Tumor Necrosis Factor-α Suppresses Adipocyte-Specific Genes and Activates Expression of Preadipocyte Genes in 3T3-L1 Adipocytes

Nuclear Factor-κB Activation by TNF-α Is Obligatory

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Tumor necrosis factor-α (TNF-α) is a contributing cause of the insulin resistance seen in obesity and obesity-linked type 2 diabetes, but the mechanism(s) by which TNF-α induces insulin resistance is not understood. By using 3T3-L1 adipocytes and oligonucleotide microarrays, we identified 142 known genes reproducibly upregulated by at least threefold after 4 h and/or 24 h of TNF-α treatment, and 78 known genes downregulated by at least twofold after 24 h of TNF-α incubation. TNF-α−induced genes include transcription factors implicated in preadipocyte gene expression or NF-κB activation, cytokines and cytokine-induced proteins, growth factors, enzymes, and signaling molecules. Importantly, a number of adipocyte-abundant genes, including GLUT4, hormone sensitive lipase, long-chain fatty acyl-CoA synthase, adipocyte complement-related protein of 30 kDa, and transcription factors CCAAT/enhancer binding protein-α, receptor retinoid X receptor-α, and peroxisome profilerator−activated receptor γ were significantly downregulated by TNF-α treatment. Correspondingly, 24-h exposure of 3T3-L1 adipocytes to TNF-α resulted in reduced protein levels of GLUT4 and several insulin signaling proteins, including the insulin receptor, insulin receptor substrate 1 (IRS-1), and protein kinase B (AKT). Nuclear factor-κB (NF-κB) was activated within 15 min of TNF-α addition. 3T3-L1 adipocytes expressing IκBα-DN, a nondegradable NF-κB inhibitor, exhibited normal morphology, global gene expression, and insulin responses. However, absence of NF-κB activation abolished suppression of >98% of the genes normally suppressed by TNF-α and induction of 60–70% of the genes normally induced by TNF-α. Moreover, extensive cell death occurred in IκBα-DN−expressing adipocytes after 2 h of TNF-α treatment. Thus the changes in adipocyte gene expression induced by TNF-α could lead to insulin resistance. Further, NF-κB is an obligatory mediator of most of these TNF-α responses. *Diabetes* 51:1319–1336, 2002

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ACRP30, adipocyte complement-related protein of 30 kDa; Adm, adrenomedullin; AKT, protein kinase B; AP-1, activator protein 1; Bcl-3, B-cell leukemia/lymphoma-3; CDK, cyclin-dependent kinase; CEBP-α, CCAAT/enhancer binding protein-α; EST, expressed sequence tag; Fra-1, Fos-related antigen-1; GATA-6, GATA-binding protein 6; GFP, green fluorescent protein; HMGP-1C, high mobility group protein-1 isoform C; HSL, hormone-sensitive lipase; IKK, IκB kinase; IKKi, inducible IKK; IL-6, interleukin 6; IR, insulin receptor; IRS-1, IR substrate 1; LPSBP, lipopolysaccharide binding protein; LTR, long terminal repeat; MAPKKK, mitogen-activated protein kinase kinase kinase; MMP-3, matrix metalloproteinase 3; NF-κB, nuclear factor-κB; PAI-1, plasminogen activator inhibitor 1; pMIG, pMSCV-IRES-GFP; pIκBα-DN, pMSCV-IκBα__DN-IRES-GFP; PPAR-γ, peroxisome profilerator−activated receptor γ; RXR, receptor retinoid X receptor-α; Spi2-1, serine protease inhibitor 2-1; TNF, tumor necrosis factor-α; VCAM-1, vascular cell adhesion molecule 1.

Insulin resistance is a major defect frequently seen in obesity and obesity-linked type 2 diabetes (1–3). Tumor necrosis factor-α (TNF-α) has been implicated as a contributing cause of insulin resistance associated with the above diseases by its expression pattern as well as by provocative in vitro and in vivo models (4–7). TNF-α is expressed in adipose tissue, and its mRNA and protein levels are significantly increased in adipose tissue of obese animals (8–10) and humans (11,12). Long-term exposure of cultured cells (1–5 days) (13) or animals (14) to TNF-α induces insulin resistance, whereas neutralization of TNF-α in *fa/fa* Zucker rats for 3 days increases insulin sensitivity, reduces hyperinsulinemia, and increases insulin receptor (IR) tyrosine kinase activity in adipose tissue (5). Moreover, data from gene knockout animals indicate that the absence of TNF-α or TNF receptors significantly improves insulin sensitivity in genetic (*ob/ob*) and dietary (high-fat diet) models of rodent obesity (15,16). Thus, TNF-α clearly contributes to the development of insulin resistance in the above settings.

Although the effects of TNF-α are well known, the mechanisms by which TNF-α induces insulin resistance are not fully understood. Because the tyrosine kinase activity of the IR is critical for insulin action (17), the effects of TNF-α on insulin-induced tyrosine phosphorylation of the IR and IR substrate 1 (IRS-1) have been the focus of intensive studies. TNF-α treatment of a number of cell lines, including primary human adipocytes, rat hepatoma Fao cells, human embryonic kidney 293 cells, and NIH-3T3 fibroblasts, has been shown to result in immediate inhibition of insulin-induced tyrosine phosphorylation of IR and IRS-1 (18–20). In contrast, short-term TNF-α treatment (15 min to 2 h) of 3T3-L1 adipocytes promotes insulin-mediated tyrosine phosphorylation of IRS-1 (21),

whereas long-term TNF-α exposure (6 h to 5 days) diminishes tyrosine phosphorylation of both IR and IRS-1 (22,23). Thus, the effects of TNF-α on insulin signaling appear to be related to the cell type and duration of TNF-α treatment.

Chronic (6 h to 5 days) TNF-α treatment of 3T3-L1 adipocytes leads to decreased tyrosine phosphorylation of IRS-1, with a concomitant increase in serine phosphorylation (13,22,23). The increased serine phosphorylation of IRS-1 inhibits the tyrosine kinase activity of the IR and has been postulated as a mechanism by which long-term TNF-α treatment impairs insulin action (23,24). Because phosphorylation and dephosphorylation are rapid events that take place in seconds to minutes, an increase in serine phosphorylation and a decrease in tyrosine phosphorylation of IRS-1 after a 5-day exposure to TNF-α seems unlikely to be caused by a direct effect of TNF-α. Supporting this notion, Stephens et al. (25) showed that insulin resistance induced by chronic (4-day) TNF-α treatment is associated with a loss of IRS-1 and GLUT4 proteins rather than a defect in IR signaling. Thus, the effects of TNF-α on tyrosine and/or serine phosphorylation of IRS-1, and its subsequent impact on insulin signaling and induction of insulin resistance have not been established.

TNF-α is a well-known transcriptional regulator, which, through the activation of nuclear factor-κB (NF-κB), activator protein 1 (AP-1), and possibly other DNA binding proteins, regulates the expression of diverse immune and inflammatory response genes in many cell types, including monocytes, macrophages, lymphoid cells, and fibroblasts (26–28). Consequently, TNF-α affects multiple processes, including proliferation, differentiation, apoptosis, and survival in many types of cells, and modulates inflammation, immunity, and energy metabolism in vivo (29,30). In 3T3-L1 adipocytes, the loss of insulin responsiveness requires chronic TNF-α treatment, indicating that changes at the level of gene transcription might be required for TNF-α induction of insulin resistance as well. Based on these observations, we hypothesized that transcriptional regulation might constitute at least a subset of TNF-α effects, distinct from interfering with IR signaling in 3T3-L1 adipocytes.

In this study, we demonstrated that TNF-α has major and rapid effects on 3T3-L1 adipocyte gene expression, including suppression of adipocyte-abundant genes essential for insulin responsiveness and selective induction of preadipocyte genes. Further, we demonstrated that TNF-α acts via NF-κB activation, as most aspects of TNF-α–induced changes in gene expression were abolished through the expression of a nondegradable mutant of NF-κB inhibitor, IκBα-DN.

Our data are intriguing because other components of the NF-κB signaling pathway have been implicated in the induction of insulin resistance. The IκB kinase (IKK) superfamily comprises IKK-α, -β, and -γ, together with their upstream activating kinases (protein kinase C isozymes, mitogen-activated protein kinase kinase kinases [MAPKKKs]), mediate signaling to NF-κB from a diverse array of stimuli, including TNF-α (31). TNF-α activates IKKs, which can in turn phosphorylate IκBs (the inhibitors of NF-κB) and activate NF-κB. In human embryonic kidney of 293 cells, forced expression of IKKβ or its stimulator NF-κB inducing kinase (a MAPKKK) induces responses typical of impaired insulin signaling, such as attenuated insulin activation of the IR, IRS-2, and AKT. Conversely, inhibition of IKKβ increases systemic insulin sensitivity in *ob/ob* mice or during high-fat feeding (32). Taken together, these data implicate NF-κB as an obligatory mediator of TNF-α induction of insulin resistance in 3T3-L1 adipocytes, and suggest that adipocyte NF-κB may be a reasonable target for drug development.

RESEARCH DESIGN AND METHODS

Cell culture. 3T3-L1 cells were purchased from American *Type Culture* Collection (Rockville, MD), maintained as fibroblasts, and differentiated into adipocytes, as previously described (25). TNF-α and insulin treatments were performed as described in figure legends.

Stable cell lines. The retroviral expression vector pMSCV-IRES-GFP (green fluorescent protein, pMIG), as well as the construct pMSCV-IκBα__DN-IRES-GFP (pIκBα-DN) encoding the dominant negative IκBα (a nondegradable mutant of IκBα) and GFP were gifts from L. Van Parijs (Massachusetts Institute of Technology, Cambridge, MA). To generate retroviruses, Phoenix packaging cells were transfected with 8 μg pMIG or pIκBα-DN plasmid cDNA using Polyfect (Qiagen, Chatsworth, CA). The supernatant containing the retroviruses was collected 48 h after transfection and used to infect 3T3-L1 preadipocytes in the presence of 4 μg/ml polybrene. Then, 48 h after infection, cells were selected for GFP expression by flow cytometry. Because both GFP and IκBα-DN are under control of the retroviral long terminal repeat (LTR) promoter, protein levels of GFP are therefore indicative of IκBα-DN expression. GFP-positive cells were amplified once and were sorted again by flow cytometry to select cells with the top 1% of GFP expression.

Northern blot analysis. Blots containing 20 μg total RNA per lane isolated from indicated cells were hybridized with ^{32}P-labeled cDNAs encoding mouse plasminogen activator inhibitor-1 (PAI-1), adipocyte complement-related protein of 30 kDa (ACRP30), and GLUT4. The filters were exposed to an imager screen (Fuji Scientific), after which filters were stripped and reprobed with ^{32}P-labeled β-actin cDNA. All hybridization signals were scanned on a phosphoimager and normalized to β-actin signals.

Microarrays. Total RNA was isolated from indicated sources by using an RNeasy kit (Qiagen). cDNA was synthesized from equal amounts of total RNA by using the Superscript System (Life Technologies, Rockville, MD) with an oligo-dT primer containing T7 promoter sequences. Biotinylated cRNA was generated by in vitro transcription, purified by the RNeasy kit (Qiagen), and quantified by ultraviolet spectrometry. Hybridization to murine U74A or U74Av2 oligonucleotide arrays (Affymetrix, Santa Clara, CA) was performed according to vendor's protocols and scanned on Affymetrix Gene-Array Scanners. Three independent experiments were performed to examine gene expression after 0, 4, and 24 h of TNF-α incubation. In addition, data were collected at multiple time points (0, 0.5, 1, and 2 h) after TNF-α stimulation of wild-type adipocytes or adipocytes expressing IκBα-DN to assess the functional involvement of NF-κB in the kinetics of TNF-α–induced changes in gene expression. Because extensive cell death occurred in IκBα-DN adipocytes after 2 h of TNF-α stimulation, we performed this experiment in the two cell types up to 2 h of TNF-α addition.

The quality of the hybridization on each array was first evaluated by the percent of P-calls (P-calls according to an Affymetrix algorithm) present on each array. Because nonspecific hybridization to mismatch oligonucleotides and severe truncation of mRNAs will substantially lower the percentage of P-calls, arrays with <25% of P-calls were discarded. The average of P-calls for all arrays used in this study was 40.1 ± 1.4% (means ± SE; $n = 17$). Analysis of differential gene expression was performed by using GeneSpring software (version 4.07; Silicon Genetics, Redwood City, CA). Array measurements for all samples were normalized with arrays hybridized with cRNA prepared from the appropriate control cells by using the median of the hybridization signals of all genes with P-calls as a scaling factor. An arbitrary value of 50 was allocated for genes whose expression levels were undetectable or scan readouts were below 50 to facilitate calculation of the fold changes compared with the control condition. Identification of genes up- or downregulated by TNF-α treatment was determined by selecting only those significantly (greater than threefold for upregulation and twofold for downregulation) and reproducibly increased or decreased in at least two independent experiments. We then classified each of the TNF-α–regulated genes into functional categories according to the putative biological functions of the encoded protein, as determined by searches of public databases. Gene expression profiles were confirmed by semi-quantitative RT-PCR analysis of 23 potentially important mRNAs regulated by TNF-α.

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RT-PCR. Total RNAs were isolated from 3T3-L1 adipocytes treated with TNF-α (1 nmol/l) for 0, 4, and 24 h. Each sample of first-strand cDNA was synthesized from 1 μg total RNA. Then, various gene-specific primers were used to amplify target genes using equal amounts of the first-strand cDNA as template. PCR reactions were performed for 30 and 35 cycles, unless otherwise indicated, to identify the linear range for PCR. PCR products were separated on a 1.5% agarose gel and photographed.

Western blotting. 3T3-L1 adipocytes were incubated in growth medium with or without TNF-α (1 nmol/l) for various times. At the end of the incubation, cells were washed three times with ice-cold PBS (pH 7.4) and solubilized. The indicated cell lysates were separated by SDS-PAGE and electroblotted onto a nitrocellulose membrane (Amersham, Arlington Heights, IL). The filter was incubated for 1 h at room temperature with various primary antibodies (as indicated in the figure legends), washed, and incubated with the appropriate horseradish peroxidase–conjugated secondary antibody. Bound antibodies were detected using the enhanced chemiluminescence Western blotting analysis system (Amersham). Blots were stained with Ponceau S solution to visualize the amount of total protein in each lane.

Electrophoretic mobility shift assay and electrophoretic mobility supershift assay. Nuclear extracts were prepared from the indicated sources as described by Wiegmann et al. (33). The NF-κB–specific oligonucleotide was purchased from Santa Cruz (Santa Cruz, CA) and was end-labeled with [γ-^{32}P]ATP (Amersham) using polynucleotide kinase. Electrophoretic mobility shift assays were performed essentially as previously described (33). For the electrophoretic mobility supershift assay, the nuclear extract was preincubated with an appropriate amount of anti-RelA/p65 antibody (Santa Cruz), as suggested by the vendor, at room temperature for 20 min.

Oil Red O staining. 3T3-L1 adipocytes were grown in six-well plates, fixed with 4% paraformaldehyde, and stained with 0.2% Oil Red O for 10 min at room temperature. Cells were washed exhaustively with PBS and water and examined using an inverted microscope and photographed.

RESULTS

Time- and dosage-dependent effects of TNF-α on gene expression in 3T3-L1 adipocytes. To determine the amount of TNF-α needed to elicit maximal changes in gene expression in 3T3-L1 adipocytes, we examined the dosage-dependent effect of TNF-α on mRNA levels of several adipocyte-specific genes. A 24-h treatment of 3T3-L1 adipocytes with insulin (100 nmol/l) caused a 20% increase in PAI-1 mRNA (Fig. 1*A*, *lane 2*, and Fig. 1*C*, *top panel*), but did not affect the level of mRNA encoding ACRP30, an adipocyte-derived hormone that decreases gluconeogenesis in liver, increases free fatty acid oxidation in skeletal muscle, and reduces body weight without affecting food intake (34,35) (Fig. 1*B*, *lane 2*, and Fig. 1*C*, bottom panel). In contrast, incubation of 3T3-L1 adipocytes with 0.04 nmol/l TNF-α for 24 h resulted in a 27% reduction in ACRP30 gene expression, with maximal suppression (49%) occurring at 1 nmol/l of TNF-α (Fig. 1*B*, *lanes 3–6*, and Fig. 1*C*, bottom panel). A 24-h exposure to TNF-α at a concentration as low as 0.04 nmol/l caused a 23% increase in PAI-1 mRNA, with maximal induction of PAI-1 mRNA occurring at 0.2 nmol/l of TNF-α (167% that of control) (Fig. 1*A*, *lanes 3–6*, and Fig. 1*C*, top panel). Thus, when using PAI-1 and ACRP30 as models, the maximal induction or suppression of gene expression in 3T3-L1 adipocytes by 24-h TNF-α treatment occurs at concentrations of 0.2–1.0 nmol/l.

Figure 2 shows that TNF-α–induced changes in gene expression in 3T3-L1 adipocytes can be detected by Northern blot analysis as early as 4 h. 3T3-L1 adipocytes were incubated with insulin, TNF-α, or both for various times, as indicated in the figure legend. Insulin alone did not significantly alter the mRNA levels of either ACRP30 or GLUT4 at any time point examined (Fig. 2*A*, *lanes 2*, *6*, and *10*, and Fig. 2*B*). In contrast, although 1-h TNF-α incubation had no detectable effect on ACRP30 and GLUT4 mRNA levels, 4 h exposure to TNF-α suppressed ACRP30 mRNA by 33% (Fig. 2*A*, *lane 7*, and Fig. 2*B*, top panel), and a 24-h TNF-α incubation reduced ACRP30 mRNA levels by 79% (Fig. 2*A*, *lane 11*, and Fig. 2*B*, top panel). Similarly, 4- and 24-h TNF-α treatment of 3T3-L1 adipocytes reduced GLUT4 mRNA by 47 and 78%, respectively (Fig. 2*A*, *lane 7* and *11*, and Fig. 2*B*, bottom panel). The addition of insulin (100 nmol/l) partially blocked the inhibitory effect of 24-h TNF-α incubation on GLUT4 and ACRP30 mRNA levels. As shown in Fig. 2*A* (*lane 12*) and Fig. 2*B*, insulin reduced TNF-α–triggered inhibition from 78 to 36% for GLUT4 mRNA, and from 79 to 59% for the ACRP30 transcript.

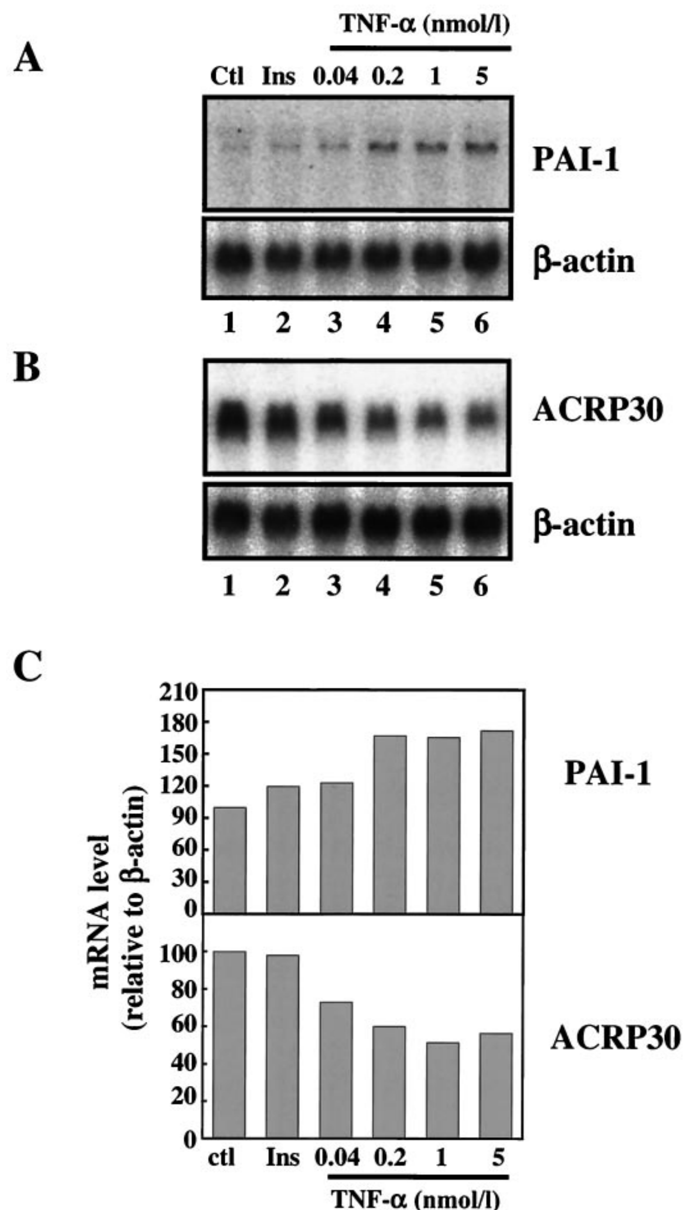

FIG. 1. Dosage-dependent effects of TNF-α on PAI-1 and ACRP30 gene expression in 3T3-L1 adipocytes. 3T3-L1 adipocytes were untreated (ctl; *lane 1*) or treated with 100 nmol/l insulin (Ins; *lane 2*), or TNF-α at 0.04, 0.2, 1, or 5 nmol/l (*lanes 3*, *4*, *5*, and *6*, respectively) for 24 h. Total RNA was prepared, and mRNA levels of PAI-1 (*A*) and ACRP30 (*B*) were analyzed by Northern blot as described in RESEARCH DESIGN AND METHODS. Each blot was stripped and reprobed with β-actin cDNA to normalize each sample. *C*: Blots were quantitated with a densitometer, and the sample mRNA levels were normalized to β-actin mRNA; control value was set at 100%.

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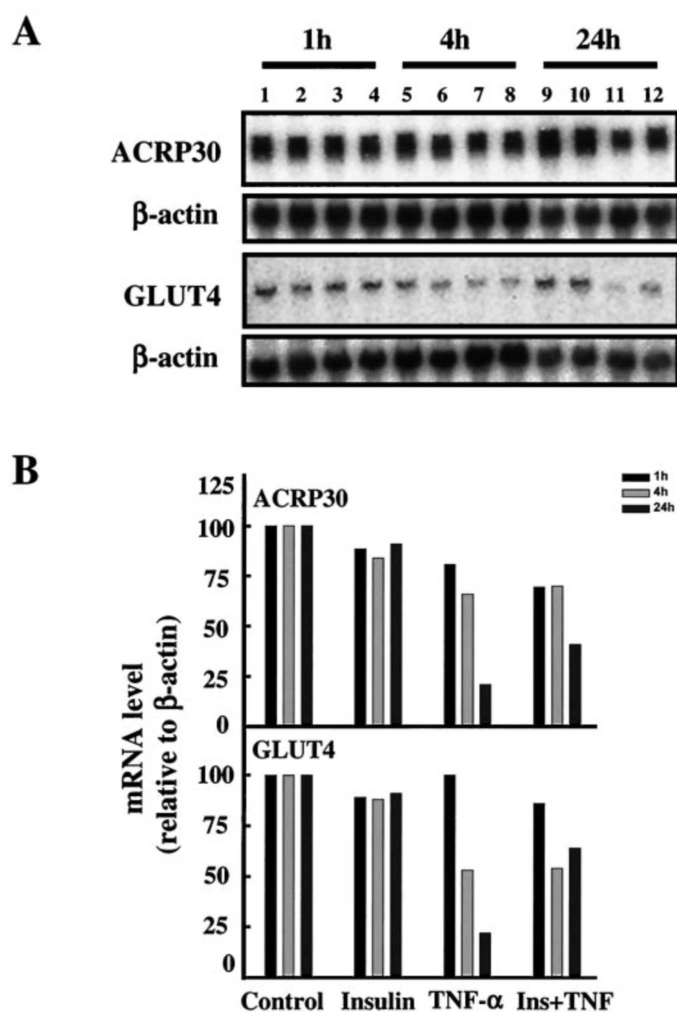

FIG. 2. Time-dependent effects of TNF-α on ACRP30 and GLUT4 gene expression in 3T3-L1 adipocytes. *A*: 3T3-L1 adipocytes were untreated (Control) or treated with insulin (100 nmol/l), TNF-α (0.2 nmol/l), or insulin + TNF-α for 1, 4, and 24 h. Total RNAs were extracted, and mRNA levels of ACRP30 and GLUT4 were examined by Northern blot. Blots were stripped and reprobed with β-actin cDNA. *B*: Northern blots were quantitated using a densitometer, and the sample mRNA levels were normalized to β-actin mRNA. The control value was set to 100%. Control: *lanes 1*, *5*, and *9*; insulin: *lanes 2*, *6*, and *10*; TNF-α: *lanes 3*, *7*, and *11*; insulin + TNF-α: *lanes 4*, *8*, and *12*.

Oligonucleotide microarrays reveal a global reduction of genes essential for adipocyte function and induction of preadipocyte genes and genes implicated in immune response in TNF-α−treated 3T3-L1 adipocytes. To determine whether adipocyte gene expression is extensively altered during TNF-α treatment, we performed oligonucleotide microarray analysis 4 and 24 h after TNF-α addition. By comparing TNF-α−treated 3T3-L1 adipocytes with control cells, we identified 59 and 130 known genes that were reproducibly upregulated by at least threefold after 4 and 24 h of TNF-α treatment, respectively. In addition, 78 known genes were downregulated at least twofold by 24 h exposure to TNF-α. The TNF-α responsive genes were then grouped according to the biological functions of their encoded proteins (Table 1).

To confirm that TNF-α indeed affected the levels of several potentially important mRNAs, we performed RT-PCR analysis. Specifically, we designed 23 pairs of different gene-specific primers and compared the amount of RT-PCR products amplified from each experimental condition after 30 and 35 PCR cycles. All of the primers yielded PCR products of the expected sizes. Although changes in the expression of 20 genes (87%) were confirmed by RT-PCR, changes in the expression of 3 genes (13%) were not. Figure 3 shows 18 representative genes. Among them, 12 genes were induced by 4 or 24 h of TNF-α treatment, including serine protease inhibitor 2-1 (Spi2-1), matrix metalloproteinase 3 (MMP3), interleukin 6 (IL-6), inducible IKK, Fas antigen, NF-κB p105, engrailed-1, Fos-related antigen-1 (Fra-1), lipopolysaccharide binding protein (LPSBP), high mobility group protein-1 isoform C (HMGP-1C), adrenomedullin (Adm), and B-cell leukemia/lymphoma-3 (Bcl-3). In contrast, mRNAs encoding CCAAT/enhancer binding protein-α (CEBP-α), glycerol-3-phosphate dehydrogenase, nuclear receptor 4A1, nuclear receptor retinoid X receptor-α (RXR-α), and hormone-sensitive lipase (HSL), were suppressed by TNF-α treatment of 3T3-L1 adipocytes (Fig. 3). Tubulin was used as an internal control for an mRNA whose level did not change. It is important to note that some changes in mRNA levels may be masked during the exponential amplification of PCR, and therefore RT-PCR is likely to yield false negative results when used to detect changes in gene expression, but unlikely to yield false positive results.

As judged by the microarray analysis, TNF-α incubation caused profound changes in gene expression in 3T3-L1 adipocytes. Several adipocyte-related transcription factors, including CEBP-α (−3.4-fold), RXR-α (−2.3-fold), and peroxisome proliferator−activated receptor-γ (PPAR-γ; −2.0 fold) were significantly downregulated by TNF-α (Table 1). In addition, TNF-α suppressed the expression of GLUT4 (−5.7-fold), glycogen synthase (−1.8-fold), and fatty acid synthase (−5.0 fold), which are essential for insulin-mediated glucose uptake and their subsequent conversion to glycogen or fatty acids. Notably, genes involved in the uptake of free fatty acids and conversion to triglyceride, such as lipoprotein lipase (−1.6-fold), long-chain fatty acyl-CoA synthetase (−2.8-fold), and diacylglycerol acyltransferase (−2.6-fold), were also downregulated by TNF-α. Moreover, a number of genes encoding adipocyte-abundant proteins, including HSL (−5.8-fold), glycerol phosphate dehydrogenase 1 (−5.2-fold), and pyruvate decarboxylase (−2.5-fold), as well as adipocyte-secreted factors such as angiotensinogen (−3.7-fold) and ACRP30 (−1.4-fold), were suppressed by TNF-α as well. This indicated that TNF-α induces insulin resistance in 3T3-L1 adipocytes at least in part through suppression of genes that are essential for the metabolic functions of adipocytes, including energy uptake and storage in response to insulin and protein secretion.

As opposed to the suppressive effects of TNF-α on adipocyte-abundant genes, TNF-α induced a number of genes that are normally enriched in preadipocytes, including CEBP-β (1.6-fold), Fra-1 (12-fold at 4 h, 5.8-fold at 24 h), GLUT1 (4.4-fold at 24 h), prostaglandin E synthase (3.2-fold at 4 h, 5.2-fold at 24 h), Spi-2 (30.5-fold at 4 h, 95.9-fold at 24 h), PAI-1 (11-fold at 4 h, 52-fold at 24 h), secreted/truncated form of vascular cell adhesion molecule 1 (VCAM-1; 9.4-fold at 4 h, 10.2-fold at 24 h), and fibronectin (2.2-fold at 24 h). Notably, many of the TNF-

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TABLE 1
Genes induced or repressed by 4- and 24-h TNF-α treatment of 3T3-L1 adipocytes

Accession no.	Gene name	Fold	Description
Transcription factors and chromatin binding proteins			
AF017128	Fra-1	12.0, 5.8	Fos-like antigen 1
X99915	Hmgic	6.8, 18.0	High mobility group protein I, isoform C
M57999	Nfkb 1	5.8, 6.3	Nuclear factor of kappa light chain gene enhancer, p105
M90397	Bcl3	8.2, 10.8	B-cell leukemia/lymphoma 3
M61007	Cebpb	1.6, 1.6	CEBP-β
M21065	Irf-1	6.7, 5.0	Interferon regulatory factor 1
L36829	*AlphaA-crybp1*	*5.4*	*Human immunodeficiency virus type I enhancer-binding protein 1*
Y15907	*Mibp-1*	*3.9*	*Human immunodeficiency virus type I enhancer-binding protein 2*
D50416	*Arec3*	*13.2*	*Sine oculis homeobox (Drosophila) homolog 4*
U36340	*Tef-2*	*5.1*	*Kruppel-like factor 3*
L27990	*Ro52*	*5.0*	*Sjogren syndrome antigen A1*
AA529583	Mrgx-pending	4.0	MORF-related gene X
L12703	En1	3.6	Engrailed 1
AF060076	Edr2	5.8	Early development regulator 2 (homolog of polyhomeotic 2)
U10374	Pparg	−2.0	PPAR-γ
X16995	Nr4a1	−5.6	Nuclear receptor subfamily 4, group A, member 1
M62362	Cebpa	−3.4	CCAAT/enhancer binding protein, alpha
X66223	Rxra	−2.3	Retinoid X receptor alpha
U51335	Gata6	−2.4	GATA-binding protein 6
Y12783	Rnf2	−3.0	Ring finger protein 2 (RING 1B protien)
X72805	H1f3	−2.8	Histone 1, family 3
AF053062	Nrip1	−2.8	Nuclear receptor interacting protein 1
AB029448	Dlxin1	−2.1	Melanoma antigen, family D, 1
M31885	Id1	−20.0	Inhibitor of DNA binding 1, dominant negative helix-loop-helix protein
D16464	Hes-1	−2.7	Hairy (Drosophila)-homolog
D31967	Jumonji	−2.1	Jumonji protein
AB024427	Sid1669	−2.2	Ring finger protein 11
X95279	Spot14	−3.2	Thyroid hormone responsive SPOT14 homolog
Secreted Protein			
U77630	Adm	7.3, 29.6	Adrenomedullin
AB011030	Prdc-pending	4.5, 8.8	Protein related to DAC and cerberus
X99347	Lbp	17.0	Lipopolysaccharide binding protein
M91380	Fstl	4.8	Follistatin-like protein
D14077	Msgp-2	3.9	Sulfated glycoprotein-2
M96827	Hp	4.4	Haptoglobin
AF045887	Aogen	−3.7	Angiotensinogen
U49915	Acrp30	−1.4	ACRP30 (adipoQ, adiponectin)
Extracellular matrix proteins and proteoglycans			
X56304	Tnc	7.4	Tenascin C
X13986	Spp1	14.0	Secreted phosphoprotein 1
M18194	Fn1	2.2	Fibronectin
AF013262	Ldc	−3.2	Lumican
Cell adhesion molecules			
U12884	Vcam 1	9.4, 10.2	Vascular cell adhesion molecule-1 (VCAM-1), truncated form
M84487	Vcam1	14, 15.4	VCAM-1
U14135	*Cd51*	*4.1*	*Integrin alpha V*
U89915	*Jam*	*3.7*	*Junction cell adhesion molecule*
AI846077	Pscd3	7.2	Pleckstrin homology, general receptor of phosphoinositides 1
X69902	Cd49f	−2.2	Integrin alpha 6 subunit
Acute-phase proteins			
X83601	Ptx3	23.5, 58.6	Pentraxin related gene
U60438	Saa2	8.1	Serum amyloid A2
Signaling molecules			
AJ242778	Abinl	7.2, 4.8	A20 binding inhibitor of NF-κB activation
U57524	Nfkbi	8.0, 5.5	IκB-α
M97590	*Ptp-1b*	*3.7*	*Protein tyrosine phosphatase, non-receptor type 1*
AF033186	*Wsb1*	*5.2*	*Wsb1, suppressor of cytokine signaling*

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TABLE 1
Continued

Accession no.	Gene name	Fold	Description
AF075461	*Shag1*	*4.1*	*Development- and differentiation-enhancing factor 2*
U47737	Tsa-1	5.2	Lymphocyte antigen 6 complex
AF074714	Mmsk2	3.5	Mitogen- and stress-activated protein kinase-2
AB016589	Ikki	8.4	Inducible IkappaB kinase
M80739	Ptpn2	4.9	Protein tyrosine phosphatase, non-receptor type 2
AI842277	Igfbp3	10.6	Insulin-like growth factor binding protein 3
U18869	Dab2	2.0	Disabled homolog 2 (Drosophila)
M60474	Macs	8.8	Myristoylated alanine rich protein kinase C substrate
L16956	Jak2	10.4	Janus kinase 2
U90926	LOC57425	9.6	Putative TNF-resistance related protein
AF033186	Wsb1	3.4	WSB-1, WD-40 repeats containing protein
AF020313	Apbblip-pending	9.3	Amyloid beta (A4) precursor protein-binding protein (FE65)
M73748	Gp38	12.7	Glycoprotein 38
J05149	Insr	Not detected	Insulin receptor
X69722	Irs1	Not detected	Insulin receptor substrate-1
AF090738	Irs2	Not detected	Insulin receptor substrate-2
Y13569	Pik3r2	No change	Phosphatidylinositol-3-kinase, p85 subunit
X65687	Akt	1.3	Protein kinase B (AKT)
U22445	Akt2	−3.6	Protein kinase B (AKT2)
U43585	Ksr	−4.8	Kinase suppressor of ras
AV367375	Pkcz	−2.4	Protein kinase C, zeta
AW123750	Gng2	−3.0	Guanine nucleotide binding protein (G protien), gamma 2 subunit
Y11092	Mknk2	−5.7	MAP kinase interacting serine/threonine kinase 2
X99963	RhoB	−2.5	Aplysia ras-related homolog B (RhoB)
AB025048	Sid6061	−2.9	RAB9, member RAS oncogene family
U67187	Rgs2	−5.4	Regulator of G-protein signaling 2
AF069954	Gng3lg	−3.3	G-protein gamma 3 linked gene
AF077659	Hipk2	−5.2	Homeodomain interacting protein kinase 2
U28656	Phas-1	−3.2	Insulin-stimulated eIF-4E binding protein
AF004927	Msigmar1	−2.6	Opioid receptor, sigma 1
U58883	Sh3d2a	−2.1	SH3 domain protein 2A
RNA synthesis and RNA binding			
U37500	Rpo2-1	5.2, 15.4	RNA polymerase II 1
AB017020	Hnrpdl	7.0	Heterogeneous nuclear ribonucleoprotein D-like
L23971	Fmr1	−2.4	Fragile X mental retardation 1
Metabolism			
AI060798	Ptges	3.2, 5.2	Prostaglandin E synthase
X78445	Cyp1b1	7.0, 4.0	Cytochrome P450, 1b1
AF017175	Cpt1a	2.7	Carnitine palmitoyltransferase 1
M22998	Slc2a1	4.4	Facilitated glucose transporter, member 1
D88994	Ampd3	22.0	AMP deaminase 3
M31690	Ass1	9.7	Argininosuccinate synthetase 1
U75215	Slc1a4	4.2	Neutral amino acid transporter
AI838274	Slc29a1	4.6	Nucleoside transporter, member 1
U21489	Acad1	3.7	Acetyl-coenzyme A dehydrogenase, long chain
AB011451	Chst2	5.1	Carbohydrate sulfotransferase 2
U79523	Pam	13.6	Peptidylglycine alpha-amidating monooxygenase
Z14132	Asmase	3.8	Sphingomyelin phosphodiesterase
X75129	Xd	4.2	Xanthine dehydrogenase
U60020	Ham1	4.9	ATP-binding cassette, sub-family B, member 2
U85247		3.6	Lysosomal alpha-N-acetylglucosaminidase
M63335	Lipoprotein	−1.6	Lipoprotein lipase
U15977	Facl2	−2.8	Long chain fatty acyl CoA synthetase
AF078752	Dgat	−2.6	Diacylglycerol acyltransferase
M23383	Slc2a4	−5.7	Facilitated glucose transporter, member 4 (GLUT4)
U53218	Gys	−1.8	Glycogen synthase
X13135	Fasn	−5.0	Fatty acid synthase
M25558	Gdc1	−5.2	Glycerol-3-phosphate dehydrogenase 1
U69543	Lipe	−5.8	Hormone sensitive lipase
L09192	Pcx	−2.5	Pyruvate decarboxylase
U25051	Pempt	−2.8	Phosphatidylethanolamine N-methyltransferase

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TABLE 1
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Accession no.	Gene name	Fold	Description
U00978	Impdh1	−2.8	Inosine 5-phosphate dehydrogenase 1
AA683883	Slc25a10	−3.6	Adenine nucleotide translocator
AJ223066	Fabpe	−2.5	Fatty acid binding protein 5, epidermal
X83202	11beta-hsd-1	−5.3	11beta-hydroxysteroid dehydrogenase type 1
U96116	Erab	−2.5	Hydroxysteroid (17-beta) dehydrogenase 10
AJ006474	Car3	−6.0	Carbonic anhydrase 3
M25944	Car2	−4.2	Carbonic anhydrase 2
M29394	Cas-1	−2.6	Catalase
X01756	Cycs	−4.4	Cytochrome c
X51905	Ldh-2	−2.9	Lactate dehydrogenase-B
X13752	Lv	−2.2	Delta-aminolevulinate dehydratase
M28723	Mer5	−2.7	Anti-oxidant protein 1
J02652	Mod-1	−2.2	Malate NADP oxidoreductase
L28835	Pmp22	−11.0	Peroxisomal membrane protein 2, 22 kDa
M91458	Scpx	−2.8	Sterol carrier protein 2
U67611	Taldol	−3.0	Transaldolase
U35741	Tst	−3.0	Thiosulfate sulfurtransferase, mitochondrial
U00978	Impdh1	−4.1	Inosine 5′-phosphate dehydrogenase 1
U96401	aldhpb	−2.7	Aldehyde dehydrogenase family 1, subfamily A4
L31783	Umpk	−2.2	Uridine monophosphate kinase
Protease and peptidase			
X66402	Mmp3	8.9, 148.0	Matrix metalloproteinase 3
X66473	Mmp13	8.5	Matrix metalloproteinase 13
D13139	Dpep1	9.8	Dipeptidase
Protease inhibitors			
M64085	Spi2-1	30.5, 95.9	Serine protease inhibitor 2-1
AF002719	Slpi	8.1, 45.3	Secretory leukocyte protease inhibitor
M33960	Planh1	11, 52	Plasminogen activator inhibitor, type 1
X70296	Spi4	3.8	Serine protease inhibitor 4
Blood coagulation			
M38337	Mfgm	4.0	Coagulation factor VIII; milk fat globule membrane protein E8
M62470	Thbs1	−6.0	Thrombospondin 1
M26071	Cf3	−2.4	Coagulation factor III
Protein modification and degradation			
U16163	P4ha2	8.2	Proline 4-hydroxylase, alpha II polypeptide
M85153	Ggta 1	5.0	Glycoprotein galactosyltransferase alpha 1, 3
AB022022	Psmd10	5.1	Proteasome 26S subunit, non-ATPase 10
Y10875	mECL-1	6.3	Proteasome subunit MECL-1
U22033	Lmp7	15.7	Large multifunctional protease 7 (proteasome subunit)
Apoptosis			
U75506	Bid	4.8, 7.1	BH3 interacting domain death agonist
Y13089	Casp 11	6.6	Caspase 11
AJ001633	Anxa3	8.2	Annexin A3
U88909	Birc3	10.2	Inhibitor of apoptosis protein 2 (Baculoviral IAP repeat-containing 3)
M61737	Fsp27	−7.4	Fat specific gene 27
AF033115	Siva	−3.2	CD27-binding (Siva) protein
AF022223	Bag-1	−2.2	Bcl2-associated athanogene 1, long form
Cytokines, cytokine receptors, and cytokine-induced genes			
X54542	Il6	9.9, 30.3	Interleukin 6
M33266	Scyb10	9.8, 18	Small inducible cytokine B subfamily, member 10
M34815	Scyb9	7, 16	Small inducible cytokine B subfamily, member 9
U27267	Scyb5	6.5, 11.0	Small inducible cytokine B subfamily, member 5
M19681	Scya2	10.8, 11.8	Small inducible cytokine A2
AF065947	Scya5	8.7, 58	Small inducible cytokine A5
X70058	Scya7	10.1, 12.2	Small inducible cytokine A7
U49513	Scya9	5.3, 24	Small inducible cytokine A9
U92565	Scyd1	5.6, 12.8	Small inducible cytokine subfamily D, 1
M89641	Ifnar	5.9, 3.8	Interferon α/β receptor
M31418	Ifi202	4.8, 7.6	Interferon-activated protein 202
M31419	Ifi204	3.6, 5.0	Interferon-activated protein 204

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TABLE 1
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Accession no.	Gene name	Fold	Description
AJ007972	Gtpi	5.4, 4.0	Interferon-γ induced GTPase
AF013486	Ifnar2	2.1, 4.6	Interferon (α and β) receptor 2
AB015978	Osmr	2.8, 6.9	Oncostatin receptor
M83649	Fas	9, 16.9	Fas antigen
M63630	Ifi47	3.6, 15.0	Interferon gamma inducible protein, 47 kDa
AA914345	Iigp-pending	2.3, 2.7	Interferon-inducible GTPase
U43084	Ifit 1	3.3, 6.4	Interferon-induced protein with tetratricopeptide repeats 1
AJ007970	Gbp2	5.0, 6.5	Guanylate necleotide binding protein 2
AW047476	Gbp3	5.6, 5.8	Guanylate nucleotide binding protein 3
AI323667	Irg1	2.0, 8.0	Immunoresponsive gene 1
M21952	Csf1	3.4, 3.9	Colony stimulating factor 1
V00755	Interferon	2.8, 9.9	Interferon beta
M31418	Ifi202a	4.8, 6.6	Interferon activated gene 202A
M31419	Ifi204	3.6, 5.0	Interferon activated gene 204
L12030	SDF-1-beta	4.8	SDF-1-β cytokine
AF061346	Edp1	5.0	TNF-α induced protein 1
U43085	Ifit2	5.0	Interferon-induced protein with tetratricopeptide repeats 2
AF022371	Ifi203	5.0	Interferon activated gene 203
M55544	Gbp1	4.6	Guanylate nucleotide binding protein 1
M21038	Mx1	6.5	Myxovirus resistance 1
Growth factors, growth factor receptors, and cell-division related proteins			
K03235	Plf2	4.3, 3.2	Proliferin
M32490	*Igfbp10*	*3.3*	*Cysteine-rich, angiogenic inducer, 61*
D30782	Ereg	8.0	Epiregulin
AF099973	Slfn2	17.3	Schlafen 2
X98471	Emp1	4.2	Epithelial membrane protein 1
X56045	Ranbp1	3.2	RAN binding protein 1
X65128	Gas1	−3.6	Growth arrest specific 1
AF055638	Gadd45g	−2.8	Growth arrest and DNA-damage-inducible, gamma
AB016080	Kip2	−3.4	Cyclin-dependent kinase inhibitor 1C
X95280	G0s2	−5.8	G0/G1 switch gene 2
D90225	Osf-1	−6.0	Pleiotrophin
U19596	Ink4c	−2.6	Cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)
Immune-related genes and cell surface antigen (MHC)			
X13333	Cd14	2.1, 4.5	CD 14 antigen
U19271	Cd205	5.8	CD 205, lymphocyte antigen 75
L38444	Tgtp	3.9	T-cell specific GTPase
U19271	Ly75	7.8	Lymphocyte antigen 75
X00496	Ii	4.1	Ia-associated invariant chain
X00246	H2-D	3.6	Histocompatibility 2, D region locus 1
X58609	H2-Q2	3.4	Histocompatibility 2, Q region locus 2
M18837	Beta2-m	5.1	MHC β2-microglobulin
M58156	K-f	4.9	MHC (A.CA/J(H-2K-f) class I antigen
M35244	H-2T10	4.1	MHC H2-TL-T10-129
X58609	MHC Q2-k	6.8	MHC (Qa) Q2-k gene for class I antigen
U21906	H2-BI	4.0	MHC class Ib gene
AF037260	Ack	−3.9	Anti-Corynebacterium kutscheri
Cytoskeleton, cytoskeleton binding protein, and motor protein			
AF001871	*Grp1*	*3.7*	*General receptor for phosphoinositides 1-associated scaffold protein*
AF063231	Dncic2	3.8	Dynein, intermediate chain 2
AW121844	Add1	3.4	Adducin 1 (alpha)
AF013715	Ppl	−2.4	Periplakin
AL021127	Calt	−2.3	Caltractin
Ion or heavy metal binding protein and ion channel			
U89889	Hpxn	5.6, 22.8	Hemopexin
U49430	Cp	10.0	Ceruloplasmin

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TABLE 1
Continued

Accession no.	Gene name	Fold	Description
K02236	Mt-2	4.1	Metallothionien II
M83218	Mrp8	5.3	Calgranulin A, intracellular calcium-binding protein
AF004666	Slc8a1	9.2	Sodium/calcium exchanger, member 1
M73696	Slc20a1	5.8	Solute carrier family 20, member 1
AF014010	Pkd2	3.4	Polycystic kidney disease 2 (polycystin-2, cation channel)
X78874	Clcn3	5.9	Chloride channel 3
AF089751	P2x4	5.4	Purinergic receptor P2X, ligand-gated ion channel, 4
AB025217	Sid470	−2.1	Calcium binding protein P22
U29530	Atp5f1	−2.6	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit b, isoform 1
Intracellular protein trafficking			
AF079901	Gs28	−4.1	Golgi SNAP receptor complex member 1
AF053550	Mtx2	−2.5	Metaxin 2
Plasma membrane phospholipids transport			
D78354	Plscr1	13.0, 21.8	Phospholipid scramblase 1
Other			
AF072697	Shyc	5.0	Selective hybridizing clone

Raw data were normalized with arrays hybridized with cRNAs prepared from the appropriate control cells by using the median of the hybridization signals of all genes with P-calls (according to Affymetrix) as a scaling factor. An arbitrary value of 50 was allocated for genes whose expression levels were undetectable or <50 to facilitate calculation of the fold changes compared with the control. Genes were grouped according to their biological functions, as determined by public database searching. Data presented are fold-changes for the indicated gene. Two numbers were included for genes that were upregulated at both 4- and 24 h TNF-α treatment, with the first number denoting fold changes at the end of 4 h and the second number indicating fold changes at the end of 24 h of TNF-α incubation. Italic type, ↑ at 4 but not 24 h of TNF-α incubation; plain type, ↑ at 24 but not 4 h of TNF-α incubation. – indicates downregulation of gene expression at 24 h of TNF-α incubation.

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α−induced genes encode secreted proteins, such as PAI-1, secreted VCAM-1, fibronectin, tenascin C (7.4-fold at 24 h), adrenomedullin (7.3-fold at 4 h, 29.6-fold at 24 h), and ceruloplasmin (10-fold at 24 h), whose overexpressions are associated with type 2 diabetes. Thus, TNF-α may also contribute to at least some of the phenotypes associated with obesity and obesity-linked type 2 diabetes by modulating the endocrine function of the adipocytes, including selective induction of preadipocyte genes as well as suppression of adipocyte-secreted factors.

Another striking result is that a number of chemokines, cytokines and their receptors, and cytokine-induced proteins were immediately induced by TNF-α in 3T3-L1 adipocytes. For example, IL-6 mRNA was upregulated nearly 10-fold after 4 h of TNF-α incubation and 30-fold after 24 h of TNF-α incubation (Table 1 and Fig. 3). Many chemokines and cytokine-induced proteins were also strongly upregulated 4 and 24 h after TNF-α incubation (Table 1), indicating a direct induction by TNF-α.

TNF-α induces mRNAs encoding DNA-binding proteins implicated in NF-κB activation and general transcriptional regulation in 3T3-L1 adipocytes. TNF-α treatment of adipocytes induced or suppressed the expression of a number of transcription factors and their associated regulatory proteins. Among them, mRNAs that encode Bcl-3 and p105-NF-κB were significantly upregulated after 4 h exposure to TNF-α and remained 6.3-fold (p105-NF-κB) and 10.8-fold (Bcl-3) of control cells after 24 h of TNF-α incubation. P105-NF-κB encodes a precursor for both the p50-NF-κB transcription factor and the NF-κB repressor I-κBγ (5), whereas Bcl-3 is a DNA binding protein that has been implicated as a modulator of the activity of NF-κB (6–8). In the absence of TNF-α, NF-κB is sequestered in the cytoplasm by binding to I-κBs (9). Stimulation by TNF-α leads to degradation of I-κB proteins and the translocation of NF-κB to the nucleus. Importantly, the expression of inducible-IKK (IKKi), which mediates the rate-limiting step of phosphorylation and degradation of the NF-κB inhibitor (IκBs) (10), was also upregulated 8.4-fold after 24 h incubation with TNF-α. Increased IKKi expression or activity may enhance the rate of IκB degradation and the subsequent release and activation of NF-κB. In addition, TNF-α stimulation resulted in a 60% increase in the mRNA levels of CEBP-β, which can also dimerize with subunits of NF-κB and activate gene transcription (11). Thus, TNF-α rapidly induced the synthesis of several mRNAs encoding proteins implicated in NF-κB activation in 3T3-L1 adipocytes, including p105-NF-κB, IKKi, and other NF-κB–associated proteins such as Bcl-3 and CEBP-β (Table 1).

TNF-α coordinately regulates expression of genes implicated in cell cycle arrest and progression in terminally differentiated 3T3-L1 adipocytes. TNF-α also upregulated several growth factors and proteins associated with cell cycle progression, but downregulated proteins associated with cell cycle inhibition, including CEBP-α (Table 1) (18), cyclin-dependent kinase inhibitor 1C, cyclin-dependent kinase inhibitor 2C (p18), growth-arrest specific 1 (19,20), and growth-arrest and DNA-damage–inducible-γ gene (21). CEBP-α is an important cell growth and differentiation transcriptional regulator that is expressed in many tissues (22). In fully differentiated 3T3-L1 adipocytes, CEBP-α is highly induced, whereas CEBP-β is suppressed (23). CEBP-α not only maintains growth arrest in terminally differentiated 3T3-L1 adipocytes (24), but also regulates the expression of adipocyte-specific genes and energy metabolism (25). These coordinate

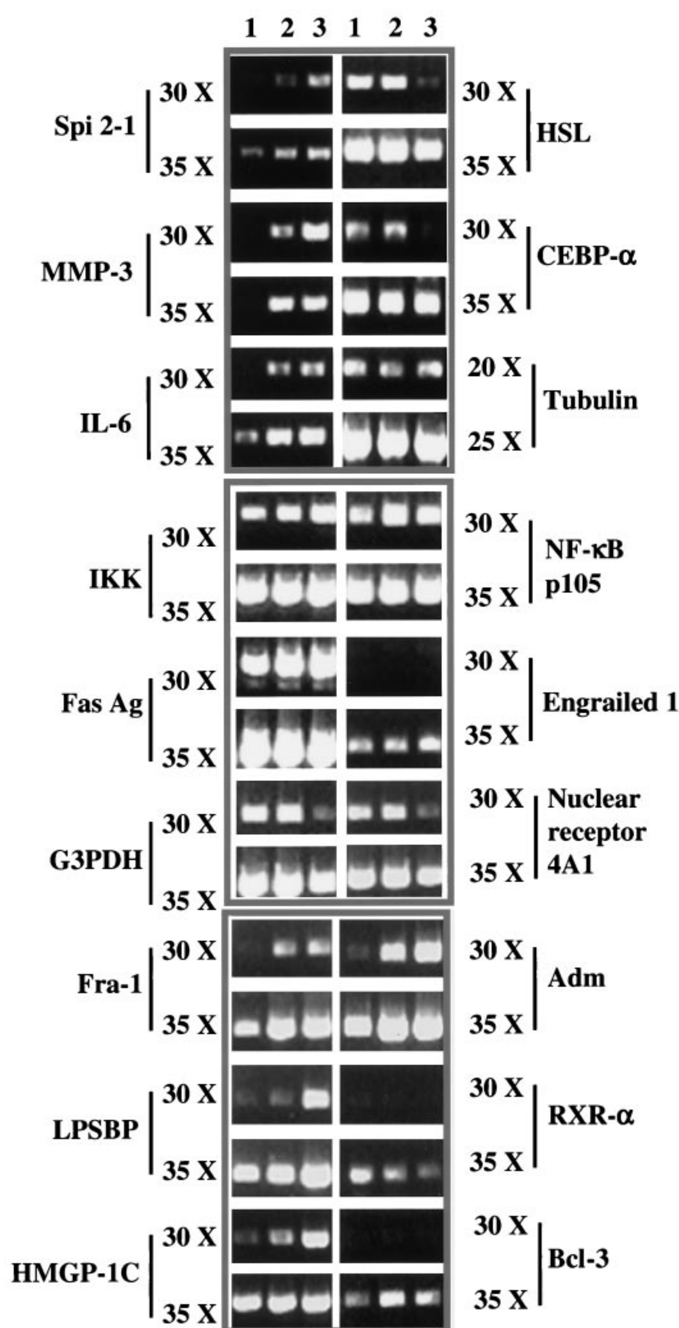

FIG. 3. Semi-quantitative RT-PCR. 3T3-L1 adipocytes were treated with TNF-α (1 nmol/l) for 0, 4, and 24 h. Total RNAs were extracted, and a sample of first-strand cDNA was synthesized from 1 μg total RNA. Then various gene-specific primers were used to amplify target genes using equal amounts of the first strand cDNA as template. PCR reaction was performed for 30 and 35 cycles, unless otherwise indicated, to identify the linear range for PCR. *Lane 1*, control condition; *lane 2*, TNF-α 4 h; *lane 3*, TNF-α 24 h. Fas Ag, Fas antigen; G3PDH, glycerol-3-phosphate dehydrogenase; NF-κB p105, NF-κB p105 subunit.

changes in gene expression might cause 3T3-L1 adipocytes to reenter the cell cycle.

TNF-α reduces protein levels of GLUT4 and several insulin signaling proteins. To assess suppression of gene expression as a potential mechanism for TNF-α−induced insulin resistance, we revisited the effects of TNF-α treatment on the levels of several proteins. TNF-α induced a progressive reduction in the levels of GLUT4, IR, and IRS-1 proteins (Fig. 4*A* and *B*), confirming results in a previous report (26). In addition, TNF-α suppressed protein levels of AKT, a key enzyme necessary for insulin-stimulated glucose uptake in adipocytes (Fig. 4*C* and *D*). Conversely, the level of GLUT1 protein increased 60% after TNF-α treatment for up to 48 h (Fig. 4*A* and *B*). Thus, our data clearly indicated that TNF-α modulates 3T3-L1 adipocyte function at the level of gene transcription, and that TNF-α−induced characteristic changes in gene expression provide a molecular basis for the development of insulin resistance.

TNF-α treatment of 3T3-L1 adipocytes leads to rapid NF-κB nuclear translocation. To determine whether TNF-α affects gene transcription through activation of NF-κB, we first assessed NF-κB expression and its translocation into the nucleus during TNF-α stimulation. NFκB1/p105, RelB, RelA/p65, and c-Rel (a COOH-terminal truncated form of 53 kDa) were readily detectable in nuclear extracts of 3T3-L1 adipocytes (Fig. 5*A*, *lane 1*). The levels of NF-κB1/p105, Rel B, and c-Rel protein were not changed 15 or 60 min after TNF-α stimulation, and no proteolytic cleavage of NF-κB1/p105 occurred. Nuclear accumulation of p65, however, increased substantially 15 and 60 min after TNF-α addition (Fig. 5*A*, *lanes 2* and *3*).

We then used a gel mobility shift assay to confirm that TNF-α acutely activates NF-κB nuclear translocation, a prerequisite for its having a functional involvement in TNF-α action in 3T3-L1 adipocytes. After TNF-α stimulation, NF-κB RelA/p65 activation and nuclear translocation occurred within 15 min and lasted at least 60 min, consistent with results in other cell types (36) (Fig. 5*B*). This indicated that NF-κB is immediately involved in TNF-α action at the gene transcription level in 3T3-L1 adipocytes.

NF-κB mediates most aspects of selective reactivation of preadipocyte gene expression and suppression of adipocyte-abundant genes by TNF-α. To ascertain whether NF-κB activation is essential to initiating changes in gene expression typical of insulin-resistant adipocytes, we established a 3T3-L1 cell line stably expressing IκBα-DN, a nondegradable mutant of NF-κB inhibitor IκB-α (37–39). 3T3-L1 cells expressing IκBα-DN differentiate normally into adipocytes (Fig. 6*A*), and respond to insulin to the same extent as do wild-type adipocytes, as measured by [^{3}H]2-deoxy-glucose uptake (data not shown). We confirmed the function of the IκBα-DN protein in fully differentiated 3T3-L1 adipocytes by incubating it with TNF-α, and monitoring the phosphorylation and degradation of both the endogenous and the mutant IκB-α proteins. As shown in Fig. 6*B*, in wild-type adipocytes, TNF-α efficiently stimulated phosphorylation of endogenous IκB-α as early as 5 min, resulting in rapid and complete degradation of the wild-type IκB-α protein within 15 min. In contrast, no TNF-α−mediated phosphorylation and degradation of the IκBα-DN protein occurred in adipocytes expressing the mutant protein. After 60 min of TNF-α addition, IκB-α protein reaccumulated in the wild-type adipocytes, as activation of NF-κB also stimulates transcription of IκB-α. The absence of reaccumulation of endogenous IκB-α protein in cells expressing IκBα-DN again indicated the complete inhibition of NF-κB activation in these cells.

Extensive cell death occurred in 3T3-L1 adipocytes

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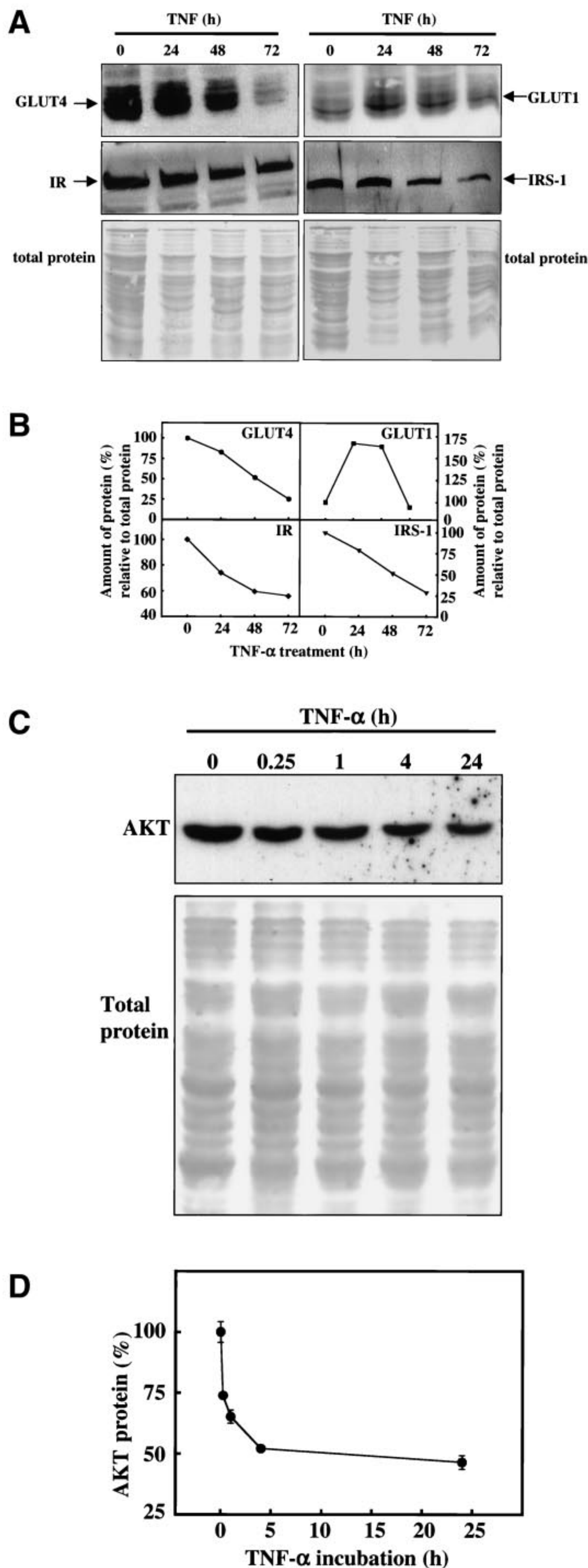

expressing IκBα-DN after 2 h of TNF-α addition, indicating a critical role of NF-κB activation in protecting against TNF-α–induced apoptosis. We collected total RNA from wild-type and IκBα-DN adipocytes stimulated for up to 2 h by TNF-α, and performed oligonucleotide microarray analysis of the global gene expression profiles.

We first compared the global gene expression profile in wild-type 3T3-L1 adipocytes and adipocytes expressing IκBα-DN. In the scatter plot shown in Fig. 7, the position of each gene on the plot is determined by its expression level in both wild-type adipocytes (x-axis) and IκBα-DN adipocytes (y-axis). Genes with identical or similar expression levels in both cell types cluster along the diagonal, whereas genes whose expression levels are higher or lower in wild-type adipocytes cluster below or above the diagonal line, respectively. Despite the similar global gene expression patterns in the two cell-types (Fig. 7), differences did exist in a subset of genes. We identified 200 genes (1.6% of 12,488 genes examined, including both known genes and ESTs) whose basal expression levels in wild-type adipocytes are at least twofold higher than those in IκBα-DN adipocytes, and 20 genes (0.16%, including both known genes and ESTs) whose expression levels are at least twofold lower than those in IκBα-DN adipocytes. Thus, basal NF-κB activity is required for the expression or suppression of a subset of genes in 3T3-L1 adipocytes. Because the differences in the basal gene expression did not affect adipocyte differentiation or the response to insulin, we did not further investigate their functional significance.

Next, we assessed the kinetics of TNF-α–induced changes in gene expression. In wild-type 3T3-L1 adipocytes, TNF-α suppressed the mRNA levels of 61, 193, and 264 genes (including known genes and ESTs) at least twofold at the 0.5-, 1-, and 2-h time points. In adipocytes expressing IκBα-DN, however, only 1.6% (1 of 61 genes), 0.5% (1 of 193), and 1.9% (5 of 264) of the genes that are normally suppressed by TNF-α were downregulated at the 0.5-, 1-, and 2-h time points, respectively (Fig. 8*A*). On the other hand, TNF-α induced 52, 33, and 39 genes (including known genes and ESTs) by at least twofold in 3T3-L1 adipocytes after 0.5-, 1-, and 2-h incubation, respectively. Notably, ~27% (14 of 52), 39% (13 of 33), and 31% (12 of 39) of the genes that are normally induced by TNF-α were still upregulated by TNF-α in IκBα-DN adipocytes (Fig. 8*B*). Moreover, in IκBα-DN adipocytes, TNF-α induced and suppressed many genes that were not affected by TNF-α in wild-type adipocytes. In particular, TNF-α suppressed 35, 27, and 27 genes (known genes and ESTs) and induced 110, 214, and 266 genes (known genes and ESTs) in adipocytes expressing IκBα-DN protein at 0.5-, 1-, and 2-h time points, respectively. Thus, the absence of NF-κB activation abolished suppression of 98–99% of the genes

FIG. 4. Effects of TNF-α on protein levels of IR, IRS-1, AKT, GLUT1, and GLUT4 in 3T3-L1 adipocytes. *A*: 3T3-L1 adipocytes were treated with TNF-α (1 nmol/l) for 0, 24, 48, and 72 h. Total cellular proteins were analyzed by SDS-PAGE and immunoblotted with antibodies as indicated in the figure. *B*: Signals on Western blots were scanned using a densitometer and plotted. *C*: 3T3-L1 adipocytes were incubated with TNF-α (1 nmol/l) for 0, 0.25, 1, 4 h, and 24 h. Total cellular proteins were separated by SDS-PAGE, and immunoblotted with anti-AKT antibody. *D*: Signals on Western blot were scanned and analyzed using a densitometer and plotted. Equal sample loading was verified by staining the blots with Ponceau S. The control value was set at 100%.

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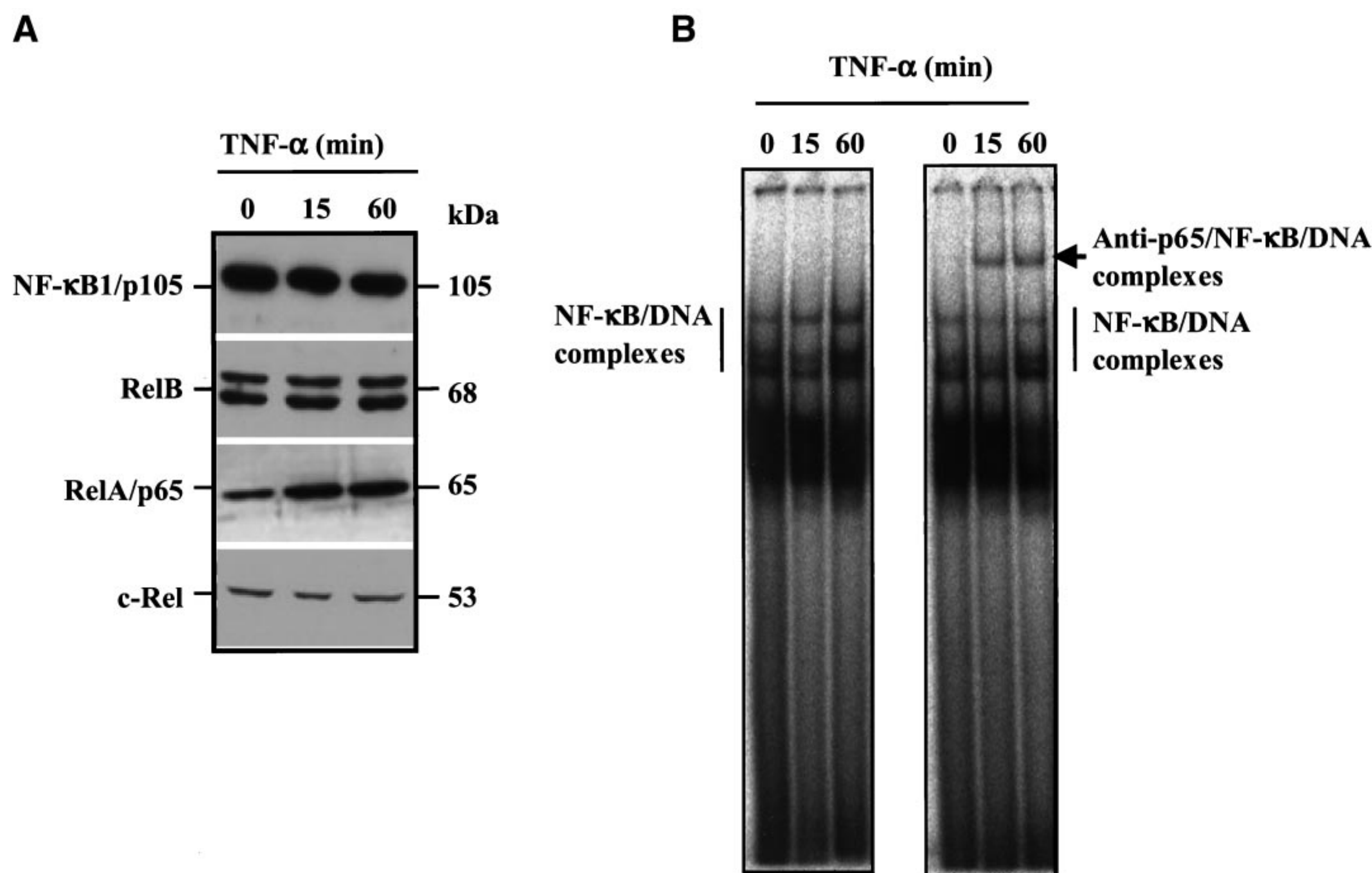

FIG. 5. NF-κB expression and nuclear translocation during TNF-α stimulation of 3T3-L1 adipocytes. *A*: 3T3-L1 adipocytes were treated with TNF-α (1 nmol/l) for 0, 15, and 60 min. Nuclear extracts were analyzed by SDS-PAGE and immunoblotted with the indicated antibodies. *B*: 3T3-L1 adipocytes were treated as above, and equal amounts of nuclear extracts were incubated with [^{32}P]-labeled NF-κB consensus sequence, in the absence (*left panel*) or presence (*right panel*) of anti-RelA/p65.

normally suppressed by TNF-α and induction of 60–70% of the genes normally induced by TNF-α, and produced a distinct gene expression profile in response to TNF-α.

We then compared the kinetics of TNF-α treatment up to 2 h on the expression of several key adipocyte transcription factors, as well as genes involved in glucose and free fatty acid uptake and storage (Fig. 9). There was no significant difference in the basal expression levels of the genes we examined in wild-type and IκBα-DN adipocytes. By 2 h of TNF-α treatment of wild-type adipocytes, the mRNA levels of PPAR-γ, CEBP-α, GLUT4, 1-acyl-glycerol-3-phosphate acyltransferase, and diacylglycerol acyltransferase were decreased 50% or more. In contrast, their levels were unchanged or slightly increased after TNF-α treatment of IκBα-DN adipocytes. The decrease in mRNA levels was likely attributable to both inhibition of gene transcription and the short half-life of the mRNAs examined. The levels of abundant and stable mRNAs, such as those encoding ACRP30 and long-chain fatty acyl CoA synthase, were not affected at the 2-h time point, but were decreased after 24 h of TNF-α treatment (Fig. 1*B* and C; Table 1). Because of apoptosis of the IκBα-DN adipocytes after 2 h of TNF-α treatment, we could not determine whether TNF-α suppression of their expression depended on NF-κB.

DISCUSSION

Insulin resistance refers to a state in which physiological concentrations of insulin produce a less than normal response. Major metabolic consequences of insulin resistance include elevated plasma concentrations of circulating fuel molecules (glucose and free fatty acids) and in some cases, triglycerides and cholesterol as well. Although decreased receptor tyrosine kinase activity and impaired insulin receptor signal transduction contribute to the development of insulin resistance, these factors are insufficient to explain the complex phenotype. Insulin resistance in obesity and obesity-linked type 2 diabetes is often accompanied by significant changes in gene expression in major insulin-responsive tissues. Thus, it can be informative to identify mediators that elicit the insulin-resistance phenotype. TNF-α is increasingly recognized as a link between obesity and insulin resistance, and thus has been the focus of intensive study.

Here, we reported three major findings that, when taken together, describe one of the mechanisms by which TNF-α treatment causes insulin resistance in 3T3-L1 adipocytes. First, we found that TNF-α immediately alters the expression of many genes in 3T3-L1 adipocytes. It specifically downregulates many adipocyte-abundant genes that are critical for insulin responsiveness and selectively reacti-

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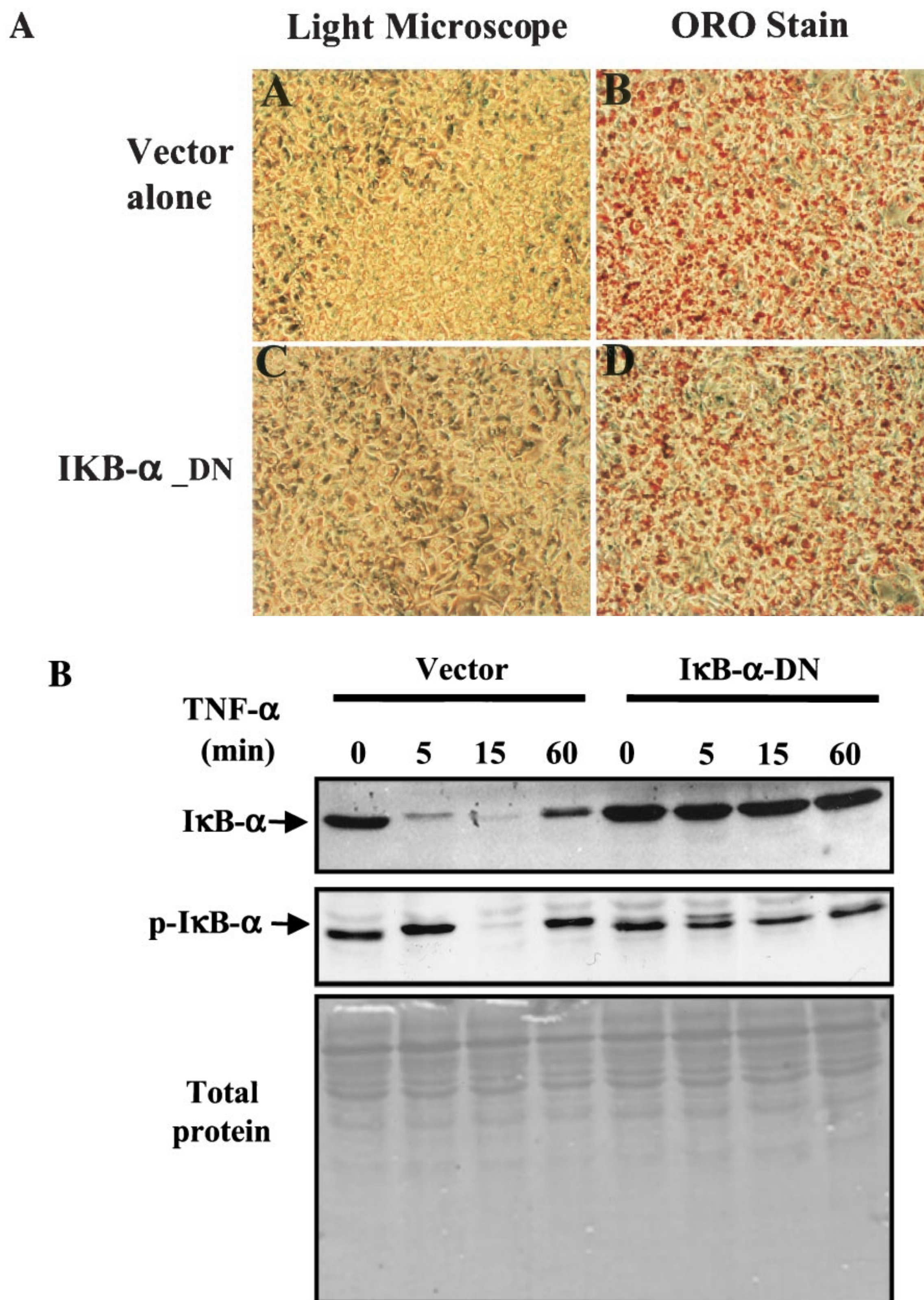

FIG. 6. Expression of IκBα-DN does not affect adipocyte differentiation but IκBα-DN is resistant to TNF-α–induced phosphorylation and degradation. *A*: Stable 3T3-L1 cell lines expressing the retroviral vector or mutant IκBα-DN protein were established as described in RESEARCH DESIGN AND METHODS, and were induced to differentiate into adipocytes according to standard protocols. Unstained cells or cells stained with Oil Red O (ORO) were examined using an inverted-phase contrast microscope and photographed. *B*: 3T3-L1 adipocytes expressing vector or IκB-DN were treated with TNF-α (1 nmol/l) for 0, 5, 15, and 60 min. Total cell lysate were prepared, and 40 μg total cellular protein was analyzed by SDS-PAGE and immunoblotted with anti–IκB-α and anti–phospho-IκB-α antibodies. The blot was stained by Ponceau S to verify sample equal loading.

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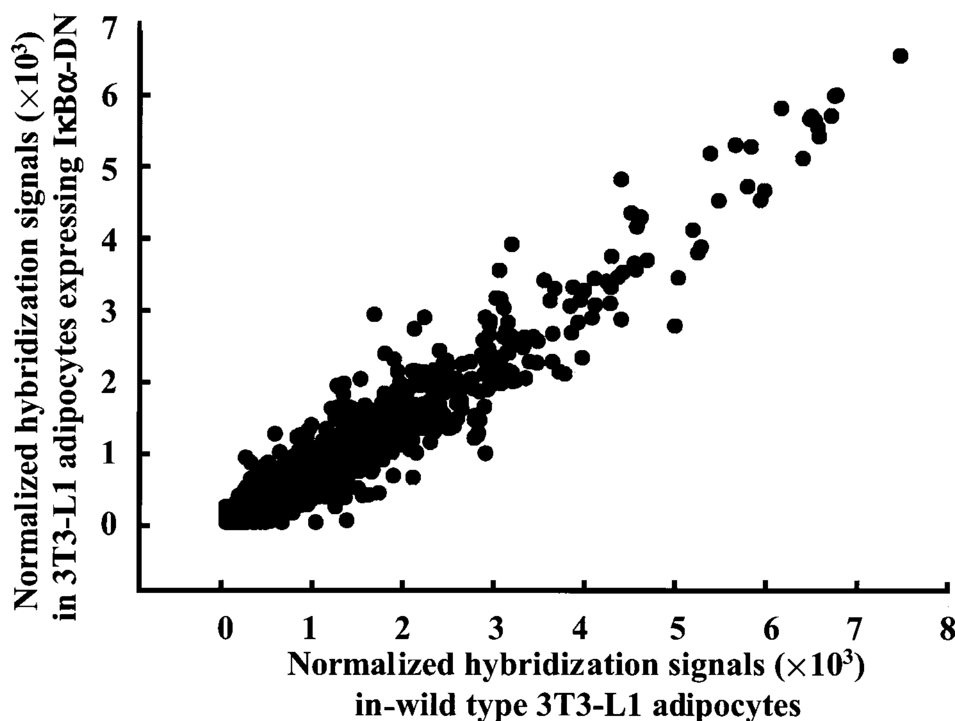

FIG. 7. Global gene expression profile in wild-type 3T3-L1 adipocytes and adipocytes expressing IκBα-DN. The global gene expression profiles of wild-type 3T3-L1 adipocytes or 3T3-L1 adipocytes expressing IκBα-DN were assessed using oligonucleotide microarray analysis as described in RESEARCH DESIGN AND METHODS. A total of 12,488 genes and ESTs present on the murine U74av2 array were plotted using a scatter plot. The position of each gene on the plot is determined by its expression levels in wild-type adipocytes (*x*-axis) and IκBα-DN (*y*-axis).

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vates many preadipocyte genes. TNF-α also induces several transcription factors and genes implicated in NF-κB activation, as well as many genes involved in cell growth, proliferation, and inflammation. Second, we identified NF-κB as an obligatory mediator of the TNF-α−induced changes in gene expression that could lead to insulin resistance in 3T3-L1 adipocytes. Third, we found that NF-κB is also specifically required for protection against TNF-α−induced apoptosis in 3T3-L1 adipocytes. Thus, our findings implicate adipocyte NF-κB as a potential target for pharmaceutical interventions of the insulin resistance seen in obesity and obesity-linked type 2 diabetes.

TNF-α induced coordinate changes in gene expression that closely resemble many of the characteristics seen in insulin-resistant adipocytes. TNF-α has direct effects on gene expression in 3T3-L1 adipocytes, as changes in mRNA levels can be seen as early as 0.5 h after TNF-α addition. Notably, TNF-α suppressed the expression of a number of transcription factors essential for the adipocyte phenotype, including PPAR-γ, RXR-α, CEBP-α, and Nrip-1. The PPAR-γ/RXR-α heterodimer induces many adipocyte-specific genes implicated in adipocyte differentiation and affects systemic glucose and lipid homeostasis (27). During 3T3-L1 adipocyte differentiation, CEBP-α is highly induced, whereas CEBP-β and CEBP-γ are significantly downregulated (23). A high CEBP-α/CEBP-β ratio is therefore a marker of adipocyte differentiation. CEBP-α acts synergistically with PPAR-γ and is critical for maintenance of the adipocyte phenotype as well as the expression of adipocyte-specific genes essential for insulin action (27). TNF-α potently induced the expression of CEBP-β (Table 1) and completely reversed the normal adipocyte CEBP-α/CEBP-β ratio. In addition, Nrip-1, a coactivator for many nuclear receptors, was also significantly suppressed. Downregulation of Nrip-1 mRNA might therefore potentiate the inhibitory effects of TNF-α on PPAR-γ, RXR-α, and CEBP-α actions.

Furthermore, TNF-α suppressed many adipocyte-abundant genes that encode proteins essential for insulin responsiveness (Table 1 and Fig. 4). Among them, IRS-1 and AKT are critical for insulin signal transduction, and lipoprotein lipase, long-chain fatty acyl CoA synthase, diacylglycerol acyltransferase, GLUT4, glycogen synthase, and fatty acid synthase are required for insulin-stimulated glucose and fatty acid uptake and their conversion to glycogen and triglyceride (28,29). Because the overall physiological effects of insulin on energy metabolism are to promote energy storage and inhibit energy mobilization, TNF-α may thus antagonize the action of insulin by shutting down the expression of genes that are essential for insulin responsiveness.

In addition to inhibiting adipocyte-specific gene expression, TNF-α also induced many growth factors and proteins associated with cell cycle progression, and in parallel downregulated cell cycle inhibitor proteins, including CEBP-α, GATA-6, cyclin-dependent kinase (CDK) inhibitor 1C, CDK inhibitor 2C (p18), growth-arrest specific 1, and growth-arrest and DNA-damage–inducible-γ gene. CEBP-α not only regulates the expression of adipocyte-specific genes and energy metabolism, but also inhibits the transcription of proteins necessary for cell cycle progression, such as cyclins or CDKs, and stimulates the expression of cell cycle inhibitors that inactivate specific CDKs (40). Although direct evidence for GATA-6 playing a role in the maintenance of adipocyte growth-arrest state is lacking, GATA-6 has been implicated in the regulation of cell proliferation and differentiation in other cell types. GATA-6 mRNA is downregulated in proliferating vascular smooth muscle cells, and forced expression of GATA-6 in vascular smooth muscle cells induces growth arrest that is associated with enhanced expression of CDK inhibitor p21 (41, 42). Thus, downregulation of mRNAs encoding CEBP-α and other growth arrest−inducing proteins, which is also enhanced by the induction of growth factors, might re-

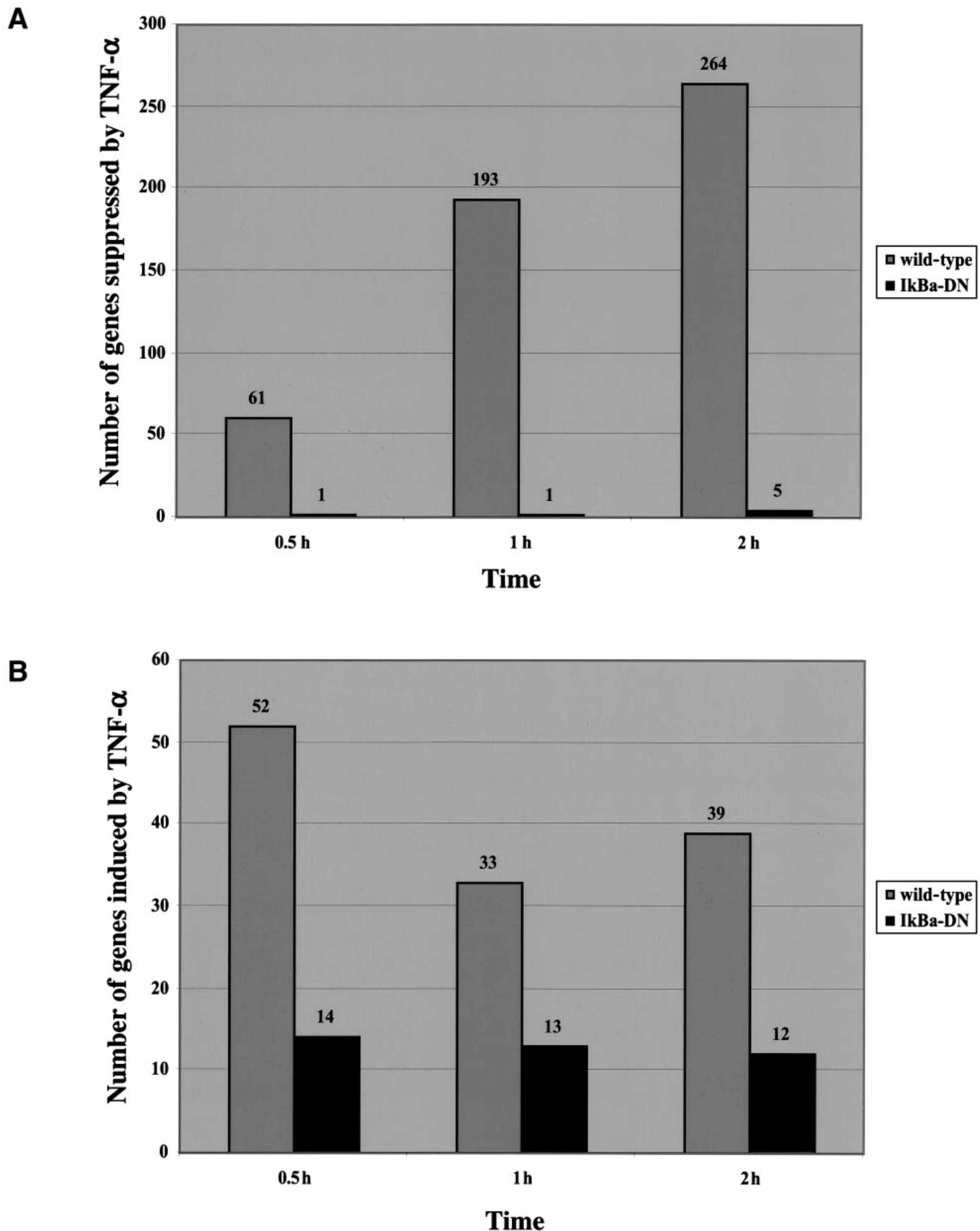

FIG. 8. TNF-α−induced changes in gene expression in wild-type 3T3-L1 adipocytes and adipocytes expressing IκBα-DN. Total RNAs were isolated from wild-type or IκBα-DN 3T3-L1 adipocytes incubated with TNF-α (1nmol/l) for 0, 0.5, 1, and 2 h. The kinetics of TNF-α induced changes in gene expression was assessed by microarray analysis as described in RESEARCH DESIGN AND METHODS. *A*: Total number of TNF-α−suppressed genes, including both known genes and ESTs. *B*: Total number of TNF-α−induced genes, including both known genes and ESTs.

lease cell cycle constraints and result in the activation of cell cycle and repression of adipocyte-specific genes in 3T3-L1 adipocytes. These changes, together with the suppression of adipocyte-specific proteins, may contribute to the loss of the adipocyte phenotype and insulin responsiveness seen after long-term TNF-α treatment of 3T3-L1 adipocytes.

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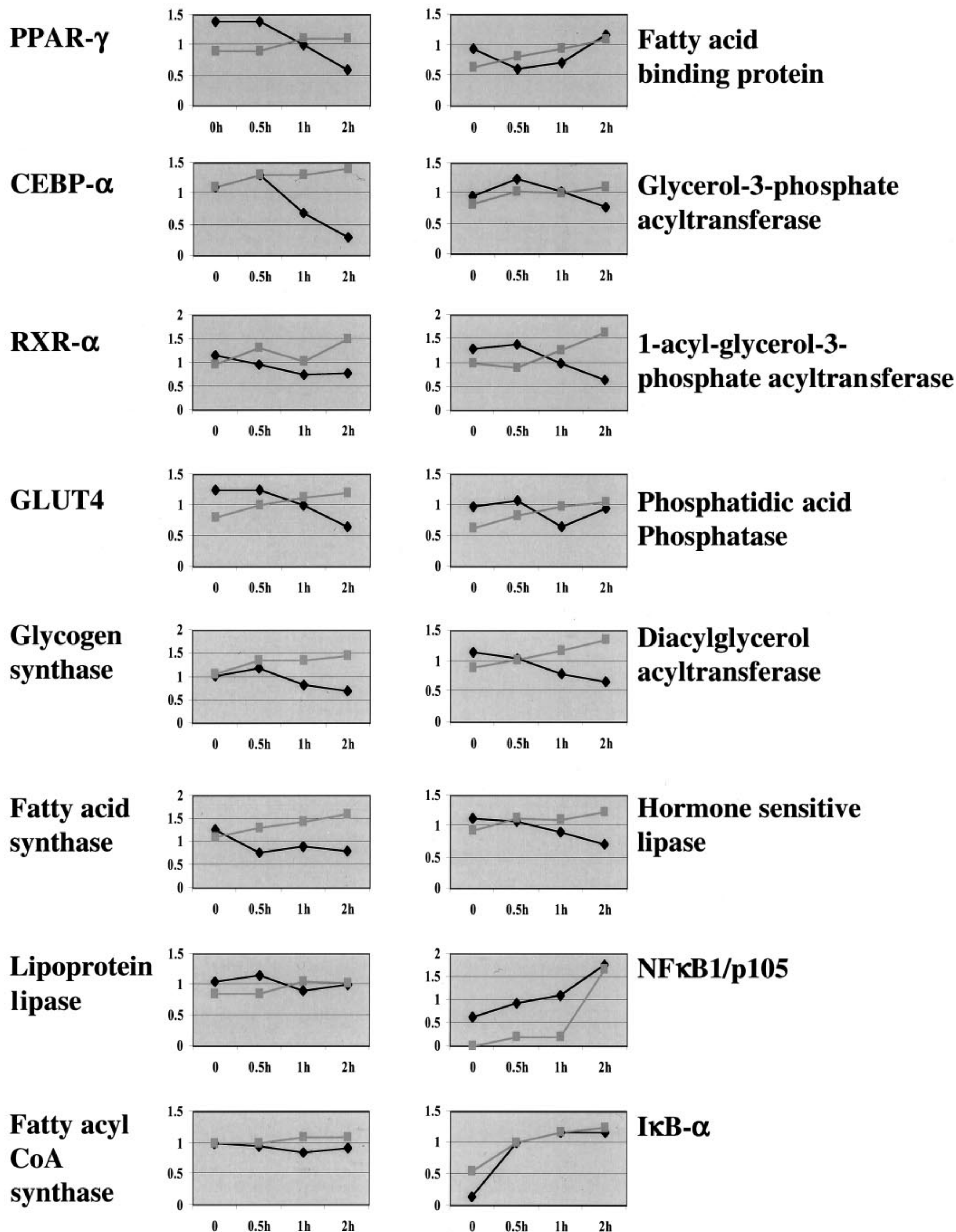

FIG. 9. Comparison of the effects of TNF-α on mRNA levels of several adipocyte-abundant genes in wild-type and IκBα-DN 3T3-L1 adipocytes. Wild-type (black line) or IκBα-DN 3T3-L1 (gray line) adipocytes were treated with TNF-α (1 nmol/l) for 0, 0.5, 1, and 2 h. Total RNAs isolated from each time point were analyzed by microarray analysis as described in RESEARCH DESIGN AND METHODS. Normalized hybridization signals of the indicated genes were plotted as a function of time.

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TNF-α modulates secretion of free fatty acids, cytokines, and hormones from adipocytes that may act as systemic mediators to antagonize the actions of insulin. Although TNF-α mRNA and protein levels are upregulated in adipose tissue and skeletal muscle in obese animal and human subjects (8,9,11,12), the plasma concentration of TNF-α is undetectably low (11,43). Thus, TNF-α is likely to act locally via an autocrine effect in adipose tissue and/or skeletal muscle, and may also act indirectly through mediators produced by these tissues in response to TNF-α.

Because TNF-α increases lipolysis in adipocytes (43–45), plasma free fatty acid is a potential candidate for a systemic mediator of TNF-α action. In support of this notion, it has been shown that infusion of TNF-α in rats increases plasma free fatty acid levels (6), and neutralization of TNF-α in Zucker rats has been associated with both increased insulin sensitivity and decreased plasma free fatty acid levels (5). Moreover, circulating free fatty acid and triglyceride levels are significantly lower in TNF-α knockout mice than their wild-type littermates (15). Excess plasma free fatty acids antagonize the actions of insulin in insulin-sensitive tissues (46) and may thus enhance the effects of TNF-α in the induction and aggravation of insulin resistance seen in obesity and obesity-linked type 2 diabetes.

Cytokines are other potential systemic mediators of TNF-α action. Table 1 lists a number of cytokines, cytokine-induced proteins, and chemokines induced by TNF-α. Among them, IL-6 and interferon-α have been implicated in the induction of insulin resistance in a number of pathological settings, including infection and cancer-induced cachexia (47–50). In addition, interferon-γ has been shown to induce IL-6 secretion and may thus enhance the metabolic effects of IL-6. Therefore, 3T3-L1 adipocytes produce several cytokines and chemokines in response to TNF-α that may potentially, at least in cell culture, contribute to the development of insulin resistance.

TNF-α also modulates protein secretion from adipocytes. Among the genes induced by TNF-α, secreted VCAM-1, fibronectin, tenascin C, ceruloplasmin, PAI-1, and adrenomedullin have been associated with type 2 diabetes or an excessive rate of cardiovascular diseases (51–53). In addition, TNF-α significantly suppressed mRNA levels of the gene encoding ACRP30, an adipocyte-derived hormone that inhibits gluconeogenesis in liver (34), enhances free fatty acid oxidation in skeletal muscle, and reduces body weight in mice (35). Because ACRP30 promotes both fatty acid and glucose utilization in skeletal muscle, downregulation of ACRP30 gene expression may mediate, at least in part, the effects of TNF-α in inducing insulin resistance in vivo. Hence, in addition to the direct effects on adipocyte gene expression, the metabolic effects of TNF-α may also be mediated and/or amplified by the systemic protein and lipid mediators produced by adipose tissue in response to TNF-α.

NF-κB is indispensable for TNF-α suppression of genes essential for insulin action. The blockade of NF-κB activation through the expression of IκBα-DN abolished the suppression of >98% of the genes normally suppressed by TNF-α, and induction of 60–70% of the genes normally induced by TNF-α. Strikingly, the absence of NF-κB activation significantly altered the global changes in gene expression in response to TNF-α, indicating NF-κB is a master regulator of gene expression and a key mediator of TNF-α action in 3T3-L1 adipocytes. Moreover, NF-κB is essential for protection against TNF-α−induced apoptosis in 3T3-L1 adipocytes. Whether NF-κB is essential for the initiation of changes in adipocyte gene expression and the induction of insulin resistance in vivo cannot be answered by our 3T3-L1 adipocyte model alone. Yet, 3T3-L1 adipocytes expressing IκBα-DN are useful as an initial step to define the molecular mediators in TNF-α−induced insulin resistance, and may provide a basis for pharmaceutical intervention in type 2 diabetes.

In summary, we conclude that TNF-α immediately causes changes in gene expression that provide a basis for the induction of insulin resistance in 3T3-L1 adipocytes, and that NF-κB activation is obligatory for TNF-α action. Further studies are required to identify the particular NF-κB subunits that mediate the suppression and/or induction of the adipocyte genes, as well as the molecular pathways mediating NF-κB activation in 3T3-L1 adipocytes.

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