine oxidase, uncoupled eNOS) (for review see (Daiber *et al.*, 2014).

Amplification of cellular oxidative stress by mitochondria

The efflux from mitochondrial matrix is facilitated for hydrogen peroxide which can cross phospholipid membranes more easily. There are, however, also a number of mitochondrial pores (mPTP, aquaporins, inner membrane anion channel (IMAC) and others) that could be involved in this efflux of mitochondrial hydrogen peroxide from the matrix, especially for mitochondrial superoxide which cannot cross phospholipid membranes (Aon *et al.*, 2003; Drose and Brandt, 2008; Almasalmeh *et al.*, 2014; Kroller-Schon *et al.*, 2014) (Figure 4). Although the majority of mitochondrial superoxide is efficiently dismutated to hydrogen peroxide by MnSOD, it was recently shown that cyclosporine A significantly decreases flashes of superoxide formation (Wang *et al.*, 2008; Jian *et al.*, 2014) and mPTP directly stimulates superoxide release (Hou *et al.*, 2014) suggesting a role of mPTP for mitochondrial superoxide efflux. Alternatively, Lustgarten *et al.* showed that mitochondrial superoxide is released from the matrix through voltage dependent anion channels (Lustgarten *et al.*, 2012). Likewise, mitochondrial superoxide/hydrogen peroxide are directly produced in the intermembrane space facilitating their escape to the cytosol (e.g. complex III, p66shc, MAO) (Cadenas and Davies, 2000; Drose and Brandt, 2008; Gertz and Steegborn, 2010; Kaludercic *et al.*, 2014b) (Figure 4). It is also unclear, whether mitochondrial superoxide/hydrogen peroxide can directly cause redox signalling in the cytosol without an amplification mechanism, which according to the crosstalk hypothesis is simply based on the ROS-mediated activation of secondary ROS sources. There is also some evidence that the ROS involved in this crosstalk may include peroxynitrite since angiotensin-II mediated activation of mitochondrial superoxide/hydrogen peroxide formation and endothelial dysfunction was prevented by the NOS inhibitor L-NAME and the scavenger of peroxynitrite-derived free radicals, uric acid (Doughan *et al.*, 2008). There is evidence that almost any ROS source can interact with each other while the mitochondria could represent indeed a central amplification mechanism in this network since they are present in almost any cell and tissue (Figure 4). They also play an important role in oxygen sensing, energy metabolism

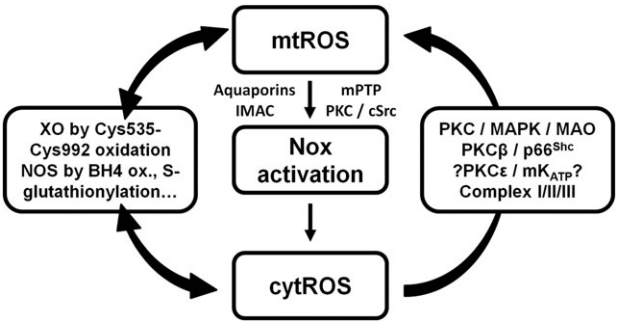

Figure 4

Crosstalk between different sources of ROS and RNS (mitochondria, NADPH oxidases, xanthine oxidase and NO synthase). Xanthine oxidase (XO) originates from oxidative stress-mediated conversion of the xanthine dehydrogenase via oxidation of critical thiols in cysteine535/992. NO synthases (mainly eNOS) are uncoupled upon oxidative depletion of tetrahydrobiopterin (BH_4), S-glutathionylation (−SSG) and other redox switches (see Figure 2). Mitochondrial superoxide/hydrogen peroxide formation may is triggered by oxidative stress from all ROS sources (including other damaged/activated mitochondria) via redox-activation of PKC, MAPK, other kinase pathways and potential involvement of redox-sensitive mitochondrial ATP-sensitive potassium channels (mtK_{ATP}) with subsequent $p66^{Shc}$, monoamine oxidase (MAO), respiratory complex activation or impairment of mitochondrial antioxidant defence. Mitochondrial superoxide/hydrogen peroxide is released to the cytosol via mitochondrial pores and channels (e.g. redox-sensitive mitochondrial permeability transition pore (mPTP), inner membrane anion channel (IMAC) or aquaporins) or by diffusion due to increased mitochondrial permeability under pro-inflammatory conditions. In the cytosol these species (along with released calcium) cause activation of redox-sensitive protein kinases (PKC) and tyrosine kinases (cSrc) with subsequent activation of NADPH oxidases and amplification of the cellular oxidative stress. Modified from Daiber, *Biochim. Biophys. Acta* 2010 (Daiber, 2010). With permission of Elsevier. Copyright 2010.

and translation of redox signals (Dehne and Brune, 2014; Ullrich and Schildknecht, 2014; Yin *et al.*, 2014). Mitochondrial superoxide/hydrogen peroxide formation and release by a certain dysfunctional/activated population of mitochondria can trigger mitochondrial superoxide/hydrogen peroxide formation and release from surrounding mitochondria initiating synchronized whole cell oscillations in mitochondrial metabolism (for review see (Zorov *et al.*, 2014)).

Redox activation of eNOS, xanthine oxidase and NADPH oxidase as superoxide sources

The redox-dependent conversion of eNOS from a nitric oxide synthase to a superoxide generating enzyme and the redox-triggered conversion of xanthine dehydrogenase to the oxidase, was described in detail in previous review articles (Munzel *et al.*, 2005; Forstermann and Munzel, 2006; Schulz *et al.*, 2008; Karbach *et al.*, 2013; Schulz *et al.*, 2014) (Figure 2). The most likely redox switches in Nox2 and to a minor extend Nox1 activation comprises the oxidation of a zinc-cysteine complex in the phorbol ester and diacylglycerol binding domain of classical PKC isoforms (Lin and Takemoto, 2005), which will lead to its activation and phosphorylation of cytosolic regulatory subunits such as $p47^{phox}$ or the direct interaction of protein disulphide isomerases with these subunits (Laurindo *et al.*, 2008) (summarized in (Schulz *et al.*, 2014)). In addition, all Nox isoforms are redox regulated at the transcriptional level (Peshavariya *et al.*, 2009; Lassegue and Griendling, 2010) (summarized in (Schulz *et al.*, 2014)).

Redox activation of mitochondrial superoxide/hydrogen peroxide formation

Finally, the redox trigger for mitochondrial superoxide/hydrogen peroxide formation and release may compromise several pathways (Figures 4, 5): 1) Previous data has shown that H_2O_2 activates redox-sensitive PKCβ leading to phosphorylation of $p66^{Shc}$ at serine 36 leading to mitochondrial accumulation of the protein, mtROS formation and subsequent mPTP opening leading to decreased mitochondrial calcium accumulation in the presence of ATP (Pinton *et al.*, 2007). This sequence of events was prevented by cyclosporine A dependent inhibition of the mPTP, genetic deletion of $p66^{Shc}$ and aggravated by PKCβ overexpression; 2) Cytosolic superoxide/hydrogen peroxide formation directly activates redox-sensitive PKC/MAPK signalling pathways (Lin and Takemoto, 2005; Liu *et al.*, 2011) (or the inhibitory MAPK phosphatases are suppressed in a redox-dependent fashion (Kim *et al.*, 2012)) leading to activation of c-Jun. and Egr-1 transcription factors and increased expression of monoamine oxidase-B (Wong *et al.*, 2002). This will result in increased hydrogen peroxide production in the intermembrane space or in the cytosolic space (Kaludercic *et al.*, 2014a). Of note, MAO-B activation in a cardiac dysfunction model caused severe induction of mitochondrial hydrogen peroxide formation, loss in mitochondrial membrane potential and ATP synthase switches to the reverse mode in order to stabilize the proton gradient and membrane potential (Kaludercic *et al.*, 2014b). In addition, genetic or pharmacological inhibition of both MAO isoforms were shown to prevent cardiac maladaptive remodelling induced by thoracic aortic constriction (Kaludercic *et al.*, 2010; Kaludercic *et al.*, 2014b) and endothelial dysfunction induced by lipopolysaccharide and angiotensin II (Sturza *et al.*, 2013); 3) The involvement of redox-sensitive mitochondrial K_{ATP} channels (Doughan *et al.*, 2008; Dikalov *et al.*, 2014). Of note, this pathway is only based on effects of pharmacological inhibitors so far (for details see below). 4) Depletion of mitochondrial defence system by shift from NADH/NADPH to $NAD^+/NADP^+$ in depolarized mitochondria (with low $\Delta\Psi_m$) and loss of glutathione peroxidase and peroxiredoxin systems that are coupled to NADPH-dependent reduction leads to increased mitochondrial superoxide/hydrogen peroxide formation despite lower formation rate of these ROS by respiratory complexes (for details see below) (Nickel *et al.*, 2014).

Concepts of increased mitochondrial permeability, reactivity and release pathways of mitochondrial oxidants

Once the superoxide is formed in the matrix it undergoes fast conversion to hydrogen peroxide, which may cross the mitochondrial membrane (Forman and Kennedy, 1974) or mitochondrial superoxide and hydrogen peroxide are released to the cytosol which could involve mitochondrial

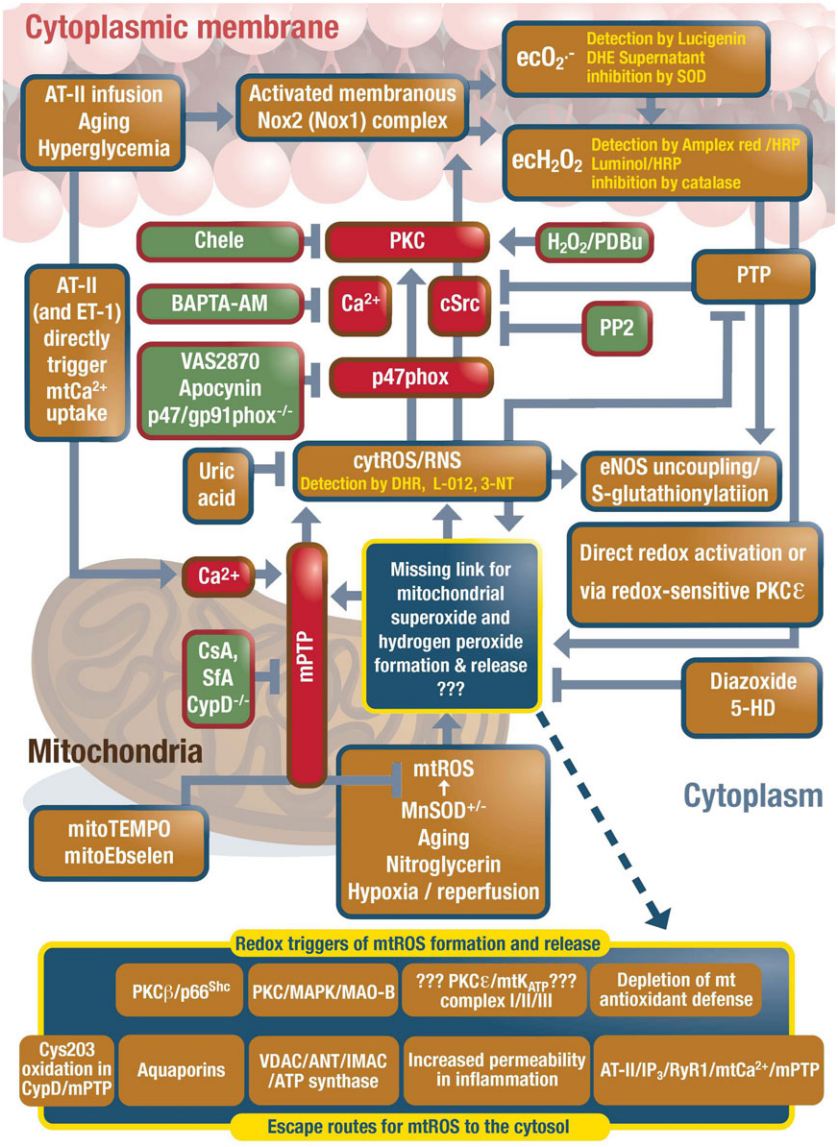

Figure 5

Redox crosstalk between mitochondria and NADPH oxidase through reactive oxygen and nitrogen species. Based on our own and others observations, we favour the crosstalk between mitochondria and Nox2 (in white blood cells and the vasculature). Mitochondrial superoxide/hydrogen peroxide formation is induced by the aging process, nitroglycerin treatment, hypoxia/reperfusion or stimulated by extramitochondrial ROS causing redox-activation of PKC, MAPK, other kinase pathways and potential involvement of redox-sensitive mitochondrial ATP-sensitive potassium channels (mtK_{ATP}) with subsequent $p66^{Shc}$, monoamine oxidase (MAO), respiratory complex activation or impairment of mitochondrial antioxidant defence. Evidence for involvement of redox-sensitive mitochondrial permeability transition pore (mPTP) is based on its pharmacological and genetic inhibition and normalization of the phenotype in various disease and pharmacological complications (e.g. eNOS S-glutathionylation and uncoupling). Previous data on redox-sensitive cysteine residues in cyclophilin D, a regulatory subunit of the mPTP support opening of the pore under oxidative stress conditions. Other redox-regulated parts of the pore may constitute of the voltage-dependent anion channel (VDAC) and adenine nucleotide translocase (ANT) or it may be even formed by dimerised mitochondrial ATP synthase in the inner mitochondrial membrane. Direct angiotensin-II (AT-II) triggered mitochondrial calcium uptake could contribute to mPTP opening. Other routes for release of mitochondrial superoxide/hydrogen peroxide may involve the inner membrane anion channel (IMAC), aquaporins or diffusion due to increased mitochondrial permeability under pro-inflammatory conditions. The contribution of mK_{ATP} channels to this crosstalk is so far only based on inhibitors and openers of mK_{ATP} channels (e.g. 5-HD, glibenclamide and diazoxide). Evidence for involvement of redox-sensitive protein kinases (PKC, cSrc) is based on pharmacological and genetic inhibition of these kinases and inhibition of the crosstalk and rescue of the adverse effects. The role of mitochondrial superoxide/hydrogen peroxide and calcium in this process was also shown by specific scavengers and chelators as well as MnSOD deficiency for mitochondrial superoxide importance. PTP means protein tyrosine phosphatase. This scheme includes the concepts of previous work on "ROS-induced ROS" (for review see (Brandes, 2005; Di Lisa and Bernardi, 2006; Daiber, 2010; Dikalov, 2011; Nickel *et al.*, 2014; Schulz *et al.*, 2014; Zorov *et al.*, 2014)). Modified from Kröller-Schön *et al.* and Daiber, *Antioxid. Redox Signal.* 2014 (Kroller-Schon *et al.*, 2014). With permission of Mary Ann Liebert, Inc. Copyright 2014.

pores such as the permeability transition pore (mPTP) (Brandes, 2005; Di Lisa *et al.*, 2011; Jian *et al.*, 2014) and mPTP directly stimulates superoxide release (Hou *et al.*, 2014). Alternatively, aquaporins were identified as mitochondrial channels that conduct small uncharged molecules such as water but also hydrogen peroxide facilitating the release of H_2O_2 to the cytosol (Almasalmeh *et al.*, 2014). In addition, under inflammatory conditions such as sepsis mitochondrial permeability is largely increased allowing direct release of mitochondrial superoxide to the cytosol (Piskernik *et al.*, 2008).

Although mitochondrial hydrogen peroxide can confer most of the redox chemical reactions required for the here described signalling pathways (e.g. oxidation of zinc-sulphur complexes, thiol oxidations), superoxide (it remains to be elucidated whether formed from primary mitochondrial source or secondary crosstalk-activated sources such as Nox) has at least two specific reactivities not shared by hydrogen peroxide (summarized in (Ullrich and Kissner, 2006)): 1) Interaction with metal centers as observed in the redox-regulation of calcineurin. 2) Consumption of nitric oxide under formation of peroxynitrite. Without detailed discussion here about the higher chemical reactivity of peroxynitrite (and its derived free radicals) over hydrogen peroxide it should be kept in mind that a small portion of superoxide could elicit effects different from hydrogen peroxide (via peroxynitrite formation or direct interaction with transition metal centres), even when the latter is present at higher concentrations (for review see (Bachschmid *et al.*, 2005; Ullrich and Kissner, 2006)).

Role for cyclophilin D, calcium and other redox-regulated structures for increased mitochondrial permeability by the mPTP

In 2011, redox sensitive cysteine 203 in the regulator of mPTP, cyclophilin D, was shown to act as a redox switch of mPTP by conferring increased opening probability of the pore under oxidative stress conditions (Figure 5) (Nguyen *et al.*, 2011). Transfection of cells with the C203S mutant of cyclophilin D decreased hydrogen peroxide-dependent mPTP opening and cell death. Interestingly, S-nitrosation of cysteine 203 also decreased H_2O_2-induced mPTP opening to the level of cyclophilin D deficient cells. Many other redox-sensitive regulatory structures related to mPTP have been suggested (e.g. nitration of the voltage-dependent anion channel [VDAC] and oxidation of vicinal thiols in the adenine nucleotide translocase [ANT] summarized in (Radi *et al.*, 2002; Daiber, 2010; Schulz *et al.*, 2014)). For instance, peroxynitrite leads to mPTP opening (Vieira *et al.*, 2001). However, even the pore forming subunits of mPTP are not finally identified, leaving the exact molecular nature of the mPTP elusive (Bernardi and Di Lisa, 2015).

According to very recent findings the IP_3 agonists angiotensin-II and endothelin-1 cause activation of a sarcoplasmic IP_3-receptor (type 3) that colocalises with mitochondria and subsequent activation of the mitochondrial ryanodine receptor (mRyR1) to increase mitochondrial calcium levels causing opening of the mPTP in an ATP-dependent fashion with subsequent, time-dependent loss of mitochondrial calcium (Seidlmayer *et al.*, 2014). Similar observations were made in ischemia reperfusion triggered mPTP opening (Seidlmayer *et al.*, 2015). The involved receptors might be located in the so-called mitochondrial calcium microdomain (Nickel *et al.*, 2014).

Mitochondria-associated membranes, ER calcium trafficking and impact on mPTP

Another emerging concept of mitochondrial calcium homeostasis is the "mitochondria-associated membranes" one. Several contact sites ("microdomains") between ER and mitochondria were identified and subsequent studies have identified an IP_3R-GRP75-VDAC1 complex is localized in these microdomains and contributes to calcium homeostasis (Rizzuto *et al.*, 1993; Szabadkai *et al.*, 2006). IP_3R1-dependent Ca^{2+} release in the environment of mitochondria-associated membranes is tightly regulated by the ER oxidoreductase Ero1-Lα (Anelli *et al.*, 2012) and contributes to the opening of mPTP since some of its subunits are probably localised in mitochondria-associated membranes (Bonora *et al.*, 2013). Interestingly, the content of $p66^{Shc}$ in mitochondria-associated membranes increases with age (Lebiedzinska *et al.*, 2009), a condition for which we have recently reported on a mitochondria-Nox crosstalk in wild type and glutathione peroxidase-1 knockout mice at different age (Oelze *et al.*, 2014).

A more speculative redox pathway for increased mitochondrial permeability and oxidant formation – the mtK_{ATP}-mPTP-axis

Based on a more speculative hypothesis, the redox trigger for mitochondrial superoxide/hydrogen peroxide formation could involve the mitochondrial ATP-sensitive potassium channels (mtK_{ATP}) upon direct activation by ROS and RNS (most probably via thiol oxidation/nitrosation) (Queliconi *et al.*, 2011) or can be opened by redox-based activation of PKC(ε) isoforms (Ohnuma *et al.*, 2002). According to many reports opening of the mtK_{ATP} channel during ischemia/reperfusion may confer highly protective effects (e.g. in the setting of myocardial infarction) (Di Lisa *et al.*, 2007). Therefore, it might be complicated to explain the opening of mPTP by ROS-induced opening of mtK_{ATP} with simultaneous depolarization of the mitochondrial membrane potential and mitochondrial superoxide/hydrogen peroxide formation since previous work just showed the opposite (Costa *et al.*, 2006). Decrease in $\Delta\Psi_m$ would impair mitochondrial ATP synthesis leading to diminished calcium uptake, an essential activator of mPTP opening, which makes opening of the pore unlikely in deenergised mitochondria unless another stronger trigger such as redox-driven mPTP opening would compensate for the lack of calcium (Bernardi and Di Lisa, 2015; Zorov *et al.*, 2014). In summary of these reports in favour of a role for mtK_{ATP} in the redox crosstalk of NADPH oxidase and mitochondria one could say that activation of mitochondrial superoxide/hydrogen peroxide formation is sensitive to mtK_{ATP} inhibitors and openers.

Likewise, mPTP opening could be triggered in deenergised and depolarized mitochondria by alternative regulation of mitochondrial calcium levels (Saotome *et al.*, 2005). In various reports, a collapse of $\Delta\Psi_m$ was observed prior to mitochondrial swelling and opening of mPTP (reviewed in (Zorov *et al.*, 2014)). Alternatively, the direct stimulation of mitochondrial calcium uptake by AT-II via ryanodine receptors in the mitochondrial calcium microdomain may

compensate for missing ATP (Seidlmayer *et al.*, 2014). Likewise, the inhibitor of signal transducer and activator of transcription 3 (STAT3) stattic allowed mPTP opening at lower calcium levels, which was associated with increased mitochondrial hydrogen peroxide release (Boengler *et al.*, 2013). Regulation by uncoupling proteins (Chen *et al.*, 2015) and connexin 43 (Schulz *et al.*, 2007) represent other important pathways associated with mPTP opening and mtROS release.

The concept of "redox-optimized ROS balance" as a possible explanation of paradox in mitochondrial redox signalling

However, the concept of "redox-optimized ROS balance" challenges the dogma that mitochondrial superoxide/hydrogen peroxide formation and release is a direct function of mitochondrial respiration and membrane potential with the general assumption that loss of $\Delta\Psi_m$ results in decreased oxidative stress (for review see (Nickel *et al.*, 2014)). Regulation of glutathione peroxidase and peroxiredoxin activity by NADH/NADPH ratio and nicotinamide nucleotide transhydrogenase (Nnt) activity plays a major role in this process. Whereas in isolated mitochondria uncoupled respiration was associated with decreased mitochondrial superoxide/hydrogen peroxide formation and release (Starkov and Fiskum, 2003), opposite observations were made in isolated cardiomyocytes with uncoupled mitochondria (Aon *et al.*, 2003; Aon *et al.*, 2010). Based on the concept of "ROS-induced ROS release" extramitochondrial ROS can lead to loss of $\Delta\Psi_m$ and consumption of NADH/NADPH with subsequent burst-like release of mitochondrial superoxide to the cytosol associated with the opening of channels in the inner mitochondrial membrane, namely mPTP (Zorov *et al.*, 2000) or IMAC (Aon *et al.*, 2003).

Mechanism of redox crosstalk from mitochondria to NADPH oxidases and vice versa to induce vascular dysfunction

Evidence for this redox crosstalk

The interaction of mitochondrial superoxide/hydrogen peroxide with NADPH oxidases and vice versa may represent an essential redox axis for the pathogenesis of various disease (for review see (Kimura *et al.*, 2005; Daiber, 2010; Dikalov, 2011; Nickel *et al.*, 2014; Schulz *et al.*, 2014)). The kindling radicals for initiation of the crosstalk can be formed by mitochondrial dysregulation in the aging process (Oelze *et al.*, 2014), in response to nitroglycerin therapy, in the animal model of MnSOD deficiency (Wenzel *et al.*, 2008c) or by angiotensin II-dependent induction of the NADPH xoxidases (for summary see Table 3). Mitochondrial superoxide/hydrogen peroxide was also implicated in redox signalling events in response to β-amyloid, lipopolysaccharide induced sepsis, or in the setting of hyperglycaemia, cancer as well as redox regulation of transcription (Schulz *et al.*, 2014). Here we focus on recent publications and will provide the most actual overview on the detailed mechanism of this crosstalk.

Redox crosstalk from NADPH oxidases to mitochondria – role of mitochondrial pores and channels

This crosstalk can either start at the level of the NADPH oxidase (most likely the phagocytic isoform Nox2 or the Nox1, which can use the same cytosolic regulatory subunits as Nox2 and use the same redox triggers such as redox-sensitive PKC or related kinases such as cSrc) or at the mitochondrial site – depending on the disease or stress condition (Figure 5) (Kimura *et al.*, 2005; Wenzel *et al.*, 2008b; Daiber, 2010; Kroller-Schon *et al.*, 2014; Schulz *et al.*, 2014). Angiotensin II infusion activates Nox2 via formation of the classical PKC activator diacylglycerol. Likewise hyperglycaemia via advanced glycation end product (AGE)/AGE receptor (RAGE) signalling, activation of the renin-angiotensin-aldosterone system (RAAS), low-grade inflammation and the aging process via similar mechanisms lead to activation of different NADPH oxidase isoforms, which will generate the kindling radicals. These initially formed ROS contribute either directly or indirectly via redox-sensitive PKCε (and other isoforms) or MAPK to mitochondrial alkalinisation, impaired calcium haemostasis, change in mitochondrial membrane potential and finally increased mitochondrial superoxide/hydrogen peroxide formation/release (Heinzel *et al.*, 2005; Andrukhiv *et al.*, 2006; Di Lisa *et al.*, 2007), which may involve a number of mitochondrial sources of ROS (Figure 5).

So far, the involvement of mtK_{ATP} channels was only indirectly proven by the effects of inhibitors such as glibenclamide or 5-hydroxydecanoic acid (5-HD) and openers such as diazoxide (Kimura *et al.*, 2005; Doughan *et al.*, 2008; Wenzel *et al.*, 2008b; Dikalov *et al.*, 2014). However, the mtK_{ATP} openers diazoxide or pinacidil increased mitochondrial ROS in a 5-HD-sensitive manner, whereas a potassium ionophore (valinomycin) increased mitochondrial ROS formation to a similar extent but without being affected by 5-HD (Krenz *et al.*, 2002). mtK_{ATP} opening and mitochondrial ROS formation in response to acetylcholine involved a tyrosine (Src) kinase (Oldenburg *et al.*, 2003a). Likewise, the putative sarcolemma-selective K_{ATP} channel opener P1075 caused mtK_{ATP} channel opening and mitochondrial ROS formation in isolated cardiac myocytes (Oldenburg *et al.*, 2003b).

In contrast, mtK_{ATP} activation has been also shown to prevent cellular ROS formation and cell death in cultured neurons, and mtK_{ATP} opening directly and significantly prevented mitochondrial H_2O_2 release in isolated brain mitochondria (Fornazari *et al.*, 2008), although a well-known therapeutic feature of mtK_{ATP} opening in ischemic preconditioning in cardiac tissue is based on initial mild ROS formation in response to mtK_{ATP} opening leading to subsequent induction of redox-sensitive transcription factor and up-regulation of antioxidant and protective genes (Garlid, 2000; Philipp *et al.*, 2006). In line with this observation, vitamin C therapy blocked cardioprotection by ischemic preconditioning in pigs supporting a role of ROS in this process (Skyschally *et al.*, 2003). However, protection by mtK_{ATP} opening usually requires timely restricted activation of mitochondrial pores (e.g. by exact temporal periods of ischemia and reperfusion or repeated short-time challenges with organic nitrates) (Hausenloy *et al.*, 2004; Lim *et al.*, 2007), which could represent a major difference to the adverse effects of continuous opening of this pore. Even tolerance to the protective effects of ischemic preconditioning has been described (Gori *et al.*, 2010; Lisi *et al.*, 2012). Likewise, the duration of ischemia has large impact on the role of mPTP opening for I/R

damage (Ruiz-Meana *et al.*, 2011) and short-term openings could have protective whereas long-term openings could lead to detrimental effects (reviewed in (Bernardi and Di Lisa, 2015)). Recently, it was shown that hyperlipidemia leads to inhibition of cardioprotection by mtK_{ATP} channels (Csonka *et al.*, 2014).

Signalling and damage by mitochondrial oxidants in the cytosol

Likewise, these kindling radicals can directly contribute to the dysregulation of eNOS, sGC, ET-1 pathways or prostanoid synthesis (by the above described redox signalling events, see also Figure 2) with functional consequences (e.g. endothelial and cardiac dysfunction, increased vascular tone and hypertension). The vicious circle starts when the mitochondrial superoxide/hydrogen peroxide are released from the mitochondrial matrix to the cytosol, a process that is thought to be associated with a number of mitochondrial pores and channels or with increased mitochondrial permeability under pro-inflammatory conditions (Figure 5). mPTP inhibition by cyclosporine A, sanglifehrin A or cyclophilin D deficiency, respectively, or mitochondria-targeted antioxidants mitoTEMPO (superoxide scavenger) or mitoEbselen (hydrogen peroxide scavenger) prevented the activation of NADPH oxidases, normalized ROS formation and improved disease related complications (Doughan *et al.*, 2008; Wenzel *et al.*, 2008b; Dikalova *et al.*, 2010; Dikalov *et al.*, 2014; Kroller-Schon *et al.*, 2014). Once released to the cytosol these mitochondrial superoxide/hydrogen peroxide may activate secondary ROS sources such as Nox2 (Nox1) via PKC or tyrosine kinase cSrc (which is either up-stream of PKC or more important for ROS-triggered Nox2 activation than PKC (Dikalov *et al.*, 2014; Kroller-Schon *et al.*, 2014)) mediated translocation of regulatory cytosolic Nox subunits, which was further substantiated by the effects of various Nox inhibitors, genetic deletions or silencing of Nox2 related subunits.

According to recent data, Src family kinase (SFK) Lyn functions as a redox sensor in leukocytes to detect H_2O_2 at wounds in zebrafish larvae (Yoo *et al.*, 2011, 2012). The role of tyrosine kinases in the redox crosstalk between mitochondria and NADPH oxidases was also established in a model of cellular starvation by widespread increase in tyrosine kinase activity and impaired protein tyrosine phosphatase activity leading to a positive feedback loop involving generation of ROS by NADPH oxidase and mitochondria (Graham *et al.*, 2012). The importance of calcium in these specific processes (e.g. for activation of protein kinases (Tsuda *et al.*, 1986) or opening of mPTP (Di Lisa *et al.*, 2007)) and the crosstalk in general is summarized in Table 4. According to a recent review by Zorov *et al.* calcium might play no role at all for mPTP opening in intact cells due to replacement by some other cytosolic factors but there seems to be an interconnection between ROS and calcium dependent down-stream signalling (Juhaszova *et al.*, 2004; Zorov *et al.*, 2014).

Redox crosstalk from mitochondria to NADPH oxidases

The crosstalk can also originate from the mitochondrial site when mitochondrial superoxide/hydrogen peroxide are directly induced by the aging process (Wenzel *et al.*, 2008c; Oelze *et al.*, 2014), hypoxia/reperfusion, nitroglycerin treatment or manganese superoxide dismutase deficiency (Figure 5, Table 3). In this case the mitochondrial superoxide/hydrogen peroxide serve as kindling radicals and activate Nox2 (Nox1) leading to more potent cytosolic ROS formation by the NADPH oxidases and eventually xanthine oxidase (upon redox conversion from the xanthine dehydrogenase form) with subsequent dysregulation and uncoupling of eNOS (Kroller-Schon *et al.*, 2014). Indeed, we have recently shown that the aging process increases mitochondrial superoxide/hydrogen peroxide formation, induces low-grade inflammation, leads to activation of NADPH oxidases and finally results in endothelial/vascular dysfunction, all of which is significantly aggravated in the absence of MnSOD (increased mitochondrial ROS) (Wenzel *et al.*, 2008c) but also deficiency in the glutathione peroxide-1 (increased cytosolic ROS) (Oelze *et al.*, 2014).

Table 4

Evidence for an essential role of calcium for the redox crosstalk between mitochondria and NADPH oxidases.

Model and calcium signaling site	Ref.
Superoxide Flux in Endothelial Cells via the Chloride Channel-3 Mediates Intracellular Calcium/ROS Signaling.	(Hawkins *et al.*, 2007)
Hypoxia-triggered Nox1 activation contributes to an increase in cytosolic calcium levels.	(Rathore *et al.*, 2008)
Hypoxia-triggered mtROS formation induces accumulation of cytoplasmic calcium via dissociation of FK506-binding protein 12.6 from ryanodine receptor 2.	(Wang and Zheng, 2010; Liao *et al.*, 2011)
Ischemia leads to closed mPTP and calcium accumulation that is released to the cytosol due to mtROS formation and pH increase with subsequent mPTP opening during the reperfusion phase with collapse of $\Delta\Psi_m$, matrix swelling and NAD^+ release.	(Di Lisa *et al.*, 2011)
ROS trigger oscillations of $\Delta\Psi_m$ involving mitochondrial calcium uptake that can induce reperfusion arrhythmias and modulate the opening probability of the mPTP.	(Maack and Bohm, 2011)
mtROS increase cytosolic calcium levels and modulators of $[Ca^{2+}]_i$ such as BAPTA-AM or thapsigargin impair the mtROS-Nox2 crosstalk in human B lymphoblasts.	(Dikalov *et al.*, 2012)
The chelator of intracellular calcium, BAPTA-AM, completely prevented the mitochondrial superoxide/hydrogen peroxide-Nox2 crosstalk in isolated leukocytes.	(Kroller-Schon *et al.*, 2014)

Which NADPH oxidase is the target of this redox crosstalk?

The Nox2 isoform and to a minor extent also Nox1 isoform exert several redox switches (e.g. redox sensitive kinases, protein disulphide isomerases) making them a preferred target of redox regulation (Kroller-Schon *et al.*, 2014; Schulz *et al.*, 2014). The Nox2 isoform of immune cells plays an important role for development of endothelial dysfunction and hypertension, an early vascular disturbance that leads to the development of atherosclerosis (Guzik *et al.*, 2007; Wenzel *et al.*, 2011). The silencing of Nox2, but not of Nox1, Nox4 and Nox5 normalizes angiotensin II-induced mitochondrial and cytosolic ROS formation as well as cSrc activation in cultured endothelial cells and genetic Nox2 deletion improves hypertension in response to angiotensin II (Dikalov *et al.*, 2014). Nox2 deficiency prevents eNOS S-glutathionylation and uncoupling in aortic tissue upon in vivo angiotensin II treatment (Kroller-Schon *et al.*, 2014). Although Nox4 silencing normalized ROS formation and eNOS function as well as upregulation of Nox4 expression in mesangial cells treated with angiotensin II (Lee *et al.*, 2013), a recent yeast two-hybrid-based study on the interaction of Nox4 with complex I revealed no relevance of Nox4 for basal mtROS formation (Hirschhauser *et al.*, 2015). In addition, there is only limited evidence for direct redox-based activation pathways of Nox4 (besides the transcriptional control) and for a role of mitochondrial superoxide/hydrogen peroxide in this process. Of note, microRNA-mediated NOX4 upregulation in hyperlipidemia is responsible for oxidative and nitrosative stress and deterioration of cardiac function pointing towards epigenetic regulation of Nox4 expression (Varga *et al.*, 2013).

Conclusions and clinical implications

The present review highlights the mechanisms underlying increased oxidative stress in cardiovascular disease. Although it is in general accepted that oxidative stress plays a crucial role in the initiation and continuation of the atherosclerotic process, therapies to fight oxidative stress such as supplementation with "classical antioxidants" such as vitamins yielded disappointing results. A so far underestimated reason may be among others the more complex nature of ROS formation e.g. via redox crosstalk mainly between the vascular and phagocytic NADPH oxidase and mitochondria resulting in the production of so called kindling radicals ultimately leading to eNOS uncoupling. More recently, the involvement of the immune system in the development of vascular dysfunction (Karbach *et al.*, 2014) and the "ROS-induced ROS concept" further contributed to the complexity of this crosstalk hypothesis but also provided new explanations on so far unanswered questions (Zorov *et al.*, 2014). Of note, mitochondrial ROS were reported to activate the NLRP3 inflammasome (Zhou *et al.*, 2011), promote the expression of proinflammatory cytokines in LPS-induced sepsis (Bulua *et al.*, 2011) and improve bactericidal activity (West *et al.*, 2011) (for review see (Ortona *et al.*, 2014)). Putting together these lines of evidence with recent data on redox activation of immune cells by mitochondrial superoxide/hydrogen peroxide formation (Dikalov *et al.*, 2012; Kroller-Schon *et al.*, 2014) provides the basis for tight association between redox regulatory pathways and inflammation. The discovery of the players in this crosstalk, potential mechanisms and in addition the more and more complete understanding why and how eNOS uncoupling occurs will help to develop more specific and therefore more powerful antioxidants which may ultimately be more successful than current therapeutic approaches.

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Conflict of interest

We certify that there is no conflict of interest with any financial organizations regarding the materials discussed in the manuscript.

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