Current Perspective

Development of Genetically Engineered Mice Lacking All Three Nitric Oxide Synthases

Masato Tsutsui[1],*, Hiroaki Shimokawa[2], Tsuyoshi Morishita[3], Yasuhide Nakashima[3], and Nobuyuki Yanagihara[1]

[1]*Department of Pharmacology, School of Medicine, University of Occupational and Environmental Health,*
1-1 Iseigaoka, Yahatanishi-ku, Kitakyushu 807-8555, Japan
[2]*Department of Cardiovascular Medicine, Tohoku University Graduate School of Medicine,*
1-1 Seiryo-machi, Aoba-ku, Sendai 980-8574, Japan
[3]*Second Department of Internal Medicine, School of Medicine, University of Occupational and Environmental Health,*
1-1 Iseigaoka, Yahatanishi-ku, Kitakyushu 807-8555, Japan

Abstract. Nitric oxide (NO) is produced in almost all tissues and organs, exerting multiple biological actions under both physiological and pathological conditions. NO is synthesized by three different isoforms of NO synthase (NOS): neuronal, inducible, and endothelial NOSs. Due to the substantial compensatory interactions among the NOS isoforms, the ultimate roles of endogenous NO in our body still remain to be fully elucidated. To address this point, we have successfully developed mice in which all three NOS genes are completely disrupted. NOS expression and activities were totally absent in the triply n/i/eNOS$^{-/-}$ mice before and after treatment with lipopolysaccharide. While the triply n/i/eNOS$^{-/-}$ mice were viable, their survival and fertility rates were markedly reduced as compared with wild-type mice. The phenotypes of those mice that we first noticed were polyuria, polydipsia, and renal unresponsiveness to vasopressin, characteristics consistent with nephrogenic diabetes insipidus. We subsequently observed that in those mice, arteriosclerosis is spontaneously developed with a clustering of cardiovascular risk factors. These results provide the first evidence that the systemic deletion of all three NOSs causes a variety of cardiovascular diseases in mice, demonstrating a critical role of the endogenous NOSs system in maintaining cardiovascular homeostasis.

Keywords: nitric oxide synthase, knockout mouse, cardiovascular disease, nephrogenic diabetes insipidus, metabolic syndrome

Introduction

Nitric oxide (NO), an important intracellular messenger molecule, plays important roles in maintaining homeostasis (1 – 6). NO is formed from its precursor L-arginine by a family of NO synthases (NOSs) with stoichiometric production of L-citrulline. NO is a gaseous free radical that can freely pass through plasma membranes and then elicits multiple actions without receptor coupling either directly or via the soluble guanylate cyclase/cGMP-mediated pathway. Three distinct NOS isoforms exist that are encoded by three distinct genes: neuronal (nNOS, NOS1), inducible (iNOS, NOS2), and endothelial NOS (eNOS, NOS3) (1 – 6). 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. In contrast, iNOS is induced when stimulated by microbial endotoxins or certain proinflammatory cytokines, producing a larger amount of NO in a calcium-independent manner (1 – 6).

Genetically engineered animals are a powerful experimental tool for studying the function of target genes

in vivo. Animals bearing targeted deletions of singly and doubly NOS isoforms have thus been developed (7 – 17). nNOS-deficient (nNOS$^{-/-}$) mice exhibit hypertrophy of the pyloric sphincter (9), increased aggressive behavior (13), and protection from cerebral ischemia (18). eNOS$^{-/-}$ mice are hypertensive (10), susceptible to cerebral ischemia (19), and show accelerated vascular lesion formation (20). The principle phenotype of iNOS$^{-/-}$ mice is an increased susceptibility to infection with pathogens and resistance to the hypotensive response when exposed to bacterial lipopolysaccharide (LPS) (17). In doubly n/eNOS$^{-/-}$ mice, but not in singly nNOS$^{-/-}$ or eNOS$^{-/-}$ mice, hippocampal long-term potentiation is blunted (15). Thus, the roles of individual NOS have been extensively studied. However, due to the substantial compensatory interactions among the NOS isoforms (15), the ultimate roles of NO derived from the whole NOS system remain to be fully elucidated.

We have recently succeeded in developing mice in which all three NOS isoforms are totally deficient (triply n/i/eNOS$^{-/-}$ mice) (Fig. 1A) (21). In this review, we first introduce our previous studies on NO that led us to develop the triply n/i/eNOS$^{-/-}$ mice and then describe in detail the generation and the initial functional analysis of those mice.

Our previous studies on NO

First, we examined the molecular mechanism for endothelial NO synthesis in pigs and humans. There are at least two different signal transduction pathways involved in the synthesis of NO by endothelial cells, Gi-protein– and Gq-protein–mediated pathways. We revealed that the Gi-protein–mediated NO production is selectively impaired in arteriosclerosis (22, 23), partly due to reduced expression of endothelial Gi protein (24, 25). This notion was subsequently confirmed in humans (26).

Secondly, we examined the effect of increasing intracellular tetrahydrobiopterin concentrations on endothelial NO synthesis in isolated canine cerebral arteries. We showed that tetrahydrobiopterin has dual effects, one is the inhibitory effect on NO production via superoxide generation induced by auto-oxidation of tetrahydrobiopterin, and the other is the stimulatory effect on NO production as a cofactor of eNOS activation (27, 28). These results suggest that the balance between intracellular tetrahydrobiopterin levels and the activity of antioxidant enzymes (e.g., superoxide dismutases) may be an important determinant of endothelial NO production (27, 28).

Thirdly, we studied the potential usefulness of eNOS gene therapy in cerebral arteries ex vivo and in vivo. We demonstrated that adventitial expression of recombinant eNOS gene restores NO production in arteries without endothelium, causing adventitia-dependent endothelium-independent relaxations (29 – 32). Our findings provide a novel concept that the vascular adventitia could play a role in the regulation of vascular tone after successful transfer and expression of recombinant eNOS gene
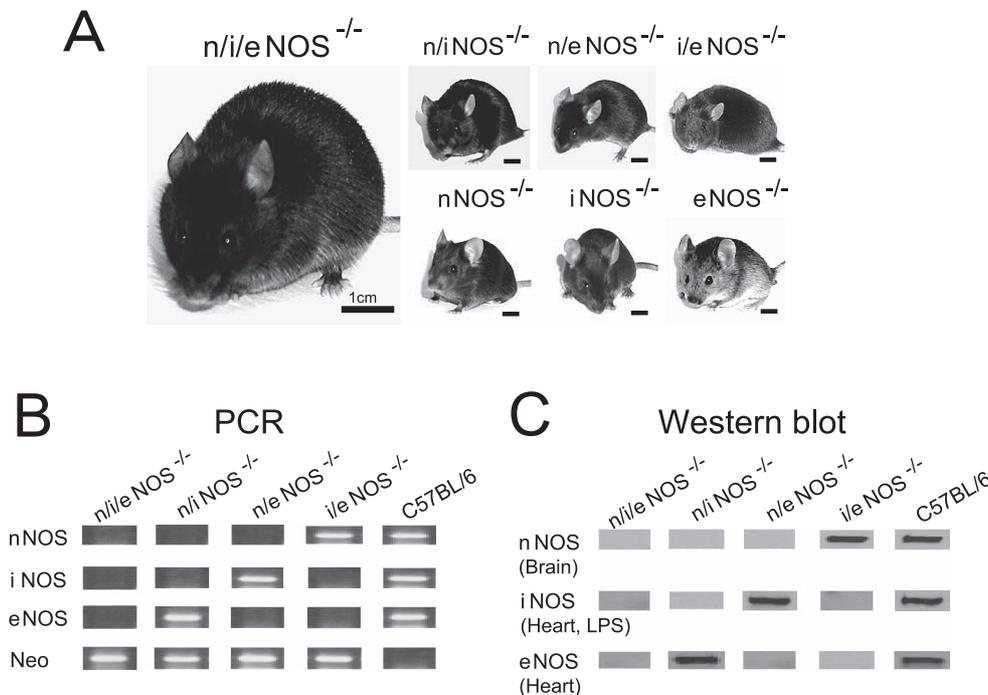
Fig. 1. Appearance (A), PCR analysis (B), and Western blot analysis (C) of wild-type and NOS$^{-/-}$ mice. A: We first produced 3 kinds of doubly NOS$^{-/-}$ mice by crossbreeding singly NOS$^{-/-}$ mice. The doubly NOS$^{-/-}$ mice were then crossed, and triply n/i/eNOS$^{-/-}$ mice were finally generated. B: PCR analysis in tail genomic DNA indicated the presence of all three NOS genes in the wild-type C57BL/6 mice, but the absence of the genes in the triply n/i/eNOS$^{-/-}$ mice. C: Western blot analysis also revealed the absence of all three NOS proteins in the brain and the heart of triply n/i/eNOS$^{-/-}$ mice before and after treatment with lipopolysaccharide (LPS, 20 mg /kg IP, 12 h). Modified from ref. 21.

(29 – 32). It is also implicated that perivascular eNOS gene delivery is a feasible new therapeutic strategy for cerebrovascular diseases.

Fourthly, we clarified the NO-independent vascular actions of L-arginine analogues (e.g., a synthetic analogue, N^{ω}-nitro-L-arginine methyl ester, and an endogenous analogue, asymmetric dimethylarginine). Although long-term treatment with L-arginine analogues had long been believed without doubt to simply inhibit endothelial NO synthesis and cause arteriosclerotic vascular lesion formation, we found that the long-term vascular effects of L-arginine analogues are not mediated by simple inhibition of endothelial NO synthesis as those analogues caused similar cardio-vascular lesions in both wild-type and eNOS$^{-/-}$ mice (33, 34). Indeed, 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 those analogues (33, 34). Our findings questioned the previous theory regarding the pro-atherogenic effects of L-arginine analogues and warranted re-evaluation of previous studies with those analogues (33, 34).

Fifthly, we performed lines of studies regarding the vasculoprotective roles of nNOS. Although the regulatory roles of eNOS and iNOS on the development of arteriosclerosis have been extensively investigated, little is known about the role of nNOS. We showed that in a carotid artery ligation model, nNOS$^{-/-}$ mice exhibited accelerated neointimal formation and constrictive vascular remodeling caused by blood flow disruption and that in a rat balloon injury model, selective inhibition of nNOS activity significantly enhanced vaso-

constrictor responses to calcium-mobilizing stimuli and exacerbated neointimal formation (35). These results provide the first evidence that nNOS plays important roles in suppressing arteriosclerotic vascular lesion formation (35, 36). In line with our findings, a recent study reported the anti-atherogenic effect of nNOS in apolipoprotein E$^{-/-}$ mice (37). We further showed that nNOS could act as an "inducible" enzyme in the vascular system, in contrast to the nervous system, and that vascular nNOS expression is up-regulated in response to growth factors (e.g., angiotensin II and platelet-derived growth factor), inflammatory cytokines (e.g., interleukin-1β), and HMG-CoA reductase inhibitors (35, 36, 38).

In the series of experiments mentioned above, we were able to demonstrate that all three NOS isoforms play roles in the development of arteriosclerosis, but due to the compensated interactions among the three NOS isoforms, the role of the whole NO/NOSs system remains to be elucidated. Therefore, in order to address this point, we decided to establish genetically engineered mice lacking all NOSs.

Generation of triply n/i/eNOS$^{-/-}$ mice

We first produced three kinds of doubly NOS$^{-/-}$ mice (n/iNOS$^{-/-}$, n/eNOS$^{-/-}$, and i/eNOS$^{-/-}$ mice) by crossing singly NOS$^{-/-}$ mice (nNOS$^{-/-}$, iNOS$^{-/-}$, and eNOS$^{-/-}$ mice). The doubly NOS$^{-/-}$ mice were then interbred over 4 to 5 generations, and triply n/i/eNOS$^{-/-}$ mice were finally developed (Fig. 1A) (21).

NO has been implicated in many biological phenomena (1 – 6). Therefore, when we started this project, we
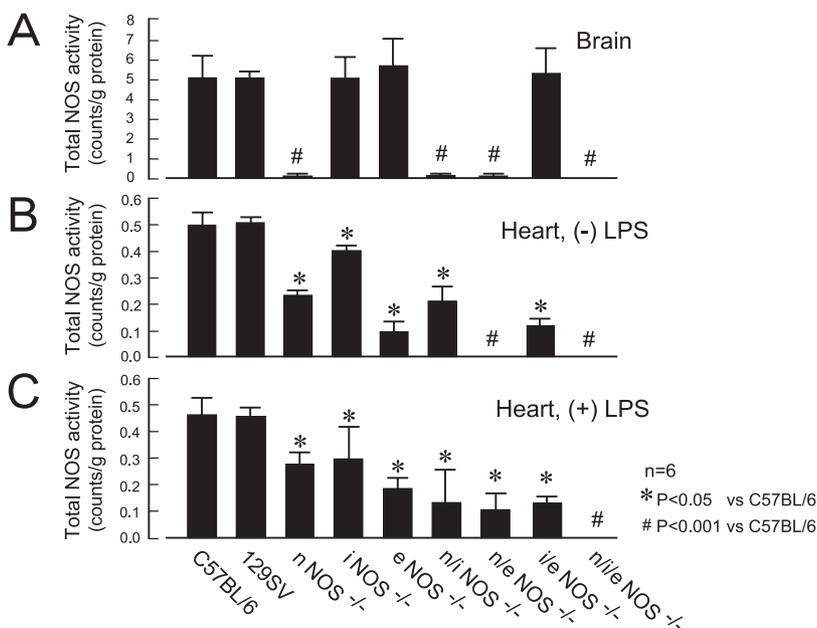
Fig. 2. Total NOS activity in the brain and the heart of wild-type and NOS$^{-/-}$ mice. A: In the normal brain, total NOS activity was markedly reduced in nNOS$^{-/-}$ mice as compared with the wild-type mice, and it was completely absent in the triply n/i/eNOS$^{-/-}$ mice. B: In the normal heart, total NOS activity was significantly reduced in nNOS$^{-/-}$, iNOS$^{-/-}$, and eNOS$^{-/-}$ mice, and it was also completely deficient in the triply n/i/eNOS$^{-/-}$ mice. C: In addition, in the heart treated with lipopolysaccharide, total NOS activity was again undetected in the triply n/i/eNOS$^{-/-}$ mice. Modified from ref. 21.
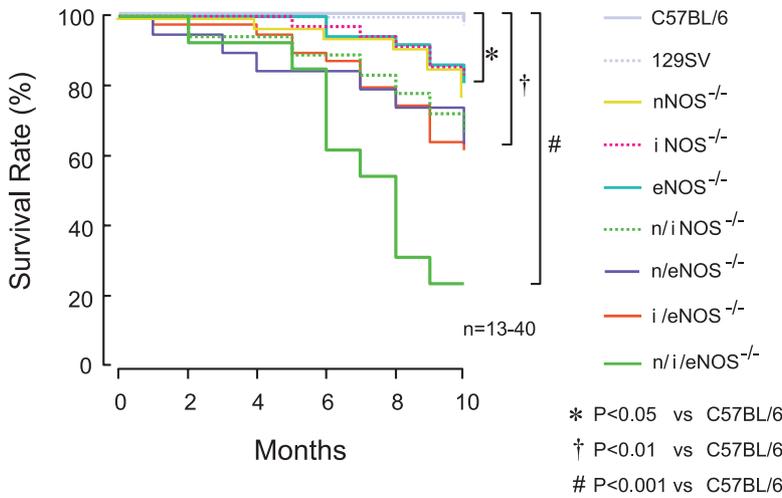
Fig. 3. Survival rate in both genders of wild-type and NOS$^{-/-}$ mice. During the 10 months of follow-up, all the wild-type mice lived, whereas approximately 80% of the triply n/i/eNOS$^{-/-}$ mice died. Survival rate was significantly worse in proportion to the number of disrupted NOS isoforms in the order of singly, doubly, and triply NOS$^{-/-}$ mice. Modified from ref. 21.
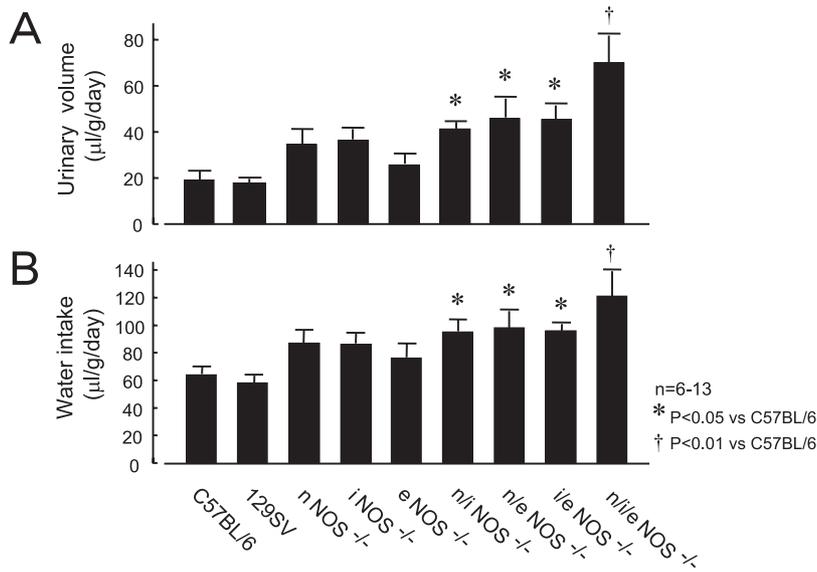
Fig. 4. Urinary volume (A) and water intake (B) in wild-type and NOS$^{-/-}$ mice. A: Urinary volume was significantly increased in accordance with the number of disrupted NOS genes in the order of singly, doubly, and triply NOS$^{-/-}$ mice; and the triply n/i/eNOS$^{-/-}$ mice exhibited approximately 3-fold increase in urinary volume as compared with the wild-type mice. B: Water intake was also significantly enhanced in the order of singly, doubly, and triply NOS$^{-/-}$ mice. Modified from ref. 21.
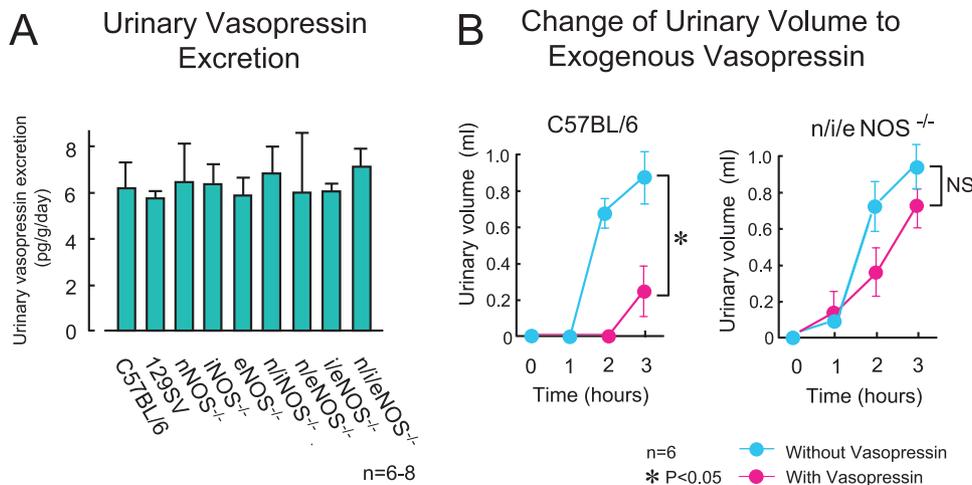
Fig. 5. Central vasopressin release (A) and renal sensitivity to vasopressin (B) in wild-type and NOS$^{-/-}$ mice. A: Central vasopressin secretion, as assessed by urinary vasopressin excretion, was normal in the triply n/i/eNOS$^{-/-}$ mice as compared with the wild-type mice. B: Administration of synthetic vasopressin (0.002 unit, IP) significantly reduced urinary volume in the wild-type C57BL/6 mice, whereas this anti-diuretic effect was markedly impaired in the triply n/i/eNOS$^{-/-}$ mice. From these results, we identified that the triply n/i/eNOS$^{-/-}$ mice suffer from nephrogenic diabetes insipidus. Modified from ref. 21.

expected that triply n/i/eNOS$^{-/-}$ mice could be embryo-lethal, which, however, was not the case. The triply n/i/eNOS$^{-/-}$ mice were fertile and indistinguishable from wild-type animals in appearance (Fig. 1A). They developed normally and were able to mate with normal increase in body weight. The triply n/i/eNOS$^{-/-}$ mice were devoid of all NOSs protein expression and total NOS enzymatic activity (Fig. 1: B and C). Because alternatively spliced forms of nNOS and iNOS have been identified (39), we paid a special attention to the possible remaining NOS expression and function. In the triply n/i/eNOS$^{-/-}$ mice, however, we did not detect any expression (Fig. 1: B and C) or enzyme activity of nNOS or iNOS in the brain, heart (Fig. 2), or kidney (data not shown) either before or after administration of lipopolysaccharide (Fig. 2). Thus, the NOS system is completely disrupted in our triply n/i/eNOS$^{-/-}$ mice.

We next studied whether systemic NO production was also completely defective in the triply n/i/eNOS$^{-/-}$ mice. As expected, both plasma NOx concentrations and urinary NOx excretion, markers of systemic NO production, were extremely low (approx. 3% of normal value) in the triply n/i/eNOS$^{-/-}$ mice, indicating that most of endogenous NO is derived from NOSs. The remaining NO production in the triply NOS$^{-/-}$ mice could be due to a NOS-independent mechanism (40). Indeed, bacteria, especially enterobacterium, synthesize NOx via active nitric acid metabolism, and myoglobin in turn generates NO from NOx (41). In addition, catalase could also produce NO from hydroxylamine or sodium azide (42).

Survival and fertility in triply n/i/eNOS$^{-/-}$ mice

We then examined the survival and the fertility of the triply NOS$^{-/-}$ mice. Experiments were performed in both genders of the following 9 strains: wild-type C57BL and 129SV mice; singly nNOS$^{-/-}$, iNOS$^{-/-}$, and eNOS$^{-/-}$; doubly n/iNOS$^{-/-}$, n/eNOS$^{-/-}$, and i/eNOS$^{-/-}$; and triply n/i/eNOS$^{-/-}$ mice. During the 10-months follow-up, all wild-type mice lived, whereas only 3 of the 13 triply NOS$^{-/-}$ mice (23%) survived (Fig. 3) (21). Survival rate was significantly worse in accordance with the number of disrupted NOS genes (Fig. 3). The number of offspring produced from breeding pairs was also significantly smaller in the same manner (21). These results indicate an essential role of the NOS system in survival and fertility. The findings also suggest that each NOS isoform individually plays an important role in survival and fertility. We have recently revealed that the survival rate was significantly better in female than in male triply NOS$^{-/-}$ mice (unpublished observation). It is thus conceivable that female hormone plays an important role in the survival of the triply NOS$^{-/-}$ mice.

We next investigated the cause of death in the triply n/i/eNOS$^{-/-}$ mice. Our unpublished observation revealed that more than half of the male triply n/i/eNOS$^{-/-}$ mice died due to myocardial infarction associated with severe arteriosclerotic coronary lesions. In all the dead triply n/i/eNOS$^{-/-}$ mice, adventitial mast cell infiltration of the coronary arteries was noted (21). Since previous studies have revealed that histamine released from mast cells causes coronary artery spasm in the arteriosclerotic coronary artery, myocardial ischemia induced by coronary artery spasm may be involved, at least in part, in the cause of death in the triply n/i/eNOS$^{-/-}$ mice (21).

The mouse with homozygous null mutations in the genes for both the high-density lipoprotein (HDL) receptor SR-BI and apolipoprotein E (apoE) is known as a murine model of spontaneous myocardial infarction (43). However, the longevity of this SR-BI/apoE double knockout mouse is extremely short, and even when fed a standard chow diet, all mice die before 2 months of age. To the best of our knowledge, our triply n/i/eNOS$^{-/-}$ mouse is the first murine model of spontaneous myocardial infarction, and thus can be a useful model to study the pathogenesis of the disorder.

Nephrogenic diabetes insipidus in triply n/i/eNOS$^{-/-}$ mice

Since the triply n/i/eNOS$^{-/-}$ mice were very precious because of poor survival and fertility, we first performed non-invasive analysis without killing the animals. Because our initial observation suggested the presence of polyuria in the triply n/i/eNOS$^{-/-}$ mice, we examined body water balance. The triply n/i/eNOS$^{-/-}$ mice showed prominent polyuria, polydipsia, and dehydration as compared with wild-type mice (Fig. 4) (21). Regarding the pathogenesis of the polyuria, osmotic diuresis, or water diuresis should be differentiated. In the triply n/i/eNOS$^{-/-}$ mice, the urine was hypotonic, low ionic, and without glucose, excluding osmotic diuresis due to natriuresis and diabetes mellitus (21). Water diuresis is caused by either central or nephrogenic diabetes insipidus or psychogenic polydipsia. Psychogenic polydipsia can be excluded because the triply n/i/eNOS$^{-/-}$ mice had dehydration. Next, to distinguish between central and nephrogenic diabetes insipidus, we examined the release of and the renal sensitivity to vasopressin. In the triply n/i/eNOS$^{-/-}$ mice, renal responsiveness to exogenous vasopressin was markedly impaired, while vasopressin release was unaltered, identifying the nephrogenic origin of diabetes insipidus in those mice (Fig. 5) (21). Nephrogenic diabetes insipidus is classified into two major categories: hereditary (or congenital) and acquired. Acquired nephrogenic diabetes insipidus

is induced by electrolyte abnormalities (e.g., hyper-calcemia or hypokalemia) or renal insufficiency of any cause. In the triply NOS$^{-/-}$ mice, plasma Ca^{2+} and K$^+$ concentrations were normal, while renal tubular apoptosis and regeneration, glomerulosclerosis, and glomerular thrombi were noted (21). These results suggest that the deletion of the whole NOS system is the primary cause of renal structural changes that result in nephrogenic diabetes insipidus.

The extent of tubular ischemic injury by glomerular thrombi is usually more severe in proximal tubules than in distal or collecting tubules. However, tubular lesions in the triply n/i/eNOS$^{-/-}$ mice were detected predominantly in the distal and collecting tubules rather than in the proximal tubules (21). It is thus conceivable that the tubular lesions are not caused by glomerular thrombi-induced ischemia.

Under physiological conditions, vasopressin stimulates adenylate cyclase, increases cAMP production, and activates cAMP-dependent protein kinase via the V$_2$ receptor in renal collecting duct principal cells. Phosphorylation of aquaporin-2 by the kinase in turn leads to translocation of aquaporin-2 from cytoplasmic vesicles to the apical plasma membrane, thereby increasing water permeability and reabsorption. Nephrogenic diabetes insipidus is characterized by an inability to concentrate urine despite normal or elevated plasma concentrations of vasopressin. In inherited nephrogenic diabetes insipidus, renal responsiveness to vasopressin is impaired by abnormalities of the vasopressin V$_2$ receptor or by those of the vasopressin-regulated water channel, aquaporin-2. To further clarify the mechanism involved, we examined cAMP accumulation and localization of aquaporin-2 in the collecting ducts of 1-week old triply n/i/eNOS$^{-/-}$ mice before the development of structural abnormalities. Vasopressin-stimulated cAMP accumulation, but not basal cAMP accumulation, was significantly reduced in the collecting ducts of the triply n/i/eNOS$^{-/-}$ mice, as compared with wild-type mice (21). Furthermore, expression of aquaporin-2 in collecting duct plasma membrane was more significantly decreased in the triply NOS$^{-/-}$ than in wild-type mice (21). These results suggest that reduced cAMP production and decreased aquaporin-2 expression are also involved in the pathogenesis of nephrogenic diabetes insipidus in the triply n/i/eNOS$^{-/-}$ mice. Consistent with our notion, previous studies revealed that NO stimulates cAMP production via cGMP-dependent activation of adenylate cyclase in isolated rat kidney and that NO promotes the membrane insertion of aquaporin-2 in collecting duct (44, 45). Importantly, the occurrence of functional abnormalities preceded that of structural abnormalities. Thus, the triply n/i/eNOS$^{-/-}$ mice appear

to possess the feature of inherited, rather than acquired, nephrogenic diabetes insipidus.

Cardiovascular phenotypes in triply n/i/eNOS$^{-/-}$ mice

We have recently tested our hypothesis that vascular lesion formation is spontaneously developed in the triply n/i/eNOS$^{-/-}$ mice (46). Experiments were performed in male wild-type; singly nNOS$^{-/-}$, iNOS$^{-/-}$, and eNOS$^{-/-}$; and triply n/i/eNOS$^{-/-}$ mice at 2 and 5 months of age maintained on regular diet. At 2 months of age, no significant vascular lesions were detected in any strains studied. However, at 5 months of age, significant wall thickening and perivascular fibrosis were noted only in the triply NOS$^{-/-}$ mice, but not in other strains, in both large epicardial coronary arteries and coronary micro-vessels (46). Furthermore, similar vascular lesions were also noted in cerebral and renal arteries and aortas, only in the triply NOS$^{-/-}$ mice. Notably, vascular angiotensin-converting enzyme immunoreactivities and cardiac superoxide production (by lucigenin chemiluminescence) were markedly enhanced only in the triply NOS$^{-/-}$ mice. These results indicate that the complete deletion of the whole NOS system accelerates a spontaneous development of systemic arteriosclerosis, associated with upregulation of angiotensin-converting enzyme and increased oxidative stress (46).

We further examined whether or not cardiovascular risk factors are present in the triply NOS$^{-/-}$ mice (47). Triply n/i/eNOS$^{-/-}$ mice showed hypertension and hypertriglyceridemia as compared with wild-type mice. Those mice also exhibited impaired glucose tolerance and severe insulin resistance compared with wild-type mice. Furthermore, visceral obesity associated with adipocyte hypertrophy was noted in the triply n/i/eNOS$^{-/-}$ mice; these are all characteristics consistent with human metabolic syndrome. Importantly, supplementation of NO by long-term dermal application of isosorbide dinitrate normalized those metabolic phenotypes in the triply n/i/eNOS$^{-/-}$ mice (the authors' unpublished observations). These results suggest an important role of defective NO production in the pathogenesis of metabolic syndrome (47).

Concluding remarks

We have successfully developed genetically engineered animals deficient in all three NOSs. In the functional analysis, we were able to demonstrate that the triply n/i/eNOS$^{-/-}$ mice manifested phenotypes of aging, arteriosclerosis, coronary artery spasm, metabolic syndrome, and nephrogenic diabetes insipidus (Fig. 6). These findings provide the first evidence that genetic

Cardiovascular Phenotypes in Mice Lacking All NOSs
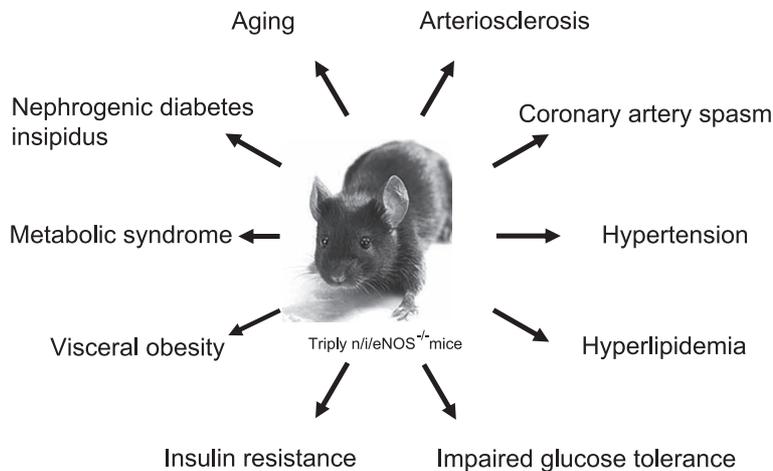
Fig. 6. Schematic diagram showing the cardiovascular phenotypes in mice lacking all NOSs. The triply n/i/eNOS$^{-/-}$ mice manifest a variety of cardiovascular phenotypes, including aging, arteriosclerosis, coronary artery spasm, metabolic syndrome, and nephrogenic diabetes insipidus. These findings demonstrate a central role of NO derived from the whole NOS system in the pathogenesis of the cardiovascular diseases. Our triply mutant mouse is a new animal model of the disorders.

disruption of the whole NOS system causes a variety of cardiovascular diseases in mice in vivo, demonstrating the central role of defective NO production in the pathogenesis of those disorders.

The roles of NO have so far been extensively investigated in pharmacological experiments or in experiments using mice deficient in each NOS isoform. However, in pharmacological experiments, there are always problems regarding the selectivity and specificity of drugs, while in the mice deficient in each NOS isoform, compensatory mechanisms by other NOSs are apparently involved. Our triply n/i/eNOS$^{-/-}$ mouse is a useful experimental tool to solve those problems and to investigate the roles of the whole NOS system.

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