

Endothelial Function

A Critical Determinant in Atherosclerosis?

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Abstract—Common conditions predisposing to atherosclerosis, such as hypercholesterolemia, hypertension, diabetes, and smoking, are associated with endothelial dysfunction. Endothelial function has largely been assessed as endothelium-dependent vasomotion, at least in part based on the assumption that impaired endothelium-dependent vasodilation also reflects the alteration of other important functions of the endothelium. An important rationale for this approach has been the observation that endothelium-derived nitric oxide (NO), a major mediator of endothelium-dependent vasodilation, has important anti-inflammatory and antithrombotic properties, ie, inhibiting leukocyte adhesion, limiting platelet adhesion and aggregation, and the expression of plasminogen activator inhibitor-1 (PAI-1), a prothrombotic protein. Accumulating data suggest that the degree of impairment of endothelium-dependent vasomotion has profound and independent prognostic implications. A common mechanism underlying endothelial dysfunction relates to increased vascular production of reactive oxygen species. Recent studies also suggest that inflammation per se and C-reactive protein in particular may directly contribute to endothelial dysfunction. These findings raise the question of whether assessment of endothelial function can be used in the clinical setting to identify patients at high risk. New insights into mechanisms of endothelial dysfunction, such as a better understanding of the regulation of important vascular sources of oxygen radicals, may lead to novel therapeutic strategies with the potential to improve prognosis. (*Circulation*. 2004; 109[suppl II]:II-27–II-33.)

Key Words: atherosclerosis ■ C-reactive protein ■ endothelium ■ inflammation ■ nitric oxide ■ thrombosis

Over the last two decades it has become evident that the endothelium is not an inert, single-cell lining covering the internal surface of blood vessels, but in fact plays a crucial role in regulating vascular tone and structure. Importantly, a healthy endothelium inhibits platelet and leukocyte adhesion to the vascular surface and maintains a balance of profibrinolytic and prothrombotic activity (Figure 1).¹ Common conditions predisposing to atherosclerosis, such as hypercholesterolemia, hypertension, diabetes, and smoking, are associated with endothelial dysfunction, leading to a proinflammatory and prothrombotic phenotype of the endothelium. The advanced understanding of the pathobiology of atherosclerosis suggests that these alterations of endothelial function may play a pivotal role in the development and progression of atherosclerosis and its clinical complications.

Endothelial function has been assessed primarily in terms of endothelium-dependent vasomotion, largely based on the assumption that impaired endothelium-dependent vasomotion reflects alterations of other functions of the endothelium as well. An important rationale for this approach has been the observation that endothelium-derived nitric oxide (NO), synthesized by the endothelial NO synthase (eNOS) from the precursor L-arginine, is not only a major mediator of endothelium-dependent vasodilation but also is critically involved

in the regulation of other protective properties of the healthy endothelium.

Endothelium-Derived NO: An Antiatherosclerotic Molecule?

Recent advances have established a pivotal role for inflammation in all stages of atherosclerosis, including initiation, progression, and the complicated advanced lesion.^{1,2} Increasing evidence suggests an important anti-inflammatory role of endothelium-derived NO. Experimental studies have demonstrated that leukocyte adhesion and infiltration into the arterial wall, regulated by leukocyte-adhesion molecules and chemokines, represent an essential step in atherosclerotic lesion formation.^{1–6} Pharmacologic inhibition of endothelium-derived NO production leads to a marked increase of the endothelial adhesiveness for monocytes,^{7,8} and this effect is attenuated by dietary L-arginine, the substrate of eNOS. Similarly, increased leukocyte endothelial cell interactions have been observed in eNOS-deficient mice.⁹ At a molecular level, inhibition of eNOS results in increased expression of leukocyte-adhesion molecules and critical chemokines, such as monocyte chemoattractant protein-1,^{10,11} which is thought to be responsible for the migration of monocytes into the intima at sites of atherosclerotic lesion formation.^{2–4} Con-

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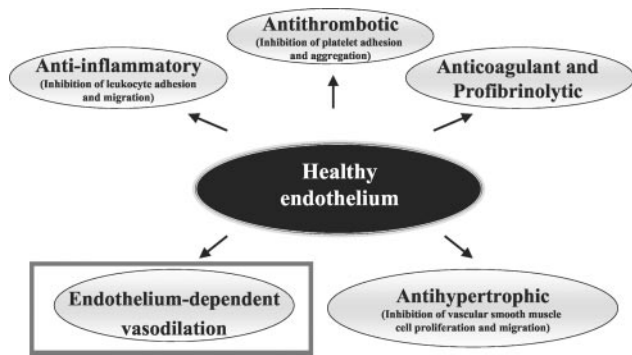


Figure 1. The healthy endothelium not only mediates endothelium-dependent vasodilation, but also actively suppresses thrombosis, vascular inflammation, and hypertrophy. Nitric oxide is a particularly important mediator of both endothelium-dependent vasodilation and anti-inflammatory and antithrombotic effects of the endothelium, and endothelium-dependent vasomotion is therefore thought to represent a “read-out” of other important functions of the endothelium.

versely, NO synthase gene therapy rapidly reduces hypercholesterolemia-induced leukocyte-adhesion molecule expression (ie, vascular cell adhesion molecule-1) and ameliorates monocyte infiltration into the arterial wall of cholesterol-fed rabbits.¹²

These observations have prompted studies to determine whether endothelium-derived NO production has an impact on atherosclerotic lesion formation. In mice lacking the low-density-lipoprotein (LDL) receptor, an animal model of familial hypercholesterolemia, inhibition of endothelial NO production accelerated atherosclerotic lesion formation, whereas L-arginine treatment decreased lesion development.¹³ Moreover, it has recently been demonstrated that specific removal of the eNOS from mice prone to develop atherosclerosis, ie, the apolipoprotein E (apoE)-knockout mouse model, resulted in a marked acceleration of atherosclerotic lesion formation in the aorta, and in significant coronary atherosclerosis.¹⁴ This effect could not be attributed to the moderate hypertension of the apoE/eNOS double knockout mice, because a subsequent study in such mice showed a similar degree of accelerated atherosclerosis in coronary arteries and the aorta after normalization of blood pressure using hydralazine, thereby providing further evidence for antiatherosclerotic properties of endothelium-derived NO.¹⁵

In patients with coronary disease, endothelium-dependent vasomotion at the forearm is related to serum levels of a systemic marker of inflammation, the high-sensitive C-reactive protein (CRP).¹⁶ Recent studies suggest that CRP, besides being a marker of inflammation, may also directly contribute to endothelial dysfunction.¹⁷ Exposure of endothelial cells to CRP decreases endothelial NO production and downregulates eNOS expression due to decreased eNOS mRNA stability.^{18,19} Hingorani et al²⁰ have recently provided further evidence that systemic inflammation per se may promote endothelial dysfunction and thereby contribute to a vicious cycle. These authors demonstrated that the inflammatory reaction in response to vaccination with *Salmonella typhi* was associated with a temporary but profound dysfunction of

the arterial endothelium in both resistance and conduit vessels.

Endothelium-Derived NO: An Antithrombotic Molecule?

Apart from its impact on leukocyte adhesion, endothelium-derived NO also limits platelet activation, adhesion, and aggregation.²¹ In addition, antithrombotic effects of endothelium-derived NO may relate to inhibition of plasminogen activator inhibitor-1 expression, a prothrombotic protein.²² eNOS has also been identified in platelets, making them capable of producing NO.^{23,24} In patients with coronary disease, reduced platelet-derived NO production was an independent predictor of acute coronary events.²⁵

How to Assess Endothelial Function in Patients?

In 1980, Furchgott and Zawadzki discovered the obligatory role of the endothelium in arterial relaxation in response to the administration of acetylcholine.²⁶ In endothelium-denuded vessels, acetylcholine causes vasoconstriction due to a direct effect on vascular smooth muscle cells.²⁶ In 1986, Ludmer and colleagues adapted this “endothelial function test” to the catheter laboratory and for the first time demonstrated endothelial dysfunction in epicardial coronary arteries of patients with coronary disease.²⁷ Whereas acetylcholine caused a dose-dependent dilation of coronary arteries in subjects without coronary disease, in patients with coronary disease, a “paradoxical” vasoconstriction was observed in response to acetylcholine, indicating an impaired endothelium-dependent vasomotion.²⁷ Coronary endothelial dysfunction has since been shown to be progressive, so that the vasoconstriction caused by acetylcholine is more pronounced in patients with established coronary disease as compared with patients with hypercholesterolemia.²⁸ Treatment with the substrate of the NO-synthase, L-arginine, could improve acetylcholine-tested coronary vasomotion in patients with hypercholesterolemia.²⁹ Quyyumi et al later confirmed that the impaired response to acetylcholine in patients with coronary disease or cardiovascular (CV) risk factors was largely due to a reduced coronary availability of endothelium-derived NO.³⁰

The measurement of flow-dependent dilation of the brachial artery as a noninvasive endothelial function test was introduced in 1992 by Celermajer et al,³¹ and this approach has now been used by numerous groups throughout the world to monitor endothelial function. This method uses a stimulus that is particularly relevant physiologically for endothelium-dependent vasodilation (ie, increases in laminar shear stress), the tangential force exerted by blood flow over the surface of the endothelium.³² Increases in shear stress lead to a rapid activation of eNOS, and over a longer period, to increased eNOS expression.³² Accordingly, we and others have been able to demonstrate that flow-dependent dilation of the radial and brachial arteries is largely inhibited after NO synthase inhibition in humans,³³ and therefore provides a valuable “read-out” of vascular NO availability. Using this approach, it was shown that the major CV risk factors impair flow-dependent dilation in a progressive manner, so that a more

Studies on Prognostic Implications of Endothelial Function

	<i>n</i>	Patient Population	Vessel	Endothelial Function Test	Follow-up (month)	Result(s)
Coronary vessel						
Al Suwaidi et al ³⁹	157	Coronary disease	Coronary microcirculation	ACh	28	CV events only in lowest tertile of ACh response
Schächinger et al ⁴⁰	147	Diagnostic catheterization (or PTCA 57% CAD)	Epicardial coronary artery	ACh FDD	82	ACh/FDD were independent predictors of CV events
Halcox et al ⁴¹	308	Diagnostic catheterization (57% CAD)	Epicardial coronary artery; coronary microcirculation	ACh	46	ACh was independent predictor of CV events
Peripheral vessel						
Heitzer et al ⁴³	281	Coronary disease	Forearm microcirculation	ACh	54	ACh was independent predictor of CV events
Perticone et al ⁴²	225	Hypertension (untreated)	Forearm microcirculation	ACh	31	ACh was independent predictor of CV events
Gokce et al ⁴⁵	187	Vascular surgery (preop.)	<i>A. brachialis</i>	FDD	1	FDD was independent predictor of postop. CV events
Neunteufl et al ⁴⁴	77	Chest pain	<i>A. brachialis</i>	FDD	60	FDD was independent predictor of CV events

Ach indicates acetylcholine response; CAD, coronary artery disease; CV, cardiovascular; FDD, flow-dependent dilation; and PTCA, percutaneous transluminal coronary angioplasty.

severe impairment of flow-dependent vasodilation is observed with increasing numbers of risk factors.³⁴ Interestingly, in a recent study, Hill et al³⁵ demonstrated that the number of circulating endothelial progenitor cells was related to flow-dependent, endothelium-mediated dilation of the brachial artery and to the CV risk-factor score, raising the possibility that the capacity for endothelial repair may be reduced with increasing numbers of CV risk factors.

Furthermore, it has been postulated that beneficial effects of regular aerobic training, including its antiatherogenic properties, may be mediated in part through shear-induced increases in NO secretion and eNOS expression. Stimulation of eNOS expression induced by shear stress is largely dependent on activation of the tyrosine kinase c-Src in cultured endothelial cells.³⁶ Interestingly, in c-Src heterozygous mice, the beneficial effect of exercise training on vascular eNOS expression is lost, suggesting an important role of this pathway in exercise training.³⁷

We have found that regular physical training improves peripheral endothelium-dependent, NO-mediated vasodilation in patients with chronic heart failure.³³ Hambrecht et al demonstrated improved coronary endothelium-dependent vasomotion after physical training in patients with coronary disease.³⁸

Prognostic Implications of Endothelial Dysfunction

Working from the assumption that measurement of endothelium-dependent vasomotion represents a surrogate of endothelial NO availability and relates to other important functions of the endothelium, several groups have recently addressed the important question of whether endothelium-dependent vasomotion provides prognostic information in humans—a highly relevant issue from a clinical standpoint

(Table).^{39–45} Suwaidi et al³⁹ monitored 157 patients with mildly diseased coronary arteries for an average of 28 months and observed cardiac events only in the patients showing the lowest tertile of coronary responses to acetylcholine. Similarly, in a study of 147 patients, Schächinger et al⁴⁰ used 3 different stimuli for endothelial release of NO: acetylcholine, cold pressor testing, and increased blood flow. During a follow-up period of about 8 years, the authors showed that responses to each of these stimuli were independent predictors of CV events. In an even larger study from the National Institutes of Health, Halcox et al⁴¹ monitored 308 patients and showed that both epicardial and microvascular coronary endothelium-dependent vasodilator function were independent predictors of CV events.

Given the systemic nature of endothelial dysfunction, the question arises as to whether peripheral vascular function may also serve as a prognostic marker. Four clinical studies have now addressed this question and have demonstrated that peripheral endothelium-dependent vasodilation, measured in response to acetylcholine or as flow-dependent vasodilation, has profound and independent prognostic implications (Table).^{42–45} Gokce et al⁴⁵ performed a prospective study and examined brachial artery vasodilation in 187 patients undergoing vascular surgery. Patients were monitored for 30 days after surgery. Preoperative, flow-dependent, endothelium-mediated dilation was significantly lower in patients with a postoperative event, compared with those without an event, and was an independent predictor of outcome. In another study, Heitzer et al⁴³ analyzed forearm blood flow in response to acetylcholine in 276 patients with coronary disease. In addition, in a subset of these subjects, investigators repeated acetylcholine response measures during intra-arterial infusion of the antioxidant vitamin C. Subjects were monitored for up to 87 months. As in earlier studies, the investigators found

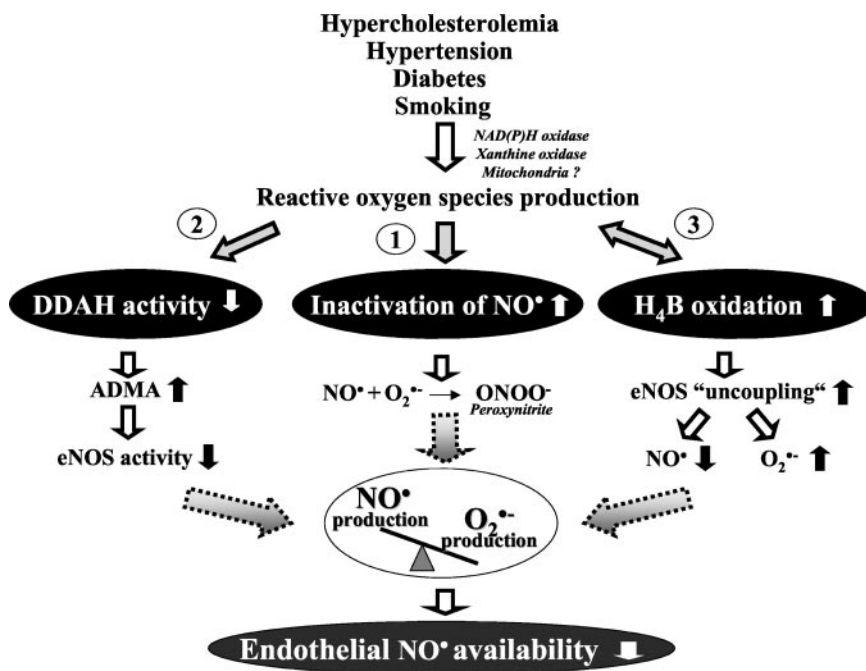


Figure 2. The major cardiovascular risk factors increase vascular production of reactive oxygen species, which may reduce endothelial NO availability via at least 3 pathways: direct inactivation of NO by superoxide ($O_2^{\cdot-}$), resulting in loss of the bioactivity of NO; reduced NO-synthase activity due to increased levels of ADMA, an endogenous NO-synthase inhibitor, resulting from redox-sensitive inhibition of DDAH; and eNOS “uncoupling,” due to increased oxidation of H₄B. ONOO⁻ indicates peroxynitrite.

that the amount of increase in forearm blood flow in response to acetylcholine was an excellent prognostic indicator, ie, the subsequent event rate was high in those with blunted responses to acetylcholine. An important novel mechanistic aspect of this study was that the abnormal responses to acetylcholine could be dramatically improved by the intra-arterial administration of antioxidant vitamin C only in the group with CV events, suggesting an important role of reduced NO availability due to increased reactive oxygen species production.

Mechanistic Insight into Endothelial Dysfunction

Taken together, these studies suggest a prognostic relevance of endothelial dysfunction and have further stimulated interest in understanding the underlying mechanisms that cause impaired endothelium-dependent vasomotion and endothelial NO availability in the clinical setting. Whereas endothelial dysfunction is likely a multifactorial process,⁴⁶ there is accumulating evidence that increased vascular production of reactive oxygen species plays an important role.^{47,48} Increased vascular superoxide production has been demonstrated in all major conditions predisposing to atherosclerosis.⁴⁷ Furthermore, Sorescu et al⁴⁹ have recently directly shown that there is increased superoxide production in coronary arteries in patients with coronary disease. In particular, superoxide reacts rapidly with NO, resulting in formation of the peroxynitrite anion and loss of bioactivity of NO. Furthermore, it has recently been demonstrated that increased vascular reactive oxygen species production promotes the oxidative degradation of the critical eNOS cofactor tetrahydrobiopterin (H₄B) leading to eNOS “uncoupling” with reduced NO• production and increased O₂^{•-} production from the enzyme.^{50–54} In addition, recent data suggest that there is redox-sensitive inhibition of the enzyme dimethylarginine dimethylaminohydrolase (DDAH), leading to increased lev-

els of the endogenous NO-synthase inhibitor asymmetric dimethylarginine (ADMA) and reduced eNOS activity.⁵⁵ Therefore, changes of the endothelial redox state may have a profound impact on endothelial NO• availability (Figure 2).^{50–54}

The nicotinamide adenine dinucleotide phosphate (NADPH) oxidase has been identified as an important vascular source of superoxide, an enzyme that is stimulated by proatherosclerotic stimuli such as angiotensin II, mechanical stretch, and proinflammatory cytokines.⁵⁶ Furthermore, genetic deficiency of the NADPH oxidase subunit p47^{phox} results in a dramatic reduction of atherosclerotic lesion development in the descending aorta of apoE-deficient mice.⁵⁷ Using electron spin resonance spectroscopy and in cooperation with David Harrison’s laboratory, we have recently presented evidence that the coronary activity of the NADPH oxidase is significantly increased in patients with coronary disease (Figure 3).⁵⁸ Another potential vascular source of superoxide is xanthine oxidase.^{59–61} In patients with coronary disease, increased coronary and endothelium-bound xanthine oxidase activity has recently been observed and was related inversely to endothelium-dependent vasodilation, suggesting that increased xanthine oxidase activity contributes to endothelial dysfunction (Figure 3).⁵⁸ Activation of these superoxide-producing enzymes, in particular the NADPH oxidase, may subsequently oxidize tetrahydrobiopterin and thereby profoundly alter the function of eNOS as noted above.⁵¹ These reactions are likely to form a vicious cycle of ongoing production of oxygen radicals in the endothelium. In patients with coronary disease, intracoronary infusion of tetrahydrobiopterin improves acetylcholine-tested coronary vasomotor function, thereby providing indirect evidence for eNOS uncoupling in these patients.⁶²

Importantly, vascular superoxide levels and NO bioactivity are determined not only by the rate of superoxide production but also by the rate of superoxide degradation. The major

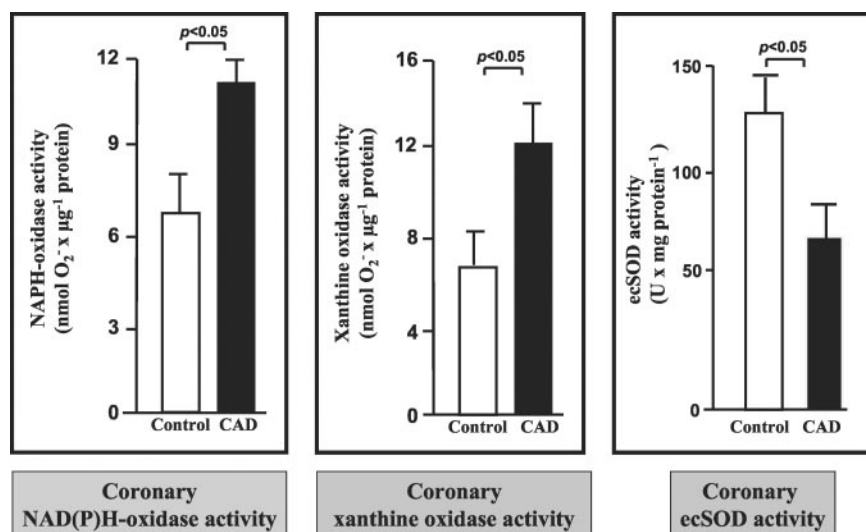


Figure 3. Potential mechanisms leading to increased vascular superoxide (O_2^-) production in patients with coronary artery disease. Coronary activities of NAD(P)H-oxidase and xanthine oxidase, both potent O_2^- -producing enzymes, are increased in patients with coronary disease, as determined by electron spin resonance spectroscopy. In contrast, the coronary activity of ecSOD, a major vascular O_2^- -degrading enzyme system, is markedly reduced in these patients. CAD indicates coronary artery disease. Reproduced and modified from Spiekermann S et al⁶⁸ and Landmesser U et al.⁶⁶

superoxide-degrading enzyme system is superoxide dismutase, and the extracellular form of superoxide dismutase (ecSOD) is of particular interest in the vessel wall because it is highly expressed and strategically located between endothelium and vascular smooth muscle cells (Figure 4).^{63–65} Fukui et al⁶² have put forward the concept that eNOS-derived NO induces ecSOD expression in the arterial wall as an important feed-forward mechanism to increase its biological effect; conversely, reduced NO availability would then reduce vascular ecSOD activity and further augment endothelial dysfunction. Indeed, observations have recently been made in patients with coronary disease that support this concept; in coronary arteries from patients with coronary disease, ecSOD activity was profoundly reduced (Figure 3).⁶⁶ Furthermore, endothelium-bound ecSOD activity determined in vivo (ie, released from the endothelium by heparin bolus injection) was shown to be reduced and closely related to

endothelium-dependent, NO-mediated vasodilation in patients with coronary disease, suggesting that reduced ecSOD activity contributes to endothelial dysfunction.⁶⁶

Given the accumulating evidence for an important role of increased oxygen-radical production for endothelial dysfunction, but also for oxidation of LDL cholesterol, LOX-1 receptor expression, and proinflammatory signaling in the endothelium, the negative outcome of most but not all of the recent clinical trials using vitamin E may come as a surprise. The negative studies by no means invalidate the role of oxidative stress in vascular biology. Rather, a number of facts would suggest that vitamin E may not represent an optimal strategy to effectively prevent vascular oxygen radical formation.⁴⁸ First, vitamin E reacts poorly with superoxide, the oxidant thought to be particularly important for many processes, including endothelial dysfunction.⁴⁸ Second, on scavenging a radical, vitamin E becomes the tocopheroxyl radical that may even augment lipid peroxidation under certain circumstances.^{67–69} Third, vitamin E is concentrated in lipid membranes and may not be able to intervene in oxidative processes in the cytoplasm or extracellular space. Therefore, a more promising approach to the reduction of ambient levels of superoxide in the endothelium and thus the improvement of endothelial function may be to prevent the activation of pro-oxidant enzymes such as the NADPH oxidase, rather than trying to scavenge radicals using antioxidant vitamins. Notably, experimental studies suggest that angiotensin-converting enzyme (ACE) inhibition and statin treatment reduce the vascular activation of the NADPH oxidase, which may make an important contribution to the beneficial effect on endothelial function.^{70,71} Indirect evidence suggests that this “antioxidant” effect may be involved in the effect of ACE inhibition on endothelial function in patients with coronary disease.⁷² Local administration of the antioxidant vitamin C improved endothelium-dependent, NO-mediated vasodilation in these patients before ACE-inhibitor treatment, an effect that was lost after 4 weeks of ACE-inhibitor therapy.⁷²

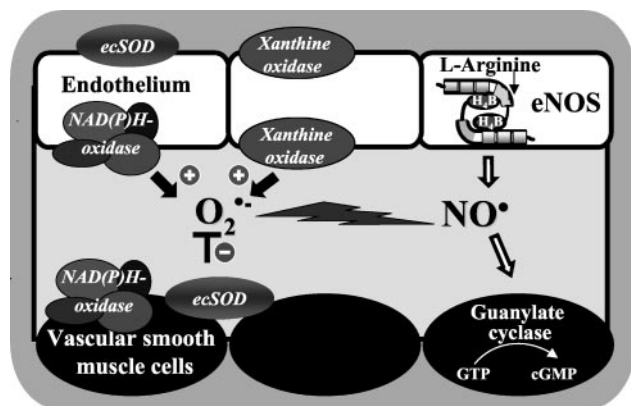


Figure 4. The bioactivity of eNOS is dependent on vascular levels of superoxide (O_2^-), ie, the balance between O_2^- production by enzymes such as the NADPH-oxidase and xanthine oxidase, and O_2^- degradation by antioxidant enzymes, such as the ecSOD. O_2^- reacts with NO at an almost diffusion-controlled rate, leading to peroxynitrite formation and loss of the bioavailability of NO. Oxidant stress may further reduce the bioavailability of NO by the mechanisms shown in Figure 2. cGMP indicates cyclic guanosine monophosphate; GTP, guanosine triphosphate; and H₄B, oxidation of tetrahydrobiopterin.

Conclusions

Beyond the impairment of endothelium-dependent vasomotion, endothelial dysfunction promotes key aspects of the

atherosclerotic disease process, ie, vascular inflammation and thrombosis, that are relevant at all stages of the disease. Conversely, a systemic inflammatory response that may involve detrimental effects of C-reactive protein on the endothelium augments endothelial dysfunction.⁷³ Accumulating evidence suggests profound prognostic implications of the degree of coronary and peripheral endothelial dysfunction, as assessed in response to acetylcholine or flow-dependent dilation (FDD). The observation that endothelial dysfunction represents an independent predictor of CV events in recent studies (Table) suggests that assessment of endothelial function integrates variables beyond conventional risk factors that may include the “pathogen burden,”⁷⁴ genetic predisposition, and other not-yet-identified factors causing endothelial activation.

These findings, however, raise the question of whether assessment of endothelial function can be used in the clinical setting to identify patients at high risk. In this respect, a first short-term prospective study supports the concept that non-invasive assessment of endothelial function (FDD of brachial artery) may represent a future strategy to identify high-risk patients.⁴⁵ However, before the clinical use of such an approach can be recommended, several issues need to be addressed by ongoing and future studies, and prospective studies will be needed to provide more information on the sensitivity and specificity for individual patients.

New insights into mechanisms of endothelial dysfunction, such as a better understanding of the regulation of important vascular sources of oxygen radicals, may lead to novel therapeutic strategies with the potential to improve prognosis. Notably, treatment strategies that improve prognosis in high-risk patients, such as statin or ACE-inhibitor therapy, as well as physical training, are effective in improving endothelial function, which likely contributes to their therapeutic benefit. Furthermore, within the next few years we expect to learn more about the function and pathophysiologic relevance of endothelial progenitor cells, first described in 1997 by Asahara et al,⁷⁵ and to better understand the role they play in endothelial-cell repair.^{76,77}

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