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Crucial vasculoprotective role of the whole nitric oxide synthase system in vascular lesion formation in mice: Involvement of bone marrow-derived cells

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ABSTRACT

Although all three nitric oxide (NO) synthases (nNOS, iNOS, and eNOS) are expressed in injured arteries, it remains to be elucidated the role of the NOSs in their entirety in the vascular lesion formation. We addressed this issue in mice deficient in all NOS genes. Vascular injury was induced by permanent ligation of a unilateral carotid artery in wild-type (WT), singly, and triply $NOS^{-/-}$ mice. Two weeks after the procedure, constrictive vascular remodeling and neointimal formation were recognized in the ligated arteries. While constrictive remodeling was noted in the $nNOS^{-/-}$ and $iNOS^{-/-}$ genotypes, it was most accelerated in the $n/i/eNOS^{-/-}$ genotype. While neointimal formation was evident in the $eNOS^{-/-}$ and $nNOS^{-/-}$ genotypes, it was also most aggravated in the $n/i/eNOS^{-/-}$ genotype. Those lesions were reversed by long-term treatment with isosorbide dinitrate, a NO donor. Finally, we examined the involvement of bone marrow-derived cells in the vascular lesion formation. Bone marrow from the WT, singly, or triply $NOS^{-/-}$ mice was transplanted into the WT mice, and then the carotid ligation was performed. Intriguingly, constrictive remodeling and neointimal formation were both similarly most exacerbated in the case of the $n/i/eNOS^{-/-}$ bone marrow transplantation. These results indicate that the complete disruption of all the NOS genes causes markedly accelerated vascular lesion formation caused by blood flow disruption in mice in vivo, demonstrating the crucial vasculoprotective role of the whole endogenous NOS system. Our findings also suggest that the NOS system in bone marrow-derived cells may be involved in this vasculoprotective mechanism.

Introduction

Nitric oxide (NO) plays an important role in maintaining vascular homeostasis [1–3]. NO is synthesized by three distinct isoforms of NO synthase (NOS), including neuronal (nNOS), inducible (iNOS), and endothelial NOS (eNOS), all of which have been reported to be expressed in both early and advanced arteriosclerotic vascular lesions in humans [4]. Genetically manipulated animals are powerful experimental tools to study the function of specific genes in vivo. The role of individual NOS isoforms in vascular lesion formation has been investigated in animals bearing the targeted deletion of each NOS gene [5]. A model of unilateral carotid artery ligation is widely used to induce arteriosclerotic vascular lesions in mice [6]. By using the NOS isoform-deficient mice and the ligation model, we previously showed that eNOS inhibits neointimal formation, that iNOS attenuates the development of constrictive vascular remodeling [7], and that nNOS suppresses both

Abbreviations: NO, nitric oxide; NOS, nitric oxide synthase; nNOS, neuronal nitric oxide synthase; iNOS, inducible nitric oxide synthase; eNOS, endothelial nitric oxide synthase; WT, wild-type; NOx, nitrite plus nitrate; ISDN, isosorbide dinitrate; GFP, green fluorescent protein; LPS, lipopolysaccharide; INF-γ, interferon-γ; α-MEM, α-minimum essential medium.

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neointimal formation and constrictive vascular remodeling [8,9]. Although nNOS was originally identified as a constitutively expressed enzyme, we revealed that vascular nNOS expression is regulated by inflammatory and proliferative stimuli in the injured vascular lesions [10,11].

Based on these lines of evidence, we conceived an ensuing project to explore the role of the NOSs in their entirety in vascular lesion formation. To this end, we developed mice in which all the NOS genes are totally disrupted (triply n/i/eNOS$^{-/-}$ mice) [12,13]. In this study, we investigated neointimal formation and constrictive vascular remodeling in response to ligation-induced vascular injury in the n/i/eNOS$^{-/-}$ mice. We previously indicated that bone marrow-derived vascular progenitor cells may contribute substantially to vascular lesion formation in the mouse carotid artery ligation model [14]. In this study, we further tested our hypothesis that transplantation of NOS-deficient bone marrow may enhance neointimal formation and constrictive vascular remodeling after carotid artery ligation.

Materials and methods

Animals

This study was reviewed and approved by the Ethics Committee of Animal Care and Experimentation, University of Occupational and Environmental Health, 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. The investigation conforms 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). Experiments were performed in 8-week-old male wild-type (WT) C57BL/6 (Kyudo, Co., Ltd., Tosu, Japan), singly nNOS$^{-/-}$, iNOS$^{-/-}$, and eNOS$^{-/-}$, and triply n/i/eNOS$^{-/-}$ mice weighing 20–25 g [12]. They were maintained on a regular diet (CE-2, CLEA Japan, Inc., Tokyo, Japan). The genotype for the NOS genes was determined by PCR of tail genomic DNA [12]. Surgical procedures were performed under general anesthesia with pentobarbital (60 mg/kg, IP) (Kyoritsu Seiyaku Corporation, Tokyo, Japan). The left common carotid artery was permanently ligated with a 6–0 silk suture at the site just proximal to the carotid bifurcation. In the experiment in which the effect of a NO donor on vascular lesion formation was examined, the following three groups were studied: WT mice receiving drinking water, n/i/eNOS$^{-/-}$ mice receiving drinking water, and n/i/eNOS$^{-/-}$ mice receiving isosorbide dinitrate (ISDN, 0.6 mg/dl, Eisai Co., Ltd., Tokyo, Japan) in drinking water for 14 days after the carotid artery ligation [15]. Systolic blood pressure was measured by the tail-cuff method under conscious conditions (Model MK-2000, Muromachi Kikai Co., Ltd., Tokyo, Japan).

Morphometry

The animals were euthanized by an overdose intraperitoneal injection of pentobarbital. The aorta was cannulated and perfused with a 3% formaldehyde solution under physiological pressure, and then the carotid arteries were removed and embedded in paraffin. The sections were stained with hematoxylin and eosin. Histological analysis was performed with a light microscope equipped with a 2-dimensional analysis system (Axiovert 135TV, Carl Zeiss, Jena, Germany) [16]. The extents of constrictive vascular remodeling (reduction of cross-sectional vascular area) and neointimal formation were evaluated at 1 mm proximal to the ligation site [7,8]. The extent of constrictive vascular remodeling was analyzed by the reduction in the external elastic lamina length in the ligated left carotid artery as compared to the control right carotid artery. The extent of neointimal formation was assessed by the ratio of neointima area to media area in the ligated left carotid artery. Lumen diameter (D) was determined from the equation D = the circumferential length of vessel lumen/π, assuming that the vessel cross-sections were circular in vivo [17].

Immunostaining

On days 0, 3, and 14 after the carotid artery ligation, the carotid arteries were quickly removed and fixed with 3% paraformaldehyde. The vessels were embedded in paraffin and were made into 4 μm-thick sections. Endogenous peroxidase was inhibited with 5 mM periodic acid solution. The sections were incubated with protein-blocking serum to minimize spurious background staining. They were reacted with rabbit polyclonal antibody (Transduction Laboratories, Franklin Lakes, NJ) at a dilution of 1:500 for nNOS, 1:250 for eNOS, and 1:100 for iNOS, for 2–3 h at room temperature and overnight at 4 °C, and then treated with an Envision horse radish peroxidase polymer reagent (Dako, Tokyo, Japan) for 1–2 h at room temperature. An avidin biotin immunoperoxidase system was used to detect the antigen. The staining with nonimmune IgG was negative, confirming the specificity of our staining (data not shown).

NOx measurement

The diet and tap water contain NOx (nitrite plus nitrate). To minimize the influence of the NOx, blood and urine sampling was performed 24 h after fasting and replacement of the tap water with ultrapure water purified with the Milli-Q system (Millipore Corporation, Billerica, MA). The urine was collected with metabolic cages. Vacuum tubes with sodium EDTA were used for obtaining the plasma. The blood samples were immediately centrifuged at 3000 rpm, 4 °C for 15 min, and the supernatants were stored at −80 °C. Plasma NOx concentrations were assessed by the Griess method with the use of the ENO-20 NOx analysis system (Eicom, Kyoto, Japan), as we previously reported [12,16,18].

In another set of experiments, bone marrow cells were freshly and aseptically isolated from the tibias and fibulas of the WT and n/i/eNOS$^{-/-}$ mice, and plated at a density of 3×10^6 cells per well in IWAKI 12-well microplates (Asahi Glass, Tokyo, Japan). The cells were maintained in α-minimum essential medium (α-MEM, Grand Island, NY) supplemented with 15% fetal bovine serum (FBS) and without phenol red, which might affect NOx measurement, in the presence or absence of lipopolysaccharide (LPS, 1 μg/ml, Sigma, St. Louis, MO) [19] plus interferon-γ (INF-γ, 150 unit/ml, Wako, Osaka, Japan) [19] at 37C for 24–72 h in a CO_2 incubator. NOx levels in culture medium were assayed. Since considerable NOx levels are detected in FBS, all the data were subtracted from NOx levels of FBS-containing α-MEM in the absence of bone marrow cells.

Bone marrow transplantation

Bone marrow cells were prepared as we previously reported [14,20]. The donor mice (WT, singly, or triply NOS$^{-/-}$ mice) were sacrificed by diethylether, and the tibias and fibulas were aseptically removed. Fresh bone marrow was pushed out from the bones by using a 25G needle syringe containing 1 ml saline. The dissociated bone marrow cells were passed through a cell strainer (70 μm nylon, BD Falcon, Bedford, MA), and washed with saline. The recipient WT or triply NOS$^{-/-}$ mice were subjected to lethal irradiation (9.17 Gy) in order to destroy their bone marrow. On the next day, bone marrow obtained from WT, singly, or triply NOS$^{-/-}$ mice was transplanted into the irradiated WT or triply NOS$^{-/-}$ recipients by tail vain injection. Four weeks after the transplantation, permanent ligation of a unilateral carotid artery was performed, and 2 weeks

later, histopathological examinations were carried out. All the irradiated WT mice that did not receive bone marrow transplantation died, whereas most of the irradiated WT mice that received bone marrow transplantation lived (data not shown), suggesting successful bone marrow engraftment by our procedure.

NOS activity

Total NOS activity in the ligated carotid artery was measured by a NOS activity assay kit (Cayman, Ann Arbor, MI). Tissue homogenate of the ligated carotid artery was incubated with a reaction buffer at 37 °C for 24 h.

Statistical analysis

Results are expressed as mean ± SEM. Statistical analyses were performed by one-way ANOVA followed by Fisher's post hoc test. A value of $P < 0.05$ was considered to be statistically significant.

Results

Markedly accelerated vascular lesion formation after carotid artery ligation in n/i/eNOS$^{-/-}$ mice

We examined the effect of the targeted deletion of the NOS genes on vascular lesion formation in a model of unilateral carotid artery ligation in 8-week-old wild-type (WT), singly, and triply NOS$^{-/-}$ mice. In the control non-ligated carotid artery, no pathological findings were seen in any of the genotypes (Fig. 1A). In contrast, on day 14 after the carotid artery ligation, constrictive vascular remodeling, neointimal formation, and luminal narrowing were noted in the ligated arteries of all the genotypes studied (Fig. 1B). Intriguingly, the extents of those vascular structural changes were greatest by far in the triply n/i/eNOS$^{-/-}$ genotype. Quantitative analysis indicated that while significant constrictive vascular remodeling (as assessed by reduction in external elastic lamina length in the ligated carotid artery as compared to the control carotid artery) was seen in the nNOS$^{-/-}$ and iNOS$^{-/-}$ genotypes, it was most accelerated in the n/i/eNOS$^{-/-}$ genotype (Fig. 2A). While significant neointimal formation (as evaluated by the intima-to-media ratio in the ligated carotid artery) was observed in the nNOS$^{-/-}$ and eNOS$^{-/-}$ genotypes, it was most aggravated in the n/i/eNOS$^{-/-}$ genotype (Fig. 2B). While significant luminal narrowing (as analyzed by lumen diameter in the ligated carotid artery) was noted in each of the singly NOS$^{-/-}$ mice, it, too, was most exacerbated in the n/i/eNOS$^{-/-}$ genotype (Fig. 2C).

Markedly enhanced vascular inflammatory cell infiltration after carotid artery ligation in n/i/eNOS$^{-/-}$ mice

We next examined the involvement of inflammation in the vascular lesion formation after the carotid artery ligation in the n/i/eNOS$^{-/-}$ genotype. On day 14 after the carotid artery ligation, while significant infiltration of inflammatory cells, most of which were neutrophils, was detected in the adventitia of the ligated arteries in all the NOS$^{-/-}$ genotypes, it was again most augmented in the n/i/eNOS$^{-/-}$ genotype (Figs. 1B and 2D).

Expression of three NOS isoforms in vascular lesions after carotid artery ligation

In the WT mice, immunoreactivity for nNOS was almost absent in the control artery before the carotid artery ligation, whereas it was observed faintly in the adventitial inflammatory cells on day 3 after the procedure, and became evident on day 14 in the neointima and medial smooth muscle cells, and to a lesser extent, in the endothelial cells (Fig. 3A) [8]. The immunoreactivity for iNOS was induced in the adventitial inflammatory cells on day 3 after the carotid artery ligation [8], and in the neointima and medial smooth muscle cells on day 14 in the WT mice (Fig. 3B). The eNOS immunoreactivity was limited to the endothelium both before and after the carotid artery ligation in the WT mice (Fig. 3C) [8]. In contrast, no immunoreactivity was noted in the carotid artery of the n/i/eNOS$^{-/-}$ mice throughout the experimental periods (Fig. 3A-C).

Blood pressure in n/i/eNOS$^{-/-}$ mice before and after carotid artery ligation

Arterial blood pressure was significantly elevated in the eNOS$^{-/-}$ and n/i/eNOS$^{-/-}$ genotypes to a similar extent both before and 14 days after the carotid artery ligation (Fig. 4). Those changes in blood pressure were not correlated with the differences in the extent of the vascular lesion formation among the five genotypes studied (Figs. 1 and 2).

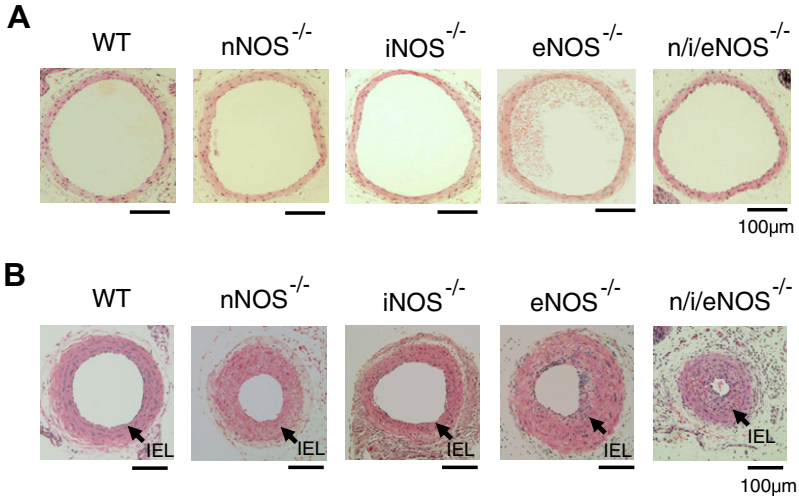

Fig. 1. Histology of mouse carotid artery in the wild-type and NOS$^{-/-}$ mice before and 14 days after the carotid artery ligation (hematoxylin-eosin staining). (A) Non-ligated control carotid arteries of the wild-type (WT) and NOS$^{-/-}$ mice. (B) Ligated carotid arteries of the wild-type and NOS$^{-/-}$ mice on day 14 after carotid artery ligation. Arrows indicate the internal elastic lamina. IEL, internal elastic lamina.

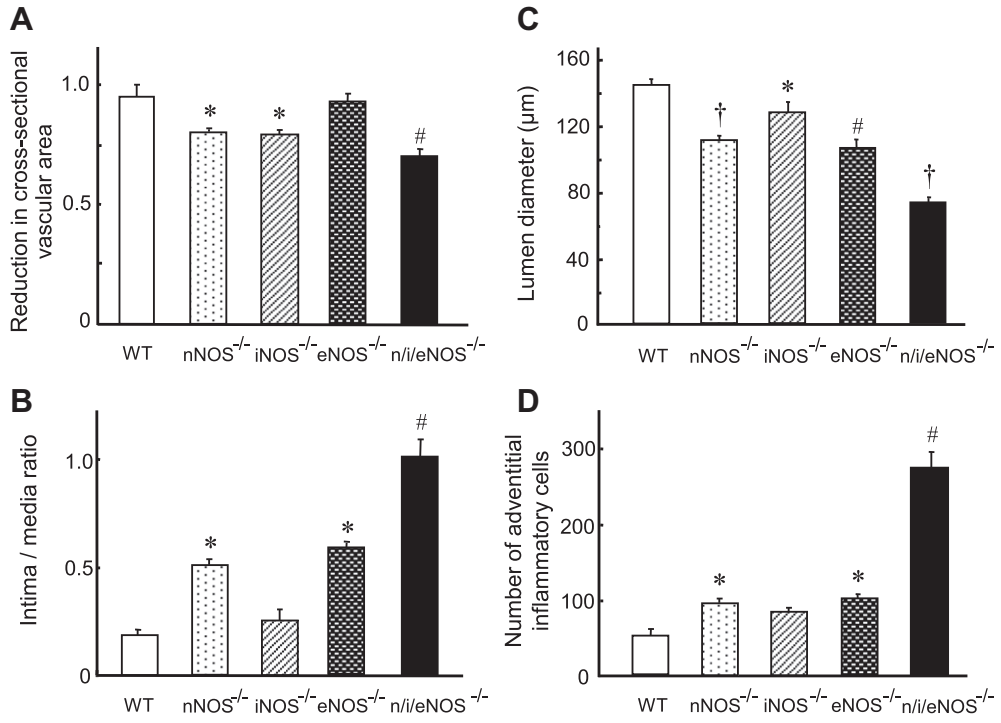

Fig. 2. Quantitative analysis of vascular lesion formation and inflammatory cell infiltration in ligated carotid arteries of the WT and NOS$^{-/-}$ mice on day 14 after the carotid artery ligation (n = 10–16). (A) Reduction in vascular cross-sectional area in the ligated carotid artery as compared to the control carotid artery (constrictive vascular remodeling). $^{*}P < 0.05$ vs. WT; $^{\#}P < 0.01$ vs. WT. (B) The intima-to-media ratio in the ligated carotid artery (neointimal formation). $^{*}P < 0.05$ vs. WT; $^{\#}P < 0.001$ vs. WT. (C) Lumen diameter in the ligated carotid arteries (luminal narrowing). $^{*}P < 0.05$ vs. WT; $^{\#}P < 0.001$ and $^{\dagger}P < 0.0001$ vs. WT. (D) The number of adventitial inflammatory cells (mostly neutrophils) in the ligated carotid arteries. $^{*}P < 0.05$ vs. WT; $^{\#}P < 0.001$ vs. WT.

Reversal of the accelerated vascular lesion formation after carotid artery ligation in n/i/eNOS$^{-/-}$ mice by long-term treatment with a NO donor

We next investigated the effect of NO supplementation on the carotid artery ligation-induced vascular lesion formation in the n/i/eNOS$^{-/-}$ genotype. Serum and urinary NOx (nitrite plus nitrate) levels, markers of NO production, were both markedly reduced in the n/i/eNOS$^{-/-}$ genotype as compared with the WT genotype (Fig. 5B and C). Long-term oral treatment with ISDN (NO donor, 14 days) significantly restored the reduced NOx levels in the n/i/eNOS$^{-/-}$ genotype to the levels in the WT genotype (Fig. 5B and C). The long-term treatment with ISDN significantly prevented the progression of constrictive vascular remodeling (Fig. 5A and D), neointimal formation (Fig. 5A and E), and luminal narrowing (Fig. 5A and F), along with the amelioration of inflammatory cell infiltration (Fig. 5A and G), in the ligated carotid arteries of the n/i/eNOS$^{-/-}$ genotype.

Transplantation of n/i/eNOS$^{-/-}$ mouse bone marrow causes markedly accelerated vascular lesion formation after carotid artery ligation

To examine the involvement of the bone marrow NOS system in vascular lesion formation after carotid artery ligation, we then explored the effect of bone marrow transplantation from NOS$^{-/-}$ mice on the vascular lesion formation in WT mice. The bone marrow of WT, singly, or triply NOS$^{-/-}$ mice was transplanted into the WT mice, and then the carotid artery ligation was performed. Interestingly, the extents of constrictive vascular remodeling (Fig. 6A), neointimal formation (Fig. 6B), and luminal narrowing (Fig. 6C) were all the highest in the case of the n/i/eNOS$^{-/-}$ bone marrow transplantation. The extent of inflammatory cell infiltration was also the largest in the case of the n/i/eNOS$^{-/-}$ bone marrow transplantation (Fig. 6D).

The extents of vascular lesion formation after the carotid artery ligation in the WT mice with the n/i/eNOS$^{-/-}$ bone marrow transplantation were not so different in comparison to the n/i/eNOS$^{-/-}$ mice without the transplantation (Fig. 6A–C vs. Fig. 2A–C). Therefore, we further inquired about the possibility of the involvement of the vascular NOS system in this process. In the n/i/eNOS$^{-/-}$ mice that underwent the WT bone marrow transplantation, as compared with the WT that underwent the WT bone marrow transplantation, the ligation-induced constrictive vascular remodeling (Fig. 7A) or luminal narrowing (Fig. 7C) was not significantly worsened, while the ligation-induced neointimal formation was significantly exacerbated to a small extent (Fig. 7B).

Transplantation of n/i/eNOS$^{-/-}$ mouse bone marrow causes marked reductions in NOS activity and expression in vascular lesions after carotid artery ligation

The NOS activity in the ligated carotid artery was significantly lower in the WT mice that received the WT bone marrow transplantation than in the non-irradiated control WT mice that did not receive the bone marrow transplantation (Fig. 7D), suggesting that the bone marrow transplantation or irradiation may decrease the NOS activity in the ligated carotid artery. When compared with the WT mice that received the WT bone marrow transplantation, the NOS activity in the ligated carotid artery was significantly reduced in the WT mice that received the n/i/eNOS$^{-/-}$ bone marrow transplantation, but not in the n/i/eNOS$^{-/-}$ mice that received the WT bone marrow transplantation (Fig. 7D). A significant difference in the NOS activity in the ligated carotid artery was noted between the WT mice that received the n/i/eNOS$^{-/-}$ bone marrow

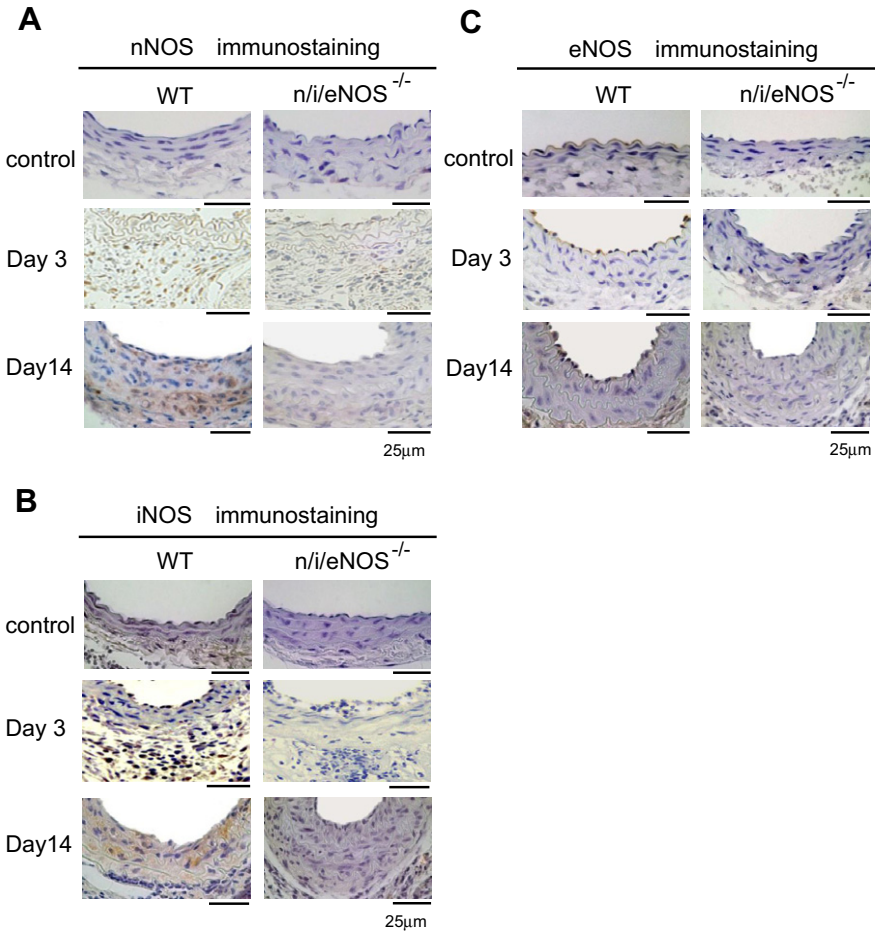

Fig. 3. Immunostaining for nNOS, iNOS, and eNOS in the carotid arteries of the WT and n/i/eNOS$^{-/-}$ mice before and on day 3 and 14 after the carotid artery ligation. Brown color was positive for the staining. (A) nNOS immunostaining. (B) iNOS immunostaining. (C) eNOS immunostaining.

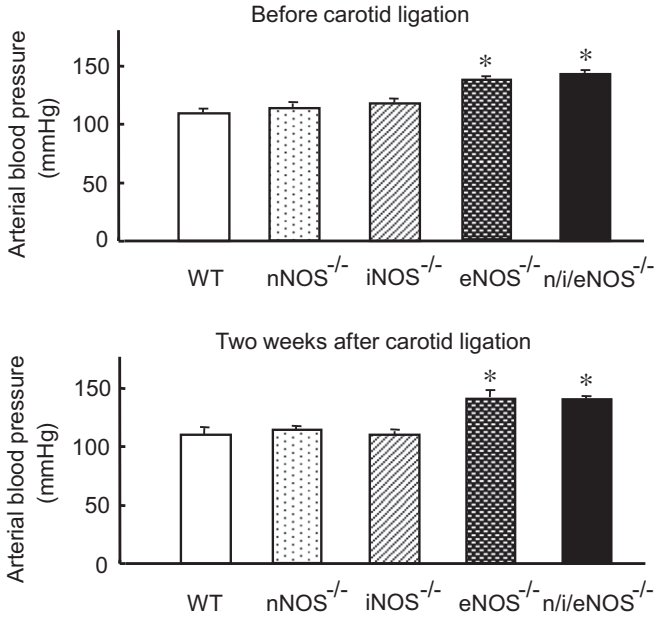

Fig. 4. Arterial blood pressure in the WT and NOS$^{-/-}$ mice before and 2 weeks after the carotid artery ligation. Arterial blood pressure was measured by the tail-cuff method under conscious conditions (n = 10–16). $^{*}P < 0.05$ vs. WT.

transplantation and the n/i/eNOS$^{-/-}$ mice that received the WT bone marrow transplantation.

When compared with the WT mice that received the WT bone marrow transplantation, the extents of nNOS, iNOS, and eNOS immunoreactivities in the ligated carotid arteries were all similar in the n/i/eNOS$^{-/-}$ mice that received the WT bone marrow transplantation, but markedly decreased in the WT mice that received the n/i/eNOS$^{-/-}$ bone marrow transplantation (Fig. 8), which findings were consistent with the level of the NOS activity in each group.

NOx production in bone marrow cells

To assess the ability of NO production in bone marrow cells, bone marrow cells of the WT and n/i/eNOS$^{-/-}$ mice were cultured in the presence or absence of LPS plus INF-γ for 24–72 h. In bone marrow cells of the WT mice, treatment with LPS plus INF-γ caused marked increases in NOx levels in culture medium in a time-dependent manner (Fig. 9). In contrast, in bone marrow cells of the n/i/eNOS$^{-/-}$ mice, this treatment did not induce any increase in NOx levels in culture medium (Fig. 9).

Discussion

The principle novel findings of the current study were that the n/i/eNOS$^{-/-}$ mice showed markedly accelerated inflammatory vascular lesion formation induced by carotid artery ligation, which findings were prevented by long-term treatment with the NO donor ISDN, and that the WT mice that received the transplantation of bone marrow from the n/i/eNOS$^{-/-}$ mice exhibited similar exac-

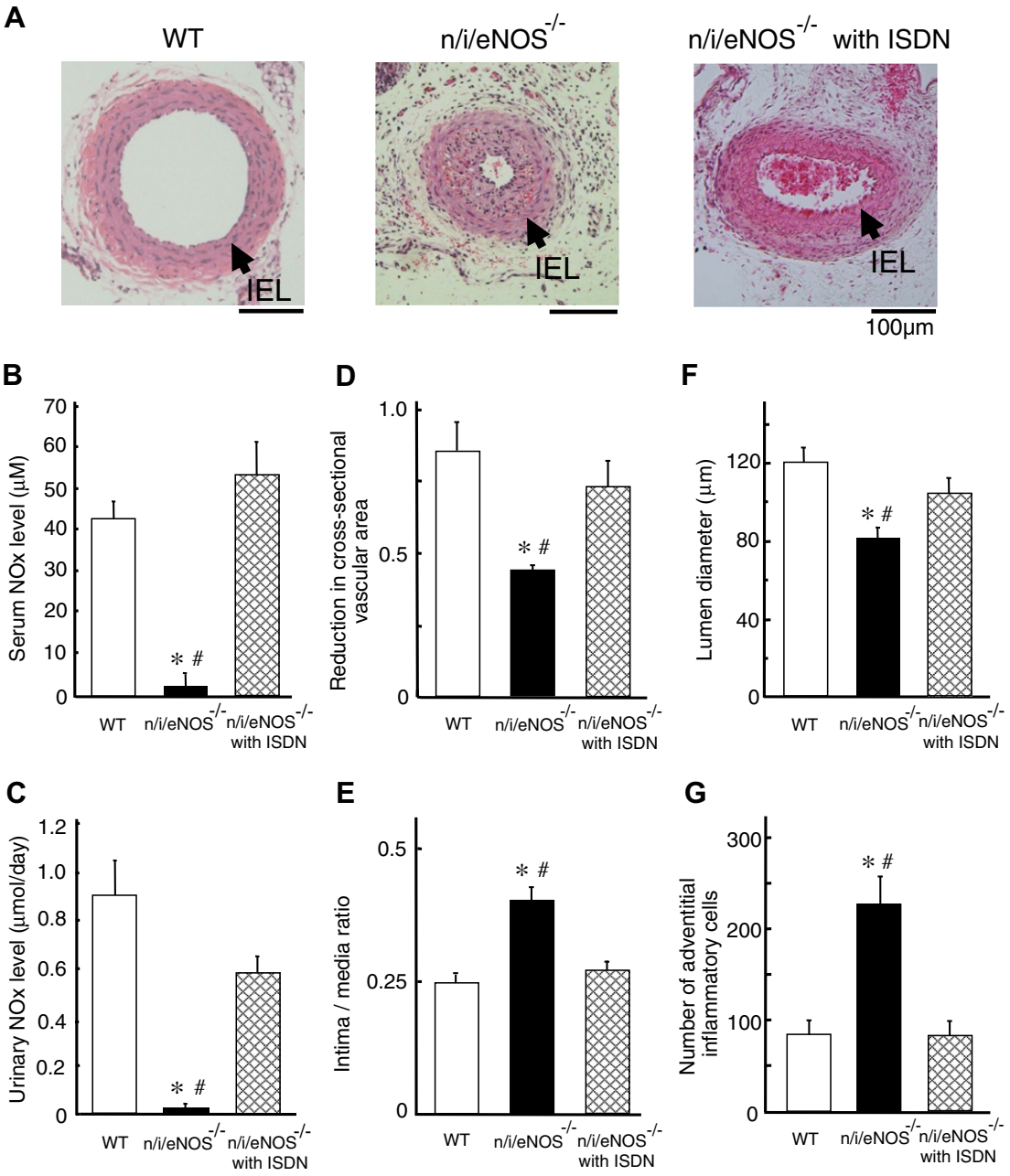

Fig. 5. The effects of long-term treatment with isosorbide dinitrate (ISDN), a NO donor, on plasma and urinary NOx levels, vascular lesion formation, and inflammatory cell infiltration after carotid artery ligation in the n/i/eNOS$^{-/-}$ mice. ISDN was dissolved in drinking water (0.6 mg/ml), and administered orally in the n/i/eNOS$^{-/-}$ mice for 14 days. (A) Histology of the ligated carotid arteries. (B,C) Serum and urinary NOx levels (n = 9–18). $^{*}P < 0.01$ vs. WT; $^{\#}P < 0.01$ vs. n/i/eNOS$^{-/-}$ with ISDN. (D) Reduction in cross-sectional vascular area in the ligated carotid as compared to the control carotid artery (n = 6–10). $^{*}P < 0.01$ vs. WT; $^{\#}P < 0.05$ vs. n/i/eNOS$^{-/-}$ with ISDN. (E) The intima-to-media ratio in the ligated carotid artery (n = 12–14). $^{*}P < 0.001$ vs. WT; $^{\#}P < 0.001$ vs. n/i/eNOS$^{-/-}$ with ISDN. (F) Lumen diameter in the ligated carotid arteries (n = 12–18). $^{*}P < 0.001$ vs. WT; $^{\#}P < 0.001$ vs. n/i/eNOS$^{-/-}$ with ISDN. (G) The number of adventitial inflammatory cells in the ligated carotid arteries. $^{*}P < 0.001$ vs. WT; $^{\#}P < 0.0001$ vs. n/i/eNOS$^{-/-}$ with ISDN.

erbation of the inflammatory vascular lesion formation. These results provide the first direct evidence for the important vasculoprotective role of the whole endogenous NOS system. Our findings also suggest that the NOS system in bone marrow-derived cells may be involved in this vasculoprotective mechanism.

Carotid artery ligation model

In the present study, we used a well-characterized animal model of vascular lesion formation. In the unilateral carotid artery ligation model, one of the major causes of vascular lesion formation is the reduction of shear stress and the turbulence of blood flow in the artery proximal to the ligation site. This model may thus represent the process of vascular lesion formation in the stenosed or occluded artery in humans [6].

Vasculoprotective role of the whole NOS system

We previously reported that at 4 weeks after carotid artery ligation, eNOS$^{-/-}$ mice show increased neointimal formation, that iNOS$^{-/-}$ mice exhibit enhanced constrictive vascular remodeling, and that nNOS$^{-/-}$ mice denote both pathological vascular struc-

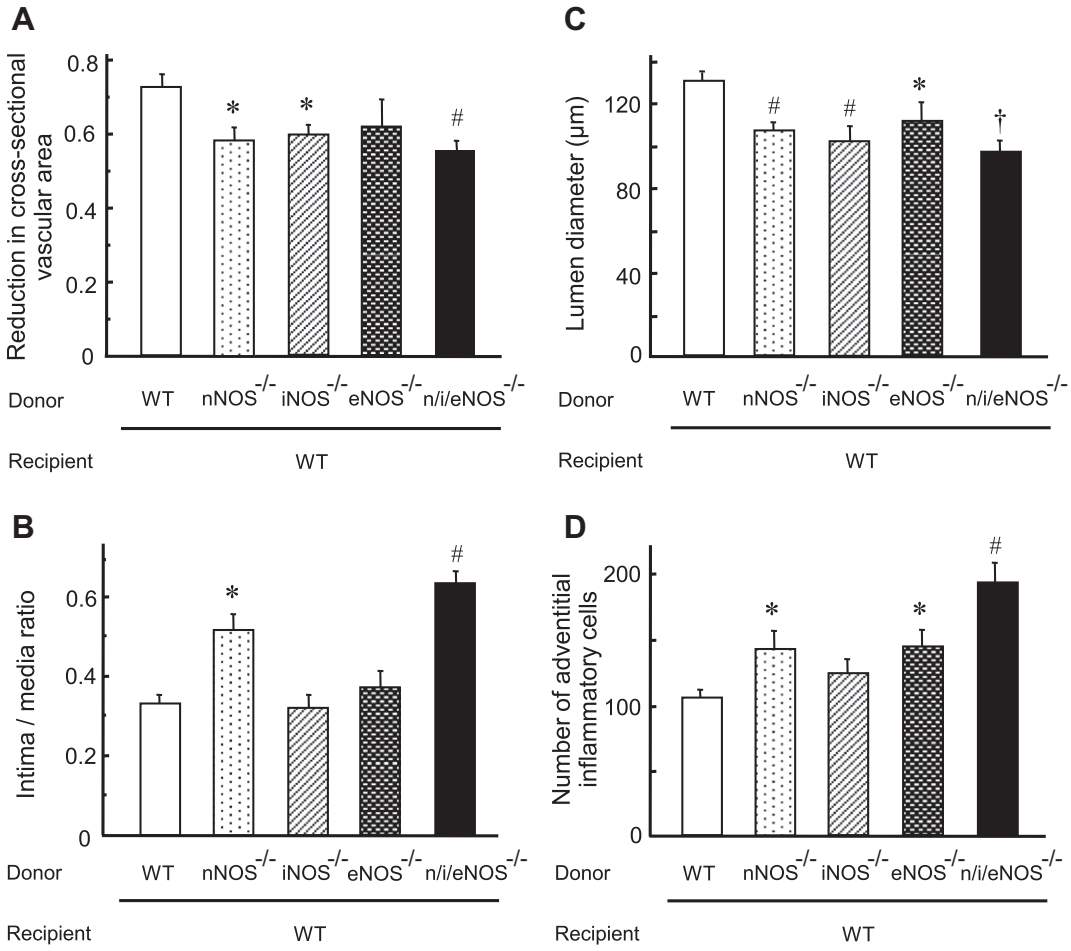

Fig. 6. Vascular lesion formation and inflammatory cell infiltration in the ligated carotid arteries of the WT mice that received transplanted bone marrow from WT or NOS$^{-/-}$ mice on day 14 after the carotid artery ligation. (A) Reduction in vascular cross-sectional area in the ligated carotid as compared to the control carotid artery (n = 5–7). $^{*}P < 0.01$, $^{\#}P < 0.001$ vs. WT. (B) The intima-to-media ratio in the ligated carotid artery (n = 7–13). $^{*}P < 0.001$, $^{\#}P < 0.0001$ vs. WT. (C) Lumen diameter in the ligated carotid arteries (n = 7–13). $^{*}P < 0.05$, $^{\#}P < 0.005$, $^{\dagger}P < 0.0001$ vs. WT. (D) The number of adventitial inflammatory cells in the ligated carotid arteries (n = 8–13). $^{*}P < 0.005$, $^{\#}P < 0.001$, $^{\dagger}P < 0.0001$ vs. WT.

tural changes [7,8]. These results suggest that individual NOS isoforms have different vasculoprotective actions against vascular lesion formation in vivo. We confirmed those findings at 2 weeks after the carotid artery ligation in the present study. Importantly, the n/i/eNOS$^{-/-}$ mice represented the highest extents of constrictive vascular remodeling and neointimal formation, concomitant with conspicuous luminal narrowing. Furthermore, long-term NO supplementation with the NO donor ISDN prevented those alterations in the vascular architecture in the n/i/eNOS$^{-/-}$ mice. Thus, it is conceivable that NO derived from the whole NOS system has important vasculoprotective actions against vascular lesion formation.

As we reported previously [8] and presently, in the early stage of vascular lesion formation (on day 3 after the carotid artery ligation), nNOS and iNOS immunoreactivities emerged in adventitial inflammatory cells, suggesting that nNOS and iNOS inhibit the development of constrictive vascular remodeling. In the advanced stage of vascular lesion formation (on day 14 after the procedure), nNOS was expressed in the neointima, medial smooth muscle cells, and endothelial cells, and iNOS emerged in the neointima and medial smooth muscle cells. On the other hand, eNOS expression was limited to endothelial cells of the ligated carotid artery throughout the experimental periods. It is likely that the nNOS, iNOS, and eNOS in those cells suppress neointimal formation. In line with the hypotheses, antiatherogenic actions of NO have been suggested as follows: NO inhibits smooth muscle cell proliferation and migration, matrix production, platelet adhesion and aggregation, leukocyte adhesion, and oxidation of low-density lipoproteins in vitro [21–23]. Although nNOS was regarded as a constitutively expressed enzyme, we reported that vascular nNOS expression is up-regulated by inflammatory and proliferative stimuli (e.g., angiotensin II, interleukin-1β, and platelet-derived growth factor) and a statin.

Mildly elevated blood pressure was noted in the eNOS$^{-/-}$ and n/i/eNOS$^{-/-}$ genotypes, whereas those changes were not correlated with the differences in the extent of vascular lesion formation among the five genotypes. These results suggest that hypertension plays a minor role in the accelerated vascular lesion formation in the n/i/eNOS$^{-/-}$ mice.

Vasculoprotective role of the bone marrow whole NOS system

Although the "Response to Injury" hypothesis that the intimal smooth muscle cells migrate from the medial layer has been widely and long accepted [24], recent studies indicate the possibility that the neointimal cells are derived from circulating progenitor cells of bone marrow origin [25–28]. In agreement with this new concept, we also showed that human intimal smooth muscle and endothelial cells express hematopoietic lineage markers such as stem cell factor and c-kit [29–31]. We further reported that in

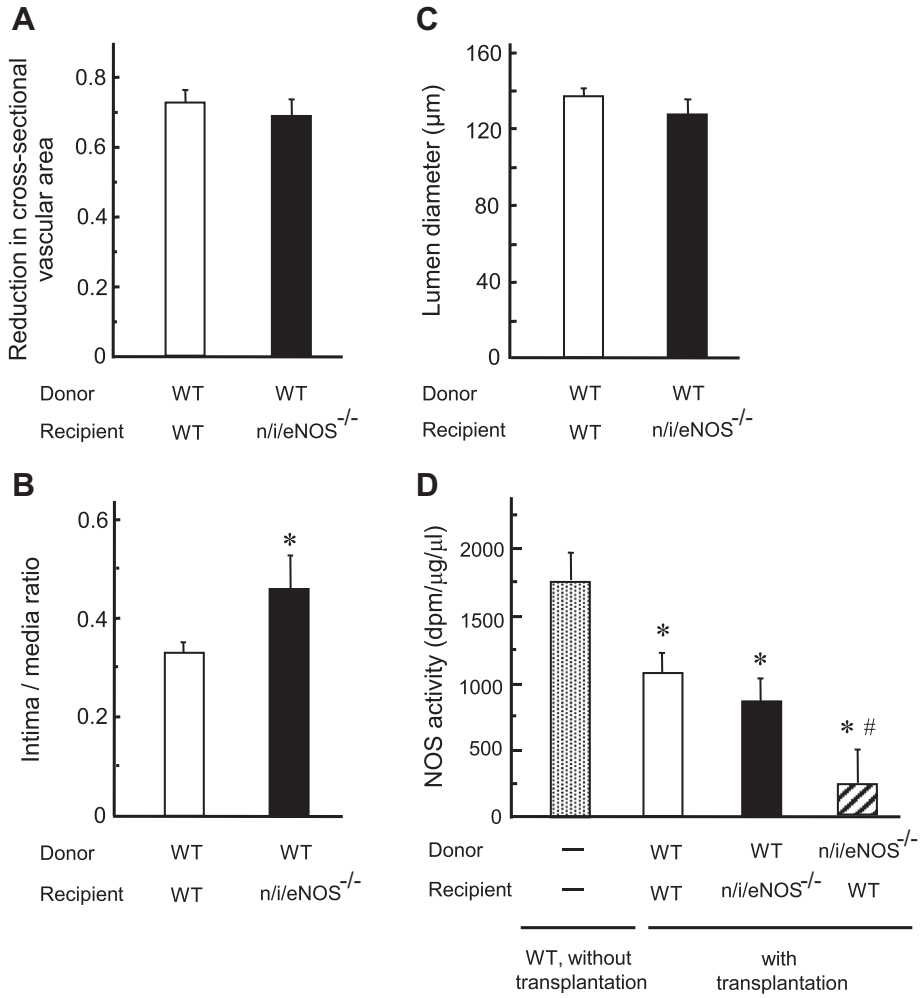

Fig. 7. Vascular lesion formation and total NOS activity in the ligated carotid arteries of the WT or n/i/eNOS$^{-/-}$ mice that received transplanted bone marrow from WT or n/i/eNOS$^{-/-}$ mice on day 14 after the carotid artery ligation. (A) Reduction in vascular cross-sectional area in the ligated carotid as compared to the control carotid artery (n = 10–11). (B) The intima-to-media ratio in the ligated carotid artery (n = 11–14). $^{*}P < 0.05$. (C) Lumen diameter in the ligated carotid arteries (n = 11–15). (D) Total NOS activity in the ligated carotid arteries (n = 5–6). $^{*}P < 0.05$ vs. WT mice without bone marrow transplantation. $^{\#}P < 0.05$ vs. WT mice with WT bone marrow transplantation. dpm, disintegrations per minute.

WT mice that were subjected to ligation-induced vascular injury after bone marrow transplantation from green fluorescent protein (GFP)-transgenic mice, positive green fluorescence (i.e., bone marrow-derived cells) was extensively detected in the neointima, the medial smooth muscle cells, the adventitia, and the endothelium in the ligated arteries [14]. These results suggest the involvement of bone marrow-derived vascular progenitor cells in the ligation-induced vascular lesion formation. Based on these lines of evidence, in this study we investigated whether the transplantation of bone marrow from NOS$^{-/-}$ mice may affect the vascular lesion formation in the injured arteries. Intriguingly, the WT mice that received transplanted bone marrow from the n/i/eNOS$^{-/-}$ mice manifested prominently progressed vascular lesion formation associated with enhanced inflammatory cell infiltration after the carotid artery ligation, which mimicked the results observed in the ligated arteries of the n/i/eNOS$^{-/-}$ mice. It is thus possible that the NO produced from bone marrow-derived cells may play an important vasculoprotective role against the ligation-induced vascular lesion formation. To the best of our knowledge, this is the first demonstration showing the importance of the bone marrow NOS system in the maintenance of vascular architecture.

The NOS activity and the immunoreactivities for nNOS, iNOS, and eNOS in the ligated carotid artery were comparable between the WT mice that underwent the WT bone marrow transplantation and the n/i/eNOS$^{-/-}$ mice that underwent the WT bone marrow transplantation, whereas they were markedly decreased in the WT mice that underwent the n/i/eNOS$^{-/-}$ bone marrow transplantation as compared with the WT mice that underwent the WT bone marrow transplantation. These findings are in agreement with the result that the extents of vascular lesion formation in the ligated carotid artery were larger in the WT mice that underwent the n/i/eNOS$^{-/-}$ bone marrow transplantation than in the n/i/eNOS$^{-/-}$ mice that underwent the WT bone marrow transplantation. It is possible that the n/i/eNOS$^{-/-}$ bone marrow-derived vascular progenitor cells may be transformed into vascular wall cells, and contribute to the exacerbation of the ligation-induced vascular lesion formation and the decrease in the NOS activity in the ligated carotid artery.

Involvement of vascular inflammation

Prominent infiltration of adventitial inflammatory cells (mostly neutrophils) was noted in the ligation-induced vascular lesions in both the n/i/eNOS$^{-/-}$ mice without bone marrow transplantation and the WT mice with the n/i/eNOS$^{-/-}$ bone marrow transplantation. Furthermore, in the n/i/eNOS$^{-/-}$ mice, suppression of the vascular lesion formation by long-term treatment with ISDN was associated with attenuation of the inflammatory cell infiltration.

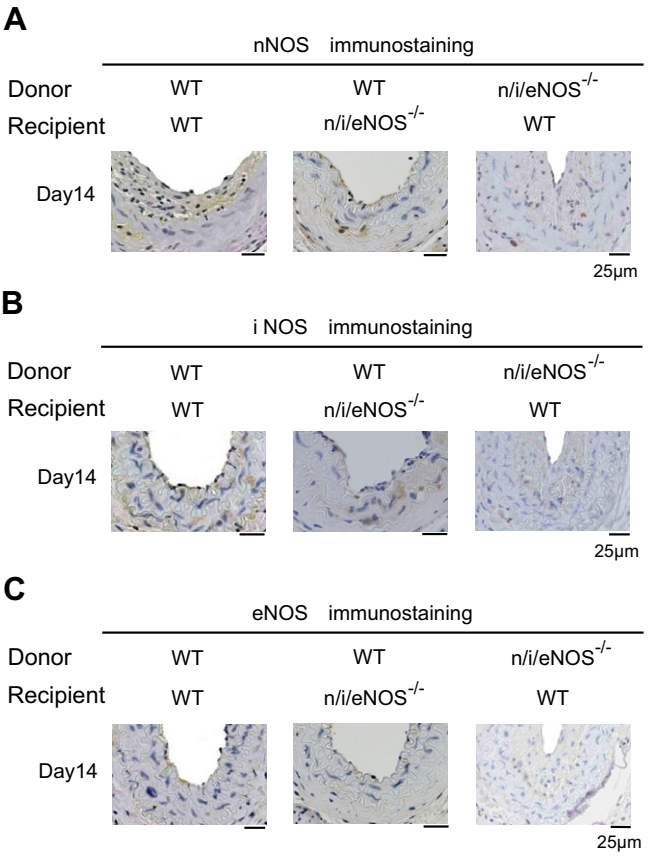

Fig. 8. Immunostaining for nNOS, iNOS, and eNOS in the ligated carotid arteries of the WT mice that underwent the WT bone marrow transplantation, the n/i/eNOS$^{-/-}$ mice that underwent the WT bone marrow transplantation, and the WT mice that underwent the n/i/eNOS$^{-/-}$ bone marrow transplantation on day 14 after the carotid artery ligation. (A) nNOS immunostaining. (B) iNOS immunostaining. (C) eNOS immunostaining.

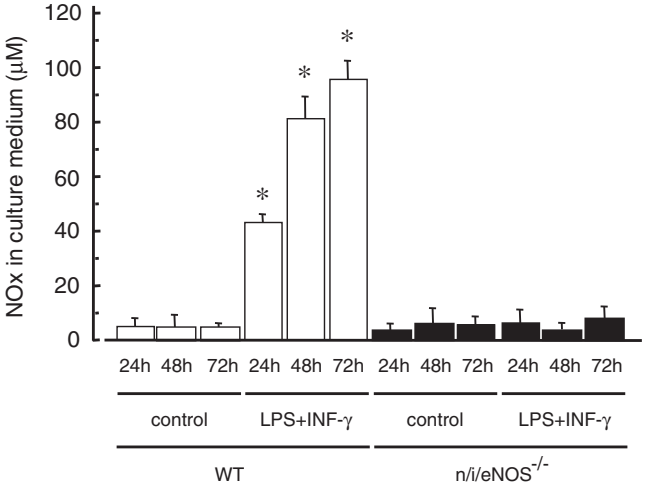

Fig. 9. NOx levels accumulated in culture medium of the WT and n/i/eNOS$^{-/-}$ mouse bone marrow cells in the presence or absence of lipopolysaccharide (LPS, 1 μg/ml) plus interferon-γ (INF-γ, 150 unit/ml) for 24–72 h (n = 5 each). *P < 0.05 vs. control.

These findings are consistent with previously recognized anti-inflammatory properties of NO [1–3]. Thus, vascular inflammation appears to be involved in the accelerated vascular lesion formation in these particular animal models.

Bone marrow cells of the WT mice were capable of generating a large amount of NOx in response to LPS plus INF-γ, indicating that bone marrow cells of the WT mice can produce high levels of NO when stimulated by the immune system. In contrast, no such NOx production was noted in bone marrow cells of the n/i/eNOS$^{-/-}$ mice. Thus, the inability of NO production in the n/i/eNOS$^{-/-}$ bone marrow-derived cells could be one of the reasons to cause increased inflammatory cell infiltration.

In conclusion, we were able to prove that the complete disruption of all NOS genes results in markedly accelerated inflammatory vascular lesion formation caused by blood flow disruption in mice in vivo, indicating the crucial vasculoprotective role of NO derived from the NOSs in their entirety. We were also able to demonstrate that the transplantation of bone marrow lacking all NOS genes causes a similar acceleration of inflammatory vascular lesion formation, suggesting the involvement of NO derived from the bone marrow NOSs in the vasculoprotective machinery. The present findings should contribute to a better understanding of the role of the entire endogenous NO/NOS system in arteriosclerotic vascular lesion formation.

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