Prevention of Obesity and Insulin Resistance in Mice Lacking Plasminogen Activator Inhibitor 1

Li-Jun Ma,[1] Su-Li Mao,[1] Kevin L. Taylor,[1] Talerngsak Kanjanabuch,[1] YouFei Guan,[2] YaHua Zhang,[2] Nancy J. Brown,[2] Larry L. Swift,[1] Owen P. McGuinness,[3] David H. Wasserman,[3] Douglas E. Vaughan,[2] and Agnes B. Fogo[1]

Increased plasminogen activator inhibitor 1 (PAI-1) has been linked to not only thrombosis and fibrosis but also to obesity and insulin resistance. Increased PAI-1 levels have been presumed to be consequent to obesity. We investigated the interrelationships of PAI-1, obesity, and insulin resistance in a high-fat/high-carbohydrate (HF) diet–induced obesity model in wild-type (WT) and PAI-1–deficient mice (PAI-1$^{-/-}$). Obesity and insulin resistance developing in WT mice on an HF diet were completely prevented in mice lacking PAI-1. PAI-1$^{-/-}$ mice on an HF diet had increased resting metabolic rates and total energy expenditure compared with WT mice, along with a marked increase in uncoupling protein 3 mRNA expression in skeletal muscle, likely mechanisms contributing to the prevention of obesity. In addition, insulin sensitivity was enhanced significantly in PAI-1$^{-/-}$ mice on an HF diet, as shown by euglycemic-hyperinsulinemic clamp studies. Peroxisome proliferator–activated receptor (PPAR)-γ and adiponectin mRNA, key control molecules in lipid metabolism and insulin sensitivity, were maintained in response to an HF diet in white adipose tissue in PAI-1$^{-/-}$ mice, contrasting with downregulation in WT mice. This maintenance of PPAR-γ and adiponectin may also contribute to the observed maintenance of body weight and insulin sensitivity in PAI-1$^{-/-}$ mice. Treatment in WT mice on an HF diet with the angiotensin type 1 receptor antagonist to downregulate PAI-1 indeed inhibited PAI-1 increases and ameliorated diet-induced obesity, hyperglycemia, and hyperinsulinemia. PAI-1 deficiency also enhanced basal and insulin-stimulated glucose uptake in adipose cells in vitro. Our data suggest that PAI-1 may not merely increase in response to obesity and insulin resistance, but may have a direct causal role in obesity and insulin resistance. Inhibition of PAI-1 might provide a novel anti-obesity and anti–insulin resistance treatment. *Diabetes* 53:336–346, 2004

From the [1]Department of Pathology, Vanderbilt University Medical Center, Nashville, Tennessee; the [2]Department of Medicine, Vanderbilt University Medical Center, Nashville, Tennessee; and the [3]Department of Molecular Physiology and Biophysics, Vanderbilt University Medical Center, Nashville, Tennessee.

Address correspondence and reprint requests to Agnes B. Fogo, MD, MCN C3310, Department of Pathology, Vanderbilt University Medical Center, 21st and Garland Ave., Nashville, TN 37232-2561. E-mail: agnes.fogo@vanderbilt.edu.

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AT1RA, angiotensin type 1 receptor antagonist; ECM, extracellular matrix; HF, high fat/high carbohydrate; KRP, Krebs-Ringer phosphate; PAI-1, plasminogen activator inhibitor 1; PPAR, peroxisome proliferator–activated receptor; RAS, renin-angiotensin system; UCP, uncoupling protein.

Obesity is reaching epidemic proportions worldwide. More than half of the adults in the U.S. are overweight or obese (1). Obesity is a strong risk factor for the development of insulin resistance and type 2 diabetes (2). Type 2 diabetes affects ~17 million adults in the U.S. with increased morbidity and mortality due to increased micro- and macrovascular complications (3,4). Aggressive intervention in the early course of the disease can decrease many of the above consequences. Importantly, the prevalence of obesity-related disorders emphasizes the need for concerted efforts to prevent obesity rather than just treatment of its associated diseases.

Although environmental factors play a role in the development of type 2 diabetes, there are also significant genetic risks. Plasminogen activator inhibitor 1 (PAI-1) is the primary physiological inhibitor of tissue-type plasminogen activator and urokinase-like plasminogen activator and inhibits both fibrinolysis and proteolysis (5,6). Increased PAI-1 has been linked to not only thrombosis and fibrosis but also insulin resistance. Circulating PAI-1 levels in humans are increased in obesity and the insulin resistance syndrome and correlate strongly with the degree of insulinemia (7–12). For the last decade, the role of adipose tissue, an endocrine organ, in the development of obesity and insulin resistance has attracted attention. Adipose tissue produces and secretes a large number of hormones, cytokines, and proteins that affect glucose homeostasis and insulin sensitivity, including tumor necrosis factor-α, PAI-1, leptin, peroxisome proliferator–activated receptor (PPAR)-γ, resistin, and adiponectin (13–15). PAI-1 is overexpressed in adipose tissue of obese mice and humans (16,17), and adipose tissue itself may directly contribute to the elevated PAI-1 levels. Obese mice, humans with type 2 diabetes (18–20), and even the offspring of patients with type 2 diabetes (21) have elevated plasma PAI-1. Thus, the elevated PAI-1 associated with obesity has been considered to be a consequence of obesity and to be a marker of risk of type 2 diabetes (22).

PAI-1 is induced both in vitro and in vivo by angiotensin via the angiotensin type 1 receptor (23,24), and high levels are decreased by angiotensin inhibition (25). Adipose tissue itself is an important source of not only PAI-1, but also angiotensinogen. Obesity results in increased activity and/or expression of genes of the renin-angiotensin system (RAS) in adipocytes (26–28). In turn, activated RAS and

increased insulin in obese and hyperinsulinemic states may further upregulate PAI-1 expression in adipocytes (29,30), mainly mediated through the angiotensin type 1 receptor (31).

Effects of modification of PAI-1, through genetic knockout or overexpression of PAI-1, on the development of obesity have been reported, but with controversial results (32–34). We hypothesized that PAI-1 is not merely a product of obesity, but contributes to obesity and insulin resistance. We therefore studied a high-fat/high-carbohydrate (HF) diet–induced model of obesity and insulin resistance in wild-type (WT) and PAI-$1^{-/-}$ mice on a C57BL/6 background. Our findings show that PAI-$1^{-/-}$ mice were protected from obesity and insulin resistance in response to the HF diet. Inhibition of PAI-1 might provide a novel anti-obesity and anti-diabetes treatment and thus has marked beneficial effects on both obesity and type 2 diabetes.

RESEARCH DESIGN AND METHODS

Mice. Adult (10-week-old) male PAI-1–deficient mice (PAI-$1^{-/-}$) on a C57BL/6J background and C57BL/6J wild-type (WT) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were housed in a pathogen-free barrier facility (12-h light/12-h dark cycle). Mice were placed on either a normal chow (Purina Rodent "5001" meal, 23.4% protein, 4.5% fat, 6.0% fiber, 0.40% sodium; Tusculum Feed Center, Nashville, TN) or an HF diet for 12 weeks (Diet F1850; Bioserv Industries, Frenchtown, NJ) in which 58% of total calories are derived from fat and 27% of total calories are from carbohydrate. This diet has been shown to induce obesity, hyperglycemia, and insulin resistance in C57BL/6 mice (35). One additional group of WT mice was given both an HF diet and angiotensin type 1 receptor antagonist (AT1RA) (losartan, 80 mg/l drinking water) for 12 weeks. The dose chosen for AT1RA was found to be effective in treating sclerosis and inhibiting PAI-1 in rats (25,36). Groups of mice were killed under anesthesia with sodium pentobarbital (50 mg/kg body wt i.p.) at baseline (baseline control), 1 week, or 12 weeks after initiation of study (10, 11, and 22 weeks of age, respectively). White adipose tissue (obtained from epididymal fat pads), skeletal muscle (a mix of soleus, gastrocnemius, and vastus lateralis), heart, liver, and kidney were harvested for molecular analysis. Food intake was weighed daily, and body weights were measured every 2 weeks. Metabolic studies were performed as indicated below. All animal protocols were approved by the Vanderbilt University Institutional Animal Care and Use Committee.

Blood and plasma measurements. Blood glucose and plasma measurements were done 6 h after daytime food withdrawal at 0, 4, 8, and 12 weeks. Fasting glucose levels were determined using an Accu-Check glucose monitor (Roche Diagnostics, Boehringer Mannheim, Indianapolis, IN). Fasting plasma insulin and leptin were measured by radioimmunoassay (Linco Research, St. Louis, MO). Active plasma PAI-1 was assessed with an enzyme-linked immunosorbent assay kit specific for active murine PAI-1 (MPAIKT; Molecular Innovations).

Tissue and serum lipid analysis. Triglycerides in tissues were determined by chromatography after extraction of lipids with chloroform and methanol (37,38). Serum triglycerides were measured using an enzymatic assay adapted to microtiter plates (Raicham, San Diego, CA)

Euglycemic-hyperinsulinemic clamp studies. The euglycemic-hyperinsulinemic clamp studies were performed as described previously (39) at 12 weeks after an HF diet in chronically catheterized conscious mice in the Mouse Metabolic Phenotyping Center at Vanderbilt University Medical Center. Briefly, catheterization of the jugular vein and carotid artery was carried out 7 days before study. Each animal was fasted for 6 h on the morning of the experiment. After basal sampling at time 0, insulin was continuously infused at 4 $mU \cdot kg^{-1} \cdot min^{-1}$ for the duration of the 2-h study. Blood glucose levels were measured from 8 μl blood every 5–10 min using a Hemocue glucose analyzer. Glucose was infused at a variable rate to maintain clamped blood glucose levels at 140 mg/dl.

Body temperature. Temperature of mice at baseline and 12 weeks after an HF diet was measured with a rectal thermometer at a depth of 1.5 cm (Yellow Springs Instruments, Yellow Springs, OH).

Indirect calorimetry. Oxygen consumption (V_{O_2}) and the respiratory exchange ratio were measured by an Oxymax indirect calorimeter (Columbus Instruments, Columbus, OH) with an air flow of 0.75 l/min. V_{O_2} is expressed as the volume of O_2 consumed per $kilogram^{0.75}$ body weight per hour. After 1 h, to allow for adaptation to the metabolic chamber, V_{O_2} was measured, starting at 10:00 A.M., in individual mice for 1 min at 15-min intervals for a total of 22 h under a consistent environmental temperature (22°C). The respiratory exchange ratio is the ratio of the volume of CO_2 produced to the volume of O_2 consumed. Energy expenditure was calculated as EE = $(3.815 + 1.232 \times V_{CO_2}/V_{O_2}) \times V_{O_2}$ (40). Mice ambulatory activity was simultaneously estimated by the number of laser beams broken in both X and Y directions.

Northern blot analysis. A 388-bp cDNA probe for uncoupling protein (UCP)-3 (GenBank accession number AF019883) was generated from total murine skeletal muscle RNA by RT-PCR with the specific primers 5′-CAACG GTTGTGAAGTTCCTG-3′ (forward) and 5′-AATCGGACCTTCACCAC-ATC-3′ (reverse) (41). Northern blot experiments were done as previously described (25) with the following probes: mouse PAI-1 (365 bp), PPAR-γ (280 bp), adiponectin (780 bp, provided by Dr. Harvey Lodish, Massachusetts Institute of Technology, Boston, MA), UCP-1, and UCP-2 (provided by Dr. Se-Jin Lee, Johns Hopkins University School of Medicine, Baltimore, MD). The ratio of specific message to the housekeeping gene GAPDH mRNA or 28S rRNA was used to quantify expression for each tissue sample.

Real-time quantitative PCR. Total RNA preparation and the RT reaction were carried out as described previously (42). PCRs were performed using an ABI PRISM 7700 sequence detection system instrument and software (PE Applied Biosystems, Foster City, CA). The primer sequences for the mouse adiponectin genes were as follows: 5′-TGTTGGAATGACAGGAGCTGAA-3′ (forward) and 5′-CACACTGAAGCCTGAGCGA-TAC-3′ (reverse). The TaqMan probe for adiponectin (5′-CATAAGCGGCTTCTCCAGGCTCTCCT-3′) was labeled at the 5′ end with the reporter dye FAM and at the 3′ end with the quencher TAMRA. RNA samples were normalized to the level of 18S rRNA (Applied Biosystems). The probe for 18S rRNA was labeled with *V*IC (reporter dye) and TAMRA (quencher dye).

Isolation and culture of adipose cells. Isolated white adipose cells were obtained from 4-week-old male C57BL/6J WT or PAI-$1^{-/-}$ mice as previously described (43,44). Briefly, epididymal fat pads were dissected, minced, and digested using collagenase type I (Sigma, St. Louis, MO) in Krebs-Ringer HEPES buffer supplemented with 20 mg/ml BSA at 37°C for 2 h. Preadipocytes were isolated and cultured in Dulbecco's modified Eagle's medium containing 15 mmol/l $NaHCO_3$, 15 mmol/l HEPES, 33 μmol/l biotin, 17 μmol/l pantothenate, 0.5 μmol/l human insulin, and 0.2 nmol/l triiodothyronine. Differentiation of preadipocytes to adipocytes was induced by addition of a hormonal cocktail (1 μg/ml insulin, 0.25 μmol/l dexamethasone, and 0.5 mmol/l isobutylmethylxanthine) and confirmed morphologically by multiple oil red O–stained fat droplets in the cytoplasm (45).

Analysis of adipocyte size. Histological sections of epididymal fat pads from WT and PAI-$1^{-/-}$ mice were stained with hematoxylin and eosin and studied under 200× magnification to compare adipocyte size.

Glucose uptake. 2-Deoxyglucose uptake was measured (46). Primary mouse WT and PAI-$1^{-/-}$ adipocytes (day 10 after differentiation) in six-well plates were cultured overnight in 10% fetal serum–Dulbecco's modified Eagle's medium with low glucose (1 g/l). After Krebs-Ringer phosphate (KRP) buffer wash (containing 136 mmol/l NaCl, 4.7 mmol/l KCl, 1 mmol/l $CaCl_2$, 1 mmol/l $MgSO_4$, 5 mmol/l sodium pyrophosphate, 20 mmol/l 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 (10 nmol/l). [2-^{3}H]-deoxyglucose was added for a final concentration of 0.1 mmol/l (23.70 Ci/mmol, Perkin Elmer 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 fold increase compared with WT baseline after protein concentration was normalized in each sample.

Statistical analysis. Continuous data are expressed as means ± SE. Differences between the groups were tested using ANOVA. Overall differences in the euglycemic-hyperinsulinemic clamp study and oxygen consumption study between PAI-$1^{-/-}$ and WT were analyzed using the restricted/residual maximum likelihood–based mixed effect model to adjust for the intracorrelation effect for the mice that had multiple results across different time points. A P value <0.05 was considered significant.

RESULTS

PAI-$1^{-/-}$ mice are protected from diet-induced obesity. When fed with normal chow, PAI-$1^{-/-}$ and WT mice gained weight similarly (Fig. 1*A*). In response to an HF diet, WT mice gained more weight from 4 to 12 weeks, whereas the body weight of PAI-$1^{-/-}$ mice on an HF diet was indistinguishable from that of PAI-$1^{-/-}$ mice on normal chow (at 12 weeks: PAI-$1^{-/-}$ + HF 32.1 ± 1.0 vs.

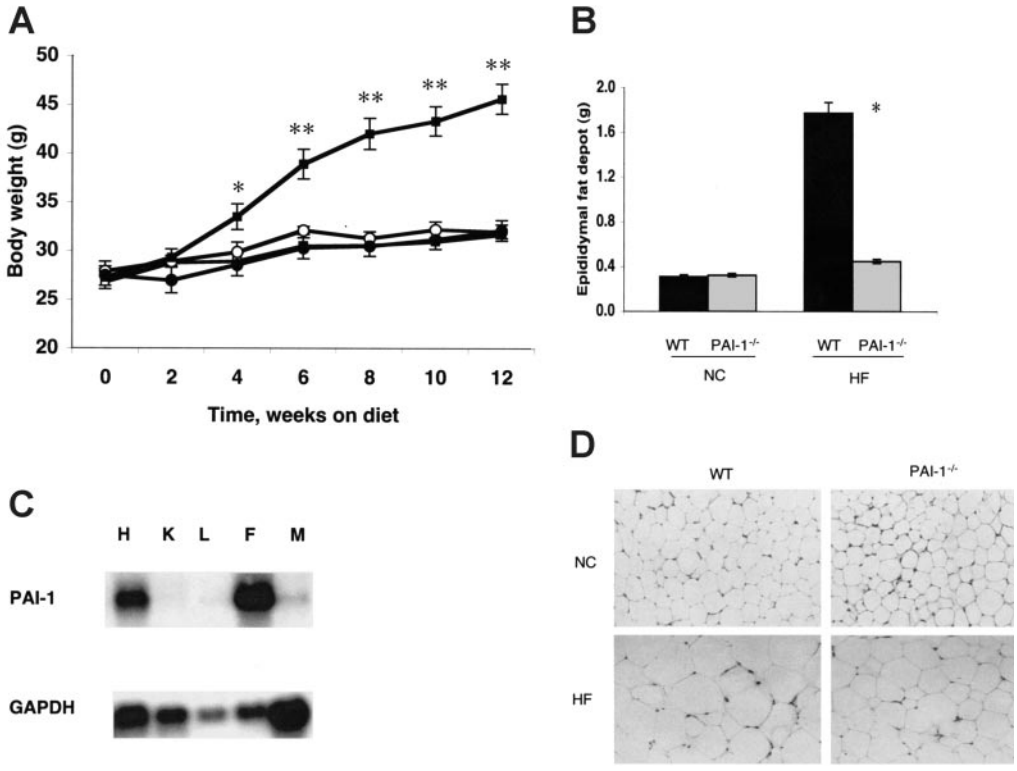

FIG. 1. Effects of PAI-1 deficiency on body weight and fat accumulation. *A*: Body weight gain over time in WT and PAI-1$^{-/-}$ mice on normal chow (NC) and an HF diet (n = 19–21 mice in each group). WT (□) and PAI-1$^{-/-}$ (○) at baseline and after 12 weeks of HF diet (WT, ■; PAI-1$^{-/-}$, ●). *B*: Epididymal fat pad weights of WT and PAI-1$^{-/-}$ mice at baseline on normal chow and after 12 weeks of an HF diet (n = 19–21 mice in each group). *P < 0.01, **P < 0.001. *C*: Representative Northern blot analysis of PAI-1 mRNA expression in different organs in WT mice after 12 weeks of an HF diet (H, heart; K, kidney; L, liver; F, white adipose tissue; M, skeletal muscle). *D*: Histological sections of epididymal fat pads (all hematoxylin and eosin, ×200) of WT and PAI-1$^{-/-}$ mice at baseline on normal chow and after 12 weeks of an HF diet (representative of n = 3 in each group).

WT + HF 45.6 ± 1.5 g, $P < 0.001$; PAI-1$^{-/-}$ + HF 32.1± 1.0 vs. PAI-1$^{-/-}$ + normal chow 32 ± 0.7 g, NS, Fig. 1*A*).

Reduced body fat mass and lipid levels in PAI-1$^{-/-}$ mice. We assessed whether the differences in weight gain were associated with alterations in adiposity. Baseline epididymal fat pad mass in PAI-1$^{-/-}$ mice was similar to that seen in WT mice fed normal chow (0.33 ± 0.02 vs. 0.32 ± 0.02 g, NS, Fig. 1*B*). However, at 12 weeks after the HF diet, the epididymal fat pad mass in PAI-1$^{-/-}$ mice was only ~25% of that in WT mice (0.45 ± 0.02 vs. 1.77 ± 0.10 g, $P < 0.01$, Fig. 1*B*). Subcutaneous white fat pad mass in PAI-1$^{-/-}$ mice was also markedly less than in WT mice in response to an HF diet (data not shown). PAI-1 mRNA was highly expressed in epididymal fat in WT mice after 12 weeks of HF feeding compared with other organs (Fig. 1*C*). Sections of white adipose tissue revealed that PAI-1$^{-/-}$ mice had decreased adipocyte size compared with WT controls both at baseline and 12 weeks after an HF diet (Fig. 1*D*).

Baseline triglyceride contents in skeletal muscle and liver were comparable between PAI-1$^{-/-}$ and WT mice (Table 1), although PAI-1$^{-/-}$ mice tended to have lower triglyceride levels. Triglyceride contents in both skeletal muscle and liver in WT mice were markedly increased at 12 weeks in response to the HF diet. In contrast, the PAI-1$^{-/-}$ mice had significantly lower triglyceride levels in both tissues at 12 weeks (Table 1). No significant differences were observed in plasma triglyceride concentrations between genotypes.

Increased metabolic rates in PAI-1$^{-/-}$ mice. We examined whether the decreased body weight resulted from decreased food intake or increased energy expenditure. Food intake in PAI-1$^{-/-}$ and WT mice on an HF diet was similar (2.6 ± 0.1 vs. 2.5 ± 0.1 g/day). The oxygen consumption rates of PAI-1$^{-/-}$ and WT mice at baseline on normal chow were similar and showed a normal pattern during the 22-h measurement, with higher rates during the night (active state) and lower rates during the day (resting state) (Fig. 2*A*). However, after 12 weeks on HF diet feeding, PAI-1$^{-/-}$ mice exhibited significantly higher rates of total and resting O_2 consumption than WT mice (Fig. 2*B*, $P < 0.001$, across all time points). Interestingly, the pattern of oxygen consumption in response to an HF diet was shifted, with PAI-1$^{-/-}$ mice maintaining higher metabolic rates during daytime resting state. In contrast, WT mice displayed lower metabolic rates during the day and at night (Fig. 2*B*). In parallel, the energy expenditure of PAI-1$^{-/-}$ and WT mice at baseline was similar (Fig. 2*C*). However, the energy expenditure in PAI-1$^{-/-}$ mice at 12 weeks in response to HF feeding was also increased compared with WT mice ($P < 0.001$) (Fig. 2*D*). The respiratory exchange ratios of PAI-1$^{-/-}$ and WT mice on

TABLE 1
Effects of PAI-1 deficiency on lipid content at baseline and 12 weeks after an HF diet

	WT + NC	PAI-1$^{-/-}$ + NC	WT + HF	PAI-1$^{-/-}$ + HF
Muscle TG content (mg/g tissue)	6.9 ± 1.9	3.0 ± 1.1	19.3 ± 3.4	11.3 ± 2.4*
Liver TG content (mg/g tissue)	6.6 ± 1.5	4.1 ± 0.4	29.3 ± 4.8	8.1 ± 2.3*
Serum G content (mg/dl)	97.5 ± 4.3	86.3 ± 6.0	87.8 ± 7.0	83.8 ± 6.0

Data are means ± SE. *$P < 0.05$, PAI-1$^{-/-}$ + HF vs. WT + HF (n = 8–10 in each group). NC, normal chow; TG, triglyceride.

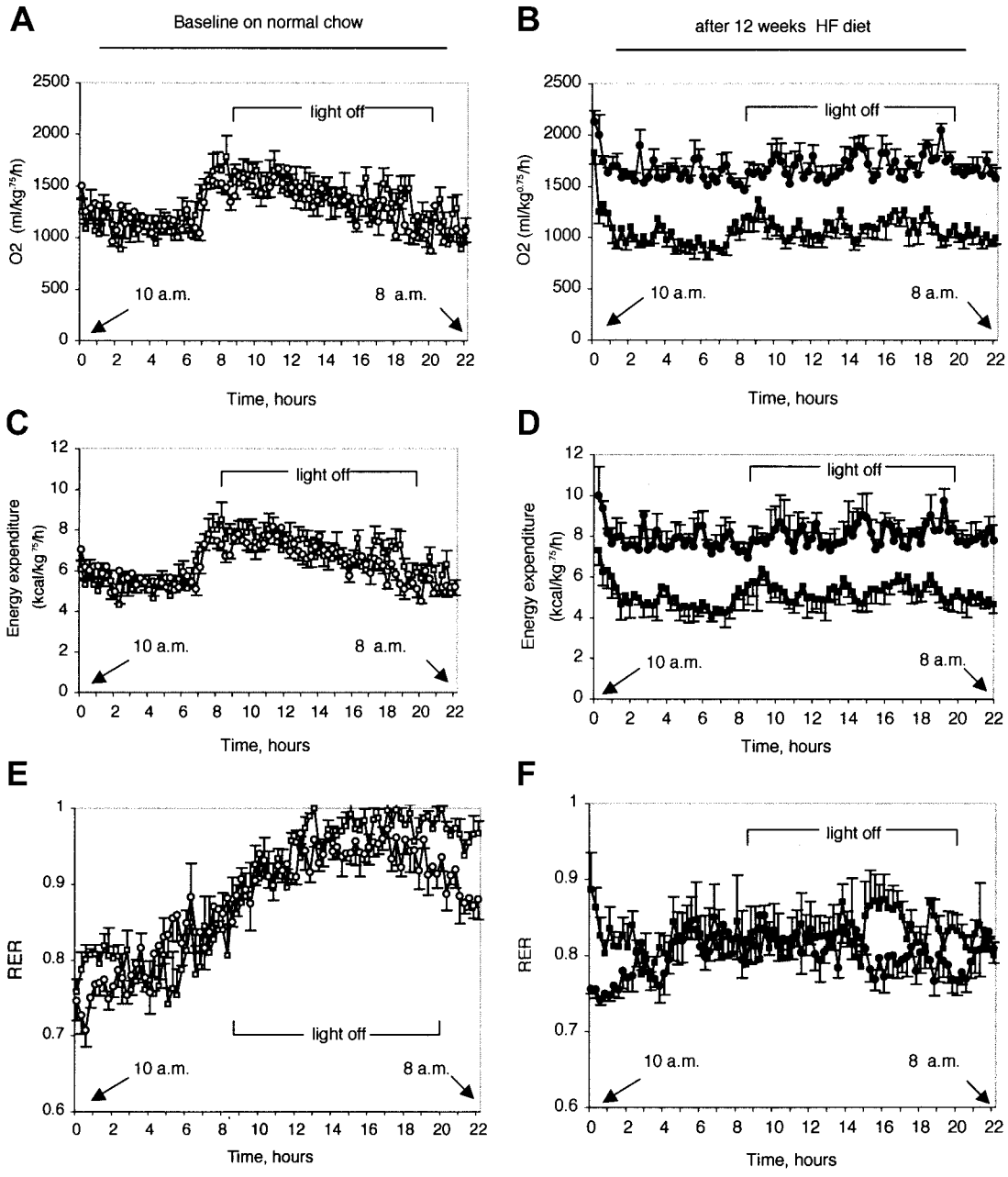

FIG. 2. Increased metabolic rates in PAI-1$^{-/-}$ mice. Effects of PAI-1 deficiency on whole-body oxygen consumption (*A* and *B*), energy expenditure (*C* and *D*), and respiratory exchange ratio (RER) (*E* and *F*) were measured by indirect calorimetry. WT (□) and PAI-1$^{-/-}$ (○) at baseline and at 12 weeks after an HF diet (WT, ■; PAI-1$^{-/-}$, ●). $P < 0.001$, across all time points for both oxygen consumption rates and energy expenditure, WT vs. PAI-1$^{-/-}$ after 12 weeks of an HF diet ($n = 4$ for each group).

both normal chow and an HF diet were similar (Fig. 2*E* and *F*). Consistent with their elevated metabolic rates, PAI-1$^{-/-}$ mice displayed an increase (0.7°F) in core body temperature at 12 weeks after the HF diet compared with WT mice (at baseline: WT 102.3 ± 0.23°F, PAI-1$^{-/-}$ 102.1 ± 0.27°F; at 12 weeks after HF diet: WT 101.4 ± 0.29°F, PAI-1$^{-/-}$ 102.1 ± 0.21°F). Surprisingly, PAI-1$^{-/-}$ mice had similar physical activity compared with WT mice at baseline (Fig. 3*A*) and even slightly lower activity at 12 weeks after the HF diet (Fig. 3*B*).

Expression of UCPs. UCP-1 mRNA in brown adipose tissue was expressed similarly in WT and PAI-1$^{-/-}$ mice at baseline and after the HF diet (Fig. 4*A* and *C*). There was only trace UCP-2 mRNA expression in skeletal muscle in WT and PAI-1$^{-/-}$ mice at baseline. After 12 weeks of the HF diet, UCP-2 mRNA expression in skeletal muscle was significantly increased, especially in PAI-1$^{-/-}$ mice (Fig. 4*B* and *D*). UCP-3 mRNA in skeletal muscle was expressed at similarly high levels in WT and PAI-1$^{-/-}$ mice at baseline. In response to HF feeding, PAI-1$^{-/-}$ mice significantly increased UCP-3 mRNA compared with WT mice, which showed no change in UCP-3 (Fig. 4*B* and *E*).

Enhanced insulin sensitivity in PAI-1$^{-/-}$ mice. The impact of an HF diet on metabolic effects in WT mice was established after only 1 week of HF feeding (Table 2). After 12 weeks of HF feeding, WT mice developed hyperglycemia (WT 207.3 ± 11.6 vs. PAI-1$^{-/-}$ 116.7 ± 4.2 mg/dl, $P < 0.001$, Fig. 5*A*), hyperinsulinemia (WT 5.0 ± 0.8 vs.

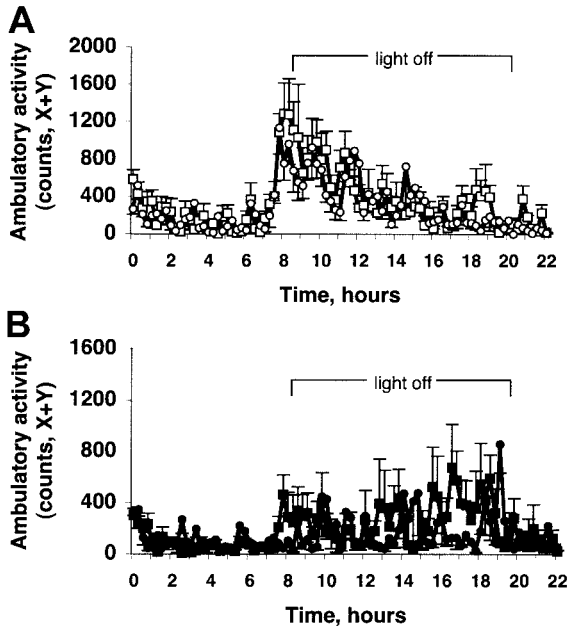

FIG. 3. Mice physiology activity. Activity at baseline on normal chow (*A*) and at 12 weeks after an HF diet (*B*) in WT and PAI-1$^{-/-}$ mice were recorded using the Oxymax System during a 22-h period. Data for WT (□) and PAI-1$^{-/-}$ (○) mice at baseline and at 12 weeks after an HF diet (WT, ■; PAI-1$^{-/-}$, ●) are shown.

PAI-1$^{-/-}$ 0.5 ± 0.1 ng/ml, $P < 0.001$, Fig. 5*B*), and hyperleptinemia (WT 76.0 ± 7.0 vs. PAI-1$^{-/-}$ 2.5 ± 0.8 ng/ml, $P < 0.001$, Fig. 5*C*). In contrast, the levels of blood glucose, plasma insulin, and leptin in PAI-1$^{-/-}$ mice were indistinguishable from baseline after 12 weeks of an HF diet (Fig. 5*A*–*C*). During the euglycemic-hyperinsulinemic clamp study, insulin-stimulated whole-body glucose infusion rates to maintain euglycemia (Fig. 6*A*) were significantly higher in PAI-1$^{-/-}$ mice than in WT mice (Fig. 6*B*, overall difference $P = 0.0039$), indicating increased insulin sensitivity in PAI-1$^{-/-}$ mice.

TABLE 2
Metabolic effects of an HF diet on WT mice

	Day 0	Day 7 after HF diet
n	24	24
Blood glucose (mg/dl)	137 ± 3.4	162 ± 6.0†
Plasma insulin (ng/ml)	0.37 ± 0.03	0.54 ± 0.04*
Plasma leptin (ng/ml)	1.55 ± 0.34	3.7 ± 1.0*

Data are means ± SE. *$P < 0.05$, †$P < 0.01$ vs. day 0.

Expression of adipocyte-derived genes in PAI-1$^{-/-}$ mice. The development of obesity and insulin resistance in WT mice on an HF diet was associated with a 2.5-fold increase in PAI-1 mRNA in white adipose tissue compared with WT mice on normal chow (Fig. 7*B* and *E*). PPAR-γ mRNA expression in white adipose tissue in PAI-1$^{-/-}$ mice on normal chow was lower than that in WT mice on normal chow. At 12 weeks after HF feeding, PPAR-γ mRNA was downregulated in WT mice but maintained in PAI-1$^{-/-}$ mice (Fig. 7*A* and *C*; note that RNA loading verified by 28S rRNA in an ethidium bromide–containing agarose gel was parallel to GAPDH; therefore, values are expressed as ratio vs. GAPDH). Adiponectin mRNA was similarly highly expressed in WT and PAI-1$^{-/-}$ mice on normal chow. After 12 weeks of the HF diet, adiponectin

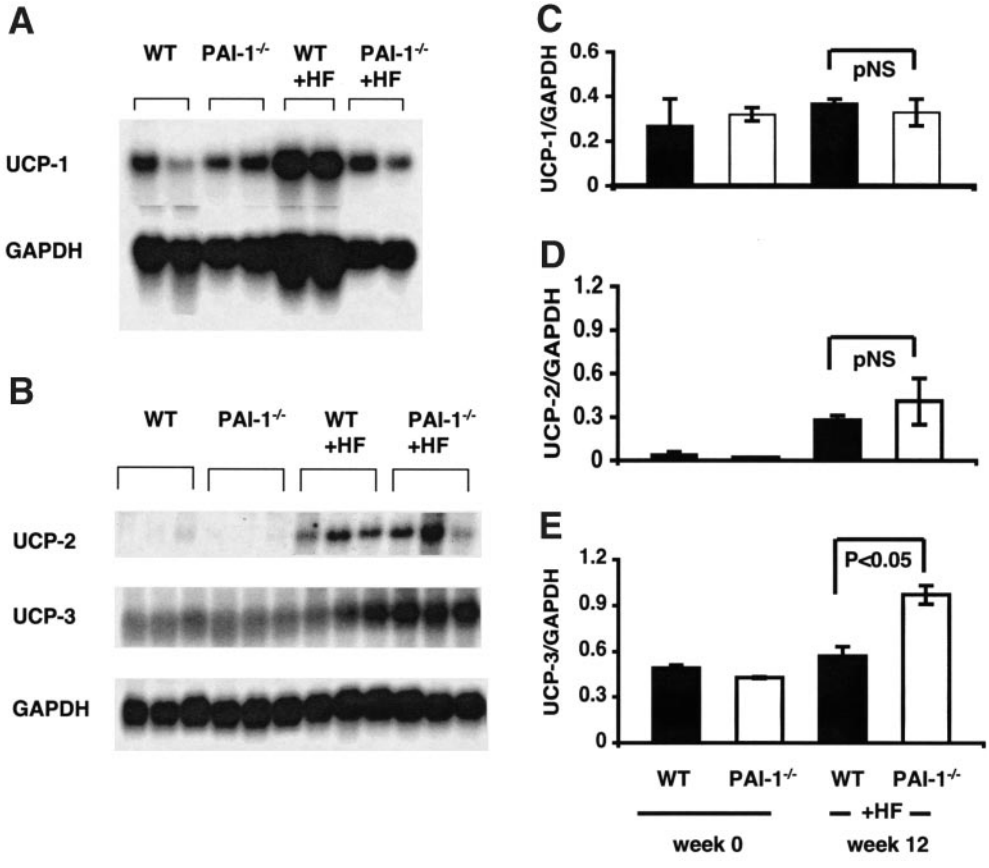

FIG. 4. Expression of UCPs. Relative amounts of UCP-1 mRNA expression in brown adipose tissues (*A* and *C*) and UCP-2 mRNA (*B* and *D*) and UCP-3 mRNA (*B* and *E*) expressions in skeletal muscles in WT (■) and PAI-1$^{-/-}$ mice (□) were determined by Northern blot analysis and normalized to GAPDH mRNA.

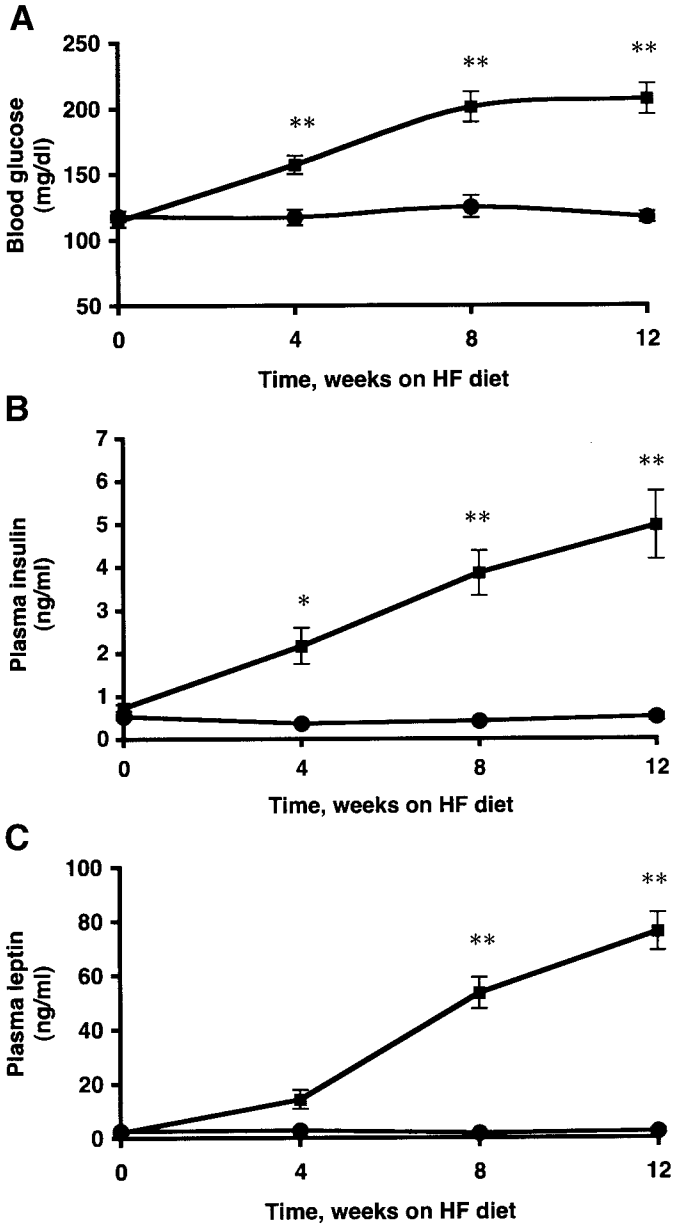

FIG. 5. Prevention of insulin resistance in PAI-1–deficient mice. Fasting glucose (*A*), fasting plasma insulin (*B*), and fasting plasma leptin (*C*) levels in response to an HF diet in WT (■, $n = 21$) and PAI-$1^{-/-}$ (●, $n = 19$) mice. *$P < 0.01$, **$P < 0.001$, WT vs. PAI-$1^{-/-}$.

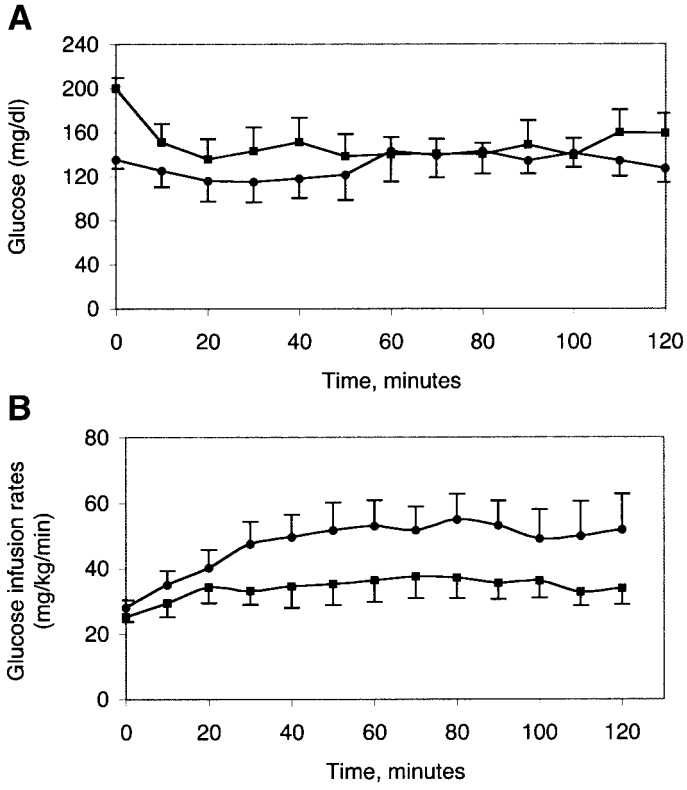

FIG. 6. Euglycemic-hyperinsulinemic clamp study. Blood glucose concentrations (*A*) and glucose infusion rates (*B*) during the euglycemic-hyperinsulinemic clamp at 12 weeks after an HF diet in WT (■, $n = 10$) and PAI-$1^{-/-}$ (●, $n = 9$) mice. $P = 0.0039$, overall difference of glucose infusion rates across the time points between groups.

mRNA was markedly suppressed in WT mice, but was maintained in PAI-$1^{-/-}$ mice (Fig. 7*B* and *D*). Interestingly, adiponectin mRNA level changes in white adipose tissue (downregulated in WT mice but maintained in PAI-$1^{-/-}$ mice) were observed after only 1 week of the HF diet (Fig. 8), a time point when metabolic abnormality was already established in WT mice (Table 2).

Attenuation of obesity and insulin resistance with the angiotensin receptor antagonist linked to inhibition of PAI-1. To test whether inhibition of angiotensin achieved by AT1RA can prevent the development of obesity and insulin resistance in a manner similar to the genetic absence of PAI-1, AT1RA (80 mg/l losartan) was administered to WT mice along with an HF diet. Food intake in mice with or without AT1RA was comparable (WT + HF + AT1RA, 2.4 ± 0.1 g/day). These AT1RA + HF–treated mice had lower weight gain than WT mice on an HF diet alone (Fig. 9*A*). Hyperglycemia and hyperinsulinemia were also markedly attenuated after treatment with AT1RA compared with WT mice on an HF diet alone (Fig. 9*B* and *C*). At 12 weeks after an HF diet, in parallel with the 2.5-fold increase of PAI-1 mRNA in white adipose tissue in WT mice, active plasma PAI-1 levels in WT mice increased ~2.5-fold compared with baseline in WT mice. AT1RA treatment for 12 weeks in WT mice on an HF diet markedly inhibited plasma active PAI-1 to levels only half of those observed in WT mice on an HF diet alone (Fig. 10).

PAI-1 deficiency enhanced glucose uptake in primary adipose cells. Baseline glucose uptake without insulin stimulation, determined by [2-^{3}H]-deoxyglucose incorporation, was higher in PAI-$1^{-/-}$ adipocytes versus WT (threefold increase vs. WT baseline, $P < 0.01$). Insulin-stimulated glucose uptake was further markedly increased in PAI-$1^{-/-}$ adipocytes (10-fold increase vs. WT baseline, $P < 0.01$) compared with WT adipocytes (fourfold increase vs. WT baseline, $P < 0.01$) (Fig. 11).

DISCUSSION

This study demonstrates that HF diet–induced obesity, hyperglycemia, and hyperinsulinemia were prevented in mice lacking PAI-1. The protection against obesity in PAI-$1^{-/-}$ mice was linked to increased metabolic rates, energy expenditure, and body temperature, but similar physical activity, compared with WT mice. Furthermore, PAI-$1^{-/-}$ mice did not have increased tissue triglyceride in

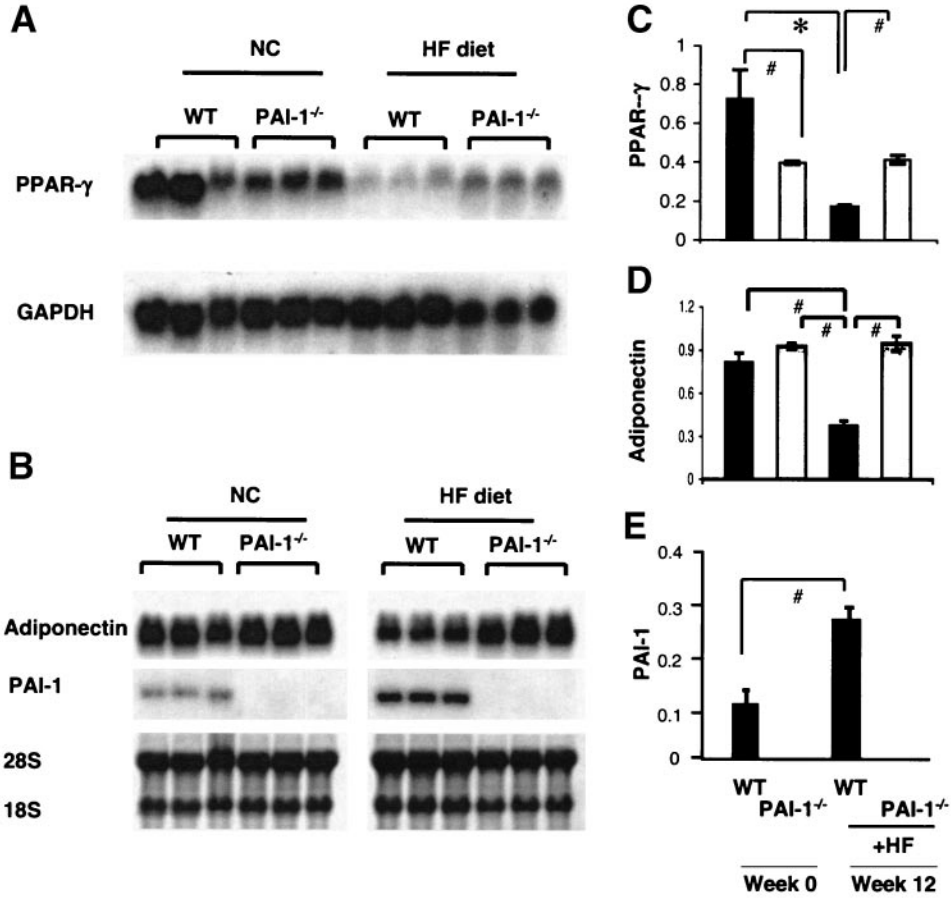

FIG. 7. Expression of adipocyte-derived genes in white adipose tissue. Relative amounts of PPAR-γ (*A* and *C*), adiponectin (*B* and *D*), and PAI-1 mRNA expressions (*B* and *E*) in WT (■, $n = 9$) and PAI-1$^{-/-}$ (□, $n = 9$) mice at baseline and at 12 weeks after an HF diet were determined by Northern blot analysis and normalized to GAPDH mRNA or 28S rRNA. #$P < 0.05$, *$P < 0.01$.

skeletal muscle and liver and had increased insulin sensitivity compared with WT mice. The mechanisms for protection against insulin resistance in PAI-1$^{-/-}$ mice may involve maintained expression of PPAR-γ and adiponectin, key adipocyte-derived hormones. Obesity and insulin resistance were also attenuated by inhibition of angiotensin, an effect linked to inhibition of PAI-1, suggesting interactions of angiotensin and PAI-1 in both obesity and insulin resistance.

Morange et al. (32) previously observed that PAI-1 deficiency had no beneficial effects on obesity. Overexpression of PAI-1 in mice surprisingly attenuated nutritionally induced obesity (34). These reports are different from the current study and the results of others (33). Schafer et al. (33) demonstrated that the absence of PAI-1 reduced adiposity and improved the metabolic profiles in genetically obese mice (PAI-1$^{-/-}$ *ob/ob*), which were on a C57BL/6 background. Using WT C57BL/6 mice has allowed us to produce a reliable and overwhelming model of obesity and insulin resistance with an HF diet. In contrast, Morange et al. studied young PAI-1$^{-/-}$ mice (4 weeks old) on a mixed genetic background (81% C57BL/6 and 19% 129SV) and showed milder hyperinsulinemia in the corresponding WT mice after 17 weeks on an HF diet (32). Thus, differences in genetic background, animal age, and/or high-fat food formula between the studies of Morange et al. (32), the results of Schafer et al. (33), and the present study possibly account for the different outcomes. These varying results in different mice strains mirror the key influence of genetic susceptibilities observed in a myriad of human diseases, such as the observation that only 30–40% of diabetic patients will develop diabetic nephropathy.

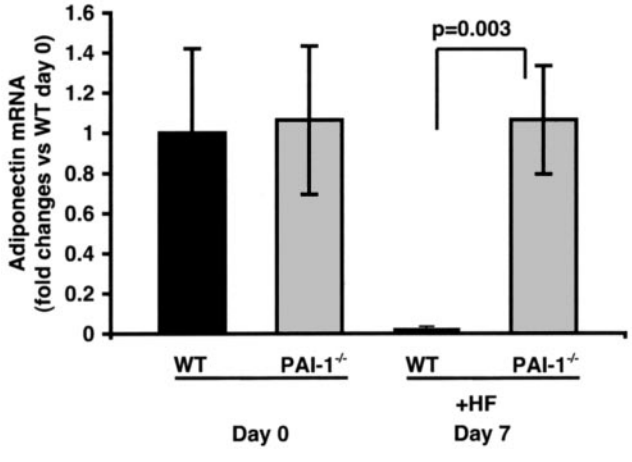

FIG. 8. Effects of short time exposure of an HF diet on expression of adiponectin mRNA in white adipose tissue. Adiponectin mRNA expressions in WT (■, $n = 5$) and PAI-1$^{-/-}$ (□, $n = 5$) mice were determined by real-time PCR and normalized to 18S rRNA.

Obesity results from an imbalance between energy intake and energy expenditure, including that linked to resting metabolic rates (47–49). The UCPs are mitochondrial inner membrane proteins that play important roles in whole-body energy expenditure (46,47,50,51). UCP-1 is exclusively expressed in brown adipose tissue, whereas UCP-2 is widely expressed in many tissues and UCP-3 is primarily expressed in skeletal muscle. Overexpression of

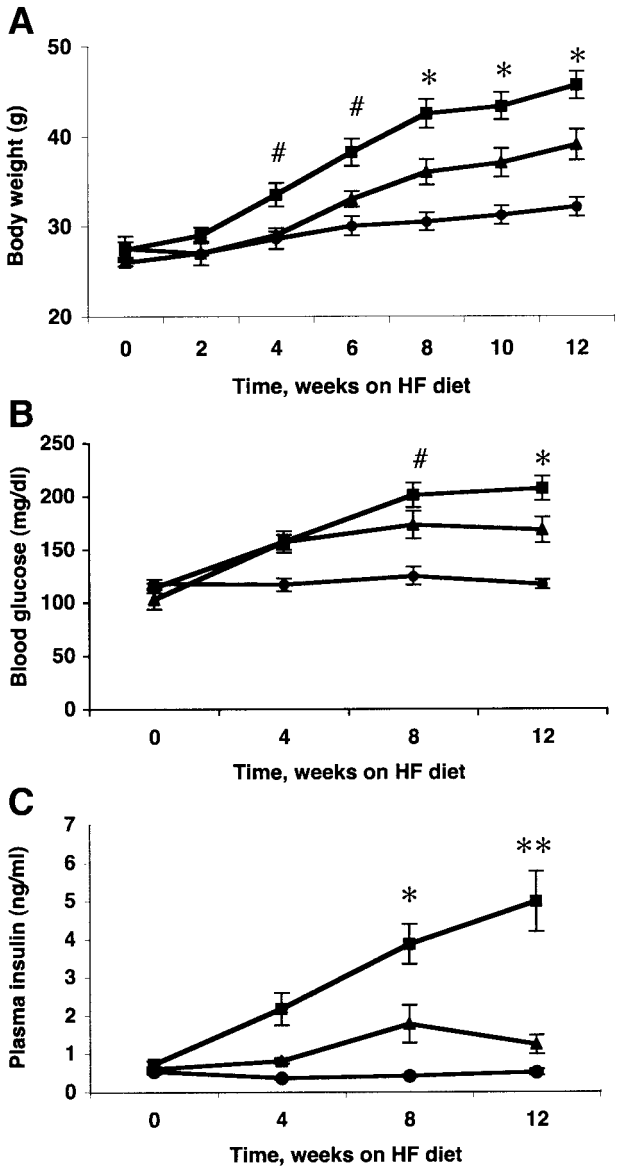

FIG. 9. Amelioration of obesity and insulin resistance by AT1RA. Body weight (*A*), fasting blood glucose (*B*), and fasting plasma insulin (*C*) in WT mice on an HF diet without (■, $n = 21$) or with (▲, $n = 8$) AT1RA and PAI-$1^{-/-}$ mice on an HF diet (●, $n = 19$). #$P < 0.05$, *$P < 0.01$, **$P < 0.001$, WT + AT1RA on HF vs. WT on HF alone ($n = 21$).

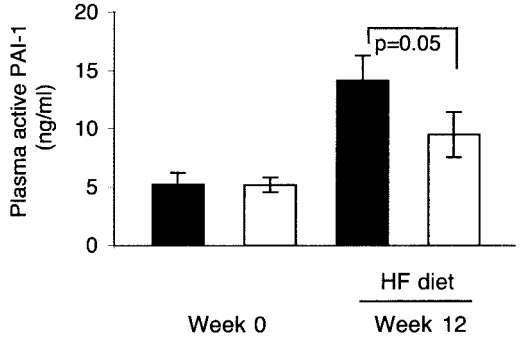

FIG. 10. Inhibition of plasma PAI-1 by AT1RA. Effects of AT1RA treatment for 12 weeks on plasma active PAI-1 levels in WT mice with an HF diet alone (■) and with HF diet + AT1RA (□) ($n = 3$–5 in each group).

UCP-1 or UCP-3 in skeletal muscle in mice protects against diet-induced obesity and its metabolic consequences (52,53). Because skeletal muscle is the principal tissue responsible for insulin-stimulated glucose disposal, increased uncoupling activity due to overexpression of UCP could result in increased glucose metabolism. Expression of UCP-3 in skeletal muscle cells in vitro stimulates glucose uptake (54). In contrast, in patients with type 2 diabetes, UCP-3 was decreased in skeletal muscle, as whole-body insulin-mediated glucose utilization was also decreased (55). Consistent with previous studies, our data support that increased skeletal muscle UCP-2 and UCP-3 may contribute to increased metabolic rates and energy expenditure in PAI-$1^{-/-}$ mice. Although we do not have evidence of a direct interaction of PAI-1 with UCPs, we speculate that PAI-1 deficiency in skeletal muscle may indirectly regulate UCP-2 and UCP-3 through activated PPAR-γ. Interestingly, recent studies have shown a direct interaction between PPAR-γ and UCP-2, suggesting that PPAR-γ activation could increase glucose metabolism in part by direct upregulation of muscle UCP-2 expression (56,57).

Our euglycemic-hyperinsulinemic clamp studies show that mice lacking PAI-1 have increased insulin sensitivity. A strong correlation between intracellular triglyceride content and insulin resistance has been well established in both human and animal studies of obesity-related insulin resistance and type 2 diabetes (58). Decreased tissue triglyceride level in skeletal muscle and liver in response to an HF diet in PAI-$1^{-/-}$ mice is potentially a protective mechanism contributing to the observed increased insulin sensitivity. Adipocytes were also smaller in PAI-$1^{-/-}$ mice compared with WT mice after an HF diet, an additional potential contributor to increased insulin sensitivity (59). Our data from the primary adipose cell culture further provide direct evidence indicating that PAI-1 deficiency enhances glucose uptake in white adipose tissue, which may play an important role in increased whole-body insulin sensitivity in PAI-$1^{-/-}$ mice.

We next examined potential molecular mechanisms that might underlie the protection from insulin resistance in the PAI-$1^{-/-}$ mice. PPAR-γ and adiponectin are key proteins implicated in the development of obesity and insulin resistance. PPAR-γ, a member of the nuclear receptor superfamily of ligand-activated transcriptional factors, has

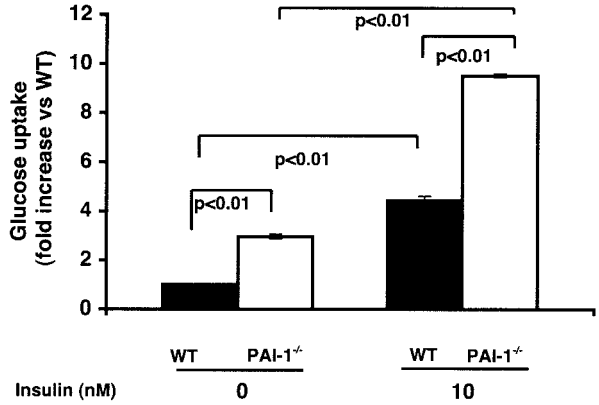

FIG. 11. PAI-1 deficiency enhanced glucose uptake in adipocytes in vitro. Representative glucose uptake in WT (■) and PAI-$1^{-/-}$ (□) adipocytes in vitro ($n = 3$ in each group).

been implicated in metabolic diseases including type 2 diabetes (15). PPAR-γ is highly enriched in adipose tissue and has effects on genes involved in adipocyte differentiation and glucose homeostasis. Activation of PPAR-γ transcriptional activity by thiazolidinediones reduces insulin resistance and hyperglycemia in type 2 diabetes (60). Adiponectin is another adipocyte-derived hormone produced inversely to the amount of fat stores (14,61,62). Adiponectin is reduced in obese mice and humans as well as in type 2 diabetes (62–64). Decreased circulating adiponectin is strongly associated with progression of insulin resistance in humans (65). Conversely, direct treatment with recombinant adiponectin reversed insulin resistance in mice, whether associated with lipoatrophy or obesity (66), whereas mice lacking adiponectin exhibited severe insulin resistance (67,68). Interestingly, activation of PPAR-γ increases adiponectin, indicating that one of the insulin-sensitizing mechanisms of PPAR-γ may be via regulation of adiponectin (69,70). In this study, we found that PPAR-γ and adiponectin mRNA expressions were downregulated in WT mice in response to the HF diet, associated with the development of obesity and insulin resistance. In contrast, PPAR-γ and adiponectin mRNAs were maintained after an HF diet in the protected PAI-$1^{-/-}$ mice. Our results are consistent with the hypothesis that stimulation of adiponectin production and/or activation of PPAR-γ expression enhance whole-body insulin sensitivity (70,71).

Although there is no evidence that PAI-1 can directly affect PPAR-γ and adiponectin, indirect interactions of PAI-1 with PPAR-γ and adiponectin through remodeling of the extracellular matrix (ECM) component are possible. We speculate that PAI-1 deficiency might modulate the microenvironment and network of ECM surrounding adipocytes, which can affect cell-ECM interactions and transduction of extracellular signals to intracellular components such as PPAR-γ. Furthermore, although PAI-$1^{-/-}$ mice have no light microscopic–detectable phenotype, subtle changes of the microvasculature could contribute to changes in diffusion distance in skeletal muscle and thus affect glucose sensitivity.

Recent studies of interaction of PAI-1 with the adhesive ECM glycoprotein vitronectin have provided additional insight into the effects of PAI-1 deficiency on insulin resistance. PAI-1 circulates in plasma as a complex with vitronectin (72), stabilizing PAI-1 in an active conformation. PAI-1 can competitively inhibit the urokinase-like plasminogen activator receptor–dependent attachment of cells to vitronectin (73). Furthermore, binding of PAI-1 to vitronectin may also affect integrin-mediated cell adhesion to vitronectin (66,67,74). PAI-1 can also inhibit insulin signaling by competing with αvβ3 integrin for vitronectin binding (75). Thus, deficiency of PAI-1 may enhance insulin-signaling pathways.

Previous studies have established the connection between obesity, insulin resistance, and increased PAI-1. In this study, we observed total protection against diet-induced obesity and insulin resistance when PAI-1 was absent. Our data suggest that increased PAI-1 may not merely be secondary to obesity, but has direct effects on the development of obesity and insulin resistance. We therefore assessed whether inhibiting PAI-1 indirectly by angiotensin receptor antagonism would also be effective in preventing obesity and insulin resistance. Of interest, obesity itself is associated with activation of the RAS. RAS inhibition improves insulin resistance in humans and mice (76) and decreases plasma PAI-1 in hypertensive type 2 diabetic patients (77). The effects of RAS inhibition on the prevention of type 2 diabetes by ACE inhibitors and angiotensin type 1 receptor antagonists have been evaluated in recent clinical trials. The Captopril Prevention Project (78) was the first controlled clinical trial to demonstrate a reduction in the development of type 2 diabetes in hypertensive patients treated with ACE inhibitors. This decreased development of type 2 diabetes in patients receiving angiotensin inhibition was confirmed in two other clinical trials: the Heart Outcomes Prevention Evaluation and the Losartan Intervention for Endpoint Reduction in Hypertension Study (79–81). A new prospective Diabetes Reduction Assessment with Ramipril and Rosiglitazone Medications trial is also being evaluated (82). RAS inhibition has been proposed to prevent type 2 diabetes by promoting recruitment and differentiation of adipocytes (83). Our data show that the AT1RA losartan attenuated the development of obesity and insulin resistance in WT mice induced by an HF diet. Further, our data show that active plasma PAI-1 was decreased by AT1RA. We thus propose that an additional key mechanism for the beneficial effects of RAS inhibition on the development of obesity and diabetes may be mediated through regulation of PAI-1.

In summary, HF diet–induced obesity and insulin resistance are prevented in mice lacking PAI-1. This protection is linked to maintenance of the key metabolic molecules PPAR-γ and adiponectin and an increased metabolic rate, which may be due to modulation of UCPs. AT1RA attenuated development of diet-induced obesity and insulin resistance, linked to inhibition of PAI-1. Our data suggest that inhibition of PAI-1 might prove to be a novel anti-obesity and anti–insulin resistance treatment.

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