From the Society for Vascular Surgery

# Cigarette smoking increases aortic dilatation without affecting matrix metalloproteinase-9 and -12 expression in a modified mouse model of aneurysm formation

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***Objective:*** **The development of abdominal aortic aneurysms (AAA) is presumed to result from multiple genetic and environmental factors, with exposure to tobacco smoke the single largest known factor predisposing to aneurysm growth. We have attempted to adapt the elastase-perfused animal model to determine whether tobacco exposure can lower the threshold of aortic injury necessary for AAA development.**

***Methods:*** **Adult C57BL/6 mice underwent transient perfusion of the infrarenal aorta with an active solution of elastase: high-dose (HDE, 0.19 U/mL, n = 9), standard-dose (SDE, 0.16 U/mL, n = 21) or low-dose (LDE, 0.07 U/mL, n = 24). Control animals (n = 24) were treated with heat inactivated elastase (HIE). Twenty LDE perfused mice were exposed to cigarette smoke (LDE-S) beginning 2 weeks before perfusion and continuing until aortic harvest. Aortic diameter (AD) was measured preperfusion, postperfusion, and at harvest on day 14. AAA was defined as %ΔAD ≥100% between preperfusion and harvest. Aortas from each group (except HDE) were analyzed for matrix metalloproteinase-9 (MMP-9) and MMP-12 expression by real-time polymerase chain reaction normalized to glyceraldehyde-3-phosphate dehydrogenase.**

***Results:*** **All SDE mice developed large AAA by %ΔAD (189.3% ± 16.9%, mean ± standard error of the mean), but control mice had only a small dilatation (69.7% ± 3.7%, $P < .01$). Higher doses of elastase did not produce larger aneurysms in HDE mice. In contrast, only 63% of LDE mice showed aneurysmal dilatation, and these were significantly smaller (104.3% ± 4.2%, $P < .01$). When exposed to cigarette smoke, LDE animals developed significantly larger aneurysms (%ΔAD, 134.5% ± 7.9%, $P = .0021$). There was no difference in normalized aortic MMP-9 and MMP-12 expression between elastase doses or between smoke-exposed and unexposed animals. Histologic analysis revealed that smoking increased the extent of aortic elastin degradation when compared with LDE-S animals.**

***Conclusion:*** **Aneurysm development in the elastase model is dependent on the quantity of active elastase infused. Exposure of animals to tobacco smoke after a relatively minor aortic elastase injury produces increases in elastin degradation and aneurysm size without affecting MMP-9 or MMP-12 expression. To our knowledge, this is the first demonstration in an animal model that smoking can act as a synergistic factor in AAA development. Further understanding of the relationship between smoking and AAA in this model may help unveil the pathophysiologic pathways involved between cigarette smoke and AAAs. (J Vasc Surg 2007;45:1217-27.)**

***Clinical Relevance:*** **Cigarette smoking is causally associated with abdominal aortic aneurysm; however, its mechanism of action remains unknown. The development of an animal model on which to study this relationship might lead to the development of therapies that could inhibit aneurysm formation in these patients.**

Abdominal aortic aneurysms (AAAs) are thought to arise through a combination of multiple genetic and environmental factors, including advanced age, male gender, family history, hypertension, and atherosclerosis.[1-3] The strongest epidemiologic risk factor known for the development and expansion of AAAs is a history of long-term cigarette smoking[4-7]: smokers have a threefold to sixfold increased

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risk of death from AAA compared with nonsmokers.[7,8] The effect of cigarette smoking on the development of AAAs is far greater than its effect on either coronary artery or cerebrovascular disease.[7,8] Based on these and other data, the United States Surgeon General in 2004 made the determination that cigarette smoking is causally related to aortic aneurysm disease.[9] Although little is yet known about the specific mechanisms linking cigarette smoking and AAAs, a better understanding of this relationship will yield valuable insights into the complex pathogenesis and pathophysiology of aneurysm disease.[7,10-13]

Much of our current understanding of the pathophysiology of AAAs has come from studies using animal models of disease, such as the aneurysmal aortic lesions that typically develop after transient perfusion of the abdominal aorta with porcine pancreatic elastase (PPE).[14] This experimental model has been well-characterized in rats and mice, where it has been used to clarify molecular mechanisms of the role of inflammatory mediators and elastin-degrading matrix metalloproteinases (MMPs) in AAAs, as well as the potential for pharmacologic treatment to suppress aneurysm formation.

Another advantage of the murine experimental model is its potential to be concurrently exposed to cigarette smoke in the same manner that has been used to model the development of tobacco-associated pulmonary diseases, such as lung cancer and emphysema. This approach may be particularly fruitful in examining potential similarities between the mechanisms underlying destructive remodeling of elastin-rich extracellular matrix in experimental pulmonary emphysema and AAAs, which are both characterized by chronic inflammation and increased tissue expression of elastolytic MMPs.[14,15]

In a recent preliminary study, we demonstrated that mice exposed to cigarette smoke exhibited a relative increase in the size of elastase-induced experimental AAAs during a period of 3 months, but that smoke exposure had no significant effects on aneurysm development or size during the initial 2 weeks after elastase perfusion.[16] This was an important limitation to investigating the role of cigarette smoking in AAAs, in that the processes of aortic wall inflammation, proteinase expression, degradation of medial elastin, and aneurysm formation are typically most pronounced or completed ≤14 days after elastase perfusion.

To address this concern, the goal of the present investigation was to create a more pathophysiologically relevant model of aneurysmal degeneration in which the effects of cigarette smoke exposure could be examined during the initial phase of aortic inflammation and degeneration of the elastic media. We initially evaluated whether modifying the elastase perfusion procedure with a reduced concentration of elastase might result in submaximal aortic injury and partial aneurysm formation. We then tested the hypothesis that cigarette smoke exposure in animals undergoing submaximal elastase-induced injury would result in pathologic changes comparable to those induced by standard elastase-induced injury in nonsmoking animals. We also predicted that the effects of cigarette smoking during aneurysm development might be mediated by increased local expression of elastolytic matrix-degrading proteases, such as MMP-9 and MMP-12, and sought to specifically examine this possibility.

## METHODS

**Elastase perfusion model of AAA.** Adult male C57BL/6J (Jackson Laboratory, Bar Harbor, Me) mice underwent transient perfusion of the abdominal aorta according to a protocol approved by the Animal Studies Committee at Washington University School of Medicine as described in the Expanded Methods in the Appendix (online only) and in previous reports.[16] Three different concentrations of type I PPE (E-1250, Sigma, St. Louis, Mo) were used, designated as high-dose elastase (HDE, 0.19 U/mL), standard-dose elastase (SDE, 0.16 U/mL), and low-dose elastase (LDE, 0.07 U/mL). An additional group of mice underwent aortic perfusion with heat-inactivated elastase (HIE) as a negative control, in which the standard dose elastase solution was heated to 100°C for 30 minutes to eliminate enzymatic activity.

Because commercial PPE preparations exhibit significant batch-to-batch variability in measured protease activity as well the capacity to induce AAAs,[17,18] the standard dose of elastase that is required to reproducibly induce AAAs in this experimental model is determined empirically for each commercial lot of PPE. After preliminary experiments for this study determined that there was satisfactory induction of AAAs at an elastase concentration of 0.16 U/mL, which was thereafter designated as the standard concentration, all of the experiments conducted here were performed with a single PPE preparation derived from the same commercial source and lot.

Preperfusion aortic diameter (AD) was measured before isolation of the abdominal aorta at the time of elastase perfusion, and postperfusion AD was measured at least 5 minutes after successful restoration of arterial flow. Final AD measurements were obtained in vivo immediately before euthanasia. For each animal, the extent of aortic dilation was calculated as the percentage increase between the preperfusion and postperfusion AD (immediate ΔAD), the percentage increase between postperfusion and final AD (interval ΔAD), and the percentage increase between final and preperfusion AD measurements (overall ΔAD). Most mice were euthanized 2 weeks after elastase perfusion, except 11 LDE perfused mice that were euthanized 3 weeks after perfusion.

**Cigarette exposure of mice.** Mice were subjected to cigarette smoke according to a protocol designed to induce the development of experimental pulmonary emphysema, as previously described.[19] Specially designed cages were used to expose the animals to smoke from three University of Kentucky 2R4F research cigarettes (filter removed) for 1 hour daily, 6 days per week. This level of smoke exposure is effectively equivalent to that produced with two University of Kentucky 2R1 cigarettes (which are no longer pro-

duced), and results in minimal systemic toxicity even when used for the prolonged period of 3 to 6 months required to induce development of pulmonary emphysema.[19]

Mice underwent the cigarette smoke exposure protocol for 2 weeks before the elastase perfusion procedure, and the cigarette smoke exposure continued on the same schedule until the time of euthanasia at 2 weeks. Littermate mice were maintained with identical diets during the same period of time and served as controls.

During the course of these experiments we observed no differences in periprocedural technical complications, tolerance for anesthesia, or immediate death between the smoking and nonsmoking mice that had undergone the elastase perfusion procedure.

**Light microscopy.** After euthanasia, a subset of mice underwent systemic perfusion and fixation with 10% neutral buffered formalin. The abdominal aorta was excised and immersed in 10% neutral buffered formalin for 24 hours and then embedded in paraffin. Aortic 5-μm cross sections were mounted and stained with hematoxylin and eosin for regular histology or Verhoeff van Gieson stain for elastin. Representative sections were obtained from animals in each treatment group and were imaged. Six individuals unaware of the experimental group scored these photomicrographs, as previously described.[20] The elastin content (absent elastin to normal elastin) and the degree of inflammatory infiltration (absent inflammation to severe inflammation) were evaluated on a scale from 0 to 4, and the mean ± standard error score was calculated.

**RNA extraction for real-time polymerase chain reaction and gene expression analysis.** Fresh aortic specimens immediately after harvest were disrupted and homogenized in 400 μL of Trizol reagent (Invitrogen Corp, Carlsbad, Calif), and then chloroform (20% vol) was added and the sample was spun in a microcentrifuge at 12,000*g* for 15 minutes. The resultant supernatant was collected and an equal volume of ethanol (70% vol) added. The mixture was then loaded into an RNEasy Micro Kit spin column (Qiagen Inc, Valencia, Calif), and total RNA was collected in 14 μL of RNase-free water.

Synthesis of complementary DNA (cDNA) was performed on a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, Calif) with 100 μL of standard reaction mixture as provided in the GeneAmp RNA PCR kit (Applied Biosystems). The reaction products served as a template for real-time polymerase chain reaction (RT-PCR) amplification on a 7500 Fast Real-Time PCR System (Applied Biosystems) in 25 μL total reaction volume with Power Syber Green Master Mix (Applied Biosystems), 10 pmol each forward primer and reverse complement primer, and 0.2 μg of cDNA. The reaction was incubated at 95°C for 10 minutes for DNA polymerase (AmpliTaq Gold, Applied Biosystems) activation, then 40 cycles of denature for 15 seconds at 95°C and annealing/extend at 60°C for 1 minute. Results of MMP-9 and MMP-12 were normalized and expressed as pg/pg glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

**Noninvasive blood pressure measurements.** Noninvasive determinations of systolic and diastolic blood pressure were made in conscious mice using a tail-cuff system (RTBP2005; Kent Scientific, Litchfield, Conn). Pressures in smoke-exposed and non-exposed mice were measured at least 7 days after perfusion and preceding harvest during 4 consecutive days. Blood pressure results represent the mean of the last three sessions for each mouse on consecutive days. Mean arterial pressure was calculated as described elsewhere.[21]

**Urine cotinine measurements.** Urine was collected from a subset of mice exposed to tobacco smoke by squeezing the bladder while the mouse was anesthetized at the time of aortic perfusion. Urine was again collected at the time of euthanasia by bladder puncture and aspiration. Urine cotinine concentration was evaluated using a modification of a cotinine enzyme-linked immunosorbent assay (ELISA) originally developed to assay cotinine in saliva (Salimetrics, State College, Pa). The standard protocol was followed, with the exception that the samples and the standards were diluted in a synthetic urine matrix, Surine (Dyna-Tek, Lenexa, Kan). Samples were assayed in duplicate.

**Statistical analysis.** The results are expressed as mean ± standard error of the mean. Logarithmic transformation of the MMP expression data was necessary to create a normal data distribution for statistical analysis. Statistics used for multiple sample experiments included one-way analysis of variance (ANOVA) or Welch ANOVA for unequal variances when appropriate and Tukey-Kramer multiple comparison post-test. Two-sample analysis was performed using the unpaired *t* test, assuming unequal variances. Presence of an aneurysm at harvest was defined as an increase of ≥100% over the preperfusion aortic diameter. The $\chi^2$ (or the Fisher exact test when average cell size was <5) was used to test for differences in incidence of AAA between groups. All analyses were performed with JMP 5.1 statistical software (SAS Institute Inc, Cary, NC) for Windows (Microsoft, Redmond, Wash), and $P < .05$ was considered significant.

## RESULTS

**Effect of varied elastase concentrations on standard model of aneurysm development.** To determine the effect of different concentrations of PPE on experimental aneurysm development, three experimental groups were treated with successful perfusions of standard-dose (SDE, n = 12), high-dose (HDE, n = 9), and low-dose elastase (LDE, n = 24) concentrations, and a fourth control group was treated with heat-inactivated elastase (HIE, n = 24). Aortas from all groups were harvested at 2 weeks postperfusion.

No significant difference was found in preperfusion AD among the groups, and immediately postperfusion there were also no significant differences in the immediate %ΔAD among the groups, with the exception of the comparison between the HDE and HIE mice (HDE, 72.5% ± 2.7%; SDE, 64.3% ± 3.2%; LDE, 62.2% ± 1.9%; HIE, 56.8% ±

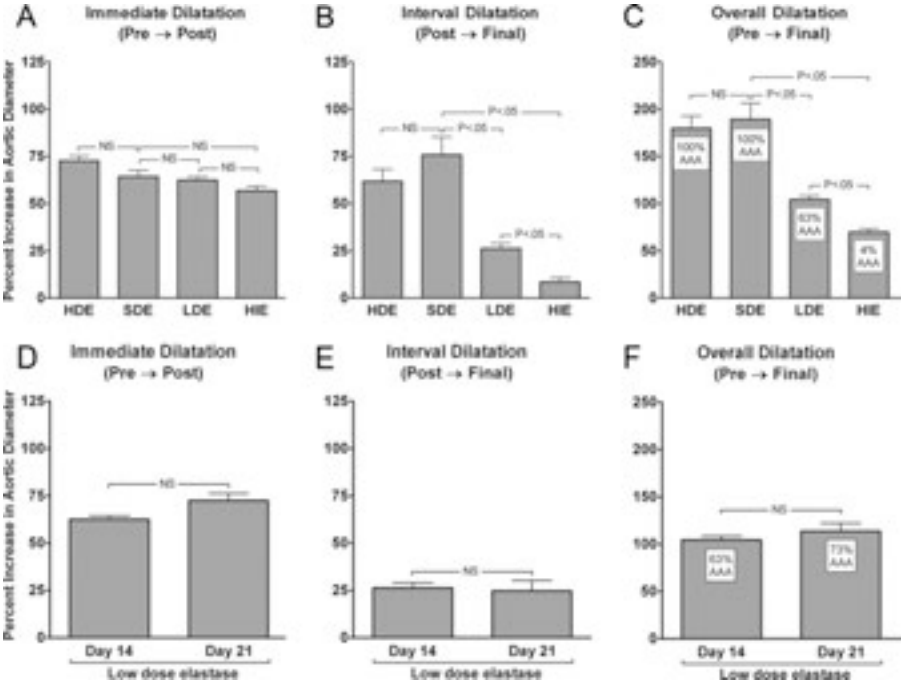



**Fig 1. A-C,** Effect of varying doses of elastase on aortic aneurysm *(AAA)* dilatation. C57BL/6 mice underwent transient aortic perfusion with a solution of high-dose *(HDE)*, standard-dose *(SDE)* or low-dose active elastase *(LDE)*. Heat-inactivated elastase *(HIE)* was used as a control. **A,** Immediate aortic dilatation, **B,** interval aortic dilatation, and **C,** overall aortic dilatation. Comparisons were calculated with one-way analysis of variance and the Tukey multiple comparisons test. **D-F,** Effect of varying incubation time (postperfusion to harvest) in LDE-perfused mice. C57BL/6 mice were subjected to transient aortic perfusion with a solution of LDE. Mice were then harvested at the standard 14-day time-point or 21 days after perfusion (LDE D21). **D,** Immediate aortic dilatation, **E,** interval aortic dilatation, and **F,** and overall aortic dilatation. Comparisons were calculated with unpaired *t* test assuming unequal variances. *NS,* Not significant.

2.3%; ANOVA $P = .0020$; Fig 1, *A*). On histology, similar to prior reports comparing SDE and HIE perfusion, no significant difference was found in the immediate postperfusion elastin content between aortas in LDE and HIE groups.

As expected, a minimal increase occurred in the interval %ΔAD (8.4% ± 2.2%) in the animals treated with the HIE, but continued dilatation (75.9% ± 9.5%) was noted in animals treated with SDE, with all mice developing aneurysms by 2 weeks (Fig 1, *B and C*). Histologically, the aortas of mice treated with HIE had intact elastic lamina and minimal inflammatory infiltrate, whereas the SDE mice had minimal or absent elastic lamellae and extensive inflammatory infiltrates.

Although the immediate postperfusion diameter of the HDE-perfused mice was significantly larger than that seen in HIE-perfused mice, no additive effect was seen on the interval or overall %ΔAD compared with the SDE-treated mice (179.8% ± 12.8% vs 189.3% ± 16.9%; $P$ = NS). There was also no difference in the histologic changes at harvest with HDE perfusion compared with SDE.

Reducing the concentration of elastase infused significantly altered the development of aortic dilatation at 2 weeks, essentially creating intermediate changes between HIE and SDE perfusion. Perfusion of the murine aorta with LDE resulted in an overall %ΔAD that was significantly smaller than SDE-perfused mice (104.3% ± 4.2% vs 189.3% ± 16.9%, $P < .05$), but was significantly larger than the HIE-perfused mice (69.7% ± 3.7%, $P < .05$). Aneurysms developed in all SDE or HDE mice, whereas aneurysms were present in only 63% of the LDE mice and 4% of the HIE mice ($P < .017$ LDE vs SDE; $P < .0001$ LDE vs HIE).

The histology of the aortas from the LDE mice demonstrated significant elastin loss and increased inflammatory infiltration compared with HIE mice. Yet when compared with SDE-perfused aortas, the preservation of the elastic lamellae was significantly better, whereas the degree of inflammatory cell infiltration was similar (Fig 4, *C*).

**Effect of extended aneurysm incubation in mice perfused with low-dose elastase.** To determine whether the smaller mean interval and overall %ΔAD seen in the LDE mice simply reflected a prolonged time course for aneurysm development when a lower dose of elastase was used, we increased incubation time (perfusion to harvest) to a total of 3 weeks in a subgroup of 11 LDE mice. We found that samples from animals harvested 3 weeks after LDE perfusion were not different than those harvested at 2

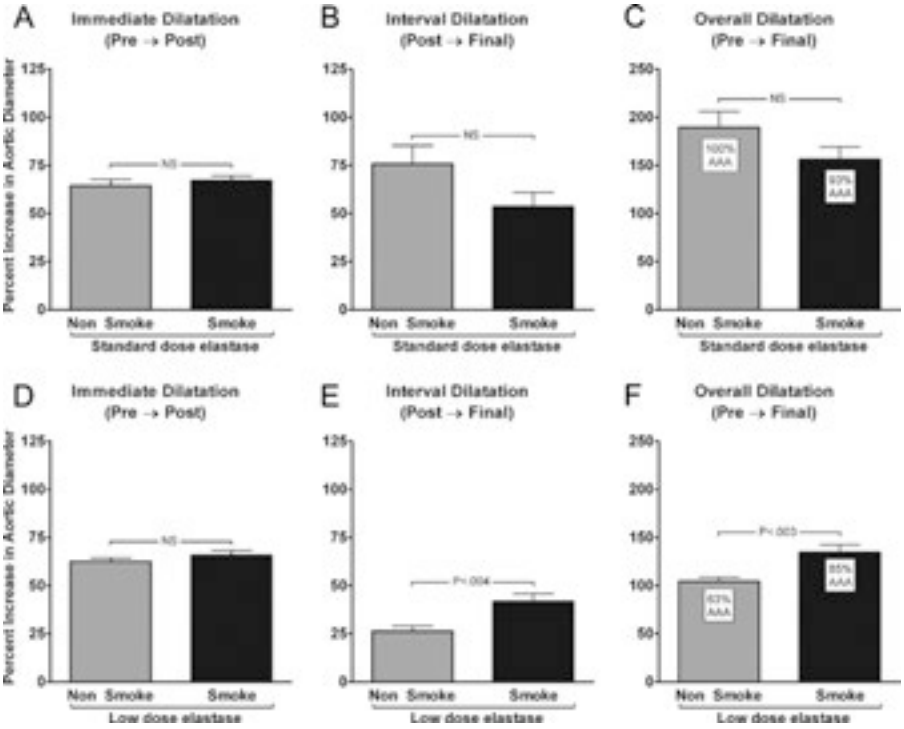



**Fig 2.** Effect of smoking on abdominal aortic aneurysm *(AAA)* dilatation. C57BL/6 mice underwent transient aortic perfusion with a solution of standard-dose *(SDE)* or low-dose active elastase *(LDE)*. SDE mice (n = 14) and LDE mice (n = 20) were exposed to cigarette smoke for 2 weeks before surgery and then continued smoking until aortic harvest. **A-C,** Effect of smoking on SDE-perfused mice. **A,** Immediate aortic dilatation, **B,** interval aortic dilatation, and **C,** overall aortic dilatation. Comparisons were calculated with unpaired *t* test assuming unequal variances. **D-F,** Effect of smoking on LDE-perfused mice. **D,** Immediate aortic dilatation, **E,** interval aortic dilatation, and **F,** overall aortic dilatation. Statistical comparisons were calculated on the mean change in aortic size with the unpaired t-test assuming unequal variances. *NS,* Not significant.

weeks with respect to interval %ΔAD (24.4% ± 5.5% vs 26.2% ± 2.7%, $P$ = NS; Fig 1, *E*) and overall %ΔAD (113.8% ± 8.4% vs 104.2% ± 4.2%, $P$ = NS; Fig 1, *F*). The incidence of aneurysms was also similar in each group (63% vs 73%; $P$ = NS).

**Effect of tobacco smoke exposure on model aneurysm development.** To evaluate the effect of cigarette smoke on model aneurysms, mice were exposed to cigarette smoke for 2 weeks before elastase perfusion, and exposure was continued until harvest. Littermate mice were maintained with identical diets during the same period of time and served as controls. LDE was perfused in 23 smoke-exposed mice (LDE-S) and SDE was perfused in 14 smoke-exposed mice (SDE-S), and their aortas were harvested after an additional 2-week incubation.

Animals exposed to preoperative tobacco smoke did not demonstrate a different initial response to elastase perfusion. The immediate %ΔAD (Fig 2, *A and D*) induced by aortic perfusion was not different between smoke-exposed and unexposed animals (SDE, 66.8% ± 2.6% vs 64.3% ± 11.1%, $P$ = NS; LDE, 64.2% ± 3.2% vs 62.2% ± 1.9%, $P$ = NS). There were also no grossly apparent changes in the technical aspects of the model or survivability of the animals.

In the SDE animals, at harvest there was no significant effect on either interval %ΔAD (53.6% ± 7.4% vs 75.9% ± 9.5%, $P$ = NS) or overall %ΔAD (156.3% ± 13% vs 189.3% ± 16.9%, $P$ = NS) when comparing SDE-S and control animals (Fig 2, *A to C*). There was also no impact on the incidence of AAA (93% vs 100%, $P$ = NS). Cigarette smoke exposure in the LDE mice, however, resulted in a nearly a 60% increase in the aortic mean interval %ΔAD (41.5% ± 4.0% vs 26.2% ± 2.7%, $P < .004$) compared with nonsmoking animals (Fig 2, *E*). On the whole, there was a 30% increase in the overall %ΔAD in LDE-S mice compared with control animals (134.5% ± 7.9% vs 104.2% ± 4.2%, $P < .003$).

There were also more aneurysms among the mice exposed to smoke, although the difference did not quite reach the threshold for statistical significance (85% vs 63%, $P$ = .08). Concordant with the changes seen in absolute aortic diameter were increases in elastic fiber loss in the LDE-S animals compared with the LDE animals. Furthermore, animals exposed to tobacco smoke for 4 weeks before LDE

**Table.** Noninvasive arterial blood pressure measurements*

| *Treatment*† | *Mice (n)* | *Systolic (mm Hg)* | *Diastolic (mm Hg)* | *Mean (mm Hg)* |
|---|---|---|---|---|
| No smoke | 24 | 89.4 ± 2.9 | 76.2 ± 2.8 | 80.6 ± 2.8 |
| Smoke | 16 | 90.9 ± 3.6 | 76.5 ± 3.2 | 81.3 ± 3.3 |

*Noninvasive systolic and diastolic arterial blood pressure was measured in conscious smoke-exposed and control mice, at least 7 days after perfusion and before harvest at day 14. Mean arterial pressure was calculated as described elsewhere.

†Two-way comparisons were calculated with unpaired *t* test assuming unequal variances, and results were not significant.

perfusion resulted in similar sized aneurysms compared with animals exposed for only 2 weeks (data not shown.)

**Effect of tobacco smoke exposure on blood pressure.** To determine whether smoke-induced hypertension might be related to the increased aneurysm size in the LDE-S animals, we evaluated the blood pressure in smoke-exposed (n = 24) and control (n = 16) mice after aortic perfusion. Noninvasive tail-cuff measurements of systolic and diastolic blood pressure were performed in mice after aortic perfusion. In these mice, cigarette smoking had no effect on systolic or diastolic blood pressure, or mean arterial pressure (Table).

**Urinary cotinine in smoke-exposed animals.** To assess the consistency of smoke exposure in the animals, urine was obtained from animals both at the time of aortic perfusion and at euthanasia. Quantification of urinary cotinine concentration was performed using a commercially available ELISA. All smoke-exposed animals had similarly elevated levels of urinary cotinine at harvest. Mean urinary concentration of cotinine did not vary when measured at the time of surgery or at harvest.

**Matrix metalloproteinase-9 and -12 expression in murine aortas.** To determine whether changes in production of elastolytic proteases MMP-9 or MMP-12 may play a role in the differences in aortic aneurysm formation seen between elastase doses and with smoke exposure, aortas from each group (SDE, n = 8; SDE-S, n = 12; LDE, n = 12; LDE-S, n = 17, and HIE, n = 15) were analyzed for MMP-9 and MMP-12 expression. Results of MMP-9 and MMP-12 analysis are expressed after normalization to GAPDH.

Despite the large differences in aortic diameters based on the quantity of elastase perfused, aortic MMP-9 expression levels at 14 days were not significantly affected by elastase dose or activity (SDE, $1.3 \times 10^{-2} \pm 3.3 \times 10^{-3}$; LDE, $2.1 \times 10^{-2} \pm 6.9 \times 10^{-3}$; HIE, $1.2 \times 10^{-2} \pm 2.8 \times 10^{-3}$; $P$ = NS; Fig 3, *A*). Likewise, aortic MMP-12 messenger RNA (mRNA) levels were also similar irrespective of the perfusion dose of elastase (SDE, $4.3 \times 10^{-1} \pm 1.6 \times 10^{-1}$; LDE, $4.1 \times 10^{-1} \pm 1.1 \times 10^{-1}$; HIE, $1.2 \times 10^{-1} \pm 2.5 \times 10^{-2}$; ANOVA $P$ = NS; Fig 3, *B*).

We also compared the production of MMP-9 and MMP-12 based on tobacco smoke exposure in the SDE and LDE mice. In this analysis, smoking had no influence on aortic MMP-9 mRNA levels (SDE-S, $1.9 \times 10^{-2} \pm 4.8 \times 10^{-3}$ vs $1.3 \times 10^{-2} \pm 3.3 \times 10^{-3}$, $P$ = NS; LDE-S, $8.5 \times 10^{-3} \pm 1.4 \times 10^{-3}$ vs $2.1 \times 10^{-2} \pm 6.9 \times 10^{-3}$, $P$ = NS; Fig 3, *C*). We were also unable to demonstrate any significant difference in normalized MMP-12 levels when comparing the smoke-exposed and unexposed mice. (SDE-S, $3.7 \times 10^{-1} \pm 6.3 \times 10^{-2}$ vs $4.3 \times 10^{-1} \pm 1.6 \times 10^{-1}$, $P$ = NS; LDE-S, $2.5 \times 10^{-1} \pm 4.8 \times 10^{-2}$ vs $4.1 \times 10^{-1} \pm 1.1 \times 10^{-1}$, $P$ = NS; Fig 3, *D*).

## DISCUSSION

AAA is a distinct pathologic condition that is believed to require a convergence of multiple predisposing factors. Both genetic and environmental elements have been identified in the pathogenesis of this disease, of which tobacco smoke exposure is the largest epidemiologic factor predisposing to aneurysm development and growth.[5-7,11,22] Histologically, the disease is characterized by the development of aortic wall inflammation and accompanied by significant medial elastic fiber degeneration attributed to increased local production of several elastolytic metalloproteases.[23-27] These histologic changes have been recapitulated in several animal models including the murine elastase-perfusion model.

These models have been useful in advancing our understanding the pathophysiology of AAA, yet they offer little insight into the processes that may initiate aneurysm formation in humans. Modeling the arterial changes induced by tobacco smoke may offer our first means by which to study the events that lead to aneurysm formation. The success of tobacco smoke exposure in animals to model pulmonary disease suggests that such exposure in mice may have similar pathologic effects to the changes induced by smoke exposure in humans. It is remarkable to note that the matrix changes of a model of emphysema includes chronic inflammatory infiltration with upregulation of expression of MMP-2, MMP-9, and MMP-12.[28-34]

The only previously published study on cigarette smoke and model aneurysms was not able to show any effect of tobacco smoke on initial aneurysm development.[16] However, mild progressive dilatation was noted after a prolonged postperfusion interval with tobacco smoke. Although this conforms to the human epidemiologic finding that AAA expansion is accelerated with ongoing smoke exposure,[35,36] their model did not mimic the extensively documented effects of tobacco smoke on the risk of aneurysm development.[5,37-40]

Further, there were multiple other limitations of that model to determining the effect of tobacco smoke on AAA development. Unlike nearly all other published studies on the elastase-perfused murine AAA that have used the C57/Bl6 strain, the mice used were of the 129/SvEv strain, a strain recognized to be more resistant to aneurysm development than the C57 strain.[41] Based on the timing of the changes, it is most likely that the difference seen was related to an effect of tobacco smoke on the repair and stabilization of the injured aorta. This may be more related to the unique features of the model AAA rather than representative of the changes seen in

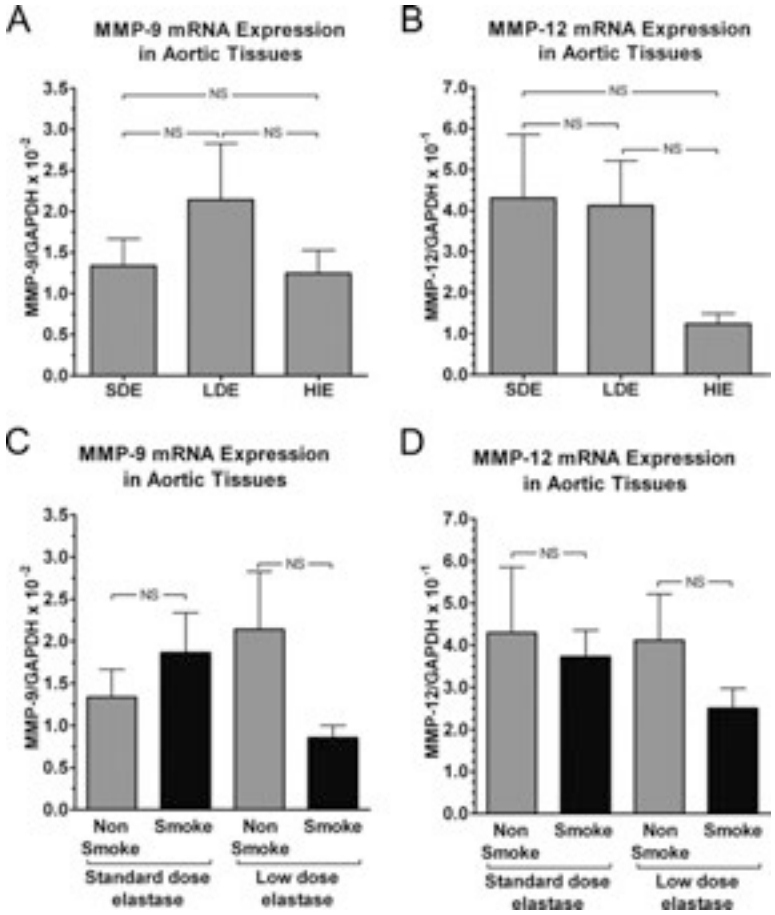
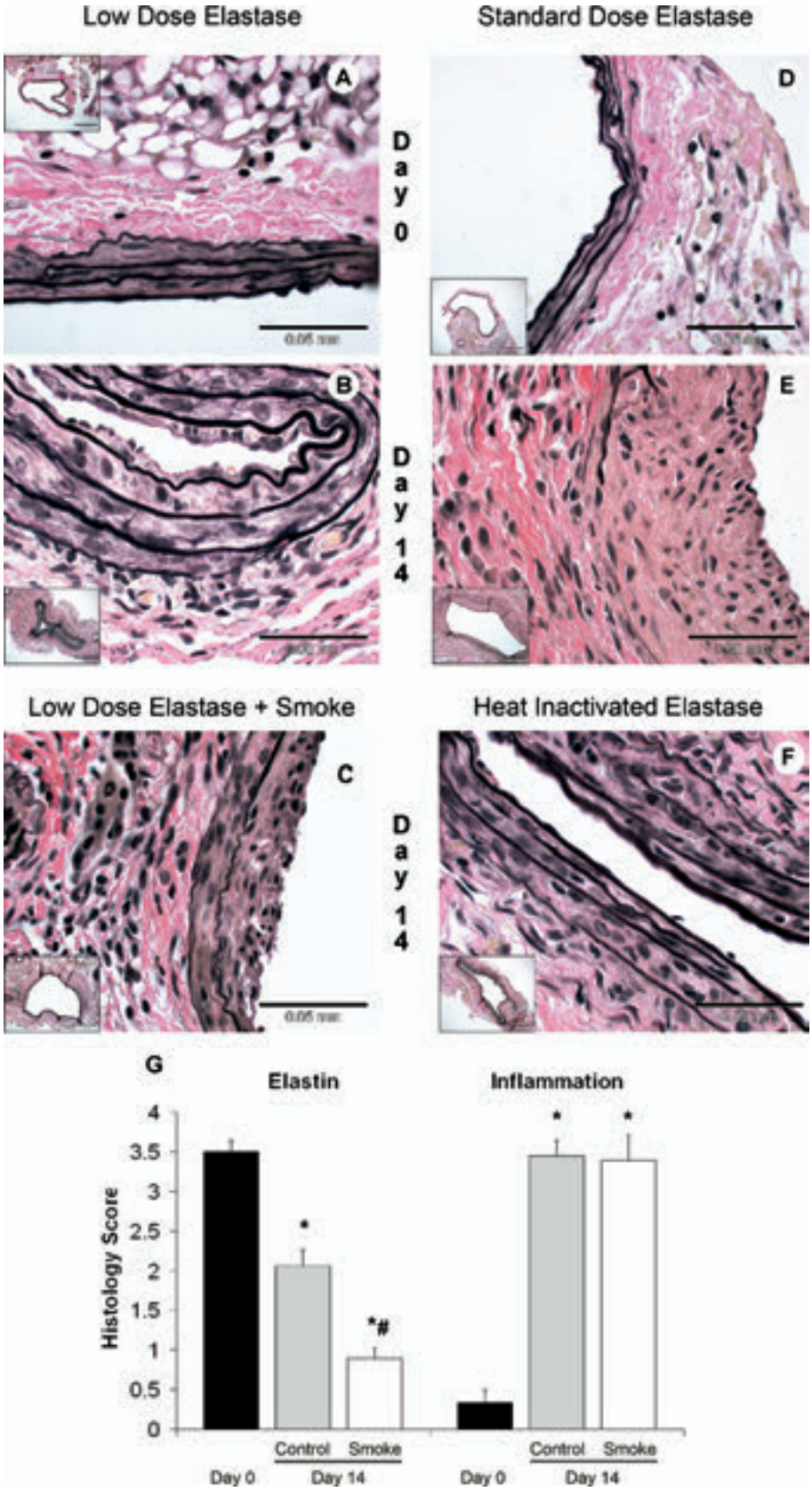



**Fig 3. A and B,** Effect of varying doses elastase on matrix metalloproteinase *(MMP)* expression. C57BL/6 mice underwent transient aortic perfusion with a solution of standard-dose *(SDE)* or low-dose active elastase *(LDE)*. Heat-inactivated elastase *(HIE)* was used as control. At harvest aortic tissue was promptly processed for messenger RNA *(mRNA)* extraction. **A,** MMP-9 expression. **B,** MMP-12 expression. Results were normalized to log base 10 for analysis and differences were calculated with one-way analysis of variance and Tukey-Kramer multiple comparison post test. **C and D,** Effect of smoking on MMP expression. C57BL/6 mice were subjected to transient aortic perfusion with a solution of SDE or LDE. SDE mice ( n = 14) and LDE mice (n = 20) were exposed to cigarette smoke for 2 weeks before surgery and then continued smoking until aortic harvest. At harvest aortic tissue was promptly processed for mRNA extraction. **C,** MMP-9 expression in SDE and LDE mice. **D,** MMP-12 expression in SDE and LDE mice. Results were normalized to log base 10 for analysis, and differences were calculated with unpaired *t* test assuming unequal variances. *NS,* Not significant.

human AAA, because model AAAs not exposed to tobacco smoke do not continue to dilate after 2 weeks, whereas clinical AAA nearly invariably demonstrate progressive dilatation.[42] Finally, that study did not describe the histologic or any biochemical changes that accompanied the increase in diameter, making it unclear what effect tobacco smoke exposure was having on the aortic wall.

These preliminary results were promising, but the utility was limited, and we desired to model the effects of tobacco smoke on the critical initiating events of aneurysm formation. We hypothesized that the standard model of elastase-induced aneurysm formation creates a maximal aortic injury response pattern, effectively masking any effect of additional inflammatory or matrix degrading stimuli, including that potentially borne with tobacco smoke. This was confirmed by the lack of additional dilatation at harvest in mice perfused with the highest concentration of elastase.

The perfusion model was then modified with a lower concentration of elastase in the perfusate. The result in these animals with LDE perfusion was an aneurysm intermediate both by diameter measurement and histology between control and SDE perfusion. When these LDE-perfused mice were euthanized at 3 weeks postperfusion rather than at the standard 2 weeks, no further increase in AAA size was noted, suggesting that LDE perfusion creates a submaximal aortic dilatation and matrix injury compared


Low Dose Elastase
Standard Dose Elastase
A
D
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B
E
Day 14
Low Dose Elastase + Smoke
Heat Inactivated Elastase
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Day 14

with SDE perfusion, not simply a slower increase in aortic dilation.

To determine whether smoke exposure would significantly alter the development of model AAA in these submaximally injured aortas, we exposed mice to cigarette smoke for 2 weeks before aortic perfusion with LDE. The mice were then continuously exposed to cigarette smoke during the next 2 weeks before harvest. This smoke exposure in the LDE-perfused mice resulted in significantly larger aneurysms when compared with the LDE-perfused mice that were not exposed to smoke.

On histology, the aneurysms in the LDE-S mice demonstrated elastin degradation similar to that seen in the SDE-perfused mice (Fig 4). Given the lack of increase in aortic diameter at 3 weeks in the LDE-perfused mice, this effect of smoking does not appear to be an acceleration of an ongoing injury induced by the LDE perfusion. We also demonstrated that there was no effect on mean blood pressure, a potential mediator of increased aortic dilatation, in the LDE-S mice after the relatively short exposures to cigarette smoke used in this study.

Of interest was that we did not see a dose-response relationship between pre-elastase smoke exposure and aneurysm size. This suggests that the effect of smoke exposure in this model may be a permissive one, such that smoke exposure enhances the matrix-degrading process activated by a second phenomenon (ie, low-dose elastase injury). This may be consistent with human epidemiology where the quantity of tobacco exposure is only roughly correlated with the incidence of aneurysms, although more rapid growth of aneurysms occurs in those who continue to smoke.[35,36]

In the mice not exposed to smoke, we found no relationship between elastase dose or aneurysm size and MMP-9 or MMP-12 production in the aortic wall. This confirms the results of a recent study that used microarrays to compare aortic expression of MMPs at harvest in animals perfused with SDE and HIE.[43] Nevertheless, prior studies in genetically modified animals demonstrate that MMP-9 is critical to aneurysm formation in the murine model of AAA.[14] Further studies will be necessary to clarify the relationship between the expression of these proteases and their activity within the aortic wall.

We also did not find a significant difference in the local production of either MMP-9 or MMP-12 in the mice exposed to smoke compared with the control mice. Given the local upregulation of MMP-12 expression in the lungs of mice exposed to cigarette smoke, we anticipated that effect might carryover in the macrophages recruited to the aorta during the formation of the aneurysm. Our findings here suggest a more indirect relationship between matrix changes in the lungs and the aorta in mice exposed to cigarette smoke. To fully evaluate the impact of protease changes on the development of aneurysms in smoke-exposed mice, analysis of the protease activity of the aortic tissues will also be required. Future studies examining the time-course of MMP expression will also need to be done in the smoke-exposed mice as well as in genetically altered mice.

Alternatively, the effect of tobacco smoke on AAA development may lie in factors not directly related to MMP production, such as other inflammatory mediators or in changes in the arterial inflammation response profile. For example, Paik et al[44] showed that nitrite ion, a byproduct of cigarette smoke, is capable of binding under physiologic conditions to insoluble elastin. They also showed that segments of human aortic wall treated with nitrite ion resulted in structural disruption of elastin.

## CONCLUSIONS

To our knowledge, this is the first demonstration in an animal model that smoking can act as a synergistic factor in AAA formation, confirming the effect of cigarette smoke exposure on the human disease and opening exciting new possibilities in aneurysm research. This study is the first step in understanding the in vivo effects of tobacco smoke exposure on the arterial wall. Of importance is that it may also allow us to begin to understand the changes that may initiate AAA development. Future studies are needed to further delineate the changes in the arterial wall and the inflammatory response induced by cigarette smoke exposure. We anticipate extensive use of this model to further our understanding of the pathophysiology of aneurysm disease.

**Fig 4.** Murine aortic wall histology. **A–F,** Staining of aortic sections was performed with Verhoeff-van Gieson stain to emphasize the elastic fibers in the tissue. Aortas shown in panels **A, B,** and **C** were all perfused with low dose elastase and panels **D** and **E** were perfused with standard-dose elastase (SDE). Panel **F** was perfused with SDE that underwent heat inactivation. Panel **G** plots the mean ± standard error of the blinded grading of sections from low-dose elastase (LDE) perfused aortas. Both elastin and inflammation were graded on a scale from 0 to 4, with higher numbers representing greater amounts of elastin or inflammation, respectively. Samples in **A** and **D** were fixed immediately after perfusion and demonstrate intact elastin fibers and no appreciable cellular infiltrate. **D,** After 2 weeks, marked inflammatory infiltration and severe elastin damage is seen in SDE-perfused animals. **B,** Relatively modest elastin damage is seen 2 weeks after LDE perfusion, despite a significant inflammatory infiltrate. **C and G,** There is significantly more inflammation and elastin degradation in LDE-perfused animals that are also exposed to cigarette smoke. All photomicrographs are high-power views (original magnification ×100), and insets are at low-power view (original magnification ×20). *$P < .001$ compared with day 0; #$P < .001$ compared with control.

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## AUTHOR CONTRIBUTIONS

Conception and design: JC, RT, MB
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Data collection: JC, MB, BA, TE
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Statistical analysis: JC, MB
Obtained funding: JC
Overall responsibility: JC

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## Appendix (online only)

### Extended Methods

**Elastase perfusion model and aortic diameter measurements.** Adult male C57BL/6J mice underwent transient perfusion of the abdominal aorta according to a protocol approved by the Animal Studies Committee at Washington University School of Medicine. Mice were anesthetized with intraperitoneal sodium pentobarbital (55 to 60 mg/kg) and a laparotomy was performed under sterile conditions. The abdominal aorta was exposed with the assistance of an operating stereomicroscope (Leica Microsystems, Wetzlar, Germany) from the level of the left crossing renal vein to the bifurcation, and the lumbar branches were ligated with 9-0 sutures.

Preperfusion aortic diameter (AD) was measured with a calibrated ocular grid, and temporary 6-0 silk ligatures were placed around the proximal and distal aorta. An aortotomy was created at the level of the bifurcation using the tip of a 30-gauge needle. A heat-tapered segment of PE-10 polyethylene tubing (Baxter, McGraw Park, Ill) was introduced through the aortotomy and secured in place with a 6-0 silk tie, and the aorta was perfused for 5 minutes with a saline solution containing active or inactive type I porcine pancreatic elastase (PPE; 7.8 U/mg; catalog E-1250, Sigma Chemical Co, St. Louis, Mo). The concentrations of PPE were varied as follows: high-dose elastase (HDE, 0.19 U/mL), standard-dose elastase (SDE, 0.16 U/mL), low-dose elastase (LDE, 0.07 U/mL), and heat-inactivated elastase (HIE, 0.16 U/mL, 100°C for 30 minutes). After perfusion, the catheter was removed, and the aortotomy was closed with a 10-0 suture.

Postperfusion AD was measured at least 5 minutes after successful restoration of arterial flow. Final AD measurements were obtained in vivo during a repeat laparotomy before the animal was killed and tissue procured.

For each animal, the extent of aortic dilation was calculated as the percentage increase between the preperfusion and postperfusion AD (immediate %ΔAD), the percentage increase between postperfusion and final AD (interval %ΔAD), and the percentage increase between final and preperfusion AD measurements (overall %ΔAD). Abdominal aortic aneurysm (AAA) was defined as an overall %ΔAD of at least 100%. All mice were euthanized 2 weeks after elastase perfusion, except 11 LDE-perfused mice, whose aortas were harvested 3 weeks after the initial procedure (LDE D21).

During and immediately after aortic perfusion, overall perioperative mortality (surgery to 48 hours including animals euthanized because of anatomic variations or technical complications) was 30.3% and did not significantly vary based on the perfusate elastase concentration ($\chi^2$ analysis). Similarly, the perioperative mortality in the mice exposed to cigarette smoke before the procedure was 27.3%, which also did not vary based on perfusate ($\chi^2$ analysis). Mortality among the mice was rare in both treatment groups beyond 48 hours after elastase perfusion (no smoke, 9.2%; smoke, 7.5%; $\chi^2$ $P$ = NS).

**Mouse model of cigarette smoking.** Mice were exposed to cigarette smoke according to a previously described protocol to induce the development of experimental pulmonary emphysema.[17] Using specially designed cages and a smoking apparatus adapted for mice, animals were exposed to smoke from 3 University of Kentucky 2R4F research cigarettes (filter removed) daily for 6 days per week. It has been observed that for periods of up to 6 months, blood carbon monoxide does not reach toxic levels, and mice appear grossly normal throughout the entire experimental procedure.[17]

**Light microscopy.** After death, mice underwent systemic perfusion and fixation with 10% neutral buffered formalin. The abdominal aorta was excised and immersed in 10% neutral buffer formalin for 24 hours and then embedded in paraffin. Aortic cross sections (5 μm thick) were then mounted and stained with hematoxylin and eosin for regular histology or Verhoeff van Gieson stain for elastin. Representative cross-sectional areas from each specimen were photographed. Aortas from normal nonperfused SDE day 0 and LDE day 0 (without smoke exposure) mice were used as controls.

**RNA extraction for real-time polymerase chain reaction and gene expression analysis.** Fresh aortic specimens immediately after harvest were disrupted and homogenized in 400 μL of Trizol reagent (Invitrogen Corp, Carlsbad, Calif) and then chloroform (20% vol) was added and the sample was spun in a microcentrifuge at 12,000*g* for 15 minutes. The resultant supernatant was collected, and an equal volume of ethanol (70% vol) was added.

The mixture was then loaded into an RNEasy Micro Kit spin column (Qiagen Inc, Valencia, Calif), and total RNA was collected in 14 μL of RNase-free water. Synthesis of complimentary DNA (cDNA) was performed on a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, Calif) with 100 μL reaction volume using 2 μg of total RNA, 1 U/μL RNase inhibitor, 1 U/μL recombinant Moloney murine leukemia virus reverse transcriptase, 2.5 μM random hexamers, 1 mM 2'-deoxyribonucleoside 5'-triphosphate, 5 mM magnesium chloride solution, and 1× polymerase chain reaction (PCR) buffer as provided in the GeneAmp RNA PCR kit (Applied Biosystems). Reactions were incubated at 25°C for 10 minutes. Reverse transcription was initiated by heating to 48°C for 30 minutes and terminated by heating to 95°C for 10 minutes.

The reaction products served as a template for real-time PCR amplification using the following mouse MMP and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward primers (FP) and reverse complement primers (RCP), all from Integrated DNA Technology Inc; Coraville, Iowa:

MMP-9: FP 5′-ACAATCCTTGCAATGTGGATGTT-3′, RCP 5′-CGCCCTGGATCTCAGGAATA-3′;

MMP-12: FP 5′-TTAACCCCAGCACATTTCGC-3′, RCP 5′-ACTGAATGTTACGTATGTCATCAGCA-3′;

GAPDH: FP 5′-CATTGTGGAAGGGCTCATGA-3′, RCP 5′-TCTTCTGGGTGGCAGTGATG-3′.

The reactions were performed on a 7500 Fast Real-Time PCR System (Applied Biosystems) in 25 μL of total reaction volume with Power Syber Green Master Mix (Applied Biosystems), 10 pmol each FP and RCP, and 0.2 μg of cDNA. The reaction was incubated at 95°C for 10 minutes for DNA polymerase (AmpliTaq Gold, Applied Biosystems) activation, then 40 cycles of denature for 15 seconds at 95°C and annealing/extend at 60°C for 1 minute. The resultant product was normalized with GAPDH, and absolute quantitation was calculated using a standard curve.

**Noninvasive blood pressure measurements.** Noninvasive determinations of systolic and diastolic blood pressure were made in conscious mice using a tail-cuff system (RTBP2005; Kent Scientific, Litchfield, Conn). Pressures in smoke-exposed and control animals were measured at least 7 days after perfusion and preceding harvest at day 14 during 4 consecutive days. To optimize the pulse signal, mice were warmed to 38°C at each session. Six measurements were recorded for each mouse per session. Blood pressure results represent the mean of the last three sessions for each mouse on consecutive days. Mean arterial pressure was calculated as described elsewhere.[18]

**Statistical analysis.** The results are expressed as mean ± standard error of the mean. Logarithmic transformation of the MMP expression data was necessary to create a normal data distribution for statistical analysis. Statistics used for multiple sample experiments include one-way analysis of variance (ANOVA) or Welch ANOVA for unequal variances, when appropriate, and Tukey-Kramer multiple comparison post-test. Two-sample analysis was performed using the unpaired *t* test assuming unequal variances. Presence of an aneurysm at harvest was defined as an increase of ≥100% over the preperfusion aortic diameter. The $\chi^2$ (or Fisher exact test when average cell size was less than 5) was used to test for differences in incidence of AAA between groups. All analyses were performed with JMP 5.1 statistical software (SAS Institute Inc, Cary, NC, 1989-2003) for Windows (Microsoft, Redmond, Wash). $P < .05$ was considered significant.