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REVIEW

Therapeutic effect of enhancing endothelial nitric oxide synthase (eNOS) expression and preventing eNOS uncoupling

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Nitric oxide (NO) produced by the endothelium is an important protective molecule in the vasculature. It is generated by the enzyme endothelial NO synthase (eNOS). Similar to all NOS isoforms, functional eNOS transfers electrons from nicotinamide adenine dinucleotide phosphate (NADPH), via the flavins flavin adenine dinucleotide and flavin mononucleotide in the carboxy-terminal reductase domain, to the heme in the amino-terminal oxygenase domain. Here, the substrate L-arginine is oxidized to L-citrulline and NO. Cardiovascular risk factors such as diabetes mellitus, hypertension, hypercholesterolaemia or cigarette smoking reduce bioactive NO. These risk factors lead to an enhanced production of reactive oxygen species (ROS) in the vessel wall. NADPH oxidases represent major sources of this ROS and have been found upregulated in the presence of cardiovascular risk factors. NADPH-oxidase-derived superoxide avidly reacts with eNOS-derived NO to form peroxynitrite ($ONOO^-$). The essential NOS cofactor (6*R*-)5,6,7,8-tetrahydrobiopterin (BH_4) is highly sensitive to oxidation by this $ONOO^-$. In BH_4 deficiency, oxygen reduction uncouples from NO synthesis, thereby converting NOS to a superoxide-producing enzyme. Among conventional drugs, compounds interfering with the renin-angiotensin-aldosterone system and statins can reduce vascular oxidative stress and increase bioactive NO. In recent years, we have identified a number of small molecules that have the potential to prevent eNOS uncoupling and, at the same time, enhance eNOS expression. These include the protein kinase C inhibitor midostaurin, the pentacyclic triterpenoids ursolic acid and betulinic acid, the eNOS enhancing compounds AVE9488 and AVE3085, and the polyphenolic phytoalexin *trans*-resveratrol. Such compounds enhance NO production from eNOS also under pathophysiological conditions and may thus have therapeutic potential.

Abbreviations

ACEI, angiotensin-converting enzyme inhibitor; ARB, AT_1 receptor blocker; BH_4 (6R-)5,6,7,8-tetrahydrobiopterin; DOCA, deoxycorticosterone acetate; eNOS, endothelial nitric oxide synthase; GCH1, guanosine-5′-triphosphate cyclohydrolase 1; GPx1, glutathione peroxidase 1; NO, nitric oxide; Nox, homolog protein of the nicotinamide adenine dinucleotide phosphate oxidase subunit gp91phox; $O_2^{-}\cdot$, superoxide anion; $ONOO^-$, peroxynitrite; ROS, reactive oxygen species; SHR, spontaneously hypertensive rats; SOD, superoxide dismutase

Introduction

The signalling molecule nitric oxide (NO) regulates vital functions such as neurotransmission or vascular tone (via activation of soluble guanylyl cyclase), gene transcription and mRNA translation (via iron-responsive elements), and post-translational modifications of proteins via ADP-ribosylation (Förstermann *et al.*, 1994). However, NO can also react with superoxide anion ($O_2^{-}\cdot$), forming the potent cytotoxin peroxynitrite ($ONOO^-$). $ONOO^-$ causes oxidative damage, nitration and S-nitrosylation of biomolecules including proteins, lipids and DNA (Ridnour *et al.*, 2004).

Three isoforms of NO synthase (NOS; EC 1.14.13.39) have been found in mammals. Neuronal 'n'NOS (or NOS I) is a low-output enzyme that is constitutively expressed in neurons and some other cell types. Inducible 'i'NOS (or NOS II) is a high-output enzyme, whose expression can be induced by cytokines and other agents in almost any cell type. Endothelial 'e'NOS (or NOS III) is also a low-output enzyme that is constitutively expressed in endothelial cells and few other cell types. The nature of nNOS and eNOS as low-output enzymes and iNOS as a high-output enzyme depends not so much on the conversion rate of the different isozymes, but rather reflects the short-lasting, pulsatile, Ca^{2+}-activated NO production of nNOS and eNOS versus the continuous, Ca^{2+}-independent NO production by iNOS. Whereas eNOS-derived NO is a protective principle in the vasculature (see below), the large amounts of NO produced by iNOS are mainly responsible for the fall in blood pressure in septic shock (MacMicking *et al.*, 1995; Liu and Huang, 2008). iNOS-derived NO may also contribute to vascular peroxynitrite formation (Upmacis *et al.*, 2007) and has been shown to be proatherogenic (Kuhlencordt *et al.*, 2001).

eNOS, an enzyme of major importance in the vasculature

In blood vessels, eNOS is the predominant NOS isoforms; it is responsible for most of the NO produced in this tissue (Schwarz *et al.*, 1999; Li and Förstermann, 2000b). NO dilates blood vessels by directly stimulating soluble guanylyl cyclase and increasing cyclic GMP in smooth muscle cells (Rapoport *et al.*, 1983; Förstermann *et al.*, 1986; Ignarro *et al.*, 1986). NO released towards the lumen of a blood vessel is a potent inhibitor of platelet aggregation and adhesion to the vascular wall (Alheid *et al.*, 1987; Busse *et al.*, 1987; Radomski *et al.*, 1987). NO can also inhibit leukocyte adhesion to the vessel wall by either interfering with the ability of the leukocyte adhesion molecule CD11/CD18 to bind to the endothelial cell surface or by suppressing CD11/CD18 expression on leukocytes (Kubes *et al.*, 1991; Arndt *et al.*, 1993). Leukocyte adherence is an early event in the development of atherosclerosis and therefore, NO may protect against the onset of atherogenesis. Furthermore, NO has been shown to inhibit DNA synthesis, mitogenesis and proliferation of vascular smooth muscle cells (Garg and Hassid, 1989; Nakaki *et al.*, 1990; Hogan *et al.*, 1992; Nunokawa and Tanaka, 1992). These antiproliferative effects are mostly mediated by cGMP (Garg and Hassid, 1989; Nakaki *et al.*, 1990; Nunokawa and Tanaka, 1992; Southgate and Newby, 1990). The inhibition of platelet aggregation and adhesion protects smooth muscle from exposure to platelet-derived growth factor(s). Therefore, NO also prevents a later step in atherogenesis, fibrous plaque formation. Finally, eNOS is probably essential for the function of endothelial progenitor cells involved in vascular repair (Aicher *et al.*, 2003; Sasaki *et al.*, 2006).

It is not surprising that a loss of function of such an enzyme in vascular disease has major pathophysiologic consequences. Pharmacological approaches to maintain or restore eNOS functionality (ability of the eNOS enzyme to produce NO) are thus warranted.

Normal function of eNOS and regulation of enzyme activity

Functional NOS enzymes are homodimers. The C-terminal reductase domain of one monomer [that binds nicotinamide adenine dinucleotide phosphate (NADPH), flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD)] is linked to the N-terminal oxygenase domain of the other monomer. This oxygenase domain carries a prosthetic heme group. The oxygenase domain also binds (6R-)5,6,7,8-tetrahydrobiopterin (BH_4), molecular oxygen and the substrate L-arginine (Crane *et al.*, 1998; Alderton *et al.*, 2001). All NOS isozymes catalyse flavin-mediated electron transfer from the C-terminally bound NADPH to the heme on the N-terminus. Calmodulin (CaM), upon calcium-induced binding to the NOS, increases both the electron transfer within the reductase domain (from NADPH to the flavins) and also the electron transfer from the reductase domain to the heme center in the oxygenase domain (Hemmens and Mayer, 1998). At the heme, the electrons are used to reduce and activate O_2. In order to synthesize NO, the enzyme needs to cycle twice. In a first step, NOS hydroxylates L-arginine to N^{ω}-hydroxy-L-arginine (which remains largely bound to the enzyme). In a second step, NOS oxidizes N-hydroxy-L-arginine to citrulline and NO (Noble *et al.*, 1999; Stuehr *et al.*, 2001).

eNOS synthesizes NO in a pulsatile, Ca^{2+}/CaM-dependent manner with eNOS activity markedly increasing when intracellular Ca^{2+} rises. However, eNOS can be activated by stimuli that do not produce sustained increases in intracellular Ca^{2+}, but still induce a long-lasting release of NO. Such best established stimulus is the shear stress of the flowing blood, which can increase enzyme activity at resting Ca^{2+} levels. This activation is mediated by phosphorylation of the enzyme (Fleming and Busse, 2003). The eNOS protein can be phosphorylated on several serine, threonine and tyrosine residues; however, major changes in enzyme function have been reported for the phosphorylation of serine 1177 (activation) and threonine 495 (inhibition) in the human eNOS sequence (Fleming, 2010). These mechanisms, together with eNOS expression levels and eNOS functionality, are determining factors of vascular NO production.

Cardiovascular risk factors lead to an enhanced production of reactive oxygen species (ROS) in the vascular wall

Many cardiovascular diseases are associated with increased levels of ROS in the vessel wall (Förstermann, 2008) (Figure 1). Several enzyme systems can potentially produce these ROS; however, four enzyme systems seem to predominate. These include the NADPH oxidases, xanthine oxidase, enzymes of the mitochondrial respiratory chain and uncoupled eNOS (Mueller *et al.*, 2005). Of these, NADPH oxidases seem to be the predominant source of ROS in the vasculature (Förstermann, 2008).

Different isoforms of O_2^{-}·-producing NADPH oxidase exist in the vascular wall. They are expressed in endothelial and

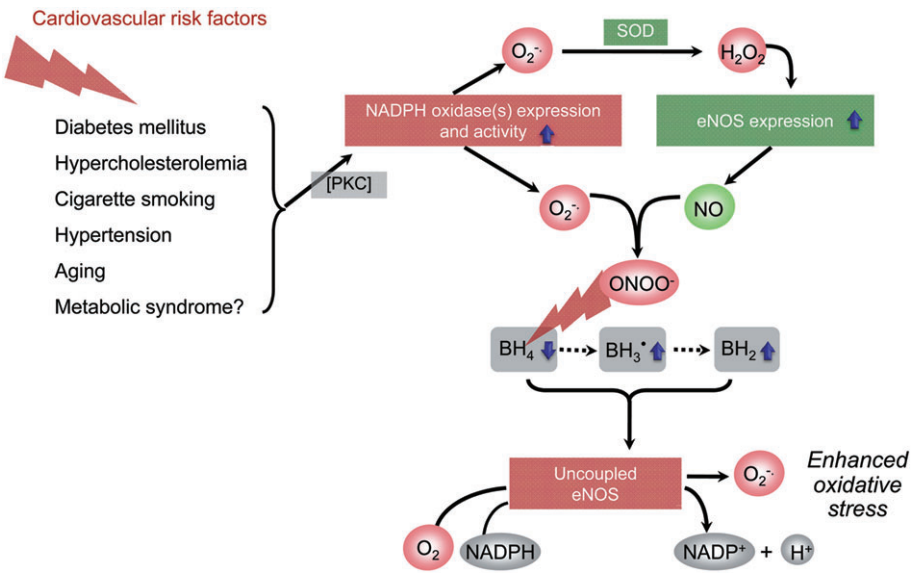

Figure 1

Potential mechanisms by which risk factors for atherosclerosis and cardiovascular disease lead to endothelial nitric oxide synthase (eNOS) uncoupling and endothelial dysfunction. In many types of vascular disease, nicotinamide adenine dinucleotide phosphate (NADPH) oxidases are upregulated in the vascular wall and generate superoxide ($O_2^{\cdot-}$). At least in diabetes and angiotensin II-induced hypertension the increased NADPH oxidase expression and activity seem to be a consequence of PKC activation. NADPH oxidases derived $O_2^{\cdot-}$ can be dismutated (or reduced) by superoxide dismutase (SOD) (or superoxide reductase). The resulting hydrogen peroxide (H_2O_2) can increase the expression eNOS, and, indeed, eNOS expression is increased in most types of vascular disease. The products of NADPH oxidases and eNOS, $O_2^{\cdot-}$ and nitric oxide (NO), respectively, rapidly recombine to form peroxynitrite ($ONOO^-$). This can oxidize the essential cofactor of eNOS (6*R*-)5,6,7,8-tetrahydrobiopterin (BH_4) to trihydrobiopterin radical ($BH_3\cdot$). $BH_3\cdot$ can be converted to the quinonoid 6,7-[8H]-H_2-biopterin, which is non-enzymatically rearranged to 7,8-dihydrobiopterin (BH_2). As a consequence, oxygen (O_2) reduction by eNOS is uncoupled from NO formation, and a functional eNOS is converted into a dysfunctional $O_2^{\cdot-}$-generating enzyme that contributes to vascular oxidative stress. The enhanced eNOS expression (see above) aggravates the situation.

smooth muscle cells, as well as the adventitia. Cardiovascular risk factors increase the expression and/or activity of these enzymes, leading to increased ROS production. Evidence for an activation of NADPH oxidase in the vasculature has been provided in animal models of hypertension [such as angiotensin II infusion (Rajagopalan *et al.*, 1996; Fukui *et al.*, 1997) or spontaneously hypertensive rats (SHR) (Morawietz *et al.*, 2001; Li *et al.*, 2006)] and for different forms of diabetes mellitus (Hink *et al.*, 2001). Also experimental hypercholesterolaemia is associated with an activation of NADPH oxidase (Warnholtz *et al.*, 1999). In atherosclerotic arteries, there is evidence for increased expression of the NADPH oxidase subunits gp91phox (Nox2) and Nox4, both of which can contribute to increased oxidative stress (Sorescu *et al.*, 2002).

In hypercholesterolaemia, both systemic and local renin angiotensin systems may be activated. Angiotensin-converting enzyme activity, as well as local angiotensin II concentrations are increased in atherosclerotic plaques (Diet *et al.*, 1996; Ohishi *et al.*, 1997), and the inflammatory cells present in the atherosclerotic vessel wall are capable of producing large amounts of angiotensin II. The stimulating effects of angiotensin II on the activity of NADPH oxidases suggest that an activated renin angiotensin system could cause increased vascular $O_2^{\cdot-}$ production and thus vascular dysfunction (Griendling *et al.*, 2000). In vessels from hypercholesterolaemic animals (Vergnani *et al.*, 2000) and in platelets from hypercholesterolaemic patients (Nickenig *et al.*, 1999), the angiotensin II receptor subtype AT_1 has been found to be upregulated, probably in response to low-density lipoprotein (Nickenig *et al.*, 1997). Thus, studies in laboratory animals and man have provided evidence for a stimulation of the renin angiotensin system in atherosclerosis and for a (subsequent) activation of NADPH oxidases in the vascular wall. In certain types of pathophysiology, other enzymes may also become important sources of ROS.

One such enzyme is xanthine oxidase (Landmesser *et al.*, 2007). However, its role in cardiovascular disease is controversial. Whereas some investigators reported an improvement of endothelial dysfunction in hypercholesterolaemic and diabetic patients with xanthine oxidase inhibitors such as oxypurinol and allopurinol (Cardillo *et al.*, 1997; Butler *et al.*, 2000), others failed to show an effect with allopurinol (O'Driscoll *et al.*, 1999).

Increased ROS formation in the vascular wall reduces bioactive NO by promoting NO inactivation (by reaction of superoxide with NO forming peroxynitrite) and by decreasing NO production (eNOS uncoupling, see below).

eNOS itself can be a source of superoxide

As described above, a functional eNOS transfers electrons from NADPH, via the flavins FAD and FMN in the carboxy-terminal reductase domain, to the heme in the amino-terminal oxyge-

nase domain, where the substrate L-arginine is oxidized to L-citrulline and NO. The flow of electrons within NOS is tightly regulated, and if disturbed the chemical reduction of oxygen and the generation of NO are uncoupled and $O_2^{-}\cdot$ is generated from the oxygenase domain (Stuehr *et al.*, 2001).

eNOS uncoupling has been seen *in vitro* in endothelial cells treated with low-density lipoprotein (Pritchard *et al.*, 1995), in peroxynitrite-treated rat aorta (Laursen *et al.*, 2001), and in isolated blood vessels from animals with pathophysiological conditions such as SHR (Cosentino and Luscher, 1998), stroke-prone spontaneously hypertensive rats (Kerr *et al.*, 1999), angiotensin II-induced hypertension (Mollnau *et al.*, 2002), deoxycorticosterone acetate (DOCA)-salt hypertension (Landmesser *et al.*, 2003), streptozotocin-induced diabetes (Hink *et al.*, 2001) or nitroglycerin tolerance (Münzel *et al.*, 2000). eNOS uncoupling has also been observed in patients with endothelial dysfunction due to diabetes mellitus (Heitzer *et al.*, 2000b), essential hypertension (Higashi *et al.*, 2002), hypercholesterolaemia (Stroes *et al.*, 1997) and in chronic smokers (Heitzer *et al.*, 2000a).

eNOS uncoupling may have major consequences on endothelial function. On the one hand, it reduces or abolishes formation, and on the other hand, it boosts pre-existing oxidative stress.

Oxidative stress produced by NADPH oxidase induces eNOS uncoupling

Due to the enhanced oxidative stress (see above), an increased degradation of NO by its reaction with $O_2^{-}\cdot$ is likely to occur in vascular disease. This, in turn, can lead to eNOS uncoupling by $ONOO^{-}$ and thus endothelial dysfunction.

There is a growing body of evidence that vascular NADPH oxidase plays a crucial role in the phenomenon of eNOS uncoupling. The important hint came from experiments with NADPH oxidase (p47phox) knockout animals (Landmesser *et al.*, 2003). When hypertension was induced in normal mice with a combination of the mineralocorticoid DOCA and salt, these animals showed an increased production of vascular ROS. This was significantly reduced by the NOS inhibitor N^{G}-nitro-L-arginine methyl ester (L-NAME), demonstrating a marked contribution of uncoupled eNOS to oxidative stress in vascular tissue. p47phox knockout animals showed much less oxidative stress upon DOCA-salt treatment, and levels of ROS could no longer be reduced with L-NAME (Landmesser *et al.*, 2003).

These findings demonstrate that NADPH oxidase-derived ROS can indeed represent the trigger leading to eNOS uncoupling, and that uncoupled eNOS significantly contributes to oxidative stress (Landmesser *et al.*, 2003).

Protein kinase C (PKC) is involved in some types of endothelial dysfunction

In some types of vascular disease, PKC activation is involved in the induction of oxidative stress. Vascular PKC activity has been found to be elevated in models of diabetes and angiotensin II-induced hypertension (Honing *et al.*, 1998; Hink *et al.*, 2001; Mollnau *et al.*, 2002). In these models, the increased NADPH oxidase expression and activity in the vascular wall, the enhanced $O_2^{-}\cdot$ generation and the eNOS uncoupling seem to be a consequence of PKC activity. PKC inhibitors reduced $O_2^{-}\cdot$ production, inhibited upregulation of NADPH oxidase *in vivo* and reversed eNOS uncoupling. Thus, increases in the expression and activity of NADPH oxidases are at least, in part, PKC-dependent. PKC activation also leads to an enhanced eNOS expression (Li *et al.*, 1998; Hink *et al.*, 2001), which, if the enzyme becomes uncoupled, aggravates the pathophysiological situation.

Oxidation of BH_4 can trigger eNOS uncoupling

NO and L-citrulline production by eNOS in endothelial cells correlates closely with the intracellular concentration of BH_4 (Werner-Felmayer *et al.*, 1993; Rosenkranz-Weiss *et al.*, 1994). In isolated arteries (Cosentino and Katusic, 1995) or rats *in vivo* (Yamashiro *et al.*, 2002), a BH_4 depletion produced endothelial dysfunction in a short period of time (Figure 1). In isolated aortas from pre-hypertensive SHR, BH_4 supplementation diminished the NOS-dependent generation of $O_2^{-}\cdot$ (Cosentino and Luscher, 1998). Administration of BH_4 restored endothelial function in animal models of diabetes (Pieper, 1997) and insulin-resistance (Shinozaki *et al.*, 2000), as well as in patients with hypercholesterolaemia (Stroes *et al.*, 1997), diabetes mellitus (Heitzer *et al.*, 2000b), essential hypertension (Higashi *et al.*, 2002) and in chronic smokers (Heitzer *et al.*, 2000a).

Intracellular BH_4 levels depend on the balance of its de novo synthesis and its degradation/oxidation. BH_4 is one of the most potent naturally occurring reducing agents. It is therefore reasonable to hypothesize that oxidative stress may lead to excessive oxidation and depletion of BH_4 (Milstien and Katusic, 1999; Laursen *et al.*, 2001). As oxidative stress occurs in cardiovascular pathophysiology (see above), oxidation of BH_4 may be the common cause of eNOS dysfunction in these situations. In agreement with this concept, BH_4 levels have been found decreased in the aorta from insulin-resistant rats (Shinozaki *et al.*, 1999), in plasma of SHR compared with age-matched WKY rats (Hong *et al.*, 2001), in aorta of hypercholesterolaemic apolipoprotein E- (apoE)-knockout mice (Laursen *et al.*, 2001) and in DOCA-salt-treated hypertensive rats (Landmesser *et al.*, 2003). Conversely, an infusion of the eNOS cofactor BH_4 can restore eNOS functionality, as demonstrated by studies in chronic smokers (Heitzer *et al.*, 2000a), oral glucose challenge (Ihlemann *et al.*, 2003), diabetics (Heitzer *et al.*, 2000b), hypercholesterolaemic patients (Stroes *et al.*, 1997) and hypertensive individuals (Higashi *et al.*, 2002).

It is particularly $ONOO^{-}$, the direct reaction product of NO and $O_2^{-}\cdot$, that oxidizes BH_4 to $BH_3\cdot$ radical. $BH_3\cdot$ can be re-reduced to BH_4 by NOS itself or non-enzymatically when enough reducing equivalents such as vitamin C are available (Kuzkaya *et al.*, 2003; Werner *et al.*, 2003). $BH_3\cdot$ radical can also be disproportionate to the quinonoid 6,7-[8H]-H_2-biopterin, which again can be reduced by vitamin C back to BH_4 (Werner *et al.*, 2003; Heller *et al.*, 2006).

Therapeutic effects of enhancing eNOS expression and preventing eNOS uncoupling

Strategies to increase eNOS protein without a concomitant augmentation of endothelial BH_4 levels may lead to eNOS uncoupling, enhanced oxidative stress and progression of vascular diseases. Therefore, compounds that increase eNOS protein levels are only beneficial when guaranteeing eNOS functionality (Li and Forstermann, 2009a). In the past, we have found compounds that maintain eNOS functionality in disease, and at the same time, upregulate expression of the enzyme.

Compounds AVE9488 and AVE3085

Two small-molecular-weight eNOS transcription enhancers, 4-fluoro-*N*-indan-2-yl-benzamide (AVE9488) and 2,2-difluoro-benzo[1,3]dioxole-5-carboxylic acid indan-2-ylamide (AVE3085), have been identified in a high throughput screening (Wohlfart *et al.*, 2008). These compounds stimulate eNOS transcription in endothelial cells *in vitro* and in vascular tissues *in vivo*. Importantly, treatment of apoE knockout mice with AVE9488 enhances vascular BH_4 levels and reverses eNOS uncoupling. In apoE knockout mice, but not in eNOS-knockout mice, treatment with AVE9488 reduces cuff-induced neointima formation. A 12-week treatment with AVE9488 or AVE3085 reduces atherosclerotic plaque formation in apoE knockout mice, but not in apoE/eNOS-double knockout mice (Wohlfart *et al.*, 2008). Moreover, AVE9488 reverses impaired functional activity of endothelial progenitor cells from patients with ischaemic cardiomyopathy (Sasaki *et al.*, 2006), and improves cardiac remodeling and heart failure after experimental myocardial infarction (Fraccarollo *et al.*, 2008). Despite the promising results in the above *in vivo* experiments, the long-term therapeutic benefit of AVE9488 is not (yet) known.

The protein kinase C inhibitor midostaurin

As mentioned above, PKC activation is involved in the induction of oxidative stress in several types of vascular disease. The ROS H_2O_2 in turn, enhances eNOS expression (Cai *et al.*, 2001). This may be an attempt of the organism to compensate for the reduced NO bioactivity. However, this compensation mechanism is often futile, because the eNOS enzyme becomes or remains uncoupled under pathological conditions. In fact, the upregulation of eNOS expression makes the situation even worse, because an uncoupled eNOS aggravates oxidative stress.

Midostaurin (4′-N-benzoyl staurosporine, CGP41251, PKC-412) is a glycosidic indolocarbazole analog of staurosporine that, via PKC inhibition, reduces NADPH oxidase expression (Li *et al.*, 2006). As a consequence of reduced oxidative stress, midostaurin increases BH_4 levels and reverses eNOS uncoupling in SHR and in atherosclerosis-prone apoE knockout mice (Li *et al.*, 2005, 2006).

However, based on the above mechanisms, inhibition of PKC also leads to a normalization of eNOS expression (Hink *et al.*, 2001). The result is a functional, NO-producing eNOS enzyme, yet at normal expression levels (Hink *et al.*, 2001). These effects are seen at low doses of midostaurin, used to inhibit PKC. At higher doses, however, midostaurin not only reverses eNOS uncoupling (via PKC inhibition), but also upregulates eNOS expression by a PKC-independent mechanism (Li and Forstermann, 2000a; Li *et al.*, 2005, 2006). The result is a NO-mediated vasodilation and a reduction in blood pressure (Li *et al.*, 2005, 2006).

Unfortunately, the therapeutic usefulness of the PKC inhibitor is limited by significant systemic toxicity *in vivo*.

Betulinic acid and ursolic acid

Recently, we have described vascular effects of two pentacyclic triterpenoid acids that occur in various plants: betulinic acid (Steinkamp-Fenske *et al.*, 2007b) and ursolic acid (Steinkamp-Fenske *et al.*, 2007a). Both compounds are secondary plant metabolites widespread in fruit peel, leaves and stem bark. They are also important components of oriental and traditional medicine herbs widely distributed all over the world (Ovesna *et al.*, 2004; Jäger *et al.*, 2009). So far, both betulinic acid (Steinkamp-Fenske *et al.*, 2007b) and ursolic acid are mainly known for their anti-tumour activity (Ovesna *et al.*, 2004; Mullauer *et al.*, 2010). We found that these triterpenoid acids upregulate eNOS expression, and at the same time, reduce NADPH oxidase expression in human endothelial cells through PKC-independent mechanisms (Steinkamp-Fenske *et al.*, 2007a,b) (Figure 2). The triterpenoids thus have the potential to reverse eNOS uncoupling. In addition, betulinic acid also enhances eNOS enzymatic activity by phosphorylation of eNOS at serine 1177 and dephosphorylation of eNOS at threonine 495 (N. Hohmann *et al.*, unpubl. data). Both compounds are devoid of prominent *in vivo* toxicity (at least in rodents) (Jäger *et al.*, 2009; Mullauer *et al.*, 2010). Their therapeutic potential in cardiovascular disease needs to be further investigated in *in vivo* studies.

Trans-*resveratrol*

Trans-resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) is a polyphenolic phytoalexin found in red grapes and several other plants (Li and Forstermann, 2009). *Trans*-resveratrol has been shown to prevent or slow the progression of a wide variety of diseases including cancer and cardiovascular diseases (Bradamante *et al.*, 2004). *Trans*-resveratrol also extends the lifespan of various organisms from yeast to vertebrates (Baur and Sinclair, 2006). This is true despite its low bioavailability. *Trans*-resveratrol can accumulate in tissues resulting in a ~30-fold enrichment over serum concentrations. In addition, *in vivo* concentrations of bioactive metabolites can be more than 10 times higher than the native compound (Baur and Sinclair, 2006). No significant *in vivo* toxicity has been reported for *trans*-resveratrol (Cottart *et al.*, 2010) which allows the use of the compound at high doses.

We have previously demonstrated that *trans*-resveratrol increases eNOS expression in human endothelial cells (Wallerath *et al.*, 2002) (Figure 2). In addition, others have shown that *trans*-resveratrol stimulates a complicated signalling pathway in endothelial cells that increases the interaction between oestrogen receptor-α, caveolin-1 and c-Src, and stimulates phosphorylation of caveolin-1, c-Src and eNOS (at serine 1177) (Klinge *et al.*, 2008). This results in an increased NO production by eNOS (Klinge *et al.*, 2008) (Figure 2).

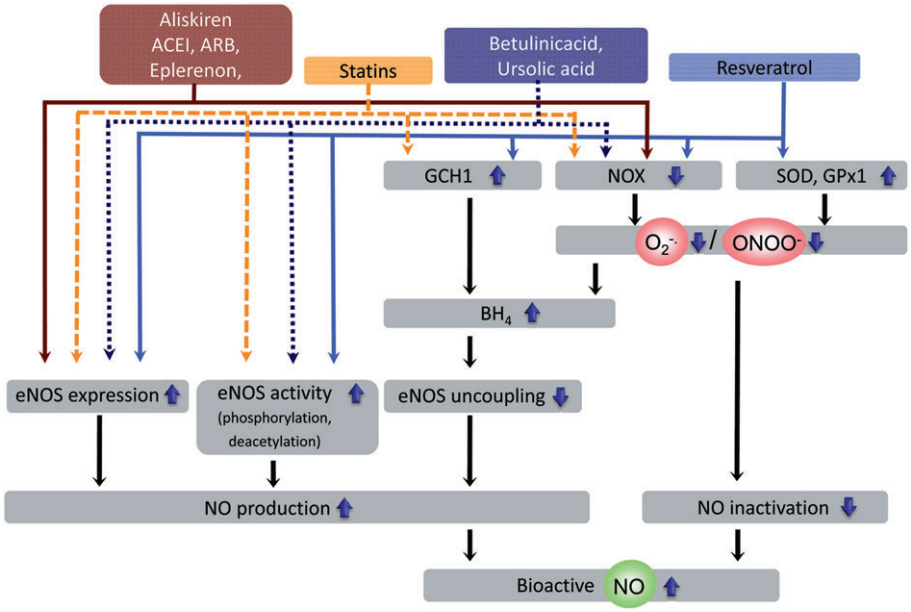

Figure 2

Therapeutic effects of enhancing endothelial nitric oxide synthase (eNOS) expression and preventing eNOS uncoupling. The renin inhibitor aliskiren, angiotensin-converting enzyme inhibitors (ACEI), angiotensin II receptor 1 blockers (ARB), as well as the selective aldosterone antagonist eplerenone enhance the expression of eNOS. In addition, these drugs prevent eNOS uncoupling by downregulating nicotinamide adenine dinucleotide phosphate oxidase (NOX) expression and activity, and by preventing (6*R*-)5,6,7,8-tetrahydrobiopterin (BH_4) oxidation (see text). Statins (3-hydroxy-3-methylgulutaryl-coenzyme A [HMG-CoA] reductase inhibitors) stabilize eNOS mRNA, downregulate NOX and increase BH_4 biosynthesis by upregulating GTP cyclohydrolase 1 (GCH1). Betulinic acid and ursolic acid are compounds that enhance eNOS expression and, at the same time, downregulate NOX expression. *Trans*-resveratrol stimulates the expression of eNOS. It also enhances enzyme activity by increasing phosphorylation at serine 1177 and by activating the protein deacetylase SIRT1, which in turn deacetylates eNOS at lysines 496 and 506 (in the calmodulin-binding domain), thereby stimulating eNOS activity. In addition, it upregulates antioxidant enzymes (such as superoxide dismutases (SOD) and glutathione peroxidase 1 (GPx1) and downregulates NOX. This leads to a reduction of peroxynitrite ($ONOO^-$)-mediated BH_4 oxidation and NO inactivation by superoxide $O_2^{\cdot-}$. *Trans*-resveratrol also stimulates the expression of GCH1.
The enhanced nitric oxide (NO) bioactivity resulting from increased NO production and reduced NO inactivation is likely to mediate/contribute to the vasoprotective effects of the aforementioned compounds.

Furthermore, eNOS has been shown to co-localize (and co-precipitate) with the protein deacetylase SIRT1 (silent mating type information regulation 2 homolog 1) (Mattagajasingh *et al.*, 2007). *Trans*-resveratrol can activate SIRT1, which in turn, deacetylates eNOS at lysines 496 and 506 in the calmodulin-binding domain, thereby stimulating eNOS activity and increasing endothelial NO production (Mattagajasingh *et al.*, 2007) (Figure 2).

Recent data from our own laboratory demonstrate that *trans*-resveratrol can also reverse eNOS uncoupling (Xia *et al.*, 2010). As a polyphenolic compound, *trans*-resveratrol has been shown to scavenge several types of radicals (including hydroxyl, superoxide and metal-induced radicals (Bradamante *et al.*, 2004). However, the direct antioxidant effect of *trans*-resveratrol is poor; the protective effects of trans-resveratrol against oxidative injury are likely to be attributed mostly to the upregulation of endogenous cellular antioxidant system, rather than its direct ROS scavenging activity. Treatment with *trans*-resveratrol leads to an upregulation of superoxide dismutases 1 (SOD1), SOD2, SOD3, glutathione peroxidase 1 and catalase in the hypercholesterolaemic apoE-knockout mice (Xia *et al.*, 2010), as well as in cultured human endothelial cells (Spanier *et al.*, 2009; Xia *et al.*, 2010) (Figure 2). At the same time, the expression and activity of NADPH oxidases are downregulated. As a result, peroxynitrite levels and BH_4 oxidation are reduced by *trans*-resveratrol (Xia *et al.*, 2010). Importantly, *trans*-resveratrol also increases BH_4 levels by upregulating GTP cyclohydrolase 1 (GCH1), the rate-limiting enzyme for BH_4 biosynthesis (Xia *et al.*, 2010) (Figure 2). The resulting reversal of eNOS uncoupling, along with the enhanced expression levels (Wallerath *et al.*, 2002) and enzymatic activity (eNOS phosphorylation and eNOS deacetylation) (Li and Forstermann, 2009), is likely to contribute to the protective effects of *trans*-resveratrol.

Statins

Statins (3-hydroxy-3-methylgulutaryl-coenzyme A reductase inhibitors) are a group of lipid-lowering drugs used in the prevention and treatment of cardiovascular disease. Although it is widely accepted that most of the clinical benefit obtained with statins is a direct result of their lipid-lowering properties, these agents appear to display additional cholesterol-independent or pleiotropic effects on various aspects of cardiovascular disease (Liao, 2002). These include improvement of endothelial function, stabilization of atherosclerotic plaques, inhibition of oxidative stress and inflammation, and reduction of thrombogenic responses (Liao and Laufs, 2005). These beneficial effects of statins are, in part, mediated by an

effect on eNOS because they can be inhibited by eNOS inhibitors (John *et al.*, 1998) and are absent in eNOS-deficient mice (Landmesser *et al.*, 2004).

Statins increase the expression of eNOS by a post-transcriptional mechanism involving inhibition of geranylgeranylation of Rho GTPase, and stabilization of eNOS mRNA (Laufs and Liao, 1998; Laufs *et al.*, 1998a) (Figure 2). Statins can also enhance eNOS activity by decreasing caveolin abundance (Feron *et al.*, 2001) and by post-translational mechanisms involving activation of the phosphatidylinositol 3-kinase/Akt pathway (Kureishi *et al.*, 2000) (Figure 2).

In addition, several statins inhibit endothelial $O_2^{-}\cdot$ formation by reducing the expression and/or activity of NADPH oxidase and by preventing the isoprenylation of p21 Rac, which is critical for the assembly of NADPH oxidase (Wagner *et al.*, 2000) (Figure 2). Extracellular SOD activity was more than doubled by simvastatin. Simvastatin treatment also increased the number of functionally active endothelial progenitor cells (Landmesser *et al.*, 2005).

Statins have also been shown to increase GCH1 mRNA expression in endothelial cells and to elevate intracellular BH_4 levels (Hattori *et al.*, 2003) (Figure 2). In streptozotocin-induced diabetic rats, atorvastatin normalizes endothelial function, reduces oxidative stress by inhibiting vascular NADPH oxidases and prevents eNOS uncoupling by an upregulation of GCH1 (Wenzel *et al.*, 2008).

The aforementioned effects may be responsible for part of the anti-atherogenic action of statins (Nissen *et al.*, 2006; Patel *et al.*, 2007).

Drugs interfering with the renin–angiotensin–aldosterone system

The renin–angiotensin–aldosterone system is upregulated in the vasculature of atherosclerotic vessels. Angiotensin II and aldosterone both promote endothelial dysfunction and atherosclerosis (Imanishi *et al.*, 2008a). Angiotensin II activates NADPH oxidases via AT_1 stimulation (Griendling *et al.*, 2000). In addition, the AT_1 receptor is upregulated *in vitro* by low-density lipoprotein (Nickenig *et al.*, 1997). Accordingly, drugs interfering with the renin–angiotensin–aldosterone system decrease vascular oxidative stress and improve bioavailability of vascular NO by various mechanisms.

The renin inhibitor aliskiren increases eNOS expression, enhances eNOS phosphorylation at serine 1177 (thereby increasing activity), decreases NADPH oxidase expression, augments vascular BH_4 levels and restores eNOS uncoupling in Watanabe heritable hyperlipidaemic rabbits (Imanishi *et al.*, 2008b) (Figure 2). The anti-atherosclerotic effect of aliskiren (Verma and Gupta, 2008) is comparable with the AT_1 receptor blocker (ARB) valsartan (Imanishi *et al.*, 2008b) or irbesartan (Nussberger *et al.*, 2008). Combination therapy of aliskiren and valsartan had an additive effect on endothelial function, BH_4 content, NO release and plaque volume reduction (Imanishi *et al.*, 2008b).

Angiotensin-converting enzyme inhibitors (ACEI) and ARB have indirect antioxidant effects by preventing the activation NADPH oxidase (Mancini *et al.*, 1996; Warnholtz *et al.*, 1999; Wassmann *et al.*, 2002; Klingbeil *et al.*, 2003) (Figure 2). In addition, they can also increase the activity of extracellular SOD (SOD3) (Hornig *et al.*, 2001). ACEI significantly reduce cardiovascular events in patients with established coronary artery disease or at high risk for the disease (Bauersachs and Fraccarollo, 2008). ARB can improve eNOS functionality; losartan restored glomerular NO production by increasing GCH1 protein expression and elevating BH_4 bioavailability in diabetic rats (Satoh *et al.*, 2008).

Eplerenone, a selective aldosterone antagonist, has been shown to attenuate atherosclerosis in cholesterol-fed monkeys (Takai *et al.*, 2005). Imanishi *et al.* investigated the effect of eplerenone and enalapril, alone or in combination, on atherosclerotic changes in genetically hyperlipidaemic rabbits (Imanishi *et al.*, 2008a). Both eplerenone and enalapril reduce NADPH oxidase activity, elevate vascular BH_4 levels (and thus limit eNOS uncoupling), and enhance eNOS expression and NO bioavailability (Figure 2). Eplerenone also increases eNOS phosphorylation at serine 1177. Both drugs decrease atherosclerotic plaque formation and the combination leads to an additive reduction (Imanishi *et al.*, 2008a).

These multiple pleiotropic effects of compounds interfering with the renin–angiotensin–aldosterone system may make important contributions to the therapeutic benefit of such drugs.

Conclusions

The pathophysiological causes of oxidative stress are likely to involve changes in a number of different enzyme systems; most importantly, there is an upregulation of NADPH oxidases and eNOS. Together they lead to an increased production of $ONOO^{-}$. This conveys oxidative damage to eNOS and/or its cofactor BH_4, leading to 'uncoupling' of the enzyme. As consequence, an increased production of ROS by uncoupled eNOS is likely to contribute significantly to vascular oxidative stress and endothelial dysfunction. Several drugs in clinical use have pleiotropic actions that improve endothelial function, and novel pharmacological approaches to prevent or reverse endothelial dysfunction are being investigated.

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Conflicts of interest

None.

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