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Review

Endothelial NADH/NADPH-dependent enzymatic sources of superoxide production: relationship to endothelial dysfunction★✪

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There is growing evidence that endothelial dysfunction, which is often defined as the decreased endothelial-derived nitric oxide (NO) bioavailability, is a crucial factor leading to vascular disease states such as hypertension, diabetes, atherosclerosis, heart failure and cigarette smoking. This is due to the fact that the lack of NO in endothelium-dependent vascular disorders contributes to impaired vascular relaxation, platelet aggregation, increased vascular smooth muscle proliferation, and enhanced leukocyte adhesion to the endothelium. During the last several years, it has become clear that reduction of NO bioavailability in the endothelium-impaired function disorders is associated with an increase in endothelial production of superoxide ($O_2^{\cdot -}$). Because $O_2^{\cdot -}$ rapidly scavenges NO within the endothelium, a reduction of bioactive NO might occur despite an increased NO generation. Among many enzymatic systems that are capable of producing $O_2^{\cdot -}$, NAD(P)H oxidase and uncoupled

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Abbreviations: BH4, tetrahydrobiopterin; eNOS, endothelial NO synthase; LDL, low density lipoproteins; L-NAME, *Nω*-nitro-L-arginine methyl ester; ROS, reactive oxygen species.

endothelial NO synthase (eNOS) apparently are the main sources of $O_2^{\cdot -}$ in the endothelial cells. It seems that $O_2^{\cdot -}$ generated by NAD(P)H oxidase may trigger eNOS uncoupling and contribute to the endothelial balance between NO and $O_2^{\cdot -}$. That is maintained at diverse levels.

ENDOTHELIAL DYSFUNCTION

Endothelial dysfunction is a hallmark of the diseases comprehensively called endothelium-impaired function disorders, e.g. atherosclerosis, essential hypertension, diabetes and heart failure. The term 'endothelial dysfunction' may refer to the impairment of endothelium-dependent vasodilation and dysregulation of endothelial–blood cell interactions which may lead to localized inflammation and ultimately to severe vascular lesions and thrombosis. However, in a large majority of scientific literature endothelial dysfunction is defined as an impairment of endothelium-dependent vasorelaxation caused by a decrease of nitric oxide (NO) bioavailability in the vessel wall. Indeed, some of the most important effects that NO exerts in the vascular wall are potentially vasoprotective, because these effects maintain important physiological functions such as vasodilation, anticoagulation, leukocyte adhesion, smooth muscle proliferation, and the antioxidative capacity (Gewaltig & Kojda, 2002; Fenster *et al.*, 2003). In laboratory practice, this form of endothelial abnormality is commonly bioassayed by impairment of endothelium-dependent vasorelaxation (Vita *et al.*, 1990). Acetylcholine was found to be an ideal stimulus for assessment of endothelial dysfunction as it causes muscarinic receptor-mediated, endothelium-dependent NO release. However, acetylcholine and other stimuli leading to receptor-mediated vasodilation *via* NO release can also cause vasoconstriction by directly activating smooth muscle cells. Therefore, the net effect results in either vasodilation or vasoconstriction, depending on the functional integrity of the endothelium. Thus, exercise, mental stress and exposure to cold, all of which are associated with sympathetic activation, usually cause vasodilation in order to provide oxygen supply during metabolic demand; but in contrast can also cause vasoconstriction when endothelial vasodilator function is impaired.

The pivotal role of endothelial NO in protecting against the development of vascular atherosclerotic lesions was demonstrated by observations that targeted deletion of the eNOS gene in mice resulted in hypertension (Huang *et al.*, 1995), vascular remodeling (Rudic *et al.*, 1998) and typical atherosclerotic lesions (Moroi *et al.*, 1998). By contrast, local delivery of eNOS was observed to induce regression of atherosclerotic lesions and reduce neointimal proliferation (Channon *et al.*, 2000). Mounting evidence from clinical and experimental studies indicate that traditional risk factors for atherosclerosis also predispose one to endothelial dysfunction. Importantly, it has been documented that endothelium dysfunction due to reduction of NO bioavailability in the vessel wall is one of the earliest manifestations of endothelium-impaired function disorders (Cooke & Dzau, 1997; Harrison, 1997) and correlates with a risk factor profile (Vita *et al.*, 1990). Also, prospective studies in humans clearly elicited that deficient NO-dependent endothelial function is a quantitative, independent predictor of adverse cardiovascular events and long-term outcome (Schachinger *et al.*, 2000; Heitzer *et al.*, 2001).

BIOAVAILABILITY OF NO

The availability of NO *in vivo* is regulated by a combination of synthesis and breakdown of NO. NO is continuously released from endothelial cells in response to stimulation of endothelial NO synthase (eNOS) by both receptor-independent stimuli, such as shear stress, as well as receptor-dependent agonists, such

as acetylcholine, thrombin, serotonin and bradykinin. A depletion of NO production in endothelium-impaired function disorders may result from a decreased amount of eNOS protein, a deficiency of substrate or cofactors for eNOS, and changes in cellular signaling that finally lead to inappropriate eNOS activation. In line with these regulatory mechanisms, oxidized LDL, but not native LDL, has been found to result in a decrease in eNOS mRNA stability, which coincides with a decrease in the enzyme activity (Liao *et al.*, 1995; Jessup, 1996). However, others have demonstrated that low concentrations of LDL or lysophosphatidylcholine are capable of up-regulating eNOS mRNA expression, suggesting a dose-dependent effect (Zembowicz *et al.*, 1995; Jessup, 1996). It should be emphasized that some endothelium-impaired function disorders may be associated with a decrease in NO bioavailability despite increased NO synthesis. In contrast to the decreased eNOS expression in atherosclerosis (Oemar *et al.*, 1998), the expression of eNOS is up-regulated in hypertension (Kerr *et al.*, 1999), diabetes (De Vriese *et al.*, 2001) and aging (van der Loo *et al.*, 2000), indicating that NO degradation process is mostly responsible for a loss of NO bioavailability in the vessel wall. A number of models of endothelial dysfunction in experimental animals together with clinical data provided evidence that NO bioavailability is reduced by increased production of reactive oxygen species (ROS) in the vessel wall (Cai & Harrison, 2000; Hamilton *et al.*, 2004). Of ROS, superoxide ($O_2^{\cdot -}$) is the key molecule as many other ROS are formed secondary to the reactions involving $O_2^{\cdot -}$. Because $O_2^{\cdot -}$ and $NO^{\cdot}$ are both radicals and contain unpaired electrons in their outer orbitals, they undergo an extremely rapid, diffusion limited radical-radical reaction, leading to the formation of peroxynitrite ($ONOO^-$), a much stronger oxidant than $O_2^{\cdot -}$ itself. The reaction rate for the formation of $ONOO^-$ has been determined to be $6.7 \pm 0.9 \times 10^9$ mol $\cdot$ L^{-1} $\cdot$ s^{-1} (Huie & Padmaja, 1993), which is approximately three-times faster than the scavenging of $O_2^{\cdot -}$ with superoxide dismutases. Thus, alterations in the amounts of either $O_2^{\cdot -}$ or superoxide dismutase could markedly change levels of $NO^{\cdot}$. The reaction between $O_2^{\cdot -}$ and $NO^{\cdot}$ not only results in removing the beneficial effects of NO, but also increasing the damaging effects of $ONOO^-$, which can be protonated to peroxynitrous acid – the cleavage products of which are among the most reactive oxygen species in the biological system. Although $O_2^{\cdot -}$ is primarily a chemical antagonist of NO, $O_2^{\cdot -}$ may also act as a vasoconstrictor through mobilization of cytosolic Ca^{2+} in vascular smooth muscle cells or promote Ca^{2+} sensitization of the contractile elements (Jin *et al.*, 1991; Suzuki *et al.*, 1992). Enhanced formation of $O_2^{\cdot -}$ and other secondary ROS can stimulate smooth muscle cell hypertrophy and hyperplasia. Activation of NF-$\kappa\beta$ by ROS is also critical in initiating expression of proinflammatory molecules such as vascular cell adhesion molecule (VCAM-1) and monocyte chemotactic protein-1 (MCP-1), both of which are involved in early steps in atherogenesis (Wolin, 2000; Landmesser & Harrison, 2001).

VASCULAR SOURCES OF $O_2^{\cdot -}$

Although marked $O_2^{\cdot -}$ production has been revealed in all layers of human blood vessels, denudation of endothelium in the early stages of atherosclerosis was found to normalize $O_2^{\cdot -}$ increased production (Munzel *et al.*, 1995), implicating the endothelium itself as a main source for oxidative stress. Similar observations were made in studies on vessels from patients with non insulin-dependent diabetes mellitus. In contrast to non-diabetic vessels, removal of endothelium in vessels from diabetic patients resulted in reduction of increased $O_2^{\cdot -}$ release (Guzik *et al.*, 2002). Also, nitrate tolerance, developed during long-term treatment with nitrates, was associ-

ated with an increase in endothelial $O_2^{\cdot -}$ production and endothelial dysfunction (Munzel *et al.*, 2000; Schulz *et al.*, 2002).

The major source of $O_2^{\cdot -}$ in endothelium (and in both adventitia and vascular smooth muscle cells) are membrane-bound oxidases which utilize NADH and NADPH as substrates (Cai & Harrison, 2000; Griendling *et al.*, 2000). Exposure of cultured vascular smooth muscle cells to angiotensin II and tumor necrosis factor alpha (TNFα) increased the activity of NAD(P)H cytochrome P-450 oxidoreductase, commonly termed NAD(P)H oxidase (Griendling *et al.*, 1994; De Keulenaer *et al.*, 1998). NAD(P)H oxidase(s) generate $O_2^{\cdot -}$ through the assembly of a multi-subunit protein complex. The complex consists of a membrane-integrated cytochrome b_{558}, which is itself composed of 2 subunits (gp91phox or its NOX analogues and p22phox) and at least three cytosolic proteins (p47phox, p67phox and p21rac) (Griendling *et al.*, 2000). Angiotensin II infusion in rats increased vascular production independent of the concomitant hypertension, as revealed by parallel experiments using infusion of noradrenaline (Rajagopalan *et al.*, 1996). In accordance, enhanced NAD(P)H oxidase protein subunit levels have been found in human vascular endothelium in atherosclerosis (Guzik *et al.*, 2000; Rueckschloss *et al.*, 2001), hypertension (Hamilton *et al.*, 2002) and diabetes (Guzik *et al.*, 2002) in association with increased $O_2^{\cdot -}$ production. These observations suggest that up-regulated gene expression or post-trancriptional increases in protein levels accounted for increased NAD(P)H oxidase activity and contribute to the enzyme-dependent oxidative stress in endothelium-impaired function disorders in humans. In addition, it has been shown that common genetic polymorphisms within the *CYBA* gene, encoding the NAD(P)H oxidase p22phox subunit, is associated with differences in gene expression, enzyme activity and vascular $O_2^{\cdot -}$ generation, suggesting the presence of genetic variation within human population in modulating vascular oxidative stress and ultimately availability of bioactive NO (Cahilly *et al.*, 2000; Schachinger *et al.*, 2001).

Another NAD(P)H oxidoreductase activity catalyses the oxidation of hypoxanthine and xanthine in purine metabolic pathways. The xanthine oxidoreductase can exist in two interconvertible forms, either as xanthine dehydrogenase that reduces NAD^+ or as xanthine oxidase that results in production of both $O_2^{\cdot -}$ and hydrogen peroxide. In endothelial cells, the activity and expression of xanthine oxidase is enhanced by interferon-γ (Dupont *et al.*, 1992). It has been demonstrated that the early stages of atherosclerosis in rabbits are associated with increased oxopurinol-inhibitable $O_2^{\cdot -}$ production derived from endothelium (Ohara *et al.*, 1993; White *et al.*, 1996). The same observation has been made in spontaneously hypertensive rats (SHRs) either in aorta (Nakazono *et al.*, 1991) or microvessels (Suzuki *et al.*, 1995). In humans, the role of vascular xanthine oxidoreductase in $O_2^{\cdot -}$ production and as a consequence of modification in NO bioavailability remain poorly defined (Guzik *et al.*, 2002). Interestingly, xanthine oxidoreductase was identified on the outer surface of human endothelial cells in culture (Rouquette *et al.*, 1998) and the enzyme displacement from the heparin binding site by heparin infusion ameliorated the impairment of endothelium-dependent vasorelaxation (Nakazono *et al.*, 1991; White *et al.*, 1996; Houston *et al.*, 1999). The enzyme in a molybdenum-deficient form is not inhibited by oxypurinol and can use NADH as a substrate for reduction of oxygen to $O_2^{\cdot -}$ (Sanders *et al.*, 1997). In this state, xanthine oxidase activity is identified as a typical activity of NADH oxidase.

A third source of vascular NADPH-dependent $O_2^{\cdot -}$ production that has received growing attention is eNOS.

eNOS AS A SOURCE OF VASCULAR $O_2^{\cdot -}$

Like the other two isoforms of the NO synthase family, eNOS contains two functionally distinct domains, i.e. an N-terminal oxygenase, where haem, BH4 and L-arginine bind, and a C-terminal reductase comprising binding sites for FAD, FMN and NADPH. These two domains are linked by a calmodulin-binding site where, upon calcium -induced binding, calmodulin increases the rate of electron transfer from NADPH to the reductase domain flavins and from the reductase domain to the haem centre for the oxidation of the substrate, L-arginine (Palmer *et al.*, 1988; Hemmens & Mayer, 1998). During the synthesis of $NO^{\cdot}$, eNOS receives and stores enough electrons from NADPH to transform the co-substrates O_2 and L-arginine into the products NO and L-citrulline. However, under conditions of substrate or cofactor deficiency, eNOS, when activated, cannot catalyze the five electron oxidation of L-arginine into $NO^{\cdot}$. Under these circumstances, eNOS can still receive electrons from NADPH and store them in its bound flavins in the reductase domain, and then can donate electrons one-at-a-time to its other substrate, O_2, in the oxygenase domain resulting in a one electron reduction to form $O_2^{\cdot -}$ (Wever *et al.*, 1997; Raman *et al.*, 1998; Vasquez-Vivar *et al.*, 1998). It should not be surprising that eNOS in its 'uncoupled' stage may generate $O_2^{\cdot -}$ instead of $NO^{\cdot}$ as the reductase (C-terminal) domain of each NOS isoform reveals significant homology with NAD(P)H cytochrome P-450 reductase (Bredt *et al.*, 1991). Indeed, NO synthases were believed to belong to the P-450 superfamily of enzymes (White & Marletta, 1992) and were capable of reducing certain cytochromes and O_2 to H_2O_2 in a calmodulin- and NAD(P)H-dependent manner (Mayer *et al.*, 1992). This phenomenon of eNOS uncoupling such that $O_2^{\cdot -}$ was formed rather than $NO^{\cdot}$ has been demonstrated in studies of purified enzyme (Wever *et al.*, 1997; Raman *et al.*, 1998; Vasquez-Vivar *et al.*, 1998). There is indirect evidence to suggesting that eNOS uncoupling contributes to endothelium dysfunction and increased $O_2^{\cdot -}$ production in oxidative stress of ischemia/reperfusion injury (Huk *et al.*, 1997), hypercholesterolemia (Pritchard *et al.*, 1995), hypertension (Mollnau *et al.*, 2002), diabetes (Guzik *et al.*, 2002) and heart failure (Dixon *et al.*, 2003).

The beneficial effect of L-arginine supplementation has been documented repeatedly both in animals and in humans in several conditions, including hypercholesterolemia, hypertension and diabetes (Cheng *et al.*, 2001). However, the *in vitro* studies documented that the possibility of reduced availability of L-arginine may be unlikely because endothelial cells contain the amino acid in concentrations a thousand times greater (millimolar range) than those required for the activity of eNOS (micromolar range). This contradiction may be explained by the fact that native endothelial cells *in vivo* are continuously exposed to hormonal (e.g. acetylcholine) and mechanical (shear stress) stimuli that might lead to relative intracellular deficiency of L-arginine, especially in the close proximity of eNOS. These stimuli affect eNOS activity and could be the reason why the half-maximal substrate concentration (K_m) of L-arginine is higher *in vivo* (Toutouzas *et al.*, 1998). Also, it is believed that the beneficial effects of L-arginine administration are partially caused by a competition of this amino acid with the derivative asymmetric dimethyl L-arginine (ADMA), which is an endogenous inhibitor of eNOS activity (Sydow *et al.*, 2003).

Among the cofactors for eNOS, BH4 is critical for eNOS activity. When endothelial cells are presented with sub-optimal concentrations of BH4, eNOS generates $O_2^{\cdot -}$, instead of $NO^{\cdot}$ (Klatt *et al.*, 1992; Stroes *et al.*, 1998). Thus, restoration of BH4 endothelial cells should restore the activity of eNOS and lead to an increased formation of $NO^{\cdot}$. Administration of BH4 has been shown to enhance

NO production in pre-hypertensive rats (Cosentino *et al.*, 1998), improve endothelium-dependent vasodilation in coronary arteries following reperfusion injury (Tiefenbacher *et al.*, 1996) as well as in aorta from diabetic rats (Pieper, 1997), coronary resistance vessels from rats (JCR:LA-corpulent) with model of human vascular disease (Brunner *et al.*, 2000) and aorta from insulin-resistant rats (Shinozaki *et al.*, 2000). BH4 supplementation improved endothelium-dependent relaxation in patients with hypercholesterolemia (Stroes *et al.*, 1997), endothelium-mediated relaxation in venous conduits used for coronary artery bypass graft surgery (Verma *et al.*, 2000), patients with type II diabetes (Heitzer *et al.*, 2000b; Verma *et al.*, 2000), in normal epicardial coronary arteries (Setoguchi *et al.*, 2001) and in smokers (Heitzer *et al.*, 2000a). BH4 administration has also been demonstrated to improve functional recovery following ischemia and reperfusion; an effect ascribed to improved coronary endothelial function and reduced oxidative stress (Verma *et al.*, 2002). However, the beneficial effects of supplementation with BH4 or biopterin analogues remain uncertain in vascular disease states in which oxidative stress was increased (Tarpey, 2002; Vasquez-Vivar *et al.*, 2002). Similar to L-arginine treatment, it is still unclear as to the true mechanistic relationship between endothelial BH4 concentrations and eNOS regulation *in vivo*, because high extracellular BH4 concentrations may result in nonspecific antioxidant effects that indirectly increase NO bioactivity by $O_2^{\cdot -}$ and other oxidative radicals scavenging rather than by modulation of eNOS activity. On the other hand, high tissue concentrations of BH4 have been reported to be pro-oxidant, leading directly to $O_2^{\cdot -}$ generation that reduced NO bioavailability (Kinoshita & Katusic, 1996; Tsutsui *et al.*, 1996).

Although there is growing evidence that eNOS can make $O_2^{\cdot -}$, depending on the conditions, it is at present unclear whether this occurs physiologically. Because of the very rapid reaction of $O_2^{\cdot -}$ with NO, synthesis of both species by the same enzyme is likely to result in $ONOO^-$ formation within the active site. Indeed, our recent studies have shown that eNOS activation in human endothelial cells implicates not only NO production, but also concomitant formation of both $O_2^{\cdot -}$ and $ONOO^-$ (Kalinowski *et al.*, 2004). This was demonstrated in the most accurate method by using $NO/O_2^{\cdot -}/ONOO^-$-electrosensors miniaturized to nanometers that offer the advantage of allowing measurements to be made in a single cell in the close proximity of the enzyme and in real-time with very high sensitivity. Inhibition of NO production by L-NAME with concurrent inhibition of $O_2^{\cdot -}$ and $ONOO^-$ generation during activation of eNOS additionally confirmed that eNOS is partially uncoupled in intact endothelial cells. It seems that the extent of eNOS uncoupling is dependent on $ONOO^-$ produced initially in the reaction between NO and $O_2^{\cdot -}$ generated by NAD(P)H oxidase. Intriguingly, the degree of enzyme uncoupling was different in the human umbilical endothelial cells depending on the ethnic group. We have found that the steady-state of $NO/O_2^{\cdot -}/ONOO^-$ balance in the endothelial cells from blacks compared with whites is maintained closer to the redox state that had been documented in the endothelium-impaired function disorders. Our observation related to ethnic differences in endothelial $NO/O_2^{\cdot -}/ONOO^-$ balance may explain the existence of predisposition to endothelium dysfunction and cardiovascular disorders prevalent in blacks.

CONCLUSIONS AND PERSPECTIVES

Constitutively expressed eNOS produces low concentrations of NO, which is necessary for good endothelial function and integrity. It appears the eNOS produces not only NO, but also may be a source of marked amounts of $O_2^{\cdot -}$ even in intact endothelial cells. Of note,

the extent of eNOS uncoupling is dependent on another significant enzymatic source of $O_2^{\cdot -}$ in the endothelial cells – NAD(P)H oxidase. In addition, there is substantial evidence that in certain disease conditions, NO production is not altered or even increased, but its bioavailability is reduced because of $O_2^{\cdot -}$ within endothelium. Loss of endothelial NO bioavailability caused by its enhanced biodegradation in reaction with $O_2^{\cdot -}$, yielding $ONOO^-$, is the key feature of such diverse vascular disease states as hypertension, diabetes, atherosclerosis, heart failure, and cigarette smoking. Thus, the endothelium is a novel therapeutic target for the treatment of cardiovascular diseases associated with endothelial dysfunction. However, future goals for cardioprotective therapy must be based on the understanding that the balance between NO and $O_2^{\cdot -}$ in endothelial cells is functionally much more important than the absolute level of NO alone.

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