

Recent Advances in Understanding Endothelial Dysfunction in Atherosclerosis

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Over the last two decades, it has become evident that decreased bioavailability of endothelial nitric oxide (NO) produced from endothelial NO synthase (eNOS), referred to as endothelial dysfunction, plays a crucial role in the development and progression of atherosclerosis. Much progress has been made in understanding the mechanisms of decreased endothelial NO bioavailability at the levels of regulation of eNOS gene expression, eNOS enzymatic activity and NO inactivation. Initial studies suggest that increasing eNOS gene expression would improve endothelial NO release in the hope of inhibiting the progression of atherosclerosis. Recent experimental studies, however, do not always support this therapeutic concept and show some evidence that overexpression of eNOS in atherosclerosis may be even harmful for the disease progression. Thus, recent research to improve endothelial function in atherosclerosis has focused on regulation of eNOS enzymatic activity and prevention of NO inactivation by oxidative stress. Since the role of oxidative stress in endothelial NO bioavailability has been reviewed in a large number of comprehensive articles, this article focuses on the relevant regulatory mechanisms of eNOS enzymatic activity that are emerging to play a role in endothelial dysfunction in atherosclerosis.

Keywords: Arginase; Atherosclerosis; BH4; eNOS; L-arginine; Oxidative stress

The endothelium-derived nitric oxide (NO) is synthesized from the substrate L-arginine via endothelial NO synthase (eNOS) and plays a crucial role in regulating a wide spectrum of functions in the cardiovascular system, including vasorelaxation, inhibition of leukocyte-endothelial adhesion, vascular smooth muscle cell (SMC) migration and proliferation, as well as platelet aggregation.¹ Physical or biochemical injury to the endothelium impairs production and/or function of endothelium-derived vasoprotective mediators of vascular health, such as NO, resulting in increased vascular contractions to vasoconstrictors such as endothelin-1, thromboxanes and serotonin,^{2,3} enhanced thrombus formation and exacerbated SMC proliferation and migration.¹ It is, therefore, not surprising that loss of endothelial NO function is associated with several cardiovascular disorders, including atherosclerosis, which is due either to decreased production or to increased degradation of NO.⁴ Experimental and clinical studies provide evidence that defects of endothelial NO function, referred to as endothelial dysfunction, is not only associated with all major cardiovascular

risk factors, such as hyperlipidemia, diabetes, hypertension, smoking and severity of atherosclerosis, but also has a profound predictive value for the future atherosclerotic disease progression.⁵⁻⁹ Therefore, the dysfunctional eNOS/NO pathway is considered as an early marker or a common mechanism for various cardiovascular disorders.

Although the underlying mechanisms of endothelial eNOS/NO dysfunction in atherosclerosis have been intensively studied and various mechanisms responsible for decreased endothelial NO bioactivity under the disease condition have been suggested, no single mechanism can fully explain the endothelial dysfunction. This may simply be due to the fact that atherosclerosis is a complex disease procedure and that multiple regulatory mechanisms are involved in endothelial NO bioactivity, particularly at the eNOS enzymatic level. Pathophysiologically, endothelial NO bioactivity is simply determined by the balance between synthesis and degradation of the molecule. Biochemically, the endothelial NO production is regulated at three different

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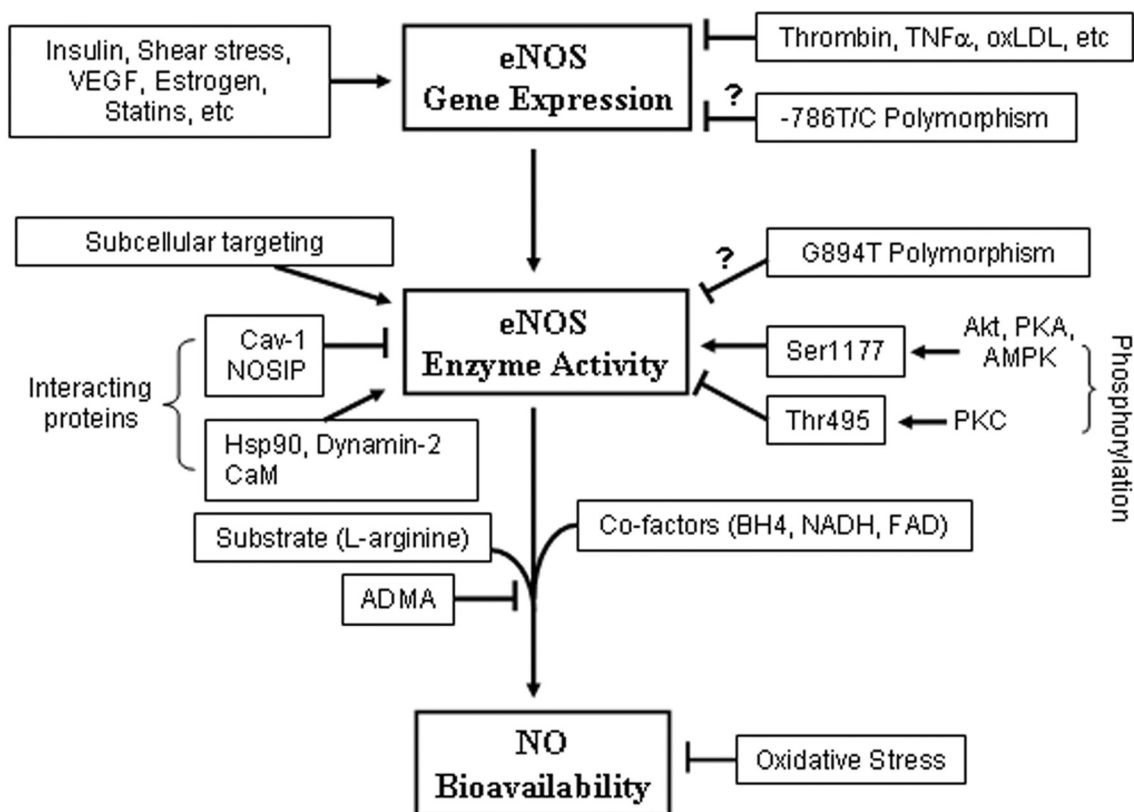


Figure 1. Regulatory mechanisms of endothelial NO production at three different levels. While shear stress, insulin, VEGF, estrogen, and statins increase eNOS gene expression, several factors that are involved in atherosclerotic disease, such as thrombin, TNF- α and Ox-LDL, suppress eNOS gene expression. Whether the -786T/C substitution in the promoter region reduces eNOS gene expression is not clear. Subcellular targeting of eNOS regulated by co- or post-translational modifications, such as N-myristoylation and cysteine, palmitoylation is critical for optimal NO production. The mechanism by which localization influences eNOS activation is not fully understood. Interaction of eNOS with caveolin-1 (Cav-1) and NOSIP proteins reduces eNOS enzymatic activity, and interaction with heat shock protein 90 (Hsp90) and dynamin-2 increases eNOS enzymatic activity. Increase in intracellular Ca²⁺ concentration forms the Ca²⁺-calmodulin (CaM) complex, which also interacts with eNOS and stimulates eNOS enzymatic activity. Whether the eNOS Glu298Asp variant (G894T polymorphism) affects eNOS enzymatic activity remains obscure. Phosphorylation of human eNOS at serine 1177 (Ser1177) by protein kinases Akt, PKA or AMPK enhances, whereas phosphorylation of the enzyme at threonine 495 (Thr495) by PKC inhibits eNOS enzymatic activity. The eNOS enzymatic activity is also dependent on the availability of co-factors, such as BH₄, NADH, FAD, and substrate L-arginine. Increase in production of endogenous ADMA reduces NO production. Increase in oxidative stress inactivates NO resulting in decreased NO bioavailability.

levels: 1) eNOS gene expression, 2) eNOS enzymatic activity and 3) degradation of NO (figure 1). Dysfunction of any of these mechanisms can cause endothelial dysfunction. This review article discusses the current knowledge of endothelial dysfunction in atherosclerosis, focusing on the mechanisms of deregulation of eNOS gene expression and enzymatic activity and also discusses future therapeutic perspectives to improve endothelial function in atherosclerosis. The detailed mechanisms of endothelial NO degradation by oxidative stress are comprehensively reviewed elsewhere.^{10,11}

eNOS GENE EXPRESSION IN ATHEROSCLEROSIS

eNOS Gene Polymorphisms and Atherosclerosis

Human eNOS is encoded by a 26-exon gene on chromosome 7.¹² Several clinical genetic studies identified different common eNOS gene polymorphisms that might be associated with atherosclerotic coronary artery disease. Among the identified eNOS polymorphisms, the G894T substitution within exon 7 resulting in the conversion of glutamate to

aspartate at position 298 (Glu298Asp variant) is the one most frequently studied and has been reported to be associated with atherosclerotic coronary artery disease in two independent cohorts recruited from the Cambridge Heart AntiOxidant Studies (CHAOS-I and CHAOS-II).¹³ The Glu298Asp variant was also reported to be associated with an enhanced systemic pressor response to phenylephrine in a French cohort,¹⁴ a lower basal blood flow but a preserved response to adenosine in coronary arteries in Caucasians of German descent¹⁵ and vasospastic angina pectoris in a Japanese population,¹⁶ as well as with essential hypertension resistant to conventional antihypertensive therapy.¹⁷ While these studies showed association of the Glu298Asp variant with atherosclerotic coronary artery disease, other studies do not support these findings. No association of the eNOS variant with presence or severity of coronary artery disease or with myocardial infarction has been reported in several studies.¹⁸⁻²¹ No adverse effect of the Glu298Asp variant on survival was found in patients with ischemic heart failure.²² The reason for the

controversy among the studies remains unclear. It could be due to selection of patient populations from different ethnic groups^{23,24} or different interactions of the genetic background with the environmental factors (i.e., cardiovascular risk factors present in the specific patient populations).²⁵ One of the considerable limitations of these population studies is the lack of information on whether this polymorphism is functional, that is, whether the Glu298Asp variant is associated with reduced NO synthesis. It has been postulated that the Glu298Asp variant is associated with an increased susceptibility to proteolytic cleavage,²⁶ but other studies suggest that this could have resulted from an artifact of experimental sample preparation.^{27,28} It is important to note that in 104 Caucasians undergoing coronary bypass graft surgery, the endothelial function as assessed by endothelium-dependent relaxations to various agonists in saphenous vein segments is strongly negatively associated with increased atherosclerotic risk factor profiles, and the presence of Glu298Asp variant exerts no additional effect.²⁹ Similar results on the function of the Glu298Asp variant was also reported in Caucasian hypertensive patients by Rossi et al.³⁰ Their study demonstrated that the Glu298Asp polymorphism does not affect the forearm blood flow responses to acetylcholine.³⁰ These results of functional analysis suggest that the Glu298Asp polymorphism does not have a major direct functional effect on eNOS activity in atherosclerosis and may simply be an indirect genetic marker associated with the disease.

Other eNOS polymorphisms that are present in the eNOS promoter region and speculated to influence mRNA transcription and reduce eNOS gene expression are also described. The T to C substitution in the promoter region (-786T/C) has been reported to be able to influence transcriptional activity *in vitro* and found to associate with coronary artery vasospasm in a Japanese population.³¹ Most recently, Cattaruzza and colleagues³² reported that the -786T/C genotype, which results in the exchange of a cytosine for a thymidine at position -786, is associated with loss of eNOS mRNA and protein expression in cultured endothelial cells in response to shear stress, with reduced endothelium-dependent relaxations in saphenous veins obtained at surgery from carriers of the gene and with a higher frequency of coronary artery disease as assessed by quantitative angiography. Unfortunately, eNOS protein level in blood vessels from patients carrying the -786T/C polymorphism was not analyzed in this study. The question whether the -786T/C polymorphism affects eNOS expression *in vivo* remains unanswered. Similar to the Glu298Asp variant, inconsistent associations of the -786T/C polymorphism with endothelial functional measures and with clinical endpoints, such as myocardial infarction and cardiac death, were also shown.^{30,32} A meta-analysis of 26 studies involving 23,028 subjects showed only marginal increased risk of ischemic heart disease in individuals homozygous to Glu298Asp variant, and no significant association was found with the -786C allele.³³ It seems that eNOS polymorphisms might represent only an indirect genetic marker for atherosclerosis. It remains possible that an assessment of the

eNOS polymorphisms integrated into cardiovascular risk factors might increase the predictive value for clinical outcomes of coronary artery disease. It has also been suggested that very large scale genetic association studies of endothelial function or clinical outcomes are required, as are molecular studies, to obtain significant power to detect a statistical significance in such population studies.

eNOS Protein Expression in Atherosclerosis

In contrast to the association studies of eNOS gene polymorphisms, much more is known about eNOS mRNA and/or protein expression in atherosclerosis, particularly in animal models. Past studies provide substantial *in vitro* evidence that the protein expression level of eNOS can be altered by a number of hormonal substances or therapeutic drugs (figure 1).³⁴⁻³⁹ Although *in vitro* experiments demonstrate that a variety of atherogenic stimuli or mediators, such as oxidized low density lipoprotein (LDL), tumor necrosis factor (TNF)- α , thrombin and serum from patients with severe heart failure are able to suppress eNOS gene expression in cultured endothelial cells,³⁴⁻³⁹ there is not much information available for eNOS gene expression *in vivo* in human atherosclerosis. In 1998, Oemar and colleagues⁴⁰ reported that arteries with advanced atherosclerotic plaques have intact endothelial coverage and that eNOS protein is not detectable in endothelial cells over the advanced atherosclerotic lesions as demonstrated by immunohistochemistry. One limitation of the study is that diseased carotid arterial specimens were compared with the atherosclerosis-resistant internal thoracic arteries.⁴¹ Studies with human aortic and coronary arterial tissues obtained from autopsy or from transplant donors found a significant decrease in eNOS gene expression in endothelial cells overlying advanced atherosclerotic lesions, but not in those of early atherosclerotic samples.^{42,43} In line with this report, most studies in atherosclerotic animal models demonstrate unchanged or even augmented expression of eNOS in atherosclerotic arteries, despite the presence of endothelial dysfunction.⁴⁴⁻⁴⁷ A most recent study with human coronary atherectomy specimens showed a higher eNOS gene expression in patients with acute coronary syndromes than those with stable angina.⁴⁸ These results suggest that endothelial dysfunction in atherosclerosis, at least at the early disease stage, is not attributable to a decrease in eNOS gene expression.

Although various studies have shown that inhibition of eNOS either by pharmacological inhibitor or by eNOS gene knockout on the apolipoprotein E (ApoE^{-/-}) background promotes atherogenesis,⁴⁹⁻⁵² and eNOS gene transfer showed improvement of endothelial function and inhibition or regression of atherosclerotic lesions in animal models,^{53,54} controversial results are, however, reported in ApoE^{-/-} mice which overexpress the eNOS gene (eNOS transgenic mice). In this mouse model, acceleration of atherosclerotic lesion formation was observed.⁵⁵ This study contrasts with the results by van Haperen et al,⁵⁶ who showed a reduction of atherosclerotic lesions using the same experimental approach

(i.e., in ApoE^{-/-} mice overexpressing eNOS gene). The controversy between the two studies is not clear. Nevertheless, one can assert that endothelial dysfunction in atherosclerosis is not primarily caused by decreased eNOS gene expression. Substantial evidence implicates that dysfunctional eNOS enzymatic activity and/or increased oxidative stress play predominant roles in atherosclerotic endothelial dysfunction. It is emerging that under certain conditions, eNOS can become pro-atherogenic, likely through production of reactive oxygen species – a so called “uncoupling of eNOS” (discussed in detail below). Accordingly, in animal models of atherosclerosis, removal of endothelial cells or infusion of eNOS inhibitor, not only prevented NO formation, but also reduced superoxide anion production.^{57,58} This hypothesis is supported by a recent study by Shi et al⁵⁹ who showed that eNOS-deficient mice fed a high fat and high cholesterol diet developed much smaller aortic lesions than did wild-type control mice. It remains, therefore, a challenge to understand the mechanisms of deregulation of eNOS enzymatic activity in atherosclerosis.

eNOS ENZYMATIC DYSFUNCTION IN ATHEROSCLEROSIS

The enzyme activity of eNOS is affected by multiple factors (figure 1), including 1) the Glu298Asp variant that is speculated to influence eNOS enzymatic activity (discussed above), 2) post-translational modification associated with subcellular localization, 3) interacting proteins, such as caveolin-1 (Cav-1) and heat shock protein 90 (Hsp90), 4) co-factors, such as flavins, NADPH and tetrahydrobiopterin (BH₄), 5) activation through Ca²⁺, 6) phosphorylation/dephosphorylation by protein kinases stimulated by hormonal agonists and 7) availability of substrate L-arginine. In the following section, we summarize and discuss the mechanisms that are emerging to play roles in eNOS enzymatic dysfunction in atherosclerosis.

eNOS-Protein Interaction

Concepts related to the regulation of intracellular signaling by alterations in the localization and association of distinct protein mediators are continually changing. Whereas the activation of eNOS has long been known to be dependent on protein-protein interactions, especially between calmodulin and eNOS, numerous additional eNOS-associated proteins have been identified over the last 5 years. It is now evident that endothelial NO production is not simply dependent on the expression of the eNOS enzyme, but is determined by an eNOS signaling complex that consists of the enzyme and a conglomerate of adaptor proteins, structural proteins, kinases, phosphatases and potentially also motor proteins that affect complex associations and determine intracellular localization.^{60,61}

Caveolin-eNOS Interaction

Biochemical studies provide evidence that eNOS activity can be regulated by its localization in caveolae, the plasmalemmal vesicles in the cells.⁶² eNOS in the caveolae interacts with the

major caveolae coat protein, Cav-1. This interaction tonically inhibits eNOS enzymatic activity.^{63,64} The formation of the Ca²⁺-calmodulin complex in endothelial cells upon stimulation with agonists can disrupt the interaction between eNOS and Cav-1 leading to enhanced eNOS enzymatic activity.⁶⁴ Bucci et al⁶⁵ demonstrated that a chimeric peptide with a cellular internalization sequence fused to Cav-1 scaffolding domain was efficiently incorporated into blood vessels and endothelial cells resulting in selective inhibition of acetylcholine-induced vasodilation and NO production. Consistent with its role in inhibiting eNOS activity, Cav-1 deficient mice demonstrates a higher eNOS activity.^{66,67} Moreover, Cav-1^{-/-} and ApoE^{-/-} double knockout mice, which are defective in hepatic LDL cholesterol clearance and have significantly higher levels of total cholesterol and triglycerides with no change in high density lipoprotein levels as compared with those which are ApoE^{-/-} alone, either fed normal chow or Western high fat diet, have 70% to 80% reduction in atherosclerotic lesion burden despite the pro-atherogenic lipid profile.⁶⁸ This result suggests that an increase in NO production in ApoE^{-/-}/Cav-1^{-/-} double knockout mice may protect against atherogenesis. The possible role of Cav-1/eNOS interaction in atherogenesis is also supported by the observation that serum from hypercholesterolemic patients and LDL upregulate Cav-1 expression without affecting eNOS protein level, augment caveolin-eNOS heterocomplex formation, and thereby attenuate NO production from the endothelial cells.⁶⁹ Recently, an increase in Cav-1 protein level without change in eNOS expression in aortas was found in a type 1 diabetes mouse model, which is coupled with impaired endothelium-dependent relaxations.⁷⁰ This result may implicate a role of increased Cav-1 expression in linking diabetes mellitus and atherosclerosis. Moreover, treatment of endothelial cells or ApoE^{-/-} mice with statins decreases Cav-1 level and promotes eNOS activity.⁷¹⁻⁷³ It remains to be determined, however, whether Cav-1/eNOS interaction indeed plays a role in atherogenesis in humans.

Hsp90-eNOS Interaction

Another intermediate protein that plays crucial roles in eNOS regulation is the 90 kDa heat shock protein (Hsp90).⁷⁴⁻⁷⁶ The interaction between Hsp90 and eNOS occurs in basal conditions and can be further enhanced by a variety of stimuli which trigger NO production.^{74,77} Binding with Hsp90 significantly increases eNOS activity⁷⁴ which is mediated, in part, by the enhancement of calmodulin binding affinity to eNOS^{78,79} and also by facilitation of Ca²⁺/calmodulin to dissociate the interaction between Cav-1 and eNOS, thereby reversing the inhibitory action of Cav-1 on eNOS.⁸⁰ In addition, Hsp90 was found to be crucial in eNOS serine 1179/1177 (bovine/human) phosphorylation. Hsp90 was shown to recruit serine/threonine protein kinase, Akt, to phosphorylate eNOS at serine 1179^{81,82} and thereby increase eNOS activity. There is no direct evidence demonstrating that a change in Hsp90-eNOS interaction is involved in atherogenesis. The only indirect evidence was provided by an *in vitro* study in cultured endothelial cells showing that atorvastatin activates or phosphorylates eNOS on Ser1177, which is dependent on the ability of Hsp90 to recruit Akt in the eNOS complex.⁷³ Whether this effect also contributes to

the statin's beneficial effects on vascular functions remains unknown.

Dynamin-2

Dynamin-2 is another protein which interacts with eNOS and positively regulates eNOS enzymatic activity.^{83,84} The mechanisms and the physiological significance of the regulatory mechanism of eNOS have not been studied, yet.

NOSIP-eNOS Interaction

NOSIP, eNOS-interacting protein, is a recently discovered novel protein with a molecular weight of 34 kDa. It is widely distributed in the cardiovascular system,⁸⁵ the gastrointestinal tract⁸⁶ and the nervous system of the rat,⁸⁷ where it co-localizes with eNOS and/or neuronal NO synthase (nNOS). It interacts with eNOS and also nNOS.⁸⁵ NOSIP displaces eNOS from the cell plasma membrane and relocates the enzyme to intracellular compartments resulting in the reduction of eNOS activity.⁸⁵ The functional role of NOSIP under (physiol)-pathological conditions remains unknown.

eNOS PHOSPHORYLATION

In addition to modulation by protein-protein interaction, multiple signal transduction pathways converge to regulate eNOS by a phosphorylation process. The activation of the enzyme in response to multiple hormonal agonists such as estradiol, bradykinin and vascular endothelial growth factor (VEGF) occurs in association with elevations in cytosolic calcium concentrations.⁸⁸⁻⁹⁰ In contrast, eNOS activation by shear stress, isometric vessel contraction and insulin occurs independently of changes in intracellular calcium levels.⁹¹⁻⁹³ Shear stress-induced enzyme activation is regulated by potassium channels, and it is prevented by tyrosine kinase inhibition indicating that the process also entails tyrosine phosphorylation.⁹⁴⁻⁹⁶ In addition to regulation by calcium and via tyrosine phosphorylation, multiple protein kinases modify eNOS activity through effects on phosphorylation of serine 1177 or 1179 in human or bovine endothelial cells, respectively. The responsible kinases include AMP-activated protein kinase (AMPK), protein kinase C (PKC), cAMP-dependent protein kinase (PKA) and Akt, which is also known as protein kinase B (PKB). Among the kinases, Akt/PKB seems the most well studied enzyme in regulation of eNOS activity.⁶⁰ Factors that activate eNOS through Akt/PKB-mediated phosphorylation of Ser1177 include estradiol,⁹⁷ shear stress,⁹² VEGF,⁹⁸ insulin⁹³ and high density lipoprotein.⁹⁹ In contrast to the activation upon phosphorylation of Ser1177, phosphorylation of the threonine at position 495 yields attenuated eNOS activity.¹⁰⁰ There seems to be evidence of coordinated regulation of eNOS activity by agonists, such as VEGF, which cause both phosphorylation of Ser1177 and dephosphorylation of Thr495.¹⁰¹ Recent work has further demonstrated that PKA signaling leads to eNOS phosphorylation at Ser1179 in bovine endothelial cells and dephosphorylation of Thr495, thereby enhancing enzymatic activity, whereas PKC promotes both the dephosphorylation of Ser1177 and the

phosphorylation at Thr495, resulting in attenuated enzymatic activity.¹⁰¹ Furthermore, the dephosphorylation processes are mediated by phosphatases PP2A and PP1 acting selectively at Ser1177 and Thr495,^{102,103} respectively. Thus, eNOS activity is regulated by a complex combination of protein-protein interactions and signal transduction cascades involving regulation and phosphorylation events.

There is so far only indirect evidence for an involvement of functional changes in Akt/eNOS signaling in atherosclerotic endothelial dysfunction. The defect of Akt/eNOS signaling may play a primary role in endothelial dysfunction in type-2 diabetes mellitus. In aorta from diabetic animals, as well as in type 2 diabetic patients, Akt/eNOS phosphorylation has been shown to be decreased,^{104,105} at least partly via hyperglycemia-induced GlcNAc modification of the enzyme, which may explain the development and progression of diabetes-associated atherosclerosis.¹⁰⁴ Hambrecht et al¹⁰⁶ recently showed a significant increase in eNOS gene and protein level after only 4 weeks of regular physical exercise training, paralleled with a more pronounced eNOS phosphorylation on Ser1177 in patients with coronary artery disease,¹⁰⁶ implicating a possible role of defective Akt/eNOS pathway in atherosclerosis.

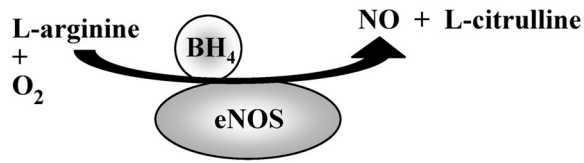
eNOS UNCOUPLING

Recent research reveals a potential role of "eNOS uncoupling" in endothelial dysfunction in atherosclerosis in animal models. In "eNOS uncoupling," electrons flowing from the reductase domain to the heme are diverted to molecular oxygen rather than to the substrate L-arginine, thereby resulting in production of superoxide instead of NO (figure 2).^{107,108} This mechanism also seems important for endothelial dysfunction associated with diabetes mellitus.^{109,110} Several biochemical mechanisms are proposed to be involved in eNOS uncoupling in atherosclerosis, such as BH₄ deficiency, and increase in endogenous asymmetric dimethylarginine (ADMA), L-arginine deficiency and oxidative stress.

BH₄ Deficiency

The co-factor BH₄ deficiency is proposed by most researchers as the mechanism involved in eNOS uncoupling which plays a role in endothelial dysfunction under various pathological conditions, including atherosclerosis.^{111,112} A normal endothelial NO generation by eNOS is dependent on the optimal concentration of the co-factor BH₄, and a suboptimal concentration of BH₄ leads to eNOS uncoupling.¹¹³ It has been demonstrated in clinical and animal studies that acute administration of BH₄ or overexpression of GTPCH-1, the rate-limiting enzyme for BH₄ synthesis, improves endothelial function associated with hypercholesterolemia, atherosclerosis and hyperglycemia.^{112,114-116} There are several studies demonstrating a decreased BH₄ synthesis in the atherosclerotic vascular wall of atherosclerotic rabbits and ApoE^{-/-} mice.^{55,117} Some controversial results have also been reported.¹¹⁸ The mechanism of decreased BH₄ synthesis is unclear. It is suggested by several groups that oxidative stress causes BH₄

Physiological condition



Atherosclerosis

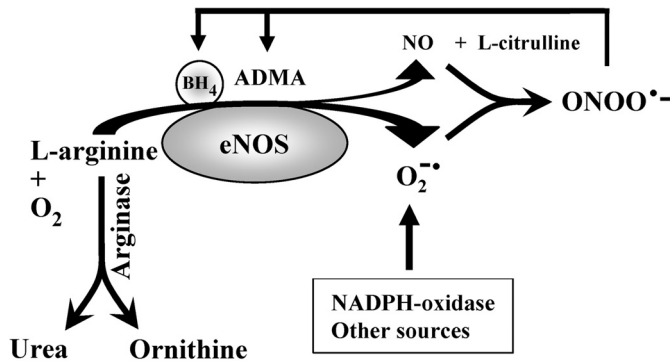


Figure 2. Mechanisms of eNOS uncoupling in atherosclerotic endothelial dysfunction. (A) Under physiological conditions, endothelial cells produce NO from L-arginine in the presence of optimal concentration of the co-factor tetrahydrobiopterin (BH₄). (B) Under pathological conditions, such as atherosclerosis, a decrease in endothelial BH₄ production, an increase in formation of endogenous eNOS inhibitor ADMA and an increase in arginase activity which metabolizes L-arginine into urea and ornithine, causes eNOS uncoupling, a condition that leads to superoxide anion (O₂⁻) production and less NO release. O₂⁻ produced from eNOS uncoupling and other enzymes reacts with NO to generate a more potent oxidant peroxynitrite (ONOO⁻) which further inactivates BH₄ and increases ADMA accumulation in endothelial cells, leading to endothelial dysfunction and may promote atherogenesis.

deficiency by oxidation of BH₄ in the cells.¹¹⁹⁻¹²¹ BH₄ deficiency induces eNOS uncoupling, resulting in the generation of superoxide anions from uncoupled eNOS, which decreases BH₄ levels further – a vicious cycle causing endothelial dysfunction (figure 2). This hypothesis was supported by the study of Alp et al. Either supplementation of BH₄ or overexpression of the BH₄ synthesis rate-limiting enzyme GTPCH-1 in the ApoE^{-/-}/eNOS transgenic mice reduced atherosclerotic lesion formation paralleled with an increase in NO formation and decrease in superoxide anion production.¹¹² In line with this hypothesis, the antioxidant vitamin C has been shown to prevent oxidation and stabilization of BH₄.¹²² Long term vitamin C administration improves endothelial function in a BH₄-dependent manner *in vivo* in mice.¹¹⁸

An Endogenous Inhibitor of eNOS: Asymmetric Dimethylarginine (ADMA)

ADMA is a naturally occurring amino acid resulting from proteolysis of methylated arginine residues in proteins.¹²³ The methylation of arginine is catalyzed by the enzyme protein arginine methyltransferase (PRMT) type I.¹²⁴ No direct route of synthesizing ADMA from free arginine has been identified. ADMA is an endogenous inhibitor of eNOS. It competes with L-arginine to inhibit eNOS for NO production.¹²⁵ Since the first discovery as an endogenous inhibitor by Vallance et al in 1992,¹²⁶ the significant role of ADMA in endothelial dysfunction has been described in various pathological conditions, including atherosclerosis.^{127,128} A high concentration of plasma ADMA has been associated with several risk factors for atherosclerosis and elevated risk for acute coronary events.¹²⁸⁻¹³⁰ A recent study showed that plasma ADMA concentrations in patients who had newly diagnosed acute coronary syndromes are higher than the age-matched healthy control subjects.¹³¹ The amount of ADMA generated within a cell is dependent on the extent of arginine methylation in proteins and the rates of protein turnover. The mechanism that leads to an increase in ADMA plasma concentration in atherosclerosis is not clear. In vascular endothelial cells, type 1 PRMT is expressed and upregulated by LDL.¹³² Ninety percent of ADMA is metabolized by dimethylarginine dimethylaminohydrolase (DDAH).¹³³ The enzyme can be inhibited by nitrosation caused by a potent oxidant peroxynitrate under the high-output NO production from eNOS expression, for example, under the condition of atherosclerosis.¹³⁴ As a consequence, ADMA will be accumulated. A similar hypothesis to BH₄ deficiency that causes eNOS uncoupling has been also proposed for ADMA accumulation: in the presence of high concentrations of ADMA, eNOS produces superoxide instead of NO, which leads to further oxidation of DDAH and further accumulation of ADMA.¹³⁵ This hypothesis warrants further investigation.

Increased arginase activity

L-arginine is the exclusive substrate of eNOS for NO production.⁴ In the early 1990s, several groups demonstrated that acute and chronic supplementation of L-arginine improves endothelial vasodilator responses in cholesterol fed animals and in patients with hypercholesterolemia and atherosclerosis.¹³⁶⁻¹³⁹ L-arginine supplementation therapy as anti-atherosclerotic approach was supported by numerous studies,^{50,140-145} but is recently challenged by increasing the number of other studies showing no such effect or no sustained effect on endothelial function.^{50,145-149} Some studies even showed a harmful effect on atherosclerotic lesion formation¹⁵⁰ in animal models, such as in ApoE^{-/-}/iNOS^{-/-} double knockout mice which are treated chronically with L-arginine supplementation. An increase in production of superoxide anion in atherosclerotic rabbit aortas treated with L-arginine has been recently described.¹⁵¹

The inconsistent results obtained with L-arginine in experimental and clinical studies may be due to the complex biochemical metabolisms of L-arginine.^{152,153} There is

recently increasing evidence suggesting a potential role of arginase in regulation of endothelial NO production by competing with eNOS for the substrate L-arginine, whereby L-arginine is metabolized to urea and L-ornithine by arginase.¹⁵² There are two types of mammalian arginase, arginase I and II, encoded by different genes.¹⁵⁴ Arginase I is located in the cytoplasm, expressed most abundantly in the liver, whereas arginase II is a mitochondrial enzyme, expressed primarily in extrahepatic tissues.¹⁵⁵ The primary function of arginase I is thought to be involved in ammonia detoxification, whereas that of arginase II, in biosynthesis of polyamines and the amino acids, ornithine, proline and glutamate,¹⁵⁶ plays a primary role of regulation of endothelial NO production.^{47,157} Interestingly, expression and activity of arginase II were found to be increased in human diabetic corpus cavernosum and inhibition of the enzyme enhances NO-dependent relaxation of corpus cavernosum smooth muscle^{158,159} suggesting a potential role of arginase II in negative regulation of NO production in diabetic erectile dysfunction. Most recently, an increase in arginase II expression or activity has been implicated in endothelial dysfunction associated with pulmonary hypertension,¹⁶⁰ aging,^{161,162} ischemia-reperfusion-induced endothelial dysfunction,¹⁶³ aortic coarctation hypertension,¹⁶⁴ salt-induced hypertension¹⁶⁵ and atherosclerosis.⁴⁷

In contrast to the observation in rats and humans in which L-arginine evokes vascular relaxation by producing NO, it causes vasoconstriction in the mouse aorta.⁴⁷ Of particular interest and importance is that the contraction induced by L-arginine is much more pronounced in atherosclerotic ApoE^{-/-} mice compared with control animals, which is converted to a greater relaxation by arginase inhibitors in atherosclerotic ApoE^{-/-} mice than wild-type animals, demonstrating a dominant role of increased arginase activity in atherosclerotic endothelial dysfunction.⁴⁷ The greater relaxation induced by L-arginine under the inhibition of arginase in atherosclerotic aortas is paralleled with the higher eNOS expression in the atherosclerotic aortas. The results further support the concept that endothelial dysfunction in atherosclerosis is mainly due to decreased NO bioavailability rather than eNOS gene expression as discussed above.⁴⁶ The simple administration of L-arginine alone as anti-atherosclerotic therapy may therefore not be suitable for some individuals and could even be harmful under certain conditions. Future research designed to target arginase specifically in the vasculature may provide a novel therapeutic approach to treat atherosclerosis and perhaps other cardiovascular disorders.

There is little information on regulatory mechanisms of arginase gene expression or activity in endothelial cells under disease conditions, including atherosclerosis. Arginase II enzymatic activity can be upregulated by thrombin and inflammatory cytokines that are important for atherothrombosis.^{47,157} Our study⁴⁷ provided the first evidence, suggesting a role of RhoA/ROCK pathway in upregulation of arginase activity in human endothelial cells.

In line with the results obtained from cultured cells, the increase in arginase II activity, but not protein expression, in atherosclerotic blood vessels of ApoE^{-/-} mice is associated with increased RhoA protein level,⁴⁷ suggesting that increased enzymatic activity of arginase II plays a predominant role in atherosclerotic endothelial dysfunction. This regulatory model of arginase II in human endothelial cells is further supported by the study of Bachetti and colleagues¹⁵⁷ who showed that arginase activity, but not gene expression, in the cells was increased after 24-hour stimulation with an inflammatory cytokine mixture.¹⁵⁷ The exact regulatory mechanisms of arginase activity in the cells and in atherosclerotic blood vessels remain elusive. Whether increased arginase activity contributes to eNOS uncoupling also remains an interesting topic for future research.

OXIDATIVE STRESS AND NO BIOACTIVITY IN ATHEROSCLEROSIS

There is considerable evidence showing the importance of oxidative stress in the pathogenesis of atherosclerosis. This aspect has been comprehensively reviewed in several articles.^{10,11} In this article we would like to point out that in atherosclerotic blood vessels there is an increase in enzyme expression that produces superoxide, such as the subcomponents of NADPH oxidase.¹⁶⁶⁻¹⁶⁸ It is emerging that besides several sources that generate superoxide (e.g., mitochondria, NADPH oxidase, xanthine oxidase, cytochrome p450-type enzymes, cyclooxygenase and lipoxygenase), eNOS seems to be an important source of superoxide generation via eNOS uncoupling mechanisms in the absence of substrate L-arginine or co-factors BH₄ or in the presence of endogenous inhibitor ADMA as discussed above. It is also noteworthy to point out that an increase in superoxide generation not only quenches NO, but also enforces eNOS uncoupling, a vicious cycle which further enhances oxidative stress, resulting in further endothelial dysfunction (figure 2). Superoxide generation via eNOS uncoupling is emerging to play an important role in endothelial dysfunction in cardiovascular disorders, including atherosclerosis.¹¹¹ Future research focusing on eNOS uncoupling would provide an interesting aspect for understanding the pathophysiology of endothelial dysfunction and also therapeutic strategy for atherosclerosis.

CONCLUSIONS

Endothelial dysfunction plays an important role in the pathogenesis of atherosclerotic coronary artery disease. Many mechanisms have been proposed to be responsible for endothelial dysfunction in atherosclerosis. Clinical genomic studies showed inconsistent results on the association of eNOS gene polymorphisms with endothelial dysfunction or clinical outcomes in patients with coronary artery disease. Meta-analysis of clinical studies suggests that eNOS polymorphisms are not a major risk but rather an indirect genetic marker for atherosclerosis. It is still possible that the eNOS polymorphisms could have more predictive value in certain ethnic populations with specified cardiovascular risk

factor profiles. Substantial insight into mechanisms of endothelial dysfunction in atherosclerosis is provided by experimental studies in animal models and by clinical pathophysiological and pharmacological studies *ex vivo* and *in vivo*. However, one should be aware of species differences in response to pharmacological interventions, for example, L-arginine supplementation, when conclusions are based on the experiments conducted in different animal models. Results from different animal models may explain some controversial findings among different studies. Extrapolation of the results obtained from animal models to humans should be cautious. Experiments with *ex vivo* specimens from animal models, particularly from humans could provide the best information for translational medicine research. It is now becoming apparent that atherosclerotic endothelial dysfunction, particularly at the early disease stages is primarily caused by deregulation of eNOS enzymatic activity and inactivation of NO through oxidative stress rather than eNOS gene down-regulation. Besides various enzymes that produce oxidative stress, eNOS uncoupling appears to be an important mechanism contributing to increased oxidative stress in atherosclerosis. eNOS uncoupling can be induced by decreased production of co-factor BH₄ or relative deficiency of the substrate L-arginine due either to increased arginase activity or accumulation of the endogenous eNOS inhibitor ADMA. It also seems to play an important role in endothelial dysfunction in atherosclerosis. Studies on regulation of eNOS enzymatic activity and maintenance of eNOS in the “coupled” state or on therapeutic approach for “re-coupling” of eNOS in atherosclerosis would be interesting areas for future research.

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