Angiotensin II–Induced Hypertension Accelerates the Development of Atherosclerosis in ApoE-Deficient Mice

Daiana Weiss, MD; John J. Kools, MS; W. Robert Taylor, MD, PhD

Background—Angiotensin II may contribute to the development and progression of atherosclerotic lesions because of its growth and proinflammatory effects. We sought to determine whether angiotensin II–induced hypertension would augment and accelerate the development of atherosclerotic lesions in apoE-deficient mice.

Methods and Results—Angiotensin II (0.7 mg·kg⁻¹·d⁻¹ SC) was administered to apoE-deficient mice via osmotic minipumps. The animals were placed on either standard chow or an atherogenic diet. After 8 weeks, the mean atherosclerotic lesion area in the descending thoracic and abdominal aortas of animals on a standard chow diet was 0.4±0.1% compared with 5.2±1.2% in those animals maintained on an atherogenic diet (P<0.0001). In angiotensin II–treated animals on standard chow, the mean lesion area was increased to 11.0±2.3%, which was further increased to 69.9±9.4% (P<0.0001) in angiotensin II–treated animals on an atherogenic diet. Similar findings were obtained when tissues from the ascending aorta were analyzed. At 8 weeks in mice receiving a standard chow diet, angiotensin II dramatically increased the atherosclerotic lesion area by 840±83 μm² (P<0.0001). Animals on a high-fat diet had a similar marked increase in lesion area in response to angiotensin II (217±19 μm², P<0.0001). In contrast, when hypertension was induced with norepinephrine, only a modest effect on the atherosclerotic lesion area was observed.

Conclusions—Angiotensin II–induced hypertension specifically increased the development of atherosclerosis in apoE knockout mice. This response was seen in animals receiving either standard chow or an atherogenic diet. These studies demonstrate the profound effect of angiotensin II on the development of atherosclerosis. (Circulation. 2001;103:448-454.)

Key Words: apolipoproteins ■ angiotensin ■ hypertension ■ atherosclerosis

Numerous clinical and epidemiological studies have identified systemic arterial hypertension as an independent and potent risk factor for the development of atherosclerotic disease.1,2 Despite the enormous progress in our understanding of the mechanisms of atherosclerosis, the molecular and cellular linkages between hypertension and atherosclerosis have yet to be clearly defined.

Hypertension presents a complex biological stimulus to the arterial wall. The ultimate response of the arterial wall to hypertension is likely the synthesis of both mechanical and humoral stimuli. The mechanical effects of elevated blood pressure on the arterial wall are not well understood. Elevated blood pressure increases wall stress in a nonuniform distribution.3,4 Increased wall stress has been proposed to be a proinflammatory stimulus,5 as evidenced by the association between mechanical strain and the production of reactive oxygen species6,7 and the expression of inflammatory gene products.8–10 Indeed, wall stress has been shown to be a biologically relevant stimulus for the development of atherosclerosis.11

In addition, the cellular components of the arterial wall are exposed to multiple neurohumoral signals that are undoubtedly altered in hypertension. Several lines of evidence have specifically implicated the renin–angiotensin II system in the pathogenesis of atherosclerosis.12–15 First, the cellular consequences of angiotensin II stimulation are potentially proatherogenic. Angiotensin II stimulation results in the production of reactive oxygen species as well as increased expression of proinflammatory gene products.14,16 Given the acknowledged role of oxidative stress and vascular inflammation in atherosclerosis,17 these effects of angiotensin II are likely mechanistically relevant to the development and progression of atherosclerotic lesions. Second, studies performed in several experimental models of atherosclerosis indicate that either inhibition of ACE activity or blockade of angiotensin type 1 receptors results in a decrease in the development of atherosclerotic lesions.18–21 Finally, numerous studies in humans have shown that high renin forms of hypertension are particularly proatherogenic and that patients with high renin hypertension are at increased risk of myocardial infarction.22–25 The recently presented results of the Heart Outcomes Prevention Evaluation (HOPE) trial demonstrated a remarkable decrease in cardiovascular morbidity and mortality when normotensive individuals at increased risk for...
cardiovascular events were treated with an ACE inhibitor.26
Taken together, these data specifically implicate the renin–angiotensin II system in the pathogenesis of atherosclerosis.

The primary goal of the present study was to define the specific importance of angiotensin II–induced hypertension in the development of atherosclerosis. We used apoE-deficient mice that were made hypertensive through the use of a continuous infusion of angiotensin II. We found that angiotensin II–induced hypertension dramatically accelerated the initiation and progression of atherosclerotic lesions. Conversely, when hypertension was induced to a similar level by the administration of norepinephrine, we found only a modest increase in atherosclerosis. These data demonstrate the extremely potent proatherogenic effects of angiotensin II in apoE-deficient mice and provide further support for the hypothesis that angiotensin II is mechanistically relevant in the pathogenesis of atherosclerosis.

Methods

Animals and Diets
Control C57BL/6 and homozygous apoE-deficient (C57BL/6 back- ground) male mice were purchased from the Jackson Laboratory (Bar Harbor, Me). Mice aged between 4 and 6 weeks were used in the present study. Eighty control mice and 48 apoE-deficient mice were each divided into 4 groups: (1) standard chow diet (Purina, Certified Rodent Chow 5001), (2) standard chow diet with angiotensin II treatment (0.7 mg · kg$^{-1}$ · d$^{-1}$ SC), (3) atherogenic diet (Research Diets, Inc), and (4) atherogenic diet with angiotensin II infusion. The atherogenic diet was made of purified components and was designed to match the original “Pigden’s Atherogenic Rodent Diet.” The components per kilogram as listed by the manufacturer include the following: 75 g casein, 130 g soy protein, 2 g β-methionine, 275 g corn starch, 150 g maltodextrin 10, 30 g sucrose, 90 g cellulose, 50 g soy bean oil, 75 g cocoa butter, 35 g coconut oil, 35 g salt mix S10001, 5.5 g calcium carbonate, 8 g sodium chloride, 10 g potassium citrate, 10 g vitamin mix V10001, 2 g choline bitartrate, 12.5 g cholesterol USP, and 5 g sodium cholic acid.

The animals were fed ad libitum and had free access to water. The animals were housed and cared for according to the guidelines proposed by the National Institutes of Health (NIH) for the care and use of experimental animals (NIH publication No. 85-23).

Some mice received angiotensin II infusions via a subcutaneously implanted osmotic minipump (Alzet, model 1002) for 4 or 8 weeks. The mice were anesthetized with 375 mg/kg 2,2,2-tribromoethanol (Avertin, Sigma Chemical Co). An osmotic pump containing angiotensin II dissolved in a solution of 0.15 mol/L NaCl and 0.01N acetic acid at a concentration calculated to deliver ~0.7 mg · kg$^{-1}$ · d$^{-1}$ of drug was inserted into a subcutaneous pocket. The dose of angiotensin II was selected on the basis of previous studies in our laboratories10 and provides a plasma concentration of angiotensin II similar to that reported in patients with renovascular hypertension.

An additional group of animals was treated with norepinephrine in a similar manner.10 The concentration of norepinephrine in the osmotic pump was adjusted to yield a dose of 5.6 mg · kg$^{-1}$ · d$^{-1}$.

Systolic blood pressure28 was measured during treatment and before euthanizing the animals at the end of each experiment by using a computerized, noninvasive, tail-cuff system (BP 2000 Visitech Systems). One set of 10 measurements was obtained for each animal, and the mean blood pressure was calculated. Animals were habituated to the device before measuring the pressures to ensure accurate measurements.

Evaluation of Atherosclerotic Lesions and Histological Analysis
The animals were euthanized by CO$_2$ inhalation at the prescribed time points. The heart and aorta were pressure-perfused with 0.9% sodium chloride solution, followed by pressure fixation at ~100 mm Hg with a 4% formaldehyde solution.

To evaluate the extent of atherosclerotic lesion formation, 2 approaches were used. The descending thoracic and abdominal aorta was analyzed en face, whereas the ascending aorta was reserved for microscopic analysis.29 These complementary approaches allowed us to obtain information about disease extent as well as lesion complexity. The descending thoracic and abdominal aortas were opened longitudinally and stained with oil red O (Sigma) as previously described.30 Photographs of the stained specimens were digitized for data analysis. The luminal lesion surface area was quantified by using NIH Image software (version 1.65) with custom macros. Data were expressed as the percentage of the descending aorta with positive oil red O staining.

The hearts and aortas were embedded in paraffin, and 5-μm-thick serial sections were prepared. Every 10th section was stained with hematoxylin and eosin. For quantification of luminal cross-sectional area involved by atherosclerotic plaque in the ascending aorta, digital images of the stained sections obtained at the level of the aortic valve were obtained. The atherosclerotic lesions were manually traced on the computer. Care was taken to exclude normal-appearing media and to include only the intimal/subintimal atherosclerotic lesions. Data were expressed as total lesion area of the cross section.

Statistical Analysis
All data are given as mean ± SEM. Statistical significance was determined by ANOVA. Post hoc analysis was performed by the Duncan New Multiple Range Test.

Results
Hypertension was persistent through up to 8 weeks of continuous angiotensin II infusion. The mean systolic arterial blood pressures obtained are presented in Table 1. There was no significant effect of diet on systolic blood pressure. Mean total cholesterol data are presented in Table 2.

Effects of Angiotensin II Infusion on the Development and Extent of Atherosclerosis in ApoE-Deficient Animals
Angiotensin II–induced hypertension had a dramatic effect on the development of atherosclerosis in the descending thoracic and abdominal aorta. Representative examples of en face preparations of the descending thoracoabdominal aorta after 8 weeks of treatment are shown in Figure 1. Note that regions of atherosclerosis appear white because these images were obtained before staining with oil red O. In the angiotensin

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<th>TABLE 1. Mean Blood Pressures in Angiotensin II–Treated Animals</th>
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<td>Control</td>
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<td>Atherogenic diet</td>
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<td>Angiotensin II</td>
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<td>Angiotensin II+</td>
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<td>atherogenic diet</td>
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Values are mean ± SEM. Numbers in parentheses indicate number of animals in each group.

*P<0.0001 vs control.
II–treated animals on the atherogenic diet, the descending thoracic and abdominal aortas were almost completely covered with atherosclerotic lesions. Importantly, compared with the control animals, the angiotensin II–treated animals on the normal chow diet also exhibited a marked worsening in the degree of atherosclerosis (Figure 2).

The proximal ascending aorta was also examined for evidence of accelerated atherogenesis in the angiotensin II–treated apoE-deficient animals. Compared with no treatment, the angiotensin II treatment resulted in a dramatic increase in atherosclerosis of the ascending aorta in the apoE-deficient animals on regular chow or the atherogenic diet (Figure 3).

**Effects of Angiotensin II Infusion on Atherosclerosis in Wild-Type Animals**

Wild-type animals made hypertensive with angiotensin II that were fed either a standard chow or atherogenic diet did not develop atherosclerotic lesions after 8 weeks of treatment. Therefore, additional animals were treated for a total of 12 weeks. However, even after 12 weeks, angiotensin II treatment resulted in a marked worsening in the degree of atherosclerosis (Figure 2).

**Effect of Norepinephrine-Induced Hypertension on Development and Extent of Atherosclerosis in ApoE-Deficient Animals**

To gain insight into the relative contributions of high blood pressure and angiotensin II, per se, we used norepinephrine infusion as an alternate model of hypertension. Mean systolic blood pressures for these animals are given in Table 3. Note that the degree of hypertension induced in the norepinephrine-treated animals was similar to that obtained in the angiotensin II–treated animals.

We found that compared with angiotensin II–induced hypertension, norepinephrine-induced hypertension had a modest effect on the development of atherosclerosis. Figure 5 shows a representative example of the descending thoracic and abdominal aorta of an apoE-deficient animal made hypertensive with norepinephrine and maintained on an atherogenic diet for 8 weeks. In the ascending aorta, there was no significant effect of norepinephrine-induced hypertension on the atherosclerotic lesion area (Figure 6). Whereas norepinephrine significantly increased the development of athero-

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**Table 2. Mean Total Cholesterol Levels**

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<tr>
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<th>ApoE Deficient</th>
<th>Wild Type</th>
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<tr>
<td>Control</td>
<td>533±96</td>
<td>71±3</td>
</tr>
<tr>
<td>Standard diet+angiotensin II</td>
<td>440±14</td>
<td>79±5</td>
</tr>
<tr>
<td>Atherogenic Diet</td>
<td>2971±250*</td>
<td>310±21*</td>
</tr>
<tr>
<td>Atherogenic Diet+angiotensin II</td>
<td>2571±122*</td>
<td>312±5*</td>
</tr>
<tr>
<td>Atherogenic Diet+norepinephrine</td>
<td>2476±139*</td>
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</table>

Values are mean±SEM. *P<0.0001 vs animals maintained on control diet.

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**Tables**

**Figure 2.** Effect of Ang II–induced hypertension on atherosclerotic lesion development in descending thoracic and abdominal aorta of apoE-deficient mice on either standard chow (Std diet) or atherogenic diet. Data are expressed as percent of total lumenal surface occupied by lesions. *P<0.0001 compared with Std diet.

**Figure 3.** Effect of Ang II–induced hypertension on atherosclerotic lesion development in proximal ascending thoracic aorta of apoE-deficient mice on either standard (Std) diet or atherogenic diet. Atherosclerotic lesion severity is expressed as total cross-sectional area of all lesions.* P<0.0001 compared with Std diet.
sclerosis in the descending thoracic and abdominal aorta, the magnitude of the increase was far less than that seen with angiotensin II–induced hypertension (Figure 2).

To gain insight into the potential role of mononuclear inflammation in this process, we stained paraffin-embedded sections of the ascending aorta with a murine macrophage–specific antibody (Figure 7). The most intense staining occurred in the angiotensin II–treated animals. There was evidence of a marked macrophage infiltration of the atherosclerotic lesions as well as the adventitia.

Discussion

We have shown that angiotensin II–induced hypertension dramatically increases the development of atherosclerosis in apoE-deficient mice. This response was seen in animals that received either an atherogenic diet or standard chow. Lesions developed after a relatively short period of treatment, and the extent of lesion development was markedly increased in hypertensive animals. The most dramatic results were seen in the descending thoracoabdominal aorta, where the luminal surface of the aortas in angiotensin II–treated animals on an atherogenic diet was almost completely covered with atherosclerotic lesions after only 8 weeks of treatment. These results demonstrate the very potent proatherogenic effect of short-term administration of angiotensin II in apoE-deficient mice.

The effects of angiotensin II administration on atherosclerotic lesion development in both the ascending and descending aorta were evident after only 4 weeks of treatment. In normotensive apoE-deficient animals receiving regular chow, it has been previously reported that fatty streak formation occurs in the proximal ascending aorta at no earlier than 10 weeks of age.31 The advanced, complicated, fibroproliferative plaque associated with severe luminal stenosis of coronary arteries has been reported to appear at 25 to 40 weeks of age in apoE-deficient mice fed the western-type diet. 31 Clearly, the development of complex atherosclerotic lesions in the angiotensin II–treated animals after only 4 to 8 weeks of treatment represents a marked acceleration of the disease process.

Previous studies have implicated the renin-angiotensin system in the pathogenesis of atherosclerosis. Observational studies in humans have demonstrated that patients with high levels of circulating renin are at increased risk for myocardial infarction and other atherosclerotic cardiovascular events.1,22–25,32 In addition, studies in several different models of atherosclerosis have shown that ACE inhibitors and angiotensin–type 1 receptor antagonists inhibit atherosclerotic lesion formation.18–21

The mechanism whereby angiotensin II–induced hypertension accelerates and worsens atherosclerosis is probably multifactorial. Angiotensin II has direct effects on the cellular components of the arterial wall.15 In addition, there may be effects on the arterial wall related to the resultant mechanical forces that occur as a result of elevated blood pressure.5 We have demonstrated that although norepinephrine-induced hypertension did accelerate the development of atherosclerosis in apoE-deficient animals, the magnitude of the response was
markedly less than that seen with angiotensin II–induced hypertension. Therefore, the data in the present study indicate that the dramatic proatherogenic effects of angiotensin II infusion are largely due to the humoral effects of angiotensin II and not the mechanical effects of elevated blood pressure.

Angiotensin II has several relevant direct and indirect humoral effects that may be involved in the pathogenesis of atherosclerosis. Indirect mechanisms include alterations in sympathetic outflow, aldosterone secretion, and prostaglandins.33 A central component of angiotensin II signaling is the generation of reactive oxygen species. Angiotensin II activates the NAD(P)H oxidase in cultured vascular smooth muscle cells,16 which results in the generation of reactive oxygen species. This oxidase has been functionally linked to the development of vascular hypertrophy in response to angiotensin II44 and the generation of an inflammatory response in vascular smooth muscle cells.15,35 Our finding of a dense macrophage infiltration of the arterial wall in the angiotensin II–treated animals suggests that this process may be relevant to the findings reported in the present study. Angiotensin II also upregulates thrombin receptor expression in the aortic tissues of rats via a redox-sensitive mechanism.36 This appears to occur via the generation of reactive oxygen species and is independent of the pressor effects of angiotensin II. Angiotensin II upregulates expression of monocyte chemoattractant protein-1 (MCP-1) in cultured vascular smooth muscle cells,37 which may occur through a redox-sensitive pathway. Thus, there are multiple potential humoral mechanisms involving the generation of reactive oxygen species through which angiotensin II could be proatherogenic.

Although our data demonstrate a dominant effect of the humoral components of angiotensin II stimulation on the development of atherosclerosis, the data obtained with norepinephrine-induced hypertension suggest that mechanical factors may also contribute significantly, albeit to a lesser degree. Mechanical deformation of vascular cells results in the upregulation of inflammatory mediators, such as MCP-1.8–10 Furthermore, in both angiotensin II and norepinephrine models of hypertension, expression of MCP-1 is upregulated in the aortic tissues.10 This is associated with increased infiltration of the arterial wall with monocytes/macrophages. Mechanical strain applied to both endothelial and vascular smooth muscle cells results in an increase in the production of reactive oxygen species,6,7 which have been implicated in the development of atherosclerosis.38–40 Mechanoregulation of reactive oxygen species may be an independent mechanism for the proatherogenic effect of hypertension, or reactive oxygen species may function through the upregulation of inflammatory gene products.2,41 Thus, there are several potential mechanisms whereby mechanical effects can contribute to the proatherogenic effects of hypertension.

The present study was performed with the use of an angiotensin II infusion model of hypertension. It is important to note that this is a model of a relatively rare form of hypertension. Thus, the relevance of the findings reported in the present study to the clinical syndrome of hypertension...
depends on part in the relative contributions of the humoral and mechanical aspects of this model. A potential mitigating factor is the role of the tissue-based components of the renin-angiotensin system. As evidenced by the antiatherogenic effects of agents that modulate the renin-angiotensin system,\textsuperscript{18–21} even in the absence of high systemic levels of angiotensin II, locally generated angiotensin II may have a significant impact on the development of atherosclerosis. The recent results of the HOPE trial suggest that angiotensin II may be involved in the development of atherosclerosis in individuals without high renin hypertension.\textsuperscript{26}

After submission of the present article, Daugherty et al\textsuperscript{42} presented data demonstrating a similar proatherogenic effect of angiotensin II in apoE-deficient mice. Their results differ from ours in that they found that doses of angiotensin II similar to those that we used did not result in any increase in blood pressure. This discrepancy may be due to the use of general anesthesia during blood pressure measurements, the exclusive use of female animals, or the older age of the animals used in the studies of Daugherty et al. In addition, they reported the presence of abdominal aortic aneurysms in the animals studied. This difference may also be due to the difference in the age and sex of the animals studied. They noted occasional aneurysm formation but with a lower incidence. The pathophysiology of human atherosclerotic disease is clearly multifactorial.\textsuperscript{43} Hypertension is a well-known risk factor for the development of atherosclerosis. We have shown that angiotensin II–induced hypertension specifically accelerates the development of aortic atherosclerosis in apoE-deficient animals. These findings may provide insight into the relative importance of the renin-angiotensin system in the pathogenesis of atherosclerosis and provide a foundation for future studies to explore the mechanisms through which hypertension promotes the development of atherosclerosis.

Acknowledgments

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References


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