

Troglitazone attenuates epidermal growth factor receptor signaling independently of peroxisome proliferator-activated receptor in PC-3 cells

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Abstract. The role of the peroxisome proliferator-activated receptor- γ (PPAR γ) in cell differentiation, cell cycle arrest and apoptosis has attracted increasing attention. Troglitazone (TGZ), a thiazolidinedione ligand of PPAR γ , has been recently described as possessing antitumoral properties. We studied the effects of TGZ on proliferation, growth arrest and apoptosis in PC-3 prostate carcinoma cells and its interaction with the signaling pathways of the activated EGF receptor (EGFR). We observed that TGZ, in a dose- and time-dependent manner, inhibited the growth of PC-3 cells independent of PPAR γ . In addition, TGZ induced cell cycle arrest and apoptosis. We demonstrated that cell proliferation was stimulated by EGF in a dose- and time-dependent manner and was inhibited by TGZ. The analysis of the main intracellular signaling pathways downstream of the activated EGFR, PI3K-Akt, ERK1/2 cascades and I κ B α revealed that TGZ reduced the phosphorylation levels of EGFR and of their downstream inter-mediators which mediate EGF stimulated proliferation. In conclusion, simultaneous targeting of EGFR, PI3K-Akt, ERK1/2 and NF- κ B by TGZ could be the molecular mechanism by which TGZ exerts its additive inhibitory effects on PC-3 cell proliferation.

Introduction

Peroxisome proliferator-activated receptors (PPARs) are a family of ligand-activated transcription factors belonging to the nuclear receptor superfamily (1). The three isoforms of PPAR, designated α , β and γ , are members of ligand-dependent transcription factor family that has been shown to regulate

gene networks involved in controlling cell growth, differentiation and homeostasis (2). Among PPARs, PPAR γ is primarily expressed in adipose tissue and is required for its development through regulation of the expression of adipocyte-specific genes. In recent years PPAR γ has attracted a significant amount of attention in the drug development field due to its alleged involvement in the antitumorigenic process. This association has been primarily due to the distinct mechanisms including growth arrest, apoptosis and induction of differentiation observed in the presence of PPAR γ agonists. It is evident that PPAR γ agonists may have potent anticancer potential and may serve as a rational basis for therapy of some tumors as shown *in vitro* studies focused on breast cancer (3), liver cancer (4), prostate cancer (5), pancreatic cancer (6), gastric cancer (7) and other human cancer cells. A number of PPAR γ ligands have been identified, including natural prostaglandins, such as 15-deoxy-D12, 14-prostaglandin J2 (PGJ2), and the synthetic antidiabetic thiazolidinediones (TZD), such as troglitazone (TGZ), rosiglitazone, ciglitazone, pioglitazone and certain polyunsaturated fatty acids. Troglitazone, rosiglitazone and pioglitazone have already been used clinically to treat type 2 diabetes, making use of the ability of synthetic PPAR γ ligands to sensitize insulin and to lower blood glucose concentration (8). In addition to antidiabetic effects, troglitazone has been shown to cause growth inhibition, induce differentiation and trigger apoptosis of various human malignant cells (6,9-11). Little is known, however, concerning the underlying molecular mechanisms for troglitazone's antiproliferative and pro-apoptotic activities.

Prostate cancer (PC) is the sixth most commonly diagnosed cancer in the world and the metastatic PC forms represent the second cause of mortality. The clinical course of patients after given diagnosis of prostate cancer is highly variable and the underlying reasons for such variability remain elusive. Although androgen ablation is initially found to be successful to control prostate cancer, tumors will overcome androgen blockade and will develop a hormone-unresponsive phenotype that become resistant to therapy. To better understand the pathophysiology of this disease, efforts have been made to elucidate the molecular mechanisms that mediate the development and progression of prostate cancer.

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PPAR γ is expressed in both normal and malignant prostate cancer tissues as well as in prostate cancer cell lines and the role of this receptor in carcinoma progression has been widely studied using the family of high affinity thiazolidinedione PPAR γ agonists (12,13). For example, troglitazone can inhibit the growth of human prostate cancer cells both *in vitro* as well as when proliferating as xenografts in triple immunodeficient mice (11). However, it has recently been demonstrated that the antitumorigenic effects of TZD can be ligand-dependent, and can not always be attributed to PPAR γ activation (5,14). That is to say, the effect of TZD on cell cycle and apoptosis in prostate cancer cells is via PPAR γ -dependent and PPAR γ -independent intracellular pathways.

Epidermal growth factor receptor (EGFR), also known as HER-1 or c-erbB-1, belongs to the c-erbB family of receptor tyrosine kinases. It is a 170-kD glycoprotein, which like many other tyrosine kinase receptors bears an extracellular ligand binding domain, a transmembrane domain and intracellular domain possessing tyrosine kinase activity (15). It has been proved that EGFR-mediated signaling is indispensable to maintain the orderly growth and functions of the prostate gland in humans (16,17). In addition, the activation of EGFR by its ligands of autocrine and paracrine manner appears to contribute to prostate cancer cell growth and progression, including proliferation, angiogenesis, invasion, metastasis and inhibition of apoptosis. More recently, many of the major new therapeutic approaches for prostate cancer are directed against growth factor signaling pathways involving the EGFR family of receptors and the downstream components that transduce signals to the cell nucleus (18,19). Activated EGFR may induce the stimulation of some distinct signal transduction cascades. The two most well defined pathways are the classical erbB1-Shc-Grb2/SOS-Ras-Raf-ERK1/2 signaling cascade pathway and the phosphatidylinositol (PI) 3-kinase-AKT pathway (15,20). However, receptor activation can also occur in the absence of physiological ligands via a mechanism termed EGFR 'transactivation'. Proteolytic cleavage of EGF-like ligands by matrix metalloproteinases, non-receptor tyrosine kinases, stress factors, cell adhesion, G-protein coupled receptors and cytokine receptor have all been associated with EGFR transactivation (21). This ligand-independent EGFR 'transactivation' has been implicated as a central integrator by which multiple endogenous and synthetic compounds activate intracellular kinases leading to a variety of cellular responses.

Therefore, identification of endogenous factors that may inhibit EGFR activation and its signaling pathways is of paramount therapeutic importance. Recently it has been demonstrated that activation of EGFR through tyrosine phosphorylation also occurs in response to PPAR γ agonists (22-26), suggesting possible cross-talk between EGFR and PPAR ligand-induced signaling. To further determine whether PPAR γ agonists inhibition of growth of prostate cancer cells could be attributed to EGFR signaling, changes in EGFR activation, and the downstream signaling events of the receptor, particularly activation of Akt and ERK1/2, are examined.

In the present study, we have shown that treatment with TGZ induces growth arrest and apoptosis in PC-3 cells and the treatment has a very potent inhibitory effect on the

phosphorylation and activation of the EGFR and NF- κ B pathway. Moreover, we further provide evidence that TGZ-activated EGFR signaling pathway is independent of PPAR γ . Our results clearly show that treatment of PC-3 cells with TGZ may thus offer therapeutic advantages in the treatment and prevention of prostate cancer.

Materials and methods

Cell lines and reagents. PC-3 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were maintained in DMEM supplemented with 10% FBS (Gibco BRL, Rockville, MD, USA), 100 U/ml of penicillin and 100 μ g/ml of streptomycin in a humidified 5% CO $_2$ atmosphere at 37°C. The media were changed every 3 days and the cells were separated via trypsinization, using trypsin/EDTA when they reached subconfluence. Troglitazone (TGZ), GW9662 and ZD1839 were from Cayman Chemical Co. (Ann Arbor, MI, USA). EGF was from Sigma Chemical (St. Louis, MO, USA). Antibodies specific to p-Erk1/2 (p44/p42), p-Akt (S473), p-EGFR (Y1068), p-PI3K (p85), p-I κ B α , total Erk1/2, total Akt, total EGFR, total PI3K and total I κ B α were from Cell Signaling Technology (Beverly, MA, USA). Anti-actin was a product of Santa Cruz Biotechnology (Santa Cruz, CA).

Analysis of cell proliferation inhibition. Inhibition of cell growth in response to TGZ was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells were dispersed by trypsin-EDTA treatment and 2.5 \times 10 4 cells/ml were resuspended in RPMI-1640 containing 10% of FBS and seeded into 96-well culture plates with six replicates. After 24 h of plating, the medium was replaced with one that contained 2.5% of FBS, and the incubation was continued by treatment with different doses of TGZ (0-100 μ M) or control vehicle (0.1% DMSO) in the presence or absence of GW9662 (5-50 μ M) for 24, 48, 72 and 96 h. In some experiments, growth was induced by EGF (100 ng/ml) in the absence or presence of TGZ (0-100 μ M). In all experiments, the reaction was terminated by adding of 20 μ l of 5 mg/ml stock of MTT to each well. The reaction was allowed to proceed for 3-4 h at 37°C. The culture medium was then removed. The formazan crystals were then dissolved by adding 0.1 ml DMSO. The intensity of the color developed, which is the reflection of number of live cells, was measured at a wavelength of 570 nm. All values were compared with the corresponding controls. All assays were done with six replicates.

Cell cycle analysis. Cells were seeded in six 10 cm dishes (1 \times 10 5 cells/plate) and after 24 h media was replaced with fresh media containing TGZ (0-100 μ M) treatment or control vehicle (0.1% DMSO) for 48 h. Floating and adherent cells were combined, pelleted (400 g), washed in phosphate-buffered saline (PBS) and resuspended in 200 μ l PBS containing 0.1% FBS. The cell suspension was diluted 10-fold in ice-cold 90% ethanol and placed at -20°C for 15 min. Cells were then washed twice with 0.1% FBS in PBS and resuspended in RNase (2 μ g/ml) and 5 mg/ml propidium iodide (Sigma). The percentage of cells at different cell cycle stages (G0/G1, G2/M

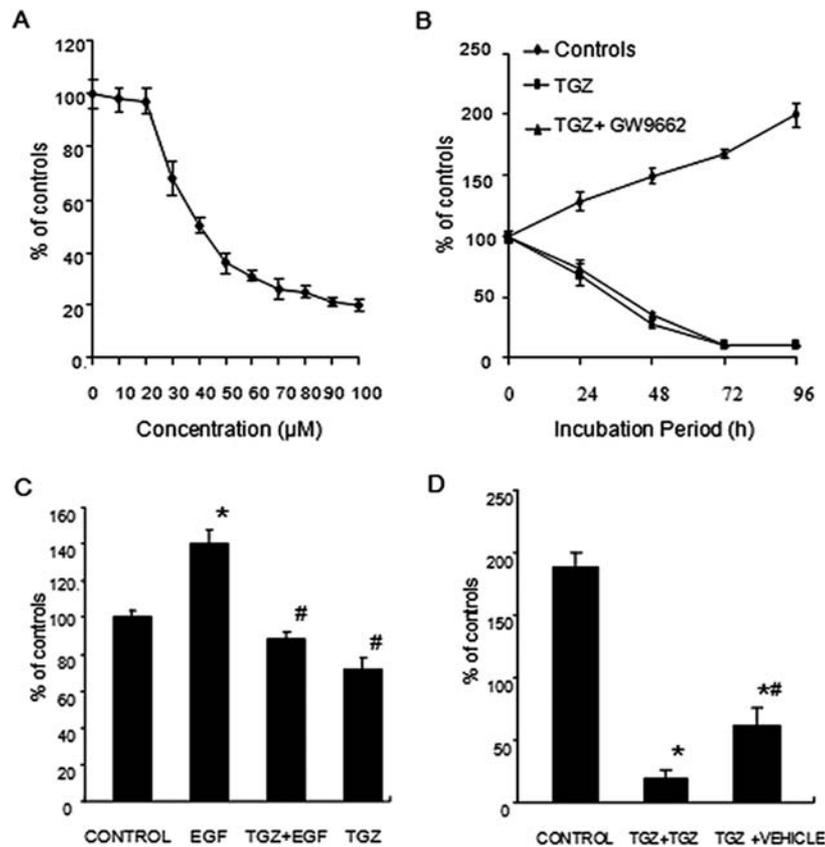


Figure 1. The effects of TGZ on PC-3 cell growth. (A) The dose-dependent effect of TGZ on PC-3 cell growth as assessed by MTT assay. Note that the increasing concentrations of TGZ (0-100 μM) resulted in a dose-dependent inhibition of the PC-3 cell growth after 96 h incubation. A 50% inhibition of growth of PC-3 cells was evident at concentrations of 40 μM of TGZ. Results are expressed as percentage of controls in six experiments. (B) The time-dependent effect of TGZ on PC-3 cell growth as assessed by MTT assay. Note that in the absence of TGZ (controls) cell growth increased gradually over the 96-h incubation period reaching a value of $\sim 200\%$ of the initial control, whereas in the presence of TGZ (70 μM) cell growth declined to $\sim 10\%$ of the initial value at 72 h and remained at that level and the growth inhibitory effects of TGZ on cell number were not reversed by GW9662. (C) The effect of TGZ on EGF-induced growth of PC-3 cells. Cells were incubated with 100 ng/ml EGF in the absence or presence of TGZ (70 μM) for 48 h. Control incubations contained neither EGF nor TGZ. (D) The proliferation of PC-3 cells in the presence and after removal of TGZ. Cells were incubated for 48 h in the absence (controls) or presence of TGZ (70 μM). After 48 h, the medium was replaced to contain TGZ (70 μM) or vehicle, and the incubation was continued for another 48 h. Negative controls were incubated for 96 h in the absence of TGZ. Columns, mean of six observations; bars, SE. * $P < 0.01$, compared with the control; ** $P < 0.01$, compared with the values from cells that were incubated continuously for 96 h.

and S phase) was determined on the basis of DNA content by flow cytometry using ModFit LT2.0 software.

Apoptosis assay. For routine analysis of apoptosis, treated cells were examined for apoptotic morphology using a fluorescence staining technique as described previously (27). Briefly, cells were exposed to DMSO or differing doses of TGZ (0-100 μM) for 48 h and were harvested by trypsinization. After staining with a mixed dye solution containing 100 mg/ml each acridine orange and ethidium bromide the morphology of the cells was observed by fluorescence microscopy, and the number of apoptotic cells was quantified. In all cases a minimum of 200 cells were counted for each sample. Using Annexin V staining to detect apoptosis, treated cells were harvested by trypsinization and rinsed with cold PBS once. After centrifugation for 5 min, cells were resuspended in 500 μl of 1X Annexin V binding buffer (BD Biosciences, San Diego, CA) and then added 1 μl of Annexin V-FITC and 1 μl of propidium iodide (Sigma). After incubation for 5 min at room temperature in the dark, the samples were analyzed by flow cytometry.

Western blot analysis. These studies were done using 6-well plates. Aliquots (10^5 cells/well) of PC-3 cells in RPMI-10% FBS were plated, and after 24 h, they were serum starved for 48 h to minimize the contributions of growth factors, particularly EGF family of peptides, and to synchronize the cells. Following treatments, cells were washed with cold PBS and immediately lysed with 1 ml lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 1 mM sodium orthovanadate, 30 mM p-nitrophenyl phosphate, 10 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin and 10 mg/ml leupeptin). Lysates were electrophoresed in SDS-PAGE, and separated proteins transferred to nitrocellulose and probed with the appropriate antibodies using the conditions recommended by the suppliers.

Immunofluorescence. PC-3 cells grown on cover glass were fixed in 3% paraformaldehyde in PBS (pH 7.4) for 10 min, permeabilized with 0.5% Triton X-100 for 5 min, blocked with 10% goat serum for 1 h and then incubated with primary antibodies overnight at 4°C. This was followed by incubation

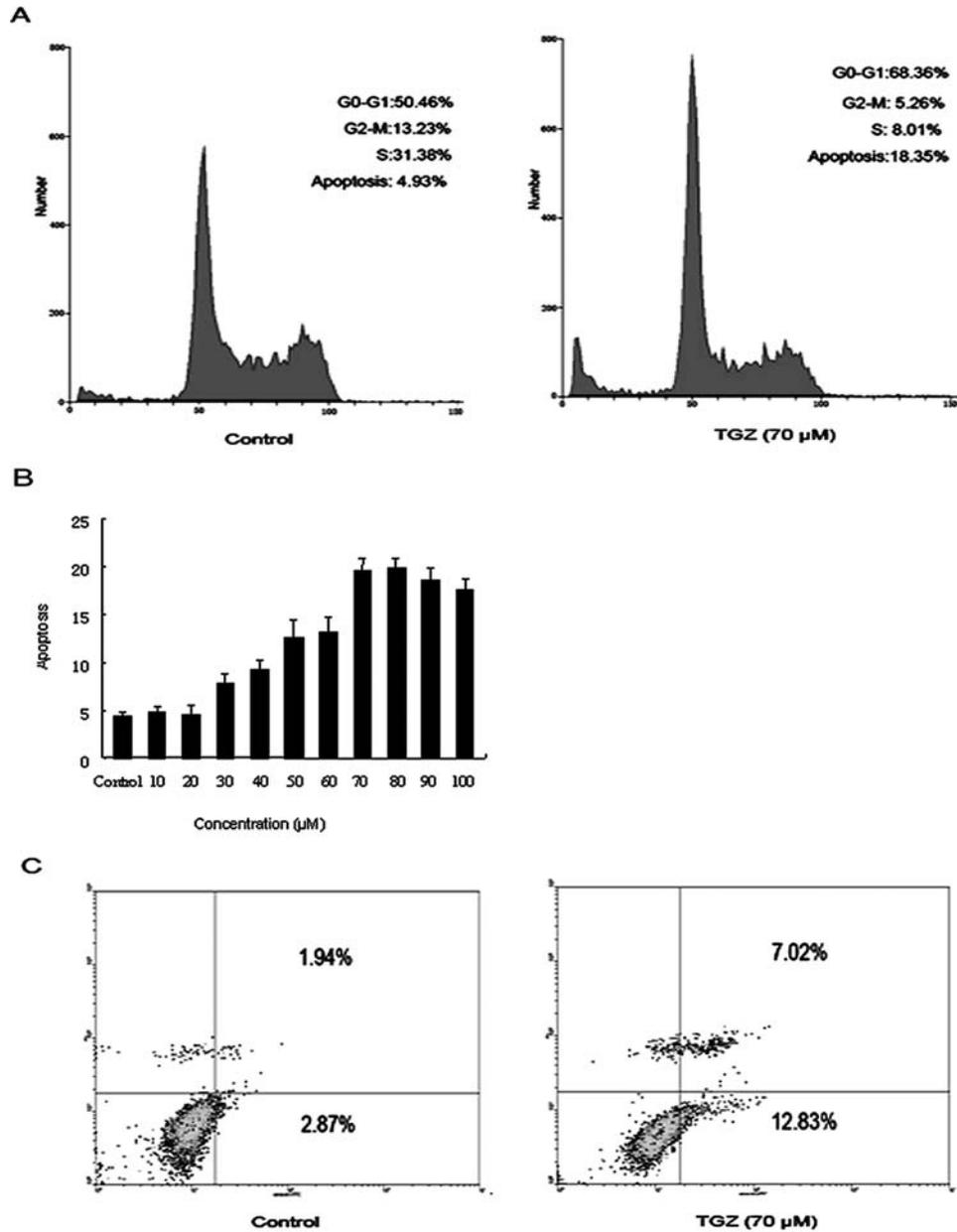


Figure 2. The effects of TGZ-induced cell cycle arrest and apoptosis of PC-3 cells. (A) Cells were cultured for 48 h with control vehicle or TGZ. The effect of TGZ on the proportion of cells in each phase of the cell cycle was determined. TGZ (70 μM) produced a cell cycle arrest in G0/G1 of PC-3 cells by increasing the distribution of PC-3 cells into the G0/G1 and subG1 phase. In addition to the increased cells in the G0-G1 phase, a corresponding reduction of cells in S and G2-M phases was also noted. Data shown is representative of two experiments. (B) PC-3 cells were treated with the indicated doses of TGZ for 48 h, and apoptosis was detected as morphologic change by fluorescent microscopy. Treatment of PC-3 cells with TGZ produced a dose-dependent apoptosis. Values are the means of three independent experiments ± SD. Control cells were treated with DMSO vehicle. (C) An example of TGZ-induced apoptosis of PC-3 cells as assessed by flow cytometry. Cells were cultured for 48 h with control vehicle or TGZ. The effect of TGZ on apoptosis was determined. Flow cytometric analysis of apoptotic fraction based on propidium iodide (y-axis) and Annexin V staining (x-axis) showed 18% of apoptosis was induced by TGZ (70 μM). Data shown is representative of three experiments.

with FITC-conjugated goat anti-rabbit and DAPI for 1 h. Sample images were taken with a fluorescence microscope (Nikon, Eclipse E 600 system), and the images were processed using Photoshop 8.0 (Adobe Systems, San Jose, CA).

Results

Response of cultured PC-3 cells to troglitazone (TGZ). The effects of TGZ on PC-3 cells were examined with different doses of troglitazone in the presence or absence of the highly selective PPAR_γ antagonist GW9662. TGZ had no effect on

the cell number at a lower dose (below 20 μM), however, TGZ showed a dose-dependent inhibition of the PC-3 cells as assessed by MTT assays. A 50% inhibition of the PC-3 cell growth was achieved by 40 μM concentrations of TGZ (Fig. 1A). For the time course changes in cell growth of PC-3 cells, the results revealed that in the absence of TGZ (controls) cell growth increased gradually over the 96-h incubation period reaching a value of ~200% of the initial control, whereas in the presence of TGZ (70 μM) cell growth declined to ~10% of the initial value at 72 h and remained at that level for the rest of the experimental period (Fig. 1B). The reason

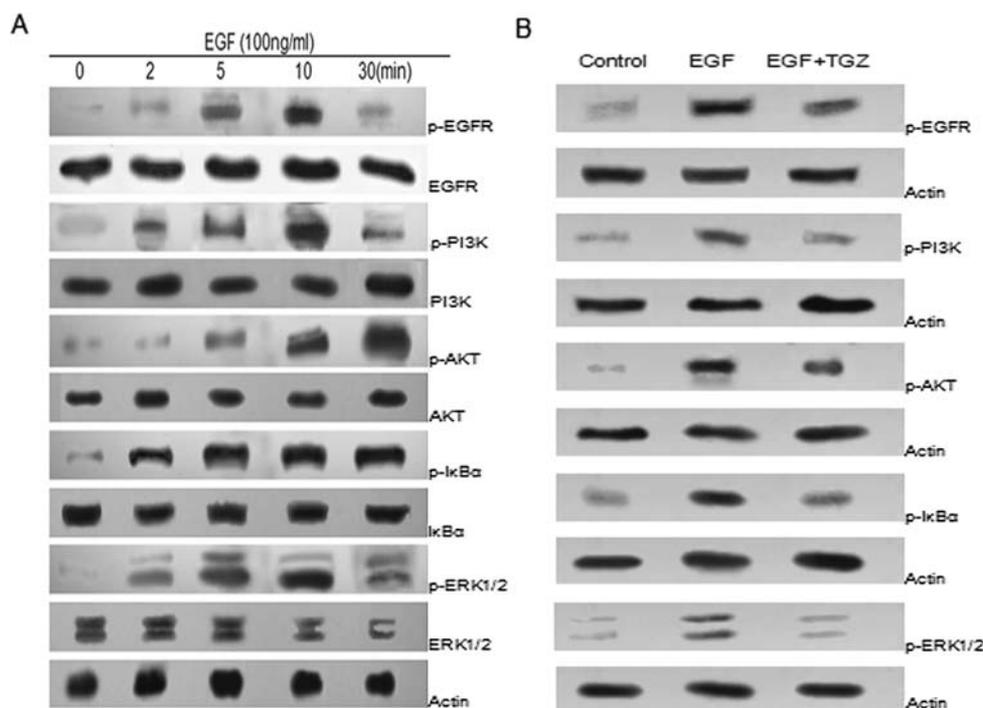


Figure 3. Effects of EGF on EGFR signaling and inhibition of EGFR signaling proteins by TGZ in PC-3 cells. (A) PC-3 cells were incubated with EGF (100 ng/ml) in serum-free media for the indicated times. Representative EGFR signaling proteins (p-EGFR, p-PI3K, p-Akt, p-ERK1/2 and p-I κ B α) were measured using phospho-specific antibodies, which detect phosphorylation at specific residue. Anti-EGFR, anti-PI3K, anti-Akt, anti-ERK1/2 and anti-I κ B α antibodies were used to determine the total expression levels of each protein. Actin was used to ensure equal protein loading. (B) PC-3 cells were stimulated with EGF (100 ng/ml) for 10 min after a 5-min pretreatment with TGZ in serum-free media. Activated EGFR signaling proteins [p-EGFR, p-PI3K (p85), p-Akt, p-ERK1/2 and p-I κ B α] were measured using phospho-specific antibodies by Western blotting. Actin was used to ensure equal protein loading.

for which we chose a TGZ dose of 70 μ M as the marker was that the dose-response study has shown a 75% inhibition of cell growth at a dose of 70 μ M TGZ. Moreover, the growth inhibitory effects of TGZ on cell numbers were not reversed by GW9662 (Fig. 1B). At doses ranging from 0 to 100 μ M, GW9662 had no effect on PC-3 cells (data not shown).

To determine whether TGZ would inhibit EGF growth of PC-3 cells, 48 h serum-starved cells were incubated with TGZ (70 μ M) in the absence or presence of 100 ng/ml EGF for 48 h. As expected, EGF caused ~50% stimulation of growth of PC-3 cells over the controls (Fig. 1C). This stimulation was abrogated by TGZ. In the presence of TGZ, there was a reduction in cell growth when compared with the controls (Fig. 1C). TGZ by itself decreased proliferation of PC-3 cells when compared with the controls (Fig. 1C).

To determine whether the TGZ action would be reversible, PC-3 cells were incubated for 48 h in the absence (controls) or presence of TGZ (70 μ M), whereafter the incubation medium was replaced with that containing either TGZ (70 μ M) or an equivalent volume of vehicle (control). The incubation was continued for another 48 h. The presence of TGZ throughout the 96 h incubation period resulted in ~90% reduction in cell growth (Fig. 1D). However, when TGZ was removed from the medium after 48 h and incubation continued for another 48 h in the absence of TGZ, there was ~30% increase in cell growth compared with that noted in cells maintained in the continuous presence of TGZ (Fig. 1D).

Effects of troglitazone (TGZ) on cell cycle and apoptosis. TGZ produced a dose-dependent cell cycle arrest in G0/G1

of PC-3 cells lines by increasing the distribution of PC-3 cells into the G0/G1 and subG1 phase. The cells located in the subG1 portion are considered as being apoptotic (28,29). In addition to the increased cells in the G0-G1 phase, a corresponding reduction of cells in S and G2-M phases was also noted (Fig. 2A).

We next examined whether apoptosis is involved in the inhibition of PC-3 cell proliferation by TGZ. Cells were treated with DMSO and increasing doses of TGZ (0-100 μ M) for 48 h. Apoptosis quantified by morphologic changes was induced in a dose-dependent manner (Fig. 2B). These morphologic changes were confirmed by Annexin V staining (Fig. 2C).

EGFR and NF- κ B signaling is activated by EGF. Activation of EGF signaling results in diverse effects, including stimulation of cell growth and inhibition of apoptosis (30). To investigate the effects of EGF on PC-3 cells, we analyzed signaling molecules related to the EGFR pathway. The phosphorylation of EGFR, PI3K (p85), Akt and ERK was detected by Western blotting with antibodies specific to the phosphorylated proteins. As shown in Fig. 3A, all of these proteins were activated after EGF treatment. EGF rapidly activated EGFR by phosphorylation at 2 min (lane 2), with maximal activation at 10 min (lane 4). The level of phosphorylated EGFR was declining by 30 min (lane 5). PI3K, a functional subunit of PI3K, was activated within 2 min of EGF administration and gradually decreased by 30 min. One of the downstream targets of EGFR-PI3K is Akt, and phosphorylated Akt gradually increased and peaked at 30 min. At the same time, exposure to EGF caused a marked stimu-

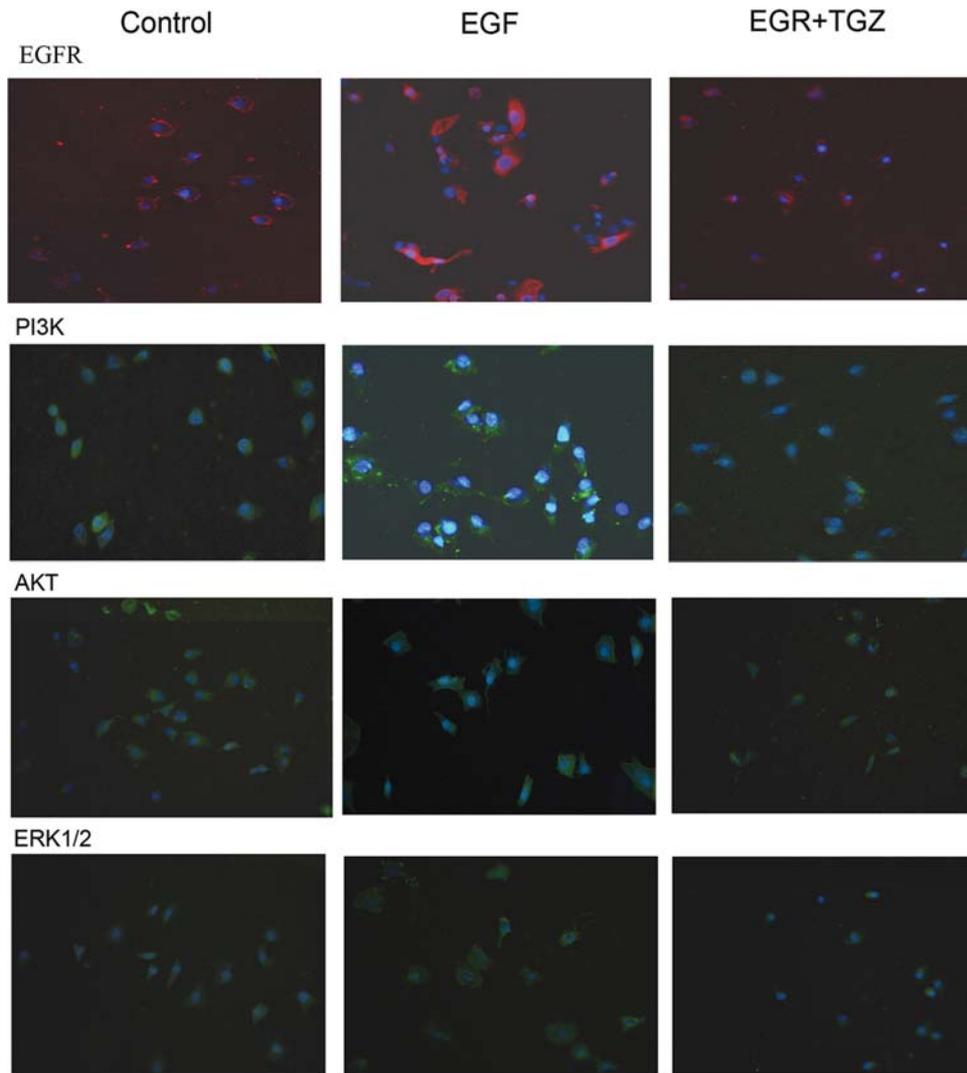


Figure 4. Inhibition of EGFR signaling in PC-3 cells by TGZ. PC-3 cells were treated with 0.2% DMSO or 70 μ M TGZ for 5 min followed by a 10-min incubation with EGF (100 ng/ml). p-EGFR (red), nuclei (green); p-PI3K (blue), nuclei (green); p-Akt (blue), nuclei (green) and p-ERK1/2 (blue), nuclei (green) were detected by immunofluorescence analysis using anti-p-EGFR, anti-p-PI3K, anti-p-Akt, anti-p-ERK1/2 and DAPI dye, respectively. Pictures with red/green and blue/green color were merged. Magnitude: 400-fold.

lation of phosphorylation of ERK1/2 and gradually decreased by 30 min.

Previously, it has been shown that NF- κ B is activated by EGFR signaling (31,32) and Akt-induced stimulation of cell survival partly involves activation of Nuclear Factor- κ B [NF- κ B; (33,34)]. Consistent with this observation, we found that treatment of PC-3 cells with EGF activated the phosphorylation of I κ B α as early as 2 min after treatment, and the increased phosphorylation persisted for up to 30 min. Under the present experimental conditions, the levels of total (non-phosphorylated forms) EGFR, PI3K, Akt, ERK and I κ B α were not affected by EGF.

TGZ reduces the phosphorylation levels of EGFR and the downstream inter-mediators in PC-3 cells. To elucidate the intracellular mechanism by which TGZ affects the EGFR pathway in PC-3 cells, we examined the effect of TGZ on EGF-induced activation of EGFR as well as its two main intracellular downstream signaling pathways engaged by the

activated EGF, namely, the phosphatidylinositol 3 kinase (PI3K)-Akt cascade and the extracellular signal-regulated (ERK) signaling (35). PC-3 cells were pretreated with TGZ (70 μ M) for 5 min, followed by EGF (100 ng/ml) treatment for 10 min. As shown in Fig. 3B, TGZ had an inhibitory effect on EGF-induced EGFR activation. We next examined EGF-stimulated phosphorylation of PI3K, a downstream target of the EGFR signaling pathway and found that it was inhibited dramatically by the treatment of TGZ. In addition, when we examined the downstream signaling molecules ERK, Akt and I κ B α , levels of p-Akt, p-ERK 1/2 and p-I κ B α were all significantly inhibited by TGZ. Densitometry analysis shows that p-Akt protein levels are decreased by TGZ by 48% as compared with EGF alone. Furthermore, studies showed that levels of p-ERK 1/2 were significantly reduced by TGZ by 46% as compared with EGF. Under the present experimental conditions, the levels of total (non-phosphorylated forms) EGFR, Akt, ERK and I κ B α were not affected by either EGF or TGZ (data not shown). Immunofluorescence studies after

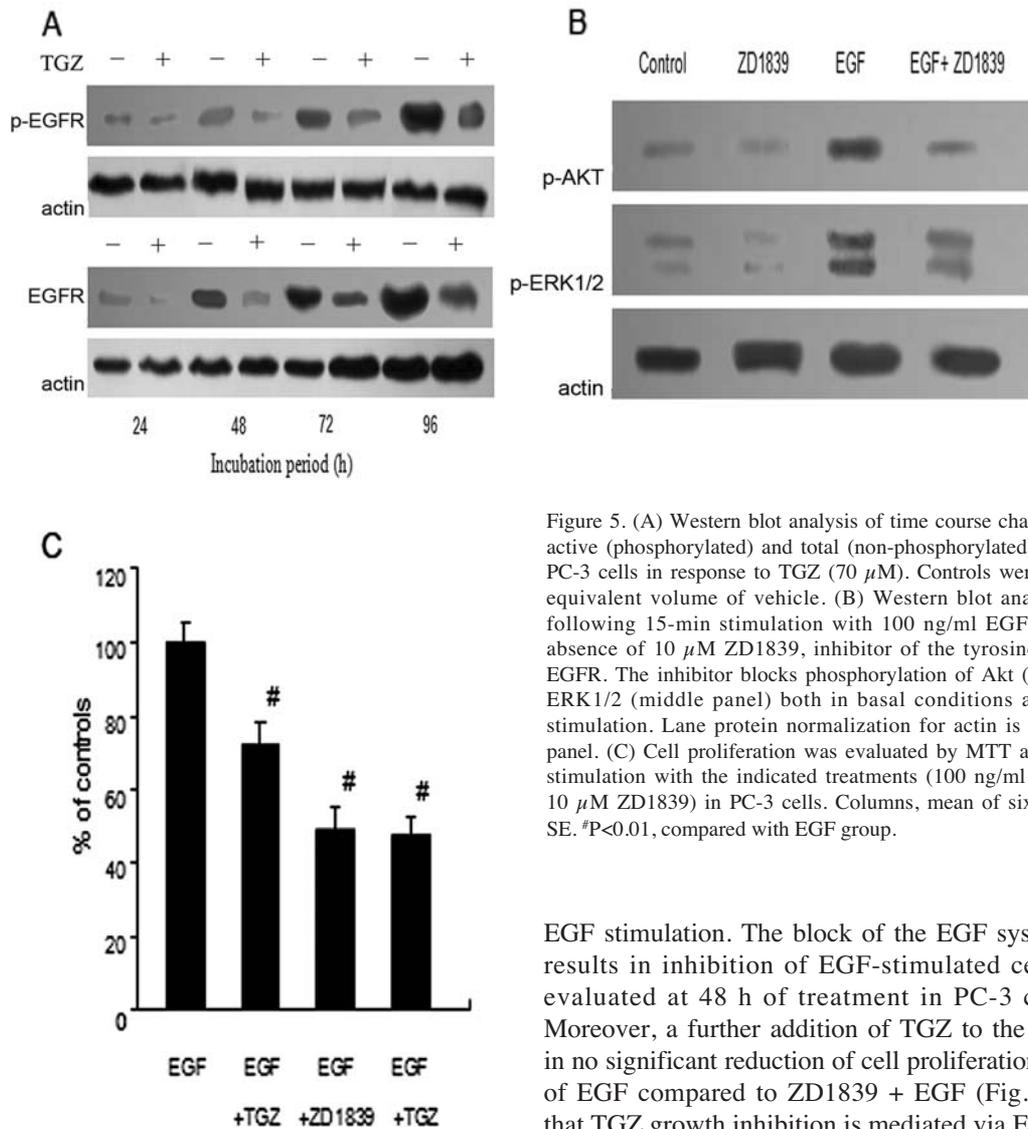


Figure 5. (A) Western blot analysis of time course changes in the levels of active (phosphorylated) and total (non-phosphorylated) forms of EGFR in PC-3 cells in response to TGZ (70 μ M). Controls were incubated with an equivalent volume of vehicle. (B) Western blot analysis of PC-3 cells following 15-min stimulation with 100 ng/ml EGF in the presence or absence of 10 μ M ZD1839, inhibitor of the tyrosine kinase activity of EGFR. The inhibitor blocks phosphorylation of Akt (upper panel) and of ERK1/2 (middle panel) both in basal conditions and following EGF stimulation. Lane protein normalization for actin is shown in the lower panel. (C) Cell proliferation was evaluated by MTT assay following 48 h stimulation with the indicated treatments (100 ng/ml EGF, 70 μ M TGZ, 10 μ M ZD1839) in PC-3 cells. Columns, mean of six observations; bars, SE. #P<0.01, compared with EGF group.

TGZ treatment confirmed that TGZ significantly inhibits EGFR and its downstream signal (Fig. 4). These results demonstrated that in addition to suppression of EGFR expression, TGF interrupted the signaling pathways for EGF in PC-3 cells by reducing the phosphorylation levels of EGFR and their downstream inter-mediators.

To determine whether and to what extent TGZ affects constitutively active EGFR in PC-3 cells, the levels of total (non-phosphorylated) and active (phosphorylated) forms of EGFR were measured in PC-3 cells over a period of 96 h following exposure to either TGZ (70 μ M) or vehicle (controls). In the absence of TGZ, the basal levels of both phosphorylated and non-phosphorylated forms of EGFR increased gradually over the 96-h incubation period, whereas in the presence of TGZ these increases were attenuated (Fig. 5A).

In order to further demonstrate the involvement of downstream inter-mediators of the EGFR in mediating cell proliferation by TGZ, we used ZD1839, a specific EGFR kinase inhibitor. As shown in Fig. 5B, ZD1839 is able to block phosphorylation of Akt (upper panel) and ERK1/2 (middle panel) both in the basal conditions and following 15-min

EGF stimulation. The block of the EGF system by ZD1839 results in inhibition of EGF-stimulated cell proliferation evaluated at 48 h of treatment in PC-3 cells (Fig. 5C). Moreover, a further addition of TGZ to the inhibitor results in no significant reduction of cell proliferation in the presence of EGF compared to ZD1839 + EGF (Fig. 5C) suggesting that TGZ growth inhibition is mediated via EGFR signaling.

Discussion

Prostate cancer (PC) is the most common malignancy diagnosed in men and the metastatic PC forms represent the second cause of mortality (36,37). As the causes of PC remain poorly understood and the mortality rate of prostate cancer continues unabated, the development of new therapeutic agents is highly desirable. Recently, PPAR γ ligands have been described as suppressing tumor cell proliferation as well as inducing apoptosis and a more differentiated phenotype in several types of cancers, including prostate cancer (11,13), thus suggesting the use of these drugs as a potential new anticancer therapy. It is well known that TGZ is a synthetic ligand for PPAR γ and the inhibition of cell proliferation and growth by TGZ has been documented in several types of human cancer cells in either a PPAR γ -dependent or a PPAR γ -independent manner (38-40). Recently, a large number of studies suggest that the anticancer effects of PPAR γ ligands on prostate cancer cells are PPAR γ -independent and that the PPAR γ -independent action supersedes their well-established PPAR γ -dependent action (41,42).

The current study investigated the inhibitory response to TGZ in prostate carcinoma PC-3 cell line. We found that

TGZ showed a dose- and time-dependent inhibition of the PC-3 cells as assessed by MTT assays. Although no *in vivo* studies have been done to determine the effectiveness of TGZ in prostate cancer, the fact that the growth of PC-3 cells remained markedly attenuated throughout the 96-h incubation period in the presence of TGZ suggests that PC-3 cells, which are non-responsive to androgen, are responsive to TGZ. This, together with the observation that removal of TGZ does not fully restore the growth of these cells, further suggests that the antiproliferative effect of TGZ on PC-3 cells is long lasting. In addition, TGZ produced a dose-dependent cell cycle arrest in G0/G1 of PC-3 cells lines by increasing the distribution of PC-3 cells into the G0/G1 and subG1 phase. This is in line with the findings of other investigators, suggesting that PPAR γ ligands promote PC-3 cell differentiation (G1/G0 arrest) by inhibiting β catenin and c-myc expression (43).

Further study demonstrated that TGZ-induced growth inhibition was PPAR γ -independent. In order to make clear the involvement of PPAR γ in the study, we have utilized the selective PPAR γ antagonist GW9662 to elucidate the involvement of PPAR γ receptor in the growth inhibitory effects and observed. We found that GW9662 did not reverse the growth inhibitory effects of TGZ in PC-3 cell line examined. As a result, our finding demonstrated that the effects induced by TGZ were PPAR γ -independent. Whilst this finding is consistent with PPAR γ -independent growth inhibition observed in some cancer types, it has been clearly demonstrated in other cases that thiazolidinedione agonists-induced growth inhibition is PPAR γ -dependent (44-46). Therefore, further studies for the mechanism are indispensable.

Further to the PPAR γ -independent effects of TGZ discovered in prostate carcinoma, our study also demonstrated that TGZ induced growth inhibition via inducing apoptosis. Recently, an independent study by Shiao *et al* (14) also reported that TGZ treatment led to reduced association of Bcl-2 and Bcl-xL with Bak, leading to caspase-dependent apoptosis in PC-3 cells. The capability of TGZ to induce apoptosis in this system may be reflective of the larger percentage of control cells in G2/M phase of the cell cycle. Thus, TGZ may be able to inhibit the growth of both actively proliferating tumors and tumors with a lower mitotic rate more typically observed in prostate cancer. However, other experiment showed that rosiglitazone arrested PC-3 cell growth without inducing the apoptosis of PC-3 cells. Thus, the anti-apoptotic role of PPAR γ agonists in prostate cancer cells appears conflicting (47).

Factors contributing to prostate cancer may involve genetic changes, activated oncogenes, growth factors, hormones and/or dietary factors. EGFR is an attractive growth factor for the development of cancer therapeutics (48,49). In fact, the progressive and metastatic growth of prostate cancer has been associated with a significant increase in the expression of EGFR and one of its ligands (50,51). In this experiment, EGF was able to stimulate cell proliferation in PC-3 cells and this stimulation was abrogated by TGZ. That is to say, the role of TGZ on the inhibition of the cell proliferation in PC-3 cells is related to the blocking of EGF signaling. This result is similar to the finding in other experiment that TGZ inhibits cell proliferation by attenuation of EGFR signaling (52). In order to further elucidate at which

level TGZ interferes with the activated EGF axis both in basal conditions (EGFR activated by endogenously produced EGF) and following a further activation of the receptor by exogenous addition of EGF, we investigated the signaling pathways acting downstream of the receptor. Our results demonstrate that TGZ is an effective inhibitor of EGFR signal transduction pathways in prostate cancer cells. In addition, we have observed that TGZ was able to block the activation of the PI3K-Akt axis induced by EGF. TGZ also affected the ERKs pathway through inhibition of the phosphorylation activation of ERK1 and 2 isoforms. Because induction of ERKs and Akt signaling pathways has often been shown to result in stimulation of cell growth and increase in survival of cells, respectively (33,34), attenuation of these processes by TGZ in PC-3 cells suggests that TGZ not only inhibits cell growth but also stimulates apoptosis of prostate cancer cells. A similar effect of TGZ on phosphorylation of ERKs has been recently described in the aorta endothelial cells (52).

It is well known that NF- κ B, a transcription factor whose induction is often associated with increased cell survival, are frequently overexpressed in several types of cancers, including prostate (33,34). NF- κ B activation in cancer cells has been intensively studied by many groups and constitutive or improper activation of NF- κ B in prostate cancer cells *in vitro* and *in vivo* has been recently recognized (53). In this experiment, further support for TGZ induction of growth inhibition for PC-3 cells came from the observation that TGZ inhibited EGF-induced stimulation of NF- κ B. Indeed, the effect of TGZ may thus be explained by its positive impact on cell death in prostate cancer: the inhibition of EGFR activity and the inhibition of NF- κ B activity.

In summary, our current data shed new light on the molecular mechanisms underlying cell proliferation in prostate cancer, demonstrating that the inhibitory effect exerted by TGZ on cell growth is due to the TGZ interferences with the two main signaling pathways downstream of the activated EGFR and the interferences with the transcription factor NF- κ B. In this pathway, TGZ may interfere with the EGFR signaling independently of PPAR γ ligand genomic actions. These results provided a novel insight into the roles and mechanisms of TGZ in inhibition of prostate cancer cell growth, and potential therapeutic strategies for the treatment of prostate cancer.

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