Tpl2 Kinase Is Upregulated in Adipose Tissue in Obesity and May Mediate Interleukin-1β and Tumor Necrosis Factor-α Effects on Extracellular Signal–Regulated Kinase Activation and Lipolysis

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OBJECTIVE—Activation of extracellular signal–regulated kinase-(ERK)-1/2 by cytokines in adipocytes is involved in the alterations of adipose tissue functions participating in insulin resistance. This study aims at identifying proteins regulating ERK1/2 activity, specifically in response to inflammatory cytokines, to provide new insights into mechanisms leading to abnormal adipose tissue function.

RESEARCH DESIGN AND METHODS—Kinase activities were inhibited with pharmacological inhibitors or siRNA. Lipolysis was monitored through glycerol production. Gene expression in adipocytes and adipose tissue of obese mice and subjects was measured by real-time PCR.

RESULTS—IκB kinase-(IKK)-β inhibition prevented mitogen-activated protein (MAP) kinase kinase (MEK)/ERK1/2 activation in response to interleukin (IL)-1β and tumor necrosis factor (TNF)-α but not insulin in 3T3-L1 and human adipocytes, suggesting that IKKβ regulated a MAP kinase kinase kinase (MAP3K) involved in ERK1/2 activation induced by inflammatory cytokines. We show that the MAP3K8 called Tpl2 was expressed in adipocytes and that IL-1β and TNF-α activated Tpl2 and regulated its expression through an IKKβ pathway. Pharmacological inhibition or silencing of Tpl2 prevented MEK/ERK1/2 activation by these cytokines but not by insulin, demonstrating its involvement in ERK1/2 activation specifically in response to inflammatory stimuli. Importantly, Tpl2 was implicated in cytokine-induced lipolysis and in insulin receptor substrate-1 serine phosphorylation. Tpl2 mRNA expression was upregulated in adipose tissue of obese mice and patients and correlated with TNF-α expression.

CONCLUSIONS—Tpl2 is selectively involved in inflammatory cytokine–induced ERK1/2 activation in adipocytes and is implicated in their deleterious effects on adipocyte functions. The deregulated expression of Tpl2 in adipose tissue suggests that Tpl2 may be a new actor in adipose tissue dysfunction in obesity.
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Obesity and type 2 diabetes are characterized by an insulin-resistant state that could be due to the development of an inflammatory state in the adipose tissue (1,2). Indeed, adipose tissue from obese subjects is infiltrated by bone marrow–derived macrophages that largely contribute to the increased level of proinflammatory cytokines, including tumor necrosis factor (TNF)-α and interleukin (IL)-1β. These cytokines could act locally to impinge insulin signaling and action in adipocytes and could alter insulin action in liver and muscles (2). Furthermore, TNF-α and IL-1β exert lipolytic effects on adipocytes that participate in the increased free fatty acid (FFA) level during obesity. A paracrine loop involving FFAs and inflammatory cytokines between adipocytes and macrophages would establish a vicious circle that aggravates inflammatory changes in adipose tissue and that worsens insulin resistance (3).

Although the exact mechanisms by which increased inflammatory cytokines contribute to insulin resistance and lipolysis are still unknown, it is now accepted that activation of protein kinases such as IκB kinase (IKK) and mitogen-activated protein (MAP) kinases including extracellular signal–regulated kinase (ERK)-1/2 plays an important role (2,4,5). Elevated activity of ERK is found in adipose tissue or muscles of obese and insulin-resistant rodents and humans (6,7). The ERK signaling pathway is activated by various inflammatory cytokines including TNF-α and IL-1β and is involved in insulin resistance in adipocytes through an increase in insulin receptor substrate (IRS)-1 serine phosphorylation and/or a decrease in its expression (7–9). The ERK pathway is also involved in cytokine-induced lipolysis in adipocytes (10–12). An important clue for the physiological importance of the ERK pathway in insulin resistance came from the study of genetically modified mice. Indeed, mice lacking the MAP kinase ERK1 are protected from obesity and insulin resistance when challenged on a high-fat diet (13), and overexpression of the MAP kinase phosphatase-4/dual-specificity phosphatase (MKP-4/DUSP-9) that dephosphorylates ERK1/2 protects against stress-induced insulin resistance (14). Conversely, mice deficient in p62, an ERK inhibitor, have a high basal level of ERK activity and develop mature-onset obesity and insulin resistance (15). However, depending on the stimuli, the ERK outcome response is totally different, and this pathway is involved in numerous effects in addition to inflam-

TABLE 1
Characteristics of the lean, obese, obese and diabetic, and morbidly obese subjects in the fasting state

	Lean population 1	Obese population 1	Obese and type 2 diabetic population 1	Lean population 2	Morbidly obese population 2
n	11	11	11	4	6
Sex (F/M)	3/8	2/9	2/9	4/0	6/0
BMI (kg/m^2)	22.4 ± 0.6	32.3 ± 1.4*	32.6 ± 0.9*	20.9 ± 0.5	44.3 ± 7.2†
Age (years)	44 ± 4	45 ± 5	54 ± 2	37.2 ± 11.3	32.0 ± 8.5
Glucose (mmol/l)	4.91 ± 0.19	5.50 ± 0.11*	11.08 ± 1.00*‡	5.08 ± 1.44	4.99 ± 0.49
Insulin (mU/l)	6.70 ± 0.90	13.05 ± 1.89*	13.70 ± 1.81*	ND	10.67 ± 3.11

Data are means ± SE and were compared using the nonparametric Mann-Whitney test. *$P < 0.05$; †$P < 0.01$ vs. lean subjects; ‡$P < 0.05$ vs. obese subjects. ND, not determined.

mation and insulin resistance. Thus, the identification of regulatory proteins that govern the activity of ERK specifically in response to inflammatory cytokines may provide important insights into mechanisms that promote metabolic diseases, and these proteins could be potential targets to alleviate these diseases.

MAP kinase and IKK/nuclear factor (NF)-κB pathways often act synergistically to mediate cytokine action (16). It is therefore possible that in adipocytes, proteins that control cytokine-induced ERK activation are regulated by the IKK/NF-κB pathway. One interesting candidate could be MAP kinase kinase kinase (MAP3K), which regulates ERK through the phosphorylation and activation of MAP kinase kinase (MEK) (17), because some of these pathways have been involved in ERK activation selectively downstream of innate immunoreceptors (18).

Therefore, the aim of the present study was to identify kinases specifically involved in ERK activation by inflammatory cytokines in adipocytes and to address their implication in the alteration in adipocyte biology in obesity. We report for the first time that the MAP3K8 called tumor progression locus 2 (Tpl2) in mouse or Cancer Osaka thyroid (Cot) in human (19) is expressed in adipocytes and is specifically involved in ERK pathway activation by IL-1β and TNF-α, whereas it is dispensable for ERK activation by insulin. We provide the first evidence that the Tpl2 signaling pathway is implicated in cytokine-induced lipolysis and IRS-1 serine phosphorylation. We showed that Tpl2 mRNA expression is upregulated in adipose tissue of obese subjects and rodents and that inflammatory stimuli regulated Tpl2 expression.

RESEARCH DESIGN AND METHODS

Reagents. Dulbecco's modified Eagle's medium (DMEM) and FCS were obtained from Invitrogen SARL (Cergy Pontoise, France). Insulin was obtained from Lilly (Paris, France). Murine and human IL-1β and TNF-α were from PreProtech (Neuilly, France). U0126, Tpl2 kinase inhibitor [4-(3-chloro-4-fluorophenylamino)-6-(pyridine-3-yl-methylamino)-3-cyano-(1,7)-napthyridine] (20,21), and IKKβ inhibitor (InSolution IKK-2 Inhibitor IV) were obtained from Calbiochem (La Jolla, CA). Tpl2 inhibitor acts as a potent, reversible, and ATP-competitive inhibitor of Tpl2 with an IC_{50} of 50 nmol/l. It displays significant selectivity over other related kinases such as EGF receptor, MEK, mitogen-activated protein kinase–activated protein kinase-2 (MK2), p38, Src, and protein kinase C. This inhibitor blocks lipopolysaccharide (LPS)-induced TNF-α production in human monocytes with an IC_{50} of 700 nmol/l (21). IKKβ inhibitor (2-[{ami-nocarbonyl}amino]-5-[4-fluorophenyl]-3-thiophenecarboxamide) is a potent, selective, and ATP-competitive inhibitor that inhibits IKKβ activity in vitro (IC_{50} = 18 nmol/l) and prevents LPS-induced cytokines production in monocytes. This inhibitor is at least 550-fold more selective for IKKβ versus other kinases, including p38, c-Jun NH_2-terminal kinase (JNK), and ERK2 (22,23). Proteasome inhibitor MG132 was obtained from Sigma-Aldrich (St. Louis, MO). siRNA against Tpl2 and p65/NF-κB were purchased from Dharmacon (Thermo Fisher Scientific, Waltham, MA). Polyvinylidene difluoride (PVDF) membranes were purchased from Millipore (Bedford, MA). Bicinchoninic reagent was obtained from Pierce Biotechnology (Rockford, IL). Enhanced chemiluminescence reagent was purchased from PerkinElmer Life Sciences (Boston, MA). All other chemical reagents were purchased from Sigma-Aldrich.

Antibodies. Antibodies against Tpl2 and IκB were obtained from Santa Cruz Biotechnology (Tebu, France). Antibody against IRS-1 was purchased from Upstate Biotechnology (Waltham, MA). All other antibodies were purchased from Cell Signaling Technology (Beverly, MA). Horseradish peroxidase–conjugated secondary antibodies were obtained from Jackson Immunoresearch Laboratories (West Grove, PA).

Cells culture and differentiation. 3T3-L1 fibroblasts and human adipocytes were grown and induced to differentiate in adipocytes as described (8,24).

Lipolysis. Glycerol content of the incubation medium was determined as an index of lipolysis using a colorimetric assay (GPO-Trinder; Sigma, St. Louis, MO). Lipolysis data were calculated as micrograms of glycerol per milligram of protein.

Animals. Male *ob/ob*, *db/db* mice and their lean littermates (*ob*/$^+$ and *db*/$^+$) (Charles River Laboratories, St. Aubin les Elbeuf, France) were maintained on a 12-h light, 12-h dark cycle and were provided free access to water and standard rodent diet. Seven- to 10-week-old male C57BL/6 mice (Janvier, Le Genest-St-Isle, France) were fed a standard diet (TD2016; Harlan) or a high-fat diet (36% fat, TD99249; Harlan) for 15 weeks. Mice were killed by cervical dislocation and epididymal fat pads were removed, freeze-clamped in liquid nitrogen, and stored at −80°C. Principles of laboratory animal care were followed, and the ethical committee of the Faculty of Medicine approved the animal experiments.

Subjects population. Two populations were studied and their clinical and biological characteristics are listed in Table 1.

Obese subjects without or with type 2 diabetes and healthy lean subjects participated in the study. None of the lean subjects had impaired glucose tolerance or a history of diabetes, obesity, dyslipidemia, or hypertension. The type 2 diabetic patients interrupted, under medical control, their usual antidiabetes treatment at least 1 week before the investigation. All studies were performed after an overnight fast.

Morbidly obese women were selected through the Department of Digestive Surgery (Nice Hospital), where they underwent an elective bariatric surgery. Control subcutaneous adipose tissue was obtained from lean women undergoing lipectomy for cosmetic purposes.

Subcutaneous adipose tissue biopsies were taken during surgery or under local anesthesia, immediately frozen in liquid nitrogen, and stored at −80°C. The study was performed according to the French legislation regarding ethics and human research (Huriet-Serusclat Law).

siRNA transfection. siRNA transfection in differentiated 3T3-L1 adipocytes was performed by electroporation using a Nucleofector II system (Amaxa Biosystems). Seven days–differentiated 3T3-L1 adipocytes were trypsinized with 5 × trypsin/EDTA for 2 min at 37°C, and trypsinization was stopped with DMEM, 10% calf serum supplemented with 4% glycerol. Cells (2×10^6 per nucleofection sample) were centrifuged at 900*g* for 5 min. Pellet was resuspended in Nucleofector Solution L (100 μl), mixed with siRNA (100 pmol), and transferred into an Amaxa cuvette, and nucleofection was done using the program A-033. Then cells were seeded in a 12-well precoated plate (collagen type I; Sigma) in DMEM and 10% FCS and were used 72 h after nucleofection.

Western blot analysis. Proteins from lysates were separated by SDS-PAGE and transferred to PVDF membranes as previously described (8). Membranes were incubated with the indicated antibody, and horseradish peroxidase–coupled anti-species antibodies were then added and chemiluminescence was

detected using a Fujifilm Las-3000 apparatus (Fujifilm Life Science, F.S.V.T Courbevoie, France). Some membranes were subsequently reprobed with the indicated antibody as a loading control. Quantifications were realized using MultiGauge software (Fujifilm Life Science).

Real-time RT-PCR. RNAs were prepared using the RNeasy Total RNA Kit (Qiagen, Courteboeuf, France), treated with DNase (Applied Biosystems), and used to synthesize cDNAs using a Transcriptor First Strand cDNA Synthesis Kit (Roche, France). Real-time quantitative PCR was performed with sequence detection systems (ABI PRISM 7500; Applied Biosystems) and SYBR green dye as described (8). Levels of mRNA were expressed relative to mouse or human RPLP0. The relative amount of mRNA between two groups was determined by using the second derivative maximum method. The results were expressed relative to the mean of the group of controls, which was arbitrarily assigned to a value of 1. Primers used (a list is available upon request at tanti@unice.fr) were designed using Primer Express software (Applied Biosystems, Austin, TX) and synthesized by Eurogentec (Seraing, Belgium).

Statistical analysis. Statistical analysis was performed by Student t or Mann-Whitney test. Correlation between two variables was analyzed using Spearman rank-correlation test. The analyses were performed with MINITAB software. A P value <0.05 was considered significant.

RESULTS

IKKβ is involved in MEK and ERK1/2 activation specifically in response to IL-1β and TNF-α but not in response to insulin. Inflammatory cytokines induce alteration of adipocytes biology that may involve activation of both ERK and IKKβ/NF-κB pathways (25). To determine whether there is a cross-talk between these two pathways, the effect of pharmacological inhibition of IKKβ on IL-1β and TNF-α–induced MEK and ERK1/2 activation was determined. IKKβ inhibitor (22,23) prevented both IL-1β and TNF-α–induced MEK and ERK1/2 phosphorylation. Importantly, insulin effect was unaltered (Fig. 1*A* and *B*). This inhibition was not due to modification in the time course of activation (data not shown). The same results were obtained in human adipocytes (Fig. 1*C*).

Tpl2 is activated by IL-1β and TNF-α through an IKKβ pathway. The results described above suggested that inflammatory signals and insulin regulated the ERK pathway differently and that IKKβ could regulate an MAP3K upstream of MEK, which is involved in ERK1/2 activation specifically in response to inflammatory cytokines. In macrophages, Tpl2 is an MAP3K that activates the MEK/ERK pathways in response to LPS through an IKKβ-dependent pathway (26,27). In 3T3-L1 adipocytes, we found that an anti-Tpl2 antibody detected two bands of 58 and 52 kd (Fig. 2*A*) that likely correspond to the long ($Tpl2_L$) and the short forms ($Tpl2_S$) of Tpl2 that arise from alternative translational initiation (28). The mRNA and protein expression of Tpl2 was increased in 3T3-L1 cells following differentiation in adipocytes, and the expression level of both isoforms in 3T3-L1 adipocytes was similar to the level found in macrophages (Fig. 2*A*). We then investigated whether Tpl2 was activated by TNF-α and IL-1β by monitoring its degradation, which is tightly coupled to its activation (29,30). The two cytokines significantly decreased total Tpl2 protein amount after 30 min of treatment and for at least 90 min (Fig. 2*B*), and $Tpl2_L$ was preferentially prone to degradation. Pharmacological inhibition of IKKβ or proteasome abolished Tpl2 degradation (Fig. 2*C*).

Tpl2 is involved in ERK1/2 activation specifically in response to IL-1β and TNF-α and is implicated in IRS-1 serine phosphorylation. To demonstrate that Tpl2 was involved in ERK1/2 activation in response to IL-1β or TNF-α, we treated 3T3-L1 adipocytes with a Tpl2 inhibitor (20) for 1 h before cytokines or insulin stimulation. Tpl2 inhibition markedly blunted the effects of IL-1β and TNF-α

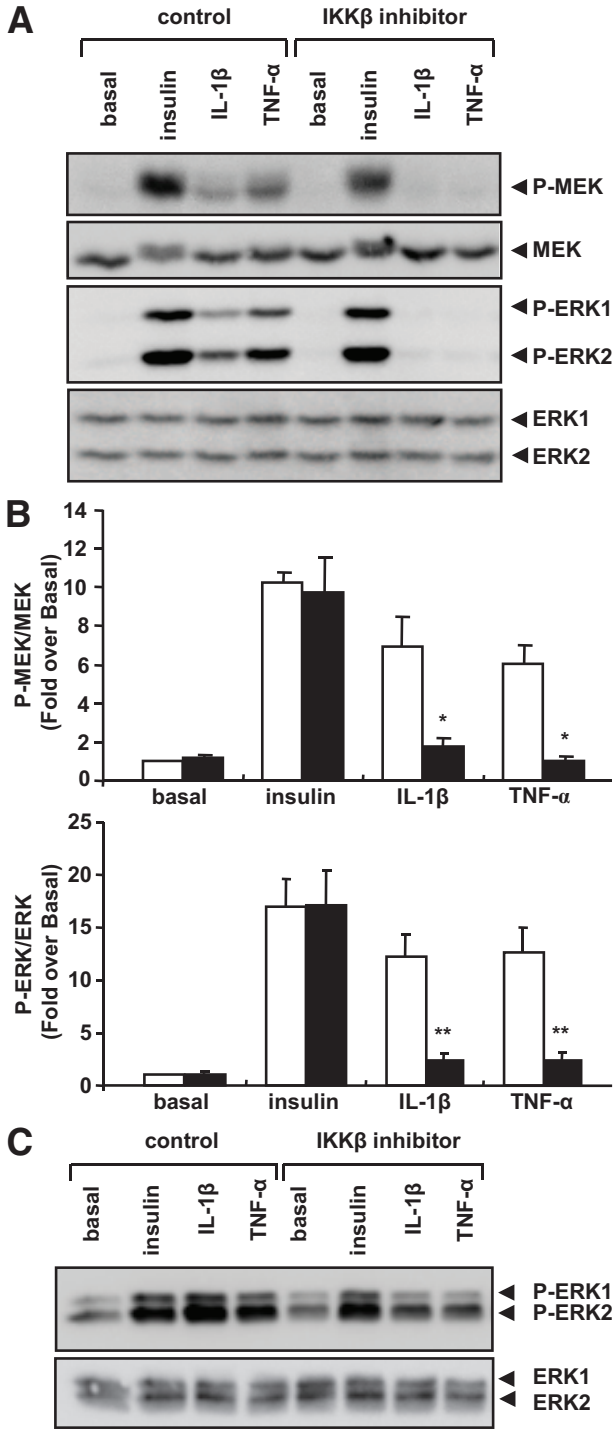

FIG. 1. Pharmacological inhibition of IKKβ prevents MEK and ERK1/2 activation in response to IL-1β and TNF-α but not in response to insulin in adipocytes. *A* and *B*: 3T3-L1 adipocytes were treated without (□) or with (■) an IKKβ inhibitor (5 μmol/l) for 1 h and then stimulated or not with IL-1β or TNF-α (20 ng/ml) for 20 min or with insulin (100 nmol/l) for 10 min. Lysates were subjected to Western blotting with antibodies against phosphorylated or total MEK or ERK1/2. Representative immunoblots and quantification of five independent experiments are shown. Data are expressed as fold of MEK and ERK1/2 phosphorylation over basal in cells without inhibitor treatment and presented as the means ± SE. *$P < 0.05$ and **$P < 0.01$ vs. stimulus effect in control cells. *C*: Human adipocytes were treated or not with an IKKβ inhibitor (2.5 μmol/l) for 30 min and then stimulated or not as described above. ERK1/2 phosphorylation and ERK1/2 total protein amount were analyzed as described above. Representative immunoblots of three independent experiments are shown.

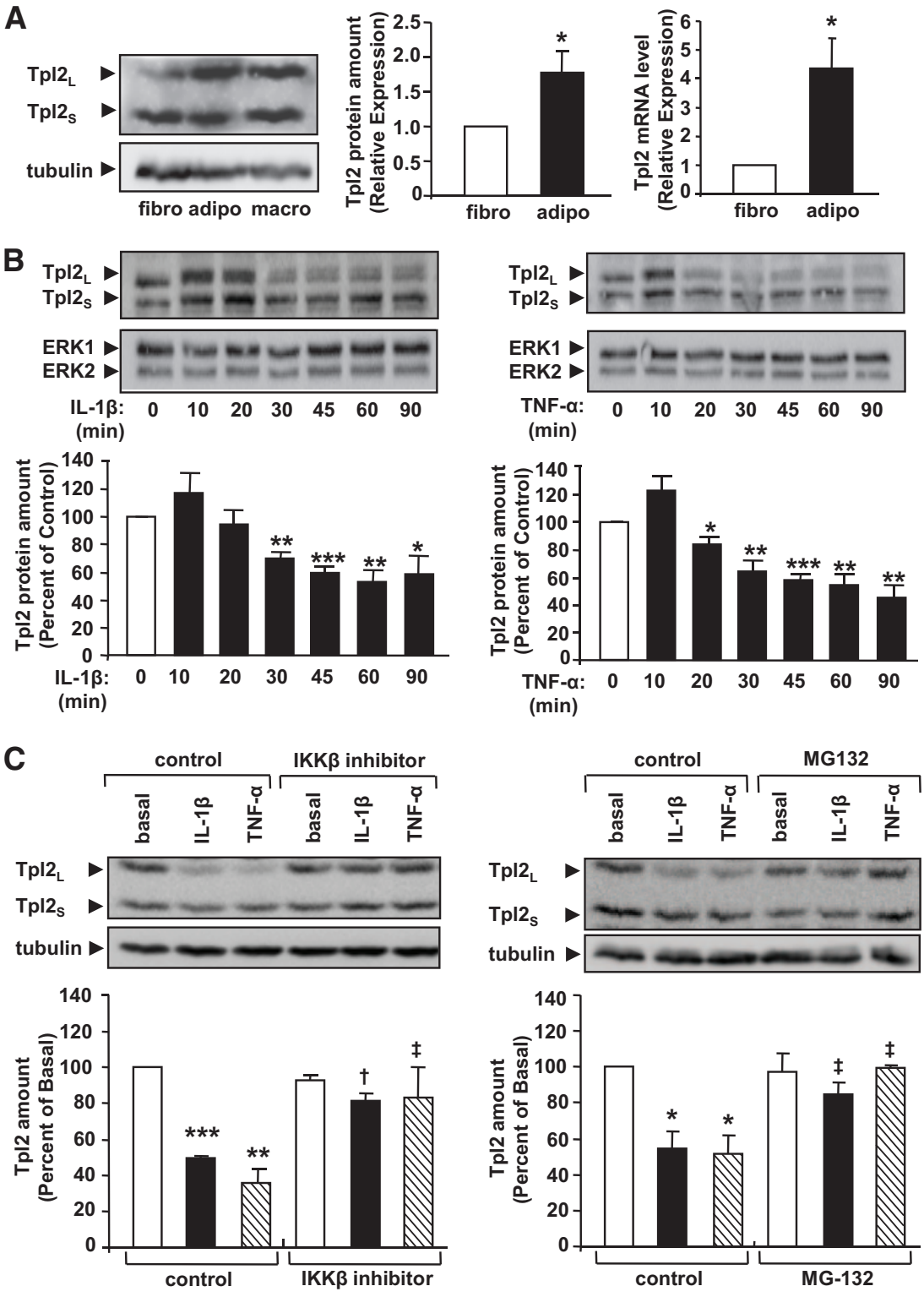

FIG. 2. Tpl2 is expressed and activated by IL-1β and TNF-α in 3T3-L1 adipocytes. *A*: Proteins from cell lysates were prepared from 3T3-L1 confluent fibroblasts (fibro), 3T3-L1 differentiated adipocytes (adipo), and RAW264.7 macrophages (macro). Lysates were subjected to Western blotting with an antibody against Tpl2. A representative immunoblot and a quantification of three independent experiments are shown. Total mRNA were prepared from 3T3-L1 confluent fibroblasts (fibro, □) and 3T3-L1 differentiated adipocytes (adipo, ■), and the relative amount of Tpl2 mRNA was determined by real-time PCR. Tpl2 mRNA expression was normalized using mouse RPLP0 RNA level. Results are expressed in arbitrary units, with the control value taken as 1, and are the means ± SE of four independent experiments. *B*: 3T3-L1 adipocytes were stimulated for the indicated times with IL-1β or with TNF-α (20 ng/ml). Tpl2 protein expression was detected using a specific antibody. Representative immunoblots and a quantification of five independent experiments are shown. Data are expressed as a percentage of Tpl2 protein amount in untreated cells and presented as the means ± SE. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ vs. untreated cells. *C*: 3T3-L1 adipocytes were treated or not with an IKKβ inhibitor (5 μmol/l) for 1 h (*left panel*) or with MG132, a proteasome inhibitor (10 μmol/l), for 5 h (*right panel*) and then stimulated without (□) or with (■) 20 ng/ml of IL-1β or TNF-α (▨) for 90 min. Tpl2 protein expression was determined using a specific antibody. Representative immunoblots and the quantification of three independent experiments are shown. Data are expressed as a percentage of Tpl2 protein amount in untreated cells and presented as the means ± SE. *$P < 0.05$ and **$P < 0.01$ vs. untreated cells, effect of IKKβ or proteasome inhibitors significant with †$P < 0.01$ or with ‡$P < 0.05$.

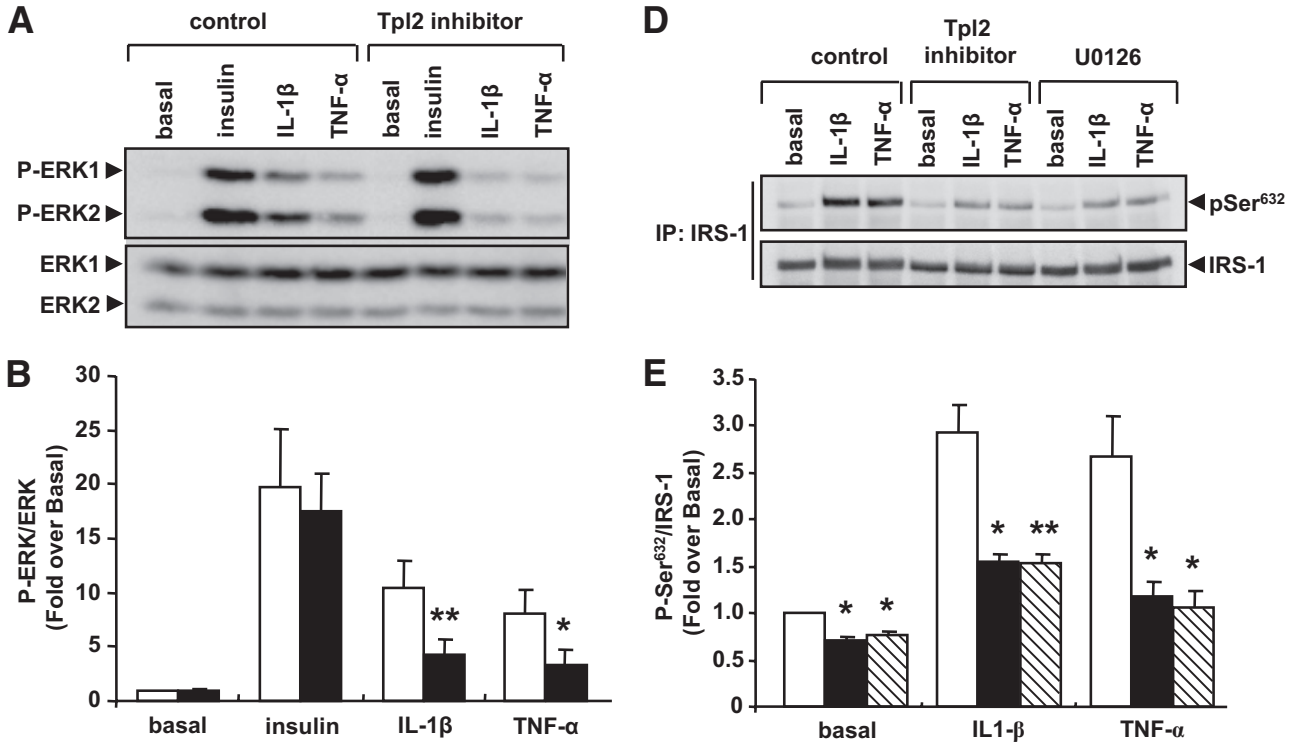

FIG. 3. Pharmacological inhibition of Tpl2 decreases ERK1/2 phosphorylation and IRS-1 serine phosphorylation in response to IL-1β and TNF-α in adipocytes. *A* and *B*: 3T3-L1 adipocytes were treated without (□) or with (■) a Tpl2 inhibitor (30 μmol/l) for 1 h and then stimulated or not with IL-1β or TNF-α (20 ng/ml) for 20 min or insulin (100 nmol/l) for 10 min. Lysates were subjected to Western blotting with antibodies against phosphorylated or total ERK1/2. Representative immunoblots and quantification of five independent experiments are shown. Data are expressed as fold of ERK1/2 phosphorylation over basal in control cells and presented as the means ± SE. *$P < 0.01$ and **$P < 0.001$ vs. stimulus effect in control cells. *C*: Human adipocytes were treated or not with a Tpl2 inhibitor (20 μmol/l) for 30 min and then stimulated or not with IL-1β, TNF-α, or insulin as described in *A*. ERK1/2 phosphorylation and ERK1/2 total protein amount were analyzed as described above. Representative immunoblots of three independent experiments are shown. *D* and *E*: 3T3-L1 adipocytes were treated without (□) or with (■) a Tpl2 inhibitor (30 μmol/l), or with a MEK inhibitor U0126 (10 μmol/l, ▨) for 1 h and then stimulated or not with IL-1β or TNF-α (20 ng/ml) for 20 min. Proteins were immunoprecipited (IP) with anti–IRS-1 antibody, resolved by SDS-PAGE, and immunoblotted with a phosphospecific antibody against serine 632 (αpSer^{632}). The membrane was stripped and probed using anti–IRS-1 antibody. Representative immunoblots and quantification of three independent experiments are shown. Results were normalized for the amount of IRS-1 present in the immunoprecipitation and are the means ± SE. *$P < 0.05$ and **$P < 0.01$ vs. stimulus effect in control cells.

on ERK1/2 phosphorylation (Fig. 3*A* and *B*). The same results were obtained for MEK phosphorylation (data not shown). In contrast, activation of JNK1/2 and p38 were not modified (supplementary Fig. S1*A* [available at http://diabetes.diabetesjournals.org/cgi/content/full/db09-0470/DC1]). Importantly, insulin effects on ERK1/2 (Fig. 3*A* and *B*) and on protein kinase B (PKB) phosphorylation were not modified (supplementary Fig. S1*A*). In human adipocytes, pharmacological inhibition of Tpl2 also inhibited ERK1/2 activation induced by IL-1β and TNF-α, whereas insulin effect was not significantly modified (Fig. 3*C*).

Activation of ERK1/2 promotes IRS-1 serine phosphorylation (31), and, among the different serine residues, serine 632 is located in a MAP kinase consensus phosphorylation site. We showed that the phosphorylation of IRS-1 on serine 632 induced by IL-1β or TNF-α treatment was strongly prevented when cells were pretreated with the Tpl2 inhibitor (Fig. 3*D* and *E*). As expected, U0126 inhibited cytokine-induced IRS-1 serine phosphorylation (Fig. 3*D* and *E*).

We then used siRNA against Tpl2 to confirm its implication in ERK1/2 activation in response to inflammatory cytokines. Transfection of siRNA against Tpl2 achieved >80% efficiency in reducing endogenous Tpl2 protein levels (Fig. 4*A*). Tpl2 knockdown markedly decreased MEK and ERK1/2 phosphorylation induced by IL-1β or TNF-α (Fig. 4*B*). In contrast, Tpl2 silencing did not modify cytokine-induced IκB degradation (Fig. 4*B*) or JNK1/2 or p38 phosphorylation (supplementary Fig. S1*B*), indicating that the observed effects did not result from a general inhibitory effect on cytokine signaling. Furthermore, Tpl2 siRNA did not affect the ability of insulin to induce MEK/ERK phosphorylation (Fig. 4*B* and *C*) or PKB phosphorylation (supplementary Fig. S1*B*).

Tpl2 is involved in IL-1β and TNF-α–induced lipolysis. Proinflammatory cytokines increase lipolysis in adipocytes via activation of the MAP kinase family (10). We determined whether Tpl2 inhibition modified the lipolytic effect of TNF-α or IL-1β by measuring glycerol release as an index of lipolysis. The absolute stimulatory effect of IL-1β and TNF-α on glycerol release was decreased by 56 and 63%, respectively, in 3T3-L1 adipocytes (Fig. 5*A*) and by 85% in human adipocytes (Fig. 5*B*). MEK inhibition by U0126 treatment slightly decreased basal lipolysis and

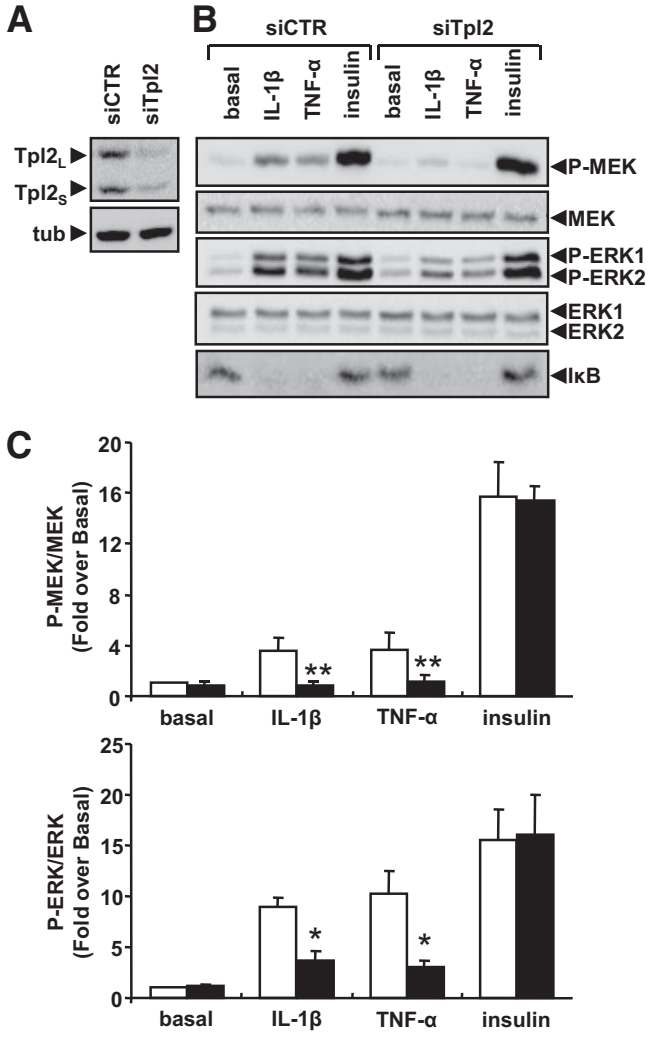

FIG. 4. siRNA-mediated silencing of Tpl2 decreases MEK and ERK1/2 phosphorylation in response to IL-1β and TNF-α in 3T3-L1 adipocytes. 3T3-L1 adipocytes were transfected with 100 pmol of control (□) or Tpl2 (■) siRNA by nucleofection using the Amaxa nucleofector, and 72 h after nucleofection, the cells were stimulated or not with IL-1β or TNF-α (20 ng/ml) for 20 min or insulin (100 nmol/l) for 10 min. Lysates were subjected to Western blotting with antibodies against phosphorylated MEK or ERK1/2 or antibodies against Tpl2, IκB, MEK, and ERK1/2 proteins. Representative immunoblots (*A* and *B*) and quantification of four independent experiments (*C*) are shown (tub: tubulin). Data are expressed as fold of MEK and ERK1/2 phosphorylation over basal in control siRNA nucleofected cells and presented as the means ± SE. **P* < 0.05 and ***P* < 0.01 vs. stimulus effect in control siRNA nucleofected cells.

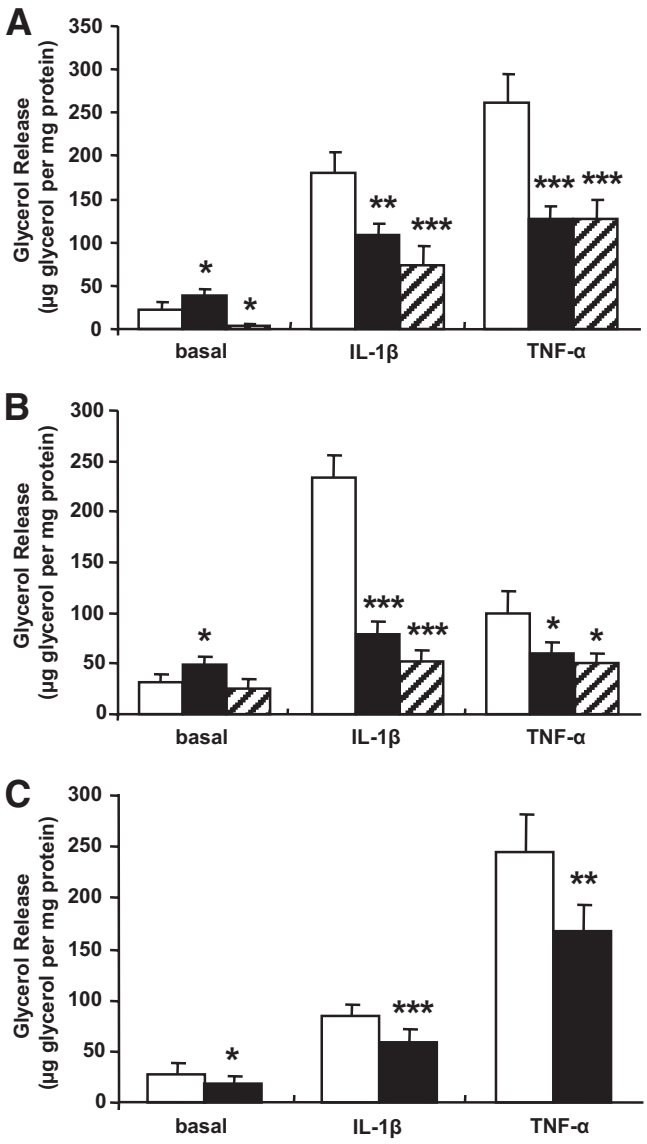

FIG. 5. Tpl2 inhibition decreases lipolysis in response to IL-1β and TNF-α in adipocytes. 3T3-L1 adipocytes (*A*) or human adipocytes (*B*) were treated without (□) or with (■) a Tpl2 inhibitor (10 µmol/l, ▨) for 1 h and then stimulated or not with IL-1β or TNF-α (20 ng/ml) for 24 h. Glycerol release was measured in the culture medium as an index of lipolysis. *C*: 3T3-L1 adipocytes were transfected with control siRNA (□) or Tpl2 (■) siRNA by electroporation using the Amaxa nucleofector, and 72 h after electroporation, the cells were stimulated or not with IL-1β or TNF-α (20 ng/ml) for 24 h and glycerol release was measured. Data are expressed as micrograms of glycerol released in the culture medium per milligram of protein and presented as the means ± SE of 4–8 independent experiments. Percent of inhibition was calculated by subtracting the value of the stimulated cytokine conditions to the value of the appropriate control without cytokines. **P* < 0.05, ***P* < 0.01, and * *P* < 0.001 vs. stimulus effect in control cells or in control siRNA electropored cells.**

inhibited cytokines effect to a level comparable to the effect observed following Tpl2 inhibition (Fig. 5*A* and *B*). siRNA-mediated silencing of Tpl2 also reduced IL-1β and TNF-α–induced glycerol release (Fig. 5*C*). These results suggest that Tpl2 is involved in the lipolytic effect of IL-1β and TNF-α in both rodent and human adipocytes through activation of the MEK/ERK pathway.

Tpl2 mRNA level is increased in adipose tissue of obese mice and subjects. ERK activity and lipolysis are increased in adipose tissue of obese rodents and obese subjects (5). We therefore investigated whether Tpl2 expression could be altered in adipose tissue in obesity. We showed that Tpl2 mRNA expression was increased in epididymal adipose tissue of *ob/ob*, *db/db*, and high-fat diet obese mice compared with their lean control littermates (Fig. 6*A*). Moreover, Tpl2 mRNA expression was positively correlated with TNF-α mRNA expression (Fig. 6*B*). A positive correlation was also found with IL-1β mRNA for adipose tissue of genetically obese mice and their lean controls (data not shown). We then examined the expression of Tpl2 mRNA in subcutaneous adipose tissue of obese patients without or with type 2 diabetes and morbidly obese subjects (Table 1). Tpl2 mRNA expression was increased in subcutaneous adipose tissue of obese subjects independently of diabetes and in adipose tissue

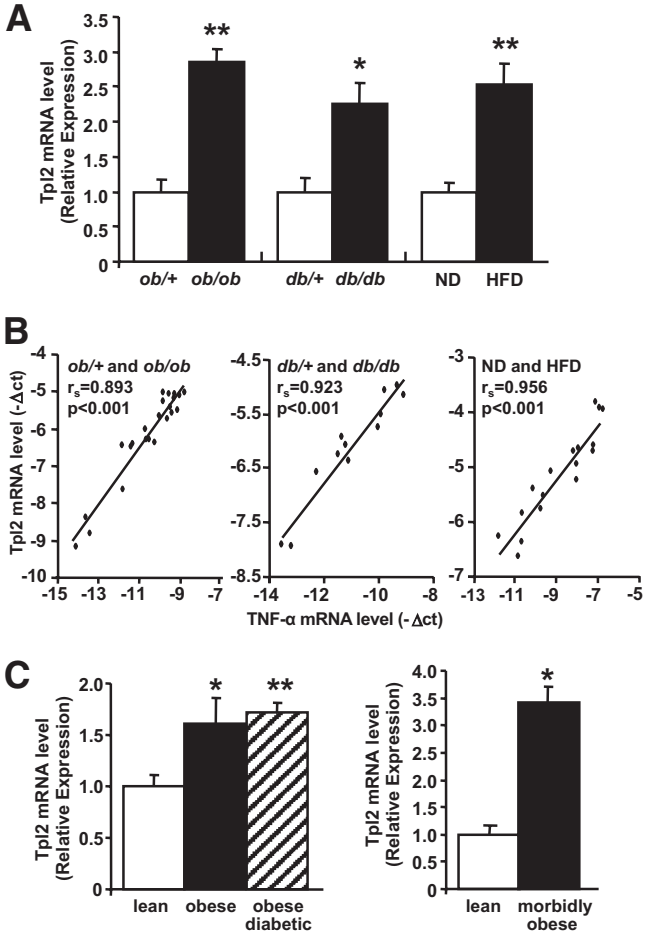

FIG. 6. Tpl2 mRNA expression is increased in adipose tissue of obese mice and subjects. *A*: Epididymal fat pads were isolated from *ob/ob*, *db/db*, and high-fat diet (HFD) mice (n = 10, 6, and 8, respectively; ■) and their lean control littermates (*ob/+* n = 10, *db/+* n = 6, and normal diet [ND] n = 8; □). Total RNAs were extracted and the relative amount of Tpl2 mRNA was determined by real-time PCR. Tpl2 mRNA expression was normalized using mouse RPLP0 RNA level. Results are expressed in arbitrary units with the control value (*ob/+*, *db/+*, or ND) taken as 1 and are the means ± SE of the number of mice in each group. *P < 0.05 and **P < 0.001 vs. lean control mice. *B*: Correlations between Tpl2 and TNF-α mRNA expression levels ($-\Delta C_t$) in *ob/ob*, *db/db*, and HFD mice and their lean control littermates were analyzed using Spearman's rank correlation test. *C*: Biopsies of abdominal adipose tissue were obtained from obese subjects without or with diabetes (n = 11 per group) and lean subjects (n = 11) or morbidly obese subjects (n = 6) and lean subjects (n = 4). Total RNAs were extracted and the relative amount of Tpl2 mRNA was determined by real-time PCR. Tpl2 mRNA expression was normalized using human RPLP0 RNA level. Results are expressed in arbitrary units with the control value (lean subjects) taken as 1 and are the means ± SE of the number of subjects in each group. *P < 0.05; **P < 0.001 vs. lean control subjects.

of morbidly obese subjects compared with lean subjects (Fig. 6*C*).

Tpl2 expression is increased by chronic IL-1β and TNF-α treatment via an IKKβ/NF-κB pathway. Because Tpl2 mRNA expression was positively correlated with TNF-α and IL-1β mRNA in mice adipose tissues, we investigated whether these inflammatory cytokines could increase the expression of Tpl2 in adipocytes. Whereas acute treatment with IL-1β or TNF-α (45 min) induced Tpl2 degradation in 3T3-L1 adipocytes, prolonged treatment with IL-1β (18 h) increased Tpl2 protein amount by threefold, whereas TNF-α (18 h) restored Tpl2 protein amount to the level of unstimulated adipocytes (Fig. 7*A*).

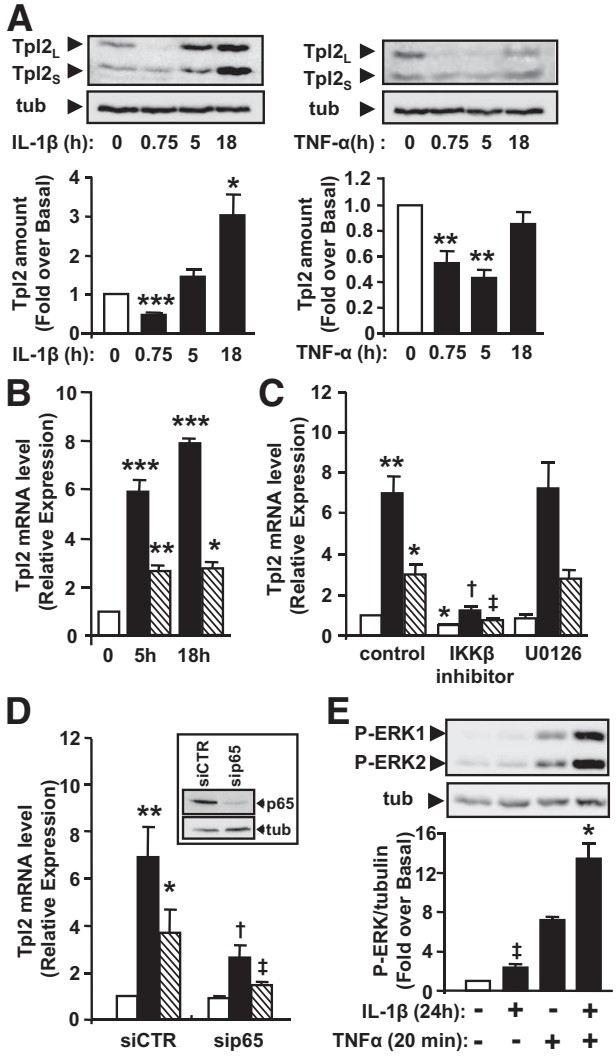

FIG. 7. Tpl2 expression is increased by long-term IL-1β and TNF-α via an IKKβ/NF-κB pathway in 3T3-L1 adipocytes. *A*: 3T3-L1 adipocytes were stimulated for the indicated times with IL-1β or with TNF-α (20 ng/ml). Tpl2 protein expression was determined using a specific antibody. Representative immunoblots and quantification of three independent experiments are shown. Data are expressed as percentage of Tpl2 protein amount in untreated cells and presented as the means ± SE. *B*: 3T3-L1 adipocytes were stimulated for 0, 5, and 18 h with 20 ng/ml of IL-1β (■) or TNF-α (▨). Total RNAs were extracted and the relative amount of Tpl2 mRNA was determined by real-time PCR. Tpl2 mRNA expression was normalized using mouse RPLP0 RNA level. Results are expressed in arbitrary units with the control value taken as 1 and are the means ± SE of three independent experiments. *C*: 3T3-L1 adipocytes were pretreated or not with an IKKβ inhibitor (5 μmol/l) or with an MEK inhibitor U0126 (10 μmol/l) for 1 h and then treated without (□) or with (■) 20 ng/ml of IL-1β or TNF-α (▨) for 18 h. mRNA expression was determined and expressed as described in *B* and is the means ± SE of three independent experiments. *D*: 3T3-L1 adipocytes were transfected with control siRNA or p65/NFκB siRNA by electroporation using the Amaxa nucleofector and 72 h after electroporation, the cells were stimulated or not with IL-1β (■) or TNF-α (▨) (20 ng/ml) for 18 h. Representative immunoblots of p65/NF-κB downregulation in three independent experiments is shown in the inset. mRNA expression was determined and expressed as described in *B* and is the means ± SE of three independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001 vs. untreated cells; †P < 0.05 vs. 18 h of IL-1β; ‡P < 0.05 vs. 18 h of TNF-α. *E*: 3T3-L1 adipocytes were treated without or with IL-1β (20 ng/ml) for 24 h and then stimulated or not with TNF-α (20 ng/ml) for 20 min. Lysates were subjected to Western blotting with antibodies against phosphorylated forms of ERK1/2 and tubulin. Representative immunoblots and quantification of four independent experiments are shown. Data are expressed as fold of ERK1/2 phosphorylation over basal and are the means ± SE. ‡P < 0.05 vs. untreated cells; *P < 0.05 vs. cells treated with TNF-α.

IL-1β or TNF-α treatment for 18 h increased Tpl2 mRNA level by 7.9- and 2.7-fold, respectively (Fig. 7*B*). IKKβ inhibition or p65/NF-κB silencing (Fig. 7*C* and *D*) prevented the increase in Tpl2 mRNA level, whereas the MEK inhibitor U0126 had no effect. We then showed that the increase in Tpl2 abundance in response to a long time of stimulation with IL-1β (18 h) resulted in an enhancement in ERK1/2 phosphorylation induced by a further acute TNF-α stimulation (Fig. 7*E*, *lanes 3* and *4*).

DISCUSSION

ERK pathway is constitutively activated in inflamed adipose tissue of obese patients and rodents and participates in the deregulation of adipocyte functions (5). Identification of regulatory proteins that govern ERK activity, specifically in response to inflammatory cytokines, may thus provide new insight into mechanisms involved in abnormal adipose tissue function.

We found that IKKβ inhibition prevented MEK/ERK1/2 activation in response to IL-1β and TNF-α, but not insulin, in both 3T3-L1 and human adipocytes. These data suggested that IKKβ regulated an MAP3K involved in ERK1/2 activation selectively by inflammatory cytokines. Among the MAP3Ks that regulate the ERK pathway, Tpl2 is more specifically activated by inflammatory stimuli (26,32). Tpl2 is expressed primarily in immune cells (33) and is involved in TNF-α production (34) following ERK1/2 activation by immunoreceptors (16,35–37). In macrophages, short and long Tpl2 isoforms are expressed due to alternative translational initiation (28,30). We found that these two isoforms were expressed in 3T3-L1 adipocytes. Expression of the long form, which is involved in MEK/ERK activation (29), was increased following adipocyte differentiation. Apart from their role in energy homesotasis, adipocytes also produce inflammatory mediators and contribute to the innate immune response (38). Thus, it makes sense that the signaling machinery that mediates this proinflammatory response is positively regulated during adipocyte differentiation.

In 3T3-L1 adipocytes, TNF-α and IL-1β rapidly decreased the amount of the long isoform of Tpl2 through a proteasome-dependent process. These data strongly support an activation of Tpl2, because Tpl2 degradation by the proteasome is tightly coupled to its activation in macrophages (29,30). Furthermore, we showed that Tpl2 degradation was prevented following IKKβ inhibition, indicating that IKKβ is an essential component of the Tpl2 signaling pathway in adipocytes. This is in agreement with studies performed in immune cells (29,30). Indeed, in nonstimulated macrophages, Tpl2 is stabilized and is inactive due to its binding to NF-κB1/p105 (29,30). Inflammatory stimuli activate IKKβ, which in turn phosphorylates NF-κB1/p105, triggering its proteolysis and the release and activation of Tpl2.

In both 3T3-L1 and human adipocytes, we demonstrated that Tpl2 was involved in MEK/ERK1/2 activation in response to IL-1β and TNF-α. In contrast, we found that Tpl2 was not involved in p38MAP kinase or JNK activation. This result is similar to what has been found in macrophages and β-cells (34,35) but differs from MEF cells, in which Tpl2 is involved in both ERK1/2 and JNK activation (39). Inhibition of IKKβ in adipocytes nearly suppressed the cytokine-induced MEK or ERK1/2 phosphorylation, whereas after inhibition of Tpl2, some phosphorylation remained. This could be because siRNA knockdown or pharmacological inhibition of Tpl2 was not sufficient to completely shutdown Tpl2 activity. Alternatively, we cannot exclude that another kinase in addition to Tpl2 is involved in ERK activation. One important finding of our study is that Tpl2 was not required for the activation of ERK or PKB pathways by insulin. Similarly, Tpl2 is dispensable for ERK activation induced by phorbol myristate acetate (PMA) (29). Thus, Tpl2 does not respond to mitogens, and its activation in adipocytes seems to be selectively restricted to inflammatory stimuli. Tpl2 could thus be an attractive target against the deleterious effects of inflammatory cytokines on adipocyte functions.

Inflammatory cytokines, such as TNF-α, stimulate lipolysis (10–12), and free fatty acids would have proinflammatory effects on adipose tissue macrophages, worsening inflammation and insulin resistance (3). By using both pharmacological and siRNA approaches, we showed that Tpl2 activation was required for cytokine-induced lipolysis. One important molecular event in TNF-α–induced lipolysis is the downregulation of perilipin through, at least in part, ERK activation (10), suggesting that the Tpl2/ERK signaling pathway could negatively regulate perilipin expression. However, Tpl2 inhibition did not totally block the lipolytic effect of TNF-α or IL-1β. It seems unlikely that this could be due to the residual ERK activity because the MEK inhibitor U0126, which completely prevents ERK activation, had a similar effect. It is more conceivable that additional pathways besides the ERK pathway could mediate the lipolytic effect and remain active following Tpl2 inhibition. Potential candidates are JNK and NF-κB, which have been shown to participate in the lipolytic effect of TNF-α (40,41). Furthermore, TNF-α, through activation of NF-κB, negatively regulates the transcription of the peroxisome proliferator–activated receptor γ (42). The consequence is the downregulation of lipid droplet–associated proteins such as CIDEA and FSP27, which contributes to the increase in lipolysis (43,44).

Abnormal ERK activation in adipocytes is also involved in alteration of insulin signaling through, at least in part, IRS-1 serine phosphorylation (7,31). We showed that inhibition of Tpl2 markedly reduced IL-1β or TNF-α–induced phosphorylation of IRS-1 on serine 632. This serine site is located in a consensus sequence for MAP kinase phosphorylation, and its phosphorylation is increased in obese and diabetic patients (45) and rodents (7). This phosphorylation negatively regulates the association of IRS-1 with phosphoinositide 3-kinase (46), suggesting that Tpl2 could also be involved in the downregulation of insulin signaling induced by inflammatory cytokines. This hypothesis deserves further future investigations.

The implication of Tpl2 in cytokine-induced lipolysis and IRS-1 serine phosphorylation suggested that abnormal activation and/or expression of Tpl2 could be involved in adipose tissue dysfunction in obesity. Interestingly, we found that Tpl2 mRNA expression was increased and was positively correlated with TNF-α mRNA levels in adipose tissue of obese rodents and subjects. This correlation strongly suggests that chronic inflammation may be involved in increased Tpl2 mRNA expression. In agreement with this possibility, a long-lasting treatment of 3T3-L1 adipocytes with TNF-α or IL-1β increased Tpl2 mRNA and restored or even increased the pool of Tpl2 protein. An elevated expression of most of the kinases in the MAP kinase pathway does not necessary result in an increase in the activity of the pathway. However, the results presented

in Fig. 7*E*, and the observation that Tpl2 overexpression increases ERK1/2 activity (32), suggest that Tpl2 expression could be rate-limiting for the activation of ERK pathway. The increase in Tpl2 mRNA level was prevented by both IKKβ inhibition and NF-κB/p65 gene silencing, and informatics analysis of the Tpl2 promoter suggests a potential NF-κB binding site. Thus, it is likely that the IKKβ/NF-κB pathway is involved in the upregulation of Tpl2 mRNA, whereas IKKβ regulates Tpl2 activation and stability through the phosphorylation and degradation of NF-κB1/p105. In agreement with this hypothesis, Tpl2 gene expression is not modified in nfκb1$^{-/-}$ macrophages, whereas Tpl2 protein expression is markedly reduced due to its degradation (29). Thus, inflammation linked to obesity could promote both the activation of Tpl2 that is coupled to its rapid degradation and the stimulation of Tpl2 gene transcription. This coordinated molecular mechanism could allow the rapid replenishment of the Tpl2 pool in inflamed adipocytes and could be responsible for the elevated activity of ERK found in adipose tissue of obese and insulin-resistant rodents and humans (7).

The pharmacological targeting of inflammatory kinases such as IKKβ has demonstrated beneficial effects in obesity. Our data suggest that Tpl2 could be also a new target to improve adipose dysfunction. Furthermore, compared with Tpl2 inhibitors, drugs that inhibit IKKβ would be expected to have more unwanted effects because the activation of NF-κB would be suppressed. Indeed, as recently discussed (26), NF-κB has many roles outside the immune system, and IKKβ is activated by many stimuli in addition to inflammatory mediators. Furthermore, whereas IKKβ knockout mice are embryonic lethal, the invalidation of Tpl2 is well tolerated with no obvious severe defects of the mice (34).

In conclusion, our work demonstrates that Tpl2 is expressed in adipocytes and is specifically involved in ERK pathway activation by IL-1β and TNF-α, whereas it is dispensable for insulin signaling. We demonstrate that inflammatory cytokines regulate both the activity and the expression of Tpl2, and this latter seems dependent on NF-κB. Finally, we provide evidence that Tpl2 signaling pathway is implicated in adipocyte lipolysis induced by these cytokines and in IRS-1 serine phosphorylation. The deregulated expression of Tpl2 in adipose tissue of obese subjects suggests that Tpl2 may be a new actor in abnormal adipose tissue function.

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