Tumor necrosis factor-α-induced production of plasminogen activator inhibitor 1 and its regulation by pioglitazone and cerivastatin in a nonmalignant human hepatocyte cell line

Tumor necrosis factor-α-induced production of plasminogen activator inhibitor-1 and its regulation by pioglitazone and cerivastatin in a non-malignant human hepatocyte cell line

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Abstract

Plasminogen activator inhibitor (PAI)-1 is an important mediator of atherosclerosis and liver fibrosis in insulin resistance. Circulating levels of PAI-1 are elevated in obese individuals, and PAI-1 mRNA is significantly higher in the livers of obese type 2 diabetic individuals than in non-obese type 2 diabetics. To address the mechanism underlying the up-regulation of hepatic PAI-1 in obesity, we tested the effects of TNF-α, an important link between obesity and insulin resistance, on PAI-1 production in the non-malignant human hepatocyte cell line, THLE-5b. Incubation of THLE-5b cells with TNF-α stimulated PAI-1 production via PKC-, MAP kinase-, protein tyrosine kinase- and NF-κB-dependent pathways. A thiazolizinedione, pioglitazone, reduced TNF-α-induced PAI-1 production by 32%, via PKC- and NF-κB-dependent pathways. The HMG-CoA reductase inhibitor cerivastatin inhibited TNF-α-induced PAI-1 production by 59%, which was reversed by coincubation with mevalonic acid. In conclusion, obesity and TNF-α up-regulation of PAI-1 expression in human hepatocytes may contribute to the impairment of the fibrinolytic system, leading to the development of atherosclerosis and liver fibrosis in insulin resistant individuals. A thiazolidinedione and a HMG-CoA reductase inhibitor may thus be candidate

drugs to inhibit obesity-associated hepatic PAI-1 production.

1. Introduction

The liver plays a central role in glucose and lipid homeostasis, and type 2 diabetes is characterized by excessive hepatic production of glucose. The long duration of diabetes and obesity causes systemic vascular complications, such as micro- and macro-angiopathy. Moreover, type 2 diabetes, together with obesity and insulin resistance, is often associated with non-alcoholic fatty liver disease (NAFLD). Histologically, NAFLD begins as simple steatosis of hepatocytes in fatty liver, but may progress to the inflammation and fibrosis characteristic of non-alcoholic steatohepatitis (NASH). The liver is also a major source of angiogenic factors and cytokines, such as plasminogen activator inhibitor (PAI)-1 (1), vascular endothelial growth factor (VEGF) and transforming growth factor (TGF)-β (2, 3). Many of these proteins may contribute to the development of atherosclerosis and NASH (4).

The common pathophysiology between obesity and type 2 diabetes is insulin resistance. We recently found that fatty liver is closely associated with insulin resistance and elevated plasma levels of PAI-1 (5), in agreement with previous observations that the plasma concentration of PAI-1 is elevated in obese individuals (6). Moreover, plasma PAI-1 levels were found to be more closely related to liver steatosis than to adipose tissue accumulation in a murine model of genetic obesity (7). In addition, PAI-1 was shown to be important in the development of atherosclerosis (8), as well as being a key participant in organ fibrosis (9). Thus, PAI-1 may constitute a link between insulin resistance and its related disorders. Although the liver has been reported to be a source of PAI-1 (10, 11), there is no in vivo or in vitro evidence of regulated PAI-1 expression in human hepatocytes.

In individuals with visceral obesity, the liver is exposed to abundant TNF-α secreted by adipocytes (12), leading to insulin resistance. TNF-α has been reported to decrease the expression of glucose transporter 4 in adipocytes (13). Moreover, in a human hepatoma cell line, TNF-α has been found to increase the serine phosphorylation of insulin receptor substrate-1, resulting in the reduction of insulin signaling (14).

Here, we assayed the expression of PAI-1 mRNA in the livers of individuals with type 2 diabetes by real-time PCR, and we found that obesity up-regulates hepatic PAI-1 expression in individuals with type 2 diabetes. To address the mechanism underlying the up-regulation of hepatic PAI-1 in obesity, we tested the effects of TNF-α, an important link between obesity and insulin resistance (15), on PAI-1 production and its signal transduction pathways in a non-malignant human hepatocyte cell line, THLE-5b. We also tested the effects of a thiazolidinedione and a 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor (statin), which are potent agents in the treatment of insulin resistance and dyslipidemia in obese type 2 diabetic patients, on the TNF-α-induced production of PAI-1 in THLE-5b cells.

2. Materials and methods

2.1. Gene expression analysis in the liver

Liver biopsy specimens were obtained from 21 individuals with type 2 diabetes (15 males, 6 females; mean age 53 ± 2 years; mean body mass index (BMI) 24.4 ± 0.9 kg/m^2; mean fasting plasma glucose (FPG) 143 ± 11 mg/dl, mean HbA_{1c} 7.3 ± 0.3%, mean ALT 34 ± 6 IU/L, The duration of diabetes was 6.3 ± 1.6 (means ± SE) years) and 11 non-diabetic individuals (6 males, 5 females; mean age 44 ± 4 years; mean BMI 26.1 ± 1.4 kg/m^2; mean FPG 91 ± 2 mg/dl; mean HbA_{1c} 5.1 ± 0.1%; mean ALT 31 ± 11 IU/L). Fasting blood samples were obtained from the antecubital vein. Blood samples were allowed to clot and were centrifuged. Serum was separated and frozen at -70°C until the time of the assay. Serum samples were assayed for lipids, TNF-α and PAI-1. The biopsy samples were immediately frozen in liquid nitrogen and stored at –80°C until use. All 32 subjects tested negative for hepatitis B and C viruses, and each consumed less than 20 grams of alcohol per day. No subjects experienced body weight loss or malnutrition before liver biopsies. All liver biopsies were performed in the fasting state. No biopsies were performed in a fed state. Diagnoses of all patients were based on standard criteria (16). The patients with diabetes were being treated with diet therapy alone or insulin, and none had been prescribed any other oral hypoglycemic agent. Any individual with a BMI greater than the median BMI in each group (24.4 kg/m^2 and 26.1 kg/m^2 in the diabetes and non-diabetes groups, respectively) was defined as obese or overweight. None of the individuals in either group was being treated with statins, angiotensin-converting enzyme inhibitors or angiotensin II receptor blockers.

Informed consent was obtained from all individuals, both for this study and for histological examination of liver diseases, including NASH, which often complicate diabetes (17). The experimental protocol was approved by the relevant ethics committee in our institution, and was carried out in accordance with the Declaration of Helsinki.

Liver biopsy specimens were histologically examined using hematoxylin-eosin and silver reticulin stains.

2.2 Cell lines and culture conditions

We used a simian virus 40 large-T (SV40-T) antigen immortalized non-malignant human hepatocyte cell line, THLE-5b. Cells at the third to fourth passage were seeded at 1.0 x 10^6/well in 6-well dishes and grown to confluence in conditioned PFMR-4 medium (Pasadena Foundation for Medical Research Medium No.4), supplemented with 4.2 μg/ml insulin and 0.2 μM hydrocortisone, at 37°C in a humidified atmosphere containing 5% CO_2, changing the medium three times per week. All experiments were performed using 80-90% confluent THLE-5b cells. Where indicated, these cells were cultured in the presence of recombinant human TNF-α (Pierce Endogen, Rockford, IL) and other agents for 24 hours. Cell culture supernatants were collected at indicated times and stored at -20°C until assayed.

The thiazolidinedione compound pioglitazone was supplied by Takeda Chemical Industries (Osaka, Japan) and dissolved to 1 mM solution in ethanol. The HMG-CoA reductase inhibitor, cerivastatin sodium, was donated by Bayer Pharmaceuticals (Osaka, Japan). Mevalonic acid was purchased from ICN Biomedicals (Zoetermeer, The Netherlands), and 2'-amino-3'-methoxyflavone (PD98059), emodin, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole (SB203580), calphostin C, and genistein were purchased from Calbiochem (La Jolla, CA).

2.3. PAI-1 Assays

The concentration of PAI-1 antigens in THLE-5b cell culture supernatants was determined by ELISA (TintElize PAI-1; Biopool International, Umea, Sweden) as described (18), with a detection limit of 0.5 ng/ml. The within- and between-assay coefficients of variation were 1.9% and 2.4%, respectively.

2.4. Real-Time Quantitative PCR

Total RNA was extracted from cells using a RNeasy mini kit (QIAGEN, West Sussex, UK), as recommended by the manufacturer, and dissolved in 50 μl RNase-free water. RNA concentration was determined spectrophotometrically at 260 nm. Complementary DNA (cDNA) was synthesized from 100 ng of total RNA as described (4) using a High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). The cDNA was used as a template in real time quantitative PCR, which was performed using an ABI Prism 7700 Sequence detection system (Applied Biosystems) as described (19). Primer sets and TaqMan probes for PAI-1, tissue-type plasminogen activator (tPA) and TNF-α were proprietary to Applied Biosystems. To control for variation in the amount of cDNA loaded, expression of PAI-1, tPA and TNF-α mRNA was normalized relative to the expression of an endogenous control, β-actin (β-actin TaqMan control reagent kit; Applied Biosystems). The PCR conditions were 1 cycle at 50°C for 2 min and 95°C for 10 min, followed by 50 cycles at 95°C for 15 s and 60°C for 1 min.

2.5. Statistical analysis of the data

All data are presented as means ± SEM. Differences between groups were determined using one-way ANOVA and the Kruskal-Wallis test, followed by a Fisher protected least significant test for pair-wise differences. $P < 0.05$ was considered statistically significant. All calculations were performed with the computer program StatView, version 4.0, for Macintosh (Abacus Concepts, Berkeley, CA).

3. Results

3.1. Hepatic expression of PAI-1 in patients with type 2 diabetes

Baseline characteristics of the patients are shown in Table 1. Obese patients in the diabetic group had higher ALT values, but there were no significant differences in age, FPG, HbA_{1c} levels, serum levels of lipids and histological scores of the liver between obese and non-obese individuals in either group.

We found that hepatic expression of PAI-1 mRNA was significantly higher in the livers of obese type 2 diabetics than in non-obese type 2 diabetics (Fig. 1). There were no significant between-group differences in tPA mRNA expression.

3.2. Hepatic expression of TNF-α in patients with type 2 diabetes_

Protein levels of TNF-α is found to be elevated in plasma as well as in the adipose tissue of obese subjects (20, 21) In our study, obesity up-regulated plasma levels of TNF-α and the hepatic expression of TNF-α mRNA in patients with type 2 diabetes(Fig. 2).

3.3. Effect of TNF-α on PAI-1 secretion from THLE-5b cells

We found that unstimulated THLE-5b cells secreted PAI-1 into the culture medium (Fig. 3A). After incubation with 1.0 ng/ml TNF-α, PAI-1 concentrations were elevated in a time-dependent manner, peaking at 24 hours to 2.23 ± 0.15-fold of the control value ($P < 0.0001$ versus control). Control cultures had a PAI-1 concentration of 360 ± 40 ng/ml medium, whereas, following incubation with 2.0 ng/ml TNF-α for 24 hours, the PAI-1 concentration was 1180 ± 10 ng/ml medium. The effect of TNF-α on PAI-1 secretion was also dose-dependent, from 0.5 to 2.0 ng/ml (Fig. 3B).

3.4. Effect of TNF-α on PAI-1 mRNA and the tPA/PAI-1 mRNA ratio in THLE-5b cells

To determine the effect of TNF-α on PAI-1 at the transcriptional level, we quantitatively evaluated PAI-1 mRNA expression by THLE-5b cells in the absence or presence of TNF-α by real-time PCR. We found that 1.0 ng/ml TNF-α induced a time-dependent increase in PAI-1 mRNA, with a maximum after 6 hours, corresponding to a 12.0 ± 4.3-fold increase relative to the control value ($P < 0.05$ versus control; Fig. 4A). Since the balance between PAI-1 and tPA has been reported to determine fibrinolytic activity (22), we also assayed the effect of TNF-α on t-PA mRNA expression in these THLE-5b cells. Although TNF-α did not affect tPA mRNA levels for 24 hours (Fig. 4B), it decreased the tPA/PAI-1 mRNA ratio in a time-dependent manner (Fig. 4C), with a maximum effect at 6 hours (16.2 ± 7.2%, $P < 0.005$ versus control).

3.5. Effect of signal transduction inhibitors on TNF-α-induced PAI-1 production in

hepatocytes

To determine which of the TNF-α-induced signal transduction pathways leads to PAI-1 production, we tested the effects of various pathway inhibitors on TNF-α-induced PAI-1 secretion (Fig. 5A). The signal transduction inhibitors were used at concentrations found to be most effective and not cytotoxic in a pilot study (data not shown), as described (23). We found that the protein kinase C (PKC) inhibitor calphostin C, the specific inhibitor of p38 mitogen-activated protein (MAP) kinase SB 203580, the MAP kinase/extracellular signal-regulated kinase (ERK)-specific inhibitor PD98059, the protein tyrosine kinase (PTK) inhibitor genistein, and the NF-κB inhibitor emodin all reduced TNF-α-induced PAI-1 secretion in a dose-dependent manner. While calphostin C, SB203580, PD98059 and emodin partially inhibited TNF-α-induced PAI-1 secretion, genistein reduced PAI-1 secretion to the control level ($P < 0.001$).

To determine whether each of these inhibitors acts at the transcriptional level, we also measured steady state PAI-1 mRNA levels in these cells by real-time quantitative PCR (Figure 5B). We found that each of these inhibitors reduced TNF-α-induced PAI-1 mRNA in a dose-dependent manner, with a maximum effect at 6 hours, to an extent almost the same as that of PAI-1 secretion.

3.6. Effects of a thiazolidinedione and an HMG-CoA reductase inhibitor on TNF-α-induced PAI-1 secretion

When we tested the effects of a thiazolidinedione, pioglitazone, and an HMG-CoA reductase inhibitor, cerivastatin, on TNF-α induced PAI-1 secretion in THLE-5b cells, we found that each inhibited TNF-α-induced PAI-1 secretion in a dose-dependent manner (Fig. 6).

Cholesterol synthesis includes the conversion of HMG-CoA to mevalonic acid by the enzyme HMG-CoA reductase. We therefore tested the effects of mevalonic acid on the cerivastatin inhibition of TNF-α-stimulated PAI-1 production in THLE-5b cells. We found that co-incubation with mevalonic acid completely reversed the inhibitory effects of cerivastatin in a dose-dependent manner (Fig. 7).

3.7. Target of pioglitazone in the signal transduction pathway leading to TNF-α-induced PAI-1 production in hepatocytes

To identify the target of pioglitazone in the signal transduction pathways leading to TNF-α-induced PAI-1 production, we investigated the effects of the signal transduction inhibitors calphostein C, SB203580, PD98059, genistein and emodin on TNF-α-induced PAI-1 production in the presence or absence of pioglitazone (Fig. 8). We found that SB203580, PD98059 and genistein inhibited TNF-α-induced PAI-1 secretion in the presence of pioglitazone by 42.1 ± 2.6%, 37.7 ± 3.1% and 84.0 ± 5.6%, respectively. While SB203580, PD98059 and genistein significantly added to the inhibitory effects of pioglitazone, calphostin C and emodin did not have additive effects.

4. Discussion

Most in vitro studies of hepatocytes have been performed using cell lines derived from hepatomas, such as the HepG2 and Huh7 cell lines. We previously observed, however, that gene expression profiles differed among hepatocyte cell lines, especially with respect to α–fetoprotein production (24). We therefore used THLE-5b cells, a non-malignant human hepatocyte cell line immortalized with SV40-T antigen. These cells maintain a non-tumorigenic phenotype and do not have telomerase activity or oncogenic ras (24). In addition, angiogenic and growth factors, such as ephrin-A1 and TGF-β_2, are not up-regulated in THLE-5b cells as they are in hepatoma cell lines (24), suggesting that THLE-5b cells are a suitable hepatocyte cell line for the study of gene expression stimulated by cytokines and growth factors.

When we evaluated gene expression profiles in the livers of patients with type 2 diabetes using real-time quantitative PCR, we found that PAI-1 gene expression was up-regulated in obese compared with non-obese individuals, suggesting that obesity may promote PAI-1 gene expression in the livers, as well as in the adipose tissue (6), of diabetic individuals.

Protein levels of TNF-α is found to be elevated in plasma as well as in the adipose tissue of obese subjects (20, 21). In our study, obesity up-regulated plasma levels of

TNF-α and the hepatic expression of TNF-α mRNA in patients with type 2 diabetes. To model the regulation of PAI-1 production by the liver during insulin resistance, we assayed the in vitro effect of TNF-α on PAI-1 production by THLE-5b cells and on its signal transduction pathways. We found that TNF-α stimulated PAI-1 secretion and PAI-1 mRNA expression in these hepatocytes, suggesting that TNF-α may be an important factor stimulating PAI-1 overproduction in the livers of patients with obesity and insulin resistance. In addition, TNF-α may inhibit the net fibrinolytic system in hepatocytes, as shown by the decrease in the tPA/PAI-1 mRNA ratio. These findings support previous in vivo results, showing that intravenous injection of TNF-α induced PAI-1 gene expression in mouse livers (25).

Our findings also indicate that the PKC, p38, ERK, protein tyrosine kinase and NF-κB pathways are involved in TNF-α-induced PAI-1 production in the liver. In contrast, the PKC and p38 pathways were reported not to be involved in TNF-α-induced PAI-1 production in HUVECs (23). Both in HUVECs and THLE-5b cells, TNF-α-induced PAI-1 production is only partly dependent on the NF-κB pathway but is completely dependent on the protein tyrosine kinase pathway, suggesting that both NF-κB-dependent and -independent pathways may be involved in TNF-α-induced PAI-1 production in hepatocytes. It is not known, however, whether other transcription factors, such as activator protein-1 and signal transducers and activators of transcription, are involved in the NF-κB-independent pathway. Since protein tyrosine kinase plays an important role in the platelet-derived growth factor-induced proliferation and activation of hepatic stellate cells *in vitro*, genistein may have therapeutic potential against liver fibrosis (26).

The thiazolidinedione, pioglitazone, has been found to improve glucose intolerance, insulin resistance and dyslipidemia (27), and may inhibit the progression of atherosclerosis (28). We previously found that troglitazone directly inhibited cytokine-induced monocyte chemoattractant protein-1 expression in human mesangial (29) and endothelial cells (30). These findings suggested that a thiazolidinedione may inhibit atherosclerosis not only by improving glucose intolerance, insulin resistance and dyslipidemia, but also through pleiotropic effects. We have shown here that pioglitazone inhibited TNF-α-stimulated PAI-1 production in hepatocytes, similar to its effect in HUVECs (23). These effects are consistent with the clinical observation that plasma concentrations of PAI-1 in patients with type 2 diabetes are reduced after troglitazone administration (31). Thus, thiazolidinediones may inhibit the development of diabetes-associated macro- and microangiopathy by reducing hepatic fibrinolytic activity, as well as by inhibiting the development of NASH (32, 33).

Concerning the maximal effect of pioglitazone, pioglitazone at a dose above 10 μM seems to be pharmacological and out of range of treatment dose in a clinical setting (34). Most in vitro experiments in the previous reports were performed by using PPAR gamma agonist at a dose under 10 μM（23, 29, 30, 35）. As shown in Figure 6, pioglitazone at 10 μM did not abolish TNF-α-induced PAI-1 production completely. Actually, we observed additive inhibitory effects of SB203580, PD98059 and genistein with pioglitazone on TNF-α-induced PAI-1 production. TNF-α may induce PAI-1 via both PPAR-γ-dependent and –independent pathway.

Although the target signal transduction pathway of thiazolidinediones in the regulation of PAI-1 production in hepatocytes has not yet been clarified, we have shown here that the inhibitory effects of pioglitazone on PAI-1 production are not additive with calphostin C and emodin. These findings suggest that, in hepatocytes, pioglitazone inhibits TNF-α-induced PAI-1 production via pathways involving PKC and NF-κB. We previously observed that the ERK kinase and NF-κB pathways are also involved in the effects of troglitazone on PAI-1 production in HUVECs (23). In mesangial cells, troglitazone has been found to inhibit the activation of the diacylglycerol-PKC-ERK pathway by high glucose concentrations (36). Furthermore, in obese individuals, troglitazone therapy has been reported to reduce the intranuclear and total cellular amounts of NF-κB in mononuclear cells of obese individuals in whom plasma PAI-1 levels also decreased (37). Thus, the PKC and NF-κB pathways may be common targets of pioglitazone action in various tissues.

The statins, a class of HMG-CoA reductase inhibitors, are the most effective agents currently available for lowering serum concentrations of low density lipoprotein cholesterol. Statins have also been shown to inhibit the progression of atherosclerosis and cardiovascular disease, and there is increasing evidence that both thiazolidinediones and statins have pleiotropic effects on the pathogenesis of obesity-associated metabolic syndrome (19, 23). Statins have been shown to downregulate thrombin generation and the thrombotic process, as well as to up-regulate plasmin and thrombolytic processes (38). In addition, statins may prevent the TNF-α-induced secretion of PAI-1 and PAI-1 mRNA expression by human peritoneal mesothelial (39) and endothelial (40) cells. We have shown here that hepatocytes are also target cells in the statin inhibition of TNF-α-stimulated PAI-1 production. Our finding, that co-incubation of mevalonic acid with cerivastatin reversed the inhibitory effect of the latter, suggests that this statin down-regulates PAI-1 production through the mevalonic acid pathway.

We previously reported that statins prevent the development of sepsis in a mouse model by inhibiting the lipopolysaccharide-induced production of TNF-α, interleukin 1β and nitric oxide (41). We also found that cerivastatin inhibits the progression of diabetic nephropathy through the suppression of enhanced expression of extracellular matrix genes (19). All of these findings indicate that statins have pleiotropic effects and indicate that clinical investigations of statins in the prevention of atherosclerosis and/or NASH in patients with metabolic syndrome are important.

In conclusion, we have shown here that PAI-1 expression in the liver was higher in obese patients with type 2 diabetes than in non-obese patients, suggesting that the liver may be a major source of circulating PAI-1 in obese diabetics. We also found that TNF-α promotes the production of PAI-1 mRNA and protein in a normal human hepatocyte cell line through pathways involving PKC, p38, ERK, protein tyrosine kinase and NF-κB, and that thiazolidinediones and statins inhibited the TNF-α-induced PAI-1 production by hepatocytes. Our results suggest that thiazolidinediones and statins may inhibit the development of atherosclerosis and NASH in obese diabetic patients.

References

(1) Leyland H, Gentry J, Arthur MJ, et al: The plasminogen-activating system in hepatic stellate cells. Hepatology 24:1172-1178, 1996

(2) Pertovaara L, Kaipainen A, Mustonen T, et al: Vascular endothelial growth factor is induced in response to transforming growth factor-beta in fibroblastic and epithelial cells. J Biol Chem 269:6271-6274, 1994

(3) Larsson J, Goumans MJ, Sjostrand LJ, et al: Abnormal angiogenesis but intact hematopoietic potential in TGF-beta type I receptor-deficient mice. EMBO J 20:1663-1673, 2001

(4) Takamura T, Sakurai M, Ota T, et al: Genes for systemic vascular complications are differentially expressed in the livers of type 2 diabetic patients. Diabetologia 47:638-647, 2004

(5) Sakurai M, Takamura T, Akahori H, et al: A pathophysiological link between insulin resistance and fatty liver-associated metabolic syndrome. Diabetologia 2004;46:Suppl 2,A195 (Abstract).

(6) Shimomura I, Funahashi T, Takahashi M, et al: Enhanced expression of PAI-1 in visceral fat: possible contributor to vascular disease in obesity. Nat Med 2:800-803, 1996

(7) Alessi MC, Bastelica D, Mavri A, et al: Plasma PAI-1 levels are more strongly related to liver steatosis than to adipose tissue accumulation. Arterioscler Thromb Vasc Biol 23:1262-1268, 2003

(8) Festa A, D'Agostino R Jr, Tracy RP, et al: Elevated levels of acute-phase proteins and plasminogen activator inhibitor-1 predict the development of type 2 diabetes: the insulin resistance atherosclerosis study. Diabetes 51:1131-1137, 2002

(9) Chapman HA: Disorders of lung matrix remodeling. J Clin Invest 113:148-157, 2004

(10) Seki T, Gelehrter TD: Interleukin-1 induction of type-1 plasminogen activator inhibitor (PAI-1) gene expression in the mouse hepatocyte line, AML 12. J Cell Physiol 168:648-656, 1996

(11) Fearns C, Loskutoff DJ: Induction of plasminogen activator inhibitor 1 gene expression in murine liver by lipopolysaccharide. Cellular localization and role of endogenous tumor necrosis factor-alpha. Am J Pathol 150:579-590, 1997

(12) Hotamisligil GS, Shargill NS, Spiegelman BM: Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. Science 259:87-91, 1993

(13) Das UN: GLUT-4, tumour necrosis factor, essential fatty acids and daf-genes and

their role in glucose homeostasis, insulin resistance, non-insulin dependent diabetes mellitus, and longevity. J Assoc Physicians India 47:431-435, 1999

(14) Kanety H, Feinstein R, Papa MZ, et al: Tumor necrosis factor alpha-induced phosphorylation of insulin receptor substrate-1 (IRS-1). Possible mechanism for suppression of insulin-stimulated tyrosine phosphorylation of IRS-1. J Biol Chem 270:23780-23784, 1995

(15) Kahn BB, Flier JS: Obesity and insulin resistance. J Clin Invest 106:473-481, 2000

(16) Expert Committee on the Diagnosis and Classification of Diabetes Mellitus Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. Diabetes Care 25:S5-S20, 2003

(17) Reid AE: Nonalcoholic steatohepatitis. Gastroenterology 121:710-723, 2001

(18) Declerck PJ, Alessi MC, Verstreken M, et al: Measurement of plasminogen activator inhibitor 1 in biologic fluids with a murine monoclonal antibody-based enzyme-linked immunosorbent assay. Blood 71:220-225, 1988

(19) Ota T, Takamura T, Ando H, et al: Preventive effect of cerivastatin on diabetic nephropathy through suppression of glomerular macrophage recruitment in a rat model. Diabetologia 46:843-851, 2003

(20) Bastard JP, Jardel C, Bruckert E, et al: Elevated levels of interleukin 6 are reduced

in serum and ssubcutaneous adipose tissue of obese women after weight loss. J Clin Endocrinol Metab 85:3338-3342, 2000

(21) Bruun JM, Verdich C, Toubro S, et al: Association between measures of insulin sensitivity and circulating levels of interleukin-8, interleukin-6 and tumor necrosis factor-alpha. Effect of weight loss in obese men. Eur J Endocrinol 148:535-42, 2003

(22) Schleef RR, Bevilacqua MP, Sawdey M, et al: Cytokine activation of vascular endothelium. Effects on tissue-type plasminogen activator and type 1 plasminogen activator inhibitor. J Biol Chem 263:5797-5803, 1998

(23) Hamaguchi E, Takamura T, Shimizu A, et al: Tumor necrosis factor-alpha and troglitazone regulate plasminogen activator inhibitor type 1 production through extracellular signal-regulated kinase- and nuclear factor-κB-dependent pathways in cultured human umbilical vein endothelial cells. J Pharmacol Exp Ther 307:987-994, 2003

(24) Kawai HF, Kaneko S, Honda M, et al: Alpha-fetoprotein-producing hepatoma cell lines share common expression profiles of genes in various categories demonstrated by cDNA microarray analysis. Hepatology 33:676-691, 2001

(25) Sawdey MS, Loskutoff DJ: Regulation of murine type 1 plasminogen activator inhibitor gene expression in vivo. Tissue specificity and induction by

lipopolysaccharide, tumor necrosis factor-alpha, and transforming growth factor-beta. J Clin Invest 88:1346-1353, 1991

(26) Liu XJ, Yang L, Mao YQ, et al: Effects of the tyrosine protein kinase inhibitor genistein on the proliferation, activation of cultured rat hepatic stellate cells. World J Gastroenterol 8:739-745, 2002

(27) Mimura K, Umeda F, Hiramatsu S et al. Effects of a new oral hypoglycaemic agent (CS-045) on metabolic abnormalities and insulin resistance in type 2 diabetes. Diabet Med 11:685-691, 1994

(28) Dormandy JA, Charbonnel B, Eckland DJ et al: Secondary prevention of macrovascular events in patients with type 2 diabetes in the PROactive Study (PROspective pioglitAzone Clinical Trial In macroVascular Events): a randomised controlled trial. Lancet 366:1279-1289, 2005

(29) Ohta MY, Nagai Y, Takamura T, et al: Inhibitory effect of troglitazone on tumor necrosis factor alpha-induced expression of monocyte chemoattractant protein-1 in human mesangial cells. Metabolism 49:163-166, 2000

(30) Ohta MY, Nagai Y, Takamura T, et al: Inhibitory effect of troglitazone on TNF-α-induced expression of monocyte chemoattractant protein-1 (MCP-1) in human endothelial cells. Diabetes Res Clin Pract 48:171-176, 2000

(31) Fonseca VA, Reynolds T, Hemphill D, et al: Effect of troglitazone on fibrinolysis

and activated coagulation in patients with non-insulin-dependent diabetes mellitus. J Diabetes Complications 12:181-186, 1998

(32) Kawaguchi K, Sakaida I, Tsuchiya M, et al: Pioglitazone prevents hepatic steatosis, fibrosis, and enzyme-altered lesions in rat liver cirrhosis induced by a choline-deficient L-amino acid-defined diet. Biochem Biophys Res Commun 315:187-195, 2004

(33) Promrat K, Lutchman G, Uwaifo GI, et al: A pilot study of pioglitazone treatment for nonalcoholic steatohepatitis. Hepatology 39:188-196, 2004

(34) Kobayashi M, Iwanishi M, Egawa K, et al: Pioglitazone increases insulin sensitivity by activating insulin receptor kinase. Diabetes 41:476-483, 1992

(35) Iwata M, Haruta T, Usui I, et al: Pioglitazone ameliorates tumor necrosis factor-alpha-induced insulin resistance by a mechanism independent of adipogenic activity of peroxisome proliferator--activated receptor-gamma. Diabetes 50:1083-1092, 2001

(36) Isshiki K, Haneda M, Koya D, et al: Thiazolidinedione compounds ameliorate glomerular dysfunction independent of their insulin-sensitizing action in diabetic rats. Diabetes 49:1022-1032, 2000

(37) Ghanim H, Garg R, Aljada A, et al: Suppression of nuclear factor-kappaB and stimulation of inhibitor kappaB by troglitazone: evidence for an anti-inflammatory

effect and a potential antiatherosclerotic effect in the obese. J Clin Endocrinol Metab 86:1306-1312, 2001

(38) Fenton JW 2nd, Jeske WP, Catalfamo JL, et al: Statin drugs and dietary isoprenoids downregulate protein prenylation in signal transduction and are antithrombotic and prothrombolytic agents. Biochemistry (Mosc) 67:85-91, 2002

(39) Haslinger B, Kleemann R, Toet KH, et al: Simvastatin suppresses tissue factor expression and increases fibrinolytic activity in tumor necrosis factor-α-activated human peritoneal mesothelial cells. Kidney Int 63:2065-2074, 2003

(40) Swiatkowska M, Pawlowska Z, Szemraj J, et al: Cerivastatin, a HMG-CoA reductase inhibitor, reduces plasminogen activator inhibitor-1 (PAI-1) expression in endothelial cells by down-regulation of cellular signaling and the inhibition of PAI-1 promoter activity. Jpn J Pharmacol 90:337-344, 2002

(41) Ando H, Takamura T, Ota T, et al: Cerivastatin improves survival of mice with lipopolysaccharide-induced sepsis. J Pharmacol Exp Ther 294:1043-1046, 2000

Legends for figures

Fig. 1. Levels of PAI-1 and tPA gene transcripts by real-time quantitative PCR. The expression of each target sequence was normalized relative to that of β-actin. Each bar represents means ± SEM. (* $P < 0.05$; ** $P < 0.005$).

Fig. 2. Levels of TNF-α gene transcripts by real-time quantitative PCR. The expression of each target sequence was normalized relative to that of β-actin. Each bar represents means ± SEM. (* $P < 0.05$).

Fig. 3. Effect of TNF-α on PAI-1 secretion by THLE-5b cells. (A) Time course of TNF-α-induced PAI-1 secretion. THLE-5b cells were cultured with (filled bars) or without (open bars) 1.0 ng/ml TNF-α in 6-well dishes for 24 hours, and PAI-1 secretion was measured by ELISA. (B) Dose-dependency of TNF-α on PAI-1 secretion, as determined by ELISA. Data are expressed as means ± SEM ($n = 3$; * $P < 0.001$ vs. control).

Fig. 4. Effect of TNF-α on PAI-1 mRNA expression in THLE-5b cells. Time course of TNF-α (1.0ng/ml)-induced expression of PAI-1 (A), tPA (B) and tPA/PAI-1 (C) mRNA expression in THLE-5b cells. Expression of tPA and PAI-1 mRNA were quantified by real-time PCR and normalized relative to the expression of β-actin.

Data are expressed as means ± SEM (n = 3; * P < 0.05; ** P < 0.001 vs. control).

Fig. 5. (A) Effect of signal transduction inhibitors on TNF-α-induced PAI-1 secretion. Cells were incubated with Calphostin C, SB 203580, PD98059, genistein or emodin, and PAI-1 concentrations in the supernatants were measured by ELISA. All of these signal transduction inhibitors reduced TNF-α-induced PAI-1 production in a dose-dependent manner. Data are expressed as means ± SEM (n = 3; * P < 0.05; ** P < 0.01; *** P < 0.001 versus TNF-α alone). (B) Effect of signal transduction inhibitors on TNF-α-induced PAI-1mRNA contents in normal human hepatocytes evaluated by real-time PCR method. PAI-1 mRNA levels were normalized with an endogeneous control, β-actin mRNA. Data are expressed as means ± SEM (n = 3; * P < 0.05; ** P < 0.01; *** P < 0.001 versus TNF-α alone).

Fig. 6. Inhibitory effects of pioglitazone or cerivastatin on TNF-α-induced PAI-1 secretion. THLE-5b cells were cultured with or without pioglitazone (1 or 10 μM) or cerivastatin (0.1 or 1.0 μM) in the presence of 1.0 ng/ml TNF-α for 24 hours, and PAI-1 concentrations in the supernatants were measured by ELISA. Data are expressed as means ± SEM (n = 3; * P < 0.05; ** P < 0.001 versus TNF-α alone).

Fig. 7. Mevalonic acid reverses the inhibitory effect of cerivastatin on TNF-α-induced PAI-1 secretion. THLE-5b cells were cultured with cerivastatin (1.0 μM) with mevalonic acid (1.0 mM or 2.0 mM) for 24 hours, and PAI-1 contents in the supernatant were measured by ELISA. Data are expressed as means ± SEM (n = 3; * $P < 0.05$; ** $P < 0.001$ versus TNF-α +cerivastatin).

Fig. 8. Effect of signal transduction inhibitors on TNF-α-induced PAI-1 secretion in the presence or absence of pioglitazone. PAI-1 contents in the supernatant were measured by ELISA. SB203580 and genistein had significant additive inhibitory effects with pioglitazone, whereas Calphostin C, PD98059 and Emodin did not. Data are expressed as means ± SEM (n = 3; * $P < 0.05$; ** $P < 0.001$ versus TNF-α + pioglitazone)

Fig. 9. A summary figure for signaling pathways tested in this study. In hepatocytes, pioglitazone inhibits TNF-α-induced PAI-1 production via pathways involving PKC and NF-κB.

Table 1 Clinical characteristics of study

	Non-diabete		Diabete	
	Non-obesity (n = 6)	Obesity (n = 5)	Non-obesity (n = 12)	Obesity (n = 9)
Age (y)	43 ± 4	45 ± 7	55 ± 2[a]	50 ± 4
Sex (n: female/male)	3 / 3	2 / 3	3 / 9	3 / 6
BMI (kg/m^2)	22.7 ± 0.7	30.3 ± 1.3[b]	21.9 ± 0.4[d]	27.7 ± 1.3[a,f]
FPG (mg/dl)	93 ± 2	89 ± 5	152 ± 17[a]	131 ± 11[a,c]
HbA_{1c} (%)	5.2 ± 0.2	5.0 ± 0.2	7.5 ± 0.5[a]	7.0 ± 0.4[a,c]
HOMA-IR	2.0 ± 0.5	2.7 ± 0.6	1.8 ±0.2	4.0 ± 1.0
Triglyceride (mg/dl)	154 ± 46	132 ± 46	123 ± 27	140 ± 20
Total cholesterol (mg/dl)	212 ± 21	214 ± 26	202 ± 12	196 ± 6
HDL-cholesterol (mg/dl)	51 ± 7	44 ± 4	48 ± 4	45 ± 3
ALT (IU/L)	18 ± 7	40 ± 17[a]	24 ± 2	48 ± 11
PAI-1 (ng/ml)	18.6± 3.4	29.8 ± 9.3	35.8 ± 9.5[a]	41.6 ± 8.7[a]
TNF-α (pg/ml)	1.0± 0.2	1.3 ± 0.1	1.1± 0.1	1.5 ± 0.1[a,e]

Values are means ± SEM. ; a, $P < 0.05$, b, $P < 0.001$ versus Non-diabetes Non-obesity group ; c, $P < 0.05$, d, $P < 0.001$ versus Non-diabetes Obesity group ; e, $P < 0.05$, f, $P < 0.001$ versus Diabetes Non-obesity group ;