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Thrombospondin-1 Is an Adipokine Associated With Obesity, Adipose Inflammation, and Insulin Resistance

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Abstract

OBJECTIVE—We examined the relationship between the expression of thrombospondin (TSP)1, an antiangiogenic factor and regulator of transforming growth factor-β activity, obesity, adipose inflammation, and insulin resistance.

RESEARCH DESIGN AND METHODS—TSP1 gene expression was quantified in subcutaneous adipose tissue (SAT) of 86 nondiabetic subjects covering a wide range of BMI and insulin sensitivity, from visceral adipose (VAT) and SAT from 14 surgical patients and from 38 subjects with impaired glucose tolerance randomized to receive either pioglitazone or metformin for 10 weeks. An adipocyte culture system was also used to assess the effects of pioglitazone and coculture with macrophages on TSP1 gene expression.

RESULTS—TSP1 mRNA was significantly associated with obesity (BMI) and insulin resistance (low insulin sensitivity index). Relatively strong positive associations were seen with markers of inflammation, including CD68, macrophage chemoattractant protein-1, and plasminogen activator inhibitor (PAI)-1 mRNA ($r \geq 0.46$, $P = 0.001$ for each), that remained significant after controlling for BMI and S_i. However, TSP1 mRNA was preferentially expressed in adipocyte fraction, whereas inflammatory markers predominated in stromal vascular fraction. Coculture of adipocytes and macrophages augmented TSP1 gene expression and secretion from both cell types. Pioglitazone (not metformin) treatment resulted in a 54% decrease ($P < 0.04$) in adipose TSP gene expression, as did in vitro pioglitazone treatment of adipocytes.

CONCLUSIONS—TSP1 is a true adipokine that is highly expressed in obese, insulin-resistant subjects; is highly correlated with adipose inflammation; and is decreased by pioglitazone. TSP1 is an important link between adipocytes and macrophage-driven adipose tissue inflammation and may mediate the elevation of PAI-1 that promotes a prothrombotic state.

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Adipose tissue is an active secretory organ, and adipokines influence energy homeostasis, inflammation, insulin resistance, and cardiovascular diseases. Recent studies have demonstrated that the cell of origin of many adipokines is the macrophage, which infiltrates adipose tissue during obesity (1,2) and secretes many cytokines such as tumor necrosis factor-α, interleukin-6 and −1, and plasminogen activator inhibitor (PAI)-1 (3). On the other hand, adipokines such as leptin and adiponectin are secreted primarily from adipocytes. However, not all adipose depots secrete the same relative proportion of adipokines, and a number of differences in adipokine expression have been described between visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT) (4). One such protein of interest is thrombospondin (TSP)1, which in one brief study was preferentially expressed in VAT compared with SAT (5). Increased TSP1 expression has been reported in adipose tissue of obese rodents (6,7); however, the cell of origin in adipose has not been determined. Although other studies (8–11) have shown elevated TSP1 levels in plasma and in the renal tissue of diabetic patients and animal models, the role of TSP1 in obesity or type 2 diabetes has not been clearly defined.

TSP1 was initially isolated from platelets and megakaryocytes (12) and was later detected in many other cell types, including macrophages and adipocytes (13–16). TSP1 is a multifunctional protein and displays diverse biological activities. It is an endogenous inhibitor of angiogenesis, regulates cell proliferation, and has a role in inflammation and wound healing (17,18). TSP1 is a major regulator of transforming growth factor (TGF)-β activity due to its ability to convert latent TGF-β procytokine to its biologically active form (19,20). PAI-1 is one of the best characterized downstream targets of TGF-β in many cell systems, including adipose tissue in vivo and ex vivo (21,22). Both increased TGF-β activity and elevated PAI-1 levels have been demonstrated to be associated with obesity, insulin resistance, and metabolic syndrome (23–25).

The purpose of this study was to characterize TSP1 gene expression in adipose tissue in detail and to examine TSP1 gene expression from adipose tissue of normal and impaired glucose tolerant (IGT) subjects and from IGT subjects before and after pioglitazone treatment. To further examine adipocyte-macrophage interactions, we also utilized an in vitro adipocyte culture system to assess the effects of pioglitazone and to further characterize TSP1 gene expression in response to coculture with macrophages.

RESEARCH DESIGN AND METHODS

Human subjects

Group 1: adipose tissue from biopsies—Nondiabetic healthy subjects with no history of coronary artery disease were recruited to the General Clinical Research Center by local advertisement. Subjects were provided written, informed consent under protocols that were approved by the local institutional review board. Subjects were included if fasting glucose was <126 mg/dl and 2-h postchallenge glucose was <200 mg/dl, as determined by an initial 75-g oral glucose tolerance test. Based on the oral glucose tolerance test, subjects were defined as either normal glucose tolerant (2-h glucose <140 mg/dl) or impaired glucose tolerant (IGT) (2-h glucose 140–199 mg/dl). A total of 86 subjects were recruited (70 women and 16 men; aged 21–66 years). Subjects were not taking any anti-inflammatory medications, ACE inhibitors, or angiotensin II receptor blockers during the study. This study group had a wide range of BMI (19–55 kg/m^2) and insulin sensitivity (insulin sensitivity index [S_i] = 1.02 –26.77 × 10^{-5} × min per pmol/l). S_i was measured using the frequently sampled intravenous glucose tolerance test. The test involves intravenous injection of glucose (11.4 g/m^2) at time zero followed by insulin bolus (0.04 IU/kg) at 20 min and frequent blood sampling (26). Insulin sensitivity was calculated according to the insulin and glucose data using the MinMod program (27). Plasma insulin was determined using a chemoluminescent assay (Molecular Light Technology Research, Cardiff, Wales, U.K.), and plasma glucose was measured by a glucose

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oxidase assay in duplicate. All subjects underwent an incisional SAT biopsy from the lower abdominal wall. IGT subjects (n = 38) were also randomized to receive either metformin or pioglitazone. Each drug was administered in a 2-week dose escalation followed by 8 weeks at a maximum dose (1,000 mg of metformin twice daily or 45 mg of pioglitazone daily). After 10 weeks of treatment, the glucose tolerance tests, insulin sensitivity measurements, and biopsy were performed again. The mean BMI of the IGT subjects was 33 kg/m^2, and mean age was 48 years. There was no significant difference in the baseline characteristics between the two drug treatment groups.

Group 2: adipose tissue from surgery—Paired samples of VAT and SAT were obtained from subjects undergoing elective abdominal surgery, and the subjects signed consent for removal of fat during surgery by a protocol approved by the University of Maryland Institutional Review Board. The surgical procedures included gastric bypass or restriction for obesity, cholecystectomy, abdominal hysterectomy, hernia repair, and other routine procedures. Adipose tissue specimens were obtained early in the surgical procedure, and samples for RNA extraction were frozen on dry ice immediately after excision. Adipose tissue samples were obtained from a total of 14 subjects who ranged in age from 24 to 62 years, and BMI ranged from 29 to 76 kg/m^2. All subjects were nondiabetic and free of any neoplastic or major disease by medical history.

Separation of adipocytes and stromal vascular fraction from whole adipose tissue: Adipocyte and stromal vascular fraction (SVF) were isolated from adipose tissue obtained by biopsy. Briefly, the SAT was digested with an equal volume of collagenase type I (Sigma, St. Louis, MO) containing 1% BSA for 30 min at 37°C in a shaking water bath. Following complete digestion, the adipocytes were separated from the SVF by centrifugation at 300*g* for 5 min. The floating adipocytes were transferred to a fresh tube, and the remaining medium was aspirated. RNA lysis buffer was added to the pellet containing the SVF and also to the transferred adipocyte fraction, and RNA was isolated using the Lipid RNeasy kit according the manufacturers instructions.

Cell culture

Adipocytes from stem cells—Cultured human adipocytes were obtained by the induction of differentiation of adult-derived human adipocyte stem cells (ADHASCs) isolated from discarded adipose tissue from normal women undergoing liposuction, based on the method of Halvorsen et al. (28) and as described by us previously (29). Although stem cell cultures are not pure, >60% of cells differentiated into adipocytes. Subjects provided informed consent under protocols that were approved by the local institutional review board

Simpson-Golabi-Behmel syndrome adipocytes—An additional source of human adipocytes studied was a cell line derived from the stromal vascular fraction of an infant with Simpson-Golabi-Behmel syndrome (SGBS), as described previously (30). SGBS cells were maintained and differentiated into adipocytes, as described previously (31). For pioglitazone treatment, differentiated adipocytes were treated with 1.5 μmol/l pioglitazone for 48 h, following which the medium was discarded and the adipocytes were collected using RNA lysis buffer. RNA was obtained using the RNeasy lipid isolation kit as per the manufacturer's instruction.

THP1 macrophages—THP1 cells, a human monocyte cell line, were maintained in RPMI medium (ATCC, Manassas, VA) with 10% fetal bovine serum and 1% penicillin/streptomycin. To obtain macrophages, cells were plated at 14×10^6 cells/100-mm culture dish in serum-free medium with 1% penicillin/ streptomycin and 250 nmol/l phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate for 3 days to differentiate the THP1 monocytes to

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macrophages. The THP1-derived macrophages were then scraped, counted using trypan blue, and plated for 48 h in the absence of 12-*O*-tetradecanoylphorbol-13-acetate, following which they were collected with RNA lysis buffer.

ADHASC-THP1 macrophage coculture—ADHASCs were grown on polyester membrane inserts with a 0.4-μm pore size and pore density $4 \times 10^6/cm^2$ for six-well culture dishes (Corning, Sigma) and differentiated as described above. THP1 cells were differentiated to macrophages as described above. The THP1-derived macrophages were then scraped, counted using trypan blue, and seeded in the wells of the six-well companion plate, corresponding to the adipocytes on inserts, at 30% of the confluent adipocyte numbers. The coculture was set up when the adipocytes were at least 60% differentiated. The adipocytes and macrophages were separated by 0.9 mm (membrane to bottom of well) in the same well but free to exchange medium. The adipocytes and macrophages were cocultured for 48 h in alpha minimum essential medium (Invitrogen) containing 5 mmol/l glutamine (Invitrogen), 1 × pencillin streptomycin (Invitrogen), and 2% fetal bovine serum, along with individual controls of adipocytes and macrophages cultured alone. Coculture experiments were performed in duplicate and the experiment repeated twice. Following coculture, the cells from the inserts and wells were collected separately with RNA lysis buffer.

Total RNA isolation and real-time RT-PCR: Total RNA from adipose tissue and cultured adipocytes were isolated using an RNAeasy Lipid Tissue Mini Kit from Qiagen (Valencia, CA) and from cultured THP1 macrophages using an RNAqueous Kit (Ambion, Austin, TX), per the manufacturer's instruction. The quantity and quality of the isolated RNA were determined by an Agilent 2100 Bioanalyser (Palo Alto, CA). One microgram of total RNA was reverse transcribed using random hexamer primers with TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA). The resultant complementary DNA (cDNA) was amplified with a 1 × SYBR Green PCR Master Mix (Applied Biosystems) plus 0.3 μmol/l of gene-specific upstream and downstream primers during 40 cycles on an Applied Biosystems 7500 Fast Realtime Cycler. A standard curve was generated using pooled cDNA from the experimental samples included in each set of experiments. For every real-time run, 8 μl of the each standard was used to generate a standard curve that ranged from 20 to 0.02 ng total cDNA sample (final concentration). Similarly, 8 μl of experimental samples (diluted at 1 ng/μl) was used with the range of standards described above to detect either 18S or the specific gene of interest, respectively. The amount of 18s or the specific gene was expressed relative to the standard curve for each. As the relative abundance of 18s compared with any specific gene examined in the same amount of sample or standard is higher, 18s was always detected at an earlier cycle (lower cycle threshold [C_t] value between 6 and 8) compared with the specific genes of interest (higher C_t value between 15 and 30). In every case, the C_t values were within the linear portion of the amplification range. The expression of the specific genes of interest was reported as relative arbitrary units, as the value obtained for the specific gene was normalized to the value obtained for 18s. Hence, the reported value for each specific gene of interest is obtained by relative quantitation in comparison with 18s. Thus, the data that are arbitrary units accurately compare samples within each set but not necessarily between different sets. The C_t values of the PCR were generally between 15 and 30 for all assays to detect the gene of interest but between 6 and 8 for the detection of 18s. In every case, the C_t values were taken when the efficiency of the reaction was ≥90%. In every run, product purity was confirmed by examining the melt curve that showed a single peak. All the real-time experiment runs that we present were repeated more than once. The primers used were as follows: human TSP1: 5' TCAGGACCCATCTATGATAAAACCTA 3' (forward), 5' TCAGGTCAGAGAAGAACACCATTTC 3' (reverse); human PAI-1: 5' GGT-GGAGAGAGCCAGATTCA 3' (forward), 5' GCTCCTTTCCCAAGCAAGTT 3'; and human leptin: 5' CCAAACCGGTGACTTTCTGT 3' (forward), 5' TGACAC-CAAAACCCTCATCA

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3' (reverse). Primers for 18s, CD68, and macrophage chemoattractant protein (MCP)-1 have been published earlier (29). All primers used are designed to span intron/exon borders.

Western blot analysis for TSP1 protein in cell lysates and medium—Adipocytes and THP1 macrophages were cultured or cocultured for 48 h as described above. Cells were then harvested using M-Per Mammalian protein extraction reagent (Pierce, Rockford, IL) containing protease inhibitor cocktail mix (1:100) (Sigma). When cocultured, the adipocytes were on inserts and THP1 macrophages on plates so that cells could be harvested separately. Medium from adipocyte (Ad) or macrophage (Mc) cultures and adipocyte-macrophage cocultures (AdMc) were collected with protease inhihibitors.

Thirty-five micrograms of protein from cell lysates or equal volumes of medium were denatured in sample buffer and electrophoresed in 7% SDSPAGE and transferred onto nitrocellulose membrane at 100 V for 1 h at 4°C. Membranes were blocked for 30 min using Casein Blocker (Pierce) and immunobloted with mouse monoclonal TSP1 antibody anti-TSP1 antibody (Ab-11) (Neomarkers, Fermont, CA) overnight at 4°C with gentle rocking. Following washing, the blots were incubated for 1 h at room temperature with goat anti-mouse secondary Ig (Pierce). Bands were visualized using SuperSignal West Dura Extended Duration substrate (Pierce), followed by exposure to X-ray film. Densitometric analysis was performed using ImageQuant software (Molecular Dynamics) and expressed as arbitrary densitometric value. Loading controls were detected by staining with ponceu S (Sigma).

Plasma PAI-1 measurements: PAI-1 concentration in plasma was quantified using a Zymutest PAI-1 Antigen ELISA Kit (HYPHEN BioMed Andresy, France, distributed by DiaPharma Group) according to the manufacturer's instructions.

Statistical analyses: Student's two-sample *t* tests were used to compare groups with respect to continuous variables, and paired *t* tests were used to compare baseline and treatment measurements within a group. Pearson's correlation coefficients were used to describe the linear association between variables. The distributions of the variables of interest were examined using quantile-qualtile plots. Where the data were nonnormally distributed (S_i, PAI-1 mRNA, CD68 mRNA, MCP-1 mRNA, and plasma PAI-1), natural logarithm transformations were used to attain approximate normality before analysis. All data from samples were expressed as mean ± SE. A *P* value ≤0.05 was taken to indicate statistical significance.

RESULTS

TSP1 gene is expressed by adipocytes

TSP1 expression from adipose tissue has been noted previously; however, the specific cell source has not been determined. To address this issue, TSP1 gene expression was examined in the adipocyte fraction and SVF isolated from SAT (Table 1). TSP1 mRNA was found to be approximately fourfold higher in the adipocyte fraction compared with the SVF. In contrast, CD68 was expressed predominantly in the SVF, consistent with its expression by adipose tissue macrophages, and leptin was expressed over 20-fold higher in adipocytes than in the SVF. We further examined the expression of TSP1 mRNA in differentiated SGBS and ADHASC adipocytes compared with THP1-derived macrophages. The expression in differentiated adipocytes was very high compared with the nearly undetectable expression by macrophages, a profile similar to the expression of leptin. It should be noted that preadipocytes in vitro also expressed TSP1 at levels comparable with that of differentiated adipocytes (2.60 ± 0.21 and 3.10 ± 0.74, respectively).

A previous study reported the expression of TSP1 to be higher in VAT compared with SAT in two obese subjects (5). To examine the differential expression of TSP1 between these two

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depots, adipose tissue was obtained from 14 subjects undergoing elective abdominal surgery, as described in RESEARCH DESIGN AND METHODS (Table 2). Although the expression of TSP1 mRNA was slightly higher in VAT compared with SAT, there was no significant difference in depot-specific expression (Table 2).

TSP1 gene expression is correlated positively with obesity and inversely with insulin sensitivity

To determine whether TSP1 expression was associated with obesity or insulin resistance, we examined the correlation between TSP1 expression and markers of obesity and insulin resistance. For this study, we used SAT from 86 subjects who were either normal glucose tolerant or IGT, displaying a wide range of BMI (19–55 kg/m^2) and S_i (1.02–22.67 × 10^{-5} × min per pmol/l). As shown in Fig. 1*A*, TSP1 gene expression was higher in subjects with higher BMI ($r = 0.29$, $P = 0.008$, $n = 82$). When we examined the correlation of TSP1 mRNA with S_i (Fig. 1*B*), a significant negative association was seen ($r = -0.35$, $P = 0.002$, $n = 73$). As expected, BMI was negatively associated with S_i in this group ($r = -0.61$, $P < 0.0001$, $n = 77$).

TSP1 is associated positively with PAI-1, a marker of metabolic syndrome

Previous studies have established an important link between PAI-1 and metabolic syndrome, and PAI-1 is a downstream target of TSP1 via TGF-β. Hence, we examined the association between adipose tissue TSP1 and PAI-1 mRNAs. As depicted in Fig. 2*A*, adipose tissue expression of TSP1 and PAI-1 mRNAs associated positively ($r = 0.48$, $P < 0.0001$, $n = 80$). Interestingly, PAI-1 gene expression was higher in the SVF compared with the adipocyte fraction (1.76 ± 0.32 and 0.49 ± 0.14, respectively), consistent with previous reports (32), that is reciprocal to the expression of the TSP1 gene (shown in Table 1), which predominates in the adipocyte fraction. A positive correlation was also observed on examining the association of adipose tissue TSP1 mRNA and circulating PAI-1 levels ($r = 0.36$, $P < 0.02$, $n = 41$, Fig. 2*B*). The association of TSP1 mRNA to PAI-1 mRNA remained significant after controlling for BMI and S_i ($r = 0.34$, $P < 0.005$, $n = 71$), whereas the association with plasma PAI-1 was no longer statistically significant, suggesting that circulating PAI-1 may also be derived from sources other than adipose tissue or may be controlled through obesity-dependent degradation.

TSP1 is associated positively with markers of inflammation

With increasing obesity, there is an increase in circulating inflammatory markers and an increased accumulation of macrophages in the adipose tissue. To determine whether adipocyte-derived TSP1 gene expression correlated with additional inflammatory markers in adipose tissue, we examined the association of TSP1 with CD68, a transmembrane glycoprotein that is highly expressed in human monocytes and tissue macrophages (Fig. 3*A*) and MCP-1 (Fig. 3*B*) gene expression. As seen in Fig. 3, both CD68 and MCP-1 mRNAs showed significant positive correlation with TSP1 mRNA ($r = 0.46$, $P < 0.0001$, $n = 81$; and $r = 0.53$, $P < 0.0001$, $n = 81$; respectively). As BMI and S_i are also associated with these inflammatory markers, we examined the correlation of TSP1 with the inflammatory markers after controlling for BMI and S_i. The relationship between TSP1 and CD68 and MCP-1 remained significant even after statistically adjusting for BMI and S_i ($r = 0.37$, $P = 0.02$, $n = 39$; and $r = 0.57$, $P < 0.0003$, $n = 39$; respectively). Thus, as with PAI-1, there were significant associations between adipose tissue TSP1 and inflammatory markers, supporting the idea that there is cross-talk between adipocytes and macrophages in adipose.

Adipocyte-macrophage interaction in coculture augments TSP1 gene and protein expression

The tight correlation among TSP1 gene expression and inflammatory markers, particularly PAI-1, prompted us to dissect the interaction between these two cell types. Differentiated

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ADHASC adipocytes were cocultured with THP-1–derived macrophages on inserts and plates, respectively, for 48 h, such that communication via soluble mediators was possible. Gene expression was compared with standalone cultures of each cell type. As shown in Table 3, TSP1 gene expression was augmented in adipocytes following coculture with macrophages, suggesting that, in adipose tissue, soluble factors produced by infiltrating macrophages may directly alter adipocyte function. Although TSP1 gene expression in THP-1 macrophages was low, its expression was also increased following coculture with adipocytes. Analysis of protein accumulation in cell lysates by Western blots (Fig. 4*A*) indicated that TSP1 protein abundance increased in response to coculture in both adipocytes and macrophages compared with either cell type alone, reflecting the changes in gene expression. As shown in Fig. 4*B*, significantly more TSP1 protein was secreted into the medium when the adipocytes and macrophages were cocultured. Together, these data suggest a bidirectional communication between adipocytes and macrophages, leading to increased TSP1 expression and secretion.

Pioglitazone treatment decreases TSP1 expression in whole adipose tissue and isolated adipocytes

Treatment of IGT subjects with pioglitazone has been shown to improve insulin sensitivity and decrease the accumulation of macrophages in adipose tissue. We examined the effect of pioglitazone on TSP1 gene expression. Thirty-eight subjects were randomly chosen to be treated with either pioglitazone or another glucose-lowering drug, metformin, for a period of 10 weeks. As we described previously (33), treatment with pioglitazone (but not metformin) improved the S_i from 2.93 to 4.04×10^{-5} × min per pmol/l ($P = 0.005$). TSP1 gene expression was examined in subjects before and following the drug treatment as depicted in Fig. 5*A* Pioglitazone significantly decreased the expression of TSP1 mRNA, as well as circulating PAI-1 plasma levels (Fig. 5 *B*). Metformin treatment had no significant effect on either TSP1 expression or PAI-1 plasma levels. To determine whether the decrease in TSP1 was a direct effect of the pioglitazone or secondary to changes in S_i, SGBS adipocytes were treated with pioglitazone in vitro. A significant decrease in TSP1 mRNA at 48 h following pioglitazone treatment was seen (Fig. 5*C*). This was associated with a concomitant decrease in PAI-1 gene expression (Fig. 5*C*), consistent with previous results on adipose PAI-1 expression following thiazolidinedione (TZD) treatment (34).

DISCUSSION

A number of functions have been attributed to TSP1, including antiangiogenic activity (17, 18,35) and, more recently, as a proatherogenic protein that may provide an important link between diabetes and vascular complications (36,37). Studies (5–7) have reported the expression of TSP1 in adipose tissue, identifying TSP1 as a gene differentially expressed in different adipose depots of morbidly obese humans (5) and overexpressed in obese Zucker rats (6). However, the expression of TSP1 in subjects with varying degrees of obesity or the potential association of TSP1 to insulin resistance or metabolic syndrome has not yet been explored. This is the first study to examine the association of TSP1 to obesity, insulin resistance, inflammation, and metabolic syndrome and to establish TSP1 as an adipokine.

Whole adipose tissue is a complex tissue that, in addition to adipocytes, contains the SVF comprised of endo-thelial cells, monocytes, macrophages, pericytes, fibroblasts, and pluripotent stem cells, including preadipo-cytes, many of which are known sources of TSP1 (12–15). Therefore, we first determined whether TSP1 is expressed by adipocytes. Separating whole adipose tissue into adi-pocyte and SVF revealed that the expression of the TSP1 gene was significantly higher in the adipocyte fraction compared with the SVF. Our observation, made from whole adipose tissue and its fractions, was further confirmed by examining the accumulation of TSP1 mRNA in adipocytes differentiated in vitro from preadipocytes of

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ADHASC and SGBS cells compared with THP-1–derived macrophages. As was observed in whole adipose tissue and its fractions, the mRNA expression in differentiated adipocytes was very high compared with the expression by macrophages. A similar profile was seen on examining TSP1 protein expression in cell lysates, suggesting that the TSP1 secreted into the medium was primarily derived from adipocytes, identifying TSP1 as an adipokine. The TSP1 gene was expressed in preadipocytes in vitro at comparable levels to differentiated adipocytes, suggesting that some of the observed expression of TSP1 in the SVF derives from preadipocytes. Previous studies have described complex regulation of TSP1 during adipocyte differentiation, with decreased TSP1 expression at day 2 and increased expression at day 6 (16). The demonstrated antiproliferative properties of TSP1 (38) may play a role in this regulation.

To better understand the role of TSP1 in humans with varying degrees of obesity and insulin resistance, we measured TSP1 mRNA in humans covering a wide range of BMI and S_i. TSP1 showed positive association with BMI and negative association with S_i, suggesting that it plays a role directly or indirectly in insulin resistance. Obesity-induced insulin resistance may be due to numerous factors such as increased adipose-derived circulating free fatty acids, increased secretion of inflammatory adipokines (39,40), or decreased secretion of adiponectin (41). Many adipose tissue inflammatory factors are secreted predominantly from adipose-derived macrophages as opposed to adipocytes (1,2,42). Although our data demonstrate that TSP1 was primarily expressed in adipocytes, we found a significant positive association between TSP1 gene expression and the inflammatory markers MCP-1 and CD68, both of which are derived predominantly from macrophages (29). MCP-1 is a chemoattractant whose increased production in obesity aids in the infiltration of macrophages into adipose tissue contributing to the inflammatory state. TSP1 also has chemotactic properties with smooth muscle cells and macrophages and is responsible for macrophage migration into the site of injury aiding in wound healing (17). Moreover, TSP1 gene and protein expression were augmented in both adipocytes and macrophages following coculture, as was TSP1 secretion, demonstrating a direct interaction between these cells that may exacerbate the inflammatory state during obesity. Taken together, these results suggest that TSP1 is an important link between macrophage-driven inflammation and altered adipocyte function in metabolic syndrome. However, our results did not confirm a previous finding that the TSP1 gene is preferentially expressed in VAT compared with SAT (5). Although expression in VAT was slightly higher than SAT, the difference was not significant. While the initial report was based on mRNA from VAT and SAT of two obese individuals, our study included 14 obese subjects.

Elevated plasma PAI-1 is recognized as a core feature of metabolic syndrome, contributing to the prothrombotic state (23,25,43). PAI-1 is produced by endothelial cells and adipose tissue (21,44) and, as demonstrated for CD68 and MCP-1, is preferentially expressed in the SVF of adipose tissue. PAI-1 gene expression is controlled by TGF-β through SMAD phosphorylation and subsequent binding to the PAI-1 promoter (45). PAI-1 is a serine protease inhibitor that influences fibrinolysis by inhibiting both tissuetype plasminogen activator (t-PA) and urokinase type plasminogen activator (u-PA) (46). We found that TSP1 gene expression correlated positively both with PAI-1 mRNA in adipose tissue and with plasma PAI-1. It is possible that this correlation is functional in adipose through TSP1-dependent TGF-β activation leading to upregulation of PAI-1 gene expression, which may contribute to the elevated circulating PAI-1 in insulin resistance and metabolic syndrome.

TZDs are insulin-sensitizing drugs that act as antidiabetes and anti-inflammatory agents. We examined the effect of the TZD pioglitazone on TSP1 gene expression in IGT subjects compared with another glucose-lowering drug, metformin. Pioglitazone-treated IGT subjects showed a significant decrease in TSP1 gene expression, whereas there was no significant change in TSP1 gene expression in subjects treated with metformin. SGBS adipocytes that

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were treated with pioglitazone also showed significant decrease in TSP1, as well as PAI-1 gene expression. These results are consistent with data showing that TSP1 is downregulated in response to TZDs during adipogenic differentiation in 3T3L1 cells (16) and that PAI-1 expression is attenuated in adipose tissue in response to TZDs (34). Recent work suggests that nuclear corepressors may repress peroxisome proliferator–activated receptor γ–mediated transcription on specific promoters in the adipocyte, providing a potential mechanism for downregulation (47). It has also been reported that in macrophages, TZDs downregulate transcriptional activation through sumoylation of the peroxisome proliferator–activated receptor γ ligand-binding domain (48), which may be relevant to inhibition of SVF gene expression. Thus, TZDs have the potential to downregulate PAI-1 gene expression directly in both macrophages and adipocytes. In addition, TZDs induced macrophage apoptosis and may thus reduce PAI-1 expression (31). Finally, TZDs downregulated TSP1 expression in adipocytes that may interfere with TGF-β–dependent expression of PAI-1 in both macrophages and adipocytes.

In summary, TSP1 is an adipokine positively associated with BMI, PAI-1 levels, and markers of inflammation and negatively associated with S_i. TSP1 has diverse biological functions, and, therefore, downregulation of TSP1 may mediate many of the wide range of beneficial effects of pioglitazone in metabolic syndrome and reduction of inflammation.

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Glossary

ADHASC	adult-derived human adipocyte stem cell
cDNA	complementary DNA
C_t	cycle threshold
IGT	impaired glucose tolerant
MCP	macrophage chemoattractant protein
PAI	plasminogen activator inhibitor
SAT	subcutaneous adipose tissue
SGBS	Simpson-Golabi-Behmel syndrome
SVF	stromal vascular fraction
TGF	transforming growth factor
TSP	thrombospondin
TZD	thiazoladinediones
VAT	visceral adipose tissue

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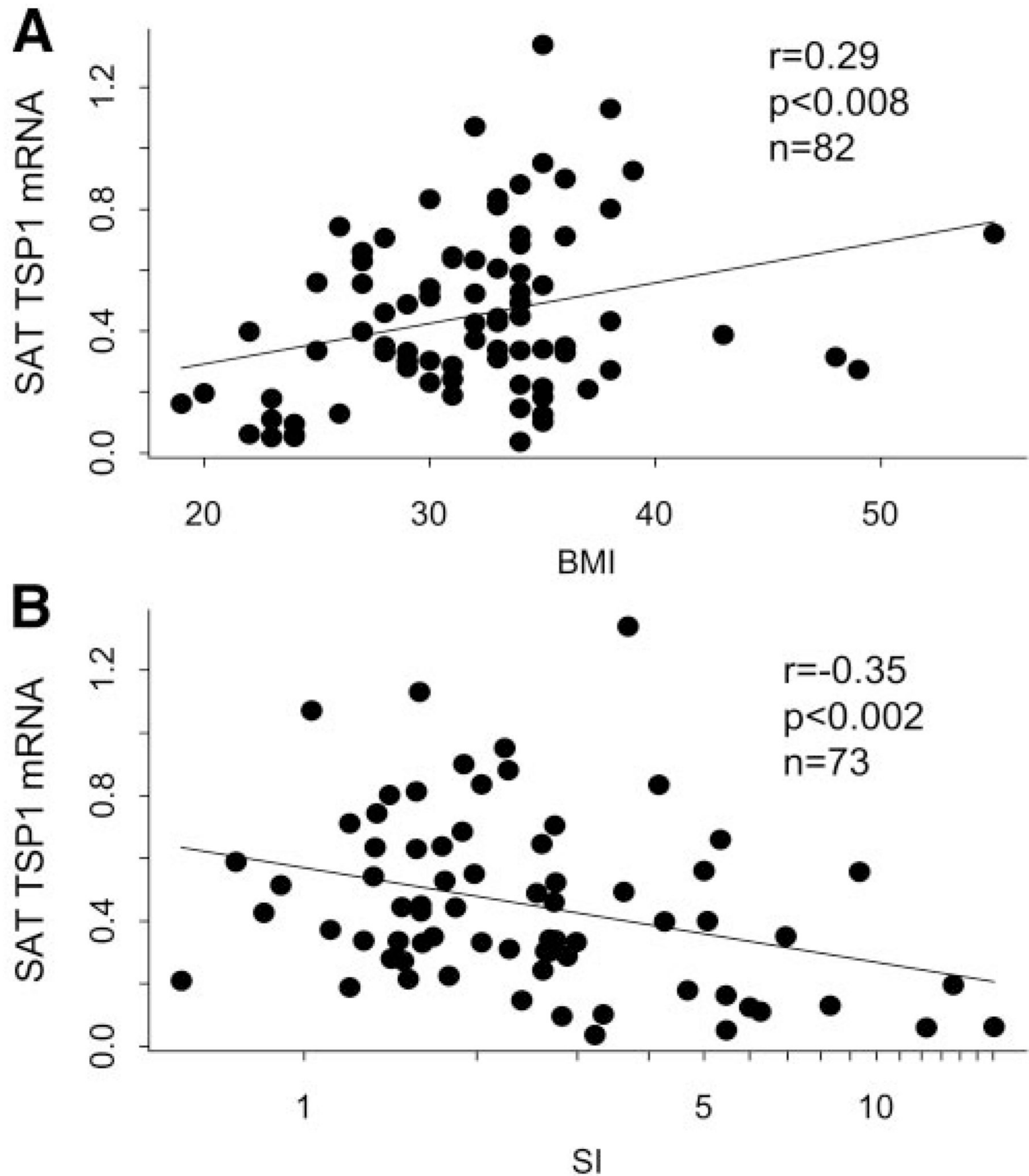

FIG. 1.

Correlations between SAT TSP1 mRNA and BMI (n = 82) (A) andS_i(n=73) (B). TSP1 mRNA was quantified by real-time RT-PCR analysis and was expressed relative to endogenous 18S RNA. S_i was determined by the frequently sampled intravenous glucose tolerance test method. TSP1 mRNA was plotted against the natural log of S_i. TSP mRNA is positively correlated with BMI and negatively correlated with S_i. Elimination of the four subjects in Fig. 1A with BMI >40 kg/m^2 yields r = 0.39, P = 0.0004, n = 78.

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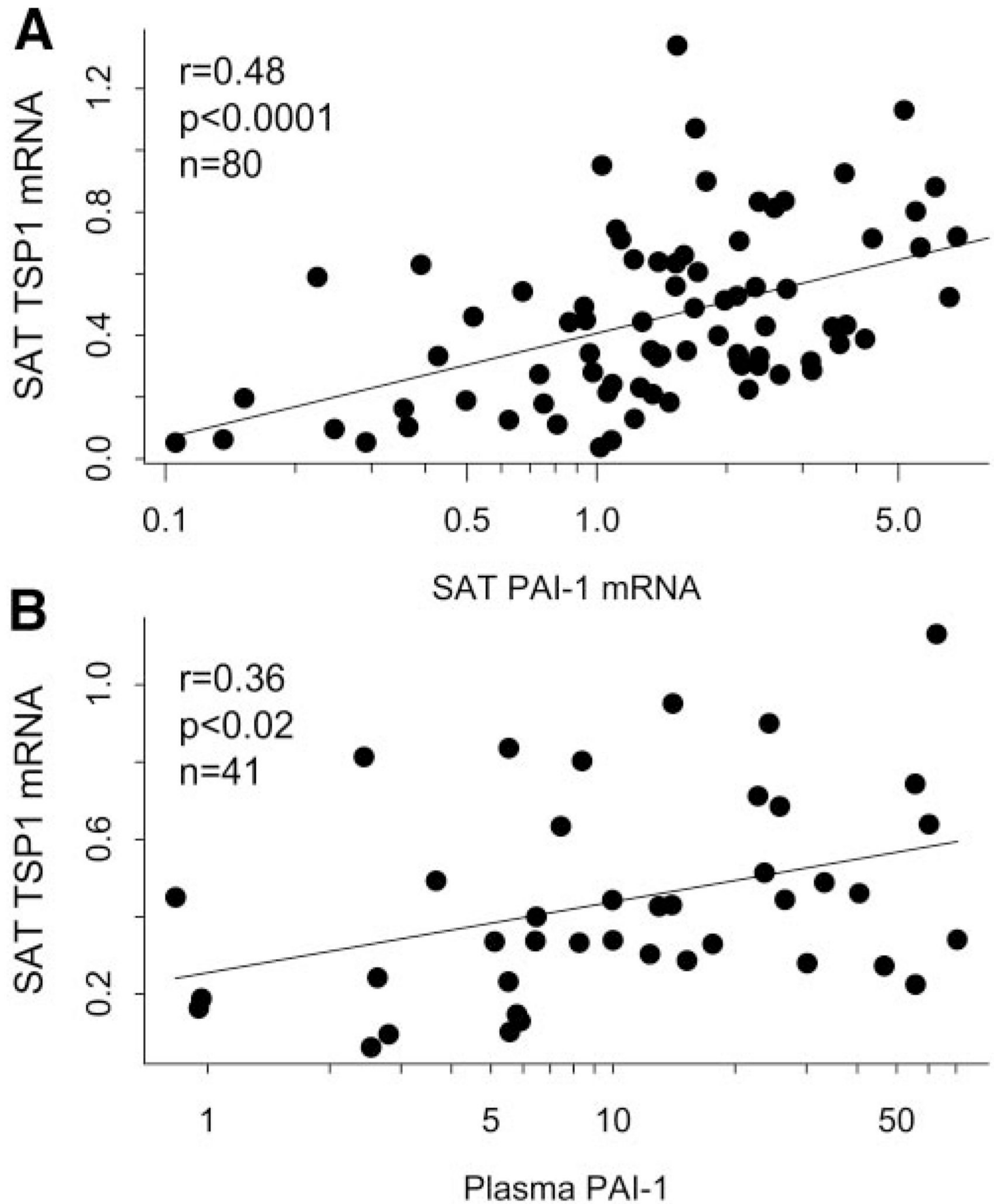

FIG. 2.

Correlation between SAT TSP1 mRNA and PAI-1 mRNA (*n* = 80) (*A*) and plasma PAI-1 (*n* = 41) (*B*). TSP1 and PAI-1 mRNA levels were quantified by real-time RT-PCR analysis and were expressed relative to endogenous 18S RNA. Plasma TSP1 was measured by enzyme-linked immunosorbent assay as described in RESEARCH DESIGN AND METHODS. TSP1 mRNA was plotted against the natural log of PAI-1 mRNA and plasma PAI-1 levels. TSP1 mRNA is positively correlated to PAI-1 mRNA and plasma levels.

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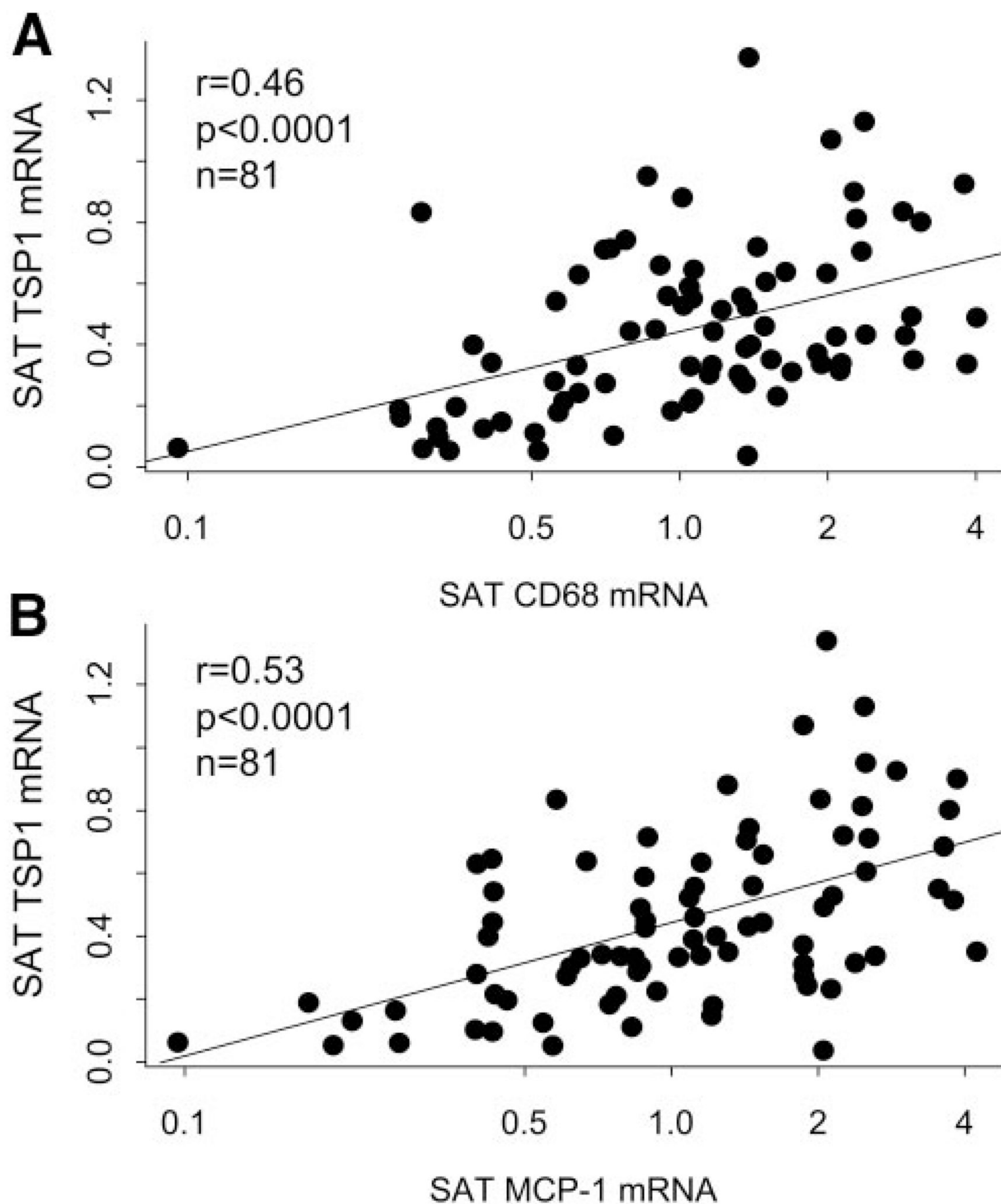

FIG. 3.

Correlation between SAT TSP1 mRNA and mRNAs encoding adipose markers of inflammation CD68 (n = 81) (*A*) and MCP-1 (n = 81) (*B*). mRNA levels were quantified by real-time RT-PCR analysis and were expressed relative to endogenous 18S RNA. The TSP1 mRNA was plotted against the natural log of CD68 mRNA and MCP-1 mRNA. TSP1 mRNA was positively correlated with both CD68 and MCP-1 mRNAs.

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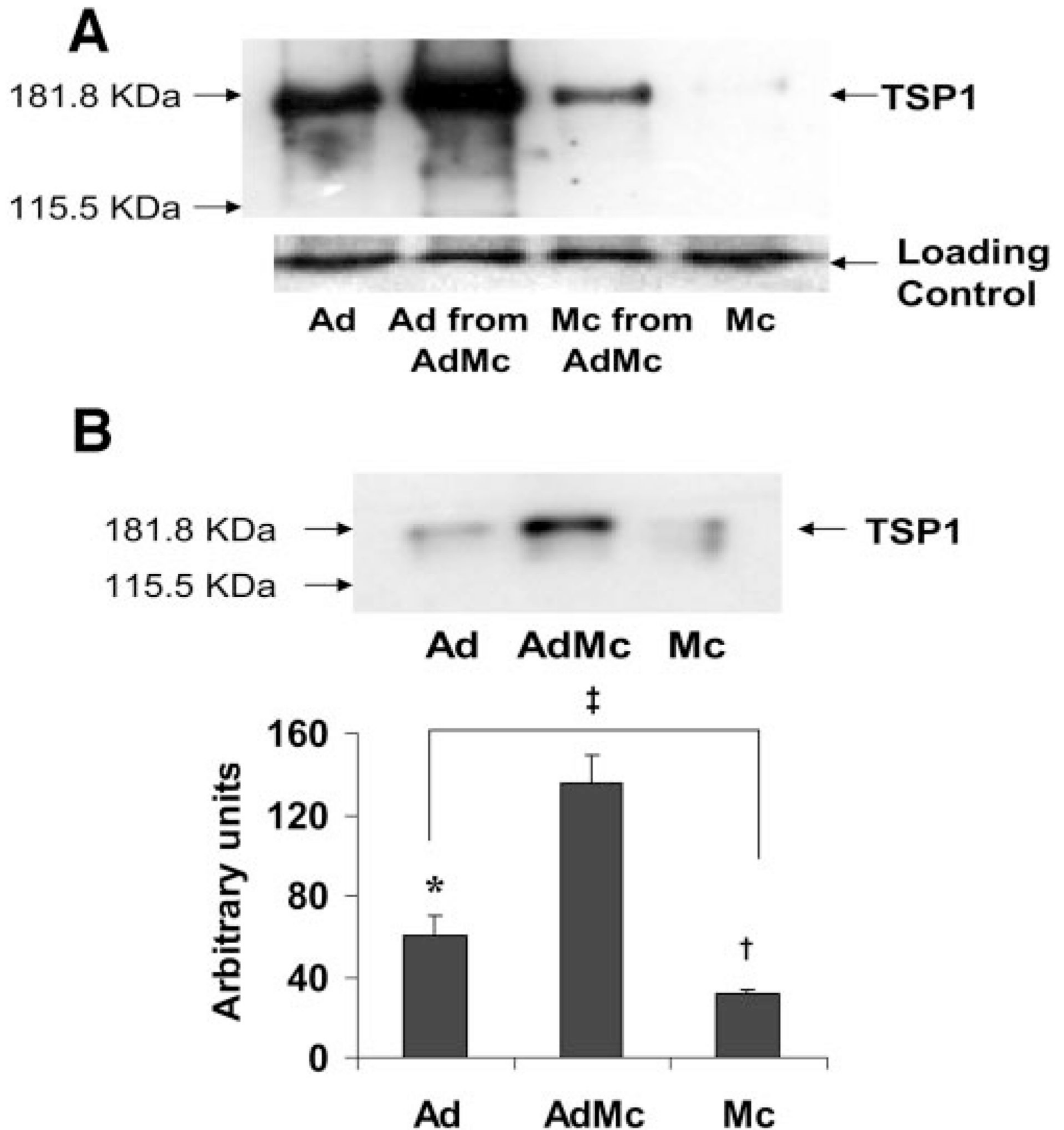

FIG. 4.

TSP1 protein expression and secretion in differentiated ADHASC and macrophages is augmented in coculture. *A*: Representative blot of TSP1 protein expression in cell lysates and loading control for cell lysates of differentiated ADHASC alone (Ad), cocultured differentiated ADHASC (Ad from AdMc coculture), cocultured THP-1 macrophages (Mc from AdMc coculture), and THP-1 macrophages alone (Mc). *B*: Representative blot of secreted TSP1 in medium from cultures of differentiated ADHASC alone (Ad), differentiated ADHASC cocultured with macrophages (AdMc), and THP-1 macrophages alone. Cells were cultured for 48 h. Equal volumes of medium was resolved under reducing conditions and subjected to Western blot to detect TSP1. Densitometric analysis of Western blots of medium for TSP1.

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The densitometric values are a means ± SE of three independent experiments. *$P < 0.01$ vs. AdMc; †$P < 0.02$ vs. AdMc; ‡$P < 0.05$ (Ad vs. Mc).

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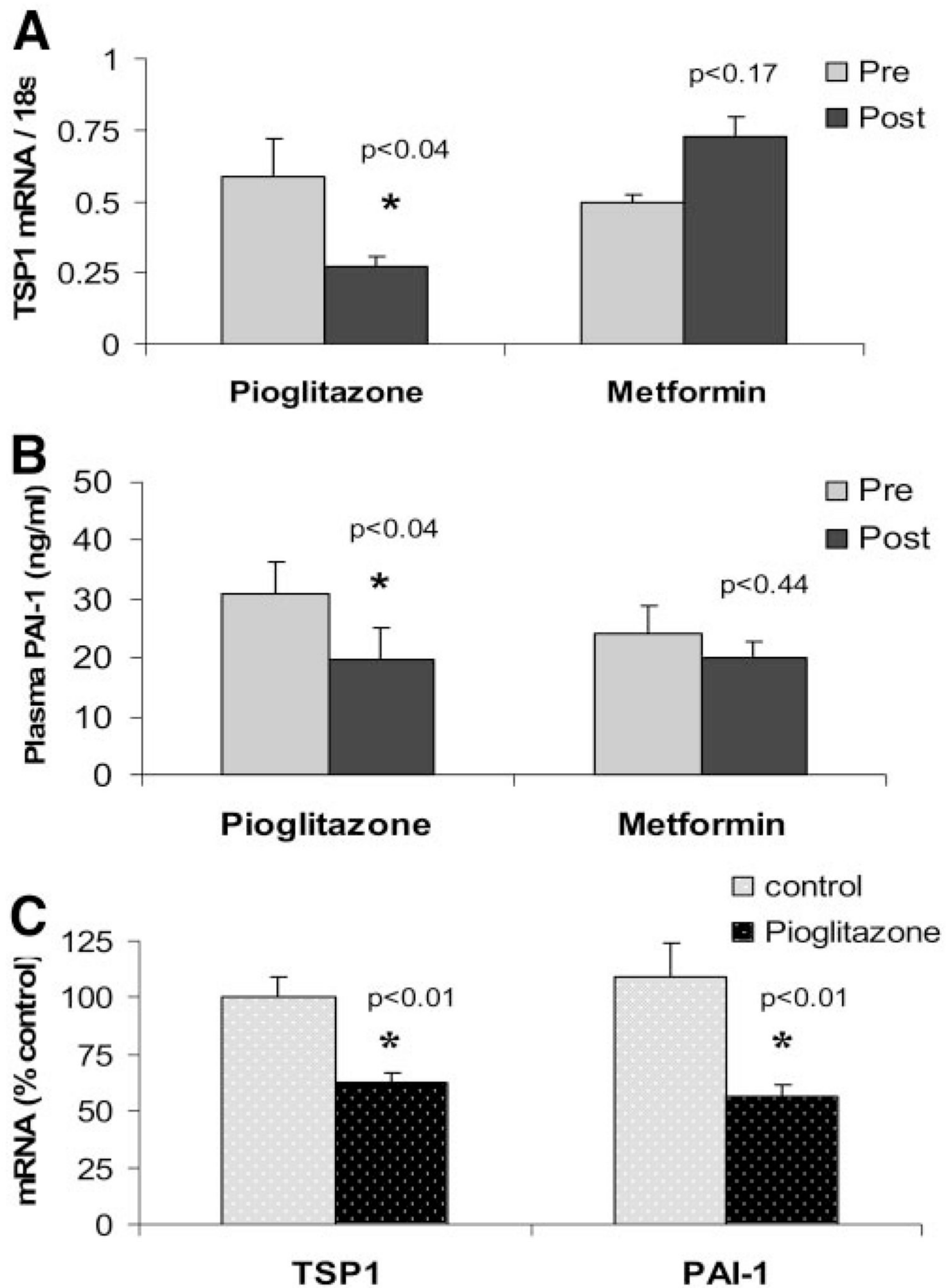

FIG. 5.

Effect of pioglitazone on TSP1 and PAI-1 expression. *A:*IGT subjects were treated with pioglitazone (n= 17) or metformin (n= 21) for 10 weeks, and TSP1 mRNA expression was quantified in adipose tissue by real-time RT-PCR. *B:*Effect of pioglitazone (n= 12) and metformin (n= 15) treatment of IGT subjects on plasma PAI-1. *C:*Effect of 48 h pioglitazone treatment of SGBS adipocytes in vitro on TSP1 and PAI-1 mRNA expression. The treatment of SGBS adipocytes was performed in duplicate and repeated twice. mRNA levels were quantified by real-time RT-PCR analysis and were expressed relative to endogenous 18S RNA.

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TABLE 1

mRNA expression in whole subcutaneous adipose tissue, its fractions, and cultured cells

Tissue	TSP1	CD68	Leptin
SAT (*n* = 14)	1.09 ± 0.12	0.88 ±0.11	1.09 ± 0.09
Adipocytes from SAT (*n* = 14)	1.23 ± 0.21	0.23 ± 0.03	2.33 ± 0.47
Stromal fraction from SAT (*n* = 14)	0.33 ± 0.07*	1.22 ± 0.15*	0.10 ± 0.01*
Cultured cells			
SGBS adipocytes	1.21 ± 0.11†	0.27 ± 0.00	2.3 ± 0.31†
THP-1 macrophages	0.01 ± 0.00	0.87 ± 0.17	0.06 ± 0.04
ADHASC adipocytes	1.03 ± 0.01†	0.88 ± 0.05	1.16 ± 0.06
THP-1 macrophages	0.01 ± 0.00	1.46 ± 0.09	ND

Data are means ± SE, normalized to 18s rRNA expression. RNA for the tissue samples of SAT and its fractions was analyzed separately from cultured cells with a different standard curve generated from pooled cDNA

* $P < 0.001$ vs. adipocytes from SAT.

† $P < 0.001$ vs. THP1 macrophages.

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TABLE 2

mRNA in different adipose depots

Surgical adipose tissue depots	TSP1	Leptin
SAT (*n* = 14)	0.86 ± 0.13	1.74 ± 0.21
VAT (*n* = 14)	1.36 ± 0.41	0.68 ± 0.12*

Data are means ± SE, normalized to 18s rRNA expression.

*$P < 0.005$ vs. SAT.

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TABLE 3

TSP1 mRNA expression in 48-h cocultured adipocytes and macrophages

Cultured cells	TSP1
ADHASC adipocytes from coculture	2.53 ± 0.14
ADHASC adipocytes alone	1.41 ± 0.05*
THP-1 macrophages from coculture	0.31 ± 0.01
THP-1 macrophages alone	0.01 ± 0.00†

Data are means ± SE, normalized to 18s rRNA expression.

* $P < 0.05$ vs. cocultured ADHASC adipocytes

† $P < 0.03$ vs. cocultured THP-1 macrophages.