TNF-α, but not IL-6, stimulates plasminogen activator inhibitor-1 expression in human subcutaneous adipose tissue

Peter Plomgaard, Pernille Keller, Charlotte Keller, and Bente Klarlund Pedersen

The Copenhagen Muscle Research Centre, and The Department of Infectious Diseases, Rigshospitalet, Denmark

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PLASMINOGEN ACTIVATOR INHIBITOR-1 (PAI-1), an inhibitor of fibrinolysis, is associated with cardiovascular disease and the metabolic syndrome (11, 16). The total amount of visceral adipose tissue is correlated with PAI-1 activity (14, 27), and weight reduction is associated with a decline in the expression of PAI-1 in the abdominal subcutaneous adipose tissue (24). Increased amounts of PAI-1 contribute to a prothrombotic state, which may promote atherogenesis and increase the risk of cardiovascular disease (5, 26). Of note, the thiazolidinediones reduce PAI-1 levels (53), which may improve cardiovascular disease outcomes.

The cytokines interleukin (IL)-6 and tumor necrosis factor (TNF)-α are produced by various cells, including the adipose tissue (6, 10, 13, 28) and macrophages (3, 30), and exert major regulatory effects on the acute-phase protein C-reactive protein (36). Overexpression of TNF-α occurs in the adipose tissue of both obese animals with insulin resistance (19) and obese humans (20). Adipose tissue secretes IL-6 (28), which is a primary regulator of the acute-phase response (36). TNF-α may stimulate PAI-1 secretion in human adipose tissue fragments (18), and circulating plasma IL-6 is associated with PAI-1 plasma levels (42). Increased levels of both TNF-α and IL-6 are observed in obese individuals (20), in Type 2 diabetes (29), and in patients with atherosclerosis (1). In two population-based studies, plasma concentrations of IL-6 predict all-cause mortality as well as cardiovascular mortality (17, 49).

Furthermore, plasma concentrations of IL-6 and TNF-α predict the risk of myocardial infarction in several studies (2, 15, 37). Recently, it was shown that the C-reactive protein level (which is induced by TNF-α and IL-6) is a stronger predictor of cardiovascular events than the LDL cholesterol level and that C-reactive protein adds prognostic information to that conveyed by the Framingham risk score (38).

The facts that TNF-α, IL-6, and PAI-1 are all associated with the metabolic syndrome (22, 52), that TNF-α induces PAI-1 expression in vitro, and that IL-6 is associated with elevated PAI-1 plasma levels (32) stimulated us to test the hypothesis that elevations of TNF-α and IL-6 were involved in the in vivo regulation of PAI-1 in human adipose tissue.

MATERIALS AND METHODS

Subjects. Twenty healthy men (mean ± SE: age 26 ± 1 yr, height 185 ± 1 cm, weight 83 ± 3 kg, body mass index 25 ± 1 kg/m²) participated in either an infusion of recombinant human (rh) TNF-α (n = 8), rhIL-6 (n = 6), or vehicle (n = 6). The groups did not differ with regard to age or body mass index. The study was approved by the Ethical Committee of the Copenhagen and Frederiksborg Communities, Denmark, and performed according to the Declaration of Helsinki. Subjects were informed about possible risks and discomfort before they gave their informed, written consent to participate. Subjects had no medical history, and physical examination revealed no abnormalities. The volunteers did not use any medication and did not have any febrile illness in the fortnight preceding the study. Furthermore, subjects abstained from heavy exercise 2 days before the experiments.

Protocol. On the day of experiment, subjects arrived at 0800 after an overnight fast.

Eight subjects were infused with rhTNF-α (Beronum) 700 ng·m⁻²·h⁻¹ (Boeringer-Ingelheim, Biberach an der Riss, Germany) for 3 h; six subjects were infused with rhIL-6 for 3 h with a constant infusion rate at 5 μg/h (Sandoz, Basle, Switzerland). The rhTNF-α and rhIL-6 were administered in 20% human albumin (Statens Serum Institut, Copenhagen, Denmark). The controls were infused with vehicle (20% human albumin) for 3 h.

Biopsies were obtained from abdominal subcutaneous adipose tissue before infusion, after 1, 2, and 3 h of infusion, and 2 h postinfusion. Biopsies were obtained using the Bergstrom cannula; the skin was anesthetized using Lidokain (20 mg/ml; Sygehus Apotekkerne Danmark, Copenhagen, Denmark). The anesthetic did not contain epinephrine to avoid unintended effects on gene expression. A 5-mm incision was made, and the cannula was introduced into the subcutaneous adipose tissue. Suction was applied, and 5–10 cuts were made.

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The biopsy was rinsed using physiological saline to remove blood contamination. The cleaned biopsy was quickly frozen in liquid nitrogen and stored at -80°C. Blood samples were collected before infusion and at 0.5, 1, 2, 3, 4, and 5 h after infusion. The blood was spun, and plasma was stored at -80°C. Subjects were permitted to consume only water during the experiment.

RNA extraction. Total RNA was extracted from ~100 mg of adipose tissue following the procedure for the TRIzol Reagent kit from In Vitrogen. The adipose tissue was homogenized using a Brinkman Polytron (version PT 2100), and a layer of triglycerides collected on the surface was removed. Chloroform was added, and tubes were spun to separate the phenol and aqueous phase. The aqueous phase was transferred to a new tube, and RNA was precipitated by adding isopropyl alcohol and centrifugation. The RNA pellet was washed in 75% ethanol and redissolved in 15 μl of diethyl pyrocarbonate-treated water. The amount of RNA was determined spectrophotometrically at 260 nm.

Reverse transcription. One microgram of RNA was reverse transcribed to single-stranded cDNA using TaqMan reverse-transcription reagents from Applied Biosystems. Random hexamers were used as primers, and the conditions were in agreement with the directions from the manufacturer.

Semi-quantitative real-time PCR. The expression of mRNA was measured using an ABI PRISM 7900HT Sequence Detection System from Applied Biosystem. 18S rRNA was used as a housekeeping reference gene. To determine the 18S rRNA and PAI-1 mRNA levels, a predeveloped primer limited assay from Applied Biosystems was used. The reagents used for the PCR reaction were all obtained from Applied Biosystems, and each sample was run in triplicate in a volume of 10 μl for 50 cycles using standard, real-time cycling conditions. The cycle threshold values were normalized to a relative standard curve run at the same plate as the samples. The standard curves confirmed an acceptable efficiency, and the ratio of amount of PAI-1 mRNA to amount of 18S rRNA was calculated (see Fig. 2).

Plasma analysis. TNF-α and IL-6 were measured using high sensitivity kits from R & D Systems (Minneapolis, MN). PAI-1 was measured using the Biopool kit from UMEÅ, and cortisol was measured using an enhanced immunoassay kit from Diagnostic Systems Laboratories. All samples were run in duplicates.

Statistics. All data were logarithmically transformed to obtain a normal distribution. The means are geometric means ± SE. Due to the nonlinear shape of the curves of plasma TNF-α, IL-6, and PAI-1 mRNA, the area under the curve was calculated, and an ANOVA was performed to evaluate the difference between the three groups. The analysis was performed using Excel 2000 from Microsoft. A repeated-measurement ANOVA was used to evaluate the effect of time and difference between groups. Systat version 8.0 for Windows (SPSS) was used for this analysis.

RESULTS

Plasma TNF-α and IL-6 levels in response to rhTNF-α and rhIL-6 infusions appear in Fig. 1. The concentration of plasma
TNF-α increased in response to rhTNF-α infusion, only reaching a level of ~20 pg/ml after 30 min (Fig. 1A). This level was stable until the cessation of the infusion, after which a steep decline was seen. Likewise, plasma concentrations of IL-6 increased only during the rhIL-6 infusion and reached a level of ~100 pg/ml (Fig. 1B). TNF-α and IL-6 concentrations did not change in the control trial. The subjects had unchanged temperature, blood pressure, and heart rate during the cytokine infusions (data not shown).

PAI-1 mRNA increased gradually during rhTNF-α infusion, reaching a maximal level of approximately ninefold \((P = 0.004)\) at the end of the infusion (Fig. 2). Two hours after cessation of rhTNF-α infusion, the level of PAI-1 mRNA had almost returned to baseline. Neither rhIL-6 infusion nor vehicle infusion had any effect on the level of PAI-1 mRNA.

PAI-1 plasma levels showed high interindividual variation. PAI-1 plasma levels declined with time. This decline was abolished by TNF-α infusion (2-way, repeated-measures, time \(\times\) group ANOVA; \(P = 0.001\)) but not by IL-6 (2-way, repeated-measures, time \(\times\) group ANOVA; \(P = \) not significant) (Fig. 3). Plasma cortisol levels increased during rhIL-6 infusion compared with placebo \((P = 0.000)\) but did not change during TNF-α infusion \((P = 0.24)\) (data not shown).

**DISCUSSION**

The present study demonstrated that supraphysiological concentrations of TNF-α induce PAI-1 mRNA expression in human adipose tissue in vivo and further induce a relative increase in the systemic levels of PAI-1. Thus the decline in plasma PAI-1, owing to circadian changes \((8, 43)\), was counteracted by TNF-α. The finding of a regulatory role of TNF-α on PAI-1 is in accordance with in vitro studies \((18, 31, 40)\). Circulating cortisol levels increased during the IL-6 infusion, as previously demonstrated \((44)\), but did not change in response to TNF-α administration. Therefore, cortisol does not seem to mediate the effect of TNF-α on PAI-1 expression.

Previous clinical studies find a correlation between plasma cytokines and PAI-1 \((45)\). However, because cytokines appear in cascades, it has not previously been possible to define the independent role of each cytokine.

PAI-1 is considered to be a risk marker in the metabolic syndrome \((22, 25, 34)\), but indications exist that PAI-1 is also directly involved in the pathogenesis of this disorder. Studies using genetic models of PAI-1 deletion \((41)\) strongly indicate that PAI-1 is involved in adipose tissue accumulation. PAI-1 knock-out mice were protected against high-fat diet, induced obesity, and insulin resistance compared with wild-type mice \((23)\). Recent observations indicate that the p75 TNF receptor plays a role in attenuating TNF-α-induced PAI-1 mRNA expression in acute inflammatory conditions \((31)\). The latter study is compatible with the fact that TNF-α is directly involved in the regulation of PAI-1 and is in accordance with the findings in the present study.

Both TNF-α and IL-6 are elevated in individuals with the metabolic syndrome \((22, 52)\) and in nonacute inflammatory conditions. The major source of these cytokines may be adipose tissue \((28)\). TNF-α is a strong inducer of IL-6 release from adipocytes \((39)\), and circulating levels of IL-6 are associated with markers of the metabolic syndrome \((12, 35)\). Given that TNF-α mainly works locally, TNF-α transcription may or may not be reflected in enhanced systemic levels of TNF-α. However, TNF-α may stimulate IL-6 production and consequently other inflammatory markers. Therefore, chronically elevated levels of IL-6 are likely to reflect local ongoing TNF-α production \((33)\).

The metabolic effect of IL-6 suggests, however, that IL-6 may not be the underlying cause of metabolic disturbances. Thus IL-6 knock-out mice developed late-onset obesity and impaired glucose tolerance \((50)\), and rhIL-6 infusion to healthy people induced lipolysis and fat oxidation \((47)\). In contrast, TNF-α has been shown to impair insulin-stimulated rates of glucose storage in cultured human muscle cells \((4)\) and to impair insulin-mediated glucose uptake in rats \((51)\). Also, obese mice with a gene knock-out of TNF-α are protected from insulin resistance \((46)\). In vitro studies suggest that TNF-α inhibits insulin signaling through inhibition of serine phosphorylation of insulin receptor substrate-1 \((21)\), suppressor of cytokine signaling 3 \((7)\), and STAT5b \((48)\). Data in the literature pointing to TNF-α as being a direct player in insulin resistance are therefore accumulating, whereas IL-6 may have the opposite effect \((9, 50)\). The conclusion from the present data is limited to the acute effect of TNF-α in lean healthy humans. However, the fact that TNF-α, and not IL-6, induces PAI-1 expression may support the theory that TNF-α, rather than IL-6, is the actual “driver” behind the metabolic syndrome.

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