Spontaneous Myocardial Infarction in Mice Lacking All Nitric Oxide Synthase Isoforms

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Background—The roles of nitric oxide (NO) in the cardiovascular system have been investigated extensively in pharmacological studies with NO synthase (NOS) inhibitors and in studies with NOS isoform–deficient mice. However, because of the nonspecificity of the NOS inhibitors and the compensatory interactions among NOS isoforms (nNOS, iNOS, and eNOS), the ultimate roles of endogenous NO derived from the entire NOS system are still poorly understood. In this study, we examined this point in mice deficient in all 3 NOS isoforms (triply n/i/eNOS$^{-/-}$ mice) that we have recently developed.

Methods and Results—The triply n/i/eNOS$^{-/-}$ mice, but not singly eNOS$^{-/-}$ mice, exhibited markedly reduced survival, possibly due to spontaneous myocardial infarction accompanied by severe coronary arteriosclerotic lesions. Furthermore, the triply n/i/eNOS$^{-/-}$ mice manifested phenotypes that resembled metabolic syndrome in humans, including visceral obesity, hypertension, hypertriglyceridemia, and impaired glucose tolerance. Importantly, activation of the renin-angiotensin system was noted in the triply n/i/eNOS$^{-/-}$ mice, and long-term oral treatment with an angiotensin II type 1 receptor blocker significantly suppressed coronary arteriosclerotic lesion formation and the occurrence of spontaneous myocardial infarction and improved the prognosis of those mice, along with ameliorating the metabolic abnormalities.

Conclusions—These results provide the first direct evidence that genetic disruption of the whole NOS system causes spontaneous myocardial infarction associated with multiple cardiovascular risk factors of metabolic origin in mice in vivo through the angiotensin II type 1 receptor pathway, demonstrating the critical role of the endogenous NOS system in maintaining cardiovascular and metabolic homeostasis. **(*Circulation*. 2008;117:2211-2223.)**

Key Words: nitric oxide synthase ■ myocardial infarction ■ arteriosclerosis ■ angiotensin

Nitric oxide (NO) is produced in almost all tissues and organs, exerting a variety of biological actions under both physiological and pathological conditions.[1–6] NO is synthesized by 3 different isoforms of NO synthase (NOS), including neuronal (nNOS), inducible (iNOS), and endothelial (eNOS) NOS.[1–6] The roles of NO in the cardiovascular system have been widely examined in pharmacological studies with NOS inhibitors.[1–6] However, the NOS inhibitors possess multiple nonspecific actions, including antagonism of muscarinic acetylcholine receptors,[7] generation of superoxide anions,[8] inhibition of cytochrome c reduction,[9] and inhibition of endothelium-independent relaxation induced by amiloride or cAMP.[10] In addition, we have recently clarified that vascular lesion formation caused by long-term treatment with either N^{ω}-nitro-L-arginine methyl ester, N^{ω}-nitro-L-arginine, or N^{G}-monomethyl-L-arginine is not mediated by simple inhibition of endothelial NO synthesis.[11,12] Thus, the specificity of the NOS inhibitors, especially the nonselective NOS inhibitors, continues to be an issue of debate.

Clinical Perspective p 2223

The roles of NO in the cardiovascular system have also been investigated extensively in studies with mice that lack each NOS isoform.[1–6] However, although singly eNOS-deficient mice manifest accumulation of cardiovascular risk factors that mimic human metabolic syndrome,[13,14] and al-

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The online-only Data Supplement, consisting of an expanded Methods section, figures, and a table, is available with this article at http://circ.ahajournals.org/cgi/content/full/CIRCULATIONAHA.107.742692/DC1.

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though it is well established that eNOS exerts antiatherosclerotic effects,[1–6] singly eNOS-deficient mice do not spontaneously develop arteriosclerotic/atherosclerotic vascular lesion formation.[15] This inconsistency may be due to a compensatory mechanism by other NOS that are not disrupted genetically.[16] Indeed, in the singly eNOS$^{-/-}$ mice, upregulation of vascular nNOS expression has been indicated.[17,18] Furthermore, we have also revealed that NOS activity and NOx (nitrite plus nitrate) production are fairly well preserved in the genotype.[19] Thus, the ultimate roles of endogenous NO derived from the whole NOS system remain to be fully elucidated.

To address this important issue, we have recently developed mice in which all 3 NOS isoforms are completely disrupted.[19] The triply nNOS/iNOS/eNOS-deficient (n/i/eNOS$^{-/-}$) mice showed markedly reduced survival and renal abnormalities; however, the exact cause of death remains to be clarified.[19] Here, we report that triply n/i/eNOS$^{-/-}$ mice, but not singly eNOS$^{-/-}$ mice, possibly die because of spontaneous myocardial infarction associated with a variety of metabolic diseases and that long-term blockade of the angiotensin II type 1 (AT_1) receptor suppresses the development of those abnormal phenotypes. These results provide the first direct evidence that the endogenous NOS system plays a critical role in suppressing cardiovascular and metabolic disorders in vivo, in which inhibition of the renin-angiotensin system may be involved.

Methods

Mice

Experiments were performed in males of the following 9 strains: wild-type (WT) C57BL/6J and 129SV (Charles River, Yokohama, Japan); singly deficient nNOS$^{-/-}$, iNOS$^{-/-}$, and eNOS$^{-/-}$ mice; doubly deficient n/iNOS$^{-/-}$, n/eNOS$^{-/-}$, and i/eNOS$^{-/-}$ mice; and triply deficient n/i/eNOS$^{-/-}$ mice[19] maintained on a standard diet. More detailed information on the mice is provided in the online-only Data Supplement.

Morphology

The animals were euthanized by inhalation of an overdose of diethyl ether (Wako Pure Chemical Industries, Osaka, Japan). Details of the methods used are provided in the online-only Data Supplement.

Blood Pressure

Blood pressures were measured by the tail-cuff method under conscious conditions (MK-2000, Muromachi Kikai, Tokyo, Japan), as we have reported previously.[12]

Lipid Metabolism

Plasma lipid profile was assessed by a Dri-Chem autoanalyzer (Fuji Film Co, Tokyo, Japan). The size of plasma low-density lipoprotein (LDL) cholesterol particles was determined by use of a high-sensitivity lipoprotein profiling system with high-performance liquid chromatography.[20]

Glucose Tolerance Test

One gram of glucose per body weight was injected into the mice via the tail vein.[21] Plasma glucose concentration was assessed by a portable plasma glucose analyzer (Sanwa Kagaku Kenkyusho Co Ltd, Nagoya, Japan).

Adiponectin Level

Plasma adiponectin concentrations were determined with an ELISA kit (Otsuka Pharmaceutical Co Ltd, Tokyo, Japan).[22]

Other Measurements and Analyses

Methods for immunostaining, Western blot analysis, echocardiography, organ-chamber experiments, kinetics of the renin-angiotensin system, and drug treatment are described in the online-only Data Supplement.

Statistical Analysis

The online-only Data Supplement contains a complete description of the methods used for statistical analysis.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Markedly Reduced Survival in Triply Deficient n/i/eNOS$^{-/-}$ Mice

During the 11 months of follow-up, all (100%) of the male WT C57BL/6J mice lived, whereas only 15% of the male n/i/eNOS$^{-/-}$ mice survived (Figure 1A). The survival rate in male mice was significantly worse in accordance with the number of disrupted NOS genes, in the order of singly, doubly, and triply deficient NOS$^{-/-}$ mice (Figure 1A). Results on plasma and urinary NOx levels in the WT and NOS$^{-/-}$ mice and the compensatory expressions of other NOS in the singly NOS-deficient mice are described in the online-only Data Supplement.

Death Due to Myocardial Infarction in Triply Deficient n/i/eNOS$^{-/-}$ Mice

Intriguingly, postmortem examination revealed that more than half (55%) of the n/i/eNOS$^{-/-}$ mice possibly died because of spontaneous myocardial infarction accompanied by severe coronary arteriosclerotic lesions (Figure 1B and 1C). In the infarct-related coronary arteries of the dead triply NOS-deficient mice, severe coronary arteriosclerotic lesions were noted (Figure 1D). Furthermore, a significant mast cell infiltration at the coronary artery adventitia was also observed in the dead n/i/eNOS$^{-/-}$ mice (Figure 1E). On the other hand, coronary atherosclerosis (eg, infiltration of foamy macrophages and extracellular lipid accumulation) and plaque rupture were rarely observed (Figure 1D).

We have previously demonstrated that triply n/i/eNOS$^{-/-}$ mice manifest nephrogenic diabetes insipidus associated with renal tubuloglomerular lesion formation.[19] In some dead n/i/eNOS$^{-/-}$ mice, we detected severe renal tubuloglomerular lesions without any other causative pathological abnormalities. In this case, we judged that the mice probably died due to renal disease (Figure 1B).

Vascular Lesion Formation in Triply Deficient n/i/eNOS$^{-/-}$ Mice

We next investigated vascular lesion formation in male triply NOS-deficient mice at 2 and 5 months of age. At 2 months of age, no significant vascular lesions were seen in any of the genotypes studied (data not shown). However, at 5 months of age, significant neointimal formation, medial thickening, and perivascular fibrosis were noted in the triply deficient n/i/

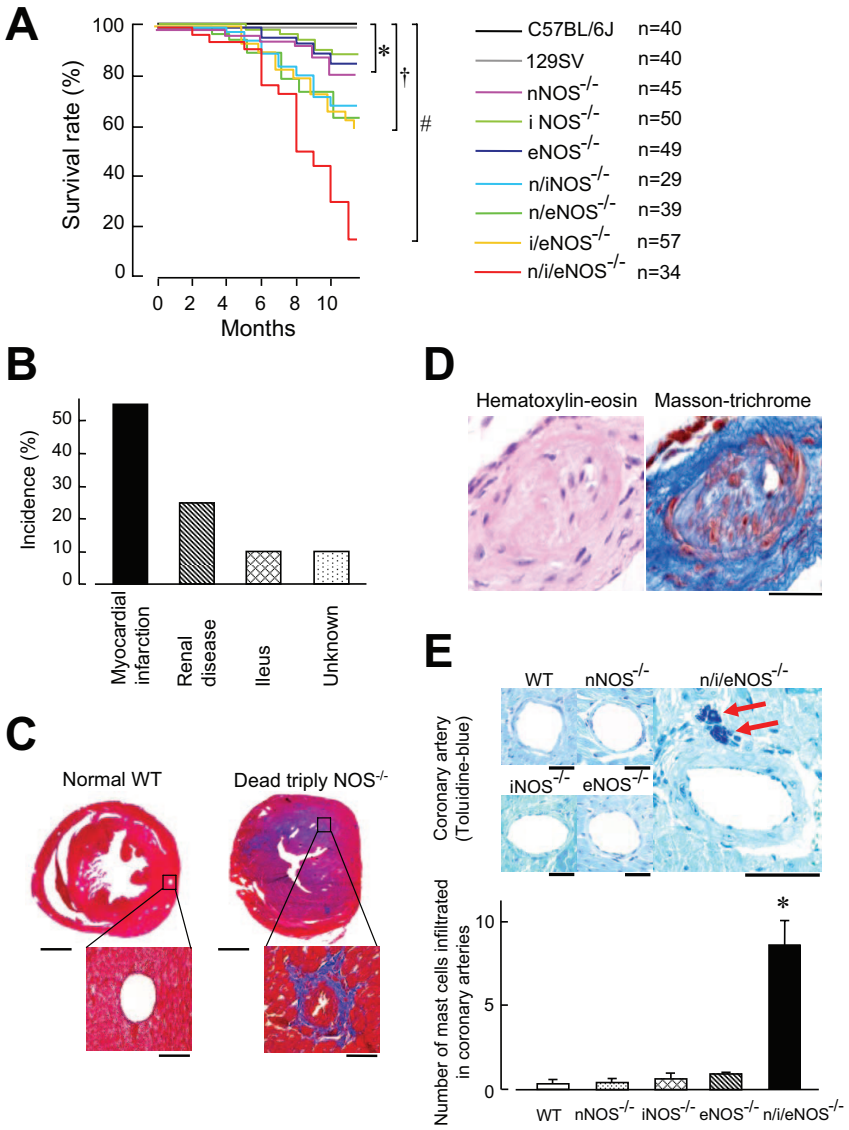

Figure 1. Decreased survival rate, spontaneous myocardial infarction, and coronary arteriosclerotic lesion formation in male triply NOS-deficient (n/i/eNOS$^{-/-}$) mice. A, Survival rate (n=29 to 57). *P<0.05 between singly deficient NOS$^{-/-}$ and C57BL/6J mice; †P<0.05 between doubly deficient NOS$^{-/-}$ and C57BL/6J mice; #P<0.05 between triply deficient n/i/eNOS$^{-/-}$ and C57BL/6J mice. B, Causes of death (n=20). C, Acute myocardial infarction and coronary arteriosclerotic lesion formation in male n/i/eNOS$^{-/-}$ mouse that died at 8 months of age (Masson's trichrome staining). Blue in the heart cross section of the dead n/i/eNOS$^{-/-}$ mouse indicates anteroseptal acute myocardial infarction (upper right panel). Adjacent coronary artery shows marked luminal narrowing, wall thickening, and perivascular fibrosis (blue; lower right panel). WT indicates WT C57BL/6J mice. D, Serial sections of the infarct-related coronary artery (hematoxylin-and-eosin and Masson's trichrome staining). E, Mast cell infiltration at the coronary artery adventitia (toluidine blue staining; n=10 to 33). Red arrows indicate mast cells. *P<0.05 vs WT. Scale bars in the heart of panel C=1 mm. Scale bars in the coronary artery of panels C through E=100 μm. In panels C and E, we used living WT mice. n represents the number of mice used in each group.

eNOS$^{-/-}$ mice but not in any singly deficient NOS$^{-/-}$ mice compared with WT mice, in both large epicardial coronary arteries (Figure 2A) and coronary microvessels (Figure 2B). This was also the case for renal vascular lesions (Figure 2C). Although vascular lesion formation in cerebral arteries also tended to be accelerated in the n/i/eNOS$^{-/-}$ mice, this did not reach statistically significant levels (data not shown). Arteriosclerosis was seen in most of the vasculature in the n/i/eNOS$^{-/-}$ mice, whereas atherosclerosis with lipid accumulation was observed in the aorta (Figure 2D).

Visceral Obesity in Triply Deficient n/i/eNOS$^{-/-}$ Mice

Because coronary arteriosclerotic vascular lesions were noted in the triply NOS-deficient mice, we then examined what cardiovascular risk factors were present in those mice at 3 months of age. We have previously reported that no significant differences are present in body weight among WT, singly NOS-deficient, and triply NOS-deficient mice.[19] A body composition study also revealed similar composition among those genotypes (data not shown). However, the epididymal and perirenal white adipose tissue weights were significantly increased in the singly eNOS$^{-/-}$ and triply n/i/eNOS$^{-/-}$ mice to a comparable extent (Figure 3A and 3B). Epididymal white adipose cells were also significantly larger in the singly eNOS$^{-/-}$ and triply n/i/eNOS$^{-/-}$ mice (Figure 3C).

Hypertension in Triply Deficient n/i/eNOS$^{-/-}$ Mice

Systolic, diastolic, and mean arterial blood pressures were all significantly elevated to a comparable extent in the singly deficient eNOS$^{-/-}$ and triply deficient n/i/eNOS$^{-/-}$ mice compared with the WT mice (Figure 3D through 3F).

Dyslipidemia in Triply Deficient n/i/eNOS$^{-/-}$ Mice

Plasma triglyceride levels were significantly higher in all the singly and triply NOS-deficient mice (Figure 4A). On the other hand, plasma LDL cholesterol and small dense LDL particle levels were markedly increased only in the triply deficient n/i/eNOS$^{-/-}$ mice (Figure 4B and 4C).

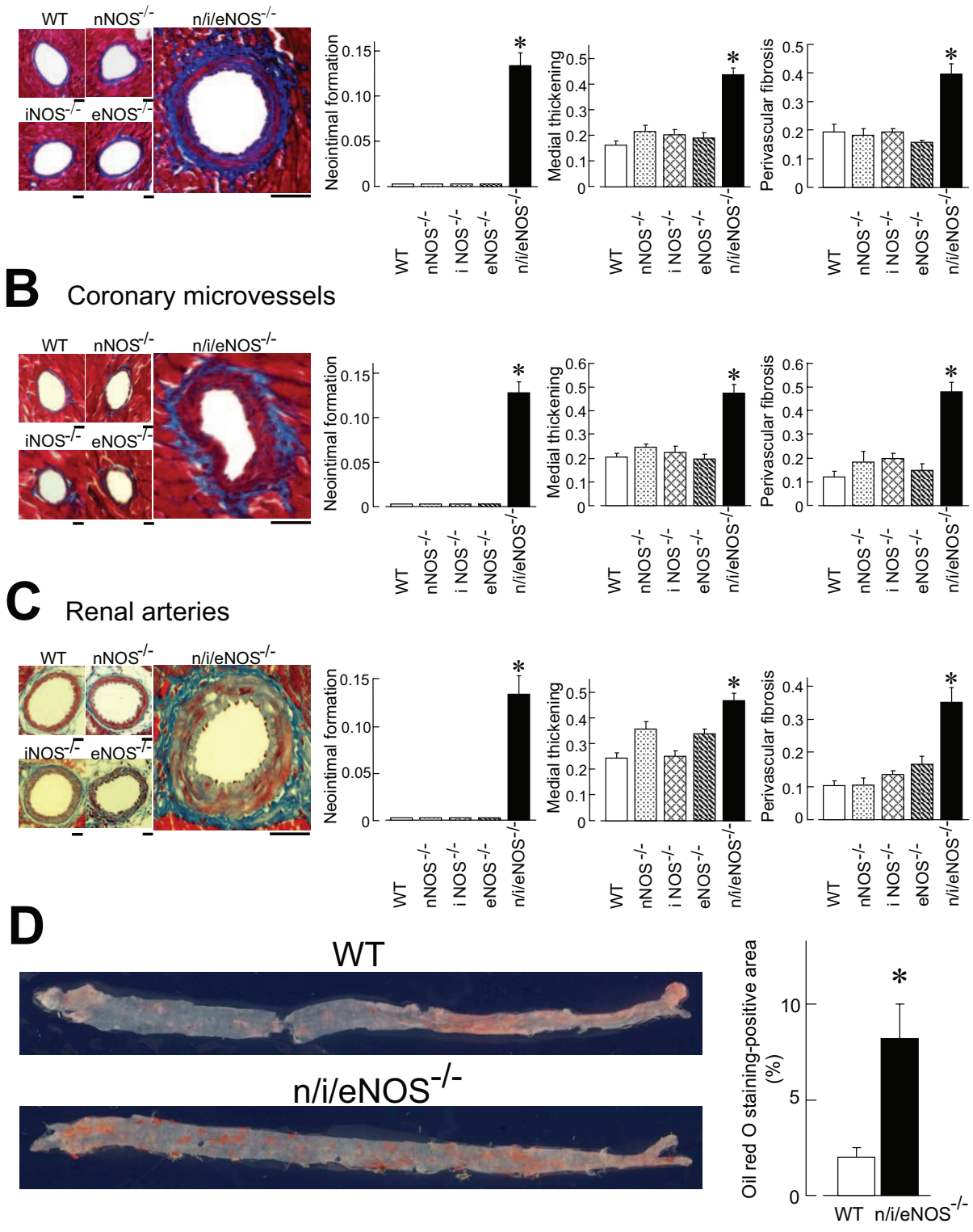

Figure 2. Coronary, renal, and aortic lesion formation in n/i/eNOS$^{-/-}$ mice. Experiments were performed in 5-month-old male mice. A through C, Neointimal formation (the ratio of intima area to media area), medial thickening (the ratio of media area to total vascular area), and perivascular fibrosis (the ratio of perivascular fibrosis area to total vascular area) in large epicardial coronary arteries (A), coronary microvessels (B), and renal arteries (C; n=6 each). D, Longitudinally opened, oil red O–stained aortas (n=6 each). *$P<0.05$ vs WT C57BL/6J. Scale bars in panels: A, 50 μm; B, 10 μm; C, 50 μm.

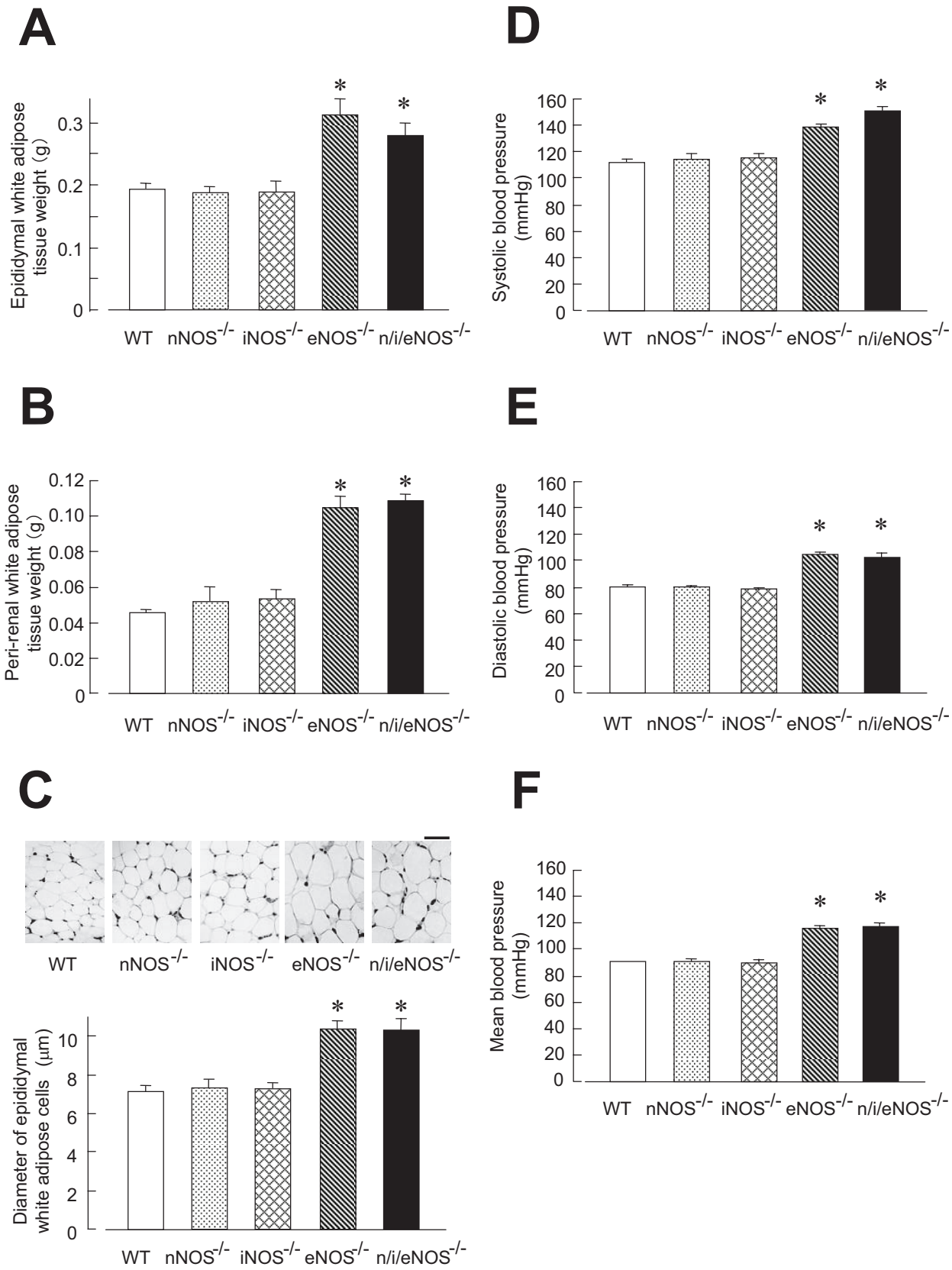

Figure 3. Visceral obesity and hypertension in n/i/eNOS$^{-/-}$ mice. Experiments were performed in 3-month-old male mice. A, Epididymal white adipose tissue weight (n=9 to 12). B, Perirenal white adipose tissue weight (n=9 to 12). C, Diameter of epididymal white adipose cells (hematoxylin-and-eosin staining; n=9 to 12). Scale bar=10 μm. D through F, Systolic, diastolic, and mean blood pressure (n=9 to 10). *$P<0.05$ vs WT C57BL/6J.

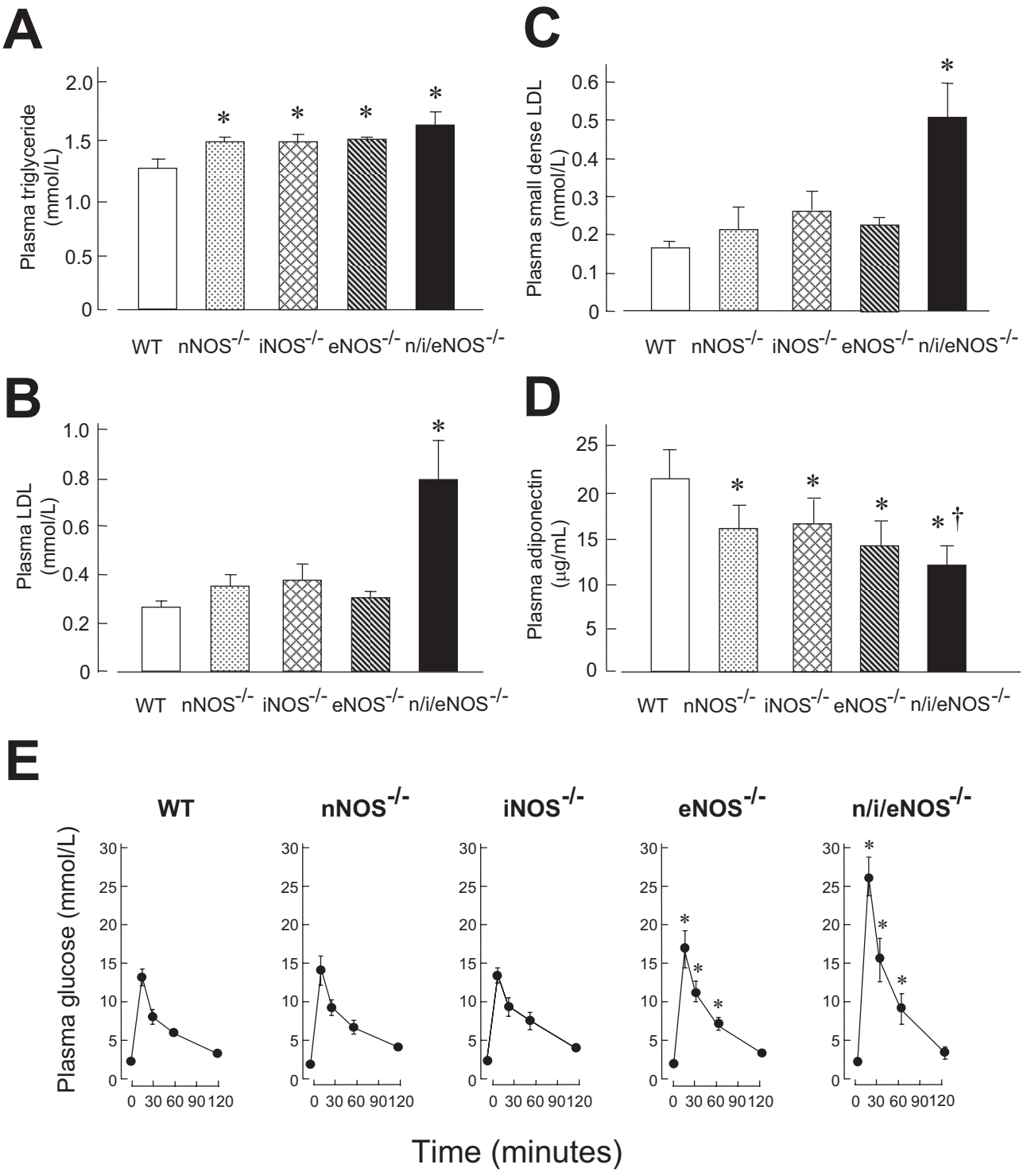

Figure 4. Hyperlipidemia, impaired glucose tolerance, and hypoadiponectinemia in n/i/eNOS$^{-/-}$ mice. Experiments were performed in 3-month-old male mice. A through C, Plasma levels of triglyceride (A), LDL cholesterol (B), and small dense LDL particles (C; n=8 to 12). *$P<0.05$ vs WT C57BL/6J. D, Plasma adiponectin levels (n=5 to 7). *$P<0.05$ vs WT. †$P<0.05$ vs nNOS$^{-/-}$, iNOS$^{-/-}$, and eNOS$^{-/-}$. E, Plasma glucose concentrations after intravenous glucose infusion (1 g/kg; n=5 to 7). *$P<0.05$ vs WT.

Impaired Glucose Tolerance in Triply Deficient n/i/eNOS$^{-/-}$ Mice

Plasma glucose concentrations after intravenous glucose injection were significantly higher in the singly deficient eNOS$^{-/-}$ and triply deficient n/i/eNOS$^{-/-}$ mice (Figure 4E). In the present study, in addition to C57BL/6J mice, we also used 129SV mice as a WT control. No significant differences were noted in the amount of visceral adipose tissue, blood pressure levels, plasma lipid profile, or glucose metabolism between the C57BL/6 and 129SV mice at 3 months of age (Figure IIIA through IIIK in the online-only Data Supplement). Furthermore, no significant difference was noted in

the extent of coronary vascular lesion formation between the 2 normal genotypes at 5 months of age (online-only Data Supplement Figure IIIL).

Hypoadiponectinemia in Triply Deficient n/i/eNOS$^{-/-}$ Mice

Plasma adiponectin concentrations were reduced significantly in all the singly and triply NOS-deficient mice, and the triply NOS-deficient mice showed the lowest levels of plasma adiponectin (Figure 4D).

Echocardiographic Abnormalities in Triply Deficient n/i/eNOS$^{-/-}$ Mice

Among the five 8-month-old n/i/eNOS$^{-/-}$ mice studied, 1 mouse showed hypokinesis and thinning (0.80 mm) of the anterior wall and a reduction in fractional shortening (38.2%), which is compatible with old myocardial infarction (Table I, online-only Data Supplement). However, the difference in fractional shortening, left ventricular (LV) end-diastolic diameter, and LV end-systolic diameter among the 5 genotypes did not reach a statistically significant level (online-only Data Supplement Table I). The thickness of both the anterior and posterior walls was increased significantly in the triply deficient n/i/eNOS$^{-/-}$ genotype compared with the WT genotype (online-only Data Supplement Table I). Although singly deficient eNOS$^{-/-}$ mice also exhibited LV hypertrophy, its extent was significantly larger in the triply deficient n/i/eNOS$^{-/-}$ genotype (online-only Data Supplement Table I).

Vascular Dysfunction in Triply Deficient n/i/eNOS$^{-/-}$ Mice

Endothelium-independent relaxations to forskolin were comparable among the 5 genotypes studied (online-only Data Supplement Figure IA). In contrast, endothelium-independent relaxations to diethylamine NONOate were significantly enhanced in triply deficient n/i/eNOS$^{-/-}$ and singly deficient eNOS$^{-/-}$ mice compared with WT mice (online-only Data Supplement Figure IB). In triply deficient n/i/eNOS$^{-/-}$ and singly deficient eNOS$^{-/-}$ mice, endothelium-dependent relaxations to acetylcholine were completely lacking (online-only Data Supplement Figure IC), and contractions to phenylephrine were markedly potentiated (online-only Data Supplement Figure ID).

Activation of the Renin-Angiotensin System in Triply Deficient n/i/eNOS$^{-/-}$ Mice

We next investigated the molecular mechanism(s) for the cardiovascular and metabolic abnormalities of triply deficient n/i/eNOS$^{-/-}$ mice. Upregulation (Figure 5A and 5B) and activation (Figure 5C) of angiotensin-converting enzyme (ACE) in the heart and the coronary artery were noted in triply deficient n/i/eNOS$^{-/-}$ mice but not in any singly NOS-deficient mice compared with WT mice at 5 months of age. Upregulation of AT_1 receptor in the heart and the coronary artery was also noted only in triply deficient n/i/eNOS$^{-/-}$ mice (Figure 5D and 5E). Furthermore, plasma renin activity (Figure IIA, online-only Data Supplement) and plasma concentrations of angiotensin II (Figure 5F) and aldosterone (Figure IIB, online-only Data supplement) were all increased significantly in this genotype.

Beneficial Effects of an AT_1 Receptor Blocker on Cardiovascular and Metabolic Abnormalities of Triply Deficient n/i/eNOS$^{-/-}$ Mice

To show the involvement of the AT_1 receptor–mediated mechanism, we examined the effects of long-term treatment with an AT_1 receptor blocker on cardiovascular and metabolic phenotypes of the triply deficient n/i/eNOS$^{-/-}$ mice. Treatment with olmesartan, a selective and potent AT_1 receptor blocker,[23] for 10 months significantly ameliorated the survival rate in triply NOS-deficient mice (Figure 6A). Although treatment with hydralazine, an antihypertensive drug, for 10 months also tended to ameliorate the survival rate in triply NOS-deficient mice, it did not reach a statistically significant level (Figure 6A). We then killed and examined the triply NOS-deficient mice that survived the 10-month experimental period at 14 months of age. Myocardial infarction (Figure 6B), coronary vascular lesion formation (Figure 6C), and coronary mast cell infiltration (Figure 6D) were all seen in the untreated triply NOS-deficient mice. Treatment with olmesartan significantly reduced the incidence of myocardial infarction (Figure 6B), the extent of coronary vascular lesion formation (Figure 6C), and the extent of coronary mast cell infiltration (Figure 6D). Treatment with hydralazine elicited similar effects; however, the effects of olmesartan were significantly greater than those of hydralazine (Figure 6B through 6D).

In addition, treatment with olmesartan for 1.5 months significantly ameliorated all metabolic abnormalities in the triply NOS-deficient mice (Figure 7A through 7E), in association with an improvement of hypoadiponectinemia (Figure 7F). Although treatment with hydralazine for 1.5 months also significantly reduced blood pressure in triply NOS-deficient mice to the same extent as olmesartan (Figure 7B), it did not significantly affect other metabolic parameters (Figure 7A and 7C through 7F).

Discussion

Clinical Significance

Several lines of evidence suggest the association of defective NOS systems with coronary arteriosclerosis, myocardial infarction, and metabolic diseases in humans. First, it has been reported that plasma and/or urinary NOx levels, which are markers of NO production derived from all 3 types of NOS in vivo, are reduced in patients with coronary arteriosclerosis, metabolic syndrome, and each component of the metabolic syndrome.[24–28] Second, plasma concentrations of asymmetric dimethylarginine, which is an endogenous NOS inhibitor, have been shown to be elevated in patients with arteriosclerosis, patients at risk of myocardial infarction, and patients with metabolic syndrome.[29] Finally, it has been revealed in humans that NOS gene polymorphisms are associated with arteriosclerosis, risk of myocardial infarction, metabolic syndrome, and low plasma NOx levels.[30] These results may imply the clinical significance of the present findings with triply NOS-deficient mice.

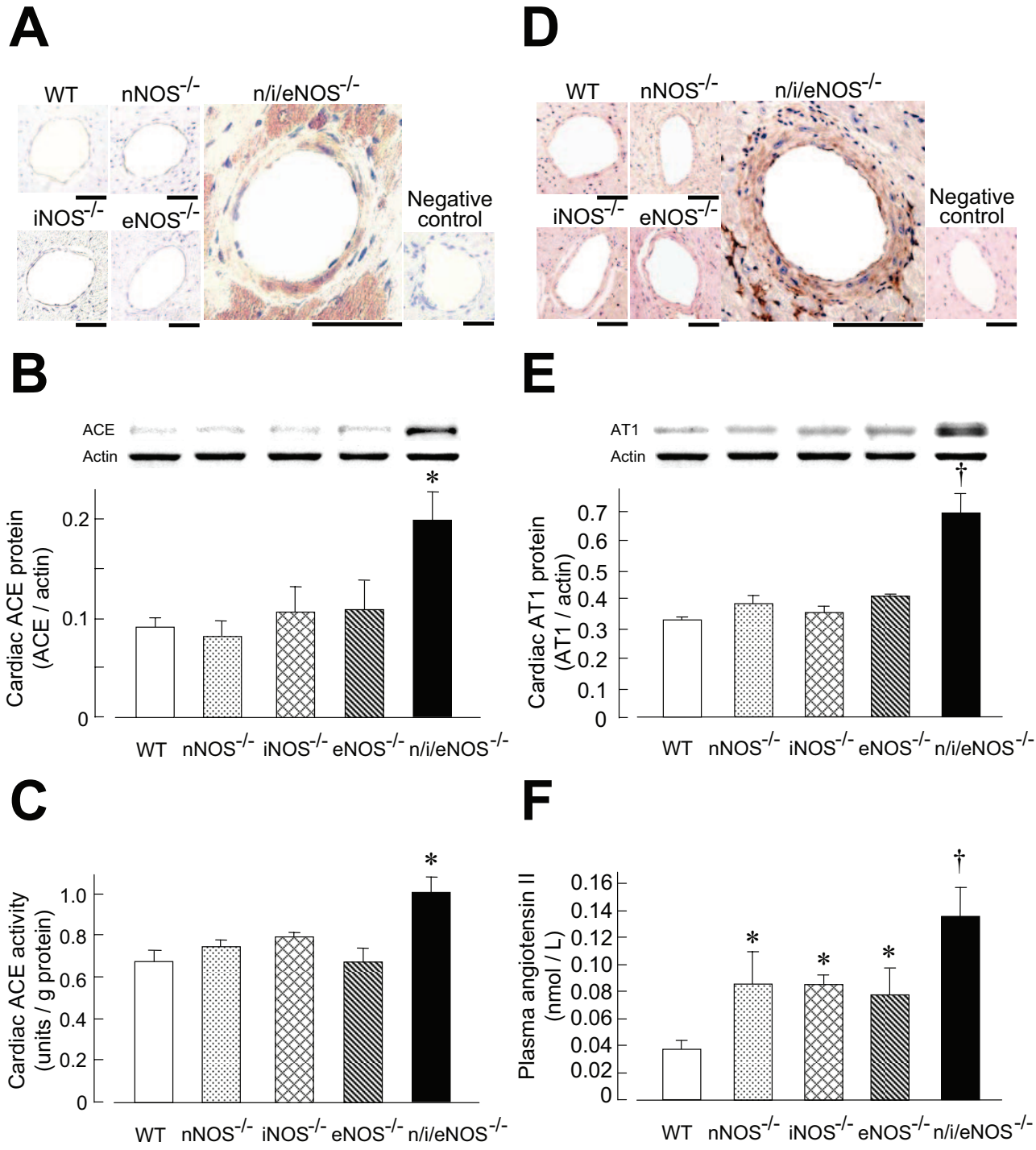

Figure 5. Activation of the renin-angiotensin system in n/i/eNOS$^{-/-}$ mice. Experiments were performed in 5-month-old male mice. A, Coronary ACE immunostaining (n=5 each). B, Cardiac ACE protein levels (n=5 to 6). C, Cardiac ACE activity (n=5 to 6). D, Coronary AT_1 receptor immunostaining (n=5 each). E, Cardiac AT_1 receptor protein levels (n=5 to 6). F, Plasma angiotensin II levels (n=6 to 7). *$P<0.05$, †$P<0.01$ vs WT C57BL/6J. Scale bars in panels A and D=50 μm.

Murine Model of Spontaneous Myocardial Infarction

In the present study, survival rate was impaired in accordance with the number of disrupted NOS genes, which suggests the essential role of the endogenous NOS system in survival. Interestingly, more than half of the male triply NOS-deficient mice possibly died of spontaneous myocardial infarction, although we could not exclude the possibility of a mere association between myocardial infarction and death. The mouse with homozygous null mutations in the genes for both the high-density lipoprotein receptor SR-B1 and apolipoprotein (apo) E is known as the first and sole murine model of spontaneous myocardial infarction.[31] However, the longevity of this SR-B1/apoE$^{-/-}$ mouse is extremely short, and even when fed a standard chow diet, all such mice die before 2 months of age.[31] Thus, in this regard, the present triply

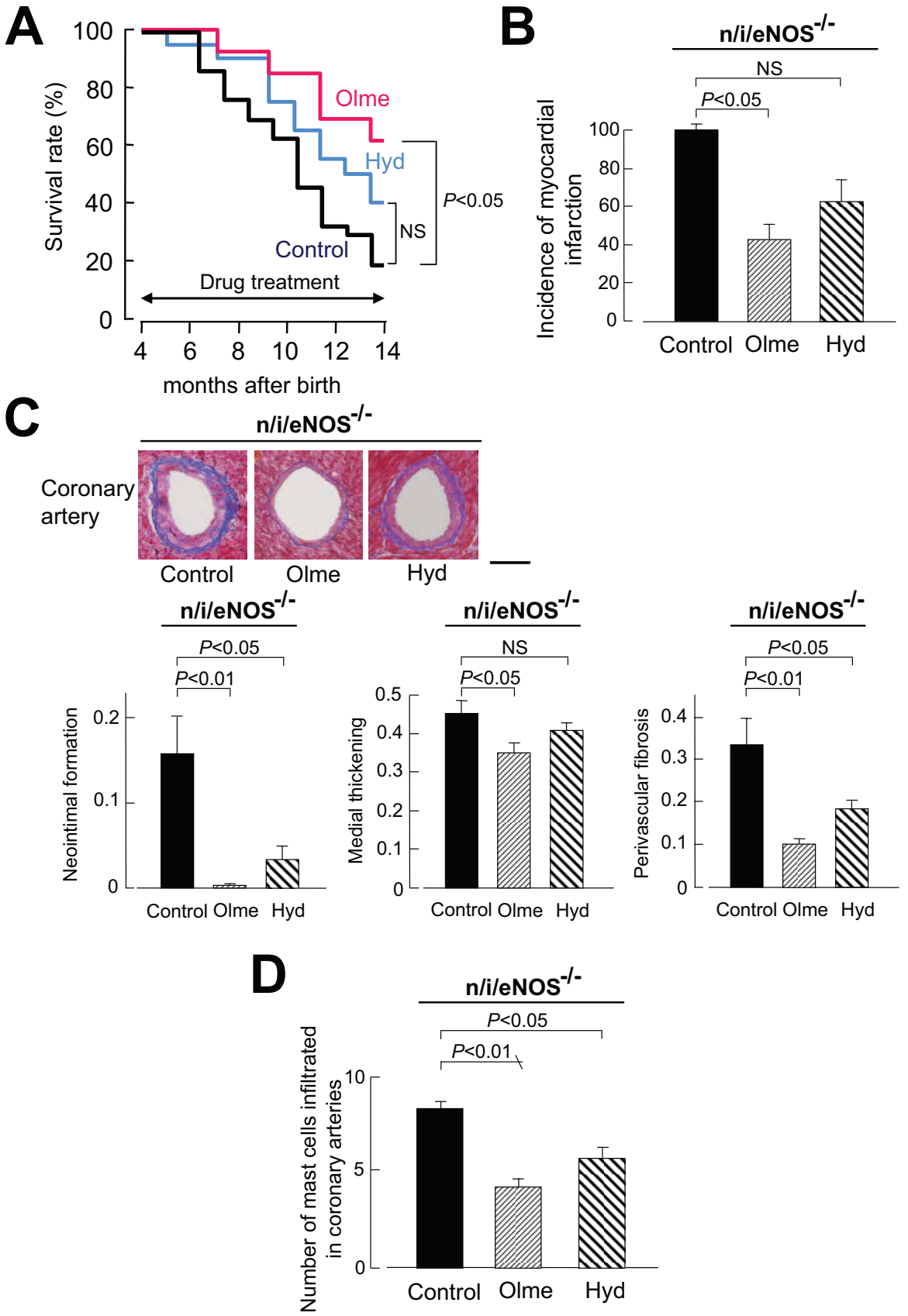

Figure 6. Beneficial effects of an AT_1 receptor blocker on cardiovascular abnormalities of n/i/eNOS$^{-/-}$ mice. A–D, Effects of treatment with either olmesartan (Olme, 5 mg · /kg^{-1} · d^{-1}) or hydralazine (Hyd, 0.05 mg/mL) for 10 months on survival rate (n=20 to 22; A), incidence of myocardial infarction (n=6 to 12; B), coronary vascular lesion formation (n=6 to 12; C), and coronary mast cell infiltration (n=6 to 12; D) in 14-month-old male mice. Scale bar in panel C=50 μm.

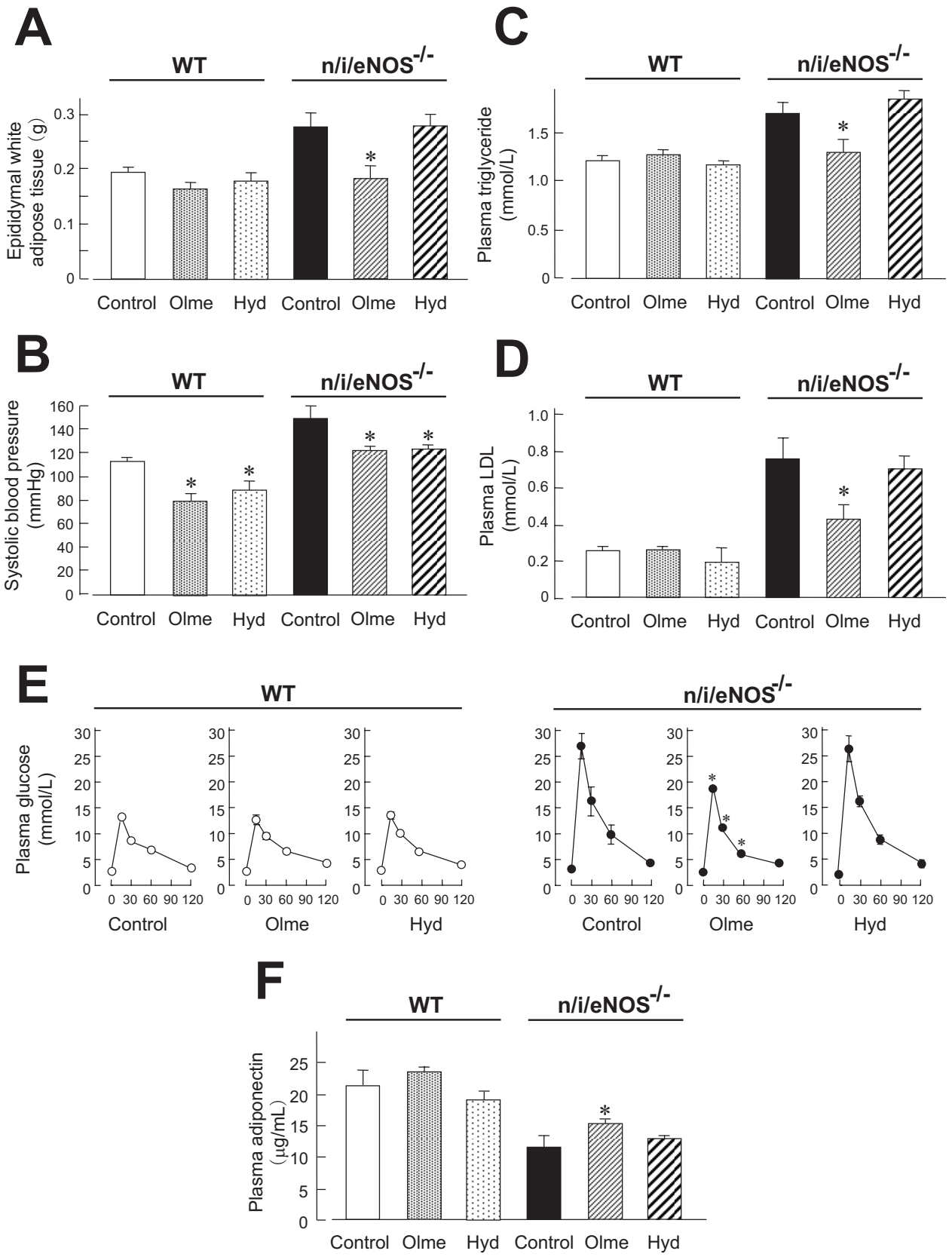

Figure 7. Beneficial effects of an AT_1 receptor blocker on metabolic abnormalities of n/i/eNOS$^{-/-}$ mice. A through F, Effects of treatment with either olmesartan (Olme) or hydralazine (Hyd) for 1.5 months on visceral obesity (A), hypertension (B), hypertriglyceridemia (C), hyper-LDL-cholesterolemia (D), impaired glucose tolerance (E), and hypoadiponectinemia (F) in 3-month-old male mice (n=6 to 12). *$P<0.05$ vs untreated control.

NOS-deficient mouse may be a more useful murine model of spontaneous myocardial infarction.

Arteriosclerosis was seen in most of the vasculature in triply NOS-deficient mice, whereas atherosclerosis was observed in the aorta alone. This finding is in agreement with the previous study demonstrating that in apoE$^{-/-}$ mice, atherosclerosis is dominant in the aorta but not in the coronary artery.[32] Human myocardial infarction results not only from coronary atherosclerosis but also from other causes, including coronary intimal hyperplasia, medial thickening, and coronary vasospasm.[33,34] In the triply NOS-deficient mice that died of myocardial infarction, marked coronary intimal hyperplasia and medial thickening were noted. Furthermore, in the dead triply NOS-deficient mice, marked infiltration of mast cells at the coronary artery adventitia was also observed. Histamine released from adventitial mast cells is thought to cause coronary vasospasm, with resultant myocardial infarction, in humans.[35] Thus, it is possible that coronary arteriosclerosis and coronary vasospasm are involved in the cause of death in triply NOS-deficient mice. The present triply NOS-deficient mouse might be a model of such nonatherosclerotic forms of myocardial infarction in humans.

Cardiovascular Risk Factors in Triply Deficient NOS$^{-/-}$ Mice

Importantly, the triply NOS-deficient mice manifested characteristics resembling the metabolic syndrome in humans.[36,37] A clustering of those cardiovascular risk factors could contribute to the development of vascular lesion formation in the triply NOS-deficient mouse.[33] Although the singly eNOS-deficient mouse also showed similar metabolic phenotypes, this genotype did not develop coronary arteriosclerosis and myocardial infarction. These results suggest that compensation by the remaining NOS may operate in singly eNOS-deficient mice and that only triply NOS-deficient mice have the cardiovascular phenotypes associated with metabolic abnormalities. Plasma levels of LDL and small dense LDL, both of which are independent cardiovascular risk factors,[38] were increased only in the triply NOS-deficient mice, which suggests that those alterations are also involved in vascular lesion formation in these mice.

Adiponectin Deficiency in Triply Deficient NOS$^{-/-}$ Mice

Adiponectin is an antiatherogenic adipocytokine that improves hypertriglyceridemia, glucose metabolism, and insulin resistance and inhibits the progression of arteriosclerosis.[39] In the case of obesity with adipocyte hypertrophy, synthesis of adiponectin is decreased, not increased, and in patients with metabolic syndrome, circulating levels of adiponectin are reduced in contrast to the increases in levels of other adipocytokines.[39] The deficiency of adiponectin is thought to play a pivotal role in the pathogenesis of metabolic syndrome and its vascular complications.[39] In the triply NOS-deficient mice in the present study, plasma adiponectin levels were reduced, and restoration of hypoadiponectinemia was linked to amelioration of metabolic phenotypes. Thus, adiponectin deficiency may also contribute to the development of metabolic abnormalities and vascular lesion formation seen in the triply NOS-deficient mice.

Cardiac Abnormalities in Triply Deficient n/i/eNOS$^{-/-}$ Mice

Although one 8-month-old triply NOS-deficient mouse exhibited local wall-motion abnormality compatible with old myocardial infarction, no significant differences were noted in markers of LV function, such as fractional shortening, LV end-diastolic diameter, or LV end-systolic diameter, among the 5 genotypes. On the other hand, LV hypertrophy was noted in triply NOS-deficient and singly eNOS-deficient mice. Its extent was greater in the triply deficient NOS$^{-/-}$ genotype than in the eNOS$^{-/-}$ genotype, whereas the extent of hypertension was comparable in the 2 genotypes, which suggests that mechanisms other than hypertension may also mediate LV hypertrophy in the n/i/eNOS$^{-/-}$ genotype.

Vascular Dysfunction in Triply Deficient n/i/eNOS$^{-/-}$ Mice

Endothelium-independent relaxations to forskolin, which is an activator of adenylate cyclase, were comparable among the 5 genotypes, which suggests that the function of vascular smooth muscle cells is intact in triply NOS-deficient mice. In contrast, endothelium-independent relaxations to diethylamine NONOate, which is an NO donor, were enhanced in the n/i/eNOS$^{-/-}$ and eNOS$^{-/-}$ mice. These results suggest hypersensitivity to exogenous NO in the blood vessels of the n/i/eNOS$^{-/-}$ mice. On the other hand, in the n/i/eNOS$^{-/-}$ and eNOS$^{-/-}$ mice, endothelium-dependent relaxations to acetylcholine, which is a physiological eNOS activator, were completely lacking, and contractions to phenylephrine, which is an α_1-adrenergic agonist, were markedly potentiated. Thus, these vascular dysfunctions could also be involved in the pathogenesis of myocardial infarction in the n/i/eNOS$^{-/-}$ mice.

NOS-Independent NO Generation

The biological activity of NO is terminated by oxidation to nitrite, whereas nitrite can be recycled back to bioactive NO in blood and tissues.[40] At least 2 sources of nitrite are available to mammals. First, nitrite is present in the diet. Second, nitrite is synthesized by bacteria, especially enterobacterium, by means of active nitric acid metabolism. Nitrite reduction to NO can occur via several routes that involve enzymes, proteins, vitamins, or even simple protons.[40] Although it is highly surprising that the triply deficient NOS$^{-/-}$ mice can survive for months, these mechanisms may contribute to the survival of the n/i/eNOS$^{-/-}$ mice. Indeed, we previously demonstrated that plasma and urinary levels of NOx were not zero (≈3% of normal values) in triply NOS-deficient mice.

Role of the AT$_1$ Receptor Pathway in the Development of Cardiovascular and Metabolic Abnormalities in Triply Deficient NOS$^{-/-}$ Mice

The renin-angiotensin system plays an important role in the pathogenesis of cardiovascular and metabolic diseases.[41,42] In the n/i/eNOS$^{-/-}$ mice, the renin-angiotensin system, as eval-

uated by tissue levels of ACE and AT_1 receptor and plasma levels of renin, angiotensin II, and aldosterone, was activated. On the basis of these results, we further examined our hypothesis that the AT_1 receptor–mediated mechanism is involved in cardiovascular and metabolic abnormalities of triply NOS-deficient mice. Notably, long-term treatment with the AT_1 receptor blocker olmesartan potently inhibited coronary arteriosclerotic lesion formation, adventitial mast cell infiltration, and the occurrence of myocardial infarction in the triply NOS-deficient mice, with a resultant improvement of the prognosis. Furthermore, treatment with olmesartan reversed all the abnormal metabolic phenotypes and ameliorated the hypoadiponectinemia. These results suggest that the AT_1 receptor pathway plays a central role in the pathogenesis of cardiovascular and metabolic disorders in the triply NOS-deficient mouse. Although long-term treatment with hydralazine lowered blood pressure levels of the n/i/eNOS$^{-/-}$ mice to the same extent as olmesartan, the beneficial cardiovascular and metabolic effects of hydralazine were significantly less than those of olmesartan in terms of coronary lesion formation, mast cell infiltration, morbidity of myocardial infarction, survival rate, and metabolic phenotypes. Thus, it is conceivable that the beneficial effects of olmesartan are mediated not only by a reduction in blood pressure but also by blockade of the AT_1 receptor.

Mechanisms for Beneficial Effects of AT_1 Receptor Blockade on Metabolic Abnormalities of Triply Deficient NOS$^{-/-}$ Mice

Although the underlying mechanisms for human metabolic syndrome have not been elucidated completely, visceral obesity and insulin resistance are thought to be the cardinal basis of the syndrome.[43] In visceral obesity, hypertrophied adipose cells secrete an excessive amount of free fatty acids and proatherogenic adipocytokines, all of which inhibit the signal transduction of insulin and cause insulin resistance.[42] Insulin resistance, in turn, induces hypertension, hyperlipidemia, diabetes mellitus, and atherosclerosis. On the other hand, reduced secretion of the antiatherogenic adiponectin in the hypertrophied adipocytes may also accelerate the metabolic syndrome.[44] Importantly, stimulation of the AT_1 receptor causes hypertrophy of human adipocytes.[42] Thus, AT_1 receptor blockers may prevent adipocyte hypertrophy, ameliorating the abnormal secretion of free fatty acids and adipocytokines with a resultant improvement of metabolic syndrome.[42] In addition, stimulation of the AT_1 receptor induces a number of other proatherogenic metabolic effects, including vasoconstriction, secretion of triglycerides from the liver, glycogenolysis in the liver, reduced delivery of glucose in skeletal muscles, induction of inflammation, and activation of the sympathetic nervous system.[42] Thus, these mechanisms may also be involved in the beneficial effects of AT_1 receptor blockers in the treatment of metabolic disorders in the triply NOS-deficient mouse.[42,43]

In conclusion, we were able to demonstrate that complete deletion of the entire endogenous NOS system causes spontaneous myocardial infarction associated with metabolic abnormalities in mice in vivo via the AT_1 receptor pathway, demonstrating the critical role of the endogenous NOS system in maintaining cardiovascular and metabolic homeostasis.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Although it is well established that endothelial nitric oxide synthase (eNOS) exerts powerful antiarteriosclerotic effects, eNOS-deficient mice do not spontaneously develop arteriosclerosis. This inconsistency may be due to a compensatory mechanism by other nitric oxide synthases (NOS) that are not genetically disrupted. Thus, it remains to be fully elucidated whether or not nitric oxide derived from the NOS system indeed plays a pathogenetic role in arteriosclerotic/atherosclerotic cardiovascular diseases. To address this issue, we have recently developed mice in which the NOS system is completely deleted (triply n/i/eNOS$^{-/-}$ mice). Here, we show that the triply deficient mice exhibit markedly reduced survival due to spontaneous myocardial infarction accompanied by severe coronary arteriosclerotic lesions. Furthermore, the triply NOS-deficient mice manifested metabolic syndrome, including visceral obesity, hypertension, hypertriglyceridemia, and glucose intolerance. These results provide the first direct evidence that genetic disruption of the entire NOS system causes spontaneous myocardial infarction associated with multiple cardiovascular risk factors in vivo, which demonstrates the critical role of the endogenous NO/NOS system in maintaining cardiovascular homeostasis. Importantly, activation of the renin-angiotensin system was noted in the triply NOS-deficient mice, and long-term oral treatment with an angiotensin II type 1 receptor blocker significantly suppressed both coronary arteriosclerotic lesion formation and the occurrence of spontaneous myocardial infarction and improved the prognosis of those mice, along with ameliorating the metabolic syndrome. These findings indicate that activation of the renin-angiotensin system plays a pivotal role in the pathogenesis of myocardial infarction in conditions of defective NO production, which suggests the therapeutic importance of renin-angiotensin system inhibitors to prevent myocardial infarction in humans.

Spontaneous Myocardial Infarction in Mice Lacking All Nitric Oxide Synthase Isoforms

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