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Troglitazone is an estrogen-related receptor α and γ inverse agonist

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ABSTRACT

As a ligand for peroxisome proliferators-activated receptor γ (PPARγ), troglitazone inhibits cell growth by mechanisms besides activating PPARγ. In this study, we found that troglitazone interfered with the interactions between estrogen-related receptor α and γ (ERRα and ERRγ) and their coactivator PPARγ coactivator-1α (PGC-1α) functioning as an inverse agonist. Additionally, troglitazone suppressed the expressions of PGC-1α and its related member PGC-1β which are key regulators of mitochondrial function. Consequently, troglitazone reduced mitochondrial mass and suppressed the expressions of superoxide dismutases to elevate reactive oxygen species (ROS) production. The increase in ROS in turn induced the expression of cell cycle inhibitor $p21^{WAF1}$. We therefore propose that ERRα and ERRγ are alternative targets of troglitazone important for mediating its growth suppressive effect.

1. Introduction

Troglitazone was sold as an anti-diabetic agent until its withdrawal due to liver toxicity issues [1]. In addition to its therapeutic use in treating diabetes, troglitazone has also been investigated as a chemotherapeutic agent. Specifically, troglitazone arrests cell growth or induces apoptosis in a variety of cancer cell lines through enhancing the expressions of cell cycle inhibitor $p21^{WAF1}$ and DNA repair enzyme GADD45α [2,3].

Since troglitazone binds to and activates peroxisome proliferators-activated receptor γ (PPARγ), PPARγ has been suggested to be a potential therapeutic target not only for diabetes but also for cancers [4]. PPARγ is a member of the nuclear hormone receptor super-family of transcription factors that regulate the expression of target genes in DNA sequence specific and ligand selective manners [5]. PPARγ binds to structurally distinctive ligands such as troglitazone through its C-terminally located ligand binding domain (LBD) [6].

However, the role of PPARγ in cancer is still incompletely understood and controversial. First of all, the potencies of different ligands to activate PPARγ do not directly correlate to their abilities to inhibit cancer cell growth. Namely, more potent agonists do not necessary inhibit cancer cell growth strongly. Secondly, an antagonist of PPARγ GW9662 does not block the abilities of PPARγ agonists to inhibit growth in certain cancer cell lines [7,8]. Thirdly, in PPARγ knock out mouse embryonic fibroblast cells, troglitazone still strongly induces cell cycle arrest [9]. Moreover, troglitazone actually increases the number of small intestinal polyps in a genetic colon cancer model [10,11]. Importantly, a Phase II clinical trial using PPARγ agonist rosiglitazone to treat breast cancer patient was prematurely terminated due to unsatisfactory results [12]. Collectively, these evidences raise the question whether the anti-cancer effects of certain PPARγ ligands are primarily mediated through PPARγ. Intriguingly, the thiazolidinedione class of PPARγ ligand such as ciglitazone, rosiglitazone, and troglitazone suppress cell growth through inducing reactive oxygen species (ROS) production [13]. However, the mechanism responsible for this thiazolidinedione-induced ROS production is not well understood.

Estrogen-related receptor α and γ (ERRα and ERRγ) are constitutively active members of the nuclear hormone receptor super-family [14]. ERRα and ERRγ are thought to regulate mitochondrial biogenesis and energy homeostasis together with their coactivators PPARγ coactivator-1α and -1β (PGC-1α and PGC-1β) [15]. As their names suggested PGC-1α and PGC-1β are coactivators for PPARγ in addition to ERRα and ERRγ [16,17], implying that these receptors can crosstalk at the level of their common coactivators. In fact, competition for a limiting amount of coactivators also known as "squelching" may account for one of the mechanisms behind the crosstalk among different members of the nuclear hormone receptor super-family. In this study, we examined if troglitazone would affect ERRα and ERRγ activities by measuring its effects on ERR-mediated transcriptional regulation, coactivator interaction, and mitochondrial function. Instead of relying simply on coactivator squelching, we found that

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troglitazone suppressed the activities of ERRα and ERRγ by directly functioning as their inverse agonists.

2. Materials and methods

2.1. Plasmids and chemicals

The pGL3-PGC-1α- and PGC-1β-promoter reporter plasmids were cloned by PCR amplification of their 2 kb genomic regions upstream of their respective transcriptional start sites into pGL3-luciferase vector (Promega, Madison, WI, USA). Troglitazone, estrogen, 4-OH tamoxifen, and GW9662 were purchased from Cayman Chemical (Ann Arbor, MI, USA). XCT-790 was synthesized and purified as described [18]. Manganese(III) 5,10,15,20-tetrakis(4-benzoic acid)porphyrin (MnTBAP) was purchased from Calbiochem (San Diego, CA, USA).

2.2. Cell culture and cell growth assay

Human lung adenocarcinoma A549 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and grown in RPMI-1640 medium (GIBCO, Carlsbad, CA, USA) supplemented with 5% fetal bovine serum (FBS) (HyClone, Guangzhou, China), 1× penicillin/streptomycin (GIBCO, Carlsbad, CA, USA), and maintained at 37 °C and 5% CO_2. For cell growth assay, cells were seeded in 96-well plates at a density of 5000 cells/well with or without compounds added. After treatment of indicated time, cell growth was measured by using CellTiter-Glo™ Kit (Promega, Madison, WI, USA).

2.3. Transfections

Transient transfections were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. For luciferase reporter assays, cells at 85–95% confluency in 96-well plates were cotransfected with reporter plasmids (25 ng/well), Renilla luciferase (3 ng/well) as an internal control for transfection efficiency and appropriate mammalian expression vectors (25 ng/well). Six hours after transfection, cells were treated with drugs for 24 h. Luciferase activity was measured as described [19,20].

2.4. Receptor and coactivator interaction analysis

Interactions between His_6-tagged-ERRα- or ERRγ-LBD with PGC-1α or SRC3 coactivator peptides in the presence of different ligands were analyzed by a BIAcore 3000 system as described [19,20]. Human His_6-tagged-ERRα- or ERRγ-LBD protein was expressed in *Escherichia coli* BL21(DE3) with 0.5 mM IPTG induction at 18 or 15 °C for 20 h and purified to more than 95% pure by a Ni-NTA column (QIAgene, Valencia, CA, USA). Biotinylated PGC-1α peptides: EEPSLAKKAALAPAN (NR2 negative control); ENEANLLAVLTETLD (NR1); EEPSLLKKLLLAPAN (NR2); and RPCSELLKYLTTNDD (NR3) were captured onto streptavidin immobilized surface of SA chip in flow cells 1, 2, 3, and 4 respectively. ERRα- or ERRγ-LBD in running buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1 mM $MgCl_2$, 0.002% NP-40, 0.2 mM DTT) were injected into flow cells for 3 min at a flow rate of 20 μL/min at 25 °C. Changes in resonance units (RU) were monitored simultaneously in all flow cells. Surface was regenerated at the end of each cycle by an injection of 0.05% SDS. Sensorgrams were generated by BIAcontrol software 4.1 using double-referencing to eliminate responses from the reference surface and buffer-only control. Specific changes in RU were generated with non-specific interaction between LBD and NR2 negative control mutant peptide deducted.

2.5. Quantitative real-time PCR

Total RNA extraction, first-strand cDNA generation, and quantitative real-time PCR analysis were performed as described [21]. The sequences of primers are listed in Supplemental data.

2.6. Mitochondrial mass assay

Mitochondrial mass measured by staining with mitotracker green (Invitrogen, Carlsbad, CA, USA) was performed as described [22]. Briefly, cells were incubated in serum free medium (pre-warmed to 37 °C) with 150 nM Mitotracker Green FM for 20 min in the dark. After staining, cells were washed twice with cold phosphate-buffered saline (PBS) and suspended in 200 μl PBS. Subsequently, cells were analyzed on a FAC-SCalibur (BD Biosciences, San Jose, CA, USA) flow cytometer with excitation at 490 nm and emission at 516 nm. Data were processed by using the CellQuest program (BD Biosciences, San Jose, CA, USA).

2.7. Reactive oxidant species (ROS) assay

The determination of ROS was based on the oxidation of 2,7-dichlorodihydrofluorescin (DCHF-DA) (Beyotime Jiangsu China) by peroxide as described [23]. In brief, cells were washed and incubated with DCFH-DA for 20 min at 37 °C in the dark. Cells were then washed twice and harvested in PBS. The fluorescence of 2,7-dichlorofluorescein (DCF) was detected with a flow cytometer (FAC-SCalibur, BD Biosciences) with excitation at 488 nm and emission at 530 nm. Data were processed by using the CellQuest program (BD Biosciences).

2.8. Western blot analysis

Cells were lysed using RIPA reagent (Shenneng, Shanghai, China) according to the manufacturer's protocol and protein extracts were analyzed by 10% SDS-PAGE and blotted onto PVDF membrane. Membranes were incubated with rabbit anti-human-PGC-1α or -1β antibodies (Cell signaling technology, Boston, MA, USA) or anti-β-actin antibody (Boster, Wuhan, China) followed by horseradish peroxidase-conjugated secondary antibody (Amersham, Piscataway, NJ, USA) and developed with BeyoECL Plus reagent (Beyotime, Guangzhou, China).

2.9. Statistical analysis

Assays done in triplicate were repeated at least three times. Data are presented as mean ± SE and analyzed by a variance test (ANOVA).

3. Results

3.1. Troglitazone induces growth arrest in a ROS-dependent manner

Although several clinical trials using troglitazone to treat advanced stage cancers were not effective [24], a large scale retrospective study nonetheless suggested that troglitazone reduces lung cancer risk [25]. We therefore first investigated if the troglitazone-mediated growth inhibition was dependent on activating PPARγ in lung cancer A549 cells. We found that troglitazone dose-dependently inhibited the growth of A549 (Fig. 1A); however, the PPARγ antagonist GW9662 failed to block this growth suppressive effect of troglitazone (Fig. 1A) even though it reversed the PPARγ transcriptional activity induced by troglitazone (Supplemental data Fig. S1). On the other hand, troglitazone dose-dependently induced the accumulation of ROS which could be blocked by an anti-oxidant MnTBAP (Fig. 1B) but

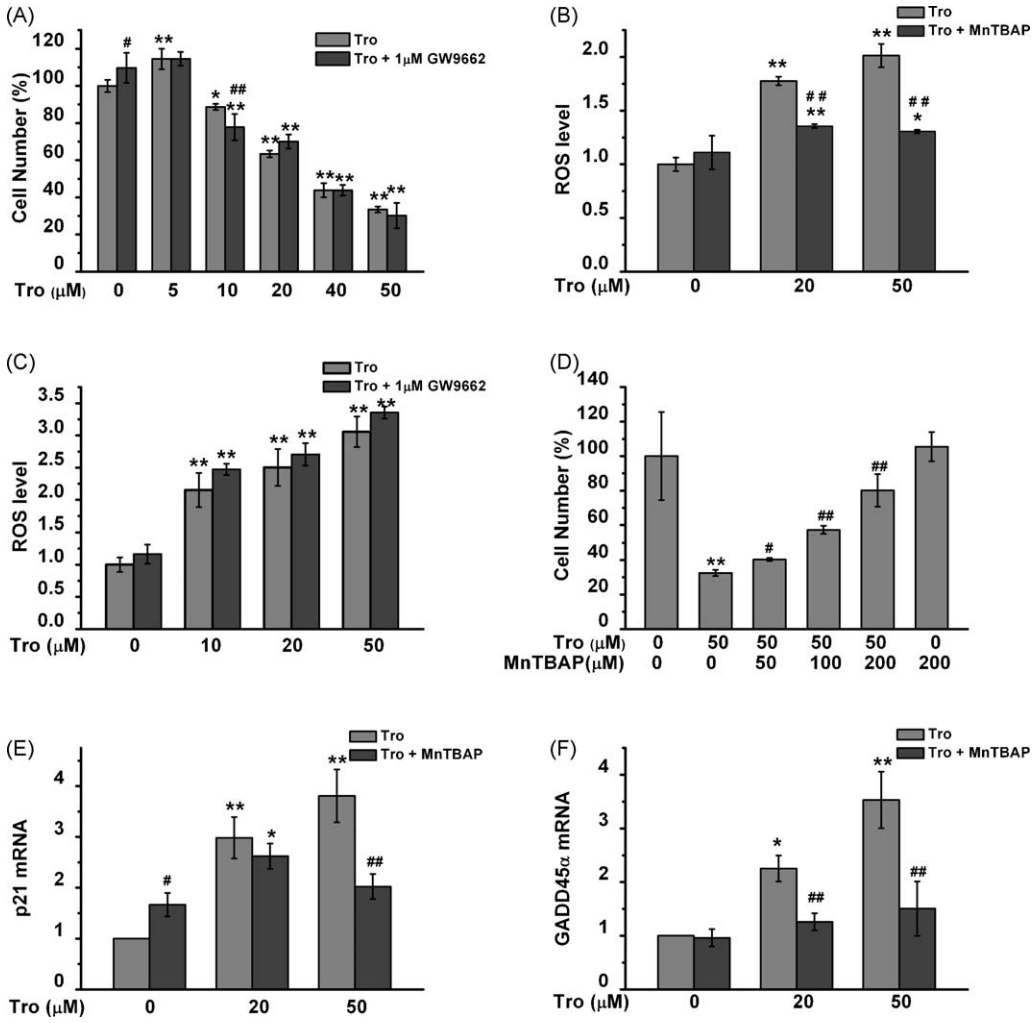

Fig. 1. Troglitazone induces growth arrest in a ROS-dependent manner. (A) A549 cells were pretreated with or without 1 μM GW9662 for 3 h prior to the addition of different doses of troglitazone or DMSO as a control for another 48 h before cell growth assay. DMSO treatment was set as 100%. (B and C) After a pre-treatment with 200 μM MnTBAP (B) or 10 μM GW9662 (C) for 3 h, different doses of troglitazone or DMSO as a control were added for another 24 h before ROS assay. The result of DMSO treatment was set as 1. (D) Different doses of MnTBAP were added for 3 h prior to the addition of 50 μM troglitazone or DMSO as a control for another 48 h before cell growth assay. (E and F) After a pre-treatment with 200 μM MnTBAP, different doses of troglitazone or DMSO as a control were added in the presence or absence of MnTBAP for another 24 h before RNA collection. The expression levels of $p21^{WAF1}$ (E) and GADD45α (F) were measured by real-time PCR with 18S rRNA as a control. The relative expression level of gene with DMSO was set at one. (A–F) Results represent mean ± SD of three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ for troglitazone compared to DMSO; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ for GW9662 or MnTBAP addition compared to troglitazone alone.

not by the PPARγ antagonist GW9662 (Fig. 1C). Importantly, the troglitazone-mediated growth inhibition was reversed by MnTBAP (Fig. 1D). Consistently, the ability of troglitazone to induce the mRNA expression levels of cell cycle inhibitor $p21^{WAF1}$ and DNA repair enzyme GADD45α were blocked by MnTBAP (Fig. 1E and F). These data collectively suggested that the mechanism of troglitazone-mediated growth inhibition is not solely dependent on activating PPARγ but on inducing ROS instead.

3.2. Troglitazone alters mitochondrial function

Since MnTBAP functions as an anti-oxidant through mimicking the action of superoxide dismutase, we then tested if aberrant expressions of superoxide dismutases 1 and 2 (SOD1 and SOD2) are related to the ability of troglitazone to induce ROS. We measured and found that troglitazone reduced the mRNA expression levels of SOD1 and SOD2 (Fig. 2A). We then investigated into the mechanism responsible for the down-regulation of these enzymes. The mRNA expression levels of SOD1 and SOD2 are in part controlled by coactivator PGC-1α [26]. We then found that the mRNA expression level of PGC-1α was suppressed by 50 μM troglitazone (Fig. 2B); whereas, the mRNA expression level of a related family member PGC-1β was suppressed by troglitazone in a dose-dependent manner (Fig. 2B). Consistently, the protein level of PGC-1β was more strongly reduced by troglitazone compared to PGC-1α and β-actin as a control (Fig. 2C). These transcriptional coactivators are key controllers of mitochondrial biogenesis [27], down-regulation of their expression would be expected to affect mitochondrial mass. We indeed found that troglitazone dose-dependently suppressed mitochondrial mass (Fig. 2D). This reduction of mitochondria mass was not blocked by the anti-oxidant MnTBAP (Fig. 2D), suggesting that the reduction is not a result of the increase in ROS. Importantly, this suppressive effect on mitochondrial mass was not blocked by PPARγ antagonist GW9662 (Fig. 2E), indicating that this troglitazone-mediated suppression is independent of activating PPARγ.

3.3. Troglitazone suppresses the transcriptional activities of ERRα and ERRγ

Since the troglitazone-suppressed PGC-1α and PGC-1β expression is correlated to its effects on inducing ROS and arresting

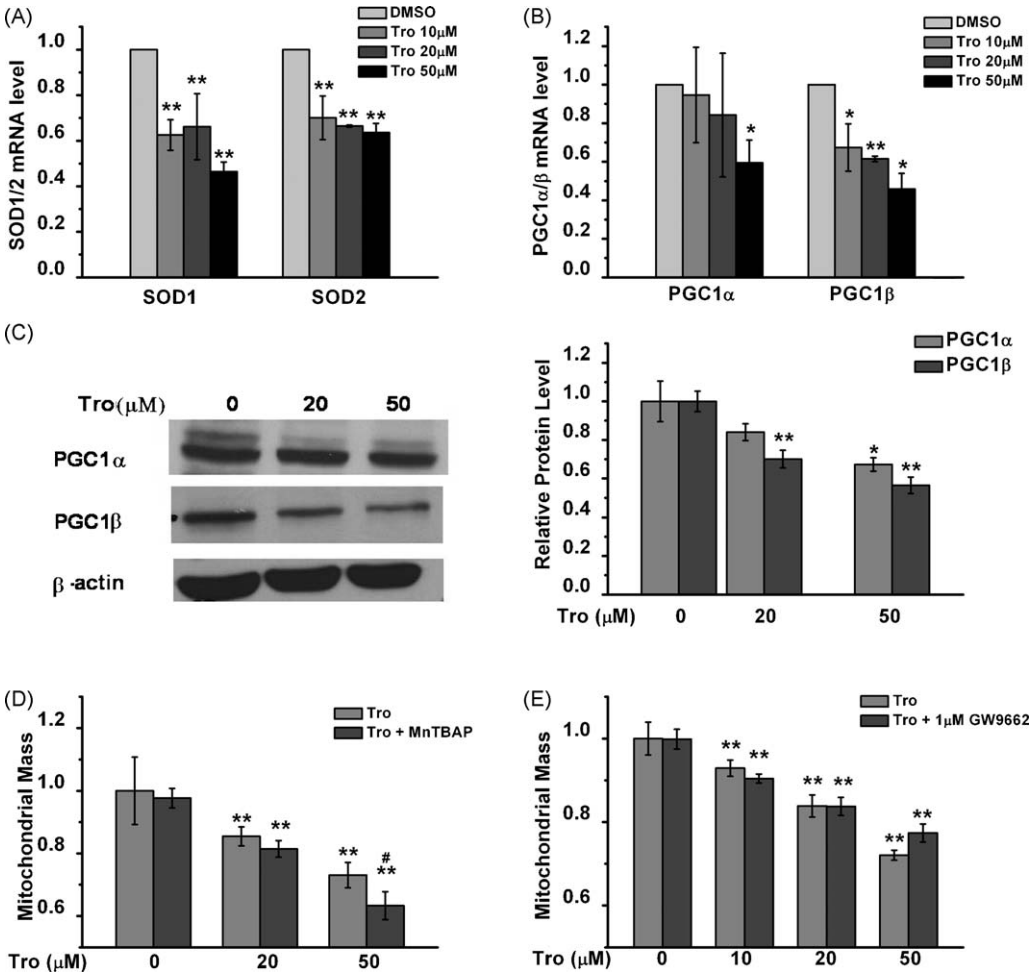

Fig. 2. Troglitazone alters mitochondrial function. (A and B) A549 cells were treated with different doses of troglitazone or DMSO as a control for 24 h before RNA collection. The expression levels of SOD1 and SOD2 (A) and PGC-1α and PGC-1β (B) were measured by real-time PCR with 18S rRNA as a control. Relative expression level with DMSO as control was set at 1. (C) Western blots of PGC-1α, PGC-1β, and β-actin as a control were shown (left panel). The relative levels of PGC-1α and PGC-1β compared to β-actin were quantified (right panel). (D and E) After pre-treatments with 200 μM MnTBAP (D) or 1 μM GW9662 (E) for 3 h, different doses of troglitazone or DMSO as a control were added for another 24 h before mitochondrial mass assay. Relative mitochondrial mass of DMSO treatment was set as 1. Results represent mean ± SD of three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ for troglitazone compared to DMSO; $^{\#}P < 0.05$ for GW9662 or MnTBAP addition compared to troglitazone alone.

growth, we next investigated into the mechanism behind suppressing their expression levels. The expression levels of a luciferase reporter under the control of PGC-1α- and PGC-1β-promoters were enhanced by ERRα [28]. Troglitazone may suppress the activity of ERRα to reduce the expressions of PGC-1α and PGC-1β. We found that over-expressing ERRα enhanced the expression of a PGC-1α-promoter luciferase reporter and this ERRα-driven expression was dose-dependently suppressed by troglitazone (Fig. 3A). Since the DNA binding specificity of ERRγ is similar to that of ERRα, we also found that over-expressing ERRγ enhanced the expression of the PGC-1α-promoter luciferase reporter although to a lesser extent compared to ERRα and troglitazone dose-dependently inhibited this ERRγ-driven expression (Fig. 3A). Intriguingly, ERRγ enhanced the expression of a PGC-1β-promoter luciferase reporter better than ERRα (Fig. 3B). Both ERRα- and ERRγ-driven PGC-1β-promoter luciferase reporter expressions were dose-dependently suppressed by troglitazone with the ERRγ-driven expression being more sensitive (Fig. 3B). These troglitazone-mediated inhibitions were not due to a non-specific effect on transcription in general when cells are undergoing arrest as troglitazone did not significantly affect the estradiol-induced transcriptional activity of estrogen receptor β (Supplemental data Fig. S2). These evidences suggested that troglitazone specifically down-modulates the activities of both ERRα and ERRγ. Indeed, using another promoter which can be regulated by both ERRα and ERRγ, we also confirmed that troglitazone dose-dependently suppressed the ERRα- and ERRγ-driven expressions (Supplemental data Fig. S3).

3.4. Troglitazone suppresses the interactions between ERRα and ERRγ with coactivators

Since troglitazone suppressed the activities of ERRα and ERRγ in a similar manner to their respective inverse agonists XCT-790 [18] and 4-OH tamoxifen [29], we next asked if troglitazone would affect the abilities of ERRα- and ERRγ-LBD to interaction with coactivators. By a surface plasmon resonance (SPR) assay, we previously established that flavonoids apigenin and kaempferol are ERRα and ERRγ inverse agonists [19,20]. Using this SPR assay, we found that troglitazone inhibited the interaction between ERRγ with the NR2 receptor interacting motif of PGC-1α similar to 4-OH tamoxifen as a positive control (Fig. 4A and Supplemental data Fig. S4). In addition, we also found that troglitazone inhibited the interaction between ERRγ with another receptor interacting motif on coactivator SRC-3 (Fig. 4B). Using XCT-790 as a positive control, similar observation was obtained with ERRα (Fig. 4C). Since this *in vitro* analysis only utilized purified receptor ligand binding domain and coactivator receptor interacting motif peptide, we concluded

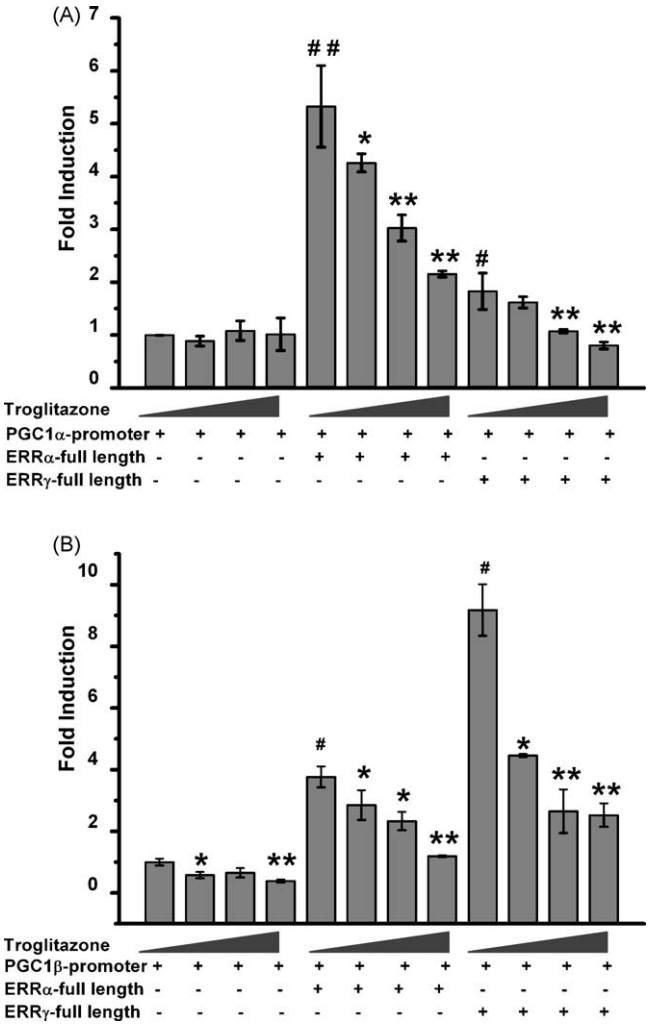

Fig. 3. Troglitazone suppresses the transcriptional activities of ERRα and ERRγ. (A and B) A549 cells were transiently transfected with a PGC-1α- (A) or PGC-1β- (B) promoter luciferase reporter plasmid, expression plasmids of ERRα or ERRγ, and an internal control *Renilla* luciferase plasmid. DMSO, 10, 20, or 50 μM of troglitazone was added for 24 h before luciferase assay. The activity of luciferase was normalized to that of *Renilla* luciferase to obtain relative activity. Fold inductions of the relative activity with DMSO as a control were shown. All transfection experiments were performed in triplicate wells and each experiment had been repeated at least three times. Results represent mean ± SD. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ for ERRα or ERRγ expression plasmid compared to control expression plasmid; $^{*}P < 0.05$ and $^{**}P < 0.01$ for troglitazone compared to DMSO.

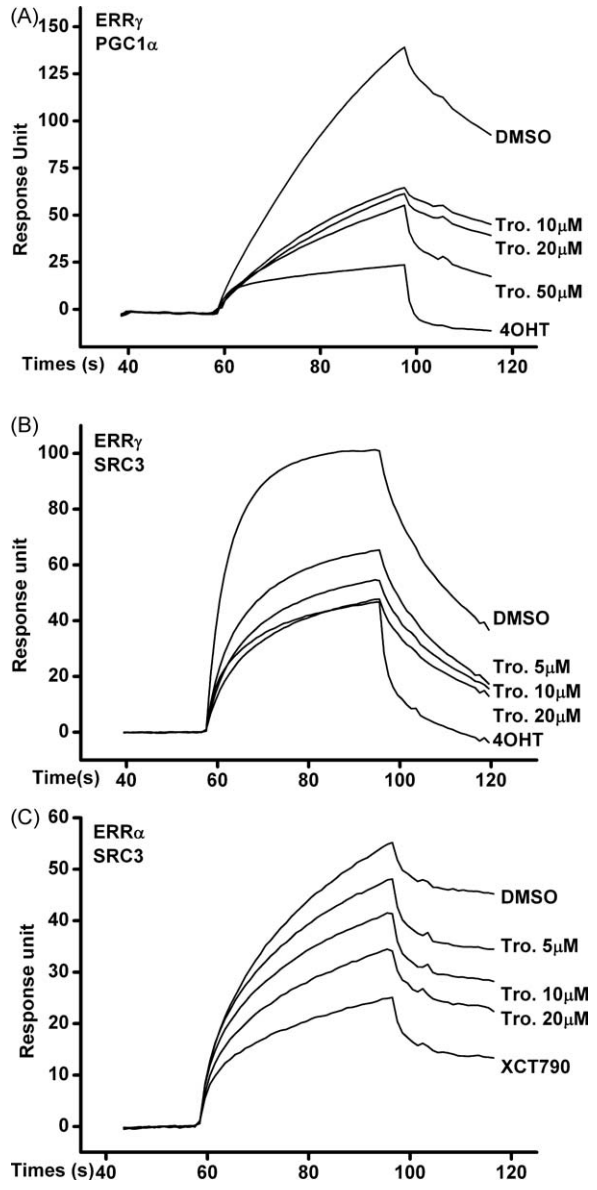

Fig. 4. Troglitazone suppresses the interactions between ERRα and ERRγ with coactivators. (A) DMSO, 1 μM 4-OH tamoxifen, or different doses of troglitazone was incubated with 50 nM ERRγ-LBD before detecting its interaction with the receptor interacting motif (NR2) of PGC-1α. (B) DMSO, 1 μM 4-OH tamoxifen, or different doses of troglitazone was incubated with 75 nM ERRγ-LBD before detecting its interaction with the receptor interacting motif (NR1) of SRC3. (C) DMSO, 20 μM XCT-790, or different doses of troglitazone was incubated with 500 nM ERRα-LBD before detecting its interaction with SRC3 NR1 motif.

that troglitazone binds to the ligand binding domain directly and inhibits the abilities of these receptors to interact with coactivators. In other words, troglitazone is an inverse agonist of ERRα and ERRγ.

4. Discussion

Troglitazone and other thiazolidinediones (TZDs) have been shown to be effective therapeutic agents for diabetes [1]. Recently, several of these TZDs have been suggested to be potential therapeutic agents for cancers due to their growth suppressive effects *in vitro* and in some cases *in vivo* [4]. Since TZDs are thought to mediate their therapeutic effects through specifically activating PPARγ, PPARγ has then been suggested to suppress tumor growth [4]. Although the role of PPARγ in TZD-mediated cancer cell growth inhibition is controversial, TZDs seem to arrest cell growth through inducing ROS [13]. Our results confirmed that ROS is a key to the troglitazone-mediated growth inhibition. Importantly, we uncovered another facet of the mechanistic action of troglitazone; i.e., suppression of ERRα and ERRγ activities.

While troglitazone has been clearly demonstrated to activate PPARγ, we demonstrated that troglitazone also suppresses the activities of ERRα and ERRγ. Besides affecting ERRα and ERRγ activities, troglitazone has also been demonstrated to down-regulate estrogen receptor signaling in breast cancer cells [30,31]. The mechanism behind this effect appears be related to troglitazone's ability to promote estrogen receptor alpha protein degradation in a PPARγ independent manner [32]. Alternatively, transcriptional coactivator squelching has been suggested to be a mechanism by which nuclear hormone receptors mediate their transrepressive effects on each other. It is conceivable that troglitazone activates PPARγ and squelches limiting amount of coactivator away from ERRα and ERRγ, resulting in a suppression

of their activities. However, the fact that troglitazone does not significantly affect the activity of estrogen receptor β, which also utilizes a similar set of coactivators [33], suggests that squelching does not play an important role in troglitazone's suppressive effects on ERRα and ERRγ activities.

Instead, using a highly purified *in vitro* system, we demonstrated that troglitazone inhibits the abilities of these receptors to interact with their coactivators making troglitazone an inverse agonist for both ERRα and ERRγ. As a dual ERRα and ERRγ inverse agonist, troglitazone suppresses not only the ERRα- and ERRγ-driven PGC-1α and PGC-1β expressions but also the PGC-1α- and PGC-1β-governed mitochondrial biogenesis and anti-oxidative enzyme expression, promoting the production of ROS which induces the expressions of cell cycle inhibitor $p21^{WAF1}$ and DNA repair enzyme GADD45α. These observations are similar to what we had established for ERRα inverse agonist XCT-790 [34]. Noticeably, over-expression of ERRα is associated with adverse clinical outcomes in several different types of cancer [35]; thus, suppressing its activity may represent a novel way of treating cancer. Therefore, we suggest that troglitazone inhibits cell growth in part through suppressing the activities of ERRα and ERRγ.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2010.03.013.

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