Prevention of Accelerated Atherosclerosis by Angiotensin-Converting Enzyme Inhibition in Diabetic Apolipoprotein E–Deficient Mice

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Background—Atherosclerosis is a major complication of diabetes, but the mechanisms by which diabetes promotes macrovascular disease have not been fully delineated. Although several animal studies have demonstrated that inhibition of ACE results in a decrease in the development of atherosclerotic lesions, information about the potential benefits of these agents on complex and advanced atherosclerotic lesions as observed in long-term diabetes is lacking. The aim of this study was to evaluate whether treatment with the ACE inhibitor perindopril affects diabetes-induced plaque formation in the apolipoprotein E (apoE)-deficient mouse.

Methods and Results—Diabetes was induced by injection of streptozotocin in 6-week-old apoE-deficient mice. Diabetic animals received treatment with perindopril (4 mg · kg$^{-1}$ · d$^{-1}$) or no treatment for 20 weeks. Nondiabetic apoE-deficient mice were used as controls. Induction of diabetes was associated with a 4-fold increase in plaque area compared with nondiabetic animals. This accelerated atherosclerosis was associated with a significant increase in aortic ACE expression and activity and connective tissue growth factor and vascular cell adhesion molecule-1 expression. Perindopril treatment inhibited the development of atherosclerotic lesions and diabetes-induced ACE, connective tissue growth factor, and vascular cell adhesion molecule-1 overexpression in the aorta.

Conclusions—The activation of the local renin-angiotensin system in the diabetic aorta and the reduction in atherosclerosis with ACE inhibitor treatment provides further evidence that the renin-angiotensin system plays a pivotal role in the development and acceleration of atherosclerosis in diabetes. (Circulation. 2002;106:246-253.)

Key Words: atherosclerosis ■ diabetes mellitus ■ angiotensin ■ vessels

Patients with diabetes are at greater risk of developing atherosclerosis than nondiabetic subjects.1 Dyslipidemia, hypertension, obesity, and hyperglycemia only partly explain the increased incidence of macrovascular complications in diabetes.2,3 Indeed, the mechanisms underlying the accelerated progression of atherosclerotic lesions in diabetic arteries remain to be clarified. Furthermore, controversy remains as to whether diabetes-associated atherosclerosis is essentially an accelerated form of atherosclerosis or whether it represents a specific form of atherosclerosis.

A variety of experimental studies have suggested a role for angiotensin II in plaque formation and development, albeit in a nondiabetic context.4 Diet et al5 observed increased ACE protein accumulation within the atherosclerotic plaque in human coronary arteries, and it has been hypothesized that this leads to an increased production of local angiotensin II, which may participate in the pathophysiology of artery disease. A large range of experimental studies and clinical trials have shown that ACE inhibitors prevent or retard the development of diabetic microangiopathy and macroangiopathy.6,7 It has not yet been determined, however, whether the renin-angiotensin system (RAS) plays a key role in the pathogenesis of diabetic complications and specifically in accelerated atherosclerosis, as observed in diabetes.

The apolipoprotein E (apoE)-deficient mouse has increasingly been used as an experimental model of atherosclerosis, developing lesions ranging from lipid-laden fatty streaks to advanced fibroproliferative lesions by the age of 30 weeks.8 Moreover, Park et al9 recently observed that the induction of diabetes in these mice for the relatively short period of 6 weeks was associated with accelerated atherosclerosis in the aortic arch.

Recent evidence suggests that the expression of connective tissue growth factor (CTGF) is absent in normal human arteries but highly enhanced in endothelial cells of atherosclerotic lesions.10 Moreover, CTGF has been shown to promote the adhesion, migration, and proliferation of both
fibroblasts and endothelial cells and to induce neovascularization in vivo. These findings suggest that CTGF may be involved in the pathogenesis and progression of advanced atherosclerosis, as observed in diabetes. The importance of this growth factor, particularly in diabetes, has been further suggested from subtractive hybridization studies reporting that CTGF is differentially expressed in response to hyperglycemia in both in vitro and in vivo contexts. In addition, we assessed expression of vascular cell adhesion molecule-1 (VCAM-1), an adhesion molecule implicated in the pathogenesis of atherosclerosis and reported to be upregulated in diabetic vessels.

The aims of our study were (1) to evaluate the effects of long-term diabetes on the development of atherosclerotic lesions; (2) to investigate the molecular mechanisms underlying diabetes-associated atherosclerosis, including evaluation of the local RAS; and (3) to determine the effects of treatment with an ACE inhibitor on the formation of atherosclerotic plaques in the aorta of diabetic apoE-deficient mice.

Methods
Six-week-old homozygous apoE-deficient male mice (back-crossed 20 times from the C57BL/6 strain; Animal Resource Center, Cannong Vale, WA, Australia) were housed at the Biological Research Laboratory at the Austin and Repatriation Medical Center and were studied according to the principles devised by the Animal Welfare Committee of the Austin and Repatriation Medical Center. Sixty mice were rendered diabetic by 6 daily intraperitoneal injections of streptozotocin (Boehringer-Mannheim) at a dose of 55 mg/kg in citrate buffer. Control mice (n = 30) received citrate buffer alone. The animals had unrestricted access to water and standard mouse chow (Barastoc). After the induction of diabetes, the animals were further randomized to be treated with the ACE inhibitor perindopril (n = 30) at a dose of 4 mg · kg body wt · d⁻¹ in drinking water for 20 weeks or to receive no treatment (n = 30).

Systolic blood pressure was assessed by a computerized, noninvasive tail-cuff system in conscious mice every 4 weeks. At the end of the study, glycosylated hemoglobin was measured by high-performance liquid chromatography (Biorad), and total cholesterol, HDL, and triglyceride concentrations were measured by the autoanalyzer technique (Hitachi 917). LDL concentration was calculated by use of the Friedewald formula. After 20 weeks, the animals were anesthetized by an intraperitoneal injection of pentobarbital sodium (60 mg/kg body wt; Nembutal, Boehringer Ingelheim). Blood was collected from the left ventral and centrifuged, and plasma and red blood cells were stored at −20 °C and 4 °C, respectively, for analysis. For each mouse, the aorta was rapidly dissected out, snap-frozen in liquid nitrogen, and stored at −80 °C. A subset of aortas from each group (n = 15 mice per group) was removed and used to obtain information about distribution and extent of atherosclerotic lesion area, in situ hybridization, and immunohistochemical studies.

Evaluation of Atherosclerotic Lesions
To evaluate the atherosclerotic lesions, 2 approaches were used: en face whole and histological section analyses. The en face approach was used to obtain information about distribution and extent of atherosclerosis in the aorta, whereas microscopic analysis was used to evaluate the lesion complexity. The entire aorta was cleaned of adventitial, Boehringer Ingelheim. Blood was collected from the left neal injection of pentobarbital sodium (60 mg/kg body wt; Nembu- citrate buffer. Control mice (n = 30) were randomized to be treated with the ACE inhibitor perindopril (n = 30) at a dose of 4 mg · kg body wt · d⁻¹ in drinking water for 20 weeks or to receive no treatment (n = 30).

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The aortic segments were then embedded in paraffin. Cross-sectional serial sections 5 μm thick were prepared and stained with hematoxylin and eosin to evaluate the atherosclerotic lesion complexity or with Masson’s trichrome to evaluate the presence of collagen. Collagen was quantified by calculation of the ratio of lesion area occupied by the Masson trichrome staining within the plaques by use of the Imaging Analysis System (AIS, Imaging Research) associated with a videocamera and computer.

Reverse Transcription–Polymerase Chain Reaction
Total RNA (3 μg) extracted from each aorta was used to synthesize cDNA with the Superscript First Strand synthesis system for reverse transcription–polymerase chain reaction (RT-PCR) (Gibco BRL). ACE, CTGF, and VCAM-1 gene expression were analyzed by real-time quantitative RT-PCR using the TaqMan system based on real-time detection of accumulated fluorescence (ABI Prism 7700, Perkin-Elmer Inc). Fluorescence for each cycle was quantitatively analyzed with an ABI Prism 7700 Sequence Detection System (Perkin-Elmer Inc, PE Biosystems). To control for variation in the amount of DNA available for PCR in the different samples, 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 ACE, CTGF, and VCAM-1 and the endogenous reference 18S rRNA were constructed with the help of Primer Express (ABI Prism 7700, Perkin-Elmer Inc). For amplification of the ACE cDNA, the forward primer was 5′-CAGAATCTACTCCAGCTGGCAAGGT-3′, and the reverse primer was 5′-TCGGTGAAGAACAGGTGTG-3′. The probe specific to ACE was 6-carboxyfluorescein (FAM)-5′-CAAACACAGC- TGCCACCTGCTGGTCC-3′-6-carboxy-tetramethylrhodamine (TAMRA) (quencher). For the CTGF cDNA, the forward primer was 5′-GAGGAAACATTTAAGGAGGCAA-3′, and the reverse primer was 5′-CGGCACAGGGCTTGATGA-3′. The probe specific to CTGF was FAM-5′-TTGAGCCTTTCTGCTGACACAGGT-3′-TAMRA. For the VCAM-1 cDNA, the forward primer was 5′-AAGCTGTTGGTTGCTTACA-3′, and the reverse primer was 5′-ACATCGTTGAGTTACACCTGATATG-3′. The probe specific to VCAM-1 was FAM-5′-AGGCCACAGCTGCAAGGA TCGCC-3′-TAMRA.

In Situ Hybridization
The site-specific expression of CTGF and VCAM-1 mRNA was determined by in situ hybridization as previously described. In brief, 4-μm paraffin aortic sections were hybridized after digestion with PronaseE at 37 °C. The hybridization buffer containing 2×10⁴ cpm/μL 32P-labeled riboprobe, 0.72 mg/mL yeast RNA, 50% deionized formamide, 100 mmol/L DTT, 10% dextran sulfate, 0.3 mol/L NaCl, 10 mmol/L Na2 HPO4, 10 mmol/L Tris HCl (pH 7.5), 5 mmol/L EDTA (pH 8.0), 0.02% BSA, 0.02% Ficoll 400, and 0.02% polyvinyl pyrrolidone (PVP) was added to each section and incubated at 60 °C overnight. After stringent washing with 50% formamide, 2×SSC, at 55 °C, the slides were air-dried and exposed to BioMax MR film (Kodak) for 3 to 5 days. Slides were coated in Amersham LM-1 emulsion in a darkroom, then incubated at 4 °C in a light-proof container with desiccant for a period of 2 to 4 weeks, according to the autoradiography results. The slides were developed with Kodak D19 developer for 4 minutes, 1% acetic acid for 1 minute, and Illford Hype fixative for 4 minutes, followed by rinsing in distilled water for 3 minutes. The sections were then fixed in 4% paraformaldehyde and treated with a progressive hematoxylin–eosin stain.

Quantitative Measurement of ACE by In Vitro Autoradiography
Sections of frozen aorta tissue (20 μm) were cut on a cryostat, thaw-mounted onto gelatin-coated slides, and dehydrated overnight
under reduced pressure at 4°C. The radioligand used for autoradiography was $^{125}$I-351A, a tyrosyl derivative of lisinopril (MSD Research Laboratories) and a potent competitive inhibitor of ACE. All sections were preincubated for 15 minutes in 10 mmol/L sodium phosphate buffer (pH 7.4) containing 150 mmol/L NaCl, 0.02% NaN$_3$, and then incubated for 60 minutes in buffer containing 0.2% mCi/mL of the radioligand, $^{125}$I-351A, and 0.2% BSA. To determine nonspecific binding, 1 µmol/L unlabelled 351A was added into the incubation buffer. After incubation, sections were washed in ice-cold fresh buffer without BSA to remove nonspecific bound radioligand. The slides were dried and then loaded into x-ray cassettes and exposed to Agfascopix CR3B X-ray films (Agfa Gevaert) for 48 to 72 hours. In each cassette, a set of $^{125}$I radioactivity standards was included. The x-ray films were developed, and autoradiographs were computerically by light microscopy for cellular localization of ACE radioligand.

### Immunohistochemistry

Serial frozen aortic sections were used to immunostain for CTGF and macrophages. The primary antibodies used were a polyclonal rabbit anti-human CTGF antibody (Serotec; diluted 1:200) and a monoclonal rat anti-mouse CD68 antibody (Chemicon; diluted 1:200), a monoclonal mouse anti-human smooth muscle cell α-actin antibody (Dako; diluted 1:50), and a monoclonal mouse anti-rat PCNA antibody (Dako; diluted 1:50). In brief, 20-μm frozen aortic sections were fixed with cold acetone, and endogenous peroxidase was inactivated with 0.1% H$_2$O$_2$ in PBS. The sections were incubated with protein blocking agent, and endogenous nonspecific binding for biotin-avidin was blocked with a biotin-avidin blocking kit (Vector Laboratories). The aortic sections were incubated with primary antibodies for 1 hour at room temperature for CTGF and overnight at 4°C for CD68. Biotinylated anti-rabbit immunoglobulin diluted 1:100 (Dako) or biotinylated anti-rat immunoglobulin diluted 1:200 (Vector Laboratories) was used as secondary antibody for 30 minutes, followed by horseradish peroxidase–conjugated streptavidin, diluted 1:500 (Dako) for 30 minutes. Peroxidase activity was identified by reaction with 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemical Co) substrate. The slides were then counterstained with hematoxylin, dehydrated, and fixed.

Serial paraffin sections of aorta were also used to stain for ACE, smooth muscle cells, and proliferating cell nuclear antigen (PCNA). The primary antibodies used were a monoclonal mouse anti-ACE antibody (Chemicon; diluted 1:200), a monoclonal mouse anti-human smooth muscle cell α-actin antibody (Dako; diluted 1:50), and a monoclonal mouse anti-rat PCNA antibody (Dako; diluted 1:50). In brief, the endogenous peroxidase was neutralized with H$_2$O$_2$ (0.3% vol/vol) for 20 minutes. The sections were incubated with primary antibodies for 1 hour at room temperature for ACE and overnight at 4°C for smooth muscle cells and PCNA. Biotinylated anti-human immunoglobulin, diluted 1:25 for ACE or diluted 1:200 for smooth muscle cells and PCNA, was then applied as a secondary antibody for 30 minutes, followed by horseradish peroxidase–conjugated streptavidin (Dako; diluted 1:500). The staining was visualized by reaction with 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemical Co). The slides were then counterstained with hematoxylin, dehydrated, and fixed. All the sections were examined by light microscopy (Olympus BX-50, Olympus Optical) and digitized with a high-resolution camera (Fujix HC-2000, Fujiﬁlm).

### Statistical Analysis

Data were analyzed by ANOVA using 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 $P<0.05$ 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 and glycosylated hemoglobin were increased in the diabetic apoE-deficient mice compared with nondiabetic control mice (Table 1). Perindopril treatment did not influence body weight or glycemic control (Table 1). There was no signiﬁcant difference in mean systolic blood pressure between untreated diabetic mice and control animals over the 20-week period (Table 1). Treatment with the ACE inhibitor perindopril was associated with a modest (7 to 8 mm Hg) but statistically significant reduction in blood pressure compared with control or diabetic untreated apoE-deficient mice (Table 1).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control (n=30)</th>
<th>Diabetic (n=30)</th>
<th>Diabetic + Perindopril (n=30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>29.7±0.5</td>
<td>21.5±0.3*</td>
<td>23.1±0.5*</td>
</tr>
<tr>
<td>Mean SBP, mm Hg</td>
<td>117±3</td>
<td>118±3</td>
<td>110±2‡</td>
</tr>
<tr>
<td>Serum glucose, mmol/L</td>
<td>8.4±0.7</td>
<td>26.1±3.3*</td>
<td>22.4±4.3*</td>
</tr>
<tr>
<td>HbA$_1c$, %</td>
<td>3.7±0.1</td>
<td>12.6±0.4*</td>
<td>12.1±0.5*</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>14.3±0.4</td>
<td>35.6±2.4*</td>
<td>32.4±3.5*</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L</td>
<td>9.4±0.4</td>
<td>26.6±2.1*</td>
<td>22.4±3.8*</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>4.5±0.2</td>
<td>8.1±0.7*</td>
<td>6.8±0.5*</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>0.9±0.1</td>
<td>1.6±0.3*</td>
<td>1.5±0.2*</td>
</tr>
</tbody>
</table>

SBP indicates systolic blood pressure of weeks 4–20; HbA$_1c$, glycosylated hemoglobin. Data are expressed as mean±SEM. *$P<0.01$ vs control group; ‡$P<0.05$ vs control and diabetic groups.

### TABLE 2. Aortic Molecular, Cellular, and Pathological Parameters of Mice at the End of the Study

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control (n=7–15)</th>
<th>Diabetic (n=7–15)</th>
<th>Diabetic + Perindopril (n=7–15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lesion area, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Entire aorta</td>
<td>4.6±0.5</td>
<td>19.1±1.5*</td>
<td>6.5±0.9†</td>
</tr>
<tr>
<td>Arch</td>
<td>10.1±1.7‡</td>
<td>18.1±1.7*</td>
<td>5.3±0.8‡</td>
</tr>
<tr>
<td>Thoracic</td>
<td>3.0±0.4</td>
<td>20.1±2.1*</td>
<td>6.2±1.1†</td>
</tr>
<tr>
<td>Abdominal</td>
<td>3.0±0.5</td>
<td>19.2±2.4*</td>
<td>7.4±1.5†</td>
</tr>
<tr>
<td>Macrophage infiltration, %</td>
<td>7.3±0.8</td>
<td>15.3±1.1*</td>
<td>6.3±1.0†</td>
</tr>
<tr>
<td>Aortic plaque</td>
<td>0.7±0.2</td>
<td>3.3±0.6*</td>
<td>1.3±0.3‡</td>
</tr>
<tr>
<td>Aortic media</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCNA, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aortic plaque</td>
<td>9.7±1.8</td>
<td>16.7±1.1*</td>
<td>10.8±1.2†</td>
</tr>
<tr>
<td>Aortic media</td>
<td>2.7±0.6</td>
<td>7.4±1.2*</td>
<td>2.3±0.6†</td>
</tr>
<tr>
<td>Lesion collagen content, %</td>
<td>4.4±1.0</td>
<td>28.8±4.3*</td>
<td>6.1±2.0†</td>
</tr>
<tr>
<td>ACE mRNA, arbitrary units</td>
<td>1.0±0.6</td>
<td>6.1±1.1*</td>
<td>0.4±0.1†</td>
</tr>
<tr>
<td>ACE binding, dpm/mm$^2$</td>
<td>49±26</td>
<td>417±77†</td>
<td>79±9†</td>
</tr>
<tr>
<td>CTGF mRNA, arbitrary units</td>
<td>1.0±0.2</td>
<td>10.5±3.2*</td>
<td>1.2±0.2†</td>
</tr>
<tr>
<td>VCAM-1 mRNA, arbitrary units</td>
<td>1.0±0.6</td>
<td>19.4±6.0*</td>
<td>0.6±0.2†</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SEM. *$P<0.01$ vs control group; †$P<0.01$ vs control group; ‡$P<0.01$ vs thoracic and abdominal segments in control group.
Assessment of Aortic Atherosclerotic Lesions

Induction of diabetes was associated with a 4-fold increase in plaque area in the entire aorta compared with nondiabetic animals (Table 2; Figure 1, A and B). Evaluation of specific areas within the aorta indicated that this increase in atherosclerosis was observed in diabetic animals in the aortic arch, thoracic aortic, and abdominal aortic regions (Table 2; Figure 1B). An increase in plaque formation in the aortic arch versus descending thoracic and abdominal regions was observed in control mice (Table 2). By contrast, in the untreated diabetic mice, the increase in plaque area resulted in no difference in extent of atherosclerosis among the 3 aortic regions (Table 2). In nondiabetic control mice, most plaques were fatty streaks (Figure 1D), and complex fibrous plaques were seen only occasionally at the aortic arch. In diabetic mice, the individual lesions were predominantly complex fibrous plaques (Figure 1E), which were present in all segments of the aorta. Fatty streaks in diabetic mice were also increased compared with control mice. The prevalent pathological characteristics of the lesions in diabetic animal were an asymmetrically thickened intima composed of a fibrous cap with smooth muscle cells, foam macrophages, and lipid-rich necrotic core with cholesterol clefts within the extracellular matrix (Figure 1E). In some cases, the vascular media was thinned because of fragmentation and loss of elastic laminae and disappearance of medial smooth muscle cells. Perindopril treatment ameliorated the development as well as the severity of atherosclerotic lesions along the entire aorta, similar to the level seen in control mice (Table 2; Figure 1, C and F).

Macrophage/Monocyte Infiltration

Diabetes induction resulted in increased CD68 staining in the aorta, with a >4-fold increase in CD68-positive cells in the aortic medial layer and a 2-fold increase in CD68-positive cells within the plaques compared with control nondiabetic apoE-deficient mice (Table 2). Treatment with perindopril was associated with a significant decrease in macrophage infiltration both in the aortic medial layer and within the atherosclerotic lesions (Table 2).

Proliferating Cell Nuclear Antigen

Immunohistochemical staining for PCNA revealed a marked increase in diabetic animals compared with control apoE-deficient mice (Table 2). PCNA protein expression was reduced by perindopril treatment (Table 2).

α-Smooth Muscle Actin

α-Smooth muscle actin staining was significantly increased within the plaques of diabetic apoE-deficient mice compared with control nondiabetic apoE-deficient mice.
with control animals (Figure 2, A and B). The chronic administration of perindopril led to a significant reduction in α-smooth muscle actin–positive cells within the atherosclerotic lesions (Figure 2C).

Collagen Content in Lesions
Collagen content was markedly increased in the lesions of diabetic apoE-deficient mice (Figure 2E) compared with control animals (Table 2; Figure 2D). Perindopril-treated diabetic mice had reduced collagen content within the plaque (Table 2; Figure 2F).

ACE Expression and Activity in the Aorta
ACE gene expression was significantly increased (5-fold) after 20 weeks of diabetes compared with nondiabetic mice, and perindopril treatment completely prevented this upregulation (Table 2).

In vitro autoradiography demonstrated that ACE was present in the normal vessel wall of nondiabetic control mice and was located mainly in the media and intimal layers. There was an ≈9-fold increase in ACE radioligand binding in diabetic apoE-deficient mice compared with the nondiabetic animals (Table 2), paralleling the changes in ACE mRNA expression. In the diabetic apoE-deficient mice, ACE radioligand binding was located in the media and extended into the atherosclerotic plaques. The chronic administration of perindopril in diabetic animals induced a significant reduction in ACE radioligand binding compared with untreated diabetic mice (Table 2).

Immunohistochemical staining for ACE demonstrated the same pattern as seen with respect to ACE gene expression and ACE activity. The induction of diabetes was associated with a significant increase in aortic staining for ACE compared with control mice (Figure 3, A and B). Perindopril treatment in diabetic mice was associated with a reduction in staining for ACE (Figure 3C). ACE immunoreactivity was consistently found at the site of macrophage accumulation.

CTGF Expression
Diabetic mice had an increase in CTGF gene expression in the aorta compared with nondiabetic control animals (Table 2). In situ hybridization confirmed the increased gene expression of CTGF in the aortas of diabetic mice (Figure 4, A and B). This increase in CTGF gene expression was observed not only in the atherosclerotic plaques but also in the aortic medial layer in the diabetic animals (Figure 4, B and E). Consistent with the mRNA findings, increased CTGF protein expression was detected in both the atherosclerotic lesions and the aortic media of diabetic animals (Figure 5B) compared with controls (Figure 5A). CTGF protein expression within the plaques was localized predominantly to the shoulder region, in the microvessels, and in the areas of extracellular matrix accumulation. Perindopril treatment in diabetic animals was associated with a reduction in the CTGF gene and protein expression as assessed by RT-PCR (Table 2), in situ hybridization (Figure 4C), and immunostaining (Figure 5C).
VCAM-1 Expression

Diabetic mice displayed a marked increase in gene expression for VCAM-1 in aortic tissue compared with nondiabetic control mice, and this increase was significantly attenuated by perindopril (Table 2). The overexpression of VCAM-1 was confirmed by in situ hybridization in aortas from untreated diabetic apoE-deficient mice (Figure 6B). An increased expression of VCAM-1 gene was found not only in the atherosclerotic plaques but also in the aortic medial layer of diabetic animals compared with control mice and diabetic mice treated with perindopril (Figure 6).

Discussion

In the present study, the cellular and molecular mechanisms of diabetes-associated atherosclerosis and the effects of ACE inhibition on these pathological processes have been examined. The major findings in our study were that induction of diabetes in apoE-deficient mice leads to a significant acceleration of atherosclerosis in all aortic segments after 20 weeks and that chronic treatment with the ACE inhibitor perindopril prevented the development of this accelerated atherosclerosis. Of particular interest, activation of the local RAS within the aorta has been identified in these diabetic apoE-deficient mice, predominantly within the plaques and the medial layer. This activation of the local RAS within the plaques of diabetic apoE-deficient mice was associated with increased macrophage/monocyte infiltration, fibrosis, cellular proliferation, and expression of growth factors and inflammatory molecules. This association between activation of the RAS and these various cellular processes is consistent with the known in vivo and in vitro actions of angiotensin II. Blockade of the RAS with the ACE inhibitor perindopril, despite modest effects on blood pressure and no significant influence on the lipid profile or glycemic control, was demonstrated in this study to have effects on the RAS within the aorta, with evidence of effective inhibition of aortic ACE activity within the vessel wall.

A major limiting factor in diabetes-related research into macrovascular disease has been the lack of an appropriate animal model. Indeed, the induction of experimental diabetes has resulted in less rather than more atherosclerosis in different animal species, including the rat and rabbit.22 Other groups have shown that streptozotocin-induced diabetes in apoE-deficient mice is followed by accelerated atherosclerosis.9,23 In the present study, we demonstrated increased atherosclerotic lesion formation in the entire aorta, and this increase was more pronounced in the thoracic and abdominal segments.

Diet et al5 demonstrated that in early- and intermediate-stage human atherosclerotic lesions, ACE was expressed predominantly in lipid-laden macrophages, whereas in advanced lesions, it was localized throughout the plaque microvasculature. These authors suggested that this increase in local ACE contributes to increased production of local angiotensin II, which participates in the pathophysiology of vascular disease. It was recently demonstrated, in a primate model of atherosclerosis, that lipid-laden macrophages...
contain angiotensin II. The present study extends these earlier findings to the accelerated and more advanced atherosclerotic lesions observed in diabetes. The induction of diabetes in apoE-deficient mice was followed by a significant increase in aortic ACE gene and protein expression as well as ACE binding density, reflecting local enzyme activity in both atherosclerotic plaques and the medial layer.

In the present study, 20 weeks of treatment with perindopril prevented diabetes-associated plaque formation and complexity in all the aortic segments studied. It appears that mechanisms other than reduction of serum glucose or cholesterol levels are involved in the antiatherosclerotic effects of ACE inhibition. The likelihood that these antiatherosclerotic actions of ACE inhibitors are predominantly blood pressure independent is suggested by other groups who have previously shown that in the absence of diabetes, ACE inhibitors attenuate atherosclerosis without affecting blood pressure. Further evidence suggesting a specific effect of angiotensin II rather than blood pressure per se is supported by studies that compared the effect of angiotensin II- and norepinephrine-induced hypertension in apoE-deficient mice. Despite similar effects on blood pressure, only angiotensin II-induced hypertension was associated with development of atherosclerosis in the ascending aorta. Moreover, in that study, the angiotensin II-induced atherosclerotic lesions in the different aortic regions had a spatial distribution very similar to that observed in diabetic mice in our study.

Perindopril treatment significantly reduced ACE gene and protein expression and activity in all aortic segments of the diabetic mice. The effect of perindopril in reducing binding of the radioligand 125I-351A to the aortic tissue is consistent with in vivo inhibition of ACE activity. The effects of perindopril on ACE gene and protein expression, however, cannot be explained as a result of the direct effects of the drug per se. It is more likely that the reduction in ACE gene and protein expression reflects a manifestation of the antiatherosclerotic action of this drug. The beneficial effects of ACE inhibitor treatment that led to an attenuation of accelerated atherosclerosis most likely occurred as a result of suppression of various actions of angiotensin II, such as vasoconstriction, smooth muscle proliferation, connective tissue synthesis, and chemotaxis of monocytes. The findings of the present study are consistent with these actions of ACE inhibition, because perindopril treatment reduced aortic smooth muscle proliferation and extracellular matrix production and prevented the increase in VCAM-1 gene expression that was observed in the aortas from the untreated diabetic mice. Previous studies have already demonstrated that monocyte infiltration is a key initial step in atheroma formation and that VCAM-1 plays a pivotal role in the initiation of atherosclerosis.

The actions of angiotensin II in promoting atherosclerosis may involve activation of cytokines and growth factors. It was recently demonstrated that angiotensin II mediates, at least in part, the overexpression of the CTGF gene observed in the arterial smooth muscle cells in an experimental model of cyclosporine-induced myocardial damage. CTGF gene expression was previously reported to be increased in the diabetic kidney, and these investigators suggested that this cytokine is implicated in the development of diabetic nephropathy. An increase in CTGF gene and protein expression was previously demonstrated in human atherosclerosis. Intriguingly, the possible link between CTGF and atherosclerosis was also suggested by Segarini et al., who demonstrated that the multiligand receptor, LDL receptor-related protein/alpha-macroglobulin receptor, is the major cell membrane receptor for CTGF, and by studies that identified an increased expression of this receptor in...
smooth muscle cells and macrophages of human atherosclerotic lesions. The strongly profibrotic actions of CTGF are consistent with the present study, in which diabetes was associated not only with an acceleration in plaque formation but also with an increase in fibrosis and extracellular matrix accumulation. Moreover, the hypothesis that CTGF may be involved in the development of atherosclerosis in our study is further strengthened by the observation that the increased gene expression of this cytokine in diabetic animals was localized not only inside the plaques but also in the aortic medial layer. Blockade of the enzyme ACE prevented the increased gene and protein expression of CTGF in the aorta of diabetic mice. These findings provide new insights into the mechanisms responsible for the antiatherosclerotic effect of ACE inhibitors. Further studies are necessary to fully elucidate the potential relevance of CTGF in the development of accelerated atherosclerosis in this model and provide the rationale to consider this cytokine as a target for new approaches to retard atherosclerosis, particularly in the diabetic context.

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