Nutritionally Induced Obesity Is Attenuated in Transgenic Mice Overexpressing Plasminogen Activator Inhibitor-1

H. Roger Lijnen,* Erik Maquoi,* Pierre Morange, Gabor Voros, Berthe Van Hoef, Francis Kopp, Désiré Collen, Irène Juhan-Vague, Marie-Christine Alessi

**Objective**—The objective of this study was to investigate the role of plasminogen activator inhibitor-1 (PAI-1) in adipose tissue development in vivo.

**Methods and Results**—Transgenic (Tg) mice overexpressing murine PAI-1 under control of the adipocyte promoter aP2 and wild-type (WT) controls were kept on standard food (SFD) or on high-fat diet (HFD) for 15 weeks. The body weight and the weight of the isolated subcutaneous and gonadal fat deposits of the Tg mice kept on the HFD were significantly lower than those of the WT mice. The number of adipocytes in the adipose tissue was similar for Tg and WT mice on the HFD, but adipocyte hypotrophy and a significantly lower ratio of stroma cells/adipocytes were observed in the Tg mice. A significant negative correlation ($P<0.01$) was observed between expression of preadipocyte factor-1, which blocks adipocyte differentiation, and adipose tissue weight. Fasting insulin and total cholesterol levels on the HFD were lower in Tg than in WT mice.

**Conclusions**—High circulating PAI-1 levels attenuate nutritionally induced obesity. This may be related to modifications in adipose tissue cellularity affecting weight and plasma metabolic parameters. *(Arterioscler Thromb Vasc Biol. 2003; 23:78-84.)*

**Key Words:** plasminogen activator inhibitor-1 ▪ obesity ▪ adipose tissue ▪ adipocyte

**Development of obesity is associated with extensive remodeling of adipose tissue and involves adipogenesis, angiogenesis, and extracellular matrix (ECM) proteolysis.** The plasminogen/plasmin (fibrinolytic) system, which contributes to tissue remodeling by degradation of ECM and basement membrane components or activation of latent growth factors, may thereby play a role in the regulation of adipose tissue growth. In mice and in humans, a significant correlation was observed between body mass index or the amount of visceral fat and plasma levels of plasminogen activator inhibitor-1 (PAI-1), the main physiological inhibitor of tissue-type (t-PA) and urokinase-type (u-PA) plasminogen activator. Several groups have reported PAI-1 synthesis by murine adipocyte cell lines, and human adipose tissue explants and primary cultures of human adipocytes. In human or murine fat, PAI-1 production was also observed in stroma cells. Adipose tissue PAI-1 mRNA levels were enhanced in obese individuals and positively correlated with the volume and lipid content of fat cells. A potential role of PAI-1 in development of obesity was suggested by the findings that PAI-1–deficient mice kept on a high fat diet developed more rapidly adipose tissue than their lean counterparts and that transgenic (Tg) mice overexpressing a stable human PAI-1 variant had virtually no intraperitoneal fat. In contrast, disruption of the PAI-1 gene in genetically obese and diabetic ob/ob mice reduced adiposity and improved the metabolic profile. To additionally elucidate the role of PAI-1 in adipose tissue development, we have studied nutritionally induced obesity in Tg mice overexpressing murine PAI-1.

**Methods**

**Generation and Characterization of Transgenic Mice**

For the construction of the transgene and the generation and characterization of the Tg mice, including PAI-1 and Pref-1 mRNA determination, please refer to http://atvb.ahajournals.org.

**Diet Model**

Five-week-old male mice weighing ~20 g were kept in microisolator cages on a 12-hour day/night cycle and fed water and a standard fat diet (SFD) (n=16 or 18 for wild-type [WT] or Tg mice) or high fat diet (HFD) (n=16 or 20 for WT or Tg mice) at libitum. The SFD (KM-04-k12, Muracon, Carfil) contains 4% (wt/wt) fat and 13% kcal as fat, corresponding to a caloric value of 10.9 kJ/g. The HFD (TD 04457.60665.DD) is available at http://www.atvbaha.org DOI: 10.1161/01.ATV.0000044457.60665.DD

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78
Blood Collection and Tissue Preparation

Blood was collected from the retroorbital sinus with or without addition of trisodium citrate (final concentration 0.01 mol/L); plasma and serum were stored at −20°C.

Intraabdominal (gonadal [GON]) and inguinal subcutaneous (SC) fat pads were removed and weighed. One portion was immediately frozen at −80°C for protein extraction; other portions were used for histology and immunocytochemistry. Therefore, 10-μm cryosections and 7-μm paraffin sections were prepared.

Other organs, including lungs, kidneys, liver, spleen, and heart, were also removed, weighed, and stored at −80°C.

Hematologic and Metabolic Parameters

PAI-1 antigen levels in plasma or tissue extracts were determined with an ELISA specific for murine PAI-1.14 PAI-1 activity levels in plasma were determined after incubation with excess human t-PA for 20 minutes at 37°C and measurement of t-PA/PAI-1 complex by ELISA using the anti-PAI-1 mAb-16F11 for coating and the anti-human t-PA mAb-62E8 for tagging.

For histopathologic examination, organs were removed (2 WT males at 13 weeks of age, 2 Tg males at 12 and 23 weeks of age each, and 3 Tg females at 19 to 20 weeks of age), and sections were stained with H&E or with an antiserum against murine fibrinogen/fibrin, as described elsewhere.32

White blood cells, red blood cells, platelets, hemoglobin, and hematocrit levels were determined using standard laboratory assays. Blood glucose concentrations were measured using Glucocard strips (Menarini Diagnostics). Triglycerides, free fatty acids, alanine aminotransferase (ALT), and total cholesterol were evaluated using routine clinical assays. Insulin was measured with a monoclonal anti-rat insulin radioimmunoassay (Linco Research, Inc).

Fibrinolytic Parameters

Extraction of adipose tissue (≈250 mg/mL) or of different organs was performed as described,13 and the protein concentration of the supernatant was determined (BCA assay, Pierce); extracts were stored at −80°C.

In situ zymography with cryosections of adipose tissue on fibrin overlays was performed at 37°C for 48 hours without or with addition to the gel of neutralizing antibodies against murine t-PA (final concentration, 200 μg/mL) or of purified murine PAI-1 (final concentration, 20 μg/mL). Lysis of the substrate gel was determined by computer-assisted image analysis (expressed in arbitrary units) and normalized to the section area.33

Adipose tissue explants were prepared and incubated as described elsewhere14; PAI-1 antigen levels secreted into the medium were measured at different time intervals (0 to 7 hours) and expressed as ng per μg DNA.

Morphometric and Immunohistochemical Analysis

The areas of at least 200 adipocyte sections were measured and the volume of each cell was calculated as described elsewhere.22 The number of fat cells in the tissue was calculated as follows: weight of total pad/mean fat cell lipid content (mean volume×lipid density). 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.

Staining of blood vessels was performed on paraffin sections using biotinylated Bandeiraea (Griffonia) Simplicifolia BSI lectin (Sigma-Aldrich).44 For each animal, at least 12 randomly selected fields in 4 different sections were analyzed by computer-assisted image analysis, and data were averaged.

Statistical Analysis

Data are expressed as mean±SEM. Statistical significance between groups was evaluated using nonparametric t testing. Values of P<0.05 were considered statistically significant.

Results

Generation and Characterization of Transgenic Mice

A construct containing 5.4 kb of the adipocyte-specific aP2 promoter driving the full-length murine PAI-1 cDNA was used to generate FVB Tg mice with PAI-1 overexpression. Of 17 live offspring from 4 different females, 4 (3 males and 1 female) had integrated the transgene. These were backcrossed with WT C57/B16 mice, and the Tg or WT littermates in the offspring were identified by polymerase chain reaction (not shown), and the plasma PAI-1 antigen level was measured by ELISA. The offspring of breeding pairs of WT and Tg mice comprised 49% WT mice (49% male and 51% female) and 51% Tg mice (43% male and 57% female). Mice from 3 or 2 different founders were used in the WT or Tg groups, respectively.

Histopathologic examination of H&E-stained crosssections through different organs of 12- to 23-week-old male and female WT or Tg mice did not show any apparent abnormalities or differences. The tissue sections included spleen, liver, lung, kidney, heart, stomach, brain, eye, reproductive organs (testis and epididymis or uterus), and SC and GON adipose tissue. Immunochemistry with an antiserum against fibrinogen/γ might not have revealed any fibrin deposits in the organs or tissues. White and red blood cells, platelets, hemoglobin, and hematocrit levels were comparable for the male WT and Tg mice used in the diet study (not shown).

Strong expression of the transgene was confirmed in adipose tissue, but weak expression in heart, kidney, and liver and stronger expression in lung and spleen of Tg mice were also observed, whereas the transgene was not detected in WT tissues (Figure 1). Adipose tissue explants of mice on SFD revealed higher PAI-1 antigen production for Tg compared with WT mice (1.6±0.40 versus 0.43±0.13 ng/μg DNA after 7 hours incubation for GON tissue [P=0.056], and 1.4±0.42 versus 0.49±0.15 ng/μg DNA for SC tissue [P=0.016]), whereas no significant difference was observed for mice on HFD (data not shown).
PAI-1 antigen levels in extracts of SC adipose tissue of Tg mice kept on SFD and in both SC and GON tissue of Tg mice on HFD were slightly elevated compared with those of WT mice, whereas significantly elevated levels were observed in lung and spleen of Tg mice, both on SFD and HFD (Table 1).

Plasma PAI-1 antigen levels at the start of the study (5 weeks of age) were about 10-fold higher in the Tg mice compared with the WT littermates (2.1±0.20 or 3.0±0.18 ng/mL for WT mice included in SFD or HFD groups, with corresponding values of 21±3.9 or 33±4.8 ng/mL for the Tg mice). At the end of the study (20 weeks of age), plasma PAI-1 antigen levels for both WT and Tg mice had not significantly changed on the SFD, whereas after 15 weeks of the HFD, they were 2- to 3-fold increased, resulting in 7-fold higher levels of Tg compared with WT mice (Table 1). PAI-1 activity on a molar basis corresponded to 30% or 25% of the corresponding values of 0.38±0.12 versus 0.61±0.16 (for GON tissue). Addition of murine PAI-1 or of polyclonal rabbit antiserum against murine t-PA to the gel resulted in inhibition of 98% of the fibrinolytic activity (data not shown).

Thus, on SFD, Tg mice with elevated plasma PAI-1 and decreased adipose tissue fibrinolytic activity had fewer stroma cells and higher Pref-1 expressions in adipose tissue. Adipose tissue development was not significantly affected, but histological tissue modifications were observed.

**Overexpression of PAI-1 Impairs Obesity in Mice on HFD**

The intake of the HFD was somewhat higher for WT than for Tg mice (4.1±0.1 g/24 hours versus 3.5±0.1 g/24 hours), and the physical activity at night was comparable (19±3000 versus 28400 cycles/12 hours).

**Overexpression of PAI-1 Does Not Affect Adipose Tissue Development in Mice on SFD**

The food intake of WT and Tg mice was comparable (0.2 g/24 hours), whereas 0.1 g/24 hours versus 4.4 (4.6 0.07 versus 0.13±0.005; *P*<0.01). Staining of blood vessels with the Bandeiraea Simplicifolia lectin did not reveal significant differences between both genotypes with respect to stained area, blood vessel size, and density (Table 3).

Fibrin overlay revealed somewhat lower overall in situ fibrinolytic activity in cryosections of SC or GON adipose tissue of Tg compared with WT mice kept on SFD (ratio of lysis area over section area of 0.48±0.08 versus 1.2±0.15 for SC tissue [mean±SEM of 5 or 6 determinations, *P*<0.005], with corresponding values of 0.38±0.12 versus 0.61±0.16 for GON tissue). Addition of murine PAI-1 or of polyclonal rabbit antiserum against murine t-PA to the gel resulted in inhibition of 98% of the fibrinolytic activity (data not shown).

Thus, on SFD, Tg mice with elevated plasma PAI-1 and decreased adipose tissue fibrinolytic activity had fewer stroma cells and higher Pref-1 expressions in adipose tissue. Adipose tissue development was not significantly affected, but histological tissue modifications were observed.

**TABLE 1. PAI-1 Antigen Levels in Plasma and Tissue Extracts of WT or Tg Mice Kept on SFD or HFD for 15 Weeks**

<table>
<thead>
<tr>
<th></th>
<th>SFD</th>
<th>HFD</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>Tg</td>
</tr>
<tr>
<td>Plasma,* ng/mL</td>
<td>2.0±0.30</td>
<td>24±3.8†</td>
</tr>
<tr>
<td>SC adipose,‡ ng/g</td>
<td>15±2.4</td>
<td>58±15</td>
</tr>
<tr>
<td>GON adipose,‡ ng/g</td>
<td>17±2.8</td>
<td>15±3.1</td>
</tr>
<tr>
<td>Lung,‡ ng/g</td>
<td>59±8.7</td>
<td>210±54‡</td>
</tr>
<tr>
<td>Kidney,‡ ng/g</td>
<td>62±3.5</td>
<td>79±5.5</td>
</tr>
<tr>
<td>Liver,‡ ng/g</td>
<td>54±4.6</td>
<td>79±17</td>
</tr>
<tr>
<td>Spleen,‡ ng/g</td>
<td>18±1.9</td>
<td>140±18‡</td>
</tr>
<tr>
<td>Heart,‡ ng/g</td>
<td>97±6.2</td>
<td>90±11</td>
</tr>
</tbody>
</table>

*Expressed as ng/mL plasma, mean±SEM of 16 to 20 determinations.
†*P*<0.0005 vs WT on the same diet.
‡Expressed as ng/g tissue, mean±SEM of 6 to 8 determinations.
§*P*<0.05 vs WT on the same diet.
versus 23,100±1870 cycles/12 hours). Because the body weight gain on the HFD was significantly lower in the Tg mice, these data suggest a lower feeding efficiency (weight gain/calories consumed).

The HFD induced marked obesity in both the WT and Tg mice. However, weight gain was lower in the Tg mice, resulting in a significantly lower body weight after 15 weeks of HFD (Figure 2 and Table 2). Also, the weight of the isolated SC or GON fat pads was lower for the Tg mice. The weight of other organs was comparable, except for a higher liver weight in WT than in Tg mice (2210±110 mg, *P<0.001); this corresponds with higher ALT levels in WT than in Tg mice on HFD (150±31 versus 63±11 IU/mL, *P<0.05). However, triglyceride levels in the liver of WT mice on HFD were not significantly elevated (1220±127 versus 840±190 mg/dL in Tg mice; *P=0.41). The number of adipocytes in the entire fat depots of WT and Tg mice was very similar for the GON tissue and was

**TABLE 2. Effect of PAI-1 Overexpression in Mice on Body Weight Gain and on Adipose Tissue Weight and Cellularity After 15 Weeks of SFD or HFD**

<table>
<thead>
<tr>
<th></th>
<th>SFD</th>
<th>HFD</th>
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<tbody>
<tr>
<td></td>
<td>WT</td>
<td>Tg</td>
</tr>
<tr>
<td>Body weight at start,* g</td>
<td>22±0.56</td>
<td>20±2.0</td>
</tr>
<tr>
<td>Body weight at end,† g</td>
<td>33±0.91</td>
<td>31±1.7</td>
</tr>
<tr>
<td>Weight gain, g</td>
<td>9.4±0.92</td>
<td>11±0.68</td>
</tr>
<tr>
<td>SC fat, g</td>
<td>0.24±0.037</td>
<td>0.24±0.027</td>
</tr>
<tr>
<td>GON fat, g</td>
<td>0.49±0.073</td>
<td>0.42±0.049</td>
</tr>
<tr>
<td>Adipocyte number, ×10⁶</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SC</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>GON</td>
<td>4.92±0.76</td>
<td>6.49±0.88</td>
</tr>
<tr>
<td>Adipocyte diameter, μm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SC</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>GON</td>
<td>62±4.1</td>
<td>55±3.1</td>
</tr>
<tr>
<td>Stroma cells/adipocytes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SC</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>GON</td>
<td>1.5±0.10</td>
<td>1.0±0.16</td>
</tr>
</tbody>
</table>

Data are mean±SEM of 12 to 20 determinations; ND indicates not determined.
*Body weight at 5 weeks of age.
†Body weight after 15 weeks of SFD or HFD.
‡P<0.0005 vs WT on the same diet.
§P<0.005 vs WT on the same diet.
somewhat higher in the SC tissue of the Tg mice (P = 0.05). Significant adipocyte hypotrophy was observed in the SC tissue, and the ratio of stroma cells versus adipocytes was lower in both SC and GON adipose tissue of the Tg mice (Table 2). Expression of Pref-1 mRNA in gonadal adipose tissue of Tg mice on HFD was about 2-fold higher than in that of WT mice (ratio Pref-1 mRNA/18S rRNA of 0.31±0.09 versus 0.14±0.05; P = 0.13).

Lectin staining did not reveal significant differences between WT and Tg mice on HFD, except for a higher average blood vessel size in the WT mice (Table 3). Fibrin overlay confirmed somewhat lower overall in situ fibrinolytic activity in SC and GON adipose tissue of Tg compared with WT mice (ratio of lysis area over section area of 0.37±0.078 versus 0.52±0.16 for SC tissue, with corresponding values of 0.29±0.058 versus 0.49±0.088 for GON tissue; mean ± SEM of 5 or 6 determinations).

After 15 weeks of HFD, WT mice presented with higher glucose and insulin levels compared with the mice on SFD. However, Tg mice on HFD had lower plasma levels of insulin (0.68±0.11 versus 1.6±0.28 ng/mL, P < 0.005) and total cholesterol (3.8±0.26 versus 5.3±0.21 mmol/L, P < 0.001) than WT controls on HFD, whereas glucose (4.5±0.30 versus 4.5±0.42 mmol/L), triglyceride (1.5±0.11 versus 1.6±0.16 mmol/L), and free fatty acids (0.89±0.05 versus 1.2±0.08 mmol/L) levels were comparable.

### Discussion

To elucidate the role of PAI-1 in development of obesity, Tg mice were generated with overexpression of murine PAI-1 under control of the adipocyte-specific promoter aP2. Strong expression of the transgene was observed in adipose tissue, lung, and spleen, and weak expression in heart, kidney, and liver. Other studies using the aP2 promoter have previously reported low-level expression of transgenes in other tissues, including muscle, heart, spleen, and thymus.35–37 In this study, 5-week-old male Tg and WT mice of the same genetic background were kept on SFD or HFD for 15 weeks.

For both WT and Tg mice on SFD or HFD for 15 weeks, the body weight was positively correlated with the weight of the SC and GON adipose tissue and with the liver weight (all P<0.0001). Tg mice on HFD had a lower body weight than WT mice. This may be explained partially by the reduced weight of SC and GON adipose tissue; it should be kept in mind that not all fat deposits were recovered from the body. Furthermore, the liver weight in the WT mice on HFD was significantly higher than in the Tg mice, and ALT levels were significantly elevated,38 although triglyceride levels were only moderately enhanced. The weight of other organs was comparable, suggesting that the observed differences in body fat are not aspecific, although effects of PAI-1 overexpression on general well being cannot be excluded.

The main findings of this study are that high circulating plasma PAI-1 levels or reduced fibrinolytic activity in adipose tissue do not affect adipose tissue development in mice kept on standard chow but result in a reduction of nutritionally induced obesity. Indeed, average body and fat pad weights of mice on SFD were not affected by the ∼12-fold higher average PAI-1 levels in Tg mice, and no significant correlation was observed between plasma PAI-1 antigen levels and body weight or SC or GON fat pad weight. The number and diameter of adipocytes in adipose tissue were not affected, but the ratio of stroma cells versus adipocytes was lower in the Tg mice. The HFD induced obesity and adipocyte hypotrophy in both WT and Tg mice. However, for Tg mice on HFD, the 7-fold higher PAI-1 levels compared with WT mice were associated with significantly lower body and fat pad weights. These findings are in agreement with a previous study showing more rapid adipose tissue development in PAI-1–deficient mice on HFD.27 Furthermore, significant adipocyte hypotrophy was observed in the SC adipose tissue of Tg mice on HFD, and the ratio of stroma cells versus adipocytes was significantly lower both in SC and GON adipose tissue of Tg compared with WT mice. In our previous study with PAI-1–deficient mice, we found that the ratio stroma cells versus adipocytes was also lower than in WT mice.27 In this study, however, only a small effect on weight gain and a decrease in endothelial cell number was observed, whereas in the present study, a strong effect on weight gain was observed but no difference in endothelial cells. Overexpression of PAI-1 may thus differentially effect cell populations compared with its deficiency.

Analysis of blood vessels did not reveal significant differences, except for a somewhat higher average vessel size but lower density in adipose tissue of WT mice on HFD. Overexpression of PAI-1 thus seems to modify the cellularity of adipose tissue, however without significantly affecting angiogenesis. Whether there is a direct relation between modification of the stroma and adipocyte hypotrophy requires additional investigation. In apparent contrast with our data, disruption of the PAI-1 gene in genetically obese and diabetic ob/ob mice resulted in reduced adiposity.29 Our model is different, because it is based on nutritionally induced obesity. The difference may be related to the absence of leptin in the ob/ob mice. The data of the present study are in agreement with our previous study in PAI-1–deficient mice27 and with a recent report that in vivo plasminogen deficiency (inducing hypofibrinolysis, as in our PAI-1 Tg mice) reduces fat accumulation.42
The Pref-1 gene product is abundant in preadipocytes but absent in adipocytes, and constitutive expression of Pref-1 blocks differentiation. Overall, for WT and Tg samples on SFD and HFD, a significant negative correlation was observed between Pref-1 mRNA expression in gonadal adipose tissue and tissue weight (P < 0.01), compatible with an inverse relationship with adipocyte differentiation. Interestingly, Pref-1 expression was significantly higher in GON adipose tissue of Tg compared with WT mice, and 2-fold higher Pref-1 mRNA levels in adipose tissue of Tg mice on HFD corresponded with a 25% reduction of GON adipose tissue weight. This may indicate a lower degree of adipocyte differentiation in the Tg adipose tissue. No correlations were observed between Pref-1 mRNA levels and adipocyte size or the ratio stroma cells/adipocytes. Interestingly, it was recently reported that differentiation of plasminogen-deficient stromal cells into mature adipocytes was reduced compared with WT cells. This supports our findings that reduced fibrinolytic activity may impair differentiation. Thus, overexpression of PAI-1 may result in enhanced expression of antidifferentiation factors such as Pref-1, which may contribute to reduce tissue mass. A similar mechanism was proposed to explain that overexpression of nuclear SREBP-1c interferes with adipocyte differentiation and development of insulin resistance.

Metabolic parameters were also affected by PAI-1 overexpression. The attenuation of obesity observed in TG mice on HFD was indeed accompanied by attenuated hyperinsulinemia, with significantly lower insulin levels compared with WT mice on HFD. Taken together, PAI-1 overexpression had little effect on adipose tissue development in mice kept on normal chow, but attenuated nutritionally induced obesity. This is the first experimental demonstration that high plasma PAI-1 levels are associated with lower body weight in obese subjects. It may be explained by an effect on the cellularity of the adipose tissue (adipocyte hypotrophy, decreased number of stroma cells, or effects on adipocyte differentiation), contributing to a reduction of adipose tissue weight with modulation of metabolic parameters.

Acknowledgments
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References


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I. Construction of the transgene

The promoter/enhancer of the mouse adipocyte P2 (aP2) gene as a 5.4 kb DNA fragment in the plasmid pBluescript SKII(+) (1) was a kind gift of Dr. B.M. Spiegelman (Dana Farber Cancer Institute, Boston, MA). The mouse PAI-1 cDNA was obtained as a 1.3 kb PvuII-DraI fragment in pBSmr1 (2). The plasmid that directs fat-specific PAI-1 expression was constructed by inserting the mouse PAI-1 cDNA into the SmaI site of the pSKII + vector downstream of the aP2 promoter/enhancer. A XbaI-HindIII fragment containing the SV40 polyadenylation signal was inserted downstream of the PAI-1 cDNA. A 7.2 kb aP2-PAI-1 construct containing the aP2 promoter, PAI-1 cDNA and the polyadenylation site, was obtained free of vector sequences by ClaI-SacII digestion and gel purification.

II. Generation and characterization of transgenic mice

The aP2-PAI-1 construct was micro-injected in the pronucleus of fertilized zygotes from FVB mice and transferred to pseudopregnant females. Offspring carrying the transgene were identified by PCR on genomic DNA extracted from tail tips with two pairs of transgene-specific (5’-GCAAGCAAGGCTGAAGACATC-3’; 5’-CAAGGGCTGAAGACATCTGC-3’) and aP2-specific (5’-CCACAATGAGGCAAATCCATAAGG-3’; 5’-CATTGCCAGGGAGAACCAAA-3’) oligonucleotides, which produce 373-bp and 265-bp DNA fragments. PCR amplification of the mouse β-globin gene (reverse, 5’-CCTTGAGGCTGTCCAAAGTGATTGAGCCCATCG-3’ and forward, 5’-
CCAATCTGCTCACACAGGATAGAGGGGAGG-3' primers) was used as an internal positive control.

Total RNA from different mouse tissues was prepared using the TRIzol reagent (Life Technologies, Gaithersburg, MD), treated with RNase-free DNase I using RNeasy mini spin columns (Qiagen, Valencia, CA) and RNA concentrations were determined with the RiboGreen RNA quantification kit (Molecular Probes Europe, Leiden, The Netherlands). To determine transgene PAI-1transcripts, a specific primer pair was designed: forward (5'-GCAAGTGATGGAGCCTTGACA-3') and reverse (5'-TTCACTGCATTCTAGTTGTGGTTTG-3'). The expression level of the mRNA was determined by semi-quantitative RT-PCR using the GeneAmp Thermostable RNA PCR kit (Applied Biosystems) as described previously (3) and was normalized to the 28S rRNA level.

Preadipocyte factor 1 (Pref-1) mRNA levels were quantified by real-time RT-PCR. Total RNA (100 ng) was reverse transcribed for 1 h at 48°C using the Taqman Reverse Transcription kit and 2.5 μmol/L random hexamers (Applied Biosystems, Foster City, CA). Quantitative real-time PCR was performed in the ABI-prism 7700 sequence detector, using the Taqman PCR core reagent kit (Applied Biosystems). Each reaction contained 300 nmol/L of both forward (5'-AACCATGGCAGTGCATCTG-3') and reverse (5'-AGCATTCGTACTGGCCTTTC-3') primers and 100 nmol/L of FAM-labeled fluorogenic probe (5'-FAM-AAATAGACGTTCGGGCTTGCACCTC-TAMRA-3') designated using the Primer Express software (Applied Biosystems). Samples were normalized using the housekeeping gene 18S rRNA. Quantitative real-time PCR for 18S rRNA was performed using the Taqman ribosomal RNA control (Applied Biosystems)
according to the manufacturer’s protocol. Each PCR amplification was performed in duplicate wells, using the following conditions: 2 min at 50°C and 10 min at 94°C, followed by 40 two-temperature cycles (15 s at 95°C and 1 min at 60°C). To quantify the results, serial dilutions of reverse transcribed total RNA extracted from a tissue with known expression of the target were used for calibration.

**III. References**

