Inhibition of inducible nitric oxide synthase limits nitric oxide production and experimental aneurysm expansion

Jason M. Johanning, MD, David P. Franklin, MD, David C. Han, MD, David J. Carey, PhD, and James R. Elmore, MD, Danville, Pa

Purpose: Nitric oxide (NO), frequently cited for its protective role, can also generate toxic metabolites known to degrade elastin. Both abdominal aortic aneurysms (AAAs) and inducible nitric oxide synthase (iNOS) are associated with inflammatory states, yet the relationship between NO production by iNOS and AAA development is unknown. The current study examines iNOS expression, NO production, and the effects of selective inhibition of iNOS by aminoguanidine in experimental AAA.

Methods: An intra-aortic elastase infusion model was used. Control rats received intra-aortic saline infusion and postoperative intraperitoneal saline injections (Group 1). In the remaining groups, intra-aortic elastase infusion was used to induce aneurysm formation. These rats were treated with intraperitoneal injections of saline postoperatively (Group 2), aminoguanidine postoperatively (Group 3), or aminoguanidine preoperatively and postoperatively (Group 4). Aortic diameter and plasma nitrite/nitrate levels were measured on the day of surgery and postoperative day 7. Aortas were harvested for biochemical and histologic analysis on postoperative day 7.

Results: Infusion of elastase produced AAAs (P < .001) with significant production of iNOS (P < .05) and nitrite/nitrate (P < .003) compared with controls. Selective inhibition of iNOS with aminoguanidine in elastase-infused aortas significantly reduced aneurysm size (P < .01) compared with elastase infusion alone. Aminoguanidine-treated rats displayed suppression of iNOS expression and plasma nitrite/nitrate production not significantly different from the control group. Histologic evaluation revealed equivalent inflammatory infiltrates in elastase-infused groups.

Conclusion: Expression of iNOS is induced and plasma nitrite/nitrate levels are increased in experimental AAA. Inhibition of iNOS limits NO production and iNOS expression, resulting in smaller aneurysm size. NO production by iNOS plays an important role with detrimental effects during experimental aneurysm development. (J Vasc Surg 2001;33:579-86.)

Nitric oxide (NO) synthesis is carried out by cells expressing either endothelial nitric oxide synthase (eNOS) or inducible nitric oxide synthase (iNOS). NO produced by eNOS in the endothelial cell is frequently cited for its vasodilating properties and protective role in vascular disease. In contrast, NO produced by iNOS within inflammatory cells generates high NO levels and toxic products known to degrade elastin. The association of inflammation and abdominal aortic aneurysms (AAAs) is well documented. However, expression of iNOS and the role of NO during AAA development have not been investigated. Indirect evidence suggests NO may play a role in aneurysm pathogenesis. Kawasaki disease, noted for its arterial inflammation and coronary artery aneurysms, is associated with increased urinary nitrite/nitrate and iNOS cofactors reflecting elevated NO levels. In addition, drugs that have been shown to limit the growth of experimental AAA, including propanolol, methylprednisolone, doxycycline, cyclosporine, and cyclooxygenase inhibitors, are known to suppress NO production.

The current study investigates the effect of NO production by iNOS on experimental AAA. Specifically, in this project we indirectly assessed NO production by means of plasma nitrite/nitrate levels and iNOS expression during experimental AAA development. Also, selective inhibition of iNOS-dependent NO production with aminoguanidine was evaluated and compared with standard experimental AAA development.

METHODS

Animal surgery. Adult male Wistar rats (300-400 g) were subjected to surgery as described by Anidjar et al and modified by Bigat et al. The described protocols were reviewed and approved by the Institutional Animal Care and Use Committee. The animal care complied with the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, Commission on Life Sciences, and National Research Council (National Academy Press, Washington, 1996). Rats were anesthetized with inhalational isoflurane (1%-2.5%) anesthesia. A laparotomy was performed, and a 1-cm segment of infrarenal aorta was isolated. The vena cava was dis-
sected free from the aorta, and all lumbar artery branches were ligated. With a cutdown on the external iliac artery, a 24-gauge intravenous catheter (Johnson & Johnson, Arlington, Tex) was introduced, and blood was collected for plasma analysis. The catheter was removed and a PE-10 polyethylene catheter (VWR Scientific Products, Bridgeport, NJ) was threaded into the aorta until its tip was resting in the isolated segment. Temporary ligatures were placed proximally and distally, and the aorta was perfused with a solution of saline or elastase dissolved in saline solution. Two milliliters was infused over 2 hours with a microinfusion pump (Bioanalytical Systems Inc, WLafayette, Ind; Harvard Apparatus, S Natick, Mass). The laparotomy was closed, and all skin wounds were stapled. The rats recovered, were individually housed, and given standard rat chow and water as desired. The animals were maintained until postoperative day (POD) 7 when they were anesthetized again. The animals were reexplored via a standard laparotomy incision, and plasma was collected through a contralateral iliac artery cutdown. Aortic diameter was measured, the aorta was harvested, and each rat was euthanized.

**Aortic infusion and drug administration.** Rats in Groups 1, 2, and 3 were randomized to aortic infusion and drug administration. Group 1 served as a control group, receiving intra-aortic saline infusion and postoperative saline. The remaining rats received an intra-aortic infusion of 12 units of elastase (Product #E-1250, Lots 18H7440 and 79H7628, unit activity determined by Succinyl-Ala-Ala-Ala-p-nitroanilide as substrate; Sigma, St Louis, Mo) mixed in 2 mL of saline. Each rat received a 1-mL intraperitoneal injection of saline (Group 2) or aminoguanidine (200 mg/kg) (Group 3) beginning on the morning of POD 1 and continuing through POD 7. A fourth group of rats underwent operation without randomization to assess the effect of preoperative and postoperative administration of aminoguanidine. These rats received intra-aortic elastase infusion identical to Groups 2 and 3 but received aminoguanidine starting 5 days preoperatively and continuing through POD 7. The dose of aminoguanidine was chosen on the basis of previous studies in animal models where inflammation was a prominent component and an intraperitoneal injection was used.

**Aortic measurement and tissue preparation.** All aortas were measured with pulsatile blood flow before and after infusion of intra-aortic solutions and at the time of reexploration. Aortic diameter was measured in situ under physiologic conditions with an ocular micrometer with 0.1-mm graduations at 10× power. At harvest, the infused segment of aorta was removed and divided into two equal pieces. The sections were snap frozen in liquid nitrogen for biochemical analysis and fixed in formaldehyde for histologic evaluation.

**Biochemical and histologic analysis.** Aortic tissue was analyzed with immunocytochemistry and Western blot analysis to assess iNOS expression and localization. Western blots were prepared as follows: Frozen aortic samples were homogenized in 5 mol/L urea, 50 mmol/L Tris, and 25 mmol/L dithiothreitol, pH 7.4 for equivalent sample concentrations (weight/volume). The samples were then mixed with electrophoresis gel sample buffer, heated, and loaded onto 7.5% sodium dodecylsulphate–
polyacrylamide gels. The gels were run until the dye front approached the gel edge, and then the proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Immobilon-P). Membranes were stained with Ponceau to ensure adequate and equivalent protein loading onto the PVDF membrane. The membrane was blocked with 2% nonfat powdered milk and then incubated with anti-iNOS antibody (Transduction Laboratories, Lexington, Ky; 1:2500). The membrane was rinsed and incubated with horseradish peroxidase–conjugated secondary antibody. Enhanced chemiluminescence was used to detect iNOS expression, and arbitrary light units (ALUs) were measured to quantify protein levels. Chemiluminescence detection was performed with a digital camera with broad dynamic range (Lumimager; Roche Molecular Biochemicals, Indianapolis, Ind), and Western blot analysis was consistent across multiple blots.

Immunocytochemistry was performed for iNOS and inflammatory cell localization. Aortic tissue was frozen in Tissue-Tek O.C.T. Compound (Miles, Inc, Elkhart, Ind) and sectioned at a thickness of 8 µm. After blocking, the specimens were incubated with anti-iNOS antibody (Transduction Laboratories; 1:200) and MAC-1 antibody (Seikagaku Corp, Tokyo, Japan; 1:100). Fluorescein isothiocyanate and Texas Red conjugated secondary antibodies were used for immunofluorescence. The slides were viewed with a Zeiss fluorescent microscope (Zeiss, Inc, Thornwood, NJ), and images were acquired with a digital camera.

Formalin-fixed specimens were processed for hematoxylin and eosin and orcein-elastin staining. Inflammation was graded with paraffin-embedded aortic sections stained with hematoxylin and eosin under light microscopy. A scoring system was developed to allow for quantitation of the findings. The levels of inflammation were assigned as follows: 5 = severe inflammation, 4 = moderate to severe inflammation, 3 = moderate inflammation, 2 = minimal to moderate inflammation, and 1 = minimal inflammation. Grading of the specimens was performed in a blinded fashion. Elastin-stained sections were evaluated for qualitative changes in elastin loss.

**Plasma analysis.** Blood (500 µL) was collected at the time of the initial laparotomy and at the time of harvest in EDTA-containing microtainers (Becton Dickinson, Franklin Lakes, NJ). The blood specimens were centrifuged for 10 minutes at 3000 rpm, and the plasma was decanted for further analysis. NO levels were indirectly quantitated by measuring total nitrite and nitrate levels in 50-µL samples with the Griess reaction. The assay sensitivity for plasma nitrite/nitrate was 0.6 to 0.9 µmol/L, and the reproducibility of the assay was ± 9% (assay performed by Oxonon, Inc, Emeryville, Calif).

**Statistics.** Comparisons of aortic diameter between the groups and absolute plasma nitrite/nitrate levels between the groups at the time of operation and on POD 7 were assessed with one-way analysis of variance (ANOVA) with Bonferroni correction. Comparing plasma nitrite/nitrate levels at the time of operation with POD 7 within each group was performed with a paired sample t

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**Figure 2.** Plasma samples were analyzed with Griess reaction to determine changes in NO levels from time of operation to POD 7. There was no change in plasma nitrite/nitrate levels for saline-infused rats. Infusion of elastase produced significant increase in plasma nitrite/nitrate levels on POD 7 for rats injected with saline but not in rats treated with aminoguanidine (AG) Post-Op, Postoperative; Pre-Op, preoperative; *P < .003.
Comparison of iNOS expression between the groups and the severity of inflammation between the groups were calculated with the Kruskal-Wallis rank sum test. Mortality rates were compared with the Fisher exact test. Data are reported as mean ± SD. The INSTAT statistical program (GraphPAD Software; Prism Inc, San Diego, Calif) and SAS statistical software package (SAS Institute, Inc, Cary, NC) were used for statistical calculations.

RESULTS

In each group, eight rats were evaluated. There was no significant change in aortic diameter in rats receiving intra-aortic saline infusion (Group 1) when comparing postinfusion with harvest diameter (Fig 1). In contrast, intra-aortic elastase infusion produced aneurysms in Groups 2, 3, and 4 (Group 1, 2.0 ± 0.3 mm vs Group 2,
8.7 ± 1.2 mm, P < .001; Group 1 vs Group 3, 6.4 ± 1.2 mm, P < .001; Group 1 vs Group 4, 4.5 ± 0.7 mm, P < .001). There was a significant reduction in aortic diameter for rats receiving aminoguanidine compared with Group 2 rats receiving saline (Group 2 vs Group 3, P < .01; Group 2 vs Group 4, P < .001). The addition of preoperative aminoguanidine administration resulted in significant reduction of aortic diameter (Group 3 vs Group 4; P < .01) compared with postoperative administration alone. There were six aortic ruptures in both Group 2 and Group 3 and no aortic ruptures in Group 1 (P < .05). Group 4 had two aortic ruptures that were not significantly different compared with Groups 1, 2, and 3.

Measurement of plasma nitrite/nitrate levels with the Griess reaction demonstrated a significant increase in nitrite/nitrate levels (P < .003) for rats receiving intra-aortic elastase and saline injections (Group 2) on POD 7 (24.4 ± 6.6 µmol/L) compared with the day of operation (18.2 ± 7.5 µmol/L). No significant increase in nitrite/nitrate levels were noted with intra-aortic saline infusion or with aminoguanidine treatment (Fig 2). ANOVA analysis between groups demonstrated no significant difference in absolute plasma nitrite/nitrate levels at the time of operation and at the time of harvest.

Sixteen aortic specimens, four from each group, were analyzed with Western blot analysis for expression of iNOS (Fig 3). Specimens from Group 1 (intra-aortic saline) demonstrated absence of iNOS expression (ALU = 0), with specimens from Group 2 (intra-aortic elastase) demonstrating iNOS expression in three of four samples (mean ALU = 4.3 × 10^5). Treatment with aminoguanidine resulted in a significant reduction of iNOS expression, with the presence of iNOS noted in one of four samples in postoperatively treated (Group 3; mean ALU = 5 × 10^4) rats and with the absence of iNOS expression in those treated preoperatively (Group 4, ALU = 0) (P < .05).

Localization of iNOS with the use of immunocytochemistry demonstrated presence of the enzyme throughout all layers of the arterial wall, with most localized to the adventitia (Fig 4). Macrophage staining with MAC-1 antibody demonstrated localization of the macrophages and other inflammatory cells within the adventitia of the arterial wall. There was noted to be co-localization of iNOS and inflammatory cells at high power; however, multiple cells other than macrophages were noted to be positive for iNOS staining (Fig 5). In addition, there was localization of iNOS within the arterial medial layer where macrophage staining was conspicuously absent (Fig 6).

Infusion of saline (Group 1) resulted in retention of elastin fibers in the medial layer and minimal thickening and disruption of the adventitia. For all rats receiving elastase (Groups 2, 3, and 4), there was virtually complete loss of elastin within the medial layer and a thickened adventitia consisting of a dense inflammatory infiltrate. Treatment with aminoguanidine (Group 3, median = 5; Group 4, median = 4.5) resulted in no significant decrease in the severity of inflammation compared with Group 2 (median = 5) (Fig 7).
DISCUSSION
The current study documents elevated plasma nitrite/nitrate levels in experimental aneurysms correlating with expression of iNOS. Selective blockade of NO production by the inhibitor aminoguanidine resulted in a significant reduction in experimental aneurysm size when given postoperatively. A further significant reduction in aneurysm size was noted with the addition of preoperative aminoguanidine compared with postoperative administration alone. The expression of iNOS was found throughout the arterial wall but localized predominantly to the inflammatory infiltrate. However, presence of iNOS within the media and its localization in the adventitia separate from macrophages suggest iNOS may be produced by smooth muscle cells and fibroblasts. The time course of iNOS induction was not directly studied; however, the significant decrease in aortic diameter with the addition of preoperative aminoguanidine suggests iNOS is induced early in aneurysm formation. These findings indicate that iNOS is induced and expressed early in the sequence of experimental aneurysm formation by multiple cells, leading to elevated NO levels whose effects are deleterious to the aorta.

Until now, only indirect evidence linked NO with aneurysm formation. In human aortic aneurysms, nitrite levels are sevenfold greater than in normal aortas, and at this physiologic concentration nitrite in vitro will degrade elastin. Kawasaki disease, a vascular inflammatory disease of childhood characterized by coronary artery aneurysms, has been associated with increased urinary nitrite/nitrate and iNOS cofactors consistent with increased NO levels. In addition, the severity and course of the disease correlated with levels of nitrite/nitrate. The current study documents a significant increase in nitrite/nitrate levels associated with experimental AAA.

A common mechanism of action in many drugs that limit aneurysm growth in the current model of experimental AAA is their ability to suppress NO production. This includes the β-blocker propranolol, the anti-inflammatory drugs methylprednisolone and cyclosporine, the antibiotic and matrix metalloproteinase (MMP) inhibitor doxycycline, and the cyclooxygenase inhibitor lornoxicam. The fact that aminoguanidine, a selective iNOS inhibitor, limited aneurysm growth to the same or greater extent as many of the above-named drugs suggests that a common mechanism of action is NO suppression. Whether the mechanism of action of these drugs is related
to NO suppression, their known primary effects, or both remains to be seen.

Aminoguanidine in vitro and in vivo selectively inhibits the activity of iNOS and is 10 to 250 times more selective for iNOS than eNOS. Aminoguanidine’s ability to inhibit eNOS in vivo at the current dosage is unknown, and blood pressure was not recorded during this experiment. However, others have shown blood pressure in anesthetized Sprague Dawley rats to significantly increase with the administration of aminoguanidine at a dose of 60 mg/kg. If aminoguanidine were to increase blood pressure at the dosage used in our study, one would expect an increase in aneurysm size and expansion rate. Thus, paradoxically, aminoguanidine may increase blood pressure while limiting aneurysm expansion. Another interesting finding is the decreased expression of iNOS with the administration of aminoguanidine. Aminoguanidine has been shown by other investigators to decrease iNOS expression in a dose-dependent fashion in vivo consistent with the current study. The mechanism of action for this finding is currently unknown.

Inflammation with a macrophage infiltrate is a consistent feature of experimental AAA. In addition, macrophages have been found to be the predominant source of NO production by iNOS. The current study supports iNOS as the source of NO in experimental AAA. With immunocytochemistry, macrophages were shown to co-localize with iNOS; however, multiple cells stained positively for iNOS within the inflammatory cell deficient medial layer. This finding is consistent with studies demonstrating that iNOS can be expressed in smooth muscle and endothelial cells. The presence of iNOS throughout the arterial wall and the inflammatory infiltrate suggests that multiple cell types in experimental AAA regulate NO production. Aminoguanidine did not decrease the severity of inflammation while limiting aneurysm expansion.

MMPs are elevated in AAA and associated with extracellular matrix degradation. Our laboratory, as well as other investigators, has shown that blockade of these MMPs limits aneurysm expansion. This suggests that NO may play a role in MMP regulation. Several studies exist to document the role of NO in MMP regulation. NO produced by eNOS gene transfer has been shown to inhibit smooth muscle cell migration and MMP-2 and MMP-9 activity in cultured rat vascular smooth muscle cells. In contrast, peroxynitrite, a reactive metabolite produced by NO and a superoxide anion, has been shown to nitrate MMP-2, resulting in collagenolytic activity. Other studies have documented a tonic inhibition of MMP-9 by NO. The current available research on NO and its relationship to MMPs suggests NO may play a role in the regulation of MMPs in AAA development.

The mechanism of action by which NO exerts its effects in experimental aneurysm formation is currently unknown. NO produced by eNOS and iNOS can act indi-
rectly through MMP regulation and as a cellular messenger through cyclic guanosine monophosphate activation. Considering direct effects, iNOS produces NO at a rate 1000-fold greater than eNOS. This, coupled with the knowledge that aminoguanidine blockade of iNOS significantly reduced aneurysm size, suggests high levels of NO are detrimental to the arterial wall. High levels of NO are associated with the production of peroxynitrite that can be directly toxic to cells through oxidative mechanisms. In addition, increased NO, peroxynitrite, and superoxide dismutase can act in combination to nitrate tyrosine amino acids. The resulting nitrotyrosine can significantly impair cytoskeletal proteins that participate in extracellular matrix remodeling. Current literature suggests multiple avenues exist by which NO could affect aneurysm development.

CONCLUSION

These results document that increased plasma nitrite/nitrate levels and expression of iNOS occur at sites of aneurysm development. iNOS is expressed early in experimental aneurysm formation by multiple cells, leading to elevated NO levels, whose effects are deleterious to the aorta. Inhibition of iNOS-dependent NO production by aminoguanidine resulted in decreased plasma nitrite/nitrate levels and iNOS expression, with a significant limitation of aneurysmal expansion.

REFERENCES