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PPARγ ligands suppress the feedback loop between E2F2 and cyclin-E1

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ABSTRACT

PPARγ is a nuclear hormone receptor that plays a key role in the induction of peroxisome proliferation. A number of studies showed that PPARγ ligands suppress cell cycle progression; however, the mechanism remains to be determined. Here, we showed that PPARγ ligand troglitazone inhibited G1/S transition in colon cancer cells, LS174T. Troglitazone did not affect on either expression of CDK inhibitor (p18) or Wnt signaling pathway, indicating that these pathways were not involved in the troglitazone-dependent cell cycle arrest. GeneChip and RT-PCR analyses revealed that troglitazone decreased mRNA levels of cell cycle regulatory factors E2F2 and cyclin-E1 whose expression is activated by E2F2. Down-regulation of E2F2 by troglitazone results in decrease of cyclin-E1 transcription, which could inhibit phosphorylation of Rb protein, and consequently evoke the suppression of E2F2 transcriptional activity. Thus, we propose that troglitazone suppresses the feedback loop containing E2F2, cyclin-E1, and Rb protein.

The peroxisome proliferator-activation receptors (PPARs) are nuclear hormone receptors, initially described as molecular targets for compounds which induce peroxisome proliferation. In PPARs, PPARγ is activated by binding certain fatty acids, eicosanoids, and insulin-sensitizing thiazolidinediones (TZD) which includes troglitazone and rosiglitazone. When PPARγ is bound with ligands, it forms heterodimeric complex with RXR and transactivates its target genes by binding to peroxisome proliferator response elements (PPRE) on the promoter/enhancer [1]. PPARγ is highly expressed in human lipocarcinomas and various other human cancers such as breast, lung, colon, prostate, bladder, and gastric cancers. It has been reported that transactivation of PPARγ with several ligands inhibits the proliferation of carcinoma cells [2,3].

Studies based on tumoral cell lines revealed that PPARγ is implicated in cell cycle withdrawal. One of the first evidence for implication of PPARγ in the cell cycle regulation is the engagement to E2F transcription factor, which is known to prompt the G1/S transition. Transactivated PPARγ suppresses the transcriptional activity of E2F/DP complex by inhibiting the complex formation [4]. Also transactivation of PPARγ is suggested to activate Rb protein by inhibiting its phosphorylation, which in turn suppresses E2F transcriptional activity [5]. Second evidence suggesting PPARγ involvement is up-regulation of cyclin-dependent kinase inhibitors (CDKI) p18 and p21 that play a crucial role in the regulation of the Rb phosphorylation [6,7]. Other evidence is that PPARγ modulates the cell cycle progression by regulating Wnt/β-catenin/TCF cascade [8,9] or by repressing cyclin-D1 in colon cancer cells [10]. Despite the increasing evidence of the mechanism of PPARγ implication in cell cycle arrest, the role for activated PPARγ to suppress carcinoma proliferation remains to be determined. In the present study we have analyzed the effect of PPARγ on transcription of cell cycle regulatory factors using colon cancer cells, and found that transactivation of PPARγ by troglitazone does not affect either CDK inhibitor (p18) or Wnt/β-catenin/TCF cascade, however decreases the mRNA level of E2F2 and its target gene cyclin-E1 that induces phosphorylation of Rb protein. We propose here the model that PPARγ arrests the cell cycle by inducing the suppression of the feedback loop between E2F2 and cyclin-E1.

Results and discussion

Troglitazone induces G1 arrest in colon cancer cell line LS174T

PPARγ is a member of the PPARs and is activated by binding certain fatty acids, eicosanoids, and insulin-sensitizing thiazolidinediones (TZD) including troglitazone and rosiglitazone. PPARγ ligands have recently been demonstrated to affect proliferation, differentiation, and apoptosis of different cell types including colon cancer [11].

Several colon cancer cell lines are known to express PPARγ. To investigate the effect of PPARγ ligands on the growth rate of colon cancer cells, two colon cancer cell lines, LS174T and SW480 were treated with PPARγ agonist troglitazone and antagonist T0070907. In Western blotting analysis using specific antibody

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against PPARγ, we detected PPARγ protein in LS174T cells but not in SW480 cells (Fig. 1A). Consistent with the PPARγ expression, treatment of LS174T cells with troglitazone significantly decreased cell growth, while the growth rate of SW480 cells were not altered by troglitazone treatment (Fig. 1B). These results indicate that PPARγ participates in the regulation of colon cancer cell growth. Next, to examine the effect of troglitazone on the cell cycle progression, we performed the fluorescence-activated cell sorting analysis. As shown in Fig. 1C, troglitazone treatment resulted in a marked accumulation of cells with G1 content. Thus, our observation indicated that troglitazone treatment arrests the cell cycle at the G1/S boundary.

Troglitazone does not affect on the Wnt signaling pathway

Recent studies indicated that PPARγ modulates colon cancer cell growth by affecting Wnt/β-catenin/TCF cascade. Wnts are a family of paracrine and autocrine factors that regulate cell growth and cell fate. Signaling is initiated when Wnt ligands bind to transmembrane receptors of the Frizzled family. In the canonical Wnt signaling pathway, Frizzleds signal through Dishevelled to inhibit the kinase activity of a complex containing glycogen synthase kinase 3 (GSK3), Axin, β-catenin, the adenomatous polyposis coli (APC), and other proteins. This complex targets β-catenin for rapid degradation through phosphorylation. Thus, once hypophosphorylated due to Wnt signaling, β-catenin is stabilized and translocated to the nucleus where it binds with the TCF/LEF family of transcription factors to regulate the expression of Wnt target genes [8,9]. To examine the effect of PPARγ ligands on Wnt signaling pathway, we first examined the protein levels of APC, β-catenin, and TCF-4 in the presence or absence of PPARγ ligands. In Western blotting analysis using specific antibodies against indicated proteins, we observed no difference of indicated protein levels in cells treated with troglitazone and T0070907 (Fig. 2A). We next transfected reporter plasmid bearing Wnt response elements into LS174T cells. The transcription from the promoter containing Wnt response element was not affected by troglitazone or T0070907 treatment (Fig. 2B). Finally, we determined the mRNA levels of Wnt target genes, cyclin D1 and c-myc, by RT-PCR. The mRNA levels of cyclin D1 and c-myc were not changed by treatment with PPARγ ligands (Fig. 2C). These results indicate that Wnt/β-catenin/TCF cascade is not involved in the troglitazone-mediated growth inhibition of LS174T cells.

Troglitazone decreases the mRNA levels of E2F2 and cyclin-E1

To investigate the mechanism of troglitazone-dependent cell cycle inhibition in LS174T cells, we analyzed gene expression utilizing DNA microarray technology. As a result of DNA microarray analysis, we have found that there is a difference in expression profile of a variety of cell cycle related genes between cells treated with troglitazone and that with T0070907. The cell cycle related genes, whose expression were two fold decreased by the treatment of these ligands in LS174T cells for 6 h or 24 h, were shown in Tables 1A and B, respectively. From these genes, we have noticed that the expression of cyclin-E1 and E2F2, both of which are essential for the progression from G1 to S phase [12], are reduced by troglitazone in both the time points (Table 1C). Therefore, we further tested the expression of the cyclin-E1 and E2F2 by RT-PCR, and found that expression of the both genes was significantly reduced in LS174T cells by the treatment with troglitazone (Fig. 3A). It should be noted that the reduction of expression of cyclin-E1 was not observed in PPARγ–negative SW480 cells (Fig. 3B). Several reports mentioned that up-regulation of CDKIs such as p18 by

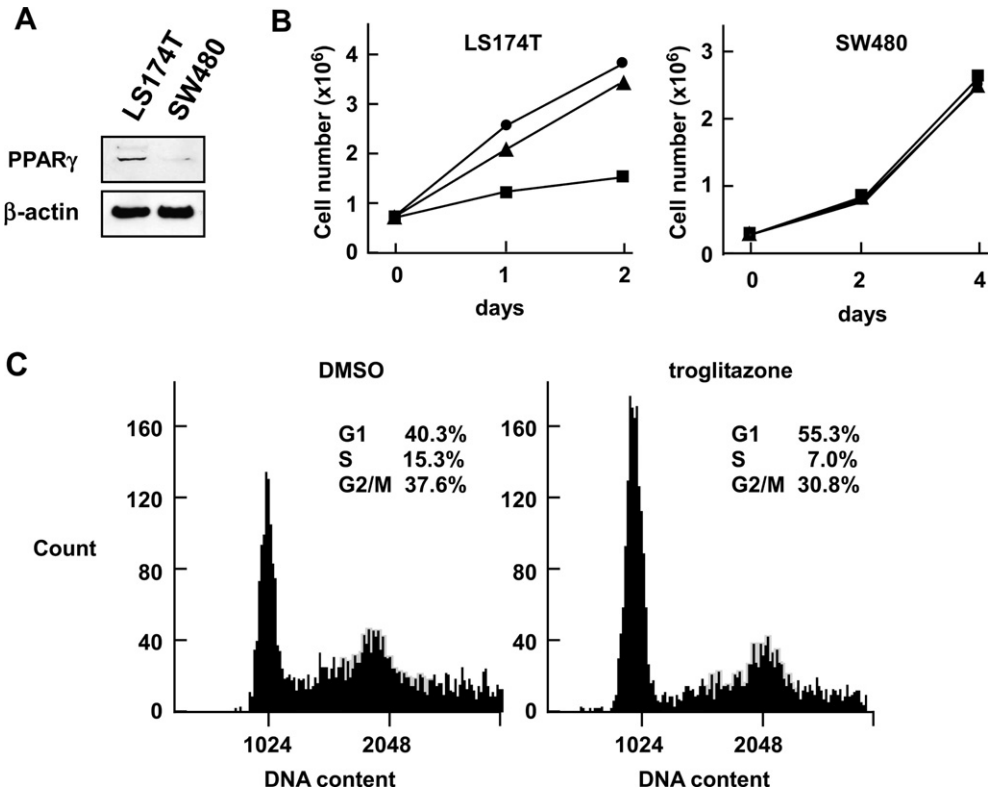

Fig. 1. Troglitazone induces G1 arrest in colon cancer cell line LS174T. (A) LS174T and SW480 cells were immunoblotted with an anti-PPARγ antibody. (B) LS174T and SW480 cells were treated with DMSO (circle), troglitazone (10 μM; square) or T0070907 (1 μM; triangle) and the cell number was counted at the indicated days. (C) LS174T cells were treated with DMSO or troglitazone (1 μM) for 24 h and DNA contents were measured using fluorescence-activated cell sorting analysis.

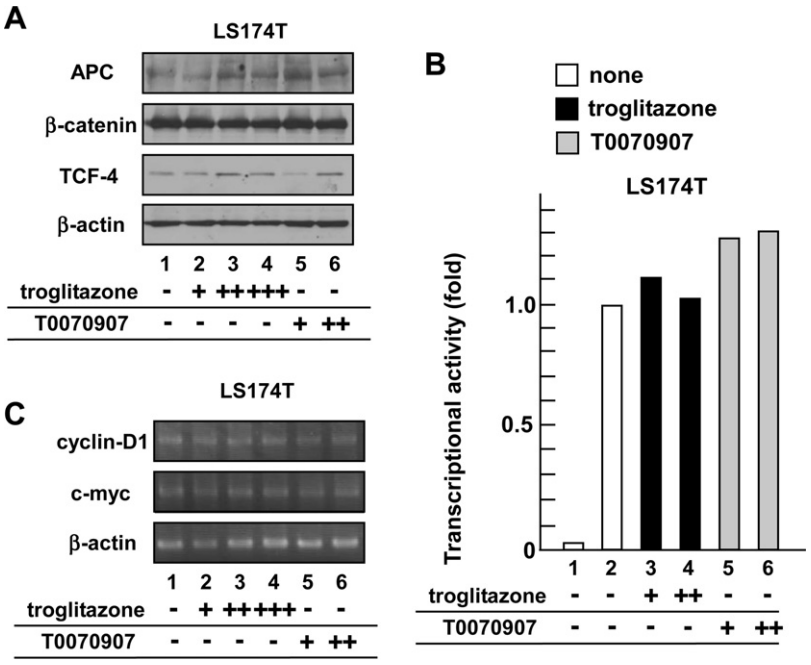

Fig. 2. Troglitazone does not affect on the Wnt signaling pathway. (A) LS174T cells were treated with DMSO, troglitazone (1, 10, or 20 μM) or T0070907 (0.1 or 1 μM) for 24 h and immunoblotted with anti-APC, anti-β-catenin, anti-TCF4, or anti-β-actin antibodies. (B) LS174T cells were transfected with either empty tk-Luc (lane 1) or TCFRE-tk-Luc (lanes 2–6) for 24 h, and then incubated with DMSO, troglitazone (1 or 10 μM) or T0070907 (0.1 or 1 μM) for 12 h to perform Luciferase assay. (C) LS174T cells were treated with DMSO, troglitazone (1, 10, or 20 μM), or T0070907 (0.1 or 1 μM) for 24 h and mRNA level of cyclin-D1, c-myc, and β-actin were analyzed by semiquantitative RT-PCR.

Table 1
Cell cycle related genes whose expression was decreased by the treatment of troglitazone for 6 h (A), 24 h (B), or both 6 and 24 h (C)

Gene name	Number[a]	Relative quantity (fold)
(A) 6 h		
Cyclin-E1	213523_at	0.40
Cyclin-E2	205034_at	0.41
E2F2	228361_at	0.45
Survivin	202094_at	0.41
CAF1 subunit B	204775_at	0.50
CDC14 homolog A	210440_s_at	0.40
(B) 24 h		
Cyclin-E1	213523_at	0.27
E2F2	228361_at	0.45
Survivin	202094_at	0.21
CAF1 subunit B	204775_at	0.31
CDK2	204252_at	0.29
CDC25 C	217010_s_at	0.34
MCM2	202107_s_at	0.10
MCM3	201555_at	0.20
MCM5	216237_s_at	0.13
MCM7	210983_s_at	0.15
DP-1	204147_s_at	0.42
DP-2	226157_at	0.31
Polo-like kinase 1	202240_at	0.33
PCNA	201202_at	0.34
(C) 6 and 24 h		
Cyclin-E1	213523_at	0.27
E2F2	228361_at	0.45
Survivin	202094_at	0.21
CAF1 subunit B	204775_at	0.31

[a] Affimetrix gene number on human genome U133 Plus 2.0 array.

PPARγ agonists causes cell cycle arrest. However, we could not observe the alteration of expression levels of p18 in LS174T cells by troglitazone treatment (Fig. 3A). Our results indicate that PPARγ inhibits growth of colon cancer cells by suppressing the expression of cyclin-E1 and E2F2.

In previous reports, it has been shown that PPARγ agonist-mediated cell cycle withdrawal was due to the down-regulation of transcriptional activity of E2F2 [4,5]. E2F family of transcriptional factors is a key regulator of the G1/S transition. During G1 phase, E2F2 remains inactive by binding with Rb protein [13]. The activity of the Rb protein is modulated by sequential phosphorylation by CDK4/6–cyclin-D and CDK2–cyclin-E complexes [13]. Hyperphosphorylated Rb proteins release E2F2 to transactivate genes required for cell cycle progression [13]. Recent studies showed that PPARγ activation inhibits the phosphorylation of Rb protein [5]. It is also reported that ligand bound PPARγ decreases the binding of E2F2/DP heterodimers to its target genes [4]. In our experiments, we revealed that PPARγ agonist decreased the mRNA levels of E2F2. Reduction of E2F2 levels resulted in the down-regulation of cyclin-E1, which is one of the target genes of E2F2. Down-regulation of cyclin-E1 levels would reduce the phosphorylation levels of Rb protein followed by the abrogation of transcriptional activity of E2F2. Thus, troglitazone suppresses the feedback loop between E2F2 and cyclin-E1, which leads to the cell cycle arrest at the G1/S transition (Fig. 3C).

Materials and methods

Cell culture and ligand treatment. LS174T and SW480 colon cancer cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Before the treatment with ligands, cells were cultured in DMEM supplemented with 4% chacolated FBS for 24 h.

Western blotting. After ligands were treated to LS74T and SW480 cells, they were lysed in TNE buffer [10 mM Tris–HCl (pH 7.8), 1% Nonidet P-40 (NP-40), 0.15 M NaCl, 1 mM ethylene diamine tetra acetic acid (EDTA), 1 μM phenylmethylsulfonylfluoride (PMSF), and 1 μg/mL aprotinin]. Extracted proteins were separated by SDS–PAGE, transferred to polyvinylidine difluoride membranes (Millipore), and probed with indicated antibodies. Specific proteins were visualized using an enhanced chemiluminescence (ECL) Western blot detection system (GE Healthcare).

Luciferase assay and transfection. The TCFRE-tk-Luc plasmid was co-transfected into LS174T cell with pRL-CMV as a reference. Transfection was performed with TransFast Transfection Reagent (Promega) according to the manufacturer's protocol. Twenty-four hours after transfection, we replaced the culture medium with fresh medium containing 4% FBS and added either vehicle alone (DMSO), troglitazone (1 or 10 μM) or T0070907 (0.1 or 1 μM) to the cells for an additional 12 h incubation. Luciferase assays were performed with Dual-Luciferase Reporter 1000 assay system

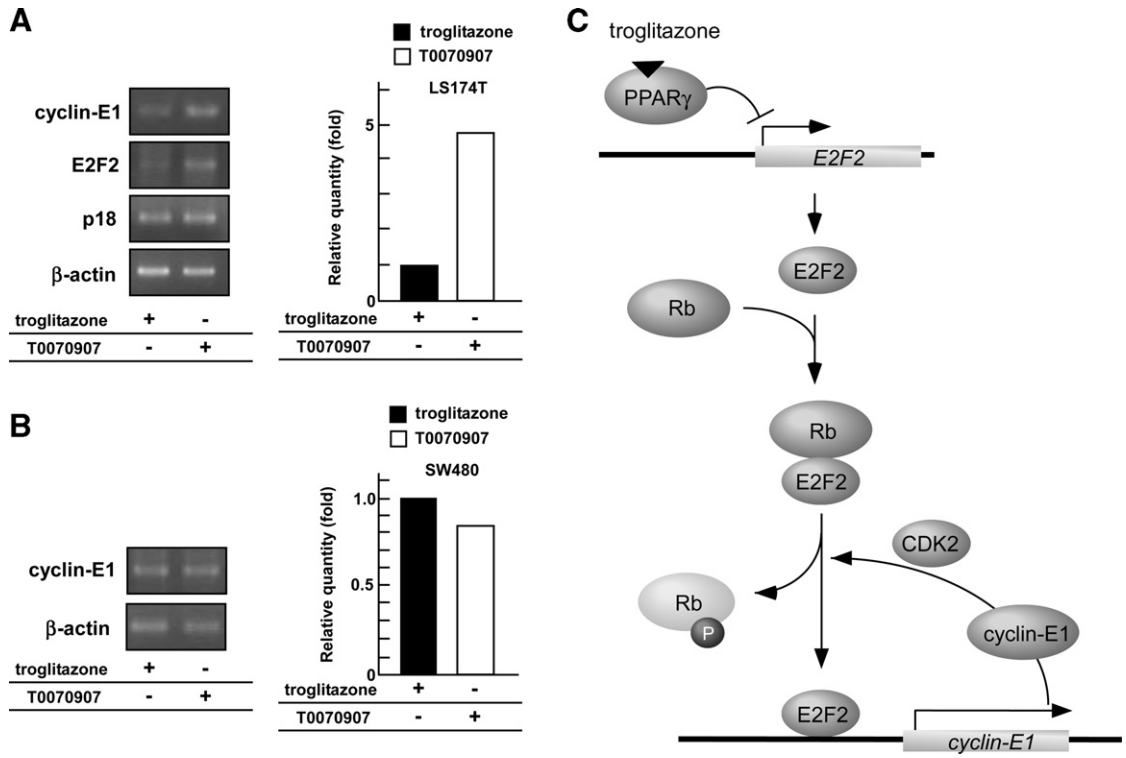

Fig. 3. Troglitazone decreases the mRNA level of E2F2 and cyclin-E1. (A) LS174T cells were treated with troglitazone or T0070907 (1 μM) for 24 h and mRNA levels of cyclin-E1, E2F2, and p18 were analyzed by semiquantitative RT-PCR (left panel) and cyclin-E1 was analyzed by quantitative RT-PCR (right panel). (B) SW480 cells were treated with troglitazone or T0070907 for 24 h and mRNA level of cyclin-E1 were identified by semiquantitative RT-PCR (left panel) and quantitative RT-PCR (right panel). (C) Possible model for suppression of feedback loop between E2F2 and cyclinE1 by PPARγ ligand. See the text in detail.

kit (Promega) on cell extracts according to the manufacturer's protocol. *Renilla* luciferase activity was measured to control for transfection efficiency. Individual transfections were assessed in triplicate and repeated at least three times.

Microarray analysis. Total RNA was prepared using an RNeasy RNA isolation kit (QIAGEN). The preparation of *in vitro* transcription products and the hybridization and scanning of the oligonucleotide array using Human Genome U133 Plus 2.0 Array (Affimetrix) were performed according to the manufacturer's protocol.

RT-PCR. Total RNA was extracted by using ISOGEN (Nippon Gene) according to the manufacturer's instructions. RNA samples were treated with DNase (Invitrogen, Carlsbad, CA) followed by the generation of cDNA using RevaTra Ace (Toyobo). The cyclin-D1, c-myc, cyclin-E1, E2F2, p18, and β-actin were amplified using to study expression of their mRNA by RT-PCR.

Cell cycle analysis. Cell cycle distribution was determined by fluorescence-activated cell sorting analysis of propidium iodide-stained ethanol-fixed cells using a Guava EasyCyte (GE Healthcare).

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