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TNF-α Induction of Lipolysis Is Mediated Through Activation of the Extracellular Signal Related Kinase Pathway in 3T3-L1 Adipocytes

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Abstract Tumor necrosis factor-α (TNF-α) increases adipocyte lipolysis after 6–12 h of incubation. TNF-α has been demonstrated to activate mitogen-activated protein (MAP) kinases including extracellular signal-related kinase (ERK) and N-terminal-c-Jun-kinase (JNK) in different cell types. To determine if the MAP kinases have a role in TNF-α-induced lipolysis, 3T3-L1 adipocytes were treated with the cytokine (10 ng/ml), in the presence or absence of PD98059 or U0126 (100 μM), specific inhibitors of ERK activity. We demonstrated that U0126 or PD98059 blocked TNF-α-induced ERK activity and decreased TNF-α-induced lipolysis by 65 or 76% respectively. The peroxisome-proliferator-activated receptor γ (PPARγ) agonists, rosiglitazone (ros), and 15-deoxy-Δ-^{12,14}- prostaglandin J_2 (PGJ2) have been demonstrated to block TNF-α-induced lipolysis. Pretreatment of adipocytes with these agents almost totally blocked TNF-α-induced ERK activation and reduced lipolysis by greater than 90%. TNF-α also stimulated JNK activity, which was not affected by PD98059 or PPARγ agonist treatment. The expression of perilipin, previously proposed to contribute to the mechanism of lipolysis, is diminished in response to TNF-α treatment. Pretreatment of adipocytes with PD98059 or ros significantly blocked the TNF-α-induced reduction of perilipin A protein level as determined by Western analysis. These data suggest that activation of the ERK pathway is an early event in the mechanism of TNF-α-induced lipolysis. J. Cell. Biochem. 89: 1077–1086, 2003.

Key words: perilipin; map kinases; PPARγ

Tumor necrosis factor-alpha (TNF-α) is a pleiotropic cytokine, which at the cellular level has been shown to regulate differentiation, cell proliferation, and apoptosis [Fiers, 1991; Vilcek and Lee, 1991; Bazzoni and Beutler, 1996]. TNF-α has also been linked to diabetes and the insulin resistance of obesity with the finding that adipose tissue and adipocytes from obese

Abbreviations used: TNF-α, tumor necrosis factor-alpha; MAP, mitogen-activated protein; ERK, extracellular signal-related kinase; JNK, c-Jun N-terminal kinase; PPARγ, peroxisome proliferator-activated receptor γ; ros, rosiglitazone; PGJ2, 15-deoxy-Δ-^{12,14}-prostaglandin J_2; FFA, free fatty acids; IP, immune complex kinase; MEK-1, mitogen-activated protein kinase kinase-1; IB, immunoblots; PKA, cyclic-AMP dependent protein kinase.

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subjects have elevated TNF-α production [Hotamisligil et al., 1993, 1995; Kern et al., 1995]. The mechanism for TNF-α function in obesity is not completely understood. Several laboratories have demonstrated that TNF-α increases adipocyte lipolysis and release of free fatty acids (FFA) [Kawakami et al., 1987; Feingold et al., 1992; Green et al., 1994; Hauner et al., 1995]. Additionally, it has been found that adipose tissue interstitial levels of TNF-α and FFA are positively correlated [Orban et al., 1999]. Importantly, in studies of animals and humans, chronic elevations of plasma FFAs have been suggested to promote insulin resistance, which is the hallmark of type II diabetes [Boden, 1997].

The mechanism(s) by which TNF-α affects lipolysis are not completely understood. Unlike catecholamines, which stimulate lipolysis within minutes, TNF-α increases lipolysis in cultured cells only after hours (>6 h) and is maximal by 12–24 h [Kawakami et al., 1987; Feingold et al., 1992; Green et al., 1994; Hauner et al., 1995]. Concurrent with an increase in lipolysis, TNF-α treatment results in decreased expression of the perilipins [Rosenbaum and Greenberg, 1998; Souza et al., 1998]. Perilipins are phosphoproteins located exclusively at the surface of the intracellular lipid droplets (LDs) in adipocytes and steroidogenic cells [Greenberg et al., 1991; Blanchette-Mackie et al., 1995; Servetnick et al., 1995]. Perilipins located around the LD have been proposed to act as a barrier to lipolysis [Londos, 1996; Souza et al., 1998; Londos et al., 1999]. Constitutive overexpression of perilipins by adenovirus infection of 3T3-L1 adipocytes blocks the ability of TNF-α to increase lipolysis [Souza et al., 1998]. Thus, regulation of perilipin expression may contribute to the mechanism of lipolysis induced by TNF-α.

The intracellular signaling mechanisms of TNF-α are complex and highly cell-type dependent, resulting in the multiple cell fates described earlier [Fiers, 1991; Vilcek and Lee, 1991; Bazzoni and Beutler, 1996]. Furthermore, the signaling mechanisms important in TNF-α regulation of adipocyte metabolism in 3T3-L1 adipocytes are completely unknown. TNF-α is a potent activator of multiple mitogen-activated protein (MAP) kinase cascades, including ERK, extracellular signal-regulated kinase, JNK, c-Jun N-terminal kinase, and p38 kinase [Wallach et al., 1999]. Activation of the MAP kinase pathways results in phosphorylation and activation of multiple transcription factor targets [Robinson and Cobb, 1997]. Interestingly, the transcription factor, peroxisome-proliferator-activated receptor γ (PPARγ), which is required for preadipocyte differentiation and induction of lipid accumulation [Spiegelman, 1998], has been shown to be phosphorylated by both ERK and JNK MAP kinases [Hu et al., 1996; Zhang et al., 1996; Adams et al., 1997]. Furthermore, phosphorylation of PPARγ by ERK and JNK blocks PPARγ-induced transcriptional activation [Hu et al., 1996; Adams et al., 1997; Camp and Tafuir, 1997]. Introduction of a phosphorylation deficient mutant PPARγ dramatically upregulates adipocyte differentiation and lipid accumulation relative to wild-type PPARγ [Hu et al., 1996; Zhang et al., 1996; Adams et al., 1997]. These data suggest that MAP kinase-induced phosphorylation of PPARγ is a negative regulator of lipid accumulation. We, therefore, hypothesized that one or more of the MAP kinases are a positive regulator of lipolysis.

EXPERIMENTAL PROCEDURES

Materials

Recombinant murine TNF-α was purchased from Genzyme (Cambridge, MA) and 3T3-L1 cells from the American Type Culture Collection (Rockville, MD). PD 98059 was purchased from Calbiochem (San Diego, CA). U0126 was purchased from Promega (Madison, WI). Myelin basic protein (MBP) and others chemicals were from Sigma Chemical Co (St. Louis, MO). Tissue culture reagents were purchased from Gibco BRL (Carlsbad, CA) and Hyclone (Logan, UT). Adenosine 5′-(γ-^{32}P) triphosphate tetra (triethyl-ammonium) salt from Dupont NEN (Boston, MA). Rosiglitazone (ros) was kindly provided by SmithKline Beecham (King of Prussia, PA) (Hamish Ross) and 15-delta^{12,14} prostaglandin J_2 (PGJ2) from Cayman Chemical (Ann Arbor, MI).

Cell Culture

3T3-L1 fibroblasts placed in 12-well plates or 100 mm dishes were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% bovine calf serum and differentiated into adipocytes using standard protocols [Kohanski et al., 1986]. Experiments were performed 10–15 days after start of the differentiation program. Adipocytes were serum-deprived overnight in

DMEM with 2% BSA. The following morning, cells were treated as described in the results.

Lipolysis

Glycerol content of the incubation medium was determined using a colorimetric assay (GPO-Trinder, Sigma). Protein content was determined using the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL).

Western Analysis

Adipocytes were rinsed briefly with 1 ml of phosphate buffered saline (PBS). Proteins were extracted as previously described [Souza et al., 1998], separated by 10% SDS–PAGE, and electrophoretically transferred to nitrocellulose membranes. Equivalent amounts of protein were loaded onto the gel for each treatment as described in the figure legends. Proteins were detected with enhanced chemiluminescence (ECL) system (Amhersham, Arlington Heights, IL). A specific rabbit polyclonal anti-perilipin A antibody was generated using peptide: PREKPARRVSDSFFRPSVC (Ab PREK) and used for Western blotting (1:1,500) [Souza et al., 1998].

Immune Complex Kinase Assay

ERK and JNK activities were measured using an immune complex kinase (IP) assay [Raingeaud et al., 1995]. Briefly, whole cell extract (50 μg of protein) was immunoprecipitated using specific antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) bound to protein A-Sepharose beads (Repligen Corp., Needham, MA). MBP (Sigma) was used as the substrate for the p44 ERK assay and N-terminal c-Jun obtained from a GST fusion protein and purified on glutathione–agarose beads (Amersham Pharmacia Biotech, Uppsala, Sweden) was used as the substrate for the p46 JNK. Activity was visualized by autoradiography of the dried 10% SDS–polyacrylamide gel, and quantitation was assessed by PhosphorImager analysis using Molecular Dynamics phosphor imaging equipment.

Immunofluorescence

For determination of perilipin A fluorescence, cells were cultured in 35 mm cover slip bottomed dishes (MatTek Corp., Ashland, MA) and infected as described above. After treatment, adipocytes were fixed in 2% paraformaldehyde for 10 min, washed, and treated with anti-perilipin A polyclonal antibody (Ab. PREK, 1:500 dilution) and a goat-anti-rabbit Alexa fluor 647 labeled antibody (2 μl/ml, Molecular Probes). Neutral lipids were stained with Nile red (1 μM) (Molecular Probes, Eugene, OR). Fluorescence imaging was assessed as described [Souza et al., 1998].

Statistics

Treatments were compared by using two-factor analysis of variance (ANOVA) with rosiglitazone or PD98059 (present/absent) and TNF-α (present/absent) as factors. When an interaction was present, the effect of rosiglitazone or PD98059 was assessed using Student's t test for independent samples. One-way ANOVA was used for statistical analyses of U0126 experiments. Results were considered statistically significant if the observed significance level (P value) was less than 0.05.

RESULTS

TNF-α-Induced Lipolysis is Regulated by ERK Activation

To determine whether TNF-α-induced MAP kinases were involved in lipolysis, we examined activation of MAP kinase pathways, by immune complex kinase (IP) assays, following TNF-α stimulation of 3T3-L1 adipocytes. Initial studies demonstrated that TNF-α stimulated ERK and JNK maximally at 15 min and by 1 h activity had returned to background levels (data not shown). Following 15 min incubation, TNF-α (10 ng/ml) potently induced ERK activity sevenfold as compared to cells incubated in the absence of TNF-α (Fig. 1A). ERK activity is regulated by the upstream kinase, MAP kinase kinase-1 (MEK-1), which phosphorylates ERK on threonine and tyrosine residues [Davis, 1993; Cobb and Goldsmith, 1995]. Preincubation of 3T3-L1 cells with the specific MEK-1 inhibitor PD98059 (100 μM) [Alessi et al., 1995], inhibited TNF-α-induced ERK activity without altering total ERK protein levels (Fig. 1A). Preincubation with U0126, another MEK-1 inhibitor, inhibited the activation of ERK as measured by the amount of phospho-ERK detected by Western analysis (data not shown). In addition, we examined TNF-α-induction of JNK activity using an IP kinase assay. JNK activity was strongly induced ~sevenfold following a 15 min stimulation with TNF-α (Fig. 1B). As expected,

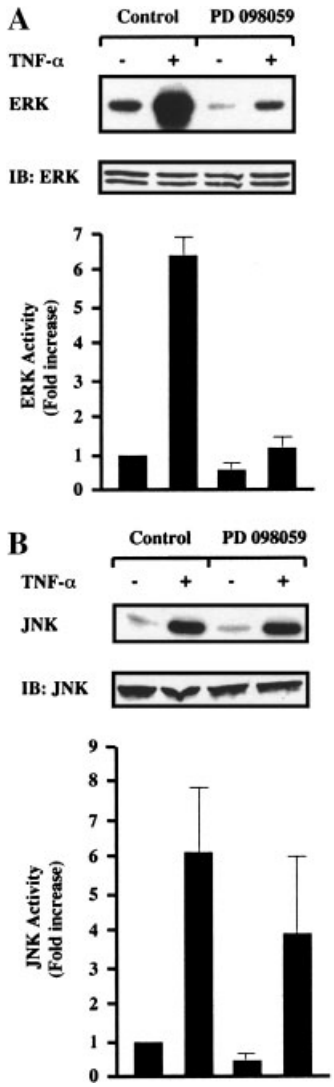

Fig. 1. TNF-α-induced activation of ERK and JNK. **A**: ERK activity; (**B**), JNK activity. 3T3-L1 adipocytes were treated with TNF-α (10 ng/ml) for 15 min following a 3 h pretreatment with PD98059 (100 μM). Kinase activities in whole cell extracts were determined by an immune complex kinase assay. Data (mean ± SE) are representative of three experiments. ERK and JNK activity are expressed as fold stimulation above basal (in the absence of TNF-α or PD98059) activity. Immunoblots (IB) were used to assess the level of ERK1/2 and JNK 1 proteins.

the MEK-1 inhibitor PD98059 did not inhibit TNF-α-inducible JNK activity (Fig. 1B). These results demonstrate that TNF-α stimulates MAP kinase pathways in 3T3-L1 cells consistent with previous studies in both 3T3-L1 cells and other cell types [Yuasa et al., 1998; Jain et al., 1999; Aguirre et al., 2000].

TNF-α is a potent, but not immediate activator of lipolysis [Kawakami et al., 1987; Feingold et al., 1992; Green et al., 1994; Hauner et al., 1995]. In order to determine whether the ERK pathway regulates TNF-α-induced lipolysis, 3T3-L1 adipocytes were pretreated for 3 h in the absence or presence of the MEK-1 inhibitor, PD98059 (100 μM), and then incubated with TNF-α for 24 h (10 ng/ml). Treatment with PD98059 significantly reduced TNF-α-stimulated lipolysis as compared to cells treated with the cytokine alone (1.7 vs. 5.2-fold, $P < 0.003$; a 76% decrease) (Fig. 2A). In the presence of PD98059, basal (i.e., ligand independent) lipolysis was not significantly different from lipolysis in the absence of the compound. The effects of PD98059 to block TNF-α-induced ERK activation and to decrease lipolysis without an effect on activation of JNK (Fig. 1B) suggest that the ERK pathway is a significant regulator of TNF-α-induced lipolysis. In addition, incubation of 3T3-L1 adipocytes with 100 μM of U0126, another MEK-1 inhibitor, resulted in a 65% decrease in TNF-α-induced lipolysis as compared to cells treated with TNF-α alone (Fig. 2B, 2.7 vs. 7.8-fold, $P < 0.001$), supporting the major role of ERK activation on TNF-α-induced lipolysis.

PPARγ Agonists Block TNF-α-Induced ERK Activation and Lipolysis

PPARγ is a nuclear transcription factor that is involved in the regulation of TNF-α-induced lipolysis and lipid accumulation in adipocytes [Hu et al., 1996; Adams et al., 1997; Souza et al., 1998]. Previously, we demonstrated that the PPARγ agonists, rosiglitazone (ros) or 15-deoxy-$\Delta^{12,14}$- prostaglandin J_2 (PGJ2) blocked TNF-α-induced lipolysis [Souza et al., 1998]. We report here that a 72 h pretreatment with these PPARγ agonists potently inhibited TNF-α-induced ERK activity (4.3 vs. 1.7 fold activation, Fig. 3A). Coincident with these effects on ERK pathway activation, ros inhibited TNF-α-induced lipolysis by greater than 90% (Fig. 3C, $P < 0.005$). Preliminary studies indicated that a 24 h preincubation with the PPARγ agonists did not block TNF-α-stimulated ERK activity, suggesting that the PPARγ agonists' effect to block TNF-α-induced ERK at 72 h is indirect. TNF-α-induced JNK pathway activation was not affected by pretreatment with ros or PGJ2 (Fig. 3B). Furthermore, these results are consistent with our PD98059 data (Figs. 1A and 2A) confirming that ERK is the predominant signal transduction pathway by which TNF-α induces lipolysis.

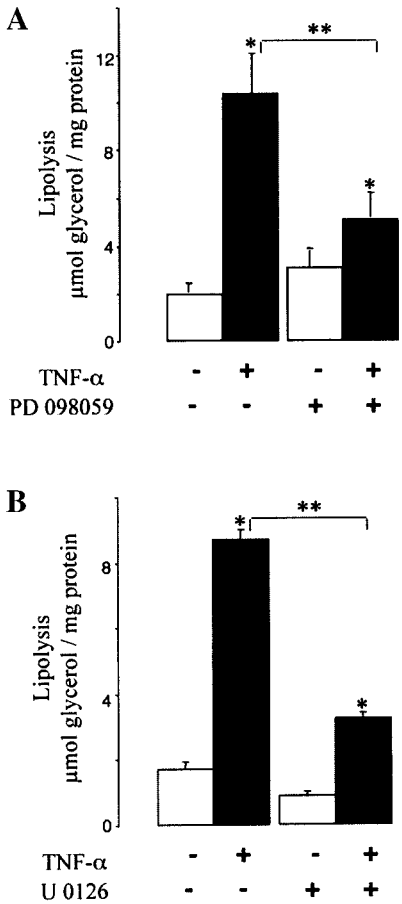

Fig. 2. PD98059 and U0126 block the ability of TNF-α to increase lipolysis. **A**: 3T3-L1 adipocytes pretreated with PD98059 (100 μM) for 3 h and treated or not with TNF-α (10 ng/ml). **B**: 3T3-L1 adipocytes were treated with U0126 (100 μM) in the presence or absence of TNF-α (10 ng/ml). Accumulation of glycerol in the media was measured as an index of lipolysis 24 h after TNF-α treatment. Data are expressed as the mean ± SE (n = 8 experiments). *$P < 0.001$ TNF-α vs. basal lipolysis (absence of TNF-α, PD98059, or U0126), **$P < 0.003$ TNF-α vs. TNF-α plus PD98059. **$P < 0.001$ TNF-α vs. TNF-α plus U0126.

A Target of TNF-α-Induced ERK Activation: Perilipin A Expression

We have previously demonstrated that TNF-α reduces the expression of perilipin A mRNA and protein expression [Rosenbaum and Greenberg, 1998; Souza et al., 1998], the most abundant perilipin isoform in adipocytes. Furthermore, we hypothesized that perilipin contributes to the mechanism of lipolysis. Perilipin A is a protein localized to the surface of intracellular LDs, which is thought to inhibit LD hydrolysis by lipases [Londos, 1996; Robinson and Cobb, 1997; Londos et al., 1999; Wallach et al., 1999]. In order to determine whether the

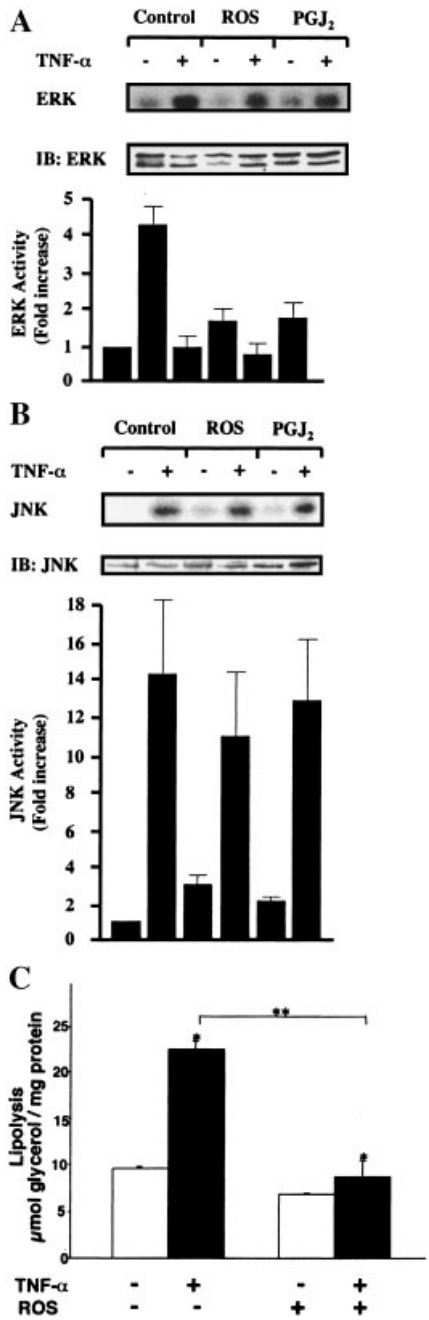

Fig. 3. PPARγ agonists block the ability of TNF-α to activate ERK and lipolysis. 3T3-L1 adipocytes were pretreated with ros (5 μM) or PGJ2 (17 μM) or neither (control) for 72 h. Media was then changed and cells were incubated with TNF-α (10 ng/ml), in the presence or absence of PPARγ agonists for 15 min to determine ERK (**A**) and JNK (**B**) activities. Data are expressed as mean ± SE (n = 3 experiments). Analysis of IB analyses were used to determine the total amount of ERK1/2 and JNK 1 proteins. **C**: Lipolysis. 3T3-L1 adipocytes were pretreated with ros (5 μM) or PGJ_2 (17 μM) for 72 h. Media was then changed and cells were incubated with TNF-α (10 ng/ml) or ros or TNF-α plus ros for 24 h (n = 3 experiments performed in triplicate). The media was then analyzed for glycerol release. *$P < 0.003$ vs. basal lipolysis (absence of ros and TNF-α). **$P < 0.005$, TNF-α vs. TNF-α plus ros.

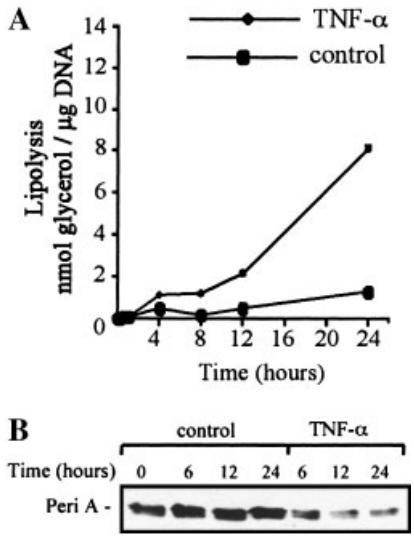

Fig. 4. Time course for TNF-α effects on perilipin expression and lipolysis. 3T3-L1 adipocytes were incubated in the absence or presence of TNF-α (10 ng/ml). Cells were harvested at the time points indicated. **A**: Accumulation of glycerol in the media at each time point was measured as an index of lipolysis. Data represents the mean of two experiments performed in triplicates. **B**: Western blotting for perilipin A protein in cell lysates collected at each time point. Data shown are representative of two experiments.

reduction in perilipin protein levels and the increase in adipocyte lipolysis occur concomitantly, we conducted time course studies. The reduction in perilipin protein expression is coincident with the onset of TNF-α-induced lipolysis (Fig. 4A,B). We observed a ~50% reduction in perilipin protein levels at 6 h after TNF-α (Fig. 4B). Importantly, at 12 h, perilipin protein levels are further reduced to ~30% of control levels, when the rate of TNF-α-induced lipolysis is increasing. These data further support the hypothesis that a reduction in perilipin protein expression is one of the mechanisms by which TNF-α increases adipocyte lipolysis.

We next investigated whether the TNF-α-induced decrease in perilipin A expression was mediated through ERK activation. Preincubation of 3T3-L1 adipocytes with the MEK-1 inhibitor, PD98059, reduced the ability of TNF-α to decrease perilipin A expression (Fig. 5A). Similarly, pretreatment with ros, for 72 h, which we previously demonstrated also blocked ERK activation (Fig. 3A), abrogated TNF-α effects on perilipin expression (Fig. 5B). Confocal studies were performed to confirm the inhibitory effect of the MEK-1 inhibitor, PD98059, on the decrease in perilipin expression in response to TNF-α (Fig. 5C). 3T3-L1 adipocytes were pretreated for 3 h in the absence or presence of PD98059 and then incubated with TNF-α for 24 h (10 ng/ml). Simultaneous fluorescent detection of Peri A and differential interference contrast (DIC) allowed us to correlate immunoreactivity of Peri A with the LD in the cell. Peri A immunostaining at the surface of the LD decreased in TNF-α-treated cells (Fig. 5C, panel b). In contrast, Peri A immunostaining in cells treated with PD98059 and TNF-α was similar to that observed in control cells (Fig. 5C, panel d). These results suggest that one mechanism by which TNF-α-induced ERK activity regulates lipolysis is, in part, by down regulation of perilipin A expression. Furthermore, these data indicate that ERK activation is an early signal for the reduction in perilipin protein expression and subsequent induction of lipolysis.

DISCUSSION

TNF-α stimulates adipocyte lipolysis, and has been hypothesized to contribute to obesity-linked insulin resistance and diabetes [Hotamisligil and Spiegelman, 1994; Hotamisligil et al., 1995; Uysal et al., 1997; Souza et al., 1998]. The present studies assessed the contributions of the ERK and JNK pathways in TNF-α induced lipolysis. IP activity assays indicate that TNF-α rapidly stimulates both the ERK and JNK pathways at concentrations that induce lipolysis. While most studies have demonstrated the ability of TNF-α to stimulate ERK and JNK pathways [Victor et al., 1993; Bazzoni and Beutler, 1996; Jain et al., 1998, 1999; Wallach et al., 1999; Valladares et al., 2000] the present study definitively links TNF-α-induced MAP kinase activation to stimulated lipolysis. Dual kinase/lipolysis experiments done in the presence of compounds that inhibit ERK activation conclusively demonstrate that the ERK pathway is a major regulator of TNF-α induced lipolysis in 3T3-L1 adipocytes (Figs. 1, 2, and 3).

We used four different compounds to inhibit the ERK pathway. Both PD98059 and U0126 are known to specifically reduce ERK activity by blocking its upstream activator, MEK-1 [Alessi et al., 1995]. PD98059 abrogated ERK activation by ~90% (Fig. 1A), and reduced TNF-α-induced lipolysis by 76% (Fig. 2). The residual lipolysis in the almost total absence of ERK activation suggests the involvement of other signal transduction pathways in TNF-α-stimu-

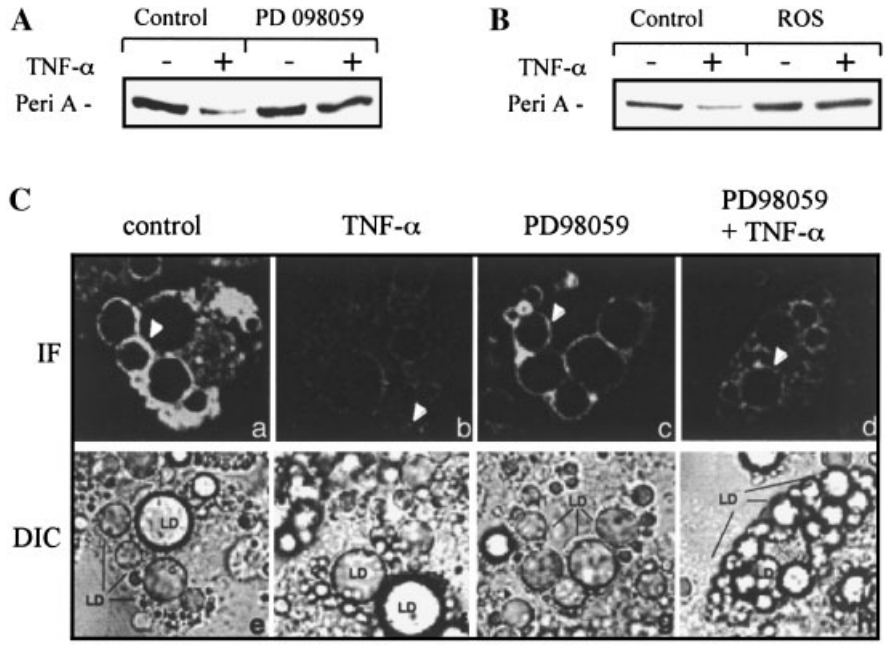

Fig. 5. PD98059 and PPARγ agonists block the ability of TNF-α to decrease perilipin A expression. **A**: Western blotting for perilipin A protein in cell lysates collected from assays described in Figure 2A. Data shown are representative of five experiments. **B**: Western blotting for perilipin A protein in cell lysates collected from assays described in Figure 3C. Data are shown representative of three experiments. **C**: 3T3-L1 adipocytes were incubated in the absence (**panels a**, **b**, **e**, and **f**) or presence of PD98059 (**panels c**, **d**, **g**, and **h**) and treated or not with TNF-α and either analyzed for perilipin A immunofluorescence (IF) by confocal microscopy or for DIC. Data shown are representative of two experiments. LD, lipid droplet. Magnification: 1,000×.

lated lipolysis. As has been suggested, one possible pathway involves the cyclic-AMP-dependent protein kinase (PKA) [Gasic et al., 1999; Landstrom et al., 2000]. TNF-α-induced lipolysis was recently demonstrated to involve the ERK pathway and PKA in differentiated human preadipocytes [Zhang et al., 2002]. However, we did not observe an elevation in cAMP levels after TNF-α treatment of 3T3-L1 adipocytes (data not shown).

The two other agents used to block ERK activity are PPARγ agonists. PPARγ agonists are known to block the ERK signal transduction cascade downstream of ERK kinase [Law et al., 1996; Camp and Tafuir, 1997; Maggi et al., 2000], but no study has previously demonstrated inhibition of ERK activity. In the present study, pretreatment with either PPARγ agonists (ros or PGJ2) blocked TNF-α-induced ERK activation. Furthermore, ros inhibited TNF-α-induced lipolysis by >90%. Notably, neither PD98059 nor ros blocked JNK activity. Thus, although TNF-α activated JNK, JNK does not appear to be a significant regulator of lipolysis in our studies. It should be noted that PPARγ agonists have been demonstrated to block IL-1 stimulated JNK activation in an insulinoma cell line [Maggi et al., 2000]. During preparation of this manuscript, Ryden et al. [2002] reported the involvement of ERK and JNK in TNF-induced lipolysis in human adipocytes using TNF-α at a very high concentration (100 ng/ml); but were unable to achieve results in 3T3-L1 cells as we report here.

ERK activation may underlie the mechanism by which other agents stimulate lipolysis. For example, leptin and epidermal growth factor are known activators of ERK and have been demonstrated to increase adipocyte lipolysis [Wiese et al., 1995; Suga et al., 1997]. In fact, treatment of cells with epidermal growth factor has been demonstrated to result in ERK phosphorylation of PPARγ [Hu et al., 1996]. In addition, β3-adrenergic agents which previously were thought to increase adipocyte lipolysis primarily by stimulating PKA, were recently demonstrated to increase ERK activation in adipocytes [Soeder et al., 1999]. Thus, ERK activation may contribute to the known lipolytic and thermogenic effects of β3-adrenergic agonists.

We have proposed that TNF-α induces lipolysis, at least in part, by downregulating perilipin protein expression. Our hypothesis is based

upon the observations that TNF-α transcriptionally represses perilipin mRNA and protein expression [Rosenbaum and Greenberg, 1998; Souza et al., 1998; Ruan et al., 2002], and that overexpression of perilipin blocks TNF-α-induced lipolysis [Souza et al., 1998]. We now extend these observations by demonstrating that ERK is the predominant signal transduction pathway by which TNF-α modulates perilipin protein expression in 3T3-L1 adipocytes. Similar to our system, TNF-α reduces perilipin protein expression in cultured human adipose tissue (personal communication from Susan K. Fried, University of Maryland, Baltimore VA Medical Center, Baltimore, MD). However, in human differentiated preadipocytes, regulation of TNF- α-induced lipolysis is associated with the phosphorylation of perilipin and PKA [Zhang et al., 2002] rather than a decrease on perilipin protein expression. We did not observe any changes in perilipin migration on SDS–PAGE (Figs. 4B and 5A,B), suggesting that perilipin is not hyperphosphorylated. Therefore, it appears that the human differentiated preadipocytes respond differently to TNF-α treatment than 3T3-L1 adipocytes and cultured human adipose tissue. Here, we specifically show that inhibitors of the ERK pathway stabilize perilipin protein levels (Fig. 5A,B,C). Furthermore, since these inhibitors blocked TNF-α-induced lipolysis, our data mechanistically link ERK activation to downregulated perilipin protein expression and TNF-α-induced lipolysis. PPARγ is a nuclear transcription factor, which is central to adipose gene regulation and metabolism [Spiegelman, 1998]. Phosphorylation of PPARγ by ERK decreases the ability of PPARγ to activate genes [Hu et al., 1996; Adams et al., 1997; Camp and Tafuir, 1997]. ERK-dependent regulation of perilipin expression in the present study is therefore consistent with the notion that perilipin is transcriptionally regulated by PPARγ, as previously suggested by our laboratory [Rosenbaum and Greenberg, 1998].

TNF-α-induced ERK activation may contribute to the development of obesity-linked insulin resistance by different mechanisms. Previous studies have suggested that TNF-α may increase insulin resistance by increasing lipolysis [Miles et al., 1997; Peraldi et al., 1997; Uysal et al., 1997]. For example, pretreatment of animals with troglitazone (a thiazolidinedione, a class of antidiabetic drugs that are PPARγ agonists) significantly blocked exogenous TNF-α's actions to increase serum FFA and insulin resistance in vivo [Miles et al., 1997]. PPARγ agonists, such as rosiglitazone and troglitazone have been demonstrated to ameliorate insulin resistance [Saltiel and Olefsky, 1996]. While a reduction in lipolysis may explain some of the antidiabetic effects of these compounds, other mechanisms may also be involved. Of potential relevance, activation of ERK has been demonstrated to phosphorylate IRS-1 and decrease insulin receptor signaling in cells [De Fea and Roth, 1997]. Thiazolidinedione treatment of adipocytes has been demonstrated to block TNF-α's inhibitory actions on the insulin signal transduction pathway [Peraldi et al., 1997]. A recent study suggests that TNF-α promotes insulin resistance through JNK activation in certain cell lines. 3T3-L1 cells were not examined and the authors did not examine the role of ERK activation [Aguirre et al., 2000]. Further studies are needed to confirm a potential link between TNF-α-induced ERK activation in adipocytes and the development of insulin resistance in animals and humans.

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REFERENCES

Adams M, Reginato M, Shao D, Lazar M, Chatterjee V. 1997. Transcriptional activation by peroxisome proliferator-activated receptor γ is inhibited by phosphorylation at a consensus mitogen-activated protein kinase site. J Biol Chem 272:5128–5132.

Aguirre V, Uchida T, Yenush L, Davis R, White M. 2000. The c-jun NH_2-terminal kinase promotes insulin resistance during association with insulin receptor substrate-1 and phosphorylation of ser^{307}. J Biol Chem 275:9047–9054.

Alessi D, Cuenda A, Cohen P, Dudley D, Saltiel A. 1995. PD 98059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase in vitro and in vivo. J Biol Chem 70:27489–27494.

Bazzoni F, Beutler B. 1996. The tumor necrosis factor ligand and receptor families. New Engl J Med 334:1717–1725.

Blanchette-Mackie EJ, Dwyer NK, Barber T, Coxey RA, Takeda T, Rondonine CM, Theodorakias JL, Greenberg AS, Londos C. 1995. Perilipin is located on the surface layer of the intracellular lipid droplets in adipocytes. J Lipid Res 36:1211–1226.

Boden G. 1997. Role of fatty acids in the pathogenesis of insulin resistance and NIDDM. Diabetes 46:3–10.

Camp H, Tafuir S. 1997. Regulation of peroxisome proliferator-activated receptor γ activity by mitogen activated protein kinase. J Biol Chem 272:10811–10816.

Cobb M, Goldsmith E. 1995. How MAP kinases are regulated? J Biol Chem 270:14843–14846.

Davis R. 1993. The mitogen-activated protein kinase signal transduction pathway. J Biol Chem 268:14553–14556.

De Fea K, Roth R. 1997. Modulation of insulin receptor substrate-1-tyrsoine phosphorylation and function by mitogen-activated kinase. J Biol Chem 272:31400–31406.

Feingold KR, Doerrler W, Dinarello CA, Fiers W, Grunfeld C. 1992. Stimulation of lipolysis in cultured fat cells by tumor necrosis factor, interleukin-1, and the interferons is blocked by inhibition of prostaglandin synthesis. Endocrinology 130:10–16.

Fiers W. 1991. Tumor necrosis factor. Characterization at the molecular, cellular, and in vivo level. FEBS Lett 285:199–212.

Gasic S, Tian B, Green A. 1999. Tumor necrosis factor α stimulates lipolysis in adipocytes by decreasing G_i protein concentrations. J Biol Chem 274:6770–6775.

Green A, Dobias SB, Walters DJA, Brasier AR. 1994. Tumor necrosis factor increases the rate of lipolysis in primary cultures of adipocytes without altering levels of hormone-sensitive lipase. Endocrinology 134:2581–2588.

Greenberg A, Egan J, Wek S, Garty N, Blanchette-Mackie E, Londos C. 1991. Perilipin, a major hormonally regulated adipocyte-specific phosphoprotein associated with the periphery of the lipid storage droplets. J Biol Chem 266(17):11341–11346.

Hauner H, Petruschke T, Russ M, Rohrig K, Eckel J. 1995. Effects of tumor necrosis factor alpha (TNFα) on glucose transport and lipid metabolism of newly-differentiated human fat cells in cell culture. Diabetologia 38:764–771.

Hauner H, Rohrig K, Petruschke T. 1995. Effects of epidermal growth factor, platelet-derived growth factor, and fibroblast growth factor on human adipocyte development and function. Eur J Clin Invest 25:90–96.

Hotamisligil GS, Spiegelman BM. 1994. Tumor necrosis factor α: A key component of the obesity–diabetes link. Diabetes 43:1271–1278.

Hotamisligil GS, Shargil NS, Spiegelman BM. 1993. Adipose expression of tumor necrosis factor-α: Direct role in obesity-linked insulin resistance. Science 259:87–91.

Hotamisligil GS, Arner P, Caro JF, Atkinson RL, Spiegelman BM. 1995. Increased adipose tissue expression of tumor necrosis factor-α in human obesity and insulin resistance. J Clin Invest 95:2409–2415.

Hu E, Kim JB, Sarraf P, Spiegelman B. 1996. Inhibition of adipogenesis through MAP kinase-mediated phosphorylation of PPARγ. Science 274:2100–2103.

Jain R, Meredith M, Pekala P. 1998. Tumor necrosis factor-α mediated activation of signal transduction cascades and transcription factors in 3T3-L1 adipocytes. Adv Enzyme Regul 38:333–347.

Jain R, Phelps K, Pekala P. 1999. Tumor necrosis factor-α initiated signal transduction in 3T3-L1 adipocytes. J Cell Physiol 179:58–66.

Kawakami M, Murase T, Ogawa H, Ishibashi S, Mori N, Takaku F, Shibata S. 1987. Human recombinant TNF suppresses lipoprotein lipase activity and stimulates lipolysis in 3T3-L1 cells. J Biochem 101:331–338.

Kern PA, Saghizadeh M, Ong JM, Bosch RJ, Deem R, Simsolo RB. 1995. The expression of tumor necrosis factor in human adipose tissue: Regulation by obesity, weight loss, and relationship to lipoprotein lipase. J Clin Invest 95:2111–2119.

Kohanski R, Frost S, Lane M. 1986. Insulin-dependent phosphorylation of the insulin receptor-protein kinase and activation of glucose transport in 3T3-L1 adipocytes. J Biol Chem 261:12272–12281.

Landstrom R, Mie J, Karlsson M, Manganiello V, Degereman E. 2000. Down-regulation of cyclic nucleotide phosphodiesterase 3B in 3T3-L1 adipocytes induced by tumor necrosis factor α and cAMP. Biochem J 346:337–343.

Law R, Meehan W, Xi XP, Graf K, Wuthrich D, Coats W, Faxon D, Hsueh W. 1996. Troglitazone inhibits vascular smooth muscle cell growth and intimal hyperplasia. J Clin Invest 98:1897–1905.

Londos C. 1996. Hormone-sensitive lipase and the control of lipolysis in adipocytes. In: LeRoith D, Taylor S, Olefsky J, editors. Diabetes mellitus: A fundamental and clinical text. Philadelphia: Lippincott-Raven. pp 223–227.

Londos C, Brasaemle D, Schultz C, Segrest J, Kimmel A. 1999. Perilipins, ADRP, and other proteins that associate with intracellular neutral lipid droplets in animal cells. Cell Dev Biol 10:51–58.

Maggi L, Sadeghi J, Weigand C, Scarim A, Heitmeier M, Corbett J. 2000. Anti-inflammatory actions of 15-deoxy-delta^{12,14}-prostaglandin J_2 and troglitazone: Evidence for heat shock-dependent and -independent inhibition of cytokine induced inducible nitric oxide synthase expression. Diabetes 49:346–355.

Miles PDG, Romeo OM, Higo K, Cohen A, Rafaat K, Olefsky J. 1997. TNF-α-induced insulin resistance in vivo and its prevention by troglitazone. Diabetes 46:1678–1683.

Orban Z, Remaley A, Sampson M, Trajanoski Z, Chrousos G. 1999. The differential effect of food intake and β-adrenergic stimulation on adipose-derived hormones and cytokines in man. J Clin Endocrinol Metab 84:2126–2133.

Peraldi P, Xu M, Spiegelman B. 1997. Thiazolidinediones block tumor necrosis factor-α-induced inhibition of insulin signaling. J Clin Invest 100:1863–1869.

Raingeaud J, Gupta S, Rogers J, Dickens M, Han J, Ulevitch R, Davis R. 1995. Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. J Biol Chem 270:7420–74216.

Robinson M, Cobb M. 1997. Mitogen-activated protein kinase pathways. Cur Opin Cell Biol 9:180–186.

Rosenbaum S, Greenberg A. 1998. The short and long-term effects of tumor necrosis factor-α and BRL 49653 on peroxisome proliferator-activated receptor (PPAR)γ2 gene expression and other adipocyte genes. Mol Endocrinol 12:1150–1159.

Ruan H, Miles P, Ladd CM, Ross K, Golub TR, Olefsky JM, Lodish HF. 2002. Profiling gene transcription in vivo reveals adipose tissue as an immediate target of tumor necrosis factor-alpha: Implications for insulin resistance. Diabetes 51:3176–3188.

Ryden M, Dicker A, van Harmelen H, Hauner M, Brunnberg L, Perbeck F, Lonnqvist P. 2002. Mapping of early signaling events in tumor necrosis factor-alpha-mediated lipolysis in human fat cells. J Biol Chem 277:1085–1091.

Saltiel A, Olefsky J. 1996. Thiazolidinediones in the treatment of insulin resistance and type II diabetes. Diabetes 45:1661–1669.

Servetnick DA, Brasaemle DL, Gruia-Gray J, Kimmel AR, Wolff J, Londos C. 1995. Perilipins are associated with cholesteryl ester droplets in steroidogenic adrenal cortical and Leydig cells. J Biol Chem 270(28):16870–16873.

Soeder K, Snedden S, Cao W, Della Rocca G, Daniel K, Luttrell L, Collins S. 1999. The beta3-adrenergic receptor activates mitogen-activated protein kinase in adipocytes through a G_i mechanism. J Biol Chem 274:12017–12022.

Souza S, Moitoso de Vargas L, Yamamato M, Line P, Franciosa M, Moss L, Greenberg A. 1998. Overexpression of perilipin A and B blocks the ability of tumor necrosis factor to increase adipocyte lipolysis in 3T3-L1 adipocytes. J Biol Chem 273:24665–24669.

Souza SC, Yamamoto M, Franciosa M, Lien P, Greenberg A. 1998. BRL blocks the lipolytic actions of tumor necrosis factor-alpha (TNF-α): A potential new insulin-sensitizing mechanism for the thiazolidinediones. Diabetes 47:691–695.

Spiegelman B. 1998. PPAR-γ: Adipogenic regulator and thiazolidinedione receptor. Diabetes 47:507–514.

Suga J, Yoshimasa Y, Yamada K, Yamamoto Y, Inoue G, Okamoto M, Hayashi T, Sigemoto M, Kosaki A, Kuzuya H, Nakao K. 1997. Differential activation of mitogen-activated protein kinase by insulin and epidermal growth factor in 3T3-L1 adipocytes: A possible involvement of P13-kinase in the activation of the map kinase by insulin. Diabetes 46:735–741.

Uysal K, Wiesbrock S, Marino M. 1997. Protection from obesity-linked insulin resistance in mice lacking TNF-α function. Nature 389:610–614.

Valladares A, Alvarez A, Ventura J, Roncero C, Benito M, Porras A. 2000. p38 mitogen-activated protein kinase mediates tumor necrosis factor-alpha-induced apoptosis in rat fetal brown adipocytes. Endocrinology 141:4383–4395.

Victor I, Schwenger P, Schlessinger J, Vilcek J. 1993. Tumor necrosis factor-induced activation and increased tyrosine phosphorylation of mitogen-activated protein (MAP) kinase in human fibroblasts. J Biol Chem 268: 18994–18999.

Vilcek J, Lee T. 1991. Tumor necrosis factor: New insights into molecular mechanisms of its multiple actions. J Biol Chem 266:7313–7316.

Wallach D, Varfolomeev E, Malini N, Goltsev Y, Kovalenko A, Boldin M. 1999. Tumor necrosis factor receptor and Fas signaling mechanisms. Annu Rev Immunol 17:331–367.

Wang M-Y, Lee Y, Unger R. 1999. Novel form of lipolysis induced by leptin. J Biol Chem 274:17541–17544.

Wiese R, Mastick C, Lazar D, Saltiel A. 1995. Activation of mitogen-activated protein kinase and phoshatidylinositol 3′-kinase is not sufficient for the hormonal stimulation of glucose uptake, lipogenesis, or glycogen synthesis in 3T3-L1 adipocytes. J Biol Chem 170:3442–3446.

Yuasa T, Ohno S, Kehrl J, Kyriakis J. 1998. Tumor necrosis factor signaling to stress-activated protein kinase (SAPK)/Jun NH_2-terminal kinase (JNK) and p38. J Biol Chem 273:22681–22692.

Zhang B, Berger J, Zhou G, Elbrecht A, Biswas S, White-Carrington S, Szalkowski D, Moller D. 1996. Insulin and mitogen-activated protein kinase mediated phosphorylation and activation of peroxisome proliferator-activated receptor-γ. J Biol Chem 271:31771–31774.

Zhang HH, Halbleib M, Ahmad F, Manganiello VC, Greenberg AS. 2002. Tumor necrosis factor-alpha stimulates lipolysis in differentiated human adipocytes through activation of extracellular signal-related kinase and elevation of intra- cellular cAMP. Diabetes 51:2929–2935.