Nitric Oxide Synthase in Atherosclerosis and Vascular Injury
Insights From Experimental Gene Therapy
Keith M. Channon, HuSheng Qian, Samuel E. George

Abstract—Gene therapy aims to intervene in a disease process by transfer and expression of specific genes in a target tissue or organ. Cardiovascular gene therapy in humans remains in its infancy, but in the last decade, experimental gene transfer has emerged as a powerful biological tool to investigate the function of specific genes in vascular disease pathobiology. Nitric oxide synthases, the enzymes that produce nitric oxide, have received considerable attention as potential candidates for vascular gene therapy because nitric oxide has pleiotropic antiatherogenic actions in the vessel wall, and abnormalities in nitric oxide biology are apparent very early in the atherogenic process. In this article, we review the use of nitric oxide synthases in experimental vascular gene therapy and assess the utility of these approaches for investigating the role of nitric oxide in atherosclerosis and their potential for human gene therapy. (Arterioscler Thromb Vasc Biol. 2000;20:1873–1881.)

Key Words: nitric oxide • atherosclerosis • gene transfer • endothelium • adenovirus

NO and Atherogenesis

NO Synthases
Nitric oxide (NO) is produced by NO synthases (NOS), which oxidize L-arginine to L-citrulline (reviewed in References 1 and 2). All 3 NOS isoforms have a similar molecular structure and require multiple cofactors, including flavins, NADPH, and tetrahydrobiopterin, that are required to maintain dimerization and NO production1,2 (the Figure). Neuronal (nNOS, or NOS I) and endothelial (eNOS, or NOS III) isoforms are constitutively expressed and are activated by calcium-calmodulin. The inducible isoforms (iNOS, or NOS II) are regulated primarily at the transcriptional level, independent of agonist stimulation and intracellular calcium levels.3,4 eNOS, expressed in endothelial cells, is the predominant NOS isoform in the vessel wall. Under basal conditions, eNOS is inactive and remains membrane bound by virtue of myristoylation, palmitoylation, and an inhibitory interaction with caveolin, the principal structural protein in caveolae. Receptor-mediated agonist stimulation (eg, bradykinin, substance P) leads to rapid enzyme activation by depalmitoylation, binding of calcium-calmodulin, displacement of caveolin, and release from the plasma membrane.5,6 Shear stress is an important physiological stimulator of eNOS activity, causing rapid membrane release and activation by Akt-dependent serine phosphorylation7,8 and upregulating eNOS gene expression by transcriptional activation of the eNOS promoter.9 After vessel injury or in disease states, iNOS expression may be induced in the media.10 atherosclerotic plaque,11 or neointima.4,12 In normal blood vessels, nNOS is present in neurons in the adventitia and may be expressed by medial smooth muscle cells (SMCs) under pathophysiological conditions. Recent data also suggest that nNOS and its splice variant μNOS may be expressed at low levels in the media and adventitia.13

Functions of Vascular NO
NO produced in the endothelium rapidly diffuses to interact with molecular targets in cells in the vascular wall and lumen.14 NO interacts with thiol groups and with metal centers in diverse protein targets, including membrane receptors, G proteins, ion channels, cytosolic enzymes, and transcription factors such as activator protein-1 and nuclear factor-κB (reviewed in Reference 15). S-Nitrosylation of thiol groups in plasma proteins such as albumin generates a circulating “pool” of NO-donating groups,16 whereas S-nitrosylation of hemoglobin in the lung provides nitrosothiol groups to the peripheral vasculature and regulates oxygen delivery.17,18

In the vascular wall, NO activates soluble guanylate cyclase in vascular smooth muscle cells (VSMCs), leading to elevation of cGMP, activation of cGMP-dependent protein kinase (PKG), and vasorelaxation, the primary basis for blood flow and pressure regulation.19 In addition to regulating vascular tone, a substantial body of evidence suggests that NO has important antiatherogenic effects20,21: (1) antiplatelet effects. NO inhibits platelet adhesion and aggregation22 and thrombin-induced expression of platelet-activating factor. (2) antiproliferative effects. NO donors potently inhibit VSMC proliferation,23,24 migration,25 and extracellular matrix synthesis.26 (3) anti-inflammatory effects. Atherogenic stimuli stimulate endothelial expression of adhesion molecules and chemokines, leading to inflammatory cell recruitment.27
NOS Dysregulation in Atherosclerosis

In addition to variations in eNOS protein levels and the interaction of NO with superoxide, regulation of the eNOS enzyme appears to be abnormal in vascular disease states, resulting in reduced NO production despite the presence of NOS protein. Furthermore, eNOS may generate superoxide when endothelial cells are subjected to stimuli such as hyperglycemia or oxidized LDL particles. This “dysregulation” of eNOS may result from several potential mechanisms.

The eNOS–caveolin 1 interaction maintains eNOS in an inactive, membrane-bound state, and normal aging. Increased superoxide production, principally by NAD(P)H oxidases and xanthine oxidase, accounts for a significant proportion of the NO deficit in several models of vascular disease, including hypercholesterolemia, atherosclerosis, hypertension, and heart failure. Correction of hypercholesterolemia reduces superoxide production and restores endothelium-dependent vasorelaxation. Antioxidants and superoxide dismutase (SOD) produce similar effects, further supporting the hypothesis that superoxide plays a role in the observed NO deficits.

NOS gene therapy aims to increase or restore vascular NO production in these NO deficiency states. NOS protein is absent or reduced in the endothelium overlying severe atherosclerotic lesions in arteries or diseased venous bypass grafts, providing an immediate rationale for gene transfer to restore NO levels in diseased endothelium or when the endothelium is lost, eg, after balloon injury. Atherogenic stimuli such as oxidized LDL may also reduce eNOS levels by inhibiting eNOS gene expression. However, in other vascular disease states in which NO bioactivity is reduced, endothelial NOS protein levels appear to be maintained or even increased, suggesting that greater complexity underlies the observed reductions in NO bioactivity. Some potential mechanisms are discussed below.

Interactions of NO With Superoxide

Increased superoxide production may account for a significant proportion of the NO deficit in atherosclerotic vessels. Superoxide reacts rapidly with NO, thereby reducing NO bioactivity and producing peroxynitrite, a strong oxidant that can have physiologically protective effects but that at high levels causes tissue damage by nitrosylation of cellular proteins and lipids. In addition, superoxide stimulates mitogenesis in VSMCs and activates other redox-sensitive signaling pathways. Increased superoxide production, primarily by NAD(P)H oxidases and xanthine oxidase, accounts for a significant proportion of the NO deficit in several models of vascular disease, including hypercholesterolemia, atherosclerosis, hypertension, and heart failure. Correction of hypercholesterolemia reduces superoxide production and restores endothelium-dependent vasorelaxation. Antioxidants and superoxide dismutase (SOD) produce similar effects, further supporting the hypothesis that superoxide plays a role in the observed NO deficits.

Rationale for NOS Gene Therapy in Vascular Disease

The observation of deficient NO-mediated vasorelaxation in hypercholesterolemia suggests that loss of NO bioactivity is an early feature of atherosclerosis. In animal models, treatment with NOS inhibitors potentiates neointimal proliferation, whereas supplemental L-arginine or NO aducts improve endothelial function and limit neointimal proliferation. Mice with targeted deletion of the eNOS gene are hypertensive and respond to vascular injury with increased intimal hyperplasia and abnormal remodeling. NO also appears to play a significant role in human atherogenesis. Atherosclerosis is associated with reduced endothelial NO production, and in patients with coronary artery disease, even angiographically normal coronary segments show paradoxical constriction to acetylcholine. Endothelial function is also deficient in preatherosclerotic conditions such as hypercholesterolemia, diabetes mellitus, hypertension, smoking, and normal aging. Treatment of hypercholesterolemia, dietary supplementation with L-arginine, or antioxidant therapy improves the functional deficit. These studies suggest that loss of normal NO bioavailability precedes progression to more advanced lesions.

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activation by calcium-calmodulin is impaired, possibly due to increased caveolin expression, increased levels of caveolin-eNOS inhibitory complexes, or disruption of normal caveolar lipid composition. In contrast to its inhibitory interaction with caveolin, activated eNOS in the cytosol is stabilized by interaction with heat shock protein 90, which tends to increase eNOS activity in response to calcium-mobilizing agonists and to shear stress–induced phosphorylation. Whether these or other eNOS regulatory mechanisms are also deranged in atherosclerosis remains to be investigated.

Tetrahydrobiopterin is a required cofactor for NOS activity: all 3 NOS isoforms require tetrahydrobiopterin for NOS homodimerization and for electron transfer during arginine oxidation. Production of NO by endothelial cells requires adequate tetrahydrobiopterin, and NOS synthesized in the absence of tetrahydrobiopterin is inactive for NO generation; exogenous tetrahydrobiopterin restores enzyme activity. More importantly, tetrahydrobiopterin-deficient NOS generates superoxide rather than NO. Recent data suggest that tetrahydrobiopterin levels are lower in the diabetic rat aorta and underlie the observed reduction in NO production. Exogenous tetrahydrobiopterin partially restores endothelial NO bioactivity and reduces NOS-dependent superoxide production in hypertension, hypercholesterolemia, and smokers, whereas inhibition of tetrahydrobiopterin synthesis impairs NO-mediated vasorelaxation. Thus, tetrahydrobiopterin may be an important factor in modulating NOS activity in vascular disease states.

Arginine availability and the relative concentration of endogenous NOS inhibitors such as asymmetric dimethylarginine can limit NO production, despite the presence of normal or high levels of NOS enzyme. High asymmetric dimethylarginine levels are associated with impaired NO-mediated vasorelaxations in hypercholesterolemia, provide a rationale for the beneficial effects of arginine supplementation in vascular disease models, and may have significant effects on NO production in vascular disease.

These and other factors causing NOS dysregulation have important implications for NOS gene therapy, because simply augmenting NOS protein levels under these conditions may not increase NO bioactivity and could increase superoxide production, resulting in detrimental rather than beneficial effects. Experimental studies of NOS gene therapy need to determine whether recombinant NOS activity is accompanied by increased superoxide production and peroxynitrite formation and to establish the optimal level of NOS expression to achieve an appropriate increase in NO production without potentially detrimental effects.

**Biological Effects of NOS Vascular Gene Transfer**

Gene transfer vectors allow targeted expression of genes in cells in the vessel wall, provide tools to investigate the function of specific genes in disease processes, and may potentially lead to gene therapy strategies for vascular disease. For NOS gene transfer, adenovirus vectors have been most widely used, with a smaller number of studies using high-efficiency plasmid-liposome gene delivery and incorporating coat proteins from hemagglutinating virus of Japan. Whereas adenovirus results in the highest efficiency of gene transfer, liposome-mediated gene transfer is a virus-free system with little host immune response and potentially longer transgene expression. Because NO diffuses from NOS-targeted cells to surrounding cells (a "bystander" effect), very high-efficiency transduction may not be an absolute requirement to exert a biological effect. Experimental studies of NOS gene transfer initially evaluated the expression and activity of recombinant NOS isoforms in cultured cells, then in isolated vascular rings after ex vivo infection and culture, and finally in several in vivo models of experimental gene therapy (the Table).

**Vasomotor Function**

The ability to restore endothelial vasomotor function in dysfunctional arteries represents a critically important test of NOS gene transfer. Ex vivo eNOS gene transfer to isolated vascular rings from normal or hypercholesterolemic rabbit carotid arteries or aorta, canine basilar arteries, porcine coronary arteries, or human saphenous veins improves vascular relaxations to a variety of vasoactive agonists, such as calcium ionophore, acetylcholine, or UTP. In vivo gene delivery of eNOS or nNOS confirms that NOS gene transfer can restore or augment regulated NO-mediated vasorelaxation in several settings, including normal, atherosclerotic, balloon-injured, or eNOS-deficient arteries. Moreover, eNOS gene transfer reduces the vasoconstrictive response to hypoxia in rat lungs and restores NO-mediated endothelial function in models of cardiac failure, hypertension, or diabetes, demonstrating that the functional effects of NOS gene transfer are not limited to pharmacological agonist stimulation ex vivo, nor to specific models of endothelial dysfunction such as hypercholesterolemia. Activation of recombinant NOS by shear stress, a major activator of eNOS in the normal vessel, has not been specifically studied after in vivo vascular gene transfer. However, hemodynamic data from the mouse lung after in vivo eNOS gene transfer suggest that recombinant eNOS is activated by increasing pulmonary blood flow, and it reduces pulmonary vascular resistance. Taken together, these studies provide important landmarks in vascular gene transfer. They demonstrate that NOS gene transfer can be used to restore or augment NOS activity in the vessel wall and can modify physiologically regulated vascular function in both normal arteries and those in a variety of pathological states.

**Intimal Hyperplasia**

NO donors or compounds that increase intracellular cGMP inhibit VSMC proliferation, VSMC migration, and collagen synthesis, all key components of intimal hyperplasia. Exogenous NO-donating compounds such as molsidomine or L-arginine also reduce intimal hyperplasia in vascular injury models in vivo. These results suggest that endothelial NO may limit VSMC proliferation and neointimal formation in vivo. Gene transfer studies provide direct evidence that local augmentation of vascular NO production by NOS isoforms inhibits intimal hyperplasia in several models of vascular injury: balloon-injured rat or pig or rabbit or canine venous bypass grafts, and rat aortic allografts. This effect seems most likely due to direct inhibition of VSMC proliferation, as evidenced by bromodeoxyuridine or proliferating cell nuclear antigen staining.
and mediated by modulation of VSMC cell phenotype or cell cycle inhibition. In addition, NOS gene transfer inhibits VSMC migration and matrix remodeling. Alternative mechanisms include increased VSMC apoptosis, reduced inflammation or platelet activation, or promotion of endothelial regrowth. These gene transfer studies strongly suggest a direct role for NO in modulating VSMC biology in the vessel wall, in response to disease states or vascular injury.

Regression of Atherosclerosis
Local gene transfer of NOS provides a powerful tool to address the importance and mechanisms underlying the “antiatherogenic” actions of NO in the vessel wall. In cholesterol-fed rabbits, nNOS gene transfer reduces endothelial adhesion molecule expression, restores endothelium-dependent vasomotor relaxation toward normal, and rapidly reduces T-lymphocyte and monocyte infiltration. These findings indicate an important role for NO in regulating endothelial cell activation and inflammatory cell infiltration in early atherosclerosis. The striking rapid changes in monocyte/macrophage infiltration suggest that foam cell formation may be a highly dynamic process in early experimental atherosclerosis. In particular, an important mediator of the antiatherogenic effect of NOS gene transfer in vivo appears to be downregulation of vascular monocyte chemotactic protein-1 expression, as previously demonstrated in cultured endothelial cells after eNOS gene delivery.

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Ang II indicates angiotensin II; CSF, cerebrospinal fluid; Ad, adenovirus; and HVJ, hemagglutinating virus of Japan liposomes.
vascular disease. All have been shown to increase vascular NO production, and all may have equivalent promise for vascular gene transfer. However, functional differences between the isoforms may affect NOS regulation after gene transfer, and further studies need to address these potential differences to make a rational choice for NOS gene therapy.

Many investigators have chosen eNOS as a candidate for vascular gene transfer on the basis that eNOS is the predominant isoform present in the normal vessel wall. eNOS gene transfer increases vascular NO production in normal and diseased arteries, whether directed at the endothelial surface or at adventitial fibroblasts. In balloon-injured arteries, eNOS is expressed in medial SMCs and increases basal cGMP levels. Thus, cells other than endothelial cells are able to support eNOS activity and therefore, represent targets for vascular NOS gene therapy. In adventitial fibroblasts, recombinant eNOS appears to be localized in caveolae and is regulated by membrane-Golgi trafficking in response to bradykinin stimulation. This approach may be an alternative to luminal delivery when the endothelium is disrupted or diseased and also provides novel routes for vascular gene delivery, eg, to the adventitia of cerebral arteries via the cerebrospinal fluid. Adventitial eNOS expression augments ex vivo vasomotor responses stimulated by pharmacological agonists in the absence of an endothelium and also increases basal cGMP levels in vivo, even though adventitial fibroblasts are not exposed to shear stress. Nevertheless, future studies need to determine how adventitial eNOS is activated in blood vessels in vivo and whether adventitial eNOS can modify vascular injury or disease states in a similar manner to luminal eNOS gene delivery.

Increasing evidence suggests that iNOS expression is increased in medial SMCs, in the neointima after vascular injury, or in allografts after transplantation, suggesting that iNOS expression might limit the thrombotic and proliferative response in injured vessels. If so, iNOS may be particularly suited to gene transfer in vascular injury states. In vivo studies in balloon-injured arteries and in arterial allografts show that iNOS gene transfer generates high-level NO production, independent of agonist stimulation or shear stress. Sustained NO production, with the higher \( V_{\text{max}} \) of the iNOS enzyme, may be an advantage over other isoforms, but concern remains that continuous high activity could also increase superoxide production, leading to peroxynitrite formation.

nNOS is abundant in neurons and skeletal muscle and appears to be expressed in medial SMCs and in the adventitia under normal and pathological conditions. Like eNOS, nNOS is expressed constitutively and regulated by calcium-calmodulin, but it has a higher \( V_{\text{max}} \) for NO production. We have used nNOS as a candidate for vascular gene transfer and have shown that nNOS acts as a functional surrogate for eNOS in normal or atherosclerotic arteries and in vein grafts and that it is activated by agonists such as bradykinin and acetylcholine in endothelial cells and SMCs. In endothelial cells, eNOS is predominantly membrane bound, whereas recombinant nNOS is cytosolic (D. McDonald and K. Channon, unpublished observations, 1999), and it remains to be established whether nNOS in endothelial cells is regulated by mechanisms such as subcellular trafficking, interactions with caveolin and other proteins such as heat shock protein 90, and shear stress–induced activation by Akt-dependent phosphorylation, as described for eNOS. Differential regulation of eNOS and nNOS in target cells in the vascular wall may provide a rationale for choosing one isoform over another for a particular application.

Future work needs to identify the relative advantages of different NOS isoforms for gene therapy in specific vascular diseases or in different target cell types; it is also possible that mutant, chimeric, or otherwise-modified NOS isoforms could offer novel approaches to increasing NO production under conditions of NOS dysregulation.

**Adjuvant Strategies in NOS Gene Transfer**

The complex mechanisms underlying reduced NO bioactivity in atherosclerosis suggest that the challenge of restoring the vascular NO system lies beyond simply increasing NOS protein in the vessel wall. Intervening in the mechanisms that limit NO production or bioactivity may be alternatives or adjuncts to NOS gene transfer.

Tetrahydrobiopterin is an important modulator of vascular NOS activity and superoxide production. In vitro, the NOS enzyme is strictly dependent on tetrahydrobiopterin for activity and produces superoxide rather than NO when tetrahydrobiopterin levels are inadequate. The activity of recombinant NOS after gene transfer may be limited by the availability of tetrahydrobiopterin in cell culture, and some data suggest that tetrahydrobiopterin levels may be limiting in vascular disease in vivo. However, NOS gene transfer elevates vascular NO production in vivo without tetrahydrobiopterin supplementation, suggesting that tetrahydrobiop terin is not a limiting factor in target cells in the vessel wall. Nevertheless, increasing vascular tetrahydrobiopterin availability, eg, by gene transfer of GTP–cytohodrolase I, may represent a powerful complementary strategy to NOS gene therapy in vascular diseases.

The SOD enzymes are a rational approach to increase NO levels. By scavenging superoxide, SOD activity is necessary for NO release from endothelial cells and subsequent interaction with molecular targets. The SODs not only remove superoxide but also by generating hydrogen peroxide (\( \text{H}_2\text{O}_2 \)) may also contribute to NO actions, eg, by activating guanylate cyclase in VSMCs. Adenoviral gene transfer of any of the 3 SOD enzymes (manganese SOD [Mn SOD], copper–zinc SOD [CuZn SOD], or extracellular SOD [EC SOD]) reduces superoxide release from endothelial cells or VSMCs and can reduce superoxide-induced LDL oxidation by endothelial cells. In the diabetic rabbit aorta, preliminary data suggested that Mn SOD gene transfer is as effective as SOD gene transfer in restoring NO-mediated vasorelaxations, but in a more detailed study, neither CuZn SOD nor EC SOD gene transfer to hypercholesterolemic rabbit aortas was sufficient to augment NO-mediated vasorelaxations, despite a reduction in vascular superoxide release. Similarly, ex vivo SOD gene transfer to diabetic rabbit carotid arteries or angiotensin II–infused rabbit aortas, in which vascular superoxide production is increased, did not enhance NO-mediated vasorelaxation, whereas eNOS gene transfer was effective. However, SOD gene transfer and superoxide reduction were most marked at
the endothelium, so that SOD gene transfer to deeper layers of the media may be inadequate to affect NO bioactivity. Nevertheless, gene transfer of SOD in combination with NOS may provide an approach to simultaneously reduce superoxide release, limit peroxynitrite formation, and potentiate NO bioactivity in vascular disease states.

Gene transfer of NO’s molecular targets is an alternative strategy to increase NO effects. Guanylate cyclase is the principal NO target in VSMCs, leading to cGMP generation and activation of PKG. Adenovirus-mediated gene transfer of both subunits of guanylate cyclase increases cGMP production in VSMCs and reduces the proliferative response to balloon injury. Similarly, PKG gene transfer increases the sensitivity of VSMCs to the antiproliferative and proapoptotic effects of NO, suggesting that these targets may be synergistic with NOS gene transfer in increasing NO-mediated effects.

Summary

Gene therapy for vascular disease remains a future possibility rather than a clinical reality. Nevertheless, the data reviewed here suggest that genetic modification of the vessel wall to increase NOS activity represents a potential approach to clinical problems such as restenosis after balloon angioplasty, venous and arterial bypass grafts, and possibly to high-risk atherosclerotic conditions or hypertension. Despite encouraging progress in providing proof of concept, however, many important questions remain to be answered to clarify the potential utility of NOS gene therapy in vascular disease. These include identifying the mechanisms of NOS dysregulation in atherosclerosis and assessing the relative importance of superoxide-NO interactions, tetrahydrobiopterin availability, and NO targets in modulating NO bioactivity. Finally, improved gene transfer vectors need to provide longer-term gene expression in vivo, without the confounding and toxic effects of vector-induced inflammation.

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