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Nitric Oxide Synthases and Cardiovascular Diseases
—— Insights From Genetically Modified Mice ——

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Nitric oxide (NO) is produced in almost all tissues and organs, exerting a variety of biological actions under both physiological and pathological conditions. NO is synthesized by 3 distinct NO synthase (NOS) isoforms (neuronal, inducible, and endothelial NOS), all of which are expressed in the human cardiovascular system. The regulatory roles of NOSs in cardiovascular diseases have been described in pharmacological studies with selective and non-selective NOS inhibitors. However, the specificity of the NOS inhibitors continues to be an issue of debate. To overcome this issue, genetically engineered animals have been used. All types of NOS gene-deficient (knockout: KO) animals, including singly, doubly, and triply NOS-KO mice, and various types of NOS gene-transgenic (TG) animals, including conditional and non-conditional TG mice bearing endothelium-specific or cardiomyocyte-specific overexpression of each NOS gene, have thus far been developed. The roles of individual NOS isoforms, as well as the entire NOS system, in the cardiovascular system have been extensively investigated in those mice, and the results provide pivotal insights into the pathophysiology of NOSs in human cardiovascular diseases. Based on studies with murine NOS genetic models, this review summarizes the latest knowledge of NOSs and cardiovascular diseases. (*Circ J* 2009; **73:** 986–993)

Key Words: Cardiovascular diseases; Knockout mice; Nitric oxide; Nitric oxide synthase; Transgenic mice

Nitric oxide (NO) research is an important academic field in which a huge number of scientists have great interest. Notably, the number of NO-related articles published annually still continues to increase even now, and more than 7000 NO-related articles are recently being published per year (**Figure 1**).

NO possesses multiple biological actions that contribute to the maintenance of cardiovascular homeostasis[1–6] NO is formed from its precursor L-arginine by a family of NO synthases (NOSs) with stoichiometric production of L-citrulline. The NOS system consists of 3 distinct NOS isoforms, encoded by 3 distinct NOS genes, including neuronal (nNOS; also known as NOS-1), inducible (iNOS; also known as NOS-2) and endothelial NOS (eNOS; also known as NOS-3).

Initial studies 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 both under 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[1–6] However, recent studies have revealed that both nNOS and eNOS are subject to expressional regulation[7–11] and that iNOS is constitutively expressed even under physiological conditions[12,13] It has also become apparent that in addition to eNOS and iNOS, nNOS also plays important roles in the cardiovascular system. Thus, NO research is taking a new turn.

Genetically engineered animals are a powerful experimental tool for studying the function of target genes in vivo. All types of NOS gene-knockout (KO) animals, including singly, doubly, and triply NOS-KO mice, have been generated (**Table 1**)[14–24] Furthermore, various types of NOS gene-transgenic (TG) animals, including conditional and non-conditional TG mice with endothelium-specific or cardiomyocyte-specific overexpression of each NOS isoform, have also been established (**Table 2**)[25–34] By using those genetically modified mice, the cardiovascular roles of NOSs have been extensively studied, and the findings provide important insights into the significance of NOSs in human cardiovascular diseases. In this review, we summarize the current knowledge of NOSs and cardiovascular diseases on the basis of research outcomes obtained from the NOS gene-modified mice.

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Arteriosclerosis and Atherosclerosis

In mice, arteriosclerotic vascular lesions are induced by either permanent ligation of the carotid artery, cuff placement around the artery or cardiac transplantation, and atherosclerotic vascular lesions are induced by crossing with apolipoprotein E (apoE)-KO mice, which manifest severe dyslipidemia. The atherosclerotic vascular lesion formation is exacerbated by a Western-type high-cholesterol diet[35,36]

Role of eNOS

Endothelium-specific eNOS-TG mice with an 8-fold

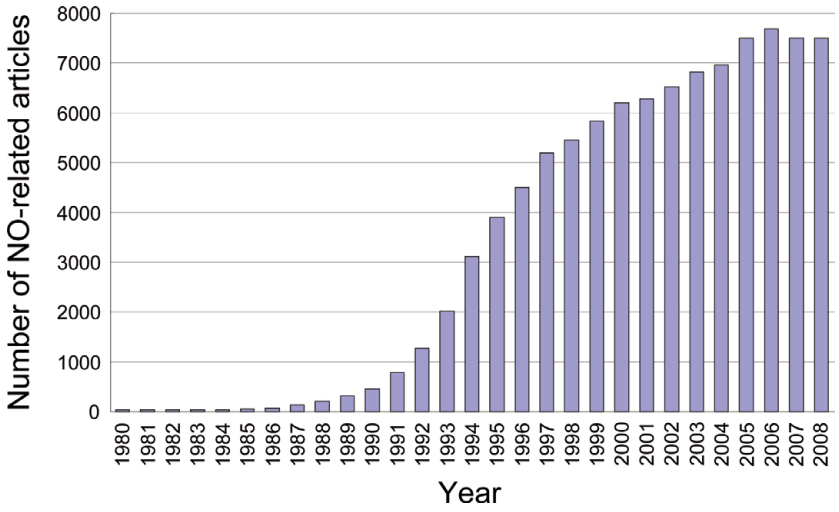

Figure 1. The annual number of nitric oxide (NO)-related articles published. The annual number of NO-related articles published still continues to increase even now, and more than 7,000 NO-related articles are recently being published per year.

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

KO mice	Site of gene deletion	Reference
nNOS-KO	Exon 2 (#1)	16
	Exon 6	15
iNOS-KO	Proximal 585 bases of promoter plus exons 1–4 (#2)	19
	Near exons 1–5	24
	Exons 12 and 13 and a part of exon 11 (#3)	18
eNOS-KO	Exons 24–26 (#4)	17
	Exon 12 (#5)	21
	Exons 24 and 25	14
n/iNOS-KO	#1 and #3	23
	#1 and #2	20
n/eNOS-KO	#1 and #4	22
	#1 and #5	23
	#1 and #4	20
i/eNOS-KO	#3 and #5	23
	#2 and #4	20
n/i/eNOS-KO	#1, #2 and #4	20

NOS, nitric oxide synthase; KO, knockout; nNOS, neuronal NOS; iNOS, inducible NOS; eNOS, endothelial NOS.

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

TG mice	Overexpression site	Promoter used	Reference
nNOS-TG	Myocardium (conditional)	α-MHC	26
	Myocardium (conditional)	α-MHC	29
	Brain	CaMKIIα	32
iNOS-TG	Myocardium (conditional)	α-MHC	30
	Myocardium	α-MHC	27
	Pancreatic β cell	Insulin	33
eNOS-TG	Endothelium	Preproendothelin-1	31
	Endothelium	eNOS	34
	Myocardium	α-MHC	25
	Myocardium	α-MHC	28

TG, transgenic; MHC, myosin heavy chain; CaMKII, calcium-calmodulin multifunctional kinase II. Other abbreviations see in Table 1.

increase in vascular NOS activity showed decreased neointimal formation after carotid artery ligation;[37] 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.[34] Consistent with those findings, eNOS-KO mice displayed increased neointimal formation, accelerated medial thickening, and abnormal vascular remodeling in response to carotid artery ligation (**Figure 2**)[38,39] and cuff placement around the femoral artery.[40] Furthermore, eNOS-KO/apoE-KO mice had worsened formation of atherosclerotic vascular lesions as compared with apoE-KO mice.[41,42] These lines of evidence indicate a vasculoprotective role of eNOS in arteriosclerosis and atherosclerosis.

In contrast, in endothelium-specific eNOS-TG mice with an 8-fold increase in vascular NOS activity, a conflicting progression of atherosclerotic vascular lesion formation elicited by crossbreeding with apoE-KO mice is reported.[43] Thus, this point needs to be examined in future studies.

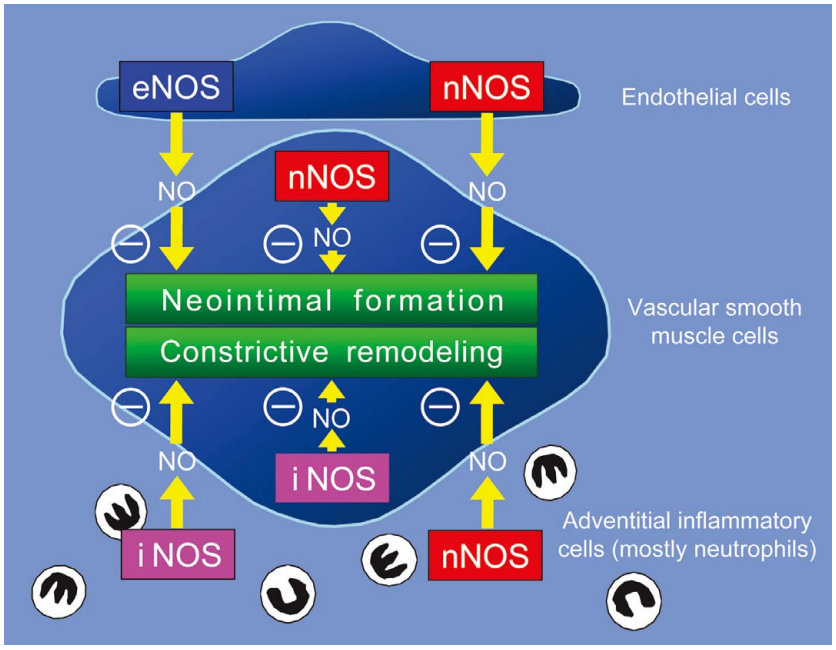

Figure 2. The different vasculoprotective roles of 3 nitric oxide synthase (NOS) isoforms in a mouse carotid artery ligation model. Studies with each NOS isoform-knockout mice have demonstrated that endothelial NOS (eNOS) inhibits neointimal formation, that inducible NOS (iNOS) attenuates constrictive vascular remodeling, and that neuronal NOS (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.

Role of iNOS

The role of iNOS in arteriosclerosis and atherosclerosis seems to be complicated. Deletion of the iNOS gene in mice exacerbated pathological vascular remodeling in a carotid artery ligation model (**Figure 2**)[39] and in a cardiac transplant model;[44] however, it conversely ameliorated neointimal formation in a carotid cuff placement model;[45] and lipid-rich atherosclerotic vascular lesion formation in apoE-KO mice.[46] Thus, iNOS appears to have 2 faces. This discrepancy may be explained in part by the oxidant and antioxidant properties of iNOS;[47] because NOS produce superoxide anions rather than NO, with resultant production of a potent oxidant peroxynitrite, under certain conditions such as deficiency of a substrate (eg, L-arginine) or a cofactor (eg, tetrahydrobiopterin) (which phenomenon is referred to as 'NOS uncoupling');[48,49]

Role of nNOS

Expression of nNOS is upregulated in the neointima, endothelial cells and macrophages in both early and advanced human atherosclerotic lesions.[50] Although the regulatory roles of eNOS and iNOS in vascular lesion formation have been widely studied, little has been 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 vascular cross-sectional area) following carotid artery ligation (**Figure 2**).[51] In agreement with our evidence, nNOS-KO/apoE-KO mice showed accelerated atherosclerotic vascular lesion formation as compared with apoE-KO mice.[52] These results suggest that nNOS also plays a role in suppressing arteriosclerotic/atherosclerotic vascular lesion formation.[11] Upregulation of nNOS may play a compensatory role in the presence of reduced eNOS activity (eg, inflammation and arteriosclerosis) to maintain vascular homeostasis.[11]

The regulatory mechanisms for vascular nNOS expression remained to be elucidated. We revealed that inflammatory and proliferative stimuli (angiotensin II, interleukin-1β, and platelet-derived growth factor) and a statin increase vascular nNOS expression.[9,10,51] It has been also reported that hypoxic conditions[53] and hypertensive situations[54,55] upregulate vascular nNOS expression.

Role of NOS System

Because all NOSs play a role in the vascular system, we next conceived a project to investigate the roles of the whole NOS system in vivo. The roles of the NOS 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 both the non-specificity of agents and compensation among NOS isoforms, the authentic roles of the NOS system were still poorly understood. To address this important issue, we have recently developed mice in which the entire NOS system is completely disrupted (triply nNOS/iNOS/eNOS-KO mice).[20,56] The triply n/i/eNOS-KO mice, but not any singly NOS-KO mice, spontaneously develop 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.[57,58] These results provided the first direct evidence for a vasculoprotective role of the entire NOS system in arteriosclerosis and atherosclerosis.

Spontaneous Myocardial Infarction (MI)

MI is the leading cause of death for both genders worldwide.[59,60] The molecular mechanisms for the pathogenesis of MI, however, remain to be fully elucidated.

Role of NOS System

It is well established that eNOS has powerful anti-arteriosclerotic and anti-atherosclerotic effects;[1–6] however, neither deletion of the eNOS gene nor pharmacological inhibition of eNOS activity induces MI in animals. On the other hand, intriguingly, our triply n/i/eNOS-KO mice had spontaneous MI and sudden cardiac death (**Figures 3A**,**B**), which is the first in-vivo demonstration of the involvement of the defective NOS system in the pathogenesis of sponta-

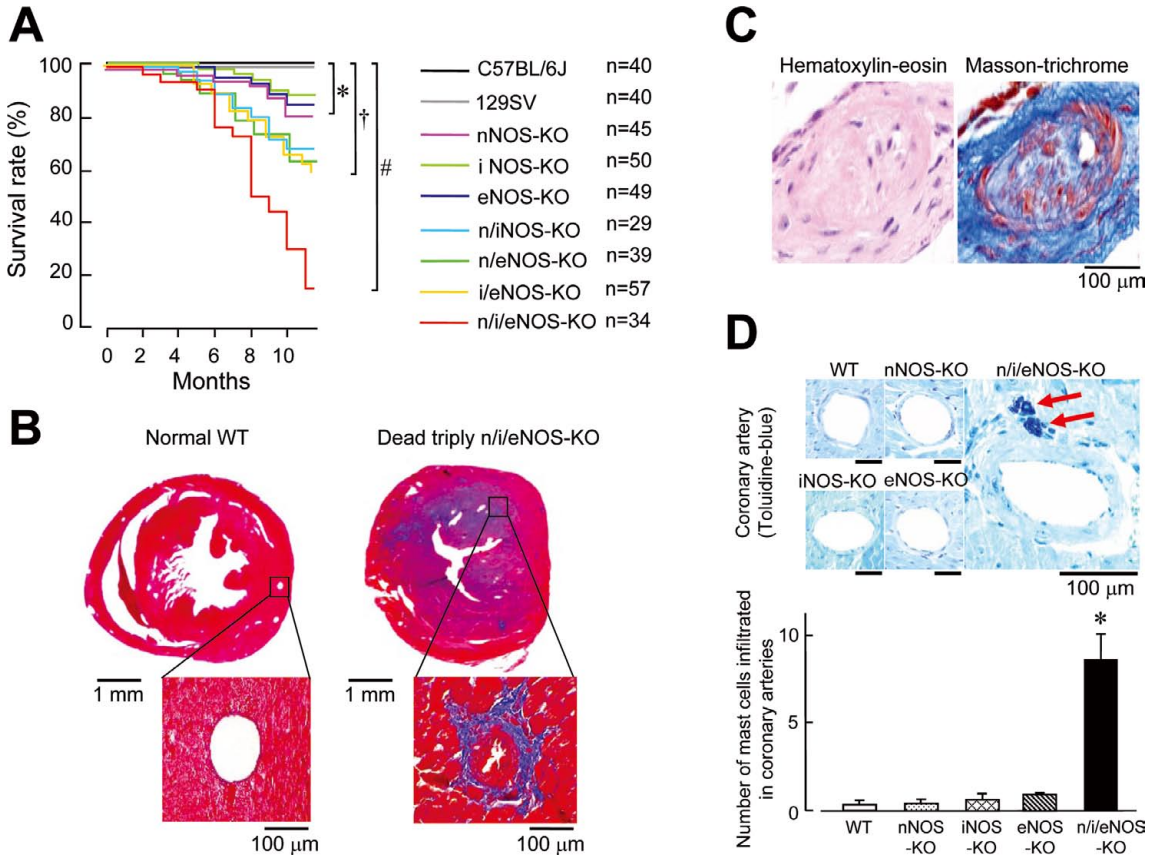

Figure 3. Decreased survival, spontaneous myocardial infarction (MI), coronary arteriosclerosis and mast cell infiltration in male triply n/i/eNOS-KO mice. (**A**) Survival rate (n=29–57). A red line represents markedly reduced survival in the triply n/i/eNOS-KO mice. *, †, #P<0.05 between wild-type (WT) C57BL/6J vs singly, doubly, and triply NOS-KO, respectively. (**B**) Acute MI and coronary arteriosclerotic lesion formation in the triply n/i/eNOS-KO mouse that died at 8 months of age (Masson-trichrome staining). Blue in the heart cross-section of the dead triply n/i/eNOS-KO 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. Data from reference 57. NOS, nitric oxide synthase; nNOS, neuronal NOS; iNOS, inducible NOS; eNOS, endothelial NOS; KO, knockout.

neous MI.

Arteriosclerosis is seen in most of the vasculature in the triply NOS-KO mice, whereas atherosclerosis is observed in the aorta alone. Human MI results not only from coronary atherosclerosis, but also from other causes, including coronary intimal hyperplasia, medial thickening, and coronary vasospasm?[59,61] In the triply n/i/eNOS-KO mice that died of MI, marked coronary intimal hyperplasia and medial thickening were noted (**Figures 3B**,**C**). Furthermore, in the dead triply n/i/eNOS-KO mice, marked infiltration of mast cells at the coronary artery adventitia was also observed (**Figure 3D**). Histamine released from adventitial mast cells is thought to cause coronary vasospasm with resultant MI in humans?[62] It is thus possible that coronary arteriosclerosis and coronary vasospasm are involved in the cause of death in the triply NOS-KO mice (**Figure 4**).

In our triply n/i/eNOS$^{-/-}$ mice, endothelium-dependent relaxation to acetylcholine, which is a physiological eNOS activator, was completely lacking, and contraction to phenylephrine, which is an α_1 adrenergic agonist, was markedly potentiated?[57] These vascular dysfunctions could also be involved in the pathogenesis of MI in the triply NOS-KO mice (**Figure 4**).

Metabolic Syndrome (MetS)

MetS is defined as a constellation of interrelated cardiovascular risk factors of metabolic origin, including visceral obesity, hypertension, hypertriglyceridemia, impaired glucose tolerance, and insulin resistance?[63,64] Notably, accumulation of 3 or more risk factors dramatically increases the risk of morbidity of arteriosclerotic cardiovascular diseases by 11-fold, indicating that MetS is an important therapeutic target for the prevention and treatment of cardiovascular diseases?[63,64]

Roles of eNOS and NOS System

eNOS-KO and our triply n/i/eNOS-KO mice manifested phenotypes that closely resemble MetS in humans. The extent of each of hypertension, hypertriglycemia, and visceral obesity was comparable in the 2 genotypes, whereas the extent of impaired glucose tolerance and of insulin resistance was greater in the triply n/i/eNOS-KO than in the eNOS-KO genotype, and hyper-low-density-lipoproteinemia was observed only in the triply n/i/eNOS-KO genotype. It is thus possible that the NOS system and eNOS play important roles in the prevention of MetS.

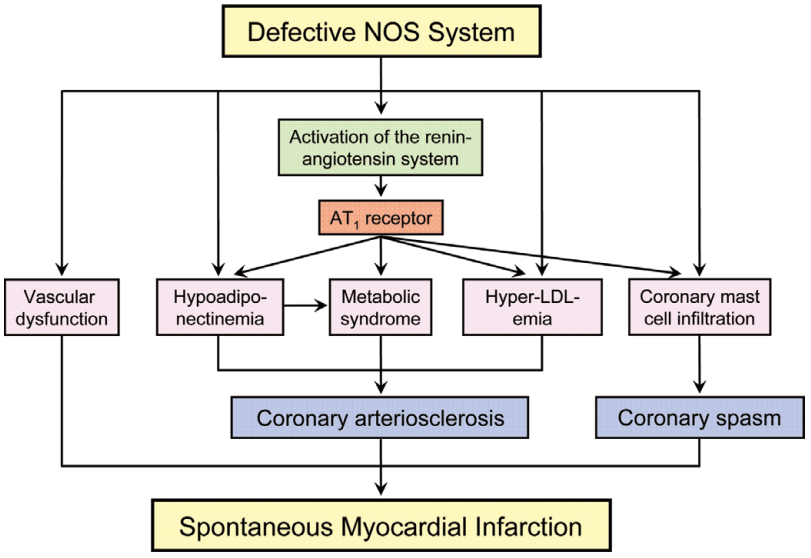

Figure 4. Mechanisms for spontaneous myocardial infarction (MI) caused by the defective nitric oxide synthase (NOS) system in mice in vivo. Genetic disruption of all NOSs caused metabolic syndrome, hypoadiponectinemia, hyper-low-density-lipoprotein (LDL)-emia, coronary adventitial mast cell infiltration, and vascular dysfunction. Those factors could contribute to the pathogenesis of spontaneous MI. Importantly, long-term pharmacological blockade of the angiotensin II type 1 (AT_1) receptor significantly reduced the incidence of MI, along with amelioration of those risk factors. It is therefore possible that the AT_1 receptor pathway is involved in its molecular mechanism.

Although metabolic risk factors were present in the 2 genotypes, spontaneous MI was noted only in the triply n/i/eNOS-KO genotype. This inconsistency may be related to a compensatory mechanism by other NOSs that are not genetically disrupted[22] Indeed, in the eNOS-KO genotype, upregulation of vascular nNOS expression has been indicated[65,66] Furthermore, we have also revealed that NOS activity and NOx production are fairly well preserved in the eNOS-KO genotype[20]

Adiponectin is an anti-atherogenic adipocytokine, improving hypertriglyceridemia, glucose metabolism, and insulin resistance, and inhibiting the progression of arteriosclerosis[67–69] Under the condition of obesity with adipocyte hypertrophy, synthesis of adiponectin is not increased, but rather decreased, and in patients with MetS, the circulating levels of adiponectin are reduced, in contrast to the increases in other adipocytokine levels. The deficiency of adiponectin is thought to play a pivotal role in the pathogenesis of MetS and its vascular complications[68] In our triply n/i/eNOS-KO mice, plasma adiponectin levels were significantly reduced[57] Thus, adiponectin deficiency may contribute to the development of metabolic abnormalities and arteriosclerotic lesion formation in the triply n/i/eNOS-KO mice (**Figure 4**).

Importantly, the renin–angiotensin system is markedly activated in the triply n/i/eNOS-KO mice, and long-term treatment with an angiotensin II type 1 (AT_1) receptor blocker, olmesartan, potently inhibited coronary arteriosclerotic lesion formation, adventitial mast cell infiltration, and the occurrence of MI in the mice, with a resultant improvement in prognosis[57] Furthermore, long-term treatment with olmesartan reversed all the abnormal metabolic phenotypes, together with amelioration of hypoadiponectinemia[57] These results suggest that the AT_1 receptor pathway is involved in the pathogenesis of MI in our triply n/i/eNOS-KO mice (**Figure 4**).

Angina Pectoris (AP)

Role of NOS System

We were unable to find any articles in which AP was studied in NOS gene-modified mice. However, as mentioned earlier, coronary arteriosclerosis and mast cell infiltration in the coronary adventitia were noted in our triply n/i/eNOS-KO mice, suggesting a potential linkage between AP (both vasospastic and organic types) and a defective NOS system. In line with our findings, a clinical study reported that NOS activity is deficient in the spasm arteries of patients with coronary spastic AP[70]

Aortic Diseases

Role of eNOS

When 12 eNOS-KO/apoE-KO mice were fed a Western-type diet for 16 weeks, 3 mice spontaneously developed abdominal aortic aneurysms and 2 developed aortic dissections (Stanford type B)[42] These results suggest that eNOS deficiency induces abdominal aortic aneurysms and aortic dissections in the presence of severe hyperlipidemia.

Role of iNOS

Aortic aneurysms can be induced in animals by perfusing the aorta with elastase. The extent of elastase-induced abdominal aneurysmal 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, the effect of which was reversed by previous ovariectomy[71] It is thus likely that iNOS deficiency also leads to the occurrence of abdominal aortic aneurysms induced by elastase solely in the female.

Heart Failure (HF)

Congestive HF can be induced by permanent ligation of the coronary artery (ie, MI) and by transverse aortic constriction (ie, pressure overload), respectively, in animals.

Role of eNOS

Cardiomyocyte-restricted eNOS-TG mice with a 30-fold increase in cardiac NOS activity showed protection against detrimental left ventricular (LV) remodeling after coronary artery ligation, exhibiting improved LV systolic and diastolic function and attenuation of LV hypertrophy[28] Endothelium-specific eNOS-TG mice with a 12-fold increase in vascular NOS activity also exhibited improved survival, LV dysfunction, and pulmonary edema following coronary ligation without affecting LV remodeling[72] Consistent with

these findings, eNOS-KO mice with HF due to either MI[73] or pressure overload[74] had reduced survival, and exacerbation of LV remodeling and LV dysfunction. It has also been reported that the presence of eNOS mediates the beneficial cardiovascular protective effects of statins,[75] angiotensin-converting enzyme inhibitors,[76] AT_1 receptor blockers,[76] and corticosteroids[77] in experimental HF. Thus, it is evident that eNOS exerts a protective role in HF.[78,79]

Role of nNOS

Conditionally targeted cardiomyocyte-specific nNOS-TG mice with a 5-fold increase in cardiac NOS activity showed delayed transition toward HF in response to pressure overload.[29] In agreement with this evidence, 2 strains of nNOS-KO mice with MI-induced HF similarly showed reduced survival, and exacerbation of pathological LV remodeling or LV dysfunction after coronary artery ligation, although the findings were not totally identical in the 2 strains.[80,81] It is thus possible that in addition to eNOS, nNOS also exerts a protective role in HF.[82]

Role of iNOS

Increased iNOS expression is noted in cardiomyocytes in septic shock, myocarditis, ischemia, and dilated cardiomyopathy, and has been implicated in the development of HF. However, cardiomyocyte-specific iNOS overexpression per se (in 2 different strains with either a 10-fold[30] or 40-fold increase[27] in cardiac NOS activity) did not result in HF, suggesting that increased iNOS expression is not the triggering factor in HF. On the other hand, iNOS-KO mice with HF induced by MI[83–85] and by pressure overload[86] showed improved survival, less LV remodeling and dysfunction, and decreased myocardial apoptosis. Furthermore, iNOS-KO mice with HF induced by cardiospecific overexpression of tumor necrosis factor-α exhibited improved β-adrenergic inotropic responsiveness. It is thus possible that, in contrast to eNOS and nNOS, iNOS exerts an opposite, unfavorable role in HF. The underlying mechanisms for the contrasting roles among NOS isoforms in HF are unclear, but may relate to differences in spatial localization, expressional regulation, NO-generating capacity, and peroxynitrite generation.[79,87,88]

Arrhythmia

Role of iNOS

The occurrence of drastic malignant arrhythmia has been reported in conditional, cardiomyocyte-specific iNOS-TG mice with a 10-fold increase in cardiac NOS activity.[30] The iNOS-TG mice displayed 2nd-degree (Mobitz type II) and 3rd-degree atrioventricular block and ventricular tachycardia, resulting in sudden cardiac death. These results indicate an arrhythmogenic role of iNOS. Because iNOS-derived superoxide-dependent peroxynitrite generation is enhanced in the iNOS-TG mice, the oxidative property of iNOS may elicit a proarrhythmic effect.

Role of eNOS

Cardiomyocyte-restricted eNOS-TG mice have a lower incidence of ectopic beats.[89] In line with this finding, eNOS-KO mice have a higher incidence of digoxin-induced ventricular tachycardia[90] and an increased susceptibility to the development of triggered activity.[91] It is thus conceivable that eNOS may protect the heart against arrhythmia.

Congenital Heart Disease

Role of eNOS

It has been reported that eNOS-KO mice develop congenital heart diseases, including atrial and ventricular septal defects,[92] a bicuspid aortic valve,[93] and defective pulmonary vasculature and airway.[94] These findings are in agreement with a clinical study showing that a single nucleotide polymorphism of the eNOS gene (894G>T) is associated with an increased risk of congenital heart diseases.[95] However, although the congenital abnormalities are seen in only one strain among three distinct eNOS-KO strains, these results should be interpreted with caution.

Conclusion

The mouse is the most ideal genetically modifiable mammalian presently available.[87] Studies in both KO and TG overexpression models provide pivotal insights into the cardiovascular pathophysiology of NOSs at the molecular level. These studies have demonstrated that, in general, eNOS and nNOS exert protective roles, while iNOS has dual roles in the cardiovascular system, and that the NOS system in its entirety plays salutary roles in a variety of cardiovascular diseases. Furthermore, the studies have indicated that the NOS uncoupling under conditions of tetrahydrobiopterin or L-arginine deficiency is an important determinant of whether or not NOSs are beneficial. Thus, observations in the genetically modified animals have greatly advanced (and will continue to improve) our understanding of the roles of NOSs in the pathogenesis of human cardiovascular diseases. Further studies are certainly needed to clarify whether these outcomes can be translated to human patients with cardiovascular diseases.

Acknowledgments

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