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Cell death is induced by ciglitazone, a peroxisome proliferator-activated receptor γ (PPARγ) agonist, independently of PPARγ in human glioma cells

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ABSTRACT

Peroxisome proliferator-activated receptor γ (PPARγ) regulates multiple signaling pathways, and its agonists induce apoptosis in various cancer cells. However, their role in cell death is unclear. In this study, the relationship between ciglitazone (CGZ) and PPARγ in CGZ-induced cell death was examined. At concentrations of greater than 30 μM, CGZ, a synthetic PPARγ agonist, activated caspase-3 and induced apoptosis in T98G cells. Treatment of T98G cells with less than 30 μM CGZ effectively induced cell death after pretreatment with 30 μM of the PPARγ antagonist GW9662, although GW9662 alone did not induce cell death. This cell death was also observed when cells were co-treated with CGZ and GW9662, but was not observed when cells were treated with CGZ prior to GW9662. In cells in which PPARγ was down-regulated cells by siRNA, lower concentrations of CGZ (<30 μM) were sufficient to induce cell death, although higher concentrations of CGZ (⩾30 μM) were required to induce cell death in control T98G cells, indicating that CGZ effectively induces cell death in T98G cells independently of PPARγ. Treatment with GW9662 followed by CGZ resulted in a down-regulation of Akt activity and the loss of mitochondrial membrane potential (MMP), which was accompanied by a decrease in Bcl-2 expression and an increase in Bid cleavage. These data suggest that CGZ is capable of inducing apoptotic cell death independently of PPARγ in glioma cells, by down-regulating Akt activity and inducing MMP collapse.

1. Introduction

Peroxisome proliferator-activated receptors (PPARs) are ligand-dependent transcription factors. They belong to the family of nuclear hormone receptors that includes the receptors for estrogen, thyroid hormone, retinoic acid, 1,25-dihydroxy vitamin D3, and retinoid X [1,2]. Activated PPARs heterodimerize with a second nuclear receptor, the retinoid X receptor, and modulate the transcription of numerous target genes after binding to specific PPAR response elements [3,4]. To date, three subtypes of PPARs have been identified (α, β/δ, and γ), which exhibit distinct tissue distribution and are associated with selective ligands. PPARγ was initially noted to be highly expressed in adipose tissue and was found to be involved in adipocyte differentiation, insulin sensitization, and lipid metabolism [1,2]. Recent evidence demonstrates that PPARγ is also widely expressed in various types of tumor cells, where it has a crucial role in suppressing cell growth and invasion and in promoting differentiation and apoptosis [5–8].

PPARγ agonists have both PPARγ-dependent and -independent effects. Naturally occurring arachidonic acid metabolites, 15-deoxy-delta prostaglandin J_2 (15d-PGJ_2), and polyunsaturated fatty acids have been identified as PPARγ ligands [9]. PPARγ can be also activated by synthetic ligands such as those belonging to the antidiabetic thiazolidinedione (TZD) class of compounds [10]. Among the TZDs, four molecules have been identified: rosiglitazone, pioglitazone, troglitazone, and ciglitazone (CGZ). Recently, it was shown that TZDs are able to induce apoptotic cell death, cell cycle arrest, and differentiation in vitro and in vivo [11,12]. CGZ is an agonist for PPARγ and its interaction with PPARγ leads to modulation of specific gene expression. Activation of PPARγ by CGZ can have either pro-apoptotic or anti-apoptotic activity in human cancer [12–15]. Previous studies demonstrate that CGZ is the most efficient TZD for the inhibition of cancer cell growth [11–13]. Recently, CGZ was also shown to inhibit the proliferation of many cancer cell lines that express high levels of PPARγ [11–13,16] On the other hand, several lines of evidence indicate that the inhibitory effect of CGZ on tumor cell proliferation was independent of PPARγ activation [11–13,16]. However, the mechanism of the anticancer effects of CGZ on glioma cells is largely unknown.

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In this study, we examined the role of PPARγ in CGZ-induced death of glioma cells. The data suggest that CGZ is capable of inducing apoptotic cell death independently of PPARγ in T98G cells, by down-regulating Akt and reducing mitochondrial membrane potential (MMP).

2. Materials and methods

2.1. Reagents

CGZ, GW9662, LY294002 and U0126 were purchased from Calbiochem (San Diego, CA). Alamar blue, Trypan blue, Lipofectamine 2000 and tetramethyrhodamine ethyl ester perchlorate (TMRE) were purchased from Gibco-Invitrogen (Rockville, MD). Hoechst 33258 was purchased from Sigma–Aldrich Chemical (St. Louis, MO). Antibodies specific for PPARγ were obtained from Abcam (San Francisco, CA). Antibodies specific for poly ADP/ribose -polymerase (PARP), caspase-3, phospho-Akt (Thr 308), phospho-Akt (Ser 473), total Akt, extracellular signal-regulated kinase (ERK) 1/2, phospho-ERK1/2, Bcl-2 and Bid were obtained from Cell Signaling Technology (Danvers, MA). Antibodies specific for β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2. Cell culture

The human non-small cell lung cancer cell line NCI-H460, human neuroblastoma cell line IMR-32, human neuroepithelioma cell line SK-N-MC, and human glioma cell lines C6, A172 and T98G were grown in non-coated T75 culture flasks (Nalge Nunc, Naperville, IL) in Dulbecco's Modified Eagle's Medium (DMEM; Gibco-Invitrogen). The human leukemia T-cell line Jurkat was grown in T75 culture flasks (Nalge Nunc) in RPMI 1640 (Gibco-Invitrogen). All cell lines were grown in media supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco-Invitrogen), 100 U/ml of penicillin (Gibco-Invitrogen), 100 mg/ml of streptomycin (Gibco-Invitrogen), 2 mM L-glutamine (Gibco-Invitrogen) and 1 mM sodium pyruvate (Gibco-Invitrogen) in a humidified 5% CO_2 atmosphere at 37 °C. The media were changed every 3 d, and the adherent cells were separated using 0.05% trypsin/0.53 mM EDTA (Gibco-Invitrogen) when they reached subconfluence.

2.3. Cell viability: Alamar blue assay

Cells (1×10^4 cells/well) were seeded on 96-well plates (Nalge Nunc) in 100 μl of DMEM containing 10% FBS in the absence of phenol red, and incubated for 24 h. Two hours prior to treatment, medium was replaced with 90 μl of DMEM containing 1% FBS without phenol red. Cells were treated for 0–72 h either with CGZ and GW9662 separately or in different combinations, as described in the individual experiments. Eleven microliters of 1 × Alamar blue was added 3 h before the end of the incubation. Absorbance at 570 nm and 600 nm was measured with an ELISA Reader (Molecular Devices, Sunnyvale, CA).

2.4. Cell viability: Trypan blue exclusion assay

Cells were harvested using 0.05% trypsin/0.53 mM EDTA, incubated with 4% Trypan blue solution, and counted using a hemocytometer under an inverted microscope (Olympus CK40; Olympus, Melville, NY). Cells failing to exclude the dye were considered to be nonviable. Data are expressed as a percentage of stained cells.

2.5. Phase contrast image

Cells were treated for 24 h either with CGZ and GW9662 separately or in different combinations, as described in the individual experiments. Cells were examined with a Nikon Eclipse TE2000-U inverted fluorescence microscope equipped with a Nikon LH-M100C-1 camera (Nikon Corporation Instruments Company, Japan). Images of each plate were captured with the digital camera.

2.6. siRNA transfection

Cells were plated 24 h before siRNA transfection to be at 50% confluence on the day of transfection. Cells were transfected with lipofectamine 2000 reagent according to the manufacturers protocol. In brief, cells treated with siRNA-lipofectamine 2000 complexes were incubated at 37 °C in a CO_2 incubator for 18 h. And then medium was changed, and the transfected cells were incubated for additional 12 h until target gene was effectively down-regulated. siRNA targeting PPARγ (sc-29455) and scrambled siRNA (sc-37007) were purchased from Santa Cruz Biotechnology.

2.7. Immunoblotting

Cells were washed with cold phosphate-buffered saline (PBS; Gibco-Invitrogen), and lysed in 300 μl of cold RIPA buffer (50 mM Tris–HCl, pH 7.5, containing 1% Triton X-100, 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), and 1% sodium deoxycholate) with a protease inhibitor cocktail (Thermo Fisher Scientific, Rockford, IL). Cell lysates were centrifuged at 3000×*g* for 15 min at 4 °C. The supernatant was harvested, and protein concentration analyzed using a BCA protein assay kit (Thermo Fisher Scientific). For electrophoresis, 50 μg protein was dissolved in sample buffer (60 mM Tris–HCl, pH 6.8, containing 14.4 mM β-mercaptoethanol, 25% glycerol, 2% SDS, and 0.1% bromophenol blue), boiled for 5 min, and separated on a 10% SDS reducing gel. Separated proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (GE Healthcare, Buckinghamshire, UK) using a trans-blot system (Gibco-Invitrogen). Blots were blocked for 1 h in Tris-buffered saline (TBS) (10 mM Tris–HCl, pH 7.5, with 150 mM NaCl) containing 5% non-fat dry milk (BD Biosciences, San Jose, CA) at room temperature, washed three times with TBS, and incubated at 4 °C overnight with primary antibodies (all antibodies, 1:1000 dilution) in TBST (10 mM Tris, pH 7.5, containing 150 mM NaCl and 0.01% Tween 20) containing 3% non-fat dry milk. The next day, blots were washed three times with TBST, and incubated for 1 h with horseradish peroxidase-conjugated secondary antibodies (1:2000 or 1:5000 dilution) in TBST containing 3% non-fat dry milk at room temperature. After washing three times with TBST, protein was visualized with an ECL detection system (GE Healthcare).

2.8. Measurement of mitochondrial membrane potential ($\Delta\Psi m$, MMP)

The $\Delta\Psi m$ was measured with the fluorescent dye, TMRE. At the end of treatment, the culture medium was removed and the cells were incubated with TMRE at a final concentration of 50 nM and Hoechst 33258 at a final concentration of 5 μg/ml in HEPES buffer (Gibco-Invitrogen) for 20 min at 37 °C in a 5% CO_2 incubator. Cells were washed with PBS and examined with a Nikon Eclipse TE2000-U inverted fluorescence microscope equipped with a Nikon LH-M100C-1 camera. Images of each plate were captured with the digital camera.

2.9. Statistical analysis

Data were presented as mean ± standard deviation (SD). Statistical significance was determined using student's *t*-test. Results were considered significant when $P < 0.05$.

3. Results

3.1. Concentrations of ciglitazone higher than 30 μM induce cell death in glioma cells

The human cell lines used in this experiment, NCI-H460, IMR-32, SK-N-MC, C6, A172, and T98G, all expressed PPARγ, although its expression in glioma cells (C6, A172, and T98G) was relatively low compared to the other cell lines (Fig. 1A). Glioma cells were treated with CGZ, a synthetic PPARγ agonist, at various doses for 24, 48, 72, or 96 h. Low concentrations of CGZ (<30 μM) had no effect on the viability of the three glioma cell lines. However, doses of CGZ higher than 30 μM effectively induced cell death in glioma cell lines (Fig. 1B–D). Higher doses of CGZ (>30 μM) activated caspase-3 in T98G cells, leading to apoptotic cell death accompanied by PARP cleavage (Fig. 2C, D).

3.2. GW9662, an antagonist of PPARγ, does not block the toxicity of ciglitazone in glioma cells

To determine whether the cell death induced by CGZ requires binding to PPARγ, cells were pretreated with GW9662, a PPARγ antagonist which blocks the binding of CGZ to PPARγ, prior to treatment with CGZ. Unexpectedly, pretreatment with GW9662 sensitized glioma cells to cell death by lower concentrations of CGZ (<30 μM) in T98G and A172 glioma cells (Fig. 1B, D). In addition, GW9662 pretreatment effectively increased cell death of C6 glioma cells in response to higher concentrations of CGZ, as compared to cells treated with 30 μM CGZ alone (Fig. 1C). The expression of PPARγ in T98G cells was not changed by treatment with CGZ (Fig. 2A). Moreover, in T98G cells treated with 20 μM CGZ, apoptotic cell death gradually increased with increasing concentrations of GW9662 pretreatment, although GW9662 alone was not cytotoxic (Fig. 2B). Caspase-3 activation and PARP cleavage, which lead to apoptotic cell death, were also increased in T98G cells treated with 20 μM CGZ after GW9662 pretreatment (Fig. 2B–D). These results indicate that pretreatment with GW9662 enhances the toxicity of CGZ and imply PPARγ is not involved in cell death induced by CGZ.

3.3. Ciglitazone induces cell death independently of PPARγ in glioma cells

Experiments were conducted to investigate whether GW9662 enhances the PPARγ-independent toxicity of CGZ by inhibiting the binding of CGZ to PPARγ. As shown in Fig. 2B, cell viability was greatly reduced when cells were pretreated with 30 μM GW9662 before treatment with 20 μM CGZ. Similar results were obtained by co-treatment with CGZ and GW9662 (Fig. 3A, B). However, when cells were treated with GW9662 after exposure to CGZ, cell viability was not significantly reduced (Fig 3A, B). To further confirm the independent effect of CGZ on the reduced viability of T98G cells, PPARγ was down-regulated using siRNA (Fig. 3C). In T98G cells in which PPARγ was down-regulated with siRNA, less than 30 μM CGZ was sufficient to induce cell death (Fig. 3D). Therefore, when PPARγ/CGZ binding was disrupted with GW9662 pretreatment or when the expression of PPARγ was down-regulated by targeting siRNA, lower concentrations of CGZ were cytotoxic, indicating that CGZ effectively induces cell death in T98G cells independently of PPARγ.

3.4. Ciglitazone decreases Akt activity and induces apoptotic cell death through the loss of MMP

Experiments were performed to elucidate the PPARγ-independent mechanism of CGZ in T98G cell death. CGZ decreased Akt activity but increased ERK1/2 activity (Fig. 4A). Pretreatment with either an Akt inhibitor (LY294002) or an ERK inhibitor (U0126)

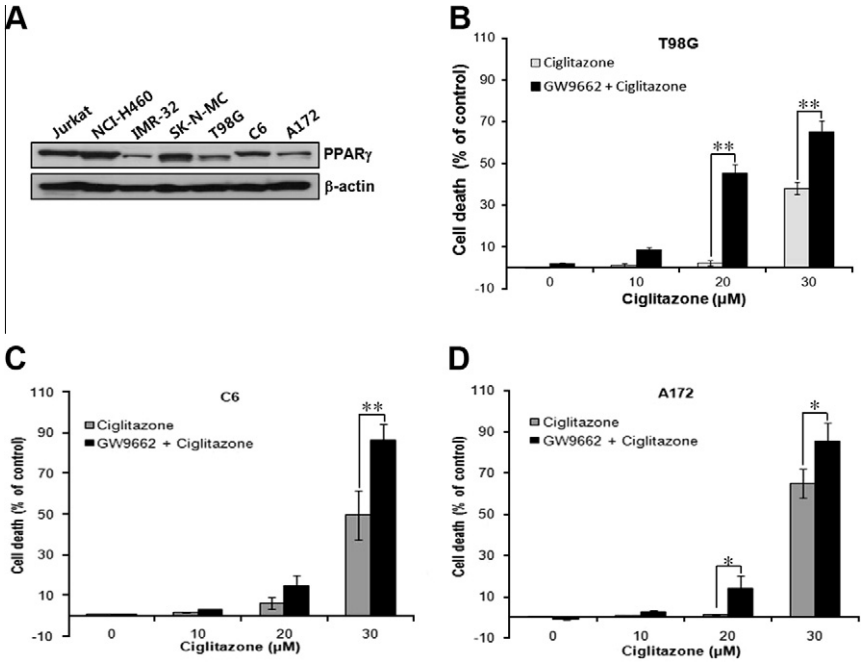

Fig. 1. The PPARγ agonist ciglitazone induces cell death in PPARγ-expressing glioma cells. (A) Analysis of PPARγ expression in human NCI-H460, IMR-32, SK-N-MC, C6, A172, and T98G cell lines by immunoblotting. Cell death, as determined by Trypan blue exclusion, following treatment with ciglitazone alone (gray bar), or with ciglitazone after pretreatment with 30 μM GW9662 (black bar), for 72 h in the glioma cell lines T98G (B), C6 (C), and A172 (D). Stained cells were counted as dead and are expressed as a percentage of the total cell number. Data are the mean ± SD from three separate experiments. $^{*}P < 0.05$; $^{**}P < 0.01$.

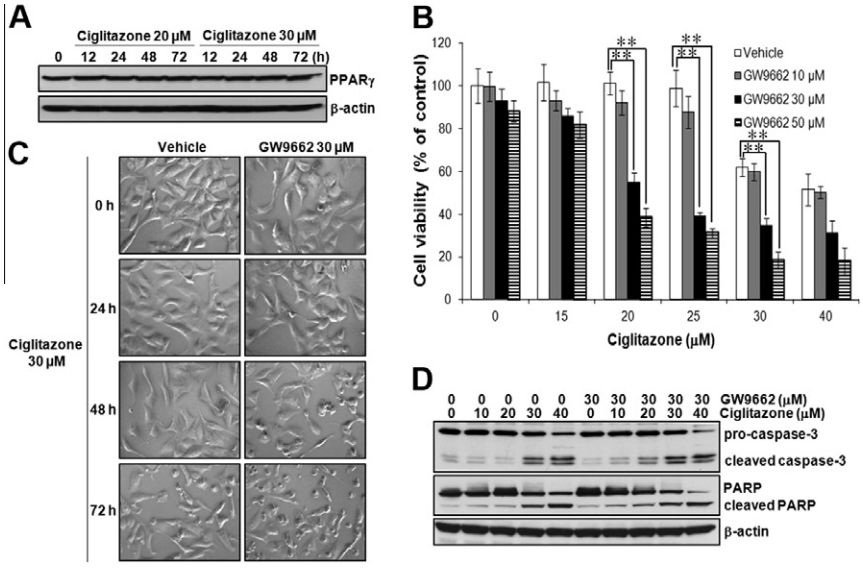

Fig. 2. GW9662, an antagonist of PPARγ, does not block the toxicity of ciglitazone in T98G cells. (A) Human glioma T98G cells were treated with 20 or 30 μM ciglitazone for 0, 12, 24, 48, or 72 h. The levels of PPARγ were analyzed by immunoblotting. (B) Viability of T98G cells, as determined by the Alamar blue assay, following treatment with ciglitazone after pretreatment with vehicle (white bar) or with 10 (gray bar), 30 (black bar), or 50 μM GW9662 (hatched bar) for 72 h. Data are expressed as a percentage of untreated control cells. Data are the mean ± SD from three separate experiments. $^{**}P < 0.01$. (C) T98G cells were treated with 30 μM ciglitazone in the absence or presence of 30 μM GW9662 for 0, 24, 48, or 72 h. Images were collected using an inverted microscope. (D) T98G cells were treated with ciglitazone in the absence or presence of 30 μM GW9662 for 48 h. Immunoblot analysis of caspase-3, PARP, and β-actin.

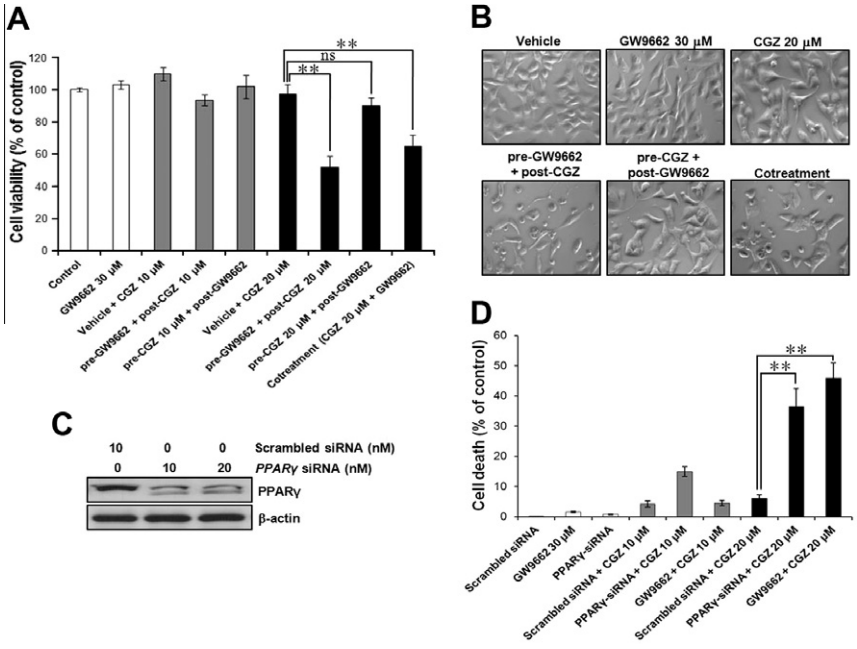

Fig. 3. Ciglitazone induces cell death independently of PPARγ in T98G cells. (A) Viability of T98G cells, as determined by the Alamar blue assay, following pretreatment with either ciglitazone (pre-CGZ) or 30 μM GW9662 (pre-GW9662) and treatment with either ciglitazone (post-CGZ) or 30 μM GW9662 (post-GW9662) for 72 h. Co-treated T98G cells were incubated with 20 μM ciglitazone and 30 μM GW9662. Data are expressed as a percentage of untreated control cells. Data are the mean ± SD from three separate experiments. Ns, not significantly different ($P > 0.05$); $^{**}P < 0.01$. (B) Images were collected using an inverted microscope. (C) T98G cells were transfected with scrambled siRNA or PPARγ siRNA (10 or 20 nm) for 18 h, and were then incubated for an additional 12 h. Immunoblot analysis of the expression of PPARγ and β-actin. (D) Trypan blue exclusion assay assessing the cell death of T98G cells following treatment with ciglitazone after transfection with scrambled siRNA or PPARγ siRNA. Stained cells were counted as dead and are expressed as a percentage of control cells incubated with scrambled siRNA. Data are the mean ± SD from three separate experiments. $^{**}P < 0.01$.

effectively increased CGZ-induced cell death in T98G cells (Fig. 4B), indicating that down-regulation of Akt activity is involved in the mechanism of cell death by CGZ, and indicating that ERK1/2 activation protects cells against CGZ-induced cytotoxicity. GW9662 treatment followed by CGZ resulted in down-regulation of Akt activity and a loss of MMP, which was accompanied by a decrease in Bcl-2 expression and an increase in Bid cleavage (Fig. 4C, E). In addition, treatment with 30 μM CGZ in Akt activity down-regulated cells by LY294002 enhanced the decrease in Bcl-2 and the increase in Bid cleavage, and increased MMP collapse, correlating with increased cell death (Fig. 4B, D, E). These data suggest that CGZ is capable of inducing apoptotic cell death with MMP collapse

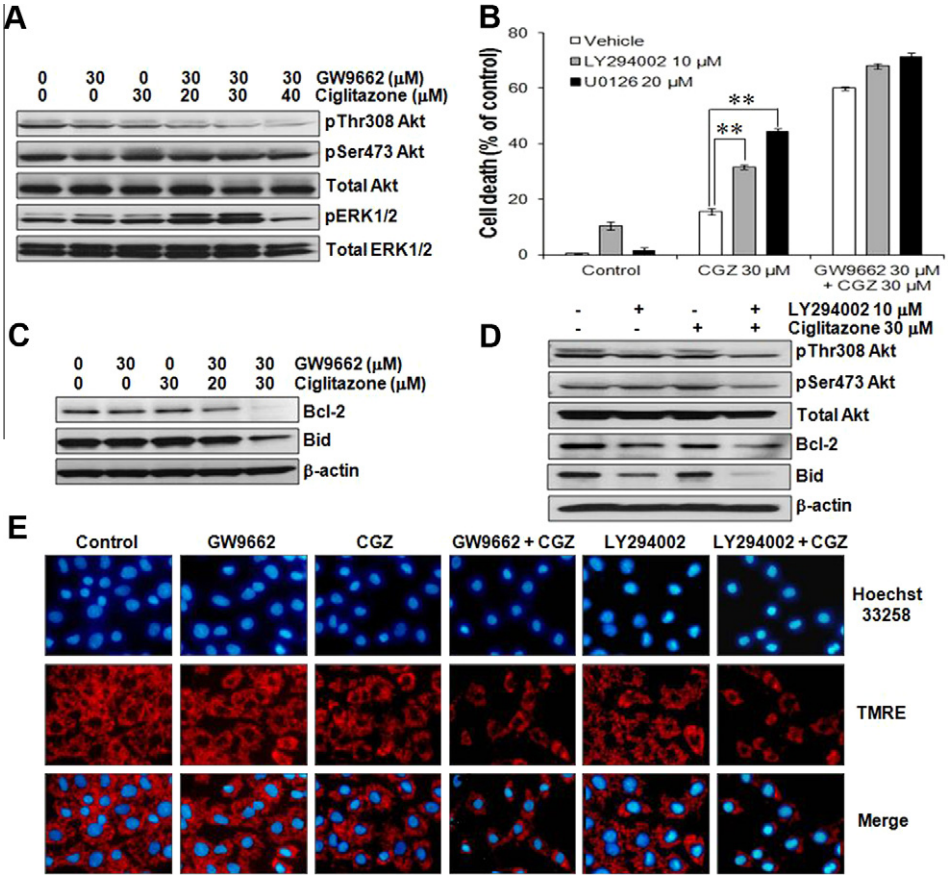

Fig. 4. Ciglitazone decreases Akt activity independently of PPARγ and induces apoptotic cell death through the loss of mitochondrial membrane potential (MMP). (A) T98G cells were left untreated or were treated with ciglitazone in the absence or presence of 30 μM GW9662 for 48 h. Immunoblot analysis of phospho-Thr308 Akt (pThr308 Akt), phospho-Ser473 Akt (pSer473 Akt), total Akt, phospho-ERK1/2 (pERK1/2), and total ERK. (B) T98G cells were left untreated, were treated with 30 μM ciglitazone, or were co-treated with 30 μM ciglitazone and 30 μM GW9662 in the absence or presence of 10 μM LY294002 or 10 μM U0126 for 72 h. Trypan blue exclusion assay assessing the death of T98G cells. Stained cells were counted as dead and are expressed as a percentage of total cells. Data are the mean ± SD from three separate experiments. $^{**}P < 0.01$. (C) T98G cells were left untreated or were treated with ciglitazone in the absence or presence of 30 μM GW9662 for 48 h. Immunoblot analysis of Bcl-2, Bid, and β-actin. (D) T98G cells were left untreated or were treated with 30 μM ciglitazone in the absence or presence of 10 μM LY294002 for 48 h. Immunoblot analysis of pThr308 Akt, pSer473 Akt, total Akt, Bcl-2, Bid, and β-actin. (E) T98G cells were left untreated or were treated with 30 μM ciglitazone in the absence or presence of either 30 μM GW9662 or 10 μM LY294002 for 48 h. Cells were then treated with 50 nm tetramethylrhodamine ethyl ester (TMRE) and 5 μg/ml Hoechst 33258 fluorescent dye for 20 min. Images were collected under an inverted fluorescence microscope.

by down-regulating Akt activity in glioma cells. In addition, at 30 μM, CGZ increased the expression of p53 and the cell cycle inhibitors $p21^{WAF1/Cip1}$ and $p27^{Kip1}$ in T98G cells (Fig. S1). Treatment with 20 μM CGZ after GW9662 induced G_2/M phase arrest, although 20 μM CGZ alone did not induce cell cycle arrest (Fig. S1).

4. Discussion

A number of studies support the suggestion that the modulation of PPARγ activation may have future therapeutic use [1–7]. PPARs are versatile and potent regulators of cellular function. They play an important role in rodent hepatocarcinogenesis, inflammation, atherosclerosis development, lipid metabolism, diabetes, and cancer. The TZDs are a family of drugs that bind to and activate PPARγ, and are used in the treatment of non-insulin-dependent type 2 diabetes. TZDs increase insulin sensitivity, decrease hepatic gluconeogenesis, and favor adipocyte differentiation, thereby limiting their growth potential. This latter observation has prompted numerous studies on the role of TZDs in the regulation of tumor cell proliferation and apoptosis. Encouraging results emerged from these studies indicating that TZDs can potentially be used as efficient anticancer drugs in various neoplasms such as prostate, colon, and lung cancers [17,18].

In this study, a low concentration of CGZ had no effect on the viability of T98G cells and did not down-regulate the activity of Akt (data not shown), which is associated with survival and protection from cytotoxicity. However, CGZ in higher concentrations decreased Akt activity and was rapidly cytotoxic. If CGZ induced cell death independently of PPARγ, the dose of CGZ required to induce cell death would be greater than the dose required to bind and antagonize PPARγ. Indeed, under our experimental conditions, 30 μM is the initiating concentration of CGZ-induced cell death in T98G glioma cells. At this concentration, CGZ partially exists in a free particle state, separate from PPARγ. In addition, when cells were pretreated with GW9662 or when PPARγ was down-regulated by siRNA, cell death could be effectively induced with a lower concentration of CGZ. Because glioma cells express a relatively low level of PPARγ compared with other cancer cell lines and the expression of PPARγ did not change in response to CGZ treatment, CGZ may have potential as a therapeutic for glioma.

However, Strakova and colleagues [19] showed that a higher concentration of CGZ (>50 μM) reduced the viability of four human glioma cell lines and arrested the cell cycle through a PPARγ-dependent mechanism. They suggested that CGZ, a PPARγ agonist, induces a PPARγ-dependent cell death because CGZ alone effectively induced cell death. However, that report did not confirm the effect of pretreatment with PPARγ antagonist

or down-regulation of PPARγ expression on CGZ-induced cell death. CGZ was also used at very high concentrations (>50 μM) for the induction of cell death, while low concentrations of CGZ did not induce cell death in glioma cells in that study. These conclusions were therefore drawn without considering the possibility that CGZ induces cell death independently of PPARγ. Although other previous studies [20] reported PPARγ-dependent cell death induced by 20 μM CGZ in A172 human glioma cells, CGZ-induced cell death in those reports was partially prevented by pretreatment with the PPARγ antagonist GW9662. These data may imply that CGZ induces cell death through PPARγ-dependent and -independent mechanisms in glioma cells. It is also suggested that 15d-PGJ_2 and CGZ induce cell death through a caspase-independent mechanism in A172 glioma cells [21]. Thus, unlike previous studies related to CGZ's toxicity in glioma cells [19–21], our results suggest that higher concentrations of CGZ mediate many cellular functions independently of PPARγ, including apoptosis and cell cycle distribution (Fig. S1).

The hallmark of glioma is increased activity of the PI3K/Akt pathway, which controls the expression of several pro-survival proteins, including nuclear factor-kappaB (NF-κB) and the Bcl-2 family members [22,23]. Bcl-2 is an anti-apoptotic protein predominantly localized in mitochondria, which regulates mitochondrial membrane integrity and cytochrome c release. The pro-apoptotic Bcl-2 family member Bid mainly resides in the cytoplasm and is cleaved into truncated Bid (tBid) in response to death stimuli [24]. tBid redistributes into the mitochondria and induces cytochrome c release into cytosol, resulting in the loss of MMP. In this study, cell death in response to CGZ treatment decreased Akt activity and enhanced the loss of MMP, an effect which was accompanied by a down-regulation of Bcl-2 expression and an increase in Bid cleavage. As yet, few studies have explored the relationship between PPARγ-independent CGZ and mitochondrial stability; therefore, further investigation on this topic is necessary.

In conclusion, CGZ may be capable of inducing cell death in T98G cells independently of PPARγ, by down-regulating Akt and inducing the collapse of MMP. These results suggest PPARγ agonists could be used for therapeutic applications in the treatment of glioma.

Acknowledgments

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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.bbrc.2011.12.001.

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