Influence of PAI-1 on Adipose Tissue Growth and Metabolic Parameters in a Murine Model of Diet-Induced Obesity

P.E. Morange, H.R. Lijnen, M.C. Alessi, F. Kopp, D. Collen, I. Juhan-Vague

Abstract—An increased plasma plasminogen activator inhibitor-1 (PAI-1) level is a risk factor for myocardial infarction, particularly when associated with visceral obesity. Although the link between PAI-1 and obesity is well documented, little is known about the physiological relevance of PAI-1 production by adipose tissue. Therefore, we have compared adipose tissue development and insulin resistance plasma parameters in PAI-1–deficient mice (PAI-1−/−) and wild-type littermates (PAI-1+/+) in a model of nutritionally induced obesity. After 17 weeks of consuming a high-fat diet (HFD), PAI-1+/+ mice showed marked obesity, with a 52% increase in body weight compared with mice that were kept on a standard fat diet (P<0.0001). This weight gain was accompanied by adipocyte hypertrophy and an increase in the number of stroma cells in the gonadal fat pad, expressed as stroma cells/adipocytes (0.67±0.05 versus 0.43±0.02; P<0.001). In plasma, the HFD induced a marked increase in PAI-1 antigen (5.1±0.56 versus 2±0.22 ng/mL; P<0.001), fasting insulinemia (1.1±0.21 versus 0.21±0.04 ng/mL; P=0.001), and glycemia (7.4±0.5 versus 5±0.3 mmol/L; P<0.001), whereas plasma triglyceride levels were not affected. When we compared PAI-1−/− and PAI-1+/+ mice on the HFD, PAI-1−/− mice gained weight faster than did PAI-1+/+ mice, with a significant difference in body weight between 3 and 8 weeks of the diet (32±1.7 versus 26±1.6 g at 6 weeks; P<0.05). After 17 weeks of the HFD, its effect on weight gain and the number and size of adipocytes was similar in PAI-1+/+ and PAI-1−/− mice. By contrast, the increase in the number of stroma cells presented by PAI-1+/+ mice was not observed in PAI-1−/− mice. In obese PAI-1−/− mice, tissue-type PA activity and antigen levels in the gonadal fat pad were significantly higher than in obese PAI-1+/+ mice (230±50 versus 47±20 arbitrary units/g; P<0.01; 40±13 versus 17±13 ng/g, P<0.05, respectively), whereas urokinase-type PA activity and antigen levels were similar in both groups. In plasma, nonobese PAI-1−/− mice displayed 62% higher insulin levels (P<0.05) than did PAI-1+/+ mice. Obese PAI-1−/− mice displayed 68% higher triglyceride levels (P<0.01) and 21% lower glucose levels (P<0.05) than did PAI-1+/+ mice. These data support an effect of PAI-1 on weight gain and adipose tissue cellularity in the induction of obesity in mice. Moreover, PAI-1 influences glucidolipidic metabolism. The elevated expression of PAI-1 observed in human obesity could be involved in mechanisms that control adipose tissue development. (Arterioscler Thromb Vasc Biol. 2000;20:1150-1154.)

Key Words: PAI-1 ■ fibrinolysis ■ obesity ■ adipose tissue ■ insulin

Epidemiological studies suggest that an increased plasma plasminogen activator inhibitor-1 (PAI-1) level is a biological risk factor for the development of atherosclerotic complications (reviewed in Reference 1). Clinical studies have shown that the insulin resistance syndrome with visceral obesity is an important determinant of plasma PAI-1 levels.1,2 Studies in rodents have underlined the role of the fat mass in explaining such a relation.3-5 Plasma PAI-1 levels in obese mice were 5-fold higher than in their lean counterparts and were associated with overexpression of the PAI-1 gene in adipose tissue.6 We have documented PAI-1 secretion by human adipose tissue and have shown that it is more pronounced in visceral than in subcutaneous fat.7 PAI-1 levels are also related to the lipid content and the volume of fat cells.8

Despite this established relation between PAI-1 and obesity, not much is known about the physiological relevance of PAI-1 in obesity and in the related insulin resistance state. PAI-1 controls the fibrinolytic system by specific inhibition of the PAs urokinase (u-PA) and tissue-type PA (t-PA). Therefore, PAI-1 has been implicated in several tissue remodeling processes such as vascular wound healing after injury, tumor invasion, and embryo implantation (reviewed in References 9 and 10). In view of the importance of the plasmin system in tissue remodeling via proteolysis of extra-cellular matrix components or activation of latent growth factors, it may be of interest to consider the elevated expression of PAI-1 not only as a marker of obesity but also as a contributior to modifications in adipose tissue involved in the development of obesity. To test this hypothesis, we have
compared adipose tissue development and plasma parameters of insulin resistance in PAI-1−/−deficient mice (PAI-1−/−) and their wild-type littermates (PAI-1+/+) by using a model of nutritionally induced obesity.

Methods

Animals and Diets

The animals used in this study were 4-week-old homozygous PAI-1−/−deficient mice (PAI-1−/−) and their wild-type littermates (PAI-1+/+) of either sex (about equal numbers of males and females in each group) (Molecular Cardiovascular Medicine Group of the University of Leuven (Belgium)) with a mixed 81% C57BL/6 19% 129SV genetic background. Half of the animals of each genotype were fed a normal standard chow (4% fat, SFD) and water ad libitum; the others were given ad libitum access to a high-fat diet (42% fat, HFD; Harlan TD88137). The mice were weighed weekly and maintained on their respective diets for 17 weeks.

Experimental Protocol

After an overnight fast, mice were euthanized by intraperitoneal injection of 60 mg/kg pentobarbital (Nembutal, Abbott Laboratories). Blood was collected from the tail vein or by vena cava puncture into tubes without or with anticoagulant (0.01 mol/L trisodium citrate, final concentration). Gonadal, retroperitoneal, and subcutaneous fat pads were removed and weighed. The tissues were immediately frozen at −20°C and used for analysis of tissue cellularity and fibrinolytic parameters. For extraction, adipose tissue (≈1 g/mL) was incubated overnight at 4°C on a tilting table in 10 mmol/L sodium phosphate buffer, pH 7.2, containing 150 mmol/L NaCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, and 0.2% NaN3. After centrifugation at 13 000 rpm for 5 minutes, the protein concentration of the supernatants was determined (BCA assay, Pierce). All experiments were performed in accordance with the guiding principles of the American Physiological Society and the International Society on Thrombosis and Haemostasis.12

Number and Size of Fat Cells

Mean adipocyte cell size was evaluated on frozen-cut adipose tissue sections of 15-μm thickness that had been stained with hematoxylin under standard conditions. Cell areas of at least 200 adipocyte sections were measured by using a computerized image analyzer (Samba 2005 TTIN-Alcatel), and the volume of each cell was calculated with the assumption that adipocytes are spherical.13,14 The method of Folch et al15 was used for lipid extraction. The number of fat cells in the adipose pads was calculated as follows: weight of lipid contained in total pad/mean fat cell lipid content (mean volume/lipid density), assuming that the volume of the adipocyte corresponds essentially to the volume of the triglyceride content.16

Mature adipocytes were defined as differentiated cells distended with lipid material. They contained peripherally inconstant nuclei. On 10 randomly selected fields, the number of stroma cell nuclei and the number of adipocytes, based on morphological criteria, were determined and the results expressed as a ratio. All histological analyses were blinded with respect to genotype (PAI-1−/− or PAI-1+/+).

Endothelial Cell Counting

To determine the number of endothelial cells in the stroma, 15-μm-thick adipose tissue sections were labeled with a primary rat anti-mouse CD31 monoclonal antibody (clone MEC 13.3, Pharmigen), and immunostaining was performed by using a biotinylated rabbit anti-rat antibody (Dako) followed by incubation with peroxidase-labeled streptavidin. The color reaction is developed by using 3-amino-9-ethylcarbazole as a chromagen. Standard immunological controls were included and showed no nonspecific labeling. The stained area of 10 randomly selected fields was measured by computer-assisted image analysis and expressed per total field area.

Insulin Resistance Parameters

Insulin was measured with a monoclonal anti-rat insulin radioimmunoassay (Linco Research). Triglyceride and free fatty acids (FFAs) were evaluated by using a routine clinical assay performed on an automatic analyzer (Hitachi 911, Roche). Blood glucose concentrations were measured by using Glucocard strips (Menarini Diagnostica).

Fibrinolytic Parameters

PAI-1, t-PA, and u-PA antigen levels were determined with specific ELISAs17 and expressed in nanograms per milliliter for plasma samples or as nanograms per gram of tissue for extracts of adipose tissue. Zymographic analysis of PA activity in gonadal adipose tissue extracts was performed after electrophoresis on a 12% acrylamide gel cast with 1% nonfat dry milk and 5 μg/mL human plasminogen under nonreducing conditions, as described elsewhere.18 The lysis of the substrate gel (area×intensity) was quantified by using Quantimet 600 image analysis software (Leica) and expressed in arbitrary units obtained per milligram of protein, which were then converted to milligrams of tissue.

Statistical Analysis

Data are expressed as mean±SEM. Statistical significance between groups was calculated by 1-way ANOVA followed by Bonferroni’s correction or by the Mann-Whitney U test, as indicated in the legends to the figure and tables. Values of P<0.05 were considered statistically significant.

Results

Body Weight

The Figure illustrates the body weight gain within the different groups of mice during the study. On the HFD, both PAI-1−/− and PAI-1+/+ mice developed marked obesity compared with mice kept on an SFD. PAI-1−/− mice gained weight faster than did PAI-1+/+ mice, the difference between SFD and HFD being significant after 4 weeks of diet feeding for PAI-1−/− mice, whereas significance was reached only after 8 weeks of diet feeding for the PAI-1+/+ mice. Consequently, body weights of HFD PAI-1−/− mice were significantly higher than those of HFD PAI-1+/+ mice between 3 and 8 weeks of diet feeding, with a maximum 23% body weight difference at 6 weeks. At 17 weeks, body weight and the mean weight of the gonadal fat pad were only slightly higher in obese PAI-1−/− mice than in obese PAI-1+/+ mice (Table 1). Similar results were obtained for the retroperitoneal and subcutaneous fat pads (data not shown).
TABLE 1. Total Body Weight and Weight and Cellularity of Gonadal Fat Pad After 17 weeks of Diet Feeding

<table>
<thead>
<tr>
<th>Genotype</th>
<th>SFD</th>
<th>HFD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PAI-1⁺/+</td>
<td>PAI-1⁻⁻</td>
</tr>
<tr>
<td>Total body weight, g*</td>
<td>28±1.4</td>
<td>30±1.4</td>
</tr>
<tr>
<td>Pad weight, g*</td>
<td>0.42±0.1</td>
<td>0.58±0.2</td>
</tr>
<tr>
<td>Fat cell diameter, μm†</td>
<td>49±4.3</td>
<td>52±4.7</td>
</tr>
<tr>
<td>Adipocyte number, ×10⁶ cells‡</td>
<td>5.1±1.2</td>
<td>5.2±1.8</td>
</tr>
<tr>
<td>Stroma cells/adipocytes§</td>
<td>0.43±0.02</td>
<td>0.45±0.02</td>
</tr>
<tr>
<td>Endothelial cells, %</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND indicates not determined. Values are expressed as mean±SEM. Statistical analysis of differences between groups was performed by using the Mann-Whitney U test.

*P<0.01 and †P<0.05 for differences between PAI-1⁺/+ mice and PAI-1⁻⁻ mice. Significant differences between SFD and HFD mice are reported in the text.

**Fat Cellularity**

Cellularity was evaluated in the gonadal pad. Results are reported in Table 1. The 17-week HFD induced in PAI-1⁺/+ and PAI-1⁻⁻ mice a similar increase in mean fat cell diameter (P<0.001), without a concomitant increase in the number of adipocytes. By contrast, the increase in stroma cell number induced by the HFD in PAI-1⁺/+ mice (P<0.01) was not observed in PAI-1⁻⁻ mice, and adipose tissue of HFD PAI-1⁻⁻ mice contained fewer endothelial cells than did that of HFD PAI-1⁺/+ animals (P<0.01).

**Plasma Metabolic Parameters**

Fasting plasma metabolic parameters after 17 weeks of diet feeding are summarized in Table 2. In PAI-1⁺/+ mice, the HFD induced a marked increase in insulinemia and glycemia (P<0.001) but no change in plasma triglyceride and FFA concentrations. In PAI-1⁻⁻ mice, the HFD induced the same increase in insulinemia (P<0.001) and also provoked an increase in triglycerides (P<0.01) and FFAs (P<0.05) but no significant change in glycemia. Significant differences were therefore observed between HFD PAI-1⁻⁻ and PAI-1⁺/+ mice: PAI-1⁺/+ animals displayed 69% higher triglycerides (P<0.01) and 21% lower glucose levels (P<0.05). Differences between PAI-1⁻⁻ and PAI-1⁺/+ mice were also observed on the SFD, with PAI-1⁻⁻ mice displaying 62% higher levels of insulin than in PAI-1⁺/+ mice (P<0.05).

**Fibrinolytic Parameters**

Plasma PAI-1 antigen levels were higher in HFD than in SFD PAI-1⁺/+ mice (3.04±0.79 ng/mL, n=7, and 1.05±0.13 ng/mL, n=11, respectively; P<0.001). PAI-1 antigen measured in gonadal adipose tissue extracts (ng/g of tissue) from HFD PAI-1⁺/+ mice was slightly but not significantly different from that of SFD PAI-1⁺/+ mice (23±3.3, n=10 and 17±4.8, n=11, respectively).

To evaluate whether a deficiency of PAI-1 caused changes in t-PA or u-PA expression in adipose tissue, t-PA and u-PA activity and antigen levels were quantified in extracts from gonadal pads of obese mice (Table 3). t-PA activity and antigen levels were significantly higher in PAI-1⁻⁻ compared with PAI-1⁺/+ mice (P<0.01 and P<0.05, respectively), whereas u-PA activity and antigen values were comparable.

**Discussion**

The effects of targeted disruption of the PAI-1 gene on body weight, adipose tissue cellularity, and plasma insulin resistance parameters were determined in nonobese and nutritionally induced obese mice to obtain insight into the physiological role of PAI-1 in the development of adipose tissue and in the regulation of glucose and lipid metabolism.

An HFD induced obesity in both PAI-1⁺/+ and PAI-1⁻⁻ mice. Cellular hypertrophy appeared to be the major mode of expansion of the intra-abdominal adipose tissue in mice as was previously shown in rats. The absence of PAI-1 resulted in an accelerated body weight gain between 3 and 8 weeks of diet feeding, indicating that PAI-1 controls the early stage of establishment of obesity in mice. After 8 weeks, the difference in body weight gain between obese PAI-1⁻⁻ and obese PAI-1⁺/+ became nonsignificant. This evolution of
TABLE 3. Plasminogen Activator Activity and Antigen Levels in Extracts of Gonadal Fat Pads of PAI-1+/+ (n=7) and PAI-1−/− (n=9) HFD Mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>t-PA act, AU/g tissue</th>
<th>u-PA act, AU/g tissue</th>
<th>t-PA ag, ng/g tissue</th>
<th>u-PA ag, ng/g tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAI-1+/+</td>
<td>47±20</td>
<td>543±107</td>
<td>17±13</td>
<td>91±13</td>
</tr>
<tr>
<td>PAI-1−/−</td>
<td>230±50†</td>
<td>580±193</td>
<td>40±13*</td>
<td>83±15</td>
</tr>
</tbody>
</table>

Act indicates activity; ag, antigen. Values are expressed as mean±SEM. Statistical analysis was performed by using the Mann-Whitney U test.

*P<0.05 and †P<0.01 for difference between PAI-1+/+ and PAI-1−/− mice.

body weight changes in PAI-1−/− mice during the diet program could be due to a compensatory mechanism that overcomes the genetic defect to control abnormal growth of adipose tissue.

The development and growth of adipose tissue require paracrine interactions between adipocytes and stroma cells. In the present study, obesity was accompanied by an increase in the number of stroma cells in PAI-1+/+ mice but not in PAI-1−/− mice, leading to a significantly lower number of stroma cells in obese PAI-1−/− mice. Because endothelial cells are the major component of the adipose tissue stroma, we have evaluated their numbers in obese PAI-1+/+ and PAI-1−/− mice. A lower number of endothelial cells was observed, suggesting that the difference in stroma cellularity was at least in part due to a difference in the number of endothelial cells. Because angiogenesis during tissue remodeling results partly from the proliferation and migration of endothelial cells, this observation is in agreement with those of Bajou et al, who showed that the bioavailability of PAI-1 to endothelial cells is essential for tumor angiogenesis. The increase in t-PA activity observed in adipose tissue of obese PAI-1−/− mice could have led to excessive extracellular matrix degradation that was not controlled by PAI-1. The results support the importance of a tightly controlled pericellular proteolysis during processes of physiological angiogenesis. There is a close relation between adipogenesis and angiogenesis, the latter maintaining an appropriate balance between blood supply and fat depot size. Chronic injection of leptin into mice led to atrophy of the fat cells and to an increase in adipose tissue vascularity. It has been hypothesized that this effect could be due to a local angiogenic signal provided by leptin that improves the efficiency of lipid release from fat stores to maintain energy homeostasis. In the light of this hypothesis, it could be suggested that PAI-1 deficiency, by inhibiting angiogenesis, impairs the efficiency of lipid release from fat stores and thereby favors fat tissue growth.

In agreement with previous results, a significant increase in PAI-1 plasma levels was observed in obese compared with nonobese PAI-1+/+ mice. PAI-1 antigen levels were not significantly increased in the fat pad of obese mice when expressed per gram of tissue. However, the amount of PAI-1 produced by adipose tissue was approximately 2 times higher in obese than in nonobese mice, when expressed per total fat pad weight (data not shown). Previous reports have shown that PAI-1 mRNA expressed per total RNA mass in adipose tissue was higher in rodents made obese either genetically or by lesioning in the ventromedial hypothalamus than in their lean counterparts. This finding suggests that the contribution of the mass of adipose tissue is at least as important as upregulation of PAI-1 synthesis in the increase in circulating PAI-1 levels observed in obese mice.

Several studies have demonstrated parallel evolutions of PAI-1 and t-PA levels in plasma. These observations have been explained by the fact that t-PA antigen levels also reflect t-PA/PAI-1 complexes, which have a delayed clearance compared with free t-PA. Coordinate regulation of t-PA and PAI-1 synthesis may, however, play a role. In our study, the absence of PAI-1 resulted in significantly higher t-PA activity, as expected, but also in higher t-PA antigen levels, suggesting that PAI-1 may downregulate t-PA synthesis. The lack of a difference in u-PA antigen and activity levels between PAI-1−/− and PAI-1+/+ mice suggests a different regulation of the 2 PAs in adipose tissue.

Concerning plasma metabolic parameters, nonobese PAI-1+/+ mice displayed higher insulin levels than did PAI-1−/− mice, indicating that the effect of PAI-1 on this parameter could be independent of obesity. It has been shown that secretion of PAs from the islets of Langerhans in the rat pancreas increases in parallel with that of insulin in the presence of glucose. In light of our results indicating that PAI-1−/− mice displayed higher insulin levels, it cannot be excluded that the plasmin system may play a role in the regulation of insulin production. After the development of obesity, PAI-1−/− mice displayed higher triglyceride and lower glucose levels compared with those of PAI-1+/+ mice. Further studies are needed to evaluate the relevance of the differences observed in these glucidolipidic parameters between PAI-1+/+ and PAI-1−/− mice.

In conclusion, this study shows that PAI-1 plays a role in adipose tissue growth and cellularity during the development of nutritionally induced obesity and affects glucidolipidic metabolism. The elevated expression of PAI-1 observed in obese human individuals could be 1 of the mechanisms involved in the control of adipose tissue development.

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References

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