Vascular Consequences of Endothelial Nitric Oxide Synthase Uncoupling for the Activity and Expression of the Soluble Guanylyl Cyclase and the cGMP-Dependent Protein Kinase

Thomas Münzel, Andreas Daiber, Volker Ullrich, Alexander Mülsch

Abstract—Endothelial dysfunction in the setting of cardiovascular risk factors, such as hypercholesterolemia, hypertension, diabetes mellitus, chronic smoking, as well as in the setting of heart failure, has been shown to be at least partly dependent on the production of reactive oxygen species (ROS), such as the superoxide radical, and the subsequent decrease in vascular bioavailability of nitric oxide (NO). Superoxide-producing enzymes involved in increased oxidative stress within vascular tissue include the NAD(P)H oxidase, the xanthine oxidase, and mitochondrial superoxide-producing enzymes. Superoxide produced by the NADPH oxidase may react with NO released by endothelial nitric oxide synthase (eNOS), thereby generating peroxynitrite. Peroxynitrite in turn has been shown to uncouple eNOS, thereby switching an antiatherosclerotic NO-producing enzyme to an enzyme that may initiate or even accelerate the atherosclerotic process by producing superoxide. 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 in the setting of hypercholesterolemia, diabetes mellitus, hypertension, congestive heart failure, and nitrate tolerance. Increased superoxide production by the endothelial and/or smooth muscle cells has important consequences with respect to signaling by the soluble guanylyl cyclase (sGC) and the cGMP-dependent protein kinase I (cGK-I), the activity and expression of which has been shown to be regulated in a redox-sensitive fashion. The present review summarizes current concepts concerning eNOS uncoupling and also focuses on the consequences for downstream signaling with respect to activity and expression of the sGC and cGK-I in various diseases. (Arterioscler Thromb Vasc Biol. 2005;25:1-7.)

Key Words:

Traditionally, the role of the endothelium was thought primarily to be that of a selective barrier to the diffusion of macromolecules from the vessel lumen to the interstitial space. During the past 20 years, numerous additional roles for the endothelium have been defined such as regulation of vascular tone, modulation of inflammation, promotion, and inhibition of vascular growth and modulation of platelet aggregation and coagulation. Endothelial dysfunction is a characteristic feature of patients with coronary atherosclerosis and more recent studies indicate that it may predict long-term atherosclerotic disease progression as well as cardiovascular event rate. Although the mechanisms underlying endothelial dysfunction may be multifactorial, there is a growing body of evidence that increased production of reactive oxygen species (ROS) may contribute considerably to this phenomenon. ROS production has been demonstrated to occur in the endothelial cell layer, but also within the media and adventitia, all of which may impair nitric oxide (NO) signaling within vascular tissue to endothelium-dependent, but also endothelium-independent, vasodilators.

More recent experimental, but also clinical, studies point to a crucial role of endothelial nitric oxide synthase (eNOS) as a superoxide-producing enzyme in the setting of atherosclerosis, hypertension, congestive heart failure, and also nitrate tolerance. Figure 1 summarizes our observations that in all these pathophysiological settings vascular oxidative stress, as visualized by dihydroethidine-dependent fluorescence, is present within the endothelium, but also the smooth muscle cell layer and the adventitia, all of which plays an important role for the development of endothelial and/or vascular dysfunction. This review briefly addresses mechanisms underlying eNOS uncoupling and focuses on the consequences with respect to the activity and/or expression of the NO target, the soluble guanylyl cyclase (sGC), and the cGMP-dependent protein kinase I (cGK-I) present in vascular smooth muscle.

The L-arginine/NO/cGMP Pathway in Vascular Tissue
The endothelium, a single-layered continuous cell sheet lining the luminal vessel wall, separates the intravascular...
and surface area (1000 m²), the endothelium is an autonomous organ. Though for long time regarded as a passive barrier for blood cells and macrosolutes, this view completely changed with the advent of endothelial autacoids like prostacyclin⁴ and NO,³ as well as with the discovery of integrins and other surface signals.⁴ It is now evident that the endothelium is at the cross-bridges of communication between blood and tissue cells and actively controls this process and the function of surrounding cells by a plethora of signaling routes. One of the prominent communication lines is established by the so-called L-arginine-NO-cyclic GMP pathway.⁵ This signaling cascade starts with eNOS (NOSIII), which generates NO and l-citrulline from L-arginine and O₂ in response to receptor-dependent agonists (bradykinin, acetylcholine, ATP) and physicochemical stimuli (shear, stretch).⁶ Reducing equivalents are provided by NADPH, and electrons are passed via a flavin chain to the catalytic center, the enzymes heme iron (Figure 2, upper). The first step of the normal NOS reaction is a classical mono-oxygenation, which consumes 1 mol of NADPH and oxygen. Molecular oxygen bound to the iron is activated and split to accomplish a hydroxylation of the substrate, the guanidino-nitrogen of L-arginine, forming N⁰-hydroxy-L-arginine. The second step is an atypical mono-oxygenation, which consumes 1 mol O₂ and 0.5 mol NADPH. It is a 3-electron oxidation of N⁰-hydroxy-L-arginine to afford the final products NO and l-citrulline. To guarantee this reaction path, the enzyme has to be homodimeric and the cofactor tetrahydrobiopterin must be present (Figure 2). For details, the reader is referred to excellent reviews on this topic.⁷ ⁸

NO diffuses to the adjacent smooth muscle where it interacts with different receptor molecules, of which the sGC is the best characterized and presumably most important one with regard to control of vessel tone and smooth muscle proliferation. The catalytically active holoenzyme exists as an atypical mono-oxygenation, which consumes 1 mol O₂ and produces NO and l-citrulline from L-arginine and O₂ in response to receptor-dependent agonists (bradykinin, acetylcholine, ATP) and physicochemical stimuli (shear, stretch).⁶ Reducing equivalents are provided by NADPH, and electrons are passed via a flavin chain to the catalytic center, the enzymes heme iron (Figure 2, upper). The first step of the normal NOS reaction is a classical mono-oxygenation, which consumes 1 mol of NADPH and oxygen. Molecular oxygen bound to the iron is activated and split to accomplish a hydroxylation of the substrate, the guanidino-nitrogen of L-arginine, forming N⁰-hydroxy-L-arginine. The second step is an atypical mono-oxygenation, which consumes 1 mol O₂ and 0.5 mol NADPH. It is a 3-electron oxidation of N⁰-hydroxy-L-arginine to afford the final products NO and l-citrulline. To guarantee this reaction path, the enzyme has to be homodimeric and the cofactor tetrahydrobiopterin must be present (Figure 2). For details, the reader is referred to excellent reviews on this topic.⁷ ⁸

Figure 1. Detection of vascular superoxide formation by the fluorescence dye dihydroethidine in aortic tissue sections from different oxidative stress animal models and in the mammary artery from nitroglycerin (NTG)-treated patients. CHF indicates congestive heart failure; hypertension: angiotensin (AT II)-infused rats vs sham-treated Wistar rats; atherosclerosis: hyperlipidemic Watanabe rabbits (WHHL) vs New Zealand White rabbits (NZWR); diabetes: streptozotin (STZ)-treated rats vs sham-treated Wistar rats; nitrate tolerance: NTG-infused patients vs patients without NTG treatment.

(blood) from the interstitial compartment and the vascular smooth muscle. Based on cell count (6 × 10¹⁰), mass (1.5 kg), and surface area (1000 m²), the endothelium is an autonomous organ. Though for long time regarded as a passive barrier for blood cells and macrosolutes, this view completely changed with the advent of endothelial autacoids like prostacyclin⁴ and NO,³ as well as with the discovery of integrins and other surface signals.⁴ It is now evident that the endothelium is at the cross-bridges of communication between blood and tissue cells and actively controls this process and the function of surrounding cells by a plethora of signaling routes. One of the prominent communication lines is established by the so-called L-arginine-NO-cyclic GMP pathway.⁵ This signaling cascade starts with eNOS (NOSIII), which generates NO and l-citrulline from L-arginine and O₂ in response to receptor-dependent agonists (bradykinin, acetylcholine, ATP) and physicochemical stimuli (shear, stretch).⁶ Reducing equivalents are provided by NADPH, and electrons are passed via a flavin chain to the catalytic center, the enzymes heme iron (Figure 2, upper). The first step of the normal NOS reaction is a classical mono-oxygenation, which consumes 1 mol of NADPH and oxygen. Molecular oxygen bound to the iron is activated and split to accomplish a hydroxylation of the substrate, the guanidino-nitrogen of L-arginine, forming N⁰-hydroxy-L-arginine. The second step is an atypical mono-oxygenation, which consumes 1 mol O₂ and 0.5 mol NADPH. It is a 3-electron oxidation of N⁰-hydroxy-L-arginine to afford the final products NO and l-citrulline. To guarantee this reaction path, the enzyme has to be homodimeric and the cofactor tetrahydrobiopterin must be present (Figure 2). For details, the reader is referred to excellent reviews on this topic.⁷ ⁸

Figure 2. Scheme depicting electron flow in coupled vs uncoupled eNOS. Electron flow starts from NADPH to flavins FAD and FMN of the reductase domain, which delivers the electrons to the iron of the heme (oxygenase domain) and to the BH₄ radical generated as an intermediate in the catalytic cycle. BH₄ seems to be essential to donate an electron and proton to versatile intermediates in the reaction cycle of l-arginine/O₂ to l-citrulline/NO. Cyclic GMP controls electron flow in eNOS. Zinc ions (Zn) bound to NOS are required for dimer formation and stability. Monomeric eNOS or BH₄/l-arginine-deficient eNOS is uncoupled and produces O₂⁻(for explanation see text).

Homologues subunits has only been detected in the human placenta (α₂)¹⁰,¹¹ and kidney (β₂).¹² At the genomic level, a dominant-negative splice variant of α₂, classified as α₂δ, has been detected.¹³ The interspecies homology of the individual subunits is high, whereas the intersubunit homology is lower.¹⁴ In contrast to α₂ and β₁, the biological significance of α₂δ, α₂β₁, and β₂β₁ is still obscure. The α₂δ subunit has recently been shown to be linked to cerebral maturation and sensory pathway refinement during postnatal development.¹⁵ A unifying concept of the molecular requisites for sGC activation has been put forth.¹⁶,¹⁷ Activation by NO requires sGC heme iron to be in the ferrous (II) state. On NO binding, the iron is moved slightly out of the porphyrin plane, thereby releasing a distal histidine (His₁⁰⁵ of the β₁ subunit) from iron coordination.¹⁸ This triggers subsequent intramolecular rearrangements influencing the catalytic center, resulting in an up to several-hundred-fold increase in cGMP formation. Depending on the cell type, cyclic GMP then elicits different biological responses, either by inhibition (cAMP-metabolizing phosphodiesterases) or by activation (cGMP-activated protein kinases, cGK, and cGMP-gated cation channels) of effector proteins.¹⁹ A comprehensive overview on cGK-I downstream signaling is provided.¹⁹ The mammalian cGK family consists of cGK-Iα, a splice variant cGK-IB β, and cGK-II, which is encoded by a separate gene.¹⁹ Both cGK-Iα isoforms, not cGK-II, are expressed in vascular cells. In rat aortic smooth muscle cells, activated cGK-1α increases the open probability of Ca²⁺-activated K⁺(BK) channels, thereby inducing a hyperpolarization of the smooth muscle cells and inhibition of agonist-induced Ca²⁺ influx. In addition, activated cGK-IB β phosphorylates the inositol triphosphate receptor-associated G-kinase substrate, thereby inhibiting agonist-induced Ca²⁺ release and smooth muscle contraction. Another cGK-I substrate found in many cell
types is the 46/50 kDa vasodilator-stimulated phosphoprotein. cGK-I phosphorylates vasodilator-stimulated phosphoprotein specifically at serine 239, and this reaction can be exploited as a biochemical monitor for the integrity and activity of the NO-cGMP pathway, as discussed. Phosphorylation and activation of cGMP-hydrolyzing phosphodiesterase 5 by cGK-I is a major mechanism to cease cGMP signaling.

**Oxidative Stress and Endothelial Dysfunction**

The endothelium-derived relaxing factor, previously identified as NO or a closely related compound, has potent anti-atherosclerotic properties. NO released from endothelial cells works in concert with prostacyclin to inhibit platelet aggregation, it inhibits the attachment of neutrophils to endothelial cells and the expression of adhesion molecules. NO in high concentrations inhibits the proliferation of smooth muscle cells. Therefore, under all conditions in which an absolute or relative NO deficit is encountered, the process of atherosclerosis is being initiated or accelerated. The half-life of NO and therefore its biological activity is decisively determined by oxygen-derived free radicals such as superoxide. Superoxide rapidly reacts with NO to form the highly reactive intermediate peroxynitrite. The rapid bimolecular reaction between NO and superoxide yielding peroxynitrite (rate constant: 5 to 10 $\times$ 10$^9$ M$^{-1}$s$^{-1}$) is 3- to 4-times faster than the dismutation of superoxide by the superoxide dismutase. Therefore, peroxynitrite 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. Recent studies also indicate that peroxynitrite may have deleterious effects on activity and function of the prostacyclin synthase and the eNOS. Other ROS such as the dismutation product of superoxide, hydrogen peroxide, and the hypochlorous acid released by activated neutrophils, are not free radicals, but have a powerful oxidizing capacity, which will further contribute to oxidative stress within vascular tissue.

**Endothelial Dysfunction and Cardiovascular Risk Factors**

It is well known that in the presence of cardiovascular risk factors endothelial dysfunction is frequently encountered. This has been shown for chronic smokers, patients with increased low-density lipoprotein levels, for patients with diabetes types I and II, for hypertensive patients, and for patients with metabolic syndrome. There are several potential abnormalities, which could account for reductions in endothelium-dependent vascular relaxation including changes in the activity and/or expression of the eNOS, decreased sensitivity of vascular smooth muscle cells to NO, or increased degradation of NO via its interaction with ROS such as superoxide. The NO degradation concept is the most attractive one because in the presence of cardiovascular risk factors, endothelial dysfunction is established and even more importantly it is markedly improved by the acute administration of the antioxidant vitamin C. Superoxide and/or peroxynitrite could also act further downstream by oxidative inactivation of sGC as well as activation of cGK-I.

**eNOS Uncoupling Contributes to Endothelial Dysfunction**

In most situations in which endothelial dysfunction caused by increased oxidative stress is encountered, the expression of the eNOS has been shown to be paradoxically increased rather than decreased. The mechanisms underlying increased expression of eNOS are likely to be secondary to increased endothelial levels of hydrogen peroxide, which has been shown to increase the expression at the transcriptional and translational level. 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. Very intriguing are observations that the eNOS itself can be a superoxide source, thereby causing endothelial dysfunction. It has become clear from studies with the purified enzyme that eNOS may become “uncoupled,” eg, in the absence of the NOS substrate L-arginine or the cofactor tetrahydrobiopterin (BH$_4$). 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, resulting in production of superoxide rather than NO (Figure 2). That eNOS-derived superoxide/ROS formation bears consequences opposite to “normal” NOS function was first shown in U937 cells transfected with wild-type eNOS, which induced increased transcription of tumor necrosis factor-$\alpha$ in an NOS inhibitor (L-NMA)-insensitive and superoxide-dismutase-sensitive fashion. We discuss several possibilities how eNOS uncoupling may occur.

**eNOS Uncoupling Caused by Increased Peroxynitrite-Mediated BH$_4$ Oxidation**

For proper function of NOS, BH$_4$ seems to be essential in several ways. BH$_4$ stabilizes the NOS dimer, facilitates its formation, and protects NOS against proteolysis. It also increases the affinity of NOS for L-arginine and affects the spin state of the heme iron, the heme redox potential, and the oxygen binding. Most importantly, however, BH$_4$ plays a decisive role for oxygen activation and the time-critical delivery of one electron and proton to the Fe(II)–O$_2$ intermediate, which converts to an iron-oxo species and releases H$_2$O$_2$ in the catalytic cycle of NOS. In the electron in the first mono-oxygenation reaction that hydroxylates L-arginine to N-hydroxy-arginine. Rapid kinetics analysis by freeze-quench EPR revealed the transient formation of a BH$_4^+$ cation radical during this reaction, which rapidly splits off a proton to form a BH$_3^-$ radical. The BH$_3^-$ radical is reduced by one electron and proton delivered by the reductase domain to BH$_4^-$, which participates in a second oxygen activation step leading to the final products NO and L-citrulline. In the absence of BH$_4^-$, the Fe(II)–O$_2$ intermediate decays under formation of superoxide and Fe(III). Dihydrobiopterin (BH$_2$) and other derivatives such as sepiapterin can bind to NOS but cannot support NO formation. Therefore, limited availability of BH$_4^-$ for NOS will inevitably result in...
increased superoxide formation at the expense of NO formation, ie, it will uncouple NOS.

What are the mechanisms leading to BH4 depletion? In vitro studies proposed that native low-density lipoprotein44 and even more pronounced oxidized low-density lipoprotein45 are able to stimulate endothelial superoxide production and that this phenomenon is inhibited by the NOS inhibitor L-NAME, pointing to a specific role of eNOS in superoxide production. Hypercholesterolemia also has shown to increase vascular formation of superoxide via activation of the NAD(P)H oxidase46 and/or xanthine oxidase.47 Superoxide derived from both enzyme sources may lead to increased formation of peroxynitrite.33,48 Peroxynitrite in turn rapidly oxidizes the active NOS cofactor BH4 to cofactor inactive molecules such as BH2.33,49 These concepts, however, also imply that the uncoupling of eNOS would invariably require a priming event such as superoxide produced by the NAD(P)H oxidase and/or the xanthine oxidase (so-called kindling radicals) leading via increased formation of peroxynitrite eNOS to produce superoxide (bonfire radical).

eNOS Uncoupling Caused by Peroxynitrite-Mediated Oxidation of the Zinc–Thiolate Complex

Another interesting concept concerning eNOS uncoupling was provided by Zou et al. These authors showed that the exposure of the isolated enzyme to peroxynitrite leads to a disruption of the zinc–thiolate cluster resulting in an uncoupling of the enzyme.28 The authors also demonstrated that a similar phenomenon occurred when endothelial cells were exposed to high concentrations of glucose. Additional experiments revealed that BH4 was oxidized at concentrations being 10- to 100-fold higher than those needed to disrupt the zinc–thiolate complex. Based on these findings, the authors suggested that the principal mechanism of uncoupling is the oxidation of the zinc–thiolate center and the subsequent release of Zn2+ ions rather than BH4 oxidation.28

eNOS Uncoupling Caused by L-Arginine Deficiency or Increased Production of Asymmetrical Methyl Arginine

Several recently published studies demonstrate that increased concentrations of asymmetrical methyl arginines (ADMA) in cultured endothelial cells or in patients with endothelial dysfunction are associated with increased ROS production in supernatants, rodents, or human plasma.50–52 The question is whether increased ROS production is the reason for increased ADMA levels or whether increased production of ADMA actually contributes to the oxidative stress burden of the vasculature via uncoupling of eNOS. Interestingly, the activity of methylation enzymes such as the S-adenosylmethionine–dependent PRMTs (type 1)51 responsible for the ADMA synthesis or the activity of ADMA hydrolyzing enzymes such as DDAH50 is redox-sensitive. Thus, oxidative stress in the vasculature should always stimulate ADMA production and/or inhibit ADMA degradation in concentrations that significantly inhibit eNOS activity or even uncouple the enzyme, which would further increase superoxide production in a positive feedback fashion.53

Which Enzyme Produces the Kindling Radical for Increased Peroxynitrite Formation Ultimately Leading to eNOS Uncoupling?

Role for NAD(P)H Oxidase

The NAD(P)H oxidase is a superoxide-producing enzyme that has been first characterized in neutrophils.54 We know that a similar enzyme exists also in endothelial and smooth muscle cells, as well as in the adventitia. The activity of the enzyme in endothelial as well as smooth muscle cells is increased on stimulation with angiotensin II.55 The stimulatory effects of angiotensin II on the activity of this enzyme would suggest that in the presence of an activated renin angiotensin system (local or circulating), vascular dysfunction caused by increased vascular superoxide production is likely to be expected. Experimental hypercholesterolemia has been shown to be associated with an activation of the NAD(P)H oxidase46 and there is a close association with endothelial dysfunction and clinical risk factors and the activity of this enzyme in human saphenous veins in patients with coronary artery disease.56 In atherosclerotic arteries there is evidence for increased expression of the NAD(P)H oxidase subunit gp91phox and nox4, all of which may contribute to increased oxidative stress within vascular tissue.57

Interestingly, there is growing body of evidence that the local renin angiotensin system is activated in the setting of hypercholesterolemia. In patients, ACE activity and therefore local angiotensin II concentrations are increased in atherosclerotic plaques58,59 and inflammatory cells are capable of producing large amounts of angiotensin II. Increased angiotensin II concentrations along with increased levels of superoxide have been shown in the shoulder region of atherosclerotic plaques.60 In vessels from hypercholesterolemic animals60 as well as in platelets from hypercholesterolemic patients,61 there is an increase in the expression of the angiotensin II receptor subtype AT1. Thus, both experimental and clinical studies have provided evidence for stimulation of the renin angiotensin system in atherosclerosis and simultaneously for an activation of the NAD(P)H oxidase in the arterial wall. Similar evidence for an activation of this enzyme in the vasculature has been provided from experimental animal models of different forms of hypertension such as angiotensin II infusion62,63 and in SHR,64 as well as in different forms of diabetes mellitus.35

The proof of concept that superoxide produced by the NADPH oxidase may indeed trigger eNOS uncoupling was provided by David Harrison’s group in the experimental animal model of DOCA-salt hypertension. With these studies, the authors showed that superoxide induced by DOCA-salt treatment caused increased vascular superoxide production, which was significantly reduced by an inhibitor of eNOS such as L-NAME. Treatment of p47phox knockout animals with DOCA-salt caused markedly reduced levels of oxidative stress and abolished superoxide effects of NOS inhibition compatible with a prevention of eNOS uncoupling.65

Role for Xanthine Oxidase

Xanthine oxidase catalyzes the sequential hydroxylation of hypoxanthine to yield xanthine and uric acid. The
enzyme can exist in 2 forms that differ primarily in their oxidizing substrate specificity. The dehydrogenase form preferentially uses \( \text{NAD}^+ \) as an electron acceptor but is also able to donate electrons to molecular oxygen. By proteolytic breakdown as well as thiol oxidation, xanthine dehydrogenase from mammalian sources can be converted to the oxidase form that readily donates electrons to molecular oxygen, thereby producing superoxide and hydrogen peroxide but does not reduce \( \text{NAD}^+ \). Oxypurinol, an inhibitor of xanthine oxidoreductase, has been shown to reduce superoxide production and to improve endothelium-dependent vascular relaxations to acetylcholine in vessels from hyperlipidemic animals.\(^{47}\) This suggests an increase in the expression or activity of xanthine oxidase in early hypercholesterolemia. The mechanisms underlying such a phenomenon remain unclear; however, it has been demonstrated that certain cytokines can stimulate the expression of xanthine oxidase by the endothelium. An alternative mechanism may be that increased cholesterol levels trigger the release of xanthine oxidase (eg, from the liver) into the circulation where it binds to endothelial glycosaminoglycans.\(^{66}\) Human studies concerning the efficacy of xanthine oxidase inhibition on endothelial dysfunction are somewhat discrepant. Although Panza et al showed that endothelial dysfunction in hypercholesterolemic patients and hypertensive diabetic subjects is improved by acute inhibition of xanthine oxidase with oxypurinol and allopurinol,\(^{67,68}\) other groups failed to show similar efficacy\(^{69}\) for allopurinol. Its role in mediating increased oxidative stress in the setting of hypertension is not quite clear. Oxypurinol has blood pressure-lowering effects comparable to heparin-binding SOD in SHR\(^{70}\) but fails to demonstrate a positive effect on endothelial dysfunction in hypertensive patients.\(^{67}\)

How Can We Assess eNOS Uncoupling?

It is important to note that eNOS-mediated superoxide production—by the isolated enzyme or in vascular tissue—is inhibited by N\(^{-}\)-nitro-L-arginine (l-NNA) and its methylester N\(^{-}\)-nitro-L-arginine methylester (l-NAME), because both substances antagonize the transfer of electrons to either \( \text{L-arginine} \) or oxygen. In contrast, l-NMMA has been previously shown to stimulate rather than to inhibit superoxide production by the isolated enzyme because of partial uncoupling of the oxidase fraction.\(^{71}\) This has been shown for inducible NOS\(^{71}\) and for neuronal NOS.\(^{72}\)

Similar phenomena may have to be expected when isolated enzymes are exposed to the structurally similar ADMA, which will prevent the oxidation of L-arginine and therefore reduce NO production or, as mentioned, will stimulate superoxide production by competing for the same binding site of the enzyme.

The l-NNA-sensitive and l-NAME-sensitive inhibition of superoxide formation is a convenient method to detect uncoupling of NOS in vascular tissues. Depending on the detection system used, addition of these NOS inhibitors to vascular cells and tissues will decrease the superoxide-derived signal, such as lucigenin-dependent (5 \( \mu \text{mol/L} \)) luminescence or the dihydroethidine fluorescence.\(^{73,74}\) The Harrison group showed that depletion of endogenous BH\(_4\) in mouse aorta by addition of exogenous peroxynitrite increased superoxide formation, and that addition of l-NAME, removal of the endothelium, or genetic knockout of eNOS prevented this radical response.\(^{33–36}\)

Modulation of the Activity and Expression of the sGC and cGK-I In Vitro by ROS and Effects of Oxidative Stress on the Activity and Expression of the sGC and cGK-I In Vivo

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Modulation of the activity and expression of the sGC and cGK-I in vitro by reactive oxygen species:

Modulation of sGC activity by ROS: The activity of the heterodimeric hemoprotein soluble guanylyl cyclase (sGC) is regulated by both, NO and superoxide, in an opposite manner. Thus, while NO can increase cGMP formation by sGC in vitro and in vivo by several hundred-fold, superoxide radicals inhibit basal and NO stimulated sGC activity. This has been shown with isolated enzyme exposed to superoxide-generating systems \(^1\), as well as with native enzyme in platelets \(^2\) and vascular cells and tissues exposed to superoxide-generating redoxcycling agents \(^3\). Though it is quite obvious that in the intact organism the enzyme activity is indirectly reduced when NO bioavailability is compromised by enhanced superoxide formation, in vitro experiments with NO-independent and -insensitive activators of sGC like carbon monoxide (CO) and YC-1 have shown that sGC activity is also directly affected by superoxide \(^2, 4\). This holds also true for cultured cells and isolated tissues exposed to oxidative stress \(^2-5\). In addition, sGC activity may be inhibited by the intermediate metabolite formed from NO and superoxide, peroxynitrite. This again has been demonstrated with the isolated enzyme (Mülsch et al. unpublished observation) and vascular tissues.\(^5\) Inhibition of sGC activity by superoxide and peroxynitrite presumably occur by different mechanisms, as suggested by the finding that the former is prevented by superoxide dismutase, is quickly reversible, and is insensitive to the presence of thiols\(^4\), whereas the latter is irreversible in vitro and is potently blocked by glutathione. Since rapid reversibility of enzyme activation is essential for participation in rapid cellular signaling, superoxide-dependent modulation of sGC activity is more likely to play a role in this context than inhibition of the enzyme by peroxynitrite.

Modulation of sGC expression by ROS: Besides these direct effects, oxidative stress may affect sGC activity also indirectly by altering expression of the sGC protein subunits. Thus, in some animal models associated with endothelial dysfunction (hypercholesterolemic rabbits; nitrate tolerant rats) sGC expression was paradoxically found to be increased, whereas in other models it was reduced (Table 1). However, the underlying signaling pathways are largely unknown. The transcription start site and the 5'-flanking region of the rat sGC \(\alpha_1\) and \(\beta_1\) genes were recently determined and sequenced (Gene bank accession No. AF327644 and AF327645). In silico analysis of the putative promoter regions (2.9 kb of \(\alpha_1\) and 1.3 kb of \(\beta_1\)) revealed the presence of several potential consensus motifs for binding of redox-sensitive transcription factors, such as AP1, NF-IL6 (C/EBP\(\beta\)), PEA3 and E2A. Similarly, putative regulatory sites for the redox-sensitive transcription factors SP-1, NFkappaB and AP-1 were identified in the mouse sGCa1 core promoter \(^6\), and SP-1 was shown to be essential for
basal promoter activity. Modulation by the MAP kinase signaling pathway is a property common to these transcription factors. Since oxidative stress activates this signalling pathway, it is quite possible that up-regulation of sGC expression in animal models of oxidative stress, which lack hypertension, occurs by transcriptional activation of sGC subunit promoters via MAP kinase(s) stimulation. On the other hand, downregulation of sGC as occurs in hypertension and aging may result from mechanical force- and growth factor-elicited transcriptional pathways linked to smooth muscle proliferation and vascular wall thickening. However, the situation is more complex, since mechanosensor- and growth factor-dependent signalling pathways partially overlap with oxidative stress-activated intracellular signalling. For instance, the transcription factors GKLF and Id3 mediate, redox-sensitive vascular smooth muscle cell proliferation in vitro and in vivo. It is too early to resolve this apparently discrepant findings until a detailed promoter analysis of sGC subunit genes is available. It is also worth to recall that transcriptional regulation of sGC in animal models is not necessarily similar in human, since promoter function may differ considerably. A first analysis demonstrated a requirement of CBF(NF-Y) and GFI1 binding to the core promoter for constitutive expression of human sGCß subunit, whereas NF1 binding decreased promoter activity.

More importantly, however, sGC expression is controlled at the post-transcriptional level by NO and cyclic nucleotides, thereby opening a pathway for superoxide to indirectly affect sGC expression: In various cell types cyclic AMP eliciting agonists decrease the expression of sGC mRNA and protein by a destabilization of the mRNA. This effect is mimicked by activation of the cGMP-signaling pathway, e.g. application of NO donors, stimulation of particulate GC by atrial natriuretic factor (ANF), and stimulation of cGK-I by the stable cGMP-analogue 8-chlorophenylthio-cGMP. The molecular mechanism underlying this phenomenon was recently identified. The 3'-untranslated regions (3'-UTR) of the rat sGCa and b1 mRNA bear several AUUUA- or AUUUAU-motifs (AU-rich elements, AREs), which target these mRNAs for rapid degradation and for trans-acting factors for regulation of mRNA stability. One of these factors is the ubiquitous 34 kDa protein HuR, also known as HuA. HuR binds to AREs present in the 3'-UTR of target mRNAs, thereby protecting these mRNA from accelerated decay. The decisive role of HuR for regulation of sGC expression was proven by siRNA-induced knock-down of HuR in rat aortic smooth muscle cells, which led to a concomitant decrease in sGC expression. Furthermore, prolonged (6 h) sGC-activation by YC-1 decreased the expression of HuR protein and HuR binding activity for sGCa mRNA in rat aortic smooth muscle cells and isolated aorta. Consequently, the expression of the sGCa subunit was decreased (Figure 3). All these effects could be blocked by an inhibitor of YC-1-stimulated sGC activity, NS 2028, indicating that they were caused by an increased sGC activity/cGMP formation. Similarly, the cAMP-induced decrease in sGC expression is accomplished by the same mechanisms, i.e., a cAMP-induced decrease of HuR expression leads to destabilization of sGC mRNA. Collectively, these findings suggest the existence of a negative feedback loop formed by sGC and HuR, e.g. increased sGC activity will decrease HuR expression, thereby leading to down-regulation of sGC and reduced cGMP formation (Figure 3). Conversely, inhibition of NO/cGMP- and PGI2/cAMP-signaling by superoxide/peroxynitrite will elicit opposite effects, i.e. up-regulation of sGC. Though this hypothesis is compatible with the finding of increased sGC expression in superoxide-generating vascular tissue, it warrants further experimental verification.
Increased superoxide and/or peroxynitrite production within the smooth muscle cells layer, in combination with an inhibition of sGC activity should consistently lead to a desensitization of the vasculature, not only to NO itself but also to endothelium independent nitrovasodilators such as NTG and SNP. It is important to note that in the setting of endothelial dysfunction due to decreased vascular NO bioavailability e.g. such as treatment with NOS inhibitor such as L-NMMA or in eNOS knockout mice, a supersensitivity to nitrovasodilators such as SNP or NTG has been shown. Thus, a demonstration of superimposable relaxation curves to NTG or SNP in the setting of endothelial dysfunction already points to a dysfunction of the vasculature at the smooth muscle levels e.g. due to increased superoxide production. In addition, manuscripts demonstrating the prognostic importance of endothelium-dependent dilation in patients with coronary artery disease also showed that an attenuation of the SNP as well as NTG responses in coronary and peripheral arteries also has some prognostic meaning.

Modulation of cGK activity by ROS: cGKI activity can be assessed by immunodetection of phosphorylation at Ser239 of the cGKI substrate protein VASP (P-VASP). This approach has been shown to be a reliable surrogate parameter of the integrity of the NO/cGMP signaling pathway in platelets, cultured endothelial and smooth muscle cells, and as shown more recently also in intact vascular tissues. The P-VASP assay appears to be sufficiently sensitive to monitor basal NO release in endothelium-intact arteries. For example, incubation of vessels with NOS inhibitors such as N^4-nitro-L-arginine (L-NNA) or with its methylester L-NAME, strongly decreased P-VASP levels. Likewise, NO donors such as SNP and NTG markedly increased P-VASP in vessels in the absence and presence of an intact endothelium. Mechanical removal of the endothelium in rat and rabbit aorta almost completely abolished "basal" VASP phosphorylation, whereas total VASP levels (phosphorylated and non-phosphorylated) were reduced by about 40-50%. These findings indicate that VASP Ser239 phosphorylation clearly depends on the presence of a functional endothelium, and that the endothelial cell layer in aortic vessels contains a considerable amount of VASP, which is lost upon removal of the endothelium. P-VASP analysis also appears suitable to monitor oxidative stress within vascular tissue. Incubation of isolated endothelium-intact vessels with diethylthiocarbamate (DETC), an inhibitor of Cu/Zn superoxide dismutase (SOD), markedly reduced P-VASP levels. Concomitantly, basal endothelial NO formation was decreased and formation of superoxide was increased.

Effects of oxidative stress on the activity and expression of the sGC and cGK-I in vivo:
1) eNOS uncoupling and consequences for the activity and expression of the sGC and cGK-I in atherosclerosis and heart failure

Hypercholesterolemia impairs endothelium-dependent vasodilation and relaxation to authentic NO, but has little effect on relaxations in response to nitrovasodilators SNP and NTG. In animals rendered hyperlipidemic by cholesterol feeding or LDL receptor deficiency, and in ApoE-knockout mice, steady-state superoxide signals were markedly higher than in control animals indicative of a key role of oxidative stress in the induction of endothelial dysfunction. Xanthine oxidase, NAD(P)H oxidase, eNOS and the mitochondria were identified as significant superoxide sources. Superoxide production was derived not only from the endothelial cell layer but also from smooth muscle cells, leading to decreased vascular NO bioavailability (as
demonstrated by the electron paramagnetic resonance (EPR) technique\textsuperscript{28} and subsequently to impaired NO/cGMP downstream signalling.

Apparently discrepant findings have been reported with regard to the expression of the NO target enzyme sGC in hypercholesterolemia. In one study diet-induced hypercholesterolemia in New Zealand White rabbits (NZWR) was associated with a 3- to 4-fold increase in expression of both sGC protein subunits $\alpha_1$ and $\beta_1$ in the aorta (mainly within neointimal lesions), whereas basal and NO-stimulated sGC activity was not modified at all\textsuperscript{20}. However, in a more recent study sGC expression was found to be reduced in the same animal model, especially in the neo-intima, which accounted for 60% of the aortic tissue, and NO-sensitive sGC activity was significantly lower compared to control vessels\textsuperscript{36}. These discrepant findings might result from the different feeding regimes applied: while in the first study the rabbits were fed for a short period with high cholesterol (8 weeks), in the latter a moderately cholesterol-enriched diet was fed for 1 year. It therefore appears that sGC expression changes during the course of atherogenesis. The discrepancy between increased expression of sGC and a lack of change in sGC activity as reported for the short term high cholesterol diet may be explained by increased ROS production within the smooth muscle cell layer. The in vivo situation (increased sGC expression, decreased activity) can be mimicked by in vitro incubation of purified\textsuperscript{4} and crude sGC\textsuperscript{5} with superoxide and peroxynitrite respectively, or by incubation of intact vascular tissue with LY83583, a redox cycler and sGC inhibitor that generates superoxide intracellularly.\textsuperscript{3}

Interestingly, increased expression of sGC in response to short term hypercholesterolemia seems to be restricted to this model. In vessels from WHHL, an animal model of hypercholesterolemia due to an LDL receptor defect, the expression of the sGC $\beta_1$ subunit was not modified at all.\textsuperscript{37} Cholesterol feeding usually results in total cholesterol levels exceeding 1500 mg/dl by far while in WHHL lipid levels are closer to the pathophysiological range (maximally up to 500 mg/dl). Thus, the lack of changes in sGC expression despite total cholesterol levels in the range of 500 mg/dl challenge the concept that high cholesterol levels, per se, increase the expression of the enzyme.\textsuperscript{37}

A similar phenomenon of increased expression of sGC has recently been observed in an animal model of chronic ischemic heart failure. In this model endothelial dysfunction was associated with increased aortic sGC $\beta_1$ protein expression,\textsuperscript{38} and an attenuated vascular cGMP increase in response to acute SNP challenges. cGMP responses to SNP were normalized by the superoxide scavenger Tiron, indicating that superoxide-mediated inactivation of NO is mainly responsible for the NO resistance of sGC. Thus, up-regulation of sGC may reflect an adaptive response, which attempts to compensate for reduced NO activation of sGC in short term experimental hypercholesterolemia and in other non-hypertensive models with increased vascular oxidative stress. Although sGC is up-regulated, the vessel is deprived of NO, therefore compensation is thwarted and endothelial and vascular dysfunction persists.

The expression of cGK-I in vessels from Watanabe heritable hyperlipidemic (WHHL) rabbits was found to be normal. However cGK activity, assessed by the P-VASP/VASP ratio, was strongly decreased compared to control, suggestive of a defect in the NO/cGMP pathway upstream of cGK.\textsuperscript{28} Incubation of vessels from control as well as hyperlipidemic animals with high concentrations of the NO donor SNP resulted in comparable maximal P-VASP levels, demonstrating that the cGMP
signaling pathway downstream of sGC per se was not impaired.\textsuperscript{28} Chronic treatment of hyperlipidemic animals with AT1 receptor blockers, PPAR-\(\gamma\)-inhibitors\textsuperscript{39} as well as with the \(\beta\)-receptorblocker nebivolol\textsuperscript{37} reduced oxidative stress within vascular tissue and improved ED. This phenomenon is likely related to the inhibitory effects of these compounds on NADPH-oxidase and/or eNOS-mediated superoxide production. The improvement of ED was associated with increased vascular NO-concentrations\textsuperscript{28} as assessed with EPR spectroscopy and a quite marked enhancement in vascular P-VASP levels, indicating the usefulness of P-VASP as a biomonitor for NO in vascular tissue.

2) eNOS uncoupling and consequences for the activity and expression of the sGC and cGK-I in hypertension

Endothelial dysfunction in different animal models of hypertension has been shown to be associated with increased expression of eNOS\textsuperscript{40,41} but decreased vascular NO bioavailability.\textsuperscript{40} Uncoupled eNOS\textsuperscript{40} and NAD(P)H oxidase\textsuperscript{42} were identified as significant superoxide sources by the observation that NOS inhibition decreased superoxide production in the tissue from hypertensive animals,\textsuperscript{40} and by the observed increases in expression and enzyme activity of NAD(P)H oxidase subunits (nox1, p22phox, p67phox and gp91phox).\textsuperscript{40,42-44} Increased superoxide production involved all vessel layers, namely the endothelium, media and adventitia\textsuperscript{40,45} and also increased vasoconstrictor tone via stimulation of the expression of endothelin-1 in the smooth muscle and endothelial cell layer\textsuperscript{46,47}. 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.

In contrast to vessels from hyperlipidemic animals, those from hypertensive animals displayed not only reduced vasodilation to endothelium-dependent vasodilators but also to endothelium-independent nitro-vasodilators SNP and NTG.\textsuperscript{42} This finding may be explained at least in part by reduced expression of sGC, since in different hypertensive animal models the expression of one or both sGC subunits (\(\alpha_1\) and \(\beta_1\)) as well as NO-dependent sGC activity was significantly decreased. This was observed in genetic hypertension, such as aged SHR\textsuperscript{48,49} stroke-prone SHR,\textsuperscript{50} type 2 diabetic, mildly hypertensive Goto-Kakizaki rats,\textsuperscript{51} and TGR(mREN2)27 renin transgenic rats,\textsuperscript{52} as well as in several models of drug-induced hypertension (Table 1).

Reduction of hypertension normalized or even enhanced sGC expression,\textsuperscript{40,41,50,53} but had differing effects on vascular superoxide production. For example, in the angiotensin II-infusion model, inhibition of protein kinase C (PKC) in vivo, reduced blood pressure and vascular superoxide formation by inhibiting activity and expression of NADPH oxidase and by preventing eNOS uncoupling\textsuperscript{40} and concomitantly prevented sGC down-regulation (unpublished observation). In contrast, other forms of antihypertensive treatment, e.g. with hydralazine significantly lowered blood pressure but failed to normalize vascular superoxide formation in chronically, NOS-inhibited rats\textsuperscript{53}. Thus, it appears that the decrease in sGC expression elicited by high blood pressure is not exclusively linked to increased superoxide formation and/or to increased levels of vasoconstrictor peptides. In contrary, vasoconstrictor peptides such as angiotensin II or endothelin 1, which are increased in hypertension, seem to trigger an up-regulation of sGC in vascular smooth muscle cells (A. Mulsch, unpublished observation). This sGC up-regulation is apparently overwhelmed by effects of yet
undetermined mediators of hypertension, which decrease sGC expression and NO/cGMP signaling, thereby promoting endothelial and smooth muscle dysfunction.

cGK-I expression in aortic tissue was not changed in two different models of hypertension. Still, cGK-I activity assessed by P-VASP analysis was markedly reduced and suggestive of a signaling defect upstream of cGK-I. In support of this, treatment with chelerythrine, an inhibitor of protein kinase C, reduced oxidative stress, increased P-VASP formation, and improved ED (T. Munzel, unpublished observation). In vitro, angiotensin II increased the activity and expression of the cGMP-specific PDE1A1 isoform in cultured smooth muscle cells (Figure 4). Thus, we cannot exclude the possibility that increased cGMP metabolism may contribute partly to the ED and decreased cGK-I activity observed in angiotensin II infusion-induced hypertension.

3) eNOS uncoupling and consequences for the activity and expression of the sGC and cGK-I in nitrate tolerance

NTG induces vasorelaxation by releasing NO or a chemically related compound via an enzymatic biotransformation process within mitochondria. Though acute application of NTG exhibits high vasodilator and anti-ischemic efficacy, this beneficial action is rapidly lost upon chronic treatment due to the development of nitrate tolerance. Chronic NTG treatment increases vascular nitrotyrosine levels within the endothelium and the subendothelial space compatible with increased peroxynitrite formation. As in the case of hypertension and hypercholesterolemia, nitrate tolerance is associated with increased expression of a dysfunctional, uncoupled eNOS and with activation of NAD(P)H oxidase. Further support for a role of ROS in nitrate tolerance was provided by the demonstration that concomitant treatment with antioxidants such as vitamin E or C, or other substances which reduce intracellular oxidative stress, such as ACE-inhibitors, AT1 receptor blockers (both, which reduce the activity of the NADPH oxidase), BH4 and folic acid (which prevents eNOS uncoupling by increasing intracellular BH4 levels), as well as peroxynitrite quenchers such as ebselen and uric acid beneficially influence tolerance and cross tolerance to other nitro-vasodilators in NTG-treated animals or human subjects. Interestingly, recent results from experimental studies indicate that ROS formed during NTG metabolism are able to inhibit the activity of the mitochondrial aldehyde dehydrogenase (ALDH-2). Accordingly, baseline cGMP levels in tolerant tissue are lower than in control vessels, and the cGMP response to acute NTG-challenges are depressed. According to a current hypothesis, reduced vascular NO bioavailability and increased production of endothelin-1 within smooth muscle cells and impaired NTG biotransformation account for tolerance. In addition, direct inhibition of NO-dependent sGC activity by superoxide and/or peroxynitrite production elicited by NTG may contribute to reduced cGMP formation. Unexpectedly, the mRNA and protein expression of sGC subunits α1 and β1 were increased 2-fold in aortas from tolerant rats and rabbits. Thus, enhanced vascular ROS levels and vasoconstrictor peptide formation elicit tolerance, despite an increase in sGC expression. The findings with this animal model of vascular dysfunction confirm our hypothesis that in the absence of established hypertension, superoxide and endothelin trigger an increase in sGC expression as part of a biological counter-regulatory mechanism.

In addition to changes in protein expression, the net function of the NO/cGMP pathway in nitrate tolerance may also be affected by post-translational
modifications of sGC, some of which, remarkably, can be induced by NO itself. There is evidence for regulation of sGC activity by ferrous to ferric heme oxidation, heme loss, cysteine residue oxidation, phosphorylation by PKA, and intracellular redistribution and association with stabilizing/destabilizing proteins. In the presence of high concentrations of NO in aerobic environments, heme-iron oxidation occurs readily with purified or crude sGC and in intact cells, causing desensitization of the enzyme to NO. However, it is not known whether the steady state levels of NO achieved in vascular smooth muscle cells of NTG-tolerant individuals are sufficiently high to elicit sGC heme oxidation and sGC inhibition as discussed recently. These authors showed that in the absence of thiols NTG directly oxidized sGC heme-iron, thereby preventing NO-dependent activation of sGC in vitro. The availability of specific activators of heme-oxidized sGC should help to clarify whether or not nitrate tolerance is mediated in part on oxidation of the sGC heme-iron in intact cells.

Whereas sGC expression in the setting of in vivo tolerance was increased, and sGC activity decreased, cGK-I expression was not changed in aortas from rabbits and rats and mammary arteries from NTG-treated animals and patients. These data contrast to those of Soff et al. which showed a significant down-regulation of cGK-I mRNA and protein in rat vascular smooth muscle cells treated in vitro with the NO donor SNAP or analogues of cGMP or cAMP, or in vascular smooth muscle from rats treated in vivo with high concentrations of the organic nitrate ISDN. The NO-donor concentration seemed to be the major determinant of whether or not chronic treatment with NO depressed cGK-I. Whereas low NTG concentrations (3-10 µg/kg/min) failed to modify cGK-I expression, high concentrations of ISDN and NTG (50-100 µg/kg/min) significantly decreased cGK-I expression by about 50%. These observations clearly demonstrate that clinically relevant NTG concentrations do not decrease expression of cGK-I, and that cGK-I levels do not significantly contribute to the development of tolerance and to cross tolerance to endothelium dependent and independent-nitrovasodilators.

Although tolerance resulting from in vivo NTG treatment did lead to changes in cGK-I expression, it significantly decreased cGK-I activity (P-VASP levels) in aortas from rats and rabbits, and in the mammary artery from patients undergoing coronary artery bypass surgery. Incubation of tolerant tissue with SNP elicited normal increases in P-VASP levels, indicating that the cGMP-signaling pathway downstream of sGC was not impaired. Further results supported the concept that ROS markedly contributed to the nitrate tolerance and cross tolerance, which was detected as inhibition of cGK-I activity. Treatment with vitamin C reduced superoxide levels, restored P-VASP levels, and partially corrected tolerance. Similar correction was observed in response to in vivo treatment with the AT1 receptor blockers, losartan and candesartan in experimental animal models and patients, respectively, all of which was paralleled by a reduction of oxidative stress in vascular tissue or in plasma thioredoxin levels. In addition, in vitro incubation with peroxynitrite quenchers such as uric acid and ebselen reversed tolerance and simultaneously restored vascular cGK activity.
Figure 3: Hypothetical scheme illustrating the effect of increased NO or O$_2^-$ levels on the expression of sGC. Constitutive expression of sGC subunits $\alpha_1$ and $\beta_1$ is the net result of "house-keeping" transcription and mRNA stability regulated by the mRNA protecting protein HuR. Increased NO levels via route 1 to 5 (red) decrease HuR expression via a cGMP/cGK-I/AP-1 activation dependent mechanism, promoting degradation of sGC subunit mRNAs and thereby reducing sGC protein synthesis. Conversely, increased superoxide levels via steps 2 to 4 (blue) activate redox-sensitive transcription factors (RSTF), increase transcription of sGC subunits, thereby leading to increased sGC protein expression. In addition, increased superoxide also increases sGC protein synthesis by inhibiting cGMP formation and stabilizing sGC mRNA via route 1 to 5 (red).
Table 1: Selected references addressing the activity and expression of the soluble guanylyl cyclase (sGC) and/or cGMP-dependent protein kinase (cGK-I) in different models of endothelial dysfunction and increased oxidative stress

<table>
<thead>
<tr>
<th>Animal Model</th>
<th>Vessel Type</th>
<th>Endothelial Dysfunction</th>
<th>NOS III Expression</th>
<th>Oxidative Stress</th>
<th>Superoxide Source</th>
<th>NO-sensitivity</th>
<th>Expression (α1, β1)</th>
<th>Activity (P-VASP)</th>
<th>Expression</th>
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<tr>
<td>Atherosclerosis</td>
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<tr>
<td>Cholesterol fed rabbits, hyperlipidemic WHHL, Apo-E knockout mouse 20, 28, 30, 31</td>
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<td>++</td>
<td>++</td>
<td>++</td>
<td>XO NOX NOS</td>
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<td>Hypertension</td>
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<td>++</td>
<td>++</td>
<td>NOX NOS</td>
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<td>not done</td>
<td>no change (β1)</td>
<td>reduced</td>
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Endothelial function, assessed with acetylcholine, oxidative stress determined with chemiluminescence techniques (lucigenin or coelenterazine) or the fluorescent dye, dihydroethidine.

Abbreviations: NOS, uncoupled endothelial nitric oxide synthase, sGC: soluble guanylyl cyclase, cGK-I: cGMP-dependent protein kinase I, VASP. Vasodilator stimulated phosphoprotein, P-VASP: phosphorylated VASP at serine239; XO: xanthine oxidase. NOX: NADPH oxidase.
Literature:


