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Review article

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MECHANISMS OF SUPEROXIDE PRODUCTION IN HUMAN BLOOD VESSELS: RELATIONSHIP TO ENDOTHELIAL DYSFUNCTION, CLINICAL AND GENETIC RISK FACTORS

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Common vascular disease states including diabetes, hypertension and atherosclerosis are associated with endothelial dysfunction, characterised by reduced bioactivity of nitric oxide (NO). Loss of the vasculoprotective effects of NO contributes to disease progression, but the mechanisms underlying endothelial dysfunction remain unclear. Increased superoxide production in animal models of vascular disease contributes to reduced NO bioavailability, endothelial dysfunction and oxidative stress. In human blood vessels, the NAD(P)H oxidase system is the principal source of superoxide, and is functionally related to clinical risk factors and systemic endothelial dysfunction. Furthermore, the C242T polymorphism in the NAD(P)H oxidase p22phox subunit is associated with significantly reduced superoxide production in patients carrying the 242T allele, suggesting a role for genetic variation in modulating vascular superoxide production. In vessels from patients with diabetes mellitus, endothelial dysfunction, NAD(P)H oxidase activity and protein subunits are significantly increased compared with matched non-diabetic vessels. Furthermore, the vascular endothelium in diabetic vessels is a net source of superoxide rather than NO production, due to dysfunction of endothelial NO synthase (eNOS). This deficit is dependent on the eNOS cofactor, tetrahydrobiopterin, and is in part mediated by protein kinase C signalling. These studies suggest an important role for both the NAD(P)H oxidases and endothelial NOS in the increased vascular superoxide production and endothelial dysfunction in human vascular disease states.

Key Words: *nitric oxide; superoxide; endothelium; tetrahydrobiopterin; human.*

NO, Superoxide and Vascular Disease: Importance of Endothelial Dysfunction

Vascular diseases including coronary artery disease, cerebrovascular and peripheral vascular diseases are the largest cause of mortality and morbidity in industrialised countries. Many common risk factors for vascular disease, such as hypertension and diabetes, remain prevalent in Western and other populations, suggesting that vascular disease will continue to impose a substantial burden on health care resources throughout the next generation. The earliest detectable changes in vascular disease states are abnormalities of the endothelium, resulting in loss of the endothelium's normal homeostatic functions that normally act to inhibit disease-related processes such as inflammation and thrombosis. In particular, nitric oxide (NO) produced by NO synthase (eNOS) in the vascular endothelium modulates blood flow and pressure (1) and has important anti-atherogenic effects on platelets, vascular smooth muscle and endothelial cells.

In humans, NO-mediated endothelial function is deficient in preatherosclerotic states such as hypercholesterolaemia, diabetes mellitus, hypertension and smoking (2,3) and correlates with risk factor profile (4). More importantly, prospective studies identify deficient NO-mediated endothelial function as a quantitative, independent predictor of adverse cardiac events (5,6). Numerous animal model studies demonstrate the important role of NO in vascular disease pathogenesis. Targeted deletion of the eNOS gene in mice results in hypertension (7) and impaired vascular remodelling, (8). whereas augmenting NO by local gene delivery of NOS improves endothelial function, limits neointimal proliferation, and induces regression of atherosclerotic lesions (Reviewed in (9)).

The mechanisms that result in loss of NO bioactivity in vascular disease have remained less clear. Data from experimental models suggest that increased production of reactive oxygen species such as superoxide anion (OO^-) rapidly react with NO and contribute to the NO deficit in vascular diseases (10-12). Recent data demonstrate that the contribution of oxidative radicals to deficient NO-mediated endothelial function is an independent prospective indicator of adverse cardiovascular risk (6). The reaction between NO and superoxide occurs at almost diffusion-limited rate, six times greater than the removal of superoxide by copper-zinc superoxide dismutase (Cu/Zn SOD). In human arteries and veins, release of superoxide is reduced by tonic scavenging of superoxide by NO produced by the endothelium; removal of the endothelium or inhibition of endothelial NOS usually results in an increase in net superoxide release, accompanied by a decrease in peroxynitrite formation (13). Human arteries such as the radial or internal mammary arteries appear to have high levels of NO bioactivity compared with veins in vitro, but also generate higher levels of superoxide under these conditions.

In addition to the effects mediated by NO scavenging, increasing evidence suggests additional roles for superoxide-NO interactions in the modulation of

vascular signalling through redox-sensitive mechanisms (14). Furthermore, peroxynitrite ($ONOO^-$) formed by the NO-superoxide reaction, has additional detrimental effects on vascular function, by oxidation of cellular proteins and lipids, LDL particles (10), or direct cell toxicity (15). However, the formation of peroxynitrite may also generate nitrosylated thiols that function as endogenous NO donors that can induce vasorelaxation (16) and inhibit platelet aggregation (17).

Sources of Vascular Superoxide Production in Human Vessels

The cellular sources of vascular superoxide vary in different vessel types from different species (18,19). Using dihydroethidium fluorescence in human blood vessels reveals marked superoxide production from the media and adventitia and a modest proportion directly from the endothelium; endothelial superoxide production appears to be prominent in vessels from patients with diabetes mellitus. Similarly, the endothelium is a more important contributor in the DOCA salt hypertensive rat (20), whereas in hypertensive, angiotensin II-infused rat or mouse aortas, the adventitia is the major source of superoxide (18,21).

Within these different cells in the vessel wall, superoxide may be produced from several different oxidase enzymes. Potential sources of vascular superoxide production include NAD(P)H-dependent oxidases, xanthine oxidase, lipoxygenase, mitochondrial oxidases and NO synthases. Several studies have examined the sources of superoxide production in human vessels, by observing the response to different oxidase inhibitors and/or substrates (22-24). Most studies have identified a membrane-associated oxidase that is inhibited by compounds such as diphenylene iodonium (a flavin oxidase inhibitor) and stimulated by NADH or NADPH, suggesting that NAD(P)H oxidases are an important source of superoxide production in human vessels as they are in several animal models of vascular disease, including diabetes (reviewed in (25)).

NAD(P)H oxidase protein subunits are present in human blood vessels including atherosclerotic coronary arteries (26), in saphenous veins and mammary arteries from patients with coronary artery disease (27), and in human vascular smooth muscle cells (28) and endothelial cells (29) in culture. The NAD(P)H oxidase system, most widely known for its role in the neutrophil respiratory burst, comprises a multisubunit enzyme complex including p47phox, p67phox, p22phox, Rac, and gp91phox or another Nox family homolog (25,30). The p22phox subunit, that is required for enzyme activity in all NAD(P)H oxidases, binds with the Nox subunit to form the membrane-bound cytochrome b558. Increased levels of the p22phox, p47phox, p67phox and Nox subunits are present in human atherosclerotic coronary arteries (26), and in diabetic vessels (27), in association with increased superoxide production. This suggests that upregulated gene expression and/or post-transcriptional increases in protein levels are important in mediating increased NAD(P)H oxidase activity in human

vascular disease. For example, Angiotensin II increases NAD(P)H oxidase activity by transcriptional upregulation of subunit expression (31). However, it is clear that the cytosolic regulatory proteins p47phox, p67phox and Rac also play an important part in regulating NAD(P)H oxidase activity in cardiovascular disease states by acute activation of the enzyme complex, for example by phosphorylation and translocation of p47phox (28).

Relationship of Vascular Superoxide Production to Endothelial Dysfunction in Atherosclerosis

The association between NO scavenging by superoxide suggests that superoxide production may in part underlie endothelial dysfunction in human atherosclerosis, as it does in some experimental models of vascular disease.

In vivo (4) and in vitro (32) data show that ACh-mediated vasorelaxations in human vessels are inversely related to the number of atherosclerotic risks factors present. However, functional studies of human vascular superoxide production have been more limited (32,33). Huraux et al. found large variability in both NO-mediated vascular relaxations and basal superoxide production in internal mammary arteries, but did not find consistent associations between these two parameters or with clinical risk factors (32). We investigated superoxide production by NAD(P)H oxidase in human vessels and the relationships between superoxide production, atherosclerotic risk factor profile and endothelial dysfunction. We observed the expected inverse correlation between risk factor profile and NO-mediated endothelium-dependent relaxations in vessel ring isometric tension studies. However, we also found that superoxide production by NAD(P)H oxidases progressively increased with increasing risk factor profile (24). Furthermore, NAD(P)H oxidase-mediated superoxide production was inversely correlated with NO-mediated vasorelaxations in individual patients, such that patients with the highest superoxide production had the most deficient endothelial function.

The association between increased vascular NAD(P)H oxidase activity and impaired endothelial vasorelaxations may be due to direct scavenging of NO by superoxide, as has been demonstrated in animal model systems. However, both could result independently from increasing exposure of endothelium, media and adventitia to factors acting through different signalling pathways. Alternatively, superoxide may directly modulate NO-mediated vascular signalling, for example by peroxynitrite-induced nitration of G proteins or other membrane components (34). Previous data suggest that G protein-coupled receptor function is deficient in atherosclerosis (35). Our observation that vasorelaxations to ACh were significantly less than maximal relaxations to the calcium ionophore A23187 is consistent with this hypothesis, and with observations in human internal mammary arteries (32). However, the significant correlation between ACh and A23187 - induced relaxations, and the association of NADH-dependent

superoxide production with both ACh and A231287-stimulated vasorelaxations suggest that a change in G protein-coupled receptor signalling is unlikely to be the sole mechanism underlying reduced NO-mediated vasorelaxations, as A23187 activates endothelial NO synthase independently of any receptor-mediated pathway. Alternatively, superoxide may impair endothelial function by direct effects on endothelial NO synthase activity, (36), possibly mediated through oxidation of the NOS cofactor, tetrahydrobiopterin (see below).

Genetic Factors Modulating Vascular Superoxide Production

Identifying NAD(P)H oxidase(s) as a major source of vascular superoxide in atherosclerosis has provoked considerable interest in the possible association of genetic variation in genes encoding NAD(P)H oxidase subunits, particularly the *CYBA* gene, encoding p22phox, with disease susceptibility. Common genetic polymorphisms within the promoter and coding sequence of the *CYBA* gene, encoding p22phox, may have important influences on gene expression, enzyme activity and on vascular superoxide generation, leading to significant functional variation between individuals in vascular oxidative stress and in NO bioactivity. The *CYBA* C242T polymorphism results in substitution of Tyr for His at residue 72 of p22phox, leading to speculation that this polymorphism may modulate enzyme activity by effects on heme binding (37). However, the initial observation of an increased prevalence of the 242T allele in control subjects *without* coronary artery disease (37) was not supported by analysis of angiographic data from the prospective Lipoprotein and Coronary Artery Study (LCAS), that suggested an association of the *CYBA* 242T allele with *increased* progression of coronary disease (38). Other case-control studies were inconsistent or found no clear associations of this polymorphism in vascular disease, likely in part due to the inherent limitations of case-control studies resulting from population bias.

More recently, functional studies have aimed to assess possible associations between *CYBA* poymorphisms and vascular function using quantitative intermediate phenotypes that may be more directly related to vascular NAD(P)H oxidase activity, and are not dependent upon classification of individuals as cases or controls. By measuring vascular superoxide production in vitro from saphenous vein segments taken from 110 patients undergoing coronary artery bypass surgery, we observed a significantly lower superoxide production in vessels from patients with the *CYBA* 242T allele that was independent of other atherosclerotic risk factors (39). Corresponding differences were also observed in another functional study of coronary artery vasomotor changes, measured in vivo (40). Whether, and if so, how, these polymorphisms are truly related to potential changes in vascular disease susceptibility remains unclear. It is equally possible that they reflect a genetic marker of other functional variants either in the same gene (e.g. in the *CYBA* promoter region (41)) or elsewhere.

Endothelial NO Synthase as a Source of Vascular Superoxide: Role of Tetrahydrobiopterin

Further complexity in the relationships between NO and superoxide in the vascular wall is suggested by the observation that eNOS itself can generate superoxide rather than NO under conditions of substrate or cofactor deficiency (42-44) or in response to atherogenic stimuli such as hyperglycaemia (45) or LDL particles (46). These findings have led to the concept of "NOS uncoupling", where the activity of the enzyme for NO production is decreased, in association with an increase in NOS-dependent superoxide production.

In vessels from patients with non insulin-dependent diabetes mellitus, total vascular superoxide production is increased, and this increase appears to be particularly associated with increased endothelial superoxide production. In contrast to non-diabetic vessels, removal of endothelium in vessels from diabetic patients reduces net superoxide release (27). Furthermore, endothelial superoxide production (but not that in the media or adventitia) is inhibited by L-NAME suggesting that uncoupled eNOS is a source of superoxide.

Tetrahydrobiopterin (BH4) is an essential cofactor for all three NOS isoforms, that require BH4 for NOS homodimerisation (47) and for electron transfer during arginine oxidation (48). Production of NO by endothelial cells requires adequate BH4, (49) and recombinant NOS synthesised in the absence of BH4 is inactive for NO generation: exogenous BH4 restores enzyme activity (47,50,51). Furthermore, BH4-deficient NOS generates superoxide by 'uncoupled' electron transfer from the reductase domain to molecular oxygen, without L-arginine oxidation.

Several studies suggest a role for BH4 in regulating vascular NO bioavailability. Endothelial dysfunction is improved by addition of BH4 to vessel rings from animals with atherosclerosis, diabetes or hypertension (43). Vascular superoxide production appears to be at least partially mediated by BH4-dependent eNOS uncoupling in several vascular disease animal models, including atheroslcerosis, (52). diabetes (53-55). and hyperhomocysteinaemia (56). In humans, BH4 administration augments NO-mediated effects on forearm blood flow in smoking, (57). diabetes (58). and hypercholesterolaemia (42,59).

Although these pharmacolgical studies may be affected by non-specific antioxidant effects of high-dose BH4 supplementation, some recent studies now provide more direct evidence for BH4-dependent NOS dysfunction in the vascular endothelium. In patients chronically treated with nitrates, nitrate tolerance is associated with endothelial dysfunction and increased vascular superoxide production (60). Nitrate-induced endothelial dysfunction in humans can be rescued by folate, potentially by effects on BH4 regeneration (61). In experimental models of atherosclerosis (52) or diabetes (55), levels of eNOS protein are maintained or increased, despite a deficit in NO production that is restored by BH4. In human blood vessels from patients with diabetes, increased endothelial superoxide generation is inhibited by the BH4 precursor sepiapterin

(27). eNOS dysfunction appears to be in part regulated by protein kinase C (PKC) signalling, since PKC inhibitors abrogate the increase in superoxide production in vessels (27,55) and improves NO-mediated endothelial function (62).

Conclusions

Increased superoxide production is an important feature of the vascular phenotype in human atherosclerosis, by effects on NO scavenging, peroxynitrite generation and redox-sensitive cell-signalling pathways. The NAD(P)H oxidases are an important source of superoxide in human vessels; the activity of this enzyme system is increased in association with atherosclerotic risk factor profile and more marked endothelial dysfunction. Genetic factors, including polymorphic variants in the genes encoding the NAD(P)H oxidases, also modulate superoxide production in human atherosclerosis. In addition to the NAD(P)H oxidase, uncoupling of endothelial NOS also contributes to vascular superoxide production, mediated by loss of BH4 availability. PKC signalling appears to be an important pathway that signals increases in NAD(P)H oxidase activity and eNOS uncoupling. These mechanisms that increase vascular superoxide production and endothelial dysfunction in human vascular disease states are key targets for therapeutic interventions.

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