Angiotensin-Converting Enzyme Inhibition Attenuates Hypofibrinolysis and Reduces Cardiac Perivascular Fibrosis in Genetically Obese Diabetic Mice

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Background—Obesity and insulin resistance are associated with accelerated macrovascular and microvascular coronary disease, cardiomyopathic phenomena, and increased concentrations and activity in blood of plasminogen activator inhibitor type 1 (PAI-1), the primary physiological inhibitor of fibrinolysis.

Methods and Results—To determine whether hypofibrinolysis in blood and tissues and its potential sequelae could be attenuated pharmacologically, we studied genetically modified obese mice. By 10 weeks of age, obese mice exhibited increases in left ventricular weight and glucose and immunoreactive insulin in blood. PAI-1 activity in blood measured spectrophotometrically was significantly elevated as well. The difference compared with values in lean controls widened by 20 weeks of age. Perivascular fibrosis in coronary arterioles and small coronary arteries was evident in obese mice 10 and 20 weeks of age, paralleling increases in PAI-1 and tissue factor expression evident by immunohistochemical image analysis, in situ hybridization, and reverse transcription–polymerase chain reaction. Inhibition of ACE activity initiated in obese mice 10 weeks of age and continued for 20 weeks arrested the increase in PAI-1 activity in blood and in cardiac PAI-1 and tissue factor mRNA as well as coronary perivascular fibrosis.

Conclusions—Thus, inhibition of proteo(fibrino)lysis and augmented tissue factor expression in the heart precede and may contribute to the coronary perivascular fibrosis seen with obesity and insulin resistance. Furthermore, inhibition of ACE activity can attenuate all 3 phenomena. (Circulation. 2001;103:3123-3128.)

Key Words: fibrinolysis ■ coronary disease ■ diabetes mellitus ■ insulin

Accelerated coronary atherosclerosis contributes to the increased mortality associated with type 2 diabetes. Its determinants include hyperglycemia, hyperlipidemia, obesity, hypertension, and insulin resistance. Coronary microvascular changes are common, often preceded by retinopathy. Perivascular arteriolar fibrosis is often present in the hearts of patients with manifestations of myocardial ischemia despite the absence of marked stenosis demonstrable angiographically. Thus, coronary microcirculatory abnormalities may be caused by insulin resistance typical of that seen with type 2 diabetes, characterized also by abnormalities in the hemostatic and fibrinolytic systems. In addition to increased concentrations in blood of plasminogen activator inhibitor type 1 (PAI-1), the primary physiological inhibitor of fibrinolysis, concentrations of tissue factor (TF), an important initiator of coagulation, are increased. Both may influence vascular remodeling. Interactions of cell surface–mediated generation of plasmin with extracellular matrix may alter both cell migration and turnover of matrix.

The present study was designed to characterize expression of proteins involved in the fibrinolytic and coagulation systems in relation to coronary microvascular changes in genetically obese mice (ob/ob) that develop insulin resistance and non–insulin-dependent diabetes mellitus and to determine whether the inhibitor of ACE activity, known to lower PAI-1 concentrations and attenuates PAI-1 expression, perivascular fibrosis, or both in obese mice.

Methods

Experimental Animals and Morphometric Analysis

Experiments were in conformity with the guidelines of Hokkaido University and were approved by the Institutional Animal Study committee. Adult male obese mice (C57BL/6J ob/ob) 8 weeks old and their lean littermates, obtained from the Jackson Laboratory (Bar Harbor, Me), had free access to chow and water. In some experiments, obese mice were given temocapril hydrochloride (Sankyo) in drinking water (20 mg · kg⁻¹ · d⁻¹) to induce ACE inhibition.

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beginning when they were 10 weeks old and continuing for 10 weeks.

In animals 10 weeks of age (10 control and 10 obese mice) and in animals 20 weeks of age (10 control, 10 obese, and 7 temocapril-treated), anesthesia was induced with ether. Blood samples were drawn from the left ventricle. The hearts were perfused with PBS, excised, rinsed in PBS, and weighed. A portion of the left ventricle was embedded into O.C.T. compound (Miles), frozen in liquid nitrogen, stored at $-80^\circ C$, cut into sections 10 $\mu m$ thick with a cryostat, and stained with elastica–Masson’s trichrome. The remaining tissue was frozen in liquid nitrogen and stored at $-80^\circ C$.

Glucose and PAI-1 activity in blood were measured as described previously. Insulin was assayed by ELISA specific for mouse insulin (Shibayagi). Left ventricular myocardium–embedded coronary luminal areas, vessel walls, and perivascular fibrosis were quantified as described previously.

Immunohistochemistry

PAI-1 and TF were detected immunohistochemically with the streptavidin–biotin immunoperoxidase method (Nichirei). In brief, frozen sections (10 $\mu m$) fixed in acetone were immersed in 3% H$_2$O$_2$ for 15 minutes and incubated with PBS containing 10% rabbit serum (for PAI-1) and 5% goat serum (for TF) to block nonspecific staining. The sections were rinsed with PBS and incubated with anti-mouse PAI-1 sheep IgG (American Diagnostica) or with anti-human TF guinea pig IgG diluted 1:250. After a washing with PBS, the sections were incubated with biotinylated anti-sheep rabbit IgG (Chemicon) for PAI-1 and anti-guinea pig IgG (Vector) for TF. They were again washed with PBS, incubated with peroxidase-labeled streptavidin, reacted with 3,3'-diaminobenzidine, and counterstained with hematoxylin. Control staining was performed with isotype IgG as the primary antibody. Qualitative assessment of intensity of staining was performed with a visual grading scale (0 to 1111). Cardiac sections were characterized by comparison with grading scale sections by 2 observers blinded to the categories of the animals. Quantitative image analysis of the sections was performed as previously described by observers blinded with respect to the groups of animals. The immunohistochemically stained slides were viewed with a light microscope, and digital gray-scale images were

Figure 1. A, Body weight, heart weight, and left ventricular weight in lean control (n=0) and 10-week-old obese (n=10) mice. B, Body weight, heart weight, and left ventricular weight in lean control (n=0), obese (n=10), and 20-week-old obese mice treated with temocapril (n=7) for 10 weeks.

Figure 2. A, Fibrosis-to-lumen and fibrosis-to-wall ratios of coronary arteries with ID <100 $\mu m$ in lean control (n=10) and 10-week-old obese (n=10) mice. Top, Representative photograph of coronary arteries from control and obese mice. B, Fibrosis-to-lumen and fibrosis-to-wall ratios of coronary arteries with ID <100 $\mu m$ in lean control (n=10), obese (n=10), and 20-week-old obese mice treated with temocapril (n=7) for 10 weeks. Top, Representative photograph of coronary arteries in control, obese, and temocapril-treated obese mice.
acquired with a CCD camera with constant settings. Image collection and analysis were performed with Microcomputer Imaging Device software (Imaging Research). Gray-scale values (pixel intensities) within the regions of interest were plotted as histograms, and minimum, maximum, and mean pixel intensity values were calculated with conventional software for comparison of intensities of immunoperoxidase reaction products. Data are expressed as intensity units above values with isotope IgG used as the primary antibody and as a control for comparison. For identification of endothelial cells, immunohistochemical staining was performed with anti–factor VIII–related antigen polyclonal antibody (Nichirei).

Expression of PAI-1 and TF mRNA
Total RNA isolated from left ventricular myocardium was used for first-strand cDNA synthesis. The reverse transcription–polymerase chain reaction (RT-PCR) with selected primers was used for amplification of PAI-1, TF, and β-actin mRNA as previously described. PAI-1 and TF sequences were amplified in a thermal cycler (Perkin-Elmer) for 35 cycles. The quality of RNA preparation and cDNA synthesis was verified by amplifying DNA coding sequences by PCR and subcloned to pBluescript II SK+ vector (Stratagene).

RT-PCR products were visualized on 2% agarose gels with ethidium bromide. Signals were digitized and evaluated with an optical scanner (GT-9500, Seiko) with density measured with the use of an NIH image program in the public domain (Research Services Branch, NIH). In situ hybridization was performed as previously described. In brief, an 856-bp fragment of mouse PAI-1 (nucleotides 475 to 1330) derived from mouse PAI-1 cDNA was amplified by PCR and subcloned to pBluescript II SK+ vector (Stratagene). Sense and antisense probes were prepared by linearizing the constructs with appropriate restriction enzymes and transcribed in vitro with either T7 or T3 RNA polymerase (Roche Diagnostic). Frozen sections were fixed in 4% paraformaldehyde/PBS and treated with 0.25% acetic anhydride. Sections were prehybridized with 200 μL hybridization buffer (5×SSC, 50% formamide, 5×Denhardt’s solution, and 500 μg/mL tRNA) and hybridized with 40 ng of digoxigenin-labeled riboprobe in 100 μL of hybridization buffer at 70°C for 16 hours. They were washed with 5×SSC at 70°C for 10 minutes, 0.2×SSC at 70°C for 60 minutes; treated with 0.1 mol/L maleic acid buffer for 5 minutes and 1% blocking reagent containing maleic buffer for 60 minutes; and incubated with alkaline phosphatase–labeled anti-digoxigenin antibody (1:5000) for 60 minutes (Roche Diagnostic). Signals were detected with X-Phosphate/nitro blue tetrazolium. Serial sections were hybridized with sense probe as negative controls.

Statistical Analysis
Comparisons between groups were performed by ANOVA with multiple comparisons (Fisher’s protected least significance t test) with Stat View (Abacus Concepts). Morphological data were analyzed with nonparametric statistics (Kruskal-Wallis for ANOVA designs). Results expressed are mean±SD. A value of P<0.05 was considered significant.

Results
Body Weight, Heart Weight, and Left Ventricular Weight
At 10 weeks of age, obese mice exhibited a >2-fold increase in body weight compared with that of controls (Figure 1). The difference was also significant in animals 20 weeks of age. Body weights of obese mice treated with temocapril were not

<table>
<thead>
<tr>
<th>Categories</th>
<th>PAI-1, AU</th>
<th>IA PAI-1, Pixel Intensity</th>
<th>TF, AU</th>
<th>IA TF, Pixel Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=10)</td>
<td>1.20±0.39</td>
<td>7.3±2.5</td>
<td>1.15±0.35</td>
<td>3.1±1.7</td>
</tr>
<tr>
<td>Obese (n=10)</td>
<td>3.32±0.62*</td>
<td>15.2±4.5*</td>
<td>3.58±0.53*</td>
<td>14.8±3.6*</td>
</tr>
<tr>
<td>Temocapril-treated (n=7)</td>
<td>2.00±0.85†</td>
<td>9.8±3.7†</td>
<td>1.68±0.51†</td>
<td>5.7±3.2†</td>
</tr>
</tbody>
</table>

IA indicates quantitative image analysis. Scores are mean±SD for immunohistochemically detected PAI-1 and TF; mean pixel intensity is based on quantitative image analysis of immunohistochemically detected PAI-1 and TF as described in Methods. The grading scales used for AU provide a range of values from 0 (minimum) to 4+ (maximum intensity and distribution).

*P<0.05 vs results in control animals.
†P<0.005 vs results in obese animals.

Figure 3. Representative photographs of cardiac cross sections stained immunohistochemically for PAI-1 and TF. Brown stain delineates PAI-1 and TF visualized with diaminobenzidine. Sections were counterstained with hematoxylin to show nuclei in dark blue. PAI-1 (A) and TF (D) stain of cardiac tissue from control 20-week-old lean mouse. PAI-1 (B) and TF (E) stain of cardiac tissue from 20-week-old obese mouse. PAI-1 (C) and TF (F) stain of cardiac tissue from 20-week-old obese mouse treated with temocapril for 10 weeks. Arrows indicate staining for PAI-1 and TF.
significantly different from those of untreated obese mice. Left ventricular weight was also increased significantly in obese mice 10 and 20 weeks of age. Heart and left ventricular weights in obese mice treated with temocapril from 10 to 20 weeks of age were significantly less than those in untreated obese mice.

Glucose and Insulin Concentrations and PAI-1 Activity in Blood

Increased concentrations of glucose in blood were evident in obese mice 10 weeks (231 ± 15 versus 103 ± 7 mg/dL, P < 0.01) and 20 weeks (242 ± 16 versus 116 ± 9 mg/dL, P < 0.01) of age. Immunoreactive insulin was increased in obese mice 10 weeks (2.98 ± 0.49 versus 0.56 ± 0.19 ng/mL, P < 0.01) and 20 weeks (3.03 ± 0.36 versus 1.02 ± 0.35 ng/mL, P < 0.01) of age as well. Temocapril did not affect the blood glucose or insulin concentrations in obese mice (results not shown). PAI-1 activity was higher in plasma from obese mice 10 weeks of age (5.5 ± 3.2 arbitrary units [AU]/mL) than in controls (2.0 ± 0.9 AU/mL, P < 0.05). A difference was also evident in animals 20 weeks of age (3.5 ± 1.9 versus 2.0 ± 0.3 AU/mL, P < 0.05). Temocapril significantly attenuated the increase in PAI-1 activity in obese mice 20 weeks of age (1.2 ± 0.6 AU/mL, P < 0.05).

Morphometric Analysis

Considerable fibrosis was seen around coronary arterioles and small arteries in obese mice 10 and 20 weeks of age (Figure 2), with a significant increase in the fibrosis-to-lumen and fibrosis-to-wall ratios at 10 and 20 weeks of age compared with values in controls. Treatment with temocapril attenuated these increases.

Immunohistochemistry

Although only faint immunoreactivity of PAI-1 and TF was detected in microvessels of control mice 20 weeks of age, strong signals were seen in endothelium in obese mice 20 weeks of age (Figure 3). Immunoreactivity of PAI-1 and TF was less in the specimens from obese mice treated with temocapril from 10 to 20 weeks of age than in those from untreated obese mice. As shown in Figure 3 and in the Table, samples from obese mice exhibited significantly more PAI-1 than those from lean controls (3.32 ± 0.62 versus 1.2 ± 0.39 AU, P < 0.05). Temocapril attenuated the increase (2.00 ± 0.85 AU, P < 0.005). Samples from obese mice exhibited significantly more TF (3.58 ± 0.53 AU) than those from lean control mice (1.15 ± 0.35 AU, P < 0.05) (Figure 3, Table). Temocapril attenuated this increase as well (1.68 ± 0.51 AU, P < 0.005). Quantitative (automated image analysis) results were consistent with these observations visually (Table). Thus, as shown in the Table and Figure 4, PAI-1 and TF were increased in the obese mouse samples, and temocapril attenuated the increases.

The abundance of PAI-1 or of TF relative to β-actin in hearts from obese and lean mice is shown in Figure 5. PAI-1 mRNA was markedly increased in obese compared with lean mice 10 weeks of age. TF mRNA was increased as well, but
less markedly. PAI-1 mRNA increased further in obese mice 20 weeks of age, as did TF mRNA. Treatment with temocapril significantly reduced the magnitude of the increase in PAI-1 mRNA and TF mRNA in obese mice, but not to control levels. Expression of β-actin mRNA was not altered. In situ hybridization demonstrated PAI-1 mRNA in medial layers within the arteries (Figure 6), consistent with our immunohistochemical results (Figures 3 and 4). No positive signals were observed in the sense control sections.

**Discussion**

**Diabetes and Hypofibrinolysis**
Genetically modified mice that are rendered leptin-deficient develop obesity, insulin resistance, hyperinsulinemia, hyperglycemia, and mild non–insulin-dependent diabetes mellitus. We found PAI-1 activity to be increased in blood as well, consistent with observations by others. Protein was increased, as judged from immunochemical analysis in the coronary endothelium of obese compared with lean mice. The RT-PCR results were consistent with increased amplification of PAI-1 and TF mRNA from obese mice. In situ hybridization showed that vascular cells were the loci of PAI-1 expression.

Fibrinolytic system activity is diminished in diabetic and insulin-resistant nondiabetic human subjects, which may be attributable to several mechanisms, including hyperinsulinemia and oxidative stress. Tumor necrosis factor, which is increased in blood and adipose tissue with diabetes, increases PAI-1 production. These factors may lead to impaired fibrinolysis well before the onset of diabetes.

**Coronary Remodeling in Obese Mice**
We found that obese mice exhibit increased perivascular fibrosis, perhaps reflecting the above alteration in local concentrations of fibrinolytic and coagulation system proteins. The increased PAI-1 is hypothesized to have attenuated generation of plasmin and reduced proteolysis of matrix. The increased TF is hypothesized to have induced procoagulant activity, thereby providing a provisional fibrin matrix augmenting cell migration. Although blood pressure was not measured in animals in this study, leptin, the product of the ob gene, increases blood pressure and is deficient in ob/ob mice. Furthermore, the blood pressure of obese-hyperglycemic C57BL/6 mice produced by breeding mice heterozygous for the obesity gene is normal. Thus, it is unlikely that elevated blood pressure and its amelioration by temocapril accounted for our results.

Diabetic patients develop a paucity of coronary collaterals, indicative of limited angiogenesis. Because altered characteristics of left ventricular capillaries in streptozotocin-induced (insulin-deficient) diabetic rats have been reported, in a preliminary study we characterized capillary profiles in obese mice. Remodeling appeared to be constrained in the obese animals (unpublished observations).

By augmenting PAI-1 expression, angiotensin influences fibrinolytic and TGF-β activity. It can also inhibit insulin signaling. In the present study, inhibition of ACE activity attenuated PAI-1 expression and microvascular remodeling. Thus, angiotensin II may increase expression of procoagulant and hypo(proteo)fibrinolytic effects in the heart associated with insulin resistance, which is potentially ameliorated by inhibition of ACE activity with attenuation of perivascular fibrosis in obese mice by reducing expression of PAI-1 and TF. These mechanisms may contribute to the favorable effects of inhibition of ACE activity seen in patients at high cardiovascular risk.

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**References**


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