Tumor necrosis factor-α (TNF-α) is a pleiotropic cytokine with a proposed role in obesity-related insulin resistance. This could be mediated by increased lipolysis in adipose tissue resulting in elevated free fatty acid levels. The early intracellular signals entailed in TNF-α-mediated lipolysis are unknown but may involve members of the mitogen-activated protein kinase (MAPK) family. We investigated the possible contribution of MAPK in TNF-α-induced lipolysis in human preadipocytes. TNF-α activated the three mammalian MAPK, p44/42, JNK, and p38, in a distinct time- and concentration-dependent manner. TNF-α also induced a concentration-dependent stimulation of lipolysis with a more than 3-fold increase at the maximal dose. Lipolysis was completely inhibited by blockers specific for p44/42 (PD98059) and JNK (dimethylaminopurine) but was not affected by the p38 blocker SB203580. Use of receptor-specific TNF-α mutants showed that activation of MAPK is entirely mediated by the TNFR1 receptor. The results in human preadipocytes differed from those obtained in murine 3T3-L1 adipocytes in which all three MAPK were constitutively active. Thus, studies of intracellular signaling pathways obtained in different cellular contexts should be interpreted with caution. In conclusion, although TNF-α activates all three known MAPK in human preadipocytes, only p44/42 and JNK appear to be involved in the regulation of lipolysis.

Obesity, peripheral insulin resistance, and non-insulin-dependent diabetes mellitus are closely related clinical conditions with an almost epidemically increasing prevalence in industrialized countries (1). The molecular mechanisms underlying this close association are still not clear but are thought to involve, among others, factors produced by the adipose tissue. A wealth of studies in recent years has implicated tumor necrosis factor-α (TNF-α)1 in this process (2). TNF-α was first described as a potent regulator of apoptosis and is well known for its central role in the immune system (3). Soluble TNF-α is a 17-kDa molecule (4) that signals through two different receptors termed TNFR1 and TNFR2 (for tumor necrosis factor receptor; in humans also termed p60 and p80 based on their apparent size in gel electrophoresis). Most cell types, including adipocytes, have the capacity to produce varying amounts of TNF-α and its receptors. The involvement of TNF-α in obesity-induced insulin resistance is based, among other things, on the following observations in cell lines, animals, and humans: (i) TNF-α mRNA and protein levels are increased in adipose tissue of overweight subjects and in animal models of obesity and insulin resistance (5). (ii) TNF-α regulates the expression of several genes associated with glucose and lipid metabolism, e.g. the glucose transporter GLUT-4, lipoprotein lipase, and the atherogenic protein plasminogen activator inhibitor-1 (6, 7). (iii) TNF-α has direct inhibitory effects on insulin receptor signaling by inhibition of autophosphorylation and/or effects on downstream signaling components (8). (iv) Administration of TNF-α-neutralizing antibodies in vivo ameliorates insulin resistance in obese rodents (5). (v) Finally, gene ablation experiments in mice have demonstrated that a lack of TNF-α enhances peripheral insulin sensitivity (9, 10).

The molecular mechanisms underlying TNF-α-mediated insulin resistance have raised considerable attention. Several lines of evidence suggest that TNF-α may at least in part act through an indirect mechanism by up-regulating circulating free fatty acid (FFA) levels (11). FFA are released by adipose tissue in a process termed lipolysis in which stored triglycerides are broken down into FFA and glycerol. FFA levels are elevated in obesity and non-insulin-dependent diabetes mellitus, and chronic elevation of FFA promotes insulin resistance (11). Prolonged treatment (6–24 h) of adipocytes with TNF-α in vitro stimulates lipolysis (12). TNF-α gene knockout mice exhibit lower circulating FFA and are protected from obesity-induced insulin resistance (9), whereas the infusion of TNF-α in rodents (13–15) or humans (16, 17) results in increased plasma FFA and systemic insulin resistance. Certain clinical conditions such as cachexia are associated with increased TNF-α levels, activated lipolysis, and high circulating FFA levels in combination with insulin resistance (18). Furthermore, recent studies have demonstrated that the novel class of insulin-sensitizing antiadipogenic agents, thiazolidinediones, inhibit TNF-α-induced lipolysis in murine adipocytes (19). Pre-
treatment of lean rats with a thiazolidinedione for 10 days prevents the increase in circulating FFAs and ensuing insulin resistance induced by TNF-α infusion (15). Taken together, these results suggest that TNF-α-induced lipolysis and thereby augmented FFA release may be one of the mechanisms by which the cytokine promotes insulin resistance. The intracellular signaling pathways involved in this regulation are, however, still unknown.

Studies in non-fat tissue have shown that TNF-α, upon binding and oligomerization of TNFR1 and TNFR2, induces several distinct signaling cascades. TNF-α regulates de novo gene expression through activation of transcription factors, and members of the family of signaling proteins termed mitogen-activated protein kinases (MAPK) play an essential role in this respect. These molecules propagate signals from the cell surface to the nucleus in defined phosphorylation cascades, and some effects of TNF-α are transduced by MAPK (20). MAPK, in turn, phosphorylate and activate an array of transcription factors. There are three distinct mammalian MAPK groups, the p44/42 (also termed extracellular signal-regulated kinases, ERK1/2), p38 (also termed stress/cytokine-activated kinases) and c-Jun-NH2-terminal kinases (JNK, also termed stress/cytokine-activated kinases, SAPK). The role of MAPK in fat cells is at present unclear. Data obtained in murine cell lines indicate that some of the MAPK can be activated by TNF-α, but their importance in lipolysis is unknown (21). We hypothesized that members of the MAPK are involved in mediating the lipolytic action of TNF-α in human adipocytes and that inhibition of one or several these pathways could abrogate the lipolytic effect of TNF-α. As a model system, we chose primary cultures of human preadipocytes, which offer the advantage of a prolonged survival when compared with mature adipocytes.

**EXPERIMENTAL PROCEDURES**

**Subjects and Adipose Tissue**—Subcutaneous adipose tissue was obtained from otherwise healthy obese subjects who underwent surgery for non-malignant disorders. None were on any regular medication. No selection was made for age (range 23–53 years), gender, or body mass index (range 26–44 kg/m2). The study was approved by the Ethics Committee at Huddinge Hospital. All subjects gave their informed consent to participate in the study. Specimens from subcutaneous adipose tissue were obtained within 30–45 min after the onset of surgery. All subjects fasted overnight, and only saline was administered until the tissue pieces were taken. In general, 25–35 g of adipose tissue was obtained from two or three different 24-well plates of preadipocytes, which yielded two or three 12-well plates of preadipocytes.

**Preadipocyte Culture**—Isolation and differentiation of preadipocytes was performed as described previously (22). Tissue specimens were transported in saline to the laboratory, where fibrous material and blood vessels were carefully dissected and discarded. The remaining tissue was cut into fragments of −5–10 mg and incubated with 0.5 g/liter collagenase (Sigma) in Krebs-Ringer phosphate buffer (pH 7.4) supplemented with 40 g/liter dialyzed bovine serum albumin (fraction V, Sigma) for 1 h at 37 °C in a shaking bath. A 5.1 volume ratio of incubation solution and fat tissue was used. The treated fat tissue was filtered through a nylon mesh with a 250-μm pore size, and remaining fibrous material was discarded. The cell suspension was centrifuged at 200 × g for 10 min at room temperature. The supernatant (containing mature adipocytes) was discarded, and the cell pellet was resuspended in 10 ml of DMEM/F12 medium (Invitrogen, Paisley, Scotland) and filtered through a nylon filter with a 70-μm pore size. After an additional centrifugation, the cell fraction was resuspended in DMEM/F12 medium supplemented with 10% fetal calf serum and 100 mg/liter penicillin/streptomycin. Cells were seeded into 12- or 24-well plates at a density of ~50,000 cells/cm2 and kept at 37 °C in 5.3 kPa of CO2 for 18–20 h. This initial incubation in fetal calf serum improves overall cell survival. Cells were then washed twice with DMEM/F12 medium and re-fed a chemically defined serum-free medium (DMEM/F12, supplemented with 100 μM cortisol, 66 μM insulin, 15 μM HEPES, 1 μM triiodothyronine, 33 μM biotin, 17 μM pantothenate, 10 μg/ml transferrin, 100 μg/liter penicillin/streptomycin, and 2.5 μg/ml amphotericin B). To differentiate the cells, a thiazolidinedione, rosiglitazone (Invitrogen, 49653, kindly provided by Smith Kline Beecham Pharmaceuticals, Harlow, Essex, UK), was added during the 14-day differentiation process to a final concentration of 10 μM (days 1–14). Cells were maintained in the medium at 37 °C and in 5.3 kPa of CO2 for a total of 16 days. Medium was changed every 3 days. After 14–16 days of culture, more than 70% of the preadipocytes displayed a round shape with a cytoplasm completely filled with multiple fat droplets. Only cultures with a differentiation density >70% were used in experiments. Plates containing more than 5% contaminating endothelial cells were discarded.

**Stimulation Experiments**—Stimulation experiments were performed using Oil-Red-O staining. Cells were fixed in 7% formaldehyde in phosphate-buffered saline, and triglyceride contents were stained with 1% Oil-Red-O in 99% isopropl alcohol. After repeated washings with water, the Oil-Red-O was dissolved in 100% isopropl alcohol, and the optical density of the solution was measured at 500 nm. An empty well treated in the same way was used as a blank. Differentiation was also determined by direct counting of differentiated cells under the microscope. Three well diameters were counted from left to right within 30 min; cells were regarded as differentiated when they displayed a round shape and the cytoplasm was completely filled with multiple fat droplets. In some experiments, differentiation was additionally determined by quantifying glycerol-3-phosphate dehydrogenase (GPDH) activity as described previously (Ref. 22 and data not shown). Expression of the type-1 (TNFR1) and type-2 (TNFR2) receptors was additionally detected and assessed by Western blot using a human-specific antibody (a gift from Dr C. Holm, Lund University, Sweden, data not shown). Viability was assessed by staining with 3-(4,5-dimethylthiazolyl)-2,5-diphenyterazolium bromide (MTT). Briefly, differentiated cells were grown in 12-well plates and incubated for 48 h in medium without or with increasing concentrations of TNF-α. Cells were then washed twice with modified Eagle’s medium (without phenol red) and subsequently incubated in 1 mg/ml MTT in modified Eagle’s medium without phenol red for 3 h at 37 °C. Supernatants were discarded, and cells were dissolved in 0.5 ml of isopropl alcohol. Absorbance was measured at 570 nm, and data from TNF-α-treated wells were expressed relative to control wells to obtain a cell survival index.

**3T3-L1 Culture**—3T3-L1 cells were cultured and differentiated as described previously (23). In brief, cells were grown to confluence in complete growth medium (DMEM/F12 Glutamax II, 45000 mg/liter glucose, 10% iron-supplemented bovine calf serum and penicillin/streptomycin). At 2–3 days after confluence, the medium was changed to differentiation medium (DM), day −2 (DMEM/F12 Glutamax II, 45000 mg/liter glucose, 10% iron-supplemented bovine calf serum, 0.5 mg/ml suberylmethylxanthine, 1 μM dexamethasone, 10 μg/ml bovine insulin and penicillin/streptomycin). After 48 h in DM, this was changed into post-differentiation medium (PF, identical to DM but without dexamethasone or isobutylmethylyxanthine). At day 7 in PF, ~90% of the cells had differentiated.

**Lipolysis Experiments**—14 days of differentiation, preadipocytes were incubated in the presence of increasing concentrations of TNF-α or in the presence of 100 ng/ml TNF-α in combination with the specific MAPK inhibitors: DMAP (dimetilaminopurine), PD98059, and SB203580 (all from Sigma) in BRL49653 free medium. All inhibitors were added 2 h before the initiation of stimulation to diminish intrinsic activity to a minimum. After 48 h (16th day of differentiation), the medium was removed, and cells were washed with DMEM/F12 medium and subsequently incubated for 3 h in DMEM/F12 supplemented with 20 g/liter BSA. The medium was removed and kept for measuring the glycerol concentration as an index of lipolysis using a bioluminescence method (Hellmer et al. (39)). Preadipocytes were lysed in a buffer containing Triton X-100, 50 mM Tris base, 150 mM NaCl, and protease inhibitors. The total amount of protein was measured using the BCA protein assay kit (Pierce). Glycerol concentrations were expressed per grams of protein.

**TNF-α Stimulation Experiments**—Preadipocytes were differentiated as described above for 14 days followed by a 48-h incubation in medium lacking BRL49653. At day 16, cells were stimulated for either 5 or 30 min in the presence of increasing concentrations of TNF-α ranging from 10 ng/ml to 100 ng/ml with or without each of the MAPK inhibitors. Inhibitors were added 2 h before stimulation to diminish intrinsic phosphorylation to a minimum. All incubations were performed in triplicates or quadruplicates. Background activity was assayed in wells lysed before TNF-α stimulation, termed t = 0. Following the incubation, cells were immediately lysed in an ice-cold buffer supplemented with protease and phosphatase inhibitors as supplied by the manufacturer (New England Biolabs, Beverly, MA). Wells incubated in the absence of TNF-α were
termed control samples. Cell lysates were centrifuged at 14,000 rpm for 30 min at +4 °C, and the supernatant was removed to new tubes. Equal amounts of cell lysates from each sample were boiled in 1× SDS loading buffer for 5 min and loaded and separated by SDS-polyacrylamide gel electrophoresis. Gels were then blotted onto polyvinylidene difluoride membranes (Amersham Biosciences, Inc., Little Chalfont, UK) by Western blotting. Blots were blocked for 1 h at room temperature in Tris-buffered saline with 0.1% Tween 20 (TBS-T) and 5% non-fat dried milk and subsequently incubated overnight at +4 °C in the presence of antibodies specific for phosphorylated forms of p38, p44/42, and JNK as described by the manufacturer (New England Biolabs). Following washing steps in TBS-T and incubation with secondary anti-rabbit antibodies conjugated to horseradish peroxidase, antigen-antibody complexes were detected by chemiluminescence (LumiGlo®, New England Biolabs) and exposed to high-performance chemiluminescence film (Amerham Biosciences, Inc.). Films were scanned, and specific bands were quantified using the NIH Image software. To control for differences in gel loading, sample concentration etc, blots were subsequently stripped and incubated with antibodies that recognized both phosphorylated and non-phosphorylated forms of p38, p44/42 and JNK, respectively. Assessment of p44/42 kinase activity was performed with a kit of reagents from New England Biolabs and used according to the instructions provided by the manufacturer.

Statistics—Values are given as means ± S.E.. Student’s t test was used where indicated. *p < 0.05 (two-sided) was regarded as statistically significant.

**RESULTS**

**Preadipocyte Cultures and TNF-α-stimulated Lipolysis—**Human preadipocytes were isolated and differentiated in a defined medium supplemented with rosiglitazone, a potent agonist of the adipocyte-specific transcription factor peroxisome proliferator-activated receptor-γ. Sixteen days after plating, more than 70% of the cells displayed classical characteristics of mature adipocytes such as expression of the adipocyte-specific enzyme GPDH, hormone-sensitive lipase, and lipid accumulation (data not shown, see “Experimental Procedures”). Differentiation rate was consistently above 70%, and contamination with endothelial cells was minimal. Prolonged stimulation with TNF-α in these cells results in enhanced lipolysis, which is first observed after 6 h (12). Adipocytes were washed and incubated in the presence of increasing amounts of TNF-α (0.1–100 ng/ml). Previous studies have demonstrated that maximum TNF-α-induced lipolysis is observed at the earliest after 6–24 h (12). We chose 48 h as a suitable incubation period in order to obtain maximum stimulation. At the end of the treatment, the medium was removed, and lipolysis was measured as the amount of released glycerol following a 3 h-incubation in cell medium. As determined by visual inspection, viability of the cell cultures at the end of each experiment was not overtly affected by the prolonged exposure to TNF-α. However, in accordance with previous studies (24), a small decrease (~15%) in cell survival, measured with the use of the vital stain MTT, could be observed at high concentrations (100 ng/ml, Fig. 1A). In contrast, when differentiation and viability was assessed as GPDH activity relative to protein content, there was no difference at any concentration of TNF-α (Fig. 1B). Thus, to control for even small differences in cell survival, glycerol release was expressed relative to total protein content in each well. A TNF-α concentration-dependent increase in lipolysis was observed with a small effect already at 1 ng/ml. A maximum stimulation was obtained between 10 and 100 ng/ml, reaching an average of 2-fold stimulation of basal lipolysis (Fig. 1A, n = 3). Taking together data from all performed lipolysis experiments (n = 13), 100 ng/ml TNF-α induced a 3.3-fold increase in lipolysis (44.26 ± 8.56 and 143.87 ± 32.5 μmol of glycerol/g of protein, control and TNF-α-treated, respectively, *p < 0.001*).

**TNF-α-stimulated Lipolysis Is Selectively Inhibited by MAPK Inhibitors—**To map signals involved in TNF-α-induced lipolysis, we focused on members of the MAPK family. We obtained well characterized inhibitors specific for the three different MAPK pathways, SB203580 for p38 (25), PD98059 for p44/42 (an inhibitor of MAP/ERK kinase 1/2, the kinase immediately upstream of p44/42) (26), and dimethylaminopurine (DMAP) for JNK/SAPK (27). These were initially added to the cells at concentrations known to be effective in other cell systems (25–27). To minimize background activity, cells were incubated for 2 h in the presence of the inhibitor prior to stimulation with 100 ng/ml TNF-α. Interestingly, although 75 μM PD98059 and 1 mM DMAP did not affect basal lipolysis, they displayed a potent inhibitory effect on TNF-α-stimulated lipolysis (Fig. 2B). In contrast, 20 μM SB203580 had no effect on basal or TNF-α-induced lipolysis rates under these conditions (Fig. 2B). The maximum effective concentration of PD98059 and DMAP was subsequently determined in separate concentration-response experiments (data not shown). Visual inspection and measurement of GPDH activity showed that the blockers did not appreciably alter the viability of the cell cultures either per se or in combination with TNF-α. Taken together, these results indicate that p44/42 and JNK (but not p38) are involved in regulating TNF-α-mediated lipolysis.

**MAPK Activation by TNF-α—**To obtain a more detailed analysis of the MAPK pathways activated by TNF-α, we obtained antibodies specific for the phosphorylated forms of p38, p44/42, and JNK. Cells were incubated in increasing concentrations of...
TNF-α for either 5 or 30 min, and Western blot analysis demonstrated that TNF-α could potently induce the phosphorylation of p38 with a half-maximal effect (EC₅₀) at ~1 ng/ml (Fig. 3A). This effect was observed already after 5 min and was sustained at 30 min of incubation (data not shown). Activation of JNK was also concentration-dependent with a half-maximal stimulation at ~0.5 ng/ml (Fig. 3B). However, in contrast to p38, JNK phosphorylation was only observed after 30 min of incubation. Finally, p44/42 appeared to be constitutively phosphorylated, and no apparent change in the degree of phosphorylation was observed at any concentration or time point (Fig. 3C). To control for differences in gel loading and sample concentration, all blots were stripped and incubated with antibodies that recognized both phosphorylated and non-phosphorylated forms of each MAPK (Fig. 3, lower panels). As our results indicated that p44/42 and JNK could be involved in TNF-α-induced lipolysis, we next focused on the effect of PD98059 and DMAP on the activity and phosphorylation status of their cognate MAPK.

**p44/42 Enzymatic Activity Is Enhanced by TNF-α**—The finding that p44/42 appeared to be constitutively phosphorylated was intriguing given that PD98059, an inhibitor of p44/42 activation, blocked TNF-α-induced lipolysis. Because phosphorylation is not equivalent with enzymatic activity, we obtained a kit specifically designed to assess kinase activity of p44/42 (see “Experimental Procedures” for details). Briefly, this method involves the immunoprecipitation of phosphorylated p44/42, which is then used in an in vitro kinase assay in the presence of recombinant elk-1, a target transcription factor for p44/42. Phosphorylated elk-1 is subsequently detected by Western blot analysis in which the degree of elk-1 phosphorylation is directly proportional to p44/42 activity. By this method, we could easily observe a potent TNF-α-induced stimulation of p44/42 enzymatic activity, which was fully inhibited by 75 μM PD98059, a concentration that completely inhibits TNF-α-induced lipolysis (Fig. 4). The specificity of PD98059 was assessed in separate experiments, demonstrating that phospho-

---

**Fig. 2. TNF-α-induced lipolysis in human preadipocytes is blocked by MAPK inhibitors.** Preadipocytes were incubated in the presence of increasing concentrations of TNF-α for 48 h (A). A concentration-dependent increase in lipolysis was observed reaching a maximum level at 10 ng/ml. Error bars indicate S.E. for n = 3 experiments. Preadipocytes were incubated as described under “Experimental Procedures” in either control (ctrl) medium or 100 ng/ml TNF-α (B). MAPK inhibitors were added 2 h prior to TNF-α stimulation. None of the inhibitors affected basal lipolysis. PD98059 (PD) and DMAP (inhibitors of p44/42 and JNK activation, respectively) but not SB203580 (SB, inhibitor of the p38-pathway) abrogated TNF-α-induced lipolysis completely. Statistical analysis was performed with Student’s t test and compared with basal values, * = , ** = p < 0.01, n.s. = non significant. Error bars indicate S.E. for n = 9 experiments.

**Fig. 3. TNF-α induces phosphorylation of MAPK.** A representative example of Western blots from preadipocytes treated with TNF-α in increasing concentrations. Antibodies specific for phosphorylated forms of each MAPK were used (upper panels). Blots were subsequently stripped and probed with antibodies recognizing both phosphorylated and non-phosphorylated forms of the respective protein (lower panels). Graphs representing densitometric scanning of the films are depicted to the right of each blot and are based on all performed experiments (n = 6). Values are expressed as percentage relative to maximum phosphorylation and did not differ by more than 10% between experiments. t = 0, cell lysates before stimulation with TNF-α; c = cells incubated in medium only. Phosphorylation of p38 was observed at 5 min reaching a maximum at 10 ng/ml (A). Phosphorylation was sustained even at 30 min (data not shown). JNK phosphorylation was only observed in the limited time-span of 30 min post-stimulation (B). Maximum response was attained at 1 ng/ml p44/42 phosphorylation was not altered at any TNF-α concentration or time point (C).
TNF-α Signaling and Lipolysis

FIG. 4. TNF-α-induced p44/42 kinase activity is blocked by PD98059. Preadipocytes were preincubated in the presence or absence of PD98059 (PD) for 2 h and subsequently stimulated with increasing concentrations of TNF-α for 30 min. A potent phosphorylation of elk-1, a p44/42 target transcription factor, was observed at the maximum TNF-α concentration (100 ng/ml). This was completely inhibited by 75 μM PD98059. Densitometric scanning of the films is shown in the graph (closed squares, without PD98059; open squares, with PD98059). Values are expressed as percentage relative to maximum phosphorylation of elk-1. These results were confirmed in 3 independent experiments and did not differ by more than 10%.

The concentration of 1 mM DMAP, which effectively inhibited the lipolytic action of TNF-α, resulted in an approximate 10-fold reduction of JNK phosphorylation (Fig. 5). Moreover, this effect was specific because DMAP did not affect the phosphorylation status of either p38 or p44/42 (data not shown).

MAPK Activation Is Mediated by the TNFR1 Receptor—Our results demonstrate that TNF-α is a potent inducer of MAPK signaling pathways in human adipocytes. To evaluate which receptor is responsible for this activation, we obtained mutated variants of TNF-α that specifically bind to either TNFR1 (TNF-α R32W/S86T) or TNFR2 (TNF-α D143N/A145R) (28).

FIG. 5. TNF-α-dependent phosphorylation of JNK is inhibited by DMAP. Preadipocytes were preincubated for 2 h in the presence or absence of DMAP and stimulated with TNF-α as indicated. 1 mM DMAP reduced the phosphorylation level of JNK almost 10-fold. Below, a graph representing scanning of the films (closed squares, without DMAP; open squares, with DMAP). Values are expressed as percentages relative to maximum phosphorylation of JNK. Results were similar in 3 independent experiments and did not differ by more than 10%.

MAPK Are Constitutively Activated in 3T3-L1 Cells—Previous studies on TNF-α signaling pathways in adipocytes have been performed on the murine adipocyte cell line 3T3-L1. To compare these data with our results obtained in human preadipocytes, 3T3-L1 cells were differentiated according to standard protocols. Phosphorylation assays were performed as described previously with increasing concentrations of TNF-α for 5 or 30 min. In our experimental setting, all MAPK were constitutively phosphorylated compared with levels in human adipocytes, and no further induction could be observed by TNF-α stimulation at any concentration or time point (Fig. 7).
strated that none had inhibitory effects on their non-cognate MAPK. It should be stressed that although our results appear to rule out the p38 pathway in TNF-α/H9251-induced lipolysis, this does not exclude other effects of p38 in adipocytes. Most if not all of the effects of TNF-α are mediated by TNFR1 and TNFR2. Although their extracellular regions are somewhat homologous, the intracellular portions are quite dissimilar. Both receptors lack domains with intrinsic catalytic activity, but results from recent years have demonstrated a complex intracellular signaling mechanism initiated upon ligand binding. Arrays of cytoplasmic adapter proteins with or without catalytic activity interact with specific domains in the intracellular portion of both receptors and couple to downstream effectors (20, 29). The two receptors are likely to mediate distinct intracellular effects. With the use of TNF-α mutants that selectively bind to either TNFR1 or TNFR2, we could demonstrate that MAPK activation is almost entirely attributed to binding and activation of the TNFR1 receptor. Taken together, our data are in line with studies in murine cells, demonstrating that human TNF-α (which only binds murine TNFR1) was as efficient in stimulating lipolysis as murine TNF-α (30). These results have recently been extended in murine preadipocyte cell lines lacking TNFR1 and/or TNFR2 in which TNF-α-induced lipolysis was completely lost only in cells lacking TNFR1 (31). However, the intracellular signaling pathways were not investigated in either of these studies.

Our results on TNF-α signaling in human preadipocytes differ substantially from those obtained in murine cells. In 3T3-L1 adipocytes we observed a constitutive phosphorylation of all MAPK that was not altered by TNF-α. Pekala and colleagues (21) have shown similar results in the same cell line, although in their experimental setup, p44/42 was weakly phosphorylated by TNF-α. The functional correlation of these findings was not closely investigated, but p44/42 and p38 did not appear to be involved in TNF-α-regulated GLUT-4 gene expression (21). Potential effects on lipolysis were not assessed. The difference in TNF-α-induced p44/42 phosphorylation in 3T3-L1 between this and the work of Pekala and colleagues (21) is at present unclear but could depend on slight differences in experimental conditions. Taken together, comparisons with the results in this study, obtained in human preadipocytes, underlines the importance of being cautious in interpreting data from different cellular contexts or transgenic animal studies. We cannot, however, exclude the possibility that the differentiation agents used for the human preadipocytes (BRL49653) and 3T3-L1 (isobutylmethylxanthine) may explain some of the observed differences between the two cell types.

**FIG. 6. Stimulation of TNFR1 is sufficient for MAPK activation.** Recombinant TNF-α molecules specific for either receptor (described under “Results”) were used to stimulate preadipocytes. An induction of p38 (5 and 30 min) and JNK (30 min) phosphorylation was observed with the TNFR1-specific agonist. Representative blots show phosphorylation at 30 min post-stimulation. Densitometric scanning of the films is depicted in the graph demonstrating p38 (closed squares) and JNK (open squares) phosphorylation by the TNFR1 agonist. No ligand-dependent phosphorylation was detected with the TNFR2-agonist at any concentration (closed circles, p38; open circles, JNK). Values are expressed as percentages relative to maximum phosphorylation.

**FIG. 7. MAPK are constitutively phosphorylated in 3T3-L1 cells.** Differentiated 3T3-L1 cells were incubated in the presence of increasing concentrations of TNF-α for 5 or 30 min. Representative Western blots are shown, probed with antibodies specific for phosphorylated or both phosphorylated and non-phosphorylated forms of JNK (30 min) (A), p38 (5 min) (B), and p44/42 (5 min) (C). No induction of phosphorylation could be observed at any concentration. t = 0, cell lysates before stimulation; c = cells incubated in medium only.
Because MAPK are activated by many soluble factors besides TNF-α, it is reasonable to question why only TNF-α results in increased adipocyte lipolysis. A possible answer to this question comes from studies in immortalized neuronal cells. In these cells, both epidermal growth factor (EGF) and nerve growth factor (NGF) mediate their biological effects through the MAPK p44/42. However, although EGF induces cell proliferation, NGF inhibits proliferation and potently activates differentiation. A series of intriguing experiments (32–34) demonstrated that this difference could be explained by the temporal activation of p44/42. Thus, although EGF activated p44/42 only transiently, NGF induced a prolonged activation of p44/42 (32). Inhibition of p44/42 activation blocked NGF-stimulated differentiation (33). The generality of this mechanism has since been confirmed in other cell systems (34). This demonstrates that the net effect of a cellular response depends not only on the activation of a signaling cascade but also on the amplitude and temporal pattern of activation. It is conceivable that the unique pattern of activation of different MAPK members by different ligands confers specificity to the cellular response, resulting in the unique signaling “fingerprint” of each ligand.

The lipolytic effect of TNF-α is most likely dependent on transcriptional regulation because a prolonged exposure to TNF-α is required to enhance basal lipolysis in fat cell cultures (12, 35). A recent study suggests that part of the TNF-α effect could be mediated by down-regulation of membrane Gα, resulting in abrogated antilipolytic signals (36). Others have suggested that TNF-α down-regulates the expression of perilipins, which are small phosphoproteins expressed at the surface of the intracellular lipid droplet (37). A decreased perilipin level is believed to facilitate the access of lipases to the lipid droplet. It remains to be established whether members of the MAPK family influence the expression of Gα or perilipins. Because of the limited availability of human adipose tissue for primary cultures, the determination of such interactions was outside the scope of this work. Moreover, TNF-α-mediated lipolysis is most likely dependent on several other downstream effectors, and further studies are warranted in order to define them.

The present data cannot exclude the involvement of other signaling pathways in TNF-α-induced lipolysis. Possible candidate signaling molecules are the transcription factor NF-κB and the lipid second messenger ceramide. The latter has been implicated in some of the direct effects of TNF-α on insulin signaling (38), but it still remains to be established whether it is involved in the regulation of lipolysis.

In conclusion, TNF-α stimulation of lipolysis in human adipocytes is dependent on the initial activation of members of the MAPK family, JNK and p44/42 but not p38. Moreover, MAPK activation is mediated by the TNFR1 receptor. We observe qualitative differences with results obtained in the murine cell line 3T3-L1. Our data suggest that elevated adipocyte TNF-α production in obesity may result in increased FFA release from fat depots through the initial activation of MAPK. Subsequent regulation of downstream effectors may contribute to an insulin-resistant state in humans.
Mapping of Early Signaling Events in Tumor Necrosis Factor-α-mediated Lipolysis in Human Fat Cells
Mikael Rydén, Andrea Dicker, Vanessa van Harmelen, Hans Hauner, Martin Brunnberg, Leif Perbeck, Fredrik Lönnqvist and Peter Arner

*J. Biol. Chem.* 2002. 277:1085-1091.  
doi: 10.1074/jbc.M109498200 originally published online November 1, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M109498200

Alerts:
- When this article is cited  
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 38 references, 16 of which can be accessed free at http://www.jbc.org/content/277/2/1085.full.html#ref-list-1