Tumor Necrosis Factor α (TNFα) Stimulates Map4k4 Expression through TNFα Receptor 1 Signaling to c-Jun and Activating Transcription Factor 2

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Tumor necrosis factor α (TNFα) is a cytokine secreted by macrophages and adipocytes that contributes to the low grade inflammation and insulin resistance observed in obesity. TNFα signaling decreases peroxisome proliferator-activated receptor γ and glucose transporter isoform 4 (GLUT4) expression in adipocytes, impairing insulin action, and this is mediated in part by the yeast Ste20 protein kinase ortholog Map4k4. Here we show that Map4k4 expression is selectively up-regulated by TNFα, whereas the expression of the protein kinases JNK1/2, ERK1/2, p38 stress-activated protein kinase, and mitogen-activated protein kinase kinases 4/7 shows little or no response. Furthermore, the cytokines interleukin 1β (IL-1β) and IL-6 as well as lipopolysaccharide fail to increase Map4k4 mRNA levels in cultured adipocytes under conditions where TNFα elicits a 3-fold effect. Using agonistic and antagonistic antibodies and small interfering RNA (siRNA) against TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2), we show that TNFR1, but not TNFR2, mediates the increase in Map4k4 expression. TNFR1, but not TNFR2, also mediates a potent effect of TNFα on the phosphorylation of JNK1/2 and p38 stress-activated protein kinase and their downstream transcription factor substrates c-Jun and activating transcription factor 2 (ATF2). siRNA-based depletion of c-Jun and ATF2 attenuated TNFα action on Map4k4 mRNA expression. Consistent with this concept, the phosphorylation of ATF2 along with the expression and phosphorylation of c-Jun by TNFα signaling was more robust and prolonged compared with that of IL-1β, which failed to modulate Map4k4. These data reveal that TNFα selectively stimulates the expression of a key component of its own signaling pathway, Map4k4, through a TNFR1-dependent mechanism that targets the transcription factors c-Jun and ATF2.

Mitogen-activated protein (MAP)2 kinases are cellular regulators of such diverse processes as apoptosis (1, 2), differentiation (3–5), and proliferation (6). MAP kinase activation by a variety of extracellular stimuli can be mediated by many types of cell surface receptors and occurs through protein kinase phosphorylation cascades (for reviews see Refs. 1 and 6). Such cascades involve the activation of a MAP kinase kinase kinase, which phosphorylates and activates a MAP kinase kinase, which in turn phosphorylates and activates an effector MAP kinase. This activation pattern is conserved from yeasts to mammals (7). In the past few years much effort has concentrated on identifying additional upstream kinases that regulate the downstream effector MAP kinases. This effort has led to the identification and characterization of the sterile 20 (Ste20p) family of protein kinases, which can act upstream of MAP kinase kinases. Sterile 20 kinases can be divided into two groups, the germinal center protein kinases and the p21-activated protein kinases. One germinal center protein kinase member, which we recently identified as a negative regulator of adipogenesis, is Map4k4 (8). Map4k4 is a member of the germinal center protein kinase IV group, which appears to control cellular events ranging from cell motility, rearrangement of the cytoskeleton, and cell proliferation (9–14). The majority of studies focusing on Map4k4 propose that Map4k4 acts as an upstream activator of the c-Jun-N-terminal kinases 1 and 2 (JNK1/2), extracellular signal-related kinase 1/2 (ERK1/2), and p38 SAP kinase (12–15).

We initially identified Map4k4 in an siRNA screen for regulators of insulin-sensitive deoxyglucose uptake in 3T3-L1 adipocytes (8). Remarkably, silencing of Map4k4 with siRNA caused an increase in the expression of peroxisome proliferator-activated receptor γ (PPARγ) along with a corresponding increase in the expression of the insulin-responsive facilitative glucose transporter isoform 4 (GLUT4) (8). PPARγ is nuclear hormone receptor that regulates the expression of numerous genes involved in adipogenesis, including those encoding GLUT4, IRS1, and PI3K, which are important in glucose and insulin signaling (16).
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Materials—Mouse recombinant tumor necrosis factor α (TNFα) was obtained from Calbiochem (catalog #654245). Mouse recombinant interleukin-1β and LPS were obtained from Sigma-Aldrich (catalog #I9646, I5271, and L6529, respectively). Antibodies against βx (sc-371), JNK1/2 (sc-7345), phospho-c-Jun (sc-1694), and phospho-JNK1/2 (sc-6254) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies against ATF2 (9226), phospho-ATF2 (9221), p38 SAP kinase (9212), and phospho-p38 SAP kinase (9215) were purchased from Cell Signaling Technology, Inc. Antibody to stimulate TNF receptor 1 (AF-425-PB) and TNF receptor 2 (ab7369) were purchased from R&D Systems, Inc. (Minneapolis, MN) and Abcam, Inc. (Cambridge, MA), respectively. TNF receptor 1 neutralizing antibody (MAB430) was also purchased from R&D Systems, Inc.

Experimental Procedures

Generation of Map4k4 Antibody—Anti-Map4k4 antibody was generated by injecting rabbits with a glutathione S-transferase fusion protein corresponding to amino acids 453–720 of Map4k4. This hydrophilic region was selected because of predicted high antigenicity and high surface probability. The region was amplified by PCR (forward primer 5′-CCCAG-GAATTCGAGAGGAGAGTGGAGAGGGAACAG-3′ and reverse primer 5′-ACGATGGCGGCCGCTCCCGCAGGCTT-GAGAGACCG-3′), cloned into a pGEX-5 vector and expressed in Escherichia coli BL21. Cultures were induced using 1 mM isopropyl 1-thio-β-D-galactopyranoside for 6 h and then lysed in STE buffer (50 mM Tris pH 7.2, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 5 mg/ml aprotinin, 5 mg/ml leupeptin, 10 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 1 mg/ml lysozyme) followed by the addition of 1% Triton, 2 μg DNase, 10 mM MgCl2, and 10 mM MnCl2, 30 min later. The Map4k4 fusion protein was then incubated with glutathione-agarose beads end over end for 1 h at 4 °C. The isolated glutathione S-transferase Map4k4 fusion peptide was shipped to Rockland for rabbit injection. 400 μg of protein was injected into two rabbits for the initial immunization followed by three 200-μg injections for immunological boosts at days 7, 14, and 21 after the initial injection. The IgG fractions were then isolated using protein A-agarose beads. To affinity purify the anti-Map4k4 antibodies, 1 mg of lysates from hemagglutinin-Map4k4 expressing COS-1 cells was resolved by SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was then cut as strips between 250 and 150 kDa and blocked with 3% bovine serum albumin, Tris-buffered saline-Tween for 1 h. The membrane strip was then incubated overnight with 2 ml of the IgG fraction and washed twice with Tris-buffered saline-Tween buffer and twice with phosphate-buffered saline. The anti-Map4k4 antibodies were eluted with the addition of 1 ml of glycine (100 mM, pH 2.6) to the strips and incubated for 10 min at room temperature, with occasional
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vortexing. The eluted antibodies were transferred to a fresh tube containing 0.1 ml of 1 m Tris buffer, pH 8.0, as well as 0.1% bovine serum albumin and 0.05% azide to bring the final pH to 7.0. Antibodies were stored at −20 °C.

Cell Culture and siRNA Transfection—3T3-L1 fibroblasts were cultured and differentiated into adipocytes as previously described (36). For siRNA transfections, cells 4 days post-induction of differentiation were used as previously described (8). Briefly, 1.125 × 10⁶ cells were electroporated using 6 nmol of siRNA and then plated in 4 wells of a 12-well plate. Cells were recovered in complete Dulbecco’s modified Eagle’s medium (10% fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin) and were cultured for 48 h after the transfection before beginning experiments.

TNFα, IL-1β, IL-6, and LPS Stimulation—3T3-L1 adipocytes, either 7 days post-differentiation or 72 h post-siRNA transfection, were washed twice with phosphate-buffered saline and treated with the indicated concentrations of TNFα in complete Dulbecco’s modified Eagle’s medium for the appropriate time intervals. For cytokine and LPS stimulations, 3T3-L1 adipocytes 4 days post-differentiation induction were harvested and resuspended in 50 ml of Dulbecco’s modified Eagle’s medium per 15-cm plate. The adipocytes were distributed such that 1 ml of the harvested adipocytes was distributed per well of a 12-plate (catalog #3515, Corning, Inc., Corning, NY). After 72 h the adipocytes were treated with the appropriate concentration of cytokine for the given period of time. In all experiments cells were washed with ice-cold phosphate-buffered saline and harvested on ice as described previously (37). Protein samples were separated on a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. Membranes were then analyzed by Western blot analysis. Changes in phosphorylation were determined through densitometry using Adobe Photoshop and normalized for loading against the non-phosphorylated kinase or transcription factor, with the exception of c-Jun, whose expression increased during the entire stimulation, and IκBα, whose expression was variable.

TNFR1 and TNFR2 Stimulation—3T3-L1 adipocytes were prepared the same as described for the cytokine treatments. 3T3-L1 adipocytes 7 days post-differentiation were treated with the indicated concentrations of antagonistic or agonistic antibodies against TNFR1 or TNFR2 in the presence or absence of TNFα for 24 h. Antibodies were used at the concentrations recommended by the manufacturers for maximal activity. Antagonistic antibodies were also tested for IL-6 induction at 10 times the concentration recommended, and no additional increase in IL-6 mRNA was detected (data not shown). After treatment, the cells were lysed and analyzed the same as for the cytokine treatments.

Isolation of RNA and Quantitative Real Time PCR—RNA isolation was performed according to the Trizol reagent protocol (catalog #15596-018, Invitrogen). Briefly, media was aspirated, and the cells were washed once with ice-cold phosphate-buffered saline. Next, 1 ml of Trizol reagent was added to each well. The concentration and the purity of the RNA were determined by measuring the absorbance at 260/280 nm. To further determine the quality of the RNA, 1 μg of total RNA was run on a 1% agarose gel, and the quality of the 28 S and 18 S ribosomal bands was inspected visually. cDNA was synthesized using 1 μg of RNA and the iScript cDNA synthesis kit (catalog #170-8891) from Bio-Rad. The cDNA was synthesized according to the protocol provided by the manufacturer in a 20-μl reaction volume. For real time PCR, 1 μl of the synthesized cDNA was loaded into 1 well of a 96-well plate for detection of a specific target gene. Primers used are listed in supplemental Fig. 1 and were designed with primer bank (38). Hypoxanthine-guanine phosphoribosyltransferase was used as an internal loading control because its expression did not change over a 24-h period with the addition of TNFα and the silencing of the genes used in this study. 10 pmol of forward and reverse primer along with 12.5 μl of the iQ SYBR Green Supermix (Bio-Rad) was added to each well along with DNase/RNase-free water for a final volume of 25 μl. Samples were run on the MyiQ Realtime PCR System (Bio-Rad). Relative gene expression was determined using the ΔCT method (39).

Statistics—The distributional characteristics of the outcomes were evaluated by both a visual inspection of histograms and the Kolmogorov-Smirnov test performed on model residuals. Transformation by natural logarithms was used in some cases to better approximate a normal distribution and to stabilize variances. The observed effects were evaluated by either one-way or multifactorial analysis of variance (ANOVA). In the presence of significant main and/or interaction effects, pairwise comparisons were made using the Tukey (HSD) multiple comparisons procedure with the exception of Fig. 3, where the Tukey-Kramer method was used due to unequal sample sizes between the groups. Computations were performed using SAS Statistical software package. The data are presented as the means ± S.E.

RESULTS

TNFα Signaling Up-regulates the Expression of Map4k4 Protein and mRNA—To better characterize the TNFα-mediated increase in Map4k4 mRNA expression, we examined the dose-response relationship and time course of TNFα action in 3T3-L1 adipocytes (Fig. 1, A and B). The fully differentiated adipocytes were treated with various concentrations of TNFα ranging from 0.1 to 100 ng/ml for 24 h. The cells were then lysed, RNA was isolated, and the Map4k4 mRNA levels were compared using quantitative real time PCR. A maximal increase in Map4k4 mRNA in response to 5–10 ng/ml TNFα was observed that reached about 3-fold (Fig 1A). This concentration of TNFα corresponds to previously reported concentrations of TNFα that caused maximal impairment of insulin signaling in 3T3-L1 adipocytes (26). A time course analysis of the increase in Map4k4 mRNA after 50 ng/ml TNFα showed a 2-fold increase in Map4k4 mRNA at 2 h that increased by 48 h to a maximal stimulation of greater than 4-fold (Fig. 1B). To determine whether the increase in Map4k4 mRNA resulted in a corresponding increase in protein, adipocytes were treated with 50 ng/ml TNFα for 24 h, lysed, and immunoblotted with an antibody we raised in rabbits. At 24 h after the addition of TNFα, a 2.5-fold increase in Map4k4 protein levels was observed (Fig. 1, C and D). Thus, treatment with TNFα significantly increases Map4k4 expression in 3T3-L1 adipocytes.
Map4k4 has been proposed to act in a cascade that stimulates JNK1/2 activity in response to TNFα through a MKK4- and MKK7-dependent mechanism (22). To determine whether TNFα increases the expression of Map4k4 mRNA selectively among the MAP kinase family, we treated 3T3-L1 adipocytes with various concentrations of TNFα over a 24 h period and then measured Map4k4, MKK4, MKK7, JNK1, JNK2, ERK1, and ERK2 protein kinase mRNA levels using quantitative real time PCR. Map4k4 mRNA levels increased 1.5, 2.6 (p < 0.01), and 3.4-fold (p < 0.01) with 1, 5, and 25 ng/ml TNFα, respectively (Fig. 2). In contrast, the expression of MKK4, MKK7, ERK2, JNK1, and JNK2 was unperturbed by TNFα signaling. ERK1 mRNA expression was found to increase only very slightly 1.2-, 1.7 (p < 0.01)-, and 1.4-fold (p < 0.05) at 1, 5, and 25 ng/ml TNFα. Furthermore, in other studies p38 SAP kinase protein expression did not change during treatment with 25 ng/ml TNFα (see Fig. 4B). Taken together, these results suggest that the increase in Map4k4 expression after TNFα treatment is unique among the MAP kinases we tested in its high responsiveness to TNFα signaling.

**Map4k4 Expression Is Specifically Augmented by TNFα and Unresponsive to IL-1β, IL-6, and LPS—TNFα and other cytokines, such as IL-1β, and LPS initiate similar intracellular signaling networks that activate p38 SAP kinase and JNK1/2 along with the transcriptional regulator NFκB (Fig. 3A) (for reviews, see Refs. 40–42). In addition, TNFα treatment of cultured and primary adipocytes increases expression and secretion of other cytokines such as IL-6, which signals through the Janus kinase signal transducer and activator of transcription pathway (Jak/Stat) (Fig. 3A) (20, 21). Thus, the increase in Map4k4 expression observed in response to TNFα may result from the secretion and actions of these secondary cytokines rather than TNFα itself. Indeed, IL-6 expression was shown in our studies to be stimulated >40-fold by TNFα in cultured adipocytes (Fig. 3C, top panel). To test whether these other cytokines also increase Map4k4 expression, we treated 3T3-L1 adipocytes with TNFα...
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Figure 4. TNFα, but not IL-1β, causes enhanced phosphorylation of p38 SAP kinase and JNK1/2 in 3T3-L1 adipocytes. 3T3-L1 adipocytes 7 days post-differentiation induction were treated with either 50 ng/ml TNFα or 100 ng/ml IL-1β. After the indicated period of time, cells were harvested, and lysates were examined by Western blot and densitometry analysis for IκBα (A), phospho (P)-p38 SAP kinase (B), phospho-JNK1 (C), and Phospho-JNK2 (D). Densitometry is representative of three independent experiments. T, total.

(50 ng/ml), IL-1β (100 ng/ml), LPS (100 ng/ml), or IL-6 (50 ng/ml) for 24 h. The concentrations of these agents tested were shown in our preliminary studies to be well in excess of the concentrations necessary for their maximal effects on expression of other genes (data not shown). In all of these experiments, only TNFα was able to increase Map4k4 expression under these conditions (Fig. 3B). That IL-1β, LPS, and IL-6 do initiate other potent biological effects under these same conditions is indicated in Fig. 3C. Thus, IL-1β and LPS stimulate IL-6 expression about 10-fold, whereas IL-6 stimulates Socs3 mRNA severalfold (Fig. 3C). Taken together, these results suggest that TNFα increases Map4k4 expression in cultured adipocytes through a signaling mechanism unique to TNFα, at least compared with the other cytokines tested in these experiments.

Signaling by TNFα, but Not IL-1β, Causes Prolonged Phosphorylation of JNK1/2 Protein Kinases, p38 SAP Kinase, c-Jun and ATF2—Because TNFα and IL-1β are thought to activate identical intracellular signaling pathways in 3T3-L1 adipocytes, we sought to compare the activation of these pathways by TNFα versus IL-1β utilizing phospho-specific antibodies targeting the downstream protein kinases and transcription factors. Cultured adipocytes were treated with TNFα (50 ng/ml) and IL-1β (100 ng/ml) for various periods of time over a 24-h period. To examine NFκB activation in parallel, we utilized antibodies recognizing total IκBα, which is rapidly degraded to initiate NFκB-mediated transcription. Interestingly, both TNFα and IL-1β caused rapid loss of the NFκB inhibitor protein, IκBα, suggesting that both cytokines activated NFκB in a similar manner (Fig. 4A). On the other hand, the activation of p38 SAP kinase was both more dramatic and more sustained in response to TNFα compared with IL-1β treatment (Fig. 4B). Specifically, TNFα caused a 70-fold increase in p38 SAP kinase phosphorylation at 10 min, whereas IL-1β caused only a 20-fold increase in p38 SAP kinase phosphorylation at this early time point. The TNFα-induced phosphorylation did subside somewhat but was maintained at levels near 20-fold over basal for >2 h and was dramatically higher than basal for >6 h (Fig. 4B). The effect of IL-1β, in contrast, returned to basal levels within 1 h. This difference in p38 SAP kinase phosphorylation caused by TNFα versus IL-1β correlated well with p38 SAP kinase activation as the phosphorylation of its sub-

strate ATF2 by TNFα, but not by IL-1β, was also sustained over the later time points (Fig. 5A).

Similar to the data obtained for p38 SAP kinase activation, both JNK1 and JNK2 protein kinases exhibited large increases in phosphorylation during the first few minutes of treatment with either TNFα or IL-1β (Fig. 4, C and D). However, TNFα-induced phosphorylations of JNK1 and JNK2 were maintained at increases of about 20- and 5-fold, respectively, for a full 24 h, whereas IL-1β-induced phosphorylations in JNK1 and JNK2 subsided to near basal levels within the first hour of stimulation (Fig. 4, C and D). The phosphorylations of JNK1 and JNK2 correlated well with the phosphorylation of their substrate transcription factors ATF2 (Fig. 5A) and c-Jun (Fig. 5B), which showed similar sustained increases in phosphorylation in response to TNFα but not to IL-1β. Furthermore, the increase in c-Jun phosphorylation was accompanied by a substantial increase in total c-Jun protein (Fig. 5C). This is consistent with previous work identifying c-Jun as a positive regulator of its own transcription (43, 44). Overall, these data suggest that although the signaling pathways activated by TNFα and IL-1β are similar, the kinetics of activation of the JNK1/2 and p38 SAP kinases are distinct.
kinase are quite different. Furthermore, the sustained phosphorylation of JNK1/2, p38 SAP kinase, c-Jun, and ATF2 in response to TNFα correlates with its action to increase Map4k4 mRNA.

Depletion of c-Jun and ATF2 Attenuates the TNFα-mediated Increase in Map4k4 mRNA Expression—c-Jun and ATF2 are among numerous transcription factors that are activated in response to TNFα/H9251 and our data described above suggested that Map4k4, c-Jun, and ATF2 are all differentially regulated by TNFα versus IL-1β. Both of these transcription factors are only transiently activated after stimulation with IL-1β, whereas TNFα provokes a robust and sustained phosphorylation of c-Jun and ATF2 in 3T3-L1 adipocytes (Fig. 5). Thus, it is plausible that the sustained activation of these transcription factors by TNFα is required to cause the increase in Map4k4 expression (Figs. 1–3). To address the role of c-Jun and ATF2 in TNFα action on the expression of Map4k4, we depleted c-Jun and ATF2 in 3T3-L1 adipocytes using siRNA and then treated the cells with various concentrations of TNFα over a 24-h period. Silencing either c-Jun or ATF2 caused a dramatic decrease in Map4k4 expression in the presence of 1, 5, and 25 ng/ml TNFα (Fig. 6A). The decrease was highly statistically significant (p < 0.01) for 5 and 25 ng/ml TNFα upon silencing c-Jun, ATF2, or both molecules in combination. Additionally, when silencing both transcription factors simultaneously, we observed a decrease in the augmentation of Map4k4 mRNA expression in response to 1 ng/ml TNFα (p < 0.05). Validation of the siRNA-based decreases in c-Jun and ATF2 mRNA and protein (not shown) revealed reductions of ~50% (Fig. 6, C and D). Interestingly, silencing of c-Jun and ATF2 did not significantly impair the up-regulation of IL-6 mRNA (Fig. 6B). This is consistent with previous work, which identified Fra-1 and JunD but not c-Jun or ATF2 as interacting with the AP-1 binding sequence in the IL-6 promoter in the presence and absence of TNFα stimulation (45, 46). Altogether, these results combined with the observed sustained activation of c-Jun and ATF2 in response to TNFα (Fig. 5, A and B) suggest that c-Jun and ATF2 are required for TNFα-enhanced levels of Map4k4.

TNFR1 Stimulation Increases Map4k4 mRNA Expression—TNFα exerts its effects through two distinct receptors, denoted type 1 (TNFR1) and type 2 receptors (TNFR2) (42). The roles of these receptors in the inhibition of insulin action, lipolysis, and the negative regulation of adipocyte-specific genes has been well characterized (34, 47, 48) and is mostly attributed to TNFR1. To determine whether TNFR1 or TNFR2 is responsible for...
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**A** Map4k4 mRNA

**B** IL-6 mRNA

**C** Map4k4 mRNA

**D** Map4k4 mRNA

increasing Map4k4 expression specifically, we first utilized antibody preparations with specific agonistic properties for TNFR1 and TNFR2 as well as antagonistic properties for TNFR1. In agreement with our previous experiments (Fig. 1, A and B, 2, and 3B), 24 h of TNFα treatment significantly increased Map4k4 mRNA expression ~3-fold in 3T3-L1 adipocytes (p < 0.01) (Fig. 7A). Incubation of the cells with TNFR1 agonistic antibodies almost completely blocked this effect of TNFα, whereas TNFR1 agonistic antibodies strongly mimicked the effect (p < 0.01) (Fig. 7A). Interestingly, agonistic antibodies against TNFR2 failed to increase Map4k4 mRNA expression (Fig. 7A). Non-immune IgG antibodies from the appropriate sources had no effect on either Map4k4 expression in TNFα-stimulated or unstimulated conditions (data not shown). These results suggest that TNFα-stimulated Map4k4 expression is mediated through TNFR1, not TNFR2.

To assess whether the antibodies were functioning properly we measured the changes in IL-6 mRNA expression, a cytokine highly up-regulated in adipocytes after TNFα stimulation (20). In agreement with previous studies (Fig. 3C and 6B), TNFα caused a 20-fold increase in IL-6 mRNA expression (p < 0.01) (Fig. 7B). Blocking the actions of TNFR1 with antagonistic antibodies significantly blunted the effect of TNFα by ~75%. However, adipocytes treated with the TNFR1 antagonistic antibodies and TNFα still showed a 4.5-fold increase in IL-6 mRNA, which presumably was due to the activity of TNFR2 (Fig. 7B). In agreement, the TNFR2 agonistic antibodies increased IL-6 mRNA expression 4-fold. Taken together, these results suggest that the TNFR1 antagonistic antibodies are potent inhibitors of TNFR1 function under the conditions of our experiments (Fig. 7B). Furthermore, TNFR1 agonistic antibodies increased IL-6 mRNA ~13-fold (p < 0.01), suggesting that unlike the increase in Map4k4 mRNA, the increase in IL-6 mRNA requires the actions of both receptors.

We also addressed the role of TNFR1 and TNFR2 in increasing Map4k4 mRNA after TNFα treatment by using siRNA to deplete the expression of these receptors in 3T3-L1 adipocytes. In agreement with the results using antagonistic and agonistic TNFα receptor antibodies, silencing of TNFR1, but not TNFR2, significantly blunted the TNFα increase in Map4k4 mRNA (Fig. 7C). Silencing was confirmed by assessing mRNA levels of TNFR2 and TNFR1, which showed ~70 and 60% depletions, respectively (Fig. 7D). Altogether, these data suggest that signaling through TNFR1 increases Map4k4 expression after TNFα treatment in cultured adipocytes.

To determine whether unique signaling pathways are activated by TNFα, which may account for the increase in Map4k4 mRNA expression, downstream signaling elements from TNFR1 and TNFR2 were dissected using agonistic antibodies. 3T3-L1 adipocytes were treated with TNFα, TNFR1, or TNFR2 agonistic antibodies for various periods of time over 12 h, and activation of JNK1/2, p38 SAP kinase, c-Jun, and ATF2 was assessed using phospho-specific antibodies. Additionally, the disappearance of IκBα was used to estimate NFκB activation. Stimulation of TNFR1 resulted in a similar increase in phosphorylation of JNK1/2, p38 SAP kinase, c-Jun, and ATF2 as well as a similar loss of IκBα and increase in total c-Jun protein when compared with TNFα treatment (Fig. 8, A–D and Fig. 9, A–C). TNFR2 stimulation did not activate most intracellular signaling pathways tested, although we did observe a slight decrease in IκBα at the later time points, suggesting a possible slight activation of NFκB (Fig. 8A). Additionally, p38 SAP kinase (Fig. 8B) and ATF2 (Fig. 9A) phosphorylation increased to 5- and 2-fold over basal, respectively, after TNFR2 stimulation. Thus, these data are similar to our previous results observed regarding the differences between IL-1β and TNFα signaling (Figs. 4 and 5), in which c-Jun and ATF2, but not...
NFκB activation, correlates with the increase in Map4k4 mRNA expression.

DISCUSSION

TNFα promotes cellular inflammatory responses by altering gene expression through NFκB and AP-1 signaling (40, 42) and appears to play significant roles in insulin resistance in obese mice (29, 30) and humans (31–33). The protein kinase Map4k4 is a proximal element in the TNFα signaling cascade (15, 22) and may mediate in part TNFα actions on PPARγ and GLUT4 that impair insulin responsiveness in adipocytes (8). Furthermore, TNFα appears to utilize Map4k4 to cause insulin resistance in muscle, as siRNA depletion of Map4k4 in muscle tissue from diabetic patients completely restored insulin sensitivity (15). In this study we document that TNFα signaling increases the expression of Map4k4 in 3T3-L1 adipocytes (Fig. 1), potentially amplifying its own acute signaling pathway. Our findings lead to the model summarized in Fig. 10 in which TNFα activates TNFR1, causing a robust and potentiated activation of JNK1/2 and p38 SAP kinase (Figs. 4 and 8). According to this model, activation of JNK1/2 and p38 SAP kinase cause increased phosphorylation of ATF2 and c-Jun along with an increase in total c-Jun protein (Figs. 5 and 9). An important finding of this study was that c-Jun and ATF2 were required for optimal stimulation of Map4k4 expression by TNFα (Fig. 6). Taken together, these results provide mechanistic insight into TNFα signaling and indicate that in addition to acutely activating Map4k4 protein kinase activity (22), the cytokine enhances the abundance of this kinase through transcriptional regulation. As previously shown, siRNA depletion of Map4k4 partially prevents the TNFα depletion of GLUT4 and adipogenesis in cultured adipocytes (8). Hence, the increased activity (data not shown here) and the increased amount of Map4k4 induced by TNFα would be expected to provide a potent stimulus to decrease adipogenesis and inhibit glucose uptake.

The kinetics of phosphorylation of JNK1/2 and p38 SAP kinase and activation of NFκB through signaling by TNFR1 or TNFR2 or by TNFα and IL-1β has not been previously characterized in detail in adipocytes. The activation of these signaling pathways is of interest because IL-1β and TNFα negatively regulate insulin signaling, and both cytokines suppress genes that enhance glucose uptake in adipocytes (20, 21, 26, 28, 34). Comparing the time frame in which TNFα regulates the expression of genes to the time course of activation of these signaling pathways provides insight regarding the mechanisms responsible for gene regulation. Using this approach we found striking correlations between the extent to which TNFα was able to maintain prolonged activation of JNK1/2 and p38 SAP kinase and its ability to increase Map4k4 expression (Figs. 1 and 4). In contrast, the other cytokines we tested exerted only transient effects on these intermediate protein kinases and failed to mimic the stimulatory effects of TNFα on Map4k4 expression (Fig. 3). Interestingly, LPS and IL-1β, but not IL-6, have also been implicated in decreasing PPARγ levels in brown adipocytes (49). Considering that these cytokines do not increase Map4k4 expression (Fig. 3), our results suggest that TNFα may utilize Map4k4 expression as a unique mechanism to augment its inhibitory actions on PPARγ levels. These data suggest that TNFα is a particularly potent cytokine in regulating adipose function and support previous work indicating it is a powerful negative regulator.
of adipogenesis and insulin signaling in intact mice and humans (20, 21, 23, 25–27, 29, 30, 32, 33).

The signaling mechanism through which TNFα regulates Map4k4 expression is mostly mediated by the TNFR1, whereas the actions of TNFRII play little or no role (Figs. 7–9). These data are consistent with previous work showing a requirement of TNFR1 for TNFα attenuation of insulin signaling and the enhancement of lipolysis (34, 48). Interestingly, TNFR2 was not required for the impairment of adipogenesis (47). Furthermore, in that study expression of a chimeric receptor containing the TNFR2 extracellular domain and the TNFR1 intracellular domain inhibited adipogenesis, suggesting that distinct intracellular signaling pathways are activated by TNFR1 and not by TNFRII in cultured adipocytes. This concept was confirmed by our findings (Figs. 8 and 9). Furthermore, in the same body of work the authors show that the presence of the chimeric receptor suppressed PPARγ levels despite a failure of this receptor to activate NFκB in the presence of TNFα. Thus, our findings confirm data from other cell systems in which TNFRII stimulation failed to produce robust JNK and NFκB activation (42, 50, 51).

Map4k4 regulation by TNFα may be relevant to functions of this protein kinase beyond its role in adipose biology, especially to its potential regulation of cell motility. For example, Map4k4 is necessary for the epithelial to mesenchymal transition during mouse embryo gastrulation, as evidenced in Map4k4 null mice (12, 52). Although these mice die in the embryonic stage, it is possible to show that the mesoderm is properly specified in these mouse embryos. Instead, the defect resides in the inability of these mesenchymal precursor cells to migrate (12). Additionally, TNFα has been proposed to positively regulate the epithelial to mesenchymal transition in a number cell systems (53, 54), potentially suggesting a role for Map4k4. Furthermore, Map4k4 was also recently shown to be necessary for the migration of cancer cells (13). Considering that malignancies like colon cancer require an epithelial to mesenchymal transition and that this transition is positively regulated by TNFα (53), a potential role for Map4k4 regulating cancer invasion is plausible. These and other recent studies indicating that Map4k4 negatively regulates cellular adhesion and promotes cellular migration (12–14) suggest that Map4k4 may in some way modulates cytoskeletal elements or other cellular components necessary for cell motility. Thus, in addition to potentially regulating cancer cell invasion, the regulation of Map4k4 may be important in cells that require motility for proper function, such as cells of myeloid lineages. These cells are known to use a transcriptional network similar to adipocytes to regulate gene expression and even the production of cytokines (55, 56). Thus, it is possible that TNFα may increase Map4k4 expression in other cell types to elicit multiple biological responses, including metastasis of cancer cells. In summary, we have shown here that TNFα is unique among a number of cytokines in its ability to increase the expression of a major element within its own acute signaling pathway, the protein kinase Map4k4. Map4k4 is in turn unique in its response to TNFα among many MAP kinases tested for this response. Furthermore, Map4k4 is distinctive among many MAP kinases tested in its ability to modulate
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Understanding the underlying molecular mechanisms whereby TNFα regulates Map4k4 expression may, thus, provide insight into new therapies for multiple disease states, including cancer and diabetes.

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FIGURE 10. Model for the increase in Map4k4 expression via TNFα signaling. Our data are consistent with the following hypothesis. Treatment of 3T3-L1 adipocytes with TNFα stimulates TNFR1 and causes enhanced activation of JNK1/2 and p38 SAP kinase. In turn, activated JNK1/2 and p38 SAP kinase cause increased phosphorylation and, thus, activation of c-Jun and ATF2. Increased activation of c-Jun and ATF2 leads to increased Map4k4 transcription, thus increasing Map4k4 expression. This increase in Map4k4 expression then negatively regulates PPARγ expression and adipogenesis in 3T3-L1 adipocytes.

insulin sensitivity in cultured adipocytes, as does TNFα (8). The increase in Map4k4 expression in response to TNFα is mediated through a signaling pathway elicited selectively by TNFR1 activation leading to c-Jun and ATF2 activation.
Regulation of Map4k4 Expression by TNFα