Nitric oxide (NO) is known to have several important vasculoprotective actions. Although NO is synthesized by 3 different isoforms of NO synthase (NOS), the vasculoprotective action of individual NOS isoforms remains to be clarified. Permanent ligation of the left common carotid artery was performed in control, endothelial NOS (eNOS) knockout (eNOS-KO), and inducible NOS (iNOS) knockout (iNOS-KO) mice. Four weeks after the procedure, neointimal formation and reduction of cross-sectional vascular area (constrictive remodeling) were noted in the left carotid artery. In the eNOS-KO mice, the extent of neointimal formation was significantly greater than in the control or iNOS-KO mice, whereas the extent of vascular remodeling was the highest in the iNOS-KO mice compared with other 2 strains. Antiplatelet therapy with aspirin or antihypertensive treatment with bunazosin failed to inhibit the accelerated neointimal formation in the eNOS-KO mice. These results indicate that eNOS and iNOS have different vasculoprotective actions against the vascular lesion formation caused by blood flow disruption in vivo: NO derived from eNOS inhibits neointimal formation, whereas NO derived from iNOS suppresses the development of constrictive remodeling. (Arterioscler Thromb Vasc Biol. 2000;20:e96-e100.)

Key Words: nitric oxide ■ nitric oxide synthase ■ arteriosclerosis ■ neointimal formation ■ vascular remodeling

Thus, the present study was designed to address this point in the carotid artery ligation model in mice.

Methods

The present study was reviewed and approved by the Scientific Committee of Kyushu University.

Animals

iNOS-KO mice were provided by Dr Mudgett (Merck Research Laboratories, Rahway, NJ), and eNOS-KO mice were provided by Dr Fishman (Harvard Medical School, Boston, Mass.). For wild genotype control, we used C57BL/6 mice. In preliminary studies, we confirmed no endothelial production of NO in eNOS-KO mice and no expression of iNOS mRNA in iNOS-KO mice after stimulation by lipopolysaccharide or after carotid artery ligation as used in the present study (data not shown). All experiments were performed in male mice except for the additional protocol with aspirin or bunazosin in eNOS-KO mice, which was performed in female mice because of the limited availability of male mice.

Carotid Artery Ligation

Animals were anesthetized with pentobarbital (50 mg/kg IV). The left common carotid artery was completely ligated by a 6-0 silk suture at the site just proximal to the carotid bifurcation. The animals were allowed to recover for 4 weeks. To examine the possible involvement of thrombus formation or mildly elevated blood pressure in the vascular lesion formation in eNOS-KO mice,
additional protocols were performed, in which eNOS-KO mice underwent the procedure with or without oral treatment with aspirin (10 mg/kg per day)9 or an α1-adrenergic receptor antagonist, bunazosin (30 mg/kg per day),10 in the drinking water. These protocols were started 1 week before the procedure and were continued for a total of 5 weeks until the end of the experiments. Systolic blood pressure was measured with the animals in conscious conditions by the tail-cuff method before and 4 weeks after the procedure.

Morphometric Analysis
Four weeks after the carotid artery ligation, the animals were euthanized by intraperitoneal injection of an overdose of pentobarbital. The aorta was cannulated and perfused with 10% formaldehyde solution under physiological pressure, and carotid arteries from both sides were removed and embedded in paraffin.8 The 4 different areas (lumen, neointima, media, and total vascular area) were measured in sections stained by Masson’s trichrome staining (KD4600, Graphtec) at 1, 3, and 5 mm proximal to the ligation site. In each specimen, the extent of neointimal formation was expressed by the intima-to-media ratio, and that of constrictive remodeling was expressed by the percent reduction of the cross-sectional vascular area in the ligated left carotid artery compared with that in the control right carotid artery.

Immunostainings
One week after the ligation, carotid arteries from both sides were removed without formaldehyde perfusion and fixed by peroxidase-lysine-paraformaldehyde. The fixed vessels were embedded in OCT compound and were made into 6-μm-thick frozen sections at −20°C. The sections were then air-dried and washed with PBS. After inhibition of internal peroxidase by 5 mmol/L periodic acid solution and blocking of nonspecific binding by normal goat serum, the sections were incubated overnight at 4°C with a rabbit polyclonal antibody against iNOS of human origin (Santa Cruz Biotechnology) at a dilution of 1:100 with PBS. According to the supplier, this antibody also reacts with iNOS of mouse origin. Subsequently, the sections were incubated with horseradish peroxidase labeled with anti-rabbit IgG(ab’2) fragment from donkey for 60 minutes at room temperature. An avidin-biotin-immunoperoxidase system was used to detect the antigen. We also performed immunostaining for neutrophils (rat monoclonal anti-mouse neutrophil antibody, Serotec).

Vascular Areas in Mice

<table>
<thead>
<tr>
<th>Strain</th>
<th>Areas, ×10^−4 μm²</th>
<th>Lumen</th>
<th>Intima</th>
<th>Media</th>
<th>Total Vascular Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (n=12)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right</td>
<td>8.93±0.29</td>
<td>0.17±0.01</td>
<td>1.56±0.07</td>
<td>10.65±0.35</td>
<td></td>
</tr>
<tr>
<td>Left</td>
<td>5.24±0.30</td>
<td>0.70±0.12</td>
<td>2.33±0.15</td>
<td>8.27±0.42</td>
<td></td>
</tr>
<tr>
<td>eNOS-KO (n=9)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right</td>
<td>7.51±0.30</td>
<td>0.16±0.01</td>
<td>1.37±0.07</td>
<td>9.04±0.37</td>
<td></td>
</tr>
<tr>
<td>Left</td>
<td>4.11±0.38*</td>
<td>1.38±0.35*</td>
<td>1.89±0.12</td>
<td>7.37±0.41</td>
<td></td>
</tr>
<tr>
<td>iNOS-KO (n=12)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right</td>
<td>8.27±0.31</td>
<td>0.17±0.01</td>
<td>1.43±0.07</td>
<td>9.87±0.36</td>
<td></td>
</tr>
<tr>
<td>Left</td>
<td>4.07±0.25*</td>
<td>0.58±0.19†</td>
<td>1.59±0.10*</td>
<td>6.24±0.27*</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean±SEM. The mean value of the area at 1, 3, and 5 mm proximal to the carotid bifurcation (ligation site in the left carotid artery) is expressed. Right and left indicate right (control) and left (ligated) carotid artery, respectively.

⁎P<0.05 vs wild-type mice; †P<0.05 vs eNOS-KO mice (by ANOVA).
Inc) and for macrophages (rat monoclonal anti-mouse macrophage antibody, provided by Dr Takeya) in a similar manner.

**Data Analysis**

All results were expressed as mean±SEM. The differences among the 3 strains were analyzed by ANOVA. A value of \( P < 0.05 \) was considered to be statistically significant.

**Results**

Before the carotid artery ligation, systolic blood pressure was slightly but significantly higher in the eNOS-KO (148±3 mm Hg, \( n=12 \)) than in the wild-type (122±6 mm Hg, \( n=9 \)) or iNOS-KO (126±6 mm Hg, \( n=12 \)) mice (\( P < 0.05 \) each). Four weeks after the procedure, the blood pressure was also slightly but significantly higher in the eNOS-KO (154±7 mm Hg) than in the wild-type (127±5 mm Hg) or iNOS-KO (122±7 mm Hg) mice (\( P < 0.05 \) each). Treatment with aspirin did not significantly alter the blood pressure (\( n=7 \), data not shown), whereas treatment with bunazosin significantly reduced the blood pressure (from 157±6 mm Hg before to 128±6 mm Hg after treatment, \( n=10 \)) in the eNOS-KO mice.

In the wild-type mice, neointimal formation and constrictive remodeling were noted in the left (ligated) but not in the right (control) carotid artery (Figure IA and IB). Neointimal formation was accelerated in the eNOS-KO mice (Figure IC), whereas the constrictive remodeling was enhanced in the iNOS-KO mice (Figure ID, Table). The iNOS immunoreactivity was absent in the intact right carotid artery of the wild-type mice (Figure IIA) or the ligated left carotid artery of the iNOS-KO mice (Figure IID), whereas it was noted in the ligated left carotid artery of the wild-type mice (Figure IIB) and, to a greater extent, in that of the eNOS-KO mice (Figure IIC). The immunoreactivity for iNOS was noted mainly in inflammatory cells at the adventitia, most of which were neutrophils. Those cells were negative for the immunostaining of macrophages or nonimmune IgG (data not shown). The number of infiltrating neutrophils (per section) was 8.6±0.8 in the wild-type mice (Figure IIE), whereas it was significantly increased in the eNOS-KO mice (26.7±1.1, \( P < 0.01 \); Figure IIF) and significantly decreased in the iNOS-KO mice (2.9±0.6, \( P < 0.01 \); Figure IIG), a finding consistent with that for iNOS immunostaining (Figure IIB through IID).

The quantitative analysis demonstrated that in the wild-type mice, the extent of neointimal formation and constrictive remodeling was larger at the most proximal (1-mm) site compared with the distal (3- and 5-mm) sites (Figure III). Furthermore, the extent of neointimal formation was signifi-
The antiplatelet treatment with aspirin or antihypertensive treatment with bunazosin failed to suppress the neointimal formation in the eNOS-KO mice; the intima-to-media ratio at the 1-mm site of the ligated left carotid artery was 0.88 ± 0.23 without any treatment (n = 7), 1.28 ± 0.34 with aspirin treatment (n = 7), and 0.85 ± 0.16 with bunazosin treatment (n = 10).

**Discussion**

The novel finding of the present study is that eNOS and iNOS play a different vasculoprotective role against the vascular lesion formation caused by blood flow disruption in mice in vivo; eNOS inhibits the neointimal formation, whereas iNOS suppresses the development of constrictive remodeling. To the best of our knowledge, this is the first report that demonstrates the different vasculoprotective roles of NOS isoforms in the same animal model of vascular lesion formation.

In the present study, we used carotid artery ligation to induce vascular lesions in mice.\(^4\) In this model, one of the major causes of vascular lesion formation is the reduction of shear stress and turbulence of blood flow in the artery proximal to the ligation site.\(^4\) Thus, this model may represent, at least to some extent, the process of vascular lesion formation in the stenosed or occluded coronary artery in humans.

Moroi et al\(^6\) recently demonstrated that the extent of neointimal formation (caused by cuff placement around the femoral artery) is greater in eNOS-KO mice than in wild-type mice. Our present finding is consistent with their observation. Furthermore, we were able to demonstrate that the accelerated neointimal formation in eNOS-KO mice was not due to a thrombus formation or mildly elevated blood pressure, because antiplatelet therapy with aspirin or antihypertensive therapy with bunazosin failed to inhibit the process. The extent of the neointimal formation tended to be smaller in the additional protocols with aspirin or bunazosin in female mice compared with the original protocol in male mice, suggesting a sex difference in this process.

Another important finding of the present study is that the development of constrictive remodeling was significantly accelerated in iNOS-KO mice. Koglin et al\(^7\) recently demonstrated that the development of transplant arteriosclerosis was enhanced in iNOS-KO mice. We also previously showed that NO derived from iNOS exerts an inhibitory effect on the development of neointimal formation and vasospastic responses caused by the long-term adventitial stimulation with interleukin-1β.\(^11\) We subsequently observed that NO derived from iNOS may also inhibit the development of constrictive remodeling in our porcine model (authors’ unpublished data, 2000). Interestingly, iNOS immunoreactivity was noted mainly in neutrophils, especially at the adventitia, and the neutrophil infiltration was reduced in the iNOS-KO mice. The stimulus for neutrophil infiltration and iNOS expression may be inflammatory cytokines,\(^11\) and the expression of the cytokines may also be reduced in the iNOS-KO mice.\(^4\)

It has been recently reported that in vivo gene transfer of either eNOS\(^12\) or neuronal NOS\(^13\) significantly inhibits neointimal formation after balloon injury, suggesting the potential usefulness of gene therapy with NOS isoforms in the treatment of vascular diseases. The present results suggest that such a gene therapy with NOS isoforms may cause different effects, depending on the site of the vascular wall transfected with the gene and also on the NOS isoform used.

**Acknowledgments**

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**References**

Different Vasculoprotective Roles of NO Synthase Isoforms in Vascular Lesion Formation in Mice

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