Decreased Neointimal Thickening After Arterial Wall Injury in Inducible Nitric Oxide Synthase Knockout Mice


Abstract—Mechanical injury in vivo results in the expression of the inducible form of nitric oxide synthase (iNOS) in vascular smooth muscle cells. However, the role of iNOS in modulating neointima formation after arterial wall injury is not clear. To determine whether the induction of iNOS gene expression promotes or attenuates the neointimal response to injury, we used a murine model of perivascular injury induced by placing a periadventitial collar around the carotid arteries in both wild-type and iNOS knockout mice (iNOS-KO mice). Periadventitial injury induced iNOS expression in the wild-type but not the iNOS-KO mice. Neointimal area and the intima/media ratio were significantly less in the iNOS-KO mice compared with the wild-type mice at 21 days. Injury-induced proliferation of medial cells and vascular cell adhesion molecule-1 expression were also attenuated in iNOS-KO mice compared with wild-type mice. The induction of iNOS and the activation of the nuclear factor-κB–mediated pathway were also demonstrated in an in vitro injury model. We conclude that mechanical injury in vivo and in vitro induces iNOS expression and that lack of iNOS expression attenuates neointima formation after perivascular arterial injury. Taken together, these findings suggest that iNOS expression after vascular injury may promote neointima formation. (Circ Res. 1999;85:1192-1198.)

Key Words: nuclear factor-κB ☐ vascular cell adhesion molecule-1 ☐ nitric oxide synthase ☐ mice, knockout ☐ neointima

Mechanical injury to the arterial walls results in an inflammatory response, as evidenced by the activation of nuclear factor-κB (NF-κB) and the expression of vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 in vascular smooth muscle cells (VSMCs).1,2 The expression of inducible nitric oxide synthase (iNOS) has also been demonstrated in injured arterial walls.3,4 Nitric oxide (NO) possesses antiproliferative properties in vitro,5,6 and its role in modulating neointima formation has been tested using either NO donors or nonspecific NOS inhibitors.7–9 Because the induction of iNOS in the injured arterial walls leads to NO production,3,4 it has been hypothesized that this NO output may reduce neointima formation and that neointima formation after injury would be more prominent in iNOS knockout mice (iNOS-KO mice). However, recent studies showed that wound repair and the regeneration of the injured liver are impaired in iNOS-KO mice.10,11 These findings suggest that iNOS induction after arterial injury may promote rather than prevent cellular proliferation and growth. iNOS and VCAM-1 are expressed in VSMCs after mechanical injury in vivo.2,3 It is not clear whether iNOS in the injured arterial wall regulates VCAM-1 expression; in vitro observation revealed that NO donors reduced VCAM-1 expression.12 A recent report showed that iNOS is involved in proinflammatory signaling and that it upregulates the expression of the inflammatory cytokines interleukin-6 and granulocyte colony-stimulating factor.13 These findings also suggest that iNOS may enhance VCAM-1 expression in the injured arterial wall.

In the present study, we used a gene-knockout strategy to test the hypothesis that a lack of iNOS expression after in vivo mechanical injury reduces neointimal thickening and VCAM-1 expression. We also tested the hypothesis that mechanical injury alone induces iNOS expression in an in vitro injury model, because it is not known whether injury itself is a stimulus for the expression of iNOS in VSMCs.

Materials and Methods

Periadventitial Collar Injury of Carotid Artery

Wild-type and iNOS-KO mice (B6/129 background; 5-week-old males; Jackson Laboratory, Bar Harbor, Me) were fed regular chow throughout the duration of the experiment. At the age of 25 weeks, mice were anesthetized with Enflurane inhalation, the right carotid artery was dissected, and a nonocclusive plastic cuff (length, 3 mm; internal diameter, 0.51 mm; Cole-Parmer Instrument Co) was placed around the artery. At 21 days after cuff placement, the mice were euthanized and the carotid arteries were excised. The effects of cuff placement were compared with those of sham-operation controls where the carotid arteries were dissected but not injured. Each group consisted of six animals. The neointimal area was calculated as the differences between the luminal and external areas of each artery. The neointimal area/media ratio was calculated as the ratio of the neointimal area to the media area. The expression of iNOS and the nuclear factor-κB–mediated pathway was measured by Western blotting and real-time reverse transcription-polymerase chain reaction (RT-PCR). The expression of inflammatory cytokines and adhesion molecules was measured by ELISA.

The results were analyzed using the Student’s t test; means ± SD are shown. A p value of <0.05 was considered statistically significant.
around the carotid artery. Mice were euthanized 3, 7, or 21 days after injury, and the carotid arteries were perfused with 0.9% saline for 10 minutes and frozen at −70°C after they were embedded in OCT compound (Tissue-Tek, Allegiance). The contralateral, noninjured carotid arteries were used as controls. This experimental protocol was approved by the Institutional Animal Care and Use Committee of our institution. Intravascular thrombosis, as determined by hematoxylin and eosin staining, was observed in only one wild-type mouse.

**Morphometric Measurement**

Sections from the middle half of the injured segment were collected. These sections were used because the anatomy of the sections from cut ends was occasionally distorted when the vessel was removed from the cuff. Serial, 4-μm-thick sections with an average 4 μm apart were obtained. Four sections were collected on each slide, and 20 to 25 slides were collected for each arterial segment. Slides were sequentially divided into 3 groups, and the first 3 slides from each group were stained with hematoxylin and eosin. Computer-assisted morphometric analysis was done with image-analysis software (Optima 5.1, Bioscan) on these sections. The measurements of sections from the slides from each animal were averaged and analyzed.

**In Vitro Injury Experiment**

Rat aortic VSMCs were cultured using DMEM/F-12 with 10% fetal bovine serum, and confluent cells were growth-arrested for 48 hours before injury. The injury was done as described previously. For Western blotting and staining for the iNOS protein, injured cells were harvested 24 or 48 hours after injury. For the electrophoretic mobility shift assay (EMSA) and the determination of cytosolic levels of NF-κB, cells were injured at 30 minutes, 2 hours, and 6 hours before harvest.

**Western Blot Analysis**

Cytosolic proteins underwent electrophoresis on a 7.5% SDS-PAGE gel for the detection of the iNOS protein or on 12% gels to detect p50, p65, and inhibitory-κB (IkB) proteins. Primary antibodies were rabbit polyclonal iNOS, p50, p65, or IkB antibody (Santa Cruz Biotechnology).

**EMSA**

The oligodeoxynucleotide used in EMSA incorporated the sequence between −113 and −92 bp in the rat iNOS promoter containing the NF-κB binding site (shown underlined; 5′-3′ CCTACTGGG-GACTCTCCCTTTG). EMSA and supershift assays were performed as previously described.

**Immunohistochemical Staining**

Primary antibodies were anti-iNOS, anti-endothelial NOS (eNOS), anti–proliferating cell nuclear antigen (PCNA) (all rabbit polyclonal, Santa Cruz Biotechnology), rat monoclonal anti–macrophages/monocytes-2 (MOMA-2) (Serotec), and rat monoclonal anti-mouse VCAM-1 antibody (Pharmingen). Mucosal cells in intestinal villi from wild-type mice were used as positive controls for PCNA staining.

The semiquantitative measurement of the VCAM-1 or MOMA-2 stained area standardized against the medial area from 4 sections per animal was done with computer-assisted analysis. Analysis by 2 observers blinded to animal groups showed an interobserver variability of <5%.

**Statistical Analysis**

Optical densities of signals from EMSA or Western blot analysis were measured by computerized densitometry and presented either in text or in graph form. Numeric data are expressed as mean±SD. The number of experiments or animals used is given in the text or figure legend. The difference among groups was determined using 2-way ANOVA for multiple comparisons followed by a Tukey-Kramer test. P<0.05 was considered statistically significant.

An expanded Materials and Methods section is available online at http://www.circresaha.org.

**Results**

**Reduced Medial Proliferation and Neointima Formation After Cuff Injury in iNOS-KO Mice**

eNOS immunoreactivity was present in the noninjured arterial segments from both groups of mice. Three days after cuff placement, eNOS immunoreactivity was absent, indicating deendothelialization in both groups of mice (Figure 1, A

![Figure 1](http://circres.ahajournals.org/).
through D). Immunohistochemical staining showed only a minimal presence of monocytes/macrophages in the media (2.8 ± 1.5% of medial area, n = 3). Concomitant expression of iNOS protein occurred in the media of injured carotid arterial segments from wild-type mice; no iNOS expression was detected in iNOS-KO mice (Figure 1, E and F). Significantly more proliferating cells existed in wild-type mice (10.2 ± 3.9 PCNA-positive cells per section; n = 6) than in iNOS-KO mice (5.3 ± 3.4 PCNA-positive cells per section; n = 7; P < 0.05).

Twenty-one days after cuff placement, a 40% reduction of the neointimal area occurred in iNOS-KO mice compared with the wild-type mice (Table, Figure 2). The intima/media ratio (I/M ratio) was also significantly smaller in iNOS-KO mice than wild-type mice. No overall difference existed in medial thickness or vessel size.

Reduced VCAM-1 Expression After Injury in iNOS-KO Mice
Littie detectable VCAM-1 existed in uninjured sections from both groups. At 3 and 7 days after injury, VCAM-1 expres-

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<th>Effect of iNOS Gene Knockout in Response to Vessel Injury</th>
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*Significantly different from wild type, P < 0.05.

Figure 2. Elastin staining of arterial segment from wild-type mouse 21 days after injury revealed significant neointima formation (A and B), whereas only scanty neointima formation was observed in iNOS-KO mouse (C and D). Arrows indicate internal elastic lamina.
ther confirms the activation of the transcription factor NF-κB (Figure 5D).

**Discussion**

This study demonstrated that a lack of iNOS expression after in vivo arterial injury in iNOS-KO mice was associated with reduced proliferation of medial VSMCs, reduced VCAM-1 expression, and decreased neointima formation. Furthermore, we also demonstrated that in vitro mechanical injury induced iNOS expression in VSMCs.

iNOS produced a sustained increase in NO compared with eNOS, and it was expressed in the arterial walls of VSMCs after mechanical injury. Because NO inhibits VSMC proliferation in vitro, the inhibition of NO output by iNOS was expected to increase neointima formation after injury. However, N-nitro-L-arginine methyl ester treatment in a rat carotid injury experiment failed to demonstrate such an increase. This chemical may possess effects other than the inhibition of NO production, which may confound the interpretation of the data. Our study in iNOS gene knockout mice avoided such problems. We observed that periadventitial cuff injury induced iNOS expression in wild-type mice, which confirmed a previous observation by Moroi et al. Twenty-one days after injury, less neointima formation existed in iNOS-KO mice than in wild-type mice. Also, less proliferation of medial VSMCs occurred in iNOS-KO mice

**Figure 3.** VCAM-1 expression in injured arterial segments from wild-type mice (A) was significantly greater than in iNOS-KO mice (B) 3 days and 7 days (data not shown) after injury. iNOS-KO mice had significantly less VCAM-1 expression 3 and 7 days after injury (C). Number of animals per group is indicated.

**Figure 4.** iNOS protein expression was significantly increased 48 hours after mechanical injury, as shown by Western blot analysis (A). Positive iNOS immunoreactivity in VSMCs was detected at injury zone (B). Incubation with non-immune rabbit IgG isotype antibody (NIA) served as negative control.
than in wild-type mice. Our findings support the hypothesis that iNOS expression in injured VSMCs promotes proliferation and neointima formation. Neointima formation has been viewed as a general wound-healing response to injury.19 Yamasaki et al10 reported that wound healing was delayed in iNOS-KO mice. Rai et al11 also reported that the proliferation of regenerating hepatocytes after liver injury was significantly less in iNOS-KO mice. Our results, together with their findings, suggest that iNOS is involved in wound healing after injury in vivo and that it contributes to neointima formation after arterial injury.

The I/M ratio in the mouse injury model reported in the literature shows substantial variation. This may be due to the method used to injure the artery and to differences in the sex or genetic background of the mice. Guidewire-induced injury of the common carotid artery did not produce neointima formation in C57BL/6J mice20; however, injuring the carotid artery by ligation showed an I/M ratio of 1.13±0.2 in mice with the same genetic background.21 Using a periadventitial cuff femoral artery injury model similar to ours, Moroi et al18 demonstrated a mean I/M volume ratio of 27% and 18%, respectively, in male and female C57BL/6 mice and 31% and 17%, respectively, in male and female SV129 mice. Our results of a mean I/M ratio of 29% in wild-type mice is comparable to their findings.

It is interesting to note that neointima formation is reportedly enhanced in eNOS KO mice,18 which is in sharp contrast to our results in iNOS-KO mice. We speculate that this difference may be due to intrinsic differences in their expression. eNOS is constitutively expressed and may continuously modulate vascular responses. Lack of eNOS, therefore, could be detrimental to vascular functions. This concept is supported by experiments in eNOS-KO mice. eNOS KO mice have higher systemic and pulmonary arterial pressures22,23 than wild-type mice. The observation that neointima formation is enhanced in eNOS-KO mice18 further strengthens this concept. In contrast, the expression of iNOS is tightly regulated. Hence, its physiological role in the vascular response to injury could be entirely different from that of eNOS. In published reports, the expression of iNOS is mostly associated with pathological states, such as atherosclerosis and transplant vasculopathy.24–26 Experiments using iNOS-KO mice have demonstrated delayed wound healing and a lower proliferative response to injury.10,11 These findings suggest that the expression of iNOS in response to injury may promote cellular proliferation. Our experimental data support this possibility. It is also interesting to note that in gene-transfer studies, eNOS or iNOS overexpression decreased neointimal thickening. We speculate that these discrepant findings may be due to the amount of NO being produced. The amount of NO produced by the overexpression of eNOS or iNOS may be much different from the amount produced by endogenous iNOS expression induced by arterial injury. NO enhances the proliferation of VSMCs at low concentrations, but it has antiproliferative effects at higher concentrations.27

Mechanical injury evokes an inflammatory response characterized by the activation of NF-κB and the expression of various adhesion molecules in the vessel wall.1,2 We demonstrated that the expression of VCAM-1 in injured carotid arteries was increased 3 and 7 days after injury in both groups of mice, which is consistent with previous reports2,28; however, VCAM-1 expression was significantly less in the iNOS-KO mice than in the wild-type mice. This finding suggests that increased iNOS expression in the injured arterial wall may promote VCAM-1 expression. These observations are further supported by the study by Hierholzer et al;13 they reported that iNOS participates in proinflammatory signaling and upregulates several inflammatory molecules in a hypoxia-reperfusion injury model, possibly via a redox-sensitive mechanism. NO generated from iNOS expression could react with superoxide and lead to the formation of
peroxynitrite, a potent oxidant. The reductase domain in iNOS is capable of generating superoxide, independent of NO production. It is therefore possible that superoxide produced by the reductase domain, coupled with peroxynitrite formation, could alter the redox state in the cells and result in a persistent activation of inflammatory signaling, thereby leading to more neointimal thickening.

In vivo experiments showed that iNOS is expressed in VSMCs in injured arteries. However it is not known whether injury itself is a stimulus for the expression of iNOS in VSMCs or whether the injury acts in concert with other stimuli, such as platelets, mononuclear cells, or serum growth factors. Hence, we tested the hypothesis that mechanical injury alone can elicit iNOS expression by using an in vitro model. Although this model does not reproduce the in vivo condition in an exact fashion, the replication and migration of VSMCs in the injury zone could mimic in vivo processes.

We observed iNOS expression in cultured rat VSMCs after injury by Western blot analysis and immunocytochemistry. Transcription factor NF-κB played an important role in the iNOS expression induced by cytokines in VSMCs. Its activation required the phosphorylation and degradation of the cytosolic inhibitor IκB to allow the nuclear translocation of p50 and p65, with subsequent initiation of gene transcription. Our observation that injury increased iNOS NF-κB binding activity with a concomitant decrease of cytosolic p50, p65, and IκB levels is consistent with this view. Other mechanical forces, such as pulsatile stretch, increase superoxide production, with subsequent activation of NF-κB in human coronary smooth muscle cells. The early appearance of superoxide 20 minutes after application of stretch is consistent with our time points as a mediator for the activation of the NF-κB system in this current study. Our observation of injury-induced iNOS expression in cultured VSMCs suggests that injury itself is a stimulus for the induction of iNOS in VSMCs. However, our results do not exclude the possibility that injury-induced growth factor expression may induce iNOS expression. The very early activation of iNOS NF-κB in our study makes this possibility less likely.

In summary, we demonstrated that mechanical injury induced reduced neointima formation in injured carotid arteries from iNOS-KO mice; this was associated with reduced VCAM-1 expression when compared with wild-type mice. These findings are in sharp contrast to those from eNOS KO mice, in which increased neointima formation after injury has been reported. Thus, our findings highlight the divergent functional roles of eNOS and iNOS genes, which is likely to be of biological significance. Molecular mechanisms responsible for this diversity remain to be defined.

Acknowledgment
This work was supported by a Research Fellowship Award from the American Heart Association, Greater Los Angeles Affiliate, to Dr Kung-Yuh Chyu.

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Circ Res. 1999;85:1192-1198
doi: 10.1161/01.RES.85.12.1192

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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