Distribution and Regulation of Plasminogen Activator Inhibitor-1 in Murine Adipose Tissue In Vivo

Induction by Tumor Necrosis Factor-α and Lipopolysaccharide

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Abstract

Although elevated plasma plasminogen activator inhibitor 1 (PAI-1) is associated with obesity, very little is known about its tissue or cellular origin, or about the events that lead to increased PAI-1 levels under obese conditions. Since TNF-α is increased in rodents both during obesity and in response to endotoxin treatment, we examined the effects of these agents on PAI-1 gene expression in the adipose tissue of CB6 mice. In untreated mice, PAI-1 mRNA was detected in both mature adipocytes and in stromal vascular cells. Both TNF-α and endotoxin significantly increased PAI-1 mRNA in the adipose tissue, peaking at 3–8 h. In situ hybridization analysis of adipose tissue from untreated mice revealed a weak signal for PAI-1 mRNA only in the smooth muscle cells within the vascular wall. In contrast, after endotoxin or TNF-α treatment, PAI-1 mRNA also was detected in adipocytes and in adventitial cells of vessels. Endotoxin also induced PAI-1 in endothelial cells, while TNF-α additionally induced it in smooth muscle cells. Mature 3T3-L1 adipocytes in culture also expressed PAI-1 mRNA, and its rate of synthesis was also upregulated by TNF-α. These studies suggest that the adipose tissue itself may be an important contributor to the elevated PAI-1 levels observed in the plasma under obese conditions. *(J. Clin. Invest.* 1996. 97:37–46.) Key words: obesity • 3T3 cells • thrombosis • endotoxin • TNF-α

Introduction

Type 1 plasminogen activator inhibitor (PAI-1)[1] plays an important role in the regulation of fibrinolysis by binding to and rapidly inactivating both tissue-type and urokinase-type PAs (1–3). Limited clinical data suggest that abnormalities in the regulation of PAI-1 itself may contribute to the development of bleeding or thromboembolic diseases (4, 5). For example, several individuals have been identified with little or no detectable functional PAI-1 in their blood and all have had lifelong bleeding problems (6–10). Moreover, disruption of the PAI-1 gene in mice was associated with a mild hyperfibrinolytic state and increased resistance to thrombosis (11). In contrast, elevations in PAI-1 activity have been associated with an increased risk for thrombotic disease. In this case, increased plasma PAI-1 levels have been detected in the plasma of patients with Gram-negative sepsis (12) and during the second and third trimesters of pregnancy (13), two conditions associated with an increased risk for thrombosis. Moreover, PAI-1 mRNA levels are elevated in severely atherosclerotic human arteries (14, 15).

Obesity is an independent risk factor for the development of atherosclerosis and cardiovascular disease (16, 17) and is associated with related metabolic disorders such as hypertriglyceridemia, hyperinsulinemia, and non–insulin-dependent diabetes (17, 18). Interestingly, in a limited number of clinical studies, significant correlations have been established between elevated PAI-1 levels and obesity (19–22). This abnormal expression of PAI-1 in obese individuals is correlated with increased risk for cardiovascular disease (23–25). Surgical treatment of morbid obesity by either gastric stapling or jejuno-ileal bypass, two treatments that lead to dramatic weight loss, also reduced PAI-1 activity and clot lysis time significantly in these patients (26). These observations raise the possibility that elevations in plasma PAI-1 levels associated with obesity may result from the constitutive synthesis of PAI-1 by the adipose tissue itself. In this regard, previous studies on the normal tissue distribution of PAI-1 in mice demonstrated high concentrations of PAI-1 mRNA in the epididymal fat pad (27). In obese animals, the mass of the epididymal fat pad typically increases severalfold, primarily because of an increase in the size and number of adipocytes. Thus, in obesity, the PAI-1 biosynthetic capacity of the adipose tissue may exceed that of all other tissues.

Studies of obese and lean rodents have demonstrated that the adipose tissue is also a major site of synthesis of TNF-α, and that this expression is elevated in several rodent models of obesity (28). Interestingly, TNF-α also stimulates PAI-1 biosynthesis by many cell types in vitro and in most murine tissues in vivo (27, 29, 30). In this study, we used a murine model system to investigate PAI-1 gene expression in the adipose tissue under basal conditions and in response to TNF-α. Endotoxin was also tested since it is known to increase endogenous levels of TNF-α and other cytokines in vivo (31). We demonstrate the presence of PAI-1 in adipocytes from control animals and show that PAI-1 is also synthesized by cultured 3T3-L1 cells, an adipocytic cell line. Furthermore, in situ hybridization and cell fractionation studies of the adipose tissues of CB6 mice revealed that PAI-1 mRNA is increased in adipocytes and in

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1. *Abbreviation used in this paper:* PAI-1, plasminogen activator inhibitor 1.

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stromal/vascular cells after LPS or TNF-α treatment. Recombinant TNF-α also induced PAI-1 mRNA expression in cultured 3T3-L1 adipocytes. Thus, the basal production of PAI-1 by cells of the adipose tissue itself may contribute to the elevated PAI-1 level observed under obese conditions. This basal expression of PAI-1 may be enhanced by TNF-α, a cytokine known to be elevated during obesity.

Methods

Tissue preparation. Adult male CB6 mice (BALBc/ByJ × C57Bl6/J, Scripps Rodent Breeding Colony), aged 2–3 mo and weighing 25–30 grams, were used for all experiments. LPS (50 μg/mouse; *Escherichia coli* serotype 0111:B4; Sigma Chemical Co., St. Louis, MO) and recombinant murine TNF-α (4 μg/mouse; kind gift of Dr. Richard Ulevitch, The Scripps Research Institute) were diluted in saline and injected intraperitoneally into mice anesthetized by inhalation of Metofane (methoxyflurane; Pitman-Moore, Mundelein, IL). Our previous studies showed that 50 μg LPS and 4 μg TNF-α gave maximum induction of PAI-1 in most murine organs (27). Control mice were anesthetized and injected with an equivalent volume of saline alone. The level of LPS contamination as determined by the Limulus Amebocyte Lysate assay (BioWhittaker, Inc., Walkersville, MD) in the recombinant murine TNF-α preparation was 0.25 ng LPS/μg TNF-α. This concentration of LPS had no measurable effect on PAI-1 gene expression in vivo. At 1, 3, 8, and 24 h after LPS or TNF-α injection, the mice were killed by overdose inhalation of Metofane and cervical dislocation. Adipose tissues were rapidly removed and immersed in chilled 4% paraformaldehyde (for in situ hybridization and immunohistochemistry) or minced and frozen in liquid nitrogen for preparation of total RNA. The paraformaldehyde-fixed (overnight) tissues were embedded in paraffin blocks and sectioned at 2–5 μm thickness using a microtome. The sections were then mounted onto Superfrost/plus microscope slides (Fisher Scientific, Pittsburgh, PA) and stored at room temperature pending analysis.

Northern blot analysis. Total RNA was extracted from the epididymal fat pads of CB6 mice by the acid guanidinium thiocyanate-phenol-chloroform method (32) and its concentration was determined by sample absorbance at 260 nm. Total RNA (15 μg) was analyzed by Northern blotting as described previously (33) using a 1-kb murine PAI-1 cDNA probe. The probe was labeled by the random primer technique (34) using [α-^{32}P]dGTP (> 3,000 Ci/mmol; Amersham Corp., Arlington Heights, IL). Autoradiography was performed at −70°C using Kodak XAR-5 film with intensifying screens. To assess variability in sample loading, Northern blots were stripped and rehybridized with a radiolabeled cDNA encoding rat 18S RNA.

Quantitative PCR. The concentration of PAI-1 mRNA was determined using a quantitative PCR procedure as described previously (35). A synthetic plasmid containing primers for PAI-1 and β-actin was used as an internal standard (Yamamoto, K., and D. J. Loskutoff, manuscript submitted for publication). An RNA standard (cRNA) was transcribed from the linearized (Kpn1) synthetic plasmid using the T7 promoter. Transcription was performed using the Riboprobe Gemini II in vitro transcription system, according to the manufacturer's instructions (Promega, Madison, WI). Reverse transcription and PCR were performed essentially as described (35) using a Gene Amp RNA PCR kit and thermal cycler (Perkin-Elmer Corp., Norwalk, CT). Briefly, total tissue RNA (1 μg) and a fixed concentration of cRNA as optimized in preliminary experiments (10^6 molecules for PAI-1; 10^7 molecules for β-actin) were combined and reverse transcribed at 42°C for 20 min in a DNA thermal cycler (Perkin-Elmer Corp.). Serial twofold dilutions of the cDNA mixture were amplified using PAI-1 or β-actin specific primers in the presence of ^{32}P–end-labeled 5′ primer (1×10^6 cpm). The mixture was amplified for 30 cycles, with denaturation at 95°C for 1 min, primer annealing at 60°C for 1 min, and extension at 72°C for 1 min. 20-μl aliquots of the PCR products were electrophoresed on 1.8% agarose gels containing ethidium bromide. The appropriate bands corresponding to the internal standard cRNA product (438 bp for PAI-1; 293 bp for β-actin) and the target mRNA product (540 bp for PAI-1; 349 bp for β-actin) were excised from the gel and the incorporated radioactivity was quantified using a scintillation counter. The amounts of radioactivity recovered were plotted against the template concentrations. A standard curve for the internal control cRNA was constructed and used to extrapolate the number of molecules of PAI-1 mRNA per microgram of total RNA. Variations in sample loading were assessed by measuring β-actin mRNA. PAI-1 mRNA levels were then corrected for the variation in β-actin mRNA. The specific activity of PAI-1 mRNA was calculated as follows:

$$\left(\frac{\text{pg PAI-1 mRNA}}{\mu\text{g total tissue RNA}}\right) =$$

$$\left(\frac{\text{No. of molecules of PAI-1 mRNA}}{\mu\text{g total RNA}}\right) \times \left(\frac{3,200 \times 321 \times 10^{12}}{6.023 \times 10^{23}}\right),$$

where 3,200 is the number of base pairs of full-length PAI-1 mRNA, 321 is the average molecular weight of a base, and 6.023×10^{23} is Avogadro's number.

Riboprobe preparation. A subclone of mouse PAI-1 cDNA containing nucleotides 1–1085 in the vector pGEM-3Z was used to prepare a riboprobe for in situ hybridization (36). This vector was linearized with EcoRI (antisense) or HindIII (sense) and was used as a template for in vitro transcription of radiolabeled antisense or sense riboprobes using SP6 or T7 RNA polymerase, respectively, in the presence of ^{35}S-UTP (> 1,200 Ci/mmol; Amersham Corp.). Templates were removed by digestion with RQ1 DNase (Promega) for 15 min at 37°C, and the riboprobes were purified by phenol extraction and ethanol precipitation.

In situ hybridization. In situ hybridization was performed as described previously (36). Briefly, paraffin-embedded tissue sections were pretreated sequentially with xylene (3 × 5 min), 2× SSC (1 × 10 min), paraformaldehyde (1 × 10 min, 4°C), and proteinase K (1 μg/ml, 1 × 10 min). The sections were prehybridized for 2 h in 100 μl of prehybridization buffer at 42°C. An additional 20 μl of prehybridization buffer containing 2.5 mg/ml of tRNA and 600,000 cpm of the ^{35}S-labeled riboprobe was added, and the sections were hybridized for 18 h at 55°C. Slides were then treated with 2× SSC (2 × 10 min), RNase A (20 μg/ml, 1 × 30 min), 2× SSC (2 × 10 min), and 0.1× SSC (2 h, 60°C). Finally, the tissue sections were washed in 0.5× SSC (2 × 10 min) and dehydrated in a graded alcohol series containing 0.3 M NH_4OAc, dried, coated with NTB2 emulsion (Kodak; 1:2 in water), and exposed in the dark at 4°C for 4–12 wk. Slides were then developed for 2 min in D19 developer (Kodak), fixed, and washed in water, and the sections were counterstained with hematoxylin and eosin. Parallel sections were hybridized using a sense probe as a control for nonspecific hybridization.

Immunohistochemistry. Rabbit anti–mouse PAI-1, used as the primary antibody, was prepared by immunizing New Zealand White rabbits with a recombinant mouse PAI-1, a kind gift from Dr. D. Lawrence and Dr. D. Ginsburg (Howard Hughes Medical Institute, University of Michigan Medical Center, Ann Arbor, MI). Caprylic acid–purified fractions (37) of this polyclonal antibody were characterized for monospecificity of the antisera by Western blot analysis. A single band of 50 kD was observed on Western blots of plasma from LPS-treated mice (data not shown).

Immunohistochemical staining was performed using the HISTOSTAIN-SP kit (Zymed Laboratories, Inc., South San Francisco, CA) as described previously (36). Briefly, paraffin-embedded, paraformaldehyde-fixed sections were deparaffinized with xylene (3 × 5 min), treated with 3% hydrogen peroxide, and rehydrated by immersion in a graded series of ethanol washes. Sections were then permeabilized by sequential treatment with 0.2% and 1% Triton X-100 in TBS (1 × 10 min each) and incubated at 37°C with prewarmed 0.23% pepsin (3,830 U/mg, Worthington Biochemical Corp., Freehold, NJ) in 0.01 N HCl for 3 min. Slides were rinsed with distilled water and with 0.2% Triton-TBS (2 × 3 min), after which time they

were incubated with 10% (wt/vol) normal goat serum in TBS for 30 min. Incubations with primary antibody (rabbit anti–mouse PAI-1; 10 μg/ml in 0.1% normal goat serum) or with normal rabbit IgG were carried out in a humid chamber at 4°C for 16–18 h, followed by incubation for 1 h at 25°C. The slides were then washed and treated sequentially with biotinylated goat anti–rabbit IgG (Zymed Laboratories, Inc.) diluted 1:100 in TBS containing 0.05% Tween 20, streptavidin–peroxidase conjugate (Zymed Laboratories, Inc.), and aminoethylcarbazole chromogen (Zymed Laboratories, Inc.) containing 0.03% hydrogen peroxide for 5–15 min. Slides were washed in distilled water, and the tissue sections were counterstained with hematoxylin, rinsed well with tap water, and mounted in GVA-mount (Zymed Laboratories, Inc.).

Cell fractionation. To verify PAI-1 gene expression in adipocytes from untreated control mice or mice treated with either LPS or TNF-α, we separated mature adipocytes from the stromal-vascular cells as described (38). Briefly, epididymal fat pads were isolated from 10–12-wk-old untreated CB6 mice and from mice at 3 and 8 h after injection with LPS (50 μg) or TNF-α (4 μg). Fat pads were washed in sterile PBS, minced, washed in KRB (pH 7.4) containing 4% albumin and 5 mM glucose, and then treated with collagenase (2 mg/ml; Sigma Chemical Co.) on a shaking platform at 37°C for 1 h. Any undigested tissue was removed with forceps. Adipocytes were then separated by their ability to float upon low-speed (200 *g*) centrifugation. To obtain total stromal-vascular fractions, the medium below the adipocyte layer was centrifuged at 500 *g* for 10 min, and the pellets were washed three times with warm KRB. Total RNA was extracted from the two fractions, and the amount of PAI-1 mRNA associated with each compartment was determined by quantitative PCR as described earlier.

Cell culture. 3T3-L1 mouse embryo fibroblasts were obtained from the American Type Culture Collection (Rockville, MD). Culture of cells and differentiation of preadipocytes to mature adipocytes were carried out as described previously (39). Total RNA was isolated from preadipocytes (at confluency) and from mature adipocytes (3 wk after confluency). For in situ hybridization and immunohistochemical analysis, cells were grown in tissue culture chamber slides (Nunc, Naperville, IL). In situ hybridization, immunohistochemistry, and quantitative PCR on preadipocytes and mature differentiated adipocytes were performed as described above. For treatment with TNF-α (recombinant human TNF-α; Boehringer Mannheim, Indianapolis, IN), preadipocytes were grown and differentiated into adipocytes in 100-mm tissue culture plates. Total RNA was then isolated from untreated adipocytes and adipocytes treated with 5 ng/ml TNF-α at 0, 1, 3, 8, and 24 h.

Quantification of murine PAI-1 protein. PAI-1 antigen levels in the conditioned media of preadipocytes and differentiated adipocytes were determined using a competitive ELISA. Microtiter wells were coated (4°C, 16 h) with purified mouse PAI-1 (100 ng/ml in PBS; kind gift from Dr. D. Lawrence and Dr. D. Ginsburg), the wells were blocked (37°C, 1 h; PBS containing 3% casein and 0.05% Tween 80) and washed (PBS). Dilutions of purified murine PAI-1 (2 μg/ml to 20 ng/ml) or appropriately diluted conditioned media were coincubated with purified rabbit anti–mouse PAI-1 (5 μg/ml) in PBS containing 1% casein and 0.1% Triton X-100 (2 h at 37°C) in the PAI-1–coated plates. After washing (PBS), bound IgG was detected using biotin-labeled anti–rabbit IgG (1:4,000 dilution; Zymed Laboratories, Inc.), followed by streptavidin alkaline phosphatase complex and *p*-nitrophenyl phosphatase (Zymed Laboratories, Inc.). Absorbance was measured at 405 nm. The concentration of PAI-1 antigen in the conditioned media was determined using a standard curve constructed from serial dilutions of purified mouse PAI-1. The standard curve was linear at PAI-1 concentrations ranging from 0.039 to 0.625 μg/ml.

Results

Regulation of PAI-1 gene expression in adipose tissue by LPS and TNF-α. LPS induces a number of cytokines in vivo, and some of these (e.g., TNF-α, IL-1, etc.) are known to modulate PAI-1 both in vitro (29, 30, 40) and in vivo (27). Additionally, TNF-α levels are also known to be elevated in several rodent models of obesity (28). Thus, we determined changes in PAI-1 mRNA expression in the adipose tissue after LPS or TNF-α treatment. Total RNA was extracted from the epididymal fat pads of CB6 mice at 1, 3, 8, and 24 h after intraperitoneal injec-

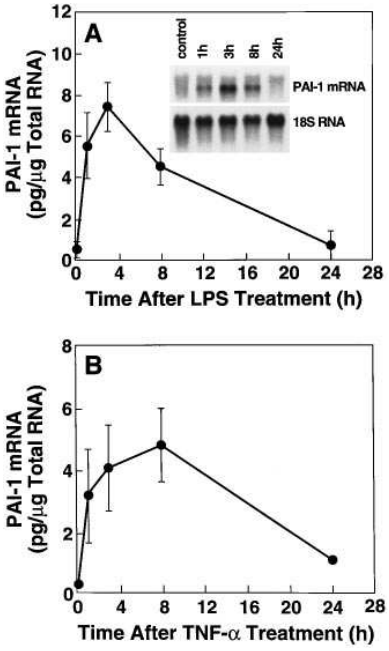

Figure 1. Kinetics of induction of PAI-1 mRNA by LPS and TNF-α in the adipose tissue of CB6 mice. Mice were injected intraperitoneally with LPS (50 μg), TNF-α (4 μg), or saline, and epididymal fat pads were removed 1, 3, 8, and 24 h later. Total RNA was analyzed for PAI-1 gene expression by quantitative PCR and Northern blot analysis as described in Methods. (*A*) Quantitative PCR analysis of PAI-1 mRNA from adipose tissue of control (time 0), and LPS-treated mice. (*Inset*) Northern blot analysis of PAI-1 mRNA in adipose tissues of control and LPS-treated mice from a representative experiment. Northern blots were evaluated for variations in sample loading by reprobing for 18 S rRNA. (*B*) Quantitative PCR analysis of PAI-1 mRNA in adipose tissues from TNF-α–treated mice. Each time point in *A* and *B* represents the mean±SD of six animals.

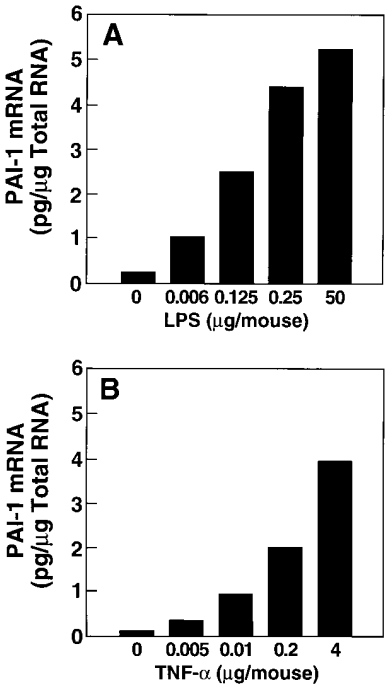

Figure 2. Dose dependency of induction of PAI-1 mRNA by LPS and TNF-α in the adipose tissue of CB6 mice. Mice were injected intraperitoneally with saline alone as the control (0 dose) or with saline containing increasing amounts of LPS (*A*) or TNF-α (*B*). Tissues were removed 3 h later, and total RNA was prepared and analyzed for PAI-1 mRNA by quantitative PCR.

tion of 50 μg LPS or 4 μg of TNF-α. Control animals were injected with saline alone. PAI-1 gene expression after LPS treatment was initially analyzed by Northern blot analysis using a murine PAI-1 cDNA probe (Fig. 1 *A*, *inset*). Increased PAI-1 mRNA was detected as early as 1 h after LPS injection, reached a maximum at 3 h, and declined to baseline values by 24 h. Quantitative PCR demonstrated the presence of 0.5± 0.21 pg PAI-1 mRNA/μg total RNA in control adipose tissue (Fig. 1) in agreement with previous results (27). This concentration increased by 15-fold within 3 h after LPS treatment, to a maximum of 7.4±1.2 pg PAI-1 mRNA/μg total RNA. Again, PAI-1 mRNA levels were significantly elevated at 1, 3, and 8 h after LPS injection, confirming the trend observed by Northern blot analysis. In separate experiments, we determined the effect of TNF-α on PAI-1 in the adipose tissue (Fig. 1 *B*). Again, quantitative PCR analysis indicated a rapid increase in PAI-1 mRNA, with significant increases observed as early as 1 h after exposure to TNF-α, with a 13-fold maximum induction by 3–8 h. The concentration of PAI-1 mRNA returned to control levels by 24 h.

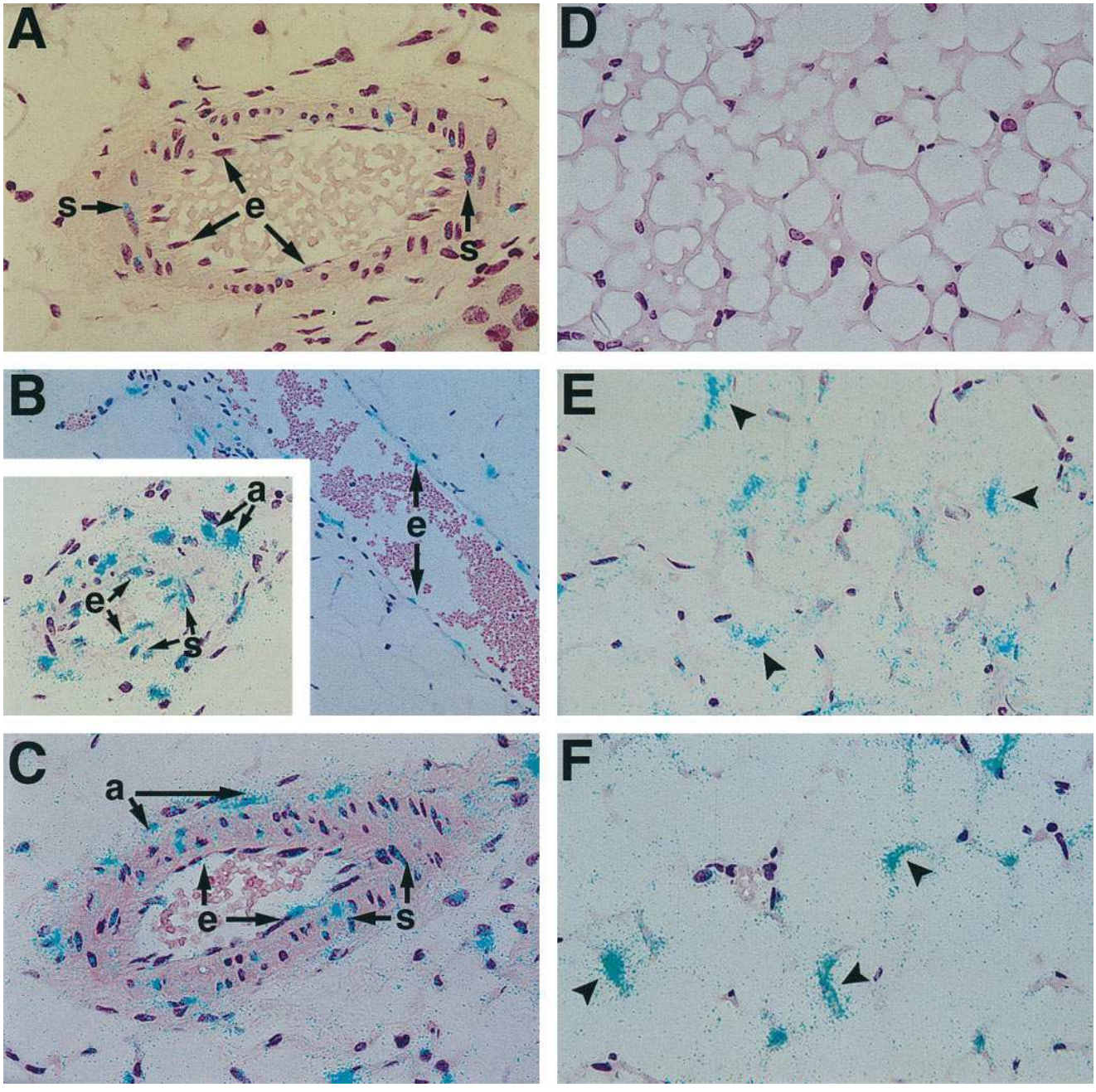

Figure 3. Localization of PAI-1 mRNA in the adipose tissue of CB6 mice after LPS or TNF-α treatment. In situ hybridization of paraffin sections showing vasculature from epididymal fat pads of untreated mice (*A*) or mice treated with LPS (*B*) or TNF-α (*C*) for 3 h. *e*, endothelial cells; *a*, adventitial cells; and *s*, cells within smooth muscle layers. In situ hybridization on sections of epididymal fat pad containing adipocytes and microvascular endothelial cells from untreated mice (*D*) or from mice treated with LPS (*E*) or TNF-α (*F*) for 3 h. Some positive cells are indicated by arrowheads. Slides were exposed for 8 wk at 4°C and stained with hematoxylin and eosin. Original magnification, ×400.

The dose dependency of the response to LPS or TNF-α was then examined. CB6 mice were injected intraperitoneally with 0.006–50 μg LPS or 0.005–4 μg TNF-α, respectively, and 3 h later epididymal fat pads were removed for analysis. Results from a representative experiment are shown in Fig. 2. Both LPS and TNF-α increased PAI-1 mRNA in a dose-dependent manner (Fig. 2, *A* and *B*). LPS increased PAI-1 mRNA by 3-fold at the minimum dose tested (6 ng), with induction increasing to 15-fold at the maximum dose (50 μg). Similarly, induction by TNF-α ranged from 2-fold at the minimum dose (5 ng) to 13-fold at the maximum dose (4 μg) tested.

Localization of PAI-1 mRNA and antigen in the adipose tissue after LPS or TNF-α treatment. Cell-specific expression of PAI-1 mRNA within the adipose tissue of control, LPS-, or TNF-α–treated mice was determined. In control mice, a relatively weak signal for PAI-1 mRNA was observed by in situ hybridization in cells of the smooth muscle layer within the arteries of the adipose tissue (Fig. 3 *A*). No specific signal was

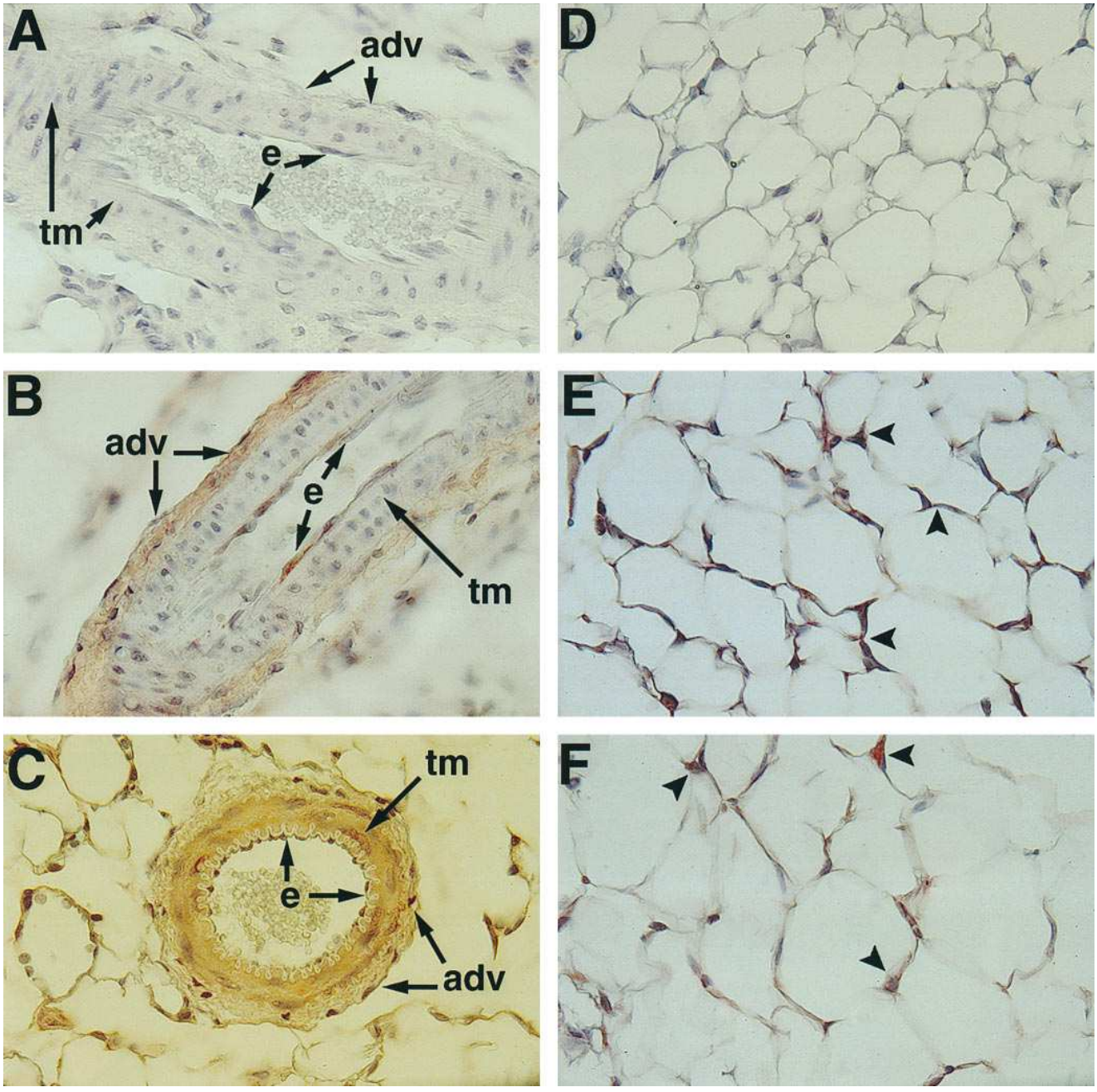

Figure 4. Localization of PAI-1 antigen in the adipose tissue of CB6 mice treated with LPS or TNF-α. Immunohistochemical analysis of paraffin sections showing vasculature from epididymal fat pads of untreated mice (*A*) or mice treated with LPS (*B*) or TNF-α (*C*) for 3 h. *e*, endothelial cells; *adv*, adventitia; *tm*, tunica media. Immunohistochemical analysis on sections of epididymal fat pads containing adipocytes and microvascular endothelial cells from control (*D*), LPS-treated (*E*), and TNF-α–treated (*F*) mice for 3 h, respectively. Some positive cells are indicated by arrowheads. Slides were counterstained with hematoxylin. Original magnification, ×400.

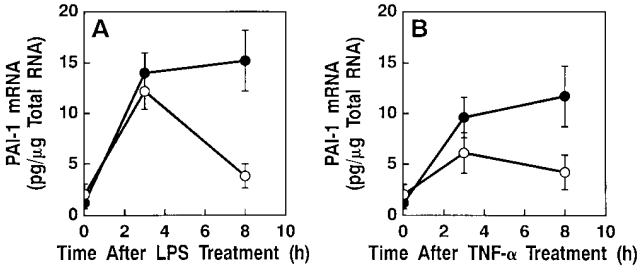

Figure 5. PAI-1 mRNA expression in cell-fractionated adipose tissue after LPS or TNF-α treatment. Epididymal fat pads were isolated from 10–12-wk-old CB6 mice (n = 3, pooled) at 0, 3, and 8 h after intraperitoneal injection of LPS (50 μg; *A*) or TNF-α (4 μg; *B*). Adipocytes and stromal vascular cells were separated by differential centrifugation. Total RNA was extracted from each fraction, and PAI-1 mRNA levels were then quantified by reverse transcription PCR. Each time point represents the mean±SD of two independent experiments. ●, adipocytes; ○, stromal vascular cells.

detected in the endothelial cells. By 3 h after LPS administration, a relatively strong hybridization signal was observed in the endothelial cells lining the arteries and veins, as well as within the adventitial cells in the tunica adventitia (Fig. 3 *B*). In addition, the cells in the smooth muscle layer continued to show a positive signal for PAI-1 mRNA. After TNF-α treatment, PAI-1 mRNA was detected primarily in cells within the smooth muscle cell layer and in adventitial cells of the tunica adventitia (Fig. 3 *C*). In contrast with adipose tissue from LPS-treated animals, the endothelial cells of TNF-α–treated animals were relatively negative or only weakly positive for PAI-1 mRNA (Fig. 3 *C*). Although not shown, this general pattern of hybridization was relatively uniform in the vasculature of the adipose tissue. By 8 h after either LPS or TNF-α administration, the signal for PAI-1 mRNA in cells within vessels of the adipose tissue (i.e., the endothelial, smooth muscle, and adventitial cells) had decreased to baseline levels (data not shown).

No PAI-1 mRNA was detected by in situ hybridization in the fat from control mice (Fig. 3 *D*). However, a strong and specific signal was observed in the fat 3 h after LPS (Fig. 3 *E*) or TNF-α treatment (Fig. 3 *F*). Morphological considerations suggest that the positive cells were adipocytes or microvascular endothelial cells, consistent with the data shown in Fig. 5. The signal in adipocytes/microvascular endothelial cells was still strongly positive even after 8 h of treatment (data not shown). This result is in contrast to the results with the vasculature where the in situ hybridization signal returned to baseline levels 8 h after either LPS or TNF-α treatment. In control experiments, no specific hybridization signal was detected using a sense PAI-1 riboprobe (data not shown).

Immunohistochemical analysis for PAI-1 antigen in the vasculature of adipose tissue, failed to reveal staining of vascular cells in control mice (Fig. 4 *A*). However, strong staining was detected within the endothelial cells as well as in the tunica adventitia 3 h after LPS treatment (Fig. 4 *B*). After TNF-α treatment (Fig. 3 *C*), PAI-1 antigen was detected mainly in the tunica media. Consistent with the in situ hybridization results, immunohistochemical analysis revealed little PAI-1 antigen in adipocytes/microvascular endothelial cells from untreated mice (Fig. 3 *D*). However, positive staining was detected in these cells 3 h after LPS (Fig. 4 *E*) or TNF-α (Fig. 4 *F*) treatment. In control experiments, no immunohistochemical staining was apparent using normal rabbit serum (data not shown).

Analysis of PAI-1 mRNA in cells from adipose tissue fractionated by differential centrifugation. To confirm PAI-1 mRNA expression in the adipocytes of control and LPS- or TNF-α–treated mice, we separated mature adipocytes from nonadipose cells (stromal-vascular fraction) by differential centrifugation and then determined the amount of PAI-1 mRNA associated with each cell fraction by quantitative PCR. Fig. 5, *A* and *B*, indicates that in the control mice, the mature adipocytes and stromal-vascular cell fractions contained 1.4±0.4 and 2±1 pg PAI-1 mRNA/μg total RNA, respectively (0 time points). 3 h after treatment with either LPS (Fig. 5 *A*) or TNF (Fig. 5 *B*), PAI-1 mRNA levels were elevated in both cell fractions. The PAI-1 response in the stromal-vascular fraction decreased by 8 h in both cases, while PAI-1 mRNA levels in mature adipocytes remained elevated at this time. These observations confirm the in situ hybridization results with intact adipose tissue which showed a positive signal in cells that morphologically resembled adipocytes at 8 h after LPS or TNF-α treatment. These results also suggest that the increase in PAI-mRNA at this later time is mainly due to an adipocytic response.

PAI-1 expression by adipocytes in culture. To better characterize PAI-1 gene expression in adipocytes, we determined whether PAI-1 could be synthesized by adipocytes in culture. 3T3-L1 cells (derived from the mouse fibroblast line 3T3) may be propagated indefinitely in culture, but if allowed to reach confluency (Fig. 6 *A*) they begin to differentiate into adipocytes (39). The process of differentiation from preadipocytes to adipocytes can be enhanced by a brief exposure to insulin and dexamethasone. In this instance, the cells accumulate triglycerides and begin to acquire the characteristic signet ring appearance of adipose cells over the next 2–3 wk (Fig. 6 *B*).

The concentrations of PAI-1 mRNA in cell extracts and of PAI-1 antigen in 24-h–conditioned medium were determined by quantitative PCR and by ELISA, respectively (Table I). Relatively low levels of both were detected in preadipocytes. PAI-1 expression was significantly higher in mature adipocytes, increasing approximately fivefold at both the mRNA and protein levels, in comparison with preadipocytes. The concentration of PAI-1 in the fully differentiated 3T3-L1 adipocytes was similar to that in adipocytes in vivo. In situ hybridization analysis failed to detect PAI-1 mRNA in preadipocytes (Fig. 6 *C*), while a positive signal was detected in mature adipocytes (Fig. 6 *D*). However, it should be noted that mature 3T3-L1 adipocytes tend to clump together, with many cells being superimposed on each other. This effect may overestimate the positive in situ hybridization signal seen within single cells. In these experiments, the cells were also stained for lipids with oil red O. Although no lipid was detected in the preadipocytes (Fig. 6 *C*), strong lipid staining was apparent in the differentiated adipocytes (Fig. 6 *D*), and the signal for PAI-1 appears to be localized to the lipid-containing cells. Finally, immunohistochemical analysis indicated that the preadipocytes were negative for PAI-1 antigen (Fig. 6 *E*), while mature adipocytes were strongly positive (Fig. 6 *F*). These cells were not counterstained with oil red O because it tended to mask the immunohistochemical signal. Immunohistochemical analysis of adipocytes using normal rabbit serum and in situ hybridization using a PAI-1 sense probe showed no specific signal (data not shown).

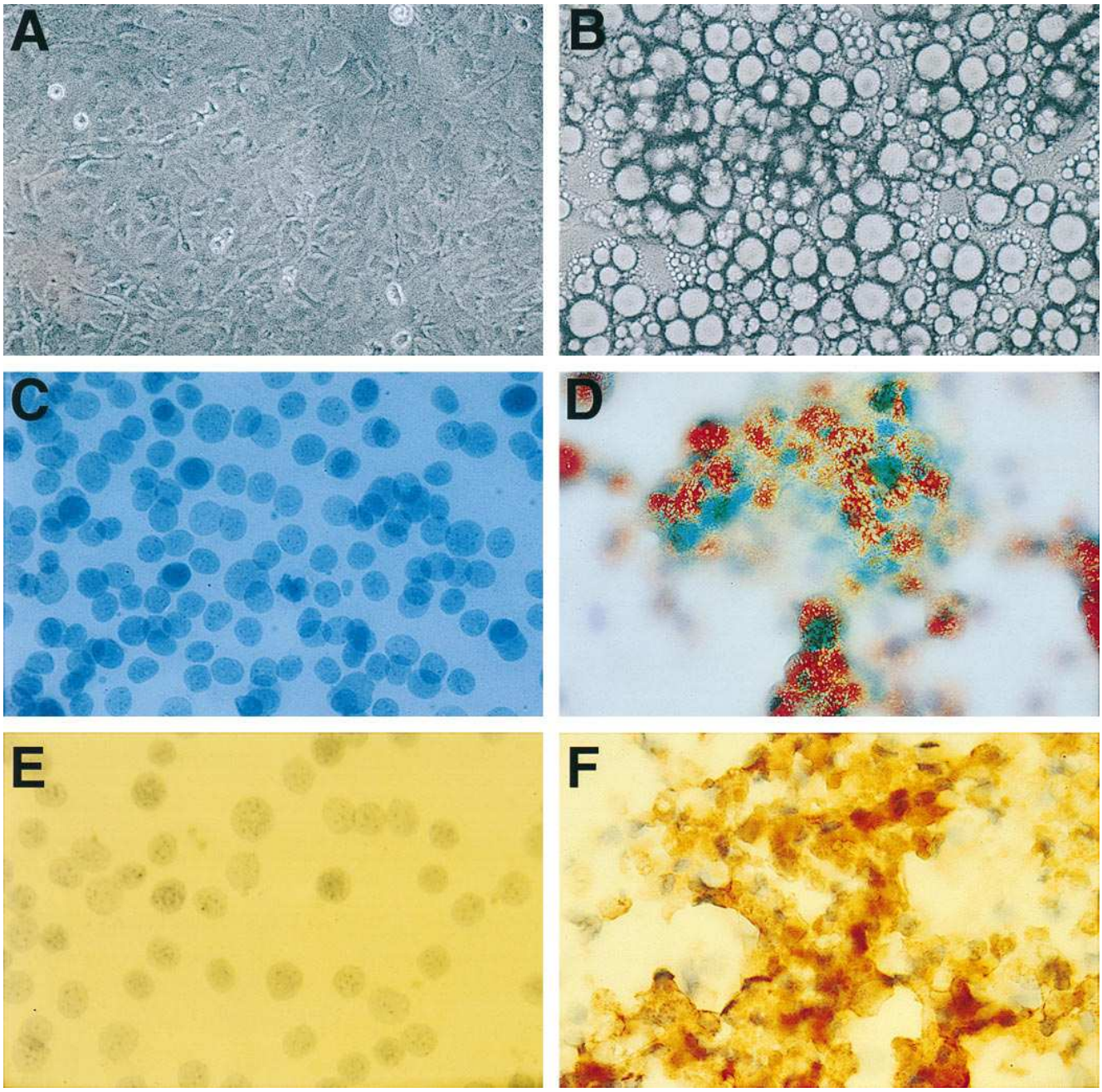

Figure 6. Localization of PAI-1 mRNA and antigen in 3T3-L1 adipocytes. Phase-contrast photomicrographs of preadipocytes (*A*) and adipocytes (*B*); original magnification, ×200. In situ hybridization for PAI-1 mRNA in preadipocytes (*C*) and adipocytes (*D*). In *C* and *D*, sections were counterstained for lipids with oil red O. Immunohistochemical analysis using anti–mouse PAI-1 antibodies in preadipocytes (*E*) and adipocytes (*F*). Sections for immunohistochemical analysis were counterstained with hematoxylin; *C–F*, original magnification, ×400.

Fig. 7 shows the effect of TNF-α (5 ng/ml; as determined by preliminary dose–response experiments) on PAI-1 mRNA expression in mature 3T3-L1 adipocytes. TNF-α increased PAI-1 mRNA expression by these cells by approximately eightfold within 3 h, with levels returning to baseline by 24 h. These changes are similar to those observed with TNF-α in vivo (Fig. 1). Therefore, while mature adipocytes in culture constitutively produce relatively high levels of PAI-1 compared with preadipocytes, the PAI-1 levels can be induced further by subsequent treatment with TNF-α.

Discussion

Obesity is a pathological condition of the adipose tissue, characterized by an increase in the number and the size of the adipocytes themselves (41). Although obesity is often associated with elevated levels of plasma PAI-1 (19–22), very little is known about the tissue origin and the events that lead to increased PAI-1 under obese conditions. Previous studies from our lab on the normal distribution of PAI-1 mRNA in tissues of the mouse demonstrated that, next to the aorta, the adipose

Table I. PAI-1 Expression in Adipocytes

Cells	PAI-1 antigen in conditioned medium	PAI-1 mRNA
	ng/ml	*pg/μg total RNA*
Preadipocytes (3T3-L1)	47±9.8	0.4±0.12
Adipocytes (3T3-L1)	225±66	2.2±0.90
Adipocytes (fractionated from adipose tissue)	—	1.4±0.40

$n = 6 \pm SD$.

tissue had the highest level of PAI-1 mRNA (27). In view of this observation, we attempted to determine whether adipocytes themselves can synthesize PAI-1 under normal conditions. Moreover, we investigated whether agents such as TNF-α, whose levels are elevated in adipose tissues of human (42, 43) and rodent models of obesity (28), also induce PAI-1 in these cells.

Quantitative PCR analysis of total RNA extracted from adipose tissues of untreated mice revealed the presence of 0.5±0.21 pg PAI-1 mRNA/μg total RNA. This result is consistent with previous data from our lab using nuclease protection assays (27). Treatment with either LPS or TNF-α increased PAI-1 mRNA in the adipose tissue ∼ 10–15-fold, with increases detected as early as 1 h after treatment and levels remaining elevated even after 8 h of treatment (Fig. 1). By 24 h of treatment, PAI-1 gene expression had returned to baseline levels. At present we do not fully understand the transient nature of the induction of PAI-1 in the adipose tissue by TNF-α. However, a similar effect on PAI-1 expression is observed in response to TNF-α, LPS, and TGF-β in several other organs in the mouse (27) and probably reflects the short half-lives of

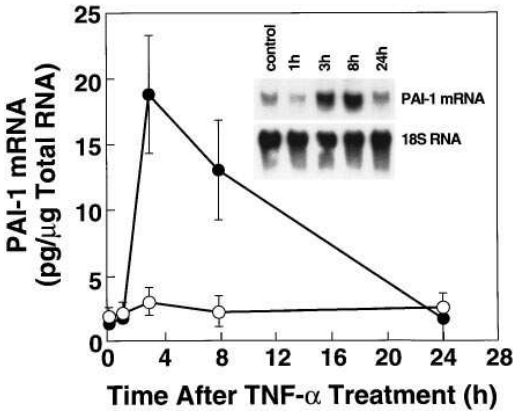

Figure 7. PAI-1 mRNA expression in 3T3-L1 adipocytes in response to TNF-α. 3T3-L1 preadipocytes were grown and differentiated into mature adipocytes in 100-mm tissue culture plates. Total RNA was isolated from untreated adipocytes or adipocytes treated with 5 ng/ml TNF-α at 0, 1, 3, 8, and 24 h after treatment. PAI-1 steady state mRNA levels were determined using quantitative PCR, $n = 6 \pm SD$. (*Inset*) A representative Northern blot showing PAI-1 mRNA from TNF-α (5 ng/ml) treated 3T3-L1 adipocytes. ●, control; ○, TNF-α–treated.

these agents in vivo (44, 45). This conclusion is supported by our observation that chronic low level exposure of 3T3-L1 adipocytes to TNF-α leads to chronic elevation of PAI-1 (data not shown). Thus chronic low-level expression of TNF-α may be sufficient for the continuous elevation of PAI-1. This possibility is under investigation.

The adipose tissue appears to be fairly sensitive to both LPS and TNF-α (Fig. 2), responding to LPS and TNF-α doses as low as 6 and 5 ng, respectively. In both instances, PAI-1 mRNA increased in response to these agents in a dose-dependent manner. Previous studies demonstrated that 6 ng of LPS caused a 24-fold increase in PAI-1 mRNA in the liver, a 3–4-fold increase in the kidney, and a 2.6-fold increase in the lung (27). PAI-1 mRNA in the heart did not increase at this dose. Therefore, significant differences in the sensitivity to LPS are evident in the PAI-1 response of various organs.

In situ hybridization and immunohistochemical analysis were used to directly identify the cell types within the adipose tissue that produce PAI-1 under basal conditions and in response to these agents. In untreated animals, a relatively weak signal for PAI-1 mRNA was observed in cells in the smooth muscle layer of vessels within the adipose tissue, while the endothelial cells appeared to be negative for PAI-1 mRNA (Fig. 3 *A*). This result is consistent with results obtained for other major mouse tissues, where a weak signal for PAI-1 mRNA was consistently detected in vascular as well as nonvascular smooth muscle cells of untreated animals (36). Treatment of mice with LPS caused a marked increase in PAI-1 mRNA levels in the endothelial cells as well as in the adventitial cells of the tunica adventitia, with a weaker response in smooth muscle cells (Fig. 2 *B*). Unlike LPS, TNF-α did not in general induce PAI-1 mRNA in endothelial cells (although there were rare positive cells; Fig. 3 *C*) and did induce PAI-1 mRNA in cells of the smooth muscle layer and adventitial cells (Fig. 2 *C*). Both agents increased PAI-1 mRNA in cells that morphologically resembled adipocytes (Fig. 3, *E* and *F*). Increased PAI-1 antigen staining was also observed in the vessel wall and adipocytes throughout the adipose tissue in response to these agents (Fig. 4).

Previous studies on the cellular localization of PAI-1 mRNA in organs taken from LPS- or TNF-α–treated mice revealed a strong signal for PAI-1 mRNA predominantly in endothelial cells at all levels of the vasculature (46). However, in the liver, in addition to induction within the sinusoidal endothelium, PAI-1 mRNA was also induced in hepatocytes (47). Thus the PAI-1 response to LPS in the adipose tissue and the liver was not restricted to the endothelium.

We also separated the mature adipocytes from stromal/vascular cells (smooth muscle cells, fibroblasts, local mast cells, macrophages, and immature adipocytes) by treatment with collagenase followed by differential centrifugation (38). Since microvascular endothelial cells are intimately associated with adipocytes in the adipose tissue, cross-contamination of the mature floating adipocyte fraction with microvascular endothelial cells was evaluated by determining the expression of vWf mRNA. PCR analysis using vWf-specific primers (sense: 5′ATGATGGAGAGGTTACACATC.3′; antisense: 5′GGCAGTTGCAGACCCTCCCTTG.3′) on total RNA from both cell fractions revealed no specific band on agarose gels from the adipocytic fraction, whereas a specific band of the appropriate size (1,130-bp) was seen from the stromal/vascular cell fraction (data not shown). Quantitative PCR analysis for

PAI-1 on total RNA showed that in untreated mice PAI-1 mRNA was present in both the mature adipocytes and in the stromal/vascular cell fraction (Fig. 5). Within 1 h of LPS or TNF-α treatment, PAI-1 mRNA was elevated in both cell fractions. Although PAI-1 mRNA in mature adipocytes remained elevated for over 8 h, the PAI-1 mRNA level in the stromal/vascular cell fraction had decreased considerably by this time. Thus, the elevated level of PAI-1 mRNA observed 8 h after treatment with these agents is predominantly due to an adipocyte response.

In separate studies, we demonstrated PAI-1 mRNA and antigen expression by cultured 3T3-L1 adipocytes (Table I and Fig. 6). We also showed that mature 3T3-L1 adipocytes respond to TNF-α with elevated PAI-1 mRNA expression (Fig. 6). This in vitro system may prove useful for studying PAI-1 gene expression during adipocyte differentiation.

In summary, both mouse adipocytes in vitro (3T3-L1 adipocytes) and in vivo (demonstrated both by in situ hybridization and cell fractionation) synthesize PAI-1 mRNA and respond to TNF-α by elevated PAI-1 mRNA expression. Thus, the synthesis of PAI-1 by cells of the adipose tissue may contribute to elevated plasma PAI-1 levels seen under obese conditions. These levels may be further increased because of the elevated levels of TNF-α in the adipose tissue which accompany obesity. Currently, we are addressing the possibility that PAI-1 levels are increased in obesity. In preliminary experiments, we have determined the level of PAI-1 expression in genetically obese rodents. Both *ob*/*ob* (28) and *db*/*db* (28) mice were shown to have elevated plasma PAI-1 activity (approximately fivefold) when compared with their lean counterparts. In addition, PAI-1 mRNA was also elevated severalfold in the adipose tissues of the obese mice when compared with their lean controls (Samad, F., and D. J. Loskutoff, unpublished observations).

It should be noted that, in addition to the importance of the adipose tissue in contributing to the plasma PAI-1 pool under normal and obese conditions, one can also speculate on the physiological function of PAI-1 in the adipose tissue itself. It is well documented from in vitro studies that newly synthesized PAI-1 is deposited into the extracellular matrix of many cell types (48, 49), where it may protect matrix components from proteolysis by limiting plasmin generation. Thus PAI-1 produced by cells in the adipose tissue may be deposited into the adipose tissue matrix in vivo, where it may function to preserve the integrity of the loose connective tissue elements that hold the adipocytes together. In this regard, PAI-1 antigen was routinely detected in matrix after LPS or TNF treatment (Fig. 4, *E* and *F*). This function may be more important in obese conditions, where adipocytes tend to be more fragile due to the severalfold increase in their size and mass. Currently, we are using an obese and lean murine model system to further elucidate the mechanisms which account for increased PAI-1 in obesity and to understand the physiological role of PAI-1 in the adipose tissue itself.

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