Irbesartan but Not Amlodipine Suppresses Diabetes-Associated Atherosclerosis

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Background—It remains controversial whether specific blockade of the renin-angiotensin system confers superior antiatherosclerotic effects over other antihypertensive agents in diabetes. Therefore, the aim of this study was to compare equihypotensive doses of the angiotensin II subtype 1 (AT_1) receptor blocker irbesartan with the calcium antagonist amlodipine on diabetes-induced plaque formation in the apolipoprotein E (apoE)-null mouse and to explore molecular and cellular mechanisms linked to vascular protection.

Methods and Results—Diabetes was induced by injection of streptozotocin in 6-week-old apoE-null mice. Diabetic animals were randomized to no treatment, irbesartan, or amlodipine for 20 weeks. Diabetes was associated with an increase in plaque area and complexity in the aorta in association with a significant increase in aortic AT_1 receptor expression, cellular proliferation, collagen content, macrophage- and α-smooth muscle actin–positive cell infiltration, as well as an increased expression of platelet-derived growth factor-B (PDGF-B), monocyte chemoattractant protein-1 (MCP-1), and vascular cell adhesion molecule-1 (VCAM-1). Irbesartan but not amlodipine treatment attenuated the development of atherosclerosis, collagen content, cellular proliferation, and macrophage infiltration as well as diabetes-induced AT_1 receptor, PDGF-B, MCP-1, and VCAM-1 overexpression in the aorta despite similar blood pressure reductions by both treatments.

Conclusions—Diabetes-associated atherosclerosis is ameliorated by AT_1 receptor blockade but not by calcium channel antagonism, providing further evidence for the vascular renin-angiotensin system playing a pivotal role in the development and acceleration of atherosclerosis in diabetes. (Circulation. 2004;109:1536-1542.)

Key Words: atherosclerosis ■ diabetes mellitus ■ angiotensin ■ vessels

Cardiovascular disease accounts for 70% to 75% of total mortality in diabetic subjects, with its major clinical manifestations more common in patients with diabetes than in nondiabetic individuals. Although hyperglycemia per se contributes to excessive cardiovascular risk in diabetes, the effect of intensive glycemic control has been demonstrated not to totally prevent cardiovascular disease. Indeed, the mechanisms underlying the accelerated progression of atherosclerotic lesions in diabetic vessels remain to be fully clarified.

Several groups have demonstrated that induction of diabetes in apolipoprotein E (apoE)-null mice leads to atherosclerotic lesions resembling in appearance and distribution those observed in humans. Our own group has shown that there was activation of angiotensin-converting enzyme (ACE) within the aorta in these diabetic apoE-null mice and that ACE inhibition attenuated atherosclerosis in this model. However, it remains controversial whether these effects of ACE inhibitors were due to their ability to reduce blood pressure, albeit modestly, in this model or were related to their action as agents that block the generation of the vasoconstrictor and proinflammatory peptide angiotensin II (Ang II). Experimental evidence suggests that calcium antagonists reduce the severity of atherosclerosis in the nondiabetic context, including in cholesterol-fed rabbits and in primates. Both Ang II subtype 1 (AT_1) receptor antagonists and calcium channel blockers have been demonstrated to have antiatherosclerotic effects in apoE-null mice. The present study was performed to compare the effects of treatment with an AT_1 receptor antagonist with treatment with a calcium channel blocker on the formation of atherosclerosis in the diabetic apoE-null mouse. Furthermore, various cellular and molecular mechanisms were evaluated to further elucidate critical pathways implicated in diabetes-associated atherosclerosis and in mediating vascular protection in this model.

Methods

Six-week-old homozygous apoE-null male mice (ARC, Canning Vale, Western Australia, Australia) were studied according to the

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principles of the Ethics Committee of the Austin and Repatriation Medical Centre. Forty-two mice were rendered diabetic by 5 daily intraperitoneal injections of streptozotocin (Boehringer Mannheim) at a dose of 55 mg/kg. Control mice (n = 14) received citrate buffer alone. Diabetic animals were further randomized to receive the AT1 receptor blocker irbesartan (Sanofi-Synthelabo) at a dose of 10 mg/kg body wt per day by gavage (n = 14) or the calcium channel antagonist amlopidine (Pfizer) at a dose of 6 mg/kg body weight per day by gavage (n = 14) or no treatment (n = 14). Furthermore, nondiabetic apoE-null mice (n = 11) were treated with irbesartan at a dose of 10 mg/kg body wt per gavage for 20 weeks. Systolic blood pressure was assessed by a computerized, noninvasive tail cuff system in conscious mice at 4-week intervals. After 20 weeks, the animals were anesthetized by an intraperitoneal injection of pentobarbital sodium (60 mg/kg body wt; Nembutal, Boehringer Ingelheim). Glycosylated hemoglobin (HbA1c) was determined by high-performance liquid chromatography (Bio-Rad), and total cholesterol, HDL, and triglyceride concentrations were measured by autoanalyzer (Hitachi 917). LDL concentration was calculated with the use of the Friedewald formula.

**Evaluation of Atherosclerotic Lesions**

To evaluate the atherosclerotic lesions, 2 approaches were used: en face whole and histological section analysis. To determine distribution and extent of atherosclerosis, aortic sections were stained with Sudan IV–Herxheimer’s solution (Sigma Chemical Co). Serial sections 4 μm thick were stained with hematoxylin-eosin to evaluate the complexity of the atherosclerotic lesions (either fatty streak, characterized by loose connective tissue matrix containing small groups of clustered macrophages, or more complex fibrous plaques, characterized by a fibrous cap with smooth muscle cells overlying an area of foam macrophages and lipid-rich necrotic core with cholesterol clefts within the extracellular matrix), or they were stained with Masson trichrome to evaluate the proportion of collagen.

**Reverse Transcription–Polymerase Chain Reaction**

Three micrograms of total RNA extracted from each aorta were used to synthesize cDNA with the Superscript First Strand synthesis system for reverse transcription–polymerase chain reaction (RT-PCR) (Gibco BRL). AT1 receptor, platelet-derived growth factor-B (PDGF-B), monocyte chemoattractant protein-1 (MCP-1), and vascular cellular adhesion molecule-1 (VCAM-1) gene expression were analyzed by real-time quantitative RT-PCR with the use of the TaqMan system on the basis of real-time detection of accumulated fluorescence (ABI Prism 7700, Perkin-Elmer Inc). Gene expression of the target sequence was normalized in relation to the expression of an endogenous control, 18S ribosomal RNA (rRNA) (18S rRNA TaqMan Control Reagent kit; ABI Prism 7700, Perkin-Elmer Inc). Primers and TaqMan probes for AT1 receptor, PDGF-B, MCP-1, and VCAM-1 and the endogenous reference 18S rRNA were constructed with the use of Primer Express (ABI Prism 7700, Perkin-Elmer Inc).

**Immunohistochemistry**

Immunostaining for CD68 (Serotec; diluted 1:50) and the AT1 receptor (Santa Cruz Biototechnology, Inc; diluted 1:200) was performed on 6-μm frozen aortic sections. Endogenous peroxidase was inactivated with the use of 0.1% hydrogen peroxide. Sections were incubated with protein blocking agent (Lipshaw-Imuncon) and an avidin/biotin blocking kit (Vector Laboratories). Biotinylated rabbit anti-rat immunoglobulin G or goat anti-rabbit immunoglobulin G (both Vector Laboratories) were used as the secondary antibody for 60 minutes, followed by Vectastain ABC Elite reagent for 30 minutes. Peroxidase activity was identified by reaction with 3,3′-diaminobenzidine tetrahydrochloride (DAB) (Sigma Chemical Co). Paraffin sections of aorta were stained for α-smooth muscle actin (α-SMA) and proliferating cell nuclear antigen (PCNA) (Dako A/S), MCP-1, and VCAM-1 (PharMingen). After incubation with the primary antibodies, biotinylated horse anti-mouse immunoglobulin G diluted 1:200 (Dako A/S) was then applied as a secondary antibody, followed by horseradish peroxidase–conjugated streptavidin (Dako A/S; diluted 1:500). The staining was visualized by reaction with DAB (Sigma Chemical Co). The sections stained for MCP-1 were pretreated with 0.2% pepsin for 20 minutes before the primary antibody was added. Immunostaining for VCAM-1 was performed with the use of the DAKO Catalyzed Signal Amplification System (Dako A/S).

PCNA within the plaques and media and CD68-positive cells within the plaques were counted manually with the use of Optimas 6.2 VideoPro-32 associated with a videocamera and computer. The CD68- and PCNA-positive cells were expressed as a percentage of the total cells (positive nuclei/total nuclei×100). Trichrome and α-SMA staining as well as immunostaining for MCP-1 were quantified with the use of Optimas 6.2 VideoPro-32, and the stained area was expressed as a percentage of total plaque area.

**In Vitro Autoradiography**

In vitro autoradiography for Ang II receptors was performed as described previously. In brief, sections of frozen aorta tissue (10 μm thick) were incubated in incubation buffer containing 0.2 mCi/mL of the radioligand I125-(Sar1)-Ang II, 10−5 mol/L of the AT1 receptor antagonist PD 123319, and 0.2% bovine serum albumin. Nonspecific binding was determined in the presence of 10−3 mol/L of the AT1 receptor antagonist valsartan. Sections were exposed to Agfascopy CR3B x-ray films (Agfa Gevaert) for 48 to 72

### TABLE 1. Characteristics of Mice at End of Study

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Diabetes</th>
<th>Diabetes + Irbesartan</th>
<th>Diabetes + Amlodipine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 14)</td>
<td>(n = 14)</td>
<td>(n = 14)</td>
<td>(n = 14)</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>32 ± 1</td>
<td>21 ± 0.3*</td>
<td>21 ± 0.4*</td>
<td>22 ± 0.8*</td>
</tr>
<tr>
<td>Mean SBP, mm Hg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weeks 8–16</td>
<td>120 ± 2</td>
<td>133 ± 3</td>
<td>103 ± 5†</td>
<td>108 ± 3†</td>
</tr>
<tr>
<td>Week 20</td>
<td>117 ± 1</td>
<td>117 ± 2</td>
<td>109 ± 3†</td>
<td>110 ± 1†</td>
</tr>
<tr>
<td>Overall</td>
<td>119 ± 1</td>
<td>123 ± 3</td>
<td>105 ± 3†</td>
<td>108 ± 1†</td>
</tr>
<tr>
<td>Serum glucose, mmol/L</td>
<td>15 ± 1</td>
<td>36 ± 2*</td>
<td>36 ± 2*</td>
<td>36 ± 2*</td>
</tr>
<tr>
<td>HbA1c, %</td>
<td>3.3 ± 0.3</td>
<td>13.5 ± 0.3*</td>
<td>13.8 ± 0.3*</td>
<td>14.6 ± 0.5*</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>15.1 ± 0.4</td>
<td>36.1 ± 2.2*</td>
<td>31.4 ± 1.6*</td>
<td>36.1 ± 1.6*</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>1.0 ± 0.1</td>
<td>1.7 ± 0.2*</td>
<td>2.0 ± 0.3*</td>
<td>1.4 ± 0.1*</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L</td>
<td>8.7 ± 0.4</td>
<td>27.2 ± 1.1*</td>
<td>22.6 ± 1.2*</td>
<td>25.8 ± 2.2*</td>
</tr>
</tbody>
</table>

SBP indicates systolic blood pressure. Data are expressed as mean ± SEM. 
*P < 0.01 vs control apoE-null mice.
†P < 0.05 vs diabetic apoE-null mice.
hours. The autoradiographs were analyzed by computerized densitometry (Optimas 6.2).

Statistical Analysis
Data were analyzed by ANOVA with the use of StatView V (Brainpower). Comparisons of group means were performed by Fisher’s least significant difference method. Data are shown as mean±SEM unless otherwise specified. A probability value of <0.05 was viewed as statistically significant.

Results
Metabolic Parameters and Systolic Blood Pressure
Diabetic animals gained less weight than did control mice (Table 1). Blood glucose, HbA1c, total cholesterol, LDL cholesterol, and triglycerides were increased in diabetic apoE-null mice (Table 1). Neither irbesartan nor amlodipine treatment significantly altered body weight, plasma lipid parameters, or glycemic control (Table 1). Blood pressure was not changed in the diabetic compared with nondiabetic mice but was significantly reduced by both treatments compared with untreated diabetic mice (Table 1).

Assessment of Aortic Atherosclerotic Lesions
Diabetes was associated with a 5-fold increase in plaque area in the entire aorta (Figure 1A and 1B, Figure 2A), and all segments of the aorta were affected, including arch and thoracic and abdominal regions (Figure 2B). Irbesartan treatment reduced plaque area most prominently in the thoracic and abdominal parts of the aorta but also in the aortic arch (Figures 1C and 2B). However, amlodipine did not alter plaque area at any of these 3 sites (Figures 1D and 2B). In nondiabetic control mice, most plaques were fatty streaks (Figure 1E), and only occasionally were complex fibrous plaques seen at the aortic arch. In diabetic mice, most lesions were complex fibrous plaques (Figure 1F), present in all segments of the aorta. Irbesartan but not amlodipine treatment ameliorated not only the development but also the severity of atherosclerotic lesions along the entire aorta (Figure 1G and 1H). Irbesartan treatment in nondiabetic mice did not attenuate total plaque area (5.2±0.5% for irbesartan versus 4.2±1.0% for control). Furthermore, there was no effect of irbesartan treatment on the different segments of the aorta (arch, 17.1±2.7% versus 11.9±1.9%; thoracic, 1.3±0.2% versus 0.9±0.2%; abdominal, 3.0±0.6% versus 3.0±1.9% for irbesartan versus control, respectively).

Total Collagen Content
Trichrome staining was significantly increased in plaques from diabetic apoE-null mice. Total collagen content was significantly reduced by irbesartan but not by amlodipine treatment (Figure 3A through 3E).

AT1 Receptor Expression
AT1 receptor gene expression was significantly increased in the aorta of diabetic mice (Table 2). Irbesartan but not amlodipine treatment was associated with a marked reduction in AT1 receptor gene expression in the aorta (Table 2). Immunohistochemistry demonstrated increased AT1 receptor expression in the plaques of diabetic mice (Figure 4A and 4B). In vitro autoradiography studies demonstrated increased radioligand binding to the AT1 receptor in the aorta of diabetic mice (Table 2). Irbesartan but not amlodipine treatment was associated with a significant reduction of radioligand binding to the AT1 receptor.

Macrophage/Monocyte Infiltration
In diabetes there was a 3-fold increase in CD68-positive cells within the plaques of diabetic apoE-null mice (Table 2). Treatment with irbesartan but not with amlodipine was associated with a significant decrease in macrophage infiltration.

Proliferating Cell Nuclear Antigen
There was a marked increase in PCNA-positive cells in the aorta of diabetic mice within the plaque and the adjacent media (Table 2). Irbesartan but not amlodipine treatment significantly reduced the number of PCNA-positive cells in the plaque and medial layer.

α-Smooth Muscle Actin
α-SMA staining was significantly increased within the plaques of diabetic mice (Figures 5A, 5B, 5E). α-SMA-
positive cells were predominantly located at the fibrous cap within the atherosclerotic plaque of these mice. Irbesartan but not amlodipine reduced \( \text{H9251} \) -SMA–positive cells within the atherosclerotic lesions (Figure 5C, 5D, 5E).

**MCP-1, VCAM, and PDGF Expression**

Gene expression of MCP-1, VCAM-1, and PDGF was increased in aortas of diabetic apoE-null mice (Table 2). These increases were ameliorated with irbesartan but not amlodipine treatment. Immunohistochemistry demonstrated increase in MCP-1 (Figure 6A through 6D, Table 2) and VCAM protein expression (Figure 6E through 6H) in the aorta of diabetic mice, which was significantly reduced by irbesartan but not by amlodipine treatment.

**Discussion**

The present study provides further evidence that the local renin-angiotensin system is activated in diabetes-associated experimental atherosclerosis. Specifically, the present study provides evidence for increased expression of the major Ang II receptor subtype, the AT\(_1\) receptor. Furthermore, the previously described antiatherosclerotic actions of the ACE inhibitor perindopril in this model have now been observed with a more direct and selective inhibitor of the renin-angiotensin system, the AT\(_1\) receptor antagonist irbesartan. Although irbesartan treatment was associated with a significant reduction in plaque formation in diabetic apoE-null mice, this occurred in the context of a significant, albeit modest, reduction in blood pressure. To investigate this issue further, a parallel group of diabetic apoE-null mice was treated with a different class of antihypertensive agent, ie, a calcium channel blocker of the dihydropyridine type (amlodipine); this agent failed to attenuate plaque area.

The superiority of agents that block the renin-angiotensin system is consistent with previous studies demonstrating that only Ang II- and not norepinephrine-induced hypertension was associated with the development of atherosclerosis in nondiabetic apoE-null mice. Ang II has several direct and indirect humoral effects that may be implicated in the pathogenesis of atherosclerosis. In vitro data have suggested that Ang II is a potent mitogen inducing accelerated influx of macrophages and monocytes into the vessel wall and inflammatory responses in cultured vascular smooth muscle cells, including upregulation of MCP-1.

Experimental evidence for an antiatherosclerotic effect of AT\(_1\) blockers in vivo has been obtained in numerous studies in hyperlipidemic animal models, but the antiatherosclerotic effect in the context of diabetes has not been previously investigated in detail. The AT\(_1\) blocker losartan was initially shown to reduce lesion size in the cholesterol-fed cynomolgus monkey. More recent studies with either high-dose losartan (25 mg/kg per day) or irbesartan (50 mg/kg per day) were reported to attenuate atherosclerosis in nondia-
betic apoE-null mice. However, another experiment in which losartan was used had findings similar to those seen in the present study, with no effect of the AT\textsubscript{1} antagonist on atherosclerosis in nondiabetic apoE-null mice.\textsuperscript{18} It remains to be determined whether the differences among all these studies relate to factors such as gender,\textsuperscript{19,20} mode of administration, or dose of the individual Ang II antagonist.

AT\textsubscript{1} receptor gene expression was significantly increased in aortas from diabetic apoE-null mice, and this increase was attenuated by treatment with irbesartan. Because AT\textsubscript{1} receptor antagonism would not be expected to directly reduce AT\textsubscript{1} receptor gene expression, these changes may reflect the antiatherosclerotic effect of irbesartan. These results were further supported by demonstrating increased AT\textsubscript{1} receptor at the protein level in diabetic apoE-null mice as well as increased radioligand binding to the AT\textsubscript{1} receptor in the aortas of diabetic mice. Radioligand binding to the AT\textsubscript{1} receptor was reduced in the irbesartan-treated group but not in the amlodipine-treated group, suggesting effective blockade of the AT\textsubscript{1} receptor at the tissue level by irbesartan.

It has been postulated that long-acting dihydropyridine calcium channel blockers have certain vasculoprotective effects.\textsuperscript{21,22} Lacidipine has been shown to reduce the development of atherosclerotic lesions in the non-diabetic apoE-null mouse.\textsuperscript{10} In the hyperlipidemic nondiabetic hamster, amlodipine limited the size and extent of atherosclerotic plaque.\textsuperscript{23} However, although irbesartan and another calcium channel blocker, lacidipine, have been shown to reduce plaque size in the non-diabetic apoE-null mouse,\textsuperscript{8,10} amlodipine failed to reduce plaque area in the diabetic context.

Diabetes-induced accelerated plaque formation in the aorta of the diabetic apoE-null mouse was associated with a significant increase in collagen content, infiltration of vascular smooth muscle cells and macrophages, and cellular proliferation. Inflammatory and proliferative changes were observed not only in the plaque but also in the adjacent aortic media. Plaque reduction by the AT\textsubscript{1} blocker irbesartan was associated with a significant decrease in fibrotic changes and in the number of infiltrating macrophages and vascular smooth muscle cells. This was associated with a reduction in expres-
sion of the proinflammatory chemoattractant MCP-1, which was overexpressed in the aortas of the diabetic apoE-null mice. This chemokine has been reported to play an important role in the development of atherosclerosis in the apoE-null mouse in the absence or presence of diabetes. The endothelial adhesion molecule VCAM-1, which is important for the adhesion of macrophages to the vascular wall, was also significantly increased in diabetes and was attenuated by irbesartan. Amlodipine did not have an effect on macrophage or vascular smooth muscle cell infiltration and proliferation or on VCAM-1 and MCP-1 expression, consistent with the failure of this agent to reduce atherosclerosis in this model.

The increased cellular proliferation in plaque and media was associated with increased expression of the proliferative cytokine PDGF in the aorta of these diabetic mice. Cellular proliferation and PDGF expression were significantly attenuated by irbesartan but not by amlodipine, suggesting a pivotal role for Ang II and its interactions with the AT₁ receptor in promoting the proliferative and inflammatory changes that ultimately result in atherosclerotic plaques in this model.

One must be cautious in extrapolating these experimental data to the clinical context. However, recent clinical studies have addressed the potential superiority of blockade of the renin-angiotensin system over other antihypertensive drugs in reducing clinical events linked to atherosclerosis. For example, in contrast to the total cohort, in which losartan was superior to atenolol predominantly in reducing the risk of stroke, within the diabetic subgroup analysis of the LIFE study, the AT₁ blocker losartan was superior to the β-blocker atenolol in reducing cardiovascular end points, including myocardial infarction. However, these findings must be considered in the light of a 2-mm Hg difference in systolic blood pressure in favor of losartan in that study. The clinical evidence for the antatherosclerotic effects of calcium channel blockers remains controversial, with the Prospective Randomized Evaluation of the Vascular Effects of Norvasc Trial (PREVENT) suggesting a possible antitherosclerotic effect of amlodipine versus placebo on the basis of effects on progression of carotid intima-media thickness. However, that study was not powered adequately to directly address cardiovascular or all-cause mortality. Furthermore, that study did not address diabetic patients.

It appears likely that in the diabetic context, the vascular renin-angiotensin system plays a critical role in mediating acceleration of atherosclerosis, and this may explain why inhibitors of the renin-angiotensin system may be superior to other antihypertensive agents such as calcium channel blockers in reducing diabetes-associated acceleration of atherosclerosis.
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References

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