Tumor Necrosis Factor-α Stimulates Lipolysis in Differentiated Human Adipocytes Through Activation of Extracellular Signal–Related Kinase and Elevation of Intracellular cAMP

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Tumor necrosis factor-α (TNF-α) stimulates lipolysis in human adipocytes. However, the mechanisms regulating this process are largely unknown. We demonstrate that TNF-α increases lipolysis in differentiated human adipocytes by activation of mitogen-activated protein kinase kinase (MEK), extracellular signal–related kinase (ERK), and elevation of intracellular cAMP. TNF-α activated ERK and increased lipolysis; these effects were inhibited by two specific MEK inhibitors, PD98059 and U0126. TNF-α treatment caused an electrophoretic shift of perilipin from 65 to 67 kDa, consistent with perilipin hyperphosphorylation by activated cAMP-dependent protein kinase A (PKA). Coincubation with TNF-α and MEK inhibitors caused perilipin to migrate as a single 65-kDa band. Consistent with the hypothesis that TNF-α induces perilipin hyperphosphorylation by activating PKA, TNF-α increased intracellular cAMP ~1.7-fold, and the increase was abrogated by PD98059. Furthermore, H89, a specific PKA inhibitor, blocked TNF-α–induced lipolysis and the electrophoretic shift of perilipin, suggesting a role for PKA in TNF-α–induced lipolysis. Finally, TNF-α decreased the expression of cyclic-nucleotide phosphodiesterase 3B (PDE3B) by ~50%, delineating a mechanism by which TNF-α could increase intracellular cAMP. Cotreatment with PD98059 restored PDE3B expression. These studies suggest that in human adipocytes, TNF-α stimulates lipolysis through activation of MEK-ERK and subsequent increase in intracellular cAMP. Diabetes 51: 2929–2935, 2002

Obesity and type 2 diabetes are associated with increased concentrations of circulating free fatty acids (FFAs) that are thought to elicit systemic insulin resistance (1). The flux of FFA is primarily dependent on lipolysis of triacylglycerol in adipocytes. Tumor necrosis factor-α (TNF-α) is known to increase adipocyte lipolysis (2–6), and its expression in adipose tissue of obese subjects is increased (7–10). This increased expression of TNF-α may enhance adipocyte lipolysis and hence increase concentrations of circulating FFAs. Indeed, adipose tissue interstitial levels of TNF-α and FFA are positively correlated (11). Infusion of TNF-α in rodents (12) and humans (13,14) results in increased plasma FFAs and systemic insulin resistance. TNF-α knockout mice exhibit lower circulating FFAs and are protected from insulin resistance of obesity (15). Furthermore, rosiglitazone, an antidiabetic agent, blocks the lipolytic effect of TNF-α in 3T3-L1 adipocytes (16), delineating a mechanism by which rosiglitazone may improve insulin resistance. In light of the accumulating data linking adipocyte lipolysis to insulin resistance, it is physiologically relevant to investigate the mechanism(s) by which TNF-α stimulates lipolysis, which to date is largely unknown.

TNF-α is a potent activator of mitogen-activated protein kinases (MAPKs), including extracellular signal–related kinase (ERK)-1 and -2 (p42/p44), c-Jun NH2-terminal kinase, and p38 kinase (17). Activation of the MAPK pathway affects many physiological processes. Of particular interest, the mitogen-activated protein kinase kinase (MEK) 1/2-ERK1/2 pathway has recently been shown to mediate TNF-α–induced serine phosphorylation of insulin receptor substrate-1, another possible mechanism by which TNF-α induces insulin resistance (18). Moreover, the MEK1/2-ERK1/2 pathway has recently been shown to regulate lipolysis in 3T3-L1 adipocytes by phosphorylating hormone-sensitive lipase (HSL), a rate-limiting enzyme in hormone-regulated lipolysis (19). We hypothesize that the MEK1/2-ERK1/2 pathway also regulates TNF-α–induced lipolysis in human adipocytes.

Perilipins are phosphophoproteins that are located at the surface of lipid droplets in adipocytes and may act as a barrier to reduce the access of lipase(s) to intracellular lipids (20–25). Phosphorylation of perilipins by cAMP-
dependent protein kinase (PKA) allows access of lipase(s) to the droplets by a modification of lipid surfaces, resulting in lipolysis (23,24,26,27). Activation of PKA has been associated with TNF-α–induced lipolysis in 3T3-L1 adipocytes and the mechanism may involve TNF-α–induced downregulation of cyclic-nucleotide phosphodiesterase 3B (PDE3B), an enzyme that hydrolyzes cAMP, which leads to increased intracellular cAMP (28). We hypothesize that TNF-α stimulates lipolysis in human adipocytes through a similar cAMP/PKA mechanism.

In this paper, we investigated the mechanisms by which TNF-α stimulates lipolysis in human fat cells. As a cell culture system, we used differentiat ed human adipocytes that offer prolonged survival compared with mature, freshly isolated adipocytes. First, we examined the potential role of the MEK2/ERK1/2 pathway. We then examined the involvement of perilipin A, the predominant form of the perilipins in human adipocytes. Finally, we studied the association of intracellular cAMP, PDE3B, and PKA with TNF-α–induced lipolysis.

RESEARCH DESIGN AND METHODS

Chemicals and reagents. All chemicals were from Sigma Chemical (St. Louis, MO) unless otherwise indicated. BSA (fraction V) was from Intergen (Purchase, NY), fetal bovine serum (FBS) was from Hyclone (Logan, UT), PD098059 was from Calbiochem (San Diego, CA), and U0126 was from Promega (Madison, WI). Tissue culture reagents were purchased from Gibco-CORBRL (Rockville, MD) and Hyclone; type I collagenase was from Worthington Biochemical (Lakewood, NJ); enhanced chemiluminescence (ECL) Western blotting detection reagents, horseradish-linked anti-rabbit and antimouse IgG were from Amersham Life Sciences (Arlington Heights, IL); concentrations were measured using an enzyme immunoassay kit from Amersham Pharmacia Biotech. Results were corrected for cellular protein content and expressed as femtomoles of cAMP per milligrams of protein.

Isolation of membrane fractions. After 24 h treatment, plasma membranes were prepared as described (31). Equal amounts of proteins were used for Western analyses of G proteins and PDE3B.

Statistical analysis. Results were expressed as means ± SE. One-way ANOVA was used for statistical analyses. P values <0.05 were considered statistically significant.

RESULTS

All experiments were performed with differentiated human adipocytes. After 2–3 weeks in culture, >80% of the preadipocytes were differentiated as assessed by microscopic lipid accumulation.

TNF-α stimulates lipolysis in differentiated human adipocytes. To investigate the mechanism(s) by which TNF-α regulates lipolysis in human adipocytes, we first examined the concentration dependency of TNF-α on lipolysis. TNF-α increased lipolysis in a dose-dependent manner beginning at 10 ng/ml. The lipolytic effect was evident only after 6 h, suggesting that this effect does not occur via a direct activation of the lipolytic machinery (Fig. 1). This finding is consistent with that of Hauner et al. (4) in human adipocytes. At 100 ng/ml, TNF-α (at 24 h) increased lipolysis by ~1.7-fold. Because of the potentially high concentrations of adipose-derived TNF-α in the adipose interstitial fluid in obese subjects (7–10), we used 100 ng/ml in subsequent experiments.

TNF-α activates ERK1/2. As a first step to determine whether the MAPK pathway is involved in TNF-α–mediated lipolysis, the time course of the effects of TNF-α on ERK1/2 activation was examined. TNF-α treatment increased ERK1/2 phosphorylation, whereas it did not affect the expression of total ERK (Fig. 2). The increase was maximal at 15–30 min, and the effect remained detectable for 24 h.

MEK1/2 inhibitors PD98059 and U0126 inhibit TNF-α–induced lipolysis. ERK1/2 activity is regulated by the upstream kinase MEK1/2, which phosphorylates ERK1/2 at threonine 183 and tyrosine 185. MEK1/2 is known to be essential for differentiation of adipocytes. After TNF-α treatment, we examined the effect of MEK1/2 inhibitors. PD98059 and U0126 each inhibited TNF-α–induced lipolysis in a dose-dependent manner. At 1 μM, PD98059 and U0126 inhibited lipolysis by ~60 and 45%, respectively. At 10 μM, PD98059 and U0126 inhibited lipolysis by ~90 and ~75%, respectively.

Conclusions. TNF-α, a pro-inflammatory cytokine, induces lipolysis in human adipocytes. This effect is mediated by increased intracellular cAMP, which activates PKA. The mechanism involves the MEK2/ERK1/2 pathway and the downregulation of PDE3B. Perilipin A, a key protein that regulates lipolysis, is involved in this process. These findings provide insights into the mechanisms of lipolysis in human adipocytes and may have implications for the treatment of obesity and related metabolic disorders.
on threonine and tyrosine residues (32,33). To study the possible role of the ERK1/2 pathway in lipolysis, two specific MEK1/2 inhibitors, PD98059 (34) and U0126 (35), were used to block activation of the MEK/ERK pathway. PD98059 inhibits MEK activation by binding to inactive MEK and preventing the phosphorylation by upstream kinases such as Raf-1. U0126 inhibits the catalytic activity of the active enzyme MEK1/2. As shown in Fig. 3A, treatment with PD98059 (6 and 24 h) inhibited TNF-α–induced and basal ERK1/2 phosphorylation without altering total ERK protein expression. Concomitant with its inhibitory effect on ERK activation, PD98059 treatment decreased lipolysis in TNF-α–treated cells to below control levels, and in non–TNF-α–treated cells (PD98059 alone), to ~50% of control (Fig. 3B).

Treatment with U0126 resulted in more potent inhibitory effects on ERK1/2 phosphorylation and lipolysis. It decreased lipolysis in both TNF-α–treated and non–TNF-α–treated cells (U0126 alone) to 9.4 and 13.6% of control, respectively (Fig. 3C) and reduced ERK1/2 phosphorylation to an undetectable level, as assessed by Western blotting (Fig. 3D).

**MEK1/2 inhibitors block the TNF-α–induced electrophoretic shift of perilipin and decrease the TNF-α–induced increase in intracellular cAMP.** TNF-α has been shown to induce lipolysis, in part, by decreasing perilipin expression in 3T3-L1 adipocytes (6,23). To determine whether TNF-α stimulates lipolysis in human adipocytes by a similar mechanism, Western blotting was performed. As shown in Fig. 4A and B, TNF-α treatment did not significantly decrease perilipin expression but caused an electrophoretic shift of perilipin from 65 to 67 kDa, consistent with PKA activation and hyperphosphorylation of perilipin. Coincubation with PD98059 or U0126 diminished this effect of TNF-α and resulted in perilipin migrating as a single 65-kDa band. These results led us to hypothesize that the MEK inhibitors PD98059 and U0126 inhibit TNF-α–induced lipolysis, at least in part, by inhibiting hyperphosphorylation of perilipin.

The rate of lipolysis in adipocytes, in general, is critically dependent on the intracellular concentration of cAMP. To examine the possibility that TNF-α–induced lipolysis could involve the regulation of intracellular cAMP, differentiated human adipocytes were treated in the absence or presence of TNF-α for 24 h. TNF-α treatment resulted in a ~1.6-fold increase in intracellular cAMP; incubation with PD98059 abolished this effect of TNF-α while decreasing basal intracellular cAMP by ~26% (Fig. 5A). These effects of TNF-α and PD98059 were consistent with their effects on lipolysis, i.e., stimulation by TNF-α and inhibition by PD98059.

**The PKA inhibitor, H89, blocks TNF-α–induced lipolysis and hyperphosphorylation of perilipin.** To further verify whether TNF-α–stimulated lipolysis is mediated by activation of cAMP-dependent PKA, cells were incubated in the presence or absence of TNF-α and the PKA inhibitor H89 (20 μmol/l) for 24 h. Treatment with H89 abrogated TNF-α–induced lipolysis (Fig. 5B), and in non–TNF-α–treated cells (H89 alone), it reduced lipolysis to ~50% of control. Concomitant with this effect, H89 reversed the TNF-α–induced electrophoretic shift of perilipin and caused it to migrate as a single 65-kDa perilipin band, suggesting that this protein was not hyperphosphorylated by PKA in the presence of H89 (Fig. 5C).

**TNF-α decreases expression of PDE3B.** In adipocytes, intracellular concentration of cAMP is determined by its synthesis controlled by adenylyl cyclase and its degradation, regulated by the hydrolytic activity of cyclic nucleotide phosphodiesterases (PDEs), especially PDE3B. To determine the mechanism(s) by which TNF-α increases intracellular cAMP in differentiated human adipocytes, Western analyses of G protein expression were performed. In rat adipocytes, TNF-α has been suggested to stimulate lipolysis by decreasing the expression of G_{i2}, which inhibits adenylyl cyclase and, hence, the production of cAMP (31). Treatment of differentiated human adipocytes with TNF-α, however, did not significantly decrease expression of G_{i1}, G_{i2}, and G_{i3}, nor did it have significant effect on G_{s} expression (data not shown). We next examined the expression of PDE3B by Western analyses using G_{s} as a loading control and both ECL- and [125I]-protein A–based detection systems (Fig. 6). Densitometry using [125I]-protein A detection indicates that TNF-α treatment for 24 h decreased expression of PDE3B by ~50%. Cotreatment with PD98059 abrogated this decrease, and treatment with PD98059 alone increased PDE3B by ~30%. Thus, the TNF-α–induced increase in intracellular cAMP is likely to be mediated by downregulation of PDE3B expression, and the regulation is downstream of ERK1/2 activation.

**DISCUSSION**

In the present study, we show that TNF-α–increased lipolysis in human adipocytes correlates with ERK activation.
The data are the first demonstration to link TNF-α activation of ERK to stimulation of PKA, resulting in increased adipocyte lipolysis.

The MEK1/2 inhibitors PD98059 and U0126 inhibited TNF-α–induced lipolysis. The reduction in lipolysis paralleled the reduction in ERK1/2 activation. In addition, PD98059 and U0126 reduced basal ERK1/2 activation and basal lipolysis, indicating that MEK1/2-ERK1/2 regulate TNF-α–induced as well as basal lipolysis in these cells. In many cells, activation of ERK triggers its translocation into the nucleus, where it phosphorylates and activates transcription factors (36). In turn, these activated transcription factors regulate the expression of specific genes. For example, the ERK pathway has been shown to mediate phosphorylation of peroxisome proliferator–activated receptor-γ, a major transcription factor in adipocytes, and reduce its transcriptional activity (37). In our study, TNF-α required 6 h to stimulate lipolysis, but the peak of ERK activation occurred within 15–30 min. The temporal discrepancy suggests that the mechanism of ERK1/2 action is more complicated than direct phosphorylation of HSL (19) and that alterations in gene and protein expression may be involved. Concomitant with the increase in lipolysis, TNF-α decreased PDE3B protein expression and increased intracellular cAMP. Both effects were restored by cotreatment with the MEK1/2 inhibitor PD98059, indicating that TNF-α may decrease PDE3B expression and thereby increase cAMP concentrations and lipolysis. We propose that TNF-α increases ERK activation within 15–30 min, which subsequently results in the alteration of gene

Results are expressed as percentages of control and are means ± SE of three individual experiments. Significantly different from untreated control: *P < 0.05, **P < 0.001. Significantly different from TNF-α–treated sample: ***P < 0.001.

C: Effect of U0126 on lipolysis. Results are means ± SE of three individual experiments. Significantly different from untreated control: *P < 0.001. Significantly different from TNF-α–treated sample: **P < 0.001. D: Effect of U0126 on ERK1/2 activation.
and protein expression over the course of hours, leading to decreased PDE3B protein expression, increased cAMP levels, and ultimately increased lipolysis. Our hypothesis is supported by previous observations that in 3T3-L1 adipocytes, treatment of 3T3-L1 adipocytes with TNF-α for 24 h reduced PDE3B mRNA and protein expression and concurrently increased lipolysis (28).

TNF-α has been shown to stimulate lipolysis in isolated rat adipocytes by decreasing the expression of G_i protein (31). In our present study, we did not observe a significant effect of TNF-α on expression of immunoreactive G_i (data not shown). The discrepancy between the two studies may be due to the differential effects of TNF-α in rat versus human adipocytes.

Increases in intracellular cAMP and resulting activation of PKA are the major pathway by which lipolytic agents stimulate lipolysis, which occurs at the surface of the intracellular lipid droplets where perilipins are specifically located (21,38). In our study, treatment with TNF-α resulted in an increase in the molecular weight of perilipin in SDS-PAGE, as evidenced by the electrophoretic shift from 65 to 67 kDa. The increase in perilipin migration was consistent and potent cAMP-dependent PKA inhibitor, inhibited TNF-α-induced lipolysis and reversed the TNF-α-induced electrophoretic shift of perilipin, causing it to migrate as a 65-kDa band. In view of the fact that hyperphosphorylation of perilipin by PKA facilitates lipase-mediated lipolysis (24), we speculate that in human adipocytes, TNF-α increases intracellular cAMP levels and PKA activation, resulting in hyperphosphorylation of perilipin and ultimately increased lipolysis. Activated PKA is known to simultaneously phosphorylate perilipin A, and the major lipase in adipocytes, HSL (39–43). Whether, the TNF-α–induced increase of intracellular cAMP in our study leads to phosphorylation of HSL awaits further study.

The TNF-α–induced electrophoretic shift of perilipin and stimulation of lipolysis were abrogated by PD98059 and U0126, suggesting that regulation of the two events is downstream of the activation of the ERK1/2-MEK1/2 pathway. In contrast to the finding of Souza and colleagues (6,23), that TNF-α increased lipolysis in 3T3-L1 adipocytes by decreasing perilipin expression, we did not observe significant reduction of perilipin by TNF-α. The difference between the two studies may reflect the differential regulatory effects of TNF-α on perilipin(s) in the two cell types, with downregulation of perilipin expression in 3T3-L1 adipocytes and the hyperphosphorylation of perilipin in human adipocytes. Both effects can result in reduced perilipin coating of the lipid droplet, leading to increased access of lipase(s) to the lipid droplet surface and increased lipolysis.

Based on our findings, we suggest a model for TNF-α–stimulated lipolysis in human adipocytes, which includes the activation of the MEK1/2-ERK1/2 pathway, resulting in downregulation of PDE3B expression with a subsequent increase in intracellular cAMP and activation of PKA, which hyperphosphorylates perilipin, leading to a modification of the lipid droplet surface and allowing lipase(s) to hydrolyze triacylglycerol. The model may represent only one of the pathways by which TNF-α stimulates lipolysis in human adipocytes, but certainly in our cell culture system, this is a major mechanism.

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REFERENCES
7. Hotamisligil GS, Arner P, Caro JF, Varsa K, Spiegelman BS: Adipose tissue tumor necrosis factor α promotes tissue and Dr. Martin Obin for critically reading the manuscript.

FIG. 6. Effect of TNF-α on PDE3B expression. Differentiated human adipocytes were incubated with or without TNF-α in the presence or absence of PD98059 for 24 h. Isolated plasma membranes were immuno-blotted for PDE3B and as a loading control, for Gαs. Protein expression was detected by ECL. Data are representative of three independent experiments.