Differential Effects of Doxycycline, a Broad-Spectrum Matrix Metalloproteinase Inhibitor, on Angiotensin II–Induced Atherosclerosis and Abdominal Aortic Aneurysms

Michael W. Manning, Lisa A. Cassis, Alan Daugherty

Objective—Angiotensin II (AngII) infusion into hyperlipidemic mice leads to the rapid formation of atherosclerotic lesions and abdominal aortic aneurysms (AAAs). To define the role of matrix metalloproteinases (MMPs) in the development of these vascular pathologies, we administered the broad-spectrum MMP inhibitor doxycycline to saline- and AngII-infused LDL receptor−/− mice.

Methods and Results—Mice were placed on a high-fat diet for 1 week before infusion with either saline or AngII (1000 ng · kg⁻¹ · min⁻¹) via osmotic pumps for 28 days. Doxycycline (30 mg · kg⁻¹ · d⁻¹) was administered in the drinking water to both saline- and AngII-infused mice. Administration of doxycycline did not significantly influence systolic blood pressure, serum cholesterol concentrations, or lipoprotein-cholesterol distribution. Doxycycline had no effect on the extent of atherosclerosis in saline- or AngII-infused mice. In contrast, doxycycline markedly reduced the incidence of AAA formation (86% vs 35%, AngII vs AngII+doxycycline, respectively; P<0.05), in addition to reducing aneurysm severity.

Conclusions—These data do not imply a role for MMPs in AngII-induced atherosclerosis but provide evidence consistent with a role in AngII-induced AAA formation. (Arterioscler Thromb Vasc Biol. 2003;23:483-488.)

Key Words: angiotensin • atherosclerosis • doxycycline • matrix metalloproteinases

Infusion of angiotensin II (AngII) to LDL receptor−/− or apoE−/− mice has been previously demonstrated to augment atherosclerotic lesion formation and promote the formation of abdominal aortic aneurysms (AAAs). Atherosclerotic lesions formed by AngII infusion are characterized by rapid formation of lipid-laden macrophages intermingled with large numbers of T lymphocytes. AngII-induced AAAs have many features of the human disease, including medial degeneration, thrombosis, and inflammation.

The vascular pathologies of atherosclerosis and AAA are both associated with a dynamic state of deposition and degradation of the extracellular matrix. Degradation of lesional extracellular matrix is likely to occur through 1 or more of the class of matrix metalloproteinases (MMPs). A role for MMPs in the atherogenic process may be inferred through the detection of MMP-1, -2, -3, -9, -11, and -14 in lesions. Based on their role in the degradation of the extracellular matrix, enhanced activity of MMPs has been considered to increase the development of atherosclerosis. However, recent studies with MMP-3 or tissue inhibitor of matrix metalloproteinases (TIMP)-1−/− mice and MMP-1−/− overexpressing mice have provided evidence to the contrary.

The MMP class of enzymes has also been implicated in the development of AAAs. This disease is defined by the medial degeneration that results from the destruction of both elastin and collagen. Several MMPs have been detected in AAAs, including 4 that degrade elastic fibers (MMP-2, -7, -9, and -12), several that degrade interstitial collagens (MMP -1, -2, -8, -13, and -14), and others that degrade denatured collagen (MMP-2 and -9). Evidence supporting a role for MMPs in animal models of AAAs includes data demonstrating resistance to AAAs in mice lacking MMP-2 or -9. In addition, a deficiency of urokinase plasminogen activator in apoE−/− mice reduced micro-AAAs, which was attributed to decreased activation of MMP-12.

The limited literature suggests that AngII exhibits cell-specific regulation of MMPs. In cardiac myocytes and pericytes, AngII promoted the synthesis of MMP-2 but decreased its presence in vascular smooth muscle cells. AngII directly promoted the synthesis of MMP-9 in cardiac myocytes. Administration of the angiotensin type 1 (AT1) receptor antagonist losartan to cardiomyopathic hamsters reduced cardiac mRNA expression of several MMPs. In addition, administration of losartan to cholesterol-fed rabbits...
reduced the mRNA expression of MMP-1 in the aorta and was suggested to contribute to the antithromogenic effects of AT1 receptor blockade. These data collectively suggest that AngII may regulate MMPs; however, the role of MMPs in AngII-induced vascular disease is not known.

One approach to the elucidation of the role of MMPs in a disease process is the use of pharmacological inhibitors. A widely used compound is doxycycline, which, though commonly known for its antibiotic properties, exerts therapeutic effects through inhibition of MMPs. An advantage of using doxycycline to study the role of MMPs in a disease process is the broad-spectrum inhibition of MMPs. We hypothesized that AngII induces the vascular pathologies of atherosclerosis and AAAs in hyperlipidemic mice by augmenting MMPs. To test this hypothesis, we determined the effect of doxycycline on the development of AngII-induced atherosclerosis and AAA formation in LDL receptor−/− mice. Results from this study provide evidence that is consistent with a role for MMPs in the development of AngII-induced AAAs and provide further support for defining the therapeutic benefit of this compound in humans afflicted with the disease.

**Methods**

**Animals**

Male LDL receptor−/− mice (8 weeks old, backcrossed 10 times onto a C57BL/6J background) were obtained from the Jackson Laboratory (Bar Harbor, Me) and housed under barrier conditions. Standard sterilized laboratory diet and water were available ad libitum. One week before pump implantation, the mice were placed on a diet containing 0.15% (wt/wt) cholesterol and 21% (wt/wt) cocoa butter fat (high-fat diet, TD 88137; Harlan Teklad). AngII (1000 ng·kg⁻¹·min⁻¹) or saline was administered subcutaneously by Alzet osmotic minipumps (model 2004) as described previously. All procedures were performed with the prior approval of the University of Kentucky Institutional Animal Care and Use Committee.

**Doxycycline Administration**

Doxycycline (Sigma) was administered daily in the drinking water at an approximate dose of 30 mg·kg⁻¹·d⁻¹ (based on the average daily water consumption). This dose has been shown previously to block MMP activity in vivo. Doxycycline treatment was begun 1 week before pump implantation and continued throughout the study. The drug was protected from light, and a fresh drug solution was provided every other day.

**Blood Pressure Measurements**

Systolic blood pressures were obtained from conscious mice by using a computerized tail-cuff method (BP-2000 Visitech Systems). Mice were acclimated to the instrument for at least 1 week before implantation of the osmotic pumps. Measurements were recorded at the same time of day throughout the study. Individual mice received 10 initial pressure readings to acclimate them to the procedure, and then 10 additional cycles were measured to obtain the daily mean systolic pressure. The criterion for acceptance of measurements was at least 5 recorded pressures per run that had a standard deviation of <30 mm Hg per animal.

**Serum Lipids and Lipoprotein Concentrations**

Serum total cholesterol concentrations were determined with enzymatic assay kits (Wako Chemical Co). Lipoprotein cholesterol distributions were evaluated in individual serum samples (50 μL) from 8 mice in each group after fractionation by size-exclusion chromatography on a single Superose 6 column. Fractions were collected, and cholesterol concentrations were determined with enzymatic kits.

**Quantification of Atherosclerosis and Characterization of Aneurysms**

Atherosclerotic lesions were quantified in the arch and thoracic aorta as described previously. The abdominal aorta was excluded from analysis owing to the presence of large AAAs. Data are expressed as percent of the intimal surface covered by grossly discernible atherosclerotic lesions. Determination of the presence of an AAA was made by 2 independent observers. The severity of AAA was based on a previously described classification in which AAAs were assigned to a group dependent on the gross appearance of the tissue.

**Statistics**

For each parameter, the mean and SEM were calculated. Blood pressure data were analyzed by 3-way repeated-measures ANOVA followed by Tukey’s post hoc test (SAS statistical by a 2-way ANOVA followed by Tukey’s post hoc test for all pairwise comparisons. Incidence of aneurysm formation was examined by using Fisher’s exact test. Values of P<0.05 were considered statistically significant.

**Results**

All mice tolerated the administration of doxycycline well, with no observable adverse reactions and no significant effects on body weight (Table 1).

**Doxycycline Did Not Alter the Development of Hypertension in Response to AngII**

LDL receptor−/− mice infused with AngII developed moderate increases (≈25 mm Hg) in systolic blood pressure

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**TABLE 1. Body Weight and Serum Cholesterol Concentrations**

<table>
<thead>
<tr>
<th>Osmotic Pump Contents</th>
<th>Drinking Water Additives</th>
<th>n</th>
<th>Body Weight, g</th>
<th>Total Cholesterol Concentrations, mg/dL</th>
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</thead>
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<tr>
<td>Saline</td>
<td>None-control</td>
<td>9</td>
<td>28.2±0.7</td>
<td>757±50</td>
</tr>
<tr>
<td>AngII</td>
<td>None-control</td>
<td>21</td>
<td>27.7±0.5</td>
<td>755±25</td>
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<tr>
<td></td>
<td>Doxycycline</td>
<td>12</td>
<td>28.5±0.6</td>
<td>704±47</td>
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<tr>
<td></td>
<td>Doxycycline</td>
<td>20</td>
<td>27.6±0.5</td>
<td>751±35</td>
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</table>

Body weights were measured weekly throughout the study, and values are represented from measurements at the conclusion of the study. Total cholesterol concentrations in serum were measured from blood recovered at the study termination. All data are represented as mean±SEM. No values were significantly different from each other.
during the 4 weeks of the study. We did not demonstrate an increase in arterial blood pressure at this dose during AngII infusion in previous studies; however, those were performed in anesthetized mice. Doxycycline did not affect systolic blood pressure in either AngII- or saline-infused mice (Figure 1).

Doxycycline Did Not Alter the Distribution of Lipoprotein Cholesterol
To determine whether doxycycline administration had any effect on lipid metabolism, plasma cholesterol concentrations were measured, and the distribution of lipoprotein cholesterol was determined by size-exclusion chromatography. Serum total cholesterol concentrations were unchanged by AngII or doxycycline administration (Table 1). Also, there were no effects of AngII or doxycycline administration on the distribution of cholesterol among lipoprotein fractions (Figure 2).

Doxycycline Did Not Alter the Extent of AngII-Induced Atherosclerosis
The extent of atherosclerosis was measured by an en face method and expressed as percent lesion covering the intimal area. Doxycycline had no effect on lesion area in saline-infused mice. As reported previously, AngII infusions produced significant increases in lesion area in the thoracic aorta (Figure 3). Increases in lesion area in the aorta in response to AngII were not influenced by doxycycline (19±1% vs 17±1%, AngII alone vs AngII + doxycycline, respectively).

Doxycycline Markedly Attenuated AngII-Induced AAAs
The incidence of AAAs in the suprarenal aorta of AngII-infused mice was 85%. Orally administered doxycycline decreased the incidence (85% vs 35%, AngII alone vs AngII + doxycycline, respectively; P<0.003; Table 2) and the severity of aneurysms formed by AngII (Figure 4). Both thrombus formation and the extensive dilation of the aorta seen in AngII-infused mice (Figure 4A) were inhibited in mice administered doxycycline. In the few AAAs that formed in AngII-infused mice given doxycycline, there was slight hypertrophy of the surrounding adventitial tissue, yet markedly less than that seen in AngII-infused mice. By using a previously described classification scheme to provide an index of pathological severity, we ascertained that AAAs formed in mice treated with doxycycline were much less

Figure 1. Systolic blood pressure measured by a computerized tail-cuff technique. Systolic blood pressure recorded during saline or AngII infusion (A) was not influenced by doxycycline administration (B). Symbols represent the mean systolic pressure (mm Hg) ± SEM per group for saline-infused (open circles) and AngII-infused (closed circles) mice. AngII induced a significant increase in systolic blood pressure that was not influenced by doxycycline administration. P<0.05 for AngII- vs saline-infused mice.

Figure 2. Serum lipoprotein distribution: cholesterol distribution was not changed by the administration of either AngII or doxycycline. Lipids were resolved by size-exclusion chromatography on a Superose 6 column. Total cholesterol concentrations are expressed as mean absorbance per fraction. Symbols represent the mean±SEM of 8 individual mice per group: saline + control (open circles); saline + doxycycline (closed circles); AngII + control (open triangles); and AngII + doxycycline (closed triangles).

Figure 3. Quantification of atherosclerosis: the extent of aortic intimal surface covered by grossly discernible lesions was determined from en face preparations of the thoracic aorta. AngII infusion increased the extent of atherosclerosis, but this was unaffected by doxycycline. Histobars represent the mean±SEM for each group (open histobars, control treated; filled histobars, doxycycline-treated). *P<0.05 for AngII compared with saline-infused mice. Doxycycline produced no significant effect.
advanced than those observed in mice infused with AngII alone (Figure 4B).3,4

Discussion

We have recently demonstrated that AngII infusion into hyperlipidemic mice greatly accelerates the extent of atherosclerosis and promotes the formation of pronounced AAAs.1–4 The present study demonstrates that administration of doxycycline had no significant effect on AngII-augmented atherosclerosis in LDL receptor−/− mice but markedly attenuated the incidence and severity of aneurysm formation. Reductions in AngII-induced AAA formation by doxycycline occurred independent of alterations in plasma lipids or systolic blood pressure.

Inhibition of MMPs by Doxycycline

Doxycycline has several pharmacological properties, but its therapeutic utility in reducing AAAs in experimental mice and humans has been ascribed to inhibition of MMPs.26 In the present study, the dose of doxycycline administered to inhibit MMPs was based on previous reports.26,40,41 Doxycycline inhibits a broad range of MMPs by proposed mechanisms that include transcriptional inhibition and a direct effect by coordination with the catalytic site.35 In preliminary studies, we found that the dose of doxycycline used in the present study inhibited surgically induced elevations in serum MMP-9 (authors’ unpublished observations). However, serum concentrations may not be indicative of the induction of augmented local proteolytic activity in discrete regions of arteries. We propose inhibition of local MMPs as the mechanism of doxycycline’s attenuation of AAA formation. This assertion is based on the drug’s known inhibition of this class of enzymes and the demonstrated role of specific MMPs in the disease process. Further studies with structural analogues of the drug may assist in establishing this mechanism.

Lack of Effect of Doxycycline on AngII-Induced Atherosclerotic Lesions

Several MMPs are present in atherosclerotic lesions, as indicated earlier. However, the mere presence of MMPs in tissue is not an indication of the extent of activity, because this is dependent on complex regulatory steps of enzyme activation and attenuation by endogenous inhibitors.15 In situ gel zymography has demonstrated that MMP activity in atherosclerotic lesions is enhanced, particularly at shoulder regions.7,42 Although MMP activity is commonly considered detrimental to the development of atherosclerotic lesions, this notion has been challenged by recent studies in mice in which MMP-1 was overexpressed in a macrophage-specific manner or in which MMP-3 was deleted.17,18 Overexpression of MMP-1, which is not normally expressed in mice, unexpectedly decreased the extent of atherosclerosis,18 whereas deletion of MMP-3 had no effect on lesion size.17 The effect of TIMP-1–mediated inhibition of MMPs is unclear, because both overexpression and deletion studies have demonstrated reduced atherosclerosis in apoE−/− mice.16,43 Further studies are needed to

<table>
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<th>TABLE 2. Incidence of AAAs Formed in AngII- and Saline-Infused LDL Receptor−/− Mice</th>
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<td>Groups</td>
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<tr>
<td>Osmotic Pump Contents</td>
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<tr>
<td>Saline</td>
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<td>AngII</td>
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<td>Saline</td>
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<td>AngII</td>
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*P=0.003 by Fishers Exact Test for comparison of AngII-infused mice with or without doxycycline.

Figure 4. Doxycycline decreases the severity of aneurysm formation. A, Photographs of 5 examples of the small number of aneurysms seen in AngII-infused mice administered doxycycline, compared with mice given AngII alone. No thrombus or dissecting were observed in AngII-infused mice that were administered doxycycline. All images are shown at the same magnification and are aligned at the renal branches (arrow). B, Aneurysms were graded according to our previously described classification scheme to provide a measure of the severity of the pathology that developed.9
Determine whether prolonged administration of doxycycline influences lesion formation in LDL receptor−/− mice.

This is the first study in which the effects of doxycycline on AngII-induced atherosclerosis have been defined. Doxycycline was used as a broad inhibitor of MMPs to effectively inhibit multiple MMPs. At the dosage used, there was no effect of doxycycline on AngII-induced atherosclerosis. These results indicate that MMPs do not contribute to the rapidly formed atherosclerotic lesions that develop in response to 1 month of AngII infusion. However, this does not discount a role for MMPs during more protracted intervals of AngII administration or a hyperlipidemic diet. Also, it does not negate a role for MMPs in advanced stages of the disease, in which their activity may be a determinant of plaque rupture.44

Doxycycline Inhibits AngII-Induced AAA Formation
MMPs are hypothesized to be important mediators in the development of AAs, although other elastolytic enzymes, such as cathepsins S and K, have also been speculated to be involved in the disease process.45 Consistent with the hypothesis that MMPs are involved in AAA formation, administration of doxycycline significantly reduced both the incidence and severity of AngII-induced AAA formation. AngII infusion into hyperlipidemic mice leads to medial degeneration that presumably underlies the development of AAs.1 Medial degeneration may be a primary event that leads to inflammation due to the chemotactic properties of elastin degradation products. Alternatively, medial degeneration may be caused by the inflammatory process. Although the sequence of biochemical and cellular events in evolving AAs has not been defined, MMP inhibition could potentially contribute to attenuation of AAA formation either by inhibiting MMPs within the arterial wall or in the infiltrating leukocytes.

The continued presence of some aneurysmal formation may have been due to incomplete MMP inhibition at the doxycycline dose used. However, the AAs in doxycycline-administered mice were minor expansions of the suprarenal aorta that lacked a significant thrombotic component. The ability of doxycycline to inhibit formation of AAs in this and other animal models of the disease indicates the importance of this group of enzymes to the disease process.40,41,46

Divergence of Effects on Atherosclerosis and AAs
It has been commonly considered that AAs are a consequence of the atherosclerotic process, although this viewpoint has been challenged.57,40 The present study demonstrated a divergent effect of doxycycline on the extent of atherosclerosis and the severity of AAs. A differential effect of manipulation of MMPs on atherosclerosis versus aortic aneurysm has been noted in mouse studies with other MMP inhibitors49 and in mice with deficiencies of either MMP-3 or TIMP-1.16,17 The AAs that form in response to AngII are highly localized to the suprarenal artery, a region of the aorta that does not typically exhibit atherosclerosis in mice of this young age.1,2,40 Moreover, in earlier studies, we have not observed atherosclerotic lesions in the region of AAA formation.2 However, previous results from our laboratory demonstrate that a hyperlipidemic environment markedly augments the formation of AAs by AngII in female mice.2 Therefore, although hyperlipidemia promotes AngII-induced AAs, the divergent effects of doxycycline on these vascular pathologies provide further evidence that atherosclerosis may not be responsible for the formation of AngII-induced AAs.

Conclusions
In summary, results from this study demonstrate that doxycycline has negligible effects on the development of AngII-induced atherosclerosis in LDL receptor−/− mice but significantly attenuates the development of AAs. The proposed mechanism for the effect of doxycycline on AngII-induced AAs is inhibition of MMPs elaborated from cells at the vascular lesion and a reduction in degradation of the extracellular matrix. Studies to further define the role of MMPs in AngII-induced AAA formation will require selective MMP inhibitors and mice with deficiencies of a specific MMP.

Acknowledgment
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