

Review

Nitric oxide and atherosclerosis: An update

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Abstract

Nitric oxide (NO) is a molecule that has gained recognition as a crucial modulator of vascular disease. NO has a number of intracellular effects that lead to vasorelaxation, endothelial regeneration, inhibition of leukocyte chemotaxis, and platelet adhesion. Endothelium damage induced by atherosclerosis leads to the reduction in bioactivity of endothelial NO synthase (eNOS) with subsequent impaired release of NO together with a local enhanced degradation of NO by increased generation of reactive oxygen species with subsequent cascade of oxidation-sensitive mechanisms in the arterial wall. Many commonly used vasculoprotective agents have their therapeutic actions through the production of NO. L-Arginine, the precursor of NO, has demonstrated beneficial effects in atherosclerosis and disturbed shear stress. Finally, eNOS gene polymorphism might be an additional risk factor that may contribute to predict cardiovascular events. However, further studies are needed to understand the possible clinical implications of these correlations.

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Nitric oxide (NO)¹ is a gas with a half-life of several seconds. It is synthesized by a family of NO synthase (NOS) enzymes producing NO and citrulline through a five-electron oxidation of the guanidine-nitrogen terminal of L-arginine. Three distinct isoforms of NOS have been identified in human beings and other organisms [65]. Two of these are

constitutively expressed: neuronal NOS (nNOS; also known as NOS-1, because it was the first isoform discovered) and endothelial NOS (eNOS; NOS-3). Both these are regulated by calcium and calmodulin and by post-translational modifications of the enzymes. The third isoform is inducible NOS (iNOS; NOS-2). It is regulated by cytokine stimulation and produces quantities of NO far exceeding those produced by the other two isoforms. These enzymes all require several cofactors for proper function, including tetrahydrobiopterin (BH4), nicotinamide-adenine-dinucleotide phosphate (NADPH), flavin adenine dinucleotide, and flavin mononucleotide. NO has a pivotal role in vascular homeostasis [65]. It plays a protective role by suppressing abnormal proliferation of vascular smooth muscle cells (VSMCs) following various pathological situations including atherosclerosis [99,66,54]. It participates in highly active metabolic and regulatory functions including control of

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¹ Abbreviations used: ADMA, asymmetric dimethylarginine; AGEs, advanced glycation end products; BH4, tetrahydrobiopterin; CHD, coronary heart disease; COX, cyclooxygenase; eNOS, endothelial nitric oxide synthase; HO, heme oxygenase; iNOS, inducible nitric oxide synthase; LDL, low-density lipoprotein; L-NMMA, N(G)-monomethyl-L-arginine monoacetate; NADPH, nicotinamide-adenine-dinucleotide phosphate; NO, nitric oxide; NOS, nitric oxide synthase; PPARs, peroxisome proliferator-activated receptors; ROS, reactive oxygen species; VSMCs, vascular smooth muscle cells.

hemostasis, fibrinolysis, platelet and leukocyte interactions with the arterial wall, presentation of histocompatibility antigens, regulation of vascular tone and growth, and homeostasis of blood pressure. Many other crucial vasoactive endogenous compounds like prostacyclin, thromboxane, endothelin, angiotensin, endothelium-derived hyperpolarizing factor, reactive oxygen species (ROS) and other free radicals, and bradykinin are formed in the endothelial cells to control the functions of VSMCs and of circulating blood cells [65,99,66,54]. However, this versatile and complex system is extremely vulnerable. Its equilibrium may be disturbed by numerous endogenous and exogenous factors including psychological and physical stress, disease states characterized by vasospasm, inflammation, leukocyte and platelet adhesion and aggregation, thrombosis, abnormal VSMC proliferation, atherosclerosis, and hypertension [65,99,66,54]. There is now abundant evidence that some pharmacological agents exert direct beneficial effects on endothelium, suggesting that at least part of their therapeutic action is associated with improvement in endothelial dysfunction [65,99,66,54,67,100]. Much of these drugs elicit their actions by improving the deleterious oxidation-sensitive mechanisms leading to vascular dysfunction and atherosclerosis [101,36].

Since its anatomic discovery, the endothelium was considered to fulfill no other purpose than that of a physical barrier between blood and tissues, until Furchgott [54] defined endothelium-dependent vasoreactivity [54]. Thus, a functional paradigm defined the balance between endothelium-derived relaxing factors and endothelium-derived contracting factors as the hallmark of both endothelial cell and arterial wall integrity. As a consequence, any reflection of a deviation from this state was defined as endothelial dysfunction [40] and recent experimental studies remark how hypercholesterolemia and hypertension have synergistic deleterious effects on coronary endothelial function [119].

NO is involved in vascular dysfunction and vascular damage leading to atherosclerosis

Physical or biochemical injury to the endothelium impairs production of homeostatic mediators of vascular health, such as NO, resulting in an intima that is characterized by enhanced thrombus formation, aberrant vessel tone, and dysregulated VSMC growth. Indeed, one of the hallmarks of a dysfunctional endothelium is diminished levels of bioavailable NO. This may result from a decrease in NO synthesis or an increase in NO inactivation owing to locally enhanced production of ROS. When present, endothelial dysfunction serves as an early marker of atherosclerosis as demonstrated by the observation that fatty streak progression is associated with increasingly impaired vascular relaxation [140]. Despite the transient compensatory increase in expression of iNOS and nNOS [42], NO bioavailability is reduced because of increased reaction rates with superoxide, yielding as by-products reactive nitrogen/oxygen species that induce protein nitration. This decreased NO bioactivity appears to be a key

contributor to vasoconstrictive remodeling and a major determinant of the occurrence of nitrative/oxidative stress. Clinically, some of the coronary heart disease (CHD) risk factors identified with endothelial dysfunction, including hypercholesterolemia, tobacco use, diabetes mellitus, and hyperhomocysteinemia, are associated with decreased bioavailable NO as evidenced by an abnormal coronary vasodilator response to acetylcholine challenge [86].

Asymmetric dimethylarginine (ADMA): a competitive inhibitor of NOS

The presence of endogenous NOS inhibitors *in vivo* has been identified as one mechanism associated with decreased NO production. A naturally occurring analog of L-arginine, asymmetric dimethylarginine (ADMA), has been identified as a competitive inhibitor of NOS [139,123]. Plasma levels of ADMA are elevated in patients with hypercholesterolemia and atherosclerotic vascular disease and, in fact, plasma ADMA levels have been correlated with the severity of endothelial dysfunction [31,133,1]. By inhibiting the production of NO, ADMA itself may be a novel risk factor for the development of atherothrombotic vascular disease [99,66,30,17]. Moreover, by inhibiting NO synthesis, plasma ADMA may reduce vascular compliance, increase vascular resistance, and limit blood flow. Furthermore, plasma ADMA may promote atherogenesis as it opposes the vasoprotective effects of NO. Thus, elevations in plasma ADMA may accelerate the progression of atherosclerosis and increase the risk of cardiovascular events. ADMA may mediate the effect of many risk factors and risk markers on the NOS pathway [65,105]. Accordingly, in patients with hypercholesterolemia, intravenous L-arginine improves NO-mediated brachial artery reactivity [17].

The role of ROS in endothelial dysfunction

In addition to decreased production of NO, endothelial dysfunction is associated with an increase in vascular oxidant stress from the production of endogenous ROS, especially superoxide radical, in excess of antioxidant capacity. Once formed, superoxide reacts with NO to form peroxynitrite. This reaction, which consumes NO, is diffusion-limited and three times faster than the catabolism of superoxide by superoxide dismutase. Under physiologic conditions, NO is probably formed at a concentration in the picomolar-to-nanomolar range, and the presence of antioxidant defenses minimizes its consumption by superoxide [65]. At very low concentrations, peroxynitrite has the same biologic activity as NO; it is only at higher levels that it exerts its toxic effects by forming the cytotoxic peroxynitrous acid, as well as resulting in protein modification by nitration of amino acids [146,9,68].

There are several important sources of ROS production in the vasculature that may be activated directly or indirectly as in the setting of endothelial dysfunction. Free radical generation by endothelial, vascular smooth muscle, or adventitial

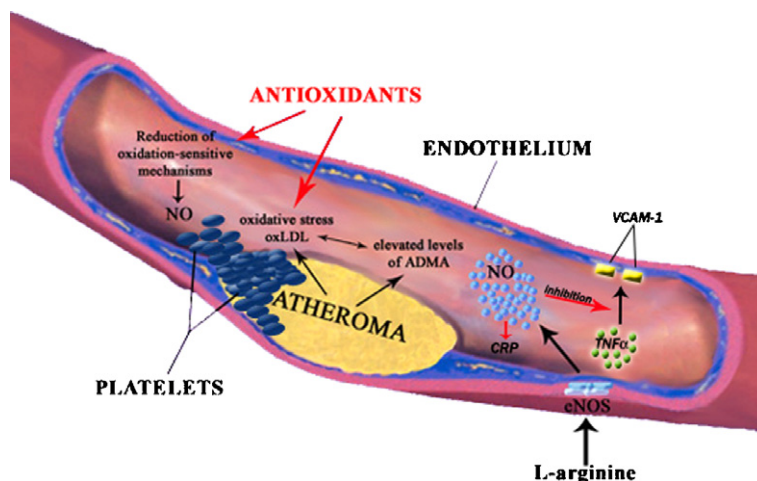


Fig. 1. Classical scheme of NO involvement in atherogenesis. Metabolic intervention with antioxidants reduces arterial oxidation-specific epitopes and systemic oxidative stress. Endothelial NO production inhibits TNF α -stimulated vascular cell adhesion molecule-1 (VCAM-1) expression in endothelial cells and reduces the circulating levels of the inflammatory marker C-reactive protein (CRP).

cells in the vessel wall may occur via activation of enzyme systems such as NAD(P)H oxidase(s), which has been identified in vascular cells and shown to produce abundant ROS in response to angiotensin II, thrombin, tumor necrosis factor- α , or elevated mechanical forces [60,11,114,138,84,99,66,146,9]. A second important source of free radical production is the enzyme xanthine oxidase. In hypercholesterolemic patients, intravenous administration of oxypurinol similarly improved the blunted vasodilator response to acetylcholine [22]. Therefore, a pathophysiological scenario involves oxidation-sensitive mechanisms, ADMA, and antioxidants in the progression of atheroma (Fig. 1).

NO and atherosclerosis

As stated, an impairment of the NOS isoform-dependent pathways is one of the earliest events in atherogenesis (reviewed in [65,99,66,54,101,36]). Thus, strategies to enhance NO bioactivity are useful in the treatment of atherosclerosis-related diseases. In order to complete the pathophysiological scenario, NOS itself is capable of producing ROS [122]. In the absence of substrate, L-arginine, or cofactors such as BH₄, NOS has been shown to synthesize superoxide in preference to NO (enzyme “uncoupling”) (Fig. 2). Thus, CHD risk factors that deplete levels of L-arginine or BH₄ may promote NOS-mediated ROS formation, and in turn, increase peroxynitrite generation [66,19]. Decreased NO bioavailability disrupts the non-thrombogenic intimal surface and promotes platelet adhesion and aggregation, as well as deposition of platelets on the abnormal endothelial surface. In the setting of established atherosclerosis, there are additional abnormalities in the coagulation system that favor thrombus formation, including an increase in circulating von Willebrand factor and a decrease in heparan sulfated glycosaminoglycans [99,66]. Using L-NMMA, a competitive inhibitor of NOS, it was shown that there was a loss of basal and flow-mediated NO production in the coronary arteries [137]. The response to L-NMMA indicated

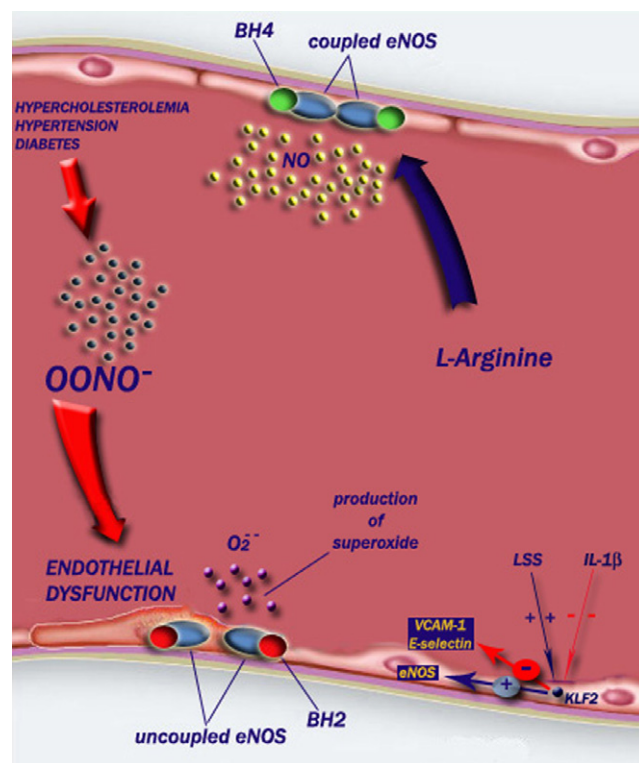


Fig. 2. The structure of NOS is a homodimer with each monomer containing an oxygenase domain and a reductase domain. The oxygenase domain contains the binding sites for heme, L-arginine (the substrate) and the cofactor tetrahydrobiopterin (BH₄), a critical determinant of eNOS activity. In vascular disease states such as diabetes, hypertension, or hypercholesterolemia, superoxide production by oxidases is markedly increased. Peroxynitrite and other reactive oxygen species oxidize BH₄ via the BH₃ radical to BH₂ and biopterin, which reduces the bioavailability of BH₄ and promotes eNOS uncoupling. This form of eNOS no longer produces NO, but instead generates superoxide. KLF2 is inhibited by the inflammatory cytokine interleukin-1 β (IL-1 β) and is induced by laminar shear stress (LSS) in endothelial cells. Overexpression of KLF2 strongly induces eNOS expression and may inhibit the proinflammatory cytokines-dependent induction of vascular cell adhesion molecule-1 (VCAM-1) and endothelial adhesion molecule E-selectin.

that the endothelium may retain basal NO production in the presence of atherosclerotic disease. L-Arginine administration partially restores endothelium-dependent vasodilation in hypercholesterolemia [33,41] and dilates coronary stenoses in patients with CHD [134]. L-Arginine supplementation for 6 months in humans also improves coronary small-vessel function in association with a significant improvement in symptoms [80]. Therefore, L-arginine administration could be a therapeutic option for patients with endothelial dysfunction and non-obstructive CHD [80,32,55].

The effects of intracoronary administration of substance P, L-NMMA, L-arginine, and nitroglycerin were studied in patients with normal coronary angiograms and in patients with CHD [135]. L-NMMA constricted and both substance P and nitroglycerin dilated normal and diseased arteries. L-Arginine reversed the effect of L-NMMA and caused greater dilation of the diseased arteries indicating that there is a deficiency of L-arginine. Moreover, there was a significant clinical improvement in more than 70% of patients, which was associated with a significant decrease in adhesion molecules and proinflammatory cytokines [15]. Thus, L-arginine may have clinical beneficial effects in patients with intractable angina. Furthermore, L-arginine improved myocardial perfusion during exercise in patients with angina and normal coronary arteries [52]. It was also investigated whether L-arginine could serve as useful adjunct therapy to improve endothelial function in patients with advanced CHD maintained on medical therapy [14]. Oral L-arginine therapy did not improve NO bioavailability in patients with advanced CHD and thus may not benefit this group of patients. However, it is possible that a more prolonged period of treatment and lesser degree of coronary lesions are necessary in order to see clinical improvement using L-arginine in CHD patients. It was also examined the effect of exogenous L- and D-arginine on coronary stenosis vasomotion in relation to stenosis morphology [136]. Intracoronary infusions of normal saline, L- and D-arginine, and glyceryl trinitrate were given in patients with CHD and stable angina. During L-arginine infusion a larger proportion of complex stenoses than smooth stenoses dilated by $\geq 10\%$ ($P < 0.01$). Irrespective of the type of morphology, there was a positive correlation ($P < 0.01$) between the severity of stenoses and the magnitude of vasodilatation to L-arginine. Thus, coronary stenoses dilate in response to L-arginine but not D-arginine. This finding is consistent with human deficiency of L-arginine at the site of coronary stenoses [136]. Novel features coming from a recent study showed beneficial effects of L-arginine on oxidation-sensitive gene expression and eNOS activity at sites of disturbed shear stress associated to atherosclerosis [37,56].

NO regulates granule exocytosis

NO may regulate exocytosis in a variety of physiological processes, including neurotransmission, vascular inflammation and thrombosis, and cytotoxic T lymphocyte cell kill-

ing [85]. NO regulates platelet activation by cGMP-dependent mechanisms and by mechanisms that are not completely defined (e.g., cGMP-independent mechanisms). Platelet activation includes exocytosis of platelet granules, releasing mediators regulating interactions between platelets, leukocytes, and endothelial cells. The process of exocytosis is mediated in part by *N*-ethylmaleimide-sensitive factor (NSF), an ATPase that disassembles complexes of soluble NSF attachment protein receptors. Exogenous and endogenous NO may inhibit platelet granule exocytosis by *S*-nitrosylation of NSF [89]. NO would inhibit the disassembly of the SNARE complex by nitrosylating critical cysteine residues of NSF [89,85]. In contrast, platelets lacking eNOS showed increased rolling on venules, and thrombosis in arterioles, as well as exocytosis in vivo. Thus, regulation of exocytosis is a mechanism by which NO may regulate thrombosis.

The role of NO as modulator of immune system

Arginine, often found in immunonutrition regimens, is also an important modulator of immune system activation [66,100,32]. However, the mechanism of how arginine may be beneficial in immunonutrition is poorly understood. The metabolism of arginine is determined by the expression of the arginine metabolizing enzymes iNOS and two arginase isoforms (arginases I and II). iNOS is induced by T helper I cytokines (interleukin-1, tumor necrosis factor and gamma-interferon), while arginases are induced by T helper II cytokines and other immune regulators such as interleukins 4, 10, and 13, transforming growth factor- β and prostaglandin E2 [66,100,32]. Endotoxin induces iNOS and arginases I and II, while arginase plays an important role in the production of ornithine, a precursor of proline and polyamines, both of which are necessary for cellular proliferation and wound healing. Arginase also induces NOS activity by competing for arginine availability in the extracellular environment, and producing polyamines, which may modulate macrophage activation. Through limitation of arginine availability in the extracellular environment, arginases also potentially regulate other “arginine-dependent” immune functions such as T-lymphocyte activation, although this hypothesis remains to be proven [66,100,32]. Thus, arginase expression appears to be essential in the regulation of the cellular immune response and the inflammatory process during critical illness and vascular diseases.

iNOS expression in human atherosclerotic lesions was studied using simultaneous in situ hybridization and immunocytochemistry [82,5,44]. In healthy arteries, iNOS was expressed at a low level in VSMCs. iNOS expression was increased in macrophages and VSMCs in the majority of early and advanced atherosclerotic lesions. Inflammation appears to play a major role in the development of atherosclerotic lesions affecting native and transplanted coronary arteries [120]. The subsequent risk of plaque rupture and acute ischemic events

correlates with the degree of inflammation and may be modified by aspirin, an anti-inflammatory cyclooxygenase inhibitor. Cyclooxygenase-2 (COX-2) and iNOS are involved in the inflammatory response via the rapid and exaggerated production of prostanoids and NO, both of which may have proatherosclerotic effects [66,7]. These effects may be mediated by the formation of peroxynitrite in the case of NO and involve “cross-talk” between the two enzyme systems. Immunocytochemical studies were performed on atherosclerotic lesions from patients with native and transplant CHD by using antibodies to COX-2, iNOS, and nitrotyrosine (an indicator of peroxynitrite production) [83]. COX-2 and iNOS colocalized predominantly in macrophages/foam cells in both type of atherosclerosis. Consistent with its anti-inflammatory properties, high levels of IL-10 expression were associated with significant decreases in iNOS expression and cellularity of lesions of advanced human atherosclerotic plaques, and this might contribute to the modulation of inflammation [83].

KLF2: a novel transcriptional regulator of endothelial proinflammatory activation

Kruppel-like factor 2 (KLF2) has been recently identified as a novel regulator of endothelial inflammatory events [124]. Transcriptional profiling studies showed the inhibition of KLF2 by interleukin-1 β and its induction by laminar shear stress in cultured endothelial cells. Overexpression of KLF2 strongly induced eNOS overexpression and enzymatic bioactivity. Moreover, KLF2 overexpression potently inhibited the proinflammatory cytokine-dependent induction of vascular cell adhesion molecule-1 (VCAM-1) and endothelial adhesion molecule E-selectin (Fig. 2). KLF2 transduced in endothelial monolayers is able to attenuate T-cell attachment and rolling. Thus, KLF2 differentially regulates key factors involved in maintaining an antithrombotic endothelial surface. In another study [81], KLF2 was found to strongly induce thrombomodulin (TM) and eNOS expression and reduce plasminogen activator inhibitor-1 (PAI-1) expression. Furthermore, overexpression of KLF2 inhibited the cytokine-mediated induction of tissue factor (TF). The functional importance of KLF2 was verified by *in vitro* clotting assays. By comparison to control infected cells, KLF2 overexpression increased blood clotting time as well as flow rates under basal and inflammatory conditions. Thus, some of these data were in contrast with siRNA-mediated knockdown of KLF2 experiments. In order to investigate the transcriptional mechanisms by which statins modulate endothelial function, it was found that statins can induce KLF2 expression and that a reduction in KLF2 expression attenuates statin-mediated accumulation of eNOS and TM levels [125]. Taken together, these observations provide a novel KLF2-mediated pathway by which statins may produce some of their beneficial effects in endothelial cells.

Induction of iNOS and HO-1 expression by AGEs in diabetic atherosclerosis

Advanced glycation end products (AGEs) are closely linked to the development of diabetic atherosclerosis. It was examined the induction of iNOS and heme oxygenase (HO)-1 expression by AGEs, as well as the signaling pathways involved and the interplay between these two enzymes [131]. The stimulation of RAW 264.7 cells with 6.64 or 33.2 $\mu\text{g/ml}$ AGEs leads to HO-1 protein expression, iNOS protein expression, and nitrite accumulation. AGEs lead to the phosphorylation of p42/44 and p38 mitogen-activated protein kinase (MAPK). The inhibition of p42/44 MAPK and protein kinase C prevented, whereas inhibition of p38 MAPK augmented, AGE-induced nitrite release and iNOS expression. In contrast, HO-1 expression was downregulated by inhibition of p38 MAPK. Furthermore, the expression of both proteins was prevented by coinubation with acetovanillone (NADPH oxidase inhibitor). AGE-induced iNOS expression was negatively regulated by stimulation of HO-1 expression with cadmium chloride or endogenous NO. Tin-protoporphyrin IX (HO-1 inhibitor) partially reversed the cadmium chloride-mediated downregulation of iNOS expression. The current study demonstrates that multiple signaling molecules are involved in AGE-stimulated iNOS and HO-1 expression. There also exists a downregulation of iNOS by its own product as well as the products of HO-1. These results suggest that HO-1 activity may have a profound effect on the balance of NO and reactive oxygen species. The authors hypothesize that stimulation of HO-1 expression may represent a new strategy for the treatment of diabetic atherosclerosis. The identification of the mechanisms by which AGEs induce HO-1 and iNOS expression, as well as the interplay between these enzymes and their product, may be important for a better understanding of diabetic atherosclerosis and nephropathy.

eNOS polymorphisms

As described, NO is synthesized from L-arginine by eNOS encoded by the NOS3 gene on chromosome 7 [65,99]. It was investigated the role of the eNOS gene variants as risk factors for early atherosclerosis and whether two polymorphisms located in the exon 7 (894 G \rightarrow T which encodes a Glu298 \rightarrow Asp amino acid substitution in eNOS) and in the promoter region (T-786 \rightarrow C) of the eNOS gene were associated with functional changes in the endothelium and carotid intima-media thickness (IMT) [113]. The study showed that the eNOS Glu298 \rightarrow Asp polymorphism affects flow-mediated brachial artery dilation (FMD) and carotid IMT, which are two markers of early atherosclerosis. This is suggestive of a genetically determined modulation of early changes in arterial structure and function related to atherogenesis. Therefore, the eNOS Glu298 \rightarrow Asp polymorphism might, in the long-term, play a significant role in atherogenesis and cardiovascular damage, raising the possibility of genotype prevention strategies.

The relationship between this Glu(298) → Asp variant and CHD was investigated using two independent case-controlled studies [64]. In the first study (CHAOS), cases consisted of 298 unrelated patients with angiographic CHD and controls were 138 unrelated healthy individuals ascertained through a population health screen. In the second study (CHAOS II), the cases were 249 patients with recent myocardial infarction, and a further 183 unrelated controls. There was an excess of homozygotes for the Asp298 variant among patients with angiographic CHD, and among patients with recent infarction when compared with their respective controls (35.9% versus 10.2%, $P < 0.0001$ in CHAOS, and 18.1% versus 8.7%, $P < 0.02$ in CHAOS II). In comparison to Glu(298) homozygotes, homozygosity for Asp(298) was associated with angiographic CHD and infarction. Thus, homozygosity for a common NOS3 polymorphism (894 G → T) which encodes a Glu298 → Asp amino acid substitution in eNOS could be a risk factor for angiographic CHD and recent infarction. However, it was studied the relationship among NO-mediated endothelial function, the presence of the eNOS Glu298 → Asp variant, and clinical risk factors for atherosclerosis [59]. Endothelium-dependent vasorelaxations were determined in human saphenous veins from patients with CHD and identified risk factors. Patients were genotyped for the eNOS G894T polymorphism.

It was determined whether T-786C, G894T, and 4a/4b eNOS genetic variants may increase the susceptibility to carotid atherosclerosis. It was proposed that the 4a allele and the eNOS combined genotypes are independent predisposing factors to carotid atherosclerosis [46].

Reduced vasorelaxations were associated with increased number of clinical risk factors for atherosclerosis ($r = -0.54$, $P < 0.001$), whereas the Glu298 → Asp variant was not associated with any differences in contractions to phenylephrine or NO-mediated vasorelaxation. Increased atherosclerotic risk factors, but not the presence of the eNOS Glu298 → Asp, are associated with impaired NO-mediated endothelial function, suggesting that this polymorphism could not have a major functional effect on vascular eNOS activity in human atherosclerosis.

A careful meta-analysis of case-control studies evaluating the association between the Glu298Asp, -786T>C, and intron-4 polymorphisms and CHD was recently performed. The principal prior hypothesis was that homozygosity for eNOS Asp298, the -786C allele in the promoter, or the intron-4 (a allele) would be associated with an increased risk of CHD. Data were available for 9867 cases and 13,161 controls from 26 studies. Homozygosity for the Asp298 was associated with an increased risk of CHD (OR, 1.31; 95% CI, 1.13–1.51). Although there was significant heterogeneity among studies of Asp298 ($P(\text{Het}) = 0.0002$), which was largely accounted for by a single study, the increase in risk was still significant after exclusion of that study from analysis. Homozygosity for the intron-4a allele was also significantly associated with higher risk of CHD (OR, 1.34; 95% CI, 1.03–1.75). However, no significant association was

found with the -786C allele (OR, 1.06; 95% CI, 0.89–1.25). Thus, individuals homozygous for the Asp298 and intron-4a alleles of eNOS are at moderately increased risk of CHD. These findings support the proposal that common genetic variations in the eNOS gene contribute to atherosclerosis susceptibility, presumably by effects on endothelial NO availability [23].

In a recent study, it was evaluated the role of these polymorphisms in the predisposition to abdominal aortic aneurysm (AAA), a chronic degenerative condition associated with atherosclerosis. Cases consisted of 250 consecutive patients with AAA compared with 250 truly healthy subjects with a negative history of vascular diseases. The study showed that the eNOS G894T polymorphism is a mild modulator of the predisposition to AAA apart from traditional risk factors, suggesting a genetic influence on the molecular mechanisms responsible for this complex disease [47].

The presence of 894T allele on eNOS gene has been also associated with impaired endothelial function and higher levels of von Willebrand factor in relatively young patients with myocardial infarction (MI). This finding implies that genetic polymorphism G894T on eNOS may affect endothelial function in patients with a history of premature myocardial infarction [3].

Also genetic variants of methylene tetrahydrofolate reductase (MTHFR) and eNOS influence homocysteine metabolism and NO synthesis, respectively, and might thus be determinants of the risk of atherosclerosis. In the Edinburgh Artery Study, the risks of peripheral arterial disease and of CHD related to MTHFR (alleles 175 and 198) and eNOS (alleles 4 and 5) polymorphisms were investigated [50]. In this population-based cohort study, 940 men and women aged 60–79 years, three groups of subjects were identified: those with peripheral arterial disease, those with CHD, and healthy controls. The distributions of the eNOS and MTHFR genotypes did not differ significantly between the groups with and without CHD. However, the eNOS-4 allele (frequency 0.13) was related to the occurrence of CHD in non-smokers. The MTHFR-175 allele (frequency 0.31) was not related to CHD, but was associated with a reduced risk of peripheral arterial disease. Neither the endothelial NOS-4 allele nor the MTHFR-175 allele was related to the ankle brachial pressure index in the whole study population. Thus, the eNOS-4 allele was associated with an increased risk of CHD in non-smokers, but otherwise the MTHFR and eNOS genotypes appeared to have little influence on peripheral arterial disease.

It was evaluated the role of three polymorphisms in the eNOS gene in relation to the existence, severity, and progression of CHD, MI, and the occurrence of ischemia in a predominantly Caucasian population [2]. Patients with CHD ($n = 760$) and age- and sex-matched population-based controls ($n = 691$) were genotyped for the -786T/C, E/D298, and 4a/b polymorphisms. Patients were randomized to pravastatin (40 mg) or placebo. Progression of atherosclerosis was evaluated by sequential angiography. Functionality was

Table 1
A selection of relevant studies showing the correlation between NOS polymorphisms and cardiovascular events

Polymorphism	Clinical feature	Reference
G894T	Abdominal aortic aneurysm	[47]
G894T	Premature myocardial infarction	[3]
T(−786)C	More advanced imbalance of autonomic activity in patients with congestive heart failure	[12]
Asp298	Coronary spastic angina	[109]
894 G→T	No evidence of a significant role in the development of CHD	[128]
Glu298→Asp	Early atherosclerosis	[113]
T(786)→C	No correlation with early atherosclerosis	
4a/4a	Acute coronary syndromes (ACS)	[46]
4a4a/−786CC	Higher predisposition to ACS	
Glu298Asp	Increased risk of CHD	[23]
T(786)→C	No significant association	
Intron-4	With CHD	
Glu298Asp	Coronary spasm	[25]
Glu(298)→Asp	Severe CHD	[29]
T(786)→C	Higher risk	
Glu(298)→Asp/T(786)→C		
T(786)→C	Severe internal carotid artery (ICA)	[57]
Asp298	Carotid atherosclerosis	[79]
[(CA), polymorphism] in intron 13	CHD	[129]
894 G→−>T	CHD and recent MI	[64]
Glu(298)→Asp	Susceptibility to acute myocardial infarction (AMI)	[63]
Glu298Asp	AMI	[127]

assessed by ST segment analysis of ambulant ECGs. The E298 ($P=0.003$) and 4a ($P=0.001$) alleles were associated with CHD. Furthermore, E298 ($P=0.009$) and −786T ($P=0.022$) alleles were associated with previous MI among patients, predominantly smokers. D/D298 homozygotes, but not −786T/C or 4a/4b mutants, had longer-lasting ischemia than others ($P<0.05$). It was found no differences in progression of atherosclerosis, irrespective of pravastatin use. Thus, E/D298 polymorphism is most consistently associated with CHD, but not with progression of atherosclerosis. The E allele is associated with CHD and MI, whereas the D allele is associated with ischemia [2]. Taken together, these data suggest that eNOS gene polymorphism might be an additional risk factor that contributes to endothelial dysfunction and atherosclerosis in many cardiovascular events. However, further studies are needed to better understand the relevance and the possible therapeutical implications of these correlations. Table 1 summarizes major studies in the field of eNOS polymorphisms and cardiovascular events.

Molecular approaches to understand pathobiology of NO and atherosclerosis

It was found that ex vivo gene transfer of eNOS to atherosclerotic rabbit aortic rings improves relaxations to acetylcholine [90]. These results suggested that reduced NO bioavailability observed in cholesterol-induced vascular dysfunction can be partially overcome by eNOS gene transfer. A correlation was also found between the VSMC infiltration in the intima and iNOS expression in the intima and the subendothelial layer of arteries from rabbits fed a long-term but low-level cholesterol-enriched diet, indicating a link between the severity of the lesion and iNOS expression [10]. NOS gene therapy also rapidly reduces adhesion mole-

cule expression and inflammatory cell infiltration in carotid arteries of cholesterol-fed rabbits [118]. Very recently, it was shown that gene transfer of endothelial NOS, but not eNOS plus inducible NOS, regressed atherosclerosis in rabbits [62].

Interestingly, supplemental L-arginine induces apoptosis of macrophages in intimal lesions by its conversion to NO, which acts through a cGMP-dependent pathway [143]. Thus, supplementation of dietary arginine may induce regression of atheroma in rabbits. The role of endogenous NO in the progression of atherosclerosis in apo E-deficient mice was also studied. Mice were treated with L-NAME, an inhibitor of NOS, or with L-arginine for 8 weeks [72]. L-NAME treatment resulted in a significant inhibition of NO-mediated vascular responses and a significant increase in the atherosclerotic plaque/surface area in the aorta of mice. L-Arginine treatment had no influence on endothelial function and did not alter lesion size. At the beginning of the study, impairment in function was apparent only in the case of L-NAME-mediated contraction, whereas ACh-induced, NO-mediated relaxation was not different between age-matched apolipoprotein E (apo E)-knockout and C57Bl/6J mice. After the 8-week treatment with the NOS inhibitor, both endothelium-dependent responses were significantly inhibited. The acceleration in lesion size concomitant to the severely impaired NO-mediated responses suggests that the lack of endogenous NO is an important progression factor of atherosclerosis in mice. Finally, there was a long-term combined beneficial effects of graduated physical training (swimming) and L-arginine treatment on atherosclerosis in hypercholesterolemic mice [106].

In a rat model in which the chronic inhibition of endothelial NO synthesis induces early vascular inflammation as well as subsequent coronary vascular remodeling,

anti-monocyte chemoattractant protein-1 (anti-MCP-1) gene therapy suppressed monocyte recruitment into the coronary vessels and the development of vascular medial thickening [43]. Thus, MCP-1 is necessary for the development of medial thickening; this new strategy may be a useful and feasible gene therapy against arteriosclerosis. More recently, to test whether deficiency in eNOS affects atherosclerosis development, a recent study compared lesion formation in apoE/eNOS-double knockout (DKO) and apoE-knockout (KO) control animals [74]. After 16 weeks of “Western-type” diet, apoE/eNOS-DKO males and females showed significant increases in lesion area of 93.6 and 59.2% compared with apoE-KO mice, respectively. All apoE/eNOS-DKO animals studied developed coronary atherosclerosis, associated with perivascular and myocardial fibrosis, whereas none of the apoE-KO mice did. Echocardiography showed a significantly increased left ventricular wall thickness and decreased fractional shortening in DKO animals. Male DKO animals developed atherosclerotic abdominal aneurysms and aortic dissection. Thus, eNOS deficiency increases atherosclerosis in Western-type diet-fed apoE-KO animals and introduces coronary disease and an array of cardiovascular complications, including spontaneous aortic aneurysm and dissection. This phenotype may constitute a valuable model to demonstrate distal coronary lesions associated with evidence of myocardial ischemia, infarction, and heart failure. It has been also recently showed that apoE-KO and LDL-receptor-deficient mice can develop spontaneous plaque rupture and thrombosis [20]. Finally, to test whether accelerated atherosclerosis and aortic aneurysms were due to hypertension, hydralazine was administered to male apoE/eNOS DKO mice to reduce blood pressure [26]. Hydralazine-treated, normotensive male apoE/eNOS DKO mice developed increased aortic lesion areas compared with male apoE KO mice. The extent of lesion was not significantly different from male apoE/eNOS DKO mice that were not given hydralazine. Thus, hypertension is not required for the accelerated atherosclerosis seen in apoE/eNOS DKO animals. Interestingly, eNOS plays also a protective role in allografts and that in eNOS-deficient allografts, early overexpression of iNOS is capable of preventing the development of allograft atherosclerosis [77].

In order to determine the role of eNOS in diet-induced fatty streak formation, it was used the eNOS-deficient mice [126]. Mice were fed an atherogenic (15% fat, 1.25% cholesterol, and 0.5% sodium cholate for 12 weeks), and atherosclerotic lesions at the aortic root were measured after oil-red O staining. Unexpectedly, eNOS-deficient mice developed much smaller aortic lesions than did wild-type control mice. This reduction in lesion formation could not be explained by changes in plasma levels of lipids and susceptibility of LDL to oxidation. To examine whether eNOS contributed to the oxidation of LDL within the arterial wall, endothelial cells were isolated from the aorta of mice and incubated with native LDL in the absence or presence of *N*-Omega-nitro-L-arginine methyl ester (L-NAME), a specific

NOS inhibitor. L-NAME significantly inhibited LDL oxidation by endothelial cells from wild-type animals ($P < 0.05$), but it had no effect on LDL oxidation by endothelial cells from eNOS-deficient mice. Thus, these data indicate that the absence of eNOS-mediated LDL oxidation may contribute to the reduction of fatty-streak formation in eNOS-deficient mice [126,91].

Proatherogenic role of ET-1

Contractions to endothelin-1 (ET-1) and/or thromboxane may be enhanced during chronic deficiency in expression or activity of NOS. It has been recently tested the hypothesis that vasoconstriction to ET-1 and/or the thromboxane mimetic, U46619, is enhanced under conditions of chronic, selective deficiency in eNOS-deficient mice by examining responses in aorta from eNOS-deficient mice compared to wild-type mice [75]. ET-1 produced dose-dependent contraction of aorta from wild-type mice that was increased twofold following acute inhibition of all NOS isoforms with *N*(G)-nitro-L-arginine (L-NNA). In eNOS-deficient mice, contractions to ET-1 were increased twofold compared to wild-type mice. L-NNA had no effect. Although contraction of the aorta to thromboxane mimetic U46619 was increased at lower concentrations, maximal contractions to U46619 were not increased following acute inhibition of wild-type or in eNOS-deficient mice. These studies provide direct evidence that vasoconstriction to ET-1 and thromboxane is augmented in the face of eNOS deficiency, demonstrating that eNOS normally inhibits vascular contractile responses [75]. These evidence may partially explain the proatherogenic role of ET-1. Recently, there is an increasing interest in NO-based therapies for pulmonary hypertension [92]. Finally, with increasing use of microarray-based technologies [103], novel genes and molecular approaches in this field will be identified and developed.

NO and oxidation-sensitive mechanisms

An extensive exploration of these oxidation-dependent mechanisms was reviewed elsewhere [99,66,101,36]. Atherogenic lipids, particularly oxidized LDL (oxLDL), are responsible for a wide range of cellular dysfunctions within the vessel wall [97]. OxLDL plays a pivotal role in human early atherogenesis [97,147,96,98]. Concerning the regulation of vascular tone, oxLDL may disturb cellular relaxation functions or act directly against vasodilating substances [130,99]. Low doses of oxLDL inhibit Gi protein function and higher doses inhibit Gq protein function, suggesting a direct interaction with NO at higher concentration [49,27]. Pritchard et al. [117] first described that native and oxLDL can uncouple eNOS, a finding which was confirmed by Vergnani [141]. OxLDL may also induce a decreased uptake of L-arginine [141]. The local depletion of the L-arginine substrate may derange the eNOS, leading to overproduction of superoxide radical from oxygen, the co-substrate of eNOS. Interestingly, physiological differences can affect arterial seg-

ments from different regions. For example, oxLDL impairs contraction and endothelium-dependent relaxation in carotid but not in basilar artery [104], suggesting that intracranial arteries may be relatively protected from atherosclerosis via endothelial resistance to oxidative injury. The relative protection of intracranial arteries was also confirmed in humans [107,34]. Interestingly, glyoxidized LDL downregulates eNOS in human coronary cells [102]. A multitude of oxidation-sensitive apoptotic signaling effects can interact with NO in the arterial wall [101,36]. Thus, the balance between NO bioactivity and oxidative stress plays an important role in the development of atherogenesis. This concept demonstrates that L-arginine hypothesis and the increased oxidative stress may actually fit together and are not two concepts which exclude each other. A new class of aspirins to which a NO-releasing moiety has been chemically bound [67,100] also exhibited antioxidant and antiatherogenic properties [95]. Indeed, we provided in such study the first evidence that NO-releasing aspirin can promote a direct reduction of atherosclerosis lesion progression. Moreover, a NO-releasing statin derivatives exerted antiproliferative and antiinflammatory properties [111]. Antioxidants present in the vascular wall decrease the release of ROS and improve the biologic activity of NO [99,24]. Vitamin C has been demonstrated to potentiate NO activity and restore vascular function in patients with CHD and associated risk factors, including hypercholesterolemia, hyperhomocysteinemia, hypertension, diabetes, and smoking [51,148]. More studies are needed in order to address the role of NO in the very early development of atherosclerosis in humans [96,112]. A lot of effects mediated by NO can affect also the efficacy of statin treatment and other anti-atherosclerotic agents on the natural history of atherosclerotic-related diseases [93,94]. Indeed, wide-ranging clinical and basic science investigations have now indicated that statins may provide beneficial effects outside reductions in LDL and triglycerides. These cholesterol-independent actions have been found to downregulate vascular inflammation and promote cardioprotection against CHD and chronic heart failure. Mechanisms of this vasculoprotection include increases in eNOS activity and a subsequent rise in NO bioavailability in the arterial wall [93,94].

NO and inflammation: implications for atherosclerosis

Progression of inflammatory conditions depends not only upon the recruitment and activation of inflammatory cells, but also on their subsequent removal from the inflammatory milieu. Programmed cell death is a fundamental process regulating inflammatory cell survival and is critically involved in ensuring the successful resolution of an inflammatory response [91]. Apoptosis results in shutdown of secretory pathways and renders effete, but potentially highly histotoxic, cells instantly recognizable for non-inflammatory clearance by phagocytes (e.g., macrophages). However, dysregulation of apoptosis and phagocytic clearance mechanisms can have drastic consequences for development and

resolution of inflammatory processes. Recruitment of inflammatory cells, particularly monocytes and macrophages, is the major driving force behind plaque growth and development. It is well established that apoptotic cells, particularly macrophages, are present in atherosclerotic plaques in both human and animal models of the disease. Apoptotic macrophages and VSMCs have been identified by TUNEL staining in sections from human plaques by various authors [13,61]. Because apoptotic cells are ingested by phagocytes without initiating any further proinflammatory response, it has been suggested that apoptosis may represent a mechanism to regress the plaque. NO is a particularly promising candidate for this strategy, because it has several other powerful anti-atherogenic characteristics including a powerful inhibitory effect on platelet and inflammatory cell activation [87,4]. Evidence is emerging in support of this hypothesis. For example, administration of L-arginine to hypercholesterolemic rabbits increases the number of apoptotic macrophages in intimal lesions by threefold. This increase in apoptosis was associated with a regression of the plaque, suggesting that manipulation of the NOS pathway may well represent a therapeutic approach to resolving the inflammatory response in the vessel wall [144,91]. Eicosanoids [namely PGE(2), PGI(2), LTB(4), and PGJ(2)] are involved in NO-induced apoptosis of VSMCs [115,132,53]. Moreover, NO can also affect mitochondrial function through its interaction with components of the electron-transport chain resulting in a physiological regulator of cell respiration, but also to augment the generation of reactive oxygen species by mitochondria, and thereby trigger mechanisms of cell survival or death [88]. Mitochondrial biogenesis is triggered by NO through activation of guanylate cyclase and generation of cyclic GMP, and yields formation of functionally active mitochondria. Thus, the concurrent action of NO at its two intracellular receptors, Cytochrome *c* oxidase and guanylate cyclase, can play a causal role in coupling energy generation with energy demand. In a very recent study, calorie restriction for either 3 or 12 months induced eNOS expression and 3',5'-cyclic guanosine monophosphate formation in various tissues of male mice [108]. This was accompanied by mitochondrial biogenesis, with increased oxygen consumption and adenosine triphosphate production, and an enhanced expression of sirtuin 1. These effects were strongly attenuated in eNOS null-mutant mice. Thus, NO plays a fundamental role in the processes induced by calorie restriction and may be also involved in prolonged lifespan in mammals. However, exceeding rates of vascular apoptosis in the arterial wall and loss of cells from the atherosclerotic fibrous cap during the latter stages of atheroma may well be detrimental, destabilizing the plaque and promoting rupture [73].

Peroxisome proliferator-activated receptors and the NO pathway

Peroxisome proliferator-activated receptors (PPARs) are a subfamily of the nuclear receptor family of transcription factors regulating the expression of some genes involved in

the regulation of metabolism, inflammation, and thrombosis [8]. Transcriptional control involves ligand activation followed by either heterodimerization with a retinoid X receptor and binding to the promoter region of target genes, or a DNA-binding independent mechanism that interferes negatively with proinflammatory signals. PPAR- α is expressed chiefly in fatty acid-oxidizing tissues including liver, skeletal muscle, and heart, but also in endothelial and VSMCs and macrophages within the arterial wall. Recently, Goya et al. [58] have shown that specific PPAR- α agonists, such as fenofibrate, regulate eNOS in endothelial cells. Fenofibrate was shown to increase the mRNA expression, protein level, and enzyme activity of eNOS in a dose-dependent manner and eNOS promoter sequence does not possess a PPAR response element showing that fenofibrate did not enhance eNOS promoter activity. However, in mRNA stability assays, fenofibrate increased the half-life of eNOS mRNA. The observation that PPAR- α agonists stabilize mRNA levels is unexpected and raises the question of whether these effects occur via PPAR- α . Further studies employing gene knock-down technology and/or in vivo analysis on PPAR- α -deficient mice are required to address this point.

PPAR- α is classically involved in the systemic regulation of lipid and lipoprotein metabolism by, for example, regulating the expression of genes controlling fatty acid β -oxidation (e.g., carnitine palmitoyl transferase-1), intravascular triglyceride lipolysis (e.g., lipoprotein lipase, apo C-III, and apo A-V), and high-density lipoprotein metabolism (e.g., apo A-I, apo A-II, and ABCA-1). PPAR- α activation can also have direct antiatherogenic effects on the different cell types of the vascular wall by decreasing the expression of adhesion molecules, tissue factor, interleukin-6 (IL-6), and endothelin-1 [69]. PPAR- α activation decreases cellular inflammation by inhibiting signaling pathways such as AP-1 complex and nuclear factor- κ B (NF- κ B), which decrease vascular oxidative stress.

Molecular mechanisms regulating eNOS

The molecular regulation of eNOS involves both genomic and non-genomic pathophysiological mechanisms. Physiological shear stress increases the abundance of eNOS [48,37], while lipoproteins (LDL), angiotensin-II, and tumor necrosis factor α decrease eNOS bioactivity by decreasing mRNA stability. The eNOS promoter gene possesses consensus sequences that are potential binding sites for transcription/nuclear factors such as AP-1 complex, NF- κ B, and IL-6 [8,36]. Hence, the suppression of inflammatory signaling pathways by PPAR- α activation provides an additional mechanism whereby fenofibrate could influence eNOS bioactivity; this concurs with the finding in the present report that the eNOS gene does not possess a PPAR response element [58].

Post-translational covalent modification of eNOS is also fundamentally important in the homeostasis of NO, chiefly by regulating the subcellular localization of the enzyme [66]. Acylation targets the localization of eNOS to plasmalemmal

caveolae, a site where the enzyme activity is inhibited through association with caveolin. In response to activation by acetylcholine of several G-protein-coupled cell surface receptors, increase in cytosolic $[Ca^{2+}]_i$ induces the allosteric binding of calmodulin to eNOS; the enzyme then dissociates from caveolin and starts to generate NO. This reaction is then acutely terminated by a deacylation/reacylation cycle of eNOS and the reassociation of the enzyme with caveolin within caveolae. Thus, PPAR- α agonists can directly influence covalent modifications of eNOS that acutely regulate its activity is still unknown. Endothelial dysfunction is predictive of clinical cardiovascular events [99,142]. There is a growing recognition that endothelial dysfunction also contributes to the later stages of the disease when patients develop clinical symptoms. Cross-sectional studies have demonstrated the most severe impairment of endothelial function in arteries containing a culprit lesion that precipitates unstable angina or myocardial infarction [110,16]. Studies in patients with type 2 diabetes and with dyslipidemia have consistently demonstrated that fibrates can improve endothelial function [45,116,21]. Correction of endothelial dysfunction may partly explain the benefit of fibrates in clinical trials [39,121], in which favorable effects were only partially correlated with changes in plasma lipids. But exactly how is the mechanism by which fibrates improve the biology of NO? One explanation relates to the inhibition of vascular inflammation that could cascade into a decrease in the oxidative catabolism of NO to peroxynitrite, improvement in the release of NO by several receptor G-protein signaling pathways, and enhancement of eNOS mRNA. Additional mechanisms might involve reduction in the endothelial cell release of endothelin-1 [38] due to a direct genomic effect or negative feedback from increased release of NO [18]. Non-genomic mechanisms that increase endothelial NO production [28] may explain clinical data from clinical studies indicating that PPAR- γ ligands improve endothelial function in human subjects [145].

eNOS is a tightly coupled enzyme system that may be easily dysregulated by perturbations in availability of substrates (e.g., L-arginine) [32,37] and cofactors (e.g., tetrahydrobiopterin) [71] as well as by competitive inhibitors such as ADMA [78]. Uncoupling of eNOS also results in increased endothelial production of superoxide and the conversion of NO to peroxynitrite, a powerful pro-oxidant that reduces eNOS bioactivity. Recoupling of eNOS with L-arginine, tetrahydrobiopterin, and antioxidant supplements are therapeutical approaches for augmenting the favorable effects of PPAR- α on eNOS. Other pharmacotherapies, such as statins [76,93], ACE inhibitors [35, 105] angiotensin-II receptor blockers [70], and calcium channel blockers [6] can regulate eNOS activity by both genomic and non-genomic mechanisms [94].

Conclusions

NO is centrally involved in cell adhesion, thrombosis, and atherosclerosis. The vasculoprotective roles of NO

include regulation of blood pressure and vascular tone, inhibition of platelet aggregation and leukocyte adhesion, and prevention of VSMC proliferation. Reduced bioavailability of NO is thought to be one of the central factors of vascular diseases, although it is unclear whether this is a cause of, or result of, endothelial dysfunction. Disturbances in NO bioavailability lead to a loss of the vasculoprotective actions and in some cases may even increase disease progression. Consequently, elucidating the mechanisms whereby NO influences atherosclerosis will directly impact on our understanding of the advantages and disadvantages of NO-based therapies. An exciting aspect of this emerging area of study is that NO research field has merged to identify a novel and clinically relevant molecular process. An emerging area suggests a mixed scenario involving eNOS and PPAR α . Pharmacological modulation of NO may restore endothelial dysfunction, inhibit oxidation-sensitive mechanisms, and reduce atherosclerosis in several clinical correlates to experimental models. However, appropriate treatment of vascular inflammation (e.g., by inhibition of iNOS induction, inhibition of iNOS activity, scavenging peroxynitrite, and other RNS) should be further explored for chemoprevention of human atherosclerosis and unstable atherosclerotic plaque. Finally, eNOS gene polymorphism might be an additional risk factor that contributes to predict atherosclerosis and many cardiovascular events. However, further studies are needed to better understand the relevance and the possible therapeutic implications of these investigations.

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