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Phosphorylation of PPARγ via Active ERK1/2 Leads to its Physical Association With p65 and Inhibition of NF-κβ

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Abstract Peroxisome proliferator-activated receptors (PPAR) are novel nuclear receptors and PPARγ ligands have been shown to produce pro-apoptotic effects in many cancer cell types, including colon cancer. PPARγ ligands exert their effect through PPARγ-dependent (genomic) and PPARγ-independent (non-genomic) mechanisms. Recent evidence suggests that PPARγ ligands exert their pro-apoptotic effects in part by directly antagonizing the NF-κβ pathway as well as through activation of the MAP kinase pathway. In this report, we have demonstrated that ciglitazone, a member of the thiazoldinedione class of PPARγ ligands induces HT-29 colon cancer cells to undergo apoptosis and prior to apoptosis, ciglitazone exposure results in a transient phosphorylation of PPARγ. This phosphorylation of PPARγ was mediated through the ciglitazone-induced activation of Erk1/2. PPARγ phosphorylation affected the genomic pathway by being inhibitory to PPARγ–DNA binding and PPRE transcriptional activity, as well as the non-genomic pathway by increasing the physical interaction of PPARγ with p65, leading to the inhibition of NF-κβ. Ciglitazone induced phosphorylation of PPARγ through the MAP kinase pathway provides a potential regulatory mechanism for PPARγ's physical interaction with p65, leading to inhibition of NF-κβ and subsequent apoptosis. J. Cell. Biochem. 90: 732–744, 2003.

Key words: PPARγ; NF-κβ; MAP kinase; apoptosis; colon cancer

Peroxisome proliferator-activated receptors (PPAR) are novel nuclear receptors that provide a direct link between fatty acid metabolism and gene transcription. The three isoforms of PPAR, designated α, β, and γ, are members of ligand-dependent transcription factor family that has been shown to regulate gene networks involved in controlling cell growth, differentiation, and homeostasis [Auwerx, 1999; Kersten et al., 2000]. The expression of each PPAR isoform varies from tissue to tissue. PPARα is expressed in hepatocytes, cardiomyocytes, and proximal tubule cells of the kidney. PPARβ is ubiquitously expressed in most tissues, while PPARγ is expressed in adipose tissues and enterocytes [Kersten et al., 2000]. The distinct tissue distribution suggests that the PPAR subtypes play different biological roles. In particular, PPARγ appears to play a pivotal role in adipogenesis, as well as cell growth and differentiation of colonocytes [Kliewer and Willson, 2000]. PPARγ is activated by an array of compounds, including fibric acid derivatives, thiazoldinediones, arachadonic acid metabolites, and fatty acids. Classically, once ligand binding occurs, activation of transcription requires PPARγ to heterodimerize with retinoid X receptor α (RxRα), followed by binding to specific DNA response elements (PPRE).

Recent data suggest that PPARγ ligands also promote their biologic effects through a non-genomic or PPARγ-independent pathways. PPAR ligands can activate MAP kinase signaling through a non-genomic mechanism [Chinetti et al., 1998; Su et al., 1999; Straus et al., 2000] and exert their pro-apoptotic effects

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by directly antagonizing the NF-κβ pathway [Camp et al., 1999; Lennon et al., 2002; Misra et al., 2002]. Interestingly, the genomic and non-genomic PPARγ pathways most likely interrelate, since activation of the MAP kinase pathway by PPARγ ligands ultimately leads to the regulation of the genomic pathway through inhibitory phosphorylation of PPARγ [Hsi et al., 2001].

The purpose of this study was to investigate the interconnection between PPARγ and the MAP kinase and NF-κβ pathways. In this report, we demonstrate that ciglitazone, a member of the thiazoldinedione class of PPARγ ligands, induces HT-29 colon cancer cells to undergo apoptosis. However, prior to apoptosis, ciglitazone exposure results in a transient phosphorylation of PPARγ through the MAP kinase signaling pathway. This phosphorylation of PPARγ is associated with decreased DNA binding and PPRE-dependent transcriptional activity. In addition, the phosphorylation of PPARγ increases its physical interaction with p65 and a subsequent decrease in κB transcriptional activity. These data suggest that PPARγ ligands exert their effect through both PPAR-dependent and independent mechanisms, which may be interrelated.

MATERIALS AND METHODS

Cell Culture and Biological Reagents

HT-29 colon cancer cells (ATCC, Manassa, VA) were maintained in McCoy's media (Gibco BRL, Rockville, MD) supplemented with 10% complement-inactivated fetal bovine serum (FBS) (Gibco BRL) at 37°C in an atmosphere of 5% CO_2. When experiments were performed in reduced serum, 0.1% FBS was added 24 h before initiating the experiment. Ciglitazone was purchased from Biomol Research Laboratories (Plymouth Meeting, PA) and dissolved in DMSO (Sigma Chemical Co., St. Louis, MO). Annexin V-FITC Apoptosis Detection Kit was purchased from PharMingen (Los Angeles, CA). Antibodies against PPARγ (sc-7196, for IB and IP), p65 (sc-109), RxRα (sc-553), c-fos (sc-7202x), c-Jun (sc-1694x), and ATF-2 (sc-187x) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). PPARγ (#2492, for IB after IP) and phospho-Erk antibodies were obtained from Cell Signaling Technology (Beverly, MA) and p50 (#06-886) was purchased from Upstate Biotechnology (Lake Placid, NY).

MTT Assay and Annexin V Staining Assay

Cell mass was determined by standard MTT assay. Specifically, 3×10^3 cells were plated in 96-well plates and treatment with ciglitazone was initiated 24 h afterwards. At the completion of treatment, MTT (200 μl, 0.5 mg/ml) was added to each well and cells were incubated for 3 h at 37°C and 5% CO_2. Formazan crystals were dissolved with DMSO (50 μl) and color intensity was measured using an ELISA reader at 570 nm (reference filter, 690 nm). Trypan blue dye exclusion assay was performed to confirm and verify cell viability. Cell apoptosis was determined using Annexin V staining kit. Briefly, FITC-conjugated annexin V and propidium iodide were added to 3×10^6 cells and fluorescence intensity was determined using a FACScan flow cytometer (Becton Dickinson, San Jose, CA) and analyzed by CellQuest software (Becton Dickinson).

Whole Cell, Cytoplasmic, and Nuclear Extracts

Whole cell extracts were prepared by lysing cell pellets with a Dounce microtip homogenizer in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.5% NP40, 50 mM NaF, 0.2 mM $NaVO_4$, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml leupeptin, 25 μg/ml aprotinin, 25 μg/ml pepstatin A). For nuclear extracts, cells were harvested, washed with cold 1× PBS, and resuspended in cytoplasmic extraction buffer (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT) for 15 min. After dounce homogenization, the cytoplasmic fraction was separated by centrifugation (14,000*g* for 5 min at 4°C). The pellet was resuspended in nuclear extraction buffer (20 mM HEPES, 1.5 mM $MgCl_2$, 25% glycerol, 420 mM NaCl, 0.2 mM EDTA, 0.5 mM DTT) for 30 min on ice and centrifuged (14,000*g* for 10 min at 4°C). Protein concentration was determined by Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA).

Western Blotting, Immunoprecipitation, and Kinase Reaction Assay

Samples for immunoblotting were prepared by mixing aliquots of the protein extracts with 3× SDS sample buffer (150 mM Tris [pH 6.8], 30% glycerol, 3% SDS, bromophenol blue dye 1.5 μg/100 ml, 100 mM DTT) and denatured by heating to 100°C for 4 min. Protein samples were then separated by SDS–PAGE (Bio-Rad

Laboratories) and transferred to a nitrocellulose membrane (Amersham, Arlington Heights, IL) by electrophoresis. The membrane was subjected to immunoblot analysis and proteins were visualized by the enhanced chemilumine scence method of detection (Amersham Pharmacia Biotech, Piscataway, NJ). For immunoprecipitation, total cellular proteins (300 μg) were incubated with the primary antibody for phospho-Erk1/2 for 2 h at 4°C followed by an incubation with protein A plus A/G agarose beads for 1 h. Samples were washed four times in protein lysis buffer, resuspended in 30 μl of 3 × SDS sample buffer, and boiled for 3 min. The proteins were then resolved in a 10% SDS–PAGE gel, transferred to a nitrocellulose membrane, and detected as described for Western blotting.

For kinase reaction assay, the cell extracts were immunoprecipitated with primary antibody as described above. The beads were washed three times in protein lysis buffer, then three times in kinase reaction buffer (Cell Signaling Technology, #9802). The kinase reaction was carried out at 30°C for 15 min in 40 μl of kinase reaction buffer containing 10 μM ATP, 10 μCi [γ-^{32}p]ATP (specific activity 6,000 Ci/mmol, NEN, Boston, MA), 10 μl of GST–PPARγ substrate (purified from *E. coli*, concentration about 100 ng/μl), then stopped by the addition of 3× SDS buffer. After size fractionation on a 10% SDS–PAGE gel, the phosphorylated GST–PPARγ was visualized by autoradiography. To confirm the presence of the kinase protein in the immunoprecipitate, the same gel was subjected to immunoblot analysis.

DNA–Protein Binding Assays

Electrophoretic mobility shift assays were performed as follows: a reaction mixture of binding buffer (50 mM KCl, 20 mM HEPES–KOH (pH 7.5), 10 mM $MgCl_2$, 10% glycerol, 0.5 mM DTT, 1% NP40), 0.2 ng of γ^{32}P-ATP labeled oligonucleotide probe or mutant oligonucleotide probe, 10 μg of sonicated salmon sperm DNA, 2 μg poly(di-DC) (Pharmacia Biotech, Piscataway, NJ), and 10 μg of nuclear protein were incubated at 25°C for 10 min and the reaction products separated on a 4% polyacrylamide gel in 0.25× TBE (22.5 mM Tris-Borate and 0.5 mM EDTA). For antibody perturbation experiments, 2.5 μg of antibody against PPARγ, RxRα, Fos, Jun, or ATF-2, was added 30 min prior to the addition of the oligonucleotide probe and incubated at 25°C. For oligonucleotide competition experiments, 50-fold excess of unlabeled competitor oligonucleotide was added 10 min prior to the addition of the oligonucleotide probe and incubated at 25°C. The double stranded DNA oligonucleotides of PPARγ and AP-1 were purchased from Santa Cruz Biotechnology (PPARγ sc-587) contains the consensus binding site for PPRE (5′-AG GTC AAA GGT CA-3′), AP-1 (sc-2501) contains the consensus binding site of 5′-CGC ATG ACT CAG CCG GAA-3′). ELISA for p65 and p50 DNA binding were performed according to manufacture's protocol (Mercury Transfactor Kit, Clontech, Palo Alto, CA).

In Vitro Protein–Protein Interaction Assay (GST Pull-Down)

GST pull-downs were performed as described elsewhere [Kodera et al., 2000]. Full length PPARγ cDNA expressed as a GST fusion protein was kindly obtained from Shigeaki Kato (Institute of Molecular and Cellular Biosciences, Saitama, Japan). GST–PPARγ fusion protein was purified using a Bulk GST Purification Module (Amersham Pharmacia Biotech Inc, #27-4570-01) following its protocol. One microgram of PPARγ–GST fusion protein was bound to glutathione sepharose beads and incubated with 500 μg of nuclear protein extract overnight. After centrifugation, beads were washed four times with wash buffer (50 mM Tris-HCl, 150 mM NaCl, 0.5% NP40, 50 mM NaF, 0.2 mM $NaVO_4$, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml leupeptin, 25 μg/ml aprotinin, 25 μg/ml pepstatin A), resuspended in Laemmli buffer, boiled for 5 min, and re-centrifuged. The supernatant was loaded on a 10% SDS–PAGE gel, electrotransferred to a nitrocellulose membrane, and the membrane was subjected to immunoblot analysis for PPARγ, RxRα, p65, and p50. To confirm phosphorylated PPARγ binding to p65 we designed a two stage pull down experiment. At the first stage, we phosphorylated the GST–PPARγ fusion protein using the kinase reaction discussed above but did not use [γ-^{32}p]ATP and increased the ATP concentration to 100 μM. After the first stage, the agarose beads were spun, and p65 or p50 were detected in the supernatant by Western blot analysis.

Transient Transfections and Luciferase Assay

The thymidine kinase driven luciferase (tk-luc) reporter plasmid with three copies of

the consensus κB site (3 κB-luc) were kindly provided by J. Cleveland (St. Judes Childrens Hospital, Memphis, TN). The pGS5–PPARγ expression plasmid was kindly provided by Ronald Evans (The Salk Institute, San Diego, CA). The PGJ3TK, a PPAR reporter plasmid in pGL3 was kindly provided by Jack Vanden Heuvel (Pennsylvania State University). For transient transfections, HT-29 cells were seeded in 10% FBS McCoy's media in 24 well plates to 60–80% confluence 24 h before transfection. Lipofectamine 2000 (Invitrogen, Carlsbad, CA, cat#11668-027) reagent was used for transfections following the manufacturer's protocol. To each well, 0.7 μg of PPAR reporter plasmid, 0.2 μg of PPARγ expression plasmid, and 0.1 μg of β-Gal DNA were used. Alternately, 0.8 μg of 3 κB-luc reporter plasmid and 0.2 μg of PPARγ expression plasmid were used. The amount of DNA was normalized by adding the reporter basic vector. Lipofectamine 2000–DNA complexes were added under serum free conditions and after a 24 h incubation, the medium was changed to fresh 0.1% FBS medium and ciglitazone was added and incubated for another 24 h. Cells were washed two times with cold PBS and lysed with 200 μl of cell culture lysis reagent (Promega, Madison, WI, #E153A), Thirty microlitres of cell extract was used for luciferase and 10 μl of cell extract was used for β-Gal assays. LumiCount was used to quantitate luciferase activity (Luciferase Assay System, Promega) and the β-Gal assay kit (Invitrogen, cat#45-0449) was used for β-Gal activities following the kit procotol. The luciferase activity was normalized to β-Gal activity.

Statistics

All experiments were performed in triplicate except where noted. Data are expressed as mean ± SEM. Analysis of continuous data was by analysis of variance (ANOVA, post hoc testing: Bonferroni) or by Student's t test. A P value ≤ 0.05 is defined as significant.

RESULTS

To determine the growth inhibitory activity of ciglitazone, HT-29 cells were treated with ciglitazone (0, 0.5, 1, 3, 5 μM) for 24 and 48 h and viable cell mass was measured by MTT assay. Exposure of ciglitazone to HT-29 colon cancer cells resulted in a significant decrease in viable cells in a time- and dose-dependent fashion (Fig. 1A). Similarly, cell viability was found to decrease under the same experiment conditions using trypan blue exclusion (data not shown). To determine whether an increase in apoptosis was associated with the observed decrease in cell number after ciglitazone treatment, HT-29 cells were treated with ciglitazone at various time points (5 μM for 1, 3, 6, and 24 h) and annexin V staining was performed (Fig. 1B,C). Exposure to ciglitazone resulted in an increase

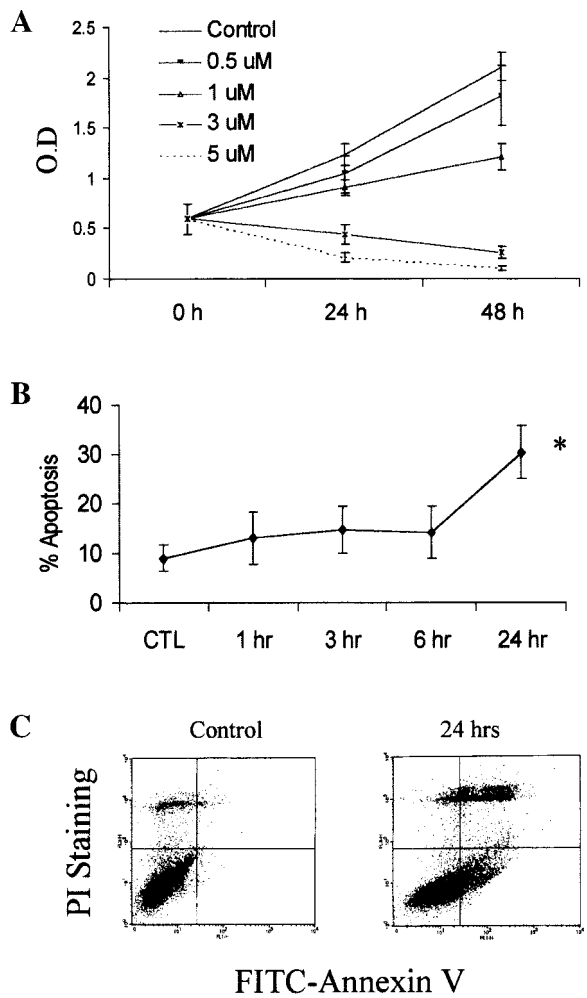

Fig. 1. Ciglitazone induces cell apoptosis in HT-29 colon cancer cells. **A**: HT-29 cells were treated with ciglitazone (0, 0.5, 1, 3, 5 μM) for 24 and 48 h and cell viability was measured using an MTT assay. **B**: HT-29 cells were treated with ciglitazone (5 μM) for 1, 3, 6, and 24 h and the percent of cells undergoing apoptosis as measured by annexin V binding was determined. Apoptosis did not significantly increase until after 6 h of ciglitazone exposure. Data are the results of triplicate experiments and presented as mean ± SEM. *$P = 0.01$. **C**: Representative of annexin staining. Cells staining positive for annexin only (**lower right quadrant**) represent early apoptosis, while those cells staining positive for both annexin and propidium iodide (PI) represent cells in late apoptosis (**upper right quadrant**).

in the number of apoptotic cells in a time-dependent fashion. While no significant increase in annexin V binding was identified by 6 h ($10.3 \pm 3.1\%$) of treatment as compared to control cells ($8.8 \pm 2.5\%$), a significant increase in apoptosis was detected after 24 h exposure ($27.7 \pm 5.1\%$) (Fig. 1B,C).

To elucidate the molecular mechanism by which ciglitazone induces HT-29 cell apoptosis, we next examined the expression of PPARγ and RxR before and after ciglitazone (5 μM) treatment. Protein levels were detected by Western blot in both cytoplasmic and nuclear fraction of HT-29 cells treated with ciglitazone (5 μM; 0, 15 min, 30 min, 1 h, and 6 h) and exposure to the PPARγ ligand did not affect the overall protein levels of either PPARγ or RxR (Fig. 2A). However, an additional band at 58 kDa was noted in the PPARγ immunoblot in the nuclear fraction, corresponding with phosphorylated PPARγ [Mukherjee et al., 1997]. The phosphorylated PPARγ band intensity transiently increased at 30 min after exposure to ciglitazone (Fig. 2A) and this increase was dose-dependent (Fig. 2B).

Since the MAPK kinase signaling pathway has been reported to regulate PPARγ phosphorylation, we next studied the expression and activity of Erk1/2 under the same experiment conditions. While there was no change in total Erk1/2 protein levels with ciglitazone treatment, a transient increase of phosphorylated Erk1/2 was observed at 30 min of ciglitazone exposure, paralleling the increase noted in the phosphorylated PPARγ. *c-fos*, a well described down stream target of Erk1/2 [Karin, 1995], was also increased at 30 min of ciglitazone exposure, which correlated with a transient increase in AP-1 DNA binding at the same time point (Fig. 2C). Finally, using a GST–PPAR fusion protein as substrate, we demonstrated that immunoprecipitated phospho-Erk1/2 was capable of phosphorylation of a GST–PPAR fusion protein in vitro and again, this phosphorylation occurred transiently at 30 min of ciglitazone exposure (Fig. 2D).

To evaluate the effects of ciglitazone on the PPARγ-dependent or genomic pathway, PPARγ–DNA binding and transcriptional activity were measured. PPARγ, heterodimerized with RxRα, was found by gel shift assay to bind PPRE element in HT-29 cell extracts (lanes 2, 5, and 7). Ciglitazone exposure decreased the constitutive DNA binding (lane 3). As controls, the cold homologous competitor oligonucleotide (50× molar excess) competed out the complex (lane 4) and ^{32}p-ATP labeling mutated oligonucleotide lost the PPARγ binding ability (lane 6) (Fig. 3A). Similar to the gel shift assay in Figure 3A, constitutive transcriptional activity was noted in cells transfected with the PPRE reporter and this activity was inhibited by ciglitazone exposure in a dose-dependent fashion. When co-transfection of PPRE-luciferase reporter plasmid with pGS5–PPARγ expression plasmid, the luciferase activity was increased (lane 5, Fig. 3B).

Since ciglitazone treatment induces apoptosis in HT-29 colon cancer cells and NFκβ is an important regulator of programmed cell death, we investigated whether ciglitazone could influence NF-κβ activation. Activation of NF-κβ in HT-29 cells may be mediated by p50 and/or p65 subunits that become liberated from the cytoplasm and rapidly translocated to the nucleus. As shown in Figure 4A, p65 was located in both cytoplasmic and nuclei fractions and overall protein expression was not affected by ciglitazone treatment. Interestingly, p50 was noted only in cytoplasm of HT-29 cells and transmigrated to nuclei following a time-dependent fashion after ciglitazone exposure. Based on the observation that ciglitazone exposure resulted in an increase in nuclear p50 levels, we predicted that p65–DNA binding would be decreased because of p50 homodimer interference. Using ELISA-based assays, we observed that p65–DNA binding was significantly decreased ($P = 0.01$) by 3 h of ciglitazone exposure and this decrease was associated with a concomitant increase in p50–DNA binding (Fig. 4B). To establish whether the decrease in NFκβ DNA binding after ciglitazone treatment correlated with in vivo κB transcriptional activity, HT-29 cells were transiently transfected with a κB-luciferase reporter plasmid. As we have previously shown [Feinman et al., 2002], HT-29 cells demonstrate constitutive κB transcriptional activity. Co-transfection with a PPARγ expression vector modestly decreased κB transcriptional activity. However, NF-κβ transcriptional activity was significantly decreased with PPARγ activation by ciglitazone in a dose-dependent fashion ($P = 0.02$, Fig. 4C).

Based on the observation that ciglitazone results in a decrease in p65 DNA binding and NF-κβ transcriptional activity and that previous reports have suggested that PPARγ can physically associate with and regulate NFκβ [Chung

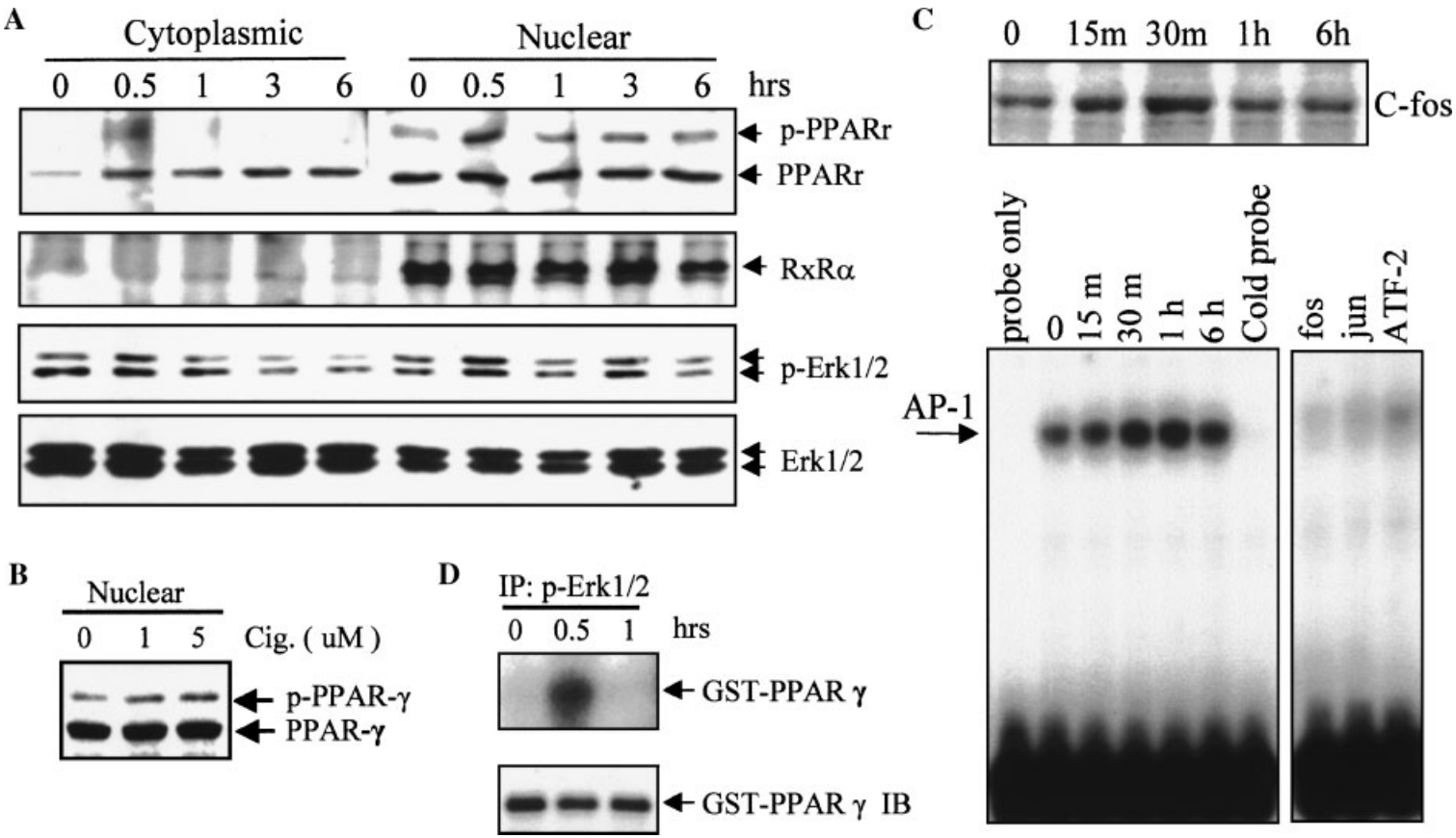

Fig. 2. Ciglitazone phosphorylates PPARγ via active Erk1/2. **A**: Cytoplasmic and nuclear fractions were prepared from HT-29 cell treated with 5 μM ciglitazone for 0–6 h. RxRα was detected only in the nuclear fraction and no changes after ciglitazone treatment were detected. PPARγ was detected in the cytoplasmic and nuclear fractions. While there were no changes in overall levels of PPARγ after ciglitazone treatment, an additional band at 58 kDa was noted in the PPARγ immunoblot of the nuclear fraction, corresponding with phosphorylated PPARγ. Phosphorylated Erk1/2 appeared to transiently increase in the cytoplasmic and nuclear fractions at 30 min exposure to ciglitazone while there were no changes detected in total Erk1/2. **B**: The PPARγ phosphorylation band intensity transiently increased at 30 min after exposure to ciglitazone and this increase was dose-dependent. **C**: HT-29 cells were treated with ciglitazone (5 μM) from 0–6 h and a transient increase of *c-fos* protein levels and AP-1 DNA binding after ciglitazone treatment at 30 min were detected, paralleling the phosphorylation of Erk1/2. **D**: Active Erk1/2 phosphorylates GST–PPARγ. HT-29 cells were treated with ciglitazone (5 μM) from 30 to 60 min. Cells were harvested and 200 μg of cell lysates were used to immunoprecipitate with phospho-Erk1/2 antibody. One portion of the immunoprecipitate was used for kinase reaction, whereas another portion was run on a 7.5% SDS–PAGE and immunoblotted to confirm the presence of GST–PPARγ. Shown are representative immunoblots of three separate experiments.

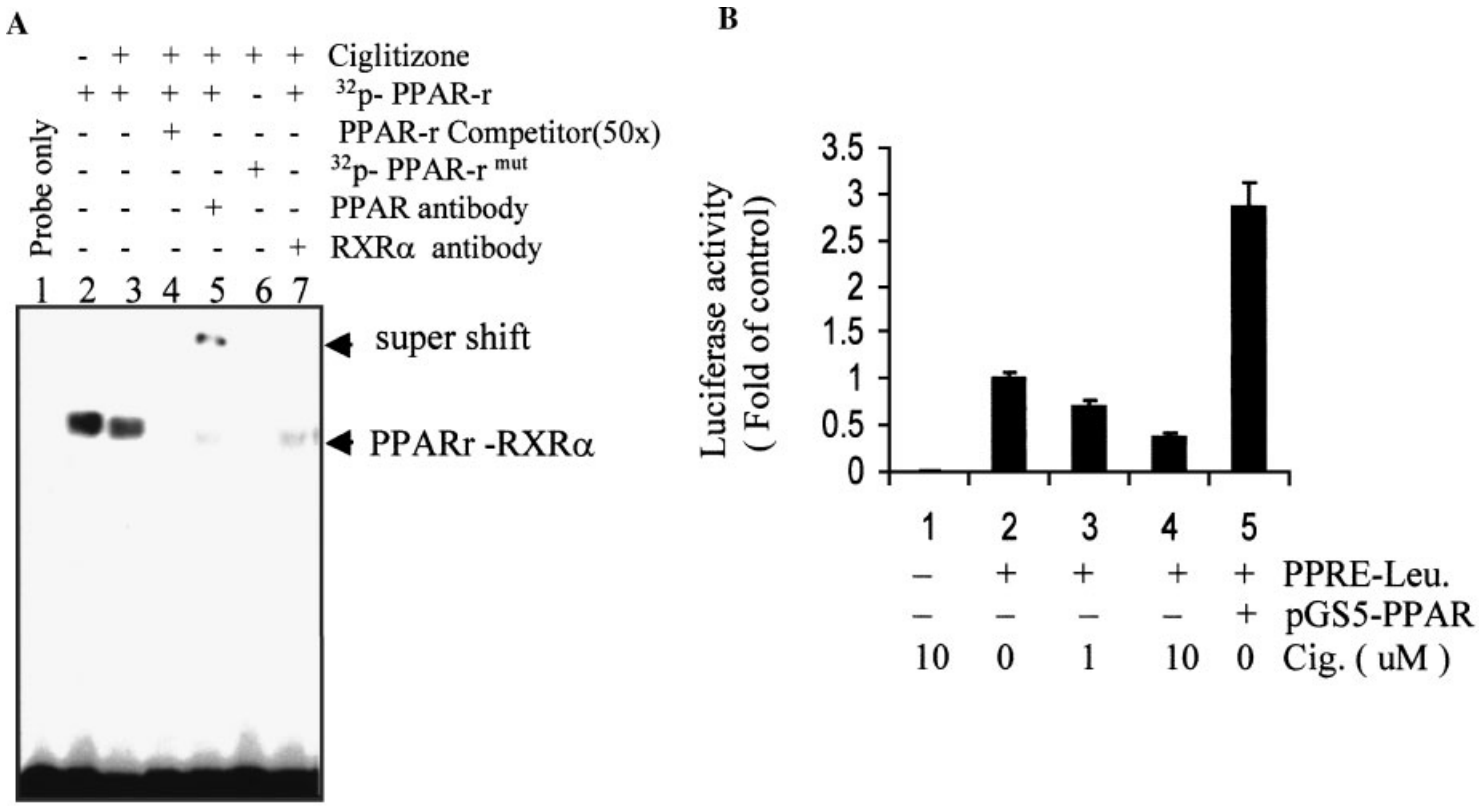

Fig. 3. Alterations in PPARγ DNA binding and transcriptional activity with ciglitazone treatment. **A**: HT-29 cells were treated without or with ciglitazone (5 μM) for 6 h. Cells were harvested, nuclear extracts prepared, and EMSA were performed using an oligonucleotide containing the consensus binding site for PPARγ as described in Materials and Methods. These experiments demonstrated that the PPARγ–RxRα heterodimer existed in HT-29 cells (**lane 2**) and that ciglitazone exposure resulted in an decrease in PPARγ–RxRα DNA binding (**lane 3**). Cold homologous competitor oligonucleotide (50× molar excess) competed with the ^{32}P labeled oligo for complex formation (**lane 4**) and a supershift was demonstrated with PPARγ antibody (**lane 5**, solid arrow), using an oligonucleotide containing an mutant site in the consensus binding site for PPARγ failed to bind to the PPARγ–RxRα complex (**lane 6**), and an antibody against RxRα block the PPARγ–RxRα DNA binding (**lane 7**). Shown are representative data from three separate experiments. **B**: Ciglitazone represses transcriptional activity of PPARγ. HT-29 cells were transiently co-transfected with pGJ3TK-luc, β-Gal expression DNA, pGL3 (lanes 2–4), or PPARγ expression plasmid as described in Materials and Methods. PPARγ transcriptional activity decrease following dosage dependence fashion after ciglitazone treatment (lanes 2–4) and this decrease can be rescued by over expression of PPARγ (lane 5).

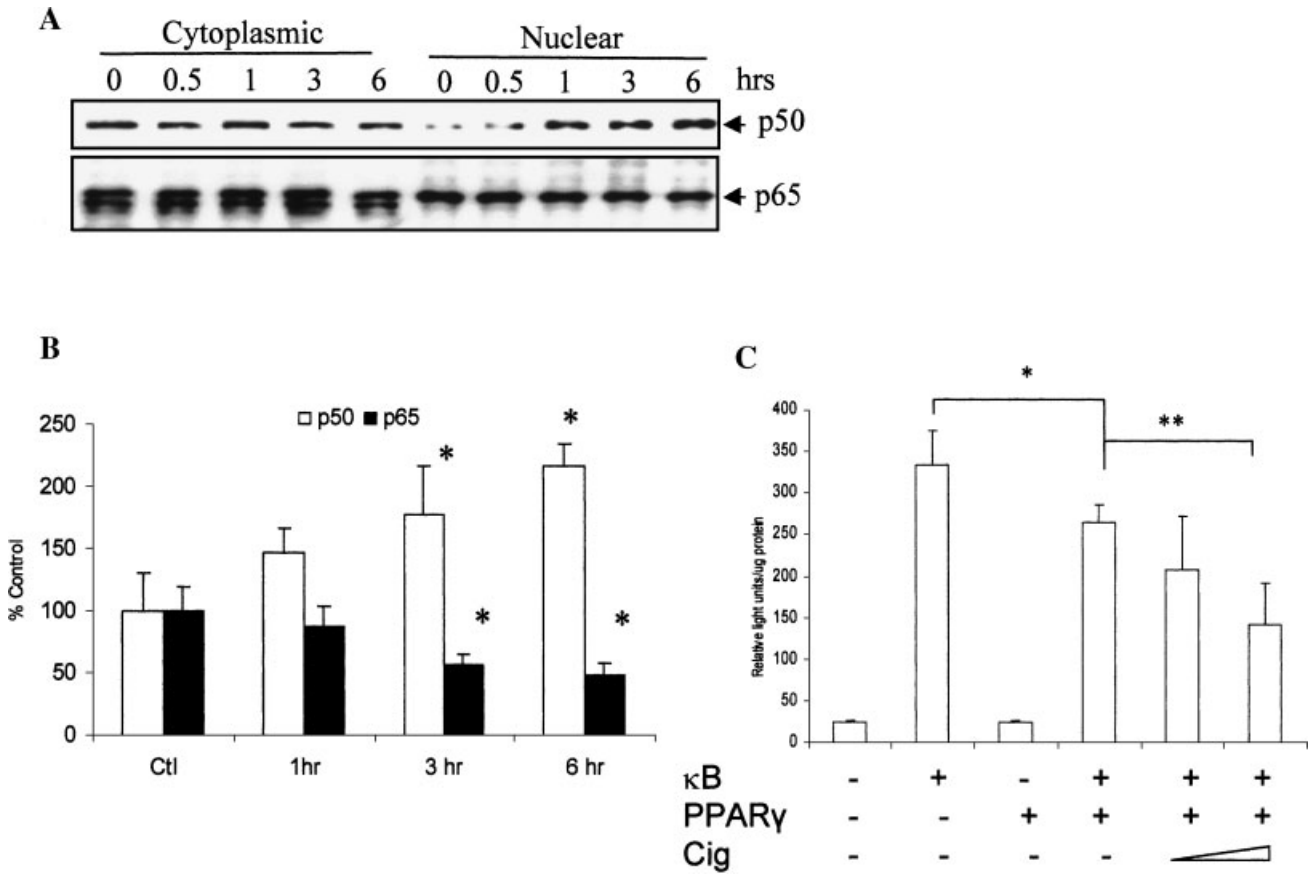

Fig. 4. Ciglitazone inhibits κB-dependent transcriptional activity. **A**: Western blot analysis of nuclear and cytoplasmic fractions for p65 show no difference in protein levels while nuclear levels of p50 increased dramatically with ciglitazone exposure. **B**: HT-29 cells were treated with ciglitazone (5 μM) and NF-κβ–DNA binding was assessed by ELISA. p65–DNA binding significantly decreased by 3 and 6 h, while p50–DNA binding significantly increased at the same time points. Data are the results of triplicate experiments and are presented as % control ±SEM. $^{*}P < 0.05$ as compared to control. **C**: Transient co-transfection of HT-29 cells with a κB reporter vector and PPARγ expression vector demonstrate that PPARγ activation is associated with NF-κβ inactivation. While the addition of the PPARγ expression vector modestly decreases κB dependent luciferase expression, activation of PPARγ with ciglitazone decreases NF-κβ transcriptional activity in a dose dependent manner. Data are the results of duplicate experiments and are presented as relative light units/μg protein ±SEM. $^{*}P = 0.02$, $^{**}P = 0.05$ as compared to control.

et al., 2000], we evaluated the effects of PPARγ activation on the physical interaction between p65 and PPARγ. Cell lysates were initially immunoprecipitated using an anti-PPARγ antibody, and the subsequent immunoblotted using an anti-p65 and p50 antibody. There was faint binding between p65 and PPARγ detected in untreated control cells. However, cells exposed to ciglitazone for 30 min revealed an obvious physical association between p65 and PPARγ and this binding decreased after 1 h exposure of ciglitazone. The same membrane was stripped and re-probed with anti-PPARγ antibody to confirm that immunoprecipitated PPARγ protein was equal (Fig. 5A). Similar experiments assessing the binding between PPARγ and p50 revealed no physical interaction (data not shown). The observation that PPARγ physically

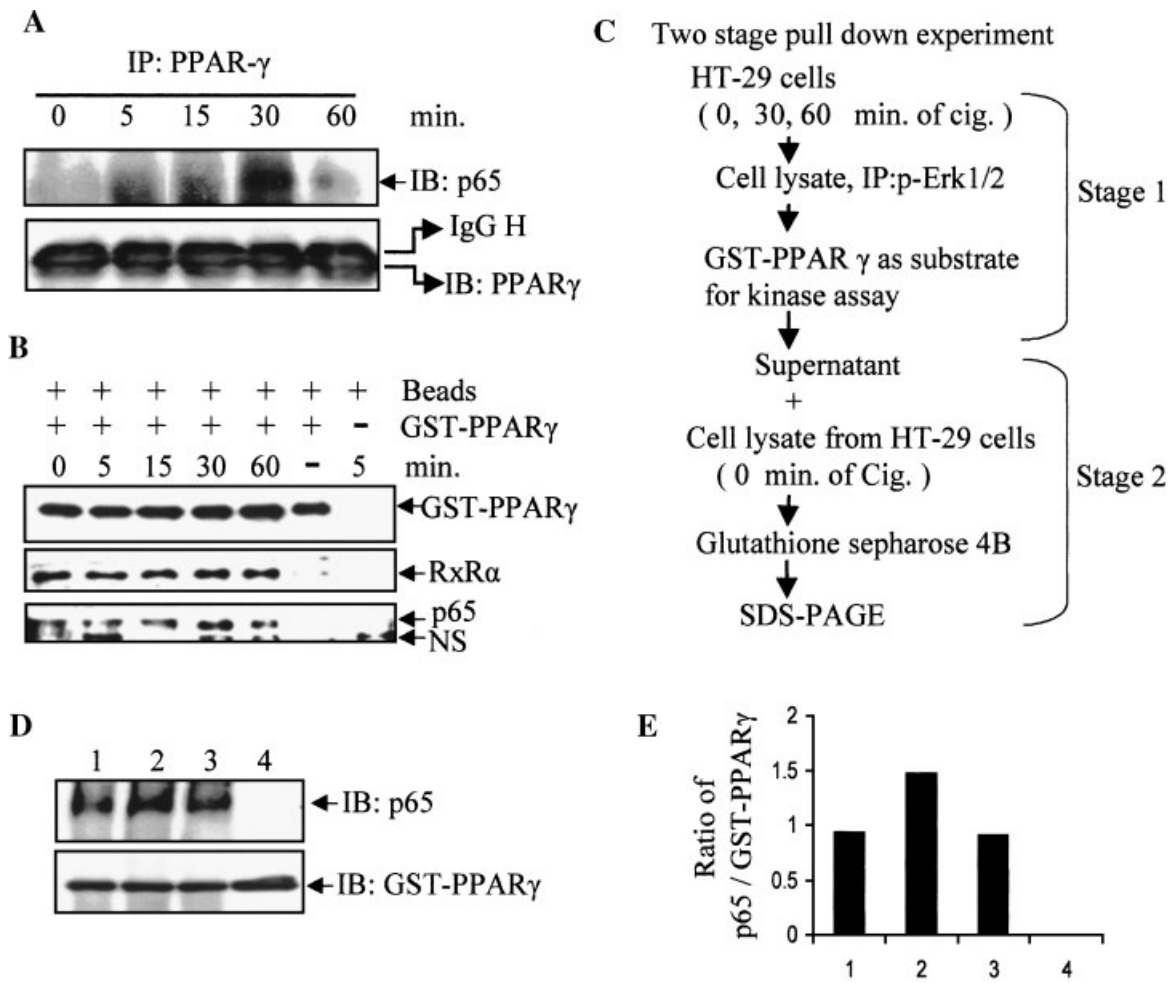

Fig. 5. The physical interaction between p65 and phosphorylated PPARγ. **A**: Nuclear fractions were prepared from HT-29 cell treated with 5 μM ciglitazone for 0–6 h. Immunoprecipitated PPARγ was determined to be physically associated with p65, which peaked after 30 min. Equal amounts of PPARγ protein was determined by reprobing the same membrane with antibody against PPARγ. No p50–PPARγ complex was detected (data not shown). Shown is a representative immunoblot of three separate experiments. **B**: In vitro GST–PPARγ pull down of RxRα and p65 from total protein extracts of HT-29 cells exposed to ciglitazone (5 μM) from 0 to 1 h. While the binding of PPARγ with RxRα was constant at all time points, the binding of PPARγ–GST to p65 increase transiently and reached the maximum binding at the 30 min point. Beads incubated with GST–PPARγ, or with nuclear fraction represent non-specific controls. NS, non-specific band. **C**: Schematic of two stage experiment. **D**: Phosphorylated PPARγ has increased binding affinity with p65 in vitro. GST–PPARγ was phosphorylated by immunoprecipitated active Erk1/2 from total extracts of HT-29 cells exposed to ciglitazone for 30 min and the phosphorylated GST–PPARγ was then used to pull down p65 from HT-29 cells (no ciglitazone treatment). While minimal p65 could be pulled down with the GST–PPARγ protein in untreated control cells (**lane 1**), the physical interaction between p65 and PPARγ transiently increased at time point at which GST–PPARγ was phosphorylated by active Erk1/2 (**lane 2**). Normal IgG was used as negative control (**lane 4**). The same membrane was stripped and reprobed with PPARγ antibody to confirm that equal amounts of GST–PPARγ were added. Shown is a representative immunoblot of two separate experiments. **E**: Densitometry and quantification of figure D.

interacted with p65 was confirmed using a GST–PPARγ pulldown experiments. As shown in Figure 5B, GST–PPARγ fusion protein with beads or nuclear extract (5 min treatment of ciglitazone) incubated with beads (no GST–PPARγ added) revealed no evidence of RxRα or p65 (negative controls). When the GST–PPARγ fusion protein was incubated with nuclear extract and glutathione beads, p65 was detected and the amount of p65 associated with PPARγ increased with ciglitazone treatment following a time-dependent fashion peaking at 30 min. The amount of RxR associated with PPARγ was unchanged.

Given the data that the maximum binding of PPARγ with p65 was at 30 min of ciglitazonetreatment, paralleling the time point of phospho-Erk1/2 and phospho-PPARγ after ciglitazone treatment, we hypothesize that the binding of PPARγ with p65 occurs with PPARγ phosphorylation. To investigate this, phospo-Erk1/2 was immunoprecipitated from untreated cells and cells exposed to ciglitazone (5 μM at 30 min and 1 h). Subsequently, GST–PPARγ fusion protein was incubated with the activated Erk1/2 and the phosphorylated GST–PPARγ fusion protein was used to pull down p65 from control HT-29 cell lysates. We demonstrated that the maximum binding of PPARγ with p65 was at 30 min of ciglitazone treatment, suggesting that phosphorylated PPAR binds with p65 (Fig. 5D).

DISCUSSION

While early investigations of PPARγ, a member of the steroid receptor super family, have centered on its effect on lipid metabolism, recent studies suggest that PPARγ may be involved in colon carcinogenesis. PPARγ is expressed at relatively high levels in normal human colon mucosa, has been shown to be aberrantly expressed in colon tumors and a growing number of PPAR ligands have been shown to have growth inhibitory effects in many cancer cell types, including colon cancer in both in vitro and in vivo models [DuBois et al., 1998; Sarraf et al., 1998; Tanaka et al., 2001]. Classically, steroid hormones exert their effect through binding and activation of their corresponding response elements, activating target gene transcription. Alternatively, there is a growing body of evidence that steroid hormones have a more rapid effect through so called non-genomic pathways in which ligand activated PPARγ nuclear receptor suppresses other gene expression by antagonizing transcriptional factors [Camp and Tafuri, 1997; Hsi et al., 2001; Takeda et al., 2001].

We demonstrate that ciglitazone, a specific PPARγ ligand, decreased cell proliferation and induced apoptosis in a colon cancer cell line. Associated with this decreased cell proliferation and induction of apoptosis was the observation that ciglitazone exposure leads to rapid phosphorylation of PPARγ via the MAPK kinase signaling pathway. This phosphorylation of PPARγ affected both the genomic pathway by inhibiting PPAR–DNA binding and PPRE transcriptional activity, as well as the non-genomic pathway by increasing the physical interaction of PPARγ with p65, leading to the decreased transcriptional activity of NF-κβ.

An important mechanism of regulation of PPARγ is through post-translational phosphorylation. The amino terminal A/B domain of PPARγ has been shown to contain a consensus MAP kinase site and phosphorylation of this site inhibits transactivational functions [Adams et al., 1997]. Growth factors such as epidermal growth factor and platelet derived growth factor have been shown to phosphorylate PPARγ via the MAP kinase signaling pathway and Camp and Tafuri report that this phosphorylation-mediated transcriptional repression is not due to a reduced capacity of the PPARγ–RxRα complex to heterodimerize or to recognize its DNA binding site, but its ability to become transcriptionally activated by the ligand [Camp and Tafuri, 1997]. Despite the fact that ciglitazone is a PPARγ ligand, we noted that both DNA binding and transcriptional activity of PPARγ was decreased with ciglitazone exposure (Fig. 3). This decrease in transcriptional activity was surprising. Others have noted that ciglitazone is a weak PPAR ligand [Qin et al., 2003] and we hypothesize that the decrease in DNA binding and transcriptional activity may in part be the result of increased inhibitory phosphorylation of PPARγ in combination with a weak genomic response. Additional possible mechanism is the recruitment of co-repressor proteins. For example, MAP kinase-dependent phosphorylation has been shown to inhibit AF-2 function via SMRT recruitment [Hong and Privalsky, 2000].

Since PPARγ activation induces apoptosis in HT-29 colon cancer cells and NF-κβ is an important regulator of programmed cell death, we

investigated the effects of ciglitazone on the NF-κβ pathway. The transcription factor NF-κβ plays a pivotal role in the regulation of apoptosis by direct regulation of genes that inhibit or promote apoptosis, through regulation of the cell cycle, which sensitizes or desensitizes a cell to apoptotic signals and lastly, through interactions with other proteins involved in cell survival [Barkett and Gilmore, 1999]. NF-κβ is a family of several structurally related proteins (p50, p52, p65, and Rel B) that form dimers and subsequently bind to the κB DNA site. IκB, a cytoplasmic inhibitor, tightly regulate NF-κβ activity by complexing with the transcription factor and trapping it in the cytoplasm. Upon phosphorylation of IκB's serine residues by IκB kinase (IKK), p65 is released, allowing nuclear transmigration. The phosphorylated IκB subsequently undergoes ubiquitination and subsequent degradation [Jobin and Sartor, 2000].

The predominant subunits of NF-κβ in HT-29 cells are p65 and p50 and similar to our previous report [Feinman et al., 2002], we detected both p50 and p65 in HT-29 colon cancer cells and demonstrated that ciglitazone treatment resulted in a decrease in the p65–DNA binding, with a concomitant increase in the p50–DNA binding (Fig. 4). This would suggest that in our model, p65 provides transcription of pro-survival genes and that ciglitazone, by inhibiting p65 binding and transcriptional activity, allows the cell to undergo apoptosis. Transcriptional activity is dependent on both DNA binding as well as the constituents of the NF-κβ dimer. In general, the p65 subunit activates κB transcriptional activity, while p50, which lacks the acidic transcriptional activation domain, is a weak activator and often at times, acts as a repressor [Schmitz and Baeuerle, 1991]. This is relevant, since constitutive expression of p65 has been shown to be important in promoting survival and oncogenesis in a variety of cancers [Bargou et al., 1997; Nakshatri et al., 1997; Visconti et al., 1997]. Supporting the role of p65 as a anti-apoptotic factor includes the fact that NF-κB regulates the expression of multiple pro-survival Bcl-2 homologues [Pahl, 2000] and that mouse p65 knockouts will die by day 10 of embryonic development and histological evaluation of these mice demonstrate massive hepatic apoptosis [Beg et al., 1995]. In addition, cells overexpressing p65 have been shown to be resistant to pro-apoptotic therapy [Anto et al., 2000].

While the function of the NF-κβ and PPARγ pathways have been separately described in colon cancer models [Brockman et al., 1998; DuBois et al., 1998; Plummer et al., 1999; Inan et al., 2000], the concept that PPAR and NF-κβ pathways interact is beginning to be explored [Chinetti et al., 1998]. Recently, it has been suggested that PPAR ligands exert their pro-apoptotic effects by antagonizing the NF-κβ pathway [Su et al., 1999]. Oxidized low-density lipoprotein (oxLDL), a molecule known to act as a PPAR ligand, has been shown to modulate transcriptional activity of PPAR and NF-κβ in an inverse manner. Exposure to oxLDL activates PPAR, while inhibiting NF-κβ activity [Han et al., 2000]. Similarly, we observed that exposure to a PPARγ-specific ligand dramatically decreases NF-κβ–DNA binding and transcriptional activity in HT-29 colon cancer cells (Fig. 4C). The mechanism of how activated PPAR inhibits NF-κβ has yet to be fully dissected. Some studies suggest that the PPAR-induced inhibition of NF-κβ is through modulation of the IκB pathway, while others suggest that this inhibition is through direct physical interaction of PPAR with NF-κβ [Delerive et al., 1999; Chung et al., 2000] and these two pathways may not be mutually exclusive. Delerive et al. [1999] reported that overexpression of PPARα represses p65 transcriptional activity and found that in vitro translated ^{35}S methionine-labeled p65 physically bound to PPARα.

Given our data that the maximum binding of PPARγ with p65 was at 30 min of ciglitazone treatment, paralleling the time point of phospho-Erk1/2 and phospho-PPARγ after ciglitazone treatment, we hypothesize that the binding of PPARγ with p65 occurs with PPARγ phosphorylation. We observed the physical interaction of PPARγ with p65 increases with PPARγ activation and we report the novel observation that the PPARγ–p65 interaction increased with phosphorylation of PPARγ (Fig. 5). This physical interaction may offer in part an explanation as to why ciglitazone exposure leads to PPRE and κB inactivation. We hypothesize that after phosphorylation by Erk1/2, PPARγ bind p65 more avidly and this PPARγ–p65 complex sequesters both proteins, therefore reducing binding to their respective response elements. An additional rationale for the observed decrease in κB transcriptional activity after ciglitazone exposure is related to the increased nuclear protein levels of p50. In untreated HT-29 cells, no

appreciable levels of p50 were noted in the nuclear protein fraction. However, immediately after ciglitazone exposure, nuclear p50 levels dramatically increased (Fig. 4A). It is likely that this increase in nuclear p50 protein levels leads to an increase in the p50:p50 inhibitory homodimer binding to the κB response element.

In summary, we have shown that prior to apoptosis, ciglitazone exposure results in a transient phosphorylation of PPARγ through the MAP kinase signaling pathway. This phosphorylation of PPARγ is associated with a decrease of DNA binding and transcriptional activity of PPRE. In addition, the phosphorylation of PPAR increases its physical interaction with p65 and a subsequent decrease of κB transcriptional activity. Ciglitazone induced phosphorylation of PPARγ through the MAP kinase pathway provides a potential regulatory mechanism of PPARγ's physical interaction with p65, leading to inhibition of NF-κβ and subsequent apoptosis.

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