Inhibition of Diet-Induced Atherosclerosis and Endothelial Dysfunction in Apolipoprotein E/Angiotensin II Type 1A Receptor Double-Knockout Mice

Sven Wassmann, MD; Thomas Czech, MS; Martin van Eickels, MD; Ingrid Fleming, PhD; Michael Böhm, MD; Georg Nickenig, MD

Background—Angiotensin II type 1 (AT1) receptor activation is potentially involved in the multifactorial pathogenesis of atherosclerosis.

Methods and Results—Apolipoprotein E–deficient (ApoE−/−) mice were crossed with AT1A receptor–deficient (AT1−/−) mice to obtain homozygous double-knockout animals (ApoE−/−-AT1−/− mice). Wild-type (C57BL/6J), ApoE−/−, AT1−/−, and ApoE−/−-AT1−/− mice were fed a high-cholesterol diet for 7 weeks. In contrast to wild-type and AT1−/− mice, this treatment led to severe atherosclerotic lesion formation in the aortic sinus and the aorta (oil red O staining) and to an impaired endothelium-dependent vasodilation (organ chamber experiments with isolated aortic segments) in ApoE−/− mice. In the age-matched ApoE−/−-AT1−/− littermates, development of diet-induced endothelial dysfunction and atherosclerotic lesion formation was profoundly inhibited. Concomitantly, aortic release of superoxide radicals was increased 2-fold in ApoE−/− mice compared with wild-type animals, whereas aortic superoxide production was normalized in ApoE−/−-AT1−/− mice (L-012 chemiluminescence). There were no significant differences in plasma cholesterol levels between ApoE−/− and ApoE−/−-AT1−/− animals. Systolic blood pressure was significantly lower in ApoE−/−-AT1−/− animals than in ApoE−/− mice (tail-cuff measurements). Oral treatment of ApoE−/− mice with either hydralazine or irbesartan reduced systolic blood pressure to the same level; however, only AT1 receptor antagonist treatment reduced atherosclerotic lesion formation and improved endothelial function.

Conclusions—Genetic disruption of the AT1A receptor leads to inhibition of vascular oxidative stress, endothelial dysfunction, and atherosclerotic lesion formation in ApoE−/− mice irrespective of blood pressure and plasma cholesterol levels. These results indicate a fundamental role of AT1 receptor activation in atherogenesis. (Circulation. 2004;110:3062-3067.)

Key Words: hypercholesterolemia ■ angiotensin ■ endothelium ■ atherosclerosis ■ free radicals

The pathogenesis of atherosclerosis involves prolonged exposure to risk factors and a poorly understood genetic predisposition.1 Oxidative stress and inflammation are decisively involved in the initiation and progression of atherosclerosis, leading to enhanced attraction, adhesion, and invasion of macrophages and lymphocytes, deposition of lipids within the vessel wall, plaque formation, and destabilization of preformed atherosclerotic lesions.2,3

Activation of the angiotensin II type 1 (AT1) receptor not only leads to vasoconstriction and neurohumoral activation, but it is one of the major sources of oxidative stress within the vasculature.4,5 Activation of the AT1 receptor and the concomitant increase of reactive oxygen species release is associated with many cellular events, such as reduced bioavailability of nitric oxide (NO), oxidative modifications of DNA and proteins, lipid oxidation, enhanced mitogenesis and apoptosis of vascular cells, and increased expression and activation of pathophysiologically important genes, such as the receptor for oxidized LDL, adhesion molecules, chemotaxis factors, proinflammatory cytokines, regulators of cell cycle progression, and matrix metalloproteinases.4,5AT1 receptor activation induces an imbalance of T-cell subtypes by increasing the Th1 cell fraction, leads to enhanced attraction and adhesion of inflammatory cells to the endothelium, and increases foam cell formation in the vessel wall.4–6 Because of these multiple interactions of AT1 receptors with vascular and white blood cells, it is thought that AT1 receptor activation is closely linked to the onset and progression of endothelial dysfunction and atherosclerosis.2,4,5,7 Treatment of animals prone to develop atherosclerosis with angiotensin...
II leads to enhanced atherosclerotic lesion formation.5,8,9 Risk factors such as hypercholesterolemia induce vascular AT1 receptor overexpression, which leads to increased oxidative stress and atherosclerosis.10–13 Therefore, it is reasonable to assume that AT1 receptor activation and regulation are involved in virtually all stages of atherogenesis.

However, this notion has so far been proven by mechanistic and pharmacological studies only. To test the role of AT1 receptor activation in atherosclerosis in a different, more specific model, we used a genetic approach. Apolipoprotein E-deficient (ApoE−/−) mice suffer from premature atherosclerosis based on a severe lipid disorder, which is augmented by cholesterol-rich diets.14 We generated double-knockout animals deficient of ApoE and AT1A receptors and investigated the effect of a cholesterol-rich diet on the development of vascular oxidative stress, endothelial dysfunction, and atherosclerotic lesion formation in this model of lipid-induced atherosclerosis.

Methods

Oil red O solution, salts, and other chemicals were purchased from Sigma Chemical. L-012 was obtained from Wako Chemicals.

Animals and Procedures
Male C57BL/6J mice (wild-type), male ApoE−/− mice (C57BL/6J genetic background; Charles River, Sulzfeld, Germany), and male, age-matched AT1A receptor knockout mice (AT1−/−) with the identical genetic background (C57BL/6J; kindly provided by Dr. Hoffman, Department of Medicine, University of North Carolina, Chapel Hill, NC) were used for this study. The AT1−/− and ApoE−/− animals were backcrossed 10 times with C57BL/6J mice before use. ApoE−/− mice were crossed with AT1−/− mice. Genotypes were determined by polymerase chain reaction amplification of tail DNA. Heterozygous animals were crossed until homozygous double-knockout mice were obtained. The animals were maintained in a 22°C room with a 12-hour light/dark cycle and received drinking water ad libitum. All mice were fed a high-fat, cholesterol-rich diet for 7 weeks that contained 21% fat, 19.5% casein, and 1.25% cholesterol (SMOOTHER, Soest, Germany), starting at the age of 12 weeks. Additional groups of male, 12-week-old ApoE−/− mice were either treated with the vasodilator hydralazine (Sigma) orally via drinking water (250 mg/L) or the AT1 receptor antagonist irbesartan (Sanofi-Synthelabo) orally via chow (50 mg/kg body weight per day) in parallel with the high-fat, cholesterol-rich diet for 7 weeks. Systolic blood pressure (SBP) and heart rate were measured by a computerized tail-cuff system (BP-2000, Visitech Systems) in conscious animals. Mice were trained for 3 consecutive days in the prewarmed tail-cuff device to accustom them to the procedure, followed by additional measurements of SBP and heart rate on 3 consecutive days. On each day of blood pressure determination, 20 measurements were obtained and averaged for each individual animal. The mean values of all 3 days were used for comparisons. Plasma lipid concentrations were determined by routine chemical methods. LDL cholesterol was calculated with the Friedewald formula. Plasma renin activity was determined with a commercially available radioimmunoassay kit (Adaltis). The mice were killed after the treatment period, and tissue samples and blood were collected immediately. Animal experiments were performed in accordance with the German animal protection law.

Measurement of Vascular Reactive Oxygen Species

Superoxide release in intact aortic segments was determined by L-012 chemiluminescence. L-012 is a luminol derivative with high sensitivity for superoxide radicals that does not exert redox cycling itself. Aortas were carefully excised and placed in chilled, modified Krebs-HEPES buffer (pH 7.4; in mmol/L: NaCl 99.01, KCl 4.69, CaCl2 1.87, MgSO4 1.20, Na HEPES 20.0, K2HPO4 1.03, NaHCO3 25.0, D(+)-glucose 11.1). Connective tissue was removed, and aortas were cut into 2-mm segments. The aortic segments were transferred into scintillation vials containing Krebs-HEPES buffer with 100 μmol/L L-012 and were incubated for 5 minutes. Chemiluminescence was then assessed over 15 minutes in a scintillation counter (Lumat LB 9501, Berthold) at 1-minute intervals. The vessel segments were then dried, and dry weight was determined. Superoxide release is expressed as relative chemiluminescence per milligram of aortic tissue.

Aortic Ring Preparations and Tension Recording

After excision of the descending aorta, the vessel was immersed in chilled buffer containing, in mmol/L: NaCl 118.0, CaCl2 2.5, KCl 4.73, MgCl2 1.2, KH2PO4 1.2, NaHCO3 25.0, Na EDTA 0.026, D(+)-glucose 5.5, pH 7.4. Adventitial tissue was carefully removed. Three-millimeter rings were mounted in organ baths filled with the above-described buffer (37°C; continuously aerated with 95% O2 and 5% CO2) and were attached to a force transducer, and isometric tension was recorded. The vessel segments were gradually stretched over 60 minutes to a resting tension of 10 mN, which was maintained throughout the experiment, and were allowed to equilibrate for another 30 minutes. Drugs were added in increasing concentrations to obtain cumulative concentration-response curves: KCI 20 and 40 mmol/L, phenylephrine 1 mmol/L, to 10 μmol/L, carbachol 10 mmol/L to 100 μmol/L, and nitroglycerin 1 mmol/L to 10 μmol/L. The drug concentration was increased when vasorestriction or vasorelaxation was completed. Drugs were washed out before the next substance was added.

Staining of Atherosclerotic Lesions and Morphometric Analysis

Hearts with ascending aortas were embedded in Tissue Tek OCT embedding medium (Miles), snap-frozen, and stored at −80°C. Samples were sectioned on a Leica cryostat (10 μm), starting at the apex and progressing through the aortic valve area into the ascending aorta, and were placed on slides. At least 25 consecutive sections per animal were used for analysis. For detection of atherosclerotic lesions, sections were fixed with 3.7% formaldehyde for 1 hour, rinsed with deionized water, stained with oil red O working solution (0.5%) for 30 minutes, and rinsed again. For morphometric analysis, hematoxylin staining was performed according to standard protocols. All sections were examined under a Nikon E600 microscope. Lucia Measurement version 4.6 software was used to measure lipid-staining area and total area of the histological sections. The descending thoracic aorta was excised, cleaned, opened longitudinally, and fixed in 3.7% formaldehyde for 1 hour. The vessel was then rinsed, stained with oil red O solution (30 minutes), rinsed again, and pinned to a wax surface. En face images were collected under the Nikon E600 microscope, and the Lucia Measurement software was used for macroscopic analysis of lipid staining and total area. Data are expressed as lipid-staining area in percent of total surface area.

Statistical Analysis

Data are presented as mean±SEM. Statistical analysis was performed with the ANOVA test followed by the Neuman-Keuls post hoc analysis. P<0.05 indicates statistical significance.

Results

Lipid Levels

Male 12-week-old C57BL/6J (wild-type), AT1−/−, ApoE−/−, and ApoE−/−-AT1−/− mice were fed a high-fat diet containing 1.25% cholesterol for 7 weeks. Table 1 shows the resulting plasma concentrations of blood lipids. In contrast to wild-type and AT1−/− mice, total cholesterol, HDL cholesterol, and LDL cholesterol plasma concentrations were significantly elevated in ApoE−/− and ApoE−/−-AT1−/− animals. There

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were no significant differences between ApoE−/− and ApoE−/−-AT1−/− mice.

**SBP, Heart Rate, and Plasma Renin Activity**

SBP and heart rate were measured in all animal groups by tail-cuff measurements. Table 1 displays SBP levels and heart rates after 7 weeks' treatment with high-cholesterol diet. SBP was significantly lower in AT1−/− and ApoE−/−-AT1−/− mice than in wild-type and ApoE−/− animals. There were no significant differences in heart rates between the animal groups. In addition, plasma renin activity was determined in all groups after the cholesterol-rich diet. No significant differences were detected between wild-type, AT1−/−, ApoE−/−, and ApoE−/−-AT1−/− mice (Table 1).

**Atherosclerotic Lesion Formation**

Development of atherosclerotic lesions was quantified after 7 weeks of cholesterol-rich diet in wild-type, AT1−/−, ApoE−/−, and ApoE−/−-AT1−/− animals by means of oil red O stainings followed by macroscopic analysis of the descending thoracic aorta and histological analysis of the aortic sinus and the ascending aorta. Figure 1 shows representative aortic preparations and aortic cross sections of all 4 animal groups. Wild-type and AT1−/− mice showed no signs of atherosclerosis in any investigated parts of the aorta. In contrast, ApoE−/− mice displayed severe atherosclerosis in the aortic sinus and the ascending aorta, and to a lesser degree in the descending thoracic aorta. In age-matched ApoE−/−-AT1−/− mice, atherosclerotic lesion formation was almost absent in all investigated parts of the aorta. Quantitative analysis of atherosclerotic lesion formation in ApoE−/− and ApoE−/−-AT1−/− mice is shown in Table 2.

**Vascular Function**

After treatment with cholesterol-enriched diet, vascular function was assessed in isolated aortic ring preparations. In contrast to wild-type and AT1−/− mice, endothelium-dependent vasodilation was significantly impaired in ApoE−/− mice, as assessed by stimulation with carbachol (Figure 2A). ApoE−/−-AT1−/− mice displayed no development of endothelial dysfunction, and endothelium-dependent vasodilation was similar to the wild-type and AT1−/− animals (Figure 2A). Endothelium-independent vasorelaxation induced by nitroglycerin was similar in all groups (Figure 2B). In addition, vasoconstriction induced by phenylephrine or KCl was similar in all groups (data not shown).

**Vascular Oxidative Stress**

Vascular release of superoxide radicals was measured by L-012 chemiluminescence assays in intact aortic segments of wild-type, AT1−/−, ApoE−/−, and ApoE−/−-AT1−/− mice. Figure 3 shows that vascular superoxide release was increased 2-fold in ApoE−/−-mice compared with wild-type animals (195±44% of wild type; P<0.05 versus wild type). Vascular oxidative stress was reduced to control levels in

### Table 1. Blood Lipids, Blood Pressure, and Renin Activity

<table>
<thead>
<tr>
<th></th>
<th>ApoE−/−</th>
<th>ApoE−/−-AT1−/−</th>
<th>Wild Type</th>
<th>AT1−/−</th>
</tr>
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<tbody>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>564±77*</td>
<td>669±65*</td>
<td>105±16</td>
<td>118±17</td>
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<tr>
<td>Triglycerides, mg/dL</td>
<td>78±8</td>
<td>91±7</td>
<td>101±21</td>
<td>57±14</td>
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<tr>
<td>HDL cholesterol, mg/dL</td>
<td>224±29*</td>
<td>248±27*</td>
<td>74±16</td>
<td>82±16</td>
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<tr>
<td>LDL cholesterol, mg/dL</td>
<td>325±49*</td>
<td>403±41*</td>
<td>13±3</td>
<td>24±2</td>
</tr>
<tr>
<td>SBP, mm Hg</td>
<td>108±3</td>
<td>83±2*</td>
<td>103±2</td>
<td>71±4*</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>540±9</td>
<td>532±8</td>
<td>524±15</td>
<td>531±8</td>
</tr>
<tr>
<td>Plasma renin activity, ng · mL⁻¹ · h⁻¹</td>
<td>7.2±1.6</td>
<td>4.5±2.5</td>
<td>6.8±1.9</td>
<td>6.2±1.6</td>
</tr>
</tbody>
</table>

SBP, heart rate, plasma concentrations of blood lipids, and plasma renin activity were determined in the 4 animal groups after 7 weeks of treatment with a high-fat diet containing 1.25% cholesterol. Values are mean±SEM, n=10 per group (n=4 per group for renin activity). *P<0.05 vs wild type.

Figure 1. Atherosclerotic lesion formation. Atherosclerotic lesion formation was determined after 7 weeks of treatment with cholesterol-rich diet in aortic sinus (A through H) and ascending aorta (E through H; histological analysis) and in descending thoracic aorta (I through L; macroscopic analysis) by oil red O staining. Representative sections of wild-type (WT), AT1−/−, ApoE−/−, and ApoE−/−-AT1−/− double-knockout mice. AT1-R− indicates AT1−/− mice.
ApoE<sup>−/−</sup>-AT1<sup>−/−</sup> mice (96±27% of wild type; P<0.05 versus ApoE<sup>−/−</sup>).

Effect of Treatment With Hydralazine or Irbesartan on Atherosclerotic Lesion Formation and Vascular Function

To elucidate the influence of blood pressure reduction on atherosclerotic lesion formation and vascular function, male 12-week-old ApoE<sup>−/−</sup> mice were treated with either the vasodilator hydralazine or the AT1 receptor antagonist irbesartan in parallel with the high-fat, cholesterol-rich diet for 7 weeks. Both treatments resulted in significant SBP reduction to the same level as in the double-knockout mice (ApoE<sup>−/−</sup>-AT1<sup>−/−</sup>, 83±2 mm Hg; ApoE<sup>−/−</sup> plus hydralazine, 87±2 mm Hg; ApoE<sup>−/−</sup> plus irbesartan, 86±3 mm Hg; all P<0.05 versus ApoE<sup>−/−</sup>). Despite significant SBP reduction, no effect on atherosclerotic lesion formation was observed in the hydralazine-treated ApoE<sup>−/−</sup> mice compared with vehicle-treated ApoE<sup>−/−</sup> animals (Figures 4A and 4C; Table 2). In contrast, irbesartan treatment resulted in marked inhibition of atherosclerotic lesion formation in the ApoE<sup>−/−</sup> mice (Figures 4B and 4D; Table 2). Moreover, treatment with the AT1 receptor antagonist led to significant improvement of endothelium-dependent vasodilation in aortic ring preparations, whereas endothelial function was impaired in hydralazine-treated ApoE<sup>−/−</sup> mice, identical to the vehicle-treated ApoE<sup>−/−</sup>-AT1<sup>−/−</sup> animals (Figure 4E). Finally, hydralazine treatment had no effect on vascular superoxide production in the ApoE<sup>−/−</sup> mice (200±38% of wild type; P<0.05 versus wild type), whereas superoxide release was significantly reduced after treatment with irbesartan (134±17% of wild type; P<0.05 versus ApoE<sup>−/−</sup>).

Discussion

Hypercholesterolemia, which is associated with accelerated atherosclerosis, leads to overexpression of AT1 receptors in the vasculature, as demonstrated in cell culture experiments, animal models, and humans. Importantantly, treatment of hypercholesterolemic rabbits, ApoE<sup>−/−</sup> mice, and nonhuman primates with AT1 receptor antagonists decreased vascular oxidative stress and inflammation, improved endothelial function, and reduced progression of atherosclerosis, even though blood pressure and plasma lipid levels remained unaltered. Improvement of endothelial function by AT1 receptor antagonists was also confirmed in hypercholesterolemic humans.

However, animal experiments and studies in humans raised the possibility that beneficial effects of AT1 receptor antagonists are not mediated by inhibition of AT1 receptor activation but rather through accumulation of angiotensin II, which

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**TABLE 2. Atherosclerotic Lesion Size**

<table>
<thead>
<tr>
<th></th>
<th>ApoE&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>ApoE&lt;sup&gt;−/−&lt;/sup&gt;-AT1&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>ApoE&lt;sup&gt;−/−&lt;/sup&gt; Plus Irbesartan</th>
<th>ApoE&lt;sup&gt;−/−&lt;/sup&gt; Plus Hydralazine</th>
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</thead>
<tbody>
<tr>
<td>Aortic sinus, μm² (% total)</td>
<td>699 344±37 831 (46.9±2.0)</td>
<td>95 386±13 248* (7.7±0.7)</td>
<td>161 775±14 528* (15.7±1.7)*</td>
<td>656 338±24 600 (51.7±2.3)</td>
</tr>
<tr>
<td>Ascending aorta, μm² (% total)</td>
<td>483 002±38 510 (55.5±2.2)</td>
<td>23 878±2 271* (4.1±0.5)*</td>
<td>28 339±4 968* (5.3±0.7)*</td>
<td>491 428±31 280 (50.1±2.4)</td>
</tr>
<tr>
<td>Descending aorta, % total</td>
<td>5.2±0.3</td>
<td>3.2±0.3*</td>
<td>2.4±0.3*</td>
<td>7.9±0.2*</td>
</tr>
</tbody>
</table>

The lipid-staining areas in the aortic sinus and ascending aorta (histological analysis) and in the descending thoracic aorta (macroscopic analysis) were determined after 7 weeks of treatment with cholesterol-rich diet by oil red O staining.

Values are mean±SEM, n=10 per group.

*P<0.05 vs ApoE<sup>−/−</sup>; †P<0.05 vs ApoE<sup>−/−</sup>-AT1<sup>−/−</sup>.

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**Figure 2.** Vascular function. After 7 weeks of treatment with cholesterol-rich diet, aortic segments of WT, AT1<sup>−/−</sup>, ApoE<sup>−/−</sup>, and ApoE<sup>−/−</sup>-AT1<sup>−/−</sup> mice were isolated, and their functional performance was assessed in organ chamber experiments. Endothelium-dependent vasodilatation induced by carbachol (A) and endothelium-independent vasorelaxation induced by nitroglycerin (B), both expressed as percent of maximal phenylephrine-induced vasoconstriction, are shown. Mean±SEM, n=6 per group. *P<0.05 vs ApoE<sup>−/−</sup>. WT indicates wild type.

**Figure 3.** Vascular superoxide production. After 7 weeks of treatment with cholesterol-rich diet, aortas of wild-type (WT), AT1<sup>−/−</sup>, ApoE<sup>−/−</sup>, and ApoE<sup>−/−</sup>-AT1<sup>−/−</sup> mice were excised, and vascular superoxide production in intact aortic segments was quantified by L-012 chemiluminescence assays. Superoxide release is expressed as relative chemiluminescence per milligram of aortic tissue. Mean±SEM, n=8 per group. *P<0.05 vs wild type; #P<0.05 vs ApoE<sup>−/−</sup>.
Atherosclerotic lesion formation and vascular function in ApoE−/− mice treated with hydralazine or irbesartan. ApoE−/− mice were treated for 7 weeks with cholesterol-rich diet and either vehicle, hydralazine, or irbesartan. A through D, Atherosclerotic lesion formation in aortic sinus (A, B) and ascending aorta (C, D), as determined by oil red O staining (histological analysis). Representative sections of hydralazine-treated and irbesartan-treated ApoE−/− mice. E. Endothelium-dependent vasodilation of aortic ring preparations induced by carbachol, expressed as percent of maximal phenylephrine-induced vasoconstriction. Mean±SEM, n=8 per group. *P<0.05 vs ApoE−/−. Irb indicates irbesartan; Hy, hydralazine.

Figure 4. Atherosclerotic lesion formation and vascular function in ApoE−/− mice treated with hydralazine or irbesartan. ApoE−/− mice were treated for 7 weeks with cholesterol-rich diet and either vehicle, hydralazine, or irbesartan. A through D, Atherosclerotic lesion formation in aortic sinus (A, B) and ascending aorta (C, D), as determined by oil red O staining (histological analysis). Representative sections of hydralazine-treated and irbesartan-treated ApoE−/− mice. E. Endothelium-dependent vasodilation of aortic ring preparations induced by carbachol, expressed as percent of maximal phenylephrine-induced vasoconstriction. Mean±SEM, n=8 per group. *P<0.05 vs ApoE−/−. Irb indicates irbesartan; Hy, hydralazine.

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As expected, blood pressure was significantly lower in ApoE−/−-AT1−−/− mice than in ApoE−/− mice, whereas heart rates were similar in both groups. To rule out a significant influence of the reduced blood pressure levels on the development of atherosclerosis, ApoE−/− mice were treated with the vasodilator hydralazine. Although blood pressure was lowered to the same level as in the double-knockout mice, atherosclerotic lesion formation and endothelial function were identical as in vehicle-treated ApoE−/− mice, which indicates that the observed blood pressure reduction has no impact on the atherosclerotic process in the model used in the present study.

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present study. In contrast, irbesartan treatment of ApoE−/− mice resulted in similar blood pressure reduction but in significant inhibition of atherosclerosis and endothelial dysfunction. These results demonstrate the specific relevance of AT1 receptor blockade independent of blood pressure lowering.

According to the findings of the presented study, the AT1 receptor is essentially involved in hypercholesterolemia-associated atherosclerosis in mice. Diminished AT1 receptor activation therefore exerts profound atheroprotection. Further studies are needed to investigate the role of AT1 receptors in atherosclerosis induced by other risk factors such as diabetes or estrogen deficiency. Finally, if confirmed in humans, the present results suggest that AT1 receptor antagonists may represent a very promising atheroprotective treatment option.

Acknowledgments

This study was supported by the Deutsche Forschungsgemeinschaft (DFG), by an unrestricted research grant from Boehringer Ingelheim, and by the European Vascular Genomics Network, a Network of Excellence granted by the European Commission (contract No. LSHM-CT-2003-502254). The excellent technical assistance of Sybille Richter is greatly appreciated.

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_Circulation_. 2004;110:3062-3067; originally published online July 26, 2004; doi: 10.1161/01.CIR.0000137970.47771.AF
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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