

Activation of Peroxisome Proliferator–Activated Receptor β/δ Inhibits Lipopolysaccharide-Induced Cytokine Production in Adipocytes by Lowering Nuclear Factor- κ B Activity via Extracellular Signal–Related Kinase 1/2

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OBJECTIVE—Chronic activation of the nuclear factor- κ B (NF- κ B) in white adipose tissue leads to increased production of pro-inflammatory cytokines, which are involved in the development of insulin resistance. It is presently unknown whether peroxisome proliferator–activated receptor (PPAR) β/δ activation prevents inflammation in adipocytes.

RESEARCH DESIGN AND METHODS AND RESULTS—First, we examined whether the PPAR β/δ agonist GW501516 prevents lipopolysaccharide (LPS)-induced cytokine production in differentiated 3T3-L1 adipocytes. Treatment with GW501516 blocked LPS-induced IL-6 expression and secretion by adipocytes and the subsequent activation of the signal transducer and activator of transcription 3 (STAT3)–Suppressor of cytokine signaling 3 (SOCS3) pathway. This effect was associated with the capacity of GW501516 to impede LPS-induced NF- κ B activation. Second, in in vivo studies, white adipose tissue from Zucker diabetic fatty (ZDF) rats, compared with that of lean rats, showed reduced PPAR β/δ expression and PPAR DNA-binding activity, which was accompanied by enhanced IL-6 expression and NF- κ B DNA-binding activity. Furthermore, IL-6 expression and NF- κ B DNA-binding activity was higher in white adipose tissue from PPAR β/δ -null mice than in wild-type mice. Because mitogen-activated protein kinase–extracellular signal–related kinase (ERK)1/2 (MEK1/2) is involved in LPS-induced NF- κ B activation in adipocytes, we explored whether PPAR β/δ prevented NF- κ B activation by inhibiting this pathway. Interestingly, GW501516 prevented ERK1/2 phosphorylation by LPS. Furthermore, white adipose tissue from animal showing constitutively increased NF- κ B activity, such as ZDF rats and PPAR β/δ -null mice, also showed enhanced phospho-ERK1/2 levels.

CONCLUSIONS—These findings indicate that activation of PPAR β/δ inhibits enhanced cytokine production in adipocytes by preventing NF- κ B activation via ERK1/2, an effect that may help prevent insulin resistance. *Diabetes* 57:2149–2157, 2008

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Accumulating evidence implicates a low-grade chronic systemic inflammatory response to nutrient excess as a key mechanism that links obesity to metabolic disorders, including insulin resistance and cardiovascular disease (1). Thus, models of diet-induced and genetic obesity show increased adipose tissue expression and content of pro-inflammatory cytokines (such as tumor necrosis factor- α [TNF- α], interleukin [IL] 1, monocyte chemo-attractant protein-1 [MCP-1], and IL-6) (2–4). Of these cytokines, IL-6 correlates most strongly with insulin resistance and type 2 diabetes (5–7); its plasma levels are increased two- to threefold in patients with obesity and type 2 diabetes compared with lean control subjects (6). At the cellular level, insulin resistance and enhanced expression of these cytokines by adipose tissue during obesity and also under a high-fat diet have been linked to activation of the pro-inflammatory transcription factor nuclear factor- κ B (NF- κ B) (4). This nuclear factor is activated by surface proteins that recognize foreign substances, the so-called pattern recognition receptors, such as toll-like receptor-4 (TLR4). This receptor is expressed on virtually all human cells and binds a wide spectrum of exogenous and endogenous ligands, including bacterial lipopolysaccharide (LPS) (8). In the presence of LPS, the TLR4 complex (including CD-14 and an accessory protein, MD-2), recruits the adaptor protein, myeloid differentiation factor-88, which in turn recruits IL-1 receptor–associated kinase, leading to NF- κ B activation and enhanced expression of several inflammatory mediators (including IL-6 and MCP-1). Of note, NF- κ B activation by LPS requires mitogen-activated protein kinase (MAPK)–extracellular signal–related kinase (ERK)1/2 (MEK1/2) activation, because inhibition of this pathway reduces LPS-induced cytokine production in adipocytes (9).

Recent evidence suggests that inflammatory processes induced by obesity and high-fat diet cause systemic insulin resistance via a mechanism involving TLR4 (10). For instance, saturated free fatty acids (FFAs) activate TLR4-mediated inflammatory signaling in adipocytes and macrophages, and this effect is blunted in the absence of this receptor (10). These observations indicate that enhanced adipose tissue lipolysis observed in insulin-resistant states may release the endogenous ligand for TLR4 to induce inflammation (11). In addition, it has been demonstrated that high-fat diets augment plasma LPS to a concentration sufficient to increase body weight, fasting glycemia and

inflammation (12). Furthermore, LPS receptor–deleted mice (CD14 mutants) are hypersensitive to insulin, and the development of insulin resistance, obesity, and diabetes in this animal model is delayed in response to a high-fat diet (12).

In recent years, peroxisome proliferator–activated receptor β/δ (PPAR β/δ) activation has been proposed as a potential treatment for insulin resistance (13). PPARs are members of the nuclear receptor superfamily of ligand-inducible transcription factors. They form heterodimers with retinoid X receptors (RXRs) and bind to consensus DNA sites composed of direct repeats of hexameric DNA sequences separated by 1 bp (DR1) (14). Ligand binding induces a conformational change in PPAR-RXR complexes, thereby releasing corepressors in exchange for coactivators, which leads to the recruitment of the basal transcription machinery and enhanced gene expression. In addition, PPARs may suppress inflammation through diverse mechanisms, such as reduced release of inflammatory factors or stabilization of repressive complexes at inflammatory gene promoters (15–18). Of the three PPAR isotypes found in mammals, PPAR α (NR1C1) (19) and PPAR γ (NR1C3) are the targets for hypolipidemic (fibrates) and antidiabetic (thiazolidinediones) drugs, respectively. Finally, activation of the third isotype, PPAR β/δ (NR1C2) by high-affinity ligands (including GW501516) enhances fatty acid catabolism in adipose tissue and skeletal muscle, thereby delaying weight gain (rev. in 13). However, there is no information available on whether PPAR β/δ ligands prevent inflammation in adipocytes. Here, we examined whether PPAR β/δ activation by GW501516 prevents LPS-induced inflammation in adipocytes. We found that this drug prevented LPS-induced IL-6 expression and secretion by adipocytes. This effect was associated with the capacity of the PPAR β/δ ligand to prevent LPS-induced NF- κ B activation. Consistent with the role of PPAR β/δ in blocking NF- κ B–induced IL-6 expression, a genetic model of obesity and insulin resistance, the ZDF rat, showed reduced PPAR β/δ expression and DNA-binding activity and enhanced IL-6 expression and NF- κ B DNA-binding activity in white adipose tissue. Likewise, IL-6 expression and NF- κ B DNA-binding activity was higher in this tissue in PPAR β/δ -null mice than in wild-type mice. Because MEK1/2 is involved in NF- κ B activation in adipocytes (9), we explored whether PPAR β/δ blocked NF- κ B activation by inhibiting this pathway. In agreement with this possibility, GW501516 prevented ERK1/2 phosphorylation by LPS. In contrast, animal models showing increased NF- κ B activity in white adipose tissue, the ZDF rat and the PPAR β/δ -null mice, showed enhanced phospho-ERK1/2 levels. Overall, on the basis of our findings, we propose that PPAR β/δ activation be considered a molecular target to prevent inflammation of adipose tissue and the metabolic alterations associated with this process, such as insulin resistance.

RESEARCH DESIGN AND METHODS

The PPAR β/δ ligand GW501516 was from Biomol Research Labs (Plymouth Meeting, PA). Other chemicals were from Sigma (St. Louis, MO).

Cell culture. 3T3-L1 preadipocytes (American Type Culture Collection) were grown to confluence in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum. Differentiation of the 3T3-L1 cells was induced 2 days after confluence (day 0) in DMEM containing 10% fetal bovine serum, 500 μ M methylisobutylxanthine, 0.25 μ M dexamethasone, and 10 μ g/ml insulin for 48 h. The cells were then incubated in 10% FBS/DMEM with insulin for 8 days. Medium was changed every 2 days. Fat droplets were observed in >90% of cells after day 10. Adipocytes were then incubated for

96 h with 0.5 μ M GW501516 and then with 100 ng/ml LPS for either 1 or 24 h. After the incubation, RNA, total proteins, and nuclear extracts were extracted from adipocytes as described below. Inhibitors were added 30 min before the incubation with LPS. Culture supernatants were collected, and the secretion of IL-6 was assessed by ELISA (Amersham Biosciences, Little Chalfont, U.K.).

Animals. Male obese ZDF rats (ZDF/Gmi, *fa/fa*) and lean litter mates (*fa/+* or *+/+*) were used. Both strains were maintained under standard light-dark cycle (12-h light/dark cycle) and temperature (21 \pm 1°C) and fed with the Purina 5008 chow. Epididymal white adipose tissue was rapidly removed, frozen in liquid nitrogen, and stored at –80°C. Blood samples were collected in EDTA tubes, and plasma was obtained by centrifugation at 2,200 \times *g* for 10 min at 4°C. Plasma glucose (Sigma), triglycerides (Sigma), and nonesterified fatty acids (Wako, Germany) levels were determined with a colorimetric test. Insulin (Amersham Biosciences) was determined by radioimmunoassay. All procedures were conducted in accordance with the principles and guidelines established by the University of Barcelona Bioethics Committee, as stated in Law 5/1995, 21st July, passed by the Generalitat de Catalunya. The generation of PPAR β/δ -null mice has been described previously (20).

Measurements of mRNA. Levels of mRNA were assessed by the RT-PCR as previously described (21). Total RNA was isolated using the Ultraspec reagent (Biotecx, Houston, TX). The total RNA isolated by this method is nondegraded and free of protein and DNA contamination. The sequences of the sense and antisense primers used for amplification were: *IL-6*, 5'-TCCAGCCAGTTGCCTTCTTGG-3' and 5'-TCTGACAGTGCATCATCGCTG-3'; *Mcp-1*, 5'-GGGCTGTTGTTACAGTTGC-3' and 5'-GGGACACCTGCTGCTGGTAT-3'; *Pdk-4*, 5'-AGGTCGAGCTGTTCTCCCGT-3' and 5'-GCGGTCAGGCAGGATGTCAAT-3'; *Cpt-1*, 5'-TTCAGTGTACCCAGACGGG-3' and 5'-AATGGACAGCCCCATGGAGA-3'; *Ppar β/δ* , 5'-GAGGAAGTGGCCACGGGTGAC-3' and 5'-CCACTGAGGCCCATCACAG-3'; *Socs-3*, 5'-TTTTCGCTGCAGAGTGACCC-3' and 5'-TGGAGGAGAGAGGTGCGCTCA-3'; and adenosyl phosphoribosyl transferase (*Aprt*), 5'-GCCTCTGGCCAGTCACTGA-3' and 5'-CCAGGCTCACACTCCACCA-3'. Amplification of each gene yielded a single band of the expected size (*IL-6*, 229 bp; *Mcp-1*, 157 bp; *Pdk-4*, 167 bp; *Cpt-1*, 222 bp; *Ppar β/δ* , 151 bp; *Socs-3*, 250 bp; and *Aprt*, 329 bp). Preliminary experiments were carried out with various amounts of cDNA to determine nonsaturating conditions of PCR amplification for all the genes studied. Therefore, under these conditions, relative quantification of mRNA was assessed by the RT-PCR method used in this study (22). Radioactive bands were quantified by video-densitometric scanning (Vilbert Lourmat imaging). The results for the expression of specific mRNAs are always presented relative to the expression of the control gene (*Aprt*).

Isolation of nuclear extracts. Nuclear extracts were isolated as previously described (21). Cells were scraped into 1.5 ml cold PBS, pelleted for 10 s, and resuspended in 400 μ l cold buffer A (10 mmol/l HEPES, pH 7.9, at 4°C, 1.5 mmol/l MgCl₂, 10 mmol/l KCl, 0.5 mmol/l dithiothreitol [DTT], 0.2 mmol/l phenylmethylsulfonyl fluoride [PMSF], and 5 μ g/ml aprotinin) by flicking the tube. Cells were allowed to swell on ice for 10 min and then vortexed for 10 s. Samples were then centrifuged for 10 s, and the supernatant fraction was discarded. Pellets were resuspended in 50 μ l cold buffer C (20 mmol/l HEPES-KOH, pH 7.9, at 4°C, 25% glycerol, 420 mmol/l NaCl, 1.5 mmol/l MgCl₂, 0.2 mmol/l EDTA, 0.5 mmol/l DTT, 0.2 mmol/l PMSF, 5 μ g/ml aprotinin, and 2 μ g/ml leupeptin) and incubated on ice for 20 min for high-salt extraction. Cellular debris was removed by centrifugation for 2 min at 4°C and the supernatant fraction (containing DNA-binding proteins) was stored at –80°C. Nuclear extract concentration was determined by the Bradford method.

Electrophoretic mobility shift assay. Electrophoretic mobility shift assay (EMSA) was performed using double-stranded oligonucleotides (Promega, Madison, WI) for the consensus binding site of the NF- κ B nucleotide (5'-AGTTGAGGGGACTTCCAGGC-3') and PPAR (5'-CAAACTAGGTCAAAGGTCA-3'). Oligonucleotides were labeled in the following reaction: 2 μ l oligonucleotide (1.75 pmol/ μ l), 2 μ l 5 \times kinase buffer, 1 μ l T4 polynucleotide kinase (10 units/ μ l), and 2.5 μ l [γ -³²P]ATP (3,000 Ci/mmol at 10 Ci/ml) incubated at 37°C for 1 h. The reaction was stopped by adding 90 μ l Tris-EDTA buffer (10 mmol/l Tris-HCl, pH 7.4, and 1 mmol/l EDTA). To separate the labeled probe from the unbound ATP, the reaction mixture was eluted in a Nick column (Amersham) following the manufacturer's instructions. Eight micrograms of crude nuclear protein was incubated for 10 min on ice in binding buffer (10 mmol/l Tris-HCl, pH 8.0, 25 mmol/l KCl, 0.5 mmol/l DTT, 0.1 mmol/l EDTA, pH 8.0, 5% glycerol, 5 mg/ml BSA, and 50 μ g/ml poly(dI-dC)), in a final volume of 15 μ l. Labeled probe (~60,000 cpm) was added, and the reaction was incubated for 15 min at 4°C (NF- κ B) or room temperature (peroxisome proliferator response element). Where indicated, specific competitor oligonucleotide was added before the labeled probe and incubated for 10 min on ice. p65 antibody was added 15 min before incubation with the labeled probe at 4°C. Protein-DNA complexes were

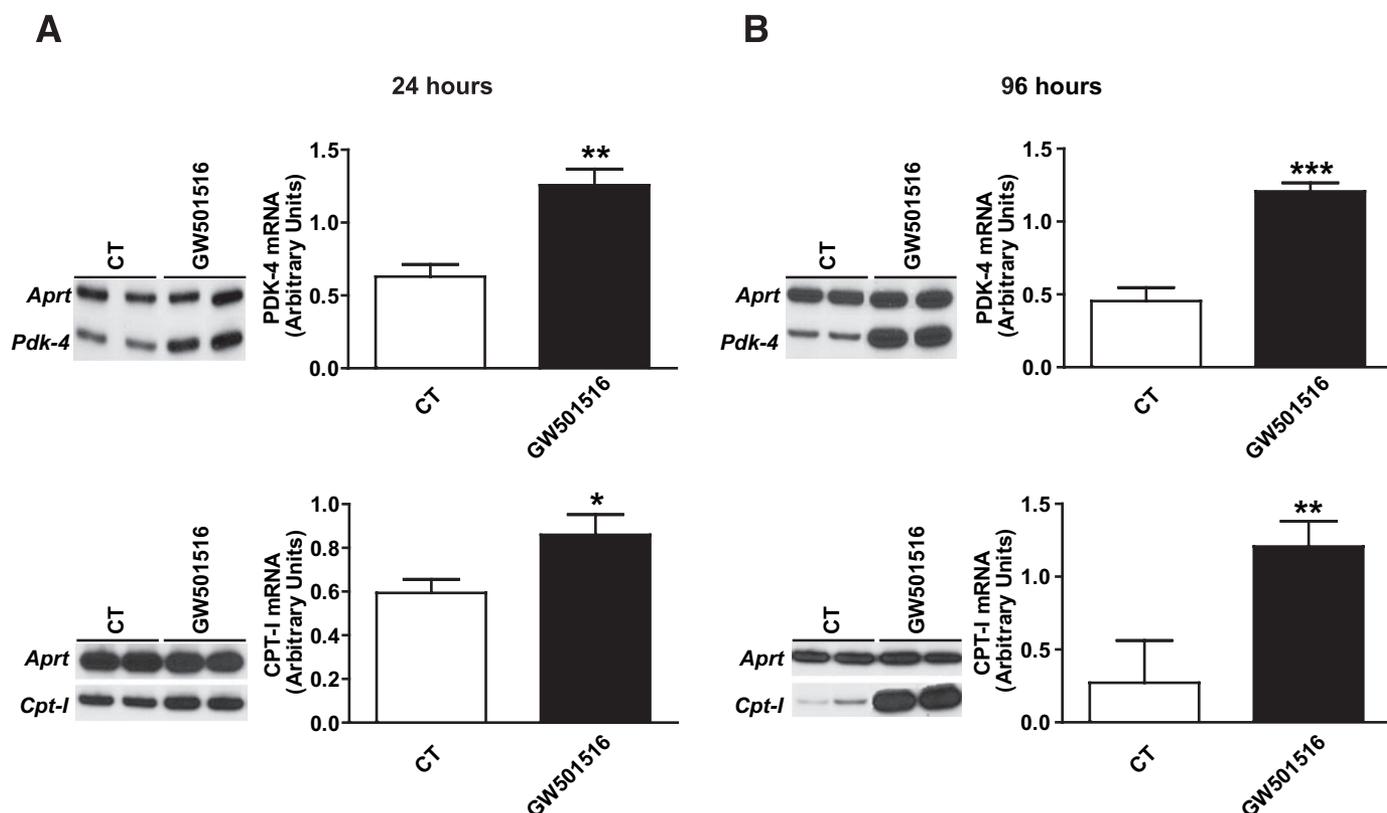


FIG. 1. The PPAR β/δ agonist GW501516 induces *Pdk-4* and *Cpt-1* expression in 3T3-L1 adipocytes. Differentiated adipocytes were incubated in the presence or in the absence of 0.5 $\mu\text{mol/l}$ GW501516 for either 24 h (A) or 96 h (B). Total RNA was isolated and analyzed by RT-PCR. A representative autoradiogram and the quantification normalized to the *Aprt* mRNA levels are shown. Data are means \pm SD of five independent experiments. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ vs. control.

resolved by electrophoresis at 4°C on a 5% acrylamide gel and subjected to autoradiography.

Immunoblotting. To obtain total protein, cells and adipose tissue were homogenized in cold lysis buffer (5 mmol/l Tris-HCl, pH 7.4, 1 mmol/l EDTA, 0.1 mmol/l PMSF, 1 mmol/l sodium orthovanadate, and 5.4 $\mu\text{g/ml}$ aprotinin). The homogenate was centrifuged at $16,700 \times g$ for 30 min at 4°C. Protein concentration was measured by the Bradford method. Proteins (30 μg) were resolved by SDS-PAGE on 10% separation gels and transferred to Immobilon polyvinylidene difluoride membranes (Millipore, Bedford, MA). Western blot analysis was performed using antibodies against total and phospho-ERK1/2 (Cell Signaling) and total (Santa Cruz) and phospho-STAT3 (Tyr⁷⁰⁵) and Ser⁷²⁷) (Cell Signaling). Detection was achieved using the EZ-ECL chemiluminescence kit (Amersham). Size of detected proteins was estimated using protein molecular-mass standards (Invitrogen, Barcelona, Spain).

Statistical analyses. Results are expressed as means \pm SD of five separate experiments. Significant differences were established by Student's *t* test or one-way ANOVA, according to the number of groups compared, using the GraphPad InStat program, v2.03 (GraphPad Software, San Diego, CA). In the latter case, when significant variations were found, the Tukey-Kramer multiple comparisons test was applied. Differences were considered significant at $P < 0.05$.

RESULTS

PPAR β/δ activation prevents LPS-induced IL-6 expression and secretion in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were exposed for 24 h to 0.5 $\mu\text{mol/l}$ GW501516, a selective ligand for PPAR β/δ with an apparent K_d value of 1 nmol/l and 1,000-fold higher affinity toward PPAR β/δ than PPAR α and PPAR γ (23). Under these conditions, we evaluated the expression of two well-known PPAR β/δ -target genes, pyruvate dehydrogenase kinase 4 (*Pdk4*) and carnitine palmitoyltransferase 1 (*Cpt-1*) (24). Ligand treatment for 24 h caused a slight increase in the mRNA levels of both *Pdk4* (100%, $P < 0.01$) and *Cpt-1* (45%, $P < 0.05$) (Fig. 1A). When adipocytes were

treated for 96 h with 0.5 $\mu\text{mol/l}$ GW501516, a greater increase was observed in the transcript levels of *Pdk4* (170%, $P < 0.001$) and *Cpt-1* (300%, $P < 0.01$) (Fig. 1B). Therefore, we selected this longer exposure to the PPAR β/δ ligand in further experiments. No changes were observed either in the expression of well-known PPAR γ -target genes (i.e., *Ob*: data not shown), confirming that the drug treatment was selective for PPAR β/δ , or in the expression of markers of adipocyte differentiation (i.e., *Pref-1*; data not shown), indicating that this latter process was unaffected. After a 96 h-incubation with GW501516, cells were exposed to 100 ng/ml LPS for 24 h to induce inflammation. LPS activates NF- κ B through TLRs in adipocytes (9,25) and induces insulin resistance (12,26). When we evaluated the mRNA levels of *Mcp-1* and IL-6, two genes that are under the control of the pro-inflammatory transcription factor NF- κ B, we observed that LPS treatment strongly increased the former (22-fold induction, $P < 0.001$), whereas cells co-incubated with GW501516 showed a significant reduction (51% reduction, $P < 0.01$ vs. LPS-exposed cells) (Fig. 2A). Furthermore, when LPS-exposed cells were co-incubated with 10 $\mu\text{mol/l}$ parthenolide, which specifically inhibits the activation of NF- κ B (27), the effect of LPS was suppressed. Treatment with LPS also led to enhanced IL-6 mRNA levels (4.2-fold induction, $P < 0.05$), but this increase was blocked in the presence of GW501516 and parthenolide (Fig. 2B). Likewise, incubation with LPS for 24 h caused a 4.2-fold increase in the levels of IL-6 protein secreted into the culture media (control 10.5 ± 3.7 vs. LPS 55 ± 11 ng/ml; Fig. 2C), whereas pretreatment with GW501516 (56% re-

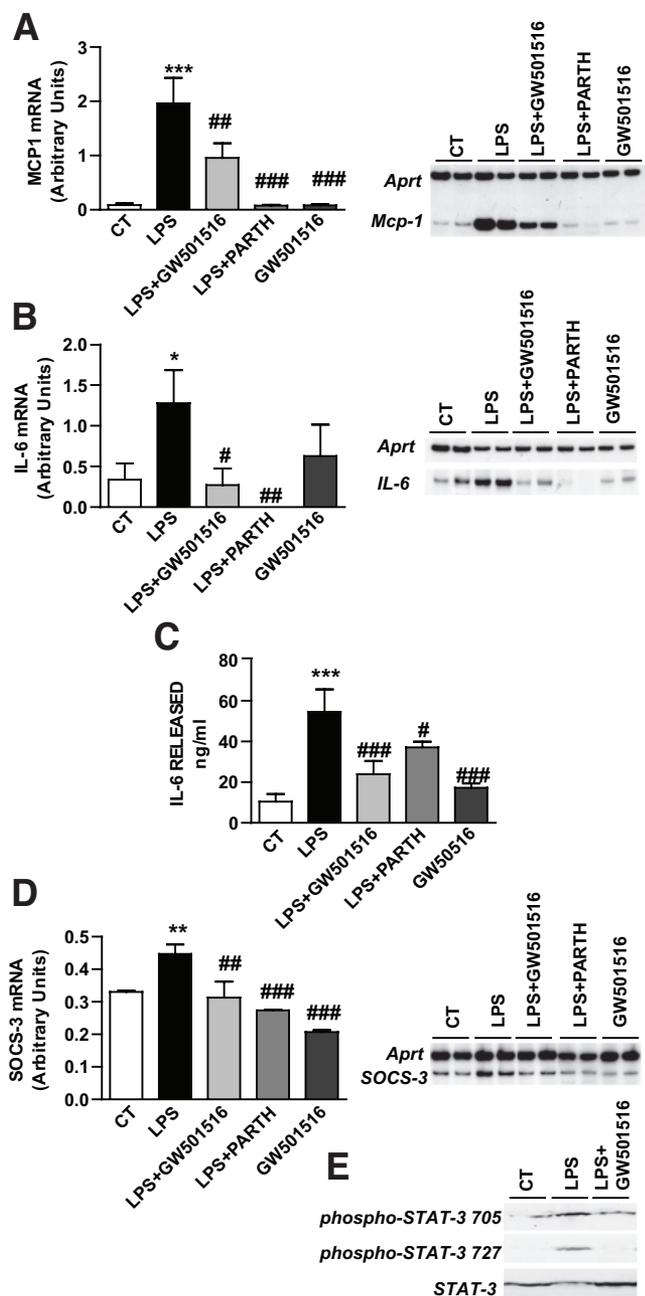


FIG. 2. The PPAR β/δ agonist GW501516 prevents LPS-induced expression and secretion of pro-inflammatory cytokines. When indicated, differentiated adipocytes were incubated with 0.5 $\mu\text{mol/l}$ GW501516 for 96 h and subsequently exposed to 100 ng/ml LPS for 24 h in the presence or in the absence of GW501516 or 10 $\mu\text{mol/l}$ NF- κB inhibitor parthenolide (PARTH). Effects of GW501516 on the expression of *Mcp-1* (A) and *IL-6* (B). Total RNA was isolated and analyzed by RT-PCR. A representative autoradiogram and the quantification normalized to the *Aprt* mRNA levels are shown. C: Effect of GW501516 on the secretion of IL-6 to the culture media, as determined by ELISA. D: Effect of GW501516 on the expression of SOCS-3. E: Analysis of STAT3 and phospho-STAT3 by immunoblotting of nuclear protein extracts from 3T3-L1 adipocytes treated with 100 ng/ml LPS for 3 h in the presence or in the absence of 0.5 $\mu\text{mol/l}$ GW501516. Data are means \pm SD of five independent experiments. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ vs. control. # $P < 0.05$, ## $P < 0.01$, and ### $P < 0.001$ vs. LPS-exposed cells.

duction, $P < 0.001$) and parthenolide (32% reduction, $P < 0.05$) significantly reduced the secretion of this cytokine. Several cytokines, including IL-6, stimulate *Socs-3* expression in adipocytes (28), a protein that inhibits insulin signaling in these cells (29). The elevated levels of mRNA

encoding *Socs-3* and the increase in Stat-3 phosphorylation (Fig. 2D and E) suggested that the increase in IL-6 release caused by LPS treatment might have local actions (30). Interestingly, GW501516 treatment prevented the increase in *Socs-3* expression and Stat-3 phosphorylation, a fact that is consistent with the reduction in IL-6 levels after drug treatment.

PPAR β/δ activation prevents LPS-mediated NF- κB activation in 3T3-L1 adipocytes. Activation of NF- κB plays a crucial role in IL-6 production in adipocytes, as demonstrated by the effects of parthenolide. To test whether GW501516 prevented LPS-induced IL-6 expression by reducing NF- κB activity, we performed EMSA. NF- κB formed three complexes with nuclear proteins (complexes I-III) (Fig. 3A). The specificity of the three DNA-binding complexes was assessed in competition experiments by adding an excess of unlabeled NF- κB oligonucleotide. NF- κB binding activity, mainly of specific complex II, increased in nuclear extracts from LPS-treated cells (Fig. 3B). In contrast, in the presence of LPS and the PPAR β/δ ligand, the binding activity of NF- κB was reduced (Fig. 3A). Addition of antibody against the p65 subunit of NF- κB completely supershifted complex II, thereby indicating that this band comprised mainly this subunit (Fig. 3C).

Reduced PPAR β/δ activity and enhanced IL-6 expression in white adipose tissue of an animal model of obesity and insulin resistance. We hypothesized that a reduction in PPAR β/δ activity and the consequent increase in NF- κB activity is responsible, at least in part, for the enhanced expression of IL-6 in white adipose tissue of animal models of obesity and insulin resistance. To check this hypothesis, we evaluated the expression of PPAR β/δ and its activity in white adipose tissue of ZDF (*fa/fa*) rats. Male ZDF and lean rats were killed at 12 weeks of age. By this age, ZDF rats presented established diabetes compared with lean animals, as demonstrated by increased plasma levels of glucose (505 ± 67 vs. 167 ± 37 mg/dl, $P < 0.0001$), triglycerides (649 ± 298 vs. 92 ± 31 mg/dl, $P < 0.01$), nonesterified fatty acids (0.62 ± 0.17 vs. 0.26 ± 0.09 mEq/l, $P < 0.01$), and insulin (19.3 ± 7.9 vs. 10.4 ± 2.0 ng/ml, $P < 0.05$). White adipose tissue of ZDF rats showed a significant reduction in *Ppar β/δ* transcripts (50% reduction, $P < 0.05$) (Fig. 4A). Similarly, the mRNA levels of its target gene, *Pdk4*, were also reduced (41%, $P < 0.05$) (Fig. 4B), suggesting that the activity of this PPAR isotype was reduced. In contrast, the expression of IL-6 was significantly enhanced (60%, $P < 0.05$) (Fig. 4C). EMSA showed that the DNA-binding activity of PPAR was reduced in white adipose tissue from ZDF rats compared with lean animals (Fig. 4D). In contrast, NF- κB DNA-binding activity was increased in ZDF rats compared with lean animals (Fig. 4E). These data suggest that the low-grade chronic systemic inflammatory associated with the development of obesity and insulin resistance involves a decreased PPAR β/δ activity and the subsequent activation of NF- κB .

To clearly demonstrate the involvement of PPAR β/δ in the control of NF- κB -mediated *IL-6* expression in white adipose tissue, we used the PPAR β/δ -null mice. The absence of *Ppar β/δ* in white adipose tissue (Fig. 5A) was associated with a reduction in the mRNA levels of *Pdk4* (65%, $P < 0.001$) (Fig. 5B) compared with wild-type mice, whereas IL-6 expression was enhanced (fourfold induction, $P < 0.05$) (Fig. 5C). In agreement with the enhanced expression of IL-6 in white adipose tissue of PPAR β/δ -null

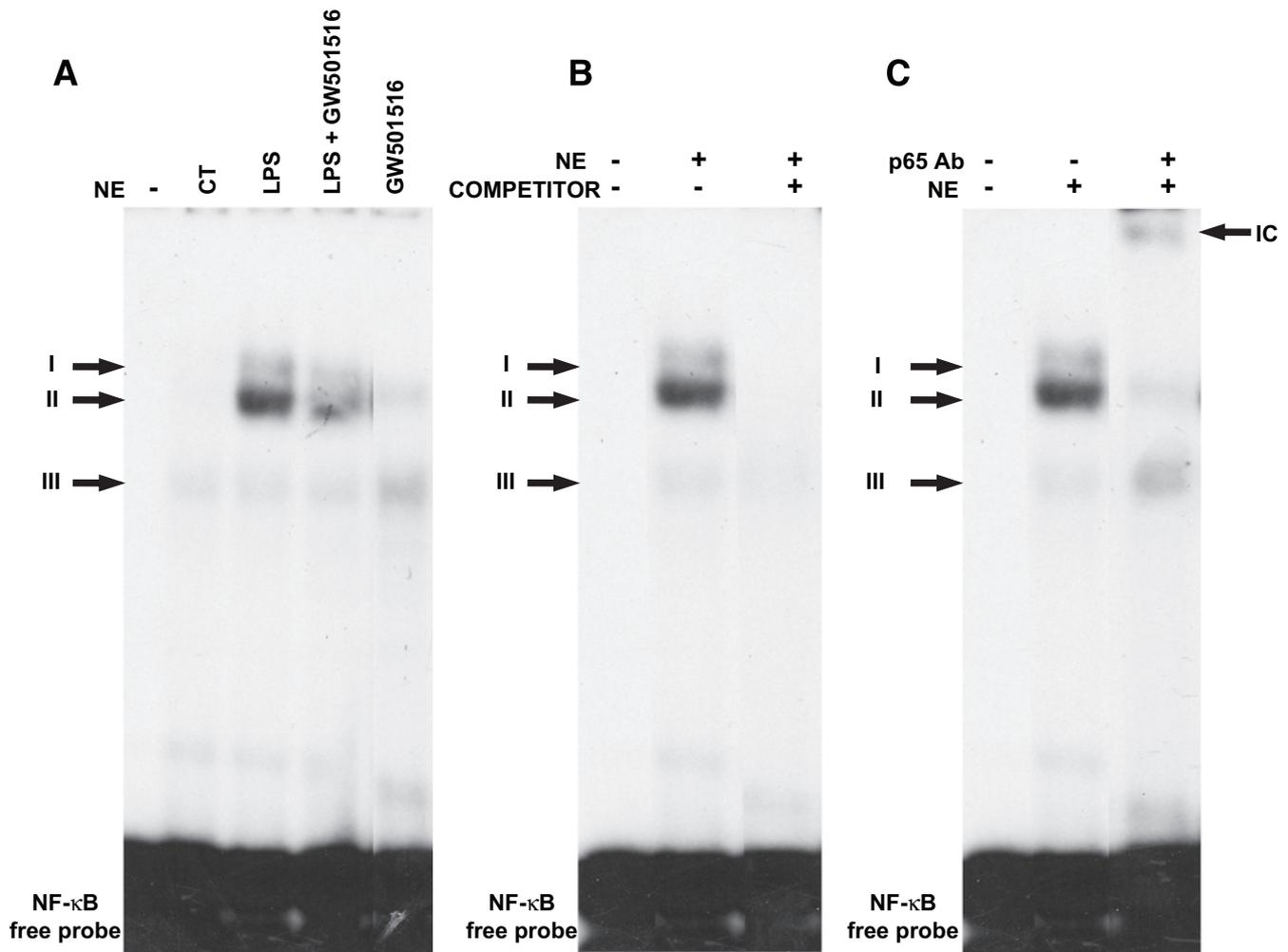


FIG. 3. The PPAR β/δ agonist GW501516 prevents LPS-induced NF- κ B activation in 3T3-L1 adipocytes. When indicated, differentiated adipocytes were incubated with 0.5 μ mol/l GW501516 for 96 h and subsequently exposed to 100 ng/ml LPS for 1 h in the presence or in the absence of GW501516. **A:** Autoradiograph of EMSA performed with a 32 P-labeled NF- κ B nucleotide and crude nuclear protein extract (NE). Three specific complexes (I–III), based on competition with a molar excess of unlabeled probe (**B**), are shown. **C:** A supershift analysis performed by incubating NE with an antibody directed against the p65 subunit of NF- κ B is also shown.

mice, NF- κ B binding activity was higher in this tissue compared with that of wild-type mice (Fig. 5D).

PPAR β/δ regulates ERK1/2 phosphorylation in adipocytes. Next, we attempted to determine the molecular mechanism by which PPAR β/δ activation inhibits NF- κ B. Although it has been reported that PPAR β/δ activation prevents LPS-induced degradation of inhibitor of κ B α (I κ B α), thus suppressing LPS-induced NF- κ B in cardiomyocytes (31), we did not observe changes in the protein levels of this NF- κ B inhibitor (data not shown) after GW501516 treatment. On the other hand, ERK1/2 activation leads to NF- κ B activation in adipocytes (9). In agreement with this hypothesis, the adipose tissue of ZDF rats showed enhanced phospho-ERK1/2 levels in the adipose tissue of ZDF rats compared with lean rats (Fig. 6A). This observation supports a potential relationship between activation of ERK1/2 and NF- κ B. To demonstrate the involvement of the ERK-MAPK cascade in LPS-induced NF- κ B activation in adipocytes, we used U0126, a potent and specific ERK1/2 inhibitor, which binds to MEK, thereby inhibiting its catalytic activity and phosphorylation of ERK1/2. Cells exposed to LPS showed increased mRNA levels of both *Mcp-1* (31-fold induction, $P < 0.001$) and *IL-6* (fourfold induction, $P < 0.05$), whereas co-

incubation with U0126 caused a 35% ($P < 0.001$) and 72% ($P < 0.05$) reduction in the LPS-mediated induction of these two genes, respectively (Fig. 6B and C). As a control, we verified that U0126 treatment inhibited LPS-induced ERK1/2 activation (Fig. 6D).

Finally, we examined whether the PPAR β/δ ligand prevented LPS-induced NF- κ B activation by reducing ERK1/2 phosphorylation in adipocytes. Immunoblotting detection of total and phosphorylated ERK1/2 (Fig. 6E) revealed that LPS treatment activated this pathway, whereas in the presence of the PPAR β/δ agonist, this pathway was blocked. These observations suggest that activation of this transcription factor prevents NF- κ B activation by inhibiting ERK1/2 phosphorylation. To demonstrate that PPAR β/δ regulates the ERK1/2 pathway, we explored the expression of this kinase in adipose tissue of the PPAR β/δ -null mice. These mice showed enhanced phospho-ERK1/2 protein levels compared with wild-type mice (Fig. 6F), indicating that PPAR β/δ regulates this pathway.

DISCUSSION

Obesity, insulin resistance, and type 2 diabetes are closely associated with low grade of chronic inflammation char-

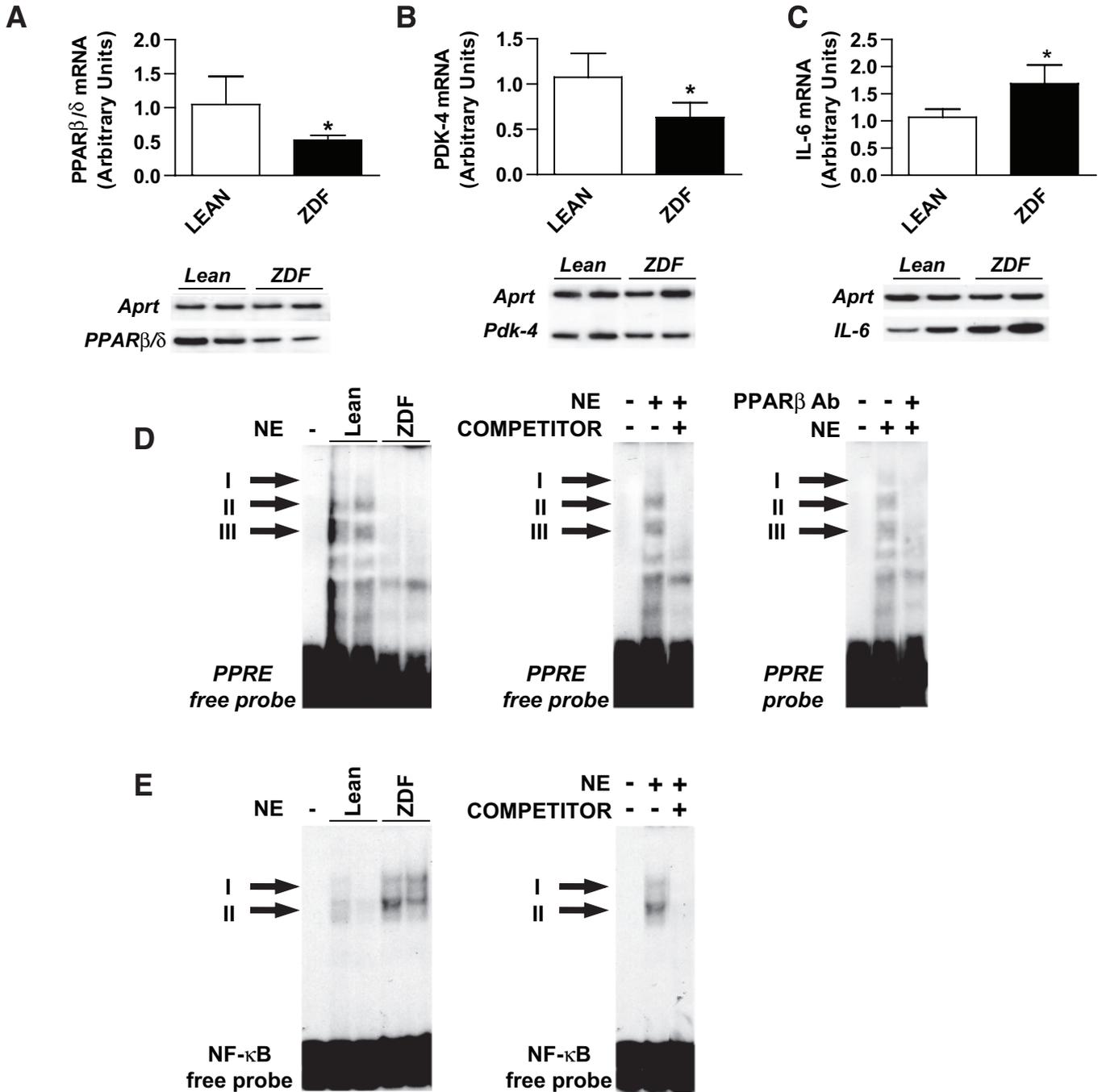


FIG. 4. In white adipose tissue of ZDF rats, PPAR β/δ expression is reduced, whereas NF- κ B activity is increased. Analysis of the mRNA levels of *Ppar β/δ* (A), *Pdk-4* (B), and *IL-6* (C) in white adipose tissue of lean and ZDF rats. Total RNA was isolated and analyzed by RT-PCR. A representative autoradiogram and the quantification normalized to the *Appt* mRNA levels are shown. Data are means \pm SD of five independent experiments. * $P < 0.05$. D: Autoradiograph of EMSA performed with a 32 P-labeled PPRE nucleotide and crude nuclear protein extract (NE). Three specific complexes (I–III), based on competition with a molar excess of unlabeled probe (right), are shown. An analysis performed by incubating NE with an antibody directed against PPAR β/δ is also shown. This antibody does not shift the complex, but prevents its binding to the PPRE. E: Autoradiograph of EMSA performed with a 32 P-labeled NF- κ B nucleotide and NE. Two specific complexes, based on competition with a molar excess of unlabeled probe (right), are shown.

acterized by abnormal cytokine production (32). The adipocyte plays a crucial role in this process, because this cell is a source of cytokines (TNF- α , IL-6, and MCP-1), which are secreted as a result of the activation of several signaling cascades involved in obesity-induced insulin resistance (33). A number of studies have implicated chronic activation of the pro-inflammatory transcription factor NF- κ B as one of these signaling pathways (34) that links inflammation with obesity and type-2 diabetes. For

instance, overexpression of the NF- κ B activator IKK β in mice results in increased inflammatory cytokine production and the onset of diabetes (35). Furthermore, in human adipose tissue, inhibition of NF- κ B suppresses the release of pro-inflammatory cytokines (36). Activation of NF- κ B can be achieved by either LPS or FFAs through TLR4 (26). Several studies have proposed that LPS participates in initiating the subclinical inflammation observed in obesity and type 2 diabetes. Thus, circulating LPS levels are higher

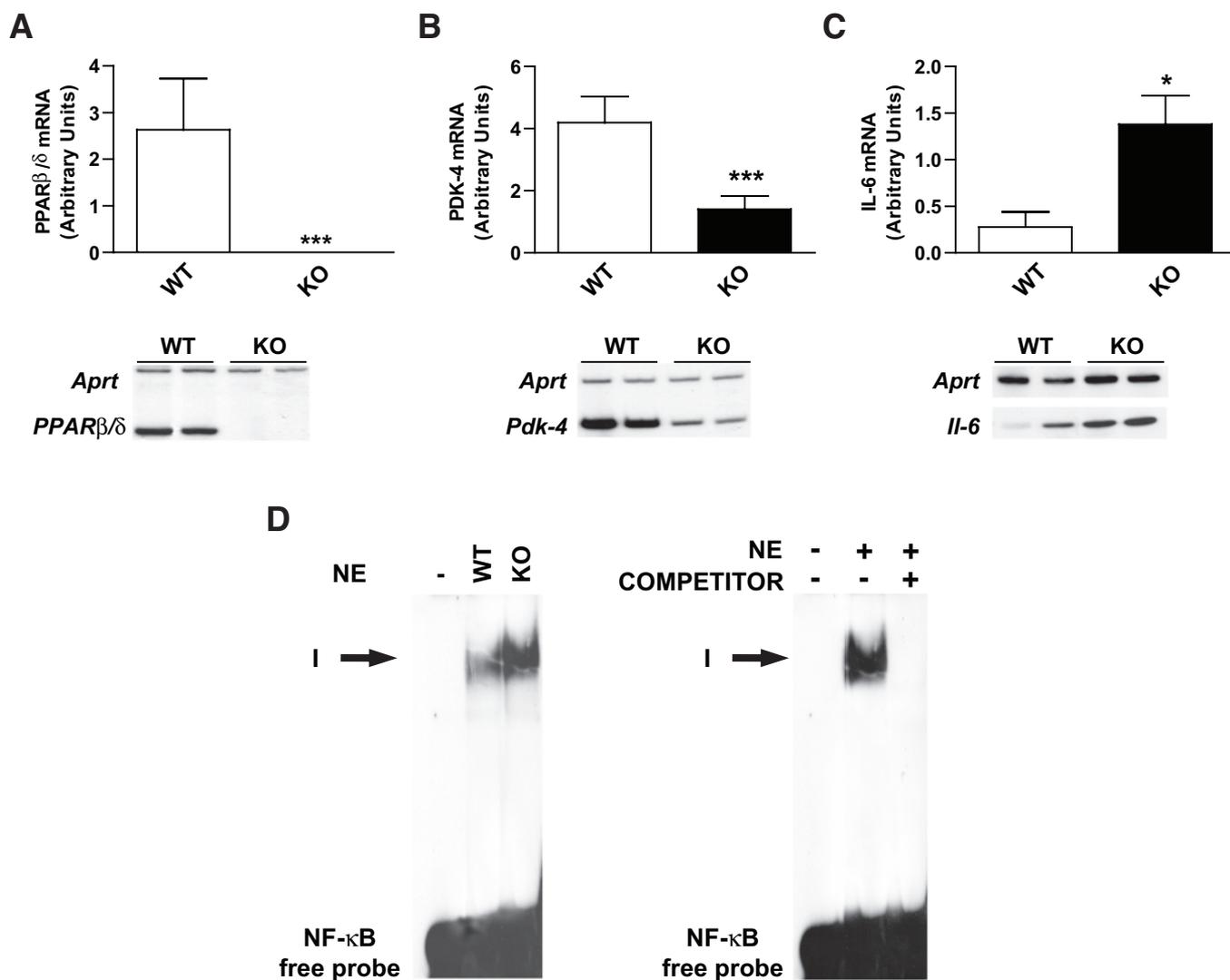


FIG. 5. The PPAR β/δ -null mouse shows increased NF- κ B activity in white adipose tissue. Analysis of the mRNA levels of *Ppar β/δ* (A), *Pdk-4* (B), and *Il-6* (C) in white adipose tissue of wild-type (wt) or PPAR β/δ -null (ko) mouse white adipose tissue. Total RNA was isolated and analyzed by RT-PCR. A representative autoradiogram and the quantification normalized to the *Apvt* mRNA levels are shown. Data are means \pm SD of five independent experiments. * $P < 0.05$. D: Autoradiograph of EMSA performed with a 32 P-labeled NF- κ B nucleotide and crude nuclear protein extract (NE). One specific complex, based on competition with a molar excess of unlabeled probe (right), is shown.

in type 2 diabetic patients than in lean healthy subjects, and LPS stimulates the secretion of pro-inflammatory cytokines in human adipocytes (37).

Here, we demonstrate for the first time that PPAR β/δ activation in adipocytes inhibits LPS-induced cytokine expression and secretion by preventing NF- κ B activation. We (38) and others (31) have previously proposed that PPAR β/δ activation inhibits NF- κ B activation in cardiac cells either by promoting a protein-protein interaction between PPAR β/δ and the p65 subunit of NF- κ B or by increasing the expression levels of the NF- κ B inhibitor I κ B α . In adipocytes, we did not observe changes either in the PPAR β/δ -p65 interaction (data not shown) or in I κ B α protein levels after GW501516 treatment. These observations suggest that these mechanisms are not involved in the anti-inflammatory effects of this drug in adipocytes. In contrast, it has been previously reported that ERK1/2 activation is crucial for the induction of inflammatory changes in adipocytes (34) and leads to enhanced NF- κ B activity (9). In agreement with this role of ERK1/2 in inflammation in adipocytes, we observed a reduction in

the expression of pro-inflammatory cytokines in these cells when exposed to LPS in the presence of the MAPK pathway inhibitor, U0126. This finding is consistent with previous studies reporting reduced cytokine expression levels in the presence of this inhibitor in human adipocytes (9). These data demonstrate that ERK1/2 inhibition prevents NF- κ B activation and the subsequent increase in cytokine expression. In addition, we demonstrate that PPAR β/δ activation inhibits ERK1/2 phosphorylation, which is consistent with the reduction in NF- κ B activation after GW501516 treatment. Furthermore, the increase in phospho-ERK1/2 levels observed in white adipose tissue from PPAR β/δ -null mice is consistent with enhanced NF- κ B activity. The involvement of PPAR β/δ in ERK1/2 regulation has been previously reported in other cell types. In fact, Burdick et al. (39) recently reported that the PPAR β/δ agonist GW0742 reduces phospho-ERK1/2 levels in human keratinocytes. In addition, phospho-ERK1/2 levels are higher in the skin of PPAR β/δ -null mice treated with 12-*O*-tetradecanoylphorbol-13-acetate than in the skin of wild-type animals (40). Overall, these data confirm that

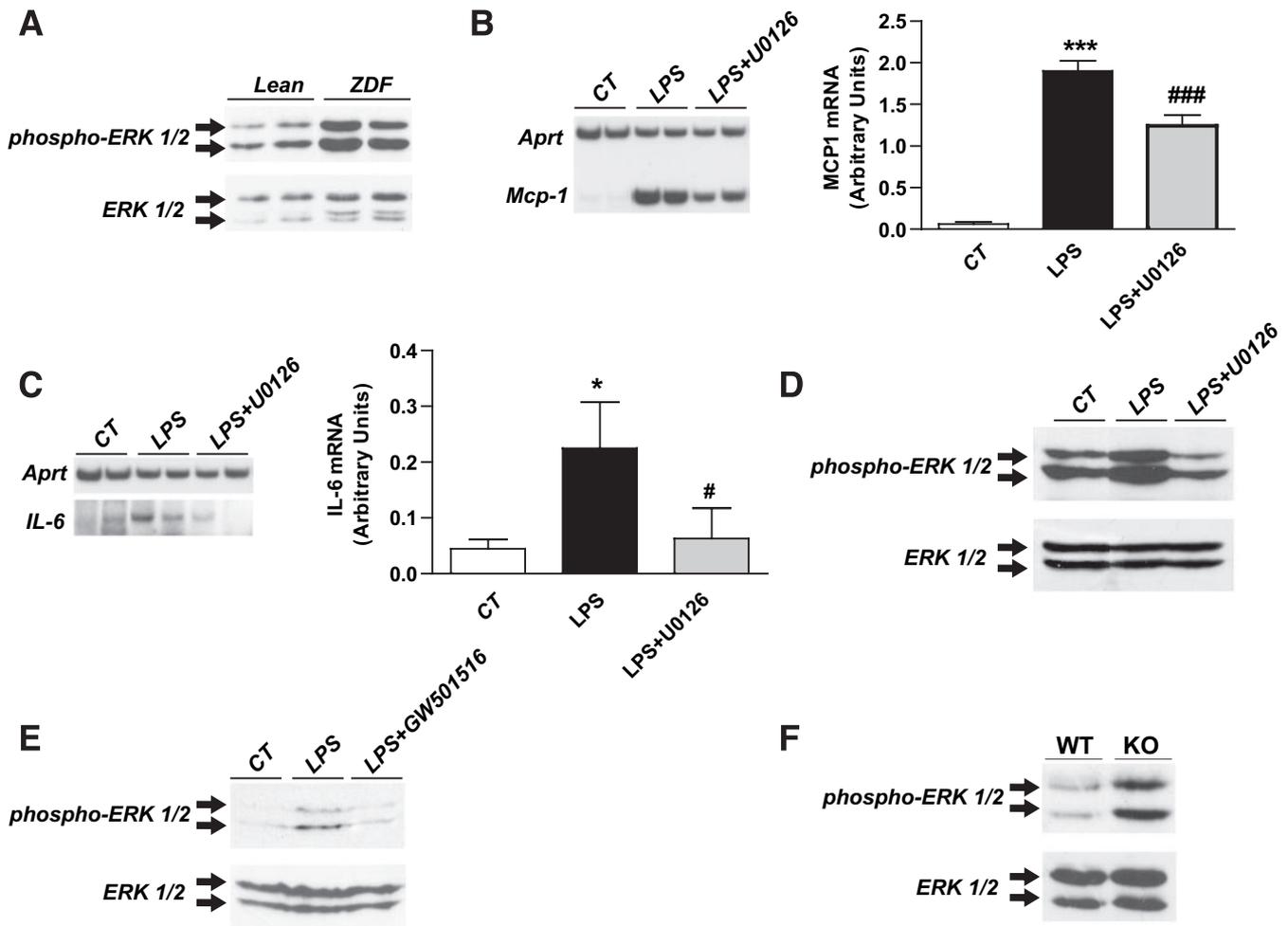


FIG. 6. PPAR β/δ regulates ERK1/2 phosphorylation in adipocytes and white adipose tissue. **A:** Analysis of ERK and phospho-ERK by immunoblotting of total protein extracts from white adipose tissue of lean and ZDF rats. The MEK1/2 inhibitor U0126 blocks NF- κ B activation by LPS. Analysis of the mRNA levels of Mcp-1 (**B**) and IL-6 (**C**) in 3T3-L1 adipocytes. Differentiated adipocytes were treated with 100 ng/ml LPS for 6 h in the presence or in the absence of 10 μ mol/l of the MEK1/2 inhibitor U0126. Total RNA was isolated and analyzed by RT-PCR. A representative autoradiogram and the quantification normalized to the Aprt mRNA levels are shown. Data are expressed as means \pm SD of five independent experiments. * P < 0.05 and *** P < 0.001 vs. control; # P < 0.05 and ### P < 0.001 vs. LPS-exposed cells. Analysis of ERK and phospho-ERK by immunoblotting of total protein extracts from 3T3-L1 adipocytes treated with 100 ng/ml LPS for 10 min in the presence or in the absence of 10 μ mol/l of U0126 (**D**) or 0.5 μ mol/l GW501516 (**E**). **F:** Wild-type (wt) or PPAR β/δ -null (ko) mouse white adipose tissue.

PPAR β/δ regulates phospho-ERK1/2 levels in several tissues. Additional studies are required to elucidate the molecular mechanism by which PPAR β/δ controls ERK1/2 activation.

Here, we demonstrate a correlation between enhanced ERK1/2 phosphorylation and NF- κ B activation in the white adipose tissue of a genetic model of obesity and diabetes, the ZDF rat. Given the low level of Ppar β/δ in the white adipose tissue of these animals, we hypothesize that a decrease in the expression of this nuclear receptor is one of the underlying mechanisms of this correlation. On the basis of our data, we propose that in this animal model, PPAR β/δ downregulation leads to enhanced ERK1/2 phosphorylation, which in turn activates NF- κ B in white adipose tissue and, as a result, induces pro-inflammatory cytokine expression. This hypothesis is supported by the observation that white adipose tissue from the PPAR β/δ -null mice shows increased phospho-ERK1/2 levels and NF- κ B activity and higher expression of IL-6 compared with wild-type mice. Although further studies are required to demonstrate this relationship between PPAR β/δ and NF- κ B in white adipose tissue, our data provide further insight into the

role of obesity-related insulin resistance and low-grade inflammation.

In summary, on the basis of our findings in adipocytes, we propose that PPAR β/δ activation prevents LPS-induced NF- κ B activation via ERK1/2, thereby reducing the production of pro-inflammatory cytokines involved in the development of insulin resistance. This action of PPAR β/δ may contribute to preventing obesity-induced insulin resistance.

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