Peroxisome Proliferator-activated Receptor γ Agonists Promote TRAIL-induced Apoptosis by Reducing Survivin Levels via Cyclin D3 Repression and Cell Cycle Arrest*

Received for publication, October 8, 2004, and in revised form, November 23, 2004
Published, JBC Papers in Press, November 29, 2004, DOI 10.1074/jbc.M411519200

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Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a promising cancer therapy that preferentially induces apoptosis in cancer cells. However, many neoplasms are resistant to TRAIL by mechanisms that are poorly understood. Here we demonstrate that human breast cancer cells, but not normal mammary epithelial cells, are dramatically sensitized to TRAIL-induced apoptosis and caspase activation by peroxisome proliferator-activated receptor γ (PPARγ) agonists of the thiazolidinedione (TZD) class. Although TZDs do not significantly alter the expression of components of the TRAIL signaling pathway, they profoundly reduce protein levels of cyclin D3, but not other D-type cyclins, by decreasing cyclin D3 mRNA levels and by inducing its proteasomal degradation. Importantly, both TRAIL sensitization and reduction in cyclin D3 protein levels induced by TZDs are likely PPARγ-independent because a dominant negative mutant of PPARγ did not antagonize these effects of TZDs, nor were they affected by the expression levels of PPARγ. TZDs also inhibit G1 to S cell cycle progression. Furthermore, silencing cyclin D3 by RNA interference inhibits S phase entry and sensitizes breast cancer cells to TRAIL, indicating a key role for cyclin D3 repression in these events. G1 cell cycle arrest sensitizes breast cancer cells to TRAIL at least in part by reducing levels of the anti-apoptotic protein survivin: ectopic expression of survivin partially suppresses apoptosis induced by TRAIL and TZDs. We also demonstrate for the first time that TZDs promote TRAIL-induced apoptosis of breast cancer in vivo, suggesting that this combination may be an effective therapy for cancer.

Several members of the tumor necrosis factor (TNF) α family of cytokines, including TNF-α, FasL/CD95L, and TNF-related apoptosis-inducing ligand (TRAIL/Apo2L), induce apoptosis in target cells by binding to cell surface receptors and activating caspases (1). Although TNF-α and FasL induce apoptosis in both normal and cancer cells, TRAIL preferentially induces apoptosis in transformed cells. TRAIL initiates apoptosis by binding to its DR4 and DR5 death receptors, which contain a cytoplasmic death domain that serves as a protein interaction module to recruit the death domain-containing adapter protein FADD to the death-inducing signaling complex (DISC) (2, 3). FADD in turn recruits procaspases-8 and -10 to the DISC via death effector domains, thereby triggering their proteolytic activation (4, 5). In type I cells, there is sufficient activation of the initiator caspases-8/10 to directly cleave and activate executioner caspases (3, 6, and 7), thereby inducing apoptosis (6). However, type II cells require the mitochondria to amplify the cell death signal: caspase-8 cleaves the pro-apoptotic Bel-2 family member BID, which triggers its translocation to the mitochondria and the release of cytochrome c and Smac into the cytoplasm (7–9). These pro-apoptotic proteins promote caspase-3 activation by activating caspase-9 (cytochrome c) or derepressing the caspase inhibition imposed by IAPs (Smac) (8–10). TRAIL also has three decoy receptors (DcR1, DcR2, and osteoprotegerin), which inhibit apoptosis by sequestering TRAIL from DR4 and DR5 death receptors (3).

TRAIL is expressed in hepatic natural killer cells and is essential for natural killer cell-mediated tumor surveillance: inhibition of TRAIL with blocking antibodies or germ-line deletion of TRAIL sensitizes mice to carcinogen-induced fibrosarcomas and promotes primary tumor growth and metastasis in experimental models (11, 12). Consistent with its normal physiologic function, recombinant soluble TRAIL potently induces apoptosis in a variety of human cancer cells in vitro and in xenograft carcinomas in vivo (13, 14). Although some recombinant forms of TRAIL (particularly fusion proteins engineered to facilitate oligomerization) have been shown to induce apoptosis in human hepatocytes, keratinocytes, and astrocytes, the native recombinant protein does not induce apoptosis in normal cells (even at 100 μg/mL) and does not appear to cause systemic toxicity in primates (13, 15, 16). Furthermore, a monoclonal antibody targeting DR5 was recently demonstrated to induce apoptosis in human hepatocellular and breast carcinomas in vitro and in xenograft tumors in vivo without causing...
liver toxicity (17, 18). Taken together, these findings strongly suggest that activation of the TRAIL signaling pathway (via ligand and/or agonistic antibodies) may be an effective cancer therapy with limited toxicity.

Unfortunately, many human cancers are resistant to TRAIL-induced apoptosis or become resistant to TRAIL when TRAIL is used as a single agent (19–22). Although the mechanisms of TRAIL resistance in most tumors are poorly understood, overexpression of the caspase-8 dominant negative inhibitor FLIP, activation of Akt, or loss of caspase-8/10 or Bax may contribute to TRAIL-resistance in some neoplasms (22–26). Clearly, the identification of drugs that sensitize tumors to TRAIL-induced apoptosis, without compromising its tumor selectivity, could substantially broaden the therapeutic impact of TRAIL. Indeed, radiation and chemotherapeutic drugs have been shown to promote TRAIL-induced apoptosis in vitro and in vivo at least in part by increasing the expression of DR5 and Bax/Bak (19–22). However, these cancer therapies have significant systemic toxicity.

In an effort to identify non-toxic agents that sensitize tumors to TRAIL-induced apoptosis, we postulated that drugs that bind to and activate PPARγ, a ligand-activated transcription factor of the nuclear hormone receptor family (27), might alter the expression of proteins that regulate TRAIL-induced apoptosis and “reprogram” TRAIL-resistant tumors to respond to TRAIL. Synthetic agonists of PPARγ, the thiazolidinediones (TZDs) troglitazone and rosiglitazone, inhibit the growth of malignant epithelial cells (Cambrex, East Rutherford, NJ) were cultured according to the manufacturer’s instructions. Fractions containing G1 arrest to TRAIL-sensitivity. In addition, we show for the first time that TZDs sensitize human breast carcinomas to TRAIL-induced apoptosis in vitro, suggesting that the combination of TZDs and TRAIL may be an effective therapy for cancer.

**EXPERIMENTAL PROCEDURES**

*Cell Culture and Reagents—Human MDA-MB-435, MDA-MB-468, and T47D breast carcinoma cells were obtained from the American Type Culture Collection (ATCC) and maintained in Dulbecco’s modified Eagle’s medium (Mediatech, Herndon, VA) supplemented with 4.5 g/liter glucose and 4 mM L-glutamine, 100 units/ml penicillin/streptomycin, and 10% fetal calf serum (Invitrogen, Carlsbad, CA). Human mammary epithelial cells (Cambrex, East Rutherford, NJ) were cultured according to the manufacturer’s instructions. Cell cultures were grown in 5% CO2 atmosphere at 37 °C. The PPARγ agonists used in these studies were troglitazone (TGZ) (kindly provided by Dr. Andrea Dunaif, Northwest University) and rosiglitazone (RSG) (Avandia, GlaxoSmithKline, Valencia, CA) according to the manufacturer’s protocol. An- nexin V-positive cells were scored as apoptotic.

**PPARγ Adenovirus Experiments—**Adenoviral constructs expressing wild-type murine PPARγ (Ad-WT PPARγ), dominant-negative murine ligand LXXLL PPARγ (Ad-DN PPARγ), or control β-gal. Systeatin (Ad-βGal) were described previously (44). MDA-MB-468 breast cancer cells were infected with Ad-βGal, Ad-WT PPARγ, or Ad-DN PPARγ at 25 plaque-forming units per cell as detailed (Ad-βGal) (44). Seven hours later, adenovirus-infected cells were treated with vehicle alone, TGZ alone, TRAIL alone, or the combination of TGZ and TRAIL, and apoptosis was scored as described above.

**Immunoblotting—**Cell lysates were prepared in modified RIPA buffer (50 mM Tris, 0.1% SDS, 150 mM NaCl, 0.5% deoxycholate, and 1% Nonidet P-40) and analyzed by immunoblotting as described (45). The following antibodies were used for immunoblotting: DR4, DR5, DcR2 (Stressgen, Victoria, Canada), tubulin, FLAG, phospho-RE (Sigma-Aldrich), caspase-3, p27, RIP, Bel-2, cyclin D3, cyclin A, CDK2, CDK4, FADD, TRADD, DR1, FLIP, caspase-8, Bak, PARP, p21 (2B Biosciences), caspase-9, -10, -2, -3, -6 (BD Biosciences), RIP, Bcl-XL, cyclin D3, cyclin A, CDK2, CDK4, FADD, TRADD, DR1, FLIP, caspase-8, Bak, PARP, p21 (BD Biosciences), caspase-9, -10, -2, -3, -6 (BD Biosciences), RIP, Bcl-XL, p21, cyclin D3, and 18 S rRNA (for normalization) were amplified by real-time PCR using iQ SYBR Green Supermix and an iCycler iQ real-time PCR detection system (Bio-Rad) according to the manufacturer’s protocol. Primers and probes for Cyclin D3 and 18 S rRNA were designed with Primer Express TM1.5 software (Applied Biosystems) set at default parameters to select optimized primer and probe sets. The cyclin D3 primers were 5′-TGGCTCTAGG-GAAGCTCAAGTG-3′ (forward) and 5′-CTGTGACACACAGGC-CCGGT-3′ (reverse). The 18 S rRNA probe was 5′-FAM-CTTCTATG- TCTGCGACCGGCTTCCTCCTGCG-3′-BHQ1′. The 18 S rRNA probe was 5′-CGGCTTACACATCCAAGGAA-3′ (forward) and 5′-GCTG- GAATTACCGCCCCTG-3′ (reverse). The 18 S rRNA probe was 5′-FAM- TCTGCGACACAGAGTCTCCTACCTGCG-3′-BHQ1′. 100 ng of total cDNA, 100 nm probe, and 200 nm primers were used in PCR reactions (40 cycles of 95°C for 15 s and 60°C for 1 min).

**Construction of pSUPER-Cyclin D3 and DNA Experiments—**pSUPER was made as described (46) and verified by DNA sequencing. To create pSUPER-cyclin D3 the following oligonucleotides were annealed: 5′-ggactgccgaGACACTCTACATGATtcagagaATCTGTA-GAGGTGCTGTTttttttgaa-3′ and 5′-aattccaaatggaaACGAC- CACTCTACATAGetctttgaaATCTGTAAGGTGCTGTTttttttgaa-3′ (the capitalized letters are the cyclin D3 target sequence corresponding to nucleotides 840–858). After annealing, the oligonucleotides were ligated into the corresponding BglII and HindIII sites in pSUPER, and the sequence of the silencing construct was confirmed by automated DNA sequencing. MDA-MB-435 breast carcinoma cells were co-transfected with 0.2 µg of pEGFP-N1 (BD Biosciences) and 0.8 µg of pSUPER-cyclin D3 or pSUPER vector using Lipofectamine Plus reagent (Invitrogen). Forty-eight hours later, GFP-positive cells were sorted by FACS analysis and analyzed for cyclin D1, cyclin D3, and survivin expression by immunoblotting. Alternatively, for apoptosis experiments, MDA-MB-435 cells were co-transfected with pEGFP-N1 and pSUPER-cyclin D3 (or pSUPER vector), and 48 h later, treated with vehicle (phosphate-buffered saline) or 2.5 µg/ml TRAIL for an additional 24 h. GFP-positive cells were identified by immunofluorescence as detailed previously (47), and nuclei were scored for apoptosis.
an expression of the "Apoptosis Experiments."  

Cell Cycle Blockade—MDA-MB-435 cells were treated with vehicle, double thymidine block (overnight 2 mM thymidine treatment on two consecutive nights with normal growth media between thymidine treatments) or nocodazole (0.1 μM/ml) for 24 h, and cells were then treated with 2.5 μg/ml TRAIL for an additional 8 h. Cell cycle distribution was analyzed by propidium iodide staining for DNA content using flow cytometry, and cell death was measured using the Annexin-V-Po

flow assay with the annexin V detection kit (BD Bioscience).  

Survivin Transfection Experiments—MDA-MB-435 cells were co-transfected with 0.2 μg of pEGFP-N1 (BD Bioscience) and 0.8 μg of pcDNA3 vector or pcDNA3-survivin using Lipofectamine Plus reagent (Invitrogen). 24 h later, transfected cells were treated with TGZ (0–50 μM) for 48 h and then treated with TRAIL (0–2.5 μg/ml) for an additional 16 h. Apoptosis was measured in transfected (GFP-positive) cells by flow cytometry using the Annexin-V-Po assay with the annexin V detection kit (BD Bioscience).  

 Xenograft Tumor Experiments—2.5×10^6 MDA-MB-435 breast carcinoma cells were implanted subcutaneously (s.c.) into the mammary fat pads of a single 4–5-week-old female athymic nude mouse (Harlan Sprague-Dawley, Madison, WI) and xenograft tumors established. 1-mm^3 pieces of the xenograft tumor were then transfected s.c. into both mammary fat pads of 4–5-week-old female athymic nude mice. Two weeks after tumor implantation, mice were randomized into 6 treatment groups (10 mice per group) for 5 weeks: 0.75% methylcellulose vehicle (Sigma-Aldrich), RSG 20 mg/kg/day by oral gavage, RSG 50 mg/kg/day by oral gavage, TRAIL 5 mg/kg/day intraperitoneally, RSG 20 mg/kg/day orally + TRAIL 5 mg/kg/day intraperitoneally, or RSG 50 mg/kg/day orally + TRAIL 5 mg/kg/day intraperitoneally. Tumors were measured weekly with Vernier calipers, and tumor volume was calculated using the equation: tumor volume (mm^3) = (length × width^2). To determine the effect of these treatments on the induction of apoptosis, female athymic nude mice with established MDA-MB-435 xenograft tumors were treated for 2 weeks with vehicle, RSG 50 mg/kg/day by oral gavage, TRAIL 10 mg/kg/day intraperitoneal, or RSG and TRAIL. Xenograft tumors were fixed in formalin, embedded in paraffin, and sectioned by standard methods. Apoptotic nuclei were identified using the TUNEL assay (in situ Cell Death Detection Kit, TMR Red, Roche Applied Science) according to the manufacturer's instructions. Tumors were also analyzed for cyclin D3 and survivin levels as de

scribed in “Immunoblotting.” All procedures involving animals were approved by the Animal Care and Use Committee of Northwestern University.  

RESULTS  

PPARγ Agonists Sensitize Human Breast Cancer Cells, but Not Normal Human Mammary Epithelial Cells, to TRAIL-induced Apoptosis in Vitro—To examine whether PPARγ agonists might restore the sensitivity of TRAIL-resistant human cancer cells to TRAIL-induced apoptosis, we incubated estrogen receptor (ER)-negative human MDA-MB-435 breast carcinoma cells with the synthetic PPARγ agonist troglitazone (TGZ, 0–50 μM) for 48 h and then added TRAIL (0–2.5 μg/ml) for an additional 16 h. MDA-MB-435 cancer cells were highly resistant to TRAIL alone (<10% of these cells were apoptotic after treatment with 2.5 μg/ml TRAIL (Fig. 1A). Similarly, TGZ alone (at concentrations ≤50 μM) did not induce apoptosis in these cancer cells. However, TGZ (50 μM but not 10 μM) dramatically sensitized MDA-MB-435 cells to TRAIL-induced apoptosis, with 50% apoptosis induction in cells treated with 50 μM TGZ and 2.5 μg/ml TRAIL. Similar results were observed when apoptosis was measured by annexin V labeling (Fig. 1B). TGZ also sensitized T47D and MDA-MB-468 breast cancer cells to TRAIL (data not shown). A second synthetic PPARγ agonist, rosiglitazone (RSG), also acted synergistically with TRAIL to promote apoptosis in human T47D breast cancer cells, an ER-positive, TRAIL-resistant breast cancer cell line (Fig. 1C). Like TGZ, RSG did not induce apoptosis when used as a single agent, but sensitized T47D breast cancer cells to TRAIL-induced apoptosis (some synergy was seen with 10 μM RSG). Taken together, these findings indicate that TGZs promote TRAIL-induced apoptosis in multiple TRAIL-resistant breast cancer cell lines.  

because much of the enthusiasm for TRAIL stems from its ability to preferentially induce apoptosis in cancer cells, we examined whether PPARγ agonists undermined the tumor-selectivity of TRAIL. To this end, we treated human mammary epithelial cells (HMECs) with vehicle or 50 μM TGZ for 48 h, and then added 2.5 μg/ml TRAIL for 16 h. Although this combination resulted in robust apoptosis in TRAIL-resistant MDA-MB-435 breast cancer cells (Fig. 1A), HMECs were resistant to TGZ and TRAIL (Fig. 1D). Hence, the TRAIL-sensitizing actions of TGZ appear to be restricted to breast cancer cells, while mammary epithelial cells remain resistant to the combination of TGZ and TRAIL.  

TGZ Sensitizes Breast Cancer Cells to TRAIL-induced Apoptosis by a PPARγ-independent Mechanism—To determine whether the TRAIL-sensitizing effects of PPARγ agonists were mediated by PPARγ, we infected human MDA-MB-468 breast carcinoma cells (which express low levels of PPARγ, Fig. 2) with adenovirus expressing β-Gal, WT PPARγ, or mutant L466A PPARγ. This latter mutant functions as a dominant negative inhibitor of PPARγ that antagonizes PPARγ-dependent transcription (44). However, the dominant negative PPARγ mutant (DN PPARγ) did not alter the sensitivity of MDA-MB-468 cells to apoptosis induced by the combination of TGZ and TRAIL compared with control cells infected with adenovirus expressing β-Gal (Fig. 2). Furthermore, infection of MDA-MB-468 cells with adenovirus expressing WT PPARγ had little effect on their sensitivity to TRAIL and TGZ, despite dramatically increasing the amount of PPARγ. These results indicate that the TRAIL-sensitizing effects of TGZ are likely PPARγ-independent.  

TGZ Promotes TRAIL-induced Caspase Activation but Does Not Significantly Alter the Expression of DISC or Apoptosis-regulating Proteins—To delineate the mechanisms by which PPARγ agonists promote TRAIL-induced apoptosis in cancer cells, we treated human MDA-MB-435 breast cancer cells with vehicle control, 50 μM TGZ alone for 64 h, 2.5 μg/ml TRAIL alone for 16 h, or with 50 μM TGZ for 48 h followed by 2.5 μg/ml TRAIL for an additional 4–16 h. Cell lysates were then analyzed by immunoblotting for proteolytic processing of procaspase-8 (upstream initiator caspase), the caspase-8 substrate BID (7), procaspase-3 (downstream effector caspase), and the caspase-3 substrate PARP (48). Although treatment with TRAIL alone resulted in some processing of procaspase-8 to its p43/p41 intermediates and the p20/p18 forms of the large subunit, there was little evidence of BID cleavage, procaspase-3 processing or PARP proteolysis (Fig. 3A). In contrast, the combination of TRAIL and TGZ resulted in robust procaspase-8 processing, BID cleavage, procaspase-3 processing (indicated by disappearance of the proenzyme), and PARP proteolysis. We postulated that TGZ promoted TRAIL-induced caspase activation by altering the expression of DISC components or other apoptosis-regulating proteins. To test this hypothesis, we examined the effects of TGZ treatment on the expression of DISC components (Fig. 3B) or apoptosis-regulating proteins (Fig. 3C). TGZ had small effects on the expression levels of a few of these proteins (e.g., FADD, FLIP, and Bak levels were increased slightly by TGZ, whereas RIP and XIAP levels were diminished slightly). Although these findings do not exclude the potential contribution of some of these proteins to the TRAIL-sensitizing actions of PPARγ agonists, they strongly suggest that these drugs have additional, unidentified targets that mediate their TRAIL-sensitizing actions.  

TGZ Represses Cyclin D3 mRNA Levels and Induces Cyclin D3 Protein Degradation by the Ubiquitin-Proteasome Pathway—To identify proteins whose expression was dramatically altered by PPARγ agonists, an unbiased proteomics screen using more than 850 mononclonal antibodies (BD PowerBlot,
BD Biosciences) was performed to analyze protein expression in MDA-MB-435 breast carcinoma cells treated with vehicle or 50 \( \mu M \) TGZ for 64 h (data not shown). Using this approach, we identified a few cell cycle regulators whose expression levels were reduced by TGZ, including cyclin D3, CDK2, CDK4, and cyclin A (Fig. 4A, two independent immunoblotting experi-

**FIG. 1.** PPAR\( \gamma \) agonists sensitize human breast cancer cells, but not normal human mammary epithelial cells, to TRAIL-induced apoptosis *in vitro*. A, MDA-MB-435 cells were treated with TGZ (0–50 \( \mu M \)) for 48 h and then treated with TRAIL (0–2.5 \( \mu g/ml \)) for an additional 16 h. B, MDA-MB-435 cells were preincubated with vehicle or 50 \( \mu M \) TGZ for 48 h followed by 16 h treatment with vehicle or 2.5 \( \mu g/ml \) TRAIL. Apoptosis was measured by annexin V labeling; the nucleic acid dye 7-amino-actinomycin D (7-AAD) was used to detect non-viable cells. C, T47D cells were treated with RSG (0–50 \( \mu M \)) for 48 h and then treated with TRAIL (0 or 2.5 \( \mu g/ml \)) for 16 h. D, HMECs were treated with TGZ (0 or 50 \( \mu M \)) for 48 h and then treated with TRAIL (0 or 2.5 \( \mu g/ml \)) for 16 h. In A, C, and D, apoptotic nuclei were scored (mean \( \pm \) S.E., \( n = 3 \)). *, \( p < 0.01 \) versus vehicle-treated cells.

**FIG. 2.** TGZ sensitizes breast cancer cells to TRAIL-induced apoptosis by a PPAR\( \gamma \)-independent mechanism. MDA-MB-468 breast cancer cells were infected with Ad-Gal, Ad-WT PPAR\( \gamma \) or Ad-DN PPAR\( \gamma \) and then treated with TGZ (0 or 25 \( \mu M \)) for 48 h and with TRAIL (0 or 0.5 \( \mu g/ml \)) for an additional 16 h. Apoptotic nuclei were scored (mean \( \pm \) S.E., \( n = 3 \)). The expression of PPAR\( \gamma \) was confirmed by immunoblotting (left panel, two independent experiments).
FIG. 3. TGZ promotes TRAIL-induced caspase activation but does not significantly alter the expression of DISC or apoptosis-regulating proteins. A, MDA-MB-435 cells were treated with vehicle control (C), 50 μM TGZ alone for 64 h, 2.5 μg/ml TRAIL alone for 16 h, or with 50 μM TGZ for 48 h followed by 2.5 μg/ml TRAIL for an additional 4–16 h. Proteolytic processing of procaspase-8, BID, procaspase-3 or PARP was determined by immunoblotting. B and C, MDA-MB-435 cells were treated with TGZ (0–50 μM) for 64 h, and the expression of DISC (B) and apoptosis-regulating (C) proteins was determined by immunoblotting.

FIG. 4. TGZ reduces cyclin D3 protein levels by a PPARγ-independent mechanism involving the proteasomal degradation of cyclin D3 protein and by decreasing cyclin D3 mRNA levels. A, immunoblot analysis of MDA-MB-435 cells that were untreated or treated with 50 μM TGZ for 64 h (two independent experiments shown). B, MDA-MB-435 cells were treated with vehicle or 50 μM TGZ for 64 h, and quantitative real-time PCR was performed to detect cyclin D3 mRNA. Human 18 S rRNA was also amplified as an internal loading control in all samples. Data are the mean ± S.E. (n = 4) of the cyclin D3 mRNA levels relative to vehicle-treated cells. *, p < 0.01 versus vehicle-treated cells. C, MDA-MB-435 cells were pretreated with vehicle or 50 μM TGZ for 48 h, and then treated for an additional 16 h with the proteasome inhibitor epoxomicin (200 nM). Cyclin D3 protein levels were determined by immunoblotting. D, MDA-MB-468 breast cancer cells were infected with Ad-βGal, Ad-WT PPARγ, or Ad-DN PPARγ and then treated with TGZ (0 or 25 μM) for 64 h. PPARγ and cyclin D3 protein levels were determined by immunoblotting.
ments shown). Of all the proteins examined, cyclin D3 levels were the most dramatically affected by TGZ, with virtual disappearance of cyclin D3 in TGZ-treated MDA-MB-435 breast cancer cells. In contrast, cyclin D1 levels were not affected by TGZ under these conditions; cyclin D2 was not expressed in MDA-MB-435 cells (data not shown). Hence, TGZ specifically regulated cyclin D3 expression and not other D-type cyclins. Importantly, many other cell cycle regulators were not affected by TGZ, including cyclin E, p27, and p21.

To determine the mechanisms by which PPARγ agonist inhibits cyclin D3 protein levels, we first examined the effect of TGZ on cyclin D3 gene expression by quantitative real-time PCR. TGZ decreased cyclin D3 mRNA levels ~4-fold in MDA-MB-435 cells (Fig. 4B). In addition, the reduction in cyclin D3
protein levels induced by TGZ was partly suppressed by 200 nm epoxomicin (Fig. 4C), a specific proteasome inhibitor (49). Similar results were obtained with two other proteasome inhibitors, MG132, and proteasome inhibitor II; treatment with proteasome inhibitors and TGZ led to the accumulation of polyubiquitinated cyclin D3 protein (data not shown). Moreover, the dominant negative PPARγ mutant (DN PPARγ) did not block the TGZ-induced reduction in cyclin D3 protein levels, nor did the expression levels of PPARγ affect TGZ-induced suppression of cyclin D3 (Fig. 4D). CDDO-Me, a pharmacologic antagonist of PPARγ (50) also had little effect on TGZ-induced reduction in cyclin D3 levels (data not shown). These findings suggest that TGZ reduces cyclin D3 protein levels by a PPARγ-independent mechanism.

Inhibition of Cyclin D3 Expression by TGZ Disrupts G1 to S Cell Cycle Progression and Promotes TRAIL-induced Apoptosis in Vitro—Consistent with the established function of cyclin D3 in promoting G1 to S cell cycle progression, the reduction in cyclin D3 and CDK4 levels by TGZ treatment led to a dramatic reduction in phosphorylated RB (Fig. 4A) and inhibited G1 to S cell cycle progression (24% reduction in S-phase entry, Fig. 5A). To examine the specific contribution of cyclin D3 to the cell cycle and TRAIL-sensitizing actions of PPARγ agonists, we selectively inhibited the expression of cyclin D3 by plasmid-based RNA interference (RNAi). MDA-MB-435 cells were co-transfected with pEGFP-N1 and pSUPER-cyclin D3 (or pSUPER vector). GFP-positive cells were sorted by FACS and analyzed by immunoblotting. A, MDA-MB-435 cells were treated with TGZ (0–50 μM) for 64 h, and survivin expression was determined by immunoblotting. B, MDA-MB-435 cells were co-transfected with pEGFP-N1 and pSUPER-cyclin D3 (or pSUPER vector). GFP-positive cells were sorted by FACS and analyzed by immunoblotting for survivin expression. C, MDA-MB-435 cells were treated with vehicle, double thymidine block (2 mM) block or nocodazole (0.1 μg/ml) and survivin expression determined by immunoblotting. D, MDA-MB-435 breast cancer cells were transiently transfected with plasmids containing empty vector or survivin. After 24 h of incubation, transfected cells were treated with TGZ (0–50 μM) for 48 h and then treated with TRAIL (0–2.5 μg/ml) for an additional 16 h. Transfected (GFP-positive) cells that were annexin V-positive were identified by FACS and scored as apoptotic (mean ± S.E., n = 3). *p < 0.05 versus vector-transfected cells.

CDDO-Me (49) is a specific proteasome inhibitor that selectively reduced survivin protein levels (Fig. 6D), but had minimal effect on the expression of the related IAP family member XIAP (Fig. 3C). In addition, silencing cyclin D3 with pSUPER-D3 decreased survivin expression to a similar degree as TGZ treatment (Fig. 6B). Furthermore, G1 arrest induced by double thymidine block, but not G2/M arrest induced by nocodazole, similarly reduced survivin levels (Fig. 6C). Collectively, these results suggest that the effects of TGZ on survivin expression are mediated at least in part by the G1 arrest induced by cyclin D3 repression. To determine whether the reduction in survivin levels were responsible for the TRAIL-sensitizing effects of TGZ, we transiently transfected MDA-MB-435 cells with plasmids containing empty vector or survivin and then treated transfected cells with the combination of TGZ and TRAIL. Ectopic expression of survivin partially protected breast cancer cells against apoptosis induced by TGZ and TRAIL compared with vector-transfected cells (Fig. 6D). These results indicate that TGZ sensitizes breast cancer cells to TRAIL, at least in part, by inducing G1 cell cycle arrest and reducing survivin levels.

RSG Sensitizes Human Breast Cancer Xenografts to TRAIL-induced Apoptosis in Vivo—To determine whether PPARγ agonists sensitize tumors to TRAIL-induced apoptosis in vivo, we treated female athymic nude mice with established MDA-MB-435 xenograft tumors with vehicle, RSG alone (20 or 50 mg/kg/day by oral gavage), TRAIL alone (5 mg/kg/day intraperitoneal), or the combination of RSG (20 or 50 mg/kg/day) and TRAIL (5 mg/kg/day) for 6 weeks. RSG was chosen for these
studies because of its current use in patients with type 2 diabetes (53). Neither RSG nor TRAIL alone inhibited xenograft tumor growth (Fig. 7A). In contrast, the combination of RSG and TRAIL suppressed tumor growth and was well tolerated by mice. Although TRAIL treatment induced low levels of apoptosis in xenograft tumors as determined by TUNEL staining (Fig. 7B), RSG dramatically sensitized tumors to TRAIL-induced apoptosis. RSG also decreased the protein levels of cyclin D3 and survivin in the breast tumors (Fig. 7C), consistent with the observed in vitro effects of PPARγ agonists. Taken together, these findings indicate that RSG promotes TRAIL-induced apoptosis in vivo, thereby suggesting that this combination may be a novel and effective therapy for cancer.

**DISCUSSION**

Many human tumors are resistant to TRAIL-induced apoptosis or acquire resistance to TRAIL during treatment (19–22), thereby potentially limiting its therapeutic utility. Hence, it is imperative to identify drugs or agents, which overcome the de novo or acquired resistance of neoplasms to TRAIL-induced apoptosis. Ideally, these agents should be non-toxic and should not undermine the tumor-specific pro-apoptotic actions of TRAIL; they should also sensitize diverse cancers to TRAIL. Although radiation and some chemotherapeutic drugs (e.g. topoisomerase inhibitors and taxanes) promote TRAIL-induced apoptosis in vitro and in vivo, these agents have significant systemic toxicity (19–22). In contrast, several non-toxic, putative chemopreventive agents, including COX-2 inhibitors, resveratrol and a number of PPARγ ligands (TZDs and triterpenoids), have been shown to promote TRAIL-induced apoptosis in vitro (35, 36, 51, 52). However, none of these agents has been shown to act synergistically with TRAIL to induce apoptosis and inhibit tumor growth in vivo, a critical issue for their clinical development.

Here we present evidence for the first time that TRAIL-resistant human carcinomas can be sensitized to TRAIL-induced apoptosis in vivo by RSG, a synthetic PPARγ agonist (TZD) that is currently used to treat type 2 diabetes (53). Importantly, RSG sensitizes established MDA-MB-435 xenograft tumors to TRAIL at doses (20–50 mg/kg/d) that are well tolerated by mice and do not suppress tumor growth in the
absence of TRAIL. The findings are particularly significant because MDA-MB-435 cancer cells have a mutation in the p53 gene and are highly aggressive (54, 55). These observations indicate that the combination of TRAIL and RSG may be a novel and effective therapy for cancer, including apoptosis-resistant tumors with defects in p53. Furthermore, because TRAIL plays a critical role in immune surveillance against tumors (11, 12), it is tempting to speculate that the chemopreventive effects of PPARγ ligands observed in mammary tumor models (31) may reflect the sensitization of these tumors to endogenous TRAIL.

We have also demonstrated that TZDs promote TRAIL-induced apoptosis, at least in part, by a novel mechanism, viz., by inhibiting the expression of cyclin D3. We initially postulated that TZDs promoted TRAIL-induced apoptosis by altering the expression of one or more DISC proteins because these drugs sensitized cancer cells to TRAIL-induced activation of caspase-8, the initiator caspase in the death receptor pathway. However, sensitized cancer cells to TRAIL-induced activation of caspase-8, inhibiting the expression of cyclin D3. We initially postulated sensitized breast cancer cells to TRAIL. These results are in agreement with studies indicating that several drugs that induce G1 arrest sensitize tumors to TRAIL in vitro (52, 58). Hence, TZD-induced G1 arrest resulting from repression of cyclin D3 (and other cell cycle regulators such as CDK4) sensitizes cancer cells to TRAIL. Because cyclin D3 is amplified and/or overexpressed in a number of human cancers, including breast carcinomas, B cell and T cell malignancies (37–39), these tumors may be particularly vulnerable to the combination of PPARγ agonists and TRAIL.

We have also demonstrated that reduction in the levels of the anti-apoptotic protein survivin directly links TZD-induced G1 arrest to TRAIL-sensitivity. Survivin is a member of the IAP family that is selectively expressed in many human neoplasms, but not normal adult tissues, and plays a dual role in apoptosis suppression and cell cycle regulation (40, 59). Indeed, survivin overexpression has been shown to confer protection against many apoptotic stimuli, including TRAIL, while inhibition of survivin by a variety of strategies sensitizes cancer cells to apoptosis (40, 52, 60–62). Although the mechanisms of its anti-apoptotic function are not clear, recent studies suggest that survivin may inhibit apoptosis by binding to Smac once it is released from mitochondria and disrupt the pro-apoptotic actions of cytosolic Smac by sequestration (63). In addition, survivin bound to its cofactor HBXIP interacts with pro-caspase-9 and prevents its recruitment to the apoptosisosome (64), thereby inhibiting caspase-9 activation. Importantly, the mitochondrial amplification of cell death signals initiated by TRAIL is critical for apoptotic execution in some cancer cells (22, 24, 26). Consistent with published reports (40), we have shown that survivin expression is indeed cell cycle-regulated with highest levels at G2/M and lowest at G1. Moreover, both TZD treatment and cyclin D3 silencing potently reduce survivin levels. Taken together, these findings suggest that the effects of TZDs on survivin expression are mediated at least in part by the G1 arrest induced by cyclin D3 repression, although we cannot exclude the possibility that TGZ may have a direct effect on survivin expression as well. Furthermore, we have shown that ectopic expression of survivin partially inhibits apoptosis induced by TZDs and TRAIL, thereby indicating that survivin is a key mediator of TRAIL resistance in cancer cells and that the reduction in survivin levels induced by G1 arrest is at least partly responsible for the TRAIL-sensitizing effects of PPARγ agonists. Interestingly, the chemopreventive agent resveratrol also causes G1 arrest-induced survivin depletion and TRAIL sensitization, although the G1 arrest triggered by resveratrol is mediated by p21 induction rather than cyclin D3 repression (52). Collectively, these results strongly suggest that G1 cell cycle arrest and resultant reduction in survivin levels may be a general strategy to sensitize cancer cells to TRAIL.

In conclusion, we have demonstrated that PPARγ agonists sensitize diverse breast cancer cells (ER-positive and ER-negative, p53 mutant), but not normal HMECs, to TRAIL-induced apoptosis by a novel PPARγ-independent mechanism, viz., by selectively repressing cyclin D3, which induces G1 cell cycle arrest and reduces survivin levels. Moreover, our findings clearly indicate for the first time that the combination of TZDs and TRAIL is an effective cancer therapy in vivo against human breast cancer that is resistant to either agent alone. In this way, these experiments provide the first “proof of principle” in vivo data for this novel combination.

Acknowledgments—We thank Dr. Andrea Dunaif for providing troglitazone and Dr. Honglin Li for the BID antibody.

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