α₁-Adrenergic Plus Angiotensin Receptor Blockade Reduces Atherosclerosis in Apolipoprotein E–Deficient Mice

Konstantinos P. Makaritsis, Haralambos Gavras, Yue Du, Aram V. Chobanian, Peter Brecher

Abstract—We have used the apolipoprotein E (apoE)–deficient mouse model to determine whether both the angiotensin II type 1 (AT₁) and the α₁-adrenergic receptors influence arteriosclerotic changes in this hyperlipidemic animal model. Mice were treated with antihypertensive drugs beginning at 9 weeks of age, and aortic atherosclerosis was measured after 12 weeks of treatment. Systolic blood pressure in the untreated apoE-deficient mouse averaged 104 mm Hg throughout the treatment period. Prazosin at a dose of 7.5 mg · kg⁻¹ · d⁻¹ was ineffective in attenuating atherosclerosis and did not significantly reduce blood pressure. Losartan, at dosages of either 20 or 30 mg · kg⁻¹ · d⁻¹, also did not influence atherosclerosis and had only a slight blood pressure–lowering effect. However, combined treatment with both prazosin and losartan markedly reduced atherosclerotic lesion development from an average lesion size per section of 2.6 to 1.5×10⁴ μm² (P<0.001) and significantly reduced blood pressure to 85±5 mm Hg. Treatment with L⁵-nitro-L-arginine methyl ester (40 mg · kg⁻¹ · d⁻¹) produced significant elevations of blood pressure (127±3.8 mm Hg) but had no effect on the development of atherosclerosis. None of the treatments used affected plasma cholesterol throughout the 12-week period. These studies suggest that the vascular changes associated with atherosclerosis are influenced by a combination of AT₁ and α₁-adrenergic receptor activation. (Hypertension. 1998;32:1044–1048.)

Key Words: atherosclerosis ■ blood pressure ■ losartan ■ prazosin ■ mice ■ hypercholesterolemia

The relationship between hypertension and atherosclerosis is well established, but the mechanisms underlying the role of hypertension as a risk factor are unclear. Angiotensin-converting enzyme (ACE) inhibitors have been shown to inhibit atherosclerosis in the cholesterol-fed rabbit,¹ the Watanabe heritable hyperlipidemic rabbit,² and hamster³ and primate⁴ models. Losartan treatment has been shown to prevent both coronary vascular injury and myocyte damage induced by continuous angiotensin II infusion in the rat.⁵ There have been surprisingly few studies on losartan effects in animal models of atherosclerosis. The angiotensin II type 1 (AT₁) antagonist SC-51316 was shown to have no effect on the progression of atherosclerosis in cholesterol-fed rabbits over a 3-month period.⁶ In contrast, losartan administration was shown to significantly reduce atherosclerotic lesion area in apolipoprotein E (apoE)–deficient mice when given for a period of 3 months.⁷

The α₁-adrenergic system also has been implicated in the development of atherosclerotic lesions. Adrenergic stimulation caused DNA replication in medial smooth muscle cells,⁷ and chronic α₁-adrenergic stimulation increased DNA synthesis in both uninjured and injured arteries in the rat.⁸ Administration of prazosin appears to suppress the mitogenic effect of catecholamines in cultured rat aortic smooth muscle cells,⁹ and doxazosin treatment reduced both serum lipid levels and the number of macrophage-derived foam cells in the aortic arch of the hypercholesterolemic hamster.¹⁰

Recently, mice lacking the gene for apoE have been developed and found to have both profound hypercholesterolemia and the propensity to develop atherosclerotic lesions with many similarities to those found in humans.¹¹ In the present study we have used the apoE-deficient mouse model to determine whether inhibition of AT₁ receptors and α₁-adrenergic receptors might prevent the development of atherosclerosis. These studies suggest that the vascular changes affecting atherosclerosis are influenced by combined effects mediated by both the AT₁ and the α₁-adrenergic receptors.

Methods

Materials

Losartan was a gift from Dupont/Merck (Wilmington, Del). Prazosin hydrochloride, L⁵-nitro-L-arginine methyl ester (L-NAME), and dl-propranolol were purchased from Sigma Chemical Co.

Animals

All mice used in these studies were purchased from the Jackson Laboratory and designated C57BL/6J-apoE. These mice were apoe-deficient female homozygotes (apoE−/−) that were backcrossed for 10 generations to C57BL/6J at the Jackson Laboratories and were delivered to our facilities at 8 weeks of age. The mice were maintained on a standard chow diet (Purina, Certified Rodent Chow 5002) containing 4.5% fat and given free access to both food and

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water throughout the study. After 1 week of acclimation to the animal quarters, drug treatment was initiated, and the animals were maintained for 12 additional weeks on the designated treatment. At 21 weeks of age the mice were killed with an overdose of sodium pentobarbital.

Blood Pressure Measurements
Systolic blood pressure and heart rate measurements were obtained in conscious mice using a computerized, noninvasive tail-cuff system (BP 2000 Visitech Systems). Mice were trained for 5 consecutive days (each session consisting of 20 unrecorded measurements) to familiarize the animals with the tail-cuff apparatus. Subsequently, blood pressure and heart rate measurements were obtained weekly for 12 consecutive weeks in all treatment groups. One set of 20 measurements was obtained for each mouse weekly, and the mean blood pressure and heart rate were calculated.

Drug Treatment
Drugs were administered to the mice in the drinking water. The solutions were prepared at 2- to 3-day intervals, and the concentrations were based on the average volume of water consumed per day (5 mL) and the body weight that increased progressively from about 19 to 22 g per mouse throughout the 12-week treatment period.

Plasma Cholesterol Measurements
Blood samples were obtained from the inferior vena cava of anesthetized animals before the heart was removed for morphometric analysis. Blood was drawn into tubes containing EDTA and immediately centrifuged; total plasma cholesterol was measured enzymatically using a commercially available kit from Sigma Diagnostics.

Evaluation of Atherosclerotic Lesions
The heart and aorta were perfused with PBS and then acetate-buffered formalin. The heart and proximal aorta were then removed, weighed, and kept in formalin overnight. The heart was then sliced with a scalpel to align the aortic root for subsequent sectioning according to the procedure described by Paigen et al. The tissue was processed and embedded in paraffin using conventional procedures. Sections (5 μm) were obtained from the region starting with the aortic sinus, and 5 sections, each spaced 50 to 60 μm apart, were saved for morphometric analysis following the guidelines outlined by Paigen et al. These sections were stained with hematoxylin and eosin and photographed at a magnification of x40. The images from these microscopic sections were scanned into a computer using a Polaroid Sprint 35 scanner. The resulting images were then used to quantify the lesion area measured using NIH Image 3.0 software by carefully outlining the intimal lesion, which could readily be distinguished from the media. The data from each of the 5 sections from each animal were averaged and expressed as the lesion area per section.

Statistical Analysis
All values are expressed as mean±SEM. Values for body and heart weights, blood pressure, heart rate, plasma cholesterol, and quantification of lesion size were compared using 1-way ANOVA. Subsequent comparisons were performed using a 2-tailed unpaired Student’s t test.

Results
Effects of Prazosin, Losartan, and Combined Treatment
Figure 1 compares data from 21-week-old untreated apoE (−/−) homozygous mice with those treated for 12 weeks with prazosin (7.5 mg · kg⁻¹ · d⁻¹), losartan (30 mg · kg⁻¹ · d⁻¹), or a combination of those drugs. Figure 1 (top) summarizes the degree of atherosclerosis found in the aortic sinus area and shows that the combined treatment clearly decreased lesion size, whereas individual treatment with either prazosin or losartan did not significantly affect the progression of atherosclerosis. These data in Figure 1 were obtained using at least 9 mice per group and included at least 2 separate prospective comparisons performed over an 8-month period. Systolic blood pressure measurements made during the last week of treatment averaged 105 mm Hg for the control group and was not affected by prazosin treatment, although animals given either losartan alone or prazosin+losartan did have a significant reduction in systolic blood pressure when compared with controls (Figure 1, middle). Total plasma cholesterol levels (Figure 1, bottom) were unaffected by any of the drugs. The data in Figure 2 show that neither heart rate, heart weight, nor the ratio of heart weight to body weight was influenced by treatment with prazosin, losartan, or prazosin+losartan at the dosages given. Body weight in all groups was similar, with the weight at the beginning of treatment (9 weeks of age) averaging 19.6 g and the final body weight at 21 weeks of age averaging 22.6 g.

Figure 3 shows representative histological sections stained with hematoxylin and eosin from 21-week-old control apoE-deficient mice and those given the combination of prazosin and losartan for 12 weeks. Consistent with other published
studies using the apoE knockout mouse, lesions were well developed by 21 weeks of age, consisting of both foam cells and fibrous plaques. The size and severity of the lesions were visibly reduced by the combined treatment. Sections from losartan- or prazosin-treated mice were indistinguishable from the sections taken from 21-week-old untreated apoE knockout mice (data not shown).

Effects of L-NAME and Propranolol Treatment

The Table summarizes measurements of lesion area and other parameters obtained after 12 weeks of treatment in mice given L-NAME and DL-propranolol. L-NAME did not affect the progression of atherosclerosis, but it increased systolic blood pressure significantly from 105 to as much as 127 mm Hg when given at a dosage of 40 mg \( \text{kg}^{-1} \cdot \text{d}^{-1} \). If given at a lower dosage (20 mg \( \text{kg}^{-1} \cdot \text{d}^{-1} \)), the blood pressure elevation was less apparent and not statistically significant. Again no change in atherosclerosis was found. Neither L-NAME nor propranolol changed plasma cholesterol or the heart weight/body weight ratio, but L-NAME at the lower dosage did produce a slight but statistically significant decrease in heart rate. Propranolol did not alter either blood pressure or heart rate in the apoE mouse, and its effect on atherosclerosis was not statistically significant.

Effect of Drug Treatment on Lesion Area, Plasma Cholesterol, Systolic Blood Pressure, Heart Rate, and Heart Weight/Body Weight in ApoE Knockout Mice at 21 Weeks of Age

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>No. of Animals</th>
<th>Lesion Area, ( \mu \text{m}^2/\text{Section} \times 1000 )</th>
<th>Plasma Cholesterol, mmol/L</th>
<th>Systolic Blood Pressure, mm Hg</th>
<th>Heart Rate, bpm</th>
<th>Heart Weight/Body Weight, ( \times 1000 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no treatment)</td>
<td>17</td>
<td>261±20</td>
<td>10.6±0.8</td>
<td>105±3.2</td>
<td>557±11</td>
<td>5.9±0.3</td>
</tr>
<tr>
<td>L-NAME, 40 mg ( \text{kg}^{-1} \cdot \text{d}^{-1} )</td>
<td>6</td>
<td>232±17</td>
<td>15.1±0.5</td>
<td>127±3.8*</td>
<td>540±24</td>
<td>6.9±0.7</td>
</tr>
<tr>
<td>L-NAME, 20 mg ( \text{kg}^{-1} \cdot \text{d}^{-1} )</td>
<td>6</td>
<td>280±55</td>
<td>10.7±1.1</td>
<td>112±1.9</td>
<td>486±15†</td>
<td>5.4±0.1</td>
</tr>
<tr>
<td>Propranolol, 40 mg ( \text{kg}^{-1} \cdot \text{d}^{-1} )</td>
<td>6</td>
<td>187±14</td>
<td>12.4±0.6</td>
<td>106±4.6</td>
<td>563±8</td>
<td>5.6±0.2</td>
</tr>
</tbody>
</table>

All data are expressed as mean±SEM.

\( *P=0.001 \) vs control; \( †P=0.004 \) vs control.
treatment with prazosin may be indicated by our findings. The combined model.

between hypertension and atherosclerosis in this animal mouse show a novel response to clinically useful agents in the in vivo studies reported here regarding the apoE knockout mouse. The treatment of hypertension and suggest a possible association affecting aortic atherosclerosis in the apoE-deficient mouse. The atherosclerosis make interspecies comparisons difficult.

Our studies on losartan are directly in conflict with a recent report showing that losartan treatment inhibited atherosclerosis in the apoE-deficient mouse. In that study, mice were treated with losartan (25 mg · kg⁻¹ · d⁻¹) for 12 weeks, and atherosclerosis measured at the age of 5 months was reduced by 80%, an effect attributed to changes in LDL lipid peroxidation. We have no explanation for the striking difference between this study and our own, but in mice from the Jackson Laboratories we have reproduced our findings with losartan treatment in 2 separate experiments conducted several months apart and found no effect of losartan at 20 or 30 mg · kg⁻¹ · d⁻¹ after a 12-week treatment period. Over a period of 2 years we have reproducibly found that the atherosclerotic index of 21-week-old female mice averaged 2.6×10⁷ µm² per section, whereas the comparable value found in the study of Keidar et al was 0.80×10⁷ µm² per section.

The relationship between ACE activity and atherosclerosis was studied recently by Krege et al, who showed that when mice with a mutation that affects ACE activity were bred with mice heterozygous for apoE gene disruption, the reduction in L-NAME treatment, even at dosages that did not elevate blood pressure, also increased atherosclerosis. Species differences between the hypercholesterolemic rabbit and apoE knockout mice with respect to the factors influencing atherosclerosis make interspecies comparisons difficult.

Discussion

A major finding in this study is that combined treatment with prazosin and losartan effectively inhibited the progression of atherosclerosis in the apoE knockout mouse. This combination of antihypertensive drugs has not been used in other animal models of atherosclerosis. In contrast to a study showing that chronic losartan treatment in the apoE-deficient mouse led to an 80% decrease in the progression of atherosclerosis, we found that losartan treatment alone did not affect aortic atherosclerosis in the apoE-deficient mouse. The in vivo studies reported here regarding the apoE knockout mouse show a novel response to clinically useful agents in the treatment of hypertension and suggest a possible association between hypertension and atherosclerosis in this animal model.

A role for blood pressure reduction in reducing atherosclerosis may be indicated by our findings. The combined treatment with prazosin+losartan produced a striking reduction in blood pressure that was both rapid and more pronounced than that seen with losartan treatment alone. Studies using ACE inhibitors in genetically hypercholesterolemic rabbits also found results that could be explained in part by blood pressure effects. Our studies with L-NAME treatment clearly showed an increased systolic pressure but no effect on the severity or size of the atherosclerotic lesion, suggesting that increased blood pressure per se does not necessarily accelerate atherosclerosis in this model. In contrast, a study using a 1-kidney, 1-clip model of renovascular hypertension in the Watanabe heritable hyperlipidemia rabbit produced blood pressure increases of 40 to 60 mm Hg over levels in normotensive controls and led to enhanced atherosclerosis after either a 3- or 6-month treatment period. Another study using hypercholesterolemic rabbits has indicated that L-NAME treatment, even at dosages that did not elevate blood pressure, also increased atherosclerosis. Species differences between the hypercholesterolemic rabbit and apoE knockout mouse with respect to the factors influencing atherosclerosis make interspecies comparisons difficult.

Our studies on losartan are directly in conflict with a recent report showing that losartan treatment inhibited atherosclerosis in the apoE-deficient mouse. In that study, mice were treated with losartan (25 mg · kg⁻¹ · d⁻¹) for 12 weeks, and atherosclerosis measured at the age of 5 months was reduced by 80%, an effect attributed to changes in LDL lipid peroxidation. We have no explanation for the striking difference between this study and our own, but in mice from the Jackson Laboratories we have reproduced our findings with losartan treatment in 2 separate experiments conducted several months apart and found no effect of losartan at 20 or 30 mg · kg⁻¹ · d⁻¹ after a 12-week treatment period. Over a period of 2 years we have reproducibly found that the atherosclerotic index of 21-week-old female mice averaged 2.6×10⁷ µm² per section, whereas the comparable value found in the study of Keidar et al was 0.80×10⁷ µm² per section.

The relationship between ACE activity and atherosclerosis was studied recently by Krege et al, who showed that when mice with a mutation that affects ACE activity were bred with mice heterozygous for apoE gene disruption, the reduction in ACE activity had no effect on the progression of atherosclerosis induced by a cholesterol-rich diet. Those studies, which suggested that genetic variation in the level of ACE does not affect the development of atherosclerosis, are consistent with our findings that losartan did not influence the course of atherosclerosis in the homozygous apoE knockout mouse fed a normal diet. ACE inhibitors have been shown to reduce atherosclerosis in both the Watanabe heritable hypercholesterolemic rabbit and the cholesterol-fed rabbit model, suggesting either a role for bradykinin or that the effect of angiotensin II in the progression of atherosclerosis may differ in mice and rabbit models. This seems to be the case for probucol treatment, which reduced atherosclerosis in the Watanabe heritable hypercholesterolemic rabbit but increased atherosclerosis in the apoE-deficient mouse model.

Other mechanistic explanations for the combined effects of an AT₁ and α₁-adrenergic antagonist could involve direct effects on the cell types often associated with the pathogenesis of atherosclerosis. Because of the complex interactions that occur in vivo over prolonged periods, it is difficult to define the precise mechanism(s) responsible for this novel in vivo effect, but our results do indicate that combined treatment with both prazosin and losartan results in a significant inhibition of atherosclerosis in a hyperlipidemic animal model.
model that has many characteristics comparable to lesion
development in humans.

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