

LIVER CANCER

Troglitazone, a peroxisome proliferator-activated receptor γ ligand, induces growth inhibition and apoptosis of HepG2 human liver cancer cells

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Supported by Grants from the State Key Basic Research Program, No. 2002CB513100

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Received: September 19, 2007 Revised: December 12, 2007

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Zhou YM, Wen YH, Kang XY, Qian HH, Yang JM, Yin ZF. Troglitazone, a peroxisome proliferator-activated receptor γ ligand, induces growth inhibition and apoptosis of HepG2 human liver cancer cells. *World J Gastroenterol* 2008; 14(14): 2168-2173 Available from: URL: <http://www.wjgnet.com/1007-9327/14/2168.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.2168>

Abstract

AIM: To examine the effect of troglitazone, a peroxisome proliferator-activated receptor γ (PPAR γ) ligand, on the proliferation and apoptosis of human liver cancer cells.

METHODS: Liver cancer cell line HepG2 was cultured and treated with troglitazone. Cell proliferation was detected by 3-(4-,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay; apoptosis was detected by flow cytometry and terminal deoxynucleotidyl transferase-mediated nick end labeling of DNA fragmentation sites (TUNEL) assay; and apoptosis-related protein was detected by immunocytochemistry and Western blotting.

RESULTS: Troglitazone inhibited growth and induced apoptosis of HepG2 cells in a dose-dependent manner, and induced activation of caspase-3 expression. Troglitazone not only drove apoptosis-inhibiting factor survivin to translocate incompletely from the nucleus to the cytoplasm, but also inhibited expression of survivin, while it did not affect expression of apoptosis-promoting factor Bax.

CONCLUSION: PPAR γ ligands inhibit growth and induce apoptosis of liver cancer cells, and may have applications for the prevention and treatment of liver cancer.

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Key words: Peroxisome proliferator-activated receptor γ ; Troglitazone; Liver neoplasms; Apoptosis

Peer reviewers: Frank A Anania, Professor, Emory University School of Medicine, Division of Division Digestive Diseases,

INTRODUCTION

Primary hepatocellular carcinoma (HCC) is the fifth most common malignant tumor worldwide, and causes more than 500 000 deaths annually^[1]. Dissemination of Hepatitis B virus (HBV) and Hepatitis C virus (HCV) causes the incidence of HCC to increase^[2]. Surgical resection is the treatment of choice for HCC, but only 10%-20% of HCC patients are resectable at the time of diagnosis^[3]. Liver transplantation is only indicated for early HCC (Milan criteria 1 tumor, < 5 cm in diameter, or up to three tumors, all < 3 cm in diameter), as far as outcome is concerned^[4]. In addition, recurrence remains high in patients who receive radical treatment^[5,6]. It is therefore urgent to seek a new approach for the treatment of HCC.

Peroxisome proliferator-activated receptor (PPAR) belongs to the nuclear hormone receptor superfamily^[7]. Three subtypes of PPAR (PPAR α , PPAR γ and PPAR δ) have been identified, among which PPAR γ has been studied most extensively. PPAR- γ exerts its effects by forming heterodimers with the 9-*cis*-retinoid X receptor (RXR) after ligand activation. The PPAR/RXR heterodimer acts as a regulatory transcription factor with peroxisome proliferator responsive element (PPRE) to regulate the expression of target genes which participate in the physiological and pathological processes of lipid metabolism, glucose metabolism, adipocyte differentiation, energy balance, inflammatory reactions and atherosclerosis^[8,9]. PPAR γ ligands are classified as natural and synthetic. The former include long-chain polyunsaturated fatty acid and eicosanoids, and the latter include thiazolidinediones (TZDs), the most commonly used drugs for diabetes, such as troglitazone, pioglitazone, rosiglitazone and LY171.833. Several recent studies have shown most human tumors

express PPAR γ , and there is evidence that PPAR γ ligands have anti-tumor activity^[10-20]. One of the important effects of these drugs is induction of apoptosis, although the exact mechanism remains elusive.

The present study used liver cancer cell line HepG2 that endogenously expresses PPAR γ as an experimental model^[15] to study the effects of PPAR γ ligand troglitazone on proliferation and apoptosis of liver cancer cells. It also analyzed the molecular mechanisms of these effects in an attempt to provide experimental clues for the use of PPAR γ ligands in the treatment of liver cancer.

MATERIALS AND METHODS

Chemicals

Troglitazone (Cayman Chemical Industry, USA) was dissolved in DMSO and then diluted to appropriate concentrations with culture medium. The final concentration of DMSO in the culture medium did not exceed 1 mL/L.

Cell culture

HepG2 cells were cultured in Dulbecco's modified Eagle's medium (GIBCO Laboratories, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, GIBCO Laboratories), 100 U/mL penicillin, and 100 g/mL streptomycin. Cells were grown at 37°C in an atmosphere of 95% air and 5% CO₂.

MTT assay

Cell proliferation was detected by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma, St Louis, MO, USA) assay. When cells were cultured to the log phase, they were seeded on a 96-well plate (2×10^4 cells/100 μ L/well) for 24 h until confluence. Cells were divided into a control (DMSO) group and a troglitazone group. The concentration of troglitazone was 5, 10, 20, 40, 80 and 100 μ mol/L. After 120 h, 10 μ L MTT (5 g/L) was added and incubated at 37°C for 4 h. DMSO (75 μ L) was added to each well, which was oscillated for 10 min until the crystals were dissolved completely. Absorbance (A) was detected with an enzyme calibrator at 560 nm. Cell viability = (A of study group/A of control group) \times 100%. The experiment was repeated twice. There were six wells for each concentration.

Flow cytometry

Cells were seeded onto a six-well plate and allowed to grow to 40% confluence. After treatment with or without the drug, both floating and adherent cells were collected. Cells were suspended and then fixed with ice-cold 70% alcohol at -20°C, followed by washing and staining with 50 μ g/mL propidium iodide in the presence of 100 μ g/mL ribonuclease A for 30 min at 37°C in the dark. DNA content was analyzed by flow cytometry using Cell Quest software (Becton Dickinson and Beckman-Coulter, San Jose, CA, USA).

TUNEL assay

HepG2 cells were grown on poly-L-lysine-coated slides in a six-well plate. After treatment with or without the drug, the slides were gently washed three times in 0.1 mol/L PBS

(pH 7.4), fixed with 80% ice-cold ethanol, and immediately transferred to a freezer until use. To study apoptosis of cultured cells, terminal deoxynucleotidyl transferase-mediated nick end labeling of DNA fragmentation sites (TUNEL) assay was performed using Fluorescein FragEL DNA Fragmentation Detection Kit (Oncogene Research Products, San Diego, CA, USA) according to the manufacturer's instructions. Ten high-power areas with even distribution of positive cells were used for calculation of the percentage of apoptotic cells under a fluorescence microscope.

Immunocytochemistry

HepG2 cells were grown on poly-L-lysine-coated slides in a six-well plate. After treatment with the drug, the slides were washed twice with PBS and fixed in 4% paraformaldehyde for 30 min at room temperature. Immunostaining was performed using the streptavidin-biotin complex method with UltraSensitive S-P Kit (Fuzhou Maxim Biotech, Fuzhou, Fujian, China). The slides were pretreated first with 0.3% hydrogen peroxide in PBS for 10 min to inactivate endogenous peroxidase, and then microwave antigen retrieval was performed with 0.01 mol/L citrate buffer at pH 6.0 for 20 min, followed by incubation with polyclonal rabbit anti-human antibody against the active caspase-3 (1:25; R&D Systems, Wiesbaden, Germany), rabbit anti-human survivin polyclonal antibody (1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight, biotinylated secondary antibody for 30 min, and peroxidase-labeled streptavidin for 30 min. Color reaction was developed with 3, 3'-diaminobenzidine as a chromogen. Finally, the slides were counterstained with hematoxylin, and dehydrated through graded alcohol. For negative controls, slides were processed as above but treated with PBS instead of the primary antibody.

Western blotting

Troglitazone-treated HepG2 cells were prepared in a cell-lysis solution. The protein concentration was determined by BCA Protein assay kit (Perbio Science Deutschland, Germany). Protein samples (50 μ g) were separated by SDS-PAGE and transferred to nitrocellulose membranes, to which rabbit anti-human survivin polyclonal antibody (1:1000), mouse anti-human Bax antibody (1:1000), mouse anti-human β -actin antibody (1:1000), and horseradish-peroxidase-labeled secondary antibody (all from Santa Cruz Biotechnology) were added. The signals were detected by enhanced chemiluminescence according to the protocol supplied with the kit.

Statistical analysis

Data were expressed as mean \pm SD. Statistical correlation of data was checked for significance by ANOVA and Student's *t* test. Differences with *P* < 0.05 were considered significant. These analyses were performed using SPSS 11.0 software (SPSS, Chicago, IL, USA).

RESULTS

Troglitazone inhibits cell growth

The MTT assay showed that cell viability was 96.8% \pm 1.22%,

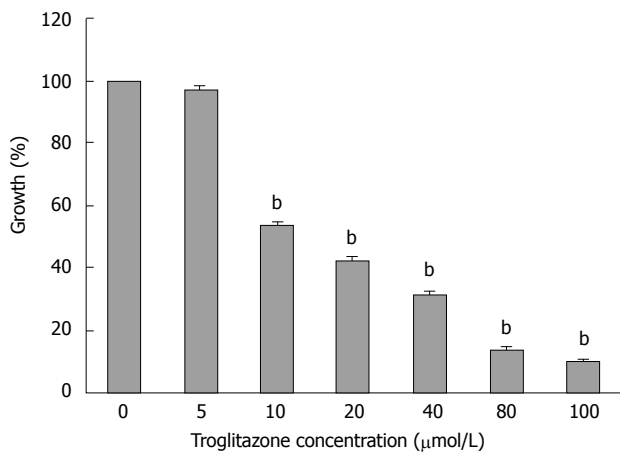


Figure 1 Effect of troglitazone on growth of HepG2 cells. Cells were incubated with different concentrations of troglitazone for 120 h. Troglitazone inhibited growth of HepG2 cells in a dose-dependent manner, as demonstrated by MTT assay. Data represent the mean \pm SD from six wells. ^b $P < 0.01$ vs corresponding control group (unpaired Student's *t* test).

53.4% \pm 1.2%, 42.3% \pm 1.2%, 31.4% \pm 1%, 13.6% \pm 0.8% and 9.6% \pm 0.7% at 5, 10, 20, 40, 80 and 100 $\mu\text{mol/L}$ troglitazone for 120 h, respectively (Figure 1), which indicated that troglitazone inhibited the growth of liver cells in a dose-dependent manner.

Troglitazone induces apoptosis

HepG2 cells were treated with troglitazone (20 and 30 $\mu\text{mol/L}$) for 24 h, and phase-contrast microscopy revealed that some cells became round, blunt and smaller in size, accompanied by karyopyknosis; light refraction was increased; and cells became detached and suspended in the medium, especially with 30 $\mu\text{mol/L}$ troglitazone. In the control group, cells were regular in morphology and grew fully in patches and confluent, rarely sloughing off (Figure 2).

Flow cytometry showed that after treatment of HepG2 cells with 20 and 30 $\mu\text{mol/L}$ troglitazone for 24 h, the percentage of sub-G₀/G₁ peak apoptotic cells was 25.5% \pm 1.1% and 43.9% \pm 1.7%, respectively. In the untreated controls, none of the cells was found in the sub-G₀/G₁ peak (Figure 3). TUNEL staining also showed that treatment with troglitazone for 24 h caused apoptosis of HepG2 cells. The apoptotic rate increased with concentration of troglitazone: 22.4% \pm 1.2% for 20 $\mu\text{mol/L}$, and 38.6% \pm 0.8% for 30 $\mu\text{mol/L}$ troglitazone. In the control group, there were only background cells, without the presence of TUNEL positive cells (Figure 4, $P < 0.001$).

Troglitazone induces activation of caspase-3 expression

The caspase family plays a very important role in mediating apoptosis, among which caspase-3 is the key executive molecule, and its activated form has multiple functions in signal transmission of apoptosis^[21,22]. Troglitazone activated caspase-3 expression. Positive staining was signified by a yellow-brown color, present in smaller cells whose plasma was condensed. After treatment of HepG2 cells with 20 and 30 $\mu\text{mol/L}$ troglitazone, the positive cell rate was 21.2% \pm 1.4% and 35.8% \pm 2.4%, respectively (Figure 5). No positive cells were found in the controls.

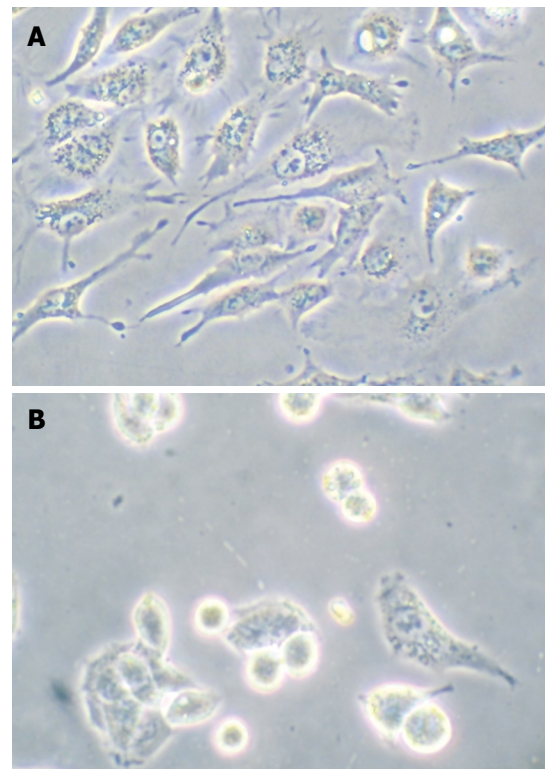


Figure 2 Morphological changes were examined by phase-contrast microscopy. A: Controls; B: 30 $\mu\text{mol/L}$ troglitazone.

Troglitazone drives survivin protein to translocate incompletely from the nucleus to the cytoplasm and inhibits its expression

Immunocytochemical staining showed that apoptosis-inhibiting factor survivin was mainly expressed in the nucleus and weakly in the cytoplasm of untreated HepG2 cells. After treatment with 20 and 30 $\mu\text{mol/L}$ troglitazone for 24 h, survivin translocated partly from the nucleus to the cytoplasm, and the intensity of its expression decreased markedly. Also, apoptotic cells were seen in the treatment group, and presented with a smaller cell size and condensed cytoplasm (Figure 6). Western blotting revealed that, in HepG2 cells treated with troglitazone for 24 h, expression of HepG2 survivin was inhibited in a dose-dependent manner, but the treatment did not significantly affect expression of apoptosis-promoting factor Bax (Figure 7).

DISCUSSION

Apoptosis is a normal phenomenon in the process of embryonic growth of all organisms and human development. Disturbance of this process is associated with the development of many severe diseases and disorders including tumors. Selective induction of tumor-cell apoptosis may become the basic strategy in the treatment of malignant tumors. Most of the available chemotherapy drugs work through destroying tumor cells by inducing apoptosis^[23]. In our study, the MTT assay showed troglitazone inhibited growth of liver cancer cells in a dose-dependent manner. To determine whether the inhibitory effect of troglitazone on the proliferation of liver cancer cells was associated with induction of apoptosis, flow cytometry and TUNEL

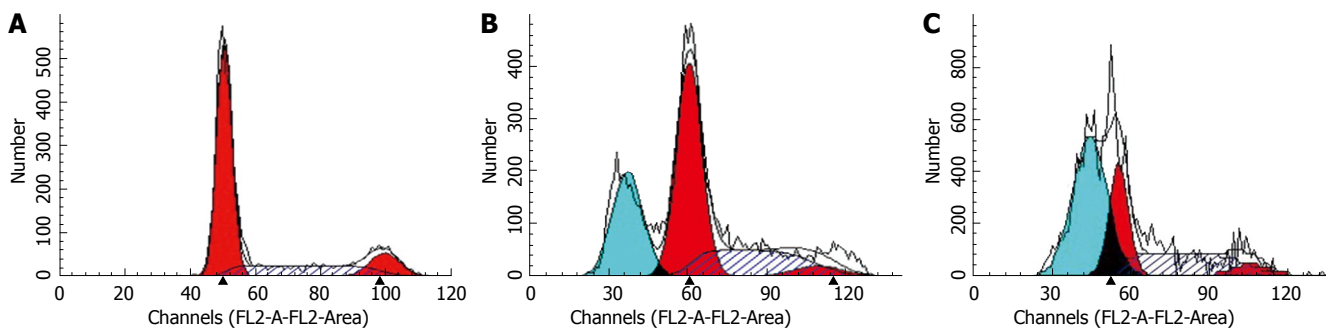


Figure 3 Cell cycle analysis by flow cytometry. A: Control cells; B: 20 $\mu\text{mol/L}$ troglitazone; C: 30 $\mu\text{mol/L}$ troglitazone.

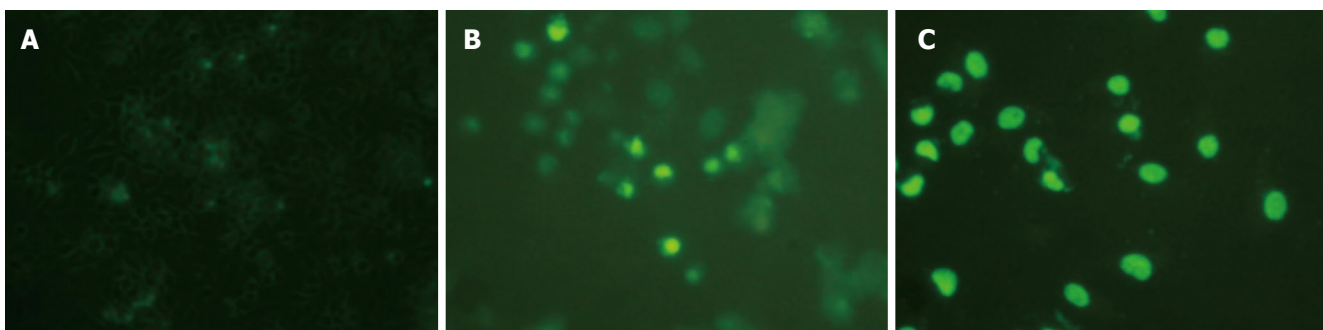


Figure 4 Troglitazone significantly increased the number of TUNEL-positive cells in a dose-dependent manner. A: Control cells; B: 20 $\mu\text{mol/L}$ troglitazone; C: 30 $\mu\text{mol/L}$ troglitazone.

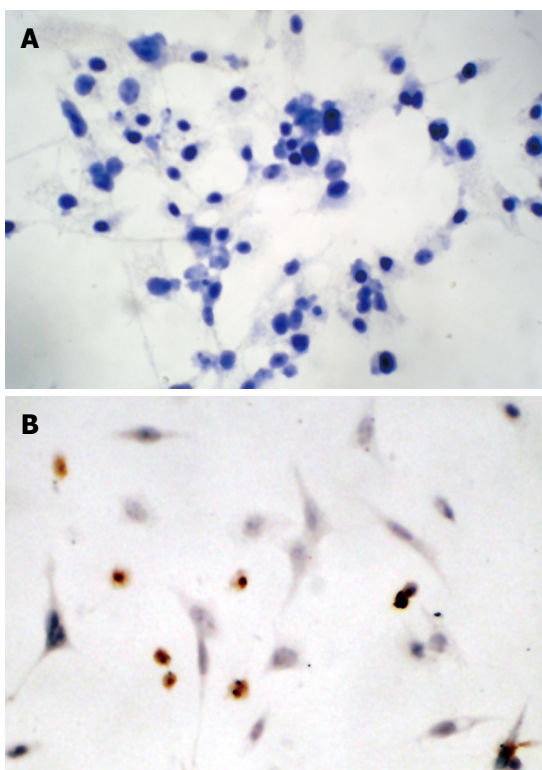


Figure 5 Immunocytochemistry showed that caspase-3 was activated. The shrunken cells were positively stained for activated caspase-3. No positive cell was detectable in the control group (A: original magnification, $\times 200$). Whereas large numbers of positive cells labeled for activated caspase-3 were observed in HepG2 cells treated with 30 $\mu\text{mol/L}$ troglitazone for 24 h (B: original magnification, $\times 400$).

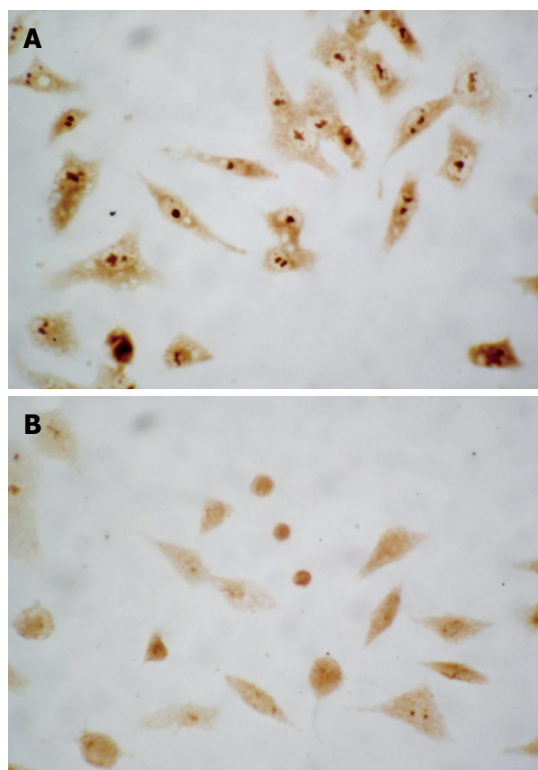


Figure 6 Survivin was present predominantly in the nucleus in untreated cells (A: original magnification, $\times 400$) as demonstrated by immunocytochemistry. After exposure of HepG2 cells to 30 $\mu\text{mol/L}$ troglitazone for 24 h, survivin incompletely translocated from the nucleus to the cytoplasm (B: original magnification, $\times 400$).

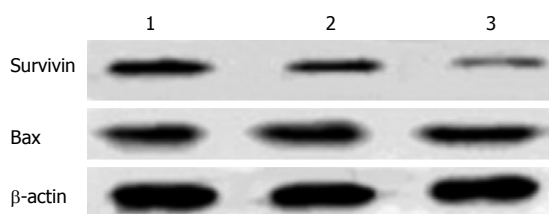


Figure 7 Effect of troglitazone on the expression of Bax and survivin. Lane 1: Control cells; lane 2: 20 $\mu\text{mol/L}$ troglitazone; lane 3: 30 $\mu\text{mol/L}$ troglitazone.

staining were conducted. The results confirmed troglitazone induced apoptosis of HepG2 cells in a dose-dependent manner. Thus, clarifying the apoptosis-related gene protein that is associated with troglitazone-induced apoptosis is of primary importance in explaining the mechanism of action of troglitazone.

Caspase is a group of cysteine hydrolytic proteinases that are able to specifically cleave the peptide chain behind the residue base of the target protein aspartate. It is the key molecule in the regulation of apoptosis. Caspase can trigger a cascade reaction under the control of apoptosis signals. According to their method of entering the apoptotic site, caspases are classified as initiator and effector caspases^[21], among which, caspase-3 is the most important terminal effector caspase in apoptosis, and plays an irreplaceable role. After initiation of the apoptotic process, caspase-3 transforms from the zymogen form to the activated form and functions by hydrolyzing proteins essential for survival of many kinds of cells^[22].

Survivin is a member of the inhibitor of apoptosis (IAP) family of negative regulators of programmed cell death, and is undetectable in normal adult tissue, but is abundantly expressed in fetal tissue and a variety of human cancers including HCC. High levels of survivin in tumors are frequently correlated with malignant parameters, which suggest a role in tumorigenesis^[24,26]. Survivin can inhibit terminal effector caspase-3 and caspase-7, and interfere with caspase-9 activity and processing. Therefore, approaches designed to counteract antiapoptotic activity may inhibit tumor growth^[27].

There has long been controversy over the subcellular location of survivin. Some researchers have found survivin expression is located in the nucleus in HCC, esophageal squamous cell carcinoma, ovarian carcinoma, mantle cell lymphoma, cholangiocarcinoma, and non-small cell lung cancer, and is associated with poor prognosis. Other researchers have pointed out that survivin expression is located in the nucleus in gastric cancer, bladder mucosa and transitional cell carcinoma, and breast cancer, and is a predictor of good prognosis^[28]. The results of our immunohistochemical staining confirmed that survivin was expressed mainly in the nucleus of HepG2 cells, and weakly in the cytoplasm. Fortugno *et al*^[29] have found two survivin pools using immunofluorescence, one in the cytoplasm and the other in the nucleus. Suzuki *et al*^[30] have found survivin protein can translocate from the cytoplasm to the nucleus. Here, it forms a complex with CDK4, which enables p21 to combine with a precursor released from the CDK4 complex and mitochondrial caspase-3, and inhibits

apoptosis by inhibiting the activity of caspase-3. The results of our study showed troglitazone induced apoptosis in a dose-dependent manner, during which survivin translocated incompletely from the nucleus to the cytoplasm, with a decrease in the intensity of expression; at the same time caspase-3 expression was activated. We presume troglitazone dissociated the survivin/CDK4 complex by inducing survivin expression and driving survivin to translocate incompletely from the nucleus to the cytoplasm. This forced p21 to form a complex with CDK4 by dissociating the combination of p21 and the precursor of mitochondrial caspase-3 and activating caspase-3. This led to cleavage of the corresponding nuclear and cytoplasmic substrates and finally caused apoptosis.

TZD induces massive apoptosis in renal cancer cells, with increased Bax expression and decreased Bcl-2 expression^[18]. However, troglitazone significantly increases the expression of c-myc mRNA, but has no effect on expression of Bcl-2 and Bax in thyroid carcinoma cells^[13]. In the current study, we also found Bax may not participate in troglitazone-induced apoptosis, suggesting that the mechanisms by which the PPAR γ ligands cause tumor cell apoptosis are different depending on the type of cancer.

In conclusion, PPAR γ ligands have the effect of inhibiting growth and inducing the apoptosis of liver cancer cells, and may have applications for the prevention and treatment of liver cancer.

COMMENTS

Background

Peroxisome proliferator-activated receptor (PPAR) belongs to the nuclear hormone receptor superfamily. Three subtypes of PPAR (PPAR α , PPAR γ and PPAR δ) have been identified, among which PPAR γ has been studied most extensively. Recent data have shown that ligands for PPAR γ exhibit growth-inhibitory effects on many types of human cancer. One of the important effects of these drugs is inducing apoptosis, although the exact mechanism remains elusive.

Research frontiers

We used liver cancer cell line HepG2 that endogenously expresses PPAR γ as an experimental model to study the effects of PPAR γ ligand troglitazone on the proliferation and apoptosis of liver cancer cells, and analyzed the molecular mechanisms of these effects.

Innovations and breakthroughs

Troglitazone inhibited growth and induced apoptosis of HepG2 cells in a dose-dependent manner, and induced activation of caspase-3 expression. Troglitazone not only drove apoptosis-inhibiting factor survivin to translocate incompletely from the nucleus to the cytoplasm, but also inhibited expression of survivin.

Applications

PPAR γ ligand has the effect of inhibiting growth and inducing apoptosis of liver cancer cells, and may have potential as a drug against liver cancer.

Peer review

This study should arouse interest in readers. It provides important information for the investigation of the anti-cancer mechanism of troglitazone and other PPAR γ ligands, and for the development of anti-cancer therapy using PPAR γ ligands.

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