

[BIO25] Ciglitazone inhibits growth and induces apoptosis in human colorectal cancer cell lines independently of PPAR γ

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Introduction

The nuclear hormone receptor peroxisome proliferator-activated receptor (PPAR) γ is a ligand-activated transcription factor that regulates several biological processes such as adipogenesis, glucose homeostasis and cell growth. Several polyunsaturated fatty acids, the thiazolidinediones (TZDs) class of antidiabetic drugs and the nuclear prostanoid 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (15d-PGJ₂) has been identified as ligands for PPAR γ (for review, see Na and Surh, 2003).

PPAR γ is expressed at significant levels in human liposarcoma and breast adenocarcinoma and treatment with PPAR γ ligands reduces the growth rate and induces terminal differentiation of these malignant cells (Tontonoz *et al.*, 1997, Mueller *et al.*, 1998). In the large intestine, PPAR γ is expressed in human colonic mucosa, colon adenocarcinoma and cultured colon cancer cells (Sarraf *et al.*, 1998). However, the exact role of PPAR γ in colon cancers has not been elucidated.

In the present study, we investigate the effect of ciglitazone, a PPAR γ ligand on the mRNA and protein expression of PPAR γ and its ability to inhibit growth and induce apoptosis in two colon cancer cell lines: HT-29 and COLO 205. We found that ciglitazone inhibited growth and induced apoptosis in both cell lines. Unexpectedly, results from this study also showed that ciglitazone activity downregulated PPAR γ mRNA and protein expression. Therefore, we suggest that the antitumour effects of ciglitazone may be independent of PPAR γ .

Materials and methods

Chemicals

Ciglitazone (Cayman Chemicals, USA) was dissolved in dimethyl sulfoxide (DMSO) (Sigma, USA) to obtain a 100 mM stock solution. Final concentration of the

DMSO in culture medium was not more than 0.03%.

Cell culture

HT-29 and COLO 205 were cultured in RPMI 1640 (Gibco BRL, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL) and 100 U/ml and 100 mg/ml Penicillin/Streptomycin solution. The cells were maintained in a humidified atmosphere in 5% CO₂ at 37°C.

Cytotoxicity Assay

As an index of cytotoxicity, lactate dehydrogenase (LDH) leakage was measured using the Cytotoxicity Detection Kit (LDH) (Roche, Germany). Cells were seeded in 24-well plates at a concentration of 1×10^5 cells/ml in RPMI (the optimum cell number for this assay was determined in preliminary experiments) until they reached about 70% confluence. The growth medium was removed and replaced with RPMI containing 2% FBS. Cells were incubated with various concentrations of ciglitazone for 6 to 72 h. To determine maximum LDH release, cells were solubilised with a final concentration of 1% (w/v) Triton X-100. Spontaneous LDH release (low control) was determined by incubating the cells with medium alone. Supernatants were removed and centrifuged. Cell-free supernatants were then transferred to clean 96-well plates. LDH activity was assayed in the supernatants by a reaction in which the tetrazolium salts, INT, was reduced to a red formazan salt. Absorbance was read at 490 nm with a microplate reader. Results were expressed as % cytotoxicity [(experimental value-low control/high control-low control) \times 100]. Effective concentration (EC₅₀) values were expressed as micromolar of ciglitazone concentration that causes a 50% growth inhibition as compared to controls.

RNA isolation and real-time quantitative polymerase chain reaction

Total RNA was isolated from cells using the RNeasy™ Total RNA Kit (Qiagen, Germany) and first strand cDNA was synthesised using the Revert Aid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, USA). Real-time quantitative PCR was performed in the ABI Prism 7000 Sequence Detection System (Applied Biosystems, USA) using Taqman probes and primers. To prepare standard samples for real-time PCR, conventional RT-PCR was performed and PCR products were cloned into the TOPO® vector (Invitrogen, USA) and transformed into *E.coli* competent cells. The recombinant plasmid was isolated and sent for sequencing to confirm the presence of PPAR γ 1 fragment. The concentration of the plasmid was adjusted to 1 pmol/ μ l and used as standard samples (ranging from 10^{-4} to 10^{-8} pmol copies) for generating a standard curve for each quantitative PCR experiment. The primer sequences for PPAR γ 1 gene were as follows: forward primer, 5'-CTT TAT GGA GCC CAA GTT TGA GTT-3' and reverse primer, 5'-GGC TTC ACA TTC AGC AAA CCT C-3' (129 bp). The sequences for PPAR γ 1 probe was 5'-CAC TGG AAT TAG ATG ACA GCG ACT TGG CA-3'. The reaction was initiated at 50°C for 2 min. This step was required for optimal AmpErase® uracyl-N-glycosylase (UNG) enzyme activity. The temperature was increased to 95°C for 10 min to activate the AmpliTaq Gold enzyme. This was followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min.

Western Blotting

Cells grown in 75 cm²-flasks were harvested and lysed in lysis buffer containing 50 mM Tris HCl, 150 mM NaCl, 1% Nonidet P-40 (NP-40), 0.2% sodium dodecyl sulfate (SDS), 1 mM phenylmethylsulfonyl flouride (PMSF), 2 μ g/ml leupeptin, 2 μ g/ml aprotinin and 1 mM sodium orthovanadate (Na₃VO₄). The cell homogenates were collected by centrifugation at 12,000 rpm at 4°C, and protein concentrations were determined using the Bio-Rad Protein Assay Kit (Bio-Rad, CA). One hundred micrograms of protein from whole cell lysates were electrophoresed through a 10% SDS-polyacrylamide gel. Proteins were transferred onto Hybond™ ECL nitrocellulose

membrane (Amersham Biosciences, UK). The membrane was blocked with 3% skim milk and incubated overnight at 4°C with 1:500 goat anti-human PPAR γ polyclonal antibody (Santa Cruz Biotechnology, CA). After three washes with 0.1% Tween 20 in TBS, the membranes were incubated for 1 h at room temperature with peroxidase conjugated rabbit anti-goat IgG (1:1000 dilution) (Dako, Denmark). The membranes were again washed, and peroxidase were detected with an enhanced chemiluminescence system (Amersham Biosciences, UK) using the Hyperfilm™ ECL (Amersham Biosciences, UK).

Detection of apoptosis

Cells grown in 25 cm²-flasks (1×10^5 cells/ml) were treated with either ciglitazone (at EC₅₀ values) or DMSO (control). After a fixed period of incubation, adherent and floating cells were combined and washed twice with PBS containing 0.1% Tween-20. Incubation of cells with M30 cytoDEATH antibody was then performed for 60 min at room temperature. The cells were washed again twice before resuspended in PBS. Analysis was done using a flow cytometer (Becton Dickinson, USA).

Calculation and statistical analysis

The experiments were carried out in triplicates for three times (n=3). Data are given as mean \pm standard error mean (sem). EC₅₀ values were obtained as half-maximum-effect concentrations from the response curves. Significance was determined by conducting an independent Student T-test, and considering p<0.01 to be statistically significant.

Results

Ciglitazone inhibits growth of colon cancer cells in vitro

The cell lines were cultured in the presence of various concentrations of ciglitazone to investigate the effect of ciglitazone on cell growth. Figure 1 shows that ciglitazone markedly inhibited cell growth in HT-29 and COLO 205 (p<0.01). The EC₅₀ values of ciglitazone at 24 h were obtained at about 20 μ M for HT-29 and about 30 μ M for COLO 205. At all exposure times, a 100 μ M of ciglitazone concentration caused more than

80% cell death. A significant inhibition of ciglitazone was seen at concentrations of 30

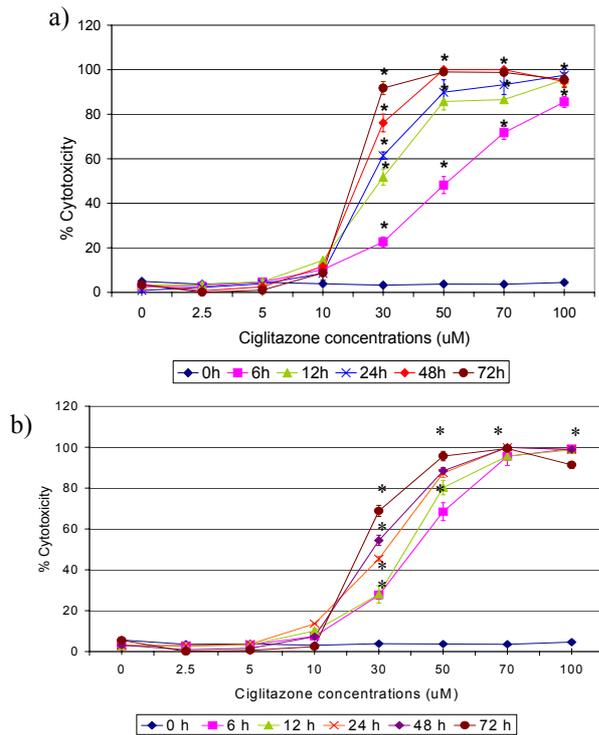


FIGURE 1 Cytotoxic effect of ciglitazone in a) HT-29 and b) COLO 205 cells. Cells were treated with ciglitazone (2.5-100 μM) or DMSO (control) for 0-72 h. Cytotoxicity was measured based on LDH release by the cells. Data are given as mean ± standard error of mean (sem) (n=3). Significance was determined by conducting an independent Student T-test. *p<0.01 were considered statistically significant (compared to control).

μM and higher, and was observed even as early as 6 h.

Ciglitazone downregulates PPARγ mRNA levels in colon cancer cell lines

TZDs are being tested in clinical trials for the treatment of human cancers expressing high levels of PPARγ because it is assumed that activation of PPARγ mediates their anticancer activity (Grommes *et al.*, 2004). In an effort to determine whether ligand treatment increases PPARγ transcriptional activity, we cultured 2 colorectal cancer cell lines with ciglitazone (at EC₅₀ values) for up to 72 h and analysed for the PPARγ mRNA levels. Real-time PCR analysis demonstrated that the mRNA level was higher in HT-29 compared to COLO 205 in both control and treated groups. Unexpectedly, the levels of mRNA were significantly reduced in

ciglitazone-treated cell lines at all time points (Figure 2). A decrease in PPARγ transcripts was observed in both cell lines as early as 6 h of ciglitazone treatment and this reduced level was maintained for up to 72 h in both cell lines.

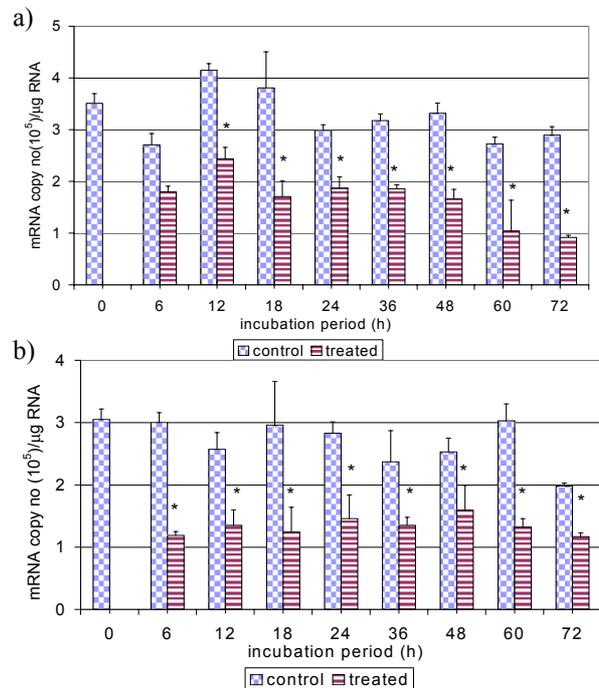


FIGURE 2 Ciglitazone effects on PPARγ1 mRNA expression in a) HT-29 and b) COLO 205 cell lines. Cells were treated with 20 μM (HT-29) or 30 μM (COLO 205) ciglitazone for 6-72 h. Gene expression was measured by quantitative real-time PCR. Data are expressed as mean ± sem (n=3). *p<0.01, significantly different from untreated control cells.

Effects of ciglitazone on PPARγ protein levels in colon cancer cell lines

Since ciglitazone activity downregulates PPARγ mRNA expression in the cell lines, it prompted us to determine whether this ligand has the same effect on PPARγ protein levels. For both HT-29 and COLO 205 (Figure 3), high PPARγ protein expression was detected in control groups at all time points. For HT-29, a decreased in protein expression can be detected as early as 12 h. PPARγ protein level remained lower in treated cells. However, for COLO 205, the effect of ciglitazone was observed after 36 h where the protein level in treated cells decreased dramatically compared to untreated cells. PPARγ has a molecular weight of about 52 kD.

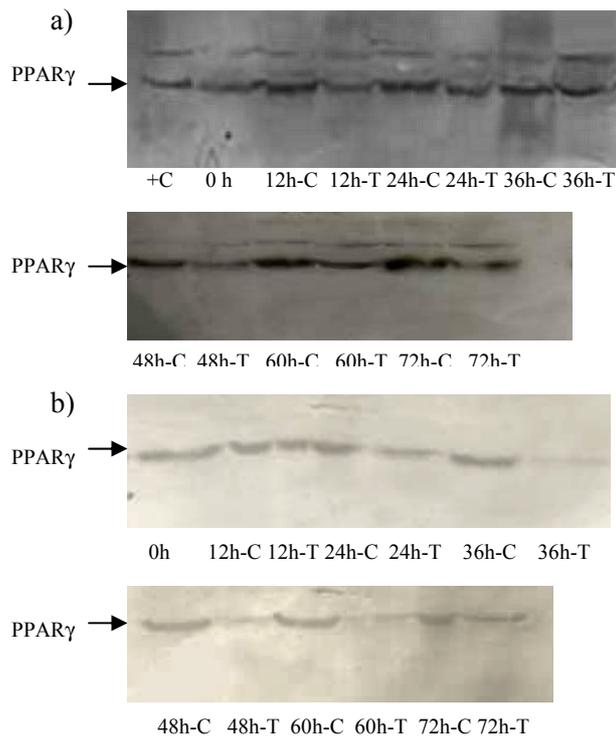


FIGURE 3 Western blot analysis for PPAR γ protein in a) HT-29 and b) COLO 205 cell lines. Cells were treated with 20 μ M (HT-29) or 30 μ M (COLO 205) ciglitazone for 12-72 h. Cell lysates electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gel (100 μ g/lane). Proteins from gels were transferred to nitrocellulose membrane, and PPAR γ was detected with goat anti-human PPAR γ polyclonal antibody and visualised with enhanced chemiluminescence. +C: positive control, C: control, T: ciglitazone-treated

Induction of apoptosis by ciglitazone in human colorectal cancer cell lines

The percentages of HT-29 and COLO 205 cells undergoing apoptosis after 6-48 h of ciglitazone treatment were shown in Figure 4. For both cell lines, there was no significant difference in the percentage of apoptotic cells between untreated cultures and treated groups for up to 12 h of incubation. A remarkable increase in the number of apoptotic cells treated with ciglitazone was observed at 24 h onwards. At 48 h of treatment, about 64% of HT-29 and 43% of COLO 205 cells underwent apoptosis. The apoptotic effect was negligible in cells treated with DMSO at all incubation periods.

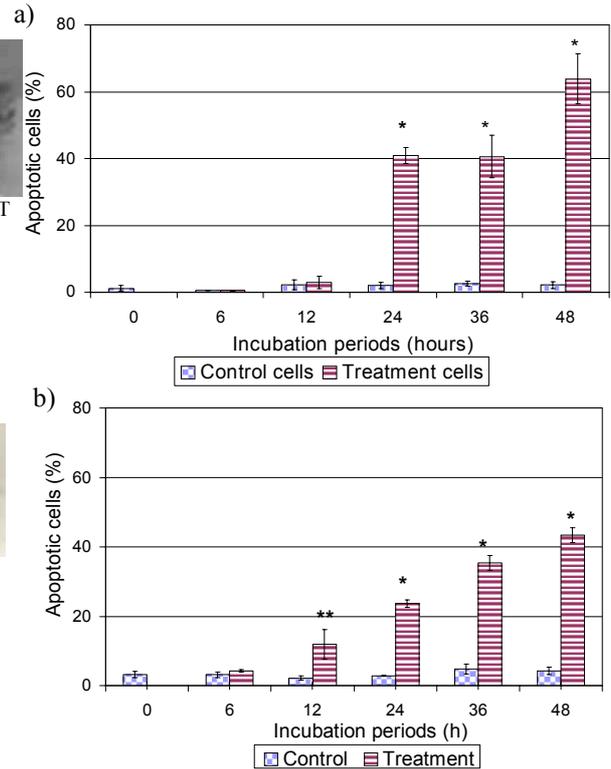


FIGURE 4 Induction of apoptosis by ciglitazone in a) HT-29 and b) COLO 205 cells. Cells were treated with either DMSO as a control or 20 μ M (HT-29) or 30 μ M (COLO 205) ciglitazone for 0-48 h. Cells were incubated with M30 cytoDEATH antibody for CK18 detection. Data shown are the mean values \pm s.e.m (n=3). Statistical analyses were performed using independent Student T-test. *p<0.01 and **p<0.05 were considered statistically significant (compared to control).

Discussion

Previous studies have reported the expression of PPAR γ and the antitumour effects of its ligands in human malignancies (for review, refer to Koeffler, 2003). In the present study, we examined the expression of PPAR γ and any antiproliferative effect of its synthetic ligand, ciglitazone, in human colorectal cancer cell lines. Real-time PCR and western blot analyses have shown that PPAR γ mRNA and protein levels were downregulated. The exact mechanism for this downregulation is unclear, however, our results are in agreement with a few previous studies.

In 1999, Camp and colleagues demonstrated that troglitazone reduced the PPAR γ mRNA and protein levels as well as the DNA binding activity of PPAR γ in 3T3-L1 fully

differentiated adipocytes. BRL49653 (also known as rosiglitazone) reportedly has similar suppressive effect on PPAR γ mRNA and protein levels in adipocytes (Rosenbaum and Greenberg, 1998). Perrey *et al.* (2001) also found that TZDs and the natural PPAR γ ligand, 15d-PGJ₂ acutely downregulated the expression of PPAR γ mRNA in mature adipocytes. These three studies, however, did not clarify the exact mechanism underlying the downregulation of PPAR γ , although Perrey *et al.* (2001) suggested that this was a phenomenon to protect the receptors from excessive consumption. In 2000, Hauser and colleagues reported that PPAR γ protein levels were significantly reduced in adipose cells and fibroblasts in response to TZDs treatment. They suggested that degradation of PPAR γ is correlated with the ability of the ligands to activate the receptor. Although ligand binding and activation of the activation function-2 (AF2) domain induce the transcriptional function of PPAR γ , these same processes also induce ubiquitination and subsequent degradation of the receptor by proteasome (Hauser *et al.*, 2001).

In this study, results have shown that although ciglitazone downregulated PPAR γ mRNA and protein expression levels, the same ligand significantly inhibited growth and induced apoptosis in the cells. These results suggest that ciglitazone suppresses the growth of human colorectal cancer cells independent of PPAR γ . In 2001, Palakurthi and colleagues reported that the antiproliferative effect of TZDs is independent of PPAR γ and mediated instead by the inhibition of translation initiation. This is due to activation of protein kinase R that phosphorylates the α subunit of eukaryotic initiation factor 2 (eIF2), thus rendering eIF2 inactive.

A study by Nakashiro and colleagues (2003) have shown that troglitazone significantly suppresses the growth of human oral squamous carcinoma (OCCC) cells, such as BHY cells, which do not express PPAR γ mRNA and protein. Although troglitazone significantly suppressed the growth of OCCC cells, it did not induce activation of PPAR γ (Nakashiro *et al.*, 2003). Furthermore, a more recent study demonstrated that 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO), another ligand for PPAR γ , had similar growth inhibitory activity in all cell lines expressing

different levels of PPAR γ expression (Melichar *et al.*, 2004). Moreover, the growth-inhibitory activity of CDDO was unaffected by PPAR γ antagonist T007 (Melichar *et al.*, 2004). These results suggest that the growth-inhibiting action of synthetic ligands may depend on some other mechanism without affecting PPAR γ activation. Likewise, Li and colleagues (2001) demonstrated that 15d-PGJ₂ induced apoptosis in human myofibroblasts independently of PPAR γ , since PPAR γ is not expressed in these cells. According to their report, the receptor ligand caused rapid production of reactive oxygen species (ROS) while inducing apoptosis.

In this study, although the mRNA expression level seems identical between both cell lines, HT-29 expresses higher level of PPAR γ mRNA expression compared to COLO 205. This may be due to the fact that, HT-29 is more differentiated than COLO 205, since it originated from a well-differentiated adenocarcinoma, compared to COLO 205 cell line, which was established from a metastatic colonic adenocarcinoma. Sasaki *et al.*, (2002) reported that there was a tendency towards higher levels of PPAR γ expression levels with higher differentiated lung cancer.

PPAR γ has been shown to play an important role in regulating cell growth and death in various cancer cells. However, many factors contribute to the expression and regulation of PPAR γ activity. Whether PPAR γ has a direct role in cancer cell growth remains to be elucidated. The anticancer activity of TZDs which appear to be independent of the levels of PPAR γ in cancer needs further evaluation.

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