Plasminogen activator inhibitor-1 modulates adipocyte differentiation

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Liang, Xiubin, Talerngsk Kanjanabuch, Su-Li Mao, Chuan-Ming Hao, Yi-Wei Tang, Paul J. Declerck, Alyssa H. Hasty, David H. Wasserman, Agnes B. Fogo, and Li-Jun Ma. Plasminogen activator inhibitor-1 modulates adipocyte differentiation. Am J Physiol Endocrinol Metab 290: E103–E113, 2006. First published September 6, 2005; doi:10.1152/ajpendo.00605.2004.—Increased plasminogen activator inhibitor-1 (PAI-1) is linked to obesity and insulin resistance. However, the functional role of PAI-1 in adipocytes is unknown. This study was designed to investigate effects and underlying mechanisms of PAI-1 on glucose uptake in adipocytes and on adipocyte differentiation. Using primary cultured adipocytes from PAI-1+/+ and PAI-1−/− mice, we found that PAI-1 deficiency promoted adipocyte differentiation, enhanced basal and insulin-stimulated glucose uptake, and protected against tumor necrosis factor-α-induced adipocyte dedifferentiation and insulin resistance. These beneficial effects were associated with upregulated glucose transporter 4 at basal and insulin-stimulated states and upregulated peroxisome proliferator-activated receptor-γ (PPARγ) and adiponectin along with downregulated resistin mRNA in differentiated PAI-1−/− vs. PAI-1+/+ adipocytes. Similarly, inhibition of PAI-1 with a neutralizing anti-PAI-1 antibody in differentiated 3T3-L1 adipocytes further promoted adipocyte differentiation and glucose uptake, which was associated with increased expression of transcription factors PPARγ, CCAAT enhancer-binding protein-α (C/EBPα), and the adipocyte-selective fatty acid-binding protein aP2, thus mimicking the phenotype in PAI-1−/− primary adipocytes. Conversely, overexpression of PAI-1 by adenovirus-mediated gene transfer in 3T3-L1 adipocytes inhibited differentiation and reduced PPARγ, C/EBPα, and aP2 expression. This was also associated with a decrease in urokinase-type plasminogen activator mRNA expression, decreased plasmin activity, and increased collagen I mRNA expression. Collectively, these results indicate that absence or inhibition of PAI-1 in adipocytes protects against insulin resistance by promoting glucose uptake and adipocyte differentiation via increased PPARγ expression. We postulate that these PAI-1 effects on adipocytes may, at least in part, be mediated via modulation of plasmin activity and extracellular matrix components.

Plasminogen activator inhibitor-1: peroxisome proliferator-activated receptor-γ; adipocyte differentiation; glucose uptake; insulin resistance

PAI-1 has been associated with fibrosis and thrombosis in experimental models and humans (20, 28, 30, 39, 62).

Emerging evidences implicate PAI-1 as a significant risk factor for macrovascular and microvascular complications of diabetes. PAI-1 is also linked to insulin resistance. Indeed, circulating PAI-1 levels are elevated at an early stage of impaired glucose tolerance and continue to be elevated as diabetes and the metabolic syndrome develop (29, 38). Plasma PAI-1 correlates strongly with the degree of insulinemia, and PAI-1 levels are further increased in type 2 diabetes even after correction for insulin levels (2, 42, 61). Increased circulating PAI-1 levels are also found in offspring and relatives of type 2 diabetics (22). Obesity also contributes significantly to elevated plasma PAI-1 levels. PAI-1 is overexpressed in adipose tissue of obese mice and humans (1, 53). In contrast, surgical fat removal or weight loss attributed to diet-mediated fat reduction is associated with a decrease in plasma PAI-1 in obese subjects (31, 45). In previous studies, increased PAI-1 levels were presumed to be consequent to obesity and insulin resistance. Our recent data indicate that PAI-1 may not merely increase in response to development of obesity and insulin resistance but may have a direct causal role (40). Using a high-fat diet-induced obesity and insulin resistance mouse model, we found that PAI-1 deficiency completely prevented development of obesity and insulin resistance (40).

Although adipose tissue only accounts for a relatively small proportion (<10%) of the peripheral glucose utilization in response to insulin in nonobese humans and animals, adipocytes may still play an important role in insulin resistance (57).

PAI-1, like tumor necrosis factor-α (TNF-α), adiponectin, and resistin, is highly expressed in adipose tissues of obese animals and human subjects (3, 14, 27, 50). Howevr, the functions of PAI-1 in differentiated adipocytes remain unknown.

Adipocyte differentiation is a complex process. The differentiation of fibroblast-like preadipocytes to mature adipocytes involves striking morphological and biochemical changes. Besides the key regulatory roles of two well-characterized adipogenic transcription factors, CCAAT enhancer-binding protein-α (C/EBPα) and peroxisome proliferator-activated receptor-γ (PPARγ; see Ref. 49), multiple events occur during adipocyte differentiation, including dynamic changes of cell-matrix interactions and extensive extracellular matrix (ECM) remodeling (11). On the basis of the established role of PAI-1 in inhibition of ECM degradation, we hypothesized that alteration of PAI-1 will affect remodeling of ECM, adipocyte differentiation, and insulin resistance in adipocytes. The present

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study was therefore designed to investigate underlying mechanisms by which PAI-1 influences differentiation and glucose homeostasis in adipocytes.

RESEARCH DESIGN AND METHODS

Cell culture. Two in vitro cell culture models were used in this study. To determine the effects of PAI-1 deficiency on adipocyte differentiation, glucose uptake, and TNF-α-induced insulin resistance, primary cultures of adipocytes were obtained from 4-wk-old male PAI-1−/− or PAI-1−/+ mice (both on C57BL/6 background) as previously described (8, 24, 40). Differentiation of preadipocytes to adipocytes was induced by addition of an adipogenic hormonal cocktail (1 μg/ml insulin, 0.25 μM dexamethasone, and 0.5 mM isobutylmethylxanthine) and confirmed morphologically by multiple oil red O-stained fat droplets in the cytoplasm (40). Primary adipocytes at day 10 after induction of differentiation were used for this study. Insulin-resistant primary adipocytes were obtained by incubating these differentiated 10-day adipocytes for an additional 3 days in the presence of 3 ng/ml TNF-α (Sigma, St. Louis, MO) with or without insulin stimulation for 10 min (52).

For studies of altered PAI-1 expression, we used murine 3T3-L1 preadipocytes (American Type Culture Collection, Manassas, VA) grown in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin at 37°C in 5% CO2. After confluence was reached (2 days), differentiation was initiated with adipogenic hormonal cocktail as described above for 2 days and then with DMEM containing insulin (1 μg/ml) alone for 2 days, followed by an additional 2 days in medium without insulin. These differentiated 3T3-L1 adipocytes at day 6 exhibited intracellular lipid droplets and were used for this study. To inhibit PAI-1, 3T3-L1 preadipocytes were treated for 6 days with a neutralizing monoclonal antibody against PAI-1 (MA-33H1F7, 10 g/ml) along with induction of differentiation (6, 7, 15). MA-33H1F7 inhibits mouse and rat PAI-1 by converting PAI-1 to a noninhibitory substrate for tPA (16). The inhibiting efficacy of this antibody on PAI-1 has been demonstrated in vivo (6). The dose of anti-PAI-1 antibody was chosen on the basis of its biological activity in inhibiting PAI-1 effects previously demonstrated in vivo (6).

Adenoviral infection of 3T3-L1 preadipocytes. Recombinant adenovirus bearing human PAI-1 (Ad-PAI-1) and control adenovirus expressing Escherichia coli β-galactosidase (Ad-lacZ) were gifts from Dr. Robert Gerard (University of Texas Southwestern Medical Center; see Refs. 10 and 33). The recombinant viruses were propagated in HEK 293 cells and purified by CsCl density gradient centrifugation. 3T3-L1 preadipocyte cultures (2 days postconfluence) were infected with Ad-PAI-1 or Ad-lacZ by 1 × 10⁹ plaque-forming units/well for 3 h before induction of differentiation. The medium containing free virus was then removed, fresh DMEM with 10% fetal bovine serum was added, and cells were induced to differentiate as above.

Oil Red O staining. Differentiation of preadipocytes to adipocytes was monitored by measurement of intracellular lipid accumulation using Oil Red O staining. After fixation with 10% formalin in PBS for 1 h, the cells were washed and stained with filtered 0.3% Oil Red O in 55% isopropanol for 1 h (40), followed by counterstaining with 0.5% methyl green (Polysciences, Warrington, PA) in 0.1 M sodium acetate, pH 7.4. Differentiation was calculated as percent cells with Oil Red O positivity of total cells, assessed under ×100 magnification.

Glucose uptake. [2-¹⁴C]Deoxyglucose uptake was measured as described previously (40, 43). Briefly, primary adipocytes (10 days postdifferentiation) and 3T3-L1 adipocytes (6 days postdifferentiation) in six-well plates were cultured overnight in serum-free DMEM with low glucose (1 g/l). After KRP buffer wash (containing 136 mM NaCl, 4.7 mM KCl, 1 mM CaCl₂, 1 mM MgSO₄, 5 mM sodium pyrophosphate, 20 mM HEPES, and 1% BSA), cells were incubated with 1 ml KRP buffer at 37°C for 20 min in the presence or absence of insulin as indicated. [2-¹⁴C]Deoxyglucose was added for a final concentration of 0.1 mM (11.0 Ci/mmol; PerkinElmer Life Sciences, Boston, MA) and incubated for 10 min at 37°C. The cells were washed with cold KRP buffer and solubilized in 0.1% SDS. The radioactivity of a 200-μl aliquot was determined in a scintillation counter. Glucose uptake was expressed as the degree of increase compared with basal PAI-1−/− or 3T3-L1 cells, normalized to protein concentration in each sample.

RNA extraction and assessment. Total RNA was extracted from cells as described previously (40, 41). Relative quantitation of expression of several murine genes in primary adipocytes and 3T3-L1 adipocytes was determined by a real-time, one-step RT-PCR assay (TaqMan) using an ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA). A 25-μl reaction mixture containing 2 μg of total RNA, 0.5 μM of each primer, and 0.2 μM TaqMan probe was mixed with 25 μl of the TaqMan One-Step RT-PCR 2× Master Mix (Applied Biosystems), as described previously (17). Primers and probes designed to target mouse PPARγ, adiponectin, resistin, PAI-1, uPA, and collagen I genes are listed in Table 1. The reaction conditions were designed as follows: RT at 48°C for 30 min and initial denaturation at 95°C for 10 min followed by 40 cycles with 15 s at 95°C for denaturing and 1 min at 60°C for annealing and extension. The threshold cycle (Ct), i.e., the cycle number at which the amount of amplified gene of interest reached a fixed threshold, was subsequently determined. Relative quantification of each target mRNA level was normalized to 18S rRNA or β-actin and calculated by the comparative Ct method described elsewhere (36).

Immunofluorescence. 3T3-L1 cells cultured on cover slips were infected with Ad-PAI-1 or Ad-lacZ or not treated as described above. After fixation in methanol-acetone (1:1) for 10 min at room temperature, the cells were permeabilized and blocked with 0.1% Triton X-100 and 5% BSA in PBS for 10 min. After being washed, the cells were then incubated with sheep anti-PAI-1 antibody (1:25; American Diagnostica, Stamford, CT) or goat anti-β-Gal antibody (1:25; Bio-

Table 1. Nucleotide sequences of oligonucleotide primers and probes (5′ to 3′) for real-time PCR

<table>
<thead>
<tr>
<th>Genes</th>
<th>5′ to 3′ Oligonucleotide Sequences</th>
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<tbody>
<tr>
<td>Mouse PPARγ</td>
<td></td>
</tr>
<tr>
<td>Sense</td>
<td>CTGTATTGCGTGAAGACATCTGGGAG</td>
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<tr>
<td>Antisense</td>
<td>ATAGCAGTGTGACATGACGGA</td>
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<tr>
<td>Probe</td>
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<tr>
<td>Mouse adiponectin</td>
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<tr>
<td>Probe</td>
<td>CATAAAGGCGTTCGGCGACTTCCTCT</td>
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<tr>
<td>Mouse resistin</td>
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</tr>
<tr>
<td>Sense</td>
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</tr>
<tr>
<td>Antisense</td>
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<tr>
<td>Probe</td>
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</tr>
<tr>
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<tr>
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<tr>
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<td></td>
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<tr>
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<tr>
<td>Probe*</td>
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<tr>
<td>Mouse collagen I</td>
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<td>Sense</td>
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</tr>
<tr>
<td>Antisense</td>
<td>CTGTCGACATCTTCGAGTTGCTGATAC</td>
</tr>
<tr>
<td>Probe*</td>
<td>TATTGGCTCGCCGCCA</td>
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</table>

*These probes were dually labeled with 6-carboxyfluorescein at the 5′ end and minor groove binder at the 3′ end.
genesis) for 1 h at room temperature. FITC-conjugated rabbit anti-sheep IgG (DakoCytomation, Carpinteria, CA) or FITC-conjugated rabbit anti-goat IgG antibodies (Dako) were then applied and incubated for 1 h. Internalization of inhibitory monoclonal PAI-1 antibody or control antibody in 3T3-L1 cells was assessed by direct staining of permeabilized cells with fluorechrome tetramethylrhodamine isothiocyanate-conjugated rabbit anti-mouse IgG (1:25; Dako). Images of immunofluorescent cells were captured with a Zeiss AxioCam camera attached to a Nikon Eclipse E400 microscope.

Western blotting. Adipocytes (primary and 3T3-L1) grown in six-well plates were induced to differentiate along with treatments indicated above. Cells were lysed in lysis buffer [containing 150 mM NaCl, 50 mM Tris·HCl, pH 7.5, 5 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 µg/ml phenylmethylsulfonyl fluoride, and 1:100 proteinase inhibitor cocktail tablet (Roche Diagnostics, Mannheim, Germany)]. Total protein (30 µg) was separated on SDS-PAGE and transferred to a nitrocellulose membrane. Western blottings were performed with polyclonal rabbit antibodies against PPARγ (catalog no. 2929; Cell Signaling Technology, Beverly, MA), C/EBPα (4AA; Santa Cruz Biotechnology, Santa Cruz, CA), or fatty acid-binding protein (aP2, C-15; Santa Cruz Biotechnology). The blots were subsequently incubated with horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG (Amersham Biosciences, Little Chalfont, UK) or HRP-conjugated bovine anti-goat IgG (Santa Cruz Biotechnology). Immunoreactive proteins were detected and visualized by using enhanced chemiluminescence detection reagents (Amersham Biosciences). The membranes were stripped for β-actin by using monoclonal anti-β-actin antibody (Sigma), as a control for normalization.

Plasmin activity. Total plasmin activity in 3T3-L1 cells lysis was measured by a modified protocol as described previously (28) using a plasmin-specific chromogenic substrate (Chromozym PL; Roche Molecular Biochemicals, Indianapolis, IN). This substance is specifically cleaved by plasmin into a residual peptide and 4-nitroaniline, which can be detected spectrophotometrically. 3T3-L1 adipocyte lysis (80 µl) and 20 µl of 3 mM Chromozym PL were added per reaction. Absorbance was measured at 405 nm. A standard linear curve was generated with serial dilutions of human plasmin (Roche). Results are expressed as units per milligram protein.

Statistical analysis. Data are presented as means ± SE, unless otherwise noted. P values were calculated by ANOVA followed by unpaired t-test as appropriate. A P value of <0.05 was considered to be significant.

RESULTS

PAI-1 deficiency or inhibition stimulates adipocyte differentiation. To address whether the absence of PAI-1 affects adipocyte differentiation, primary cultured adipocytes from PAI-1+/+ and PAI-1−/− mice were used. Preadipocytes from PAI-1−/− showed more avid differentiation (75 ± 1.2 vs. 60 ± 2.4%, P < 0.01) and smaller mature adipocytes vs. PAI-1+/+ at day 10 after induction (Fig. 1, A–D). Consistent with these morphological observations, differentiated PAI-1−/− adipocytes expressed higher levels of the adipocyte-related transcription factor C/EBPα (Fig. 2A) and adipogenic marker aP2 (Fig. 2B) compared with PAI-1+/+ at 10 days after induction (Fig. 2, A and B). Exposure of the differentiated primary PAI-1+/+ adipocytes to TNF-α (3 ng/ml) for 3 days resulted in decreased C/EBPα and aP2, consistent with dedifferentiation effects of TNF-α (47, 58). However, C/EBPα and aP2 protein levels in response to TNF-α in PAI-1−/− adipocytes were relatively more preserved with less decrease vs. PAI-1+/+ adipocytes (Fig. 2, A and B).

In the present study, we confirmed again that PAI-1 deficiency in primary adipocytes significantly enhanced basal glucose uptake vs. PAI-1+/+ (5.8-fold increase in PAI-1−/− vs. PAI-1+/+, P < 0.01, Fig. 3A; see Ref. 40). PAI-1 deficiency further enhanced insulin-stimulated glucose uptake over a range of insulin doses in differentiated adipocytes compared with differentiated PAI-1+/+ adipocytes (Fig. 3A). As ex-
compared with control-induced 3T3-L1 cells (Fig. 4). The addition of anti-PAI-1 antibody to 3T3-L1 adipocytes in the presence or absence of TNF-α stimulated glucose uptake, whereas the absence or presence of PAI-1 deficiency on total cellular GLUT4 protein in 3T3-L1 adipocytes without antibody treatment or treated with nonspecific control antibodies (Fig. 5).

Effects of PAI-1 deficiency on expression of genes related to insulin sensitivity and ECM modulation. We next examined the expression of key genes that regulate insulin sensitivity by real-time RT-PCR. The absence or presence of PAI-1 deficiency in primary preadipocytes before induction had no significant effect on mRNA expressions of PPARγ or adiponectin (Fig. 6, A and B). In contrast, when PAI-1 deficiency adipocytes were differentiated at day 10 after induction, PPARγ and adiponectin were both markedly increased compared with undifferentiated preadipocytes. Interestingly, differentiated adipocytes deficient in PAI-1 showed even further upregulation of PPARγ and adiponectin (1.8- and 1.3-fold of PAI-1+/+ differentiated cells, respectively; Fig. 6, A and B). In contrast, resistin mRNA was significantly downregulated in PAI-1−/− (~7-fold) vs. PAI-1+/+ adipocytes after differentiation (Fig. 6C).

We next examined the effects of inhibition of PAI-1 on expression of the transcription factors PPARγ and C/EBPα and the adipocyte differentiation marker aP2 in differentiated 3T3-L1 adipocytes after PAI-1 antibody treatment. Endogenous PAI-1 mRNA expression in 3T3-L1 cells was not different before or after induction of differentiation. Unexpectedly, PAI-1 antibody treatment led to a decrease of PAI-1 mRNA expression by lack of Oil Red O staining (Fig. 4, F–H).
levels in differentiated 3T3-L1 cells (data not shown). PPARγ (Fig. 7, lane 1), C/EBPα (both 42- and 30-kDa isoforms), and aP2 (Fig. 8, A and B, lane 1) were undetectable or low in undifferentiated 3T3-L1 preadipocytes at baseline as assessed by Western blot analyses. PPARγ (Fig. 7, lane 2), C/EBPα, and aP2 (Fig. 8, lane 2) were strongly induced in day 6 differentiated 3T3-L1 adipocytes compared with undifferentiated preadipocytes and were further upregulated when PAI-1 was inhibited via neutralizing anti-PAI-1 antibody (MA-33H1F7; Figs. 7 and 8, lane 6). Noninhibitory control antibody (MA-32K3) had no effects on PAI-1 (data not shown), PPARγ, C/EBPα, and aP2 expressions (Figs. 7 and 8). Our data suggest that this increased PPARγ and C/EBPα might be potential contributors to the enhanced adipogenesis in PAI-1 antibody-treated 3T3-L1 adipocytes.

As described above, differentiated 3T3-L1 cells treated with neutralized PAI-1 antibody had decreased PAI-1 mRNA levels (data not shown). We postulated that PAI-1 antibodies may
PAI-1 modulates adipocyte differentiation

Fig. 7. Representative Western blot analysis of PPARγ in 3T3-L1 adipocyte lysates. PPARγ protein expression was undetectable in undifferentiated cells alone (lane 1) or in undifferentiated cells infected with Ad-PAI-1 (lane 3) or treated with neutralizing anti-PAI-1 antibody (lane 5). PPARγ was induced in differentiated cells (lane 2). Ad-PAI-1 infection in differentiated cells inhibited PPARγ protein levels (lane 4). Conversely, PPARγ protein expression was further increased in differentiated cells after PAI-1 inhibition with neutralizing anti-PAI-1 antibody treatment (lane 6). Control Ad-lacz infection (lane 7) or control antibody treatment (lane 8) in differentiated cells had comparable PPARγ protein levels vs. normal differentiated cells (lane 2).

<table>
<thead>
<tr>
<th>Adenovirus Infection/Ab</th>
<th>Ad-PAI-1</th>
<th>Neutralizing PAI-1 Ab</th>
<th>Ad-lacz</th>
<th>CONT-Ab</th>
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<tr>
<td>Induction of differentiation</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>(lane)</td>
<td>1</td>
<td>2</td>
<td>3</td>
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PPARγ

β-actin

differentiation resulting from PAI-1 inhibition via anti-PAI-1 antibody treatment (Fig. 4B). PAI-1 overexpression inhibited adipocyte differentiation, leading to only 15 ± 5% of Ad-PAI-1-infected 3T3-L1 preadipocytes undergoing differentiation at day 6 after infection (P < 0.01; Fig. 4D). 3T3-L1 cells infected with control adenovirus carrying lacZ (Fig. 4E) had comparable levels of differentiation as those cells treated with adipogenic cocktail only (Fig. 4A). Adenovirus-infected 3T3-L1 adipocytes without induction of adipogenesis showed no differentiation, as evidenced by lack of Oil Red O staining (Fig. 4, I and J).

Effects of PAI-1 overexpression on adipogenic-related genes and ECM modulators. PAI-1 mRNA was overexpressed (4.8- and 6.1-fold, respectively) in both undifferentiated and differentiated 3T3-L1 adipocytes (6 days after differentiation) after PAI-1 adenovirus infection compared with both undifferentiated preadipocytes and differentiated 3T3-L1 adipocytes (Fig. 10A). Control Ad-lacz had no effect on PAI-1 mRNA expression. The expressions of PPARγ, C/EBPα (42- and 30-kDa isoforms), and aP2 were significantly downregulated in Ad-PAI-1-infected differentiated cells compared with noninfected differentiated cells (P < 0.01; Figs. 7 and 8).

We next determined whether genes of ECM and related proteins were modulated after PAI-1 overexpression in differentiated 3T3-L1 cells. Quantitative real-time RT-PCR analysis revealed that uPA mRNA was highly expressed in differentiated 3T3-L1 adipocytes vs. 3T3-L1 preadipocytes (Fig. 10B). PAI-1-overexpressing undifferentiated 3T3-L1 cells showed no change in uPA mRNA expression. However, overexpression of PAI-1 in differentiated 3T3-L1 adipocytes inhibited the increase of uPA mRNA expression by 35% (P < 0.05 vs. noninfected cells; Fig. 10B). In contrast, collagen I mRNA expression was significantly decreased in differentiated 3T3-L1 cells vs. 3T3-L1 preadipocytes. Overexpression of PAI-1 in differentiated 3T3-L1 adipocytes resulted in increased collagen I mRNA expression compared with noninfected cells (2.6-fold increase, P < 0.05; Fig. 10C). Of note, infection with adenovirus carrying lacZ had no effect on the expression of genes examined (Fig. 10).

have been internalized and thus interacted with PAI-1 protein, with postulated feedback effects on PAI-1 mRNA expression. Therefore, direct immunofluorescence staining was applied to localize the neutralizing monoclonal anti-PAI-1 antibody or control antibody in permeabilized 3T3-L1 cells with fluorochrome TRITC-conjugated rabbit anti-mouse IgG (1:25; Dako). 3T3-L1 cells treated with either PAI-1 antibody or control antibody displayed intracellular staining, whereas those cells without antibody treatment showed no staining (data not shown). Of note, these data do not clarify the molecular mechanism whereby intracellular PAI-1 antibody affected PAI-1 mRNA expression.

The effects of the neutralizing anti-PAI-1 antibody on intracellular plasmin activity in 3T3-L1 cells were also assessed. In contrast to the decreased plasmin activity induced by PAI-1 overexpression, total plasmin activity was significantly increased (1.6-fold) in differentiated 3T3-L1 adipocytes treated with anti-PAI-1 antibody control compared with control, differentiated 3T3-L1 adipocytes (66 ± 2 vs. 42 ± 1 U/μg protein, P < 0.01).

Overexpression of PAI-1 inhibits adipocyte differentiation. To investigate whether PAI-1 directly influences adipocyte differentiation, PAI-1 was overexpressed in 3T3-L1 adipocytes with the use of an adenovirus expression system. Low levels of PAI-1 were seen in noninfected, differentiated 3T3-L1 cells (Fig. 9A). Infection was assessed using an adenovirus expressing β-gal (Ad-lacz), confirming 70–90% infectivity at 24 h posttransfection in day 1 differentiated 3T3-L1 adipocytes by indirect immunofluorescence (Fig. 9B). Comparable infection efficiencies of 70–90% were also observed for adenovirus expressing PAI-1 (Ad-PAI-1; Fig. 9, C and E). Immunofluorescence staining results further confirmed that PAI-1 protein was markedly increased in Ad-PAI-1-infected 3T3-L1 cells at both 1 day and 6 days after differentiation (Fig. 9, C and E, respectively) vs. trace amounts in noninfected, differentiated 3T3-L1 cells (Fig. 9A). Control Ad-lacz had no effect on PAI-1 protein expressions in 3T3-L1 cells at either 1 day or 6 days after differentiation (Fig. 9, D and F, respectively).

We next assessed the effect of overexpression of PAI-1 on adipocyte differentiation. In contrast to enhanced adipocyte
Fig. 8. Effects of modulation of PAI-1 on expression of C/EBPα and aP2 in 3T3-L1 adipocyte lysates by Western blot. C/EBPα (A) and aP2 (B) were expressed at low levels or undetectable in undifferentiated cells alone (lane 1) or in undifferentiated cells with indicated treatments (lanes 3, 5, 7, and 9). C/EBPα (A) and aP2 (B) were strongly induced in day 6 differentiated adipocytes (lane 2) and were downregulated in Ad-PAI-1 infected cells (lane 4), but were upregulated in differentiated cells after PAI-1 inhibition with neutralizing anti-PAI-1 antibody treatment (lane 6). Control Ad-lacZ infection (lane 8) or control antibody treatment (lane 10) in differentiated cells had comparable C/EBPα (A) and aP2 (B) protein levels vs. normal differentiated cells (lane 2).
To test our hypothesis that PAI-1 overexpression inhibits the plasminogen system during adipocyte differentiation, plasmin activity was measured. To this end, we compared differentiated 3T3-L1 cells with or without PAI-1 adenovirus infection. PAI-1 overexpression in differentiated 3T3-L1 adipocytes inhibited total plasmin activity (26% vs. noninfected cells (31 ± 2 vs. 42 ± 6 U/μg protein, P < 0.05). Plasmin levels were comparable between noninfected and Ad-lacZ-infected differentiated 3T3-L1 cells (A). Control Ad-lacZ had no effect on PAI-1 protein expressions in cells at either 1 day or 6 days after differentiation (D and F, respectively).

**DISCUSSION**

Elevated plasma PAI-1 activity has been associated with insulin resistance and closely correlated with visceral fat accumulation in human subjects (1, 12, 23, 34). These data suggest that visceral adipose tissue can be an important contributor to the elevated plasma PAI-1 levels observed in obesity. Indeed, a large body of evidence indicates that adipose tissue produces substantial amounts of PAI-1. Despite the association between increased PAI-1 and obesity, the functional role of PAI-1 in differentiated adipocytes remains to be elucidated. To address this question, we used both PAI-1-deficient primary adipocytes and the preadipocyte 3T3-L1 cell line. In this study, we provide evidence that PAI-1 deficiency promotes adipocyte differentiation and protects against TNF-α-induced dedifferentiation and insulin resistance in primary adipocytes. Inhibition of PAI-1 by an inhibitory anti-PAI-1 antibody in differentiated 3T3-L1 cells recaptured the phenotype of PAI-1 deficiency and enhanced adipocyte differentiation of, and glucose uptake in, 3T3-L1 cells. Conversely, overexpression of PAI-1 in 3T3-L1 cells inhibited adipocyte differentiation. Our results clearly indicate that PAI-1 plays an important role in modulation of adipocyte differentiation.

In our previous studies, we found decreased fat pad weight in PAI-1−/− vs. PAI-1+/+ mice in response to a high-fat diet (40). Furthermore, adipocytes were smaller and more differentiated, with more Oil Red O staining in PAI-1−/− mice vs. the wild type. Thus PAI-1 deficiency promoted adipocyte differentiation, but there was no overall increase in the mass of adipose tissue. There are several possible explanations for the discrepancy between positive correlation of PAI-1 with obesity observed in vivo vs. PAI-1-induced inhibition of adipocyte differentiation found in the present in vitro study. First, it could reflect differences in the role of systemic PAI-1 vs. possible local effects of PAI-1 in adipocytes. As evidenced in our previous study in PAI-1−/− mice on a high-fat diet (40), systemic PAI-1 deficiency also increased resting metabolic rates and total energy expenditure, which was associated with a marked increase in uncoupling protein-3 expression in skeletal muscle, likely mechanisms contributing to prevention of obesity in vivo (40). Second, it is likely that PAI-1 is not the only factor influencing adipogenesis. Human obesity is controlled by many other factors.

Adipocyte differentiation is regulated by the coordinated expression of various transcription factors, including PPARγ.
C/EBP, we examined expression of PPAR and H9251 by adipocyte-related key transcription factors PPAR. Differentiation and glucose uptake in adipocytes are contributed to severe insulin resistance (4, 25). To investigate whether the modulating effects of PAI-1 on adipocyte differentiation and glucose uptake in adipocytes are contributed to by adipocyte-related key transcription factors PPARγ and C/EBPα, we examined expression of PPARγ, C/EBPα, and PAI-1 target genes, including αP2. In the present study, PAI-1 deficiency in primary adipocytes increased basal glucose uptake and adipocyte differentiation, which was associated with increased PPARγ and C/EBPα. Furthermore, PAI-1 inhibition (achieved by an inhibitory anti-PAI-1 antibody) resulted in enhanced adipocyte differentiation and was also associated with significantly upregulated PPARγ, C/EBPα, and αP2 expression in differentiated 3T3-L1 adipocytes. Conversely, PAI-1 overexpression in differentiated 3T3-L1 adipocytes inhibited adipocyte differentiation and was accompanied by decreases in PPARγ, C/EBPα, and αP2 levels. These observations imply that modulating effects of PAI-1 on glucose uptake and adipocyte differentiation were mediated, at least in part, by PPARγ and C/EBPα. Glucose transport across the plasma membrane is mediated by a family of glucose transporter proteins (GLUTs). Upregulated GLUT4 in PAI-1-deficient primary adipocytes may also contribute to the increased basal glucose uptake.

TNF-α is produced in and secreted by adipocytes, and has been implicated as an important mediator of insulin resistance and adipocyte differentiation (63). Blocking of TNF-α functions by either genetic deletion of the TNF-α receptor or neutralization of TNF-α in rodents protects from obesity-induced insulin resistance and increases insulin sensitivity (26, 27, 60). Interestingly, TNF-α induces PAI-1 expression in adipose tissue in rodents and humans (13, 51). In vitro, long-term exposure to TNF-α induces adipocyte dedifferentiation and suppresses insulin-stimulated glucose uptake in adipocytes (18). Negative regulation of PPARγ and C/EBPγ gene expression by TNF-α contributes to its inhibition of adipocyte differentiation, as well as its induction of insulin resistance (32, 47, 67). In this study, we have demonstrated that PAI-1 deficiency protects against TNF-α-induced adipocyte dedifferentiation, as shown by relatively maintained expression of adipocyte transcription factor C/EBPα and the adipogenic marker αP2. We have further demonstrated that PAI-1 deficiency protects against TNF-α-induced insulin resistance, as shown by the maintained high level of glucose uptake in adipocytes. Our results suggest increased PAI-1 levels promote insulin resistance in adipocytes, which may contribute to a role of PAI-1 in systemic insulin resistance (5, 40, 54). Our data also raise the interesting question that TNF-α-induced insulin resistance might, at least in part, be mediated through PAI-1. Further study will be needed to address this hypothesis.

Adipocyte differentiation is a complex process. It has been suggested recently that various factors in cell-cell and cell-matrix communications govern expression of adipocyte transcription factors and therefore regulate conversion of preadipocytes to adipocytes. During differentiation, expression of ECM components, including collagens and proteases, is changed (64, 66). Indeed, adipocytes produce and release a variety of proteolytic proteins (9, 11). Plasminogen activators tPA and uPA are serine proteinases that also play an important role in regulating adipose tissue remodeling. tPA expression and activity decreased, whereas uPA expression and activity increased during adipocyte differentiation (55). Conversely, inhibition of serine proteinase reduced adipocyte differentiation (56). Similarly, plasminogen deficiency suppressed differentiation of 3T3-L1 cells (56). The plasminogen cascade has been postulated to foster adipocyte differentiation by degradation of the fibronectin-rich preadipocyte stromal matrix (56). In the present study, our observations of decreased expression of collagen I and increased expression of uPA after 3T3-L1

![Image](http://ajpendo.physiology.org/DownloadedFrom/10.220.27.33.6/June252017)

**Fig. 10.** Effects of overexpression of PAI-1 in 3T3-L1 cells on gene expression involved in extracellular matrix turnover. 3T3-L1 preadipocytes were infected with adenovirus encoding β-gal (Ad-lacZ) or adenovirus expressing PAI-1 (Ad-PAI-1). mRNA expressions of PAI-1 (A), urokinase-type plasminogen activator (uPA; B), and collagen I (C) were analyzed by quantitative real-time RT-PCR at 6 days after induction of differentiation and compared with cells without induction. Data are means ± SE of values from 3 independent experiments.
adipocyte differentiation are consistent with previous data (55, 66). Interestingly, the inhibited adipocyte differentiation in PAI-1-overexpressing 3T3-L1 cells was associated with upregulated collagen I and downregulated uPA and plasmin. Conversely, PAI-1 inhibition via neutralizing PAI-1 antibody was associated with increased plasmin activity and increased adipocyte differentiation.

Although there is no evidence that PAI-1 can directly affect PPARγ, indirect interactions of PAI-1 with PPARγ through remodeling of ECM components are possible. We speculate that PAI-1 might modulate the microenvironment and network of ECM surrounding adipocytes via uPA and/or plasmin, affecting cell-ECM interactions and transduction of extracellular signals to intracellular components, such as PPARγ. We further conclude that PAI-1 directly modifies adipocyte differentiation and glucose uptake. We postulate that PPARγ may contribute to PAI-1-modulated adipocyte differentiation and insulin sensitivity. Our data suggest that inhibition of PAI-1 might prove to be a novel anti-insulin resistance treatment.

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PAI-1 MODULATES ADIPOCYTE DIFFERENTIATION

E113

34. Landin K, Stigendahl L, Eriksson E, Krotkiewski M, Risberg B, Livak KJ and Schmittgen TD. Lyon CJ and Hsueh WA.