

NF- κ B is important for TNF- α -induced lipolysis in human adipocytes

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Abstract Tumor necrosis factor- α (TNF- α) promotes lipolysis in mammal adipocytes via the mitogen-activated protein kinase (MAPK) family, resulting in reduced expression/function of perilipin (PLIN). The role of another pivotal intracellular messenger activated by TNF- α , nuclear factor- κ B (NF- κ B), has not been recognized. We explored the role of NF- κ B in TNF- α -induced lipolysis of human fat cells. Primary cultures of human adipocytes were incubated in the presence of a cell-permeable peptide that inhibits NF- κ B signaling (WP). Incubation with WP, but not with a biologically inactive peptide (MP), abolished the nuclear translocation of NF- κ B and effectively abrogated TNF- α -induced lipolysis in a concentration-dependent manner. Western blot analysis demonstrated that although TNF- α per se reduced mainly PLIN protein expression, TNF- α in the presence of WP resulted in a pronounced combined reduction of both hormone-sensitive lipase (HSL) and PLIN protein. The expression of a set of other lipolytic or adipocyte-specific proteins was not affected. The regulation was presumably at the transcriptional level, because mRNA expression for HSL and PLIN was markedly reduced with TNF- α in the presence of NF- κ B inhibition. This was confirmed in gene reporter assays using human PLIN and HSL promoter constructs. **We conclude that in the presence of NF- κ B inhibition, TNF- α -mediated lipolysis is reduced, which suggests that NF- κ B is essential for retained human fat cell lipolysis.**—Laucinkiene, J., V. van Harmelen, E. Arvidsson Nordström, A. Dicker, L. Blomqvist, E. Näslund, D. Langin, P. Arner, and M. Rydén. **NF- κ B is important for TNF- α -induced lipolysis in human adipocytes.** *J. Lipid Res.* 2007. 48: 1069–1077.

Supplementary key words tumor necrosis factor- α • nuclear factor- κ B • signal • differentiation

The release of energy-rich FFAs through lipolysis is a key event in adipocyte function, and increased circulating FFA levels promote insulin resistance (1). In obesity and cachexia as well as other clinical conditions, the lipolytic activity of fat cells is increased, resulting in reduced insulin sensitivity. Although a number of hormones and other external factors enhance lipolysis in fat cells, abnormal autocrine regulation could be a common pathological factor for obesity and cachexia, as reviewed (2, 3). Adipocytes as well as stromal cells in adipose tissue produce the cytokine tumor necrosis factor- α (TNF- α). In vitro studies have demonstrated that TNF- α stimulates adipocyte lipolysis in murine and human adipocytes (4). TNF- α expression is increased in obesity in both rodent and human adipose tissue (5, 6) and is also enhanced in animal models of cachexia (7).

TNF- α promotes lipolysis via several mechanisms. For instance, TNF- α downregulates phosphodiesterase-3B (PDE3B) expression in 3T3-L1 cells (8) and human adipocytes (9). PDE3B is the major hydrolytic enzyme of cAMP activated by insulin, which thereby mediates the antilipolytic effect of this hormone. Another proposed mechanism of TNF- α is downregulation of the GTP binding protein $G\alpha_i$, which mediates antilipolytic signals. However, the expression of $G\alpha_i$ is downregulated in rodent (10) but not human adipocytes (11). In both rodent and human cells, TNF- α activation promotes phosphorylation (9) and the downregulation of perilipin (PLIN) expression (11, 12). PLIN is located on the surface of the intracellular triglyceride lipid droplet and regulates the access of hormone-sensitive lipase (HSL), the main hydrolytic

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enzyme of stored triglycerides in human fat cells. The alterations at the levels of PLIN, PDE3B, and $G\alpha_i$ lead to increased basal (spontaneous) lipolysis.

To date, the PLIN effect is the best studied in human lipolysis (11). However, it is still not entirely clear how PLIN regulates lipolysis. Two different models have been proposed. In the barrier/translocation model introduced by Londos et al. (13), PLIN constitutes a physical barrier to HSL. Upon PLIN phosphorylation and downregulation, lipid surface accessibility for HSL is enhanced. This fits with data from PLIN knockout mice, which display an increased basal adipocyte lipolysis. In contrast, a series of recent studies have proposed a second model in which PLIN functions as a scaffold protein. In this model, PLIN phosphorylation leads to an altered structure and/or interaction with regulatory proteins. PLIN thereby recruits HSL and probably increases lipase access to the lipid surface (14–17). It is important to stress that neither of these models has been demonstrated in human adipocytes; rather, both are based on studies in rodent fat cells and immortalized cell lines.

The intracellular signaling pathways elicited by TNF- α form a complicated and intricate web that has mainly been characterized in nonfat cells (18). Some of the signals that mediate the TNF- α effect on lipolysis have been dissected in adipocytes (9, 19). These involve p44/42 and *c-jun* NH₂-terminal kinase (JNK) of the mitogen-activated protein kinase (MAPK) family, which phosphorylate and activate downstream transcription factors and thereby regulate the expression of genes involved in lipolysis. Most importantly, p44/42 and JNK relay the effects on PLIN (11, 20) and PDE3B (9).

Additional pathways besides MAPKs could mediate TNF- α effects on adipocyte lipolysis. One candidate is nuclear factor- κ B (NF- κ B) (21), which is a homodimer or heterodimer composed from five related transcription factors. These contain a conserved sequence that is responsible for dimerization and DNA binding; the best characterized members are p65 and p50 (22). In the cytoplasm, NF- κ B is bound to inhibitors of NF- κ B (I κ Bs), which prevent NF- κ B from entering the cell nucleus. TNF- α activates a set of proteins termed I κ B kinases (IKKs). IKKs are trimeric complexes formed by α , β , and γ subunits in which the α and β subunits phosphorylate I κ B. This leads to an altered conformation of I κ B, which releases NF- κ B to enter the nucleus while I κ B is targeted to the proteasome degradation pathway.

The function of NF- κ B in human adipocytes is unclear, although a few reports in murine cells indicate that NF- κ B inhibits adipogenesis (23) and that TNF- α downregulates adipocyte-specific genes via NF- κ B (24). Studies of NF- κ B have to some extent been hampered by the lack of selective and specific inhibitory compounds. However, a 28 amino acid long peptide derived from the IKK γ sequence was recently developed. This peptide inhibits trimerization of the IKK complex and the subsequent activation of NF- κ B (25). The peptide includes a short sequence derived from the *Drosophila antennapedia* homeo-domain protein, which enables cell membrane translo-

cation. It is an effective NF- κ B inhibitor and inflammatory antagonist in different in vitro (26) and in vivo (27) models. To study the role of NF- κ B in adipocyte lipolysis, we treated primary cultures of human adipocytes with this inhibitor (herein referred to as WP) and TNF- α . Parallel experiments were performed with a similar but biologically inactive (mutated) peptide lacking the membrane permeabilization sequence (MP).

MATERIALS AND METHODS

Subjects and adipose tissue

Subcutaneous adipose tissue was obtained from otherwise healthy subjects who underwent gastric banding surgery for obesity or abdominal liposuction for cosmetic reasons. No subject was on any regular medication. No selection was made for age, gender, or body mass index. The study was approved by the Ethics Committee at Karolinska University 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 before surgery, and only saline was administered intravenously until the tissue samples were taken. In general, 5–40 g of adipose tissue was obtained, which yielded two to three 24-well plates of preadipocytes.

Cell culture

Isolation and differentiation of preadipocytes were performed as described previously (28). After differentiation, >70% of the adipocytes displayed a round shape with a cytoplasm completely filled with multiple fat droplets. Only cultures with a differentiation density of $\geq 70\%$ were used in experiments. Differentiation was determined by quantifying glycerol-3-phosphate dehydrogenase activity as described previously (19). Viability was assessed as described previously (19) using the 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide assay. The isolation and differentiation of mesenchymal stem cell cultures from human subcutaneous adipose tissue were set up as described in detail previously (29).

RT-PCR

Cells were treated as described below. Total RNA was extracted from differentiated adipocytes using the RNeasy mini kit (Qiagen GmbH, Hilden, Germany), and the RNA concentration and purity were assessed spectrophotometrically with an Agilent Bioanalyzer. Half a microgram of total RNA from each sample was reverse-transcribed to cDNA using the Omniscript reverse transcription kit (Qiagen) and oligo(dT) primers (Invitrogen, Tåstrup, Denmark). In a final volume of 25 μ l, 5 ng of cDNA was mixed with 2 \times SYBR Green PCR Master Mix (Eurogentec S.A., Ougrée, Belgium) and primers (Invitrogen). The primer pairs were selected to yield a single amplicon based on dissociation curves and analyzed by agarose gel electrophoresis. The selected PLIN primers were sense (TGGAGACTGAGGAGAACAAG) and antisense (ATGTCACAGCCGAGATGG), spanning bases 1,059–1,178. The selected HSL primers were sense (CTCAGTGTGCTC-TCCAAGTG) and antisense (CACCCAGCGGAAGTCTC), spanning bases 2,606–2,747. The primer pair for interleukin-6 was sense (AGACAGCCACTCACCTCTTCAG) and antisense (TTTCTGCCAGTGCTCTTTGC), spanning bases 192–324. The primer pair for adipose triglyceride lipase (ATGL) was sense (GTGTCAGACGGCGAGAATG) and antisense (TGGAGGGA-

GGGAGGGATG), spanning bases 563–681. The primer pair for glyceraldehyde-3-phosphate dehydrogenase was sense (ACCCAC-TCCCTCCACCTTTGAC) and antisense (TCCACCACCCTGTTG-CTGTAG), spanning bases 943–1,052. Quantitative real-time PCR was performed in an iCycler IQ™ (Bio-Rad Laboratories, Inc., Hercules, CA). mRNA levels were determined by a comparative threshold cycle (C_t) method. 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 duplicate.

Protein expression

After 12–14 days of differentiation, adipocytes were incubated in the presence of 100 ng/ml TNF- α in combination with 100 μ M WP or MP. The peptide sequences have been described previously (25), and purified peptides were ordered from Innovagen AB (Lund, Sweden). Peptides were added 2 h before the initiation of stimulation to enable efficient membrane translocation. All incubations were performed in triplicate. Wells incubated in the absence of TNF- α and/or peptides were termed control samples. After 48 h, 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 -glycerophosphate, 1 mM Na_3VO_4 , 1 μ g/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. The total amount of protein was measured using the BCA protein assay kit (Pierce, Rockford, IL). Equal amounts of protein from each sample were boiled in 1 \times SDS loading buffer for 5 min and loaded and separated by SDS-PAGE. Gels were then blotted onto polyvinylidene difluoride 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% nonfat dried milk and subsequently incubated overnight at +4°C in the presence of antibodies specific for PLIN (Progen Biotechnik, Heidelberg, Germany), HSL (a gift from Dr C. Holm, Lund University, Sweden), peroxisome proliferator-activated receptor γ (PPAR γ ; Santa Cruz Biotechnology, Santa Cruz, CA), regulatory subunit 2 β of protein kinase A (PKAR2 β ; Transduction Laboratories), and β -actin (Sigma, St Louis, MO). After washing steps in TBS-T and incubation with secondary antibodies conjugated to horseradish peroxidase, antigen-antibody complexes were detected by chemiluminescence (Supersignal®; Pierce, Rockford, IL) and exposed to high-performance chemiluminescence film (Amersham Pharmacia, Little Chalfont, UK).

NF- κ B nuclear translocation assay

To detect NF- κ B activation, we used the TransAM NF- κ B p65 kit from Active Motif (Rixenart, Belgium), which is a sensitive

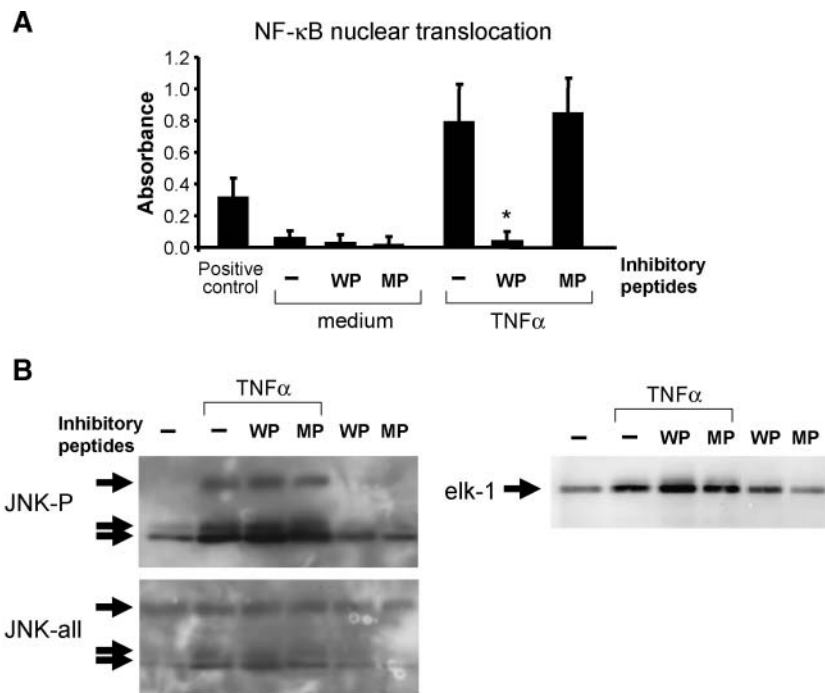


Fig. 1. Inhibitory effects of a cell-permeable peptide (WP) that inhibits NF- κ B signaling and translocation but not mitogen-activated protein kinase (MAPK) phosphorylation and activity upon TNF- α stimulation. A: Human adipocytes were treated with medium alone, WP, peptide lacking the membrane permeabilization sequence (MP), or 100 ng/ml TNF- α with or without 100 μ M WP or MP, respectively, as indicated. Nuclear extracts were isolated and analyzed with an ELISA kit, and specific binding was determined by colorimetry. Positive control cells derived from Jurkat cells were supplied by the manufacturer. Values shown are means \pm SD. * $P < 0.05$ versus TNF- α -treated cells. B: NF- κ B inhibition does not affect MAPK activity. Cells were incubated as indicated for 2 h in the presence or absence of WP or MP. TNF- α (100 ng/ml) was then added for 20 min, after which proteins were isolated and analyzed by Western blot and probed with antibodies against phospho-c-jun NH₂-terminal kinase (phospho-JNK; upper left panel) or total JNK (lower left panel). Effects on p44/42 were determined in a similar manner using activity assays with elk-1 as a substrate (right panel). Blots are representative examples from three independent experiments.

ELISA-based method. Preadipocytes were seeded on six-well plates at a density of $\sim 30,000$ cells/cm², differentiated as described above, and kept in culture until day 12. Cultures were incubated for 2 h in the presence or absence of MP or WP at a concentration of 100 or 33 μ M. Thereafter, TNF- α was added at a concentration of 100 ng/ml, and the cells were further incubated for 4 h. Nuclear extracts were prepared using the nuclear extract kit from Active Motif according to the manufacturer's instructions. Nuclear extract (2.5 μ g) was used in the binding reactions. Jurkat nuclear extract (2.5 μ g) was used as a positive control. Wild-type and mutated consensus oligonucleotides were used in the assay as a competitor for NF- κ B binding to monitor the specificity of the assay. Wild-type but not mutated consensus oligonucleotides inhibited TNF- α -induced NF- κ B p65 activation (data not shown).

Phosphorylation and enzyme activity assays

Detection of phosphorylated JNK was performed using a set of reagents from Cell Signaling as described previously (19). Briefly, adipocytes were treated with 100 ng/ml TNF- α for 20 min. Cells were subsequently lysed according to the instructions in the kit and separated by SDS-PAGE, and proteins were detected by Western blotting using antibodies directed against phosphorylated and total (phosphorylated and nonphosphorylated forms) JNK. Because p44/42 is often constitutively phosphorylated in

human adipocytes, we used a previously described kit (19) to detect p44/42 enzyme activity (Cell Signaling). Cells were treated as described above, and cellular lysates were immunoprecipitated with Sepharose-bound anti-p44/42 antibodies. After washing, elk-1, a substrate for p44/42, was added in the presence of ATP, and the kinase assay was performed at 30°C for 30 min. The reaction was stopped by a 5 min boiling step, and proteins were separated by Western blotting. Phosphorylated elk-1 was detected with phospho-specific antibodies.

Lipolysis

Experiments were conducted as described (19). In brief, after 48 h of treatment with TNF- α with or without peptide inhibitors, the medium was removed and cells were washed and subsequently incubated for 3 h at 37°C in DMEM/F12 medium supplemented with 20 g/l BSA. After incubation, medium aliquots were removed and kept at -20°C for subsequent measurement of glycerol concentration (an index of lipolysis) using a sensitive bioluminescence method (30). To control for interexperimental differences in adipocyte differentiation, release of glycerol was expressed as a percentage over control.

Transient transfections and reporter assays

The construct containing the human HSL promoter was described previously (31). The HSL promoter included a genomic

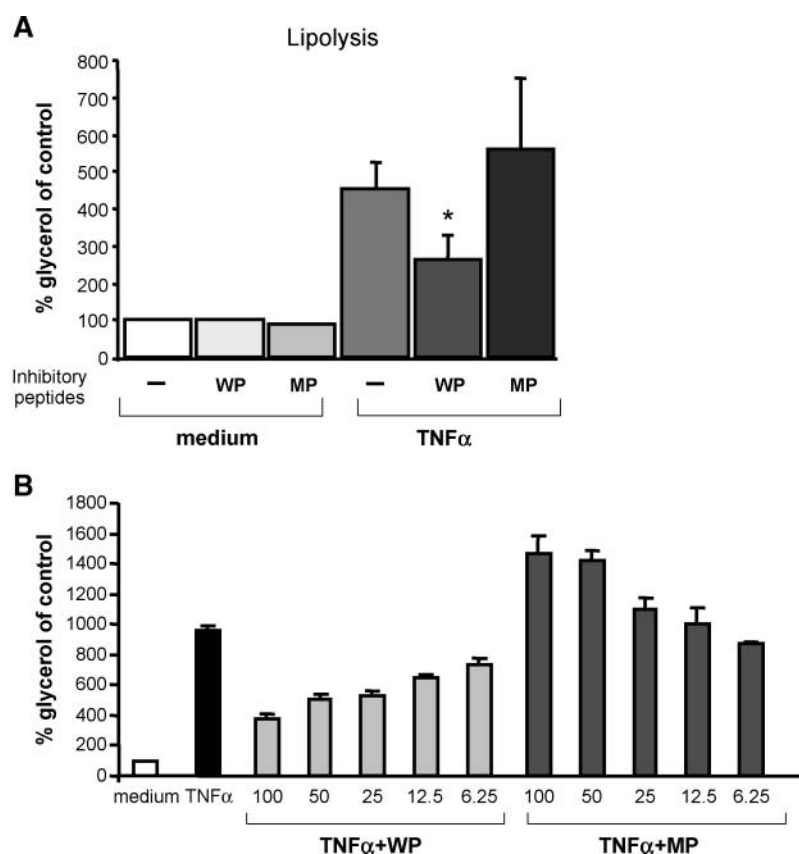


Fig. 2. Inhibitory effects of WP on TNF- α -induced lipolysis. Human adipocytes were incubated as indicated for 48 h. Glycerol release into the medium was measured. A: One hundred micromolar WP effectively reduced TNF- α -induced lipolysis, whereas MP did not influence lipolysis. Values shown are means \pm SD. * $P < 0.05$ compared with TNF- α -stimulated cells. Experiments were repeated six times. B: Increasing dilutions of WP and MP were used in separate incubations with 100 ng/ml TNF- α . WP but not MP displayed a concentration-dependent inhibitory effect on TNF- α -induced lipolysis. Experiments were repeated three times. Values shown are means \pm SD.

fragment of 2.4 kb (−2,400/+38 corresponding to the transcription start site), which was cloned upstream of the luciferase reporter gene in pGL3-basic vector (Promega). A portion (1.6 kb) of the human PLIN promoter (32) (−1,597/+41) was cloned into pGL3-basic vector. Human mesenchymal stem cell lines were plated onto 12-well plates and differentiated for 10–12 days. Cells were transfected by the Lipofectamine/Plus Reagent method (Invitrogen) according to the manufacturer's instructions. One microgram of test DNA, 8 μ l of Plus Reagent, and 4 μ l of Lipofectamine per well were used for transfection. A cytomegalo virus (CMV)- β -galactosidase-containing vector (0.05 μ g) was used as an internal control. MP or WP at a concentration of 100 μ M was added to the cultures at 24 h after transfection. TNF- α was added 2 h later at a concentration of 100 ng/ml. After an additional 24 h, cells were washed and lysed in 150 μ l of lysis buffer (containing 25 mM Tris-Acetic acid, pH 7.8, 1 mM EDTA, 10% glycerol, 1% Triton, and 2 mM DTT). Reporter assays for luciferase (Luciferase Assay System; Promega) and β -galactosidase (β -Galactosidase Enzyme Assay System; Promega) were performed according to the instructions of the manufacturer using an Infinite200 luminometer (Tecan, Salzburg, Austria). Each experimental group was tested in triplicate and the mean \pm SD was calculated. Two different mesenchymal stem cell lines were tested with similar results.

Statistical methods

Values are given as means \pm SD. The Kruskal-Wallis non-parametric test and the Wilcoxon signed rank test were used for statistical analysis. $P < 0.05$ (two-sided) was regarded as statistically significant.

RESULTS

WP inhibits NF- κ B translocation selectively in human adipocytes

To assess the effectiveness of the peptide inhibitor, we set up a nuclear translocation assay using primary cultures of human adipocytes. Stimulation with TNF- α (100 ng/ml) for 4 h resulted in a significant increase of NF- κ B in nuclear extracts at levels equivalent to those in positive controls (Jurkat cells) (Fig. 1A). Coincubation with WP effectively abrogated TNF- α -stimulated NF- κ B translocation at both 100 μ M (Fig. 1A) and 33 μ M (data not shown). In contrast, MP did not affect the ability of TNF- α to translocate NF- κ B. Incubation with either WP or MP alone did not influence nuclear translocation at any concentration. We also tested the effects of the peptides on survival and differentiation. No significant effect of the peptides alone or in combination with TNF- α could be observed after a 48 h incubation (data not shown). The selectivity of WP on NF- κ B signaling was also assessed. In particular, the MAPKs JNK and p44/42 were studied, because these have been shown to mediate TNF- α -induced lipolysis. Stimulating cells with TNF- α (100 ng/ml) for 20 min in the presence of either peptide did not affect JNK phosphorylation or p44/42 activity (Fig. 1B).

WP inhibits TNF- α -activated lipolysis in human adipocytes

The effect of NF- κ B inhibition on lipolysis was determined by assessing glycerol release into the medium. TNF-

α (100 ng/ml) stimulated basal lipolysis by 4.5-fold ($456 \pm 67\%$) (Fig. 2A) compared with control cells. Preincubation with WP (100 μ M) significantly reduced the lipolytic effect of TNF- α ($263 \pm 69\%$; $P < 0.05$ compared with TNF- α -treated cells, $n = 6$), whereas MP (100 μ M) had no significant effect ($559 \pm 191\%$). Incubation with the peptides alone did not affect lipolysis. To determine whether the effect of NF- κ B inhibition was concentration-dependent, we preincubated adipocytes with decreasing concentrations of both peptides (Fig. 2B). WP displayed a clear concentration-dependent inhibition of TNF- α -stimulated lipolysis, whereas MP had no inhibitory effect at any concentration.

WP enhances TNF- α -mediated downregulation of PLIN and HSL protein in human adipocytes

To explore whether inhibition of NF- κ B reduces TNF- α -mediated lipolysis via PLIN and/or HSL, we isolated protein from adipocytes treated with TNF- α for 48 h with or without the peptides. TNF- α alone reduced PLIN levels compared with those in control cells (Fig. 3) but had no effect on HSL protein levels. In contrast, coincubation with TNF- α and WP, but not with MP, resulted in a marked reduction of both PLIN and HSL protein. Furthermore, this effect was specific, in that the expression of the lipolysis-regulating proteins PPAR γ and PKAR2 β as well as the structural protein β -actin was not affected by NF- κ B inhibition (Fig. 3).

WP enhances TNF- α -mediated downregulation of PLIN and HSL mRNA in human adipocytes

To evaluate whether the effects on PLIN and HSL protein were secondary to changes in gene expression, mRNA

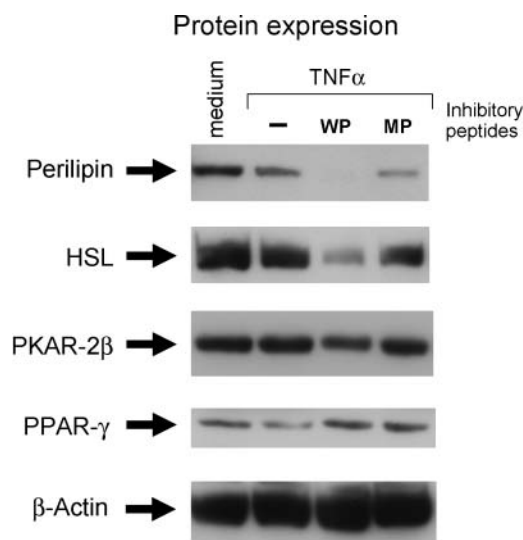


Fig. 3. Effects of WP on TNF- α -regulated protein expression. Human adipocytes were incubated as indicated for 48 h. Total protein lysates were obtained and analyzed by Western blot. The figure depicts a representative blot consecutively probed with antibodies directed against the indicated proteins. Experiments were repeated three times. PKAR2 β , regulatory subunit 2 β of protein kinase A; PPAR γ , peroxisome proliferator-activated receptor γ .

levels were determined in parallel experiments performed as described above. TNF- α reduced PLIN and HSL mRNA levels (Fig. 4A). TNF- α incubation in the presence of WP resulted in a significantly more pronounced reduction of both PLIN and HSL mRNA, reaching levels <25% of those in TNF- α -treated cells (Fig. 4A; $P < 0.05$). This effect was specific, in that no significant effect of coinubation with WP could be observed on ATGL expression ($P = 0.08$), whereas interleukin-6 mRNA levels were increased in cells treated with TNF- α and WP. Coinubation with MP did not affect TNF- α -mediated effects on mRNA (Fig. 4A), nor did incubation with the peptides alone (data not shown).

WP enhances TNF- α -mediated downregulation of PLIN and HSL via direct effects on transcription

The effects of NF- κ B inhibition on mRNA expression could be attributable to direct effects on transcriptional activity. Therefore, we performed reporter gene assays by transfecting human adipocytes derived from mesenchymal stem cells with constructs containing promoter regions of human HSL or PLIN coupled to the luciferase gene. TNF- α alone downregulated the transcriptional activity of PLIN

and HSL promoters (Fig. 4B). Coinubation with WP, but not MP, significantly enhanced this effect and further decreased transcription by 50% (Fig. 4B; $P < 0.05$), suggesting that downregulation of mRNA levels by TNF- α is modulated by NF- κ B.

DISCUSSION

In this work, we have studied the effects of NF- κ B inhibition on TNF- α -induced lipolysis in primary cultures of human adipocytes derived from preadipocytes. We demonstrate that a peptide inhibitor of IKK activation effectively blocks TNF- α -mediated nuclear translocation of NF- κ B, which in turn results in abrogated lipolysis via reduced expression of both HSL and PLIN protein. This is presumably mediated by transcriptional effects. Our results suggest that a functional NF- κ B pathway is essential for an intact lipolytic response to TNF- α . However, the fact that NF- κ B inactivation, in contrast to p44/42 or JNK inhibition, does not block TNF- α -mediated lipolysis completely indicates that NF- κ B may have a predominantly

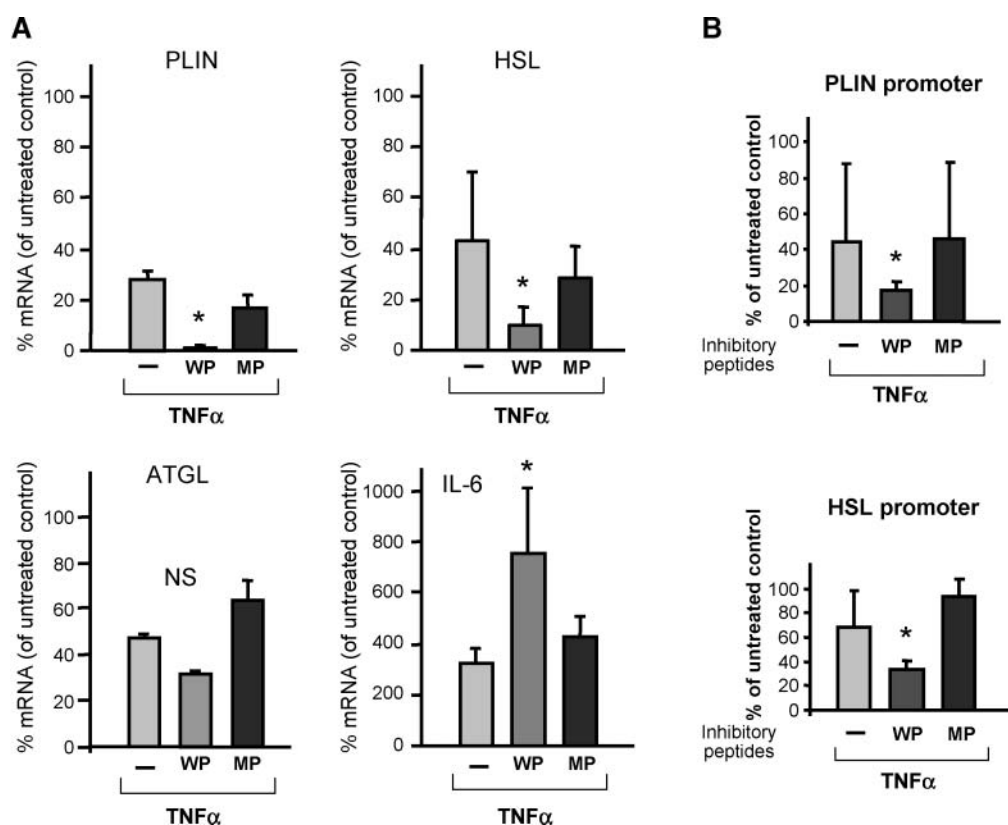


Fig. 4. Effects of WP on TNF- α -regulated mRNA expression is transcriptionally regulated. A: Human adipocytes were incubated as indicated for 48 h. Total RNA was isolated, and samples were analyzed by quantitative RT-PCR. Results are means of three independent experiments \pm SD. * $P < 0.05$ compared with TNF- α -treated cells. HSL, hormone-sensitive lipase; IL-6, interleukin-6; PLIN, perilipin. B: Gene reporter assay in differentiated adipocytes from human mesenchymal stem cells (hMSCs). Cells were transfected with human constructs of the PLIN and HSL promoters. After a 24 h incubation, cells were stimulated with TNF- α with or without the peptides as indicated. Values shown are means \pm SD. * $P < 0.05$ compared with TNF- α -treated cells.

modulatory role. Nevertheless, to the best of our knowledge, this is the first report demonstrating a putative metabolic role for NF- κ B in human adipocytes, which seems to be different from that observed in 3T3-L1 adipocytes (24).

The importance of NF- κ B in adipocytes has only been studied in murine adipocytes. In these studies, I κ B variants with mutated phosphorylation sites were overexpressed (commonly by viral infection), thereby preventing NF- κ B translocation. However, 3T3-L1 cells overexpressing non-degradable I κ B mutants followed by TNF- α stimulation survived for only 2 h after TNF- α stimulation, suggesting that some caution should be taken in interpreting the results (24). Using a similar approach in murine stromal bone marrow cells, cytokine-activated NF- κ B appeared to inhibit PPAR γ -mediated effects, leading to inhibition of adipogenesis and promotion of osteogenesis (23). Together, these reports in murine cells indicate that TNF- α may induce a dedifferentiating effect via NF- κ B. Because we wanted to study the importance of NF- κ B in human adipocytes, viral overexpression of dominant-negative I κ B was not a feasible approach given the limited survival previously shown in murine cells. We chose instead to use selective inhibitors. However, although several NF- κ B inhibitors have been presented, many of these lack selectivity. For instance, although BAY 11-7082 inhibits NF- κ B activation, it also activates p38 and JNK (33), the latter of which is involved in mediating TNF- α -induced lipolysis. The inhibitor chosen for this study of human primary adipocytes has been shown to be effective in several different cellular systems. Our results clearly indicate that the

peptide is highly efficient in inhibiting NF- κ B activation, as demonstrated by the complete lack of TNF- α -induced NF- κ B translocation in the presence of the peptide. Moreover, the peptide is selective, in that no effect on MAPK activation could be observed.

The lipolytic response to TNF- α is not immediate and is only observed after 6–24 h of stimulation, depending on the cell type (19). Therefore, it is generally regarded that TNF- α mediates its action via gene regulation. As mentioned above, previous reports of primary cultures of human adipocytes indicate that a major effect of TNF- α is increased phosphorylation and downregulation of PLIN via the MAPKs p44/42 and JNK (9, 19, 34) (for a summary, see Fig. 5A, B). Our results suggest that when NF- κ B signaling is inhibited, the concomitant TNF- α -induced activation of p44/42 and JNK results in a significantly more pronounced reduction of PLIN expression. In the “barrier/translocation” hypothesis, this would normally be expected to enhance lipolysis, whereas in the scaffold model, a significantly reduced level of PLIN would abrogate lipolysis via reduced interaction with HSL. Irrespective of which model is valid for human fat cells, NF- κ B is obviously important to retain HSL expression, because coinubation with TNF- α and WP significantly reduces the adipocyte content of this key enzyme. We cannot firmly establish whether the reduced lipolytic capacity observed after NF- κ B inhibition is primarily attributed to PLIN or HSL reduction or both. However, given the pivotal importance of HSL for human lipolysis (35), it is quite conceivable that the pronounced reduction in expression of this enzyme is the most important. Therefore, we pro-

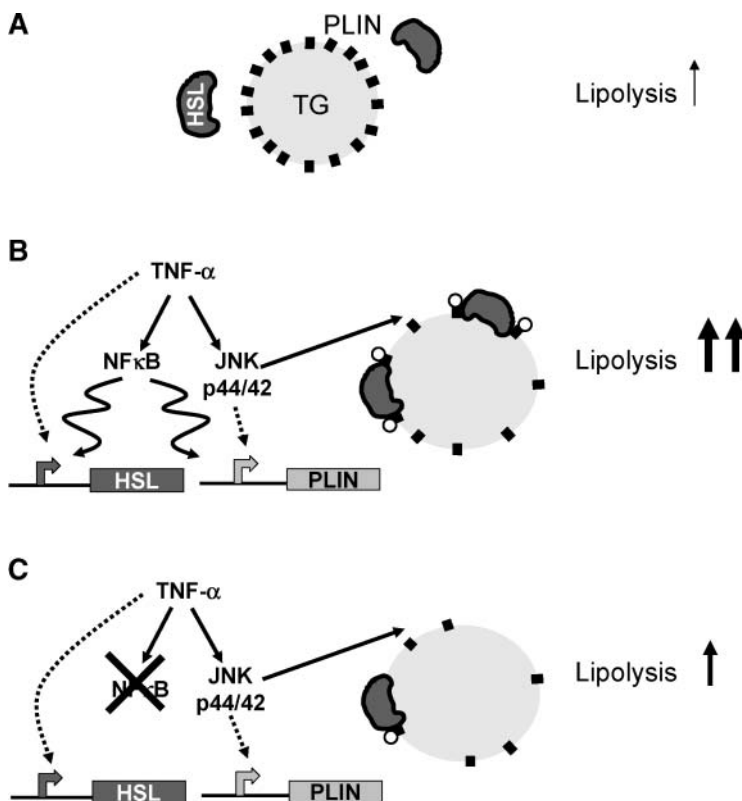


Fig. 5. Schemes of the role of NF- κ B in TNF- α -induced lipolysis. The role of PLIN in regulating lipolysis is not entirely clear. This figure is based on the recently proposed scaffold model, although our results would also fit with the barrier/translocation hypothesis. A: In the basal state, PLIN coats the intracellular lipid droplet and HSL cannot initiate hydrolysis of stored triglycerides (TGs) to any great extent. However, a low level of basal lipolysis is still present. B: TNF- α phosphorylates PLIN, which alters the conformation and/or binding to additional proteins on the lipid surface. HSL is thereby recruited and hydrolyzes stored triglycerides. PLIN mRNA and protein levels are reduced as well, and both of these effects are mediated via the MAPKs p44/42 and JNK. TNF- α also has effects on HSL transcription, although to a lesser extent than on PLIN. Dotted lines represent inhibition. The data presented herein suggest that NF- κ B modulates the transcriptional activity of both genes. This prevents TNF- α from efficiently increasing lipolysis, because the level of the rate-limiting enzyme in human lipolysis is reduced. Therefore, the net effect of TNF- α on lipolysis is dependent on the differential expression of HSL and PLIN in relation to each other.

pose that NF- κ B signaling is required to retain transcriptional activity of the PLIN and HSL genes (Fig. 5C). This is demonstrated in our gene reporter experiments, wherein a transcriptional regulation by TNF- α on HSL and PLIN promoters is shown. To our knowledge, this is the first study in fat cells indicating that HSL and PLIN mRNA levels are transcriptionally regulated by TNF- α . It could be argued that a 48 h inhibition of NF- κ B could result in compensatory mechanisms accounting for some of the effects observed in this study. However, we find this less likely, because reporter gene expression driven by PLIN or HSL promoter was already reduced at 24 h, and neither survival nor differentiation was affected. Moreover, the effect on HSL and PLIN protein expression was specific, in that the levels of a number of other proteins, including PPAR γ , were not altered.

Although we found that NF- κ B inhibition resulted in reduced TNF- α -induced lipolysis, it is not clear that such an effect should always be expected in human fat cells. The net effect of the NF- κ B-TNF- α interplay on lipolysis is dependent on how much HSL and PLIN are reduced in comparison with each other. A small effect on HSL but a large one on PLIN may actually boost TNF- α stimulation and vice versa. Detailed studies on HSL versus PLIN in human are not possible to perform, because they would require human fat cell lines (which are not yet available) that can be used for stable overexpression or depletion of HSL and PLIN.

ATGL was recently isolated and described as an important lipase in rodent fat cells (36). However, ATGL expression is downregulated by TNF- α in 3T3-L1 cells (37), suggesting that the lipolytic effect of TNF- α is not mediated via this lipase. In this work in human adipocytes, we confirmed this observation. Coincubation of TNF- α with WP did not result in any significant alteration of ATGL mRNA levels. This suggests that the NF- κ B effect observed in this study is not primarily mediated via ATGL. In agreement with these data, we recently demonstrated that, in contrast to findings in murine cells, ATGL is of minor importance in human fat cells (35, 38). In fact, ATGL appears to regulate above all a small portion of basal lipolysis in human adipocytes. Therefore, the overwhelming part of human fat cell lipolysis seems to be regulated by HSL.

In summary, we have shown that an intact NF- κ B pathway is important to retain a full lipolytic response to TNF- α . TNF- α induces a concomitant decrease in the expression of two key regulatory proteins for lipolysis, HSL and PLIN, which at least in part is attributable to effects on gene transcription. p44/42 and JNK are probably the most important mediators of TNF- α signaling to lipolysis. However, the dual activation of MAPKs and NF- κ B by TNF- α allows for lipolytic fine-tuning and may account for the fact that TNF- α accelerates lipolysis in such contrasting disorders as obesity and cachexia. Given the important role of TNF- α in insulin resistance, our data add to previous results mapping the molecular mechanisms of TNF- α -mediated lipolysis in human obesity and cachexia. ■

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