

*PPAR $\beta/\delta$  prevents NF- $\kappa$ B activation in adipocytes*

**Activation of Peroxisome Proliferator-Activated Receptor  $\beta/\delta$   
(PPAR $\beta/\delta$ ) Inhibits LPS-induced Cytokine Production in Adipocytes by  
Lowering NF- $\kappa$ B Activity via ERK1/2**

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**Objective:** Chronic activation of the nuclear factor (NF)- $\kappa$ 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) $\beta/\delta$  activation prevents inflammation in adipocytes.

**Research Design and Methods and Results:** Firstly, we examined whether the PPAR $\beta/\delta$  agonist GW501516 prevents 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 STAT3-SOCS3 pathway. This effect was associated with the capacity of GW501516 to impede LPS-induced NF- $\kappa$ B activation. Secondly, in *in vivo* studies, white adipose tissue from *Zucker Diabetic Fatty* (ZDF) rats, compared to that of lean rats, showed reduced PPAR $\beta/\delta$  expression and PPAR DNA-binding activity, which was accompanied by enhanced IL-6 expression and NF- $\kappa$ B DNA-binding activity. Furthermore, IL-6 expression and NF- $\kappa$ B DNA-binding activity was higher in white adipose tissue from PPAR $\beta/\delta$ -null mice than in wild-type mice. Since mitogen-activated protein kinase (MAPK)–extracellular signal–related kinase (ERK)1/2 (MEK1/2) is involved in LPS-induced NF- $\kappa$ B activation in adipocytes, we explored whether PPAR $\beta/\delta$  prevented NF- $\kappa$ B activation by inhibiting this pathway. Interestingly, GW501516 prevented ERK1/2-phosphorylation by LPS. Further, white adipose tissue from animal showing constitutively increased NF- $\kappa$ B activity, such as ZDF rats and PPAR $\beta/\delta$ -null mice, also showed enhanced phospho-ERK1/2 levels.

**Conclusions:** These findings indicate that activation of PPAR $\beta/\delta$  inhibits enhanced cytokine production in adipocytes by preventing NF- $\kappa$ B activation via ERK1/2, an effect that may contribute to prevent insulin resistance.

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  $\alpha$  [TNF $\alpha$ ], 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 2-3 fold 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 NF- $\kappa$ 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 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 (MyD88), which in turn recruits interleukin-1 receptor-associated kinase (IRAK), leading to NF- $\kappa$ B activation and enhanced expression of several inflammatory mediators (including IL-6

and MCP-1). Of note, NF- $\kappa$ B activation by LPS requires mitogen-activated protein kinase (MAPK)–extracellular signal–related kinase (ERK)1/2 (MEK1/2) activation, since 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 (FFA) 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  $\beta/\delta$  (PPAR $\beta/\delta$ ) 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

(DRs) of hexameric DNA sequences separated by 1 bp (DR1) (14). Ligand binding induces a conformational change in PPAR-RXR complexes, thereby releasing co-repressors in exchange for co-activators, 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 $\alpha$  (NR1C1) (19) and PPAR $\gamma$ (NR1C3) are the targets for hypolipidemic (fibrates) and anti-diabetic (thiazolidinediones) drugs, respectively. Finally, activation of the third isotype, PPAR $\beta/\delta$  (NR1C2), by high-affinity ligands (including GW501516) enhances fatty acid catabolism in adipose tissue and skeletal muscle, thereby delaying weight gain (for review see (13)). However, there is no information available on whether PPAR $\beta/\delta$  ligands prevent inflammation in adipocytes. Here we examined whether PPAR $\beta/\delta$  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 $\beta/\delta$  ligand to prevent LPS-induced NF- $\kappa$ B activation. Consistent with the role of PPAR $\beta/\delta$  in blocking NF- $\kappa$ B-induced IL-6 expression, a genetic model of obesity and insulin resistance, the ZDF rat, showed reduced PPAR $\beta/\delta$  expression and DNA-binding

activity, and enhanced IL-6 expression and NF- $\kappa$ B DNA-binding activity in white adipose tissue. Likewise, IL-6 expression and NF- $\kappa$ B DNA-binding activity was higher in this tissue in PPAR $\beta/\delta$ -null mice than in wild-type mice. Since MAPK-ERK1/2 (MEK1/2) is involved in NF- $\kappa$ B activation in adipocytes (9), we explored whether PPAR $\beta/\delta$  blocked NF- $\kappa$ 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- $\kappa$ B activity in white adipose tissue, the ZDF rat and the PPAR $\beta/\delta$ -null mice, showed enhanced phospho-ERK1/2 levels. Overall, on the basis of our findings, we propose that PPAR $\beta/\delta$  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**

**Materials.** The PPAR $\beta/\delta$  ligand GW501516 was from Biomol Research Labs Inc. (Plymouth Meeting, PA). Other chemicals were from Sigma (St. Louis, MO).

**Cell culture.** 3T3-L1 preadipocytes (ATCC) were grown to confluence in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% bovine calf serum. Two 2 days after confluence (day 0), differentiation of the 3T3-L1 cells was induced in DMEM containing 10% fetal bovine serum, methylisobutylxanthine (500  $\mu$ M), dexamethasone (0.25  $\mu$ M), and insulin

(10  $\mu$ g/ml) 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 more than 90% of cells after day 10. Adipocytes were then incubated for 96 h with 0.5  $\mu$ 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, UK).

**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 2200 *g* for 10 min at 4°C. Plasma glucose (Sigma, St. Louis, MO), triglycerides (Sigma), and non-esterified fatty acids (Wako, Germany) levels were determined with a colorimetric test. Insulin (Amersham Biosciences) was determined by RIA. 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 $\beta/\delta$  null mice was described previously (20).

**Measurements of mRNA.** Levels of mRNA were assessed by the reverse transcription-polymerase chain reaction (RT-PCR) as previously described (21). Total RNA was isolated using the Ultraspec reagent (Biotecx, Houston). The total RNA isolated by this method is non-degraded 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'-GGGCCTGTTGTTCCACAGTTGC-3' and 5'-GGGACACCTGCTGCTGGTGAT-3'; *Pdk-4*, 5'-AGGTCGAGCTGTTCTCCCGCT-3' and 5'-GCGGTCAGGCAGGATGTCAAT-3'; *Cpt-1*, 5'-TTCAGTGTGACCCCAGACGGG-3' and 5'-AATGGACCAGCCCCATGGAGA-3'; *Ppar $\beta/\delta$* , 5'-GAGGAAGTGGCCACGGGTGAC-3' and 5'-CCACCTGAGGCCCCATCACAG-3'; *Socs-3* (Suppressor of cytokine signaling 3) 5'-TTTTTCGCTGCAGAGTGACCCC-3' and 5'-TGGAGGAGAGAGGTCCGGCTCA-3' and *Aprt* (adenosyl phosphoribosyl transferase), 5'-GCCTCTTGCCAGTCACCTGA-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 $\beta/\delta$* : 151 bp, *Socs-3*: 250 bp and *Aprt*: 329 bp). Preliminary experiments were carried out with various amounts of cDNA to determine non-saturating

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 of cold phosphate-buffered saline, pelleted for 10 seconds and resuspended in 400 $\mu$ l of cold Buffer A (10mM HEPES pH 7.9 at 4°C, 1.5mM MgCl<sub>2</sub>, 10mM KCl, 0.5mM DTT, 0.2mM PMSF, and 5 $\mu$ g/ml aprotinin) by flicking the tube. Cells were allowed to swell on ice for 10 min, and then vortexed for 10 sec. Samples were then centrifuged for 10 sec and the supernatant fraction was discarded. Pellets were resuspended in 50 $\mu$ l of cold Buffer C (20mM HEPES-KOH pH 7.9 at 4°C, 25% glycerol, 420mM NaCl, 1.5mM MgCl<sub>2</sub>, 0.2mM EDTA, 0.5mM DTT, 0.2mM PMSF, 5 $\mu$ g/ml aprotinin and 2 $\mu$ 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 (EMSA).** EMSA was performed using double-stranded oligonucleotides (Promega, Madison, WI) for the

consensus binding site of the NF- $\kappa$ B nucleotide (5'-AGTTGAGGGGACTTTCCCAGGC-3') and PPAR (5'-CAAACACTAGGTCAAAGGTCA-3'). Oligonucleotides were labeled in the following reaction: 2  $\mu$ l of oligonucleotide (1.75 pmol/ $\mu$ l), 2  $\mu$ l of 5x kinase buffer, 1  $\mu$ l of T4 polynucleotide kinase (10 U./ $\mu$ l), and 2.5  $\mu$ l of [ $\gamma$ -<sup>32</sup>P] ATP (3000 Ci/mmol at 10 mCi/ml) incubated at 37°C for 1 h. The reaction was stopped by adding 90  $\mu$ l of TE buffer (10 mM Tris-HCl pH 7.4 and 1 mM 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 mM Tris-HCl pH 8.0, 25 mM KCl, 0.5 mM DTT, 0.1 mM EDTA pH 8.0, 5% glycerol, 5 mg/ml BSA and 50  $\mu$ g/ml poly(dl-dC)), in a final volume of 15  $\mu$ l. Labeled probe (approximately 60,000 cpm) was added and the reaction was incubated for 15 min at 4°C (NF- $\kappa$ B) or room temperature (PPRE). 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 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 mM

Tris-HCl (pH 7.4), 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 5.4  $\mu$ g/ml aprotinin). The homogenate was centrifuged at 16,700 g for 30 min at 4°C. Protein concentration was measured by the Bradford method. Proteins (30  $\mu$ g) 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 (signal transducer and activator of transcription 3) (Tyr<sup>705</sup> and Ser<sup>727</sup>) (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  $\pm$  S.D. of 5 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 programme (GraphPad Software V2.03) (GraphPad Software Inc., 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 $\beta/\delta$  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  $\mu$ M GW501516, a selective ligand for PPAR $\beta/\delta$  with an apparent Kd value of 1nM and 1000-fold higher affinity toward PPAR $\beta/\delta$  than PPAR $\alpha$  and PPAR $\gamma$  (23). Under these conditions, we evaluated the expression of two well-known PPAR $\beta/\delta$ -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%,  $P < 0.05$ ) (Figure 1A). When adipocytes were treated for 96 h with 0.5  $\mu$ M GW501516, a greater increase was observed in the transcript levels of *Pdk4* (170%,  $P < 0.001$ ) and *Cpt-1* (300%,  $P < 0.01$ ) (Figure 1B). Therefore, we selected this longer exposure to the PPAR $\beta/\delta$  ligand in further experiments. No changes were observed either in the expression of well-known PPAR $\gamma$ -target genes (i.e. *Ob*, data not shown), confirming that the drug treatment was selective for PPAR $\beta/\delta$ , 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- $\kappa$ 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































