Cardiovascular Research (2010) **87**, 675–682
doi:10.1093/cvr/cvq092

Severe dyslipidaemia, atherosclerosis, and sudden cardiac death in mice lacking all NO synthases fed a high-fat diet

Yasuko Yatera[1], Kiyoko Shibata[1], Yumi Furuno[1], Ken Sabanai[2], Naoya Morisada[3], Sei Nakata[1], Tsuyoshi Morishita[1], Yumiko Toyohira[2], Ke-Yong Wang[4], Akihide Tanimoto[5], Yasuyuki Sasaguri[4], Hiromi Tasaki[1], Yasuhide Nakashima[1], Hiroaki Shimokawa[6], Nobuyuki Yanagihara[2], Yutaka Otsuji[1], and Masato Tsutsui[2,7]*

[1]Second Department of Internal Medicine, University of Occupational and Environmental Health, Kitakyushu, Japan; [2]Department of Pharmacology, University of Occupational and Environmental Health, Kitakyushu, Japan; [3]Department of Pediatrics, University of Occupational and Environmental Health, Kitakyushu, Japan; [4]Department of Pathology, University of Occupational and Environmental Health, Kitakyushu, Japan; [5]Department of Molecular and Cellular Pathology, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima, Japan; [6]Department of Cardiovascular Medicine, Tohoku University Graduate School of Medicine, Sendai, Japan; and [7]Department of Pharmacology, Faculty of Medicine, University of the Ryukyus, 207 Uehara, Nishihara, Okinawa 903-0215, Japan

Received 10 December 2009; revised 2 March 2010; accepted 16 March 2010; online publish-ahead-of-print 18 March 2010

Time for primary review: 26 days

Aims The precise role of the nitric oxide synthase (NOS) system in lipid metabolism remains to be elucidated. We addressed this point in mice that we have recently developed and that lack all three NOS isoforms.

Methods and results Wild-type (WT), singly, doubly, and triply $NOS^{-/-}$ mice were fed either a regular or high-cholesterol diet for 3–5 months. The high-cholesterol diet significantly increased serum low-density lipoprotein (LDL) cholesterol levels in all the genotypes when compared with the regular diet. Importantly, when compared with the WT genotype, the serum LDL cholesterol levels in the high-cholesterol diet were significantly and markedly elevated only in the triply $NOS^{-/-}$ genotype, but not in any singly or doubly $NOS^{-/-}$ genotypes, and this was associated with remarkable atherosclerosis and sudden cardiac death, which occurred mainly in the 4–5 months after the high-cholesterol diet. Finally, hepatic LDL receptor expression was markedly reduced only in the triply $NOS^{-/-}$ genotype, accounting for the diet-induced dyslipidaemia in the genotype.

Conclusion These results provide the first direct evidence that complete disruption of all NOS genes causes severe dyslipidaemia, 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 the whole endogenous NOS system in maintaining lipid homeostasis.

Keywords Nitric oxide • Nitric oxide synthase • Atherosclerosis

1. Introduction

Nitric oxide (NO) plays an important role in maintaining cardiovascular homeostasis.[1–4] Three distinct NO synthase (NOS) isoforms exist and are encoded by three distinct genes, including neuronal (nNOS or NOS1), inducible (iNOS or NOS2), and endothelial NOS (eNOS or NOS3). Initial NO studies indicated that nNOS and eNOS are constitutively expressed mainly in the nervous system and the vascular endothelium, respectively, synthesizing a small amount of NO in a calcium-dependent manner both under basal conditions and upon stimulation, and that iNOS is induced only when stimulated by microbial endotoxins or certain proinflammatory cytokines, producing a greater amount of NO in a calcium-independent manner.[1–4] However, recent studies have revealed that both nNOS and eNOS are also subject to expressional regulation, and that iNOS is constitutively expressed even under physiological conditions.[4] Furthermore, it has become apparent that in addition to eNOS and iNOS, nNOS is also expressed in the cardiovascular system, exerting important cardiovascular actions.[4] Thus, NO research is taking a new turn.

* Corresponding author. Tel: +81 98 895 1133; fax: +81 98 895 1411, Email: tsutsui@med.u-ryukyu.ac.jp

The roles of the NOS system *in vivo* have been investigated in pharmacological studies with NOS inhibitors and in studies with NOS isoform-deficient mice. However, because of the non-specificity of the NOS inhibitors and the compensatory interactions among the NOS isoforms, the authentic roles of the entire NOS system still remain to be fully elucidated. To address this important issue, we have recently developed mice in which all three NOS isoforms are completely deleted.[5,6] The triply nNOS/iNOS/eNOS-deficient (n/i/eNOS$^{-/-}$) mice are unexpectedly viable and appear normal, but their survival and fertility rates are markedly reduced when compared with wild-type (WT) mice. The triply NOS$^{-/-}$ mice also exhibit marked hypotonic polyuria, polydipsia, and renal unresponsiveness to an antidiuretic hormone, vasopressin, all of which are characteristics consistent with nephrogenic diabetes insipidus.[5,6] In addition, we have recently revealed that the triply NOS$^{-/-}$ mice spontaneously develop myocardial infarction.[7,8] However, the role of the NOS system in the regulation of lipid metabolism is not fully understood. Thus, in this study, we examined the effect of a Western-type cholesterol-rich diet on lipid metabolism in our triply mutant mice.

2. Methods

2.1 Animal preparation

This study was reviewed and approved by the Ethics Committee of Animal Care and Experimentation, University of Occupational and Environmental Health, Japan, and University of the Ryukyus, Japan, and was carried out according to the Institutional Guidelines for Animal Experimentation and the Law (No. 105) and Notification (No. 6) of the Japanese Government. This investigation conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Male WT C57BL/6 and 129SV (Charles River, Yokohama, Japan) and NOS$^{-/-}$ mice were fed either a regular diet (0.075% cholesterol) or a high-cholesterol diet (1.25% cholesterol) for 12 weeks, from 4 to 16 weeks of age. In the experiment in which the survival rate was examined, the mice were fed either the regular diet or the high-cholesterol diet for 20 weeks, from 4 to 24 weeks of age. We generated doubly and triply NOS$^{-/-}$ mice by crossing singly NOS$^{-/-}$ mice, as reported previously.[5] Systolic blood pressure was measured by the tail-cuff method under conscious conditions (Model MK-2000, Muromachi Kikai Co., Ltd, Tokyo, Japan).

2.2 Morphology

The animals were euthanized by inhalation of an overdose of diethyl ether (Wako Pure Chemical Industries, Osaka, Japan). The aorta was cannulated and perfused with a 4% paraformaldehyde solution under physiological pressure.[7] The hearts, brains, lungs, kidneys, and spleens were embedded in paraffin, and 5 μm-thick slices were stained with haematoxylin–eosin or Masson-trichrome solutions. The sections were scanned with a light microscope equipped with a two-dimensional analysis system (IBAS, Carl Zeiss, Jena, Germany).[9]

2.3 Oil red O staining

The thoracic and abdominal aortas were used for *en face* staining to visualize the accumulation of neutral lipids (cholesteryl ester and triglycerides). The aortas were cut open with the luminal surface facing up and fixed in 10% formalin. They were thoroughly cleaned of adventitial fat using microforceps and spring iris scissors under a stereoscopic microscope. The inner aortic surface was stained with an oil red O solution for 30 min at room temperature. After rinsing with 50% isopropyl alcohol and distilled water, the oil red O-stained area was analysed by scion image beta 4.02 software (Scion, Frederick, MD, USA). The extent of lipid accumulation was evaluated by the ratio of the lipid-accumulating lesion area to the total aortic area.

2.4 Immunostaining

Sections of paraffin-embedded tissue were incubated with an anti-mouse monoclonal macrophage galactose-specific lectin-2 (Mac-2) antibody (Cedarlane Laboratories, Ontario, Canada), as we reported previously.[9]

2.5 Lipid metabolism

The blood was collected after 15 h of fasting. The serum 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 the high-sensitivity lipoprotein profiling system using high-performance liquid chromatography.[10]

2.6 Angiotensin-converting enzyme activity

Immediately after the euthanasia, the hearts were quickly dissected and cryopreserved at −80°C. Cardiac angiotensin-converting enzyme (ACE) activities were assessed by an ACE activity assay kit (Life Laboratory, Yamagata, Japan).

2.7 Eight-isoprostane

Urine was collected with metabolic cages and stored at −80°C. Urinary 8-isoprostane levels were measured with an enzyme immunoassay kit (Oxford Biomedical Research, Oxford, MI, USA).

2.8 C-reactive protein level

Serum C-reactive protein levels were analysed by a mouse C-reactive protein ELISA kit (Funakoshi Co., Ltd, Tokyo, Japan).

2.9 Western blot analysis

The livers and the serums were freshly isolated and frozen in liquid nitrogen. The liver sections were homogenized at 4°C with 500 μL of buffer containing 1.1 μmol/L leupeptin, 0.7 μmol/L aprotinin, 120 μmol/L phenylmethanesulfonyl fluoride, 0.7 μmol/L pepstatin, 1 mmol/L iodoacetamide, and 1 mmol/L diisopropylfluorophosphate. The serums were diluted with the same buffer 50 times in volume. Western blot analyses for serum apolipoprotein E (apoE) and for hepatic LDL receptor and sterol regulatory element-binding protein-2 (SREBP-2) were performed as we reported previously.[11] Activation of SREBP-2 is mediated by a post-translational cleavage in which the immature protein (120 kDa) is enzymatically truncated into a smaller mature protein (68 kDa) that enters the nucleus and increases transcription of the LDL receptor gene. Thus, the SREBP activity was assessed by the expression levels of the activated form of SREBP-2 (68 kDa), as reported previously.[12] Protein concentrations were determined by the Bradford method.[13]

2.10 Real-time polymerase chain reaction

The intestines were rinsed twice with sterile PBS, and the duodena were separated and opened longitudinally. The duodena were scrapped on ice, frozen in liquid nitrogen, and stored at −80°C. Total RNA was extracted by an Isogen reagent (Nippon Gene, Tokyo, Japan), and reverse transcription was realized with a QuantiTect Reverse Transcription kit (Qiagen, Tokyo, Japan). mRNA levels were determined by real-time quantitative polymerase chain reaction (PCR) on an ABI prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA) using a QuantiTect Probe PCR Kit (Qiagen) and primers for murine Niemann-Pick C1-like 1 (NPC1L1) (sense 5′-GCA AGG TGA TCA GGA GGT TGA-3′; antisense 5′-ATC CTC ATC CTG GGC TTT GC-3′).[14] The PCR conditions were 95°C for 15 min, followed by 40 cycles of 15 s at 94°C and 60 s at 60°C.

2.11 Statistical analysis

Results are expressed as mean ± SEM. Survival curves were analysed by the Kaplan–Meier method. Differences in other parameters were evaluated by ANOVA followed by the Scheffe *post hoc* test for multiple comparisons, or by unpaired *t*-test. A value of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1 Severe dyslipidaemia in triply n/i/eNOS$^{-/-}$ mice fed a high-cholesterol diet

We first investigated the effect of the Western-type cholesterol-rich diet for 3 months on the serum lipid profiles in the eight strains (WT C57BL/6, singly nNOS$^{-/-}$, iNOS$^{-/-}$, eNOS$^{-/-}$, doubly n/iNOS$^{-/-}$, n/eNOS$^{-/-}$, i/eNOS$^{-/-}$, and triply n/i/eNOS$^{-/-}$ mice). The high-cholesterol diet significantly increased the serum levels of total cholesterol (*Figure 1A*), LDL cholesterol (*Figure 1B*), and small dense LDL particles (*Figure 1C*) in all the genotypes studied when compared with the regular diet. Intriguingly, when compared with the WT genotype, the serum levels of total cholesterol, LDL cholesterol, and small dense LDL particles in the high-cholesterol diet were all significantly and markedly higher only in the triply n/i/eNOS$^{-/-}$ genotype, but not in any singly or doubly NOS$^{-/-}$ genotypes, and these levels were similar to those in apoE$^{-/-}$ mice that manifest severe hyperlipidaemia.[15,16] In contrast, the serum levels of high-density lipoprotein (HDL) cholesterol (*Figure 1D*) or triglyceride (*Figure 1E*) in the high-cholesterol diet did not significantly differ in any of the genotypes.

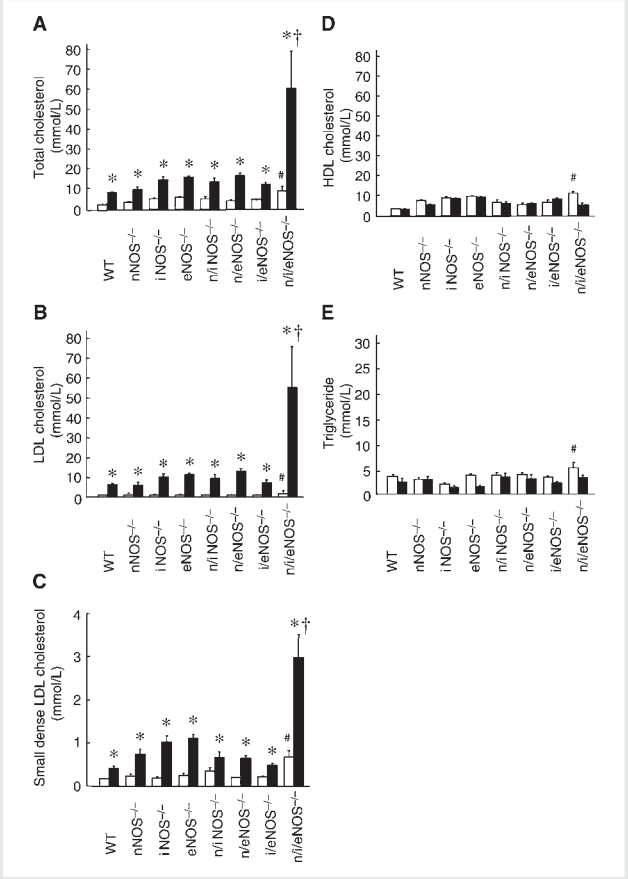

Figure 1 Serum lipid profile in WT and NOS$^{-/-}$ mice fed a regular or high-cholesterol diet for 3 months ($n = 6–11$). White and black bars indicate the regular and high-cholesterol diets, respectively. WT, C57BL/6; LDL, low-density lipoprotein; HDL, high-density lipoprotein. *$P < 0.05$ vs. the regular diet; †$P < 0.05$ vs. WT mice fed the high-cholesterol diet; #$P < 0.05$ vs. WT mice fed the regular diet.

3.2 Atherosclerosis in triply n/i/eNOS$^{-/-}$ mice fed a high-cholesterol diet

We next examined whether the cholesterol-rich diet would elicit atherosclerotic vascular lesion formation in the eight strains. Although in the WT, singly, and doubly NOS$^{-/-}$ genotypes, the high-cholesterol diet tended to induce lipid accumulation in the aortas, these effects did not reach statistically significant levels (*Figure 2*). However, in the triply n/i/eNOS$^{-/-}$ genotype, the high-cholesterol diet significantly and markedly caused aortic lipid accumulation (*Figure 2*). In addition, the high-cholesterol diet also significantly and markedly elicited atheromatous plaque formation in the aortic sinus only in the triply n/i/eNOS$^{-/-}$ genotype (see Supplementary material online, *Figure S1*). In those atheromatous plaque lesions, subendothelial aggregation of Mac-2-positive macrophage-derived foam cells (*Figure 3G–I*) and a necrotic lipid core covered by a well-formed fibrous cap (*Figure 3D–F*) were noted.

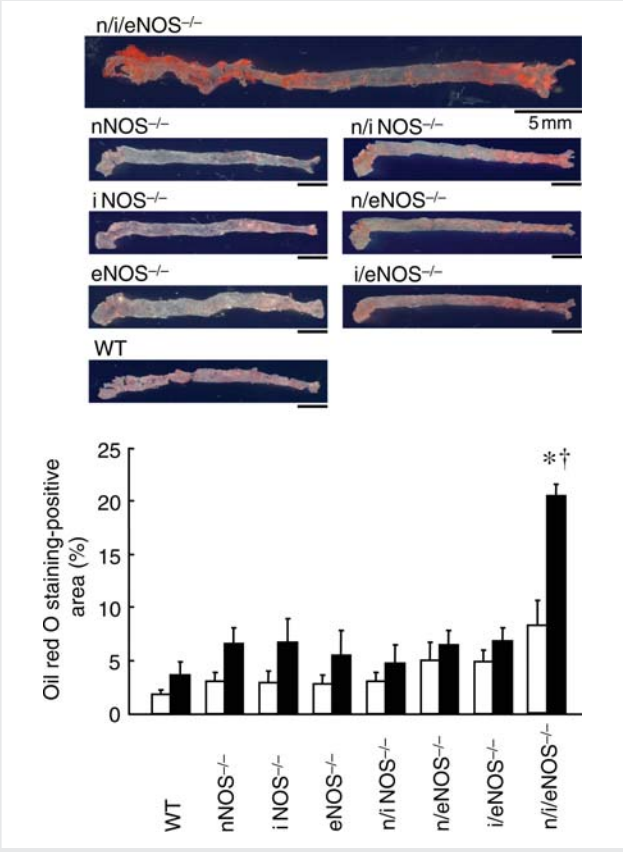

Figure 2 Lipid accumulation in longitudinally opened aortas of WT and NOS$^{-/-}$ mice fed a high-cholesterol diet (oil red O staining) ($n = 6–11$). Red colour indicates positive staining. White and black bars represent the regular and high-cholesterol diets, respectively. WT, C57BL/6. *$P < 0.05$ vs. the regular diet; †$P < 0.05$ vs. WT mice fed the high-cholesterol diet.

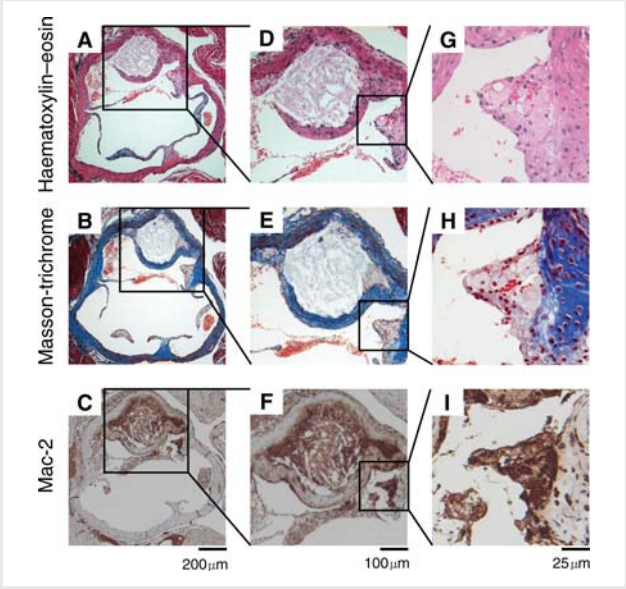

Figure 3 Subendothelial conglomeration of macrophage-derived foam cells (*G–I*) and a necrotic lipid core covered by a fibrous cap (*D–F*) in the aortic sinus of triply n/i/eNOS$^{-/-}$ mice fed a high-cholesterol diet (*A–C*). Serial sections were used in each column. Upper, middle, and lower panels indicate haematoxylin–eosin, Masson-trichrome, and Mac-2 staining, respectively. Brown colour is positive in Mac-2 immunostaining.

3.3 Reduced survival in triply n/i/eNOS$^{-/-}$ mice fed a high-cholesterol diet

Since we experienced the sudden death of the triply n/i/eNOS$^{-/-}$ mice during the cholesterol-rich feeding, we examined the survival rate. The survival rate with the high-cholesterol diet for 5 months was significantly and markedly reduced only in the triply n/i/eNOS$^{-/-}$ genotype [37.5% (15/40)] when compared with the WT genotype (*Figure 4A*). We then performed a post-mortem histopathological analysis of the 15 dead triply n/i/eNOS$^{-/-}$ mice to identify the cause of death. In all the dead mice, marked neointimal formation and perivascular fibrosis of the coronary artery were noted, and their extents were both significantly greater when compared with living control WT mice fed the regular diet and with living triply n/i/eNOS$^{-/-}$ mice fed the high-cholesterol diet (*Figure 4B*). Furthermore, old myocardial infarction was detected in one mouse (*Figure 5A*), giant organized thrombi in both the left and right ventricles were seen in two mice (*Figure 5B*), and pulmonary congestion was observed in all the dead mice (*Figure 5C*). On the other hand, no pathological finding that explains the cause of death was present in the brain, kidney, or spleen.

3.4 Blood pressure in WT and NOS$^{-/-}$ mice fed a high-cholesterol diet

Arterial blood pressure (mmHg) was significantly elevated in the eNOS$^{-/-}$ (118.4 ± 2.5), i/eNOS$^{-/-}$ (117.8 ± 1.2), n/eNOS$^{-/-}$ (123.4 ± 5.9), and n/i/eNOS$^{-/-}$ mice (126.7 ± 11.2) when compared with the WT mice (103.4 ± 5.2) ($n = 7–9$, each $P < 0.05$). There was no significant difference in the hypertension levels among those genotypes.

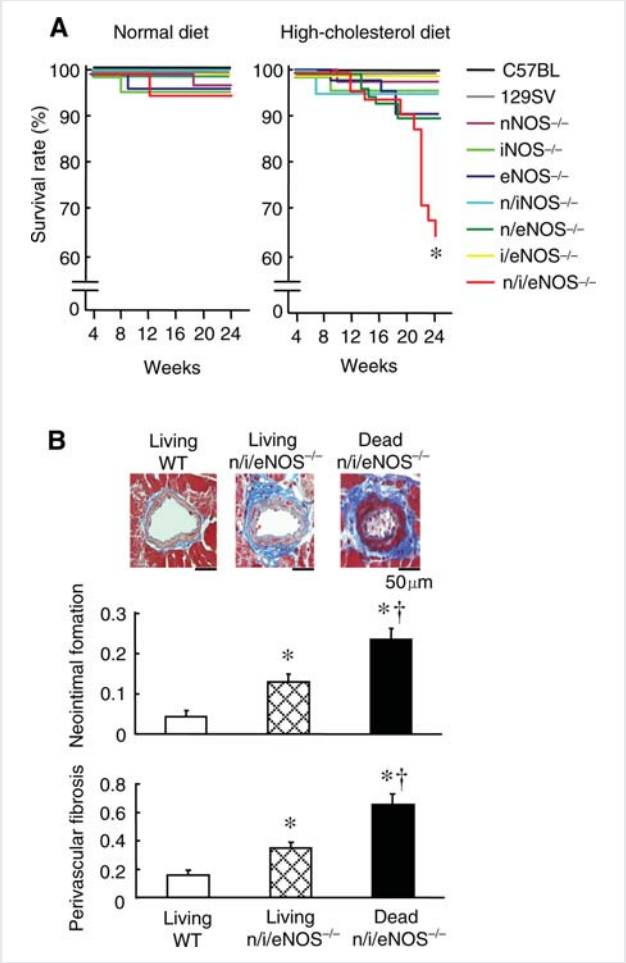

Figure 4 Survival rate (*A*) and coronary arteriosclerotic lesion formation (*B*) of triply n/i/eNOS$^{-/-}$ mice that died during a high-cholesterol diet. (*A*) Survival rate ($n = 15–40$). *$P < 0.05$ vs. WT C57BL/6. (*B*) Coronary vascular lesion formation in living control WT mice fed a regular diet and living and dead triply n/i/eNOS$^{-/-}$ mice fed the high-cholesterol diet ($n = 6–15$). WT, C57BL/6. *$P < 0.05$ vs. living WT mice fed the regular diet; †$P < 0.05$ vs. living n/i/eNOS$^{-/-}$ mice fed the high-cholesterol diet.

3.5 Cardiac ACE activity in WT and NOS$^{-/-}$ mice fed a high-cholesterol diet

We next examined whether or not the renin–angiotensin system is activated in the high-cholesterol diet-fed triply n/i/eNOS$^{-/-}$ mice. The cardiac ACE activities in the high-cholesterol diet were significantly enhanced only in the triply n/i/eNOS$^{-/-}$ genotype, but not in any singly or doubly NOS$^{-/-}$ genotypes, when compared with the WT genotype (see Supplementary material online, *Figure S2*). In the triply n/i/eNOS$^{-/-}$ genotype, the cardiac ACE activities tended to increase in the high-cholesterol diet than in the regular diet, although the difference did not reach a statistically significant level (see Supplementary material online, *Figure S2*).

3.6 Urinary 8-isoprostane level in WT and NOS$^{-/-}$ mice fed a high-cholesterol diet

The urinary levels of 8-isoprostane, a marker of oxidative stress, in the high-cholesterol diet, were also significantly increased only in the

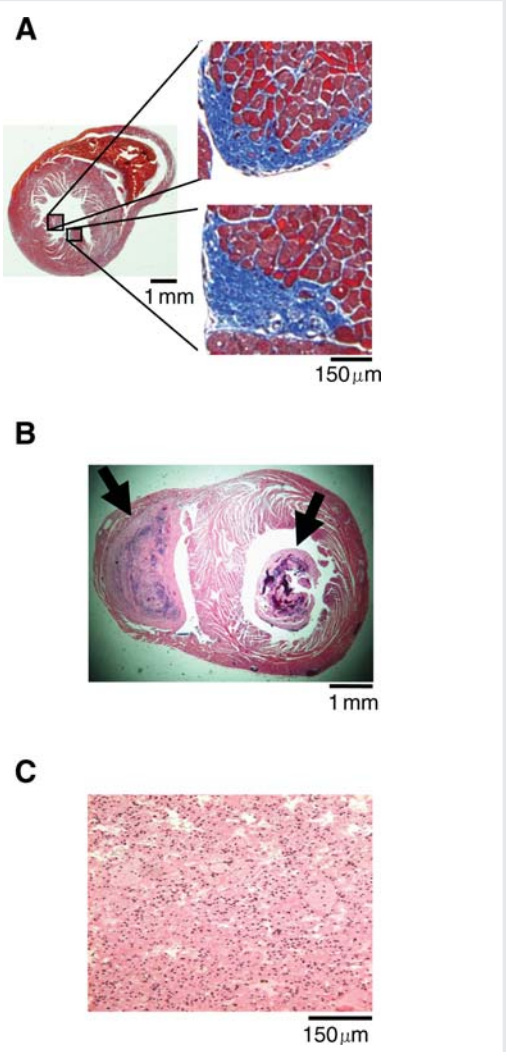

Figure 5 Post-mortem histopathological analysis of triply n/i/eNOS$^{-/-}$ mice that died during a high-cholesterol diet. (*A*) Old myocardial infarction (blue colour). (*B*) Giant organized thrombi in the left and right ventricles (arrows). (*C*) Pulmonary congestion. (*A*) Masson-trichrome staining and (*B* and *C*) haematoxylin–eosin staining.

triply n/i/eNOS$^{-/-}$ genotype compared with the WT genotype (see Supplementary material online, *Figure S3*). In the triply n/i/eNOS$^{-/-}$ genotype, the urinary 8-isoprostane levels were significantly higher in the high-cholesterol diet than in the regular diet (see Supplementary material online, *Figure S3*).

3.7 Serum C-reactive protein level in WT and NOS$^{-/-}$ mice fed a high-cholesterol diet

There was no significant difference in the serum C-reactive protein levels, a marker of inflammation, in the WT or NOS$^{-/-}$ genotypes fed the regular or high-cholesterol diet (see Supplementary material online, *Figure S4*). This negative result might be because C-reactive protein is a useful marker of inflammation in humans, but not in mice.[17]

3.8 Serum apoE levels and small intestinal cholesterol transporter expression in triply n/i/eNOS$^{-/-}$ mice fed a high-cholesterol diet

We finally studied the mechanism(s) for the diet-induced dyslipidaemia in the triply n/i/eNOS$^{-/-}$ mice. There was no significant difference in serum apoE levels in the WT and triply n/i/eNOS$^{-/-}$ genotypes. Also, no significant difference was noted in the expression levels of the cholesterol transporter NPC1L1 in the small intestine in the two genotypes (*Figure 6B*).

3.9 Hepatic LDL receptor expression and SREBP-2 activity in triply n/i/eNOS$^{-/-}$ mice fed a high-cholesterol diet

Notably, the expression levels of the hepatic LDL receptor, which introduces circulating LDL into hepatocytes, were significantly and markedly reduced only in the triply n/i/eNOS$^{-/-}$ genotype, but not in any singly or doubly NOS$^{-/-}$ genotypes, when compared with the WT genotype (*Figure 6C*). Furthermore, the activity of SREBP-2 (68 kDa, activated form), which regulates the expression of the LDL receptor, was also significantly lower only in the triply n/i/eNOS$^{-/-}$ genotype (*Figure 6D*).

4. Discussion

The major novel findings of the present study are that mice devoid of all three NOS genes develop severe diet-induced dyslipidaemia, atherosclerotic vascular lesion formation, and cardiovascular death via reduced expression of the hepatic LDL receptor. To the best of our knowledge, this is the first study that demonstrates that the defective NOS system is linked to the pathogenesis of diet-induced dyslipidaemia.

4.1 Role of the NOS system in the regulation of lipid metabolism

Non-selective L-arginine analogues, such as N^{ω}-nitro-L-arginine or N^{ω}-nitro-L-arginine methyl ester, have been widely used as pharmacological tools to inhibit NO synthesis derived from the whole NOS system. Although the role of the NOS system in lipid metabolism has been studied with those L-arginine analogues, the obtained results are conflicting, such that the NOS system has been suggested to be both essential[18] and non-essential[19] for lipid metabolism in rats. This inconsistency may be due to their multiple non-specific actions.[20–23] Indeed, we clarified the NO-independent vascular actions of L-arginine analogues. Although long-term treatment with L-arginine analogues had long been believed without doubt to simply inhibit vascular NO synthesis and cause arteriosclerotic vascular lesion formation, we found that the long-term vascular effects of L-arginine analogues are not solely mediated by the simple inhibition of NO synthesis.[9,24] 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.[9,24] These findings questioned the previous theory regarding the effects of L-arginine analogues and warranted re-evaluation of previous studies using those analogues.[9,24] Thus, the authentic role of the NOS system in lipid metabolism still remains to be fully elucidated.

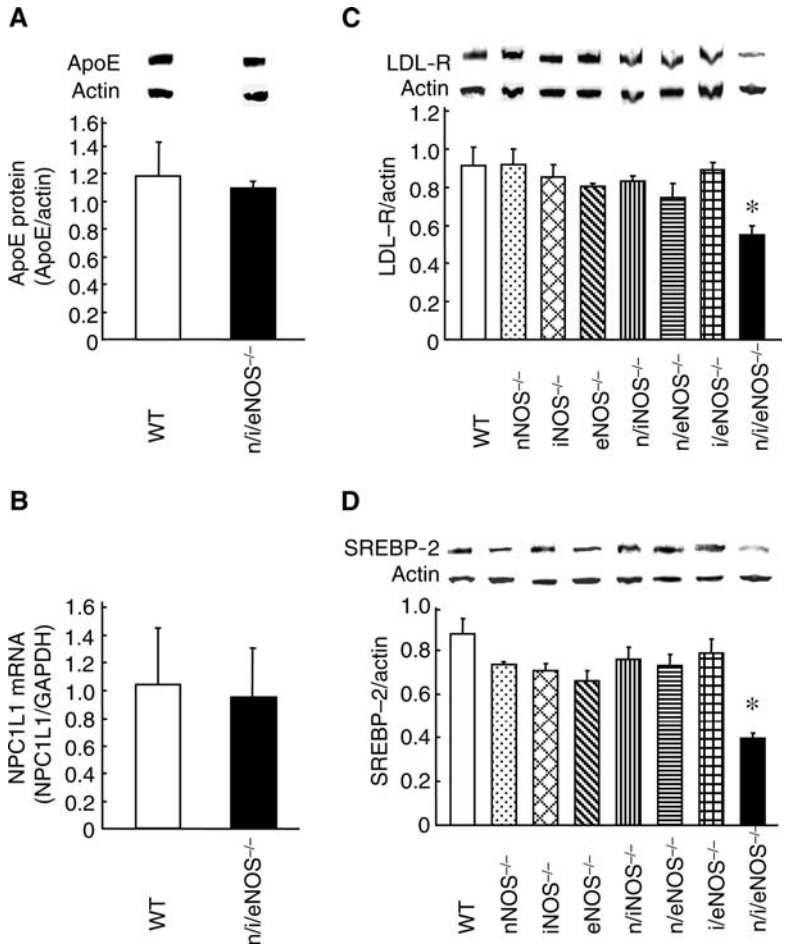

Figure 6 Serum apoE levels (*A*), cholesterol transporter NPC1L1 mRNA expression levels in the small intestine (*B*), LDL receptor expression levels in the liver (*C*), and SREBP-2 activity in the liver (*D*) in WT and NOS$^{-/-}$ mice fed a high-cholesterol diet ($n = 6–11$). WT, C57BL/6. *$P < 0.05$ vs. WT.

In this study, in response to the high-cholesterol diet, the triply NOS$^{-/-}$ mice, but not the singly or doubly NOS$^{-/-}$ mice, exhibited marked increases in serum total cholesterol levels. These increases were due to alterations in the serum levels of LDL cholesterol and small dense LDL particles, both of which are important cardiovascular risk factors,[25] but not alterations in the serum levels of HDL cholesterol or triglyceride. These results indicate that the whole NOS system plays a key role in the regulation of lipid metabolism. Consistent with the evidence, NO supplementation by overexpression of the eNOS gene in transgenic mice decreases plasma total and LDL cholesterol levels.[26]

Several lines of evidence suggest the association of the defective NOS system with dyslipidaemia in humans. First, it has been reported that plasma NO_x (nitrite plus nitrate) levels, which are markers of NO production derived from all three types of NOS *in vivo*, are reduced in patients with hyperlipidaemia.[27] Secondly, it has been revealed that lower plasma NO_x levels are significantly correlated with higher plasma total and LDL cholesterol levels.[27] Finally, plasma concentrations of asymmetric dimethylarginine, which is an endogenous NOS inhibitor, have been shown to be elevated in hypercholesterolaemic individuals.[28] These results may imply the clinical significance of the present findings with the triply mutant mice.

The reason why the singly or doubly NOS$^{-/-}$ mice fed the high-cholesterol diet did not show significant dyslipidaemia may be due to a compensatory mechanism by other NOSs that are not disrupted genetically. Indeed, we have revealed that other NOSs are expressed in the singly and doubly NOS$^{-/-}$ mice,[5,29] and that NOS activity and NOx production are fairly well preserved in those genotypes.[5]

4.2 Atherosclerosis and sudden cardiac death in triply NOS$^{-/-}$ mice fed a high-cholesterol diet

Since atherogenic lipoproteins were increased in the triply NOS$^{-/-}$ mice, we next studied atherosclerotic vascular lesion formation. Cholesterol deposits, atheromatous plaques, conglomerated foamy macrophages, and necrotic lipid cores with fibrous caps were present in the aorta of the triply NOS$^{-/-}$ mice fed the high-cholesterol diet, all of which findings are recognized in both the early and advanced stages of human atherosclerosis. Thus, the features of the atherosclerotic lesions that were developed in the triply NOS$^{-/-}$ mice are similar to those described in humans,[30] and therefore represent an important model for human dyslipidaemia and atherosclerosis.

Since some triply NOS$^{-/-}$ mice died during the cholesterol-rich feeding, we then performed a post-mortem histopathological analysis to identify the cause of death. Markedly accelerated coronary vascular lesion formation and pulmonary congestion were noted in all the dead

triply NOS$^{-/-}$ mice, and old myocardial infarction and giant organized thrombi in both ventricles were found in some of them. Thus, it is likely that the triply NOS$^{-/-}$ mice died due to the cause of cardiovascular death, such as myocardial ischaemia-evoked malignant arrhythmia or heart failure.

4.3 Considerations of statin therapy in triply NOS$^{-/-}$ mice fed a high-cholesterol diet

Statins (3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors) are potent blockers of cholesterol biosynthesis and widely used in the treatment of hypercholesterolaemia. A number of large clinical trials have demonstrated their clinical usefulness for preventing cardiovascular events, such as myocardial infarction and sudden cardiac death. Thus, it is interesting to speculate the potential effect of statin therapy in the triply NOS$^{-/-}$ mice. However, our preliminary study indicated that long-term treatment with a statin did not significantly decrease plasma LDL cholesterol levels in the triply NOS$^{-/-}$ mice (unpublished observations). Previous studies also reported that statins do not reduce, but rather elevates, plasma LDL levels in high-cholesterol diet-fed apoE$^{-/-}$ mice.[31] Whether statin therapy is beneficial in the treatment of atherosclerosis and cardiovascular death in the triply NOS$^{-/-}$ mice fed the high-cholesterol diet through lipid lowering-independent actions remains to be examined in a future study.

4.4 Mechanism for diet-induced atherosclerosis in triply n/i/eNOS$^{-/-}$ mice

Whereas blood pressure was significantly elevated in the eNOS-disrupted singly, doubly, and triply NOS$^{-/-}$ mice to a comparable extent, significant atherosclerosis was noted only in the triply NOS$^{-/-}$ genotype, suggesting a minor role of hypertension in the development of the diet-induced atherosclerosis in the genotype. On the other hand, significant increases in cardiac ACE activities and urinary 8-isoprostane levels were noted in the high-cholesterol diet-fed triply NOS$^{-/-}$ mice. Thus, it is conceivable that the activation of the renin–angiotensin system and increased oxidative stress may be involved in the diet-induced atherosclerosis.

4.5 Mechanism for diet-induced dyslipidaemia in triply n/i/eNOS$^{-/-}$ mice

Finally, we examined the mechanism(s) for dyslipidaemia in the triply NOS$^{-/-}$ mice fed the cholesterol-rich diet. Since the extent of the diet-induced dyslipidaemia in the triply NOS$^{-/-}$ mice was equivalent to that in apoE$^{-/-}$ mice, we first analysed the serum apoE levels. However, no defect of serum apoE was seen in the triply NOS$^{-/-}$ mice. We next examined the kinetics of the cholesterol absorption and degradation machineries. Dietary cholesterol is absorbed into the body through the cholesterol transporter NPC1L1 in the small intestine, and circulating LDL cholesterol in the blood is bound to the LDL receptor in the liver, taken up and broken down by hepatocytes. The expression levels of the small intestinal NPC1L1 were not altered in the triply NOS$^{-/-}$ mice, whereas the expression levels of the hepatic LDL receptor were markedly reduced only in the triply NOS$^{-/-}$ mice, in parallel with alterations in the serum LDL cholesterol levels. SREBP-2 was discovered as a transcriptional factor that controls LDL receptor gene expression.[32] The activity of SREBP-2 was also diminished only in the triply NOS$^{-/-}$ mice. Thus, it is possible that the lower expression of the hepatic LDL receptor mediated by reduced SREBP-2 activity is involved in the diet-induced dyslipidaemia in the triply NOS$^{-/-}$ genotype.

In conclusion, we were able to prove that complete disruption of the entire NOS system causes severe diet-induced dyslipidaemia, lipid-rich atherosclerotic lesion formation, and sudden cardiac death in mice *in vivo* through the down-regulation of the hepatic LDL receptor, demonstrating the critical role of the whole endogenous NO/NOS system in the regulation of lipid metabolism.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Conflict of interest: none declared.

Funding

This work was supported in part by Grants-in-Aid for Scientific Research (20390074 and 17390071) and a Grant-in-Aid for Exploratory Research (16650097) from the Japan Society for the Promotion of Science, Tokyo, Japan, and by grants from the Daiichi Sankyo Pharmaceutical Co., Tokyo, Japan, the Research Foundation for Treatment of Metabolic Abnormalities, Osaka, Japan, the Novartis Foundation for the Promotion of Science, Tokyo, Japan, the Japan Heart Foundation Grant for Research on Arteriosclerosis Update, Tokyo, Japan, and the University of Occupational and Environmental Health for Advanced Research, Japan.

References

1. Ignarro LJ. Biosynthesis and metabolism of endothelium-derived nitric oxide. *Annu Rev Pharmacol Toxicol* 1990;**30**:535–560.
2. Moncada S, Palmer RMJ, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol Rev* 1991;**43**:109–142.
3. Murad F. What are the molecular mechanisms for the antiproliferative effects of nitric oxide and cGMP in vascular smooth muscle? *Circulation* 1997;**95**:1101–1103.
4. Tsutsui M, Shimokawa H, Otsuji Y, Ueta Y, Sasaguri Y, Yanagihara N. Nitric oxide synthases and cardiovascular diseases: insights from genetically modified mice. *Circ J* 2009;**73**:986–993.
5. Morishita T, Tsutsui M, Shimokawa H, Sabanai K, Tasaki H, Suda O *et al*. Nephrogenic diabetes insipidus in mice lacking all nitric oxide synthase isoforms. *Proc Natl Acad Sci USA* 2005;**102**:10616–10621.
6. Tsutsui M, Shimokawa H, Morishita T, Nakashima Y, Yanagihara N. Development of genetically engineered mice lacking all three nitric oxide synthases. *J Pharmacol Sci* 2006;**102**:147–154.
7. Nakata S, Tsutsui M, Shimokawa H, Suda O, Morishita T, Shibata K *et al*. Spontaneous myocardial infarction in mice lacking all nitric oxide synthase isoforms. *Circulation* 2008;**117**:2211–2223.
8. Tsutsui M, Nakata S, Shimokawa H, Otsuji Y, Yanagihara N. Spontaneous myocardial infarction and nitric oxide synthase. *Trends Cardiovasc Med* 2008;**18**:275–279.
9. Suda O, Tsutsui M, Morishita T, Tanimoto A, Horiuchi M, Tasaki H *et al*. Long-term treatment with *N*(omega)-nitro-L-arginine methyl ester causes arteriosclerotic coronary lesions in endothelial nitric oxide synthase-deficient mice. *Circulation* 2002;**106**:1729–1735.
10. Usui S, Hara Y, Hosaki S, Okazaki M. A new on-line dual enzymatic method for simultaneous quantification of cholesterol and triglycerides in lipoproteins by HPLC. *J Lipid Res* 2002;**43**:805–814.
11. Morishita T, Tsutsui M, Shimokawa H, Horiuchi M, Tanimoto A, Suda O *et al*. Vasculoprotective roles of neuronal nitric oxide synthase. *FASEB J* 2002;**16**:1994–1996.
12. Gharavi NM, Baker NA, Mouillesseaux KP, Yeung W, Honda HM, Hsieh X *et al*. Role of endothelial nitric oxide synthase in the regulation of SREBP activation by oxidized phospholipids. *Circ Res* 2006;**98**:768–776.
13. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;**72**:248–254.
14. Duval C, Touche V, Tailleux A, Fruchart JC, Fievet C, Clavey V *et al*. Niemann-Pick C1 like 1 gene expression is down-regulated by LXR activators in the intestine. *Biochem Biophys Res Commun* 2006;**340**:1259–1263.
15. Plump AS, Smith JD, Hayek T, Aalto-Setala K, Walsh A, Verstuyft JG *et al*. Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells. *Cell* 1992;**71**:343–353.
16. Zhang SH, Reddick RL, Piedrahita JA, Maeda N. Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. *Science* 1992;**258**:468–471.

17. Pepys MB, Hirschfield GM. C-reactive protein: a critical update. *J Clin Invest* 2003;**111**: 1805–1812.
18. Khedara A, Kawai Y, Kayashita J, Kato N. Feeding rats the nitric oxide synthase inhibitor, L-*N*(omega)nitroarginine, elevates serum triglyceride and cholesterol and lowers hepatic fatty acid oxidation. *J Nutr* 1996;**126**:2563–2567.
19. Navarro J, Sanchez A, Saiz J, Ruilope LM, Garcia-Estan J, Romero JC *et al*. Hormonal, renal, and metabolic alterations during hypertension induced by chronic inhibition of NO in rats. *Am J Physiol* 1994;**267**:R1516–R1521.
20. Buxton ILO, Cheek DJ, Eckman D, Westfall DP, Sanders KM, Keef KD. N^G-nitro-L-arginine methyl ester and other alkyl esters of arginine are muscarinic receptor antagonists. *Circ Res* 1993;**72**:387–395.
21. Heim KF, Thomas G, Ramwell PW. Effect of substituted arginine compounds on superoxide production in the rabbit aorta. *J Pharmacol Exp Ther* 1991;**257**: 1130–1135.
22. Peterson DA, Peterson DC, Archer S, Weir EK. The nonspecificity of specific nitric oxide synthase inhibitors. *Biochem Biophys Res Commun* 1992;**187**:797–801.
23. Thomas G, Ramwell PW. N^{ω}-nitro-L-arginine benzyl ester, a potent irreversible inhibitor of endothelium dependent relaxation. *Biochem Biophys Res Commun* 1991;**179**: 1677–1682.
24. Suda O, Tsutsui M, Morishita T, Tasaki H, Ueno S, Nakata S *et al*. Asymmetric dimethylarginine produces vascular lesions in endothelial nitric oxide synthase-deficient mice. *Arterioscler Thromb Vasc Biol* 2004;**24**:1682–1688.
25. Sniderman AD, Furberg CD, Keech A, Roeters van Lennep JE, Frohlich J, Jungner I *et al*. Apolipoproteins versus lipids as indices of coronary risk and as targets for statin treatment. *Lancet* 2003;**361**:777–780.
26. van Haperen R, de Waard M, van Deel E, Mees B, Kutryk M, van Aken T *et al*. Reduction of blood pressure, plasma cholesterol, and atherosclerosis by elevated endothelial nitric oxide. *J Biol Chem* 2002;**277**:48803–48807.
27. Tanaka S, Yashiro A, Nakashima Y, Nanri H, Ikeda M, Kuroiwa A. Plasma nitrite/nitrate level is inversely correlated with plasma low-density lipoprotein cholesterol level. *Clin Cardiol* 1997;**20**:361–365.
28. Boger RH, Bode-Boger SM, Szuba A, Tsao PS, Chan JR, Tangphao O *et al*. Asymmetric dimethylarginine (ADMA): a novel risk factor for endothelial dysfunction: its role in hypercholesterolemia. *Circulation* 1998;**98**:1842–1847.
29. Takaki A, Morikawa K, Tsutsui M, Murayama Y, Tekes E, Yamagishi H *et al*. Crucial role of nitric oxide synthases system in endothelium-dependent hyperpolarization in mice. *J Exp Med* 2008;**205**:2053–2063.
30. Libby P. The vascular biology of atherosclerosis. In: Libby P, Bonow R, Mann D, Zipes D, eds. *Braunwald's Heart Disease*. Philadelphia: Saunders Elsevier; 2008. p985–1002.
31. Nachtigal P, Jamborova G, Pospisilova N, Pospechova K, Solichova D, Zdansky P *et al*. Atorvastatin has distinct effects on endothelial markers in different mouse models of atherosclerosis. *J Pharm Pharm Sci* 2006;**9**:222–230.
32. Hua X, Yokoyama C, Wu J, Briggs MR, Brown MS, Goldstein JL *et al*. SREBP-2, a second basic-helix-loop-helix-leucine zipper protein that stimulates transcription by binding to a sterol regulatory element. *Proc Natl Acad Sci USA* 1993;**90**:11603–11607.