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AIM AND SCOPES

Cell Membranes and Free Radical Research is a print and online journal that publishes original research articles, reviews and short reviews on the molecular basis of biophysical, physiological and pharmacological processes that regulate cellular function, and the control or alteration of these processes by the action of receptors, neurotransmitters, second messengers, cation, anions, drugs or disease.

Areas of particular interest are four topics. They are;

A- Ion Channels (Na^+ - K^+ Channels, Cl^- channels, Ca^{2+} channels, ADP-Ribose and metabolism of NAD^+, Patch-Clamp applications)

B- Oxidative Stress (Antioxidant vitamins, antioxidant enzymes, metabolism of nitric oxide, oxidative stress, biophysics, biochemistry and physiology of free oxygen radicals

C- Interaction Between Oxidative Stress and Ion Channels

(Effects of the oxidative stress on the activation of the voltage sensitive cation channels, effect of ADP-Ribose and NAD^+ on activation of the cation channels which are sensitive to voltage, effect of the oxidative stress on activation of the TRP channels)

D- Gene and Oxidative Stress (Gene abnormalities. Interaction between gene and free radicals. Gene anomalies and iron. Role of radiation and cancer on gene polymorphism)

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Keywords

Ion channels, cell biochemistry, biophysics, calcium signaling, cellular function, cellular physiology, metabolism, apoptosis, lipid peroxidation, nitric oxide synthase, ageing, antioxidants, neuropathy.

Oxidative Stress and Vascular Function

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List of abbreviations

RONS reactive oxygen or nitrogen species
mPTP mitochondrial permeability transition pore
NOX1
NOX2
eNOS

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Abstract

Many drug-induced complications and diseases are known to be associated with or even based on a dysequilibrium between the formation of reactive oxygen or nitrogen species (RONS) and the expression/activity of antioxidant enzymes that catalyze the breakdown of these harmful reactive species. The "kindling radical" concept is based on the initial formation of RONS that in turn activate additional sources of RONS in certain pathological conditions. Recently, we and others have demonstrated such "cross-talk" between NADPH oxidases and mitochondria in the setting of nitroglycerin-induced nitrate tolerance, the aging process and angiotensin-II triggered arterial hypertension via redox pathways compromising the mitochondrial, ATP-sensitive potassium channel (mK_{ATP}), the mitochondrial permeability transition pore (mPTP), cSrc and protein kinases and the NADPH oxidase isoform Nox2 (and eventually Nox1). This review will focus on the uncoupling of endothelial nitric oxide synthase (eNOS) by initially formed "kindling radicals" (RONS) and on the different "redox switches" that are involved in the uncoupling process of eNOS. S-glutathionylation of the eNOS reductase domain, adverse phosphorylation of eNOS, and of course the oxidative depletion of tetrahydrobiopterin (BH_4) will be highlighted as potential "redox switches" in eNOS. In addition, RONS-triggered increases in levels of asymmetric dimethylarginine (ADMA) and L-arginine depletion will be discussed as alternative reasons for dysfunctional eNOS. Finally, we present the clinical perspectives of eNOS uncoupling (and dysfunction) for the development and progression of cardiovascular disease and discuss the important prognostic value of the measurement of endothelial function (e.g. by flow-mediated dilation or forearm plethysmography) for patients with cardiovascular disease.

Keywords

Oxidative stress, superoxide, peroxynitrite, nitric oxide synthase uncoupling, NADPH oxidase, mitochondria, xanthine oxidase.

Introduction

Oxidative stress has been demonstrated to be a hallmark of most cardiovascular and neurodegenerative diseases (Griendling and FitzGerald 2003; Ischiropoulos and Beckman 2003). The most common reactive oxygen and nitrogen species (RONS) include superoxide radicals, hydrogen peroxide, hydroxyl radicals, carbon-centered peroxides and peroxyl radicals, nitric oxide radicals (•NO), nitrogen dioxide radicals, peroxynitrite, and hypochlorite. In physiological concentrations, these oxidative species work as intracellular messengers. Among these, the best understood is nitric oxide (•NO), which acts as an important vasodilator and inhibitor of platelet activation (Furchgott and Zawadzki 1980). Oxidative stress defines the state of cellular redox imbalance in favor of free radicals, produced by sources like NADPH oxidase or mitochondrial respiratory chain, and reduced protecting antioxidant enzyms such as superoxide dismutases (mitochondrial MnSOD and cytosolic/extracellular Cu,Zn-SOD) shown by Fridovich and coworkers in the 1960s (McCord et al. 1971).

Redox signaling versus oxidative stress

Peroxynitrite ($ONOO^-$), generated through a diffusion-controlled reaction between •NO and superoxide, is a much more potent oxidant than •NO and superoxide and its contribution to cardiovascular and neurodegenerative disease has long been accepted (Ischiropoulos and Beckman 2003; Turko and Murad 2002). In the absence of •NO, superoxide is either degraded through a spontaneous self-dismutation or catalytically scavenged by SODs. Due to its short half-life and limited reactivity, superoxide mainly reacts with metal centers such as iron-sulfur-clusters (present for instance in aconitase (Flint et al. 1993) or calcineurin (Namgaladze et al. 2002)). Higher concentrations of superoxide anions cause the yield of hydroxyl radicals. This phenomenon is described as the Haber-Weiss-Cyle, with an ferric ion (Fe^{3+}) as the essential katalysator. An increase of hydroxyl radicals enhances immediately the risk of DNA strand breaks and oxidation of any biomolecule. In the presence of •NO, the dismutation of superoxide is outcompeted by the formation of peroxynitrite since the reaction of •NO with $•O_2^-$ is 3- to 5-fold faster than the dismutation of $•O_2^-$ catalyzed by SODs. ONOO- confers similar redox modifications (mainly oxidation of thiols, thioethers or metal-catalyzed nitration of tyrosines), leading to an outage of the protective proteins and oxidative stress. In the longterm the consequences is death of particluar cells (apotosis/necrosis) and the hole organism. These events are discussed in detail in previous reviews (Beckman and Koppenol 1996; Daiber and Ullrich 2002; Radi 2004) and in figure 1.

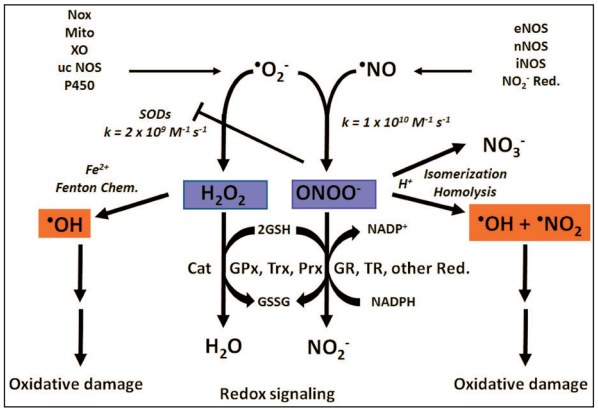

Figure 1. The chemical basis of vascular redox signaling. Superoxide formation from NADPH oxidases (Nox), the mitochondrial respiratory chain (Mito), xanthine oxidase (XO), an uncoupled NO synthase and P450 side reactions confers redox signaling mainly upon breakdown by self-dismutation or catalyzed by superoxide dismutases (SODs) to hydrogen peroxide. H_2O_2 modulates the thiol/disulfide equilibrium and thereby modifies enzymatic activities (e.g. in zinc-finger-motifs as found in transcription factors). Reaction with thiol groups is also a major route of detoxification for H_2O_2 via peroxiredoxins (Prx) and thioredoxins (Trx) or the selenol in glutathione peroxidase (GPx) – these systems require energy consuming recycling by NADPH-coupled reductases. Another route for H_2O_2 decomposition is catalyzed by catalase (Cat). When H_2O_2 accumulates it may lead to the Fenton reaction (hydroxyl radical formation) upon reaction with ferrous iron (Fe^{2+}) triggering severe oxidative damage at the protein, lipid and DNA level. Likewise, nitric oxide (previously termed EDRF) is formed from neuronal, endothelial or inducible NO synthases or reduction of nitrite from nutrition conferring the diffusion-controlled reaction with superoxide to yield peroxynitrite anion ($ONOO^-$). This rapid kinetics even outcompetes the extremely fast SOD-catalyzed breakdown of superoxide making the formation of peroxynitrite a cellular event. Once formed, peroxynitrite may inactivate SOD isoforms (via nitration/dityrosine formation and disruption of the Cu,Zn-complex) further increasing superoxide formation in a positive feedback fashion. $ONOO^-$ confers similar redox signaling mechanisms as compared to H_2O_2 but is 100-1000-fold more potent in mediating these reactions. Upon protonation, peroxynitrite undergoes either spontaneous isomerization to nitrate (representing a detoxifying route) or undergoes homolysis to form the hydroxyl radical and the nitrogen dioxide radical leading to oxidative damage comparable to the Fenton reactivity of H_2O_2. Summarized from Daiber and Ullrich et al. (Daiber and Ullrich 2002; Frein et al. 2005; Ullrich and Kissner 2006). Daiber et al., I. Laher (ed.), Systems Biology of Free Radicals and Anti-Oxidants, *Springer-Verlag Berlin Heidelberg* 2014. With permission of Spinger Verlag Heidelberg. Copyright © 2014.

Molecular proof of a damaging role of oxidative stress in cardiovascular diseases

The first reports on the role of oxidative stress in the progression and pathophysiology of cardiovascular disease were published in the early 1990's by Harrison and Ohara in an experimental model of hypercholesterolemia (Harrison and Ohara 1995; Ohara et al. 1993). Molecular proofs of the pathophysiological compound of oxidative stress in cardiovascular diseases was provided by a large number of preclinical studies using genetic tools (e.g. knockout mice) which clarified the involvement of ROS producing or degrading enzymes in the onset and progression of these diseases. Moreover, deletion of the NADPH oxidase subunits $p47^{phox}$ and Nox1 has a protective effect on blood pressure and endothelial function in angiotensin-II-induced hypertension in mice (Landmesser et al. 2002; Matsuno et al. 2005). In contrast, overexpression of Nox1 in these transgenic mice causes a further increase in blood pressure (Dikalova et al. 2005). Just as well, partial deletion of the mitochondrial superoxide dismutase ($MnSOD^{+/-}$) increased age-dependent mitochondrial oxidative stress and endothelial dysfunction (Wenzel et al. 2008c). These data (along with several other ones in the literature (Chen et al. 2012; Daiber 2010; Schulz et al. 2012)) provide molecular proof of the crucial role of oxidative stress in causing cardiovascular disease.

The "kindling radical" hypothesis

Under the terms of the concept of "kindling radicals" (or also "bonfire" hypothesis), initial formation of ROS (e.g. from NADPH oxidases) triggers further damage such as eNOS uncoupling by different mechanisms (see "redox switches" below). The ROS-induced ROS production concept can be extended to almost any kind of source of RONS as almost all of these sources contain "redox switches". Beyond cytoplasmic enzymes, there is clear evidence of a cross-talk between mitochondrial ROS formation and NADPH oxidases (Daiber 2010)(Schulz et al. 2012).

Examples for this cross-talk between mitochondrial and NADPH oxidase-derived ROS are based on the observation that chronic nitroglycerin treatment increases mitochondrial oxidative stress, a phenomenon which has been attributed a mechanistic importance in nitrate tolerance (loss of nitroglycerin bioactivation by mitochondrial aldehyde dehydrogenase [ALDH-2]), but also with NADPH oxidase-induced dysfunction of eNOS. Demonstrating the existence of a cross-talk between different cellular ROS sources, the mitochondrial permeability transition pore (mPTP) blockade or genetic deletion of subunits of the phagocytic NADPH oxidase both improved eNOS dysfunction in nitrate tolerance and angiotensin-II induced arterial hypertension (Wenzel et al. 2008b)(Kroller-Schon et al. 2013). The hypothesis underlying this cross-talk is based on induction of mitochondrial ROS formation, directly by mitochondrial dysregulation in the aging process, under nitroglycerin therapy, MnSOD deficiency, or by angiotensin-II driven activation of the NADPH oxidase (Nox2, Nox1) with subsequent impairment of the mitochondrial respiration via redox- activated mitochondrial, ATP-sensitive potassium channels (mK_{ATP}) (figure 2). Upon escape of mtROS to the cytosol, they activate redox-sensitive cSrc kinase or protein kinase pathways that lead to the activation of Nox2 (or Nox1) and this secondary burst triggers eNOS uncoupling with endothelial dysfunction (via the "redox switches" in eNOS that are described below) or further increase mitochondrial dysfunction initiating a vicious circle (Kroller-Schon et al. 2013).

Other origins of oxidative stress have similar redox switches: Consequently the uncoupling of eNOS (but also other isoforms) is based on increased formation of oxidants and various reports exist that demonstrate that eNOS function is improved and uncoupling is reversed when the sources of oxidative stress are either inhibited by pharmacological manipulations (e.g. by PKC inhibitors, NADPH oxidase inhibitors or AT_1-receptor-blockers) (Guzik et al. 2002; Knorr et al. 2011; Li et al. 2006; Loomis et al. 2005; Mollnau et al. 2002; Wenzel et al. 2008d) or there is a genetic deletion of the $p47^{phox}$ or $gp91^{phox}$ subunit resulting in dysfunctional phagocytic NADPH oxidase (Landmesser et al. 2003; Xu et al. 2006; Zhang et al. 2011). Similar observations were made when antioxidants were added acutely and at high concentrations to the system (e.g. infusion of vitamin C) (Heitzer et al. 1996a; Heitzer et al. 2001b). The interconnection between different sources of oxidative stress also explains the observation that inhibition of only one source of RONS is able to completely normalize a cardiovascular disease state. This was shown for the inhibition of NADPH oxidase by apocynin in diabetes, hypertension and ischemia/reperfusion (Dodd and Pearse 2000; Hayashi et al. 2005; Li et al. 2003).

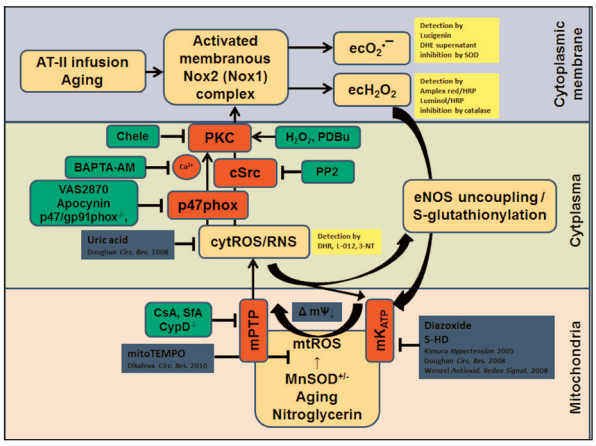

Figure 2. Postulated molecular mechanisms of the crosstalk between mitochondria and NADPH oxidase through reactive oxygen species based on studies in white blood cells and in genetic/pharmacological animal. The brown boxes represent fundamental processes involved in the process of the crosstalk between mitochondria and NADPH oxidase through reactive oxygen species and the genetic/pharmacological stress factors that trigger this crosstalk. The red boxes contain important enzymatic constituents of the mitochondrial-Nox redox signaling axis and the red circle represents the important role of cytosolic calcium levels. The green boxes represent genetic and pharmacological inhibitors and activators of this crosstalk. The yellow boxes show the detection assays used for the involved ROS and RNS. The blue boxes represent previous findings providing the basis for our understanding of the crosstalk concept (Kimura et al. 2005, Doughan et al. 2008, Wenzel et al. 2008, Dikalova et al. 2010). Modified from Kröller-Schön et al., Antioxid. Redox Signal. 2013 (Kroller-Schon et al. 2013). With permission of Mary Ann Liebert, Inc. Copyright © 2013.

Redox switches in endothelial nitric oxide synthase (eNOS)

In addition to the classical regulation of enzymatic activity of eNOS (e.g. by calcium/calmodulin, caveolin, HSP90, palmitoylation and myristoylation), other regulatory pathways such as phosphorylation and S-glutathionylation are forthrightly linked to the formation of redox-active species. These "redox switches" in eNOS confer alterations in enzymatic eNOS activity and may contribute to uncoupling of eNOS as depicted in figure 3. Uncoupling of eNOS is a condition by which electrons leak from the transport in the reductase domain (from NADPH over FMN and FAD) and are transmitted to molecular oxygen to yield superoxide instead of NO. This reversion is even more disadvantageous than inhibition of eNOS, because uncoupling will switch the enzyme from a nitric oxide to a superoxide source, changing from a protective/beneficial to a harmful/toxic phenotype (Forstermann and Munzel 2006; Munzel et al. 2005). It should be noted that not only eNOS may be uncoupled and produce superoxide but also neuronal NOS (type 1) (Miller et al. 1997; Pou et al. 1999; Vasquez-Vivar et al. 1999a; Vasquez-Vivar et al. 1999b) and inducible NOS (type 2) (Miller et al. 2000; Ungvari et al. 2003; Xia et al. 1998a; Xia and Zweier 1997b) are subject to the uncoupling phenomenon and produce superoxide in the uncoupled state.

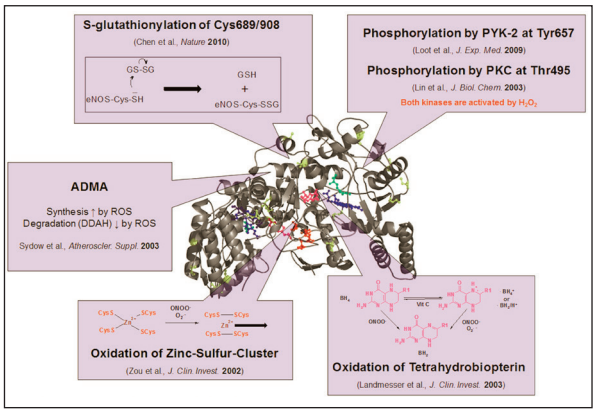

Figure 3. X-ray structure of human eNOS with the iron-porphyrin (blue), the substrate L- arginine (green), the P450-forming axial iron-thiolate ligand from a cysteine residue (yellow), the cofactor BH4 (purple), the zinc-thiolate complex forming cysteines (red, two from each subunit) and the zinc ion (brown). The blue boxes represent the "redox switches" in eNOS triggering regulatory pathways that depend on oxidants and reductants. Modified from Daiber and Münzel, Steinkopff Verlag Darmstadt 2006 (Daiber and Münzel 2006). With permission of Steinkopff Verlag Darmstadt. Copyright © 2006.

Oxidative depletion of tetrahydrobiopterin

Among the regulatory pathways ("redox switches") of eNOS, the oxidative depletion of tetrahydrobiopterin (BH_4) is the most prominent (figure 3). Several independent groups provided substantial evidence for a causative role of BH_4 depletion in the process of NOS uncoupling characterized by superoxide formation in the presence of the electron source NADPH (Vasquez-Vivar et al. 1999a; Vasquez-Vivar et al. 1998; Xia et al. 1998b). Milstein and Katusic reported highly efficient oxidative degradation of BH_4 to dihydrobiopterin (BH_2) by peroxynitrite and thereby provided an explanation of how RONS (especially peroxynitrite) may contribute to oxidative uncoupling of eNOS (Milstien and Katusic 1999). The understanding of the importance of vitamin C for the recycling or rescue of the $\bullet BH_4^+$ radicals (once BH_2 is formed only energy-consuming enzymatic reaction confers reduction to BH_4) (Baker et al. 2001; d'Uscio et al. 2003; Kuzkaya et al. 2003; Whitsett et al. 2007), provided an attractive explanation for the highly beneficial effects of vitamin C infusion

on improvement of endothelial function (measured by plethysmography) in chronic smokers (Heitzer et al. 1996a) and diabetic patients (Heitzer et al. 2001a). The extent of the effect of vitamin C on endothelial function even turned out to be a valuable prognostic marker for cardiovascular events (risk for cardiovascular disease) (Heitzer et al. 2001b).

The first direct evidence of a role of BH_4 depletion in eNOS uncoupling and subsequent endothelial dysfunction in vivo was obtained in hypertensive mice in 2003 (Landmesser et al. 2003). Clinical evidence for a role of oxidative BH_4 is based on improvement of endothelial function[1] in chronic smokers by BH_4 but not by tetrahydroneopterin (NH_4), which shares the same antioxidant properties with BH_4 but is not a cofactor for eNOS (Heitzer et al. 2000a; Heitzer et al. 2000b). Likewise, supplementation with the BH_4 analogue folic acid improves endothelial function in human subjects (Antoniades et al. 2006; Gori et al. 2001) or treatment with the BH_4 precursor sepiapterin restored endothelial function in experimental hypertension as well as atherosclerosis (Laursen et al. 2001; Schuhmacher et al. 2010).

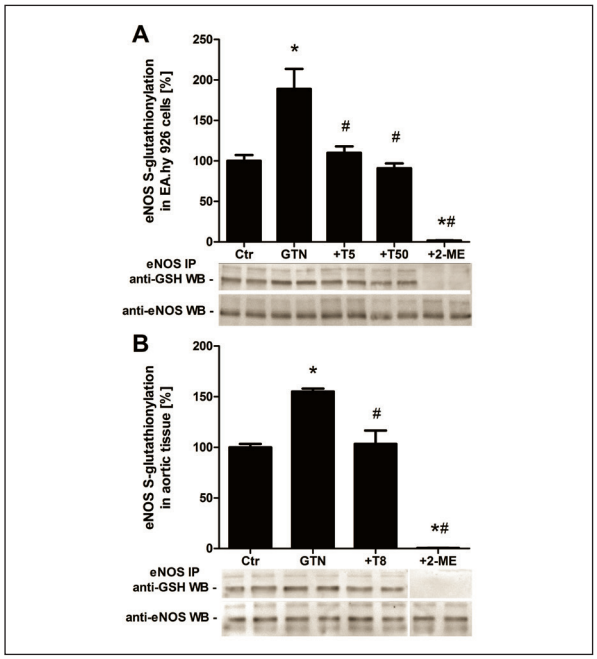

Figure 4. Effects of telmisartan treatment on S-glutathionylation of eNOS in nitroglycerin treated endothelial cells and nitrate tolerant rats. S-glutathionylation of eNOS was determined by eNOS immunoprecipitation from (A) and (B) samples, followed by anti- glutathione staining and normalization on eNOS. Disappearance of the anti-glutathione staining in the presence of 2-mercaptoethanol served as a control. Representative blots are shown at the bottom of each densitometric quantification along with the respective loading control. From Knorr et al., Arterioscler. Thromb. Vasc. Biol. 2011 (Knorr et al. 2011). With permission of Wolters Kluwer Health. Copyright © 2011.

Furthermore pharmacological inhibition of eNOS in vessels decreased superoxide formation (Munzel et al. 2000b) and forearm blood flow in arteries of GTN-treated volunteers was improved by folic acid (Gori et al. 2001; Gori et al. 2003). Finally, BH_4 levels in vessels from tolerant rabbits were significantly decreased (Ikejima et al. 2008). Likewise, Nox1 overexpression and/or angiotensin-II infusion in mice caused a decrease in vascular BH_4 levels, vascular NO bioavailability, eNOS uncoupling, loss of eNOS dimers and vascular dysfunction that were normalized by BH_4 supplementation (Dikalova et al. 2010b). Similar mechanisms were reported to be responsible for diastolic dysfunction (Silberman et al.) representing a potential therapeutic target in cardiovascular disease (Harrison et al. 2010).

S-glutathionylation of the eNOS reductase domain

S-glutathionylation represents an important redox regulatory mechanism for many enzymes (e.g. mitochondrial aldehyde dehydrogenase (Wenzel et al. 2007), or SERCA (Adachi et al. 2004)) and was also reported for eNOS (figure 3). According to a recent report by Zweier and coworkers, eNOS is adversely regulated and uncoupled by S-glutathionylation at one or more cysteine residues of the reductase domain (Chen et al. 2010) mostly at 689 and 908. In the same year, Manevich et al. have detected S-glutathionylated eNOS in response to nitrosative stress but rather under artificial conditions (Manevich et al. 2010). In a subsequent study, Chen et al. have demonstrated superoxide-induced thiyl radical formation in eNOS with subsequent intracellular disulfide formation or S-glutathionylation, both leading to uncoupling of eNOS and superoxide formation by the enzyme (Chen et al. 2011).

Based on recent observations by Knorr and colleagues, eNOS S-glutathionylation is largely increased in nitroglycerin-treated endothelial cells and aortic tissue from nitroglycerin- infused rats (figure 4), probably contributing to eNOS uncoupling and endothelial dysfunction in the setting of nitrate tolerance, which was prevented by AT_1-receptor blocker therapy with telmisartan (Knorr et al. 2011). Therefore, eNOS S-glutathionylation may represent a new "redox master switch" controlling NO versus superoxide production by this enzyme.

[1] Measured by acetylcholine-dependent increase in forearm blood flow by plethysmography. This technique is based on an increase in vessel diameter and blood flow by proximal infusion of acetylcholine in increasing doses by a catheter and simultaneous monitoring of the vessel diameter and/or blood flow using Doppler ultrasound.

Other Mechanisms

Phosphorylation represents another redox-sensitive regulatory pathway of eNOS (figure 3). There are 3 different important phosphorylation modifications of eNOS: First, the activating phosphorylation at serine1177 (Dimmeler et al. 1999), second, the inactivating phosphorylation at tyrosine657 (Loot et al. 2009) and third, the inactivating phosphorylation at threonine495 (Fleming et al. 2001; Lin et al. 2003). PKC, which is activated in endothelial cells in response to GTN treatment (Lin et al. 2003), can actually cause eNOS uncoupling via phosphorylation of eNOS at Thr495. Therefore, both regulatory pathways, s-glutathionylation and phosphorylation, may be regarded as "redox switches" in eNOS.

Another direct redox-regulatory pathway for eNOS function is the oxidative disruption of the zinc-sulfur-complex ($ZnCys_4$) in the binding region of the eNOS dimer resulting in a loss of SDS-resistant eNOS dimers, which has been first described by Zou and coworkers for peroxynitrite-mediated oxidation of eNOS (Zou et al. 2002).

Assymetric dimethylarginine (ADMA) is probably the most potent endogenous inhibitor of eNOS (Boger 2003a) and it is still a matter of debate whether or not ADMA itself may lead to uncoupling of eNOS (Sydow and Munzel 2003). Thus, oxidative stress in the vasculature may to ADMA concentrations that significantly inhibit eNOS activity (Cooke 2000) or may even uncouple the enzyme and switch it to a superoxide synthase.

Furthermore L-Arginine is the endogenous substrate of eNOS providing the nitrogen for NO synthesis. According to a number of publications, "L-arginine deficiency" may contribute to uncoupling of eNOS (Bode-Boger et al. 2007). Many studies reported a positive effect of L-arginine supplementation in different settings of endothelial dysfunction (e.g. hypercholesterolemia, hypertension, diabetes mellitus, etc) as reviewed in (Sydow and Munzel 2003), and controversy still exists on the protective effect of L-arginine (Bevers et al. 2006). To this date it is too early to decide on whether L-arginine therapy or deficiency significantly contributes to endothelial dysfunction and cardiovascular events, but the above mechanisms represent feasible hypotheses to justify further investigations on this subject.

Identification of uncoupled endothelial nitric oxide synthase (eNOS) in vascular cells and tissue

Direct detection of eNOS-derived superoxide formation

According to previous reports on nitrate tolerance (Munzel et al. 2000, Munzel et al. 2000) and experimental diabetes (Hink et al. 2001), atherosclerosis (Laursen et al. 2001, Mollnau et al. 2003) as well as hypertension (Mollnau et al. 2002, Landmesser et al. 2003) by Münzel and Harrison, superoxide formation from uncoupled eNOS is best detected by direct measurement of superoxide formation in the presence and absence of NOS inhibitors. Increase in superoxide signal in the control tissue (excluding direct antioxidant effects of the NOS inhibitors) and a decrease in superoxide signal in the diseased sample by NOS inhibitors, can be explained by the elimination of •NO formation (from intact eNOS) in the control groups since breakdown by •NO was blocked

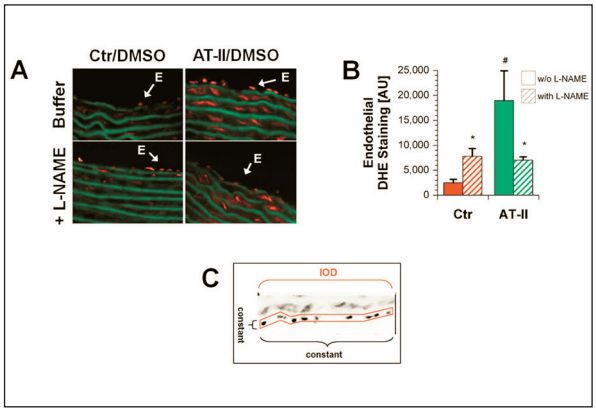

Figure 5. Detection of eNOS uncoupling by oxidative fluorescence microtopography. (A) To determine eNOS-dependent ROS formation, vessels were preincubated with the NOS inhibitor L-NAME (500 μM, lower panel), embedded in Tissue Tek resin, frozen, cryo- sectioned and stained with DHE (1 μM). (B) Densitometric data are presented as bar graphs. Pictures and data shown are representative for ≥ 4 animals per group. *, P<0.05 vs. control/DMSO. (C) eNOS uncoupling was assessed by densitometric quantification of DHE staining in the endothelial cell layer which was extracted from the whole microscope image. A fixed area was used for densitometric quantification and the procedure is shown for one representative endothelial cell layer of AT-II treatment group. eNOS uncoupling was previously assessed by the effects of L-NAME on DHE staining (Oelze et al. 2006, Wenzel et al. 2008). The method of densitometric quantification of endothelial DHE staining was adopted from the protocol of Alp et al. (Alp et al. 2003). From Schuhmacher et al., Hypertension 2010 (Schuhmacher et al. 2010). With permission of Wolters Kluwer Health. Copyright © 2010.

and superoxide may accumulate. In contrast, inhibition of uncoupled eNOS results in decreased superoxide formation in the diseased group. These studies used the chemiluminescence dye lucigenin, that undergoes superoxide specific formation of a dioxetane intermediate with subsequent decomposition to acridone and emission of chemiluminescent light (Liochev and Fridovich 1997). Lucigenin represents a valid tool for biological detection of superoxide when working at low lucigenin concentrations (5 μM) without exogenous addition of NADH (Daiber et al. 2004). Other suitable detection methods for NOS uncoupling (always in combination with a specific NOS inhibitor such as L-NAME) include the cytochrome c assay (SOD inhibitable signal) (Landmesser et al. 2003, Cai et al. 2005), HPLC-based 2-hydroxyethidium quantification (the superoxide-specific oxidation product of dihydroethidine) (Dikalova et al. 2010, Xia et al. 2010) and dihydroethidine (DHE)-dependent oxidative fluorescence microtopography in DHE-stained aortic cryo-sections (Hink et al. 2001, Oelze et al. 2006, Wenzel et al. 2008, Schuhmacher et al. 2010, Knorr et al. 2011). EPR-based measurement of superoxide-adducts with different spin probes (e.g. DMPO-OOH) would also display high specificity for superoxide but the sensitivity of these spin probes in vascular samples or cell culture is limited.

An example for detection of eNOS uncoupling by DHE-derived oxidative fluorescence microtopography is presented in figure 5 (Schuhmacher et al. 2010). Likewise, vascular NOS uncoupling may be detected by lucigenin-enhanced chemiluminescence in response to reaction with superoxide in intact aortic ring segments as presented in figure 6 (Wenzel et al. 2008).

Indirect detection of eNOS uncoupling

The indirect methods to detect eNOS uncoupling are mainly based on functional readout of eNOS activity (namely NO formation or endothelium-dependent vasodilation) in response to therapy/incubation with compounds that have been reported to improve the function (coupling state) of eNOS (e.g. folic acid,

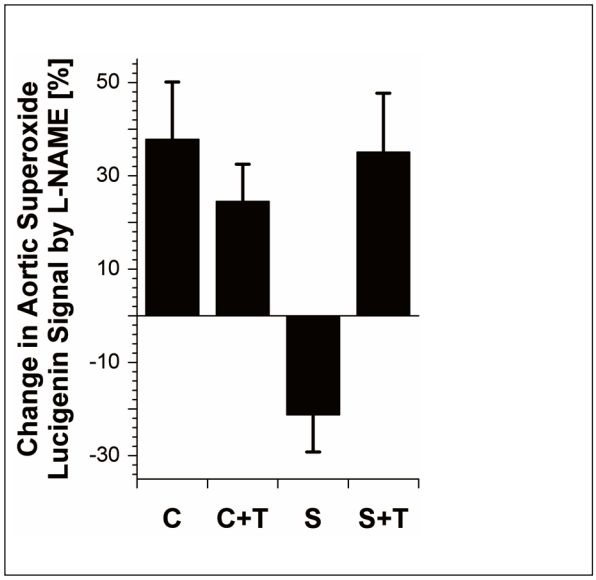

Figure 6. Effects of telmisartan treatment on vascular NOS uncoupling in aorta from diabetic (STZ-treated) rats. Lucigenin-enhanced chemiluminescence was used to assess vascular ROS formation in intact aortic ring segments. eNOS-dependent superoxide formation was assessed by substraction of lucigenin signal of L-NAME-treated aortic rings from lucigenin signal without L-NAME and expressed as percentage change of signal based on the L-NAME-free group. The data are mean±SEM of 12–20 aortic rings from 5 to 7 animals/group. *, P<0.05 vs. control group and #, P<0.05 vs. STZ-treated group. Data are shown for control (C), control/telmisartan (C+T), STZ (S), and STZ/telmisartan (S+T) animals. From Wenzel et al., Free Radic. Biol. Med. 2008 (Wenzel et al. 2008). With permission of Elsevier. Copyright © 2008.

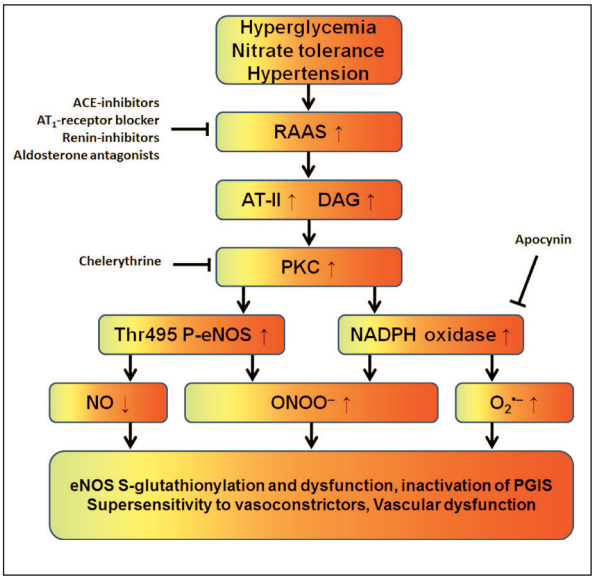

Figure 7. Sequence of events during activation of the renin-angiotensin-aldosterone system (RAAS) in the setting of hyperglycemia (diabetes mellitus type 1 and 2), nitrate tolerance or hypertension. Some therapeutic and experimental interventions are also shown. AT-II, angiotensin-II; DAG, diacylglycerol; PKC, protein kinase C; Thr495 P-eNOS, threonine495- phosphorylated endothelial NO synthase; •NO, nitric oxide; $•O_2$-, superoxide; ONOO-, peroxynitrite; PGIS, prostacyclin. From Oelze et al., Exp. Diabetes Res. 2010. With permission of the authors. Copyright © 2010.

sepiapterin, BH_4) (Gori et al. 2001, Laursen et al. 2001, Alp et al. 2003, Antoniades et al. 2006, Wang et al. 2008, Dikalova et al. 2010). A prominent example for indirect detection of eNOS uncoupling is the impaired NO synthesis in DOCA- salt induced hypertension (indicated by the decreased EPR-NO-signal) and improvement of NOS activity by supplementation with BH_4 or genetic deletion of the NADPH oxidase subunit $p47^{phox-/-}$ (eliminating the Nox2-dependent superoxide formation that could represent the "kindling radical" for NOS uncoupling. These beneficial effects of BH_4 and its precursors in various disease models were reviewed in detail by Schulz et al. (Schulz et al. 2008).

Clinical perspective on the role of endothelial dysfunction as a consequence of endothelial nitric oxide synthase (eNOS) uncoupling in cardiovascular diseases

According to a number of reports in the literature and as shown in figure 7, endothelial dysfunction, atherosclerosis and the late cardiovascular complications of these adverse phenomena are associated with a chronic activation of the local and/or circulating renin-angiotensin-aldosterone system (RAAS). Suggesting a role of an inappropriate RONS production in this setting, diabetic patients show particularly beneficial responses to mineralocorticoid receptor blockade (Pitt et al. 2003), ACE inhibitor (Mehler et al. 2003), AT_1-receptor blockade (Kintscher et al. 2007) and renin inhibitor (Parving et al. 2009) therapy. Likewise, nitrate tolerance is associated with an activation of the RAAS (Kurz et al. 1999) and experimental nitrate tolerant animals and tolerant patients take profit from AT1- receptor blockade (Hirai et al. 2003, Knorr et al. 2011) and ACE inhibitor (Heitzer et al. 1998, Watanabe et al. 1998, Berkenboom et al. 1999) therapy. The similarities between the mechanisms underlying endothelial dysfunction in diabetes mellitus and nitrate tolerance were recently discussed in detail (Oelze et al. 2010): they include the activation of a superoxide source such as the NADPH oxidase to produce the "kindling radicals" leading to uncoupling of eNOS via the above described "redox switches" and may also extend to direct uncoupling of eNOS by PKC-dependent phosphorylation of eNOS at Thr495. A number of experimental studies using transgenic or knockout mouse models have provided molecular evidence that oxidative stress leads to vascular (endothelial) dysfunction and associated cardiovascular diseases (see chapter 1.2). It has been shown that NO production in mice with deoxycorticosterone acetate (DOCA)-salt-induced hypertension approaches the normal level following genetic deletion of the NADPH oxidase subunit p47PHOX and simultaneous administration with the NOS cofactor tetrahydrobiopterin (Landmesser et al. 2003). These results demonstrate that NADPH oxidase-derived ROS contribute to the pathogenesis of hypertension and the associated endothelial dysfunction. Moreover, these results show that uncoupled NOS is a major source of ROS and that restoring eNOS function can cure hypertension in experimental systems.

Several other conditions (or risk factors) associated with cardiovascular disease are characterized by endothelial dysfunction. Endothelial dysfunction has been shown in chronic smokers (Heitzer et al. 1996, Heitzer et al. 2000), patients with increased LDL levels (Drexler and Zeiher 1991, Heitzer et al. 1996), patients with type I and II diabetes mellitus or metabolic syndrome (Ting et al. 1996, Williams et al. 1998, Butler et al. 2000, Heitzer et al. 2000, Heitzer et al. 2001, Mather et al. 2001) as well as hypertensive patients (Perticone et al. 2001, Tzemos et al. 2001). Several mechanisms could account for the reduction in endothelium-dependent vascular relaxation observed in these settings, including changes in the activity and/or expression of eNOS, decreased sensitivity of vascular smooth muscle cells to NO, or increased degradation of NO via its interaction with ROS such as superoxide. Suggesting an interaction between an inappropriately high bioavailability of ROS and endothelial dysfunction, vascular responses in these settings are improved by the antioxidant vitamin C (Heitzer et al. 1996, Duffy et al. 2001) (also reviewed in (Schulz et al. 2008)). Importantly, the extent of the improvement in endothelial function observed in response to vitamin C (i.e., a surrogate evidence of oxidative stress) has been shown to predict patients' prognosis (Heitzer et al. 1996, Heitzer et al. 2001, Warnholtz et al. 2007). In line with this evidence, there are a number of reports supporting the importance of endothelial function in predicting patients prognosis: for instance, the study by Gokce et al. in patients undergoing peripheral or coronary bypass surgery (Gokce et al. 2002). as well as a study by Perticone et al in patients with essential hypertension (Perticone et al. 2001).

Taken together, there is no doubt that measurement of endothelial function, and particularly the impact of oxidative stress on endothelial function, provides substantial prognostic information about future cardiovascular events in secondary prevention, whereas its role in primary prevention remains to be established.

Whether this can be clinically exploited remains a complicated question. As of now, there is sound evidence from clinical and experimental data for a role of uncoupled NOS in the development and progression of cardiovascular disease (Munzel et al. 2005, Forstermann and Munzel 2006, Schulz et al. 2008). More detailed information on this topic can be found in our upcoming book chapter in Systems Biology of Free Radicals and Anti-Oxidants (edited by I. Laher) and published by Springer-Verlag Berlin Heidelberg in 2014.

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