A Peroxisome Proliferator-activated Receptor-γ Agonist, Troglitazone, Facilitates Caspase-8 and -9 Activities by Increasing the Enzymatic Activity of Protein-tyrosine Phosphatase-1B on Human Glioma Cells

Despite dramatic advances in adjuvant therapies, patients with malignant glioma face a bleak prognosis. Because many adjuvant therapies seek to induce glioma apoptosis, strategies that lower thresholds for the induction of apoptosis may improve patient outcomes. Therefore, elucidation of the biological mechanisms that underlie resistance to current therapies is needed to develop new therapeutic strategies. Here we proposed a novel mechanism of pro-apoptotic effect induced by a pharmacological peroxisome proliferator-activated receptor-γ (PPARγ) agonist, troglitazone, that facilitates caspase signaling in human glioma cells. Troglitazone activates protein-tyrosine phosphatase (PTP)-1B, which subsequently reduces phosphotyrosine 705 STAT3 (pY705-STAT3) via a PPARγ-independent pathway. Reduction of pY705-STAT3 in glioma cells caused down-regulation of FLIP (FADD-like IL-1β-converting enzyme-inhibitory protein) and Bcl-2. Furthermore, troglitazone induced Ser-392 phosphorylation of p53 via a PPARγ-dependent pathway and up-regulation of Bax in a p53 wild-type glioma. When given with tumor necrosis factor-related apoptosis-inducing ligand or caspase-dependent chemotherapeutic agents, such as etoposide and paclitaxel, troglitazone exhibited a synergistic effect by facilitating caspase-8/9 activities. A PPARγ antagonist, GW9662, did not block this effect, although a PTP inhibitor abrogated it. Knockdown of STAT3 by STAT3-small interfering RNA negated the inhibitory effect of PTP inhibitor on troglitazone, indicating that troglitazone uses a STAT3 inactivation mechanism that makes caspase-8/9 activities susceptible to cytotoxic agents in glioma cells and that PTP1B plays a critical role in the down-regulation of activated STAT3, as well as FLIP and Bcl-2. When taken with caspase-dependent anti-neoplastic agents, troglitazone may be a promising drug for use against malignant gliomas because it facilitates the caspase cascade, thereby lowering thresholds for the apoptosis induction of glioma cells.

Malignant gliomas adopt the ability to bypass or disrupt fail-safe mechanisms, such as programmed cell death and host immune defense (1–3), which make current therapeutic interventions ineffective at eradicating residual tumor reservoirs. Because many adjuvant therapies for malignant tumors seek to induce tumor cell apoptosis, strategies that lower thresholds for the induction of apoptosis, which can then make other treatments more effective, may improve patient outcomes. Therefore, further understanding of the biological anti-apoptotic mechanisms that govern resistance to conventional glioma therapies is required in order to develop safer and more effective treatments.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)2 is a promising anti-neoplastic agent because it induces apoptosis in cancer cells with only a negligible effect on normal cells (4, 5). It has been known that TRAIL triggers caspase cascade through interaction with TRAIL–responsive death receptors (DR), such as DR4 and DR5, which induce cleavage of caspase-8 in the Fas-associated death domain protein-dependent mechanism (6). In this pathway, cleaved caspase-8 plays a significant role as an initiator that can process other members in the caspase cascade. Cleaved caspase-8 induces cleavage of Bid, which up-regulates mitochondrial cytochrome c (Cyt-c) release (7). Cyt-c then cooperates with apoptotic protease-activating factor-1 to activate caspase-9 (8). After these activations, both caspase-8 and -9 activate caspase-3, which is the primary activator of apoptotic DNA fragmentation and leads to cancer cell apoptosis (9).

Despite the numerous reports describing the favorable anti-tumor activities of TRAIL, malignant gliomas exhibit considerable heterogeneity in their sensitivity to TRAIL, even among those expressing DR4 and DR5 (5, 10). In this regard, it has been reported recently that several cancers, including gliomas, constitutively express FLIP (Fas-associated death domain-like IL-1β-converting enzyme-inhibitory protein) (11–13), which is a cytoplasmic protein that inhibits the recruitment and processing of caspase-8 (12), and that the overexpression of FLIP induces cancer’s resistance to DR-dependent apoptosis (14). In addition, it has also been found that Bcl-2 family proteins modulate caspase-9 activity by controlling the permeability of mitochondrial membranes and that dysregulation of these proteins in cancer cells correlates with their anti-apoptotic potential and progression (15). Specifically, the anti-apoptotic Bcl-2 family, including Bcl-2 and Bcl-xL, stabilizes the mitochondrial porin channel (voltage-dependent anion channel) and inhibits Cyt-c release (16), whereas the pro-apoptotic Bcl-2 family, including Bax and Bad, antagonizes this process by competitive heterodimerization with anti-apoptotic Bcl-2 proteins (17). Although down-regulation of FLIP and/or anti-apoptotic Bcl-2 family proteins are therefore a therapeutic target for promotion of caspase

2 The abbreviations used are: TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; rTRAIL, recombinant TRAIL; Cyt-c, cytochrome c; FLIP, FADD-like IL-1β-converting enzyme-inhibitory protein; TGD, thiazolidenediones; PTP, protein-tyrosine phosphatase; pY705-STAT3, phosphotyrosine-705 STAT3; TG, troglitazone; GW, PPARγ agonist GW9662; PTPI, PTP inhibitor; PI, propidium iodide; siRNA, small interfering RNA; DR, death receptors; SHPI, Src homology 2-containing PTP inhibitor; RTK, receptor tyrosine kinases; Ab, antibody; FACS, fluorescence-activated cell sorter; Annexin V-fluorescein isothiocyanate; JAK, Janus tyrosine kinase; STAT, signal transducers and activators of transcription; IL, interleukin.

8 The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom correspondence should be addressed: Maxine Dunitz Neurosurgical Institute, Cedars-Sinai Medical Center, Ste. 800 East, 8631 West 3rd St., Los Angeles, CA 90048. Tel.: 310-423-0875; Fax: 310-423-0810; E-mail: yuji@cshs.org.
cascade activity, the mechanism that regulates these proteins in glioma cells is not fully understood.

In this report, we demonstrate that a peroxisome proliferator-activated receptor-γ (PPARγ) agonist, troglitazone, facilitates caspase-8 and -9 actions by down-regulating FLIP and Bcl-2 in human glioma cells. Troglitazone induces inactivation of signal transducer and activator of transcription-3 (STAT3) and synergistically enhances the cytotoxic effects of TRAIL and other caspase-dependent chemotherapeutic drugs. This effect is exhibited through a PPARγ-independent mechanism, in which protein-tyrosine phosphatase 1B (PTP1B) plays a critical role in the down-regulation of activated STAT3, as well as FLIP and Bcl-2. Here we propose a novel mechanism to explain the pro-apoptotic effect induced by troglitazone in human glioma cells.

**EXPERIMENTAL PROCEDURES**

**Tumor Cells**—A human primary cultured glioma (MG-328) was established from the surgical specimen of a patient with newly diagnosed glioblastoma at Cedars-Sinai Medical Center after Institutional Review Board-approved consent was obtained. MG-328 and human glioma cell lines, U-87MG (American Type Culture Collection, Manassas, VA) and LN-18 (provided by Dr. Erwin Van Meier, Emory University, GA), were maintained at 37 °C and 5% CO2 in Dulbecco’s modified Eagle’s medium/F-12 with 10% heat-inactivated fetal bovine serum, 2 mM glutamate, 10 mM HEPES, 100 units/ml penicillin, and 100 μg/ml streptomycin.

**Reagents**—Recombinant human TRAIL was obtained from PeproTech (Rocky Hill, NJ). A PPARγ agonist, troglitazone (TG), was obtained from Biomol (Plymouth Meeting, PA). A PPARγ antagonist, GW9662 (GW), was obtained from Cayman Chemical (Ann Arbor, MI). PTP1, α-bromo-4-hydroxyacetophenone, and Src homology 2-containing PTP (SHP1; 1SH01) and rabbit polyclonal Ab of phosphotyrosine 705 STAT3 (pY705-STAT3), which is an activated receptor–STAT3 (STAT3) and synergistically enhances the cytotoxic effects induced by troglitazone in human glioma cells.

**Experimental Procedures**

**RealTime Quantitative Reverse Transcription-PCR**—Gene expression was quantified by real time quantitative reverse transcription-PCR using QuantiTect SYBR Green dye (Qiagen, Valencia, CA). DNA amplification was performed using an iCycler (Bio-Rad), and the binding of the fluorescence dye SYBR Green I to double-stranded DNA was measured. The PCRs were set up in microtubes at a volume of 25 μl. Oligonucleotide primers were designed as follows: STAT3 forward, 5’-GCC AGA GAG CCA GGA GCA-3’. STAT3 reverse, 5’-ACA CAG ATA AAC TGG GTC TTC AGG TAT G-3’. β-actin forward, 5’-TTC TAC AAT GAG CTC GTG GTG-3’. β-actin reverse, 5’-GGG GTG TTG AGG GTC TCA AA-3’. The reaction components were 2.0 μg of cDNA synthesized as above, 12.5 μl of 2× QuantiTect SYBR Green PCR Master Mix (Qiagen, Valencia, CA), and 0.4 μl each pair of oligonucleotide primers. The program was as follows: initial activation for 15 min at 95 °C, 50 cycles consisting of melting for 30 s at 95 °C, annealing for 25 s at 60 °C, and extension for 30 s at 72 °C. After cycling, relative quantification of target gene mRNA against an internal control, β-actin, was possible by the following a ΔC method, and an amplification plot of fluorescence signal versus cycle number was drawn. The difference (ΔC) between the mean values in duplicated samples of the target gene and those of β-actin was calculated using Microsoft Excel, and the relative quantification value was expressed as 2 ΔC. The relative expression of each sample in the figure was normalized by “no treatment” expression.

**Abs**—Rabbit polyclonal Abs against DR4, DR5, and PPARγ were obtained from Cayman Chemical. Mouse monoclonal Abs against STAT3, phosphotyrosine 705 STAT3 (pY705-STAT3), which is an activated form of STAT3, Bax, and Bcl-2 were obtained from Pharmingen. Rabbit polyclonal Abs against p53 and phospho-Ser-392-p53 (pS392-p53) were obtained from Cell Signaling Technology (Beverly, MA). Mouse monoclonal Abs against PTP1B (PTPase1B; AE-21) and Src homology 2-containing PTP-1 (SHP1; 1SH01) and rabbit polyclonal Ab against FLIP were obtained from Calbiochem. Mouse monoclonal Ab of β-tubulin was obtained from Sigma. Horseradish peroxidase-linked Abs of sheep anti-mouse IgG and donkey anti-rabbit IgG were obtained from Amersham Biosciences.

**Western Blot**—Samples were extracted with buffer containing 1% Triton X-100, 150 mM NaCl, 50 mM Tris (pH 7.5), and 1 mM phenylmethylsulfonyl fluoride (Roche Applied Science) and were subjected to SDS-PAGE with 10% polyacrylamide gel. Electrophoretic transfer to nitrocellulose membranes (Amersham Biosciences) was followed by immunoblotting. The signal was detected by using an ECL detection system (Amersham Biosciences).

**Apoptosis Assay and Cell Viability Assay**—Treatment of cells with TG, GW, PTP1, and/or SHPI for 24 h was followed by treatment with rTRAIL (VP16 or Taxol), and all the cells, including cells that had not adhered, were harvested after 24 h (48 h). An annexin V-fluorescein isothiocyanate apoptosis detection kit I (Pharmingen) was used for viability assay of the cells. Cells were stained with annexin V-fluorescein isothiocyanate (Ann) and propidium iodide (PI) according to the manufacturer’s protocol and were analyzed by FACScan (Pharmingen). Cells that stained negative for both Ann and PI were defined as viable cells for viability assay.

**Caspase Activity Assay**—Activity of caspase-3, -8, and -9 was measured using caspase-3/3/2, FLICE/caspase-8, and APOPCYT/Caspase-9 colorimetric assay kits, respectively, from Medical and Biological Laboratories Co. (Nagoya, Japan). Briefly, the treatment of cells with TG, GW, and/or PTP1 for 24 h was followed by treatment with rTRAIL (VP16 or Taxol). After 2 h (24 h) of treatment, all the cells were harvested, and samples were extracted with Cell Lysis Buffer. Concurrently, a sample for a negative control was extracted from the cells without apoptosis induction. A standard curve using the absorbance of p-nitroanilide standards was constructed, and then the specific activi-
ties on each sample were calculated according to the manufacturer’s protocol.

Statistics—Student’s t test was used for statistical comparison of results.

RESULTS

The Role of STAT3 in Resistance to TRAIL-induced Apoptosis—To examine the mechanism that causes resistance to TRAIL, we used glioma cell lines that are partially resistant to TRAIL despite their expression of DR4 and/or DR5. Western blot was used to detect the expression levels of DR4 and DR5 in LN-18, U-87MG, and MG-328 (Fig. 1A). The cytotoxic activity of TRAIL (18.75–300 ng/ml) was examined in each glioma (Fig. 1, B and C). As shown in Fig. 1B, which is a representative result of FACS analysis using an annexin-V apoptosis detection kit on LN-18, treatment with TRAIL (300 ng/ml) increased the percentage of Ann(+)H11001PI(+)H11002 cells, which indicates early stage of apoptosis, after 2 h of treatment (33.7%) and 24 h of treatment (60.1%) compared with no treatment control (2.2%). Treatment with 300 ng/ml of TRAIL induced 40–60% cell death after 24 h of treatment (Fig. 1C), although the viable cells maintained a proliferative ability after 72 h of cell culture with 300 ng/ml TRAIL (not shown). These findings indicate that these gliomas can acquire a resistance to TRAIL despite their expression of functionally intact DR4/5.

After this observation, we sought to determine whether STAT3 contributes to the resistance of gliomas to TRAIL by using STAT3-specific small interference RNA (STAT3-siRNA) (18). The specificity of STAT3 inhibition in LN-18 after transfection with STAT3-siRNA was investigated by real time quantitative PCR and Western blot analysis (Fig. 2A and B). Although neither nonsilencing control nor vehicle control had any significant change in expressions of STAT3 mRNA or STAT3 protein compared with the no treatment control, STAT3-siRNA transfection in glioma cells decreased both STAT3 mRNA (Fig. 2A) and STAT3 protein (Fig. 2B) expressions in a dose-dependent manner. These findings indicate that the STAT3-siRNA used in this study exhibited a STAT3-specific silencing effect. As shown in Fig. 2, B and C, Western blot analysis detected high levels of STAT3, pY705-STAT3, FLIP, and Bcl-2 in the no treatment control, nonsilencing control, and vehicle control. The pY705-STAT3 was down-regulated concomitant with the decrease in STAT3 in STAT3-siRNA-transfected glioma cells (Fig. 2, B and C). In addition, FLIP and Bcl-2 expression levels decreased in the STAT3-siRNA groups (Fig. 2, B and C), suggesting that knockdown of STAT3 induces down-regulation of FLIP and Bcl-2 in glioma cells by inhibiting the transcriptional activity of STAT3. Bax expression levels, which is a pro-apoptotic protein induced by p53, were not altered by knockdown of STAT3 (Fig. 2, B and C). To confirm further the regulatory function of STAT3 on the DR-signaling pathway, the nonsilencing control, vehicle control, and STAT3-siRNA-transfected cells were treated with 100 ng/ml TRAIL, and then cell viability (Fig. 2D) and caspase activity assays (Fig. 2E) were performed. Transfection of STAT3-siRNA to glioma cells did not induce their apoptotic death, but it significantly enhanced the cytotoxic effect of TRAIL (Fig. 2D). The specific activities of caspases-3, -8, and -9 were also significantly increased in the siRNA-transfected group (Fig. 2E). In the assessment of the 72-h cell culture, TRAIL (100 ng/ml) killed more than 95% of the cells in the siRNA-transfected group of each glioma (not shown). These results indicate that STAT3 plays a critical role in the resistance of gliomas to TRAIL. Down-regulation of FLIP and Bcl-2 in the STAT3-siRNA-transfected group may have a particular relevance to the facilitation of the activity of caspase-3, -8, and -9.
FIGURE 2. The role of STAT3 in causing resistance to TRAIL in glioma cells. A and B, STAT3-siRNA (50–600 pmol) or nonsilencing siRNA (600 pmol) was transfected to glioma cells (LN-18) with Oligofectamine reagent. Cells that transfected with Oligofectamine alone were referred to as vehicle. Cells were used for experiments 24 h after transfection. A, total RNA samples extracted from cells were subjected to real time quantitative PCR. B, protein samples extracted from cells were subjected to Western blot. C–E, STAT3-siRNA (600 pmol) or nonsilencing siRNA (600 pmol) was transfected to glioma cells. Cells that transfected with Oligofectamine alone were referred to as vehicle. C, protein samples extracted from cells were subjected to Western blot. D, visible cell (%) was determined using a trypan blue dye exclusion assay. E, Caspase activity was determined using the Caspase Colorimetric Assay Kit.
Facilitation of Caspase Activity by a PPARγ Agonist

Troglitazone Activities via PPARγ-dependent and -independent Pathways—We sought to determine whether a PPARγ agonist would enhance TRAIL-induced apoptosis. Using a Western blot analysis, PPARγ expression was observed in LN-18 and U-87 but not in MG-328 (Fig. 3A). Treatment over 48 h with TG, which is a pharmacological PPARγ agonist, did not induce apoptotic death on glioma cells but did significantly enhance the cytotoxic activity of TRAIL not only in LN-18 and U-87MG but also in MG-328 (Fig. 3B). These results suggest the possibility that the synergistic effect of TG on TRAIL is controlled by a PPARγ-independent mechanism. Although TG had no effect on STAT3 expression levels, it induced down-regulation of pY705-STAT3 (Fig. 3C). In addition, TG diminished FLIP and Bcl-2 protein expression in these cells (Fig. 3C), which was probably caused by down-regulation of pY705-STAT3. TG treatment in U-87MG, of which p53 is a wild type (20), increased Bax expression levels, although Bax levels were not altered in LN-18, which is a p53 mutant cell line (20), or in MG-328 (Fig. 3C). Although we did not confirm the sequence of p53 in MG-328, this result may indicate that TG up-regulates a transcriptional activity of p53 via a PPARγ-dependent pathway, in which wild-type p53 should induce up-regulation of Bax.

Next, we used a PPARγ antagonist, GW9662 (GW), to clarify the role of PPARγ in the effect of TG on these glioma cells. Similar to the effect of TG alone, down-regulation of pY705-STAT3 was observed on the cells treated with TG and GW (Fig. 4A), indicating that the inhibitory effect of TG on pY705-STAT3 is caused via a PPARγ-independent pathway.

In a previous report, it was demonstrated that phosphorylation of p53 at Ser-392 (pS392-p53) influenced the growth suppressor function and transcriptional activation of p53 (21). Based on this report, we confirmed the expression levels of pS392-p53 as an index of the transcriptional activity of p53. As shown in Fig. 4A, GW abrogated the up-regulation of pS392-p53 induced by TG in LN-18 and U-87MG. GW also inhibited the up-regulation of Bax found in TG-treated U-87MG. There was no significant change in the expression levels of pS392-p53 or Bax in MG-328 in either setting (Fig. 4A). These results indicate that TG induces phosphorylation of p53 at Ser-392 via a PPARγ-dependent pathway and causes up-regulation of Bax in gliomas that have a wild-type p53. Although these results suggest that TG may have the potential to control caspase cascade signaling via both PPARγ-dependent and-independent pathways, the synergistic activity of TG in TRAIL-treated glioma cells was not blocked by a PPARγ antagonist even in U-87MG, as assessed by a cell viability assay (Fig. 4B). In addition, treatment with GW did not reduce the specific activities in any caspases that we observed (Fig. 4C), indicating that the synergism of TG in TRAIL-treated glioma cells occurs through a PPARγ-independent mechanism. Taken together, these results indicate that activation of STAT3 and the subsequent down-regulation of the downstream proteins, such as FLIP and Bcl-2, by TG may have a particular relevance to the mechanism by which TG synergistically enhances the pro-apoptotic effect of TRAIL.

The Significance of PTP1B Activity in the Effect of TG—PTP1B negatively regulates tyrosine phosphorylation of JAK2 and STAT3 (22, 23). In addition, SHP-1 negatively regulates STAT3 activity by facilitating tyrosine dephosphorylation of the upstream JAK2 (24). Based on these findings, we hypothesized that the inhibitory effect of TG on pY705-STAT3 may be caused by activation of these PTP proteins. To confirm the relationship between pY705-STAT3 and PTPs, protein samples extracted at different time points after treatment with TG were subjected to Western blot in which pY705-STAT3 and PTPs, protein samples extracted at different time points after treatment with TG were subjected to Western blot. D. cells were treated with rTRAIL (100 ng/ml) for 24 h and then were stained with Ann and PI. Several groups were cultured for 24 h without rTRAIL treatment. Cells that stained negative for both Ann and PI were defined as viable cells. Data are means ± S.D. of three independent experiments; ** refers to statistical significance (p < 0.01). E. the activities of caspase-3, -8, and -9 were measured by enzyme activity assay after 2 h of the TRAIL treatment. Nonsilencing controls were used for control. The specific activities on each sample were calculated according to the manufacturer’s protocol. Data are means ± S.D. of three independent experiments; ** refers to statistical significance (p < 0.01) compared with each control.
until 32 h. SHP-1 was constitutively and highly expressed in all of these gliomas, and TG had only a negligible effect on SHP-1 expression.

To confirm the role of PTP activity in the mechanism controlling the synergistic effect of TG on TRAIL-treated glioma cells, we used \( /H9251 \)-bromo-4-hydroxyacetophenone (PTPI), which is an inhibitor for both PTP1B and SHP-1, and \( /H9251 \)-bromo-4-carboxymethoxyacetophenone (SHPI), which is a specific inhibitor for SHP-1 (27). Specifically, TG-treated glioma cells were co-treated with PTPI or SHPI and were then assessed by Western blot and by cell viability and caspase activity assays. In the Western blot analysis, PTPI abrogated the inhibitory effect of TG on pY705-STAT3 (Fig. 6A), whereas SHPI did not inhibit the down-regulation of pY705-STAT3 induced by TG (Fig. 6B). These findings indicate that activation of PTP1B, but not SHP-1, is involved in the inhibitory effect of TG on pY705-STAT3. PTPI did not inhibit the up-regulation of pS392-p53 in LN-18 and U-87MG caused by TG. Neither STAT3 nor p53 expression levels were altered in either setting (Fig. 6A).

In the cell viability (not shown) and caspase activity assays (Fig. 6C), PTPI abrogated the synergistic effect of TG on TRAIL, although SHPI did not abrogate it (not shown). Thus, these results indicate that PTP1B plays a critical role in TG’s effect that enhances the cytotoxic activity of TRAIL in glioma cells.

Furthermore, we sought to determine whether STAT3 inactivation is a key target for PTP1B in this mechanism. To confirm this, STAT3-siRNA-transfected glioma cells were treated with TG, PTPI, and TRAIL.
Facilitation of Caspase Activity by a PPARγ Agonist

The Synergism of TG with Chemotherapeutic Drugs—Given the already described ability of TG to facilitate caspase cascade in glioma cells by attenuating FLIP and Bcl-2 expression levels, TG may also exhibit a synergistic effect with chemotherapeutic drugs that activate caspase-8 and -9. Based on this hypothesis, we sought to determine whether TG enhances the cytotoxic activities of etoposide (VP16) and paclitaxel (Taxol), which are known to involve activation of caspase-2, -8, -9, and -10 during their apoptosis induction (28–30). To confirm this, treatment of U-87MG with TG and/or PTPI was followed by treatment with VP16 (0.01–10 μM) or Taxol (0.005–5 μM). The cells were then subjected to cell viability (Fig. 7, A and B) and caspase activity assays (Fig. 7, C and D). The synergistic activities of TG for both VP16 (Fig. 7, A and C) and Taxol (Fig. 7, B and D) were observed in all of the assays. Although PTPI had only a limited effect in inhibiting cytotoxic (Fig. 7, A and B) and caspase-3 activity (Fig. 7, C and D) in these assessments, PTPI completely abrogated the effect of TG on the activity of caspase-8 and -9 (Fig. 7, C and D). These results indicate that TG uses a specific mechanism that makes caspase-8 and -9 activities susceptible to cytotoxic agents in glioma cells, and that PTPI1B plays a critical role in the down-regulation of constitutively activated STAT3, as well as the downstream FLIP and Bcl-2 in this mechanism. Although the exact reason for the limited inhibitory effect of PTPI on cytotoxic or caspase-3 activity was not determined, these results suggest that TG may have other pro-apoptotic activities that are exhibited through a caspase-3-dependent and PTPI1B-independent mechanism.

Further observation of the 5-day cell cultures in these assessments found that more than 95% of the cells in the TG and TG/PTPI treatment groups were killed by VP16 (10 μM) or Taxol (5 μM), whereas the cells in the control group maintained an ability to proliferate (not shown). Therefore, TG may be a promising drug that can abrogate the mechanism that makes malignant gliomas resistant to cytotoxic agents.

Based on these results, we schematized the synergistic activities of TG in the caspase cascade (Fig. 8). Specifically, TG induces activation of PTPI1B and the subsequent tyrosine dephosphorylation of constitutively activated STAT3 in glioma cells via a PPARγ-independent pathway. This event causes down-regulation of FLIP and Bcl-2 and facilitates the activities of caspase-8 and -9 when taken with caspase-dependent anti-neoplastic agents. TG also has the ability to induce transcriptional activation of p53 via a PPARγ-dependent pathway. In the cells with wild-type p53, this event causes up-regulation of Bax, which can facilitate caspase-9 activity, although this may be only a minor effect of the synergism with TG. Thus, TG enhances the cytotoxic effect of caspase-dependent anti-neoplastic agents, such as TRAIL, VP16, and Taxol, by facilitating caspase cascade signaling in glioma cells. PTPI1B plays a critical role in this mechanism.

DISCUSSION

PPARγ is a member of the nuclear hormone receptor superfamility of ligand-activated transcription factors. Interaction of PPARγ with its agonists, such as 15-deoxy-Δ12,14-prostaglandin J2 and thiazolidenediones (TZD), exerts anti-tumor effects in a variety of cancers, indicating anti-proliferative, anti-angiogenic, and pro-differentiation effects (31). Apart from these anti-tumor activities, contradictory evidence in support of the tumor-promoting activity of PPARγ has been observed in Min mice, with a genetic predisposition to adenomatous polyposis coli (32, 33). Thus, the question of whether PPARγ is a tumor suppressor gene remains controversial. Despite the oncogenic activities of PPARγ, it is now recognized that PPARγ agonists have pro-apoptotic activities, and many of them are induced through PPARγ-independent pathways (31). Therefore, PPARγ-independent mechanisms triggered by PPARγ

and were then subjected to caspase activity assay. In this assessment, the inhibitory effect of PTPI on TG was negated in the STAT3-siRNA-transfected glioma cells (Fig. 6D). These results indicate that activation of PTPI1B by means of proteolysis of 50-kDa PTPI1B and the subsequent tyrosine dephosphorylation of pY705-STAT3 is a key mechanism in this effect.

FIGURE 6. The significance of PTPI1B in the effect of TG. A, cells were treated with TG (30 μM) and/or PTPI inhibitor (PTPI, 50 μM) for 24 h. Protein samples extracted from the cells were subjected to Western blot. B, cells were treated with TG (30 μM) and/or SHP-1 inhibitor (SHPI, 200 μM) for 24 h. Protein samples extracted from the cells were subjected to Western blot. C, treatment of cells with TG and/or PTPI was followed by treatment with TRAIL (100 ng/ml) for 2 h. Cells not treated with TG or PTPI were used for control. The activities of caspase-3, -8, and -9 were measured by enzyme activity assay. The specific activities on each sample were calculated according to the manufacturer’s protocol. Data are mean ± S.D. of three independent experiments; ** refers to statistical significance (p < 0.01) compared with each control. D, STAT3-siRNA (600 pmol) or nonsilencing siRNA (600 pmol) was transfected to glioma cells. Cells that transfected with Oligofectamine alone were referred to as vehicle. Cells were used for experiments 24 h after transfection. The activities of caspase-3, -8, and -9 were measured similar to C. Data are mean ± S.D. of three independent experiments; * refers to statistical significance (p < 0.05); ** refers to statistical significance (p < 0.01) compared with each nonsilencing control.
agonists may have particular relevance to the paradoxical findings on the effect of PPARγ in cancer cells, although the mechanisms are not fully understood. Among the PPARγ-independent effects, PPARγ agonists mediate pro-apoptotic and anti-inflammatory activities by inhibiting the transcriptional activities of the STAT family, including STAT1,-3, and -5 (34–36). Furthermore, it has been reported recently that the pro-apoptotic activity of a PPARγ agonist acting via a PPARγ-independent mechanism correlates with the inhibitory effect on FLIP and Bcl-xL/Bcl-2 functions (37, 38).

It has been shown that activated STAT3 contributes to the inhibition of Fas-mediated apoptosis signaling by affecting the expression levels of FLIP and Bcl-2 (39). Activation (i.e. tyrosine phosphorylation) of STAT3 is induced by signaling through Jak/STAT-associated receptors such as glycoprotein 130, growth hormone receptors, interferon receptors, and receptor tyrosine kinases (RTKs), of which glycoprotein 130, exemplified by the IL-6 receptor, and RTKs, exemplified by epidermal growth factor receptor and vascular endothelial growth factor receptor, have been reported as key mediators in the inappropriate activation of STAT3 in glioma cells (40–42). In our studies, STAT3 in glioma cells was constitutively activated, and FLIP and Bcl-2 were highly expressed. Knockdown of STAT3 by its specific siRNA facilitated caspase cascade signaling by attenuating FLIP and Bcl-2 expression. Furthermore, this effect was faithfully reproduced by a PPARγ agonist, troglitazone, which induced down-regulation of pY705-STAT3, FLIP, and Bcl-2 in glioma cells via a PPARγ-independent pathway.

It is well established that tyrosine phosphorylation is negatively regulated by PTPs, which represent a large and structurally diverse family of enzymes that rival the protein-tyrosine kinase family, including RTKs, in structural diversity and complexity (43, 44). Protein-tyrosine kinases, PTPs, and their corresponding substrates are integrated within elaborate signal transducing networks, in which PTPs can either antagonize or potentiate protein-tyrosine kinase-induced signaling events in vivo. Defective or inappropriate operation of these networks leads to aberrant tyrosine phosphorylation, which contributes to the development of many human diseases, including cancers (45). So far, RTK activity and the effect of RTK inhibitors on malignant glioma have been noted (46, 47), but the role of PTPs in gliomas is largely unknown.

In our report, expression levels of the cytoplasmic nontransmembrane PTP family, PTP1B and SHP-1, were analyzed after treatment with troglitazone. Expression levels of 42-kDa PTP1B, which is an acti-
Facilitation of Caspase Activity by a PPARγ Agonist

activated Ca\(^{2+}\) influx and subsequent calpain activation may be a critical mechanism by which troglitazone activates PTP1B in glioma cells via a PPARγ-independent pathway.

Here we demonstrate that inactivation of constitutively activated STAT3 concomitant with PTP1B activation plays a critical role in the synergistic effect of troglitazone on the cytotoxic activities of anti-neoplastic agents in glioma cells. Although troglitazone may have the ability to facilitate caspase-3 activity and other pro-apoptotic signals through a PTP1B-independent pathway, we note that troglitazone is a promising anti-neoplastic agent because of its synergistic ability to facilitate caspase-8- and -9 signaling in a PTP1B-dependent manner. These findings support the possibly enhanced effectiveness of using PPARγ agonists in clinical chemotherapy protocols that also use caspase-dependent anti-neoplastic agents such as TRAIL, etoposide, and paclitaxel for patients with malignant tumors as a means of facilitating the caspase cascade.

REFERENCES

1. Chakravarti, A., Zhai, G. G., Zhang, M., Mallhotra, R., Latham, D. E., Delaney, M. A., Robe, P., Nestler, U., Song, Q., and Loeffler, J. (2004) Oncogene 23, 7494–7506
2. Bobola, M. S., Emond, M. J., Blank, A., Meade, E. H., Kolsto, D. D., Berger, M. S., Rostomily, R. C., Silbergeld, D. L., Spence, A. M., and Silber, J. R. (2004) Clin. Cancer Res. 10, 7875–7883
3. Akasaki, Y., Liu, G., Chung, N. H., Ehtesham, M., Black, K. L., and Yu, J. S. (2004) J. Immunol. 173, 4325–4339
4. Ashkenazi, A., Pai, R. C., Fong, S., Leung, S., Lawrence, D. A., Marters, S. A., Blackie, C. Chang, L., McMurtrey, A. E., Hebert, A., DeForge, L., Kounenis, I. L., Lewis, D., Harris, L., Russiere, J., Koeppen, H., Shahrkoob, Z., and Schwall, R. H. (1999) J. Clin. Investig. 104, 155–162
5. Hao, C., Beguinot, F., Condorelli, G., Trecia, A., Van Meir, E. G., Yong, V. W., Parney, J. F., Roe, H. W., and Petrutti, K. C. (2001) Cancer Res. 61, 1162–1170
6. Thomas, L. R., Henson, A., Reed, J. C., Salsbury, F. R., and Thorburn, A. (2004) Oncogene 23, 5789–5798
7. Yoon, G., Kim, K. O., Lee, J., Kwon, D., Shin, J. S., Kim, S. J., and Choi, I. H. (2002) J. Neurooncol. 60, 135–141
8. Krueger, A., Baumann, S., Krammer, P. H., and Kirchhoff, S. (2001) Mol. Cell. Biol. 21, 8247–8254
9. Chang, X., Jin, T. G., Yang, H., DeWolf, W. C., Khoszvai-Far, R., and Olumi, A. F. (2000) Cancer Res. 64, 7086–7091
10. Rippo, M. R., Moretti, S., Vescovi, S., Tomasetti, M., Orecchia, S., Amici, G., Catalano, A., and Procopio, A. (2004) Oncogene 23, 7753–7760
11. Reed, J. C. (1999) Curr. Opin. Oncol. 11, 68–75
12. Shimizu, S., Koirala, M., and Tsujimoto, Y. (1999) Nature 399, 483–487
13. Adams, J. M., and Cory, S. (1998) Science 281, 1322–1326
14. Kondo, L., Kotecki, M., Kruger, M. M., and Cochran, B. H. (2003) BMC Cancer 10, 1471-2407-3-23
15. Liu, G., Ng, H., Akasaki, Y., Yuan, X., Ehtesham, M., Yin, D., Black, K. L., and Yu, J. S. (2004) Eur. J. Immunol. 34, 1680–1687
16. Van Meir, E. G., Kikuchi, T., Tada, M., Li, H., Odersen, A. C., Wojcik, B. E., Huang, H. J., Friedmann, T., de Triboulet, N., and Cavenee, W. K. (1994) Cancer Res. 54, 649–652
17. Kohn, K. W. (1999) Mol. Biol. Cell 10, 2703–2734
18. Zabotinoty, J. M., Bence-Hasencle, K. K., Stricker-Krongrad, A., Haji, F., Wang, Y., Minokoshi, Y., Kim, Y. B., Elmsquqit, J. K., Tartaglia, L. A., Kahn, B. B., and Neel, B. G. (2002) Dev. Cell 2, 489–495
19. Kazubski, W., Falik, H. D., Schaefer, V. G., Haasch, D., Frost, L., Hessler, P., Kroeger, P. E., White, D. W., Jirosuke, M. R., and Trevillan, J. M. (2002) Mol. Cell. Endocrinol. 195, 109–118
20. Bouquet, C., Susini, C., and Melmed, S. (1999) J. Clin. Investig. 104, 1277–1285
21. Frangioni, J. V., Beahm, P. H., Shifrin, V., Jost, C. A., and Neel, B. G. (1992) Cell 68, 545–560
22. Frangioni, J. V., Oda, A., Smith, M., Salzman, E. W., and Neel, B. G. (1993) EMBO J. 12, 4817–4820

FIGURE 8. Schema of synergistic activities of TG on TRAIL, VP16, and Taxol for facilitation of caspase cascade signaling.
Facilitation of Caspase Activity by a PPARγ Agonist

27. Arabaci, G., Guo, X.-C., Beebe, K. D., Coggeshall, K. M., and Pei, D. (1999) J. Am. Chem. Soc. 121, 5085–5086
28. Lin, C. F., Chen, C. L., Chang, W. T., Jan, M. S., Hsu, L. J., Wu, R. H., Tang, M. J., Chang, W. C., and Lin, Y. S. (2004) J. Biol. Chem. 279, 40755–40761
29. Perkins, C. L., Fang, G., Kim, C. N., and Bhatta, K. N. (2000) Cancer Res. 60, 1645–1653
30. Park, S. J., Wu, C. H., Gordon, J. D., Zhong, X., Emami, A., and Safa, A. R. (2004) J. Biol. Chem. 279, 51057–51067
31. Koeffler, H. P. (2003) Clin. Cancer Res. 9, 1–9
32. Saez, E., Tontonoz, P., Nelson, M. C., Alvarez, J. G., Ming, U. T., Baird, S. M., Thomazy, V. A., and Evans, R. M. (1998) Nat. Med. 4, 1058–1061
33. Lefebvre, A. M., Chen, I., Desreumaux, P., Najib, J., Fruchart, J. C., Geboes, K., Briggs, M., Heyman, R., and Auwerx, J. (1998) Nat. Med. 4, 1053–1057
34. Nikitakis, N. G., Stavash, H., Hebert, C., Reynolds, M. A., Hamburger, A. W., and Saak, J. I. (2002) Br. J. Cancer 87, 1396–1403
35. Chen, C. W., Chang, Y. H., Tsi, C. I., and Lin, W. W. (2003) J. Immunol. 171, 979–988
36. Park, E. J., Park, S. Y., Joe, E. H., and Jou, I. (2003) J. Biol. Chem. 278, 14747–14752
37. Kim, Y., Suh, N., Sporn, M., and Reed, J. C. (2002) J. Biol. Chem. 277, 22320–22329
38. Shian, C. W., Yang, C. C., Kulp, S. K., Chen, K. F., Chen, C. S., and Huang, J. W. (2005) Cancer Res. 65, 1561–1569
39. Haga, S., Terui, K., Zhang, H. Q., Enosawa, S., Ogawa, W., Inoue, H., Okuyama, T., Takeda, K., Akira, S., Ogino, T., Irani, K., and Ozaki, M. (2003) J. Clin. Invest. 112, 989–998
40. Weissenberger, J., Loeffler, S., Kappeler, A., Kopf, M., Lukes, A., Afanasieva, T. A., Aguzzi, A., and Weis, J. (2004) Oncogene 23, 3308–3316
41. Schaefer, L. K., Ren, Z., Fuller, G. N., and Schaefer, T. S. (2002) Oncogene 21, 2058–2065
42. Thomas, C. Y., Chouinard, M., Cox, M., Parsons, S., Stallings-Mann, M., Garcia, R., Jove, R., and Wharen, R. (2003) Int. J. Cancer 104, 19–27
43. Ostman, A., and Bohmer, F. D. (2001) Trends Cell Biol. 11, 258–266
44. Andersen, J. N., Mortensen, O. H., Peters, G. H., Drake, P. G., Iversen, L. F., Olsen, O. H., Jansen, P. G., Andersen, H. S., Tonks, N. K., and Moller, N. P. (2001) Mol. Cell. Biol. 21, 7117–7136
45. Hunter, T. (2000) Cell 100, 113–127
46. Li, B., Chang, C. M., Yuan, M., McKenna, W. G., and Shu, H. K. (2003) Cancer Res. 63, 7443–7450
47. Charest, A., Kheifets, V., Park, J., Lane, K., McMahon, K., Nutt, C. L., and Housman, D. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 916–921
48. Teixeira, J. E., and Mann, B. J. (2002) Infect. Immun. 70, 1816–1823
49. Hsu, S., Schmid, A., Sternfeld, L., Andrieu, L., Solis, G., Hoser, H. W., and Schulz, I. (2003) Cell. Signal. 15, 1149–1156
50. Palakurthi, S. S., Aktas, H., Grubissich, L. M., Mortensen, R. M., and Halperin, J. A. (2001) Cancer Res. 61, 6213–6218