REVIEW SERIES

Oxidative stress and endothelial dysfunction in hypertension

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Systemic arterial hypertension is a highly prevalent cardiovascular risk factor that causes significant morbidity and mortality, and is becoming an increasingly common health problem because of the increasing longevity and prevalence of predisposing factors such as sedentary lifestyle, obesity and nutritional habits. Further complicating the impact of this disease, mild and moderate hypertension are usually asymptomatic, and their presence (and the subsequent increase in cardiovascular risk) is often unrecognized. The pathophysiology of hypertension involves a complex interaction of multiple vascular effectors including the activation of the sympathetic nervous system, of the renin–angiotensin–aldosterone system and of the inflammatory mediators. Subsequent vasoconstriction and inflammation ensue, leading to vessel wall remodeling and, finally, to the formation of atherosclerotic lesions as the hallmark of advanced disease. Oxidative stress and endothelial dysfunction are consistently observed in hypertensive subjects, but emerging evidence suggests that they also have a causal role in the molecular processes leading to hypertension. Reactive oxygen species (ROS) may directly alter vascular function or cause changes in vascular tone by several mechanisms including altered nitric oxide (NO) bioavailability or signaling. ROS-producing enzymes involved in the increased vascular oxidative stress observed during hypertension include the NADPH oxidase, xanthine oxidase, the mitochondrial respiratory chain and an uncoupled endothelial NO synthase. In the current review, we will summarize our current understanding of the molecular mechanisms in the development of hypertension with an emphasis on oxidative stress and endothelial dysfunction.

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INTRODUCTION

Owing to the continuous increase in life expectancy and to the increasing prevalence of predisposing factors such as obesity or physical inactivity, hypertension is an increasing health problem in Western societies. As cardiovascular risk rises continuously with increases in blood pressure, hypertension is believed to be an important determinant of cardiovascular morbidity and mortality. Owing to its high prevalence in Western societies and increasing prevalence in developing countries, the global disease burden attributable to hypertension is substantial and estimated around 4.5% according to the World Health Organization.¹ The discovery of vascular receptors that control vessel tone and neurohumoral mediators in hypertension has led to the development of modern antihypertensive drugs such as beta-blockers, angiotensin converting enzyme inhibitors, AT-1 receptor blockers or calcium channel blockers. Although the pathophysiology of hypertension is extremely complex and multifactorial, and the role of factors such as endothelin,² cyclooxygenase-dependent vasoconstrictors³ and endothelium-derived hyperpolarizing factor⁴ needs to be acknowledged, numerous experimental animal studies suggest that this condition is associated with an increased formation of reactive oxygen

species (ROS) from all layers of the vascular wall. A relationship between elevated blood pressure and vascular oxidative stress was also shown in patients with essential,^{5,6} renovascular⁷ or malignant hypertension.⁸ However, whether oxidative stress has a causal role in the development of hypertension and whether it may contribute to elevated blood pressure itself and/or vascular remodeling remains a matter of controversy. This scenario is supported by observations that correction of increased blood pressure by antihypertensive drugs is associated with a reduction in vascular oxidative stress.^{9–11} In addition, reducing vascular oxidative stress by antioxidants or ablation of ROS-producing enzymes has been shown to decrease blood pressure in animal models.^{12,13} Oxidative stress may directly alter vascular function and tone, for example, by oxidative modification of proteins or nucleic acids. A major mechanism for the impact of oxidative stress on vascular tone is the decrease of nitric oxide (NO) bioavailability and/or signaling, leading to endothelial dysfunction, and ROS may also promote vascular cell proliferation and migration, inflammation and apoptosis, as well as extracellular matrix alterations. All of these ROS-related processes contribute to the development of hypertension (Figure 1). ROS-producing enzymes involved in increased oxidative stress within vascular tissues include the NADPH oxidase (Nox), the

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Figure 1 Mechanisms of oxidative stress-mediated changes in vascular morphology and function. Reactive oxygen species in the vasculature lead to increased vascular cell proliferation and migration, apoptosis, inflammation and extracellular matrix alterations, all of which may eventually cause vascular hypertrophy and remodeling. In addition, ROS decrease vascular nitric oxide bioavailability and cause endothelial dysfunction. All of these changes contribute to the structural and functional changes of the vasculature that are associated with the development and perpetuation of hypertension.

xanthine oxidase and the mitochondrial respiratory chain. Superoxide anions may directly react with NO, thereby stimulating the production of the NO/superoxide anion reaction product peroxynitrite (ONOO^-), which in turn has been shown to uncouple the endothelial nitric oxide synthase (eNOS), thereby causing an anti-atherosclerotic NO-producing enzyme to become a ROS-producing enzyme, thus accelerating the atherosclerotic process.¹⁴ Increased oxidative stress in the vasculature, however, is not restricted to the endothelium and has also been demonstrated to occur within the smooth muscle cell layer and the adventitia. Increased ROS production has important consequences with respect to signaling by the soluble guanylate cyclase and the cyclic guanosine monophosphate-dependent kinase I, whose activity and expression has been shown to be regulated in a redox-sensitive manner.^{15,16} In the following paragraphs, we will summarize the role of different enzymatic sources of oxidative stress that are felt to be relevant to hypertension, and we will discuss how ROS may alter vascular function and contribute to the development and progression of hypertension.

ROLE OF THE ENDOTHELIUM FOR THE DEVELOPMENT OF VASCULAR DISEASE

Traditionally, the role of the endothelium was thought primarily to be that of a selective barrier to prevent the diffusion of macromolecules from the blood lumen to the interstitial space. During the past 20 years, numerous additional roles have been defined for this tissue, including the regulation of vascular tone, modulation of inflammation, promotion, as well as inhibition of vascular growth and modulation of platelet aggregation and coagulation.¹⁷ Any distortion of these properties is termed endothelial dysfunction, a phenomenon that can be easily demonstrated as an impaired vasorelaxation in response to endothelium-dependent vasodilators such as acetylcholine.¹⁸ Endothelial dysfunction is a characteristic feature of patients with atherosclerosis and more recent studies indicate that it may predict long-term atherosclerotic disease progression as well as cardiovascular event rate.¹⁹ Although the mechanisms underlying endothelial dysfunction are multifactorial, a growing body of evidence suggests that increased ROS production contributes considerably to this

phenomenon in several ways. In particular, the interaction of ROS with NO, which is the best characterized (and likely most important) mediator of endothelial function, is felt to have an important role in determining the causal relationship between oxidative stress and hypertension. As described below, ROS may uncouple the eNOS-catalyzed reduction of molecular oxygen from the oxidation of L-arginine, resulting in the paradoxical production of the ROS superoxide anion instead of the reducing NO.²⁰ Alternatively, ROS may react with NO directly, reducing its bioavailability.^{21,22} Further, ROS production has been demonstrated to occur not only in the endothelial cell layer but also within the media and adventitia, all of which may impair NO signaling within vascular tissues.^{23,24} Finally, ROS may impair the activity of a number of other pathways, thus modifying at the same time the responsiveness to endothelium-independent vasodilators.^{25,26} Owing to spatial compartmentalization, reaction kinetics and physiological functions of low ROS concentrations, the scavenging of ROS by cellular enzyme systems (for example, superoxide dismutase, catalase) is impaired. This is in accordance with clinical studies, which could not prove any benefit for the supplementation with antioxidants such as vitamin C or vitamin E.²⁷ Therefore, a much better way to limit oxidative stress in the vasculature seems to be the selective inhibition of putative ROS sources involved in vascular pathology. In the following sections, we will discuss how oxidative stress may affect endothelial function with a particular focus on the so far identified ROS sources that are relevant to hypertension.

OXIDATIVE STRESS CAUSES ENDOTHELIAL DYSFUNCTION

The endothelium-derived relaxing factor, previously identified as NO²⁸ or a closely related compound,²⁹ has potent anti-atherosclerotic properties. Nitric oxide released from endothelial cells works in concert with prostacyclin to inhibit platelet aggregation;³⁰ it inhibits the attachment of neutrophils to endothelial cells and the expression of adhesion molecules. Nitric oxide in high concentrations inhibits the proliferation of smooth muscle cells.³¹ Therefore, under all conditions in which an absolute or relative NO deficit is encountered, the process of atherosclerosis is initiated or accelerated. The half-life of NO and therefore its biological activity is decisively determined by oxygen-derived free radicals such as superoxide anions,³² which as mentioned above rapidly bind to NO to form the highly reactive intermediate ONOO^- .²² Notably, the rate constant ($5\text{--}10 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$) of this rapid bimolecular reaction between NO and superoxide is about three to four times faster than the dismutation of superoxide by the superoxide dismutase.²¹ Therefore, ONOO^- formation represents a major potential pathway of NO reactivity pending on the rates of tissue superoxide production. Peroxynitrite in high concentrations is cytotoxic and may cause oxidative damage to proteins, lipids and DNA.²² Recent studies also indicate that ONOO^- may have deleterious effects on the activity and function of prostacyclin synthase³³ and eNOS.³⁴ There are number of lines of evidence that support the role of these pathways in the development and the progression of arterial hypertension. Numerous studies have demonstrated an impairment in the vascular responsiveness to endothelium-specific agents in patients with hypertension and/or siblings of patients with hypertension,^{35–40} and that these abnormalities can be corrected by the infusion of an antioxidant.⁴¹ The presence and severity of subclinical and clinical target organ damage is associated, in the setting of hypertension, with that of endothelial dysfunction; both an increased intima-media thickness of the common carotid artery and proteinuria appear indeed to be directly correlated with endothelium-dependent vasomotor responses.^{42–44} Finally, in a prospective study in 952 postmenopausal

women, an impairment in endothelial function was shown to be an efficient independent predictor of the development of arterial hypertension during a follow-up of 3.6 years.⁴⁵ Although the role of each individual ROS cannot be investigated *in vivo* in humans, and, although superoxide anion and ONOO⁻ are the best characterized ROS, the possible role of other oxidant compounds such as hydrogen peroxide (the dismutation product of superoxide anions) and hypochlorous acid, which cannot be considered as free radicals but have a powerful oxidizing capacity, also needs to be mentioned.

ENDOTHELIAL DYSFUNCTION AND PROGNOSIS

During the past 10 years, numerous clinical trials have demonstrated a close association between coronary and peripheral endothelial function and the likelihood of cardiovascular events. The work from Schächinger *et al.*⁴⁶ showed that a preserved responsiveness to the endothelium-dependent vasodilator acetylcholine, administered intracoronarily, is associated with a significantly lower incidence of cardiovascular events defined as a composite end point, including cardiovascular death, unstable angina, myocardial infarction, coronary revascularization, ischemic stroke and peripheral artery revascularization. Similarly, endothelial function measured at the level of the forearm vasculature has been shown to correlate with the incidence of cardiovascular events in at least two studies.^{47,48} Notably, the excess risk in patients with the worst endothelial function was still significant after controlling for individual risk markers. Further, the coexistence of left ventricular hypertrophy and endothelial dysfunction in hypertensive patients increases significantly the risk of subsequent cardiovascular events.⁴⁹ Even more interestingly, patients who showed an improvement in endothelial function after intraarterial infusion with vitamin C (showing an evidence of endothelial dysfunction caused by increased oxidative stress), had a worse prognosis as compared with patients with a low or no vitamin C effects.⁵⁰ This finding not only strengthens the concept that oxidative stress indeed is not only the key factor in determining the degree of endothelial dysfunction but also that it impacts the prognosis of patients with established atherosclerosis. These findings seem to be also valid for different localizations of atherosclerotic disease. Gokce *et al.*⁵¹ quantified endothelial function in patients undergoing peripheral or coronary bypass surgery. In all patients, flow-mediated dilation of the brachial artery was measured before surgery and the patients were grouped into three tertiles; flow-mediated dilation >8%, between 4–8% and <4%. The authors found that patients with an flow-mediated dilation >8% had almost no cardiovascular events during a follow-up period of 30 days, whereas patients with an flow-mediated dilation <8% had substantially more events.⁵¹ The results of this study confirm that measurement of endothelial function in the brachial artery may provide an information about plaque stability in coronary arteries.

Taken together, there is no doubt that measurement of endothelial function provides substantial prognostic information about future cardiovascular events in atherosclerotic disease and hypertension, whereas its role in primary prevention and whether the introduction of novel methods to assess endothelial function will further increase its prognostic potential⁵² remains to be established.

ROS SOURCES IN HYPERTENSION

NADPH oxidases

The Nox family members are enzyme complexes whose exclusive function is the production of superoxide anions by the transfer of electrons from NADPH to molecular oxygen. They consist of several transmembraneous (Nox1-5, p22phox) and regulatory cytosolic subunits (p47phox, p67phox, rac, Noxo1 and Noxa1).⁵³ NADPH oxidases

were first discovered as part of the anti-microbial defense in neutrophils, in which a Nox2-containing Nox produces large amounts of superoxide anions. Meanwhile, we know that different Nox isoforms also exist in endothelial cells, in vascular smooth muscle cells as well as in the adventitia. The activity of the enzyme complex in endothelial and smooth muscle cells is increased upon stimulation with angiotensin II (ATII).⁵⁴ In addition, increased Nox activity has been observed in different animal models of hypertension including ATII infusion,^{14,55,56} deoxycorticosterone acetate (DOCA)-salt hypertension,⁵⁷ renovascular hypertension⁵⁸ and in spontaneously hypertensive rats.⁵⁹

Whether ROS derived from the Nox activation has a causal role in the development of hypertension was investigated by several animal studies using specific knockdown of Nox subunits. A major ROS source in response to ATII is Nox1 in the vascular smooth muscle layer. Deletion of Nox1 resulted in a marked decrease of blood pressure in response to ATII treatment,⁶⁰ whereas its overexpression in vascular smooth muscle cells lead to a further blood pressure augmentation.⁶¹ Ablation of p47phox, which is an essential regulatory cytosolic subunit of several Noxs, completely blunted the blood pressure increase in response to ATII.^{62,63} To what extent ROS from invaded mononuclear cells may contribute to overall vascular oxidative stress remains controversial at the moment. However, some recently published studies support the notion that Nox from inflammatory cells may have been underestimated as a significant vascular ROS source in the past. In this respect, a recent work by Guzik *et al.*⁶⁴ could show that T-cell depletion in mice blunted the ATII effects on blood pressure, vascular superoxide production and endothelial function. Interestingly, adoptive cell transfer with T cells lacking the Nox prevented ATII-induced increase in vascular ROS production and blood pressure in this animal model.⁶⁴

The stimulatory effects of ATII on the activity of the Nox would suggest that in the presence of an activated renin-angiotensin system—a common finding during hypertension—increased vascular oxidative stress is likely to be expected. In fact, angiotensin-converting enzyme activity, and therefore local ATII concentrations are increased in atherosclerotic plaques,^{65,66} and inflammatory cells are capable of producing large amounts of ATII. In addition, there is evidence for increased expression of the Nox subunit Nox2 and Nox4 in atherosclerotic arteries, which may contribute to ATII-stimulated oxidative stress during hypertension and human atherosclerotic disease.²³ Accordingly, increased ATII concentrations, along with increased levels of superoxide anions, have been shown in the shoulder region of atherosclerotic plaques.⁶⁷ In line with a role of ATII in endothelial dysfunction and hypertension, numerous studies have now demonstrated that the therapy with angiotensin-converting enzyme inhibitors and AT-1 receptor blockers is associated with an improvements in endothelial responsiveness^{68–72} (the existence of negative reports also needs to be acknowledged).⁷³

In animal models of ATII-induced hypertension,¹⁴ increased ROS production by Nox was also associated with eNOS uncoupling. As the essential eNOS cofactor tetrahydrobiopterin (BH₄) is susceptible for oxidation, a landmark study by David Harrison's group⁶² was able to proof the concept that superoxide produced by the Nox may indeed trigger eNOS uncoupling in the experimental animal model of DOCA-salt hypertension. In this study, the authors showed that superoxide induced by DOCA-salt treatment caused increased vascular hydrogen peroxide production, which was significantly reduced by the eNOS inhibitor N^G-nitro-L-arginine methyl ester. Treatment of p47phox knockout animals with DOCA-salt markedly reduced levels of oxidative stress and abolished superoxide reducing effects of NOS inhibition compatible with a prevention of eNOS uncoupling.⁵⁷

eNOS uncoupling and altered eNOS signaling

Paradoxically, in most situations in which increased oxidative stress and endothelial dysfunction are encountered, the expression of eNOS has been shown to be increased rather than decreased.^{74–77} The underlying mechanism of increased eNOS expression likely involves increased endothelial production of hydrogen peroxide, which has been shown to increase the expression of eNOS at the transcriptional and translational level.⁷⁸ However, the demonstration of endothelial dysfunction in the presence of increased expression of eNOS indicates that the capacity of the enzyme to produce NO may be limited, and the concept that eNOS itself can be a superoxide source and thereby contributes to endothelial dysfunction provides a further explanation to this observation.¹⁷

It has become clear from studies with the purified enzyme that eNOS may become ‘uncoupled’ in the absence of the NOS substrate L-arginine or the cofactor BH₄. In such uncoupled state, electrons normally flowing from the reductase domain of one subunit to the oxygenase domain of the other subunit are diverted to molecular oxygen rather than to L-arginine,^{79,80} resulting in production of superoxide anions rather than NO. The so far identified mechanisms how eNOS uncoupling may occur are discussed below.

Tetrahydrobiopterin seems to be essential for the time-critical delivery of one electron to an intermediate in the catalytic cycle of NOS. The resulting species releases both NO and L-citrulline. The BH₃ radical is reduced by iron, completing the cycle by formation of Fe^{III} and BH₄. In the absence of BH₄, the intermediate reacts with molecular oxygen, resulting in superoxide anion formation.

The first evidence that eNOS uncoupling may be a pathophysiologically relevant phenomenon was provided by *in vitro* studies in which it was demonstrated that native low-density lipoprotein⁸¹ and even more pronounced oxidized low-density lipoprotein⁸² are able to stimulate endothelial superoxide production and that this phenomenon is inhibited by the NOS inhibitor N^G-nitro-L-arginine methyl ester, pointing to a causal role for eNOS in superoxide production. With respect to hypertension, several *in vivo* studies have provided evidence that eNOS uncoupling occurs in animal models of increased blood pressure, including ATII hypertension,¹⁴ DOCA-salt induced hypertension,⁵⁷ NOX1 upregulation⁸³ and genetically determined hypertension⁸⁴ in which an uncoupled eNOS was consistently identified as a significant superoxide source. In animal models of hypertension, supplementation of BH₄ improved blood pressure and endothelial dysfunction;⁸⁵ in humans, BH₄ improved endothelial responsiveness after intraarterial administration.⁸⁶ In the following paragraphs we will discuss the potential mechanisms leading to eNOS uncoupling during vascular disease.

BH₄ levels and expression of the CTP cyclohydrolase I in the setting of eNOS uncoupling. Strategies to improve endothelial function via increasing the expression of eNOS have provided mixed results. Ozaki *et al.*⁸⁷ reported that targeted overexpression of eNOS in apolipoprotein E knockout animals accelerated rather than decreased atherosclerotic lesion formation. The authors also observed a marked decrease in vascular BH₄ content and identified the endothelium as the pivotal superoxide source compatible with eNOS uncoupling. Treatment with BH₄ reduced lesion formation, reduced vascular superoxide and increased endothelial NO production.⁸⁷ These findings clearly indicate that the stimulation of increases in eNOS expression without simultaneously increasing vascular levels of BH₄ may lead to eNOS uncoupling because of the stoichiometric relationship between endothelial BH₄ and NOS activity.⁸⁸

One very attractive concept to explain intracellular BH₄ depletion is the oxidative modification of BH₄.⁸⁹ As BH₄ is very susceptible to oxidation, ONOO[−], resulting from the reaction of NO with superoxide anion, may oxidize BH₄ to the BH₃ radical and, thus, the resulting decrease in endothelial BH₄ can induce eNOS uncoupling.

This concept, however, also implies that the uncoupling of eNOS is mediated by ONOO[−] and would invariably require a primary superoxide anion source such as the Nox, xanthine oxidase or mitochondria. Owing to the rapid reaction between superoxide anions and NO, these so called ‘kindling radicals’ would lead to the production of ONOO[−], followed by eNOS uncoupling and subsequent eNOS-mediated superoxide production (bonfire radical, see Figure 2). Recent data support that this concept is operative *in vivo*, showing that Nox1 overexpression in vascular smooth muscle cells resulted in increased vascular oxidative stress and eNOS uncoupling, which could be reversed by BH₄ supplementation.⁸³

Oxidation of BH₄ not only reduces BH₄ bioavailability but also the oxidation products such as BH₂ may compete with BH₄ for binding to eNOS,⁹⁰ thereby leading to eNOS uncoupling. Interestingly, vitamin C was able to recycle the BH₃ radical to BH₄ but not BH₂ to BH₄. This observation may indicate that the beneficial effects of vitamin C on endothelial function in patients may be explained in part by a recycling of the BH₃ radical to BH₄ rather than by directly scavenging superoxide.⁸⁹

In addition to ONOO[−]-mediated oxidation of BH₄, there may be also an intracellular decrease because of decreased synthesis by inhibition of the GTP cyclohydrolase I (*de novo* synthetic pathway) or by inhibition of the so-called salvage pathway involving enzymes such as the sepiapterin synthase, the sepiapterin reductase or the dihydrofolate reductase. Interestingly, recent studies indicate that in the setting of ATII-induced hypertension, a downregulation of the dihydrofolate reductase was observed, which was accompanied by decreased vascular BH₄ levels and an uncoupled eNOS,⁹¹ implicating a potential role of this salvage pathway in the development of hypertension.

In the case of eNOS uncoupling due to relative intracellular BH₄ deficiency, this phenomenon could be prevented by simultaneous overexpression of the BH₄ synthesizing enzyme GTP cyclohydrolase I, which has been demonstrated in isolated cells⁹² and in atherosclerotic animal models.^{93,94} Recent clinical data also indicates that variants of GTP cyclohydrolase I are governing NO production, autonomic activity and even cardiovascular risk.⁹⁵ With respect to hypertension, an important study by Du *et al.*⁹⁶ showed that increased oxidative stress due to eNOS uncoupling may directly affect blood pressure. In a murine model of DOCA-salt hypertension, BH₄ levels were decreased but could be preserved by endothelial-specific upregulation of GTP cyclohydrolase I, which was associated with a significant reduction in blood pressure.

With respect to endothelial function, evidence to support the concept that eNOS uncoupling contributes to endothelial dysfunction was obtained by experiments demonstrating that supplementation with BH₄ or the BH₄ precursor sepiapterin was able to improve endothelial dysfunction in patients with hypertension.⁸⁶ However, it is important to note that BH₄ *per se* has antioxidant properties. Therefore, it may be difficult to differentiate whether the BH₄-induced improvements in endothelial dysfunction are either due to recoupling of eNOS or due to the antioxidant properties of BH₄ itself. One possibility to differentiate these eNOS uncoupling related vs. unspecific effects on endothelial dysfunction is the use of the pteridine analog tetrahydroneopterin, a compound with comparable antioxidant properties, but no effect on eNOS uncoupling,⁹⁷ as a negative control.

Figure 2 Schematic representation of the mechanisms by which reactive oxygen species lead to an impairment of nitric oxide (NO) bioavailability and vascular dysfunction. Under normal conditions, NO is synthesized by the endothelial nitric oxide synthase (eNOS) from its precursor L-arginine and stimulates soluble guanylate cyclase (sGC), thereby increasing cyclic guanosine monophosphate levels leading to a transient drop in cellular calcium and vasorelaxation (green arrows, left side of the figure). This pathway can, however, be inhibited at several sites. Hypertension-associated changes such as increased angiotensin II levels will stimulate superoxide production within endothelial and smooth muscle cells mainly via activation of the vascular NADPH oxidase (Nox), mitochondrial respiratory chain and xanthine oxidase (red arrows). Superoxide anions may directly inactivate NO under formation of peroxynitrite (ONOO^-) and inhibit sGC activity. Peroxynitrite in turn may also inhibit sGC directly and it uncouples eNOS by oxidizing the eNOS cofactor to BH_2 . This concept, however, also means that uncoupling of eNOS would always require a 'priming event' such as superoxide produced by the Nox, mitochondria or the xanthine oxidase (kindling radical). Antioxidants or superoxide scavenging enzyme systems such as different isoforms of the superoxide dismutase or glutathione peroxidase as depicted on the right side may prevent or even slow down the oxidative damage of the vasculature.

Another way to counteract the loss of BH_4 bioavailability involves the supplementation with folic acid or closely related compounds such as 5-methyl tetrahydrofolate. Recent experimental studies revealed that folate may improve endothelial function⁹⁸ by an enhancement of binding affinity of BH_4 to NOS by a pteridine-binding domain serving as a locus through which the active form 5-methyl tetrahydrofolate facilitates the electron transfer by BH_4 from the NOS reductase domain to heme.⁹⁹ Folate also enhances regeneration of BH_4 from inactive BH_2 by stimulating dihydrofolate reductase and it chemically stabilizes BH_4 .¹⁰⁰

eNOS uncoupling due to oxidation of the zinc–thiolate complex. Another interesting concept regarding eNOS uncoupling was provided by Zou *et al.*³⁴ The authors showed that the exposure of the isolated enzyme to the oxidant ONOO^- leads to a disruption of the zinc–thiolate cluster, resulting in an uncoupling of the enzyme. They also demonstrated that a similar phenomenon occurs when endothelial cells were exposed to high concentrations of glucose. Additional experiments revealed that BH_4 was oxidized at concentrations being 10- to 100-fold higher than those needed to disrupt the zinc–thiolate complex. On the basis of these findings, the authors suggested that the principal mechanism of uncoupling is the oxidation of the zinc–thiolate center and subsequent release of zinc rather than the BH_4 oxidation process.¹⁰¹

eNOS inhibition by asymmetric dimethyl L-arginine. Besides eNOS uncoupling, inhibition of the enzyme may also result in a significant

drop in vascular NO levels, resulting in endothelial dysfunction and alterations in vascular tone, which may lead to hypertension. Increased concentrations of asymmetric dimethyl L-arginine (ADMA) in cultured endothelial cells or in patients with endothelial dysfunction are associated with increased ROS production.^{102–104} It remains unclear at the moment whether ADMA itself may contribute to vascular oxidative stress. Interestingly, the activity of methylating enzymes such as the S-adenosylmethionine-dependent protein arginine methyltransferase (type I)¹⁰³ responsible for the ADMA synthesis or the activity of ADMA hydrolyzing enzymes such as dimethylarginine dimethylaminohydrolase¹⁰² are redox sensitive. Thus, oxidative stress in the vasculature will stimulate ADMA production and/or inhibit ADMA degradation, leading to eNOS inhibition by this endogenous molecule. Decreased NO bioavailability due to high ADMA levels may also affect blood pressure. In accordance with this concept, the increase in blood pressure in Dahl salt-sensitive hypertensive rats correlated with urinary ADMA excretion.¹⁰⁵ In another elegant study, Kielstein *et al.*¹⁰⁶ were able to show that systemic infusions of ADMA in humans resulted in a significant increase in blood pressure, supporting a possible causal link between ADMA levels and the degree of hypertension.

Disturbed NO downstream signaling by the soluble guanylyl cyclase and cyclic guanosine monophosphate-dependent protein kinase in hypertension. Endothelial dysfunction in different animal models of hypertension has been shown to be associated with increased expression of eNOS^{14,107} but decreased vascular NO bioavailability.¹⁴

Uncoupled eNOS^{14,57} and Noxs^{14,55,108} were identified as significant superoxide sources in hypertensive animals. Increased superoxide production was observed in all layers of the vascular wall, namely the endothelium, media and adventitia,¹⁴ and may also increase vasoconstrictor tone via stimulation of the expression of endothelin-1 in the smooth muscle and endothelial cell layer.^{109,110} Acute or chronic treatment with antioxidants or superoxide dismutase not only improved endothelial dysfunction but also markedly reduced blood pressure, indicating the potential role of ROS in the initiation and maintenance of hypertension.

Hypertensive animals displayed not only reduced vasodilation to endothelium-dependent vasodilators but also to endothelium-independent nitro-vasodilators sodium nitroprusside and nitroglycerin.⁵⁵ This finding may be explained at least in part by reduced expression of the soluble guanylyl cyclase (sGC), as in different hypertensive animal models, the expression of one or both sGC subunits (α_1 and β_1), as well as NO-dependent sGC activity were significantly decreased. This was observed in genetic models of hypertension, such as aged spontaneously hypertensive rats,^{111,112} stroke-prone spontaneously hypertensive rats,¹¹³ Type 2 diabetic, mildly hypertensive Goto-Kakizaki rats¹¹⁴ and TGR(mREN2)27 renin transgenic rats,¹¹⁵ as well as in several models of drug-induced hypertension.

Correction of hypertension normalized or even enhanced sGC expression, but had varying effects on vascular superoxide production. For example, in ATII-treated rats, inhibition of protein kinase C *in vivo* reduced blood pressure and vascular superoxide formation by inhibiting the activity and expression of Nox and by preventing eNOS uncoupling,¹⁴ as well as sGC downregulation (unpublished observation). In contrast, antihypertensive treatment with hydralazine significantly lowered blood pressure but failed to normalize vascular superoxide formation, despite increasing sGC expression.¹¹⁶ Thus, it appears that the decrease in sGC expression elicited by high blood pressure is not exclusively linked to oxidative stress and/or to increased levels of vasoconstrictor peptides.

Mitochondrial respiratory chain

During oxidative phosphorylation in the mitochondria, electron flow may accidentally cause an electron transfer to molecular oxygen, which will give superoxide anions as a byproduct during adenosine triphosphate generation. Under normal conditions, most of the superoxide being produced will not exit the mitochondrion or will be inactivated by the mitochondrial isoform of superoxide dismutase, namely manganese superoxide dismutase. However, during increased electron flux, superoxide production may reach a critical threshold, which may allow trafficking of superoxide anions through the mitochondrial transition pore into the cytosol, in which further damage and/or the activation of additional ROS sources may occur. Some reports exist that link mitochondrial ROS production to hypertension. In a rat model of DOCA-salt hypertension, vascular mitochondrial ROS production was markedly elevated, accompanied by the activation of other ROS sources. Also, partial deficiency of the mitochondrial superoxide dismutase isoform manganese superoxide dismutase was associated with increased blood pressure in aging mice.¹¹⁷ Several studies have provided evidence that a cross-talk among different ROS sources occurs in the vasculature, in particular between the Nox and mitochondria.¹¹⁸ In accordance with this notion, ATII was shown to increase mitochondrial ROS production in a Nox-dependent manner¹¹⁹ and *vice versa*.¹²⁰ Moreover, a recent work by Dikalova *et al.*¹²⁰ also demonstrated a causal role of mitochondrial ROS in the development of hypertension, as the mitochondrial-targeted antioxidant mitoTEMPO was able to decrease ATII-induced hypertension.

Thus, some promising evidence for a role of mitochondrial ROS in hypertension is currently available, but additional work is needed.

Xanthine oxidase

Xanthine oxidoreductase catalyzes the sequential hydroxylation of hypoxanthine to provide xanthine and uric acid. The enzyme can exist in two forms that differ primarily in their oxidizing substrate specificity. The dehydrogenase form not only preferentially uses NAD⁺ as an electron acceptor but also is able to donate electrons to molecular oxygen. Xanthine dehydrogenase can be converted into xanthine oxidase irreversibly by proteolytic breakdown as well as reversibly by direct oxidation of cysteine residues. Xanthine oxidase is mainly expressed in the endothelium and can be activated by ATII.¹²¹ However, studies on endothelial function after inhibition of xanthine oxidase have provided equivocal results in hypertension models. Although Cardillo *et al.*¹²² showed that endothelial dysfunction in hypertensive diabetic subjects is improved by acute inhibition of xanthine oxidase with oxypurinol and allopurinol,¹²³ other groups failed to show similar efficacy for allopurinol.¹²⁴ Regarding the effects of xanthine oxidase inhibition on blood pressure itself, most experimental studies described a decrease in blood pressure in hypertensive animal models,^{125–128} whereas others found no effect.¹²⁹ Taken together, the available literature is in favor of a causative role of xanthine oxidase activation for the development of hypertension, whereas the effects on hypertension-associated endothelial dysfunction remain unclear at the moment.

CONCLUSION

Taken together, ample evidence from animal and human studies in hypertension shows a clear association with increased vascular oxidative stress, attributable to the activation of several ROS sources including Nox, xanthine oxidase, mitochondrial respiratory chain and uncoupled eNOS. Moreover, the reduction in oxidative stress during effective antihypertensive treatment suggests an involvement of vascular ROS in the development of hypertension. Experimental studies in animal models of hypertension further substantiate a causal role of vascular oxidative stress in the modulation of blood pressure. In particular, several pivotal studies point to a prominent role of the Nox, xanthine oxidase and mitochondrial ROS in this process.

ABBREVIATIONS

ACE, angiotensin-converting enzyme; ACh, acetylcholine; ADMA, asymmetric dimethyl-L-arginine; ATII, angiotensin II; BH₄, tetrahydrobiopterin; cGKI, cGMP-dependent protein kinase; cGMP, cyclic guanosine monophosphate; CTP CH-I, GTP cyclohydrolase I; DDAH, dimethylarginine dimethylaminohydrolase; DETC, diethylthiocarbamate; DHFR, dihydrofolate reductase; DOCA, deoxycorticosterone acetate; eNOS, endothelial nitric oxide synthase; FMD, flow-mediated dilation; LDL, low-density lipoprotein; L-NAME, N^G-nitro-L-arginine methyl ester; NO, nitric oxide; NTG, nitroglycerin; ONOO⁻, peroxynitrite; PDE1A1, phosphodiesterase 1A1; PKC, protein kinase C; PRMT, protein arginine N-methyltransferase; ROS, reactive oxygen species; sGC, soluble guanylyl cyclase; SHR, spontaneously hypertensive rats; SNP, sodium nitroprusside; SOD, superoxide dismutase; STZ, streptozotocin; VASP, vasodilator-stimulated phosphoprotein.

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