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The peroxisome proliferator-activated receptor β/δ (PPARβ/δ) agonist GW501516 prevents TNF-α-induced NF-κB activation in human HaCaT cells by reducing p65 acetylation through AMPK and SIRT1

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ABSTRACT

Nuclear factor (NF)-κB is a ubiquitously expressed transcription factor controlling the expression of numerous genes involved in inflammation. The aim of this study was to evaluate whether activation of the peroxisome proliferator-activated receptor (PPAR) β/δ prevented TNF-α-induced NF-κB activation in human HaCaT keratinocytes and, if so, to determine the mechanism involved. The PPARβ/δ agonist GW501516 inhibited the increase caused by TNF-α in the mRNA levels of the NF-κB target genes interleukin 8 (IL-8), TNF-α and thymic stromal lymphopoietin (TSLP). Likewise, GW501516 prevented the increase in NF-κB DNA-binding activity observed in cells exposed to TNF-α. The reduction in NF-κB activity following GW501516 treatment in cells stimulated with TNF-α did not involve either increased IκBα protein levels or a reduction in the translocation of the p65 subunit of NF-κB. In contrast, GW501516 treatment decreased TNF-α-induced p65 acetylation. Acetylation of p65 is mainly regulated by p300, a transcriptional co-activator that binds to and acetylates p65. Of note, AMP kinase (AMPK) activation phosphorylates p300 and reduces its binding to p65. GW501516 increased AMPK phosphorylation and the subsequent p300 phosphorylation, leading to a marked reduction in the association between p65 and this transcriptional co-activator. In addition, treatment with the PPARβ/δ agonist increased SIRT1 protein levels. Finally, the reduction in IL-8 mRNA levels following GW501516 treatment in TNF-α-stimulated cells was abolished in the presence of the PPARβ/δ antagonist GSK0660, the AMPK inhibitor compound C and the SIRT1 inhibitor sirtinol, indicating that the effects of GW501516 on NF-κB activity were dependent on PPARβ/δ, AMPK and SIRT1, respectively.

1. Introduction

In inflammatory skin diseases, such as atopic dermatitis and psoriasis, keratinocytes can respond to various stimuli and produce inflammatory mediators (cytokines, chemokines) through activation of the ubiquitously expressed transcription factor nuclear factor κB (NF-κB). In mammals the NF-κB/Rel family includes five known members: p50 (NF-κB1), p52 (NF-κB2), p65 (RelA), c-Rel, and RelB. The most abundant form of NF-κB is a heterodimer consisting of p50 and p65. In unstimulated cells, NF-κB is sequestered in the cytoplasm in an inactive form through interaction with the IκB inhibitory proteins. In the canonical activation pathway, stimulation of cells by specific stimuli, such as the proinflammatory cytokine tumor necrosis factor α (TNFα), results in phosphorylation of IκB by the IκB kinase (IKK) complex, leading to its degradation by the 26S proteasome. This releases NF-κB, which then translocates to the nucleus, where it activates transcription of a wide variety of promoters [1].

In addition, it is now well established that NF-κB-mediated transactivation is regulated by acetylation (for a review, see [2]). Thus, acetylation at multiple lysine residues in p65 and p50, which is mainly due to the intrinsic acetyltransferase activity of the transcriptional co-activator p300 [3–8], regulates different functions of NF-κB, including transcriptional activation, DNA-binding affinity, and IκBα assembly. Likewise, NF-κB transcriptional activity can be inhibited by the NAD^+-dependent protein deacetylases, sirtuins. Indeed, it has been reported that SIRT1, the most widely studied of the sirtuins, is a potent inhibitor of NF-κB transcription [9,10]. Interestingly, AMP-activated protein kinase (AMPK) enhances SIRT1 activity [11] and phosphorylates

Abbreviations: AMPK, AMP-activated protein kinase; NF-κB, nuclear factor κB; PPAR, peroxisome proliferator-activated receptors; SIRT1, silent information regulator T1; TNFα, tumor necrosis factor α.

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p300, thus inhibiting its ability to interact with nuclear receptors [12] such as NF-κB.

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily of ligand-inducible transcription factors that form heterodimers with retinoid X receptors (RXRs) and bind to consensus DNA sites [13]. PPARs may also suppress inflammation through various mechanisms, such as reduced release of inflammatory factors or stabilization of repressive complexes at inflammatory gene promoters [14–17]. Of the three PPAR isotypes found in mammals, i.e. PPARα (NR1C1), PPARβ/δ (NR1C2), and PPARγ (NR1C3), considerable attention has been paid to the role of PPARβ/δ in skin homeostasis (for a review, see [18]). In the keratinocytes of human skin, PPARβ/δ is the predominant PPAR isotype [19] and its expression is enhanced in hyperproliferative lesional skin from psoriatic patients [19,20]. Interestingly, it has been recently reported that in skin inflammatory diseases PPARα and PPARβ/δ activators improve the disease and decrease cytokine production, although the anti-inflammatory mechanism involved was not reported [21]. In this study we evaluated the effect of the PPARβ/δ agonist GW501516 on TNF-α-induced NF-κB activation in human keratinocytes. PPARβ/δ activation by GW501516 prevented TNF-α-induced expression of several NF-κB target genes and the DNA-binding activity of this proinflammatory transcription factor. The findings also demonstrate that GW501516 reduces TNF-α-induced acetylation of the p65 subunit of NF-κB through AMPK activation, which increases p300 phosphorylation, thereby reducing the p300 and p65 interaction, and SIRT1-mediated p65 deacetylation.

2. Materials and methods

2.1. Materials

Human HaCaT cell line was obtained from ATCC. The PPARβ/δ ligand GW501516 was from Biomol Research Labs Inc. (Plymouth Meeting, PA). Other chemicals were from Sigma–Aldrich (St. Louis, MO).

2.2. Cell culture

HaCaT cells were cultured in 150 cm^2 cell culture flasks at 37 °C, 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco Invitrogen, Barcelona, Spain) containing 10% fetal bovine serum (FBS) (Sigma–Aldrich, St. Louis, MO) and penicillin G sodium, streptomycin sulfate, and gentamicin (Gibco Invitrogen, Barcelona, Spain). The cells received fresh medium every 2 days and were subcultured every 4 days. Fortieth- to seventieth-passage cells were used in all experiments. When confluence was achieved the cells were trypsinized, washed, and resuspended in DMEM with 10% FBS. Cells were cultured on 60 mm culture dishes and when they reached confluence the medium was replaced by DMEM without FBS. Cells were preincubated with or without 1 μM GW501516 for 16 h and then stimulated with TNF-α (Sigma–Aldrich, St. Louis, MO) for either 2 h (mRNA studies) or 30 min (protein studies). Inhibitors (GSK0660, compound C, and sirtinol) (Sigma–Aldrich, St. Louis, MO) were added 30 min before the incubation with GW501516. After the incubation, RNA, whole cell lysates, and cytosolic and nuclear extracts were extracted from cells as described below.

2.3. Measurements of mRNA

Levels of mRNA were assessed by the reverse transcription-polymerase chain reaction (RT-PCR) as previously described [22]. Total RNA was isolated using the Ultraspec reagent (Biotecx, Houston, TX). The total RNA isolated by this method is non-degraded and free of protein and DNA contaminations. The sequences of the sense and antisense primers (Roche Diagnostics, Sant Cugat del Vallés, Spain) used for amplification were: IL-8: 5′-ATGACTTCCAAGCTGGCCGTG-3′ and 5′-GCGCAGTGTGGTCCACTCTCA-3′; TNF-α: 5′-AAGCTGAGGGGCAGCTCCAGT-3′ and 5′-TCTGGTAGGAGACGGCGATGC-3′; thymic stromal lymphopoietin (TSLP): 5′-TAGCAATCGGCCACATTGCCT-3′ and 5′-GAAGCGACGCCACAATCCTTG-3′; and 18S: 5′-ATGACTTCCAAGCTGGCCGTG-3′ and 5′-GCGCAGTGTGGTCCACTCTCA-3′. Amplification of each gene yielded a single band of the expected size (IL-8: 185 bp; TNF-α: 204 bp; TSLP: 247 bp; and 18S: 333 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 [23]. Radioactive bands were quantified by video-densitometric scanning (Vilber Lourmat Imaging, Marne-la-Vallée, France). The results for the expression of specific mRNAs are always presented relative to the expression of the control gene (18S).

2.4. Isolation of nuclear extracts

Nuclear extracts were isolated as previously described [24]. Cells were scraped into 1.5 ml of cold phosphate-buffered saline, pelleted for 10 s and resuspended in 400 μl of cold Buffer A (10 mM HEPES pH 7.9 at 4 °C, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF, and 5 μg/ml aprotinin) by flicking the tube. Cells were allowed to swell on ice for 10 min and were then vortexed for 10 s. Samples were subsequently centrifuged for 10 s and the supernatant fraction was discarded. Pellets were resuspended in 50 μl of cold Buffer C (20 mM HEPES–KOH pH 7.9 at 4 °C, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, 5 μg/ml aprotinin, and 2 μg/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.

2.5. Electrophoretic mobility shift assay (EMSA)

EMSA was performed using double-stranded oligonucleotides (Promega, Madison, WI) for the consensus binding site of the NF-κB nucleotide (5′-AGTTGAGGGGACTTTCCCAGGC-3′). Oligonucleotides were labeled in the following reaction: 2 μl of oligonucleotide (1.75 pmol/μl), 2 μl of 5× kinase buffer, 1 μl of T4 polynucleotide kinase (10 U/μl) (Gibco Invitrogen, Barcelona, Spain), and 2.5 μl of [γ-^{32}P] ATP (3000 Ci/mmol at 10 mCi/ml) (Perkin Elmer, Waltham, MA), incubated at 37 °C for 1 h. The reaction was stopped by adding 90 μl 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 (GE Healthcare, CA) following the manufacturer's instructions. Eight micrograms of crude nuclear protein were 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 μg/ml poly(dI–dC)), in a final volume of 15 μl. Labeled probe (approximately 60,000 cpm) was added and the reaction was incubated for 15 min at 4 °C (NF-κB). Where indicated, specific competitor oligonucleotide was added before the labeled probe and incubated for 10 min on ice. Protein–DNA complexes were resolved by electrophoresis at 4 °C on a 5% acrylamide gel and subjected to autoradiography.

2.6. Antibodies, immunoprecipitation and immunoblotting

Antibodies against IκBα, p65, PPARβ/δ, total and phospho-p300 (Ser^{89}) (Santa Cruz Biotechnology, CA), SIRT1 (Millipore, MA),

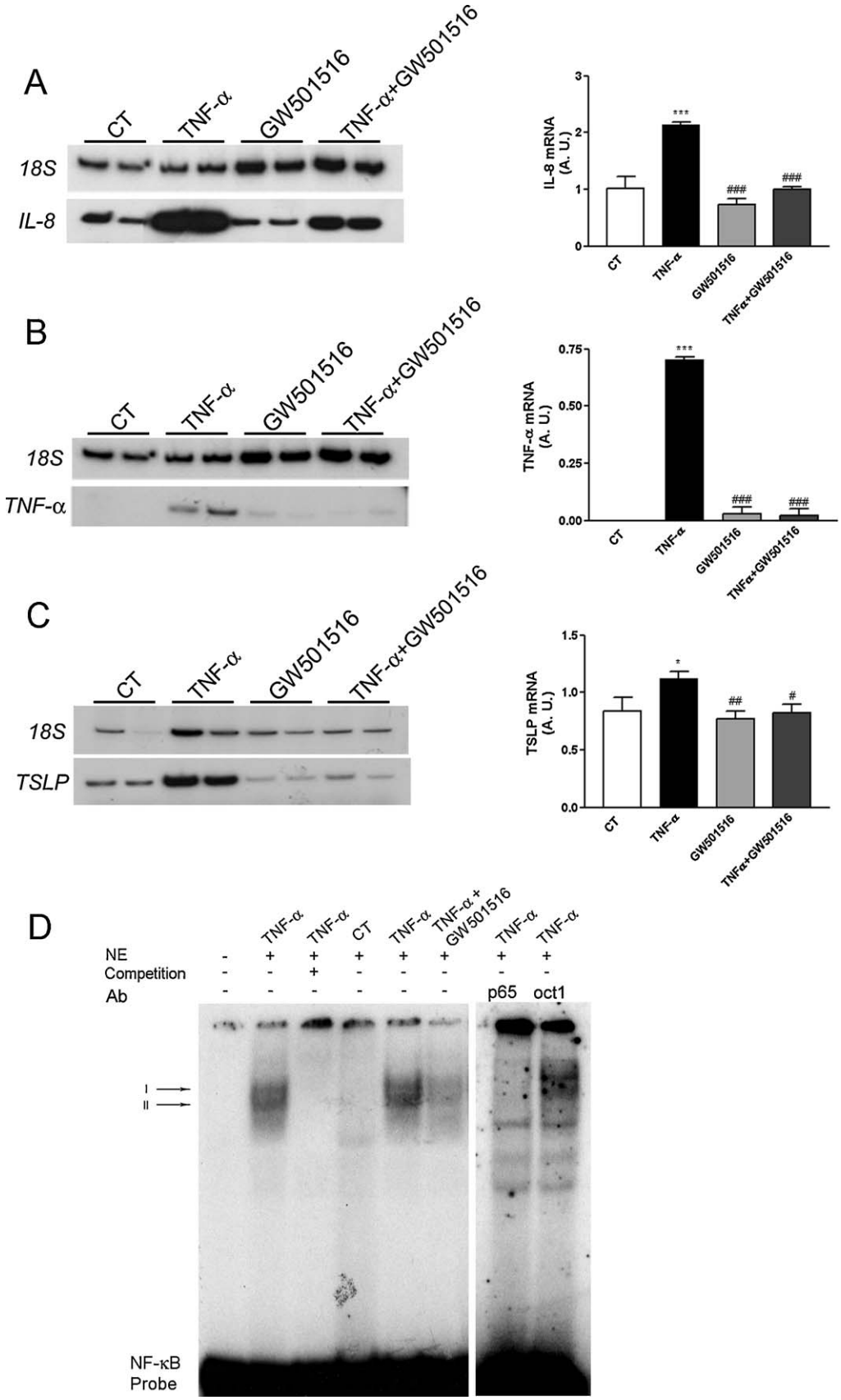

Fig. 1. The PPARβ/δ agonist GW501516 prevents TNF-α-induced cytokine expression and NF-κB activation in HaCaT cells. Analysis of the mRNA levels of IL-8 (A), TNF-α (B) and TSLP (C) in HaCaT cells untreated or treated with 1 μM GW501516 for 16 h before stimulation with 10 ng/ml TNF-α for 2 h. Total RNA was isolated and analyzed by RT-PCR. A representative autoradiogram is shown. Autoradiographs are representative of six separate experiments. (D) HaCaT cells were untreated or treated with 1 μM GW501516 for 16 h before stimulation with 10 ng/ml TNF-α for 30 min. Autoradiograph of EMSA performed with a ^{32}P-labeled NF-κB nucleotide and nuclear protein extracts (NE). Two specific complexes, based on competition with a molar excess of unlabeled probe, are shown. A supershift analysis performed by incubating NE with an antibody

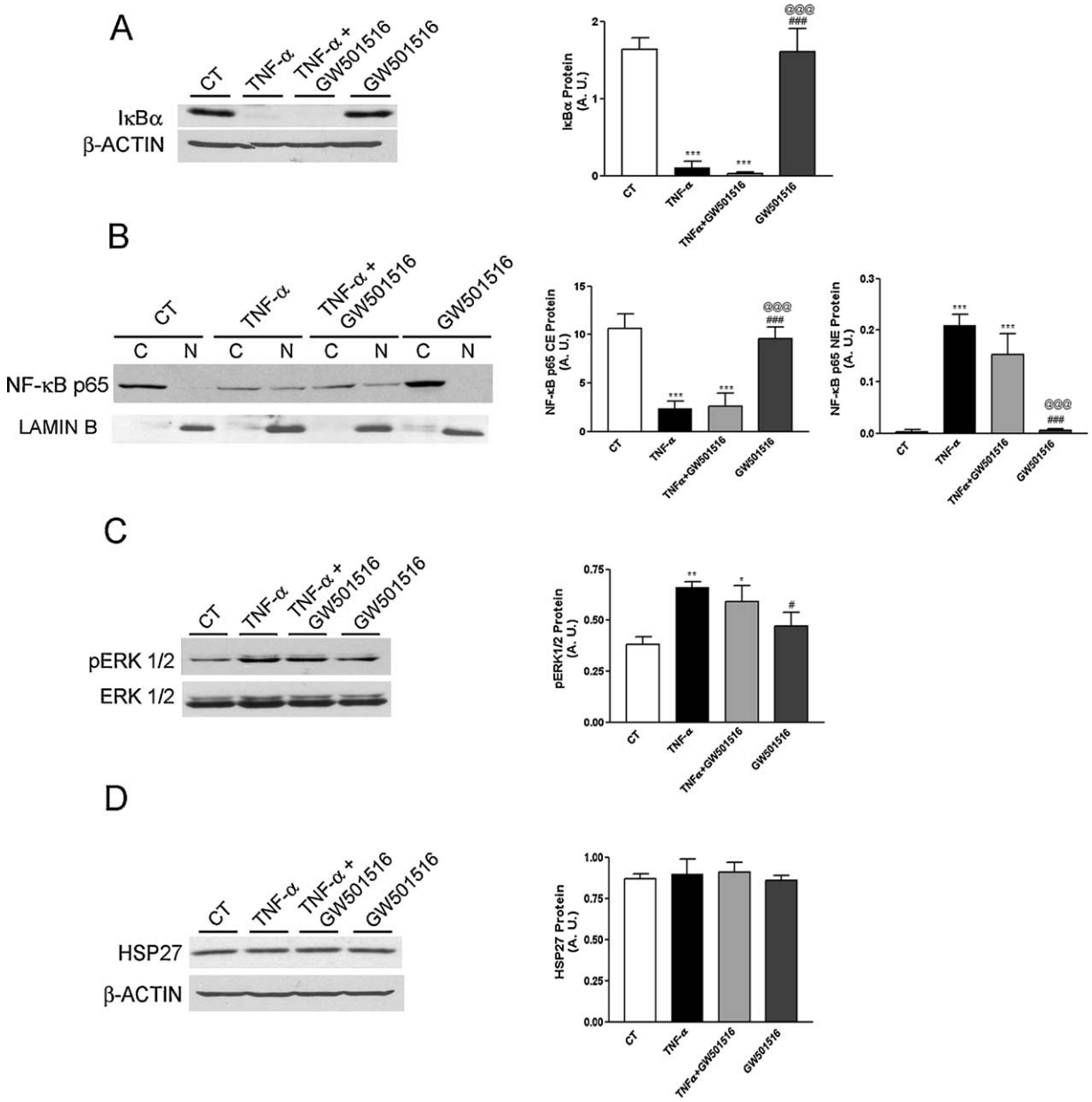

Fig. 2. The PPARβ/δ agonist GW501516 affects neither IκBα protein levels nor the translocation of the p65 subunit of NF-κB in HaCaT cells. HaCaT cells were untreated or treated with 1 μM GW501516 for 16 h before stimulation with 10 ng/ml TNF-α for 30 min. (A) Protein levels of IκBα protein extracts from HaCaT cells were assayed by Western blot analysis with IκBα and β-actin antibodies. (B) Protein levels of p65 in cytosolic (C) and nuclear (N) extracts. (C) Protein levels of total and phospho-ERK1/2 protein extracts from HaCaT cells were assayed by Western blot analysis with total and phospho-ERK1/2 antibodies. (D) Protein levels of Hsp27. Protein extracts from HaCaT cells were assayed by Western blot analysis with Hsp27 and β-actin antibodies. The blot data are representative of three separate experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control cells; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ vs. TNF-α-exposed cells; $^{@@@}p < 0.001$ vs. TNF-α-exposed cells incubated with GW501516.

HSP27 (R&D Systems, MN), total and phospho-ERK1/2 (Thr^{202}/Tyr^{204}), total and phospho-AMPK (Thr^{172}), total and phospho-ACC (Ser^{79}), acetyl-lysine (Cell Signaling Technology Inc., MA), and β-actin (Sigma–Aldrich, St. Louis, MO) were used.

Cytosolic and nuclear protein extracts were prepared as follows. Briefly, HaCaT cells grown in a 60 mm dish were rinsed with ice-cold PBS and scraped into a microfuge tube with 0.5 ml Tris–HCl (pH 7.4) containing 1 mM sodium orthovanadate, 10 μM PMSF, 2 μg/ml aprotinin, and 1 μg/ml leupeptin. The cells were pelleted by centrifugation (15,000 × *g*, 30 min at 4 °C) and both the pellet (P1) and the supernatant (S1) were processed. The cell pellet (P1) was resuspended in 0.5 ml of RIPA and homogenized in a dounce homogenizer with 20 strokes. This homogenate was incubated on ice for 30 min and then centrifuged at 13,000 × *g* for 15 min at 4 °C, with the supernatant being saved as nuclear extract. The resulting supernatant (S1) was diluted with RIPA buffer at 25% and saved as cytosolic extract.

To obtain whole-cell lysates, cells were homogenized in RIPA buffer (Sigma–Aldrich, St. Louis, MO) with phosphatase inhibitor (0.2 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 5.4 μg/ml aprotinin). The homogenate was centrifuged at 16,700 × *g* for 30 min at 4 °C. Protein concentration was measured by the Bradford method.

directed against the p65 subunit of NF-κB is also shown. Autoradiograph is representative of three separate experiments. $^{*}p < 0.05$, $^{***}p < 0.001$ vs. control cells; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ vs. TNF-α-exposed cells.

Whole-cell lysates and nuclear extracts were mixed with various antibodies (as specified in "Section 3") and protein A-coupled to agarose beads. Proteins from whole-cell lysates, cytosolic and nuclear extracts, and immunoprecipitates were separated by SDS-PAGE and transferred to immobilon polyvinylidene difluoride membranes (Millipore, Bedford, MA) and blotted with various antibodies (as specified in "Section 3"). Detection was achieved using the ECL plus chemiluminescence kit (GE Healthcare, CA). The size of detected proteins was estimated using protein molecular-mass standards (Invitrogen, Barcelona, Spain).

2.7. Statistical analyses

Results are expressed as means ± S.D. of five separate experiments. Significant differences were established by one-way ANOVA, using the GraphPad Instat program (GraphPad Software V2.03) (GraphPad Software Inc., San Diego, CA). When significant variations were found, the Tukey–Kramer multiple comparison test was applied. Differences were considered significant at $p < 0.05$.

3. Results

3.1. PPARβ/δ activation prevents TNF-α-induced expression of proinflammatory cytokines in HaCaT cells by inhibiting NF-κB

We first examined the effect of PPARβ/δ activation on the mRNA levels of three NF-κB target genes. HaCaT cells were preincubated for 16 h in the absence or in the presence of 1 μM GW501516, a selective ligand for PPARβ/δ with 1000-fold higher affinity toward PPARβ/δ than for PPARα and PPARγ [25], and then stimulated with 10 ng/ml of TNF-α for 2 h. TNF-α enhanced the expression of IL-8 and TNF-α, two-well known NF-κB-target genes [1], whereas in cells co-incubated with TNF-α plus GW501516 this increase was markedly reduced (Fig. 1A and B). Similarly, the increase caused by TNF-α in the expression of TSLP, a cytokine strongly implicated in the pathogenesis of atopic dermatitis [26] and which is under the control of NF-κB [27], was prevented in cells co-incubated with TNF-α and the PPARβ/δ agonist (Fig. 1C).

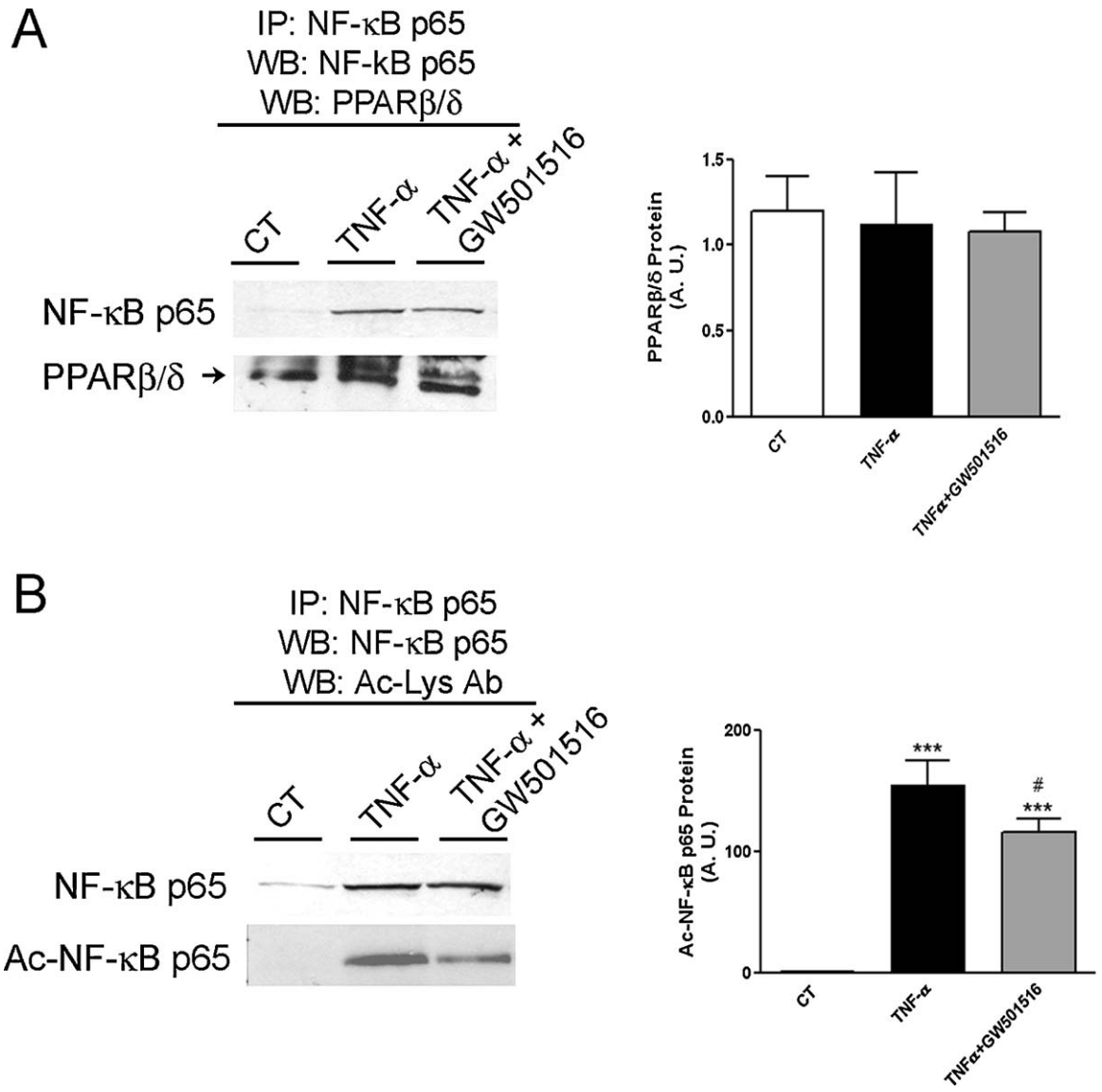

Fig. 3. The PPARβ/δ agonist GW501516 decreases acetylation of the p65 subunit of NF-κB in HaCaT cells. HaCaT cells were untreated or treated with 1 μM GW501516 for 16 h before stimulation with 10 ng/ml TNF-α for 30 min. (A) Nuclear extracts were immunoprecipitated (IP) with an antibody to the p65 subunit of NF-κB and immunoblotted (IB) with an anti-PPARβ/δ antibody. (B) HaCaT cells were treated as described in (A). Immunoprecipitation was performed with an antibody to p65 and immunoblotted with an acetyl-lysine antibody. The blot data are representative of three separate experiments.

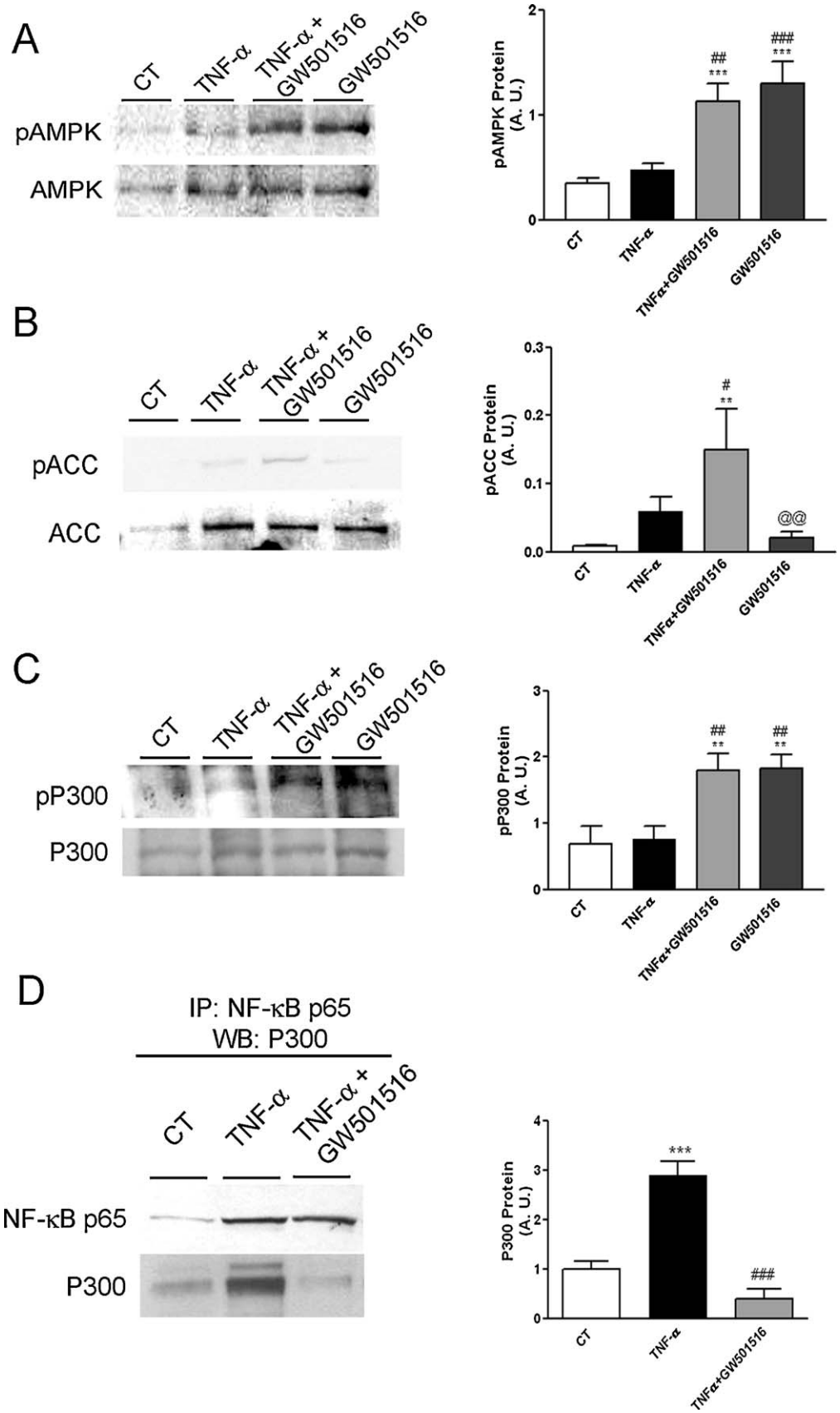

Fig. 4. The PPARβ/δ agonist GW501516 activates AMPK, phosphorylates p300 and prevents p65 and p300 association in HaCaT cells. HaCaT cells were untreated or treated with 1 μM GW501516 for 16 h before stimulation with 10 ng/ml TNF-α for 30 min. Protein levels of total and phospho-AMPK (Thr^{172}) (A), phospho-ACC (Ser^{79}) (B) and total and phospho-p300 (Ser^{89}) (C). (D) Nuclear extracts were immunoprecipitated (IP) with an antibody to the p65 subunit of NF-κB and immunoblotted (IB) with an anti-p300 antibody. The blot data are representative of three separate experiments. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control cells; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. TNF-α-exposed cells; $^{@@}p < 0.01$ vs. TNF-α-exposed cells incubated with GW501516.

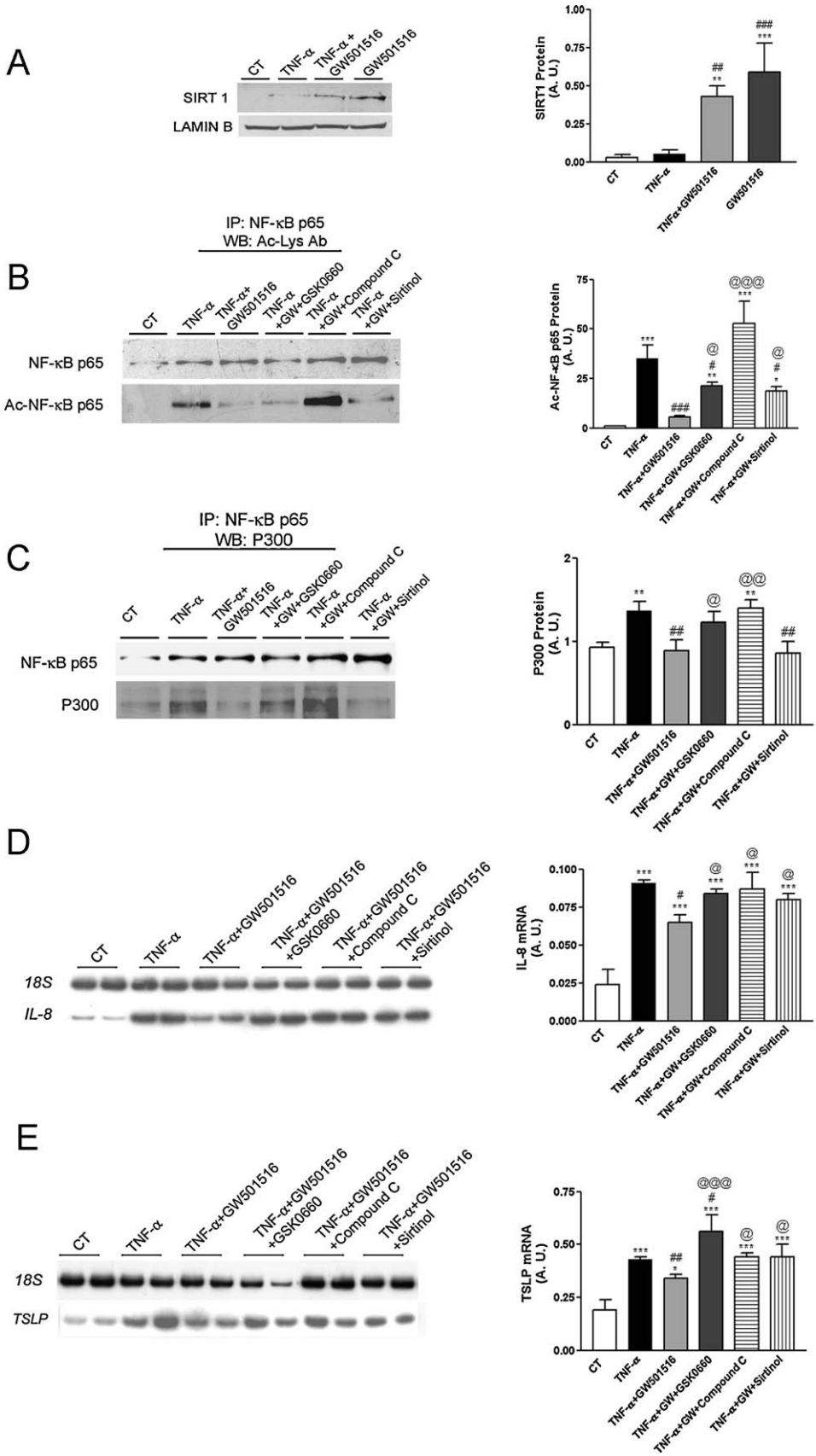

Fig. 5. The PPARβ/δ agonist GW501516 increases SIRT1 protein levels and the inhibition of TNF-α-induced cytokine expression by the PPARβ/δ agonist GW501516 is dependent on PPARβ/δ, AMPK and SIRT1 in HaCaT cells. (A) Protein levels of SIRT1. HaCaT cells were untreated or treated with 1 μM GW501516 for 16 h before stimulation with 10 ng/ml TNF-α for 30 min. The blot data are representative of three separate experiments. (B) HaCaT cells were untreated or treated with 1 μM GW501516 for 16 h in the presence or absence of GSK0660 (1 μM), compound C (10 μM), or sirtinol (20 μM) before stimulation with 10 ng/ml TNF-α for 30 min. Immunoprecipitation was performed with an antibody to p65 and immunoblotted with an acetyl-lysine antibody. (C) HaCaT cells were treated as described in (B). Nuclear extracts were

To demonstrate that GW501516 prevented TNF-α-induced NF-κB activation, we then performed an EMSA. The NF-κB probe formed two main complexes when incubated with nuclear extracts (Fig. 1D). The specificity of the DNA-binding complexes was assessed in competition experiments by adding an excess of unlabeled NF-κB oligonucleotide. Cells exposed to TNF-α showed enhanced NF-κB DNA-binding activity, whereas cells exposed to TNF-α and treated with GW501516 showed a marked reduction in binding. Addition of antibody against the p65 subunit of NF-κB reduced the intensity of the bands, whereas an unrelated antibody against Oct-1 did not, thereby indicating that these bands consisted mainly of this subunit.

3.2. PPARβ/δ activation affects neither IκBα protein levels nor p65 translocation in TNF-α-stimulated HaCaT cells

To investigate the mechanism responsible for the reduction of the TNF-α-mediated increase in proinflammatory cytokines by GW501516, we measured the protein levels of the NF-κB inhibitor IκBα, which is under the transcriptional control of PPARs [28]. Cells exposed to TNF-α showed a marked reduction in IκBα protein levels (Fig. 2A). However, drug treatment did not affect this reduction. Next, we evaluated the effects of GW501516 on p65 translocation in cytosolic and nuclear extracts (Fig. 2B). In unstimulated cells, p65 localized mainly in the cytosol and translocated to the nucleus following TNF-α stimulation. GW501516 treatment did not influence the translocation of the p65 subunit of NF-κB. Since we have previously reported that PPARβ/δ activation by GW501516 inhibited NF-κB by reducing phospho-ERK1/2 levels [29], we analyzed the phosphorylation status of this kinase. TNF-α exposure caused a slight increase in phospho-ERK1/2 levels that it was unaffected by GW501516, thereby indicating that changes in the phosphorylation status of ERK1/2 were not involved in the effects of GW501516 (Fig. 2C). Additionally, given that Hsp27 down-regulation results in increased NF-κB activity in keratinocytes [30], we measured the protein levels of this heat-shock protein. Neither TNF-α treatment nor GW501516 affected the levels of this protein, and therefore it is unlikely to be involved in the effects caused by GW501516 (Fig. 2D). One of the anti-inflammatory mechanisms of PPARβ/δ involves protein–protein interaction between PPARβ/δ and the p65 subunit of NF-κB [31]. This association prevents NF-κB from binding to its response element and thereby inhibits its ability to induce gene transcription, leading to a reduction in the expression of proinflammatory cytokines. To evaluate the contribution of this mechanism to the effects of GW501516 on NF-κB activity the interaction of PPARβ/δ with p65 was determined by immunoprecipitation of nuclear extract proteins with antibody against p65 and analysis of PPARβ/δ in the complex by Western blot (Fig. 3A). PPARβ/δ co-precipitated with p65, but no changes were observed in cells treated with GW501516, suggesting that drug treatment did not affect this association.

3.3. PPARβ/δ activation reduces p65 acetylation in TNF-α-stimulated HaCaT cells

As stated above, acetylation of different lysines in p65 regulates different functions of NF-κB, including transcriptional activation and DNA-binding affinity [2]. Therefore, we evaluated the effects of GW501516 on p65 acetylation by anti-p65 immunoprecipitation followed by anti-acetyl-lysine immunoblotting. As shown in Fig. 3B, TNF-α enhanced p65 acetylation, whereas in cells co-incubated with TNF-α plus GW501516 a marked reduction was observed. Based on the evidence that p300 acetyltransferase plays a major role in acetylation of p65 [3–8], we next determined whether p300 was involved in the inhibition of p65 acetylation caused by GW501516 in TNF-α-exposed cells. Acetylation of the p65 subunit of NF-κB by p300 requires their physical interaction and recruitment of this co-activator is a key step linking changes in the expression of NF-κB target genes in inflammatory processes [32,33]. Interestingly, phosphorylation of p300 at serine 89 by AMPK dramatically reduces its interaction with nuclear receptors [12]. Thus, we first examined whether, as reported in skeletal muscle cells [34], GW501516 increased phospho-AMPK levels in HaCaT cells. Cells exposed to GW501516 showed higher phospho-AMPK and phospho-acetyl-CoA carboxylase (ACC) levels, a downstream molecular target of AMPK, than did those treated with TNF-α (Fig. 4A and B). In agreement with the increase in phospho-AMPK levels, GW501516 enhanced p300 phosphorylation at serine 89 compared to TNF-α exposed cells (Fig. 4C). Consistent with these findings, co-immunoprecipitation studies showed that TNF-α enhanced the association between p65 and p300 compared with unstimulated cells (Fig. 4D), which is in agreement with previous studies [7], whereas GW501516 blocked this interaction. Since histone deacetylases, such as SIRT1, can reduce the acetylation level of the p65 protein and thereby inhibit the activity of NF-κB [8,9], and given the recent evidence that PPARβ/δ activation can increase the expression of SIRT1 [35], we examined the effect of GW501516 on SIRT1 protein levels. Although GW501516 exposure for 16 h did not significantly affected Sirt1 mRNA levels (data not shown), a treatment for 30 min significantly increased the protein levels of this deacetylase (Fig. 5A). Finally, to confirm that the changes observed in cells co-incubated with TNF-α and GW501516 were dependent on PPARβ/δ, AMPK and SIRT1, we used the PPARβ/δ antagonist GSK0660, the AMPK inhibitor compound C and the SIRT1 inhibitor sirtinol. As shown in Fig. 5B, the inhibition of p65 acetylation caused by GW501516 was slightly prevented by pretreatment with GSK0660 and sirtinol and specially by compound C. Similarly, GSK0660 and compound C prevented the reduction in the association between p65 and p300 caused by GW501516 (Fig. 5C). Finally, the inhibition of TNF-α-induced IL-8 and TSLP expression caused by GW501516 were blocked by GSK0660, compound C and sirtinol, indicating that the effects of GW501516 were PPARβ/δ-, AMPK- and SIRT1-dependent.

4. Discussion

Evidence has accumulated that acetylation and deacetylation are implicated in the regulation of NF-κB transcriptional activity. Although these processes occur at different levels of the NF-κB signaling pathway, direct acetylation of the NF-κB subunit p65 regulates different NF-κB functions, including transcriptional activation and DNA-binding affinity [2]. Among the acetyltransferases that can regulate NF-κB activity through p65 acetylation a major role is played by p300, a transcriptional co-activator with acetyltransferase activity [3–8]. In addition, deacetylases can also regulate NF-κB activity. Thus, SIRT1 physically interacts with and

immunoprecipitated (IP) with an antibody to the p65 subunit of NF-κB and immunoblotted (IB) with an anti-p300 antibody. HaCaT cells were untreated or treated with 1 μM GW501516 for 16 h in the presence or absence of GSK0660 (1 μM), compound C (10 μM), or sirtinol (20 μM) before stimulation with 10 ng/ml TNF-α for 2 h. Analysis of the mRNA levels of IL-8 (D) and TSLP (E) in HaCaT cells. 0.5 μg of total RNA was analyzed by RT-PCR. A representative autoradiogram normalized to 18S mRNA levels is shown. Data are expressed as mean ± S.D. of six experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control cells; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. TNF-α-exposed cells. $^{@}p < 0.05$, $^{@@}p < 0.01$ vs. cells co-incubated with TNF-α and GW501516.

deacetylates the p65 subunit of NF-κB and subsequently inhibits NF-κB transcriptional activity [9,36–38].

In this study we report that the PPARβ/δ agonist GW501516 inhibits TNF-α-induced cytokine expression through a mechanism which involves reduced p65 acetylation. Our findings also show that the anti-inflammatory effect of GW501516 is dependent on both AMPK and SIRT1 activation. AMPK is a fuel-sensing enzyme that responds to cellular energy depletion by increasing processes that generate ATP and inhibiting others that require ATP but are not acutely necessary for survival. Previous studies have demonstrated that GW501516 increases AMPK activation/phosphorylation [34,39] in skeletal muscle cells by increasing the AMP:ATP ratio [34,39]. Of note, AMPK can phosphorylate p300, inhibiting its ability to interact with nuclear receptors [12]. In agreement with this, GW501516 increased p300 phosphorylation and dramatically reduced the association between p65 and p300. In addition, AMPK activation may enhance SIRT1 activity by increasing cellular NAD^+ levels, resulting in the deacetylation and modulation of the activity of their target genes [11]. Moreover, a recent study demonstrated that PPARβ/δ regulates human SIRT1 gene transcription via Sp1 [35]. In agreement with this, we observed increased SIRT1 protein levels following PPARβ/δ agonist treatment. Indeed, several studies have indicated that SIRT1 is a potent inhibitor of NF-κB transcription [9]. Finally, the involvement of SIRT1 in the effects attained by GW501516 was clearly demonstrated by using sirtinol, a known inhibitor of SIRT1, which abolished the reduction in IL-8 and TSLP expression.

The results reported here conflict with previous studies reporting that PPARs do not repress NF-κB dependent transactivation in human keratinocytes [19,40]. The reasons for this discrepancy might involve differences in the proinflammatory stimuli used (TNF-α vs. IL-1β), the time exposure, and agonist concentration.

The present findings have implications for the potential treatment of skin inflammatory diseases with PPARβ/δ agonists. For instance, psoriasis has been characterized as an inflammatory disorder with enhanced production of cytokines in lesional psoriatic skin. Thus, in psoriatic epidermis NF-κB binding to the κB site of the IL-8 promoter is enhanced [41]. The use of a PPARβ/δ agonist may therefore improve the inflammatory process in this pathology. However, excessive PPARβ/δ activation in this context can be counterproductive since it has been demonstrated that activation of overexpressed PPARβ/δ in mice epidermis causes a psoriasis-like skin disease [42]. This is not surprising, since previous studies had already reported that overexpression of PPARs may result in deleterious effects. For instance, whereas PPARα activation improves glycemic control in diabetic monkeys [43], overexpression of this nuclear receptor causes insulin resistance [44].

In atopic dermatitis, a chronic inflammatory dermatosis, administration of PPARα and PPARβ/δ activators improved the disease and decreased cytokine production, although the anti-inflammatory mechanism involved was not reported [21]. Since NF-κB inhibition can help ameliorate atopic dermatitis [45], the inhibition of this pro-inflammatory transcription factor resulting from activation of PPARβ/δ might be involved in these effects.

Overall, our findings indicate that GW501516 inhibits TNF-α induced cytokine expression through AMPK activation, which increases p300 phosphorylation, thereby reducing the p300 and p65 interaction, and SIRT1-mediated p65 deacetylation. As a result, p65 acetylation, NF-κB DNA-binding activity, and cytokine expression are all reduced following GW501516 treatment.

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