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Pathophysiological relevance of NO signaling in the cardiovascular system: Novel insight from mice lacking all NO synthases

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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, including neuronal (nNOS), inducible (iNOS), and endothelial NOS (eNOS), all of which are expressed in the human cardiovascular system. The roles of endogenous NO in the cardiovascular system have been investigated in pharmacological studies with NOS inhibitors and in studies with mice that lack each NOS isoform. However, in the pharmacological studies, the specificity of the NOS inhibitors continues to be an issue of debate, while in each of the NOS isoform-deficient mice, a compensatory mechanism by other NOSs that are not genetically deleted is apparently involved. Thus, the authentic roles of endogenous NO are still poorly understood. To address this issue, genetically engineered mice in which all three NOS genes are completely disrupted have been developed. In the triply n/i/eNOS$^{-/-}$ mice, but not in singly eNOS$^{-/-}$ mice, several cardiovascular phenotypes, including arteriosclerosis/atherosclerosis, myocardial infarction, and dyslipidemia, have been described. Furthermore, by using the triply NOS$^{-/-}$ mice, the roles of the NOS system in endothelium-dependent hyperpolarization and stain-induced NO production have been elucidated. These results provide novel insight into the cardiovascular role of the endogenous NO/NOS system at the molecular level. This review, based on the research outcomes obtained from the triply NOS$^{-/-}$ genetic model, summarizes the latest knowledge of the pathophysiological relevance of NO signaling in the cardiovascular system.

Contents

1. Introduction

Since the endothelium-derived relaxing factor was identified as nitric oxide (NO) in 1987, NO research has achieved remarkable development. Recently, more than 7000 NO-related articles are published per year.

Abbreviations: NO, nitric oxide; NOS, nitric oxide synthase; nNOS, neuronal nitric oxide synthase; iNOS, inducible nitric oxide synthase; eNOS, endothelial nitric oxide synthase; WT, wild-type; apoE, apolipoprotein E; MI, myocardial infarction; LDL, low-density lipoprotein; EDHF, endothelium-derived hyperpolarizing factor; H_2O_2, hydrogen peroxide; BH_4, tetrahydrobiopterin; AT_1, angiotensin II type 1; ARB, angiotensin II type 1 receptor blocker.

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NO is formed from its precursor L-arginine by a family of NO synthases (NOSs) with stoichiometric production of L-citrulline (Furchgott, 1984; Ignarro, 1990; Moncada et al., 1991; Bredt & Snyder, 1994; Murad, 1997; Shimokawa, 1999; Tsutsui et al., 2009). The NOS system consists of three distinct NOS isoforms, encoded by three 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).

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 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 (Furchgott, 1984; Ignarro, 1990; Moncada et al., 1991; Bredt & Snyder, 1994; Murad, 1997; Shimokawa, 1999; Tsutsui et al., 2009). However, it was subsequently revealed that iNOS is constitutively expressed even under physiological conditions (Park et al., 1996; Buchwalow et al., 2002), and that in addition to eNOS and iNOS, nNOS also plays important roles in the cardiovascular system (Morishita et al., 2002; Nakata et al., 2005, 2007; Tsutsui, 2004).

The roles of NO in vivo have been investigated in pharmacological studies, with L-arginine analogues having been widely used as pharmacological tools to inhibit NOS activity. However, the L-arginine analogues possess multiple non-specific actions (Suda et al., 2002, 2004). Indeed, although long-term treatment with the L-arginine analogues, such as N^{ω}-nitro-L-arginine methyl ester (L-NAME), had long been believed without doubt to simply inhibit vascular NO synthesis and cause arteriosclerotic vascular lesion formation, the long-term vascular effects of the L-arginine analogues are not solely mediated by the simple inhibition of NO synthesis. Activation of the tissue renin–angiotensin system and increased oxidative stress, independent of endogenous NO inhibition, are involved in the long-term vascular effects of the L-arginine analogues. These findings warrant a re-evaluation of previous pharmacological studies using those analogues.

The roles of NO in vivo have also been investigated in studies with mice that lack each NOS isoform (Bredt & Snyder, 1994; Furchgott, 1984; Ignarro, 1990; Moncada et al., 1991; Murad, 1997; Shimokawa, 1999). However, in each of the NOS isoform-deficient ($NOS^{-/-}$) mice, a compensatory mechanism by other NOSs that are not genetically disrupted appears to operate (Son et al., 1996). Indeed, although it is well established that eNOS exerts anti-arteriosclerotic effects (Bredt & Snyder, 1994; Furchgott, 1984; Ignarro, 1990; Moncada et al., 1991; Murad, 1997; Shimokawa, 1999), the $eNOS^{-/-}$ mice do not spontaneously develop arteriosclerotic/atherosclerotic vascular lesion formation (Moroi et al., 1998). In the $eNOS^{-/-}$ mice, compensatory up-regulation of vascular nNOS is evident (Huang et al., 2002; Lamping et al., 2000; Takaki et al., 2008), and NOS activity and NOx production are fairly well preserved (Morishita et al., 2005). Thus, even at the present time when NO research has evolved strikingly, the ultimate roles of endogenous NO still remain to be fully understood.

All types of $NOS^{-/-}$ animals, including singly, doubly, and triply $NOS^{-/-}$ mice, have thus far been generated (Table 1) (Godecke et al.,

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

$NOS^{-/-}$ Mice	Sites of gene deletion	References
$nNOS^{-/-}$	Exon 2 (#1)	Huang et al. (1993)
	Exon 6	Gyurko et al. (2002)
	Exon 6	Packer et al., *PNAS* (2003)
$iNOS^{-/-}$	Proximal 585 bases of promoter plus exons 1-4 (#2)	MacMicking et al. (1995)
	Near exons 1-5	Wei et al. (1995)
	Exons 12 and 13 and a part of exon 11 (#3)	Laubach et al. (1995)
$eNOS^{-/-}$	Exons 24-26 (#4)	Huang et al. (1995)
	Exon 12 (#5)	Shesely et al. (1996)
	Exons 24 and 25	Godecke et al. (1998)
$n/iNOS^{-/-}$	#1 and #3	Tranguch and Huet-Hudson (2003)
	#1 and #2	Morishita et al. (2005)
$n/eNOS^{-/-}$	#1 and #4	Son et al. (1996)
	#1 and #5	Tranguch and Huet-Hudson (2003)
	#1 and #4	Morishita et al. (2005)
$i/eNOS^{-/-}$	#3 and #5	Tranguch and Huet-Hudson (2003)
	#2 and #4	Morishita et al. (2005)
$n/i/eNOS^{-/-}$	#1, #2 and #4	Morishita et al. (2005)

NOS, nitric oxide synthase; nNOS, neuronal NOS; iNOS, inducible NOS; eNOS, endothelial NOS; $NOS^{-/-}$, NOS-deficient.

Table 2
Studies with Triply $n/i/eNOS^{-/-}$ Mice.

Findings	References
Nephrogenic diabetes insipidus in triply $NOS^{-/-}$ mice	Morishita et al. (2005)
Pivotal role of NOS system in atorvastatin-induced vascular NO production	Nakata et al. (2007)
Spontaneous myocardial infarction in triply $NOS^{-/-}$ mice	Nakata et al. (2008)
Crucial role of NOS system in endothelium-dependent hyperpolarization	Takaki et al. (2008)
Enhanced bone mineral density and increased bone turnover in triply $NOS^{-/-}$ mice	Sabanai et al. (2008)
Dyslipidemia, atherosclerosis, and sudden death in triply $n/i/eNOS^{-/-}$ mice fed a high fat diet	(Yatera et al., 2010)

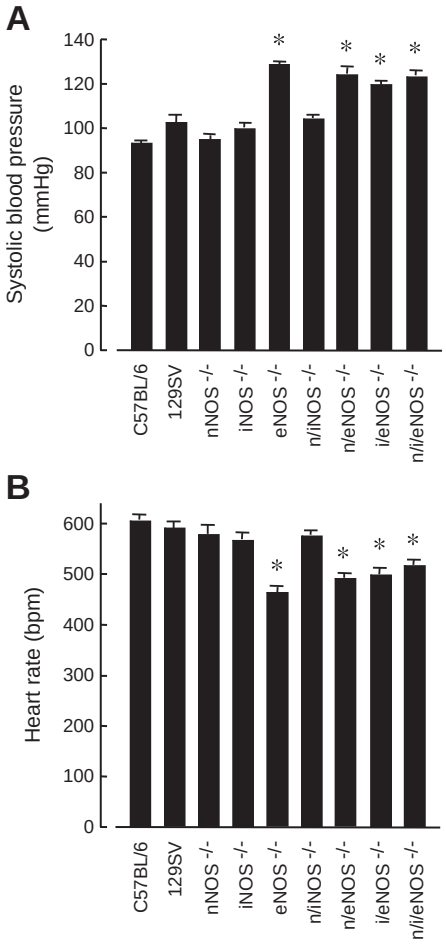

Fig. 1. Hemodynamics in wild-type (WT) and $NOS^{-/-}$ mice. (**A**) Systolic blood pressure measured by the tail-cuff method under conscious conditions (n = 9–16). *P<0.05 vs. WT C57BL/6 mice. (**B**) Heart rate measured by the tail-cuff method under conscious conditions (n = 9–16). *P<0.05 vs. WT C57BL/6 mice.
Data from Morishita et al. (2005).

1998; Gyurko et al., 2002; Huang et al., 1993, 1995; Laubach et al., 1995; MacMicking et al., 1995; Morishita et al., 2005; Shesely et al., 1996; Son et al., 1996; Tranguch & Huet-Hudson, 2003; Wei et al., 1995). Among them, the triply n/i/eNOS$^{-/-}$ mice are without the compensatory interaction of NOSs, and therefore a useful experimental tool to study the roles of endogenous NO derived from the NOS system. In the triply NOS$^{-/-}$ mice, but not in the eNOS$^{-/-}$ mice, several cardiovascular phenotypes, including arteriosclerosis/atherosclerosis, myocardial infarction, and dyslipidemia, have been described (Table 2). Furthermore, by using the triply NOS$^{-/-}$ genotype, the roles of the NOS system in endothelium-dependent hyperpolarization and in NO production induced by a statin (3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitor, cholesterol-lowering drug) have been elucidated (Table 2). These findings provide new insights into the significance of the NO/NOS system in human cardiovascular diseases. This review, based on the research outcomes obtained from the triply NOS$^{-/-}$ mutant model, summarizes the current knowledge of the pathophysiological relevance of NO signaling in the cardiovascular system.

2. Cardiovascular phenotypes in triply NOS$^{-/-}$ mice

2.1. Hypertension and bradycardia

Measurement of blood pressure by the tail-cuff method under conscious conditions showed that the triply NOS$^{-/-}$ mice were significantly hypertensive as compared with wild-type (WT) mice. The degree of hypertension in the triply NOS$^{-/-}$ mice was similar to that in the eNOS gene-disrupted singly and doubly NOS$^{-/-}$ mice (Fig. 1A). These results suggest that hypertension is a common characteristic of the eNOS gene disruption and is caused by the lack of endothelium-derived NO with the resultant increase in peripheral vascular resistance (Ortiz & Garvin, 2003).

Heart rate was significantly lower in the triply NOS$^{-/-}$ than in the WT mice, and the degree of bradycardia in the triply NOS$^{-/-}$ mice was also equivalent to that in the eNOS gene-disrupted singly and doubly NOS$^{-/-}$ mice (Fig. 1B), indicating that bradycardia, also, is a common phenotype of the eNOS gene deletion. Although there is no conclusive explanation for the decreased heart rate in association with the eNOS deletion, previous studies revealed that eNOS-derived NO could affect

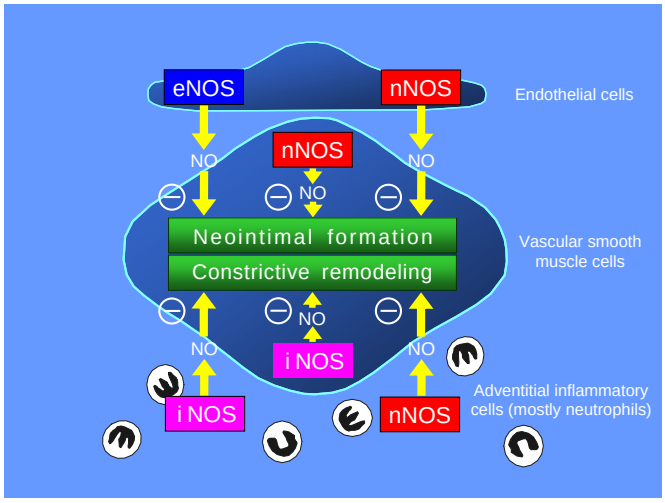

Fig. 2. The different vasculoprotective roles of three NOS isoforms in a mouse carotid artery ligation model. Studies with each NOS isoform$^{-/-}$ mice have demonstrated that eNOS inhibits neointimal formation, that iNOS attenuates constrictive vascular remodeling, and that nNOS suppresses both neointimal formation and constrictive vascular remodeling. Thus, individual NOS isoforms have different vasculoprotective actions against vascular lesion formation in mice in vivo. —, inhibition.
Diagram from Tsutsui (2004).

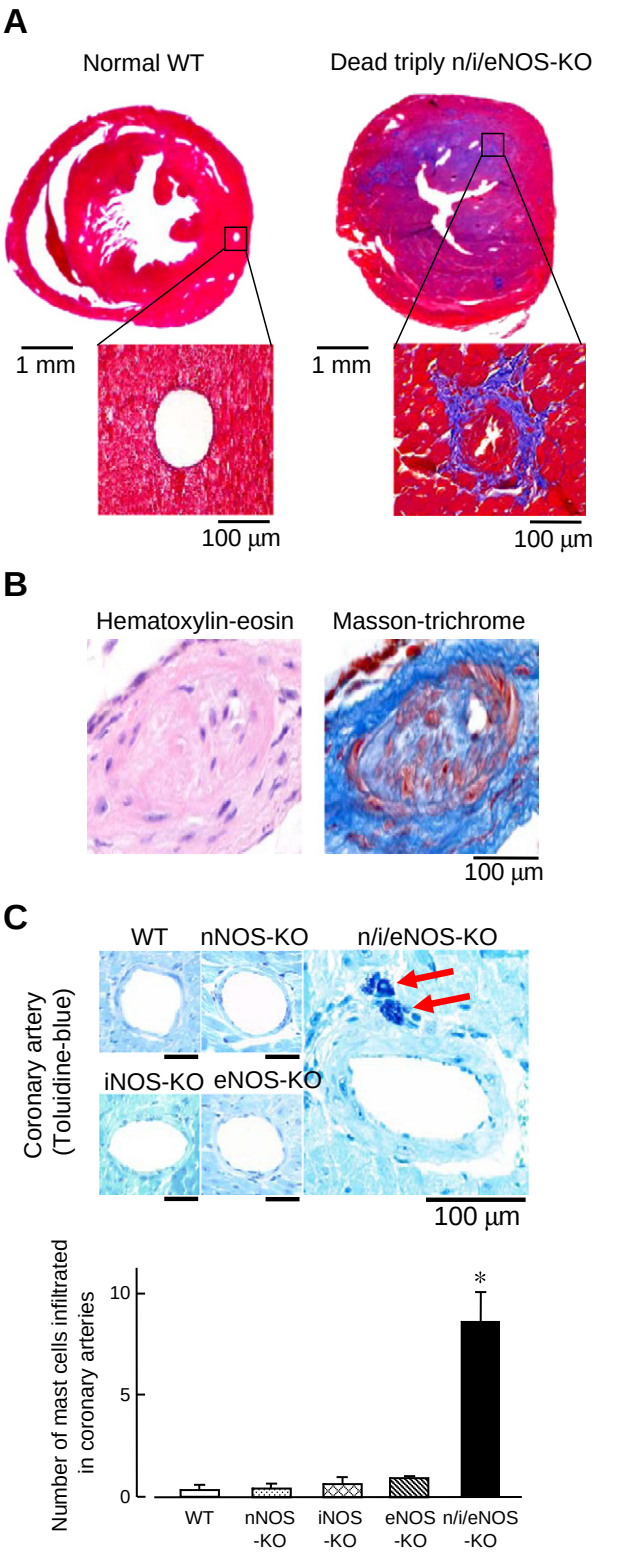

Fig. 3. Spontaneous myocardial infarction (MI), coronary arteriosclerosis and mast cell infiltration in triply NOS$^{-/-}$ mice. (**A**) Acute MI and coronary arteriosclerotic lesion formation in the triply NOS$^{-/-}$ mouse that died at 8 months of age (Masson-trichrome staining). Blue in the heart cross-section of the dead triply NOS$^{-/-}$ mouse indicates antero-septal acute MI. Adjacent coronary artery shows marked luminal narrowing, wall thickening, and perivascular fibrosis (blue). (**B**) Arteriosclerotic lesion formation in serial sections of the infarct-related coronary artery. (**C**) 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 Nakata et al. (2008).

baroreflex resetting or could be involved in establishing the baroreceptor setpoint (Ortiz & Garvin, 2003).

2.2. Vascular lesion formation

Expression of nNOS is up-regulated in the neointima, endothelial cells and macrophages in both early and advanced human atherosclerotic lesions (Wilcox et al., 1997). Although the regulatory roles of eNOS and iNOS on vascular lesion formation have been widely studied, little was known about the role of nNOS. A previous study addressed this point in nNOS$^{-/-}$ mice and demonstrated that the nNOS gene deficiency caused a worsening of neointimal formation and constrictive vascular remodeling (a reduction in vascular cross-sectional area) following carotid artery ligation (Fig. 2) (Morishita et al., 2002). In agreement with that evidence, nNOS$^{-/-}$/apolipoprotein E (apoE)$^{-/-}$ mice showed accelerated atherosclerotic vascular lesion formation as compared with apoE$^{-/-}$ mice (Kuhlencordt et al., 2006). These results suggest that nNOS also plays a role in suppressing arteriosclerotic/atherosclerotic vascular lesion formation (Tsutsui, 2004). 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. It has been reported that vascular nNOS expression is induced by inflammatory/proliferative stimuli (angiotensin II, interleukin-1β, and platelet-derived growth factor), hypoxia, hypertensive situation, and statin treatment (Boulanger et al., 1998; Ebrahimian et al., 2003; Nakata et al., 2005, 2007; Ward et al., 2005).

Arteriosclerotic vascular lesion formation was studied in the triply NOS$^{-/-}$ mice at 2 and 5 months of age. At 2 months of age, no significant vascular lesions were seen, whereas, at 5 months of age, significant arteriosclerotic vascular lesion formation (neointimal formation, medial thickening, and perivascular fibrosis) was noted in the triply NOS$^{-/-}$ mice, but not in the eNOS$^{-/-}$ mice, as compared with the WT mice, in both large epicardial coronary arteries and coronary microvessels. Atherosclerotic lesion formation with lipid accumulation was also observed in the aorta. These results indicate the protective role of the NOS system in arteriosclerosis. A comparable extent of hypertension was seen in the eNOS$^{-/-}$ and triply NOS$^{-/-}$ genotypes, whereas vascular lesion formation was observed only in the triply NOS$^{-/-}$ genotype, but not in the eNOS$^{-/-}$ genotype, indicating a minor role of hypertension in the vascular lesion formation in the triply NOS$^{-/-}$ genotype.

2.3. Myocardial infarction

Neither the deletion of the eNOS gene nor the pharmacological inhibition of eNOS activity induces MI in animals. However, the triply NOS$^{-/-}$ mice experienced spontaneous myocardial infarction (MI) (Fig. 3A). This is the first in vivo demonstration showing that the defective NOS system is involved in the occurrence of spontaneous MI. Human MI results not only from thrombotic disruption of coronary atheromatous plaques, but also from other causes, including coronary intimal hyperplasia, medial thickening, and coronary vasospasm (Antman & Braunwald, 2005; Vanhoutte & Shimokawa, 1989). In the triply NOS$^{-/-}$ mice that died of MI, marked coronary intimal hyperplasia and medial thickening were noted (Fig. 3A,B). Furthermore, in the dead triply NOS$^{-/-}$ mice, a marked infiltration of mast cells at the coronary artery adventitia was also observed (Fig. 3C). Histamine released from adventitial mast cells is thought to cause coronary vasospasm with resultant MI in humans (Laine et al., 1999). It is thus possible that coronary intimal hyperplasia, medial thickening, and vasospasm are involved in the pathogenesis of spontaneous MI in the triply NOS$^{-/-}$ mice (Fig. 4).

In the triply NOS$^{-/-}$ 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 (Nakata et al., 2008). These vascular dysfunctions could also be involved in the pathogenesis of spontaneous MI in the triply NOS$^{-/-}$ mice (Fig. 4).

2.4. Cardiovascular risk factors

Metabolic syndrome is defined as a constellation of interrelated cardiovascular risk factors of metabolic origin, including visceral obesity, hypertension, hypertriglyceridemia, impaired glucose tolerance, and

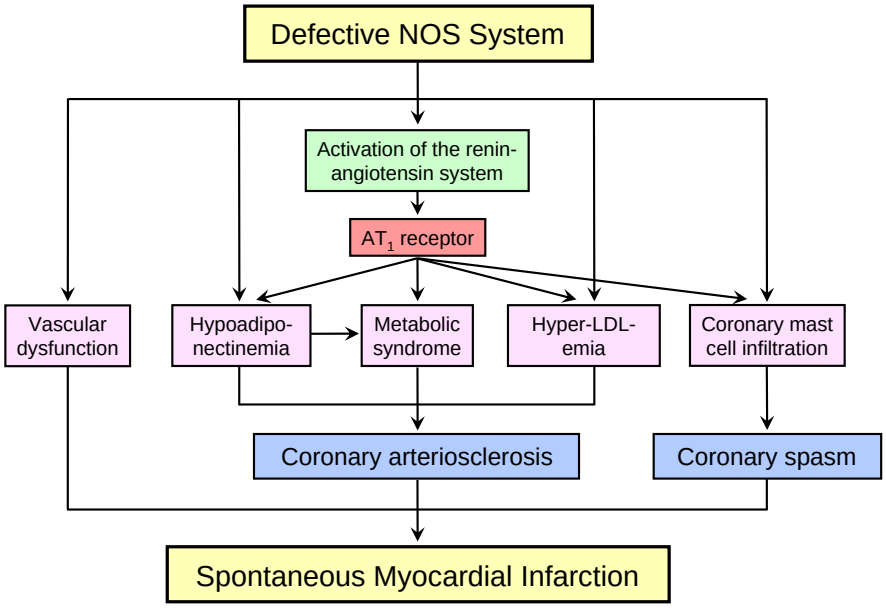

Fig. 4. Mechanisms for spontaneous MI caused by the defective NOS system in mice in vivo. Genetic disruption of all NOSs caused metabolic syndrome, hypoadiponectinemia, hyper-low-density-lipoprotein (LDL)-emia, coronary adventitial mast cell infiltration, and vascular dysfunction. Those factors could contribute to the pathogenesis of spontaneous MI. Importantly, long-term pharmacological blockade of the angiotensin II type 1 (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.
Diagram from Tsutsui et al. (2008).

insulin resistance (Nakamura et al., 2001; Takeno et al., 2008). Accumulation of 3 or more risk factors dramatically increases the risk of morbidity by arteriosclerotic cardiovascular diseases by 11-fold, indicating that metabolic syndrome is an important therapeutic target for the prevention and treatment of cardiovascular diseases (Nakamura et al., 2001; Takeno et al., 2008). The triply NOS$^{-/-}$ mice manifested phenotypes that closely resemble metabolic syndrome in humans (Nakata et al., 2008). It is therefore possible that the NOS system plays important roles in preventing metabolic syndrome.

Adiponectin is an anti-atherogenic adipocytokine, improving hypertriglyceridemia, glucose metabolism, and insulin resistance, and inhibiting the progression of arteriosclerosis (Kadowaki et al., 2006; Matsuzawa et al., 2004; Shioji et al., 2007). Under the condition of obesity with adipocyte hypertrophy, synthesis of adiponectin is not increased, but rather decreased, and in patients with metabolic syndrome, the circulating levels of adiponectin are reduced, in contrast to increases in other adipocytokines. The deficiency of adiponectin is thought to play a pivotal role in metabolic syndrome and its vascular complications (Matsuzawa et al., 2004). In the triply NOS$^{-/-}$ mice, plasma adiponectin levels were significantly reduced (Nakata et al., 2008). This suggests that the adiponectin deficiency may contribute to the development of metabolic abnormalities and vascular lesion formation in the triply NOS$^{-/-}$ mice (Fig. 4).

2.5. Sudden cardiac death

A previous study showed that during the 11 months of follow-up, all (100%) of the WT mice lived, whereas only 15% of the triply NOS$^{-/-}$ mice survived (Fig. 5A) (Nakata et al., 2008). The survival rate was significantly worse in accordance with the number of disrupted NOS genes in the order of singly, doubly, and triply NOS$^{-/-}$ mice. Postmortem histopathological analysis revealed that ~55% of the triply NOS$^{-/-}$ mice possibly died due to spontaneous MI (Fig. 5B), indicating the pivotal role of the NOS system in sudden cardiac death (Pabla & Curtis, 1995, 1996). The second and third causes of death in the triply NOS$^{-/-}$ mice were renal disease (~25%) and ileus (~10%), respectively (Fig. 5B). We have demonstrated that the triply NOS$^{-/-}$ mice manifest

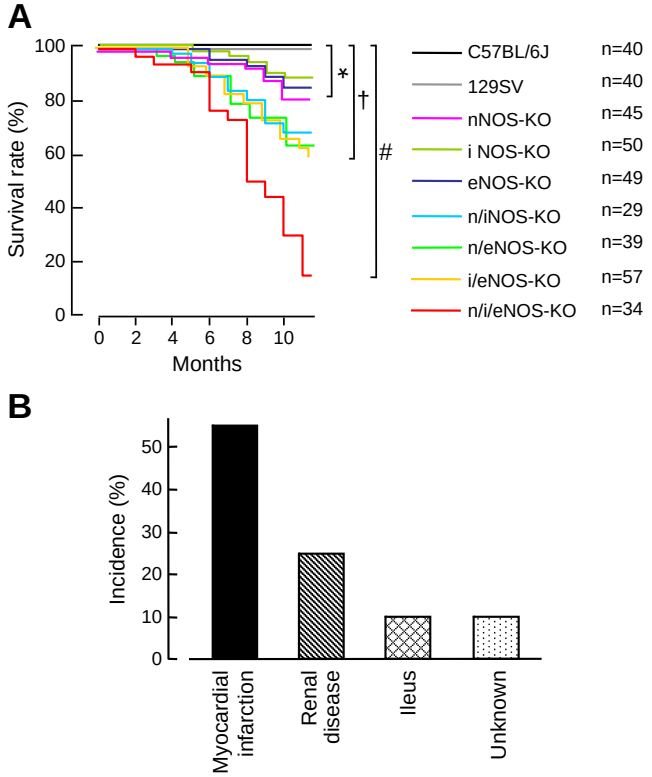

Fig. 5. Decreased survival and causes of death in triply NOS$^{-/-}$ mice. (**A**) Survival rate (n = 29–57). Red line represents markedly reduced survival in the triply NOS$^{-/-}$ mice. *, †, and #: $P<0.05$ between WT C57BL/6J vs. singly, doubly, and triply NOS$^{-/-}$, respectively. The "n" represents the number of mice used in each group. (**B**) Causes of death (n = 20). Data from Nakata et al. (2008).

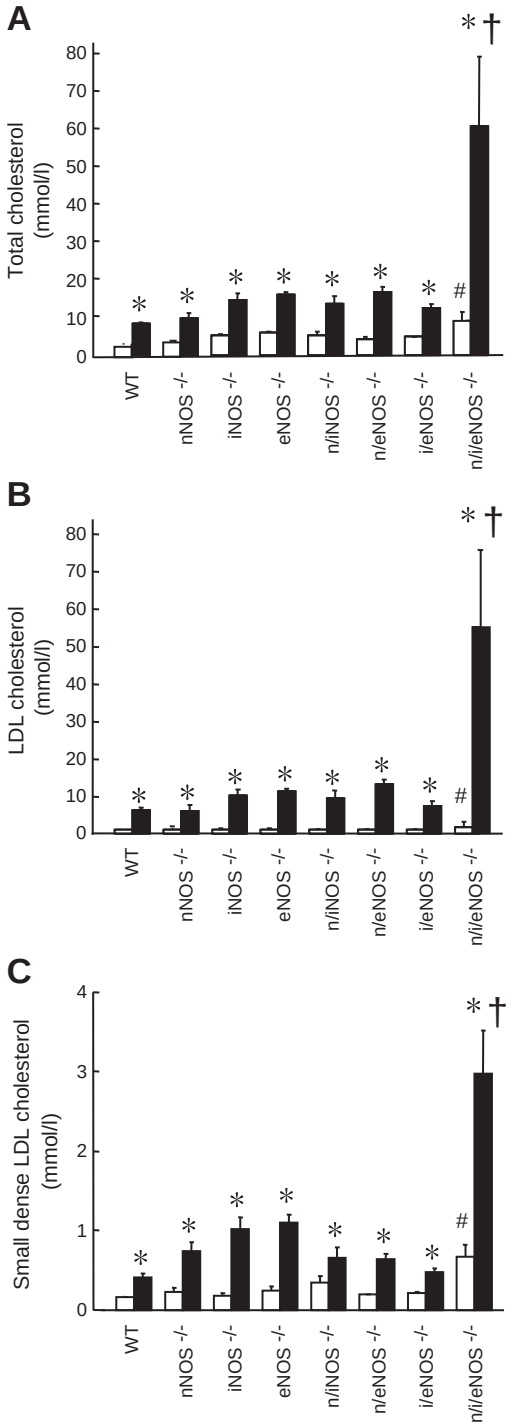

Fig. 6. Serum lipid profile in WT and NOS$^{-/-}$ mice fed a regular or high-cholesterol diet for 3 months (n = 6–11). White and black bars indicate the regular and high-cholesterol diets, respectively. WT, C57BL/6. *$P<0.05$ vs. the regular diet; †$P<0.05$ vs. WT mice fed the high-cholesterol diet; #$P<0.05$ vs. WT mice fed the regular diet. Data from Yatera et al. (2010).

nephrogenic diabetes insipidus associated with renal tubuloglomerular lesion formation (Morishita et al., 2005). In some dead triply NOS$^{-/-}$ mice, we detected severe renal tubuloglomerular lesions but no other causative pathological abnormalities. In this case, we judged that the mice died probably due to renal disease (Fig. 5B).

2.6. Diet-induced dyslipidemia

A recent study has examined the effect of the Western-type cholesterol-rich diet on lipid metabolism in triply NOS$^{-/-}$ mice (Yatera et al., 2010). In response to the high-cholesterol diet, the triply NOS$^{-/-}$ mice, but not the singly or doubly NOS$^{-/-}$ mice, exhibited marked increases in serum total cholesterol levels (Fig. 6A). These increases were due to alterations in the serum levels of low-density lipoprotein (LDL) cholesterol (Fig. 6B) and small dense LDL particles (Fig. 6C), both of which are important cardiovascular risk factors (Sniderman et al., 2003), but not due to alterations in the serum levels of high-density lipoprotein (HDL) cholesterol or triglyceride. These results suggest that the NOS system plays a key role in the regulation of lipid metabolism. Consistent with this evidence, NO supplementation by overexpression of the eNOS gene in transgenic mice decreases plasma total and LDL cholesterol levels (van Haperen et al., 2002).

It was next examined whether the cholesterol-rich diet would elicit atherosclerotic vascular lesion formation. Although in the WT, singly, and doubly NOS$^{-/-}$ genotypes, the high-cholesterol diet tended to induce lipid accumulation in the aortas, these effects did not reach statistically significant levels (Fig. 7). However, in the triply NOS$^{-/-}$ genotype, the high-cholesterol diet significantly and markedly caused aortic lipid accumulation (Fig. 7). In addition, the high-cholesterol diet also significantly and markedly elicited atheromatous plaque formation in the aortic sinus only in the triply NOS$^{-/-}$ genotype. In those atheromatous plaque lesions, conglomerated foamy macrophages and necrotic lipid cores with fibrous caps were noted. These findings are recognized in the advanced stage of human atherosclerosis. Thus, the features of the atherosclerotic lesions that were developed in the triply NOS$^{-/-}$ mice are similar to those described in humans (Libby, 2008), and therefore represent an important model for human dyslipidemia and atherosclerosis.

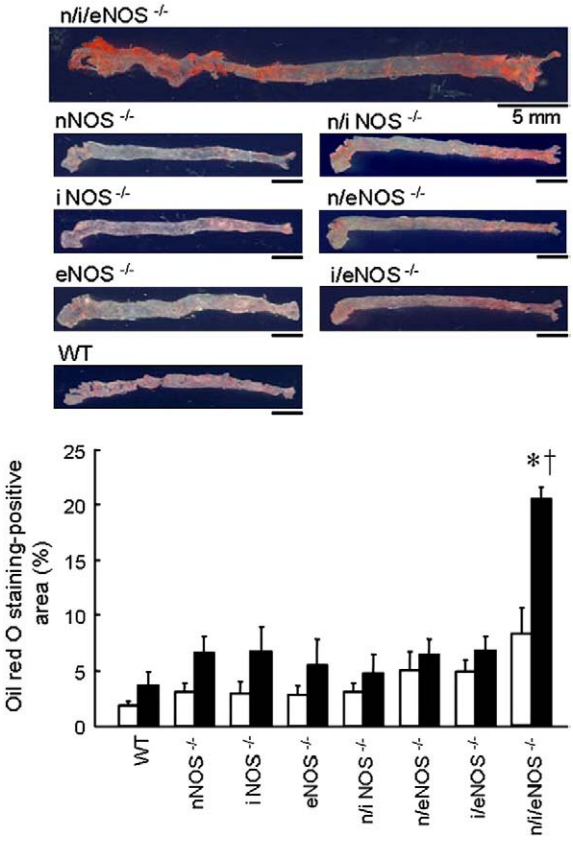

Fig. 7. Lipid accumulation in longitudinally opened aortas of WT and NOS$^{-/-}$ mice fed a high-cholesterol diet (oil red O staining) (n = 6–11). Red color indicates positive staining. White and black bars represent the regular and high-cholesterol diets, respectively. WT, C57BL/6. *P<0.05 vs. the regular diet; †P<0.05 vs. WT mice fed the high-cholesterol diet.
Data from Yatera et al. (2010).

Since some triply NOS$^{-/-}$ mice died during the cholesterol-rich feeding, a postmortem histopathological analysis was performed to identify the cause of death. Markedly accelerated coronary vascular lesion formation and pulmonary congestion were noted in all the dead triply NOS$^{-/-}$ mice, and old myocardial infarction and giant organized thrombi in both ventricles were found in some of them. Thus, it is likely that the triply NOS$^{-/-}$ mice died of cardiovascular death.

Finally, the mechanism(s) for dyslipidemia in the triply NOS$^{-/-}$ mice fed the cholesterol-rich diet was investigated. Dietary cholesterol is absorbed into the body through the cholesterol transporter Nieman-Pick C1-like 1 (NPC1L1) in the small intestine, and circulating LDL cholesterol in the blood is bound to the LDL receptor in the liver, taken up and broken down by hepatocytes. The expression levels of the small intestinal NPC1L1 were not altered in the triply NOS$^{-/-}$ mice, whereas the expression levels of the hepatic LDL receptor were markedly reduced only in the triply NOS$^{-/-}$ mice (Fig. 8A), in parallel with alterations in the serum LDL cholesterol levels. Sterol regulatory element-binding protein-2 (SREBP-2) was discovered as a transcriptional factor that controls LDL receptor gene expression (Hua et al., 1993). The activity of SREBP-2 was also diminished only in the triply NOS$^{-/-}$ mice (Fig. 8B). It is possible that the lower expression of the hepatic LDL receptor mediated by reduced SREBP-2 activity is involved in the diet-induced dyslipidemia in the triply NOS$^{-/-}$ genotype. These results demonstrate that complete disruption of the NOS system causes severe diet-induced dyslipidemia, lipid-rich atherosclerotic lesion formation, and sudden cardiac death in mice in vivo through the down-regulation of the hepatic LDL receptor, demonstrating the critical role of the whole endogenous NO/NOS system in the regulation of lipid metabolism.

2.7. Nephrogenic diabetes insipidus

The first reported phenotype in the triply NOS$^{-/-}$ mice was abnormality of the kidney (Morishita et al., 2005). The triply NOS$^{-/-}$ mice showed hypotonic polyuria, polydipsia, and reduced antidiuretic response to exogenous vasopressin. Those findings were accompanied by impaired renal cAMP production, defective membrane expression of the aquaporin-2 water channel, and tubuloglomerular lesions. These phenotypes closely resemble the human disorder nephrogenic diabetes insipidus, demonstrating a novel aspect of the important roles of endogenous NO in maintaining renal homeostasis.

3. Elucidation of the role of nitric oxide synthases in the cardiovascular system using triply NOS$^{-/-}$ mice

3.1. Role of nitric oxide synthase system in endothelium-dependent hyperpolarization

The endothelium plays an important role in maintaining vascular homeostasis by synthesizing and releasing several relaxing factors, such as prostacyclin, NO, and endothelium-derived hyperpolarizing factor (EDHF). It was previously demonstrated in animals and humans that endothelium-derived hydrogen peroxide (H_2O_2) is an EDHF that is produced in part by eNOS. On the other hand, a recent study showed that genetic disruption of all NOS isoforms abolishes EDHF responses in mice (Takaki et al., 2008). The contribution of the NOS system to

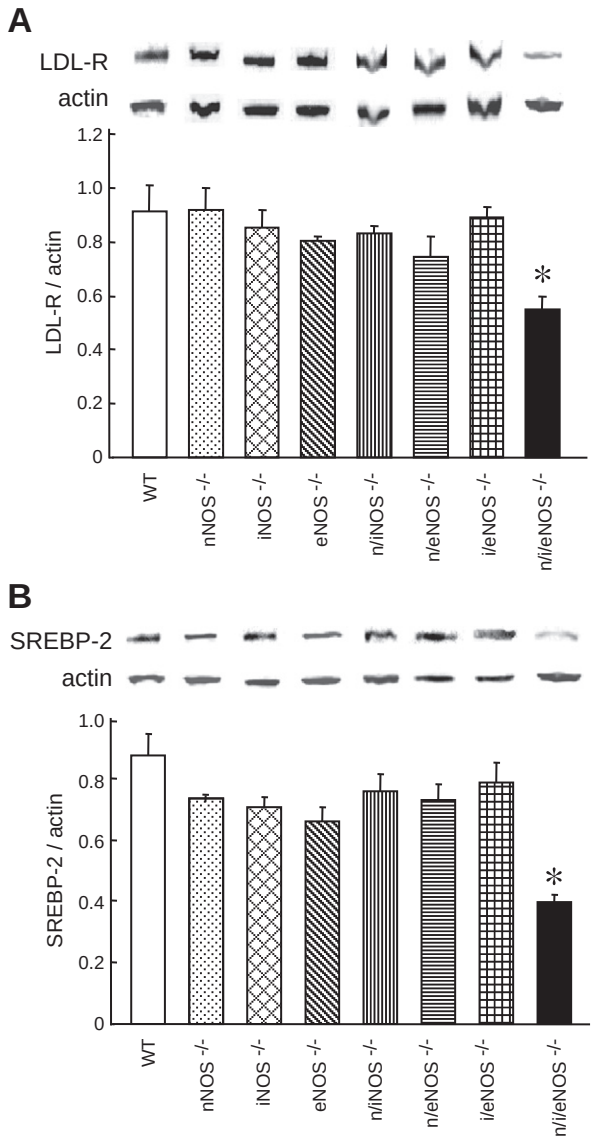

Fig. 8. LDL receptor expression levels (**A**) and sterol regulatory element-binding protein-2 (SREBP-2) activity (**B**) in the liver of WT and NOS$^{-/-}$ mice fed a high-cholesterol diet (Western blotting) (n = 6–11). WT, C57BL/6 *P<0.05 vs. WT. Data from Yatera et al. (2010).

EDHF-mediated responses was examined in the eNOS$^{-/-}$, n/eNOS$^{-/-}$, and n/i/eNOS$^{-/-}$ mice. EDHF-mediated relaxation and hyperpolarization in response to acetylcholine of mesenteric arteries were progressively reduced as the number of disrupted NOS genes increased, whereas vascular smooth muscle function was preserved. Loss of eNOS expression alone was compensated for by other NOS genes, and endothelial cell production of H_2O_2 and EDHF-mediated responses were completely absent in the triply n/i/eNOS$^{-/-}$ mice, even after antihypertensive treatment with hydralazine. NOS uncoupling, which is caused by a deficiency of tetrahydrobiopterin (BH_4), a cofactor of NOS, was not involved, as modulation of BH_4 synthesis had no effect on EDHF-mediated relaxation, and the BH_4/dihydrobiopterin (BH_2) ratio was comparable in the mesenteric arteries and the aorta. These results demonstrate that EDHF-mediated responses are totally dependent on the NOSs system in mouse mesenteric arteries. Collectively, this study provides a novel concept of the diverse roles of the endothelial NOS system mainly contributing to the EDHF/H_2O_2 responses in small-sized arteries while serving as a NO-generating system in large arteries (Fig. 9).

3.2. Role of nitric oxide synthase system in statin-induced vascular nitric oxide production

A previous study examined whether statins enhance vascular NOS expression, and if so, how much each NOS isoform-derived NO accounts for atorvastatin-induced NOx production (Nakata et al., 2007). Organ culture experiments using mouse aortas with endothelium were performed. In the isolated aortas of the WT mice, treatment with atorvastatin for 2 days significantly enhanced the protein expression of all three NOSs and NOx accumulation in a culture medium. A significant increase in atorvastatin-induced NOx accumulation in the culture medium was also seen in isolated aortas of the doubly i/eNOS$^{-/-}$ (expressing nNOS alone), the n/eNOS$^{-/-}$ (expressing iNOS alone), and the n/iNOS$^{-/-}$ mice (expressing eNOS alone), and the extent of the increase was ~25%, 25%, and 50%, respectively, as compared with the WT mice. On the other hand, no significant increase in atorvastatin-induced NOx accumulation in the culture medium was seen in the isolated aortas of the triply NOS$^{-/-}$ mice. These results suggest that atorvastatin up-regulates the vascular expression of all NOS isoforms, and that nNOS, iNOS, and eNOS account for ~25%, 25%, and 50%, respectively, of the atorvastatin-induced NOx production.

4. Therapeutic relevance

Several lines of evidence suggest an association of the defective NOS system with cardiovascular risk factors, coronary arteriosclerosis, and MI in humans. First, it has been reported that plasma and/or urinary NOx levels, which are markers of NO production derived from all three NOSs in vivo, are reduced in patients with cardiovascular risk factors and in those with coronary arteriosclerosis (Kurioka et al., 2000; Node et al., 1997; Piatti et al., 2003; Tanaka et al., 1997). Second, plasma concentrations of asymmetric dimethylarginine (ADMA), which is an endogenous NOS inhibitor, have been shown to be elevated in patients with cardiovascular risk factors, with arteriosclerosis, and with risk of MI (Cooke, 2005). Finally, it has been revealed in humans that the gene polymorphisms of individual NOS are associated with cardiovascular risk factors, arteriosclerosis, risk of MI, and low plasma NOx levels (Cook, 2006). These results may imply a clinical significance of the findings with the triply NOS$^{-/-}$ mice.

The renin–angiotensin system plays an important role in the pathogenesis of cardiovascular diseases (Dzau et al., 2002; Ernsberger & Koletsky, 2006). In the triply NOS$^{-/-}$ mice, the renin–angiotensin system, as evaluated by tissue levels of angiotensin-converting enzyme and angiotensin II type 1 (AT_1) receptor and plasma levels of renin and angiotensin II, was activated (Nakata et al., 2008). Based on these results, it was studied whether the AT_1 receptor blocker (ARB) ameliorates cardiovascular abnormalities of the triply NOS$^{-/-}$ mice. The long-term oral treatment with an ARB olmesartan potently inhibited coronary arteriosclerotic lesion formation, adventitial mast cell infiltration, and the occurrence of MI in the triply NOS$^{-/-}$ mice, with a resultant improvement of the prognosis. Furthermore, the treatment with olmesartan reversed all the abnormal metabolic phenotypes, along with amelioration of hypoadiponectinemia. These results suggest the beneficial effect of ARB on the cardiovascular abnormalities of the triply NOS$^{-/-}$ mice. Although the long-term treatment with hydralazine lowered the blood pressure levels of the triply NOS$^{-/-}$ mice to the same extent as with olmesartan, the beneficial cardiovascular effects of hydralazine were significantly less than those of olmesartan, in terms of coronary lesion formation, mast cell infiltration, morbidity of MI, survival rate, and metabolic phenotypes. It is therefore conceivable that the beneficial effects of olmesartan are mediated by not only the reduction in blood pressure but also the blockade of the AT_1 receptor. ARBs are widely used in the treatment of hypertension. Recent large randomized trials have revealed that ARBs reduce the progression of coronary atherosclerosis (Hirohata et al., 2010) and major cardiovascular

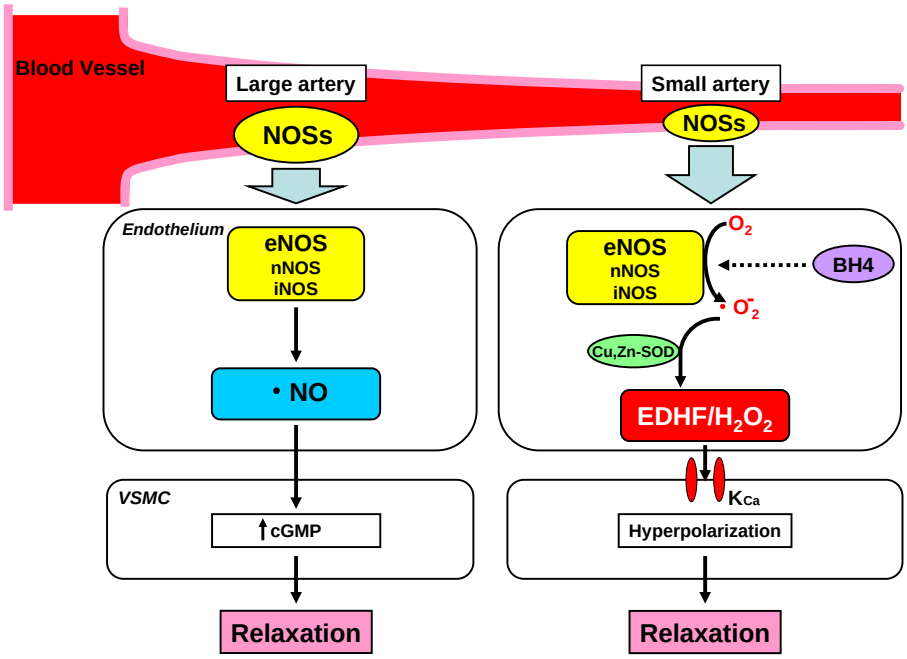

Fig. 9. Crucial role of NOS system in endothelium-dependent hyperpolarization in mice. NO mediates vascular relaxation of relatively large, conduit arteries (e.g., aorta and epicardial coronary arteries), whereas endothelium-derived hyperpolarizing factor (EDHF) plays an important role in modulating vascular tone in small, resistance arteries (e.g., small mesenteric arteries and coronary microvessels). All three NOS isoforms (nNOS, iNOS, and eNOS), especially eNOS, produce NO and superoxide anions, and the latter is dismutated by Cu,Zn-superoxide dismutase (SOD) to EDHF/hydrogen peroxide (H_2O_2). EDHF hyperpolarizes vascular smooth muscle cells (VSMCs) by opening K_{Ca} channels, and then elicits vasodilation. On the other hand, superoxide anions from uncoupled NOSs may not significantly contribute to EDHF-mediated relaxations. Collectively, this study provides a novel concept of the diverse roles of endothelial NOSs system mainly contributing to the EDHF/H_2O_2 responses in microvessels while serving as a NO-generating system in large arteries. Diagram from Takaki et al. (2008).

events (i.e., cardiovascular death and MI) (Yusuf et al., 2008). The observations in the triply $NOS^{-/-}$ mice support such a benefit of ARBs in the clinical setting.

NO donors are employed in the treatment of ischemic heart disease. A single sublingual administration of nitroglycerin relieves an anginal attack, and short-term intravenous administration of NO donors in the acute phase f MI has been shown to reduce infarct size and ameliorate cardiac function, cardiac remodeling and mortality (Bussmann et al., 1981; Jugdutt & Warnica, 1988; Rapaport, 1985). However, when NO donors are continuously applied for an extended period of time, no positive results have been obtained. Large randomized clinical trials have revealed that the long-term oral treatment with NO donors fails to improve the mortality rate in patients with acute MI (GISSI-3, 1994; ISIS-4, 1995), and small non-randomized observational studies have reported that it rather exacerbates the prognosis in patients with old MI (Ishikawa et al., 1996; Kanamasa et al., 2000). These deleterious actions of the long-term treatment with NO donors may be due to the nitrate tolerance caused by long-term continuous administration of NO donors, to the cardiac overload associated with increased neurohumoral factors, or to the rebound phenomenon induced by its abrupt cessation (Daiber et al., 2008). On the other hand, it has been reported that long-term oral treatment with nicorandil, which has actions of both a NO donor and an ATP-sensitive K^+ channel opener and becomes less likely to elicit the nitrate tolerance, significantly reduces cardiovascular death and the occurrence of MI in patients with stable angina pectoris (IONA Study Group, 2002). Whether nicorandil may be favorable in a broad spectrum of cardiovascular disorders remains to be determined in future studies.

5. Future perspectives

What are the major pending problems in this research area? Recent advances in recombinant DNA technology enable us to study the effects of the site- and time-specific knockout of target genes by the use of Cre/loxP or Flp/Frt recombination. On the other hand, our triply $NOS^{-/-}$ mice are conventional null knockout mice, and no conditional mice devoid of all subtypes of NOSs have been generated. This issue remains to be studied in future research.

How should we utilize various lines of $NOS^{-/-}$ mice in the future? There are all types of $NOS^{-/-}$ animals, including singly, doubly, and triply $NOS^{-/-}$ mice (Table 1). Furthermore, many 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 generated (Table 3) (Brunner et al., 2001; Burkard et al., 2007; Heger et al., 2002; Janssens et al., 2004; Loyer et al., 2008; Mungrue et al., 2002a; Ohashi et al., 1998; Packer et al., 2005; Takamura et al., 1998; van Haperen et al., 2002). Analysis with various combinations of those genetically manipulated mice will further improve our knowledge about the

Table 3
Mice Overexpressing the NOS Gene That Have Thus Far Been Established.

TG Mice	Overexpression site	Promoter used	References
nNOS-TG	myocardium (conditional)	α-MHC	Burkard et al. (2007)
	myocardium (conditional)	α-MHC	Loyer et al. (2008)
	brain	CaMKIIα	Packer et al. (2005)
iNOS-TG	myocardium (conditional)	α-MHC	(Mungrue et al., 2002a,b)
	myocardium	α-MHC	Heger et al. (2002)
	pancreatic β cell	insulin	Takamura et al. (1998)
eNOS-TG	endothelium	preproendothelin-1	Ohashi et al. (1998)
	endothelium	eNOS	van Haperen et al. (2002)
	myocardium	α-MHC	Brunner et al. (2001)
	myocardium	α-MHC	Janssens et al. (2004)

CaMKII, calcium-calmodulin multifunctional kinase II; MHC, myosin heavy chain; TG, transgenic.

significance of the defective NO/NOS system in human cardiovascular disorders.

6. Concluding remarks

The mouse is the most ideal genetically modifiable mammalian presently available (Mungrue et al., 2002b). Studies with the triply $NOS^{-/-}$ model provide pivotal insights into the cardiovascular roles of NOSs at the molecular level. The obtained results have demonstrated that the entire endogenous NOS system plays a pathogenetic role in a variety of cardiovascular diseases, including arteriosclerosis/atherosclerosis, myocardial infarction, and dyslipidemia. Moreover, the findings have indicated that the NOS system plays a crucial role in endothelium-dependent hyperpolarization and statin-induced vascular NO production. The evidence could contribute to a better understanding of the pathophysiological relevance of NO signaling in the cardiovascular system. In the triply $NOS^{-/-}$ mutant model, long-term treatment with the ARB significantly suppressed coronary arteriosclerotic lesion formation and the occurrence of spontaneous MI, and improved the prognosis, suggesting the therapeutic importance of ARBs to prevent cardiovascular diseases in humans. Further studies are certainly needed to clarify whether the outcomes in the triply $NOS^{-/-}$ genetic model can be translated to human patients with cardiovascular disorders.

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Conflict of interest

None declared.

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