Original Article

Troglitazone Induced Apoptosis *via* PPARγ Activated POX-induced ROS Formation in HT29 Cells*

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Abstract

Objective In order to investigate the potential mechanisms in troglitazone-induced apoptosis in HT29 cells, the effects of PPARγ and POX-induced ROS were explored.

Methods [3- (4, 5)-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assay, Annexin V and PI staining using FACS, plasmid transfection, ROS formation detected by DCFH staining, RNA interference, RT-PCR & RT-QPCR, and Western blotting analyses were employed to investigate the apoptotic effect of troglitazone and the potential role of PPARγ pathway and POX-induced ROS formation in HT29 cells.

Results Troglitazone was found to inhibit the growth of HT29 cells by induction of apoptosis. During this process, mitochondria related pathways including ROS formation, POX expression and cytochrome c release increased, which were inhibited by pretreatment with GW9662, a specific antagonist of PPARγ. These results illustrated that POX upregulation and ROS formation in apoptosis induced by troglitazone was modulated in PPARγ-dependent pattern. Furthermore, the inhibition of ROS and apoptosis after POX siRNA used in troglitazone-treated HT29 cells indicated that POX be essential in the ROS formation and PPARγ-dependent apoptosis induced by troglitazone.

Conclusion The findings from this study showed that troglitazone-induced apoptosis was mediated by POX-induced ROS formation, at least partly, *via* PPARγ activation.

Key words: Troglitazone; Apoptosis; HT29; POX; ROS formation; PPARγ; Cytochrome c release

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INTRODUCTION

Proline oxidase (POX), known as proline dehydrogenase, is a mitochondrial inner membrane enzyme that catalyzes the first step of proline degradation. POX converts proline to pyrroline 5-carboxylate (P5C) and transfers electrons into mitochondrial electron transport. The conversions form a metabolic loop between cytosol and mitochondria, coupled with oxidation of NADPH to mitochondria and serving as a mechanism of energy production. POX has been identified as a p53-induced gene since 1997; and Phang James M et al. have shown that up-regulation of POX expression in cancer cells may be sufficient to initiate apoptotic cascade[1-3].

Peroxisome proliferator-activated receptor γ

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(PPARγ) belongs to the nuclear hormone receptor superfamily and functions as a ligand-dependent transcription factor[4]. PPARγ plays important physiological roles in controlling lipid metabolism and homeostasis. PPARγ is also involved in the modulation of many cellular processes, such as apoptosis and differentiation induction[5-7]. PPARγ forms a heterodimer with the retinoid X receptor and activates target genes by binding to specific peroxisome proliferator-responsive elements (PPREs). The PPREs usually consist of a direct repeat of AGGTCA sequence separated by one or two nucleotides[5]. PPARγ is induced by diverse ligands such as natural fatty acid derivatives, antidiabetic, thiazolidinediones, and nonsteroidal anti-inflammatory drugs. Recent reports have demonstrated that PPARγ and its ligands are important in control of tumor cell growth[7]. PPARγ ligands induce terminal differentiation, cell growth inhibition and apoptosis in a variety of cancer cells, including colon, gastric, breast, prostate, and lung cancers[8-12]. PPARγ and its ligands have also been reported to induce intracellular oxidative stress, resulting in generation of reactive oxygen species (ROS)[13-15]. Since glitazones have been reported to cause depolarization of mitochondria, which has been considered to be the largest source of ROS, ROS shows the potential to modulate the growth arrest and apoptosis induced by PPARγ activation. However, the mechanism of ROS generation induced by PPARγ ligands is not clearly defined.

The significance of ROS in intracellular signaling has been well documented[16-17]. Diverse stimuli can increase intracellular oxygen radicals evoking many cellular events, such as gene activation, cell cycle arrest and apoptosis. The generation and targeting of ROS signals might be essential to apoptotic induction. Previous researchers have shown that the induction of p53 was accompanied by the induction of POX and ROS generation[18]. POX induction and ROS formation can initiate apoptosis by affecting the mitochondrial permeability transition acting on the mitochondrial permeability core complex.

To explore whether ROS generation plays a role in troglitazone-induced apoptosis and the potential mechanisms involved in HT29 cells, a representative cell line for colon cancer with a mutant p53 and wild type PPARγ, ROS formation, POX expression, and cytochrome C release were investigated after HT29 cells were treated with troglitazone. The results from current study showed that the above effects induced by troglitazone were blocked by GW9662, a specific antagonist of PPARγ, suggesting that POX activated-ROS formation in apoptosis induced by troglitazone is modulated in PPARγ-dependent pattern. Furthermore, POX siRNA inhibited the ROS and apoptosis percentage, indicating that POX may be essential in the ROS formation and PPARγ-dependent apoptosis induced by troglitazone. In conclusion, troglitazone could induce apoptosis *via* PPARγ activated POX-induced ROS formation in HT29 cells.

There has been no detailed mechanisms reported so far on the POX-mediated ROS formation as a key apoptotic mechanism by PPARγ activation in HT29 cells, except that Phang JM et al. had mentioned a similar effect may exist in HT29 from the results mainly obtained from HCT116, with wild type p53 and wild type PPARγ, which also suggested that POX was p53-induced gene to induce apoptosis through ROS generation[19]. The findings from our current study offer further insights into the molecular mechanism focusing on PPARγ activation-induced apoptosis.

MATERIALS AND METHODS

Materials

Troglitazone and GW9662 were purchased from Cayman. Co (Germany); POX-siRNA olignucleotide, negative control siRNA, TRIzol reagent, SuperScript III Reverse Transcriptase and Lipofectamine 2000 were from the Invitrogen. Antibodies of anti-Bcl-2, anti-Bax, anti-cytochrome C, and anti-β-actin were purchased from Santa Cruz Biotechnology, Inc.(CA, USA); horseradish peroxidase-conjugated secondary antibodies were purchased from Jackson Immuno-Research Laboratories, Inc. (West Grove, PA, USA). Mitochondrial tetrazolium bromide (MTT), propidium iodide, RNaseA, and other chemicals were all from Sigma Chemical Co.

Cell Culture

HT29 cells were provided by Center for Cell Resources of Shanghai Institutes for Life Sciences, Chinese Academy of Sciences/Cell bank of China Center for Type Culture Collection, Chinese Academy of Sciences (CTCCCAS). Cells were maintained in RPM*I*-1640 medium supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere of 95% air/5% CO_2 at 37 °C. A subculture of cells was processed by enzymatic digestion (trypsin/EDTA solution: 0.25/0.02%). Troglitazone was dissolved in dimethyl sulfoxide (DMSO) as a 20 mmol/L stock solution and then diluted with the phosphate buffered saline, and the final concentration of DMSO in culture medium was less than 0.1%.

MTT Assay

HT29 cells were seeded onto 96-well plates at a

concentration of 2.5×10^3 cells/well in RPMI-*1640* plus 5% FBS. Troglitazone was added to wells for desired final concentrations. After exposure to troglitazone for the desired time, 10 μL of 5 mg/mL MTT was added to each well and then the cells were incubated for 4 h. To dissolve the formazan, 100 μL of DMSO was added. The absorbance was determined with a microplate reader model 550 at the wavelength of 570 nm.

LDH Leakage Assay

Cytotoxicity induced by troglitazone was assessed by lactate dehydrogenase (LDH) leakage into the culture medium. Following exposure to troglitazone of different concentration for indicated time, the culture medium was aspirated and centrifuged at 3 000 rpm for 5 min in order to obtain a cell free supernatant. The activity of LDH in the medium was determined by using a commercially available kit from JianCheng Biotech, Nanjing, China. The assay is based on the conversion of lactate to pyruvate in the presence of LDH with parallel reduction of NAD. The formation of NADH from the above reaction resulted in a change in absorbance at 340 nm. Aliquots of media and warm reagent were mixed in a 96-well plate and absorbance was recorded with a microplate spectrophotometer system. Results were analyzed and presented as percentage of control values.

Apoptosis Assay-FACS Using Annexin V-PI Staining

Phosphatidylserine (PS) expression on the external surface of cells was measured in terms of binding of FITC-labeled annexin V. Cells were collected and washed once with cold PBS, and centrifuged to collect the cell pellet. The pellet was resuspended in cold binding buffer (10 mmol/L HEPES/NaOH, pH 7.4, 140 mmol/L NaCl, 5 mmol/L $CaCl_2$), then annexin V-FITC (final concentration 1 μg/mL) and PI (final concentration 5 μg/mL) were added and mixed gently. The tube was then incubated for about 30 min in dark prior to flow cytometry.

Measurement of Intracellular ROS Formation

Intracelluar ROS was measured by utilizing conversion of nonfluorescent 2', 7'-dichlorofluorescin (DCFH) diacetate into free DCFH. HT29 cells were treated with troglitazone of different concentrations, followed by incubation in dark for 30 min in 50 mmol/L phosphate buffer (pH7.4) containing DCFH diacetate. This agent is a nonpolar compound that readily diffuses into cells, where it is hydrolyzed to the fluorescent polar derivative DCFH and thereby trapped within cells. The quantity of DCFH fluorescence was measured at an emission wavelength of 545 nm and an excitation wavelength of 485 nm by using a fluorescence plate reader. Results were expressed as percentage of fluorescence intensity of control (nonstimulated HT29 cell).

POX Knockdown Using RNAi Oligonucleotide

The POX siRNA oligonucleotide (target Accessions No. NM 016335) was synthesized by Invitrogen (CA), and the negative control siRNA (Invitrogen) was used as a control oligonucleotide. The siRNA molecules were transfected into HT29 cells by using Lipofectamine 2000 following Invitrogen's protocols. The final concentrations of 100 nmol/L POX siRNA oligonucleotide was empirically determined to maximally suppress target RNA expression, and the siRNA oligonucleotide was transferred to the medium 48 h prior to the treatment of compounds. The ability of the siRNA oligonucleotide to knockdown POX expression was analyzed by reverse transcription-polymerase chain reaction (RT-PCR). HT29 cells transfected with each RNAi were stimulated with troglitazone for 24 h (cell viability) or 4 h (ROS).

RT-PCR and Real-time Quantitative RT-PCR Analysis

Total RNA was isolated from HT29 cells by using TRIzol reagent (Invitrogen). Reverse transcription of total RNA (1 μg) was performed with SuperScript III Reverse Transcriptase (Invitrogen). PCR primers for amplification of POX were designed based on the sequences obtained; sense, 5'-GCC ATT AAG CTC ACAGCA CTG GG-3'; antisense, 5'-CTG ATG GCC GGC TGG AAG TAG-3'. RT-PCR conditions were 40 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 1 min, followed by a 10 min extension reaction at 72 °C. Aliquots of the PCR reaction were checked by a melting curve analysis as provided by the Mx3005P QPCR system (Stratagene). The instrument settings were: POX-denaturing at 95 °C for 15 min; with 40 repeated cycles of denaturing at 94 °C for 30 s, annealing at 60 °C for 30 s, and elongation at 72 °C for 30 s. Relative abundance of mRNA was calculated after normalization to β-actin.

Western Blot Analysis

To determine the expression levels of Bcl-2, Bax, and cytochrome C, cells were lysed in buffer (150 mmol/L NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mmoL/L Tris, pH 8.0, 1 mmoL/L

phenylmethylsulfonyl fluoride (PMSF), 10 μg/mL aprotinin, 1 μg/mL leupeptin, and 10 mmol/L dithiothreitol). Protein (40-80 μg) was separated by 12%-15% SDS-polyacrylamide gel in the separation buffer (25 mmol/L Tris, 250 mmol/L Glycine, 0.1% SDS). Total proteins were transferred onto a PVDF membrane after electrophoresis. Western blot analyses using anti-Bcl-2, anti-Bax, anti-cytochrome C antibodies were performed. As an internal control, mouse monoclonal anti-β-actin antibody was used.

Statistical Analysis

All statistical analyses were performed with SPSS 10.0 statistical package for Microsoft Windows. Data were expressed as mean±SE for all measurements. $^{*}P<0.05$ and $^{**}P<0.01$ were considered statistically significant.

RESULTS

Troglitazone Induced Apoptosis in HT29 Cells

Treatment with different concentrations of troglitazone for 24 h exhibited significant cytotoxicity against HT29 cells as suggested by MTT assay and LDH leakage (Figure 1A, 1B). And the cytotoxicity was derived from its ability to induce apoptosis detected by Annexin V-PI staining using FACS (Figure 1C) and fluorescent microscopy scan with Hoechst33342 staining (Figure 1D). To test the indicator protein of

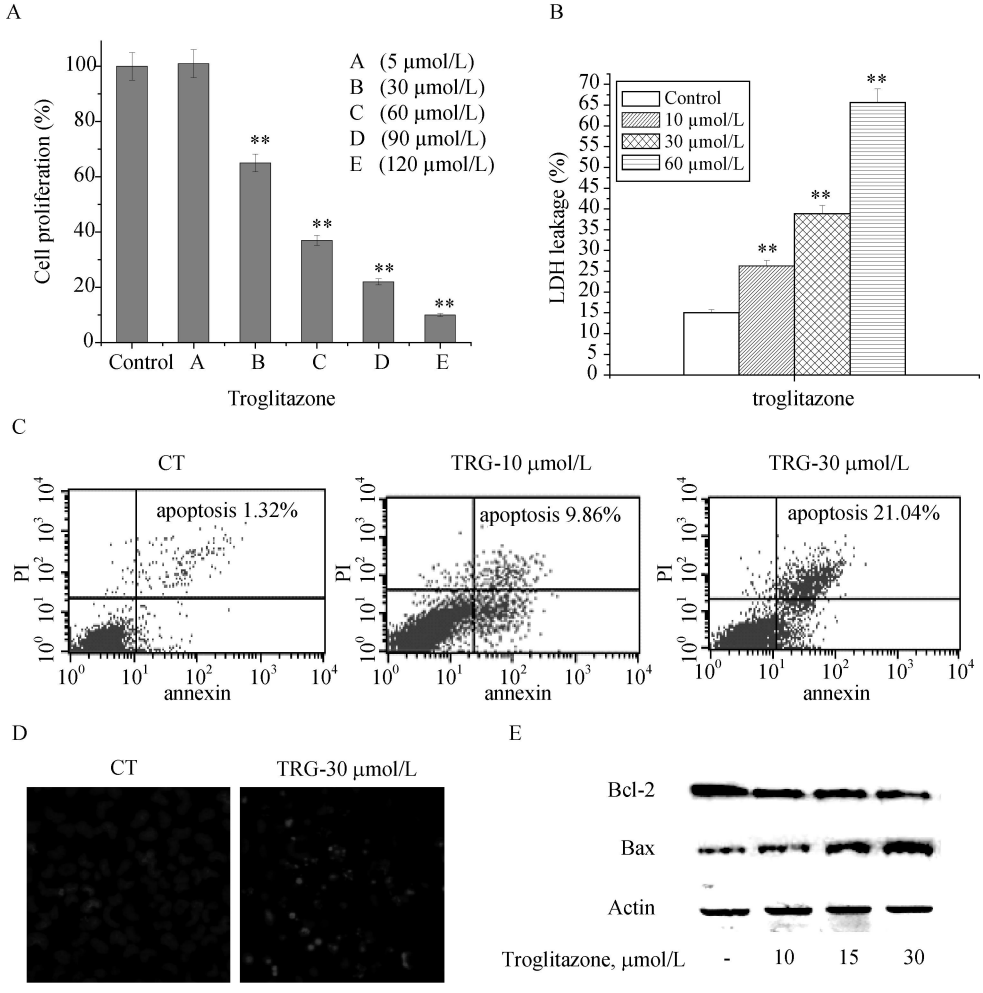

Figure 1. Troglitazone induced apoptosis in HT29 cells. Cells were incubated with the indicated concentrations of troglitazone for 24 h and then cell viability was determined with MTT assay (A) and LDH leakage assay (B). Apoptotic induction by troglitazone was measured by FACS assay with Annexin V and PI staining (C); the nuclear alteration was observed under fluorescent microscopy after cells were stained with Hoechst 33342 (D); and the apoptotic indicator protein Bcl-2/Bax were assayed by Western blot (E). Values are means±SE of two experiments with each done with quadruplicate. $^{**}P<0.01$ verus control group.

Bcl-2/Bax alteration induced by troglitazone, western blot assay was also performed. There was a significant reduction of Bcl-2 protein level by 24 h exposure of troglitazone, whereas Bax protein expression was markedly elevated (Figure 1E).

ROS Formation and Cytochrome c Release were Involved in Apoptosis Induced by Troglitazone

To test mitochondrial related pathway in apoptosis induced by troglitazone, ROS, POX, and cytochrome C release were assayed. ROS formation exhibited significant increase after treatment with troglitazone as suggested by DCFH staining (Figure 2A). Upregulation of POX expression was ascertained by RT-PCR assay (Figure 2B and 2C), and the release of cytochrome C was examined in specific cytosol fraction by western blot (Figure 2D).

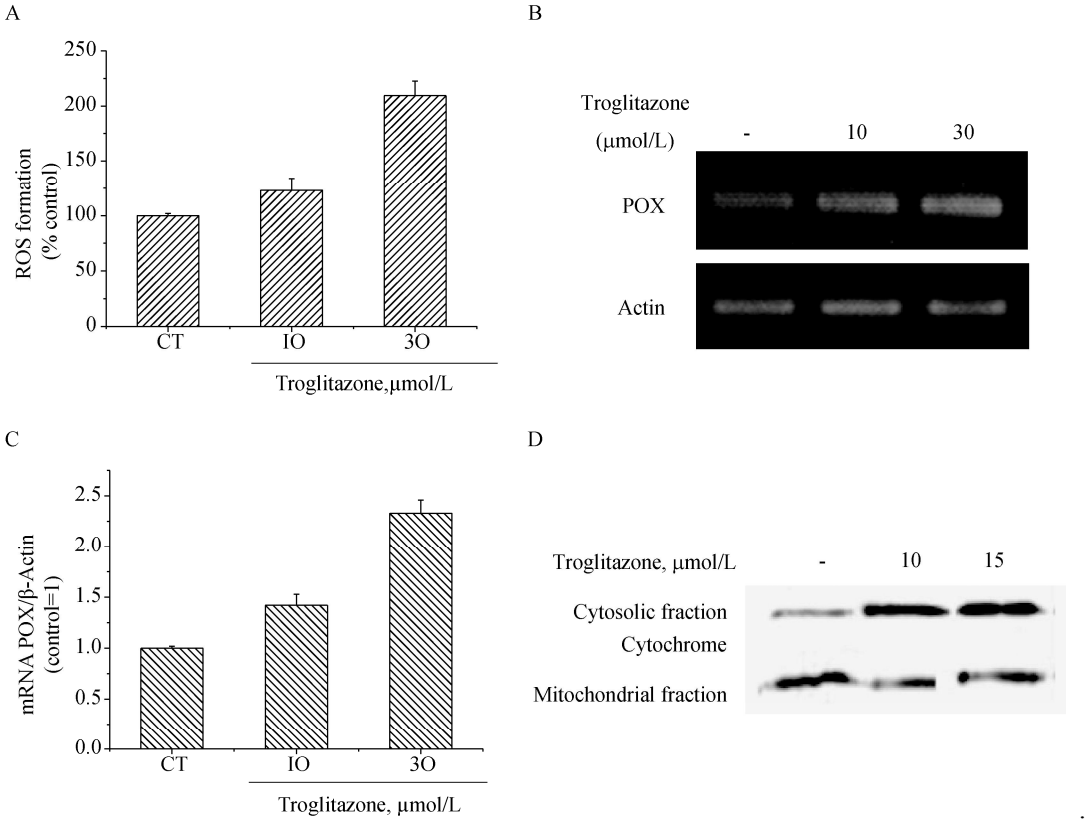

Figure 2. ROS formation and cytochrome C release were involved in troglitazone induced apoptosis. HT29 cells were treated with troglitazone for 4 h, then intracellular ROS formation was estimated by the oxidation of cell-trapped DCFH diacetate to the fluorescent compound DCF, after cells were incubated with DCFH diacetate and read by fluorescence plate reader (A); POX expression was determined by using RT-PCR (B) and real-time RT-PCR (C). HT29 cells were treated with troglitazone for 24 h, and the amount of cytochrome c in cytosolic and mitochondrial fraction was analyzed by Western blot (D).

ROS Formation in Troglitazone Induced-Apoptosis was in PPARγ-dependent Pathway

To test whether the apoptosis and ROS formation induced by troglitazone were PPARγ-dependent, GW9662, a specific antagonist of PPARγ was used. Cell viability inhibition induced by troglitazone was recovered by pretreatment with GW9662 (Figure 3A). Increase of ROS formation and POX mRNA expression was inhibited by pretreatment with GW9662, at the same time (Figure 3B, Figure 3C and 3D). These results suggested that ROS formation was, at least partly induced by troglitazone, *via* PPARγ-dependent pathway in the apoptosis.

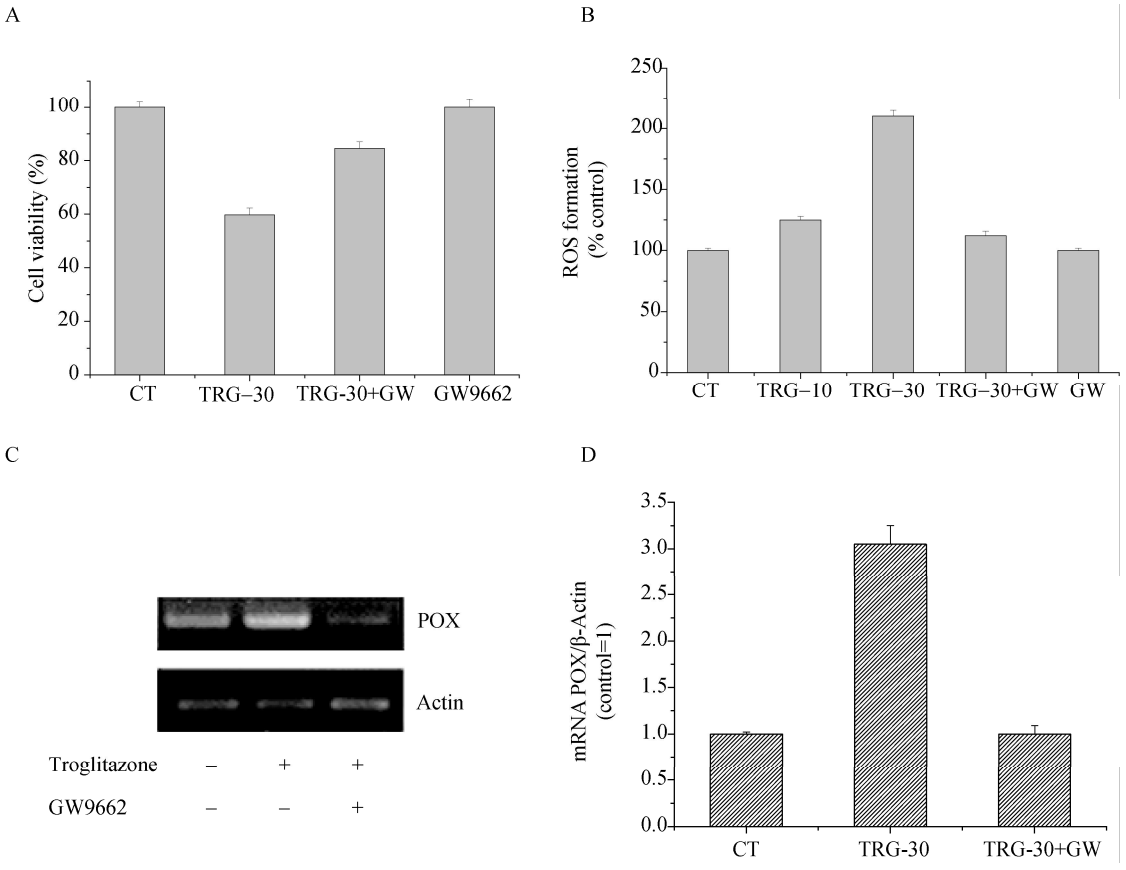

Figure 3. ROS formation in troglitazone induced-apoptotosis was in PPARγ-dependent pathway. HT29 cells were pretreated with GW9662 of 6 μmol/L for 1 h; then cells were incubated with the specific concentration of troglitazone for 24 h, followed by cell viability assay determined by MTT assay (A); Pretreated with GW9662 of 6 μmol/L for 1h, cells were first incubated with 30 μmol/L troglitazone for 4 h, then ROS formation was determined by using DCFH diacetate as substrate by fluorescence plate reader (B); and POX expression was then determined by using RT-PCR (C) or real-time RT-PCR (D).

POX siRNA Inhibited Troglitazone Induced ROS and Apoptosis

To examine the role of the POX-induced ROS in apoptotic induction by troglitazone, POX siRNA was used. The validity of POX expression treated with POX RNAi was ascertained by RT-PCR assay (Figure 4A) followed by detection of the alteration of ROS formation and apoptosis. Results showed that the increase of ROS formation and the inhibition of cell growth were blocked when HT29 cells were pretreated with POX siRNA and followed by troglitazone induction (Figure 4B and Figure4C).

DISCUSSION

The present study demonstrated that PPARγ activation by troglitazone induced apoptosis in human colon cancer HT29 cells, and that POX induction with subsequent production of ROS appeared to be a pivotal mechanism responsible for apoptotic actions. In addition, the modulation was *via* PPARγ-dependent pathway. There has been no report so far on the POX-mediated ROS formation as a key apoptotic mechanism by PPARγ activation in HT29 cells, and the findings from our study offer further insights into the molecular mechanism underlying PPARγ activation-induced apoptosis.

PPARγ is a ligand-activated transcription factor, belongs to the nuclear receptor family, and has a variety of functions including regulation of apoptosis, cell proliferation and differentiation. Since PPARγ expression has been found in various cancer types, such as liposarcoma, human breast cancer, colon cancer, prostate cancer, and lung cancer[20-23], many studies have confirmed the anti-cancer property of

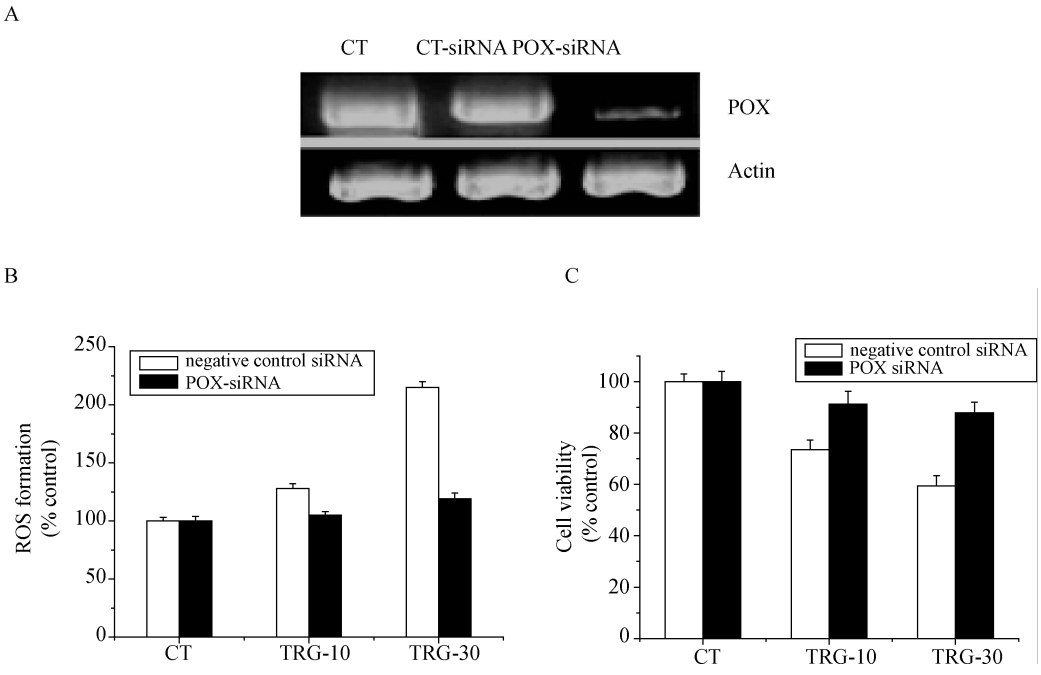

Figure 4. POX siRNA inhibited troglitazoen induced ROS formation and apoptosis. After transfection of negative control siRNA or POX-siRNA, POX mRNA expression was determined with RT-PCR (A);. Effects of troglitazone on ROS formation (B) and cell viability detected by MTT assay (C) in POX knockdown cells. Values are means±SE in two different quadruplicate experiments.

PPARγ agonists. Based on these results, PPARγ activation by synthetic ligands has been proven as an effective anti-cancer regimen by induction of apoptosis. However, the molecular mechanisms underlying these effects are still to be explored.

Results of the present study showed that troglitazone had anti-proliferative activities in HT29 cells with wild type and functional PPARγ, and its anti-proliferative activities were derived from apoptosis as shown by alteration of phosphatidylserine extroversion to external cellular membrane and the expression ratio of Bcl-2 and Bax. This finding is in agreement with previous reports demonstrating that synthetic and endogenous PPARγ ligands induce apoptosis in human colon cancers[23-26]. The apoptotic effects in the present study seem to be, at least partly, mediated by PPARγ, because GW9662, a specific antagonist of PPARγ treatment, reversed growth inhibitory effects.

In elucidation of the mechanisms producing the apoptotic cell death by PPARγ activation, we observed that ROS was involved in the apoptosis induction. ROS generation has been shown to be a common cellular mechanism for multiple cell death pathways[27], including gene activation, cell cycle arrest and apoptosis. Owing that ROS could be derived from either mitochondria or extramitochondrial origin (*i.e.* NADPH oxidase), the possible involvement of mitochondria in ROS production was investigated. The increase of cytochrome C release to cytosolic fraction accompanying with ROS formation suggested that mitochondria was a likely source of ROS. In line with this finding, ciglitazone also caused a rapid loss of mitochondrial membrane potential and induced ROS formation in glioma C6 cells[13].

Recent findings have provided substantial evidence that POX is an important source for ROS formation. And reference by Polyak et al. has shown that POX is a p53-induced gene, and troglitazone has been shown to increase POX expression in a PPARγ dependent and independent pathway in HCT116 cells with both wild type p53 and PPARγ[19]. Similarly, in our studies using HT29 cells with mutant p53 and wild type PPARγ, troglitazone increased the expression of POX in parallel with ROS formation and cell death. In addition, POX knockdown abolished the ROS generation and apoptosis induced by troglitazone, suggesting that POX played a pivotal role in troglitazone-induced apoptosis. Previous studies discovered that POX expression may be

directly regulated by PPARγ activation at the transcriptional level[19], but additional mechanism for POX regulation appeared to exist. In our present study, ROS formation underlying the apoptotic induction by troglitazone seems to be attained *via* PPARγ-mediated POX induction. We also observed in our study that both specific inhibition of POX and PPARγ could prevent the apoptotic induction treated by troglitazone. All these results reflected that the apoptosis induction by troglitazone may be mediated through PPARγ activated POX-induced ROS formation.

The significance of proline metabolism in apoptosis and carcinogenesis remains to be defined. However, it is clear that the oxidation of this amino acid can contribute to the energy supply of the cell and enhance the generation of ROS, the importance of which is increasingly recognized in the apoptotic response. Recent studies have demonstrated that POX overexpression is accompanied by the hallmarks of apoptosis, including cytochrome C release and caspase activation[28]. Thus, POX may modulate apoptosis induced by other agents and enhance apoptosis in some stress situations. Thus, it is possible to use POX as an adjunct target to inhibit cancer cell growth.

Overall, although several mechanisms for PPARγ-activated anti-proliferative activities and apoptosis induction in HT29 cells have been reported, the involvement of POX-induced ROS formation in HT29 cells may provide a novel mechanism by which troglitazone induces apoptosis. Results from the investigation of HT29 cells, with mutant p53 and wild-type PPARγ, indicated that apoptosis induction by troglitazone was mediated through PPARγ activated POX-induced ROS formation.

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