Peroxisome Proliferator-Activated Receptor γ-Independent Repression of Prostate-Specific Antigen Expression by Thiazolidinediones in Prostate Cancer Cells

Chih-Cheng Yang, Chia-Yu Ku, Shuo Wei, Chung-Wai Shiau, Chang-Shi Chen, Joseph J. Pinzone, Mathew D. Ringel, and Ching-Shih Chen

Division of Medicinal Chemistry, College of Pharmacy (C.-C.Y., C.-Y.K., S.W., C.-W.S., Cha.-S.C., Chi.-S.C.) and Divisions of Endocrinology and Oncology, Department of Internal Medicine (J.J.P., M.D.R.), The Ohio State University, Columbus, Ohio

ABSTRACT

In light of the potential use of the thiazolidinedione family of peroxisome proliferator-activated receptor-γ (PPARγ) agonists in prostate cancer treatment, this study assessed the mechanism by which these agents suppress prostate-specific antigen (PSA) secretion in prostate cancer cells. Two lines of evidence indicate that the effect of thiazolidinediones on PSA down-regulation is independent of PPARγ activation. First, this thiazolidinedione-mediated PSA down-regulation is structure-specific irrespective of the relative PPARγ agonist potency. Second, the PPARγ-inactive analogs of troglitazone and ciglitazone [Δ2TG (5-[4-(6-hydroxy-2,5,7,8-tetramethyl-chroman-2-yl-methoxy)-benzylidene]-thiazolidine-2,4-dione) and Δ2CG (5-[4-(1-methyl-cyclohexylmethoxy)-benzylidene]-thiazolidine-2,4-dione), respectively] exhibit higher potency than the parent compound in inhibiting dihydrotestosterone (DHT)-stimulated PSA secretion. Although 10 μM troglitazone and Δ2TG signifi-cantly inhibit PSA secretion, they do not alter the expression level of androgen receptor (AR) or interfere with DHT-activated nuclear translocation of AR. However, reporter gene and chromatin immunoprecipitation studies indicate that troglitazone and Δ2TG block AR recruitment to the androgen response elements within the PSA promoter. Thus, this study raises the question of whether the ability of oral troglitazone to reduce PSA levels in prostate cancer patients is therapeutically relevant. A major concern is that the concentration for troglitazone to mediate antitumor effects is severalfold higher than that of PSA down-regulation, which is difficult to attain at therapeutic doses. Nevertheless, it is noteworthy that troglitazone and Δ2TG at high doses were able to inhibit AR expression. From a translational perspective, separation of PPARγ agonist activity from AR down-regulation provides a molecular basis to use troglitazone as a platform to design AR-ablative agents.

Becauase prostate-specific antigen (PSA) is used as a sur-rogate marker for disease progression and response for pros-tate cancer treatments (for review, see Polascik et al., 1999), the effect of therapeutic agents on PSA expression warrants attention (for review, see Dixon et al., 2001). Because PSA expression and cell proliferation are independently regulated functions in prostate cancer cells (Cunha et al., 1987), a therapeutic agent may down-regulate PSA expression/secre-tion without inhibiting tumor cell growth. In such a case, a patient receiving this agent might be falsely considered to have a clinical response. Among a series of agents examined to date, the ability of thiazolidinediones (e.g., troglitazone) to down-regulate PSA is noteworthy (Hisatake et al., 2000; Mueller et al., 2000). This family of peroxisome proliferator-activated receptor γ (PPARγ) agonists increases transcrip-tion of certain insulin-sensitive genes involved in the metab-olism and transport of lipids through PPARγ activation, thereby improving insulin sensitivity. Moreover, at high doses, these agents exhibit in vitro and in vivo antitumor effects against human prostate cancer (Kubota et al., 1998; Mueller et al., 2000; Kumagai et al., 2004), although the underlying mechanism remains elusive. In light of the role of PPARγ in the regulation of prostatic epithelial proliferation

ABBREVIATIONS: PSA, prostate-specific antigen; PPARγ, peroxisome proliferator-activated receptor γ; DHT, dihydrotestosterone; AR, androgen receptor; FBS, fetal bovine serum; PPRE, peroxisome proliferator-activated receptor response element; TK, thymidine kinase; PBS, phosphate-buffered saline; TBST, Tris-buffered saline/Tween 20; ChIP, chromatin immunoprecipitation; PCR, polymerase chain reaction; ARE, androgen response element; Δ2TG, 5-[4-(6-hydroxy-2,5,7,8-tetramethyl-chroman-2-yl-methoxy)-benzylidene]-2,4-thiazolidinedione; Δ2RG, 5-{4-[2-(methyl-pyridin-2-yl-amino)-ethoxy]-benzylidene}-thiazolidine-2,4-dione; Δ2PG, 5-{4-[2-(5-ethyl-pyridin-2-yl)-ethoxy]-benzylidene}-thiazolidine-2,4-dione; Δ2CG, 5-[4-(1-methyl-cyclohexylmethoxy)-benzylidene]-thiazolidine-2,4-dione.

and differentiation, thiazolidinediones have been suggested to be useful in the setting of adjuvant and chemopreventive treatments of prostate cancer (Lieberman, 2002; Koeffler, 2003; Jiang et al., 2004). More recently, accumulating evidence suggests that the effect of thiazolidinediones on cell cycle and apoptosis in cancer cells is dissociated from their PPARγ agonist activity (Sugimura et al., 1999; Motomura et al., 2000; Okura et al., 2000; Gouni-Berthold et al., 2001; Palakurthi et al., 2001; Takeda et al., 2001; Bae and Song, 2003; Baek et al., 2003; Huang et al., 2005; Shiau et al., 2005). For example, there is discrepancy of 3 orders of magnitude between the concentration required to produce antitumor effects and that required to mediate PPARγ activation. In addition, the antitumor effect seems to be structure-specific, irrespective of potency in PPARγ activation (i.e., troglitazone and ciglitazone are active, whereas rosiglitazone and pioglitazone are not). More recently, we demonstrated that the effect of thiazolidinediones on apoptosis and cell cycle arrest in cancer cells was attributable, in part, to their ability to inhibit Bcl-xL/Bcl-2 functions and to ablate cyclin D1 expression (Huang et al., 2005; Shiau et al., 2005).

Considering the potential use of these agents in inhibiting prostate carcinogenesis, the mechanism whereby these PPARγ agonists repress PSA expression warrants investigation. By using PPARγ-inactive thiazolidinedione derivatives, we obtained evidence that the effect of troglitazone and ciglitazone on PSA down-regulation was independent of PPARγ activation. Moreover, the ability of low doses (≤10 μM) of troglitazone and ciglitazone to suppress PSA expression was caused not by reduced AR expression but by a decrease in the AR response element (ARE) activity in the PSA promoter.

Materials and Methods

Reagents. Troglitazone and ciglitazone were purchased from Sigma (St. Louis, MO) and Cayman Chemical (Ann Arbor, MI), respectively. Rosiglitazone and pioglitazone were prepared from the respective commercial capsules by solvent extraction followed by recrystallization or chromatographic purification. Δ2TG (5-[4-(6-hydroxy-2,5,7,8-tetramethyl-chroman-2-yl-methoxy)-benzylidene]-thiazolidine-2,4-dione), Δ2CG (5-[4-(1-methyl-cyclohexylmethoxy)-benzylidene]-thiazolidine-2,4-dione), Δ2RG (5-{4-[2-(methyl-pyridin-2-yl-amino)-ethoxy]-benzylidene}-thiazolidine-2,4-dione), and Δ2PG (5-{4-[2-(5-ethyl-pyridin-2-yl)-ethoxy]-benzylidene}-thiazolidine-2,4-dione) are thiazolidinedione derivatives with attenuated or unappreciable activity in PPARγ activation (Huang et al., 2005; Shiau et al., 2005). These agents were dissolved at various concentrations in DMSO and were added to cells in medium with a final DMSO concentration of 0.1%. Dihydrotestosterone (DHT; Sigma, St. Louis, MO) was dissolved in 100% ethanol (8 mg/ml) as a stock solution for serial dilutions in water. Mouse antibodies against AR, PSA, and α-tubulin were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Goat anti-rabbit and rabbit anti-mouse immunoglobulin G horseradish peroxidase conjugates were from Jackson ImmunoResearch Laboratories (West Grove, PA).

Cell Culture. LNCaP and 22RV1 cells were purchased from the American Type Culture Collection (Manassas, VA). LNCaP cells were cultured in a T-75 flask with RPMI 1640 medium containing 10% heat-inactivated FBS at 37°C in a humidified incubator containing 5% CO_2; 22RV1 cells were cultured in the same media supplemented with 4.5 mg/ml of glucose. For individual experiments, 10% FBS-supplemented RPMI 1640 medium was replaced by phenol red-free RPMI 1640 medium containing 10% charcoal/dextran-stripped FBS. The cells were cultured for 2 days before drug treatments.

PSA Immunoassay. Quantitative determinations of PSA in culture medium were performed by using a human PSA enzyme-linked immunosorbent assay kit (Anogen, Mississauga, ON, Canada). In brief, LNCaP and 22RV1 cells were plated in 96-well plates (6000 cells/well) in phenol red-free RPMI 1640 medium with 10% charcoal/dextran-stripped FBS without and with glucose, respectively, incubated for 48 h, and treated with the test agent at the indicated concentrations in the same medium in six replicates. Control cells received dimethyl sulfoxide vehicle at a concentration equal to that of drug-treated cells. At different time intervals, 20 μl of the cultured medium was collected, diluted 10-fold with the sample diluent, and the amount of PSA was determined by following the manufacturer's instructions. Absorbance at 450 nm was determined on a microtiter plate reader.

Transfections and Luciferase Assay. The 6.0-kilobase PSA-promoter-linked reporter plasmid PSA6.0-Luc and the human AR expression construct pCMVhAR were provided by Dr. Chawnshang Chang (University of Rochester Medical Center, Rochester, NY) and Dr. James Dalton (The Ohio State University, Columbus, OH), respectively. The PPRE-x3-TK-Luc reporter vector contains three copies of the PPAR-response element (PPRE) upstream of the thymidine kinase promoter-luciferase fusion gene and was kindly provided by Dr. Bruce Spiegelman (Harvard University, Cambridge, MA). LNCaP or DU145 cells were incubated in phenol red-free RPMI 1640 medium with 10% FBS until they reached 50 to 70% confluence on a 100-mm plate and were transfected with 6 μg of each of the aforementioned plasmids using Fugene 6 (Roche, Indianapolis, IN) in RPMI 1640 medium. For each transfection, herpes simplex virus thymidine kinase (TK) promoter-driven *Renilla reniformis* luciferase was used as an internal control for normalization. After transfections, cells were incubated in 10% charcoal-stripped FBS and RPMI 1640 medium, subject to different treatments for the times indicated in Figs. 1 and 6 and collected with passive lysis buffer (Promega, Madison, WI). Luciferase activity in the cell lysates was determined by luminometry. All transfection experiments were carried out in triplicate wells and repeated separately at least three times.

Immunoblotting. Cells in T-75 flasks were collected by scraping and suspended in 60 μl of phosphate-buffered saline (PBS). Two microliters of the suspension was taken for protein analysis using the Bradford assay kit (Bio-Rad, Hercules, CA). To the remaining solution was added the same volume of 2× SDS-polyacrylamide gel electrophoresis sample loading buffer (100 mM Tris-HCl, pH 6.8, 4% SDS, 5% β-mercaptoethanol, 20% glycerol, and 0.1% bromphenol blue). The mixture was sonicated briefly and then boiled for 5 min. Equal amounts of proteins were loaded onto 10% SDS-polyacrylamide gel electrophoresis gels.

After electrophoresis, protein bands were transferred to nitrocellulose membranes in a semidry transfer cell. The transblotted membrane was washed twice with Tris-buffered saline containing 0.1% Tween 20 (TBST). After blocking with TBST containing 5% nonfat milk for 40 min, the membrane was incubated with the appropriate primary antibody in TBST-1% nonfat milk at 4°C overnight. All primary antibodies were diluted 1:1000 in 1% nonfat milk-containing TBST. After treatment with the primary antibody, the membrane was washed three times with TBST for a total of 15 min, followed by incubation with goat anti-rabbit or anti-mouse IgG-horseradish peroxidase conjugates (diluted 1:5000) for 1 h at room temperature and three washes with TBST for a total of 1 h. The immunoblots were visualized by enhanced chemiluminescence.

Immunocytochemical Analysis of DHA-Stimulated AR Nuclear Localization. LNCaP cells were cultured on slides in six-well plates (200,000 cells/well) in 10% charcoal-stripped, FBS-supplemented phenol red-free RPMI 1640 and exposed to 10 nM DHT, 10 nM DHT plus 10 μM troglitazone, or Δ2TG for 48 h, washed with Dulbecco's PBS, fixed with 4% paraformaldehyde for 30 min at 37°C, and then washed with PBS twice. For staining of AR, the cells were permeabilized with 0.1% Triton X-100 in 1% FBS-containing PBS and treated with mouse monoclonal anti-AR (1:100 dilution) in PBS

containing 0.1% Triton X-100 and 0.2% bovine serum albumin at 4°C overnight and washed with PBS. For fluorescent microscopy, Alexa Fluor 488 goat anti-mouse IgG (1:200 dilution; Molecular Probes) was used for conjugating AR. The nuclear counterstaining was performed using a 4,6-diamidino-2-phenylindole–containing mounting medium (Vector Laboratories, Burlingame, CA) before examination. Images of immunocytochemically labeled samples were observed using a Nikon microscope (Eclipse E800) with an argon laser and a helium-neon, and appropriate filters (excitation wavelengths, 488 nm for AR and 543 nm for 4,6-diamidino-2-phenylindole).

Chromatin Immunoprecipitation. ChIP was performed by using an EZ-Chip kit (Upstate Biotechnology, Inc., Lake Placid, NY) according to the manufacturer's instructions. LNCaP cells were cultured in 10 ml of 10% charcoal/dextran stripped, FBS-supplemented phenol red-free RPMI 1640 medium for 48 h. After drug treatment for 12 h, cells were cross-linked with 10 ml of fresh medium containing 1% formaldehyde at room temperature for 10 min. Glycine solution (1 ml, 1.25 M) was added to stop the cross-linking reaction, and cells were washed twice with 5 ml of PBS. The cells were collected, suspended in 350 μl of SDS lysis buffer, sonicated on wet ice by using a Virtis model Sonic 300 sonicator with five sets of 10-s pulses and 8% of max power, and centrifuged at 15,000g at 4°C for 10 min. Supernatants were collected, diluted with the dilution buffer, and treated with protein G agarose at 4°C for 1 h to preclean the chromatin. After a brief centrifugation at 4000g, the supernatant was collected into a fresh 1.5-ml microcentrifuge tube. Ten microliters of the supernatant was stored away at 4°C to be used as input, and the remaining supernatant was incubated with anti-AR (Upstate Biotechnology) at 4°C overnight. After immunoprecipitation, the solution was treated with 60 μl of protein G agarose slurry at 4°C for 1 h, followed by a brief centrifuge at 4000g. The protein G beads were washed, 1 ml each in tandem, with ice-cold low-salt wash buffer, high-salt wash buffer, LiCl wash buffer, and Tris/EDTA buffer, followed by extraction with elution buffer twice. The eluted solution was added 8 μl of 5 M NaCl and incubated at 65°C overnight. A spin column provided in the kit was used to purify DNA fragments. For PCR analysis, 1 μl of input DNA extraction and 5 μl of immunoprecipitated DNA extraction were used for 36 cycles of amplification. The primers for androgen response element (ARE)I (A/B), AREII (C/D), and the middle region (E/F) (Shang et al., 2002) were obtained from Integrated DNA Technologies (Coralville, IA). The sequences were as follows: A, TCTGCCTTTGTCCCCTAGAT; B, AACCTTCAT-TCCCCAGGACT; C, AGGGATCAGGGAGTCTCACA; D, GCTAG-CACTTGCTGTTCTGC; E, CTGTGCTTGGAGTTTACCTGA; F, GCAGAGGTTGCAGTGAGCC.

Results

PPARγ-Independent Repression of PSA Secretion and Expression in LNCaP Cells. We have developed PPARγ-inactive thiazolidinediones by introducing a double bond adjoining the terminal thiazolidine-2,4-dione ring (Fig. 1A). Two lines of evidence indicate that this structural modification abrogated the PPAR activity. First, these Δ2 analogs (Δ2TG, Δ2RG, Δ2PG, and Δ2CG) were inactive in PPARγ activation according to a PPARγ transcription factor enzyme-linked immunosorbent assay (Shiau et al., 2005). Second, LNCaP and DU-145 cells were transfected with a reporter construct (PPRE-X3-TK-Luc) that contains three copies of PPRE upstream of the luciferase gene and then tested for PPARγ transactivation. Although LNCaP cells transfected with PPRE-X3-TK-Luc did not respond to any the four thiazolidinedione-PPARγ agonists regarding luciferase induction, the transfected DU-145 cells exhibited differential increase in luciferase activity in response to these agents (10 μM), ranging from 3.5-fold (ciglitazone) to 7.5-fold (rosiglita-

zone) after 24-h exposure (Fig. 1B). In contrast, none of the Δ2 derivatives elicited any significant activation of the reporter.

These resulting Δ2 analogs (Δ2TG, Δ2RG, Δ2PG, and Δ2CG), through lack of global PPAR activity, exhibited similar antiproliferative potency against prostate cancer cells as their parent compounds (Shiau et al., 2005). Among them, Δ2TG and Δ2CG were active in inducing apoptosis with IC$_{50}$ in the range between 15 and 20 μM (compared with 20–25 μM for troglitazone and ciglitazone) in serum-free medium against various prostate cancer cell lines, whereas Δ2RG and Δ2PG exhibited no significant effect on apoptosis even at 50 μM (Shiau et al., 2005). The apoptosis-inducing activity of troglitazone, ciglitazone, and their Δ2 analogs, however, was attenuated in the presence of serum because of the effects of growth factors on the activation of intracellular signaling and the high serum protein-binding affinity of these agents. As shown in Fig. 2A, no appreciable antiproliferative activity was noted with up to 50 μM troglitazone or Δ2TG in the presence of 10% FBS, whereas these agents were able to elicit significant antitumor effects as low as 10 μM in serum-free medium.

From a mechanistic perspective, these PPARγ-inactive Δ2 analogs allowed us to discern the role of PPARγ activation in various pharmacological effects of thiazolidinediones, including those on Bcl-xL/Bcl-2 inhibition and cyclin D1 repression (Huang et al., 2005; Shiau et al., 2005). In this study, we
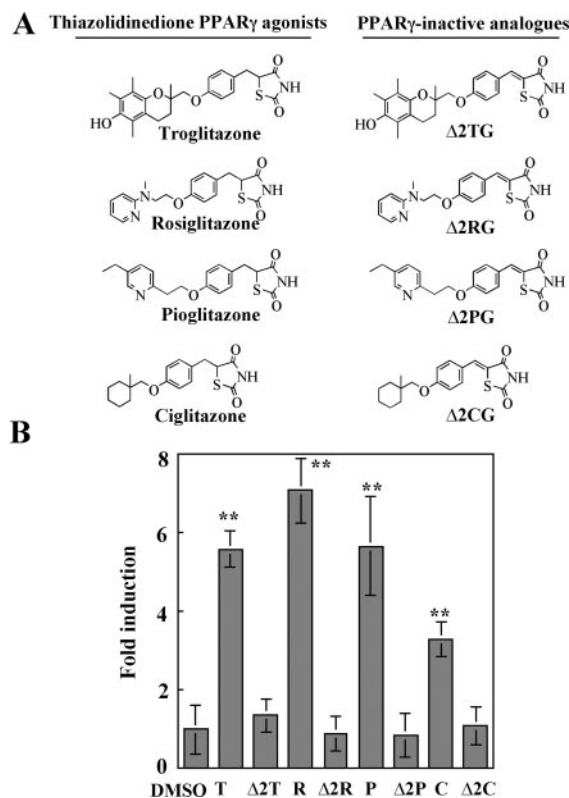
Fig. 1. Pharmacological evidence that the effect of thiazolidinediones on suppressing PSA secretion in LNCaP cells is independent of PPARγ activation. A, chemical structures of troglitazone, rosiglitazone, pioglitazone, ciglitazone, and the respective PPARγ-inactive Δ2 analogs (Δ2TG, Δ2RG, Δ2PG, and Δ2CG). B, activation of PPRE-X3-TK-Luc. DU-145 cells were transfected with PPRE-X3-TK-Luc and treated with individual test compounds (10 μM) for 24 h, and luciferase activity was determined as described under *Materials and Methods*. **, $P < 0.01$.
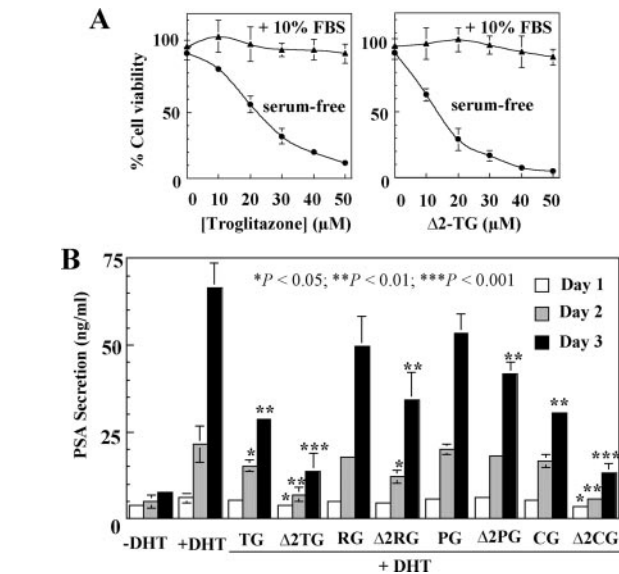
assessed the time-dependent effect of individual thiazolidinediones and their Δ2 counterparts, 10 μM each, on the secretion of PSA in LNCaP cells in 10% FBS-supplemented medium (Fig. 2B). As shown, DHT (10 nM) stimulated significant accumulations of secretary PSA in the medium by 9-fold throughout the 3-day time course, which could be differentially suppressed by individual thiazolidinediones. Although exposure to troglitazone and ciglitazone for 3 days reduced the PSA secretion by 55% ($P < 0.01$), rosiglitazone and pioglitazone, which are more potent PPARγ agonists, were only marginally effective. It is noteworthy that the
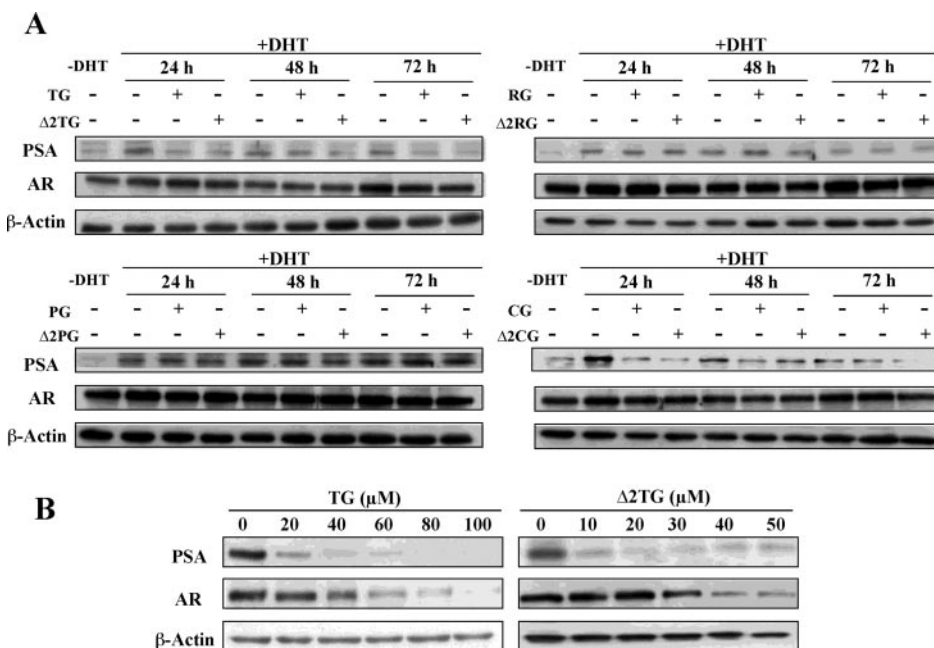

Fig. 2. A, dose-dependent effect of troglitazone (TG) and Δ2TG on the cell viability of LNCaP cells with or without 10% FBS. Cells were exposed to troglitazone or Δ2TG at the indicated concentrations in serum-free or 10% FBS-supplemented RPMI 1640 medium in 96-well plates for 24 h, and cell viability was assessed by MTT assay. Points, mean; bars, S.D. ($n = 6$). B, time-dependent effect of troglitazone (TG), Δ2TG, rosiglitazone (RG), Δ2RG, pioglitazone (PG), Δ2PG, ciglitazone (CG), and Δ2CG, each at 10 μM, on DHT-stimulated PSA secretion in LNCaP cells. Each data point represents mean ± S.D. ($n = 3$).

PPARγ-inactive Δ2 analogs were more effective than their parental thiazolidinediones, suggesting the dissociation of the effect on PSA down-regulation from PPARγ activation. Treatment of LNCaP cells with Δ2TG or Δ2CG for 3 days inhibited PSA excretion by as much as 80% ($P < 0.001$), whereas Δ2RG and Δ2PG attenuated PSA in medium by 50% and 40%, respectively ($P < 0.01$). This decrease in PSA secretion was not due to reduced cell viability because these agents at 10 μM were not able to induce appreciable apoptotic death in the presence of 10% FBS. Together, these findings suggest that troglitazone and ciglitazone mediated PSA down-regulation through a PPARγ-independent mechanism.

Figure 3A depicts the time-dependent effect of 10 μM thiazolidinediones and their Δ2 analogs on the intracellular protein levels of PSA and AR in LNCaP cells in 10% FBS-supplemented medium. As shown, DHT treatment increased PSA expression in a time-dependent manner, and the effect of individual agents on intracellular PSA production paralleled that of secretary PSA. Troglitazone, ciglitazone, Δ2TG, and Δ2CG were effective in reducing PSA expression as early as 24 h after treatment. The effect of troglitazone on repressing PSA expression is reminiscent of that reported in the literature (Hisatake et al., 2000). In contrast, rosiglitazone, pioglitazone, or their Δ2 derivatives did not give rise to appreciable reduction in PSA expression. Moreover, the expression level of AR was not altered by any of the test agents at 10 μM, suggesting that this PSA down-regulation was not attributable to decreased AR expression.

Nevertheless, it is noteworthy that troglitazone and Δ2TG at much higher concentrations were capable of lowering AR levels in LNCaP cells, although the underlying mechanism remained unclear. The IC_{50} values for suppressing AR expression were approximately 40 and 30 μM for troglitazone and Δ2TG, respectively (Fig. 3B). Together, these data indicate that the effects of troglitazone and Δ2TG on the repression of PSA and AR were mediated through distinct mechanisms.

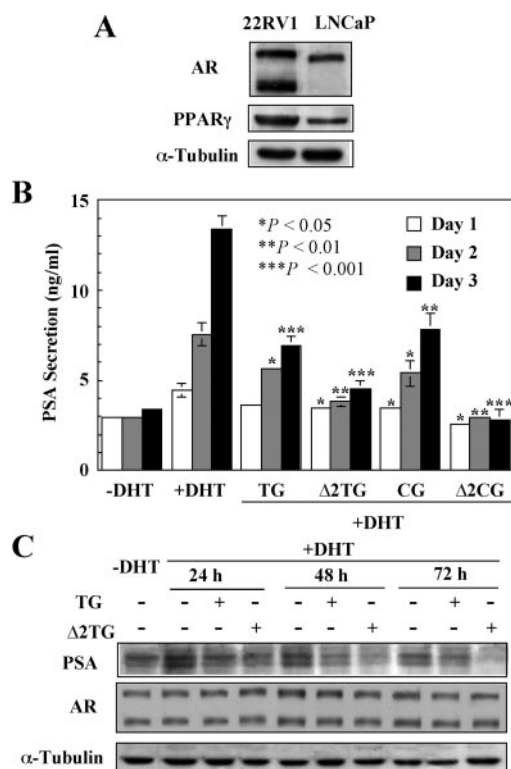**Fig. 3.** Effect of thiazolidinediones and their Δ2 analogs on intracellular protein expression levels of PSA and AR in LNCaP cells. A, time-dependent effect of troglitazone (TG), Δ2TG, rosiglitazone (RG), Δ2RG, pioglitazone (PG), Δ2PG, ciglitazone (CG), and Δ2CG, each at 10 μM, on PSA and AR expression in DHT-stimulated LNCaP cells. LNCaP cells were seeded in T-25 flasks and incubated with 10% charcoal-stripped FBS-containing RPMI 1640 medium for 2 days with or without 10 nM DHT, and exposed to individual tested agents at 10 μM, for the indicated times. Equivalent amounts of protein from cell lysates were electrophoresed and probed by Western blotting with individual antibodies. These immunoblots are representative of three independent experiments. B, dose-dependent effect of TG and Δ2TG on PSA and AR expression in DHT-stimulated LNCaP cells after a 48-h treatment. LNCaP cells were treated with the indicated doses of TG or Δ2TG as aforementioned for 48 h. Equivalent amounts of protein from cell lysates were electrophoresed and probed by Western blotting with individual antibodies. These immunoblots are representative of three independent experiments.

This PSA down-regulation was also confirmed in another androgen-dependent human prostate carcinoma cell line, 22RV1. 22RV1 cells exhibit two aberrant forms of AR (van Bokhoven et al., 2003) and expressed substantially higher levels of PPARγ compared with LNCaP cells (Fig. 4A). Al-

though 22RV1 cells secreted low levels of PSA in the presence of 10 nM DHT, treatment with these agents resulted in a significant suppression of PSA secretion, especially after 48 h (B). As observed in LNCaP cells, intracellular PSA levels in 22RV1 cells were down-regulated by 10 μM troglitazone and Δ2TG in a time-dependent manner, whereas AR expression was unaffected (C).

Troglitazone and Δ2TG Do Not Affect Nuclear Translocation of AR. Pharmacological agents might interfere with PSA expression at different stages of AR-mediated PSA transactivation, including AR expression, ligand binding, AR dimerization, and nuclear translocation, and AR binding to the androgen response elements (AREs). To discern these possibilities, we carried out immunocytochemical analysis to envisage the cellular distribution of AR in LNCaP cells treated with 10 μM troglitazone or Δ2TG. Figure 5 demonstrates that DHT treatment facilitated the translocation of AR from the cytoplasm to the nucleus. Exposure to 10 μM troglitazone or Δ2TG had no effect on this DHT-mediated AR translocation, and the total AR-staining intensity was not affected. This finding suggested that the down-regulation occurred at the level of ARE transactivation.

Troglitazone and Δ2TG Block Androgen Activation of the AREs in the PSA Promoter. To analyze the effect of troglitazone and Δ2TG on DHT-mediated transactivation of the PSA promoter, we transfected LNCaP cells with the PSA6.0-Luc vector, a luciferase reporter linked with the 6.0-

Fig. 4. Effect of troglitazone (TG), Δ2TG, ciglitazone (CG), and Δ2CG on PSA down-regulation in DHT-stimulated 22RV1 cells. A, differential expression of AR and PPARγ in 22RV1 versus LNCaP cells. B, time-dependent effect of 10 μM TG, Δ2TG, CG, and Δ2CG on PSA and AR expression in DHT-stimulated 22RV1 cells. C, time-dependent effect of TG and Δ2TG on AR expression in DHT-stimulated LNCaP cells.
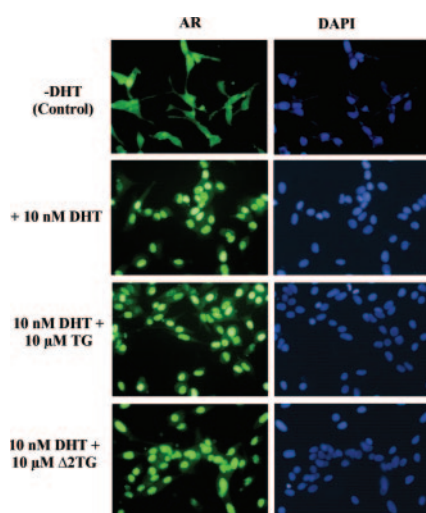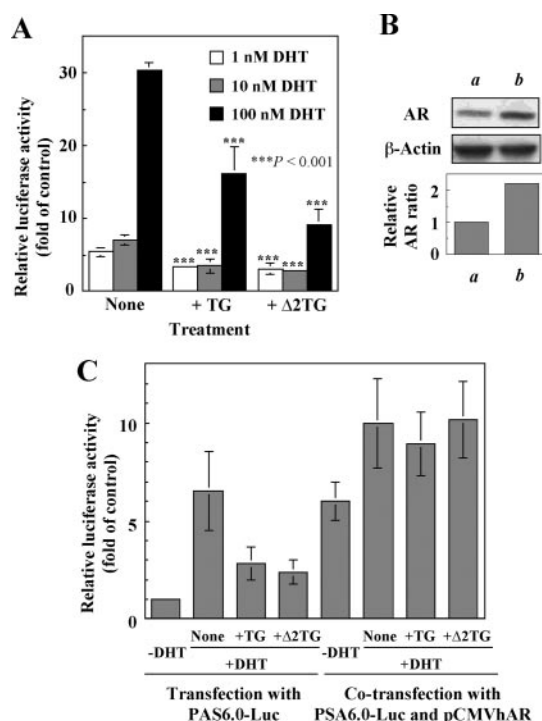

Fig. 5. Immunocytochemical evidence that troglitazone (TG) and Δ2TG at 10 μM do not interfere with DHT-stimulated nuclear localization of AR in LNCaP cells. LNCaP cells were exposed to individual treatments in 10% charcoal-stripped FBS-supplemented phenol red-free RPMI 1640 medium for 48 h, and immunocytochemistry was performed as described under *Materials and Methods*. As shown, DHT facilitated the nuclear localization of AR, which was not interfered by 10 μM TG or Δ2TG.

Fig. 6. Troglitazone (TG) and Δ2TG inhibit DHT-mediated ARE activation in LNCaP cells. A, inhibitory effect of 10 μM TG and Δ2TG on the luciferase reporter activity in PSA6.0-Luc-transfected LNCaP cells that were stimulated by different doses of DHT for 24 h. B, differential expression levels of AR in LNCaP cells transfected with PSA6.0-Luc alone (a) versus those cotransfected with PSA6.0-Luc and pCMVhAR (b). C, expression of ectopic AR protects the inhibitory effect of 10 μM TG and Δ2TG on ARE activation. Transfected LNCaP cells were exposed to 10 nM DHT alone or the combination of 10 nM DHT and 10 μM TG or Δ2TG for 24 h, and luciferase activity in the cell lysates was determined by luminometry as described under *Materials and Methods*.

kilobase PSA promoter (Zhang et al., 2002). DHT increased the reporter activity in a dose-dependent manner, and both troglitazone and Δ2TG at 10 μM could significantly suppress the luciferase activity ($P < 0.001$) (Fig. 6A). For example, in the presence of 10 nM DHT, the extent of inhibition by troglitazone and Δ2TG was 52 and 60%, respectively. To confirm this was an AR-dependent effect, LNCaP cells were cotransfected with the PSA6.0-Luc vector and a human AR expression construct (pCMVhAR), resulting in increase in AR expression by 2.2-fold (Fig. 6B). Expression of ectopic AR not only increased the basal activity of luciferase but could also rescue the suppressing effect of 10 μM troglitazone and Δ2TG on DHT-induced increase of luciferase activity in PSA6.0-Luc-transfected LNCaP cells (Fig. 6C).

To assess how troglitazone and Δ2TG affected AR-mediated transactivation of the PSA promoter, ChIP assays were performed to detect the binding of AR to ARE I and ARE II in the promoter region. LNCaP cells were cultured in phenol red-free RPMI 1640 medium containing 10% charcoal-dextran-stripped FBS for 2 days, followed by exposure to 10 nM DHT in the absence of presence of 10 μM troglitazone or Δ2TG for 12 h. After formaldehyde treatment of cells, AR antibodies were used to immunoprecipitate AR-bound genomic DNA fragments, followed by PCR analysis of the genomic DNA using pairs of primers spanning the AREs according to a published procedure (Shang et al., 2002). As shown in Fig. 7, treatment of cells with 10 nM DHT increased AR recruitment to both AREI and AREII. This DHT-induced AR recruitment was specific because no AR binding was noted to the middle control region within the PSA promoter; AR binding was not noted in the negative control in which anti-IgG was used for immunoprecipitation. Troglitazone and Δ2TG diminished the DHT-induced AR binding to AREI by 34 and 47%, respectively, and caused approximately 16 and 45% reduction, respectively, in the AR binding to AREII.

Discussion

This study assessed the mechanism by which thiazolidinediones mediated the inhibition of PSA secretion in androgen-dependent prostate cancer cells. Two lines of evidence indicate that the effect of thiazolidinediones on PSA down-regulation was independent of PPARγ activation. First, this thiazolidinedione-mediated PSA down-regulation was structure-specific irrespective of the relative potency in PPARγ activation. For example, while 10 μM troglitazone and ciglitazone were active, the more potent PPARγ agonists rosiglitazone and pioglitazone at the same concentration were not. Second, Δ2TG and Δ2CG, although devoid of PPARγ activity, exhibited higher potency than their parent molecules in suppressing PSA secretion.

Although PSA secretion was significantly inhibited by 10 μM troglitazone and Δ2TG in both LNCaP and 22RV1 cells, no appreciable changes in AR levels were noted even after 72-h exposure, refuting a possible link between PSA down-regulation and decrease in AR expression. This finding indicates that the mode of troglitazone- and Δ2TG-mediated PSA repression was different from that of vitamin E succinate (Zhang et al., 2002), even thought these molecules share the chroman substructure. Vitamin E succinate mediated PSA repression by inhibiting AR expression, whereas troglitazone and Δ2TG at 10 μM exhibited no appreciable effect on AR expression. In addition, the finding that ciglitazone and Δ2CG could also cause the down-regulation of PSA secretion argues against the involvement of the chroman moiety in mediating the PSA repressing effect of troglitazone.

Moreover, immunocytochemical analysis demonstrates that these agents did not interfere with the DHT-stimulated nuclear translocation of AR. However, reporter gene and ChIP assays revealed that troglitazone and Δ2TG inhibited AR recruitment to the AREI and AREII within the PSA
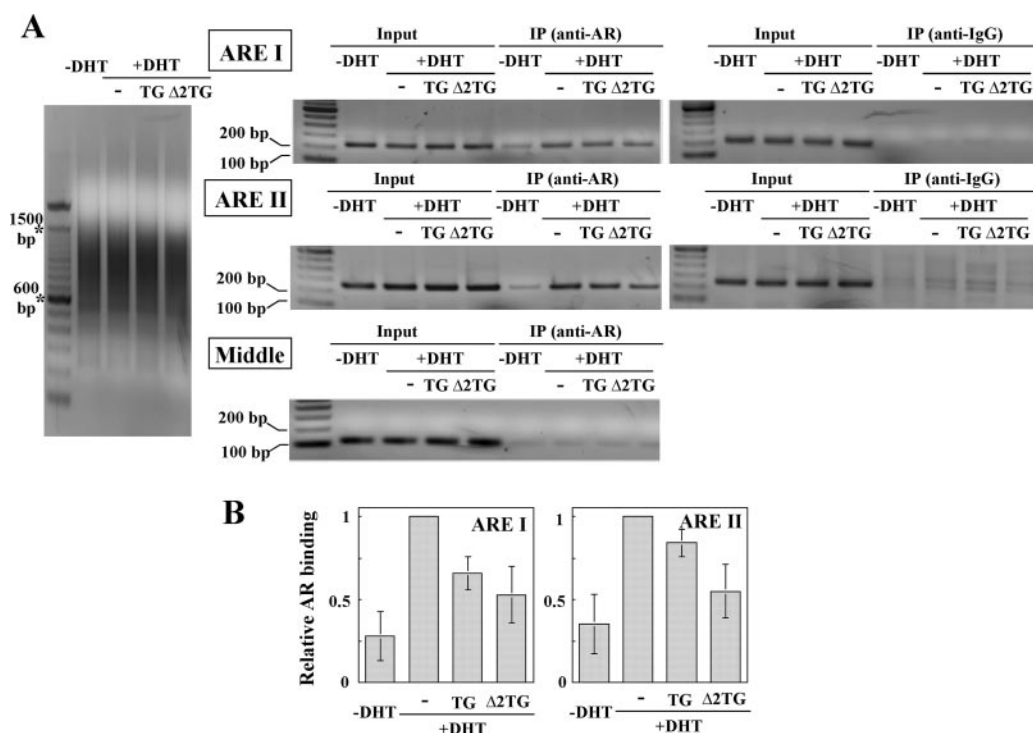
Fig. 7. ChIP analysis of the inhibitory effect of troglitazone (TG) and Δ2TG on DHT-stimulated AR recruitment to the AREI and AREII within the PSA promoter region. A, LNCaP cells were exposed to 10 nM DHT alone or the combination of 10 nM DHT and 10 μM TG or Δ2TG for 12 h. The drug-treated cells were collected, cross-linked, and sonicated, and soluble chromatin was prepared as described under *Materials and Methods*. Left, size of the genomic DNA fragments from solubilized chromatin. Right, ChIP was performed by using antibodies against AR or IgG, and PCR was conducted by using primers designed to detect the AREI binding site, the AREII binding site, and an irrelevant region within the actin promoter region (middle), as described under *Materials and Methods*. B, columns, mean of the relative AR binding, normalized to input; bars, S.D. ($n = 3$).

promoter region, thereby blocking transactivation of PSA gene expression.

Is the ability of oral troglitazone to reduce serum PSA levels in prostate cancer patients is therapeutically relevant? A major concern is that the concentration for troglitazone to mediate antitumor effects in prostate cancer cells is several-fold higher than that of PSA down-regulation, which is difficult to attain at therapeutic doses. For example, in the presence of 5 to 10% serum, neither troglitazone nor ciglitazone exhibited antiproliferative activity until the concentrations reached more than 50 μM. Therefore, decrease in serum PSA levels in response to troglitazone treatment might not truly reflect the growth status of the prostate tumor in patients.

Nevertheless, it is noteworthy that troglitazone and Δ2TG at doses higher than 30 μM were able to inhibit AR expression. Moreover, troglitazone at a very high dose (i.e., 500 mg/kg/day) has been shown to be effective in suppressing PC-3 xenograft growth in nude mice (Kubota et al., 1998). From a translational perspective, separation of these two pharmacological activities, PPARγ activation versus AR down-regulation, provides a molecular basis to use Δ2TG as a molecular platform to design AR-ablative agents. In light of the important role of AR in prostate tumorigenesis, these AR-ablative agents have the translational relevance to be developed into antitumor agents for the prevention and/or therapy of prostate cancer, which constitutes the focus of this investigation.

References

Bae MA and Song BJ (2003) Critical role of c-Jun N-terminal protein kinase activation in troglitazone-induced apoptosis of human HepG2 hepatoma cells. *Mol Pharmacol* **63:**401–408.

Baek SJ, Wilson LC, Hsi LC, and Eling TE (2003) Troglitazone, a peroxisome proliferator-activated receptor gamma (PPAR gamma) ligand, selectively induces the early growth response-1 gene independently of PPAR gamma. A novel mechanism for its anti-tumorigenic activity. *J Biol Chem* **278:**5845–5853.

Cunha GR, Donjacour AA, Cooke PS, Mee S, Bigsby RM, Higgins SJ, and Sugimura Y (1987) The endocrinology and developmental biology of the prostate. *Endocr Rev* **8:**338–362.

Day C (1999) Thiazolidinediones: a new class of antidiabetic drugs. *Diabet Med* **16:**179–192.

Dixon SC, Knopf KB, and Figg WD (2001) The control of prostate-specific antigen expression and gene regulation by pharmacological agents. *Pharmacol Rev* **53:**73–91.

Gouni-Berthold I, Berthold HK, Weber AA, Ko Y, Seul C, Vetter H, and Sachinidis A (2001) Troglitazone and rosiglitazone induce apoptosis of vascular smooth muscle cells through an extracellular signal-regulated kinase-independent pathway. *Naunyn-Schmiedeberg's Arch Pharmacol* **363:**215–221.

Hisatake JI, Ikezoe T, Carey M, Holden S, Tomoyasu S, and Koeffler HP (2000) Down-Regulation of prostate-specific antigen expression by ligands for peroxisome proliferator-activated receptor gamma in human prostate cancer. *Cancer Res* **60:**5494–5498.

Huang JW, Shiau CW, Yang YT, Kulp SK, Chen KF, Brueggemeier RW, Shapiro CL, and Chen CS (2005) Peroxisome proliferator-activated receptor γ-independent ablation of cyclin D1 by thiazolidinediones and their derivatives in breast cancer cells. *Mol Pharmacol* **67:**1342–1348.

Jiang M, Shappell SB, and Hayward SW (2004) Approaches to understanding the importance and clinical implications of peroxisome proliferator-activated receptor gamma (PPARgamma) signaling in prostate cancer. *J Cell Biochem* **91:**513–527.

Koeffler HP (2003) Peroxisome proliferator-activated receptor gamma and cancers. *Clin Cancer Res* **9:**1–9.

Kubota T, Koshizuka K, Williamson EA, Asou H, Said JW, Holden S, Miyoshi I, and Koeffler HP (1998) Ligand for peroxisome proliferator-activated receptor gamma (troglitazone) has potent antitumor effect against human prostate cancer both in vitro and in vivo. *Cancer Res* **58:**3344–3352.

Kumagai T, Ikezoe T, Gui D, O'Kelly J, Tong XJ, Cohen FJ, Said JW, and Koeffler HP (2004) RWJ-241947 (MCC-555), a unique peroxisome proliferator-activated receptor-gamma ligand with antitumor activity against human prostate cancer in vitro and in beige/nude/X-linked immunodeficient mice and enhancement of apoptosis in myeloma cells induced by arsenic trioxide. *Clin Cancer Res* **10:**1508–1520.

Lieberman R (2002) Chemoprevention of prostate cancer: current status and future directions. *Cancer Metastasis Rev* **21:**297–309.

Motomura W, Okumura T, Takahashi N, Obara T, and Kohgo Y (2000) Activation of peroxisome proliferator-activated receptor gamma by troglitazone inhibits cell growth through the increase of p27KiP1 in human. Pancreatic carcinoma cells. *Cancer Res* **60:**5558–5564.

Mueller E, Smith M, Sarraf P, Kroll T, Aiyer A, Kaufman DS, Oh W, Demetri G, Figg WD, Zhou XP, et al. (2000) Effects of ligand activation of peroxisome proliferator-activated receptor gamma in human prostate cancer. *Proc Natl Acad Sci USA* **97:**10990–10995.

Okura T, Nakamura M, Takata Y, Watanabe S, Kitami Y, and Hiwada K (2000) Troglitazone induces apoptosis via the p53 and Gadd45 pathway in vascular smooth muscle cells. *Eur J Pharmacol* **407:**227–235.

Palakurthi SS, Aktas H, Grubissich LM, Mortensen RM, and Halperin JA (2001) Anticancer effects of thiazolidinediones are independent of peroxisome proliferator-activated receptor gamma and mediated by inhibition of translation initiation. *Cancer Res* **61:**6213–6218.

Polascik TJ, Oesterling JE, and Partin AW (1999) Prostate specific antigen: a decade of discovery–what we have learned and where we are going. *J Urol* **162:**293–306.

Shang Y, Myers M, and Brown M (2002) Formation of the androgen receptor transcription complex. *Mol Cell* **9:**601–610.

Shiau CW, Yang CC, Kulp SK, Chen KF, Chen CS, and Huang JW (2005) Thiazolidinediones mediate apoptosis in prostate cancer cells in part through inhibition of Bcl-xL/Bcl-2 functions independently of PPARgamma. *Cancer Res* **65:**1561–1569.

Sugimura A, Kiriyama Y, Nochi H, Tsuchiya H, Tamoto K, Sakurada Y, Ui M, and Tokumitsu Y (1999) Troglitazone suppresses cell growth of myeloid leukemia cell lines by induction of p21WAF1/CIP1 cyclin-dependent kinase inhibitor. *Biochem Biophys Res Commun* **261:**833–837.

Takeda K, Ichiki T, Tokunou T, Iino N, and Takeshita A (2001) 15-Deoxy-delta 12,14-prostaglandin J2 and thiazolidinediones activate the MEK/ERK pathway through phosphatidylinositol 3-kinase in vascular smooth muscle cells. *J Biol Chem* **276:**48950–48955.

van Bokhoven A, Varella-Garcia M, Korch C, Johannes WU, Smith EE, Miller HL, Nordeen SK, Miller GJ, and Lucia MS (2003) Molecular characterization of human prostate carcinoma cell lines. *Prostate* **57:**205–225.

Zhang Y, Ni J, Messing EM, Chang E, Yang CR, and Yeh S (2002) Vitamin E succinate inhibits the function of androgen receptor and the expression of prostate-specific antigen in prostate cancer cells. *Proc Natl Acad Sci USA* **99:**7408–7413.

Address correspondence to: Ching-Shih Chen, College of Pharmacy, The Ohio State University, 336 L. M. Parks Hall, Columbus, OH 43210. E-mail: chen.844@osu.edu