Clinical aspects of reactive oxygen and nitrogen species

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Abstract

Endothelial dysfunction in the setting of cardiovascular risk factors, such as hypercholesterolaemia, hypertension, diabetes mellitus and 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 in endothelial and/or smooth muscle cells and the adventitia, and the subsequent decrease in vascular bioavailability of NO. Superoxide-producing enzymes involved in increased oxidative stress within vascular tissue include NAD(P)H-oxidase, xanthine oxidase and endothelial nitric oxide synthase in an uncoupled state. Recent studies indicate that endothelial dysfunction of peripheral and coronary resistance and conductance vessels represents a strong and independent risk factor for future cardiovascular events. Ways to reduce endothelial dysfunction include risk-factor modification and treatment with substances that have been shown to reduce oxidative stress and, simultaneously, to stimulate endothelial NO production, such as inhibitors of angiotensin-converting enzyme or the statins. In contrast, in conditions where increased production of reactive oxygen species, such as superoxide, in vascular tissue is established, treatment with NO, e.g. via administration of nitroglycerin, results in a rapid development of endothelial dysfunction, which may worsen the prognosis in patients with established coronary artery disease.

Introduction

Traditionally, the role of the endothelium was thought primarily to be that of a selective barrier to the diffusion of macromolecules from the blood 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 as well as 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 the rate of cardiovascular events [1]. Although the mechanisms underlying endothelial dysfunction may be multifactorial there is a growing body of evidence that increased production of free radicals may contribute considerably to this phenomenon. In addition, recent studies show that the degree of vitamin C-induced improvement of endothelial dysfunction represents a strong independent predictor of subsequent cardiovascular events, indicating that high oxidative stress in vascular tissue not only contributes to endothelial dysfunction but also decisively determines the prognosis in patients with cardiovascular risk factors [2].

This review will focus briefly on mechanisms underlying oxidative stress causing endothelial dysfunction in various diseases and will summarize the studies demonstrating the prognostic impact of endothelial dysfunction in predicting cardiovascular events in patients with coronary and peripheral artery disease as well as in patients with essential hypertension. Finally, we will address the question as to which treatment strategies successfully reduce oxidative stress in vascular tissue.

**NO, superoxide and peroxynitrite**

The endothelium-derived relaxing factor, identified as NO [3] or a closely related compound [4], has potent anti-atherosclerotic properties. NO released from endothelial cells works in concert with prostacyclin to inhibit platelet aggregation [5]; 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 [6]. Therefore, under all conditions where 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 determined decisively by oxygen-derived free radicals such as superoxide [7]. Superoxide reacts rapidly with NO to form the highly reactive intermediate peroxynitrite [8]. The rapid bimolecular reaction between NO and superoxide, yielding peroxynitrite (rate constant, $6.7 \times 10^{10} \text{ M}^{-1} \cdot \text{s}^{-1}$), is about 3–4 times faster than the dismutation of superoxide by superoxide dismutase. Therefore, peroxynitrite formation represents a major potential pathway of NO reactivity depending on the rates of tissue superoxide production. Peroxynitrite in high concentrations is cytotoxic and may cause oxidative damage to proteins, lipids and DNA [8]. Recent studies also indicate that ONOO− may have deleterious effects on the activity and function of the prostacyclin synthase [9] and endothelial NOS (nitric oxide synthase; NOS III) [10]. Other reactive oxygen species, such as the dismutation product of superoxide, $\text{H}_2\text{O}_2$ and hypochlorous acid, cannot be considered as 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 LDL (low-density lipoprotein) levels, patients with diabetes types I and II, hypertensive patients and patients with metabolic syndrome. There are several potential abnormalities that could account for reductions in endothelium-dependent vascular relaxation, including changes in the activity and/or expression of NOS III, decreased sensitivity of vascular smooth muscle cells to NO or increased degradation of NO via its interaction with reactive oxygen species such as superoxide. The NO-degradation concept is the most attractive since, in the presence of cardiovascular risk factors such as diabetes mellitus, hypertension, chronic smoking, a family history of the development of coronary artery disease as well as hyperlipidaemia, endothelial dysfunction is established, and even more importantly it is markedly improved by the acute administration of the antioxidant vitamin C [11–14].

Enzymes involved in increased vascular superoxide production

Role for xanthine oxidase

Xanthine oxidase is an enzyme that catalyses the oxidation of hypoxanthine or xanthine into uric acid, thereby producing superoxide. Xanthine oxidoreductase can exist in two interconvertible forms, either as xanthine dehydrogenase or xanthine oxidase. Oxypurinol, an inhibitor of xanthine oxidoreductase, has been shown to reduce superoxide production and to improve endothelium-dependent vascular relaxation to ACh (acetylcholine) in vessels from hyperlipidaemic animals [15]. This suggests an increase in the expression or activity of xanthine oxidase in early hypercholesterolaemia. The mechanisms underlying such a phenomenon remain unclear, although 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 (e.g. from the liver) into the circulation where it binds to endothelial glycosaminoglycans [16]. Human studies concerning the effects of xanthine oxidase inhibition on endothelial dysfunction are somewhat discrepant. Whereas some studies showed that endothelial dysfunction in hypercholesterolaemic patients and hypertensive diabetics was improved by acute inhibition of xanthine oxidase with oxypurinol [17,18], other groups failed to show similar efficacy [19] 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 with heparin-binding superoxide dismutase in spontaneously hypertensive rats (SHRs) [20] but fails to demonstrate a positive effect on endothelial dysfunction in hypertensive patients [17].
Role for NAD(P)H-oxidase

The NAD(P)H-oxidase is a superoxide-producing enzyme that was first characterized in neutrophils [21]. It is now known that a similar enzyme also exists 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 upon stimulation with angiotensin II [22]. 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 due to increased vascular superoxide production is likely to be expected. Experimental hypercholesterolaemia has been shown to be associated with an activation of NAD(P)H-oxidase [23] and there is a close association between endothelial dysfunction, clinical risk factors and the activity of this enzyme in human saphenous veins in patients with coronary artery disease [24]. In atherosclerotic arteries there is evidence for increased expression of the NAD(P)H-oxidase subunit gp91phox and nox-4, both of which may contribute to increased oxidative stress [25].

Interestingly, there is growing body of evidence that the local renin–angiotensin system is activated in the setting of hypercholesterolaemia. In patients, ACE (angiotensin-converting enzyme) activity and therefore local angiotensin II concentrations are increased in atherosclerotic plaques [26,27], 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 [28]. In vessels from hypercholesterolaemic animals [23] as well as in platelets from hypercholesterolaemic patients [29] there is an increase in the expression of the angiotensin II receptor subtype AT_1. 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 infusion [30,31] and in SHRs [32] as well as in different forms of diabetes mellitus [33].

Role for NOS

In most situations where endothelial dysfunction due to increased oxidative stress is encountered, the expression of NOS III has been shown to be increased rather than decreased [33–35]. Very intriguing are observations that NOS III itself can be a superoxide source, thereby causing endothelial dysfunction. It has become clear from studies with the purified enzyme that NOS III may become ‘uncoupled’ in the absence of the NOS substrate L-arginine or the cofactor BH_4 (tetrahydrobiopterin). In such an uncoupled state, electrons flowing from the reductase domain to the oxygenase domain are diverted to molecular oxygen rather than to L-arginine [36,37], resulting in the production of superoxide rather than NO (Figure 1).

What are the mechanisms leading to BH_4 depletion? Studies in vitro proposed that native LDL [38] and, to a greater extent, oxidized LDL [39] are able to stimulate endothelial superoxide production and that this phenomenon

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is inhibited by the NOS inhibitor L-NAME (N\textsuperscript{G}-nitro-L-arginine methyl ester), pointing to a specific role for NOS III in superoxide production. Hypercholesterolaemia has also been shown to increase vascular formation of superoxide via activation of the NAD(P)H-oxidase [23] and/or xanthine oxidase [15]. Superoxide derived from both enzyme sources may lead to increased formation of the NO/superoxide reaction product peroxynitrite [34,40]. Peroxynitrite in turn rapidly oxidizes the active NOS cofactor BH\textsubscript{4} to cofactor-inactive molecules such as dihydrobiopterin [34,41] or may attack NOS III directly causing the release of Zn from the enzyme, all of which may ultimately lead to NOS III uncoupling [10] (see Figure 1). These concepts, however, also

Figure 1 Schematic representation of the role of reactive oxygen species in causing endothelial dysfunction. Under normal conditions, NO synthesized by NOS III stimulates soluble guanylate cyclase (sGC), increasing cGMP and thus stimulating cGMP-dependent protein kinase I (cGK-I) and vasorelaxation. However, this pathway can be inhibited at several sites. Angiotensin II, hypertension, hypercholesterolaemia, chronic smoking and diabetes mellitus stimulate superoxide (O\textsubscript{2}\textsuperscript{−}) production within endothelial and smooth muscle cells and in the adventitia, which may inactivate NO, thereby diminishing cGMP-dependent protein kinase I action. Peroxynitrite (ONOO\textsuperscript{−}) may also uncouple NOS III by oxidizing zinc thiolate complexes within NOS III (1), and/or by oxidizing the NOS III cofactor BH\textsubscript{4} to dihydrobiopterin (BH\textsubscript{2}) (2). These concepts, however, also mean that uncoupling of NOS III would always require a priming event such as superoxide produced by the NAD(P)H-oxidase. Both O\textsubscript{2}\textsuperscript{−} and the NO/O\textsubscript{2}\textsuperscript{−} reaction product peroxynitrite potently inhibit soluble guanylate cyclase (3).
imply that the uncoupling of NOS III would invariably require a priming event such as superoxide produced by the NAD(P)H-oxidase or the xanthine oxidase.

NOS III uncoupling has been observed in most experimental models where oxidative stress is encountered, such as different forms of hypertension [42], diabetes mellitus [33], hypercholesterolaemia [34] and nitrate tolerance [43]. The concept of NOS III uncoupling in cardiovascular disease is further supported by the demonstration that the administration of the NOS III cofactor BH4 or substances that increase intracellular BH4 levels, such as folic acid [44], is able to improve endothelial dysfunction in patients with hypercholesterolaemia [45], chronic smokers [46], patients with diabetes mellitus [47] and patients treated chronically with nitroglycerin [48,49].

**Endothelial dysfunction and prognosis in patients with coronary artery disease**

There are several studies addressing the prognostic impact of endothelial dysfunction on subsequent cardiac events. The first study reporting that endothelial dysfunction assessed in patients with non-obstructing coronary artery disease may have prognostic implications was published by Suwaidi et al. [1]. Based on the response of their resistance vessels to intra-coronary ACh, the authors divided their patients into three groups according to the degree of endothelial dysfunction, i.e. normal endothelial function, mild dysfunction and severe dysfunction [1]. Over a 28-month follow-up, neither of the first two groups had a cardiovascular event, which included myocardial infarction, percutaneous or surgical revascularizations and/or cardiac death. In contrast, patients exhibiting severe endothelial dysfunction had 10 cardiac events, this being significantly different from the first two groups.

Similar results were provided by Schachinger et al. [50], who studied epicardial coronary arteries, thus extending previous observations to arterial conductance vessels. The authors addressed coronary vasoreactivity in 147 patients using the endothelium-dependent dilator ACh, sympathetic activation by cold pressor testing, dilator responses to increased blood flow, and dilation in response to nitroglycerin. Cardiovascular events (cardiovascular death, unstable angina, myocardial infarction, percutaneous transluminal coronary angioplasty, coronary bypass grafting, ischaemic stroke or peripheral artery revascularization) served as outcome variables over a median follow-up period of 7.7 years. Patients suffering from cardiovascular events during follow-up had significantly increased vasoconstrictor responses to ACh infusion and cold pressor testing. Impaired endothelial and endothelium-independent coronary vasoreactivity were associated with a significantly higher incidence of cardiovascular events. By multivariate analysis, all tests of coronary vasoreactivity were significant independent predictors of a poor prognosis, even after adjustment for traditional cardiovascular risk factors or the presence of atherosclerosis itself. Based on these findings the authors concluded that coronary endothelial vasodilator dysfunction of conductance vessels predicts long-term atherosclerotic disease progression and cardiovascular event rates [50].
These findings were confirmed by the study from Halcox et al. [51], who demonstrated that endothelial dysfunction of the coronary arteries at the level of resistance and conductance vessels independently predicted adverse events including cardiovascular death, myocardial infarction, stroke and unstable angina.

Excitingly, similar associations have been observed for endothelial dysfunction of peripheral conductance and resistance arteries of the forearm, again stressing the point that endothelial dysfunction is indeed a systemic disorder. In patients with essential hypertension, Perticone and colleagues [51a] demonstrated that patients with the most severe endothelial dysfunction of forearm resistance vessels had an excess cardiovascular risk for events including myocardial infarction, angina, revascularization procedures, stroke, transient ischaemic attack and aorto-iliac occlusive disease.

Using the same method of forearm plethysmography we were recently able to establish this association for patients with coronary artery disease. In these studies Heitzer et al. [2] examined endothelial function of forearm resistance vessels in a total of 281 patients with documented coronary artery disease. During a mean follow-up period of 4.5 years patients with more severe endothelial dysfunction experienced significantly more cardiovascular events, including death from cardiovascular sources, myocardial infarction, ischaemic stroke and coronary revascularization procedures. In a subgroup of patients the authors also studied the effects of vitamin C on the vasodilatory effects of the endothelium-dependent vasodilator ACh. Interestingly, patients with cardiovascular events showed a greater benefit from acute vitamin C treatment on ACh-induced vasodilation, compatible with increased oxidative stress within vascular tissue. Cox proportional regression analysis for conventional risk factors demonstrated that a blunted ACh response, the effect of vitamin C and age remained independent predictors of cardiovascular events.

Two recently published trials also showed that the non-invasively measured endothelial function of the brachial artery independently predicts cardiac events in patients undergoing peripheral and coronary bypass surgery and in patients with peripheral vascular disease [52,53]. In a prospective study Gokce et al. [52] showed that impaired flow-mediated dilation of the brachial artery (below 8%) had a sensitivity of 95%, specificity of 38% and negative predictive value of 98% for subsequent cardiovascular events. Similar observations were made for patients with peripheral vascular disease. In these patients, a flow-mediated dilation below 8.1% was associated with a significantly higher event rate, including cardiac death, myocardial infarction, unstable angina and stroke [53].

**Treatment of endothelial dysfunction**

The above-mentioned mechanisms clearly indicate that substances able to reduce oxidative stress should beneficially influence endothelial dysfunction and simultaneously improve prognosis in patients with coronary artery disease.
ACE inhibitors

Indeed, ACE inhibitors have been shown to markedly improve endothelial dysfunction and to improve prognosis in patients with coronary artery disease [54]. Regarding endothelial dysfunction, similar findings have been made with receptor-blocker therapy with the angiotensin II receptor subtype AT1 in experimental animals [23,55] and in patients with coronary artery disease [56,57]. Although the precise mechanisms of the beneficial effects of ACE inhibitors and AT1-receptor blockers remain obscure, inhibition of angiotensin II formation or AT1-receptor stimulation may inhibit NAD(P)H-oxidase-mediated superoxide production [58]. Therapy with ACE inhibitors as well as with AT1-receptor blockers has been shown to prevent NOS III uncoupling [55]. Thus ACE inhibitors as well as AT1-receptor blockers may be considered to be effective drugs against oxidative stress due to inhibitory effects on the activity of free radical-producing enzymes [58]. In addition, ACE inhibitors prevent the breakdown of bradykinin, leading to the stimulation of NO production, which will also contribute to increased vascular NO bioavailability. The HOPE (Heart Outcomes and Prevention Evaluation) trial also demonstrated clearly that in patients with increased oxidative stress, such as in diabetes mellitus, the diabetes-associated complications as well as the new onset of diabetes were reduced significantly by ACE-inhibitor treatment [54].

Statins

Similar beneficial effects have been observed in response to therapy with statins. Cerivastatin [59] and atorvastatin [60,61] were shown to improve endothelial dysfunction in patients with established coronary artery disease or cardiovascular risk factors in forearm conductance as well as resistance arteries within 3 days to 6 weeks of continuous treatment. Like ACE inhibitors, statins reduce vascular superoxide production by inhibiting the activity and/or expression of the NAD(P)H-oxidase [62,63]. Statins also stimulate the expression of NOS III [64]. Along with the reduction in oxidative stress, the resulting prevention of NOS III uncoupling will markedly increase vascular NO bioavailability, thereby improving endothelial function. Accordingly, several large-scale trials have shown that the prognosis of patients with coronary artery disease and diabetes is improved in response to long-term therapy with statins [65].

Vitamins E and C

Although the acute administration of vitamin C improves endothelial dysfunction in arterial conductance and resistance vessels in patients with overt coronary artery disease or cardiovascular risk factors [11,13,14], there is to date no study demonstrating beneficial effects of chronic vitamin C therapy with respect to prognosis. Given the conflicting data from clinical trials of antioxidants in cardiovascular disease, the emergence of quite strong beneficial acute effects of vitamin C on endothelial dysfunction in coronary artery disease patients may be quite a surprise. As mentioned above, however, it is a quite consistent finding that ascorbic acid, when given acutely in high concentrations, can improve endothelial dysfunction in diseases where oxidative stress...

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may play a role, such as hypercholesterolaemia, chronic smoking, congestive heart failure and hypertension. Therefore, we believe that ascorbic acid-induced effects on ACh-induced vasodilation can be used as a surrogate parameter reflecting oxidative stress in vascular tissue. Based on the very low rate constant of the reaction between vitamin C and superoxide, vitamin C has to be given in very high concentrations in order to compete successfully with NO for the superoxide anion. Although we can acutely improve endothelial dysfunction with this approach, it also may be easy to understand why therapies with oral vitamin C have not been very successful.

Similar effects have been observed in response to therapy with vitamin E. As demonstrated in the HOPE trial, vitamin E was not able to improve prognosis in high-risk patients [66]. Although vitamin E is quite effective in preventing LDL oxidation ex vivo, it is not active against reactive species such as superoxide, peroxynitrite and hypochlorous acid [66a], all of which have been implicated to play a role in the atherosclerotic process. Interestingly, attempts to increase the effectiveness of vitamin E with higher doses have led to an aggravation of endothelial dysfunction in experimental models of hypercholesterolaemia [67] and diabetes [68], perhaps because of the well-described requirement for co-antioxidants to achieve optimal vitamin E antioxidant activity or due to the formation of vitamin E radicals.

**Nitroglycerin**

The haemodynamic and anti-ischaemic effects of nitroglycerin are rapidly blunted due to the development of nitrate tolerance. With the initiation of nitroglycerin therapy one can detect neurohormonal activation and signs of intravascular volume expansion. These so-called pseudotolerance mechanisms may compromise nitroglycerin’s vasodilatory effects. Long-term treatment with nitroglycerin is also associated with a decreased responsiveness of the vasculature to nitroglycerin’s vasorelaxant potency, suggesting that changes in intrinsic mechanisms of the tolerant vasculature itself may also contribute to tolerance. More recent experimental work defined new mechanisms of tolerance such as increased vascular superoxide production [69]. As mentioned above, endothelial dysfunction in the setting of cardiovascular risk factors has been shown to be at least in part secondary to increased production of reactive oxygen species such as the superoxide anion [69,70]. Thus when treating these patients with nitroglycerin, NO released from this compound would combine with superoxide to form peroxynitrite, which may trigger the development of endothelial dysfunction. Treatment of experimental animals and patients with nitroglycerin leads to a marked degree of endothelial dysfunction, as indicated by a significant decrease in vascular sensitivity to the endothelium-dependent vasodilator ACh [69]. Endothelial dysfunction was associated with a broad nitrotyrosine band in the endothelial cell layer and the subendothelial space, all of which can be considered as a footprint for increased endothelial peroxynitrite formation [71]. These findings would indicate that nitroglycerin-induced peroxynitrite formation may actually cause endothelial dysfunction due to NOS III uncoupling. Recent experimental and clinical data go along with this concept. A 3-day treatment with nitroglycerin up-regulated NOS III but also
induced endothelial dysfunction and NOS III-mediated superoxide production [43]. In patients with coronary artery disease, 5-day treatment with nitroglycerin patches caused endothelial dysfunction as indicated by a more pronounced ACh-induced coronary artery constriction [70]. Using forearm plethysmography as a methodology to assess endothelial function of the forearm, Gori et al. [72] demonstrated data compatible with NOS III uncoupling in patients. In these studies, nitroglycerin treatment markedly suppressed basal and stimulated NO production [72], a phenomenon that was almost completely reversed by treatment with folic acid [49]. Mechanisms responsible for the beneficial effects of folic acid on nitrate-induced endothelial dysfunction may include an interaction with NOS III itself, e.g. stabilization of the NOS III cofactor BH₄ or serving as a substitute for BH₄ [44]. In addition, folate itself has been shown to have antioxidant properties, which are drastically less effective than those of the water-soluble antioxidant vitamin C [44]. Taken together, these data clearly indicate that although a NO deficiency is encountered in vessels exposed to certain cardiovascular risk factors, treatment with NO itself does not appear to represent a promising tool to treat endothelial dysfunction, but rather favours the formation of the highly reactive peroxynitrite, all of which in turn may cause vascular dysfunction that may at least in part be secondary to a dysfunctional NOS III and a dysfunctional prostacyclin synthase [73]. These recent experimental and clinical findings may explain observations that chronic nitroglycerin treatment may worsen prognosis in patients with ischaemic heart disease.

Taken together, there is mounting evidence that endothelial dysfunction of the coronary or peripheral circulation has important prognostic implications for future cardiovascular events. Although the mechanisms underlying endothelial dysfunction are likely to be multifactorial, it is important to note that increased production of oxygen-derived free radicals contributes markedly to this phenomenon. Given the unique role of NO as an anti-atherosclerotic molecule, the term ‘endothelial dysfunction’ as a result of diminished vascular NO bioavailability does not refer solely to diminished dilator responses to endothelium-dependent vasodilators, but may also include stimulation of processes like leucocyte adhesion and migration, platelet aggregation, smooth muscle proliferation and migration as well as pro-coagulant and anti-fibrinolytic effects. In future, assessment of endothelial function may help us to identify patients in which cardiovascular risk factors are translated into vascular damage, requiring early and adequate therapy with drugs like ACE inhibitors and statins, substances that have been shown to markedly improve vascular NO bioavailability, reduce endothelial dysfunction and therefore improve the prognosis.

References

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