Experimental Abdominal Aortic Aneurysms in Mice Lacking Expression of Inducible Nitric Oxide Synthase

Jason K. Lee, Martin Borhani, Terri L. Ennis, Gilbert R. Upchurch, Jr, Robert W. Thompson

Abstract—To determine if nitric oxide synthase (NOS) contributes to the pathophysiology of abdominal aortic aneurysms (AAAs), C57BL/6J mice underwent transient aortic injury to induce a chronic inflammatory response. Wild-type mice developed a significant increase in aortic diameter within 14 days of elastase perfusion (115±16%, 40% incidence of AAAs), along with intense and widespread staining for nitrotyrosine, mononuclear inflammation, and delayed destruction of the elastic lamellae. Expression of both endothelial and neuronal forms of NOS was substantially decreased within AAAs, whereas inducible NOS (iNOS) mRNA was increased 360%, and the enzyme was localized to infiltrating inflammatory cells. By using mice with targeted deletion of iNOS to evaluate the functional importance of this enzyme, male iNOS-/- mice developed the same extent of aneurysmal dilatation as congenic controls (121±22%, 40% incidence of AAAs) and exhibited similar structural features except for diminished nitrotyrosine staining. Aneurysmal dilatation was actually enhanced in female iNOS-/- mice (141±16%, 80% incidence of AAAs; P<0.05), but this effect was reversed by previous oophorectomy. Although extensive protein nitration and increased expression of iNOS accompany the development of elastase-induced experimental AAAs, iNOS is not required in this process and its absence may be deleterious. (Arterioscler Thromb Vasc Biol. 2001;21:1393-1401.)

Key Words: abdominal aortic aneurysm ■ animal model ■ elastase ■ inducible nitric oxide synthase ■ genetically altered mice

Abdominal aortic aneurysms (AAAs) represent a life-threatening condition characterized by pathological remodeling and segmental dilatation of the aortic wall. Tissues from human and experimental AAAs exhibit chronic transmural inflammation, medial smooth muscle cell depletion and destruction of extracellular matrix proteins, and overproduction of matrix metalloproteinases (MMPs), plasminogen activators, and cathepsins is thought to contribute to this process. Although pharmacologic strategies that use anti-inflammatory agents and proteinase inhibitors may have promise in the management of small asymptomatic AAAs, the molecular mechanisms that influence aneurysmal degeneration remain to be defined.

Nitric oxide (NO) plays an important role in cardiovascular homeostasis, atherosclerosis, and a wide variety of inflammatory conditions. Large amounts of NO are produced in atherosclerotic plaques, where it may promote tissue damage and plaque instability. One of the potential mechanisms of NO-mediated tissue damage is its capacity to amplify the effects of proinflammatory cytokines on various cell types, leading in part to increased expression of MMPs. Reactive nitrogen intermediates may also accelerate connective tissue destruction by promoting MMP activation and protein nitration causes direct oxidative damage to structural connective tissue proteins, such as elastin. The capacity of NO to induce oxidative DNA damage has led to the notion that it may also be a mediator of vascular smooth muscle cell apoptosis, potentially impairing mechanisms of connective tissue repair. Because chronic inflammation, elastin degradation, and smooth muscle cell depletion are all prominent mechanisms in aneurysmal degeneration, we postulated that elevated local production of NO might play a critical role in this process.

To begin investigating the possibility that elevated local production of NO might specifically participate in aneurysmal degeneration, we examined protein tyrosine nitration and the expression of various isoforms of nitric oxide synthase (NOS) during the evolution of elastase-induced experimental AAAs in the mouse. Aneurysmal degeneration was associated with extensive production of nitrotyrosine and a marked increase in aortic wall expression of inducible NOS (iNOS), but the expression of endothelial NOS (eNOS) and neuronal NOS (nNOS) were both decreased. We then applied the elastase-induced model of AAAs to mice with targeted gene deletion of iNOS.
disruption of the iNOS gene. These studies demonstrated the novel and unexpected finding that iNOS is not required during the development of experimental aortic aneurysms and that in ovulatory female mice its absence may be deleterious.

Methods

Experimental Animals

Wild-type C57BL/6j mice and congenic iNOS-deficient (iNOS−/−) mice were purchased from The Jackson Laboratory (Bangor, Me), and the iNOS−/− mice were confirmed by genomic analysis with polymerase chain reaction (PCR) and Southern blots.25 Homozygous wild-type or iNOS−/− mice were bred independently, and all experimental procedures were performed in animals that had reached maturity (8 to 10 weeks old), according to a protocol approved by the Animal Studies Committee at Washington University School of Medicine.

Elastase Perfusion Model and Aortic Diameter Measurements

Mice (20 to 35 g) were anesthetized with 55 to 60 mg/kg intraperitoneal sodium pentobarbital, and a laparotomy was performed under sterile conditions, as previously described.26 The abdominal aorta was isolated with the assistance of an operating stereomicroscope (Leica), and the perfusion elastase diameter (AD) was measured with an ocular grid calibrated to a resolution of 0.01 mm. After placing temporary ligatures around the proximal and distal aorta, an aortotomy was created at the bifurcation with the tip of a 30-gauge needle. A heat-tapered segment of PE-10 polyethylene tubing was introduced through the aortotomy and secured, and the aortic lumen was perfused for 5 minutes at 100 mm Hg with saline containing type I porcine pancreatic elastase (0.414 U/mL; Sigma Chemical Co.). After removing the perfusion catheter, the aortotomy was repaired without constriction of the lumen, and the post-perfusion AD was measured at least 5 minutes after restoring flow to the lower extremities. Animals were allowed free access to food and water for 14 days, when the aorta was re-exposed by laparotomy under anesthesia. Final AD measurements were obtained before euthanasia and tissue procurement.

The preperfusion, post-perfusion, and final AD measurements for each experimental group were recorded as the mean±SE and compared by using the Student t test.27 For individual animals, elastase-induced AAAs were defined as an increase in diameter (ΔAD) to at least 100% greater than the preperfusion diameter (ΔAD) = (final AD−preperfusion AD)/preperfusion AD. The prevalence of AAAs in each group was compared by using χ² analysis.27

Oophorectomy

Following our initial studies in iNOS-deficient animals, bilateral oophorectomy was performed in a separate group of female iNOS−/− mice. After the induction of anesthesia, bilateral flank incisions were made, and the ovarian pedicle was isolated from within the retroperitoneum. The ovaries were ligated and excised (or left intact for control sections, the staining procedure for Mac-3 was performed with DAB and hematoxylin. For the simultaneous detection of nitrotyrosine and macrophages in the same sections, the staining procedure for Mac-3 was performed with DAB as described, followed by nitrotyrosine staining with alkaline phosphatase.

Quantitative Real-Time RT-PCR

To quantify relative levels of NOS gene expression, aortic tissue samples were pulverized under liquid nitrogen and total RNA was isolated with Trizol reagent (Gibco BRL).26,29,30 Each sample was normalized to 1 μg of total RNA, and cDNA synthesis was performed in a 50 μL reverse-transcription reaction on a GeneAmp 2400 thermal cycler system with the following reagents provided by the manufacturer: 5.5 mmol/L MgCl₂, 0.5 mmol/L dNTPs, 1.25 U/μL MultiScribe Reverse Transcriptase, and 2.5 μM random hexamers (Applied Biosystems). The reverse-transcription products served as the template for RT-PCR analysis, with gene-specific primers, reagents, and protocols provided in the SYBR Green PCR kit and the GeneAmp 5700 Sequence Detection System (Applied Biosystems).30–32 All primers were selected by using PrimerExpress version 1.6 software (Applied Biosystems), to amplify a 50-bp product with a Tm of 60°C and target sequences that flanked or spanned an exon-exon junction in the murine mRNA transcript of interest. The primers used were as follows: eNOS (NOS I), forward primer (FP): GCG CAA TGT GAG TCC GAA A (bp 3440 to 3459), reverse primer (RP): CCT GAA GCC CCT TGC GAG (bp 3498 to 3481); iNOS (NOS II), FP: AAG GCC ACA TCG GAT TTC AC (bp 3449 to 3465), RP: GCG CAA TGT GAG TCC GAA A (bp 3498 to 3484); and β-actin, FP: CCT TAA GCC GCA CCG TGA A (bp 255 to 273), RP: GGT GAA GGT CTC AAA CAT GAT CTG (bp 306 to 283). All RT-PCR reactions were performed in duplicate and contained the following components (SYBR Green PCR Core Reagents, Applied Biosystems): 10X SYBR PCR buffer; 3 mmol/L MgCl₂; 0.2 mmol/L dATP, dCTP, and dGTP; 0.4 mmol/L dUTP; 25 μmol/L AmpliTaq Gold DNA polymerase; 10 μmol/L AmpliTagUNG; 10 ng cDNA or template standard DNA; and 300 μmol/L each of the forward and reverse primers. After initial incubations at 50°C (2 minutes) and 95°C (10 minutes) for AmpliTagase and AmpliTag Gold activation, respectively, the samples underwent 40 cycles of PCR with 95°C for 15 seconds (melting) alternating with 60°C for 1 minute (anneal/extend). Direct detection of PCR products was monitored by measuring the increase in fluorescence caused by the binding of SYBR Green I to double-stranded DNA products, along with an internal reference standard (ROX). Fluorescence signals were analyzed by using the GeneAmp 5700 Sequence Detection System software version 1.3 according to the manufacturer’s recommendations (Applied Biosystems, 1999).

For absolute quantitation of the RT-PCR products, a DNA standard template was generated for each primer pair by using wild-type murine cDNA generated from appropriate control tissues.
Results

Aneurysmal Degeneration Is Accompanied by Widespread Protein Tyrosine Nitration

Aneurysmal dilatation was consistently observed in C57Bl/6 wild-type mice 14 days after elastase perfusion, with the mean AD increasing from 0.51±0.01 mm before perfusion to 1.08±0.07 mm (P<0.05). The overall increase in AD was 115±16% in this group, with 8 (40%) of 20 animals developing AAAs (Figure 1A). Similar to our previous studies with 129/Sv mice,26 aneurysmal dilatation in C57Bl/6 mice was associated with destruction of the elastic lamellae and aortic wall infiltration by mononuclear inflammatory cells, including an abundance of Mac-3-positive macrophages (Figure 1B through 1E).

To assess the potential role of NO in aneurysmal degeneration, immunohistochemical staining was also used to detect nitrotyrosine, one of the stable end-products of protein nitration. Although there was no detectable immunoreactivity in normal aortic tissue (data not shown), intense staining for nitrotyrosine was observed in aneurysm tissue, where it appeared in a wide distribution in association with mononuclear inflammatory cells (Figure 2A through 2F), as well as smooth muscle–like cells, capillary endothelium, and regions of degenerating extracellular matrix. The intensity and broad distribution of protein tyrosine nitration was taken as evidence that development of elastase-induced AAAs is accompanied by increased local generation of reactive nitrogen intermediates.

NOS Expression in Aneurysmal Tissues

To assess the cellular mechanisms responsible for increased generation of NO within aneurysm tissue, the relative expression of three different isoforms of NOS was measured by quantitative RT-PCR. Each of the three isoforms (eNOS, iNOS, and nNOS) were expressed in the normal (preperfusion) aorta; although there were no significant differences in expression between the abdominal and thoracic segments for eNOS and iNOS, nNOS was inexplicably expressed at higher levels in the abdominal versus the thoracic aorta (Table 1). By using the nonperfused thoracic aorta from the same animals as a control, the relative expression of each NOS gene was then examined in the aneurysmal abdominal aorta 14 days after elastase perfusion. As shown in Table 1, the relative expression of all three isoforms of NOS was altered in different ways after the elastase perfusion procedure. Thus, the expression of eNOS was decreased 76% in the abdominal aorta and 26% in the thoracic aorta after elastase-induced injury, and the abdominal-to-thoracic ratio was decreased by 66% compared with unperfused (normal) animals. Similarly, the expression of nNOS was decreased 91% in the abdominal aorta and increased 74% in the thoracic aorta, with the abdominal-to-thoracic ratio decreasing by 84%. A different and more substantial pattern of altered gene expression was observed for iNOS.
TABLE 1. Expression of NOS Genes in Normal Aorta and Elastase-Induced AAAs

<table>
<thead>
<tr>
<th>Relative mRNA Abundance</th>
<th>Normal Abdominal Aorta</th>
<th>Normal Thoracic Aorta</th>
<th>Relative Expression Abdominal to Thoracic</th>
</tr>
</thead>
<tbody>
<tr>
<td>eNOS</td>
<td>10.10±5.24×10⁻⁶</td>
<td>8.34±2.20×10⁻³</td>
<td>1.25±0.51</td>
</tr>
<tr>
<td>iNOS</td>
<td>3.91±2.67×10⁻³</td>
<td>3.33±0.35×10⁻³</td>
<td>1.24±0.80</td>
</tr>
<tr>
<td>nNOS</td>
<td>4.74±3.75×10⁻³</td>
<td>0.54±0.24×10⁻³</td>
<td>8.37±3.95</td>
</tr>
</tbody>
</table>

Pre-Perfusion (Day 14) Wild-Type Mice

<table>
<thead>
<tr>
<th>Relative mRNA Abundance</th>
<th>Aneurysmal Abdominal Aorta</th>
<th>Unperfused Thoracic Aorta</th>
<th>Relative Expression Aneurysm to Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>eNOS</td>
<td>2.42±0.59×10⁻³</td>
<td>6.13±0.59×10⁻³</td>
<td>0.42±0.17</td>
</tr>
<tr>
<td>% Change</td>
<td>76% Decrease</td>
<td>26% Decrease</td>
<td>66% Decrease</td>
</tr>
<tr>
<td>iNOS</td>
<td>18.00±2.35×10⁻³</td>
<td>5.66±2.35×10⁻³</td>
<td>3.45±0.35</td>
</tr>
<tr>
<td>% Change</td>
<td>360% Increase</td>
<td>70% Increase</td>
<td>178% Increase</td>
</tr>
<tr>
<td>nNOS</td>
<td>0.41±0.40×10⁻³</td>
<td>0.94±0.40×10⁻³</td>
<td>1.32±1.14</td>
</tr>
<tr>
<td>% Change</td>
<td>91% Decrease</td>
<td>74% Increase</td>
<td>84% Decrease</td>
</tr>
</tbody>
</table>

Abdominal and thoracic aortic tissues were obtained from normal (unperfused) C57Bl/6 wild-type mice and from a second group of animals that had undergone transient elastase perfusion of the abdominal aorta 14 days earlier, as described in the text. For the animals developing elastase-induced AAAs, tissues were obtained from the aneurysmal abdominal aorta and from the uninvolved thoracic aorta as a control. The relative expression of each NOS gene was measured by real-time RT-PCR using simultaneous measurements of β-actin as an endogenous standard. Data shown represent the mean±SE for 3 animals in each group, with the alterations in elastase-perfused animals versus normal mice shown in parentheses.

Aneurysmal Degeneration in iNOS-Deficient Mice

To specifically determine if increased expression of iNOS is required in the development of AAAs, the elastase-induced mouse model was applied to an established strain of mice. Indeed, different from C57BL/6J background controls before elastase perfusion, immediately after elastase, or on day 14. Indeed, iNOS knockout mice exhibited a mean increase in AD at 131±13%, with AAAs developing in 12 (60%) of 20 animals; although this was not statistically different than wild-type controls, these data suggested a trend toward greater aneurysmal dilatation in iNOS-deficient animals. The structural morphology of the aneurysmal aorta was also equivalent in iNOS knockout mice compared with background controls, as characterized by transmural mononuclear inflammation and destruction of the medial elastic lamellae (Figure 3A through 3D), but there was a visible reduction in protein tyrosine nitration in the aneurysms from iNOS knockout mice as shown by immunohistochemistry (Figure 3E through 3H). By quantitative measures of mRNA expression, the absence of iNOS did not alter the expression of either eNOS or nNOS in the aneurysmal abdominal aorta, although both enzymes were increased in the nonperfused thoracic aorta by comparison to wild-type mice (Figure 4). Taken together, the results of these experiments indicate that iNOS expression is not required for the development of elastase-induced aneurysmal degeneration.

Differences in Male and Female Mice and Effects of Oophorectomy

Further analysis of the aortic diameter data revealed that there was no difference in aneurysmal dilatation between male iNOS knockout mice and their wild-type male counterparts, but that aneurysm development was significantly increased in iNOS knockout females compared with female wild-type controls (Table 2). In contrast, there was no significant difference in aneurysmal dilatation between male and female animals within either wild-type or iNOS knockout groups, indicating that the enhancement of aneurysm development was a peculiarity of iNOS deficiency in females. To begin examining if this phenomenon might be specifically related to the presence of circulating ovarian hormones, another series of iNOS knockout female mice underwent either oophorectomy or sham operation 3 weeks before elastase perfusion. As shown in Table 3, 100% of the control female iNOS knockout mice in this series developed large AAAs, with a mean ΔAD of 175±15%. In contrast, AAAs were present in only 43% of those that had undergone oophorectomy (mean ΔAD 100±16%; P<0.05 sham operation versus oophorectomy). Given that the extent of aneurysmal dilatation was no different in the oophorectomy group than that previously observed in wild-type mice and in iNOS knockout males, these findings demonstrate that oophorectomy reversed the accelerated aneurysm development observed in iNOS knockout females.

Discussion

The present investigations were designed to test the hypothesis that NO might be a critical molecular mediator in the
development of elastase-induced aortic aneurysms in the mouse, a recently characterized model that recapitulates many of the important features of human AAAs. The major findings of this study are that (1) elastase-induced aneurysmal degeneration is accompanied by NO-mediated tissue injury, as evidenced by extensive protein tyrosine nitration within the degenerating aortic wall; (2) elastase-induced AAAs are associated with a marked increase in aortic wall expression of iNOS, whereas the expression of other isoforms of NOS (eNOS and nNOS) is substantially decreased; and (3) the development of elastase-induced aneurysmal degeneration is unaffected in mice with targeted disruption of the iNOS gene, and includes the same morphological and molecular changes that occur in wild-type animals (ie, aortic wall infiltration by mononuclear phagocytes and destruction of the medial elastic lamellae). It was also notable that iNOS deficiency actually enhanced elastase-induced aneurysmal dilatation in female mice, but that this deleterious effect was preventable by previous oophorectomy. These findings provide evidence that protein tyrosine nitration and increased iNOS expression accompany the development of elastase-induced experimental aortic aneurysms, but that iNOS does not play a necessary role in this pathological process.

Although basal arterial wall production of NO is often attributed to other isoforms of NOS (eNOS and nNOS), the large amounts of NO produced during acute and chronic inflammation are specifically associated with increased cellular expression of iNOS. Activated tissue macrophages are most frequently responsible for iNOS expression, yet other cell types can also produce this enzyme after stimulation with proinflammatory cytokines, bacterial lipopolysaccharide, or mechanical injury. Because iNOS is localized to both macrophages and vascular smooth muscle cells in atherosclerotic tissues, expression of this enzyme is thought to play a central role in chronic inflammation and connective tissue degradation within the vessel wall. Previous experimental studies with iNOS-/- mice have also implicated this enzyme in the intimal thickening that follows vascular wall injury and in atherosclerotic lesions in hyperlipidemic apolipoprotein E-deficient mice. Furthermore, Fukuda and colleagues have demonstrated suppressed formation of saccular cerebral aneurysms in a hypertensive rat model with treatment with NOS inhibitors, and Johanning and colleagues reported that treatment with aminoguanidine reduces development of elastase-induced AAAs in the rat. Although conventional interpretation of such studies suggests that iNOS catalyzes a critical step in the inflammatory process that characterizes aneurysmal degeneration, the results of the present investigation are in obvious conflict with this conclusion. Some of these differences may be attributable to the use of animal models that emphasize distinct pathophysiological processes (ie, saccular cerebral aneurysms dependent on hemodynamic stresses versus fusiform abdominal aortic aneurysms dependent on chronic inflammation and elastin degradation). Different experimental strategies to inhibit iNOS activity may also explain these discrepancies (ie, pharmacological inhibition versus targeted gene deletion). Because our observations challenge the notion that iNOS plays a crucial role in aneurysm disease, it is evident that further study of this question will be needed.

One of the limitations of this study is that the experimental system used here may not reproduce the entire spectrum of pathological processes involved in human aneurysm disease. For example, the elastase-induced murine model of AAAs is dependent on an initial aortic wall injury with pancreatic elastase, an enzyme not known or suspected to play a role in human aneurysms. Secondly, this model does not depend on chronic inflammation and elastin degradation. Different experimental strategies to inhibit iNOS activity may also explain these discrepancies (ie, pharmacological inhibition versus targeted gene deletion). Because our observations challenge the notion that iNOS plays a crucial role in aneurysm disease, it is evident that further study of this question will be needed.
aneurysmal dilatation; rather, it appears to induce a sequence of changes similar to those observed in human AAA: transmural inflammatory cell infiltration, accelerated degradation of the medial elastic lamellae, and increased local production of elastolytic metalloproteinases (ie, MMP-2, MMP-9, and MMP-12). This model has therefore been particularly informative for investigations on the pathophysiology and potential pharmacologic treatment of aortic aneurysm disease, and its application to genetically altered mice offers a powerful approach to examine the role played by individual gene products.

\[\text{iNOS}^{-/-}\text{ mice exhibit no gross phenotypic abnormalities as adults and have therefore been useful to demonstrate the importance of this enzyme in the immune response to intracellular microbes, the hemodynamic response to bacterial sepsis, and in ischemic preconditioning.}\]

These mice have also been valuable in demonstrating that \text{iNOS} is not required in the pathogenesis of other inflammatory conditions, such as experimental autoimmune encephalomyelitis. The use of \text{iNOS}-deficient mice has distinct advantages over pharmacologic approaches to inhibit \text{iNOS} activity; for example, aminoguanidine is often used as an \text{iNOS}-specific inhibitor in experimental studies, but it is known to exhibit some degree of cross-inhibition of other isoforms of \text{NOS}. Because it may be difficult to control for secondary effects on either \text{eNOS} or \text{nNOS} during in vivo studies, pharmacologic inhibitors of \text{iNOS} do not always provide the specificity needed to clarify molecular mechanisms. Although this limitation is generally overcome by the high degree of precision achievable with gene-targeting approaches, it must be acknowledged that gene targeting abolishes \text{iNOS} expression throughout development as well as in the adult animal, and that this too may have unanticipated secondary effects.

Although the present study indicates that \text{iNOS} is not required in the development of elastase-induced AAAs, we cannot exclude the possibility that \text{iNOS} might still play a contributory role in aneurysmal degeneration. Thus, it appears unlikely that increased aortic wall production of NO and protein tyrosine nitration are innocuous events, but how they influence other molecular pathways leading to AAAs is uncertain. Despite the concern that \text{iNOS}^{-/-} mice might exhibit increased (compensatory) NO production generated by either \text{eNOS} or \text{nNOS} within the aneurysm wall, we found no evidence that these genes were upregulated during aneurysm formation in \text{iNOS}^{-/-} mice. Further studies in mice lacking \text{eNOS} and \text{nNOS} will also be helpful in addressing the role of these enzymes in AAA formation.

One of the intriguing observations in this study was that aneurysm development was accelerated in female \text{iNOS}^{-/-}.
mice, raising the possibility that iNOS might actually play a protective role in aneurysmal degeneration under certain circumstances. Because iNOS is produced primarily in activated tissue macrophages, it is possible that intracellular events responsible for its expression could also have coordinate effects on other macrophage products, such as MMP-9. Because we have recently shown that MMP-9 expression is essential in the development of elastase-induced experimental AAAs, one possibility is that simultaneous cellular production of reactive nitrogen intermediates and MMP-9 might lead to extensive nitration of the proenzyme before it can act in the extracellular space. Although others have reported that oxidative alterations can provide a mechanism for proMMP-9 activation, excessive nitration might also diminish matrix-degrading activity by promoting rapid extracellular degradation of the secreted zymogen. Finally, there is evidence that endogenous production of NO may inhibit cytokine-induced MMP-9 production, at least in cultured rat mesangial cells, and that NOS inhibitors substantially increase MMP-9 expression in cytokine-stimulated vascular smooth muscle cells in vitro. We would therefore predict that if any of these mechanisms are operative during the development of elastase-induced AAAs, the absence of iNOS might have enhanced local MMP-9 activity and promoted aneurysmal degeneration in female iNOS mice. Although it remains unclear why these effects might occur in a sex-selective fashion and how they are related to circulating ovarian hormones, there is increasing evidence that interactions between estrogen and cellular NOS expression can influence NO production in various pathophysiologic states. More comprehensive studies will be needed to elucidate the apparent interaction between ovarian hormones and NO production during aneurysm formation in female mice.

The recent interest in developing new therapeutic strategies for patients with small asymptomatic AAAs has led to greater efforts to define the molecular mechanisms that underlie aneurysmal degeneration. The results of this study add another valuable link to our understanding of vascular wall inflammation and aneurysm disease, demonstrating that iNOS does not play a requisite role in the process of elastase-induced experimental aneurysmal degeneration in the mouse; it can also be predicted that selective inhibition of iNOS might therefore have significant limitations as a therapeutic strategy to suppress the development and growth of aortic aneurysms. In light of the observation that iNOS expression actually seems to impart some degree of benefit in female mice, selective inhibition of this enzyme may even be deleterious in some circumstances. Further examination of the mechanisms underlying these findings will be therefore needed before they can be effectively translated to the clinical setting.

**Acknowledgments**

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### TABLE 2. AD Measurements in Wild-Type and iNOS<sup>−/−</sup> Mice

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>AD Pre, mm</th>
<th>AD Post, mm</th>
<th>AD day 14, mm</th>
<th>∆AD, %</th>
<th>No. of AAAs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Combined males and females</td>
<td>20</td>
<td>0.51±0.01</td>
<td>0.62±0.01</td>
<td>1.08±0.07</td>
<td>115±16</td>
<td>8/20 (40%)</td>
</tr>
<tr>
<td>Wild-type</td>
<td>20</td>
<td>0.51±0.01</td>
<td>0.65±0.01</td>
<td>1.16±0.06</td>
<td>131±13</td>
<td>12/20 (60%)</td>
</tr>
<tr>
<td>iNOS&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males only</td>
<td>10</td>
<td>0.54±0.01</td>
<td>0.83±0.01</td>
<td>1.20±0.14</td>
<td>126±31</td>
<td>4/10 (40%)</td>
</tr>
<tr>
<td>Wild-type</td>
<td>10</td>
<td>0.52±0.01</td>
<td>0.86±0.01</td>
<td>1.14±0.09</td>
<td>121±22</td>
<td>4/10 (40%)</td>
</tr>
<tr>
<td>iNOS&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Females only</td>
<td>10</td>
<td>0.47±0.01</td>
<td>0.81±0.01</td>
<td>0.97±0.05</td>
<td>105±11</td>
<td>4/10 (40%)</td>
</tr>
<tr>
<td>Wild-type</td>
<td>10</td>
<td>0.49±0.01</td>
<td>0.84±0.02</td>
<td>1.18±0.08</td>
<td>141±16</td>
<td>8/10 (80%)</td>
</tr>
<tr>
<td>iNOS&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P&lt;0.05</td>
<td></td>
</tr>
</tbody>
</table>

*Female C57Bl/6 iNOS<sup>−/−</sup> mice underwent transient aortic perfusion with elastase as described in the text. AD measurements were obtained before (AD Pre) and immediately after elastase perfusion (AD Post) and on day 14. The increase in AD (ΔAD) for each animal was determined as a percentage change from the pre-perfusion AD, with aneurysms (AAAs) defined as a ΔAD>100%. Data shown represent the mean±SE for each group (NS indicates not significant).*

### TABLE 3. Effect of Oophorectomy on Aneurysm Development in Female iNOS<sup>−/−</sup> Mice

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>AD Pre, mm</th>
<th>AD Post, mm</th>
<th>AD day 14, mm</th>
<th>∆AD %</th>
<th>No. of AAAs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham operation</td>
<td>7</td>
<td>0.43±0.01</td>
<td>0.78±0.01</td>
<td>1.19±0.07</td>
<td>175±15</td>
<td>7/7 (100%)</td>
</tr>
<tr>
<td>Oophorectomy</td>
<td>7</td>
<td>0.47±0.00</td>
<td>0.77±0.02</td>
<td>0.94±0.07</td>
<td>100±16</td>
<td>3/7 (43%)</td>
</tr>
</tbody>
</table>

*Female C57Bl/6 iNOS<sup>−/−</sup> mice underwent bilateral oophorectomy or sham operation, followed 3 weeks later by transient aortic perfusion with elastase as described in the text. AD measurements were obtained before (AD Pre) and immediately after elastase perfusion (AD Post) and on day 14. The increase in AD (ΔAD) for each animal was determined as a percentage change from the pre-perfusion AD, with aneurysms (AAAs) defined as a ΔAD>100%. Data shown represent the mean±SE for each group (NS).*
References


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