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Targets for TNF-α-induced lipolysis in human adipocytes

Mikael Rydén,[a,*] Elisabet Arvidsson,[a] Lennart Blomqvist,[b] Leif Perbeck,[b] Andrea Dicker,[a] and Peter Arner[a]

[a] *Department of Medicine M63, Karolinska Institutet, Karolinska University Hospital at Huddinge, 141 86 Stockholm, Sweden*
[b] *Department of Surgery K53, Karolinska Institutet, Karolinska University Hospital at Huddinge, 141 86 Stockholm, Sweden*

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Abstract

Background. Tumor necrosis factor-α (TNF-α)-induced lipolysis may be important for insulin resistance in both obesity and cachexia. In rodent cells TNF-α enhances lipolysis through down-regulation of the expression of the membrane proteins $G\alpha_i$ and the lipid droplet-associated protein perilipin (PLIN). In human (but not murine) adipocytes TNF-α stimulates lipolysis through the mitogen activated protein kinases (MAPKs) p44/42 and JNK although it is unclear whether this is mediated via PLIN and/or $G\alpha_i$. *Methods.* $G\alpha_i$ and PLIN as down-stream effectors of MAPKs were assessed in human adipocytes stimulated with TNF-α in the absence or presence of specific MAPK inhibitors. *Results.* A 48-h incubation with TNF-α resulted in a pronounced increase in lipolysis, which was paralleled by a decrease in the mRNA and protein expression of PLIN. Both these effects were inhibited in a concentration-dependent manner in the presence of MAPK inhibitors specific for p44/42 (PD98059) and JNK (SP600125). However, TNF-α did not affect $G\alpha_i$ mRNA or protein expression. Furthermore, experiments with pertussis toxin demonstrated that inhibition of $G\alpha_i$ signaling did not affect TNF-α-mediated lipolysis. *Conclusions.* Our results suggest that TNF-α-mediated lipolysis is dependent on down-regulation of PLIN expression via p44/42 and JNK. This could be an important mechanism for the development of insulin resistance in both obesity and cachexia. However, in contrast to findings in rodent cells, $G\alpha_i$ does not appear to be essential for TNF-α-induced lipolysis in human adipocytes.

Keywords: MAPK; TNF-α; Adipocyte; Lipolysis; Perilipin; $G\alpha_i$

Cachexia is a wasting syndrome observed in severe clinical conditions such as neoplasic diseases, physical or surgical trauma, and infections. It is characterized by pronounced weight loss mainly due to a dramatic loss of adipose tissue and muscle mass, anorexia, and insulin resistance [1]. The possible mechanisms behind loss of muscle have been studied intensively but the factors that affect adipose tissue are less well defined although increased rate of adipocyte lipolysis (depleting lipid stores in adipose tissue) has been proposed as the major cause [1]. The development of insulin resistance is of great clinical importance since it is generally difficult to treat and aggravates the prognosis. Curiously enough, cachexia displays several similarities with its opposite clinical entity obesity. Thus, both conditions are characterized by an increase in fat cell lipolysis, hyperlipidemia, marked insulin resistance, and ensuing development of diabetes mellitus. This has led researchers to hypothesize that insulin resistance in the two conditions may in fact share a common etiological mechanism [2]. Increased fat cell lipolysis could be an important factor through its release of free fatty acids (FFA) to the circulation. It is well known that elevated FFA levels decrease insulin sensitivity through effects on liver, muscle, and pancreas [3].

A candidate factor implicated in the development of both cachexia and obesity is tumor necrosis factor-α (TNF-α), a cytokine with a well-established role in immunomodulatory and inflammatory responses [4,5]. Most mammalian cells produce and respond to TNF-α. In 1985, TNF-α was identified as a polypeptide responsible for cachexia (hence the alternative name cachectin) [6]. Circulating levels of TNF-α are elevated in

* Corresponding author. Fax: +46-8-585-838-50.
E-mail address: mikael.ryden@medhs.ki.se (M. Rydén).

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serum from patients with cachectic states caused by parasitic infections [7], bacterial septicemia [8], and several cancer forms [9,10]. The involvement of TNF-α in obesity was first demonstrated in 1993 when it was shown that production in fat cells was increased in several rodent models of obesity or type II diabetes and that neutralization of TNF-α in obese mice resulted in amelioration of insulin resistance [11]. A similar overproduction of TNF-α in fat cells was later demonstrated in humans obesity and insulin resistance as well [12].

In vitro studies in both rodents and human have demonstrated that TNF-α stimulates adipocyte lipolysis [13,14]. It is not clear how TNF-α activates lipolysis but studies in 3T3-L1 cells have shown that TNF-α down-regulates the expression of the lipid droplet-associated protein perilipin (PLIN) which is thought to modulate the accession of hormone-sensitive lipase (HSL) to the surface of the fat droplet [15,16]. In rat adipocytes TNF-α also down-regulates the expression of the antilipolytic GTP-binding membrane proteins $G\alpha_i$; the protein expression of $G\alpha_i1$ and to a lesser extent $G\alpha_i3$, but not $G\alpha_i2$, is reduced by TNF-α [17]. In adipocyte cell culture, TNF-α stimulation of lipolysis is only observed after 6–12 h and is therefore most probably dependent on altered gene transcription and/or protein expression [17]. However, a stimulation of human adipocyte lipolysis through phosphorylation of PLIN was recently demonstrated [18], indicating that post-translational modification may also be of importance. It is unknown if TNF-α down-regulates expression of PLIN and $G\alpha_i$ in human fat cells.

TNF-α signals through two distinct receptors termed TNFR1 and TNFR2 (reviewed in [4,19]). The two receptors form multimeric complexes upon ligand binding and activate several distinct intracellular signals including signaling kinases such as the mitogen activated protein kinase (MAPK) family. MAPKs are a family of three distinct mammalian protein kinases termed p44/42, p38, and c-jun-NH_2-terminal kinase (JNK), which are activated in phosphorylation cascades ultimately phosphorylating and activating distinct sets of kinases and transcription factors [20,21]. JNK activates predominantly the transcription factors c-jun, Elk-1, and ATF-2, while ATF-2, c-fos, CREB, and the kinase MAPKAP2 are activated by p38. The p44/42 proteins activate predominantly the transcription factors Elk-1 and STAT1/3 as well as the kinase p90rsk. TNF-α-mediated lipolysis requires the activation of p44/42 or JNK but not p38 and these effects are specific for human, in contrast to murine, fat cells [14]. However, the downstream effectors of p44/42 and JNK-mediated lipolysis in human adipocytes (or fat cells from other species) are not clear although p44/42 have been linked to post-translational modification of PLIN in human fat cells [18].

Since available data suggest that TNF-α function differs between human and rodent adipocytes we set out to determine whether the effects of TNF-α on PLIN and $G\alpha_i$ expression that are found in rodent cells are also present in human adipocytes and if p44/42 and JNK play a role in conveying these effects. We used differentiated preadipocytes for the study since these primary cells can be cultured for considerably longer periods of time than mature fat cells.

Methods

Subjects and adipose tissue. Subcutaneous adipose tissue was obtained from otherwise healthy subjects who underwent surgery for non-malignant disorders. None were on any regular medication. No selection was made for age (range 18–57 years), gender or body mass index (range 21–54 kg/m^2). The study was approved by the Ethics Committee at Huddinge University Hospital. All subjects gave their informed consent to participate in the study. Specimens from subcutaneous adipose tissue was obtained within 30–45 min after the onset of surgery. All subjects fasted overnight prior to surgery and only saline was administered intravenously until the tissue samples were taken. In general, 10–35 g of adipose tissue were obtained which yielded two to three 24-well plates of preadipocytes.

Preadipocyte culture. Isolation and differentiation of preadipocytes were 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 approximately 5–10 mg and incubated with 0.5 g/L collagenase (Sigma, St. Louis, USA) in Krebs Ringer Phosphate buffer (pH 7.4) supplemented with 40 g/L dialyzed bovine serum albumin (fraction V, Sigma, St. Louis, USA) 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 pore size of 250 μm 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 erythrocyte lysis buffer (0.154 M NH_4Cl, 5.7 mM K_2HPO_4, and 0.1 mM EDTA, pH 7.3) for 10 min. Following a centrifugation step, cells were resuspended in 10 ml DMEM/F12 medium (Life Technologies, Paisley, Scotland) and filtered through a nylon filter with a pore size of 70 μm. After an additional centrifugation, the cell fraction was resuspended in DMEM/F12 medium supplemented with 10% fetal calf serum and 100 mg/L penicillin/streptomycin. Cells were seeded into 12 or 24-well plates at a density of approximately 50,000 cells/cm^2 and kept at 37 °C in 5.3 kPa CO_2 for 18–20 h. This initial incubation in fetal calf serum improves overall cell survival. Cells were then washed twice with DMEM/F12 and refed a chemically defined serum-free medium (DMEM/F12, supplemented with 100 nM cortisol, 66 nM insulin, 15 mM Hepes, 1 nM T_3, 33 μM biotin, 17 μM pantothenate, 10 μg/ml transferrin, 100 mg/L penicillin/streptomycin, and 2.5 μg/ml amphotericin B). To differentiate the cells, a thiazolidinedione, rosiglitazone (BRL 49653, kindly provided by SmithKline Beecham Pharmaceuticals, Harlow, Essex, UK), was added during the 14-day differentiation process to a final concentration of 10 μM (days 1–6). Cells were maintained in the medium at 37 °C and 5.3 kPa CO_2 for a total of 16 days and 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 $\geqslant$70% were used in experiments. Plates containing more than 5% contaminating endothelial cells were discarded. Differentiation was determined by quantifying glycerol-3-phosphate dehydrogenase (GPDH) activity as described previously [14]. Expression of the adipocyte-specific enzyme hormone sensitive lipase (HSL) 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).

Reverse transcription and real time polymerase chain reaction. Total RNA was extracted from differentiated preadipocytes, using the RNeasy mini kit (Qiagen GmbH, Hilden, Germany) and the RNA concentration and purity were assessed spectrophotometrically. One microgram of total RNA from each sample was reverse transcribed to cDNA using the Omniscript RT kit (Qiagen GmbH, Hilden, Germany) and oligo(dT) primers (Invitrogen, Tåstrup, Denmark). In a final volume of 25 μl, 5 ng cDNA was mixed with 2× SYBR Green PCR master mix (Eurogentec S.A., Ougrée, Belgium) and primers (Invitrogen, Tåstrup, Denmark). The primer pairs were selected to yield a single amplicon based on dissociation curves and analysis by agarose gel electrophoresis. The selected PLIN (Accession No. NM_002666) primers were sense-TGGA GACTGAGGAGAACAAG (in exon seven) and antisense-AT GTCACAGCCGAGATGG (in exon eight), spanning bases 1059–1178, yielding a fragment of 120 bp. The $G\alpha_i1$ (Accession No. NM_002069) primers were sense-TCTACAGTAACACCATCCAG TC and antisense-GCAGTCATAAAGCCTTCTTCAG spanning bases 555–699, product length 145 bp. The $G\alpha_i2$ (Accession No. NM_002070) primers were sense-AGGTGAAGTTGCTGCTGTTG and antisense-CTGGATGGTGTTGCTGTAGAC spanning bases 221–360, yielding a product of 140 bp. The primer pair for glyceraldehyde-3-phosphate dehydrogenase (GAPDH, NM_002046) was sense-CACATGGCCTCCAAGGAGTAAG and antisense-CCAG CAGTGAGGGTCTCTCT, spanning bases 1063–1144 resulting in an 82 bp long product. Quantitative real-time PCR was performed in an iCycler IQ (Bio-Rad Laboratories, Hercules, CA, USA). The thermal cycler parameters for PLIN were: 10 min at 95 °C followed by amplification of the cDNA for 40 cycles with 15 s at 95 °C and 60 s at 59 °C. For $G\alpha_i1$ and $G\alpha_i2$ the same steps were used although annealing was performed for 20 s at 58 and 59 °C, respectively, and an additional elongation step was introduced at 72 °C for 20 s. The mRNA levels were determined by a comparative C_t method (see User bulletin #2, ABI Prism 7700, pp. 11–15, available from Applied Biosystems, Foster City, CA, USA). C_t values were normalized to the reference gene GAPDH which was amplified in parallel reactions. The PCR efficiency in all runs was close to 100% and all samples were run in triplicate.

Protein expression. After 14 days of differentiation, preadipocytes were incubated in the presence of 100 ng/ml TNF-α in combination with the specific MAPK-inhibitors SP600125 [23], PD98059 [24], and SB203580 [25] (all from Sigma, St. Louis, USA). All inhibitors were added 2 h before the initiation of stimulation in order to diminish intrinsic MAPK activity to a minimum. All incubations were performed in triplicate or quadruplicate. Wells incubated in the absence of TNF-α were termed control samples. After 48 h (16th day of differentiation) the medium was removed and cells were lysed in an ice-cold buffer. For cytosolic fractions the lysis buffer contained 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, and 1 mM PMSF. Cell lysates were centrifuged at 14,000 rpm for 30 min at +4 °C, and the supernatant was removed to new tubes. For membrane fractions the protocol described by Green and co-workers [17] was followed. The total amount of protein was measured using the BCA protein assay kit (Pierce, Rockford, IL, USA). Equal amounts of protein 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 PVDF membranes (Amersham, 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 PLIN (Progen Biotechnik, Heidelberg, Germany), $G\alpha_i$ or β-actin (Sigma, St. Louis, MO, USA). Following washing steps in TBS-T and incubation with secondary antibodies conjugated to horseradish peroxidase, antigen–antibody complexes were detected by chemiluminescence (Supersignal, Pierce, Rockford, USA) and exposed to high-performance chemiluminescence film (Amersham–Pharmacia, Little Chalfont, UK). Films were scanned and specific bands were quantified using the NIH Image software and corrected for the intensity of the β-actin band in the same sample. To control for interexperimental differences the OD values from all bands were expressed relative to those from control cells, which were set at 100%.

Lipolysis. Experiments were conducted as described [14]. In brief, adipocytes were treated with 100 ng/ml TNF-α, with or without inhibitors or pertussis toxin (PTX). The toxin was pretreated as described [26] to unmask its ability to inactivate $G\alpha_i$ and used at a concentration (1 μg/ml) which has previously been shown to have a maximum effect on lipolysis in human fat cells [26]. Control cultures, incubated in medium alone, were set up in parallel. After 48 h the supernatant was removed, cells were washed and subsequently incubated for 3 h at 37 °C in medium supplemented with 20 g/L BSA. Following incubation, medium was removed and kept at −20°C for subsequent measurement of glycerol concentration (an index of lipolysis) using an ultrasensitive bioluminescence method [27]. To control for interexperimental differences in adipocyte differentiation, release of glycerol was expressed as the concentration in the medium per GPDH activity and cell number (protein content) of each sample (mM glycerol/GPDH (mU/mg)/protein/well).

Statistical methods. Values are given as mean ± standard error (SE). Analysis of variance (ANOVA) and Student's paired or unpaired *t* test were used for statistical analysis. A value of $P < 0.05$ (two-sided) was regarded as statistically significant.

Results

TNF-α reduces PLIN expression through p44/42 and JNK but not p38

Human preadipocytes were differentiated and then incubated for 48 h in the absence or presence of 100 ng/ml TNF-α. The effect on PLIN mRNA expression was assessed by real-time PCR. TNF-α treatment resulted in a reduction of PLIN mRNA to 25% of control (0.34 ± 0.044 and 0.080 ± 0.026 relative units (RU); control and TNF-α, respectively, $P = 0.0015$, $n = 6$, Fig. 1A). The effect on PLIN protein levels was determined by Western blot analysis of cytosolic protein lysates from the same set of cells using a primary antibody raised against human PLIN. TNF-α reduced PLIN expression to 50% of control (Fig. 1B). The role of individual MAPKs in affecting PLIN protein expression was determined by incubating cells in the presence of cognate inhibitors for JNK (20 μM SP600125 [23]), p44/42 (75 μM PD98059 [24]), and p38 (20 μM SB203580 [25]) with or without TNF-α (Fig. 1B). The inhibitors had no intrinsic effect on PLIN expression. SB203580 did not influence the reduction in PLIN expression induced by TNF-α. In contrast, the other two inhibitors counteracted significantly the TNF-α-mediated reduction of PLIN expression (TNF-α + PD98059 and

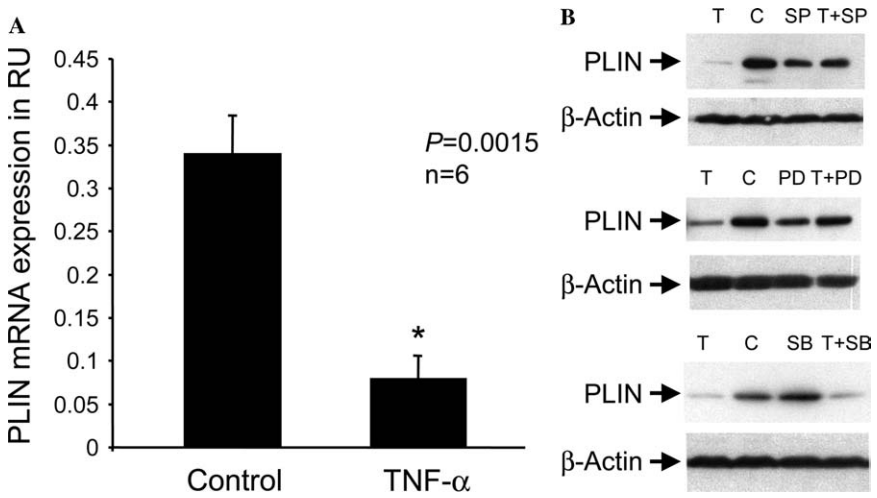

Fig. 1. TNF-α-induced reduction in PLIN mRNA and protein. (A) Real-time PCR using primers specific for human PLIN was performed on preadipocytes treated for 48 h with TNF-α or medium alone (control). TNF-α reduced PLIN mRNA significantly (* = significant difference from control). Data summarizes results from six independent experiments. (B) Representative immunoblots of PLIN expression. Human preadipocytes were incubated for 48 h in medium (C), TNF-α 100 ng/ml (T), inhibitors alone (SP = SP600125, PD = PD98059, and SB = SB203580) or concomitant presence of TNF-α and inhibitors (T + SP, T + PD, and T + SB). PLIN protein levels were determined by Western blot. TNF-α reduced PLIN protein levels by approximately 50% while incubation with the inhibitors alone did not affect PLIN expression. In the presence of TNF-α, SP, and PD, but not SB, abrogated the reduction in PLIN expression induced by TNF-α. To control for differences in loading, blots were probed with an antibody directed against β-actin.

TNF-α + SP600125, $80 \pm 9\%$ and $97 \pm 3\%$ of control, respectively, $P < 0.0001$, $n = 12$, Fig. 1B).

TNF-α-induced lipolysis is paralleled by reduced PLIN expression

To study if TNF-α-induced lipolysis is associated with reduced PLIN expression, the quantitative relationship between these two parameters was determined in experiments stimulating preadipocytes with 100 ng/ml TNF-α and different concentrations of MAPK blockers. Incubation with TNF-α for 48 h resulted in 2- to 3-fold increase in basal lipolysis ($n = 3$, Fig. 2A). Co-incubation with either 75 μM PD98059 or 20 μM SP600125 completely abrogated the lipolytic effect of TNF-α. In the case of SP600125 the effect was so pronounced that even a reduction of basal lipolysis compared to control cells was observed. Treating the cells with TNF-α and serial dilutions of each inhibitor restored the effect of TNF-α in a concentration-dependent fashion. In the case of PD98059 this was observed already at a dilution of 1:9 while SP600125 required dilution up to 1:27 before TNF-α gained full effect. Similar experiments were performed to assess PLIN protein expression. As shown above, treatment with 100 ng/ml TNF-α resulted in a 50% reduction of PLIN protein while co-incubation with either 75 μM PD98059 or 20 μM SP600125 completely abrogated this effect ($n = 12$, Fig. 2B). Serial dilutions of both inhibitors resulted in an increased effect of TNF-α on PLIN protein level, which was fully restored at dilutions of 1:9 or more.

TNF-α effects on $G\alpha_i$ expression

The effect of TNF-α on $G\alpha_i$ expression was determined by real-time PCR assays for human $G\alpha_i1$ and $G\alpha_i2$. Differentiated preadipocytes were treated with either 100 ng/ml TNF-α or medium alone followed by total RNA extraction. TNF-α did not affect the expression of $G\alpha_i1$ (0.17 ± 0.028 and 0.13 ± 0.016 RU, control and TNF-α, respectively, $P = 0.11$, $n = 6$, Fig. 3A, upper panel). Curiously, TNF-α treatment resulted in a slight although significant increase in $G\alpha_i2$ (0.31 ± 0.037 and 0.49 ± 0.046, control and TNF-α, respectively, $P = 0.0021$, $n = 6$, Fig. 3A, lower panel). In order to determine effects on protein expression, membrane fractions from the same set of cells were isolated and approximately 10 μg of membrane protein was subjected to Western blot analysis using a commercially available antibody directed against all three forms of human $G\alpha_i1$–3. There was no significant effect of TNF-α on the intensity of the band corresponding to $G\alpha_i1$–3 ($n = 3$, Fig. 3B).

TNF-α-mediated lipolysis is not influenced by $G\alpha_i$ inhibition

To study if TNF-α could stimulate lipolysis when $G\alpha_i$ was functionally inhibited we set up an assay using PTX, a well-established selective inhibitor of $G\alpha_i$. Incubation of preadipocytes with a maximum effective concentration of 1 μg/ml of PTX or TNF-α treatment alone resulted in a significant increase in basal lipolysis compared to control cells (Fig. 4). However, addition of

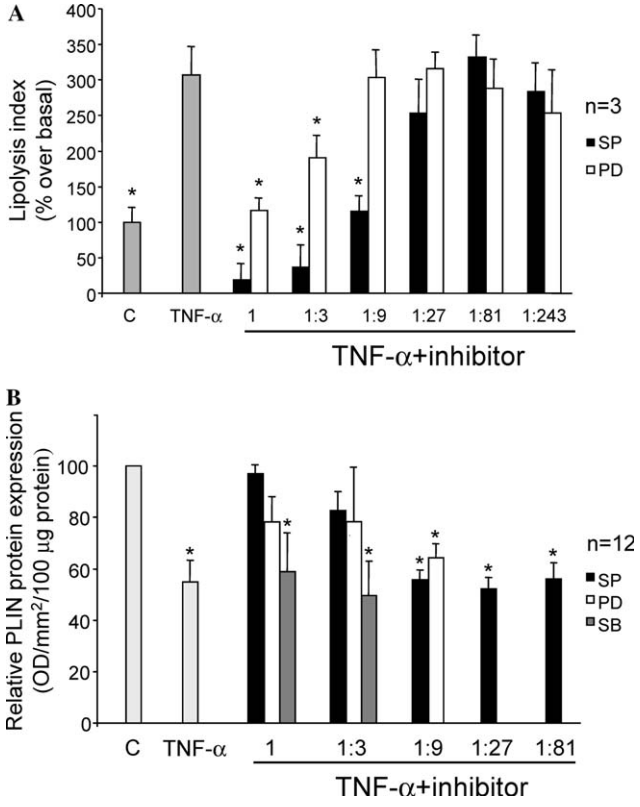

Fig. 2. TNF-α-mediated lipolysis is paralleled by a decrease in PLIN protein expression. (A) Differentiated preadipocytes were incubated for 48 h with medium alone (C), TNF-α or TNF-α in combination with dilutions of the indicated inhibitors. Basal lipolysis was measured and expressed relative to that of control cells. Basal lipolysis was activated approximately 2- to 3-fold by TNF-α. Both inhibitors were able to block the effect of TNF-α in a concentration-dependent manner. PD98059 did not show any inhibitory effect at a dilution exceeding 1:9 while SP600125 required a dilution up to 1:27 before loss of effect (* = significantly different from cells treated with TNF-α alone, $P < 0.05$). (B) Cytosolic protein fractions were isolated from preadipocytes treated as in (A). PLIN protein was detected by Western blot, films were scanned, and the densitometry was related to that of control (and corrected for β-actin). TNF-α reduced PLIN protein by approximately 50%. SB203580 (SB) did not influence this effect while PD and SP counteracted the reduction up to a dilution of 1:3 (* = significantly different from control cells, $P < 0.05$).

PTX to TNF-α incubated cells did not increase basal lipolysis above that observed with TNF-α alone where TNF-α + PTX induced a 50% higher rate of lipolysis than PTX alone ($n = 3$, $P < 0.05$).

Discussion

In this paper, we have obtained novel and species-specific insights into the mechanisms of TNF-α-mediated stimulation of lipolysis in human fat cells. TNF-α reduces PLIN expression significantly both at the mRNA and protein levels. This effect can be counteracted in the presence of inhibitors specific for JNK and p44/42. Furthermore, the reduction in PLIN protein is paralleled by an increase in basal lipolysis indicating a direct correlation between the two findings. This notion is further supported by the fact that both these effects can be abrogated in a concentration-dependent fashion by inhibition of either JNK or p44/42. In contrast to findings in rat adipocytes [17], TNF-α did not reduce $G\alpha_i$ expression in human preadipocytes. Furthermore, functional inhibition of $G\alpha_i$ with pertussis toxin did not abolish TNF-α-induced stimulation of lipolysis, suggesting that $G\alpha_i$ regulation is not a major pathway for lipolysis in human fat cells.

PLIN has emerged as an interesting protein regulating lipolysis [28]. PLIN protein coats the intracellular droplets and modulates lipolysis by regulating the substrate accessibility for the rate limiting lipolytic enzyme HSL [15]. Thus, overexpression of PLIN protects cells from lipolysis [29] while mice homozygous for a PLIN null mutation are lean and resistant to the development of obesity [30,31]. Furthermore, there is a reverse relationship between the lipolytic activity and PLIN content in human fat cells and an association between PLIN gene polymorphisms and lipolytic activity [32]. We observed a significant reduction in PLIN mRNA and protein after stimulation with TNF-α for 48 h when a maximum lipolytic effect was previously observed [14]. In a recent study performed in human preadipocytes [18], it is suggested that one lipolytic effect of TNF-α is to increase intracellular cyclic AMP levels. This may be achieved by a decreased expression of phosphodiesterase 3B and activation of cyclic AMP-dependent protein kinase (PKA) [33]. The authors observed increased PLIN phosphorylation mediated by p44/42 while total levels were not altered significantly [18]. In their experimental setting cells were stimulated for 24 h, which may be a too short period to observe reduced protein expression levels presently observed at 48 h. In a recent study in 3T3-L1 cells, PLIN levels were reduced by TNF-α via p44/42 but not JNK [16]. In our experimental setting p44/42 and JNK appear to be of equal importance in reducing PLIN levels induced by TNF-α. The mechanisms are not entirely clear but could depend on the convergence of both MAPKs in activating similar transcription factors.

TNF-α reduces the protein expression of $G\alpha_i1$ and $G\alpha_i3$ in rat adipocytes [17]. Since these proteins relay signals from antilipolytic receptors a reduced membrane concentration of $G\alpha_i$ would diminish the efficiency of antilipolytic signaling hence increasing lipolysis. We investigated $G\alpha_i1$ and $G\alpha_i2$ since the effect in the previous study was most prominent on $G\alpha_i1$ while no effect was observed on $G\alpha_i2$ expression. The latter could therefore function as a negative control. In human fat cells, TNF-α did not have any negative effect on either $G\alpha_i$ form, in the case of $G\alpha_i2$ we even observed a significant increase in mRNA. These results were subsequently confirmed at the protein level where no effect on protein expression

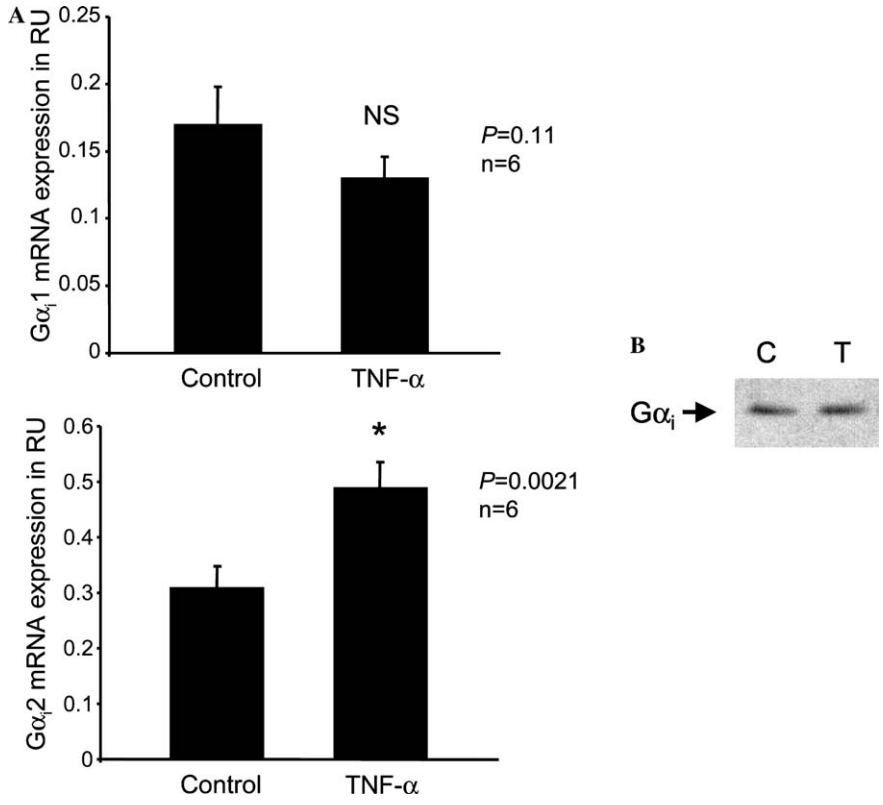

Fig. 3. TNF-α does not reduce $G\alpha_i$ mRNA or protein expression. (A) Real-time RT-PCR using primers specific for human $G\alpha_i1$ and $G\alpha_i2$ was performed on cells treated with TNF-α or medium alone. TNF-α did not affect the expression of $G\alpha_i1$ (upper panel) while $G\alpha_i2$ was even slightly increased (* = significantly different from control cells, NS = non-significant difference). Data summarize results from six independent experiments. (B) $G\alpha_i$ protein expression was not influenced by TNF-α. Experiments were repeated three times.

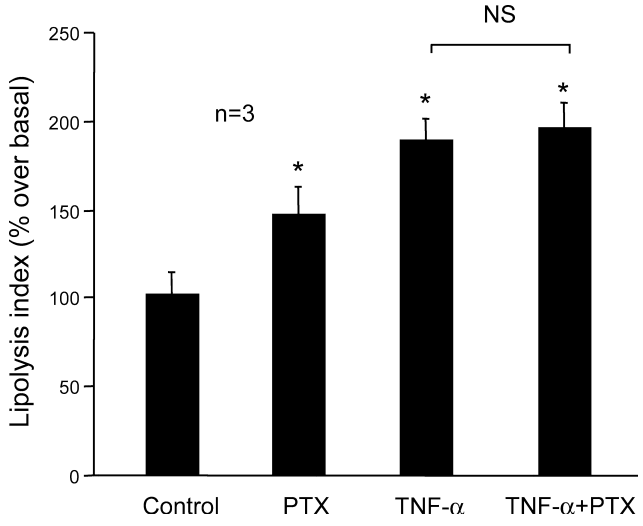

Fig. 4. TNF-α-induced lipolysis is not affected by $G\alpha_i$ inhibition. Preadipocytes were incubated for 48 h in medium (control) or 1 μg/ml of pertussis toxin (PTX) with or without 100 ng/ml TNF-α. Basal lipolysis was determined and expressed relative to control. Inhibition of $G\alpha_i$ by PTX resulted in a 50% increase in basal lipolysis while TNF-α increased it approximately 2-fold. PTX did not enhance the effect of TNF-α (* = significantly different from control cells, $P < 0.05$, NS = non-significant difference).

could be seen. However, it should be borne in mind that we used a commercially available antibody that recognizes all three forms of $G\alpha_i$. We could therefore not study selective effects of TNF-α treatment on the different protein isoforms. Nevertheless, our PTX experiments clearly show that a TNF-α-mediated lipolytic effect is present during $G\alpha_i$ inhibition. Therefore, $G\alpha_i$ is probably not a major pathway utilized by TNF-α in human fat cells, demonstrating yet another important species difference between human and rodent cells in addition to those previously described [34].

On the basis of present and previous [14,18,35] findings we propose the following novel human-specific model for TNF-α-mediated stimulation of lipolysis. The cytokine acutely activates MAPK pathways and causes an increase in cyclic AMP production and PLIN phosphorylation mediated by p44/42. Prolonged stimulation causes a transcriptionally mediated decrease in protein production which is conveyed through JNK and p44/42. The increase in lipolysis results in elevated release of free fatty acids to the circulation which in turn have a well-known deleterious effect on insulin sensitivity [3].

Interpretation of data from cell signaling studies is always dependent on the growth and differentiation conditions of the particular cell under study. Most

studies on TNF-α signaling have been performed in 3T3-L1 cells, an immortalized murine adipocyte cell line. Cell lines differ in many respects from primary cells. The unlimited cell survival and growth characterized by the former is often dependent on alterations in their signaling pathways. For instance, in our hands, 3T3-L1 cells display a constitutive MAPK phosphorylation which cannot be further enhanced by exogenous TNF-α [14]. The similarities and differences between human preadipocytes and rodent cell lines underline the importance of being cautious in interpreting data from studies in non-human cells and cell lines.

We have used MAPK inhibitors to elucidate the importance of specific pathways in primary cultures of human preadipocytes. An alternative and more direct way of assessing the importance of these pathways would be to transiently or stably overexpress different MAPKs, PLIN or $G\alpha_i$ (and/or dominant negative constructs) and evaluate their effect on TNF-α-activated lipolysis. Unfortunately, overexpression in preadipocytes is hampered by the low transfection efficiency in primary cells, the restricted availability of human tissue, and differential genetic background making interpretations difficult. Adenoviral infection with corresponding constructs is feasible but is characterized by transient expression levels as well [36]. Since no human immortalized fat cell lines are available for genetic transformation, more advanced signaling studies in human preadipocytes will have to wait.

In summary, TNF-α activates lipolysis in human adipocytes by reducing PLIN. In contrast to results in rodent fat cell lines, TNF-α does not affect $G\alpha_i$ expression or function in human cells. PLIN reduction is mediated through both p44/42 and JNK and could be of etiological importance for the development of insulin resistance in both cachexia and obesity.

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