Inducible Nitric Oxide Synthase Deficiency Does Not Affect the Susceptibility of Mice to Atherosclerosis but Increases Collagen Content in Lesions

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Background—Although endothelial nitric oxide synthase (NOS) is antiatherogenic, the role of inducible NOS (iNOS) in the development of atherosclerosis is not established.

Methods and Results—We compared the susceptibility of iNOS knockout (iNOS−/−) and wild-type (iNOS+/+) mice to the development of atherosclerosis induced by feeding an atherogenic diet for 15 weeks. Plasma lipid level, atherosclerotic lesion size, and cellular density in the lesions were all similar in the 2 strains (lesion size: iNOS+/+ 285±73×10³ μm², iNOS−/− 293±82×10³ μm², n=10). iNOS mRNA was detected in the lesions of iNOS+/+ but not iNOS−/− mice through RT-PCR. Immunohistochemically, iNOS+/+ mice showed iNOS staining in macrophages and medial smooth muscle cells in the lesions. Nitrotyrosine staining showed a similar distribution, whereas it was absent in iNOS−/− mice. There was no apparent difference in the intensity or distribution of vascular cell adhesion molecule-1 staining in the lesions of the 2 strains. However, the lesions of iNOS+/+ mice showed a markedly decreased extracellular collagen content compared with those of iNOS−/− mice

Conclusions—iNOS induction does not affect the development of atherosclerosis in mice fed an atherogenic diet, but the resulting lesions show decreased levels of extracellular collagen and may be more fragile. (Circulation. 2001;103:1115-1120.)

Key Words: mice, inbred ▪ atherosclerosis ▪ nitric oxide synthase

Chronic inhibition of NO production accelerates atherosclerosis,1,2 whereas enhanced production of NO suppresses the development of atherosclerosis or even regresses the lesions.3,4 These effects of NO in large part depend on its ability to terminate LDL oxidation,5 to inhibit the expression of adhesion molecules and chemokines,6,7 and to attenuate smooth muscle cell migration8 and are principally due to NO produced by endothelial NO synthase (eNOS). Experiments with eNOS knockout mice9,10 and eNOS gene transfer11 confirmed that eNOS is an important antiatherogenic player. However, the direct influence of inducible NOS (iNOS) on the development of atherosclerosis is unknown, although the induction of iNOS consistently occurs in atherosclerotic vessels of humans12,13 and experimental animals.14,15 The induction of iNOS may be a defense mechanism against the disease. However, a high flux of NO from iNOS may favor formation of peroxynitrite, a powerful oxidant generated by the reaction of NO and superoxide,16 and produce nitrotyrosine, which was shown to be present in human atheroma plaque.17 Thus, it is important to determine the overall impact of iNOS on atherogenesis, because this may have implications for preventive and therapeutic strategy. To examine directly the influence of iNOS on the development of atherogenesis and on the characteristics of the lesion, we compared the susceptibility of iNOS−/− and iNOS+/+ mice to the development of atherosclerosis induced with an atherogenic diet and analyzed the composition of the atherosclerotic lesions in the 2 strains.

Methods

Mice and Experimental Protocol

The iNOS−/− mice, with a mixed C57Bl/6J×129 SvEv genetic background,18 were obtained from Merck & Co, Inc. The iNOS+/+ mice were obtained by crossing 129 SvEv mice with C57 Bl/6J mice twice. The iNOS−/− and iNOS+/+ strains have similar genetic backgrounds of 75% C57Bl/6J and 25% 129/SvEv. The animals were maintained in a pathogen-free barrier facility with a 12-hour light/dark cycle and had free access to food and water. Fifteen age-matched (8-week-old) females of each strain were fed a diet that contained 15% fat, 1% cholesterol, and 0.5% sodium cholate9 for 15 weeks. Six females of each strain (8 weeks old), fed normal chow for the same period of time, served as controls. The study was approved by the Animal Care Committee of Tokai University.
Plasma Lipid Levels in inOS+/+ and inOS−/− Mice

<table>
<thead>
<tr>
<th>Lipid, mg/dL</th>
<th>Normal Diet (n=5)</th>
<th>Atherogenic Diet (n=10)</th>
</tr>
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<tbody>
<tr>
<td>Total cholesterol</td>
<td>81.0±3.0</td>
<td>85.2±4.9</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>55.5±3.4</td>
<td>51.0±3.7</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>5.0±1.3</td>
<td>6.0±3.0</td>
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Measurement of Plasma Lipid Levels
Mice were deprived of food for 16 hours, and blood was collected via the abdominal aorta into heparin-coated tubes with the animals under pentobarbital sodium–induced anesthesia. Plasma was obtained through centrifugation of the whole blood for 10 minutes at 12 000g at 4°C. Concentrations of total cholesterol, HDL cholesterol, and triglycerides were measured enzymatically with a Boehringer-Mannheim Hitachi 717 analyzer.

Tissue Preparation and Histological Analysis
After the collection of blood, the heart and proximal aorta were excised and embedded in OCT compound (Tissue-Tek), frozen on dry ice, and stored at −70°C until sectioning for histological analysis. The OCT-embedded heart with aorta was serially sectioned from the middle of the ventricle to the root of the aorta into 5-μm slices. For the quantitative evaluation of atherosclerotic lesions, 20 sections were taken every 40 μm, stained with oil red O and hematoxylin. Remaining sections were frozen at −80°C and used for other stainings. The lesion size was quantified through microscopic examination with a computer-assisted image analysis system (KS 300; Carl Zeiss). The average size of lesions in the 9 sections was taken to represent the lesion size for each animal. With sections stained with oil red O and hematoxylin H and E stainings). From this point, at level of 3-μm slices, the presence of collagen in the lesions was examined in sections stained according to the standard Van Gieson method. For evaluation of iNOS mRNA induction, the proximal aorta was snap-frozen in liquid nitrogen and stored at −80°C until use.

Immunohistochemistry
Serial cryostat sections were used to stain macrophages, smooth muscle cells, iNOS, nitrotyrosine, and vascular cell adhesion molecule (VCAM)-1. The primary antibodies that we used were a polyclonal rabbit anti-mouse iNOS antibody (017-16001; Wako Pure Chemical Industries; diluted 1:1000), a monoclonal rat anti-mouse VCAM-1 antibody (RA 140070; Antigenix America Inc; diluted 1:50), and a monoclonal mouse anti-human smooth muscle cell α-actin antibody (IgG4) (A 2547; Sigma Chemical Co; diluted 1:400).

Sections for immunohistochemical staining were fixed in acetone at −20°C for 5 minutes. All subsequent incubations were performed at room temperature, except for incubation with primary antibodies. The endogenous peroxidase activity was neutralized with H2O2 (0.3% vol/vol) for 10 minutes. An avidin-biotin blocking kit (Vector Laboratories) was used to block nonspecific endogenous biotin staining. After incubation with 4% (vol/vol) BSA (Sigma) diluted in PBS for 30 minutes, the sections were incubated with primary antibodies overnight at 4°C. Then biotinylated goat anti-rabbit IgG (for iNOS and nitrotyrosine; BA-1000, Vector Laboratories), biotinylated rabbit anti-rat IgG (for macrophages; BA-4001, Vector Laboratories), or biotinylated goat anti-mouse IgG2a antibody (for smooth muscle cells; GAM/IgG2A/BIO, Nordic Immunological Laboratories BV) was applied at 5 μg/mL and incubated for 45 minutes. Subsequently, the sections were incubated with streptavidin-peroxidase complex (Vector Laboratories) for 30 minutes. The staining was visualized with 9-amino-3-ethylene-carbazole streptavidin-peroxidase complex (Vector Laboratories) or diaminobenzidine (Vector Laboratories). Nonimmune rabbit or rat serum was used in place of the primary antibody as a negative control.

Measurements of iNOS and mRNA
To determine iNOS mRNA levels, the frozen aorta was homogenized in ISOGEN (Nippon Gene), total RNA was extracted, and RT-PCR was applied as previously described.2 Briefly, equal amounts of RNA were reverse-transcribed into cDNA. The RT products were amplified with the following primers for iNOS: 5′-CTTGCCCCCTGGAAAGTCTTC-3′ (upstream) and 3′-CTTGGTTGTCCTGGTGATGTCG-5′ (downstream), which afforded a 519-bp fragment. As an internal control, β2-microglobulin (β2M) mRNA was estimated under the same cycling conditions with...
Results

Plasma Lipid Levels
Plasma cholesterol was markedly elevated with the atherogenic diet in both iNOS\(^{+/−}\) and iNOS\(^{−/−}\) mice, but no significant difference was observed between the 2 groups (Table). Triglyceride levels were unaffected by the atherogenic diet, and there were no significant intergroup differences. Basal levels of all lipids were comparable in the 2 strains and remained in the same ranges after 15 weeks of normal chow (data not shown).

iNOS Expression in Aorta
As shown in Figure 1, iNOS mRNA was not observed in the aorta of control mice of either strain. It also was not observed in iNOS\(^{−/−}\) mice fed the atherogenic diet, as expected. However, iNOS mRNA expression was clearly demonstrated in the aorta of iNOS\(^{+/+}\) mice fed the atherogenic diet.

Atherosclerotic Lesion Formation
All mice of both strains developed atherosclerotic lesions along the proximal aortic wall and at the valve cusps after the 15-week atherogenic diet (Figure 2). Neither strain developed aortic atherosclerotic lesions when maintained on the normal chow for the same period of time. Individual lesions varied from simple fatty streaks to complex fibrous plaques, but there was no consistent difference in morphological characteristics or sites of lesions between the 2 strains. The average values of cumulative lesion size were similar in the 2 strains (iNOS\(^{+/+}\): 285±73×10\(^3\) \(\mu\)m\(^2\), iNOS\(^{−/−}\): 293±82×10\(^3\) \(\mu\)m\(^2\) [mean±SEM], n=10) (Figure 3).

Collagen Content in Lesions
As shown in Figure 4, extracellular collagen content was markedly decreased in the lesions of iNOS\(^{+/+}\) mice compared with that of comparable-sized lesions of iNOS\(^{−/−}\) mice. The cellular densities in the lesions were equal in the 2 strains (iNOS\(^{+/+}\): 3869±315 cells/mm\(^2\), iNOS\(^{−/−}\): 3568±248 cells/mm\(^2\) [mean±SEM], n=10).

Immunohistochemistry
Immunocytochemical analyses showed that lesions consisted primarily of macrophages (Figures 5A1, 5B1, and 5B5) with minimal smooth muscle cell involvement in both strains of mice (data not shown). iNOS staining was extensive in the lesions of iNOS\(^{+/+}\) mice (Figures 5B2 and 5B6) but was absent in those of iNOS\(^{−/−}\) mice (Figure 5A2). Specifically, iNOS staining was seen in macrophages (identified in adjacent sections by positive MAC-1 staining) distributed throughout the intimal lesions. Staining for iNOS was also evident in medial smooth muscle cells beneath the intimal lesions (Figure 5B2). iNOS was not detected in the aorta of either of these strains of mice maintained on normal chow (data not shown). Nitrotyrosine staining in the lesions of the iNOS\(^{+/+}\) mice showed a distribution similar to that of iNOS stainings but was more marked in the intimal area of lesions, where macrophages are the major component (Figures 5B3 and 5B7). Nitrotyrosine staining was not present in sections of aorta from iNOS\(^{−/−}\) mice fed the atherogenic diet or in such sections from both strains fed normal chow (data not shown). VCAM-1 staining was seen in the lesions of both strains, with no apparent difference in the intensity or distribution between the 2 strains (Figures 5A4 and 5B4).

Discussion
This is the first study in which iNOS knockout mice have been used to directly examine whether iNOS-mediated path-
ways promote or protect against development of atherosclerosis in vivo. iNOS was induced in the atherosclerotic lesions only in iNOS\(^{-/-}\) mice, and the sizes of atherosclerotic lesions were similar in the iNOS\(^{-/-}\) and iNOS\(^{+/+}\) mice.

The involvement of iNOS in atherosclerosis has been shown through a variety of observations, including the detection of iNOS protein or mRNA in the atherosclerotic lesions of both humans\(^{12,13}\) and experimental animals.\(^{14}\) Our results confirm and extend previous reports of the induction of iNOS in atherosclerotic vessels. iNOS may have paradoxical effects on the development of atherosclerosis due to the antiatherogenic character of NO\(^{5-8}\) and the proatherogenic property of NO-derived peroxynitrite.\(^{15,23,24}\) Our study provides direct evidence that the overall impact of iNOS on the progression of atherosclerosis is remarkably little. We know of only 1 previous study in which iNOS knockout mice were used to examine the influence of iNOS on vascular lesions.\(^{25}\) Using a transplant arteriosclerosis model in which an allogenic heart was heterotopically transplanted into an iNOS\(^{-/-}\) or iNOS\(^{+/+}\) recipient, those authors concluded that iNOS has an antiarteriosclerotic effect, based on the marked inhibition of intimal hyperplasia of coronary arteries in hearts trans-

Figure 5. Immunohistochemical staining for macrophages, iNOS, nitrotyrosine, and VCAM-1. A, Sections were obtained from iNOS\(^{-/-}\) mice fed atherogenic diet. B, Sections were obtained from iNOS\(^{+/+}\) mice fed atherogenic diet. Magnification A1 to A3, A5, B1 to B3, and B8, \(\times 1000\); B5 to B7, \(\times 2500\); and A4 and B4, \(\times 330\). A1, B1, and B5, macrophage staining. A2, B2, and B6, iNOS staining. A3, B3, and B7, nitrotyrosine staining. A4 and B4, VCAM-1 staining. A5 and B8, negative control.
planted into iNOS<sup>−/−</sup> mice. Experiments with adenovirus-mediated iNOS gene transfer also demonstrated that iNOS inhibited intimal hyperplasia in allograft arteriosclerosis<sup>26</sup> and balloon-injured arteries.<sup>27</sup> These findings are not in conflict with our result, because the pathological processes that underlie intimal hyperplasia are not the same as those in hyperlipidemic arteriosclerosis. In intimal hyperplasia, immunological reaction is the initial trigger and neointimal smooth muscle cell migration and proliferation are the major characteristics,<sup>25</sup> whereas modified LDL accumulation and monocyte/macrophage recruitment play important roles in hyperlipidemic arteriosclerosis.<sup>28</sup> As shown in Figures 5A1, 5B, and 5B5, macrophages are the predominant cells in the lesion, and the VCAM-1 induction, which is 1 of the earliest events in the development of arteriosclerosis,<sup>28</sup> was comparable in iNOS<sup>−/−</sup> and iNOS<sup>+/+</sup> mice. Thus, induced iNOS appears not to have affected the lesion size or adhesion molecule expression. However, it may increase plaque instability, because a marked decrease in extracellular collagen content was observed in the lesions of iNOS<sup>−/−</sup> mice compared with those of iNOS<sup>+/+</sup> mice. The collagen content in the lesion determines the biomechanical strength of the atherosclerotic lesion and its vulnerability to disruption.<sup>28–30</sup> NO-induced apoptosis or inhibition of cell proliferation is unlikely to account for the decreased collagen content, because lesion cellularity was similar in both strains. Other possible mechanisms are NO-induced inhibition of collagen formation<sup>31</sup> and the activation of collagen-degrading metalloproteinase.<sup>3,29,30,32,33</sup> We have recently shown that peroxynitrite is a novel activator of procollagenase,<sup>34</sup> so the finding that iNOS was colocalized with nitrotyrosine, which is formed by peroxynitrite-induced nitration,<sup>17</sup> in the lesions of iNOS<sup>+/+</sup> mice suggests that iNOS-catalyzed NO production may have enhanced peroxynitrite formation and activated MMP. It remains to be examined whether MMP induction or inhibition of collagen formation contributed to the reduced collagen content in our experiment. As a next step, it would be useful to confirm our findings by using mice crossed iNOS<sup>−/−</sup> and either apoE or LDL receptor<sup>−/−</sup> mice, which develop advanced atherosclerotic lesions that are more relevant to human disease than the current model.<sup>35</sup>

In conclusion, iNOS appeared to have neither a proatherogenic nor an antiatherogenic influence in hyperlipidemia-induced arteriosclerosis under our in vivo conditions, but it does decrease the collagen content of the lesion, which may increase plaque instability. This finding may offer a novel therapeutic approach to the prevention of acute coronary events.

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