

# PPAR $\gamma$ pathway activation results in apoptosis and COX-2 inhibition in HepG2 cells

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## Abstract

**AIM:** To investigate whether troglitazone (TGZ), the peroxisome proliferator-activated receptor (PPAR) gamma ligand, can induce apoptosis and inhibit cell proliferation in human liver cancer cell line HepG2 and to explore the molecular mechanisms.

**METHODS:** [3-(4,5)-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), [<sup>3</sup>H] Thymidine incorporation, Hoechst33258 staining, DNA ladder, enzyme-linked immunosorbent assay (ELISA), RT-PCR, Northern and Western blotting analyses were employed to investigate the effect of TGZ on HepG2 cells and related molecular mechanisms.

**RESULTS:** TGZ was found to inhibit the growth of HepG2 cells and to induce apoptosis. During the process, the expression of COX-2 mRNA and protein and Bcl-2 protein was down-regulated, while that of Bax and Bak proteins was up-regulated, and the activity of caspase-3 was elevated. Furthermore, the level of PGE<sub>2</sub> was decreased transiently after 12 h of treatment with 30  $\mu$ M troglitazone.

**CONCLUSION:** TGZ inhibits cell proliferation and induces apoptosis in HepG2 cells, which may be associated with the activation of caspase-3-like proteases, down-regulation of the expression of COX-2 mRNA and protein, Bcl-2 protein, the elevation of PGE<sub>2</sub> levels, and up-regulation of the expressions of Bax and Bak proteins.

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## INTRODUCTION

Peroxisome proliferator-activated receptors (PPARs) are transcription factors belonging to the nuclear receptor gene family. PPARs bind to specific response elements as heterodimers with the retinoid X receptor and activate

transcription in response to a variety of endogenous and exogenous ligands, including some polyunsaturated fatty acids, arachidonic acid metabolites, and some anti-diabetic drugs and non-steroidal anti-inflammatory drugs<sup>[1-6]</sup>. Recently, PPARs subfamily has been defined as PPAR $\alpha$ , PPAR $\beta$  and PPAR $\gamma$ . Three PPAR isoforms differ in their tissue distribution and ligand specificity. PPAR $\alpha$  is predominantly expressed in tissues exhibiting high catabolic rate of fatty acids, whereas PPAR $\beta$  expression is ubiquitous, and its physiological role is not clear. PPAR $\gamma$  is expressed predominantly in adipose tissue, the adrenal gland, spleen, large colon and the immune system. Several lines of evidence indicate that PPAR $\gamma$  plays an important role in regulating adipocyte differentiation and glucose homeostasis. Both PPAR $\alpha$  and PPAR $\gamma$  have been shown to be involved in anti-inflammatory reactions mediated by arachidonic acid metabolites. PPAR $\alpha$  binds to, and is activated by leukotriene B<sub>4</sub>, and its level is regulated at the transcriptional level by anti-inflammatory glucocorticoids<sup>[7-15]</sup>. PPAR $\gamma$  is activated by prostaglandin D<sub>2</sub> metabolite 15-deoxy- $\Delta^{12,14}$  prostaglandin J<sub>2</sub> (15 d-PGJ<sub>2</sub>) and synthetic anti-diabetic thiazolidinedione drugs, resulting in down-regulation of the expression of pro-inflammatory genes and inhibition of tumor cell growth<sup>[16,17]</sup>.

Cyclooxygenase (COX) is a rate-limiting enzyme, catalyzing the initial step in biosynthesis of prostaglandins (PGs) from arachidonic acid<sup>[18,19]</sup>. COX is encoded by two separate genes, COX-1 and COX-2, both of which participate in formation of a variety of eicosanoids including PGD<sub>2</sub>, PGE<sub>2</sub>, PGI<sub>2</sub>, PGF<sub>2 $\alpha$</sub> , and thromboxane A. COX-1 is expressed constitutionally in most tissues and has been proposed to be a house-keeping gene, which is involved in cytoprotection of gastric mucosa, vasodilation in kidney, and control of platelet aggregation. In contrast, COX-2 is an inducible immediate-early gene that is upregulated by various stimuli including mitogens, cytokines, growth factors, and tumor promoters. Previous studies have demonstrated that COX-2 expression is aberrantly increased in (various) human epithelial cancers in colorectum, esophagus, stomach, lung, and bladder<sup>[20-39]</sup>. These findings suggest that up-regulation of COX-2 may be a common mechanism in epithelial carcinogenesis. Recently, PPAR $\gamma$  ligands was found to suppress COX-2 expression in fetal hepatocytes<sup>[40]</sup> and in macrophage-like differentiated U937 cells<sup>[41]</sup>. However, other authors reported that 15d-PGJ<sub>2</sub> induced the expression of COX-2 in immortalized epithelial<sup>[42]</sup> and colorectal cancer cells<sup>[43]</sup>. The mechanisms for the different regulation of COX-2 expression by PPAR $\gamma$  ligands remain to be elucidated. In the present study, we wanted to investigate the effect of PPAR $\gamma$  activation on cell growth and apoptosis, and to investigate underlying mechanism in regard to the expression of COX-2 and Bcl-2 members in HepG2 cells.

## MATERIALS AND METHODS

### Cell culture

Human liver cancer cell line HepG2 was provided by the American Type Culture Collection. Cells were grown in RPMI-1640 medium supplemented with 15 % new born bovine serum, penicillin G (100 kU·L<sup>-1</sup>) and kanamycin (0.1g/L) at 37 °C in

the 5 % CO<sub>2</sub> incubator. Cells were grown on 96-well plates for MTT assay, [<sup>3</sup>H] thymidine incorporation and DNA fragmentation enzyme-linked immunosorbent assay (ELISA). For the experiment, cells were grown in fresh serum-free medium, incubated for 6 hours, and treated with experimental reagents.

#### **MTT cell viability assay**

Cell growth was assessed by a modified MTT assay. About 2×10<sup>5</sup> cells/well were plated in 96-well microtiter plates and incubated overnight. Cells were then treated with troglitazone for 48 h in various concentrations. Then 10  $\mu$ l stock MTT (0.5 g/L) was added to each well, and the cells were further incubated at 37 °C for 4 h. After supernatant was removed, 100  $\mu$ l of 0.04 M HCl in isopropanol was added to each well to solubilize the formazan products. The absorbance at the wavelength of 570 nm was measured by a micro-ELISA reader. The negative control well contained medium only. The ratios of the absorbance of treated cells relative to those of the control wells were calculated and expressed as percentage of growth inhibition.

#### **[<sup>3</sup>H] thymidine incorporation**

Cells were planted in 96-well plates and grown for 24 h after being starved by growing in the serum-free medium for 48 h. Then, they were treated with troglitazone for 48 h and labeled with 5  $\mu$ Ci of [<sup>3</sup>H] thymidine for 4 h. Radioactivity was detected using a Beckman LS counter, after the reaction was washed and stopped with 5 % trichloro acetic acid and the cells solubilized in 0.5 % of 0.25 N sodium hydroxide. Each experiment was done in quadruplicates and repeated at least three times.

#### **Hoechst 33258 staining**

Cells were fixed with 4 % formaldehyde in phosphate-buffered saline (PBS) for 10 min, stained by Hoechst 33258 (10 mg/L) for 1 hour, and subjected to fluorescence microscopy. After treatment with troglitazone, morphologic changes, including reduction in cell size and nuclear chromatin condensation, were observed.

#### **DNA ladder demonstration**

After induction of apoptosis, cells (7×10<sup>6</sup>/sample, both attached and detached cells) were lysed with 150  $\mu$ l hypotonic lysis buffer (edetic acid 10 mM, 0.5 % Triton X-100, Tris-HCl, pH7.4) for 15 min on ice and were precipitated with 2.5 % polyethylene glycol and 1 M NaCl for 15 min at 4 °C. After centrifugation at 16 000×g for 10 min at room temperature, the supernatant was treated with proteinase K (0.3 g/L) at 37 °C for 1 h and precipitated with isopropanol at -20 °C. After centrifugation, each pellet was dissolved in 10  $\mu$ l of Tris-EDTA (pH 7.6) and electrophoresed on a 1.5 % agarose gel containing ethidium bromide. DNA ladder pattern was identified under ultraviolet light.

#### **Detection of DNA fragmentation**

HepG2 cells were grown in 96-well plates. The cells were incubated with various dose of troglitazone for 48 h. DNA fragmentation was detected using an enzyme-linked immunosorbent assay (ELISA) kit (Roche). This assay was based on a quantitative sandwich enzyme-immunoassay directed against cytoplasmic histone-associated DNA fragments. Briefly, the cells were incubated in 200  $\mu$ l of lysis buffer. After centrifugation, 20  $\mu$ l of the supernatant was reacted overnight at 4 °C in streptavidin-coated wells with 80  $\mu$ l of biotinylated anti-histone antibody and peroxidase-conjugated anti-DNA antibody. After washing, the immunocomplex-bound peroxidase

was probed with 2,2'-azino-di[3-ethylbenzthiazoline sulfonate] for spectrophotometric detection at 405 nm.

#### **TUNEL reaction**

TUNEL reaction was done using apoptosis detection system (Cayman). Cells were fixed overnight at 4 °C with 4 % paraformaldehyde in PBS. The samples were washed three times with PBS and permeabilized by 0.2 % Triton X-100 in PBS for 15 min on ice. After washed twice, cells were equilibrated at room temperature for 15 to 30 min in equilibration buffer (potassium cacodylate 200 mM, dithiothreitol 0.2 mM, bovine serum albumin 0.25 g/L, and cobalt chloride 2.5 mM in 25 mM Tris-HCl, pH 6.6), and then incubated in a solution containing 5  $\mu$ M fluorescein-12-dUTP, 10  $\mu$ M dATP, 100  $\mu$ M edetic acid, and terminal deoxynucleotidyl transferase at 37 °C for 1.5 h in a dark chamber. The tailing reaction was terminated by 2×standard saline citrate (SSC). The samples were washed three times with PBS and analyzed by fluorescence microscopy. At least 1000 cells were counted, and the percentage of TUNEL-positive cells was determined.

#### **RNA isolation and northern blotting**

After incubation with different doses of troglitazone for 6 h, cells were washed with RPMI. Total RNA was extracted from adherent cells using Rneasy Mini kits (Sigma) as described previously<sup>[37]</sup>. 30 mg of total RNA from each sample was separated on agarose/formaldehyde gels and transferred to nylon membranes. The membrane was hybridized with probes for COX-2 and for GAPDH as a reference.

#### **RT-PCR for COX-2**

Total RNA was extracted from cells using TRIzol<sup>TM</sup> (Sigma). COX-2 and beta-actin mRNA were detected by polymerase-chain-reaction following reverse transcription- (RT-PCR) as described<sup>[37]</sup>. Primers for beta-actin were: sense 5' -ATCTGGCACCACACCTTCTACAATGAGCTGCG-3', antisense 5' -CGTCATACTCCTGCTTGCTGATCCACATCTGC-3'.

#### **Western blotting analysis**

The cells were lysed in a lysis buffer (hepes 25 mM, Triton X-100 1.5 %, sodium deoxycholate 1 %, SDS 0.1 %, NaCl 0.5 M, edetic acid 5 mM, NaF 50 mM, sodium vanadate 0.1 mM, phenylmethylsulfonyl fluoride (PMSF) 1 mM and leupeptin 0.1 g/L, pH7.8) at 4 °C with sonication. The lysates were centrifuged at 15 000 g for 15 min and the concentration of the protein in each lysate was determined with Coomassie brilliant blue G-250. Loading buffer (42 mM Tris-HCl, containing 10 % glycerol, 2.3 % SDS, 5 % 2-mercaptoethanol and 0.002 % bromophenol blue) was then added to each lysate, which was subsequently boiled for 3 min and then electrophoresed on a SDS-polyacrylamide gel. Proteins were transferred onto a nitrocellulose filter and incubated separately with the antibodies against Bcl-2, Bax, Bak, Bcl-x<sub>L</sub> and COX-2, and then labeled with peroxidase-conjugated secondary antibodies. The reactions were visualized using the enhanced chemiluminescence reagent (Sigma). The results were approved by repeating the reactions 2 times.

#### **Evaluation of PGE2 production**

To determine the levels of PGE2, HepG2 cells were treated with different concentrations of troglitazone for 24 h. The quantity of PGE2 in supernatants was immediately determined with the PGE2 Enzyme Immunoassay kit (Caymen Chemical) according to the manufacturer's instructions. Data were recorded using a Dynatech MR50000 microplate reader and normalized to micrograms of protein.

### Assessment of caspase-3 activity

Caspase-3 activity was evaluated using a caspase assay kit following instructions of the manufacturer. In brief, caspase-3 fluorogenic substrate (Ac-DEVD-AMC or Ac-IETD-AMC) was incubated with JTE522-treated cell for 1 h at 37 °C, then AMC released from Ac-DEVD-AMC or Ac-IETD-AMC was detected using a fluorometric plate reader with an excitation wavelength of 380nm and an emission wavelength of 420-460nm.

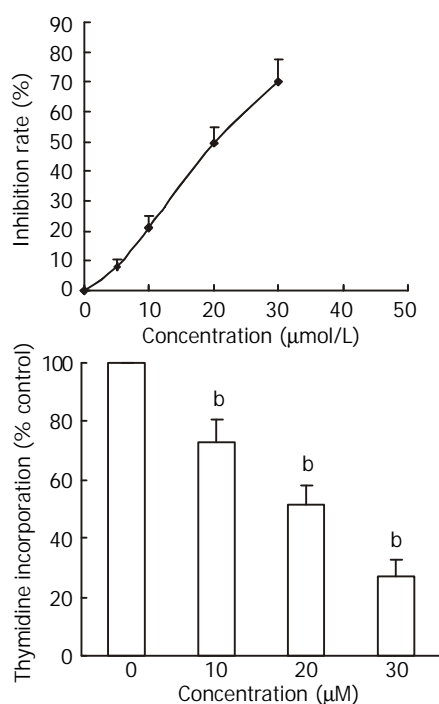
### Statistical analysis

Data were presented as the mean  $\pm$  standard error, unless otherwise indicated. Multiple comparisons were examined for significant differences using analysis of variance, followed by individual comparisons with the Bonferroni post-test. Comparisons between two groups were made with the Student *t* test.  $P < 0.05$  was considered significant.

## RESULTS

### Effects of PPAR $\gamma$ activation on cell proliferation and cell viability

HepG2 cells were incubated with various doses of troglitazone for 48 h. MTT assay showed that troglitazone significantly inhibited cell viability. The inhibition was dependent on dose of troglitazone administered (Figure 1A). Application of troglitazone also resulted in a reduction of [ $^3$ H] thymidine uptake in a dose-dependent manner (Figure 1B).

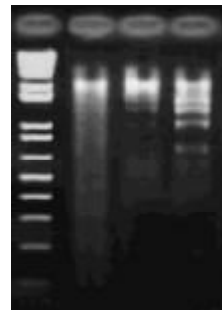


**Figure 1** Effect of TGZ on growth of HepG2 cells. HepG2 cells were incubated with various concentrations of TGZ for 48 h: (A) MTT assay; (B) [ $^3$ H] thymidine uptake assay. The value was represented as mean  $\pm$  SEM ( $n=3$ ).  $^bP < 0.01$  versus corresponding control group.

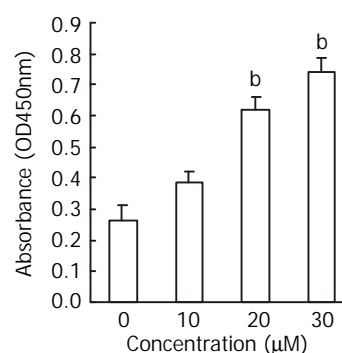
### Influence of PPAR $\gamma$ activation on apoptosis

Effect of PPAR $\gamma$  activation on apoptosis was assessed by staining with Hoechst 33258, TUNEL reaction, DNA fragmentation demonstration on an agarose gel and by ELISA. The initiating effect of PPAR $\gamma$  activation on apoptosis was confirmed in HepG2 cell, the morphologic changes included reduction in cell size and nuclear chromatin condensation visualized by Hoechst 33258 staining. The apoptotic index was also increased by treatment with different concentration of troglitazone from

3.2 $\pm$ 1.2 % to 53 $\pm$ 2.6 %. Agarose gel electrophoresis showed DNA ladder pattern in the exposed HepG2 cells (Figure 2). The PPAR $\gamma$  pathway-induced apoptosis was further demonstrated in quantitative measurement of cytoplasmic histone-associated DNA fragment by ELISA. As shown in Figure 3, troglitazone induced significant increase in DNA fragmentation in a dose-dependent manner.



**Figure 2** DNA ladder pattern formation in HepG2 cells after treatment with TGZ. Cells were treated with TGZ for 48 h and the formation of oligonucleosomal fragments was determined by 1.5 % agarose gel electrophoresis: M) DNA markers; 1) control; 2) 10  $\mu\text{M}$  TGZ; 3) 30  $\mu\text{M}$  TGZ.



**Figure 3** DNA fragmentation by ELISA assay, as measured by absorbance (OD 450 values). HepG2 cells cultured for 48 h in the presence of TGZ resulted in dose dependent DNA fragmentation.  $^aP < 0.05$ ,  $^bP < 0.01$  compared to respective control.

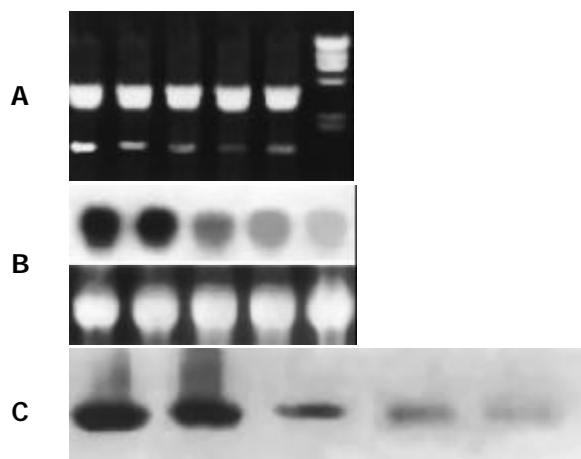
### Down-regulation of COX-2 associated with the PPAR $\gamma$ activation

The fact that the COX-2 promoter contains a PPRE indicates that COX-2 might be one of the downstream targets of the PPAR $\gamma$  pathway. In the present study, COX-2 expression was observed in HepG2 cells treated with vehicle or 30  $\mu\text{M}$  troglitazone. After 6, 12, 24 and 48 h of the treatment, cells were harvested. COX-2 mRNA was analysed by RT-PCR (4A) and Northern blotting (4B), and its translation product was demonstrated by Western blotting (4C). As shown in Figure 4, no significant change was detected during the first 6 h of treatment when compared with the control. After treatment with 30  $\mu\text{M}$  troglitazone for 12 h, the expression of COX-2 was inhibited.

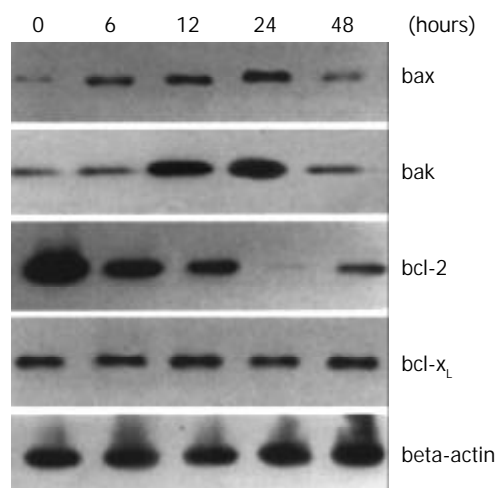
### Effects of PPAR $\gamma$ activation on expression levels of Bcl-2, Bcl-x $_L$ , Bax and Bak

To further elucidate the mechanisms of troglitazone-induced apoptosis in HepG2 cells, we assessed the involvement of bcl-2 family proteins in the process by Western blotting. Expression of Bax protein was up-regulated 6 h after 20  $\mu\text{M}$  troglitazone treatment and remained elevated to 24 h. Expression level of Bak protein was also elevated 12 h after the treatment with 30  $\mu\text{M}$  troglitazone and declined at 48 h. On the contrary, Bcl-2

protein expression was down-regulated at 6 h and undetectable at 24 h. No significant change was observed in the expression of bcl-x<sub>L</sub> protein (Figure 5).



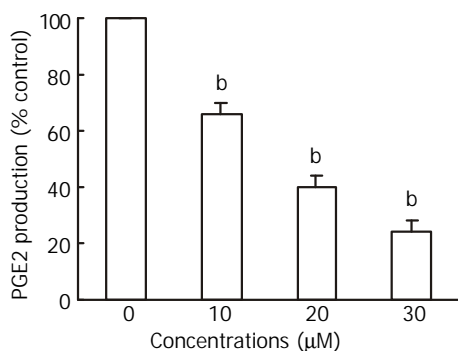
**Figure 4** Effect of TGZ on the expression of COX-2 mRNA and protein in human liver cancer cell line HepG2 cells: (A) RT-PCR; (B) Northern blot; (C): Western blot.



**Figure 5** Effects of TGZ on the levels of bcl-2, bcl-x<sub>L</sub>, bax and bak proteins in HepG2 cells at indicated time.

#### Effects of PPAR $\gamma$ activation on PGE<sub>2</sub> production

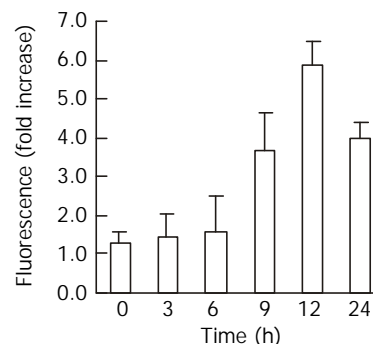
The levels of PGE<sub>2</sub> in vehicle controls were always high throughout the culture. When HepG2 cells were treated with 30  $\mu$ M troglitazone, PGE<sub>2</sub> concentration decreased transiently at 12 h (Figure 6).



**Figure 6** Effect of TGZ on the production of PGE<sub>2</sub>. The value was represented as mean  $\pm$  SEM ( $n=3$ ). <sup>b</sup> $P<0.01$  compared to respective control.

#### Change in caspase-3 activity associated with PPAR $\gamma$ activation

In consideration of frequent involvement of caspases activation in apoptosis, caspase-3 activity was assessed in HepG2 cell, treated with 20  $\mu$ M troglitazone. As shown in Figure 7, the caspase-3 activity increased with the treatment, the reaction was time-dependent.



**Figure 7** Effect of TGZ on the activity of caspase-3. The value was represented as mean  $\pm$  SEM ( $n=3$ ).

#### DISCUSSION

Potent effects of PPAR $\gamma$  on cell proliferation and cell cycling have been described. PPAR $\gamma$  ligands can trigger cell cycle arrest in NIH3T3 cells and HIB-1B cells<sup>[44]</sup>. PPAR $\gamma$  ligands can also induce terminal differentiation and withdrawal of human liposarcoma cells from the cell cycle<sup>[45]</sup>. Importantly, PPAR $\gamma$  ligands have been found to slow down the progression of advanced liposarcoma in humans<sup>[46]</sup>. Given the expression of PPAR $\gamma$  in nonadipose tissues, the effect of PPAR $\gamma$  on human breast cancer, gastric cancer, prostate cancer, colon cancer and transitional cell, bladder cancer have been explored. Treatment of cultured breast cancer cells with troglitazone results in cell growth arrest and promotes differentiation<sup>[47]</sup>. Troglitazone has also been shown to inhibit tumor growth and induce apoptosis in human breast cancer cells *in vitro* and in BNX mice. Moreover, another PPAR $\gamma$  ligand, GW7845, has been shown to decrease tumor incidence, tumor growth and tumor burden I, the NMU induced mammary carcinoma<sup>[48]</sup>. These data suggest that PPAR $\gamma$  ligands may be used as novel, nontoxic and selective chemotherapeutic agents for human breast cancers. In the present study, our results have shown that activation of PPAR $\gamma$  by troglitazone inhibits cell growth and induces apoptosis in human liver cancer HepG2 cells. We confirmed that the induction of apoptosis was mediated through down-regulation of COX-2 and Bcl-2 expression, and up-regulation of Bax and Bak expression. The down-regulation of COX-2 was coincident with down-regulation of the production of PGE<sub>2</sub>. The activity of Caspase-3 was increased after treatment with 30  $\mu$ M PPAR $\gamma$  ligand troglitazone in a time-dependent manner.

Meade *et al.* have demonstrated that COX-2 expression is enhanced by peroxisome proliferators, including some fatty acids, PGs and NSAIDs, as well as the prototypical peroxisome proliferator WY-14, 643, in mammary and colonic epithelial cells, presumably through PPAR $\alpha$ <sup>[49]</sup>. Yang *et al.* showed that activation of PPAR pathway by ciglitazone induced apoptosis and inhibition of COX-2 expression in human colon cancer cells HT-29<sup>[50]</sup>, but the result was not approved in an observation by Lefebvre *et al.*<sup>[51]</sup>. Our data showed that PPAR $\gamma$  activation inhibited the expression of COX-2. The discrepancy may be caused by different cell types used in these groups.

Overexpression of COX-2 plays important roles in cell adhesion, apoptosis and angiogenesis. Numerous epidemiological studies suggest that use of nonsteroidal anti-inflammatory drugs

(NSAIDs) decreases the incidence of gastrointestinal cancers and COX-2 is recognized as a major target of NSAIDs<sup>[52-64]</sup>. Inhibition of COX-2 by NSAIDs or COX-2 specific inhibitors causes cell death in cancer cells, indicating that COX-2 may be used as an important molecular target for prevention and therapy in gastrointestinal cancers<sup>[65-70]</sup>. The mechanism of COX-2 expression remains unclear. Subbaramaiah and colleagues have shown that PPAR $\gamma$  can inhibit COX-2 expression.

In the present study, the levels of PGE<sub>2</sub> were decreased in a time-dependent manner after the treatment with 30  $\mu$ M troglitazone, and were correlated with the change in COX-2 expression. This is in agreement with previous observations in other cell lines<sup>[71-73]</sup>. Thus, excessively synthesized PGE<sub>2</sub> mediated by overexpression of COX-2 is believed to play an important role in neoplasia formation. Inhibition of COX-2 activity may at least partly explain the chemopreventative effect of activated PPAR pathway in human liver cancer.

Apoptosis is characterized by a series of distinct morphological and biochemical changes. Several apoptosis-related genes have been found. One group of apoptosis regulatory genes is the Bcl-2 family<sup>[74-79]</sup>. Of these genes, Bcl-2, Bcl-x<sub>L</sub> are antiapoptotic, whereas Bax, Bcl-x<sub>s</sub>, Bak, Bad and Bik are proapoptotic. In this study, overexpression of Bax and Bak, and suppression of the expression of Bcl-2 were found during the apoptosis induced by PPAR $\gamma$  activation. These data confirm the role of these proteins in troglitazone-induced apoptosis in HepG2 cells. In addition, the activity of caspase-3 was also found to be elevated during the apoptotic process induced by PPAR $\gamma$  activation.

In summary, we have shown that activation of PPAR $\gamma$  by troglitazone induces apoptosis in HepG2 cells through down-regulation of the expression of COX-2 and bcl-2, up-regulation of bax and bak, and activation of caspase-3. Consistent with other potential chemopreventive agents in human liver cancer model, we believe that COX-2, bak, bax, bcl-2 and caspase-3 play some roles in the process of PPAR $\gamma$  activation-induced apoptosis. These serve as potential targets for future drugs or therapies for prevention and treatment of liver cancer.

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