

Roles of Nitric Oxide Synthases in Arteriosclerotic Vascular Disease: Insights from Murine Genetic Models

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Abstract

Nitric Oxide (NO) exerts a variety of biological actions under both physiological and pathological conditions. NO is synthesized by three distinct NO synthase (NOS) isoforms, encoded by three distinct NOS genes, including neuronal (nNOS), inducible (iNOS), and endothelial NOS (eNOS), all of which are expressed in the human vascular system. Although the roles of the NOSs in arteriosclerotic vascular diseases have been described in pharmacological studies with selective and non-selective NOS inhibitors, the selectivity and specificity of the NOS inhibitors continue to be an issue of debate. To solve this issue, genetically altered animals have been established. All types of NOS gene-deficient animals have been developed, including singly, doubly, and triply NOS-deficient mice and various types of NOS Gene-Transgenic (TG) animals have also been generated, including conditional and non-conditional TG mice bearing site-specific overexpression of each NOS gene. The roles of individual NOS isoforms as well as the entire NOSs system in arteriosclerotic vascular diseases have been extensively investigated in those mice, providing pivotal insights into an understanding of the pathophysiological significance of the NOSs in human arteriosclerotic vascular diseases. The present review, which is based on studies with the murine NOS genetic models, summarizes the latest knowledge about the NOSs and arteriosclerotic vascular diseases.

Keywords: Arteriosclerotic vascular disease; Nitric oxide synthase; Knockout mice; Transgenic mice; Myocardial infarction

Introduction

Nitric oxide (NO) exerts a variety of biological actions, and plays an important role in maintaining vascular homeostasis [1-7]. NO is synthesized by three distinct NO synthase (NOS) isoforms, encoded by three distinct NOS genes: neuronal (nNOS; also known as NOS-1), inducible (iNOS; also known as NOS-2) and endothelial NOS (eNOS; also known as NOS-3).

It was initially indicated that nNOS and eNOS are constitutively expressed mainly in the nervous system and the vascular endothelium, respectively, synthesizing a small amount of NO in a calcium-dependent manner under both basal conditions and upon stimulation, and that iNOS is induced only when stimulated by microbial endotoxins or certain proinflammatory cytokines, producing a greater amount of NO in a calcium-independent manner [6,7]. However, recent studies have revealed that both nNOS and eNOS are subject to expressional regulation and that iNOS is constitutively expressed even under physiological conditions [8-14]. All three NOS isoforms have been reported to be expressed in the vascular system under both physiological and pathological conditions [13,15].

Genetically engineered animals are a powerful experimental tool to study the function of target genes *in vivo*. All types of NOS gene-knockout (KO) animals have been generated, including singly, doubly, and triply NOS-KO mice [16-28] (Table 1). Various types of NOS Gene-Transgenic (TG) animals have also been established, including conditional and non-conditional TG mice with site-specific overexpression of each NOS isoform [29-40] (Table 2). By using those genetically engineered mice, the roles of the NOSs in the pathogenesis of arteriosclerotic vascular diseases have been extensively studied, and the findings provide pivotal insights into the significance of the NOSs in human arteriosclerotic vascular diseases. In the present review, we summarize the current knowledge of the NOSs and arteriosclerotic

vascular diseases, based on research outcomes obtained from the murine NOS genetic models.

Role of eNOS in Arteriosclerotic Vascular Disease

Endothelium-specific eNOS-TG mice with an 8-fold increase in vascular NOS activity showed decreased neointimal formation after carotid artery ligation and another strain of endothelium-specific eNOS-TG mice with a 10-fold increase in vascular NOS activity similarly exhibited a reduction in atherosclerotic vascular lesion formation induced by breeding with apoE-KO mice [38,41]. Consistent with these findings, eNOS-KO mice displayed increased neointimal formation, accelerated medial thickening, and abnormal vascular remodeling in response to carotid artery ligation and cuff placement around the femoral artery [42-44] (Figure 1). Furthermore, eNOS-KO/apoE-KO mice exhibited exacerbated formation of atherosclerotic vascular lesion as compared with apoE-KO mice [45,46]. These lines of evidence indicate a vasculoprotective role of eNOS in vascular lesion formation. On the other hand, there are also reports of inconsistent opposite results that diet-induced atherosclerotic vascular lesion formation by crossbreeding with apoE-KO mice is accelerated in endothelium-specific eNOS-TG mice with an 8-fold increase in

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NOS ^{-/-} Mice	Sites of gene deletion	References
nNOS ^{-/-}	Exon 2 (#1) Exon 6 Exon 6	<i>Cell</i> 1993;75:1273-1286 <i>Endocrinology</i> 2002;143:2767-2774 <i>PNAS</i> 2003;100:9566-9571
Renal collecting duct-specific nNOS ^{-/-}	Exon 6	<i>Hypertension</i> 2013;62:91-98
iNOS ^{-/-}	Proximal 585 bases of promoter plus exons 1-4 (#2) Near exons 1-5 Exons 12 and 13 and a part of exon 11 (#3)	<i>Cell</i> 1995;81:641-650 <i>Nature</i> 1995;375:408-411 <i>PNAS</i> 1995;92:10688-10692
eNOS ^{-/-}	Exons 24-26 (#4) Exon 12 (#5) Exons 24 and 25	<i>Nature</i> 1995;377:239-242 <i>PNAS</i> 1996;93:13176-13181 <i>Circ Res</i> 1998;82:186-194
n/iNOS ^{-/-}	#1 and #3 #1 and #2	<i>Mol Reprod Dev</i> 2003;65:175-179 <i>PNAS</i> 2005;102:10616-10621
n/eNOS ^{-/-}	#1 and #4 #1 and #5 #1 and #4	<i>Cell</i> 1996;87:1015-1023 <i>Mol Reprod Dev</i> 2003;65:175-179 <i>PNAS</i> 2005;102:10616-10621
i/eNOS ^{-/-}	#3 and #5 #2 and #4	<i>Mol Reprod Dev</i> 2003;65:175-179 <i>PNAS</i> 2005;102:10616-10621
n/i/eNOS ^{-/-}	#1, #2 and #4	<i>PNAS</i> 2005;102:10616-10621

NOS, Nitric Oxide Synthase; nNOS, Neuronal NOS; iNOS, Inducible NOS; eNOS, Endothelial NOS; NOS^{-/-}, NOS-Deficient

Table 1: Mice Lacking the NOS Genes That Have Thus Far Been Established.

TG Mice	Overexpression site	Promoter used	References
nNOS-TG	Myocardium (Conditional)	α-MHC	<i>Circ Res</i> 2007;100:e32-e44
	Myocardium (Conditional)	α-MHC	<i>Circulation</i> 2008;117:3187-3198
iNOS-TG	Brain	CaMKIIα	<i>Cell Mol Biol</i> 2005;51:269-277
	Myocardium (Conditional)	α-MHC	<i>J Clin Invest</i> 2002;109:735-743
	Myocardium	α-MHC	<i>Circ Res</i> 2002;90:93-99
	Pancreatic β Cell Retina	insulin rod opsin	<i>J Biol Chem</i> 1998;273:2493-2496 <i>PLoS One</i> 2012;7:e43089
eNOS-TG	Liver	albumin	<i>J Biol Chem</i> 2011;286:34959-34957
	Endothelium	preproendothelin-1	<i>J Clin Invest</i> 1998;109:735-743
	Endothelium	eNOS	<i>J Biol Chem</i> 2002;277:48803-48807
	Myocardium	α-MHC	<i>Circulation</i> 2001;104:3097-3102
	Myocardium	α-MHC	<i>Circ Res</i> 2004;94:1256-1262

CaMKII: Calcium-Calmodulin Multifunctional Kinase II; MHC: Myosin Heavy Chain; TG: Transgenic

Table 2: Mice Overexpressing the NOS Gene That Have Thus Far Been Established.

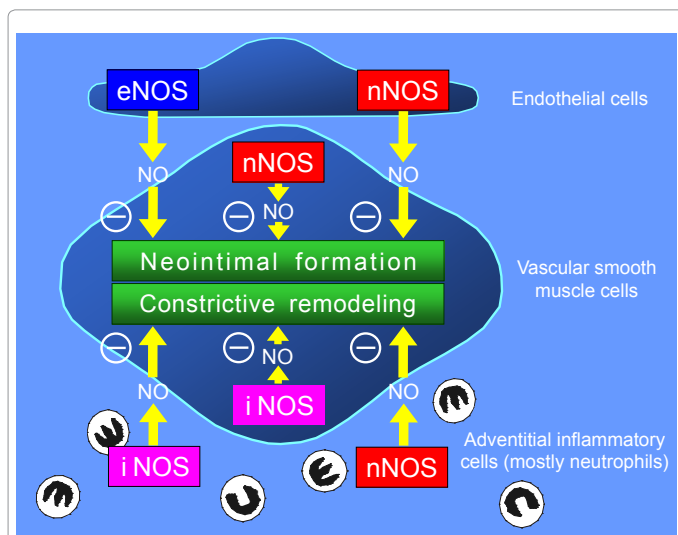


Figure 1: Different vasculoprotective roles of the three NOS isoforms in a mouse carotid artery ligation model. Studies with each NOS isoform^{-/-} mice demonstrated that eNOS inhibits neointimal formation, that iNOS attenuates constrictive vascular remodeling, and that nNOS suppresses both neointimal formation and constrictive vascular remodeling. Thus, individual NOS isoforms have different vasculoprotective actions against vascular lesion formation in mice *in vivo* inhibition [7].

vascular NOS activity and that fatty streak formation is paradoxically reduced in eNOS-KO mice [47,48]. eNOS-derived NO has multiple vasculoprotective effects, including the dilation of blood vessels and the inhibition of vascular smooth muscle cell proliferation, platelet aggregation, leukocyte-endothelial cell adhesion, and Low-Density Lipoprotein (LDL) oxidation, whereas, under certain conditions such as deficiency of a substrate (e.g., L-arginine) or a cofactor (e.g., tetrahydrobiopterin), NOSs produce superoxide anions rather than NO, with resultant production of a potent oxidant peroxynitrite (which phenomenon is referred to as 'NOS uncoupling') [49,50]. Thus, eNOS uncoupling may be partly involved in these discrepant results.

When 12 eNOS-KO/apoE-KO mice were fed on a Western-type diet for 16 weeks, 3 mice developed abdominal aortic aneurysms, and 2 developed aortic dissections (Stanford type B) spontaneously [46]. These results indicate that eNOS deficiency introduces abdominal aortic aneurysms and aortic dissections in the presence of severe hyperlipidemia, suggesting a protective role of eNOS in aortic diseases.

Role of iNOS in Arteriosclerotic Vascular Disease

The role of iNOS in vascular lesion formation seems to be complicated. Deletion of the iNOS gene in mice exacerbated pathological vascular remodeling in a carotid artery ligation model and in a cardiac transplant model; however, it conversely ameliorated neointimal formation in a carotid cuff placement model and lipid-rich

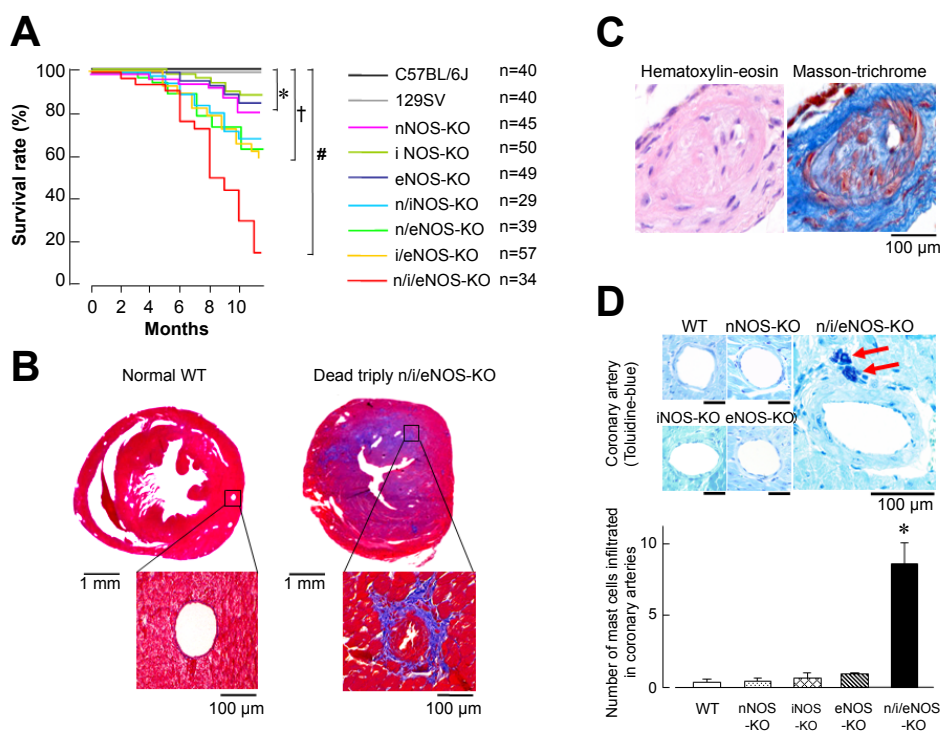


Figure 2: Decreased survival, spontaneous Myocardial Infarction (MI), coronary arteriosclerosis and mast cell infiltration in male triply *n/i/eNOSs*^{-/-} mice. (A) Survival rate (n=29-57). The red line represents markedly reduced survival in the triply *n/i/eNOSs*^{-/-} mice. *, †, and #: P<0.05 between wild-type (WT) C57BL/6J vs. singly, doubly, and triply NOS-KO, respectively. (B) Acute MI and coronary arteriosclerotic lesion formation in a triply *n/i/eNOSs*^{-/-} mouse that died at 8 months of age (Masson-trichrome staining). Blue in the heart cross-section of the dead triply *n/i/eNOSs*^{-/-} mouse indicates antero-septal acute MI. Adjacent coronary artery shows marked luminal narrowing, wall thickening, and perivascular fibrosis (blue). (C) Arteriosclerotic lesion formation in serial sections of the infarct-related coronary artery. (D) Mast cell infiltration in the coronary artery adventitia (toluidine-blue staining) (n=10-33). Red arrows indicate mast cells. *P<0.05 vs. WT [62].

atherosclerotic vascular lesion formation in apoE-KO mice [43,51-53] (Figure 1). Thus, iNOS appears to have two faces. This discrepancy may be explainable in part by the oxidant and antioxidant properties of iNOS in the presence and absence, respectively, of iNOS uncoupling [54].

The extent of elastase-induced abdominal aneurismal dilatation was comparable between male iNOS-KO and wild-type mice, whereas it was greater in female iNOS-KO than in female wild-type mice, which effect was reversed by previous ovariectomy [55]. It is thus likely that iNOS deficiency also leads to the occurrence of abdominal aortic aneurysms induced by elastase solely in the female.

Role of nNOS in Arteriosclerotic Vascular Disease

Expression of nNOS is up-regulated in the neointima, endothelial cells and macrophages in both early and advanced human atherosclerotic lesions [15]. Although the regulatory roles of eNOS and iNOS in vascular lesion formation have been widely studied, little was known about the role of nNOS. We addressed this point in nNOS-KO mice and demonstrated that nNOS gene deficiency caused a worsening of neointimal formation and constrictive vascular remodeling (a reduction in the vascular cross-sectional area) following carotid artery ligation [56] (Figure 1). In agreement with our evidence, nNOS-KO/apoE-KO mice showed accelerated atherosclerotic vascular lesion formation as compared with apoE-KO mice [57]. These results suggest that nNOS also plays a role in suppressing arteriosclerotic/atherosclerotic vascular lesion formation [12]. Up-regulation of nNOS may play a compensatory role in the presence of reduced eNOS activity (e.g. inflammation and

arteriosclerosis) to maintain vascular homeostasis [12]. We revealed that inflammatory and proliferative stimuli (angiotensin II, interleukin-1 β , and platelet-derived growth factor) and a statin increase vascular nNOS expression [10,11]. Hypoxic and hypertensive situations have also been shown to up-regulate vascular nNOS expression [58-60].

Role of the Whole NOSs System in Arteriosclerotic Vascular Disease

Because all NOSs play a role in the vascular system, we conceived a project to investigate the roles of the whole NOSs system *in vivo*. The roles of the NOSs system in the human body have been investigated in pharmacological studies with non-selective NOS inhibitors and in studies with NOS isoform-KO mice. However, because of the non-specificity of the agents and of compensation among NOS isoforms, the authentic roles of the NOSs system were still poorly understood. To address this important issue, we developed mice in which the entire NOSs system is completely disrupted (triply nNOS/iNOS/eNOS-KO mice) [22,61]. The triply *n/i/eNOSs*-KO mice, but not any singly NOS-KO mice, spontaneously developed arteriosclerotic vascular lesions (neointimal formation, medial thickening, and perivascular fibrosis) in the coronary and renal arteries, and lipid-rich atherosclerotic vascular lesions in the aorta, even on a normal chow diet, suggesting a vasculoprotective role of the entire NOSs system in vascular lesion formation [62,63] (Figure 2).

Myocardial Infarction (MI) is the leading cause of death for both genders all over the world [64,65]. The molecular mechanisms for the pathogenesis of MI, however, remain to be fully elucidated. It is well

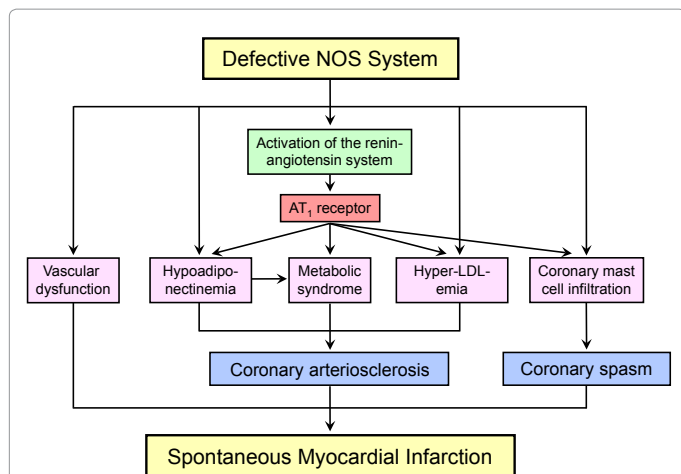


Figure 3: Mechanisms for spontaneous MI caused by the defective NOS system in mice *in vivo*.

Genetic disruption of all NOSs caused metabolic syndrome, hypoadiponectinemia, hyper-low-density-lipoprotein (LDL)-emia, coronary adventitial mast cell infiltration, and vascular dysfunction. Those factors could contribute to the pathogenesis of spontaneous MI. Importantly, long-term pharmacological blockade of the angiotensin II type 1 (AT1) receptor significantly reduced the incidence of MI, along with amelioration of those risk factors. It is therefore possible that the AT1 receptor pathway is involved in its molecular mechanism [62].

Arteriosclerosis was seen in most of the vasculature in the triply NOS-KO mice, whereas atherosclerosis was observed in the aorta alone. MI in humans results not only from coronary atherosclerosis, but also from other causes, including coronary intimal hyperplasia, medial thickening, and coronary vasospasm [64,66]. Marked coronary intimal hyperplasia and medial thickening were noted in our triply *n/i/eNOS*-KO mice that died of MI and, furthermore, marked infiltration of mast cells at the coronary artery adventitia was also observed in those mice [63] (Figure 2). Histamine released from adventitial mast cells is thought to cause coronary vasospasm with resultant MI in humans [67]. It is thus possible that coronary arteriosclerosis and coronary vasospasm are involved in the cause of death in the triply NOS-KO mice (Figure 3).

In our triply *n/i/eNOS*^{-/-} mice, endothelium-dependent relaxations to acetylcholine, which is a physiological eNOS activator, were completely lacking, and contractions to phenylephrine, which is an α_1 adrenergic agonist, were markedly potentiated [63]. These vascular dysfunctions could also be involved in the pathogenesis of MI in the triply NOSs-KO mice (Figure 3). In our triply *n/i/eNOS*^{-/-} mice, metabolic syndrome-like phenotypes, including visceral obesity, hypertension, dyslipidemia, impaired glucose tolerance, and insulin resistance were noted in association with reduced plasma levels of adiponectin, which is an anti-atherogenic adipocytokine, improving metabolic syndrome [63]. Thus, metabolic syndrome and hypoadiponectinemia could also be involved in the pathogenesis of MI in the triply NOSs-KO mice (Figure 3).

When wild-type, singly, doubly, and triply NOSs-KO mice were fed a high-cholesterol diet for 3-5 months, the serum levels of total cholesterol, LDL cholesterol, and small-dense LDL cholesterol were significantly increased in all the genotypes as compared with the regular diet. Importantly, when compared with the wild-type genotype, those levels in the high-cholesterol diet were markedly elevated only in the triply NOSs^{-/-} genotype, but not in any singly or doubly NOS^{-/-}

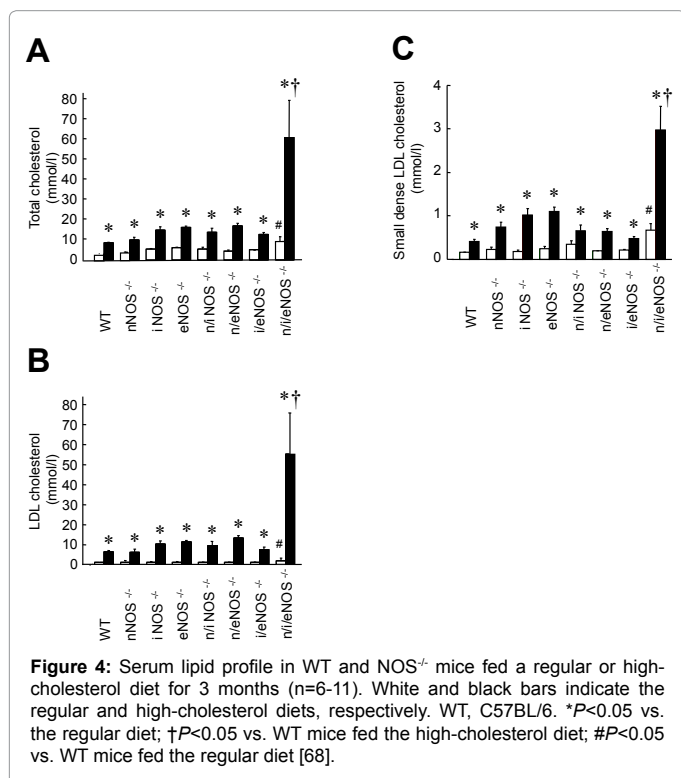


Figure 4: Serum lipid profile in WT and NOS^{-/-} mice fed a regular or high-cholesterol diet for 3 months (n=6-11). White and black bars indicate the regular and high-cholesterol diets, respectively. WT, C57BL/6. **P*<0.05 vs. the regular diet; †*P*<0.05 vs. WT mice fed the high-cholesterol diet; ‡*P*<0.05 vs. WT mice fed the regular diet [68].

established that eNOS has powerful anti-arteriosclerotic and anti-atherosclerotic effects [1-7]. However, neither deletion of the eNOS gene nor pharmacological inhibition of eNOS activity induce MI in animals. On the other hand, intriguingly, our triply *n/i/eNOS*s-KO mice experienced spontaneous MI and sudden cardiac death [63] (Figure 2). This was the first *in vivo* demonstration showing that the defective NOS system is involved in the pathogenesis of spontaneous MI.

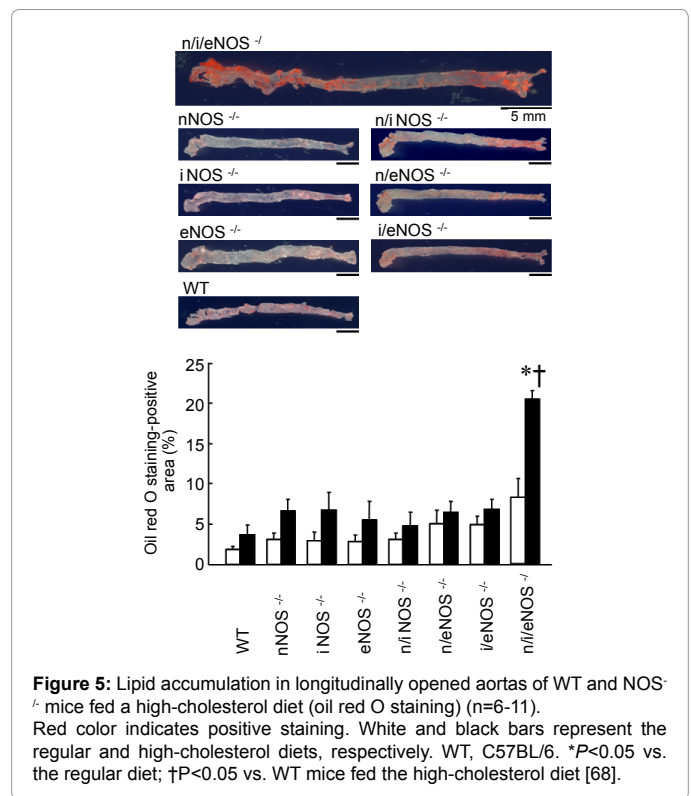


Figure 5: Lipid accumulation in longitudinally opened aortas of WT and NOS^{-/-} mice fed a high-cholesterol diet (oil red O staining) (n=6-11). Red color indicates positive staining. White and black bars represent the regular and high-cholesterol diets, respectively. WT, C57BL/6. **P*<0.05 vs. the regular diet; †*P*<0.05 vs. WT mice fed the high-cholesterol diet [68].

genotypes and this was associated with remarkable atherosclerosis and sudden cardiac death, which occurred mainly in 4-5 months after the high-cholesterol diet [68] (Figures 4 and 5). Out of 15 dead triply NOS^{-/-} mice fed the high-cholesterol diet, myocardial infarction was detected in 1 mouse, giant organized thrombi in the left and right ventricles were seen in 2 mice, and marked neointimal formation and perivascular fibrosis of the coronary artery and pulmonary congestion were noted in all the dead mice. These results suggest the protective role of the whole endogenous NOSs system in the pathogenesis of dyslipidemia and atherosclerotic vascular disease. Hepatic LDL receptor expression was markedly reduced only in the triply NOS^{-/-} genotype, accounting for the diet-induced dyslipidemia in the genotype.

Bone marrow-derived vascular progenitor cells in the blood accumulate in injured arteries, differentiate into vascular wall cells, and contribute to arteriosclerotic vascular lesion formation. All NOSs have been reported to be expressed in bone marrow cells. However, whether NOSs in bone marrow cells play a role in vascular lesion formation remained to be clarified. We addressed this point in the triply NOS^{-/-} mice and in bone marrow transplantation experiments. We previously reported that, in Wild-Type (WT) mice that underwent bone marrow transplantation from Green Fluorescent Protein (GFP)-TG mice, GFP-positive fluorescence was detected in the ligated carotid arteries, confirming the involvement of bone marrow-derived vascular progenitor cells in vascular lesion formation after carotid artery ligation [69]. In a comparison of the NOSs^{-/-} genotype that received NOSs^{-/-} bone marrow transplantation and the NOSs^{-/-} genotype that received WT bone marrow transplantation, the extent of neointimal formation and the extent of constrictive remodeling were both significantly less in those that received the WT bone marrow transplantation, along with significantly higher NOS activities in the ligated carotid arteries [70]. Furthermore, in a comparison of the WT genotype with WT bone marrow transplantation and the WT genotype with NOSs^{-/-} bone marrow transplantation, the extent of neointimal formation and the extent of constrictive remodeling were both significantly greater in the WT genotype with NOSs^{-/-} bone marrow transplantation, and this was associated with significantly lower NOS activities in the ligated carotid arteries [70]. These results indicate that NOSs in bone marrow cells exert an inhibitory effect on vascular lesion formation caused by blood flow disruption in mice *in vivo*, demonstrating a novel vasculoprotective role of NOSs in bone marrow-derived vascular progenitor cells.

Clinical Implications

Several lines of evidence suggest an association of the defective NOSs system with arteriosclerotic vascular disease in humans. First, it has been reported that plasma and/or urinary NOx levels, which are markers of NO production, are reduced in patients with the arteriosclerotic risk factors and in those with coronary arteriosclerosis [71-74]. Second, plasma concentrations of asymmetric dimethylarginine, which is an endogenous NOS inhibitor, have been shown to be elevated in patients with arteriosclerotic risk factors, with arteriosclerosis, and with risk of MI [75]. Finally, it has been revealed in humans that the gene polymorphisms of individual NOSs are associated with arteriosclerotic risk factors, arteriosclerosis, risk of MI, and low plasma NOx levels [76]. These results may imply a clinical significance of the findings with the NOSs^{-/-} mice.

Judging from the results of the murine genetic models, it is conceivable that eNOS is involved in the pathogenesis of endothelial dysfunction, arteriosclerosis, aortic dissection, and abdominal aortic aneurysm, that iNOS contributes to the pathogenesis of arteriosclerosis, aortic dissection, and abdominal aortic aneurysm, that nNOS serves

functions in the pathogenesis of arteriosclerosis, and that entire NOSs play roles in the pathogenesis of endothelial dysfunction, coronary vasospasm, arteriosclerosis, and myocardial infarction. The roles of NOSs in human arteriosclerotic vascular diseases remain to be examined in future clinical studies.

Therapeutic Potential of NOS Activators

A number of NOS activators, such as eNOS transcriptional enhancers (AVE9488 and AVE3085), tetrahydrobiopterin, statins, trans-resveratrol, vanadate, protein kinase C inhibitor midostaurin, and pentacyclic triterpenoids ursolic acid and betulinic acid, have been reported to increase NOS expression and activity or ameliorate NOS uncoupling [77-79]. These NOS activators may have therapeutic potential in the treatment of arteriosclerotic vascular diseases.

Concluding Remarks

The mouse is the most ideal genetically modifiable mammalian presently available [80]. Studies with mice that are deficient in or overexpressing NOSs provide pivotal insights into the roles of the NOSs in the pathogenesis of arteriosclerotic vascular diseases. In general, eNOS, nNOS, and the whole NOSs system exert vasculoprotective roles, while iNOS seems to exert dual effects in the vascular system. The observations with the genetically modified animals have greatly advanced our understanding of the roles of the NOSs system in the pathogenesis of arteriosclerotic vascular diseases. Further studies are certainly needed to clarify whether these observations can be translated to human patients with arteriosclerotic vascular diseases.

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