TNF-α and insulin, alone and synergistically, induce plasminogen activator inhibitor-1 expression in adipocytes

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Sakamoto, Tomohiro, J anet Woodcock-Mitchell, Kousuke Marutsuka, J ohn J. Mitchell, Burton E. Sobel, and Satoshi Fujii. TNF-α and insulin, alone and synergistically, induce plasminogen activator inhibitor-1 expression in adipocytes. Am. J. Physiol. 276 (Cell Physiol. 45): C1391–C1397, 1999.—Obesity is associated with hyperinsulinemia and elevated concentrations of tumor necrosis factor-α (TNF-α) in adipose tissue. TNF-α has been implicated as an inducer of the synthesis of plasminogen activator inhibitor-1 (PAI-1), the primary physiological inhibitor of fibrinolysis, mediated by plasminogen activators in cultured adipocytes. To identify mechanism(s) through which TNF-α induces PAI-1, 3T3-L1 preadipocytes were differentiated into adipocytes and exposed to TNF-α for 24 h. TNF-α selectively increased the synthesis of PAI-1 without increasing activity of plasminogen activators. Both superoxide (generated by xanthine oxidase plus hypoxanthine) and hydrogen peroxide were potent inducers of PAI-1, and hydroxyl radical scavengers completely abolished the TNF-α induction of PAI-1. Exposure of adipocytes to TNF-α or insulin alone over 5 days increased PAI-1 production. These agonists exert synergistic effects. Results obtained suggest that TNF-α stimulates PAI-1 production by adipocytes, an effect potentiated by insulin, and that adipocyte generation of reactive oxygen centered radicals mediates the induction of PAI-1 production by TNF-α. Because induction of PAI-1 by TNF-α is potentiated synergistically by insulin, both agonists appear likely to contribute to the impairment of fibrinolytic system capacity typical in obese, hyperinsulinemic patients.

Methods

Materials. 3T3-L1 mouse preadipocytes were obtained from the American Type Culture Collection. Penicillin, streptomycin, DMEM, trypsin, lipopolysaccharide (LPS; Escherichia coli O111:B4), and human insulin were purchased from Sigma Chemical (St. Louis, MO). Cosmic calf serum was from HyClone (Logan, UT), six-well culture plates were from Becton Dickinson Labware (Lincoln Park, NJ), human insulin ELISA kits were from Diagnostic Products (Los Angeles, CA), and recombinant mouse TNF-α and mouse TNF-α ELISA kits were from R&D Systems (Minneapolis, MN). Sheep anti-mouse PAI-1 IgG, rabbit anti-mouse u-PA antibody and anti-mouse t-PA antibody were acquired from American Diagnostica (Greenwich, CT). Western Exposure chemiluminescent detection system was from Clontech Laboratories (Palo Alto, CA). X-ray films were from Kodak (Rochester, NY), bicinchoninic acid (BCA) protein assay reagent was from Pierce (Rockford, IL), and kits to assay free fatty acids (Wako NEFA C) were from Biochemical Diagnostics (Edge-wood, NY). All chemicals were of the highest available commercial grade.

Cell culture procedures. Preadipocytes, incubated with DMEM containing 10% calf serum, 50 U/ml penicillin, and 50 μg/ml streptomycin, were grown to confluence on six-well plates and differentiated into adipocytes with dexamethasone and isobutylxanthine (ISBX) as described previously (20). In brief, confluent preadipocytes were exposed to dexamethasone (0.25 μM) and ISBX (0.5 mM) for 48 h. The cells were then placed in standard medium (without dexamethasone or ISBX), in which they accumulated small lipid droplets that grew to occupy a large fraction of total cell volume within 5 days. Seven days after initiation of differentiation (assessed by this criterion), 85–90% of the cells were judged to be differentiated.

Differentiated cells (7 days after exposure to dexamethasone) were incubated overnight in six-well plates in fresh DMEM containing 10% calf serum, 50 U/ml penicillin, and 50
μg/ml streptomycin, followed by incubation in fresh medium containing TNF-α for 24 h. In some experiments, the differentiated cells were incubated with TNF-α and/or insulin for 5 days. Media were collected and stored at −70°C.

Assays for PAI-1 activity, plasminogen activator activity by zymography, and total protein. PAI-1 activity was measured spectrophotometrically (20) and verified to be PAI-1 as judged by elimination of activity with inclusion of a PAI-1-neutralizing antibody in the medium. Samples were incubated with exogenous t-PA at room temperature for 10 min under conditions in which PAI-1 but not other low-affinity plasminogen activator inhibitors would bind to the t-PA. Subsequently, samples were acidified to eliminate nonspecific inhibition such that attributable to α2-antiplasmin activity. Residual t-PA activity was assayed by incubation with plasminogen and a chromogenic substrate, S-2251.

Total plasminogen activator activity was characterized by zymography as described previously (10). Aliquots of samples containing the same amount of total protein were subjected to electrophoresis in 9% polyacrylamide gels containing gelatin (1 mg/ml) and plasminogen (1 μg/ml). In some experiments, anti-t-PA or anti-u-PA antibodies were preincubated with the samples before zymography. After electrophoresis, the gels were washed in 2.5% Triton X-100 for 60 min and incubated in PBS for 12–16 h at 37°C. The gels were fixed in a mixture of methanol, acetic acid, and water (45:10:45) and stained with 0.5% Coomassie brilliant blue dye. Plasminogen activator activity was apparent as white zones. The molecular masses of proteins responsible for the activity were estimated with the use of molecular mass markers that were coelectrophoresed with the samples. The white-appearing zones, representing fibrinolytic activity in the gel, were semiquantified with the use of a densitometer (Bio-Rad model GS-700). The primary band at 35 kDa in control samples was defined as 100%, and percentage change in density with addition of agents was calculated with respect to that band as a reference. Total protein in conditioned media was assayed by the BCA method (Pierce).

Assay of PAI-1 protein. Concentrations of PAI-1 were determined by Western blotting with antibody specific for the antigen. Equivalent amounts of protein from conditioned media were diluted 1:1 with reduced sample buffer (0.5 mol/l Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, and 0.04% bromphenol blue), heated at 100°C for 5 min, cooled, and loaded on 10% polyacrylamide gels. Proteins were electrophoresed for 60 min and transferred to polyvinylidene difluoride membranes that were then blocked with 1% BSA and 0.1% Tween 20 in PBS (pH 7.4). Membranes were washed with 0.5% BSA and 0.1% Tween 20 in PBS several times and incubated with 1% BSA and 0.1% Tween 20 in PBS with 2 μg/ml sheep anti-mouse PAI-1 IgG containing 0.02% sodium azide for detection of PAI-1 by Western blotting. Membranes were washed with 0.5% BSA and 0.1% Tween 20 in PBS several times and incubated with alkaline phosphatase-conjugated goat anti-sheep IgG diluted 1:5,000 with 1% BSA and 0.1% Tween 20 in PBS containing 0.02% sodium azide. Membranes were incubated with chemiluminescent enhancer followed by 0.25 mM chemiluminescent substrate and exposed to X-ray film. Bands on developed film were quantified with the use of a densitometer (Bio-Rad model GS-700). Correlation between different doses of recombinant mouse PAI-1 and measured density on the X-ray films was linear up to 5 ng PAI-1 per lane.

Statistics. Data are expressed as means ± SE. Differences were assessed by ANOVA with Bonferroni least significant difference post hoc tests for comparisons within multiple groups. When appropriate, data were evaluated by ANOVA for repeated measures. Significance was defined as P < 0.05.

RESULTS

Effects of TNF-α on adipocyte PAI-1 activity. The proinflammatory cytokine TNF-α increased PAI-1 activity in the conditioned media [2.6 ± 1.0 arbitrary units (AU)/ml in control cultures compared with 8.5 ± 1.1 AU/ml with 10 ng/ml TNF-α; n = 7; Fig. 1A]. TNF-α increased PAI-1 protein accumulation (2.6 ± 0.2-fold over control with 10 ng/ml, n = 6; Fig. 1B). At 5 ng/ml TNF-α, there were no significant differences in PAI-1 protein or activity compared with control conditions in media lacking TNF-α. Accumulation of PAI-1 was evident within 6 h of exposure of cells to TNF-α, with further increases over 24 h. Cycloheximide (25 μg/ml) inhibited TNF-α-induced PAI-1 accumulation by 96 ± 10% (n = 7). LPS, in concentrations up to 100 μg/ml, did not induce PAI-1 accumulation (n = 3), indicating that endotoxin was not inducing PAI-1 in the adipocytes in vitro.

Effects of reactive oxygen species. The hydroxyl radical scavenger tetramethylthiourea (TMTU; 20 mM) almost completely inhibited both the increase in PAI-1 activity (n = 6; Fig. 2A) and the accumulation of PAI-1 protein induced by TNF-α (n = 6; Fig. 2B). Equimolar urea, used as a control, had no effect. Another hydroxyl
Fig. 2. Effects of tetramethylthiourea (TMTU; 20 mM) or urea (20 mM) as a control on TNF-α-induced (10 ng/ml) PAI-1 activity (A; n = 6) and PAI-1 protein (B; n = 6) in conditioned media of adipocytes. Confluent 3T3-L1-derived adipocytes were incubated in DMEM with 10% calf serum for 24 h and subsequently in fresh medium and serum containing TNF-α, TNF-α + TMTU, or TNF-α + urea for 24 h. PAI-1 activity and protein in medium were assayed as described in METHODS. Values are means ± SE. *P < 0.001 compared with control or 10 ng/ml TNF-α + 20 mM TMTU; †P < 0.05 compared with control; ‡P < 0.01 compared with 10 ng/ml TNF-α + 20 mM TMTU.

Fig. 3. Effects of 100 µM H2O2 and of 10 µM xanthine oxidase (XO) + 0.6 mM hypoxanthine (HX) on PAI-1 activity (A; n = 6) and PAI-1 protein (B; n = 6) in conditioned media of adipocytes. Confluent 3T3-L1-derived adipocytes were incubated in DMEM with 10% calf serum for 24 h and subsequently in fresh medium with serum with TNF-α, H2O2, or XO/HX for 24 h. PAI-1 activity and protein in media were assayed as described in METHODS. Values are means ± SE. *P < 0.001 compared with control, 10 ng/ml TNF-α, or 100 µM H2O2; †P < 0.001 compared with control; ‡P < 0.01 compared with control; ††P < 0.001 compared with control. A: control; B: control + TNF-α; C: control + H2O2; D: control + XO/HX; E: TNF-α + H2O2; F: TNF-α + XO/HX; G: H2O2 + XO/HX; H: TNF-α + H2O2 + XO/HX; I: TNF-α + H2O2 + XO/HX + TMTU; J: TNF-α + H2O2 + XO/HX + Urea.

radical scavenger, DMSO, similarly inhibited the increase of PAI-1 activity induced by TNF-α (1.5 ± 0.7 AU/ml in control media; 5.1 ± 0.6 with 10 ng/ml TNF-α; 2.2 ± 0.9 with 10 ng/ml TNF-α; 2.2 ± 0.9 with 10 ng/ml TNF-α plus 1.0% DMSO) and PAI-1 protein (100.0 ± 7.2% in control media; 186.3 ± 13.2 with 10 ng/ml TNF-α; 116.7 ± 3.5 with 10 ng/ml TNF-α plus 1.0% DMSO). When cells were incubated with hydrogen peroxide (100 µM) or a superoxide-generating system (10 µM xanthine oxidase plus 0.6 mM hypoxanthine), PAI-1 activity increased (12.3 ± 1.1 AU/ml with hydrogen peroxide and 21.1 ± 0.9 with xanthine oxidase plus hypoxanthine, n = 6; Fig. 3A), and PAI-1 protein accumulation increased as well (n = 6; Fig. 3B).

Effects of exposure of adipocytes to TNF-α, insulin, or both for 5 days. Exposure of adipocytes to TNF-α (5 and 10 ng/ml; n = 6; Fig. 4A) or insulin (20 and 100 nM; n = 6; Fig. 4B) in the media over 5 days increased production of PAI-1 protein. We also determined the levels of agent (TNF-α or insulin) left in the media during the experiment. After initial addition of 20 nM insulin to the media, levels of insulin in media had declined to 84 ± 5.3% by 24 h, to 56 ± 3.2% by 3 days, and to 33% by 5 days. After initial addition of 100 nM insulin to the media, the levels of insulin in media declined to 61.7 ± 9.9% by 3 days, and insulin levels in media remained the same at 5 days. For TNF-α added at 5 ng/ml, values of TNF-α in media increased somewhat after addition of the agent, indicating that the cells themselves may have been producing some TNF-α. After addition of 10 ng/ml TNF-α, values of TNF-α in media were 2,259 ± 269 pg/ml at 24 h, 2,937 ± 310 pg/ml at 3 days, and 2,472 ± 213 pg/ml at 5 days.

Exposure of cells to TNF-α (5 ng/ml) and insulin (20 nM) together increased PAI-1 production more than exposure to either agonist alone (n = 6; Fig. 5). The interaction between effects of insulin and TNF-α on PAI-1 was examined using combinations of individually submaximal concentrations of each. Effects with the combination of insulin and TNF-α exceeded those with each agonist alone in submaximal concentrations. Thus the interaction appeared to be synergistic. Higher concentrations of the individual agonists could not be used because of cytotoxicity. The effects of TNF-α (5 ng/ml) and insulin (20 nM) on PAI-1 production became apparent at 3 days and maximal at 5 days (n = 6; Fig. 6).

Total protein content in media was not altered by TNF-α or insulin alone or in combination. PAI-1 activity, as opposed to PAI-1 protein, did not increase in conditioned media, possibly because of spontaneous inactivation of PAI-1 in medium (19) or because of the
increase in the concentrations of plasminogen activators, counteracting PAI-1 activity.

Insulin (20 nM) and TNF-α (5 ng/ml) alone, as well as in combination, decreased plasminogen activator activity as shown by zymography (n = 5; Fig. 7). The major lytic zone observed at 38 kDa was decreased in media from cells exposed to TNF-α and insulin alone and in combination. It was diminished also by antibody to u-PA, suggesting that the primary plasminogen activator produced by the adipocytes was u-PA. The lytic band was also abrogated by use of amiloride (10 nM) in the overlying gel as a u-PA inhibitor. The minor lytic zone observed at 48 kDa was diminished by exposure of the cells to TNF-α and insulin alone. However, the combination increased this zone, as did antibody to u-PA. Although these results suggest that increased PA expression may have been responsible, the mechanism remains to be determined.

DISCUSSION

Results of this study suggest a mechanism that may contribute to exacerbation of cardiovascular disease in
gen activator activity was measured as described in METHODS. Values
medium and serum with TNF- 
DMEM with 10% calf serum for 24 h and subsequently in fresh
activity. Confluent 3T3-L1-derived adipocytes were incubated in

Second messengers involved in signal transduction pathways leading to induction of PAI-1 are not yet well characterized. Reactive oxygen intermediates, commonly produced by activated inflammatory cells (32), may function as autacoids (2). TNF-α can induce both superoxide and hydrogen peroxide production (24). In the present study, the hydroxyl radical scavengers TMTU and DMSO inhibited TNF-α-induced PAI-1 expression. Conversely, hydrogen peroxide as well as a superoxide-generating system induced PAI-1. Hydrogen peroxide is likely to have been rapidly depleted from the media. Accordingly, metabolites such as superoxide anion and hydroxyl radical may have been responsible for effects induced by exposure of the cells to agents and may have been the direct effector rather than hydrogen peroxide per se. Although both TNF-α and hydrogen peroxide induced PAI-1 production by adipocytes, TNF-α had a greater effect on PAI-1 protein relative to PAI-1 activity than did hydrogen peroxide. One explanation is that TNF-α can stimulate production of plasminogen activators as well and that this may have reduced apparent PAI-1 activity by neutralizing it.

Our results indicate that the expression of non-NADPH oxidase-dependent reactive oxygen species (3) may modify PAI-1 production by adipocytes and that TNF-α may act via reactive oxygen species that act as autacoids and may therefore contribute to impaired fibrinolysis associated with obesity. PAI-1, the primary inhibitor of t-PA and u-PA, can be induced by TNF-α, known to be increased in blood of obese subjects and those with insulin resistance (16). It appears likely that the TNF-α-induced expression of PAI-1 contributes to the exacerbation of vasculopathy in obese and insulin-resistant subjects (33).

We have shown previously that insulin and its precursors augment PAI-1 synthesis (22). The synergistic induction of PAI-1 by insulin and TNF-α demonstrated in this study is likely to impair fibrinolysis not only in obese patients but also in others with insulin resistance. Although mechanisms contributing to the apparent synergy have not yet been determined, TNF-α inhibits insulin signaling and induces insulin resistance (29). Therefore, our results suggest that TNF-α-induced adipocyte resistance to insulin will not obviate augmented adipocyte PAI-1 production. Indeed, they are consistent with the observation that PAI-1 expression in visceral fat is increased in obese rats and that

![Fig. 7. Effects of insulin and/or TNF-α on plasminogen activator activity. Confluent 3T3-L1-derived adipocytes were incubated in DMEM with 10% calf serum for 24 h and subsequently in fresh media, consistent with a lack of change in differentiation between control and agonist-treated cells. The effects of TNF-α on PAI-1 protein content were both time and concentration dependent, with a maximal effect at 10 ng/ml. A lag time of several hours was evident, suggesting that autocrine factor(s) may be involved.](http://ajpcell.physiology.org/)

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**Fig. 7.** Effects of insulin and/or TNF-α on plasminogen activator activity. Confluent 3T3-L1-derived adipocytes were incubated in DMEM with 10% calf serum for 24 h and subsequently in fresh media, consistent with a lack of change in differentiation between control and agonist-treated cells. The effects of TNF-α on PAI-1 protein content were both time and concentration dependent, with a maximal effect at 10 ng/ml. A lag time of several hours was evident, suggesting that autocrine factor(s) may be involved.

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The TNF-α-induced PAI-1 expression was diminished by cycloheximide, suggesting that the increase requires protein synthesis. It is possible that the state of differentiation of the cells affected rates of protein synthesis. Lipid breakdown is one of the effects of TNF-α on adipocytes. To determine whether the state of differentiation of the cells had changed with treatment, we assessed lipid droplet accumulation, which was comparable in cells exposed to the agonists or to control media, consistent with a lack of change in differentiation between control and agonist-treated cells.

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concentrations of PAI-1 in blood correlate closely with visceral fat mass in human subjects (28).

Insulin resistance has been implicated strongly in the pathogenesis of atherosclerosis (27). Obesity and insulin resistance are associated with endothelial cell dysfunction (29). The susceptibility of diabetic patients to vascular complications may be a function, in part, of an impaired endogenous antioxidant status (7). Our results suggest that antioxidants may limit PAI-1 induction otherwise associated with high concentrations of insulin and TNF-α in blood and in adipose tissue typical of insulin-resistant states.

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