Neuronal Nitric Oxide Synthase as a Novel Anti-Atherogenic Factor

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Nitric oxide (NO) has multiple important actions that contribute to the maintenance of vascular homeostasis. NO is synthesized by three different isoforms of NO synthase (NOS), all of which have been reported to be expressed in human atherosclerotic vascular lesions. Although the regulatory roles of endothelial NOS (eNOS) and inducible NOS (iNOS) on the development of atherosclerosis have been described, little is known about the role of neuronal NOS (nNOS). Recent studies have demonstrated that nNOS also exerts important vasculoprotective effects in vivo. In a carotid artery ligation model, nNOS-knockout mice exhibited accelerated neointimal formation and constrictive vascular remodeling caused by blood flow disruption. In a rat balloon injury model, the selective inhibition of nNOS activity potently enhanced vasoconstrictor responses to a variety of calcium-mobilizing stimuli, and exacerbated neointimal formation. Moreover, in apolipoprotein E-knockout mice, deficiency of nNOS induced progression of aortic vascular lesion formation. In these models, nNOS was up-regulated in vascular lesions, and was predominantly expressed in the neointima and medial smooth muscle cells. These results provide the first direct evidence that nNOS plays important roles in suppressing arteriosclerotic vascular lesion formation. Thus, nNOS could be regarded as a novel anti-atherogenic factor. J Atheroscler Thromb, 2004; 11: 41–48.

Key Words: Arteriosclerosis, Atherosclerosis, Neointimal formation, Vascular remodeling

Introduction

Nitric oxide (NO), synthesized by three different isoforms of NO synthase (NOS), plays an important role in maintaining vascular integrity and homeostasis (1–5). Endothelial and neuronal nitric oxide synthase (eNOS and nNOS, respectively) are constitutively expressed mainly in endothelial cells and nitrergic nerves, respectively, synthesizing a small amount of nitric oxide (NO) in a calcium-dependent manner under basal conditions as well as upon stimulation by agonists (1–5). By contrast, inducible NOS (iNOS) is expressed when stimulated by inflammatory stimuli such as microbial endotoxins and certain proinflammatory cytokines, producing a large amount of NO in a calcium-independent manner (1–5).

Accumulating evidence has indicated a vasculoprotective role of eNOS against the development of arteriosclerosis (1–4). This notion is supported by the fact that eNOS-knockout (eNOS-KO) mice exhibit accelerated neointimal formation (6, 7) and abnormal vascular remodeling (8), and that eNOS gene overexpression in transgenic mice (9) or by gene transfer (10) suppresses vascular lesion formation.

The role of iNOS in arteriosclerosis seems to be complicated. Deletion of the iNOS gene exacerbated vascular remodeling in a carotid artery ligation model (6) and in a cardiac transplant model (11) in mice; however, it conversely ameliorated neointimal thickening in a mouse...
model with carotid cuff placement (12) and lipid-rich vascular lesion formation in apolipoprotein E-knockout mice (13). This discrepancy may be explained in part by the oxidant and antioxidant properties of iNOS (14), because NOSs produce superoxide anions with resultant generation of a potent oxidant peroxynitrite rather than NO under certain conditions, such as deficiency of a substrate (e.g., L-arginine) or a cofactor (e.g., tetrahydrobiopterin) (15, 16).

nNOS can also be detected in the neointima, endothelial cells and macrophages in both early and advanced atherosclerotic lesions in humans (17). Although more than 10 years have passed since the first report on the existence of nNOS (18, 19), the role of nNOS expressed in such vascular lesions remains to be determined. We have recently demonstrated for the first time that nNOS is up-regulated in vascular lesions following vascular injury, and exerts important vasculoprotective effects in vivo (20). This review briefly summarizes current knowledge of the importance of nNOS expressed in vascular wall cells.

Expression and function of nNOS in normal blood vessels

The conventionally accepted notion of the distribution of NOS isoforms in normal blood vessels is as follows: the endothelium expresses eNOS; vascular smooth muscle cells do not express NOSs at all; and perivascular nerves in certain, but not all, blood vessels express nNOS. In the endothelium, activation of eNOS can be triggered by a variety of physiological stimuli, including shear stress, pressure, aggregating platelets, bradykinin, and acetylcholine (1–5, 21). eNOS-derived NO diffuses to the underlying vascular smooth muscle cells, and stimulates soluble guanylate cyclase, which in turn induces an increase in intracellular cGMP concentrations and subsequent activation of cGMP-dependent protein kinase (1–5, 21). eNOS-derived NO exerts relaxation of vascular smooth muscle cells, and inhibits their proliferation, platelet aggregation, adherence of blood cells (platelets and white blood cells) to the endothelium, and oxidation of low density lipoprotein (1–5, 21). These effects are all anti-atherogenic. Non-adrenergic and non-cholinergic perivascular nerves (nitrergic nerves) that innervate the adventitia of cerebral and certain peripheral arteries (e.g., mesenteric, renal, and femoral arteries), but not that of coronary arteries, contain nNOS (22–24). In perivascular nitrergic nerves, nNOS-derived NO is liberated as a neurotransmitter by electrical stimulation or nicotine, and causes relaxation of adjacent vascular smooth muscle cells, counterbalancing vasoconstrictions produced by the sympathetic nervous system (22–24). On the other hand, in normal vascular smooth muscle cells, no expression of NOS isoforms was reported in a number of studies (5, 17, 25–27). Thus, under physiological conditions, the vascular expression pattern of NOS isoforms appeared to be cell-specific.

This simple scheme, however, is now under debate. A new and ultrasensitive immunohistochemical amplification technique complemented with electron microscopic immunogold labeling and with Western blot analysis was used to examine the expression of NOS isoforms in vascular smooth muscle cells of porcine and human arteries (28). This powerful method is based on signal amplification with tyramide, which increases antigen detectability more than 1,000-fold (29–32). In the study, normal vascular smooth muscle cells of porcine and human arteries have been demonstrated to express all three NOS isoforms (Table 1), in contrast to the conventionally accepted view (28). The extent of NOSs expression in vascular smooth muscle cells was greater in muscular-type arteries (human coronary and renal arteries and an artery from the human pancreas) and in arterioles than in elastic-type arteries (human aorta and mammary artery).

Even with the use of usual immunohistochemistry, recent studies reported the presence of nNOS, but the absence of eNOS or iNOS, in normal vascular smooth muscle cells of rat carotid (33), coronary (34), and pulmonary (35) arteries, and aorta (36), in those of bovine carotid artery (37), and in cultured human aortic smooth muscle cells (38) (Table 1). Although the inconsistent results concerning NOS isoform expression in normal vascular smooth muscle cells between these studies and other reports might be due to the difference of vasculature or species examined, or the marginal levels of nNOS expression, this point is not clear. In two of these studies, the functional significance of nNOS was suggested by the evidence that a non-selective NOS inhibitor, N^ω-nitro-L-arginine (L-NNA) (39), and a selective nNOS inhibitor, 7-nitroindazole (7-NI) (40), slightly but significantly augmented contractions in response to high extracellular K^+ in endothelium-denuded rat aortas (36) and in bovine carotid arteries (37), respectively. However, another study showed no effect of L-NNA on angiotensin II-induced contractions in endothelium-denuded rat carotid arteries (33). We also found no effect of selective NOS inhibitors, s-methyl-L-thiocitrulline (SMTC) (41) and 7-NI, on contractions to angiotensin II, KCl, or endothelin-1 in normal rat carotid arteries (20). The discrepancy might be explained by the different post-translational control mechanisms, such as the loss of a protein inhibitor of NOS activity, namely PIN (42), CAPON (43) or caveolin (44–46). However, further studies are needed to determine the functional involvement of nNOS in vascular smooth muscle cells under physiological conditions.

Although in normal endothelial cells the functional expression of nNOS has been suggested in human, porcine and rat arteries (28, 47–49) (Table 1), this point may also need to be examined further in future studies.
Expression and function of nNOS in vascular lesions

It has been reported that neither nNOS mRNA nor protein was detected in normal human aortas or carotid arteries, whereas both levels were detected in early and advanced atherosclerotic lesions associated with macrophages, endothelial cells, and mesenchymal-appearing intimal cells (17). Although the functional role of nNOS in such vascular lesions is of biological interest, no study has ever addressed the potential protective roles of nNOS against vascular lesion formation. Thus, we addressed this point in two different animal models of vascular injury.

We first examined the effect of a targeted deletion of nNOS gene on vascular lesion formation in a model of unilateral carotid artery ligation in mice. In the control carotid artery, no pathological finding was noted in either the wild-type or the nNOS-KO mice. By contrast, in the ligated carotid artery, neointimal formation and constrictive vascular remodeling were noted 4 weeks after the procedure in both strains. Importantly, the extent of neointimal formation and that of constrictive vascular remodeling were significantly accelerated in the nNOS-KO mice compared with the wild-type mice. In the early stage of vascular lesion formation (on day 3 and 7 after the carotid artery ligation), nNOS immunoreactivity was first noted in neutrophils, especially at the adventitia, suggesting that nNOS inhibits the development of constrictive vascular remodeling. In the advanced stage of vascular lesion formation (on day 14 and 28 after the procedure), nNOS was expressed in the neointima, medial smooth muscle cells, and endothelial cells, suggesting that nNOS in those cells suppresses neointimal formation. Thus, it is possible that NO derived from nNOS suppresses both neointimal formation and constrictive vascular remodeling in mice in vivo (20) (Fig. 1).

It has been reported that NO derived from eNOS inhibits neointimal formation, whereas NO derived from iNOS suppresses the development of constrictive vascular remodeling in the same carotid artery ligation model using eNOS-KO and iNOS-KO mice (6). Our results thus provide the first evidence that each of the three NOS isoforms have different vasculoprotective actions against vascular lesion formation (20) (Fig. 2).

We next examined whether or not the functional up-regulation of nNOS is involved in a rat balloon injury model. Selective nNOS inhibitors, SMTC and 7-NI, significantly enhanced angiotensin II-induced contractions in the balloon-injured arteries, but not in the control arteries. The angiotensin II-induced contractions of the balloon-injured arteries were inhibited by AT_{1}-receptor antagonist losartan (50) but not by AT_{2}-receptor antagonist PD123319 (50). Stimulation with angiotensin II did not change the vascular cGMP concentrations, a marker of NO production (1), in the control arteries with endothelium, but significantly increased them in the balloon-injured arteries. SMTC and 7-NI significantly reduced the basal and angiotensin II-induced increase in tissue cGMP concentrations in the balloon-injured arteries. nNOS was absent in the control artery, whereas it was noted in neointimal and medial smooth muscle cells in the balloon-injured arteries. These results indicate that nNOS is functionally upregulated in the neointima and media after balloon injury in vivo, exerting an important inhibitory effect on AT_{1}-receptor-mediated vasoconstrictor responses by enhancing the NO/cGMP-mediated pathway (20) (Fig. 1).

Selective inhibition of nNOS activity with SMTC and 7-

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<th>Species</th>
<th>Vessel</th>
<th>Localization</th>
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<tr>
<td>Human</td>
<td>Coronary artery</td>
<td>Smooth muscle and endothelium</td>
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<td></td>
<td>Mammary artery</td>
<td>Smooth muscle and endothelium</td>
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<td>Artery, arteriole, and vein from pancreas</td>
<td>Smooth muscle and endothelium</td>
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<td>Artery from kidney</td>
<td>Smooth muscle and endothelium</td>
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<td>Aorta</td>
<td>Smooth muscle and endothelium</td>
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<td>Aorta</td>
<td>Cultured smooth muscle cells</td>
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<td>Pig</td>
<td>Carotid artery</td>
<td>Smooth muscle and endothelium</td>
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<td>Rat</td>
<td>Carotid artery</td>
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<td>Pulmonary artery</td>
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<td>Basilar artery</td>
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<td>Bovine</td>
<td>Carotid artery</td>
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NI also enhanced the contractions to KCl and endothelin-1 as well as those to angiotensin II in balloon-injured arteries. It is well established that angiotensin II (51), KCl (52), and endothelin-1 (52) all cause a large, sustained increase in intracellular calcium concentrations in vascular smooth muscle cells. Therefore, the increased concentrations of intracellular calcium in vascular smooth muscle cells stimulated by those agonists may result in both vasoconstriction and NO generation through calcium-dependent activation of nNOS with subsequent increase in tissue cGMP concentrations (20) (Fig. 1).

Long-term oral treatment with 7-NI for 14 days significantly exacerbated neointimal formation and reduced the lumen diameter in the rat model. This finding provides additional evidence that nNOS suppresses neointimal formation in the rat model of balloon injury, which partially mimics the nNOS-KO mice phenotype. Under pathological conditions associated with elevated intracellular calcium concentrations in vascular smooth muscle, such as arteriosclerosis or vasospasm, nNOS may serve as an alternative source of NO, inhibiting vascular lesion formation and spastic responses in an autocrine/paracrine manner (20) (Fig. 1).

The vasculoprotective effect of nNOS in apolipoprotein E knockout (ApoE-KO) mice has also been reported in abstract form (53). To test whether deficiency of nNOS gene contributes to atherosclerotic lesion formation, ApoE/nNOS double KO mice were generated. ApoE/nNOS double KO and ApoE-KO mice were fed a “western type” diet for 14 weeks, and the vascular lesion area of aortas were compared between the two strains. nNOS expression was detected by immunohistochemistry in the neointima of ApoE-KO mice, but not in that of ApoE/nNOS double KO mice. Male ApoE/nNOS double KO mice showed a significant increase in the vascular lesion area of aortas as compared with male ApoE-KO mice. Arterial blood pressure, body weight, and plasma cholesterol and lipoprotein levels were unchanged between the two male mice. These results indicate that nNOS may protect the development of atherosclerotic vascular lesion formation driven by chronic hyperlipidemia.

In the vascular endothelium of eNOS-KO mice, the compensatory expression and the functional involvement of calcium concentrations in vascular smooth muscle, such as arteriosclerosis or vasospasm, nNOS may serve as an alternative source of NO, inhibiting vascular lesion formation and spastic responses in an autocrine/paracrine manner (20) (Fig. 1).

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nNOS have been suggested (54–56). The functional involvement of nNOS in endothelial cells under other pathological conditions has not yet been investigated.

**Mechanisms for vascular nNOS induction**

nNOS can no longer be considered a “constitutive” enzyme, because nNOS is induced in arteriosclerotic vascular lesions. Indeed, previous studies have reported up-regulation of nNOS in a variety of diseased blood vessels, including not only in human atherosclerotic aortas and carotid arteries (17), in a mouse ligation model (20), in a rat balloon injury model (20), and in aortic lesions of ApoE-KO mice (53) as mentioned earlier, but also in carotid arteries of spontaneously hypertensive rats (33), in coronary arteries of rats infused with subpressor doses of angiotensin II for 6 days (34), in reproductive arteries (uterine and mammary arteries) of ewes infused with estrogen for 5–6 days (67), in aortic lesions of ApoE/iNOS double KO mice (58), in coronary arterial lesions of mice after long-term treatment with a synthetic L-arginine analogue, L-NAME (59), and with an endogenous L-arginine analogue, asymmetric dimethylarginine (ADMA) (60), as well as in endothelial cells of eNOS-KO mice (55) (Table 2). Thus, nNOS is subject to expressional regulation in the vascular system, while nNOS is constitutively expressed in the nervous system (61–63).

The detailed regulatory mechanisms for up-regulation of nNOS in vascular wall cells remain to be elucidated. Recently, however, we demonstrated that treatment with angiotensin II and interleukin-1β increases nNOS expression at both mRNA and protein levels in cultured rat aortic smooth muscle cells (20, 64). These results suggest that nNOS is subject to expressional regulation by inflammatory stimuli, such as angiotensin II and interleukin-1β, in rat vascular smooth muscle cells. We also examined another possibility that nNOS-containing nerves or neurons surrounding blood vessels might penetrate into vascular lesions. However, we were unable to detect any immunoreactivity of neuron-specific antibodies, such as neurofilament or protein gene product, in vascular lesions (20). In an organ culture experiment using isolated rat carotid arteries, high intraluminal pressure, which mimics the hypertensive situation, has been revealed to enhance vascular nNOS expression, which was impaired by mitogen-activated protein (MAP) kinase cascade inhibitor (extracellular signal-regulated kinase [ERK] 1/2 inhibitor) PD98059. These results show that transmural pressure increases nNOS expression in rat vascular smooth muscle cells by a mechanism involving the MAP kinase pathway (65).

**Conclusions**

Studies aiming to elucidate the role of nNOS expressed in vascular lesions have just been started. It has, so far, become evident that nNOS is functionally up-regulated in vascular lesions under pathological conditions, and exerts important vasculoprotective effects *in vivo*. Moreover, it has been demonstrated that all three NOS isoforms have different vasculoprotective actions against vascular lesion formation. These findings should lead to a better understanding of the vascular NOSs system, and may contribute to the development of new drugs and/or gene therapies for the treatment of vascular disorders. Indeed, the potential usefulness of vascular nNOS gene transfer has recently been reported (66–68). Thus, nNOS could be regarded as a novel anti-atherogenic factor. Upregulation of nNOS may play an important compensatory role in the presence of reduced eNOS activity (e.g., inflammation and arteriosclerosis) to maintain vascular

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**Table 2. Up-regulation of nNOS in vascular smooth muscle cells and in endothelial cells under pathological conditions.**

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<tr>
<th>Species</th>
<th>Vessel Localization</th>
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<tr>
<td>Human</td>
<td>Atherosclerotic aorta, Intima, endothelium, and macrophages (17)</td>
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<td></td>
<td>Atherosclerotic carotid artery, Intima, endothelium, and macrophages (17)</td>
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<tr>
<td>Mouse</td>
<td>Carotid artery of a ligation model, Neointima, media, endothelium, and adventitial inflammatory cells (20)</td>
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<td>Atherosclerotic aorta of ApoE-KO mice, Neointima (53)</td>
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<td>Atherosclerotic aorta of ApoE/iNOS double-KO mice, Neointima (58)</td>
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<td>Coronary lesion of L-NAME-treated mice, Medial smooth muscle (59)</td>
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<td>Coronary lesion of ADMA-treated mice, Medial smooth muscle (60)</td>
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<td>Coronary artery of eNOS-KO mice, Endothelium (55)</td>
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<td>Rat</td>
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<td>Ewe</td>
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<td>Mammary artery of estrogen-infused rats, Medial smooth muscle (57)</td>
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homeostasis in vivo.

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