Troglitazone Antagonizes Tumor Necrosis Factor-α-induced Reprogramming of Adipocyte Gene Expression by Inhibiting the Transcriptional Regulatory Functions of NF-κB*

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Troglitazone (TGZ), a member of the thiazolidinedione class of anti-diabetic compounds and a peroxisome proliferator activator receptor-γ (PPAR-γ) agonist, restores systemic insulin sensitivity and improves the full insulin resistance syndrome in vivo. The mechanisms underlying its in vivo function are not understood. Here we investigated the potential functional interaction between PPAR-γ and NF-κB in adipocytes. We show that TGZ selectively blocked tumor necrosis factor-α-induced and NF-κB-dependent repression of multiple adipocyte-specific genes and induction of growth phase and other genes. This occurs without interfering with NF-κB expression, activation, nuclear translocation, or DNA binding and without suppressing NF-κB-dependent survival signals. Notably, the expressions of some tumor necrosis factor-α-induced genes in adipocytes were unaffected by PPAR-γ activation. In reporter gene assays in HeLa cells, ectopic expression of PPAR-γ abolished induction of a NF-κB-responsive reporter gene by the p65 subunit (RelA) of NF-κB, and the inhibition was further enhanced in the presence of TGZ. Conversely, overexpression of p65 inhibited induction of a PPAR-γ-responsive reporter gene by activated PPAR-γ in a dose-dependent manner. The inhibitory effect was independent of the presence of NF-κB-binding sites in the promoter region. Other NF-κB family members, p50 and c-Rel as well as the S276A mutant of p65, blocked PPAR-γ-mediated gene transcription less effectively. Thus, p65 antagonizes the transcriptional regulatory activity of PPAR-γ in adipocytes, and PPAR-γ activation can at least partially override the inhibitory effects of p65 on the expression of key adipocyte genes. Our data suggest that inhibition of NF-κB activity is a mechanism by which PPAR-γ agonists improve insulin sensitivity in vivo and that adipocyte NF-κB is a potential therapeutic target for obesity-linked type 2 diabetes.

Type 2 diabetes is characterized, in part, by elevated plasma levels of free fatty acids and glucose and is associated with a cluster of abnormalities such as central obesity, dyslipidemia, hyperinsulinemia, elevated plasma inflammatory markers, impaired fibrinolysis, vascular abnormalities, and hypertension (1, 2). These abnormalities are also referred to as metabolic or insulin resistance syndrome (2–4) and are risk factors for cardiovascular and cerebrovascular diseases. In obesity-linked type 2 diabetes, decreased overall insulin sensitivity is a fundamental defect that precedes the development of the full insulin resistance syndrome and subsequent β cell failure (3, 4).

Treatments of type 2 diabetes, such as correcting relative insulin deficiency, inhibiting hepatic glucose production, and delaying glucose absorption from the gastrointestinal tract, lower plasma glucose levels but do little to improve insulin sensitivity. In time, these interventions often fail to restore metabolic homeostasis and to prevent the development of most of the complications of type 2 diabetes. Thus, there remains a great need to restore insulin responsiveness in the clinical management of type 2 diabetes.

Thiazolidinediones (TZD), a class of anti-diabetic medications and synthetic ligands for PPARγ, decrease plasma free fatty acid concentrations as well as fasting and postprandial plasma glucose levels in patients with type 2 diabetes by improving insulin sensitivity in major insulin-target tissues. In addition, TZD reduce plasma triglyceride levels, improve the plasma lipoprotein profile, lower blood pressure in diabetic hypertensive patients, and correct the proinflammatory and procoagulant state (5). Taken together, TZD target insulin resistance and restore metabolic homeostasis while improving the cluster of abnormalities that occur in type 2 diabetes. However, the direct target tissue(s) of TZD and the molecular mechanism(s) by which TZD sensitize the major insulin-responsive tissues in vivo remain elusive.

TZD are high affinity ligands for peroxisome proliferator receptor activator-γ (PPAR-γ). PPAR-γ has two protein isoforms, PPAR-γ1 and PPAR-γ2 (6, 7). Whereas PPAR-γ1 is ubiquitously expressed at low levels in many tissues including muscle, PPAR-γ2 is most highly expressed in adipose tissue (8, 9). Notably, adipose tissue is not only essential for maintaining the overall in vivo insulin sensitivity but is also a source of

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1 The abbreviations used are: TZD, thiazolidinediones; TGZ, troglitazone; TNF-α, tumor necrosis factor-α; PPAR-γ, peroxisome proliferator activator receptor-γ; ELISA, enzyme-linked immunosorbent assay; PPRE-luc, The PPAR-γ-responsive luciferase reporter gene; PEPCK, phosphoenolpyruvate carboxykinase; HSL, hormone-sensitive lipase; IκBα, inhibitor of κB protein; IL, interleukin; TG, triglyceride; FFA, free fatty acid.
endocrine activity that modulates several physiological processes that include systemic energy metabolism, inflammation, blood coagulation, blood vessel tone, and reproduction. A number of adipocyte-derived factors have been implicated in insulin resistance in obesity and obesity-linked type 2 diabetes. Thus, it is likely that TZD could directly target molecular mediator(s) of insulin resistance in adipocytes and restore the metabolic function and endocrine signals of adipose tissue, and thereby contribute to the improved systemic insulin sensitivity.

Tumor necrosis factor-α (TNF-α), an autocrine/paracrine factor that is highly expressed in adipose tissues of obese animals and human subjects, is a potential molecular mediator of insulin resistance that is of physiological importance. Although many factors may trigger the development of insulin resistance in humans (10), TNF-α-regulated autocrine or paracrine pathways in adipose tissue have been implicated in mediating the metabolic consequences of obesity to cause insulin resistance (11). One proposed mechanism for TNF-α induction of insulin resistance involves inhibition of insulin-stimulated tyrosine phosphorylation of the insulin receptor and insulin receptor substrate-1 (12–14). More recently, the inhibitor xB kinase-β has been implicated in this process (15). However, other mechanisms by which TNF-α induces insulin resistance have also been described, including down-regulation of protein levels of insulin receptor substrate-1 (16), GLUT4 (16), CEBP-α (17), PPAR-γ (18), perilipin (19, 20), and ACRP30 (21–23) in adipocytes.

We recently substantiated the critical role of TNF-α-regulated gene expression in adipocytes in the development of systemic insulin resistance by association of gene expression profiles in major insulin-responsive tissues with overall in vivo insulin sensitivity in rats infused with TNF-α (24). We also demonstrated that NF-κB activation by TNF-α is obligatory in the repression of key adipocyte genes and induction of many proinflammatory and acute phase proteins (22). These data assert the importance of TNF-α and TNF-α-induced NF-κB activity in the etiology of insulin resistance in adipocytes.

It is well established that TZD counteract a number of effects of TNF-α on adipocytes, including adipocyte differentiation (25) and gene expression (17, 18, 22, 26, 27), insulin signaling (28), insulin-stimulated glucose uptake (26), lipogenesis (29), and lipolysis (19). Yet the molecular mechanisms for these effects of TZD are not known. Interestingly, TZD-induced PPAR-γ activation in monocytes or macrophages suppresses the induction of many inflammatory and immune response mediators such as TNF-α, IL-1β, IL-6, metalloproteases, and inducible nitric-oxide synthase in part by inhibiting the activities of NF-κB, signal transducers and activators of transcription, and AP-1 (30, 31). In addition, PPAR-α agonists inhibit cytokine-induced VCAM-1 and IL-6 expression in endothelial cells and vascular smooth muscle cells through interference with NF-κB and AP-1 action by protein-protein interactions and cofactor squelching (32, 33). However, experimental data demonstrating a functional interaction between NF-κB and PPAR-γ in the context of adipocytes are lacking.

Herein we have used a number of complementary experimental approaches to determine whether ligand-induced PPAR-γ activation can override the transcription-repressive effects of NF-κB activation by TNF-α on key adipocyte genes, and whether NF-κB family members can directly inhibit the transcriptional regulatory activity of PPAR-γ.

EXPERIMENTAL PROCEDURES

Oligonucleotide Microarray Data Collection and Analysis—3T3-L1 adipocytes were untreated or stimulated with TNF-α (1 nmol/liter), troglitazone (1 μmol/liter), or both for various times. Total RNA at each time point was isolated, converted to biotin-labeled cRNA targets, hybridized to MG74Av2 oligonucleotide arrays (Affymetrix, Santa Clara, CA), and scanned on Affymetrix scanners essentially as described previously (22, 34). Gene expression data were analyzed and processed using a set of web-based analysis tools developed in the Genome Center at the Whitehead Institute for Biomedical Research (Cambridge, MA) and the Cluster and TreeView software (35).

Identification of adipocyte-abundant genes was determined by selecting those with 3-fold or higher expression levels in adipocytes than fibroblasts. We then excluded genes expressed at a very low level by setting an arbitrary threshold value for the array measurements (Average Differences, according to Affymetrix), and genes whose expression levels were below 150 at all time points and under all conditions were excluded. We also excluded genes whose differences in expression levels (maximal value-minimal value) between any two time points were less than 150. Thus, these filters allow us to include adipocyte-abundant genes that exhibit robust changes in steady state mRNA levels in response to at least one treatment.

Next we used a modified score system, originally described by Haco- hen and co-workers (36), to identify genes regulated by TNF-α, troglita- zone, or both. Briefly, let R, and C be the steady state mRNA levels of treated samples and control samples, respectively, at the i-th time point. Define μR to be the mean expression level of samples of the control time course and δi as the standard deviation of expression levels in the control samples. Then we can define a score, Si = (RI – μR)/δi, to measure the statistical significance of the changes in gene expression in the treated samples at each time point Ri. Genes with low scores are a consequence of large variation in mRNA levels in the control time course (high noise) or small differences between the control and treated samples. By setting a threshold value for Si (see below), we can exclude genes whose expression levels were not significantly affected by a treatment or fluctuated over time under control conditions.

Identification of up-regulated genes was determined by requiring one of the following: 1) Si ≥ 4 for at least 1 time point; 2) Si ≥ 1.4 for at least two consecutive time points. Down-regulated genes were selected by requiring Si ≤ –1.4 for at least three consecutive time points or Si ≤ –3 for at least one time point. We used this score system to identify TNF-α-regulated transcripts from the 175 adipocyte-abundant known genes, and we then compared the list of identified genes with our master list of 64 TNF-α-affected adipocyte-enriched genes that have been verified previously by Northern Blotting, semi-quantitative RTPCR, or literature search (22, 24). The score system identified 102 TNF-α-affect ed genes (Table I) including 51 genes from the master list. The scores of the rest of the 13 master list genes are very close to but did not pass the score threshold and were excluded from the TNF-α-regulated gene list in this study. The 13 genes are as follows: CD 36, lactate dehydrogenase 2; apoE; sterol carrier protein 2; 11-β-hydroxysteroid dehydrogenase; cytochrome P450; carbonic anhydrase 3; catalase; adeny l kinase isozyme 3; complement component C3; amyloid β (A4) precursor-like protein 2; GADD 45; and Rho B. This indicates that the score system might give false-negative results. However, the inclusion of the 13 genes would not have changed our interpretation of the results, and we therefore used this score system to assess genes regulated by other treatments.

Fold changes in gene expression in response to a treatment relative to control were calculated as the following: \( F_{up} = \frac{max(L_1, L_2, \ldots , L_n)}{min(L_1, L_2, \ldots , L_n)} \) for genes up-regulated by a treatment, and \( F_{down} = -\frac{1}{min(L_1, L_2, \ldots , L_n)} \) for down-regulated genes, where \( L_i = \text{geomean}(R_i, R_{i+1})/\text{geomean}(C_i, C_{i+1}) \).

Oligonucleotide microarray data were also collected from wild-type 3T3-L1 adipocytes and adipocytes expressing a dominant negative inhibitor of TNF-α, IxB-α-DN, treated with TNF-α (1 nmol/liter) for 0, 0.5, 1, and 2 h (22). Identification of TNF-α-repressed genes was determined by identifying one of the following: 1) mRNA levels were less than 50% or more at the end of the 2-h incubation.

Tissue Culture—3T3-L1 cells were purchased from ATCC (Manassas, VA), maintained as fibroblasts, and differentiated into adipocytes as described previously (37). HeLa cells were provided by Dr. C. C. Fuchs (Whitehead Institute, Cambridge, MA), and grown in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum.

Western Blot Analysis—3T3-L1 adipocytes were incubated in growth media or media containing TNF-α (1 nmol/liter), troglitazone (1 μmol/liter), or both together for various times. Cell lysates were separated by SDS-PAGE and electroblotted onto a nitrocellulose membrane (Amer sham, Piscataway, NJ). The filters were incubated with antibodies, washed, and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody. Bound antibodies were detected using the enhanced chemiluminescence Western blotting analysis system (Amer sham, Piscataway, NJ). Blots were stained with Ponceau
S solution to visualize the amount of total protein in each lane.

**Plasmids**—The reporter gene PEPCK-Luc was generated by subcloning the 2100-bp fragment of the PEPCK gene promoter (38) (provided by Dr. D. Granner, Vanderbilt, TN) into pTAL-Luc (Clontech, La Jolla, CA). The NF-κB-responsive luciferase reporter gene was from Clontech. The PPAR-γ-responsive luciferase reporter gene (PPRE-luc) was constructed by subcloning 6 tandem repeats of PPAR-γ-response elements into the pTAL-luc vector. The expression plasmids p65 (RelA), p50 (S276A) mutant, and p50 were provided by Dr. D. Granner (39); c-Rel was a gift from Dr. W. Tam (Whitehead Institute, Cambridge, MA). Murine full-length PPAR-γ2 was subcloned into pIRE2-GFP expression vector (Clontech), and the sequence was verified by DNA sequencing.

**Transfection Assays**—All transfecions were performed using FuGENE 6 (Roche Applied Science) according to the manufacturer’s instructions. Forty eight hours after transfection, cell lysates were prepared and analyzed for luciferase activity using a kit from Promega (Madison, WI). The luciferase activity was normalized using an internal CMV-β-Gal control plasmid. All experiments were done in triplicate and were repeated at least three times.

**ELISA**—3T3-L1 adipocytes were incubated in growth media or media containing TNF-α (1 nmol/liter), troglitazone (1 μmol/liter), or both together, for the indicated times. Nuclear extracts were prepared and assayed for NF-κB activities using an ELISA kit (Active Motif, Carlsbad, CA) following the manufacturer’s instructions. Briefly, the NF-κB consensus sequence (5′-GGGACTTTCC-3′) has been immobilized on a 96-well plate. Various nuclear extracts were then added to the plate and incubated for 1 h at room temperature. The plate was washed and incubated with anti-p65 antibody for 1 h. Following a thorough wash and incubation with a horseradish peroxidase-conjugated secondary antibody, the plate was washed extensively and developed, and the A₅₀₀ was determined by spectrophotometry. The specificity of the ELISA was verified by measuring the binding of p65 to the ELISA plate in the absence or the presence of a saturating amount (20 pmol) of oligonucleotides containing either wild-type or mutant NF-κB consensus sequences.

**Statistical Analysis**—Comparisons were performed using a two-tailed Student’s t test assuming unequal variances. p ≤ 0.05 was considered significant.

**RESULTS**

**Oligonucleotide Microarrays Reveal That TGZ Has a Moderate Effect on Adipocyte-abundant Gene Expression but Blocks, at Least Partially, TNF-α-mediated Repression of Adipocyte-abundant Genes**—To investigate the potential antagonism between TGZ and TNF-α on the expression of genes that are essential for adipocyte function, we first identified 175 known adipocyte-abundant genes, by selecting genes whose mRNA levels are 3-fold or higher in 3T3-L1 adipocytes than in 3T3-L1 preadipocytes. We then treated 3T3-L1 adipocytes with TNF-α, TGZ, and both together for up to 24 h, and we assessed the changes in the mRNA levels of these 175 adipocyte-abundant genes.

TNF-α treatment for up to 24 h significantly altered the expression levels of 102 adipocyte-abundant genes (58%, see Fig. 1 and Table I). Among them, the mRNA levels of 86 genes (84%) were repressed by TNF-α, and the expressions of 16 genes (16%) were highly induced by TNF-α treatment (Fig. 1 and Table I). In contrast, TGZ had only a moderate effect on the expression of adipocyte-abundant genes (Fig. 1 and Table I). Strikingly, the presence of TGZ decreased the maximal fold repression of 78 genes (91%) by TNF-α over a period of 24 h. Of these 78, 64 genes showed at least a 50% increase in mRNA levels compared with those in cells treated with TNF-α alone for 24 h (Table I, in boldface type). We then assessed the expression levels of the 64 genes that are protected by TGZ from TNF-α-induced down-modulation (Table I, boldface type) in adipocytes expressing a dominant inhibitor of NF-κB activation (IκBα-DN) (22) in the absence or the presence of TNF-α. We found that the mRNA levels of 28 genes were significantly repressed by TNF-α in control 3T3-L1 adipocytes after 2 h of incubation, whereas the expression levels of these genes were unaffected by TNF-α in cells expressing IκBα-DN (Table I, underlined). The mRNA levels of the rest of the 36 genes were not significantly repressed by TNF-α in control cells during the 2-h incubation. Because TNF-α induced extensive apoptosis in adipocytes expressing IκBα-DN after 2 h of incubation, we could not determine whether the repression of the other genes by TNF-α depended on NF-κB. However, the steady state mRNA levels of many of the 36 genes increased significantly in cells expressing IκBα-DN, indicating a potential NF-κB-mediated inhibitory effect on the expression of these genes. Thus, the repressive effect of TNF-α on adipocyte gene expression was NF-κB-dependent, and TGZ may protect adipocyte genes that are essential for adipocyte function through inhibition of NF-κB.

To assess the potential impact of the antagonistic actions between TGZ and TNF-α on adipocyte biology, we grouped the genes that are differentially regulated by TGZ and TNF-α according to the biological functions of their encoded proteins (Table I). These are discussed below.

**Troglitazone Prevents TNF-α-induced Down-regulation of Adipocyte Genes Implicated in the Suppression of Free Fatty Acid Release from 3T3-L1 Adipocytes**—Increased free fatty acid

![Fig. 1. Adipocyte-abundant genes repressed or induced by TNF-α. The adipocyte-abundant genes were identified as described under "Experimental Procedures." The expression profiles of control adipocytes, adipocytes treated with TNF-α (1 nmol/liter), troglitazone (1 μmol/liter), or both for 0, 1, 2, 4, 8, and 24 h were analyzed as described under "Experimental Procedures." Each single column presents a time point, and each single row of colored bars represents a gene (gene numbers are indicated to the left of the figure). The normalized gene expression data for each gene is centered to the median value, so that black color indicates gene expression at the median level, and red indicates a high level of expression, and green denotes a low level of expression. The color bar scale at top indicates relative expression in standard deviations from the median. The functional categories of the selected genes are indicated to the right of the figure.](http://jbc.org/)

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Troglitazone Inhibits NF-κB Function in Adipocytes

Regulation of adipocyte-abundant genes by troglitazone and TNF-α

The adipocyte-abundant genes and their fold changes upon adipocyte differentiation and following the indicated treatments were determined as described under “Experimental Procedures.” The identified genes were assigned into functional categories based on the putative biological functions of their encoded proteins as determined by public data base searching. The adipocyte-abundant genes induced (italic type) or repressed (roman type) by TNF-α are included in this list. NC, not changed. Fat/FB indicates the fold induction upon adipocyte differentiation. Boldface type, TGZ-protected genes whose steady state mRNA levels increased at least 50% in the presence of bothTGZ and TNF-α were blocked by TGZ. Underlined, genes whose mRNA levels were significantly repressed by TNF-α in 3T3-L1 adipocytes after 2 h of incubation but were unaffected by TNF-α in adipocytes expressing IκBα-DN. MHC, major histocompatibility complex.

| GBA | Gene name | -Fold (Fat/FB) | TNF-α | TGZ | TNF-α + TGZ |
|-----|-----------|----------------|-------|-----|-------------|
| U37799 | Scavenger receptor class B1 | 6.1 | -2.5 | 1.4 | NC |
| X89968 | 17-β-Hydroxysteroid dehydrogenase type IV | 8.8 | -2.1 | 1.4 | -1.5 |
| X75929 | ATP-binding cassette 1 | 52 | 1.8 | NC | 1.6 |

**Energy Metabolism (cholesterol and steroid)**

**Energy Metabolism (fatty acid, triglyceride, and lipoprotein)**

**Energy Metabolism (glucose)**

**Metabolism (phospholipid)**

**Metabolism (amino acid and nucleic acid)**

**Metabolism (other)**

**Stress response**

**Secreted protein (acute phase reactant)**

**Secreted protein (extracellular matrix)**
| GBA     | Gene name                                      | -Fold (Fat/FB) | TNF-α | TGZ | TNF-α + TGZ |
|---------|-----------------------------------------------|----------------|-------|-----|-------------|
| Secreted protein (hormone and plasma protein) |                  |                |       |     |             |
| AA718169 | Resistin                                       | 587            | 5.5   | NC  | -2.1        |
| U49915   | ACRP30 (AdipoQ)                                | 379            | -1.8  | -1.1| -1.4        |
| AF045887 | Angiotensinogen                                 | 135            | -4    | NC  | NC          |
| U49430   | Ceruloplasmin                                   | 5.1            | 8.4   | NC  | 5           |
| Secreted protein (immune and defense response and protease inhibitor) |                  |                |       |     |             |
| X04673   | Adipsin                                        | 370            | -1.9  | NC  | -1.4        |
| M96827   | Haptoglobin                                     | 10.5           | 5.3   | NC  | 6.6         |
| M64086   | Spi 2 proteinase inhibitor                     | 6.7            | 2.7   | NC  | 1.9         |
| Transcription factor and transcriptional regulation |                  |                |       |     |             |
| AB012273 | CEBP-γ                                         | 4              | -3.4  | NC  | NC          |
| AF053062 | Receptor interacting protein 140              | 7.7            | -5.9  | -1.7| -3.3        |
| AF085745 | Nuclear orphan receptor LXR-α                  | 64             | -1.7  | NC  | NC          |
| M62962   | CEBP-α                                         | 17             | -2.9  | 1.1 | NC          |
| U10374   | PPAR-γ                                         | 17             | -2.5  | -2.3| -5.9        |
| X95279   | Spot 14                                        | 135            | -7.3  | NC  | NC          |
| Y15001   | Iroquois homeobox protein                      | 6              | -1.7  | -1.3| -1.6        |
| U63387   | Chromobom homolog 4                            | 4              | -4.1  | NC  | -4.1        |
| AF077659 | Homeomain-interacting protein kinase 2         | 34             | -4.8  | 1.3 | -1.7        |
| AF077660 | Homeomain-interacting protein kinase 3         | 4.6            | -3.3  | 1.9 | 1.5         |
| Cell cycle and proliferation |                  |                |       |     |             |
| AF011908 | Apoptosis-associated tyrosine kinase           | 18             | -5.9  | NC  | NC          |
| D78382   | Tob                                            | 10             | -2.2  | NC  | NC          |
| X95280   | G0S2-like protein                              | 86             | -8.7  | NC  | NC          |
| U19596   | Cdk4 and Cdk6 inhibitor p18 protein            | 5.2            | -4.2  | -2.2| -2.7        |
| Apoptosis |                                               |                |       |     |             |
| M61737   | Adipocyte-specific mRNA                        | 858            | -9.6  | 1.9 | 1.5         |
| Cell adhesion |                                              |                |       |     |             |
| AF078705 | Amine oxidase, copper containing 3             | 30             | -2.3  | -1.4| -2.2        |
| X69902   | Integrin α                                      | 8.3            | -3    | NC  | NC          |
| U88915   | Junctional adhesion molecule                   | 3.6            | 1.6   | NC  | 2.8         |
| Protein degradation |                                        |                |       |     |             |
| X81323   | Tripeptidyl peptidase II                       | 3.7            | -2.7  | NC  | -1.7        |
| AB024427 | Ring finger protein 11                         | 5.8            | -2    | NC  | -1.9        |
| Signaling |                                               |                |       |     |             |
| AB016080 | Calcium-binding protein Kip2                   | 13             | -8.2  | NC  | -2.1        |
| AF009246 | Ras-related protein                            | 128            | -10.7 | 2   | -6.1        |
| AF093669 | Peroxisomal biogenesis factor                  | 6.6            | -2.5  | NC  | NC          |
| M13071   | Raf-related oncogene                           | 3              | -1.7  | NC  | -1.6        |
| U44940   | Quaking                                        | 4.7            | -2    | 1.8 | NC          |
| U588835  | c-Chl associated protein                       | 18             | -3.4  | 2.2 | NC          |
| U67187   | G protein signaling regulator 2                | 31             | -7.1  | -4.1| -7.6        |
| X72862   | Adrenergic receptor, β3                       | 43             | -13   | NC  | -10.4       |
| Y12577   | ADP-ribosylation like 4                        | 12             | -5    | 2.8 | NC          |
| U28168   | Adenomatosis polyposis coli                    | 5.1            | -2.3  | NC  | NC          |
| Transport |                                               |                |       |     |             |
| AF091390 | Phospholemman precursor                       | 6.6            | -1.8  | NC  | -1.8        |
| AB010100 | Aquaporin 7                                    | 5.8            | -2.3  | 1.5 | -1.4        |
| Z46670   | ATP-binding cassette, subfamily D, member 2    | 62             | -5    | NC  | -3.3        |
| AF098633 | GLUT4 vesicle protein                          | 5.3            | -1.5  | NC  | NC          |
| AF084575 | Adaptor protein complex-3 3A subunit                  | 4.4            | -2    | -1.2| -2.1        |
| Immune response and MHC-related |                  |                |       |     |             |
| M28233   | Interferon-γ receptor                          | 9.6            | 1.3   | NC  | 1.6         |
| U60091   | ATP-binding cassette transporter 2             | 4.7            | 4.2   | NC  | 4.3         |
| AJ007970 | mGBP-2 protein                                 | 3.8            | 15.8  | NC  | 11          |
| M27134   | Histo compatibility 2, K region locus 2        | 4.7            | 2.5   | NC  | 3.6         |
| M60609   | Histo compatibility 2, D region locus 1        | 3.4            | 3.1   | NC  | 3.6         |
| X00246   | MHC class I antigen                            | 3.6            | 3.9   | NC  | 3.6         |
| Y00292   | Histo compatibility 2, T region locus 23       | 8.6            | 3.5   | -2.1| 2.7         |
| Other |                                               |                |       |     |             |
| L268395  | Peroxisome membrane protein                    | 29             | -3.3  | NC  | -2.2        |
| AL078630 | Genomic DNA sequence from chromosome 17, containing the genes | 6.2            | 7     | 2.6 | 17          | for γ-aminobutyric acid B receptor 1, and five 7 transmembrane receptor (rhodopsin family)
release from adipocytes is a key feature in type 2 diabetes. The balance between cellular triglyceride (TG) synthesis, free fatty acid re-esterification, and lipolysis determines the amount of FFA release from adipocytes (40, 41). As we reported previously (22, 24), TNF-α significantly repressed adipocyte-abundant genes implicated in the suppression of FFA release from adipocytes. Among them, glycerol-3-phosphate acyltransferase (Fig. 2A, filled circles), 1-acylglycerol-3-phosphate acyltransferase (Fig. 2B, filled circles), diacylglycerol acyltransferase (Fig. 2C, filled circles), and long chain fatty acyl-CoA synthase (Fig. 2D, filled circles), are enzymes essential for cellular TG synthesis. Phosphoenolpyruvate carboxykinase (PEPC, Fig. 2E, filled circles), also down-regulated by TNF-α, generates glycerol 3-phosphate, a 3-carbon glycolytic intermediate essential for long chain fatty acyl CoA incorporation into TG. Although sufficient glycerol 3-phosphate can be generated from glucose, during the fasting state or lipolysis in adipocytes the supply of glucose is significantly reduced, and glycolysis is inhibited. Thus, the PEPC-dependent glycerol 3-phosphate production becomes especially important because fat cells lack the enzyme glycerol kinase that phosphorylates glycerol to generate glycerol 3-phosphate. The supply of glycerol 3-phosphate is critical for maintaining adipocyte TG synthesis, which limits the amount of free fatty acids released into plasma. Pyruvate carboxylase (Fig. 2F) is also involved in cellular lipogenesis. Transaldolase (Fig. 2G) and malic enzyme (Fig. 2H) generate NADPH required for lipid biosynthesis. S3-12 (Fig. 2I) was originally identified as a plasma membrane-associated protein that is highly induced upon 3T3-L1 adipocyte differentiation (42). Although the exact function of S3-12 remains unclear, it contains the signature domain found in the perilipin family of lipid droplet-associated proteins and thus may potentially interact with cellular fat droplets as well. Adipose differentiation-related protein (Fig. 2J) is also a member of the perilipin family and is highly induced upon adipocyte differentiation. The perilipin family of proteins has been implicated in interfering with the interaction between lipid droplets and cellular lipid hydrolases and thus reducing hormone-sensitive lipase (HSL)- or other TG hydrolase-mediated TG hydrolysis (19, 20, 43). These proteins act together favoring cellular TG synthesis and suppressing FFA mobilization and release from adipocytes, and are all repressed by TNF-α treatment.

Whereas TGZ induces the mRNA levels of PEPC (Fig. 2E, open circles) and S3-12 (Fig. 2I, open circles), it does not significantly alter the expression of the rest of the genes discussed above (Fig. 2, open circles). However, TGZ prevented, at least partially, TNF-α-induced and NF-κB-mediated repression of expression of all of these genes (Fig. 2, filled triangles). This suggests a possible molecular mechanism by which TGZ antagonizes the actions of TNF-α and suppresses FFA production from adipocytes. Notably, TGZ also blocked the down-regulation of mRNA levels of HSL by TNF-α (Table I), consistent with previous reports (19, 44). HSL is an adipocyte-specific protein that is induced 166-fold upon 3T3-L1 adipocyte differentiation (Table I). The HSL-mediated TG hydrolysis is mostly dependent on the regulation of its activity by phosphorylation state rather than HSL protein mass. Thus the down-regulation of HSL mRNA may not contribute, significantly, to the amount of FFA released from fat cells. On the other hand, the down-regulation of HSL mRNA by TNF-α and its prevention by TGZ supports our hypothesis that TGZ and TNF-α antagonize each other in the regulation of adipocyte-abundant genes.

Troglitazone Antagonizes the Effects of TNF-α on Expression of Secreted Protein and Genes Encoding Transcription Factors and Signaling Molecules That Are Essential for Adipocyte Phenotype and Function—The profile of secreted proteins changes dramatically upon 3T3-L1 adipocyte differentiation. Adipocytes secrete a variety of bioactive molecules that affect multiple biological processes such as energy homeostasis, appetite control, blood vessel tone, extracellular matrix composition, blood coagulation, and immune and inflammatory responses. To evaluate whether TGZ antagonizes the actions of TNF-α on the endocrine/paracrine functions of adipocytes, we first examined the effects of TGZ on genes encoding secreted proteins that were repressed by TNF-α. As we reported previously (22), TNF-α repressed the mRNA levels of ACRP30 (Fig. 3A), an adipocyte hormone that sensitizes the actions of insulin in liver and muscle. In addition, expression of angiotensinogen (Fig. 3B), resistin (Fig. 3C), and adipin (Fig. 3D), which are highly induced upon 3T3-L1 adipocyte differentiation and regulate blood vessel tone, energy homeostasis, and complement activation, respectively, were repressed by TNF-α as well. The presence of TGZ reversed or partially blocked the repressive effect of TNF-α on expression of these adipocyte-derived hormones.

Adipocyte differentiation is also associated with induction of many transcription factors and proteins that are essential for adipocyte phenotype and response to insulin. TNF-α down-
are shut down, whereas many anti-proliferative proteins including Cdk4 and Cdk6 inhibitor p18 and G0S2-like protein are highly induced (Table I). As a result, terminally differentiated adipocytes virtually cease proliferation and completely withdraw from cell cycle progression. TNF-α treatment of 3T3-L1 adipocytes strongly induced pro-adipocyte growth factors and repressed anti-proliferative proteins that constrain cell cycle progression, including G0S2-like protein, transducer of EBB2, p18, and apoptosis-associated tyrosine kinase (Table I). TGZ potently blocked the repression of these genes by TNF-α (Table I).

Because NF-κB activation is obligatory for TNF-α repression of these adipocyte genes, the impaired transcription repressive activity of NF-κB in the presence of TGZ specifically indicates that TGZ-induced PPAR-γ activation may directly target NF-κB in adipocytes treated with TNF-α.

Toglitzalone Inhibits NF-κB Function in Adipocytes

To determine whether TNF-α-mediated induction of gene expression in adipocytes would be affected by TGZ treatment, we examined the effects of troglitazone on the expression kinetics of TNF-α-induced secreted factors. TNF-α treatment caused a robust induction of many genes encoding cytokines, chemokines, acute phase reactants, protease inhibitors, and antioxidants. Many of the TNF-α-induced molecules such as macrophage colony-stimulating factor, monocyte chemotactic protein 1 (MCP1, Fig. 4A), monocyte chemotactic protein 3 (MCP3, Fig. 4B), vascular cell adhesion molecule-1 (VCAM-1, Fig. 4C), hemopexin, ceruloplasmin (Fig. 4D), haptoglobin, Spi 2 proteinase inhibitor, and PAI-1 are risk factors for atherosclerosis (46–49), indicating that adipocytes may be a significant contributor to these characteristic features of type 2 diabetes. Strikingly, TGZ partially prevented the induction of some of these pro-inflammatory genes (Fig. 4, A–D, and Table I) but had no significant effects on a number of genes induced by TNF-α, such as PAI-1 and haptoglobin (Table I).

To determine the effect of TGZ on TNF-α induction of pre-adipocyte-abundant transcription factors and growth phase proteins, we assessed the mRNA levels of high mobility group protein I-C (HMGI-C, Fig. 5A), CEBP-β (Fig. 5B), and Fos-like antigen-1 (Fra-1, Fig. 5C) following TGZ and/or TNF-α treatment. TNF-α treatment rapidly induced these transcription factors (Fig. 5, A–C, filled circles). Notably, TNF-α induced the expression of CEBP-β and Fra-1 in cells expressing IκB-αDN to the same extent as in control cells, indicating that the induction of these genes by TNF-α is NF-κB-independent. There was no immediate induction of HMGI-C mRNA in either control or IκB-αDN cells after 2 h of TNF-α incubation, and thus we could not determine whether the induction of HMGI-C depends on NF-κB. TGZ alone also caused a transient and small increase in the mRNA levels of CEBP-β (Fig. 5B, open circles) and Fra-1 (Fig. 5C, open circles), but it did not affect the expression of HMGI-C, nor did it impair the induction of their expression by TNF-α (Fig. 5, A–C, filled triangles).

Next, we assessed the effects of TGZ on the expression levels of three immediate early genes, IκB-α, p65, and NF-κB1 (p105/p50), which are induced by TNF-α in an NF-κB-dependent manner (Fig. 5, D–F). IκB-α is rapidly induced by TNF-α treatment, and its mRNA levels increased 3.5- and 11-fold within 1 and 2 h of TNF-α treatment, respectively (Fig. 5D). The steady state mRNA levels of IκB-α in TNF-α-treated cells were 3.5-fold of the control cells at the end of the 24-h incubation. Interestingly, TGZ did not interfere with the induction of IκB-α mRNA by TNF-α, as the mRNA levels as well as the kinetics of IκB-α expression in response to TNF-α were essentially identical in the presence or the absence of TGZ in a period of 24 h.

**Toglitzalone Blocks TNF-α-mediated and NF-κB-dependent Repression of Genes Implicated in Cell Cycle Arrest**—Upon 3T3-L1 adipocyte differentiation, the expression of many pre-adipocyte-derived growth factors such as proliferin, mitogen-regulated protein/proliferin 3, and fibroblast growth factor 7

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**Fig. 3. Steady state mRNA levels of selected genes encoding adipocyte-abundant secreted proteins, transcription factors, and signaling molecules.**

A, Acrp30, adipocyte complement-related protein of 30 kDa; B, Agt, angiotensinogen; C, Resistin; D, Adipsin; E, cebpa, CCAAT/enhancer-binding protein-α; F, Pparg, PPAR-γ; G, Cap, c-Cbl-associated protein; H, Arl4, ADP-ribosylation factor-like 4. The expression levels are presented as the percentage of the control values at the matching time points (see “Experimental Procedures”). Filled circles, TNF-α treatment; open circles, TGZ treatment; filled triangles, TNF-α and TGZ treatment.

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*p* H. Ruan and H. F. Lodish, unpublished data.
NF-κB activation, IκB-α is a major isoform of the inhibitor of κB proteins (IκBα), and is a well characterized, NF-κB-induced immediate early gene. Thus, IκB-α is a naturally occurring molecular marker of NF-κB activation. As shown in Fig. 6A, TNF-α-induced rapid reduction of IκB-α protein within 15 min. Also, no phosphorylated IκB-α was detected 15 min after TNF-α addition, indicating complete degradation of any presumably phosphorylated IκB-α proteins. After 30 min, IκB-α protein started to re-accumulate and the newly synthesized IκB-α proteins continue to increase 60 min after TNF-α addition. Some of the newly synthesized IκB-α was also phosphorylated 30 and 60 min after TNF-α addition. In contrast, TGZ did not alter the phosphorylation states or the amount of IκB-α proteins. Importantly, TGZ did not affect the degradation, re-accumulation, or phosphorylation of IκB-α proteins in response to TNF-α, consistent with the lack of effect of TGZ on the kinetics of induction of IκB-α mRNA by TNF-α (Fig. 5D).

To determine whether the DNA binding activity of NF-κB would be inhibited by TGZ, we used ELISA analysis of p65 activity in 3T3-L1 adipocytes following TNF-α stimulation. p65 was chosen because it is a major NF-κB family member that shows increased nuclear accumulation in response to TNF-α in 3T3-L1 adipocytes (22). Fig. 6B shows that upon TNF-α stimulation, p65 activity in nuclear extract increased 14.8-fold (p = 0.01) within 15 min and was maintained at the similar level until the end of the 60 min of incubation. In contrast, TGZ alone did not activate p65 nor did it impair p65 activation by TNF-α at all time points examined. The specificity of the ELISA was confirmed by measuring p65 activity in the absence or the presence of a saturating amount (20 pmol) of oligonucleotides containing either the wild-type or mutant NF-κB consensus sequences. As shown in Fig. 6C, the wild-type competitive oligonucleotides completely abolished p65 binding to the
Fig. 6. Troglitazone does not inhibit NF-κB activation and DNA binding activity in response to TNF-α. A, fully differentiated 3T3-L1 adipocytes were treated with TNF-α (1 nmol/liter), troglitazone (1 µmol/liter), or both together for 0, 15, 30, and 60 min. Total cell lysates were analyzed by SDS-PAGE and immunoblotted with anti-IκB-α and anti-phospho-IκB-α antibodies (Cell Signaling Technology, Beverly, MA). The blot was stained with Ponceau S to verify sample loading. B, 3T3-L1 adipocytes were treated with TNF-α and/or troglitazone as in A, and nuclear extracts were prepared and assayed for NF-κB activity as described under “Experimental Procedures.” The experiment was repeated using two independent batches of nuclear extracts and NF-κB consensus sequence-coated plates. C, the specificity of the ELISA was confirmed by competitive binding experiments. Briefly, nuclear extracts from TNF-α-treated 3T3-L1 adipocytes were incubated with the ELISA plate in the absence or the presence of 20 pmol of oligonucleotides containing either wild-type or mutant NF-κB consensus sequences, and the bound p65 was detected as in B. **, p < 0.01; *, p < 0.05 versus the matching controls.

ELISA plate, whereas the p65 binding was essentially unaffected by the same amount of competing oligonucleotides with mutant NF-κB consensus sequences.

We then examined the effect of TGZ-induced PPAR-γ activation on the transcription regulatory activity of p65 using reporter gene assays in HeLa cells. Fig. 7A shows that ectopic expression of p65 induced the expression of a luciferase reporter gene driven by multiple copies of NF-κB response elements (NF-κB-luc) in a dose-dependent manner, with a maximal induction of 10.5-fold (p < 0.005). p50 was ineffective (data not shown). To test whether TGZ-induced PPAR-γ activation would interfere with p65-mediated gene transcription, we co-transfected HeLa cells with NF-κB-luc, p65 (0.1 or 0.3 µg), together with PPAR-γ (0.3 µg) or the control vector (Fig. 7B).

Transfected HeLa cells were then incubated with TGZ or Me2SO for 48 h. p65 (0.1 or 0.3 µg) induced transcription of the NF-κB-luc reporter gene in HeLa cells. HeLa cells transfected with 0.3 µg of NF-κB-luc and 0.1 or 0.3 µg of p65 (solid bars), or empty vector (empty bars). Cells were also co-transfected with 0.3 µg PPAR-γ (+) or the empty expression vector (−) as indicated below the figure. The transfected cells were incubated with 1 µmol/liter troglitazone (+) or Me2SO (−) for 48 h. **, p < 0.01; *, p < 0.05 versus control cells transfected with p65 or expression vector alone.

Fig. 7. PPAR-γ inhibits NF-κB-mediated gene expression. A, p65 activates the NF-κB-luc reporter gene in HeLa cells. HeLa cells were transfected with 0.3 µg of NF-κB-luc and 0–0.3 µg of p65. Transfection efficiency was normalized by co-transfection of 100 ng of CMV-β-Gal, and total amount of DNA each well was adjusted using empty vector. ***, p < 0.001; **, p < 0.01 versus controls. B, inhibition of NF-κB-mediated trans-activation by PPAR-γ. HeLa cells were transfected with 0.3 µg of NF-κB-luc and 0.1 or 0.3 µg of p65 (solid bars), or empty vector (empty bars). Cells were also co-transfected with 0.3 µg PPAR-γ (+) or the empty expression vector (−) as indicated below the figure. The transfected cells were incubated with 1 µmol/liter troglitazone (+) or Me2SO (−) for 48 h. **, p < 0.01; *, p < 0.05 versus control cells transfected with p65 or expression vector alone.
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**Fig. 8. p65 blocks PPAR-γ-activated gene transcription.** A and B, HeLa cells were transfected with 0.3 µg of Pepck-luc (A) or 0.3 µg of PPRE-luc (B), and 0–0.3 µg of PPAR-γ. Cells were incubated with 1 µmol/liter troglitazone (solid bars) or Me2SO (empty bars) for 48 h. Transfection efficiency was normalized by co-transfection of 100 ng of CMV-β-Gal, and the total amount of DNA each well was adjusted using empty expression vector. C, HeLa cells were transfected with 0.3 µg of Pepck-luc and 0.3 µg of PPAR-γ or the empty vector. As indicated below the figure, cells were also transfected with 0.3 µg of one of the following: p50, p65, p65 (S276A), and c-Rel. Cells were incubated with 1 µmol/liter troglitazone (solid bars) or Me2SO (empty bars) for 48 h. D, transcriptional activity of PPAR-γ was inhibited by p65 in a dose-dependent manner. Cells were transfected with 0.3 µg of Pepck-luc and 0.3 µg of PPAR-γ and were also transfected with increasing amounts of p65 as indicated below the figure. Transfected cells were incubated with 1 µmol/liter troglitazone or Me2SO for 48 h. E, HeLa cells were transfected as in C, except that PPRE-luc (0.3 µg) was used as the reporter gene. Cells were then treated with 1 µmol/liter troglitazone or Me2SO for 48 h. ***, *p < 0.001; ***, *p < 0.01; *, *p < 0.05 versus controls.

(data not shown). Taken together, these data indicate that TGZ does not inhibit NF-κB activation, nuclear translocation, or gene expression but is consistent with a potential function for TGZ in antagonizing the transcriptional regulatory activity of NF-κB and/or other cofactors of NF-κB on adipocyte gene transcription.

**p65 Inhibits PPAR-γ-dependent Gene Transcription**—PPAR-γ2 is essential for the expression of adipocyte-specific genes. On the other hand, NF-κB activation is required for TNF-α-induced repression of key adipocyte genes. To confirm that NF-κB could directly inhibit PPAR-γ-dependent adipocyte-specific gene transcription, we assessed the effects of p65 on PPAR-γ-mediated gene expression using reporter gene assays in HeLa cells. As shown in Fig. 8A, transcription of a luciferase reporter gene driven by the 2100-bp PEPCCK promoter containing a functional PPAR-γ-response element (PEPCCK-luc) (50) was induced by TGZ-activated PPAR-γ in a dose-dependent manner, with a maximal induction of 11.5-fold (p < 0.02). PPAR-γ was ineffective in the absence of TGZ. Similar induction was observed when a reporter gene driven by multiple copies of PPAR-γ-response elements (PPRE-luc) was used (Fig. 8B). To determine the involvement of individual NF-κB family members on PPAR-γ-mediated transcription, we co-transfected HeLa cells with PEPCCK-luc, p50, p65, p65 (S276A) mutant, c-Rel, or the control vector. Fig. 8C shows that p65 significantly inhibited the basal activity of PEPCCK-luc, indicating that p65 represses the endogenous PPAR-γ activity and/or other endogenous PEPCCK promoter-inducing signals (left panel). In contrast, p50, another NF-κB family member, had little or no effect on basal transcription of PEPCCK-luc, whereas c-Rel caused a small but statistically significant inhibition of basal PEPCCK-luc transcription. Notably, the p65 (S276A) mutant, which contains a serine to alanine mutation at amino acid residue 276 of p65 protein, inhibited the basal PEPCCK-luc activity less effectively than its wild-type counterpart. Previous studies have established that phosphorylation of p65 on serine 276 by protein kinase A is essential for p65 interaction with the transcription co-activator CBP/p300 (51, 52); this interaction enhances the transcriptional activity and efficiency of p65. Importantly, CBP/p300 has also been implicated in positively regulating PPAR-γ and other nuclear receptor-mediated gene transcription (53). Our data thus indicate that the association of p65 with CBP/p300 is likely to be involved in the inhibitory effects of p65 on basal PEPCCK-luc transcription.

Next, we examined the effects of the above NF-κB family members on PPAR-γ-mediated gene transcription. Transcription of the PEPCCK promoter was induced 3.3-fold (p = 0.004) by PPAR-γ in the presence of TGZ, whereas PPAR-γ was ineffec-
tive in the absence of TGZ (Fig. 8C, right panel). p65 abolished induction of the PEPCK promoter by activated PPAR-γ, whereas c-Rel and p50 only partially blocked induction by activated PPAR-γ (Fig. 8C, right panel). The S276A mutant of p65 inhibited induction of the PEPCK promoter by activated PPAR-γ less effectively than did wild-type p65 but did retain some inhibitory activity. The repression by p65 was specific to the PEPCK promoter, as reporter genes driven by a TATA-like promoter or the cytomegalovirus promoter were not significantly affected by p65 (data not shown), and p65 inhibited PPAR-γ-mediated transcription in a dose-dependent manner (Fig. 8D).

To determine whether p65 inhibits PEPCK-luc transcription by directly binding to the NF-κB-response element(s) in the 2100-bp PEPCK promoter region, we constructed a PPAR-γ-responsive luciferase reporter gene driven only by multiple copies of PPAR-γ-response elements (PPRE-luc). As shown in Fig. 8E (left panel), p65 abolished basal PPRE-luc transcription, whereas c-Rel and the S276A mutant of p65 partially blocked the basal PPRE-luc transcription. p50 was ineffective. PPAR-γ up-regulated the PPRE-luc promoter 2.1-fold (p = 0.001) in the absence of TGZ, and PPRE-luc was induced 3.4-fold (p = 0.0001) by PPAR-γ when TGZ is present (Fig. 8E, right panel). When co-transfected with PPAR-γ, p65 prevented induction of the PPRE-luc promoter by PPAR-γ both in the absence and presence of TGZ (Fig. 8E, right panel). In contrast, the S276A mutant of p65 blocked the induction of PPRE-luc by activated PPAR-γ but had little effect on PPAR-γ-mediated PPRE-luc transcription in the absence of TGZ. p50 and c-Rel inhibited PPAR-γ-mediated transcription less effectively than p65. Thus, a functional NF-κB-binding site was not required for the repressive effects of NF-κB on PPAR-γ-dependent gene transcription.

DISCUSSION

We report three major findings, which collectively describe the molecular mechanisms by which TGZ antagonizes the effects of TNF-α on adipocytes. First, we demonstrated that TGZ prevents TNF-α-induced and NF-κB-dependent down-regulation of key adipocyte genes such as those implicated in the suppression of free fatty acid release and cell cycle arrest in adipocytes. Second, we showed that TGZ selectively blocks the up-regulation of a distinct subset of genes by TNF-α. Third, we showed that TGZ does not impair TNF-α-induced NF-κB activation, nuclear translocation, or DNA binding activity per se; rather, TGZ antagonizes the transcriptional regulatory activity of NF-κB. Conversely, we found that p65 potently inhibits the transcriptional activity of PPAR-γ, whereas other NF-κB family members including p50 and c-Rel are less effective.

TGZ improves insulin sensitivity in patients with type 2 diabetes and antagonizes the actions of TNF-α both in cell culture (19) and in whole animals (54). By using 3T3-L1 adipocytes expressing the non-degradable NF-κB inhibitor IκBαDN, we previously showed that NF-κB activation is obligatory for TNF-α-induced repression of most of adipocyte-specific genes, whereas only 60–70% of the genes induced by TNF-α are NF-κB-dependent (5). Those investigations demonstrated that NF-κB-mediated transcriptional inhibition is a mechanism by which TNF-α induces insulin resistance in adipocytes. The seemingly opposing effects of TGZ and TNF-α on adipocyte biology prompted us to look at their potential interactions on the expression of adipocyte-abundant genes.

Here we identified 64 known adipocyte-abundant genes that are normally repressed by TNF-α in an NF-κB-dependent manner but are protected, at least partially, by TGZ. These genes form clusters with distinct biological functions (Table I), suggesting that TGZ improves insulin sensitivity in part through antagonizing the actions of TNF-α on adipocyte gene expression. For example, Fig. 2 shows a selected group of genes encoding proteins implicated in triglyceride synthesis and/or suppression of FFA release from adipocytes. Although in many cases TGZ only partially prevented the down-regulation of the mRNA levels of these genes by TNF-α, their encoded proteins reside in a common pathway of FFA metabolism and lipogenesis. Thus, within adipocytes, the aggregate effect of even a moderate increase in each protein has a significant impact that increases FFA incorporation into triglyceride and thereby reduces FFA release.

Another group of genes regulated simultaneously by TGZ and TNF-α encodes proteins involved in cell growth and proliferation. Terminally differentiated adipocytes permanently withdraw from cell cycle progression. TNF-α-mediated reprogramming of adipocyte gene expression includes induction of genes involved in cell cycle reentry and progression and NF-κB-dependent repression of genes implicated in cell cycle arrest. TGZ is sufficient to prevent this loss of cell cycle constraints through antagonizing the transcriptional repressive activity of NF-κB on cell cycle inhibitors and thus contributes to the maintenance of the adipocyte phenotype.

Although TNF-α-induced repression of adipocyte-specific genes are mediated mainly through NF-κB (22), the induction of gene expression by TNF-α in adipocytes is mediated by multiple mediators through various pathways that may or may not be equally affected by TGZ. For example, TGZ partially blocked the induction of acute phase proteins and atherosclerotic risk factors that are normally induced by TNF-α (Fig. 4), whereas it had no effect on the induction of IκB-α (Fig. 5D), p65 (Fig. 5E), NF-κB1 (p105/p50, Fig. 5F), and several adipocyte secreted proteins including PAI-1 by TNF-α. These data reinforce the notion that TGZ is not a general inhibitor of TNF-α; rather, TGZ may specifically target and inhibit NF-κB that appears to play a major role in TNF-α-induced repression of key adipocyte genes and modulates the induction of a subset of genes by TNF-α.

By using reporter gene assays in HeLa cells, we found that p65 significantly inhibited the transcriptional activity of PPAR-γ and that the repressive effect of p65 is independent of the presence of any NF-κB-binding sites in the promoter region of PPAR-γ-responsive genes. Thus, p65 may potentially inhibit PPAR-γ activity by directly binding to PPAR-γ and inhibiting its DNA binding and/or transcriptional activity, or by sequestering key transcriptional co-activators such as CREB-binding protein (CBP/p300) or steroid receptor coactivator-1 (SRC-1). PPAR-γ requires interaction with transcription co-activators for full transcriptional activity (55), and CBP/p300 and SRC-1 have been implicated in positively regulating PPAR-γ-dependent gene transcription (53, 56). Importantly, both CBP/p300 and SRC-1 also interact with NF-κB and stimulate its transcriptional activity (51, 52, 57). Thus, a competition for limiting amounts of co-activators could result in selective inhibition of CBP/p300- and/or SRC-1-dependent gene transcription and account for the transcriptional repressive effect of NF-κB on key adipocyte genes.

As an initial step to explore the role of co-factor squelching on the inhibitory effect of p65 on adipocyte gene expression, we compared the effect of wild-type and the S276A mutant of p65, which has lost the ability to interact with co-activator CBP/p300. Our reporter gene assays in HeLa cells showed that the S276A mutant p65 blocked the PPAR-γ-mediated transcription less effectively than its wild-type counterpart, but it did retain some inhibitory activity. This indicates that co-factor squelching contributes to some of the repressive effect of p65 but other unidentified mechanisms probably also play a role. To ascer-
tain whether exogenous expression of CBP/p300 or SRC-1 can rescue the inhibitory effect of p65 on PPAR-γ-stimulated gene expression, we co-transfected CBP/p300 together with p65, PPAR-γ, and a PPAR-γ-responsive reporter gene. However, we were unable to see a significant increase in PPAR-γ-mediated gene transcription in the presence of CBP/p300. This may simply relate to the technical limitations in our assay system or the suboptimal ratios between PPAR-γ, p65, and CBP/p300. Nevertheless, our reporter gene assays using the S267A mutant p65 provided evidence, at least indirectly, that the interaction with co-factors might be involved in p65-mediated repression of PPAR-γ activity. Supporting this notion, p65 has already been shown to interact with CBP/p300 to inhibit glucocorticoid and cAMP-mediated induction of PEPCk gene expression (39).

On the other hand, PPAR-γ activation blocked p65-mediated gene transcription, and the inhibition is also independent of the presence of any PPAR-γ-response elements in the promoter region of NF-κB-activated genes. Thus, p65 and PPAR-γ antagonize the transcriptional activity of each other. The *in vivo* significance of the functional antagonism between p65 and PPAR-γ is that these two major transcription factors may lie in a common pathway that integrates multiple signals regulating adipocyte gene expression and function. The balance between the activities of p65, PPAR-γ, and possibly other DNA-binding proteins therefore is likely important for adipocyte function and response to insulin.

In this study, we identified the common target genes of PPAR-γ and NF-κB and thus provided a basis for further investigation of the exact mechanisms by which PPAR-γ and NF-κB antagonize each other. It will be intriguing to test whether endogenous NF-κB activity is truly induced in obesity and type 2 diabetes, and whether endogenous NF-κB is indeed a fundamental target for the treatment of type 2 diabetes and its complications. It will also be critical to identify the NF-κB-independent mediator(s) that are induced by TNF-α and thus contribute to the reprogramming of adipocyte gene expression and loss of insulin responsiveness.

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Troglitazone Antagonizes Tumor Necrosis Factor-α-induced Reprogramming of Adipocyte Gene Expression by Inhibiting the Transcriptional Regulatory Functions of NF-κB

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