Journal of Pharmacological Sciences 127 (2015) 42–52

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Contents lists available at ScienceDirect

Journal of Pharmacological Sciences

journal homepage: www.elsevier.com/locate/jphs

Critical review

Significance of nitric oxide synthases: Lessons from triple nitric oxide synthases null mice

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ARTICLE INFO

Article history:
Received 23 September 2014
Received in revised form
17 October 2014
Accepted 23 October 2014
Available online 18 November 2014

Keywords:
Cardiovascular disease
Null mice
Metabolic disease
Myocardial infarction
Nitric oxide synthase

ABSTRACT

Nitric oxide (NO) is synthesized by three distinct NO synthases (neuronal, inducible, and endothelial NOSs), all of which are expressed in almost all tissues and organs in humans. The regulatory roles of NOSs in vivo have been investigated in pharmacological studies with non-selective NOS inhibitors. However, the specificity of the inhibitors continues to be an issue of debate, and the authentic significance of NOSs is still poorly understood. To address this issue, we generated mice in which all three NOS genes are completely disrupted. The triple NOSs null mice exhibited cardiovascular abnormalities, including hypertension, arteriosclerosis, myocardial infarction, cardiac hypertrophy, diastolic heart failure, and reduced EDHF responses, with a shorter survival. The triple NOSs null mice also displayed metabolic abnormalities, including metabolic syndrome and high-fat diet-induced severe dyslipidemia. Furthermore, the triple NOSs null mice showed renal abnormalities (nephrogenic diabetes insipidus and pathological renal remodeling), lung abnormalities (accelerated pulmonary fibrosis), and bone abnormalities (increased bone mineral density and bone turnover). These results provide evidence that NOSs play pivotal roles in the pathogenesis of a wide variety of disorders. This review summarizes the latest knowledge on the significance of NOSs in vivo, based on lessons learned from experiments with our triple mutant model.

1. Introduction

Nitric oxide (NO) plays a crucial role in maintaining homeostasis (1–4). NO is synthesized from its precursor L-arginine by a family of NO synthases (NOSs) that include neuronal (nNOS), inducible (iNOS), and endothelial NOS (eNOS). It was initially reported that nNOS and eNOS are constitutively expressed mainly in the nervous system and the vascular endothelium, respectively, synthesizing a small amount of NO in a calcium-dependent manner under 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 (3,4). However, recent studies have revealed that nNOS and eNOS are also subject to expressional regulation (5–9), and that iNOS is expressed even under physiological conditions (10,11). Thus, it has become evident that all three NOS isoforms are expressed under both physiological and pathological conditions (10,12).

The roles of NO derived from whole NOSs have been examined in pharmacological studies with non-selective NOSs inhibitors, such as N^{ω}-nitro-L-arginine methyl ester (L-NAME) and N^{G}-monomethyl-L-arginine (L-NMMA). However, the NOS inhibitors possess multiple non-specific actions, including antagonism of muscarinic acetylcholine receptors (13), generation of superoxide anions (14),

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Peer review under responsibility of Japanese Pharmacological Society.

http://dx.doi.org/10.1016/j.jphs.2014.10.002

inhibition of cytochrome c reduction (15), and inhibition of endothelium-independent relaxation induced by amiloride or cAMP (16). We also reported that vascular lesion formation caused by long-term treatment with L-NAME or L-NMMA is not mediated by the simple inhibition of eNOS in mice, and that activation of the tissue renin-angiotensin system and increased oxidative stress are involved in the long-term vascular effects of the L-arginine analogues in an NO-independent manner (17,18).

The roles of NO derived from whole NOSs have also been investigated in studies with mice that lack each NOS isoform. However, although the single eNOS null mice manifest accumulation of cardiovascular risk factors that mimic human metabolic syndrome (19), and although it is well established that eNOS exerts anti-arteriosclerotic effects (20–25), the single eNOS null mice do not spontaneously develop arteriosclerotic/atherosclerotic vascular lesion formation (26). This inconsistency could be due to a compensatory mechanism by other NOSs that are not genetically disrupted (27). Indeed, in the singly eNOS$^{-/-}$ mice, up-regulation of vascular nNOS expression has been indicated (28,29). Furthermore, we revealed that NOS activity and NOx (nitrite plus nitrate) production are fairly well preserved in that genotype (30). Thus, the authentic roles of endogenous NO derived from entire NOSs still remain to be fully elucidated.

To address this important issue, we successfully developed mice in which all three NOS genes are completely disrupted (30). The expression and activity of NOSs are totally absent in the triple n/i/eNOSs null mice before and after administration of lipopolysaccharide. While the triple NOSs null mice were viable and appeared normal, their survival and fertility rates were markedly reduced as compared with wild-type mice. The triple NOSs null mice exhibited phenotypes in the cardiovascular, metabolic, renal, respiratory, and bone systems. These results provide evidence that NOSs play pivotal roles in the pathogenesis of a wide variety of disorders. This review summarizes the latest knowledge on the significance of NOSs in vivo, based on lessons we learned from experiments with our triple mutant model.

2. Significance of NOSs in the cardiovascular system

2.1. Hypertension

The triple NOSs null mice were significantly hypertensive as compared with the wild-type mice (30). The degree of hypertension in the triple NOSs null mice was similar to that in the eNOS null and eNOS gene-disrupted double NOSs null mice (Fig. 1A). These results suggest that hypertension is a common characteristic of the eNOS gene disruption and is caused by a lack of endothelium-derived NO with a resultant increase in peripheral vascular resistance (31). Heart rate was significantly lower in the triple NOSs null than in the wild-type mice, and the degree of bradycardia in the triple NOSs null mice was also equivalent to that in the eNOS gene-disrupted single and double NOSs null mice (Fig. 1B), indicating that bradycardia is also a common phenotype of the eNOS gene deletion. Although there is no conclusive explanation for the decreased heart rate in association with the eNOS gene deletion, previous studies revealed that eNOS-derived NO could affect baroreflex resetting or could be involved in establishing the baroreceptor setpoint (31).

2.2. Arteriosclerosis

We previously revealed that not only eNOS and iNOS but also nNOS is expressed in vascular lesions in a mouse carotid artery ligation model and a rat balloon injury model, and that all three NOSs play a role in the regulation of vascular lesion formation (7–9,32). Spontaneous development of vascular lesion formation (neointimal formation, medial thickening, and perivascular fibrosis) was noted in the large epicardial coronary arteries, coronary microvessels, and renal arteries in the triple NOSs null mice, but not in the eNOS null mice (2,33). Spontaneous lipid accumulation was also observed in the aorta of the triple NOSs null mice (2,33). These results suggest the crucial role of NOSs in inhibiting vascular lesion formation. The extent of hypertension was comparable in the triple NOSs null and eNOS null mice, whereas spontaneous vascular lesion formation was observed only in the triple

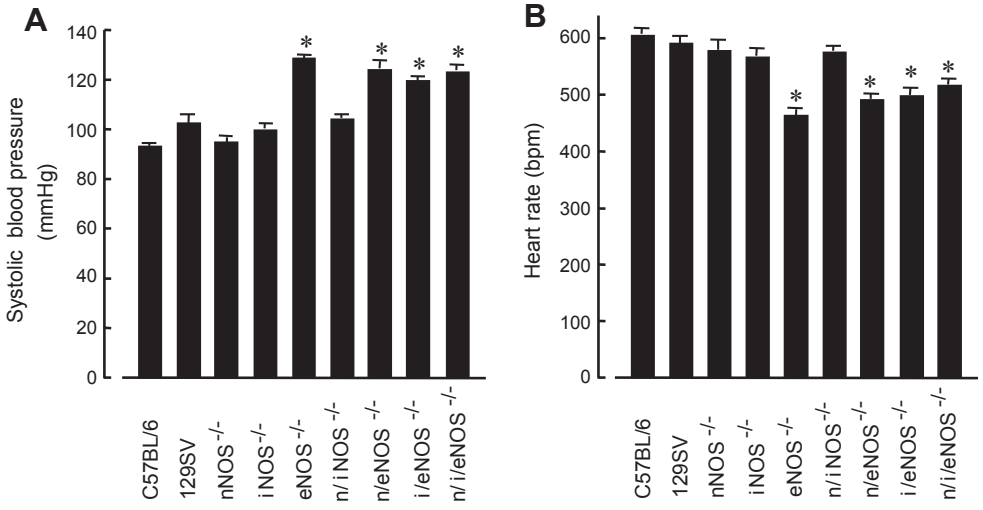

Fig. 1. Hemodynamics in wild-type and NOSs null mice. (**A**) Systolic blood pressure measured by the tail-cuff method under conscious conditions ($n = 9–16$). *$P < 0.05$ vs. wild-type C57BL/6 mice. (**B**) Heart rate measured by the tail-cuff method under conscious conditions ($n = 9–16$). *$P < 0.05$ vs. wild-type C57BL/6 mice. All single, double, and triple NOSs$^{-/-}$ mice are derived from both wild-type C57BL/6J and 129SV mice. nNOS$^{-/-}$ and eNOS$^{-/-}$ mice were backcrossed with C57BL/6J mice over five generations, while iNOS$^{-/-}$ mice were not backcrossed with any strains. Thus, we used both C57BL/6J and 129SV mice as wild genotype controls. We confirmed that there was no significant difference in blood pressure levels, heart rate, plasma lipid profile, glucose metabolism, or the amount of visceral adipose tissue between the C57BL/6 and 129SV mice at 3 months of age. Furthermore, we verified that there also was no significant difference in the extent of coronary vascular lesion formation between the 2 wild-type genotypes at 5 months of age. Results are expressed as mean ± SEM. Statistical analyses were performed by one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test for multiple comparisons. A value of $P < 0.05$ was considered to be statistically significant. Quoted from reference 30 with permission.

NOSs null mice, suggesting a minor role of hypertension in vascular lesion formation in the triple NOSs null mice (2,33).

Bone marrow-derived vascular progenitor cells in the blood accumulate in injured arteries, differentiate into vascular wall cells, and contribute to arteriosclerotic vascular lesion formation. All NOSs have been reported to be expressed in bone marrow cells. However, whether NOSs in bone marrow cells play a role in vascular lesion formation remained to be clarified. We previously reported that, in wild-type mice that underwent bone marrow transplantation from green fluorescent protein-transgenic mice, green fluorescent protein-positive fluorescence was detected in the ligated carotid arteries, confirming the involvement of bone marrow-derived vascular progenitor cells in vascular lesion formation after carotid artery ligation (34). In a comparison between the triple NOSs null genotype that received the triple NOS null bone marrow transplantation and the triple NOSs null genotype that received the wild-type bone marrow transplantation, the extent of neointimal formation and the extent of constrictive remodeling were both significantly less in those that received the wild-type bone marrow transplantation, along with significantly higher NOS activities in the ligated carotid arteries (Fig. 2) (35). Furthermore, in a comparison of the wild-type genotype with the wild-type bone marrow transplantation and the wild-type genotype with the triple NOSs null bone marrow transplantation, the extent of neointimal formation and the extent of constrictive remodeling were both significantly greater in the wild-type genotype with the triple NOSs null bone marrow transplantation, and this was associated with significantly lower NOS activities in the ligated carotid arteries (Fig. 2) (35). These results indicate that NOSs in bone marrow cells exert an inhibitory effect on vascular lesion formation caused by blood flow disruption in mice *in vivo*, demonstrating a novel vasculoprotective role of NOSs in bone marrow-derived vascular progenitor cells.

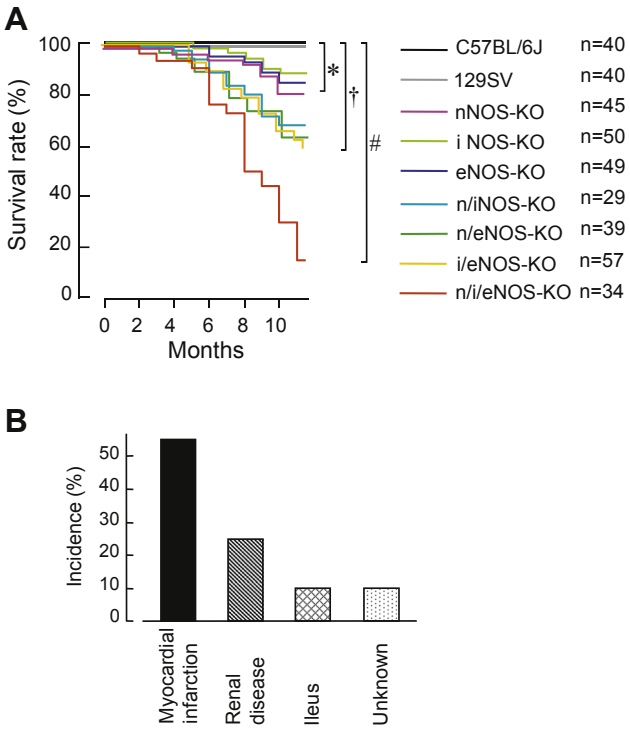

Fig. 3. Decreased survival and causes of death in triple NOSs null mice. (**A**) Survival rate ($n = 29–57$). The red line represents markedly reduced survival in the NOSs null mice. *, †, and #: $P < 0.05$ between WT C57BL/6J vs. single, double, and triple NOSs null mice, respectively. The "n" represents the number of mice used in each group. (**B**) Causes of death ($n = 20$). Results are expressed as mean ± SEM. Survival curves were analyzed by the Kaplan-Meier method. Differences in cause of death were evaluated by ANOVA followed by Scheffe post-hoc test for multiple comparisons. A value of $P < 0.05$ was considered to be statistically significant. Quoted from reference 33 with permission.

2.3. Myocardial infarction

During 11 months of follow-up, all (100%) of the wild-type mice lived, whereas only 15% of the triple NOSs null mice survived (Fig. 3A) (33). The survival rate was significantly worse in accordance with the number of disrupted NOS genes in the order of single, double, and triple NOSs null mice. Postmortem examination revealed that 55% of the triple NOSs null mice died of myocardial infarction (Figs. 3B and 4A) (33). This is the first demonstration to

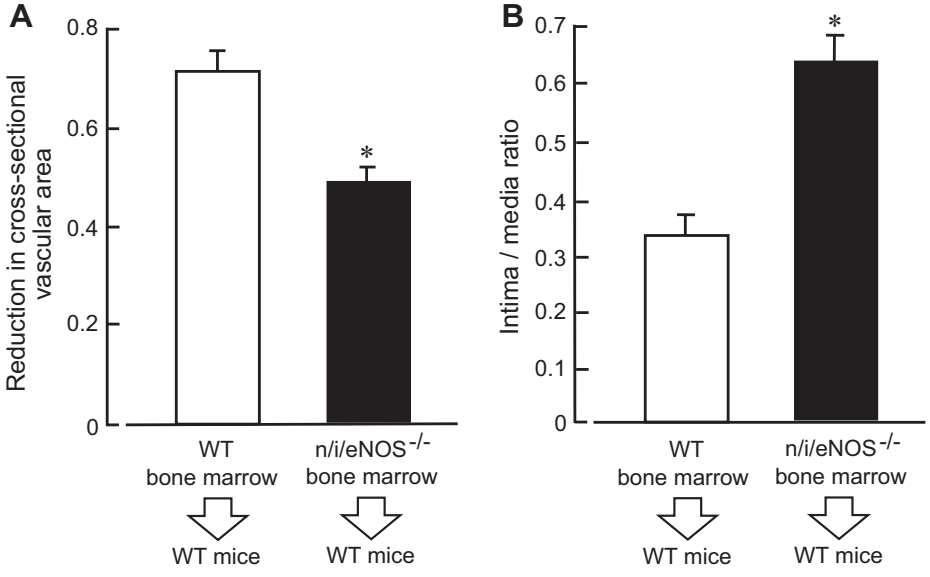

Fig. 2. Exacerbated constrictive vascular remodeling and neointimal formation in ligated carotid arteries of wild-type mice after triple NOSs null mouse bone marrow transplantation. (**A**) Constrictive vascular remodeling (reduction in cross-sectional vascular area) ($n = 5–7$). *$P < 0.001$. (**B**) Neointimal formation (intima-to-media ratio) ($n = 7–13$). *$P < 0.0001$. Results are expressed as mean ± SEM. Statistical analyses were performed by an unpaired student t-test. A value of $P < 0.05$ was considered to be statistically significant. Quoted from reference 35 with permission.

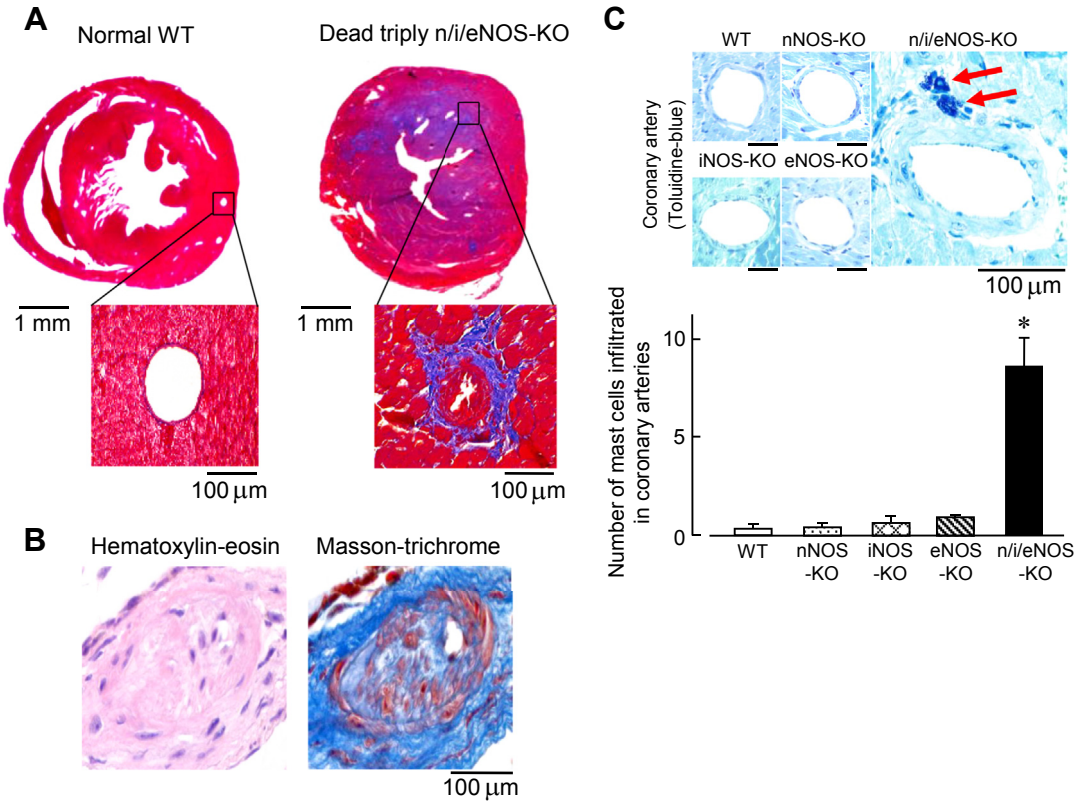

Fig. 4. Spontaneous myocardial infarction, coronary arteriosclerosis and mast cell infiltration in triple NOSs null mice. (**A**) Acute myocardial infarction and coronary arteriosclerotic lesion formation in the triple NOSs null mouse that died at 8 months of age (Masson-trichrome staining). Blue in the heart cross-section of the dead triple NOSs null mouse indicates antero-septal acute myocardial infarction. 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. WT, wild-type. *$P < 0.05$ vs. WT. Results are expressed as mean ± SEM. Survival curves were analyzed by the Kaplan-Meier method. Statistical analyses were performed by one-way ANOVA followed by Scheffe post-hoc test for multiple comparisons. A value of $P < 0.05$ was considered to be statistically significant. Quoted from reference 33 with permission.

show that a deficiency of NOSs leads to the development of spontaneous myocardial infarction. In the coronary arteries of the dead triple NOSs null mice, marked intimal formation, medial thickening, and mast cell infiltration were noted, while intra-coronary thrombus was rarely observed (Fig. 4A–C) (33). Histamine released from adventitial mast cells is thought to cause coronary vasospasm with resultant myocardial infarction in humans (36). It is thus possible that coronary intimal hyperplasia, medial thickening, and vasospasm are involved in the pathogenesis of myocardial infarction in the triple NOSs null mice. Although human myocardial infarction mainly results from rupture of atherosclerotic plaques and subsequent thrombus formation, the triple NOSs null mice seem to be a model of non-atherosclerotic forms of acute myocardial infarction in humans. In the triple NOSs null mice, there was a complete lack of endothelium-dependent relaxations to acetylcholine, which is a physiological eNOS activator, and contractions to phenylephrine, which is an α_1 adrenergic receptor agonist, were markedly potentiated (33). Thus, vascular dysfunction could also be involved in the pathogenesis of myocardial infarction in the triple NOSs null mice.

The renin-angiotensin system was markedly activated in the triple NOSs null mice, and long-term treatment with an angiotensin II type 1 (AT_1) receptor blocker olmesartan potently inhibited coronary arteriosclerotic lesion formation, vascular mast cell infiltration, and the occurrence of myocardial infarction in those mice, with a resultant improvement of the prognosis (33). These results suggest that the AT_1 receptor pathway is involved in the occurrence of spontaneous myocardial infarction in the triple NOSs null mice.

Chronic kidney disease is a condition characterized by progressive and irreversible loss of renal function. Previous epidemiological studies have indicated that the presence of chronic kidney disease significantly increases the risk of acute myocardial infarction in men, and that the impact of chronic kidney disease on the risk of cardiovascular disease is as strong as that of diabetes mellitus and pre-existing ischemic heart disease (37–39). Such a disease state is modeled in experimental animals by surgically dissecting a large part of the renal mass (40,41). On the basis of this background, we have recently investigated the effect of subtotal nephrectomy on the incidence of acute myocardial infarction in the triple NOSs null mice. Two-thirds nephrectomy (NX) caused sudden cardiac death due to acute myocardial infarction in the triple NOSs null mice as early as 4 months after the surgery (42). The 2/3NX triple NOSs null mice exhibited electrocardiographic ST-segment elevation, reduced heart rate variability, echocardiographic regional wall motion abnormality, and accelerated coronary arteriosclerotic lesion formation. Cardiovascular risk factors (hypertension, hypercholesterolemia, and hyperglycemia), an increased number of circulating bone marrow-derived vascular smooth muscle cell progenitor cells (a pro-arteriosclerotic factor), and cardiac up-regulation of stromal cell-derived factor-1α (a chemotactic factor of the progenitor cells) were noted in the 2/3NX triple NOSs null mice, and were associated with significant increases in plasma angiotensin II levels (a marker of activation of the renin-angiotensin system) and urinary 8-isoprostane levels (a marker of oxidative stress). The 2/3NX triple NOSs null mouse is a new experimentally useful

model of acute myocardial infarction. Activation of the renin-angiotensin system, oxidative stress, cardiovascular risk factors, and stromal cell-derived factor-1α–induced recruitment of bone marrow-derived vascular smooth muscle cell progenitor cells appear to be involved in the pathogenesis of acute myocardial infarction in this model. Our findings provide novel evidence that NOSs play a pivotal role in the pathogenesis of this reno-cardiac connection.

2.4. Cardiac hypertrophy

At 5 months of age, but not at 2 months of age, significant left ventricular hypertrophy (Fig. 5A), increased left ventricular weight (Fig. 5B), and cardiac myocyte hypertrophy were noted in the triple NOSs null and eNOS null mice, but not in the nNOS null or iNOS null mice, as compared with the wild-type mice (43). The extents of those structural changes were all significantly larger in the triple NOSs null than in the eNOS null mice. The left ventricular end-diastolic dimension was significantly smaller only in the triple NOSs null mice compared with the wild-type mice, indicating centripetal left ventricular hypertrophy in the triple NOSs null mice. Despite comparable blood pressure levels in the triple NOSs null and eNOS null mice, the extent of the left ventricular hypertrophy was greater in the triple NOSs null than in the eNOS null mice, and anti-hypertensive treatment with hydralazine failed to inhibit its progression, suggesting a minor role of hypertension in the pathogenesis of left ventricular hypertrophy in the triple NOSs null mice. It is thus conceivable that a lack of NOSs results in the development of left ventricular hypertrophy in mice in vivo.

Recent clinical studies have revealed that electrocardiographically determined left ventricular hypertrophy is a risk factor for cardiovascular death not only in hypertensive patients, but also in normotensive subjects (44,45). However, the underlying mechanisms remain to be elucidated. Based on our research outcomes obtained from the triple NOSs null mice, we have recently tested our hypothesis that normotensive subjects with electrocardiographically determined left ventricular hypertrophy have reduced NO production (46). The plasma NOx levels were markedly more reduced in normotensive males with electrocardiographically determined left ventricular hypertrophy than in those without. In addition, the plasma NOx levels were inversely associated with the prevalence and severity of electrocardiographically determined left ventricular hypertrophy. These findings suggest that normotensive individuals with electrocardiographically determined left ventricular hypertrophy exhibit defective NO production. Our findings may thus explain, at least in part, a potential mechanism underlying the increased risk of cardiovascular death in normotensive subjects with electrocardiographically determined left ventricular hypertrophy. It is interesting to note that the observations in the triple NOSs null mice could be translated to the human subjects.

2.5. Heart failure

Heart failure is a leading cause of morbidity and mortality in industrialized countries (47,48). There is growing recognition that not only systolic heart failure but also diastolic heart failure with normal systolic function is common and causes significant morbidity and mortality. Indeed, recent studies have revealed that as many as 30-50% of patients with congestive heart failure have diastolic heart failure, and that the morbidity and mortality rates for diastolic heart failure are nearly identical to those for systolic heart failure in aged patients (49). At 5 months of age, but not at 2 months of age, significant left ventricular diastolic dysfunction (as evaluated by echocardiographic E/A wave ratio and hemodynamic −dP/dt and Tau), with preserved left ventricular systolic function (as assessed by echocardiographic fractional shortening and hemodynamic +dP/dt) (Fig. 6), was noted only in the triple NOSs null mice, and this was associated with enhanced left ventricular end-diastolic pressure and increased lung wet weight, all of which are characteristics consistent with diastolic heart failure in humans (43). These results provide the first direct evidence that the complete disruption of all NOS genes results in diastolic dysfunction in mice in vivo, demonstrating a pivotal role of NOSs in the pathogenesis of diastolic heart failure.

2.6. Endothelium-dependent hyperpolarization

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). Shimokawa et al. demonstrated in animals and humans that endothelium-derived hydrogen peroxide (H_2O_2) is an EDHF, and that H_2O_2 is produced in part by eNOS (50,51). Shimokawa et al. subsequently examined the contribution of NOSs to EDHF-mediated responses in the single eNOS null, double n/eNOSs null, and triple n/i/eNOSs null mice (52). EDHF-mediated relaxation and hyperpolarization in response to acetylcholine of mesenteric

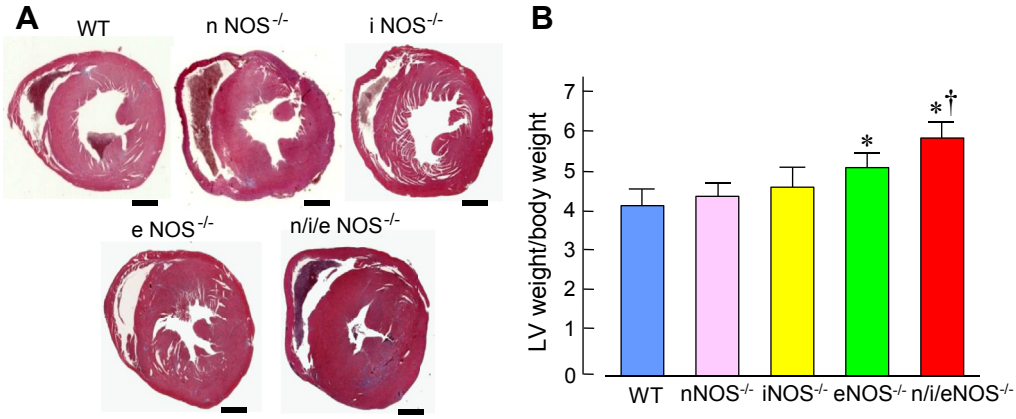

Fig. 5. Left ventricular hypertrophy in 5-month-old triple NOSs null and eNOS null mice. (**A**) Centripetal concentric left ventricular hypertrophy in the triple NOSs null and eNOS null mice. Scale bars, 1 mm. (**B**) The ratio of left ventricular weight/body weight ($n = 5$-7). *$P < 0.05$ vs. WT (wild-type), †$P < 0.05$ vs. eNOS$^{-/-}$. Results are expressed as mean ± SEM. Statistical analyses were performed by one-way ANOVA followed by Fisher's post-hoc test for multiple comparisons. A value of $P < 0.05$ was considered to be statistically significant. Quoted from reference 43 with permission.

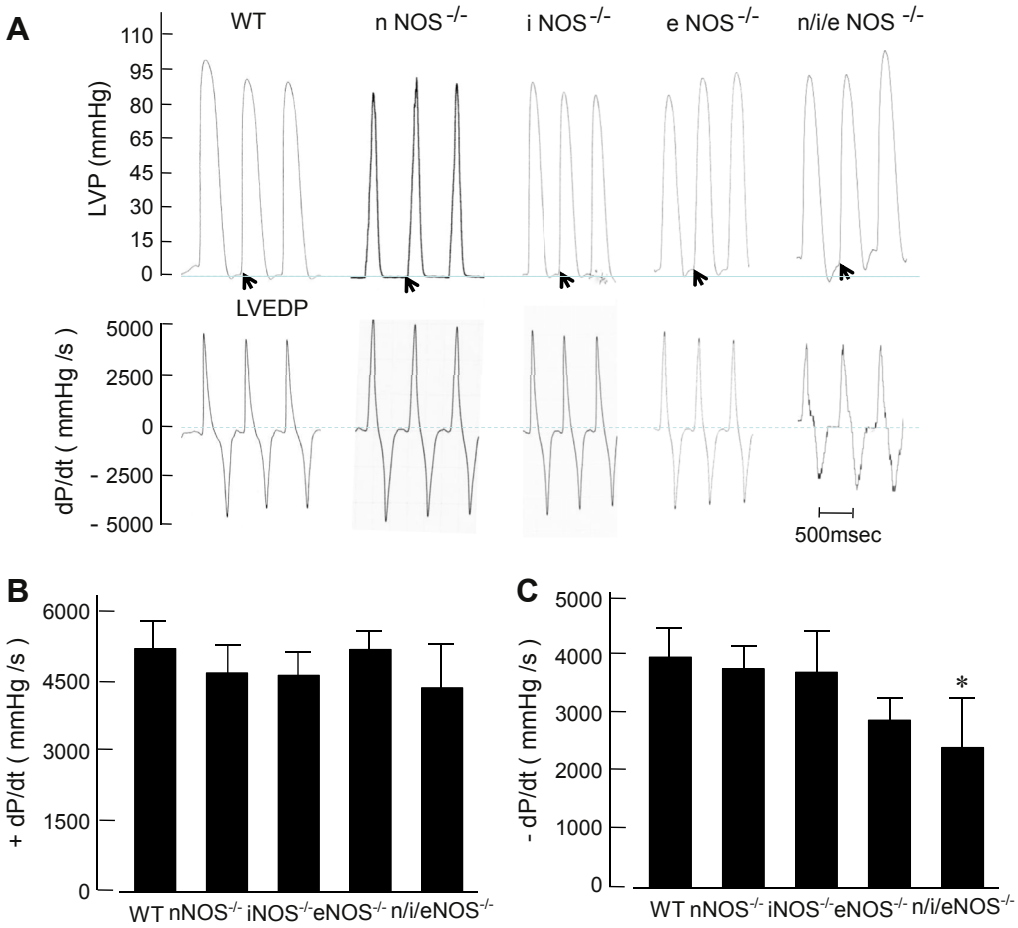

Fig. 6. Diastolic dysfunction in triple NOSs null mice assessed by cardiac catheterization. (**A**) Representative traces of left ventricular pressure and dP/dt. Arrows indicate left ventricular end-diastolic pressure. (**B**) +dP/dt, peak positive dP/dt (n = 5–6). C, -dP/dt, peak negative dP/dt (n = 5–6). WT, wild-type. *P < 0.05 vs. WT. Results are expressed as mean ± SEM. Statistical analyses were performed by one-way ANOVA followed by Fisher's post-hoc test for multiple comparisons. A value of P < 0.05 was considered to be statistically significant. Quoted from reference 43 with permission.

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 triple NOSs null mice, even after antihypertensive treatment with hydralazine. NOS uncoupling, which is caused by a deficiency of tetrahydrobiopterin, a cofactor of NOS, was not involved, as modulation of tetrahydrobiopterin synthesis had no effect on EDHF-mediated relaxation, and the tetrahydrobiopterin/dihydrobiopterin 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 on the diverse roles of the endothelial NOSs system mainly contributing to the EDHF/H_2O_2 responses in small-sized arteries while serving as a NO-generating system in large arteries.

3. Significance of NOSs in the metabolic system

3.1. Metabolic syndrome

The eNOS null and triple NOSs null mice manifested metabolic syndrome-like phenotypes, including hypertension, hypertriglycemia, visceral obesity, impaired glucose tolerance, and insulin resistance (33). The extents of hypertension, hypertriglycemia, and visceral obesity were comparable in the two genotypes, whereas the extents of impaired glucose tolerance and insulin resistance were greater in the triple NOSs null than in the eNOS null genotypes, and hyper-low-density-lipoprotein (LDL)-emia was observed only in the triple NOSs null genotype. It is thus possible that NOSs play an important role in the pathogenesis of metabolic syndrome.

Adiponectin is an anti-metabolic and anti-atherogenic adipocytokine, improving hypertriglyceridemia, glucose metabolism, and insulin resistance, and inhibiting the progression of arteriosclerosis (53–55). The deficiency of adiponectin is thought to contribute to the progression of metabolic syndrome and its vascular complications (54). In the triple NOSs null mice, plasma adiponectin levels were significantly reduced, suggesting that the adiponectin deficiency is involved in the pathogenesis of metabolic abnormalities and arteriosclerotic lesion formation in the triple NOSs null mice (33).

3.2. Severe dyslipidemia induced by a high-fat diet

We examined the effect of a Western-type cholesterol-rich diet on lipid metabolism in the triple NOSs null mice (56). The high-cholesterol diet for 3 months significantly increased serum LDL cholesterol levels in all the wild-type and single, double, and triple

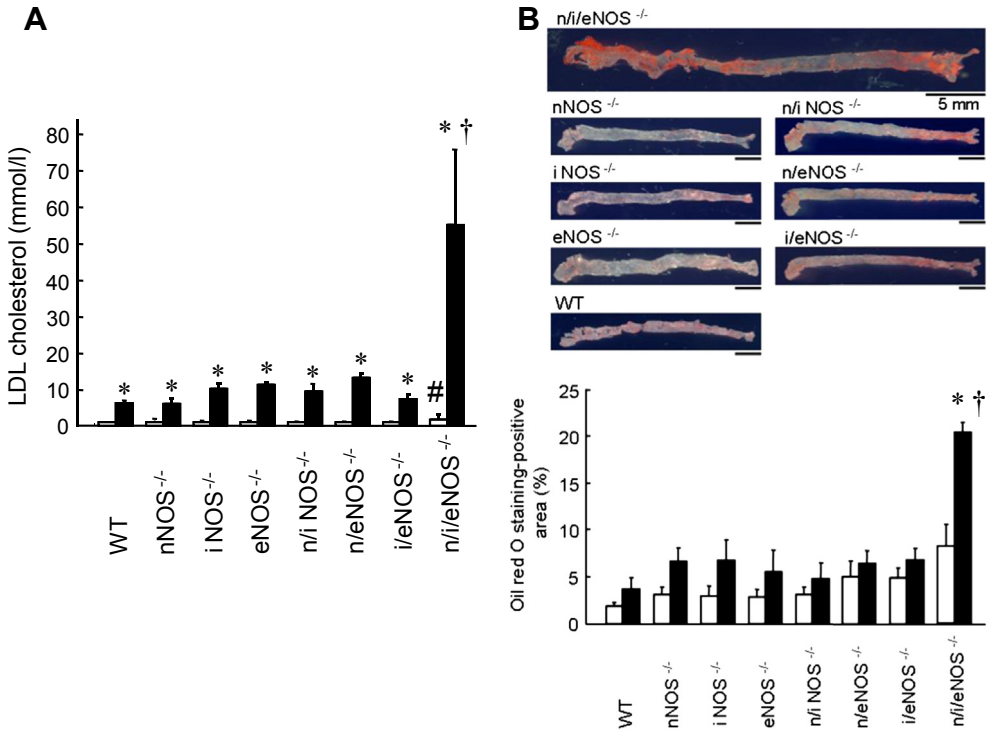

Fig. 7. Increased serum low-density lipoprotein (LDL) cholesterol levels and lipid accumulation in longitudinally opened aortas in triple NOSs null mice fed a high-cholesterol diet for 3 months. (**A**) Serum LDL cholesterol levels ($n = 6–11$). White and black bars indicate the regular and high-cholesterol diets, respectively. (**B**) Oil red O staining ($n = 6–11$). Red color indicates positive staining. WT, wild-type 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. Results are expressed as mean ± SEM. Statistical analyses were performed by two-way ANOVA followed by Scheffe's post-hoc test for multiple comparisons. A value of $P < 0.05$ was considered to be statistically significant. Quoted from reference 56 with permission.

NOSs genotypes examined as compared with a regular diet. Intriguingly, when compared with the wild-type genotype, the serum LDL cholesterol levels in the high-cholesterol diet were significantly and markedly elevated only in the triple NOSs null genotype, but not in any single or double NOSs null genotypes (Fig. 7A), and this was associated with remarkable atherosclerosis (Fig. 7B) and sudden cardiac death, which occurred mainly in 4-5 months after the high-cholesterol diet. Hepatic LDL receptor expression and hepatic levels of sterol regulatory element-binding protein-2 (SREBP-2) which is a transcriptional factor that controls LDL receptor gene expression (57) were markedly reduced only in the triple NOSs null genotype, accounting for the diet-induced dyslipidemia in the genotype. These results suggest that complete disruption of all NOSs causes severe dyslipidemia, atherosclerosis, and sudden cardiac death in response to a high-fat diet in mice in vivo through the down-regulation of the hepatic LDL receptor, demonstrating the critical role of NOSs in maintaining lipid homeostasis.

4. Significance of NOSs in the renal system

4.1. Nephrogenic diabetes insipidus

Nephrogenic diabetes insipidus is characterized by an inability to concentrate urine despite normal or elevated plasma concentrations of an anti-diuretic hormone, vasopressin. The triple NOSs null mice showed prominent polyuria, polydipsia, and blunted renal responsiveness to exogenous vasopressin (Fig. 8) (30). Vasopressin stimulates adenylate cyclase, increases cAMP production, and activates cAMP-dependent protein kinase via 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. In the kidney of the triple NOSs null mice, reduced vasopressin-induced cAMP production, decreased membranous aquaporin-2 water channel expression, and tubuloglomerular lesion formation (renal tubular apoptosis and regeneration, glomerulosclerosis, and glomerular thrombi) were noted. All of these are consistent with the characteristics of nephrogenic diabetes insipidus, suggesting a crucial role of NOSs in the pathogenesis of nephrogenic diabetes insipidus.

4.2. Pathological renal remodeling

Chronic unilateral ureteral obstruction (UUO) is a well-characterized model of experimental obstructive nephropathy, culminating in renal tubular apoptosis, interstitial fibrosis, and glomerulosclerosis (58,59). These alterations are also a common feature of a variety of kidney disorders, including chronic kidney disease (CKD) and end-stage renal disease (60). UUO caused significant renal lesion formation in the wild-type, single, and triple NOSs null mice, but the extents of renal lesion formation was markedly and most accelerated in the triple NOSs null genotype (61). UUO elicited the infiltration of inflammatory macrophages, up-regulation of transforming growth factor (TGF)-β1, and induction of epithelial mesenchymal transition (EMT) in all of the genotypes; however, the extents were again largest by far in the triple NOSs null genotype. These results suggest that the complete disruption of all NOSs results in markedly accelerated renal lesion formation in response to UUO in mice in vivo, demonstrating the critical renoprotective role of NOSs against pathological renal remodeling.

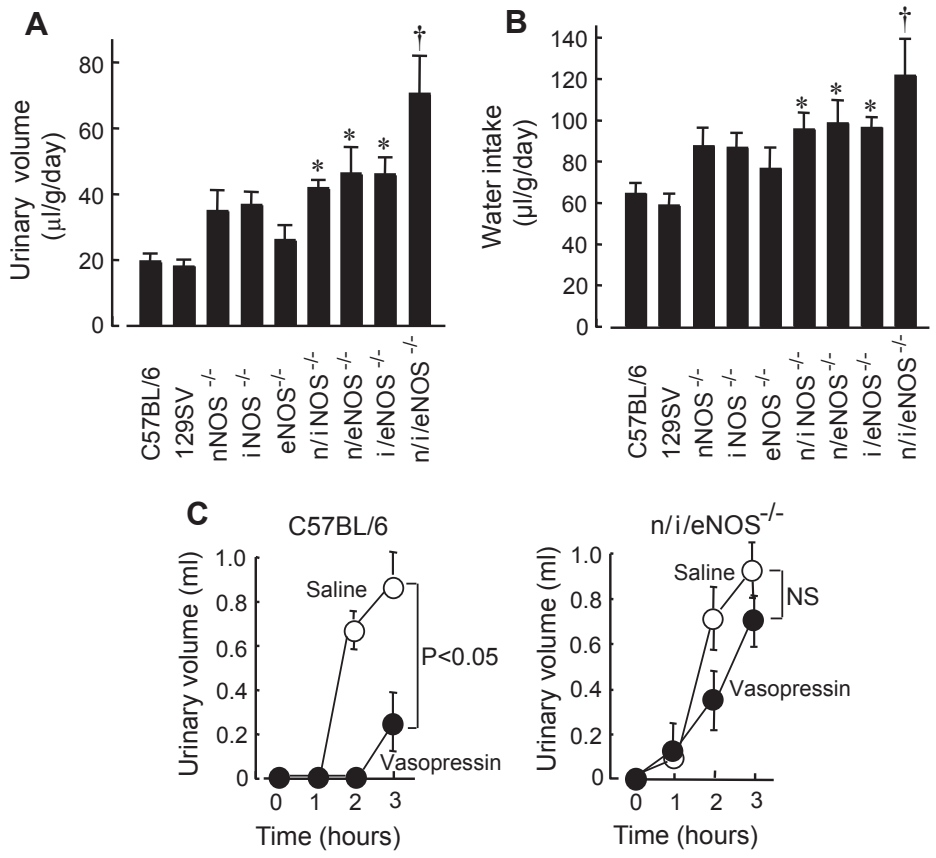

Fig. 8. Polyuria, polydipsia, and blunted renal responsiveness to exogenous vasopressin in triple NOSs null mice. (**A**) Urinary volume ($n = 6–13$). (**B**) Water intake ($n = 6–13$). (**C**) Changes of urinary volume in response to exogenous vasopressin ($n = 6$). Open circles, intraperitoneal saline; closed circles, intraperitoneal vasopressin (0.002 unit). NS, not statistically significant. *$P < 0.05$ vs. C57BL/6; †$P<0.01$ vs. C57BL/6. Results are expressed as mean ± SEM. Statistical analyses were performed by one-way or two-way ANOVA followed by Bonferroni post-hoc test for multiple comparisons. A value of $P < 0.05$ was considered to be statistically significant. Quoted from reference 30 with permission.

5. Significance of NOSs in the respiratory system

5.1. Accelerated pulmonary fibrosis

Up-regulation of NOSs and an increase in plasma NOx levels have been reported in patients with pulmonary fibrosis. However, the regulatory role of NOSs in pulmonary fibrosis remains to be clarified. Mukae et al. have recently examined the impact of bleomycin-induced pulmonary fibrosis on the triple NOSs null mice (62). Bleomycin (8 mg/kg/day) was administered intraperitoneally in the wild-type, single NOS null, and triple NOSs null mice for 10 consecutive days, and 2 weeks later, fibrotic and inflammatory changes of the lung were evaluated. The histopathological findings, collagen content, and the total cell number in bronchoalveolar lavage fluid were all most accelerated in the triple NOSs null mice (Fig. 9). Long-term treatment with a NO donor significantly prevented those pathological changes in the triple NOSs null mice. These results provide the first evidence that NOSs deficiency leads to a deterioration of pulmonary fibrosis in a bleomycin-treated murine model.

6. Significance of NOSs in the bone system

6.1. Increased bone mineral density and enhanced bone turnover

The non-specificity of the NOS inhibitors has caused conflicting results among previous pharmacological studies with the NOS inhibitors, such that NO has been suggested to be stimulatory (63) or nonessential (64) for osteoblast function and to be stimulatory (65) or inhibitory (66) for osteoclast function. We thus addressed this point in the triple NOSs null mice (67). Bone mineral density, trabecular bone thickness, and trabecular bone density were significantly higher in the triple NOSs null mice, but not in any single NOS null mice, as compared with the wild-type mice (Fig. 10). Markers of osteoblastic bone formation, including the bone formation rate, the mineral apposition rate, and the serum alkaline phosphatase concentration, were also significantly larger only in the triple NOSs null mice compared with the wild-type mice. Furthermore, markers of osteoclastic bone resorption, including the osteoclast number, the osteoclast surface, and the urinary deoxypyridinoline excretion, were again significantly greater only in the triple NOSs null mice. These results suggest that genetic disruption of NOSs enhances bone mineral density and bone turnover in mice, demonstrating the critical role of NOSs in maintaining bone homeostasis.

7. Concluding remarks

Genetically engineered mouse is one of the most useful experimental tools to study the function of target genes in vivo. The triple NOSs null model manifests abnormalities in the cardiovascular system (hypertension, arteriosclerosis, myocardial infarction, cardiac hypertrophy, diastolic heart failure, and reduced EDHF responses), the metabolic system (metabolic syndrome and high-fat diet-induced severe dyslipidemia), the renal system (nephrogenic diabetes insipidus and pathological renal remodeling), the

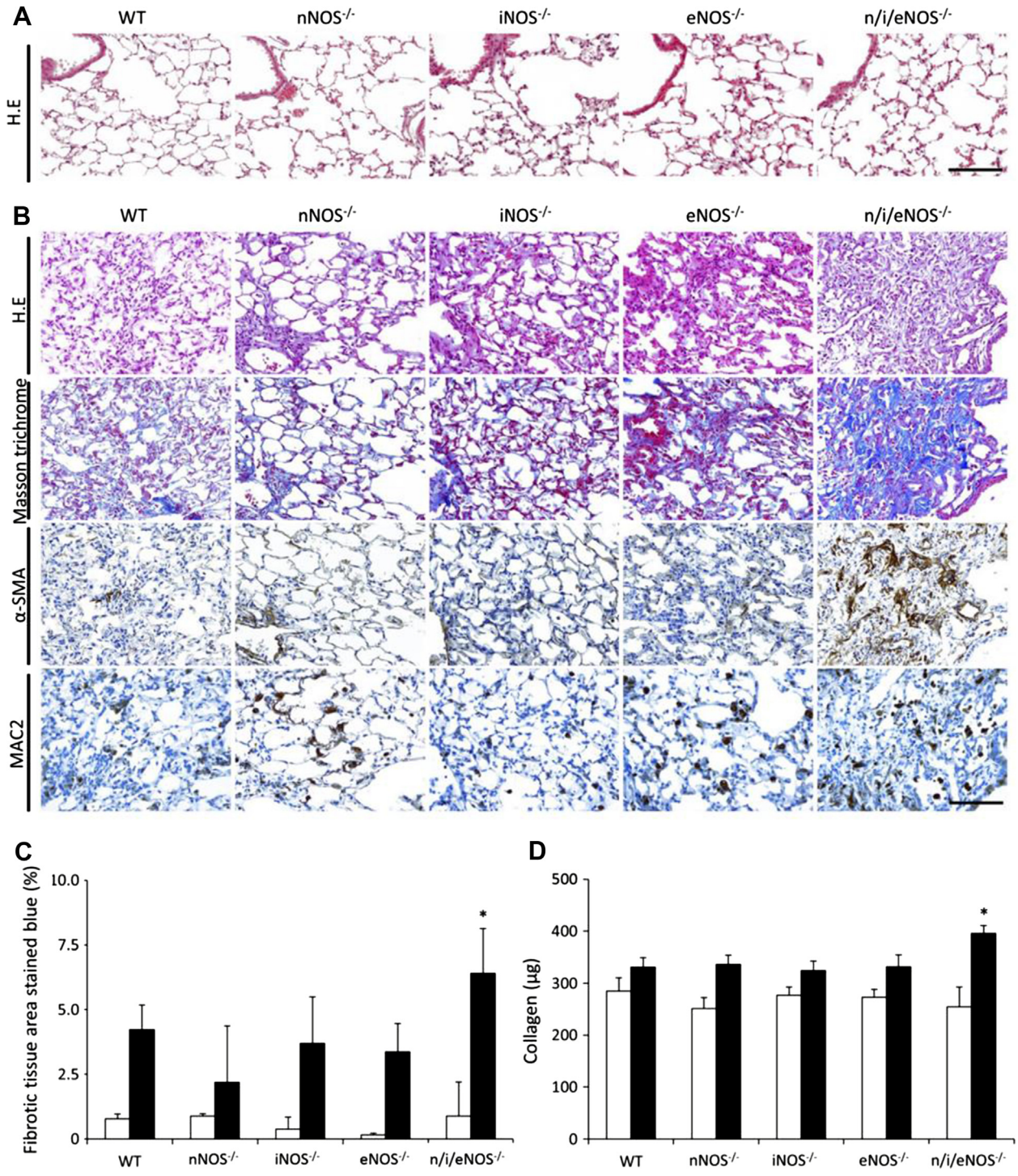

Fig. 9. Deterioration of lung fibrosis in the triple NOSs null mice at 2 weeks after bleomycin treatment. Bleomycin (8 mg/kg/day) was administered intraperitoneally in the wild-type, single NOS null, and triple NOSs null mice for 10 consecutive days, and 2 weeks later, fibrotic and inflammatory changes of the lung were evaluated. (**A**) Hematoxylin-eosin staining in normal saline (NS)-treated mice. Scale bar = 100 μm. (**B**) Hematoxylin-eosin staining, Masson-trichrome staining, α-smooth muscle actin (SMA) staining, MAC-2 staining in bleomycin-treated mice. Scale bar = 100 μm. (**C**) The fibrotic tissue area (blue-stained). (**D**) The collagen content in lung tissue. White and black bars indicate normal saline- ($n = 3$) and bleomycin- ($n = 5$) treated mice, respectively. $^{*}P < 0.05$ vs. bleomycin-treated wild-type mice. Statistical analyses were performed by the Mann-Whitney U (non-parametric) test. A value of $P < 0.05$ was considered to be statistically significant. Quoted from reference 62 with permission.

respiratory system (accelerated pulmonary fibrosis), and the bone system (increased bone mineral density and enhanced bone turnover). These findings provide the first direct evidence of the critical roles of NOSs in the pathogenesis of a wide variety of disorders.

We are currently studying the role of NOSs in cerebral infarction. Intriguingly, cerebral infarct size after middle cerebral artery occlusion was not larger, but rather markedly smaller in the triple NOSs null mice than in the wild-type mice (68). These results suggest that, in contrast to the protective role of NOSs in myocardial

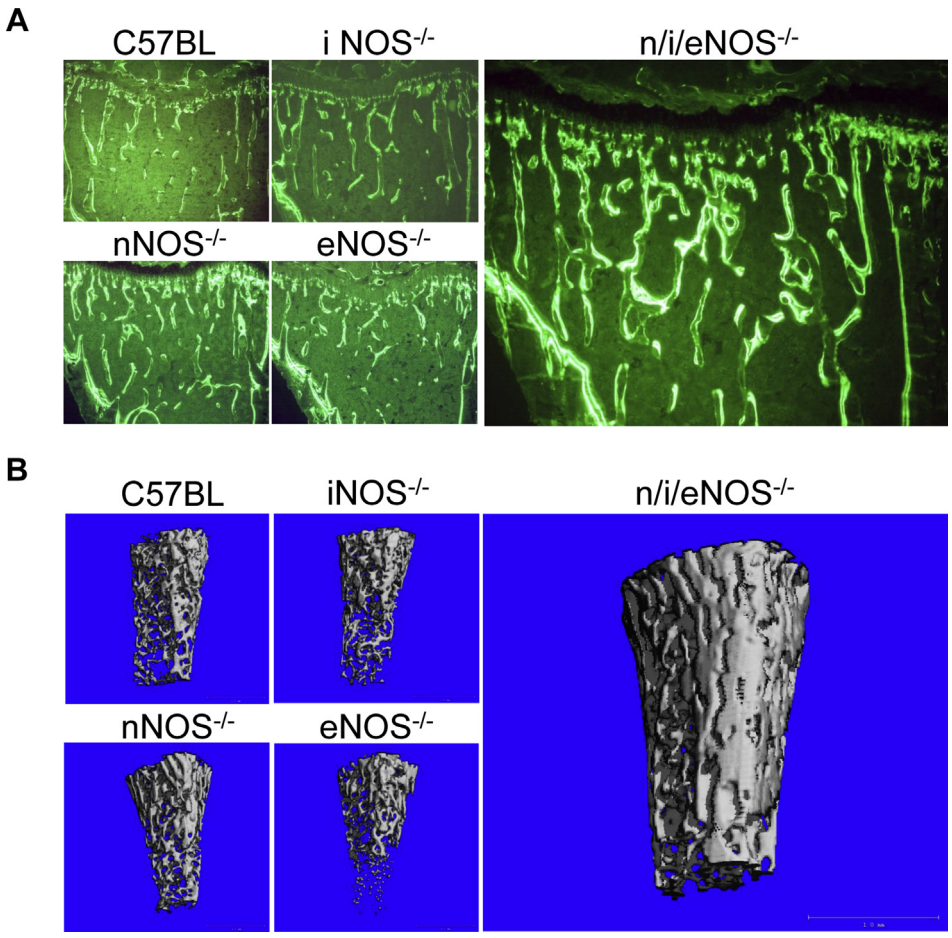

Fig. 10. Abnormal trabecular bone microstructure in triple NOSs null mice. (**A**) Calcein double labeling in the proximal tibia. (**B**) Three-dimensional micro-computed tomography of the femur. In both analyses, trabecular bone thickness and density were increased in the triple NOSs null mice. Quoted from reference s67 with permission.

infarction, NOSs may play an opposite injurious role in cerebral infarction. Thus, the roles of NOSs appear to be different in distinct organs or disease states. Further studies are certainly needed to clarify the complex roles of NOSs in humans in vivo.

Conflict of interest

None declared.

Acknowledgements

This work was supported in part by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (23590305), Special Account Budgets for Education and Research granted by the Japan Ministry of Education, Grants from the Promotion Project of Medical Clustering of Okinawa Prefecture and the University of the Ryukyus, and a Grant and Donation from the Sumitomo Dainippon Pharma Co, Japan.

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