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Effects of PPARγ Agonists on Cell Survival and Focal Adhesions in a Chinese Thyroid Carcinoma Cell Line

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Abstract Peroxisome proliferator-activated receptor γ (PPARγ) agonists cause cell death in several types of cancer cells. The aim of this study was to examine the effects of two PPARγ agonists, ciglitazone and 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 (15dPGJ2), on the survival of thyroid carcinoma CGTH W-2 cells. Both ciglitazone and 15dPGJ2 decreased cell viability in a time- and dose-dependent manner. Cell death was mainly due to apoptosis, with a minor contribution from necrosis. Increased levels of active caspase 3, cleaved poly (ADP-ribose) polymerase (PARP), and cytosolic cytochrome-*c* were noted. In addition, ciglitazone and 15dPGJ2 induced detachment of CGTH W-2 cells from the culture substratum. Both the protein levels and immunostaining signals of focal adhesion (FA) proteins, including vinculin, integrin β1, focal adhesion kinase (FAK), and paxillin were decreased after PPARγ agonist treatment. Meanwhile, reduced phosphorylation of FAK and paxillin was noted. Furthermore, PPARγ agonists induced expression of protein tyrosine phosphatase-PEST (PTP-PEST), and of phosphatase and tensin homologue deleted on chromosome ten (PTEN). The upregulation of these phosphatases might contribute to the dephosphorylation of FAK and paxillin, since pre-treatment with orthovanadate prevented PPARγ agonist-induced dephosphorylation of FAK and paxillin. Perturbation of CGTH W-2 cells with anti-integrin β1 antibodies induced FA disruption and apoptosis in the same cells, thus the downregulation of integrin β1 by PPARγ agonists resulted in FA disassembly and might induce apoptosis via anoikis. Our results suggested the presence of crosstalk between apoptosis and integrin-FA signaling. Moreover, upregulation and activation of PTEN was correlated with reduced phosphorylation of Akt, and this consequence disfavored cell survival. In conclusion, PPARγ agonists induced apoptosis of thyroid carcinoma cells via the cytochrome-*c* caspase 3 and PTEN-Akt pathways, and induced necrosis via the PARP pathway. J. Cell. Biochem. 98: 1021–1035, 2006.

Key words: PPARγ agonists; necrosis; apoptosis; focal adhesion

Activation of peroxisome proliferator-activated receptor γ (PPARγ), a member of the nuclear ligand-dependent transcription factors, leads to growth inhibition in human breast, prostate, gastric, and pancreatic cancer cells [Elstner et al., 1998; Kubota et al., 1998; Takahashi et al., 1999; Kawa et al., 2002]. PPARγ agonists induce apoptosis in several types of cancer cells, and the known mechanisms for this action include upregulation of GADD 153, c-jun, Bax, and Bad, downregulation of Bcl-2, and activation of caspase 3 in several types of cancer cells [Kim et al., 2003; Ray et al., 2004; Theocharis et al., 2004; Pignatelli et al., 2005]. Studies on thyroid

Abbreviations used: 15dPGJ2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J2; ECM, extracellular matrix; FA, focal adhesion; FAK, focal adhesion kinase; MTT, 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide; PARP, poly (ADP-ribose) polymerase; PI, propidium iodide; PPARγ, peroxisome proliferator-activated receptor γ; PTEN, phosphatase and tensin homologue deleted on chromosome ten; PTP-PEST, protein tyrosine phosphatase-PEST.

Dr. Shih-Horng Huang and Dr. Seu-Mei Wang contributed equally to this research.

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carcinoma with PPARγ agonists treatment focus mainly on the inducement of apoptosis [Ohta et al., 2001; Chung et al., 2002; Martelli et al., 2002]. In thyroid carcinoma cells, PPARγ agonist-induced apoptosis is dependent on upregulation of p21, p27, or c-myc, but independent of the expression of Bcl-2 or Bax [Ohta et al., 2001]. Caspase 3-dependent apoptosis includes the intrinsic pathway, which is initiated by cytochrome-*c* release from the mitochondria, followed by activation of caspase 9 and 3, and the extrinsic pathway, which is triggered by the binding of foreign Fas ligand to its receptor, leading to activation of caspase 8 [Riedl and Shi, 2004]. Poly (ADP-ribose) polymerase (PARP), a chromatin-associated protein involved in maintaining DNA stability and repair, can be cleaved by active caspase 3 [Ha and Snyder, 1999], and cleaved PARP has been shown to be increased in TNF-α-induced apoptosis in mouse fibroblasts [Herceg and Wang, 1999]. Whether PPARγ agonists induce apoptosis of thyroid carcinoma cells by caspase 3 activation or PARP activation remains to be determined. Little attention has been paid to the effect of PPARγ agonists on necrosis. In general, necrosis occurs when cells experience energy failure or ATP depletion [Proskuryakov et al., 2003]. Excessive PARP activation results in NAD^+ and ATP depletion, which leads to necrosis [Proskuryakov et al., 2003]. Whether PPARγ induces necrosis of CGTH W-2 cells by activating PARP needs to be elucidated.

Eibl et al. [2001] have shown that the floating cell/adherent cell ratio increases after PPARγ agonist treatment [Eibl et al., 2001]. The underlying mechanism responsible for cell detachment induced by PPARγ agonist has not been studied. Integrin, one of the transmembrane proteins, mediates the interaction between the extracellular matrix (ECM) and focal adhesion (FA), where actin stress fibers are targeted and survival signals are transduced [Burridge et al., 1988]. Integrin appears to mediate ECM-transmitted survival signal [Schlaepfer et al., 2004]. The structural integrity of FA depends on the activation of integrin and the subsequent phosphorylation of focal adhesion kinase (FAK) and paxillin [Schlaepfer et al., 2004]. Inactivation of FAK with dominant negative FAK or interference with the binding of FAK to the cytoplasmic portion of integrin β1 using competitive small peptides initiates the apoptosis of cultured fibroblasts [Hungerford et al., 1996; Ilic et al., 1998]. Activation of PPARγ upregulates the expression of phosphatase and tensin homologue deleted on chromosome ten (PTEN), which modulates cell migration by interacting with FAK and directly dephosphorylates tyrosine-phosphorylated FAK [Tamura et al., 1999; Patel et al., 2001]. Moreover, protein tyrosine phosphatase-PEST (PTP-PEST) regulates the structural integrity of FA by dephosphorylation of paxillin [Davidson and Veillette, 2001]. Whether cell detachment caused by PPARγ agonist involves PTEN and PTP-PEST deserves further study.

In this study, we first examined the effects of two PPARγ ligands, ciglitazone and 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 (15dPGJ2), on the survival of the Chinese thyroid carcinoma-derived CGTH W-2 cell line. Secondly, we asked whether PPARγ agonist-induced detachment and death of these cells was related to PTP-PEST or PTEN. Since PTEN inactivates Akt, and Akt inactivation facilitates apoptosis by activating pro-apoptotic proteins, such as Bad and caspase 9 [Datta et al., 1999], efforts were made to correlate the relationship between PTEN expression and Akt phosphorylation status.

MATERIALS AND METHODS

Cell Culture

The CGTH W-2 cell line, derived from a metastatic thyroid follicular carcinoma from a Chinese patient in Taiwan was a generous gift from Dr. Jen-Der Lin [Lin et al., 1996]. The cells have a doubling time of 18 h and have lost virtually all their iodine-concentrating ability and capacity for thyroglobulin synthesis. The cells were grown in RPMI 1640 containing 10% fetal bovine serum, 1 μM sodium pyruvate, 100 IU/ml of penicillin, and streptomycin (pH 7.2) (Gibco BRL, Grand Island, NY) in a humidified atmosphere of 5% CO_2–95% air atmosphere at 37°C.

Drug Treatment

The natural PPARγ ligand, 15dPGJ2, the synthetic ligand, ciglitazone, and the broad caspase inhibitor, z-Val-Ala-Asp(OMe)-fluoromethylketone (z-VAD-FMK), were purchased from Biolmol (Plymouth Meeting, PA). The PARP inhibitor, 3-aminobenzamide (3AB), and the general phosphatase inhibitor, sodium orthovandate, were obtained from Sigma (St. Louis, MO).

MTT Test

Thyroid carcinoma cells were plated at 2×10^4 cells in each well of 24-well plate, and incubated for 24 h with different concentrations of ciglitazone and 15dPGJ2 in the presence or absence of 10% fetal calf serum before assaying cell viability. After two washes with phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, pH 7.3; PBS), 500 μl of RPMI medium containing 0.5 mg/ml of 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT; Sigma) was added to each well, then incubation was continued for 4 h to allow the conversion of the substrate into purple formazan product. The medium was then removed and the cell lysed with 500 μl of DMSO and the absorbance at 590 nm measured with a spectrophotometer (Beckman Coulter, Inc., Fullerton, CA).

Propidium Iodide Staining for Necrotic Cells

Propidium iodide (PI) was used to detect plasma membrane disruption in necrotic cells. Cells were treated with DMSO (vehicle control), or 10 μM ciglitazone or 15dPGJ2 for 6, 18, or 24 h, then after washes with serum-free medium, were incubated for 30 min with PI (50 μg/ml in serum-free medium) in a CO_2 incubator. After a brief wash with PBS, the cells were fixed for 5 min in 10% formalin, washed with PBS, and mounted with 3% n-propyl gallate and 50% glycerol in PBS. All experiments were performed with triplicate dishes and more than 100 cells were examined for each dish.

Assay for Apoptotic Cells

Cells were treated with DMSO (vehicle control), or 10 μM ciglitazone or 15dPGJ2 for 6, 18, or 24 h, washed with PBS, and fixed for 5 min in 5% formalin and 0.5% Triton X-100 at room temperature. After a brief wash with PBS, the cells were stained for 15 min with 1 μg/ml of 4′, 6-diamidino-2-phenylindole dilactate (DAPI; Sigma) in 0.9% NaCl and mounted with 3% n-propyl gallate and 50% glycerol in PBS. Apoptotic cells were identified by the presence of chromatin condensation or apoptotic body formation. Apoptotic cells were also examined by immunofluorescence staining for activated caspase 3. All experiments were performed with triplicate dishes and more than 100 cells were examined per dish.

Immunofluorescence Microscopy

After treatments, cells were washed with PBS, then fixed for 5 min with 10% formalin in PBS, and permeabilized for 10 min with 0.1% Triton X-100 in PBS. After PBS washes (3 × 5 min), non-specific binding sites were blocked by 30 min incubation at room temperature with 5% non-fat milk in PBS. The cells were then incubated overnight at 4°C with polyclonal rabbit antibodies against phosphorylated FAK or phosphorylated paxillin (Biosource International, Inc., Camarillo, CA), or monoclonal mouse antibodies against FAK or paxillin (BD Biosciences Pharmingen, Mississauga, ON, Canada), vinculin (Sigma), or cytochrome-*c* (Promega, Madison, WI), washed with PBS, and incubated for 1 h at 37°C with Texas red- or fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (Sigma). For actin labeling, cells were incubated with FITC-phalloidin (Molecular Probe), washed, and mounted. For double labeling for necrotic cells and vinculin, cells were first vitally stained with PI, then processed for vinculin immunostaining. For double labeling for apoptotic cells and vinculin, cells were first immunostained for vinculin, then stained with DAPI. Finally, the cells were washed with PBS, mounted using 3% n-propyl gallate and 50% glycerol in PBS, and examined using a Zeiss epifluorescence microscope (Carl Zeiss, Oberkocheu, Germany) equipped with a Nikon DIX digital camera (Nikon).

Western Blotting

After treatment, CGTH W-2 thyroid carcinoma cells were washed once with PBS and homogenized in lysis buffer (10 mM EGTA, 2 mM $MgCl_2$, 60 mM PIPES, 25 mM HEPES, 0.15% Triton X-100, 1 μg/ml of pepstatin A, 1 μg/ml of leupeptin, 1 mM NaF, and 1 mM phenylmethylsulfonyl fluoride), then an equal volume of sample buffer was added and the mixture heated at 90°C for 3 min. Proteins (40 μg per lane) were electrophoresed on a 10% SDS polyacrylamide gel and transferred to a nitrocellulose membrane (Schleicher & Schuell, Inc., Keene, NH). Strips from the membrane were then blocked by incubation for 30 min at room temperature with 5% non-fat milk in Tris-buffered saline, pH 8.2, containing 0.1% Tween (TBS-Tween), and incubated overnight at 4°C with 1:1,000 dilution of monoclonal mouse antibodies against integrin β1, FAK, paxillin

(BD Biosciences Pharmingen), vinculin or β actin (Sigma), PTP-PEST (Exalpha Biologicals, Inc., Watertown, MA), PTEN (Santa Cruz Laboratory, Inc., Santa Cruz, CA), and cytochrome-*c* (Sigma) or rabbit antibodies against phosphorylated FAK or phosphorylated paxillin (Biosource International), cleaved caspase 3 (Sigma), cleaved PARP (BioVision Research Products, Mountain View, CA), phosphorylated PTEN (Cell Signalling, Beverly, MA), and phosphorylated Akt (Santa Crutz) diluted in TBS-Tween. After washes with TBS-Tween, the strips were incubated for 2 h at room temperature with 1: 7,500 dilution of alkaline phosphatase-conjugated anti-mouse or anti-rabbit IgG antibodies (Promega), and bound antibody was visualized using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma) as chromogen. The density of the bands on the nitrocellulose membrane was quantified by densitometry using Gel Pro 3.1 (Media Cybernetics, Silver Spring, MD), taking the density of the band in the control sample as 100% and expressing the density of the band in the test sample as a percentage of this. All experiments were performed at least three times, and the values are expressed as the mean ± SD.

Cell Fractionation

After treatment with PPARγ agonists, CGTH W-2 thyroid carcinoma cells were briefly washed with PBS and ultrasonicated for 2 × 10 s in RIPA buffer (50 mM Tris-HCl, 1% Triton X-100, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM NaF, 1 mM Na_3VO_4, 1 μg/mL each of aprotinin, leupeptin, and pepstatin, pH 7.4). The suspension was then centrifuged at 23,000*g* for 30 min at 4°C and the supernatant taken as the Triton-soluble portion.

Statistical Analysis

All results are expressed as the mean ± SD. Statistical differences between means were assessed using Student's *t*-test, a *P*-value less than 0.05 being considered significant.

RESULTS

Effects of Ciglitazone and 15dPGJ2 on the Viability of CGTH W-2 Cells

CGTH W-2 cell viability after treatment with ciglitazone and 15dPGJ2 was assessed using the MTT assay. Both agonists caused a dose-dependent decrease in the number of surviving cells (Fig. 1). In the vehicle control (DMSO) group, CGTH W-2 cells had a polygonal shape and adhered tightly to the culture substratum and this was unaffected by treatment with 5 μM ciglitazone (data not shown). However, after 24 h incubation with ciglitazone concentrations ≥10 μM, about 50% of the cells rounded up and became detached from the substratum. When another PPARγ agonist, 15dPGJ2, was used, the cells appeared normal when exposed to 2 or 5 μM 15dPGJ2, but about half rounded up when exposed to 10 μM 15dPGJ2. To investigate the mechanism of cell death and detachment, both agonists were, therefore, used at a concentration of 10 μM.

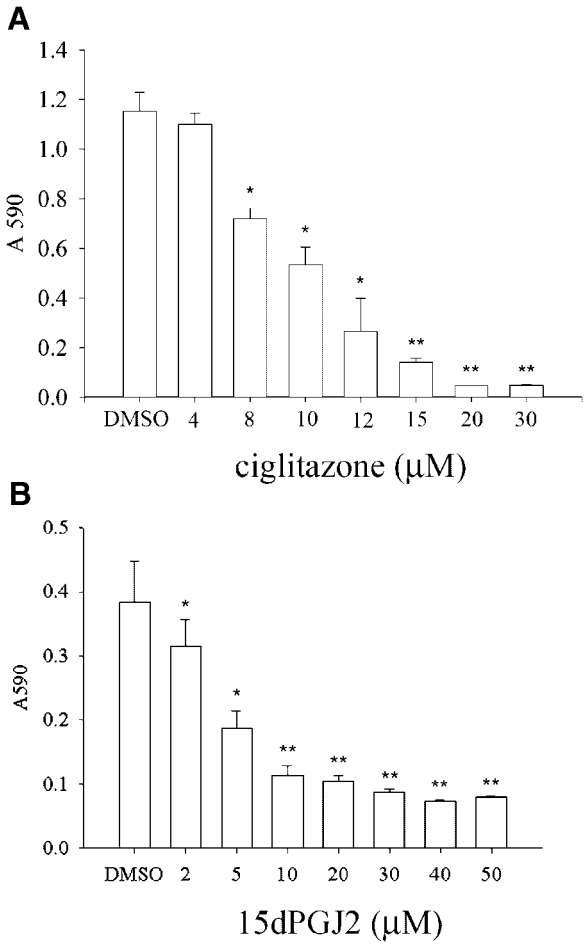

Fig. 1. Effect of ciglitazone or 15dPGJ2 on cell viability. CGTH W-2 cells were treated for 24 h with 0.1% DMSO or different concentrations of ciglitazone (**A**) or 15dPGJ2 (**B**) in serum-free medium, then assayed for cell viability by the MTT test. The results are expressed as the absorbance at 590 nm (A_{590}). *, $P < 0.05$; **, $P < 0.01$, compared to the DMSO group.

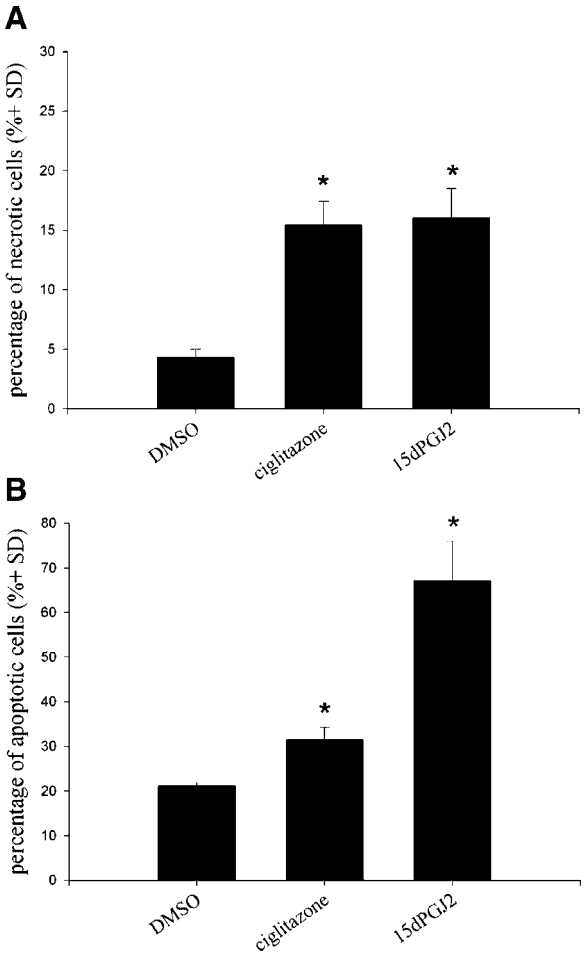

Fig. 2. Quantitative analysis of necrotic and apoptotic cells induced by treatment with ciglitazone or 15dPGJ2. CGTH W-2 cells were treated for 6 h with 0.1% DMSO or 10 μM ciglitazone or 15dPGJ2. Necrotic (**A**) or apoptotic cells (**B**) were identified by staining with PI or DAPI, respectively. n = 3. *, $P < 0.05$, compared to the DMSO group.

Induction of Necrosis and Apoptosis by Ciglitazone and 15dPGJ2

In the controls, necrotic cells made up about $4.3 \pm 0.7\%$ of the cell population (Fig. 2A). Treatment for 6 h with ciglitazone or 15dPGJ2 increased the percentage of necrotic cells to $15.4 \pm 2.0\%$ or $16.0 \pm 2.8\%$, respectively (Fig. 2A). About $21.4 \pm 0.5\%$ of the cells showed apoptotic chromatin condensation in the DMSO controls and this was increased to $34.1 \pm 1.4\%$ or $67.1 \pm 8.8\%$, respectively, by ciglitazone or 15dPGJ2 treatment (Fig. 2B).

To determine the pathways leading to apoptosis, caspase 3 activation and cytochrome-*c* release were examined by Western blotting. Both agents caused time-dependent activation of caspase 3 (Fig. 3A). Cytochrome-*c* levels in the cytosol portions increased by about 40% after 6 h of PPARγ agonist treatments (Fig. 3B). Levels of cleaved PARP, a caspase 3 substrate, also increased in a time-dependent manner, an evidence for the activation of caspase 3 (Fig. 3C). To examine changes in mitochondrial morphology, immunostaining with anti-cytochrome-*c* antibody and DAPI staining were performed. In control cells, mitochondria, immunoreactive for cytochrome-*c*, were shaped like long or short rods (Figs. 4A, B). In ciglitazone-treated cells, swollen mitochondria were frequently seen in cells with condensed chromatin (Figs. 4C, D), whereas, in $15dPGJ_2$-treated cells, the mitochondria were slender and elongated, and diffuse cytochrome-*c* staining was seen in the cytoplasm of apoptotic cells (Figs. 4E, F). PARP activity results in ATP depletion, which leads to necrosis [Ha and Snyder, 1999]. In order to determine whether PARP was involved in PPARγ agonist-induced necrosis, pre-treatment with the PARP inhibitor, 3AB, was tested. 3AB completely prevented necrosis induced by ciglitazone, whereas, simultaneous application of z-VAD-FMK and 3AB seemed to be more effective than 3AB alone in preventing 15dPGJ2-induced necrosis (Fig. 5A). Pre-treatment with z-VAD-FMK decreased the apoptosis induced by 15dPGJ2, but not by ciglitazone (Fig. 5B). Only cotreatment with 3AB and z-VAD-FMK blocked ciglitazone-induced apoptosis (Fig. 5B). Taken together, these results showed that ciglitazone and 15dPGJ2 induced caspase 3 activation, PARP cleavage, and mitochondrial swelling.

Induction of Disassembly of Focal Adhesions and Stress Fibers by Ciglitazone and 15dPGJ2

Because of the increase in the floating cell/adherent cell ratio after treatment with ciglitazone and 15dPGJ2 seen previously [Eibl et al., 2001], we examined the structure of the FA. Western blotting was performed to examine the expression of these FA proteins. Levels of vinculin, FAK, phosphorylated FAK, paxillin, and phosphorylated paxillin decreased in a time-dependent manner in response to treatment with ciglitazone (Fig. 6A) or 15dPGJ2 (Fig. 6B). Since these FA proteins are indirectly linked to actin stress fibers on the cytoplasmic side and to the ECM through transmembrane integrins [Schlaepfer et al., 2004], integrin expression was also examined. As shown in

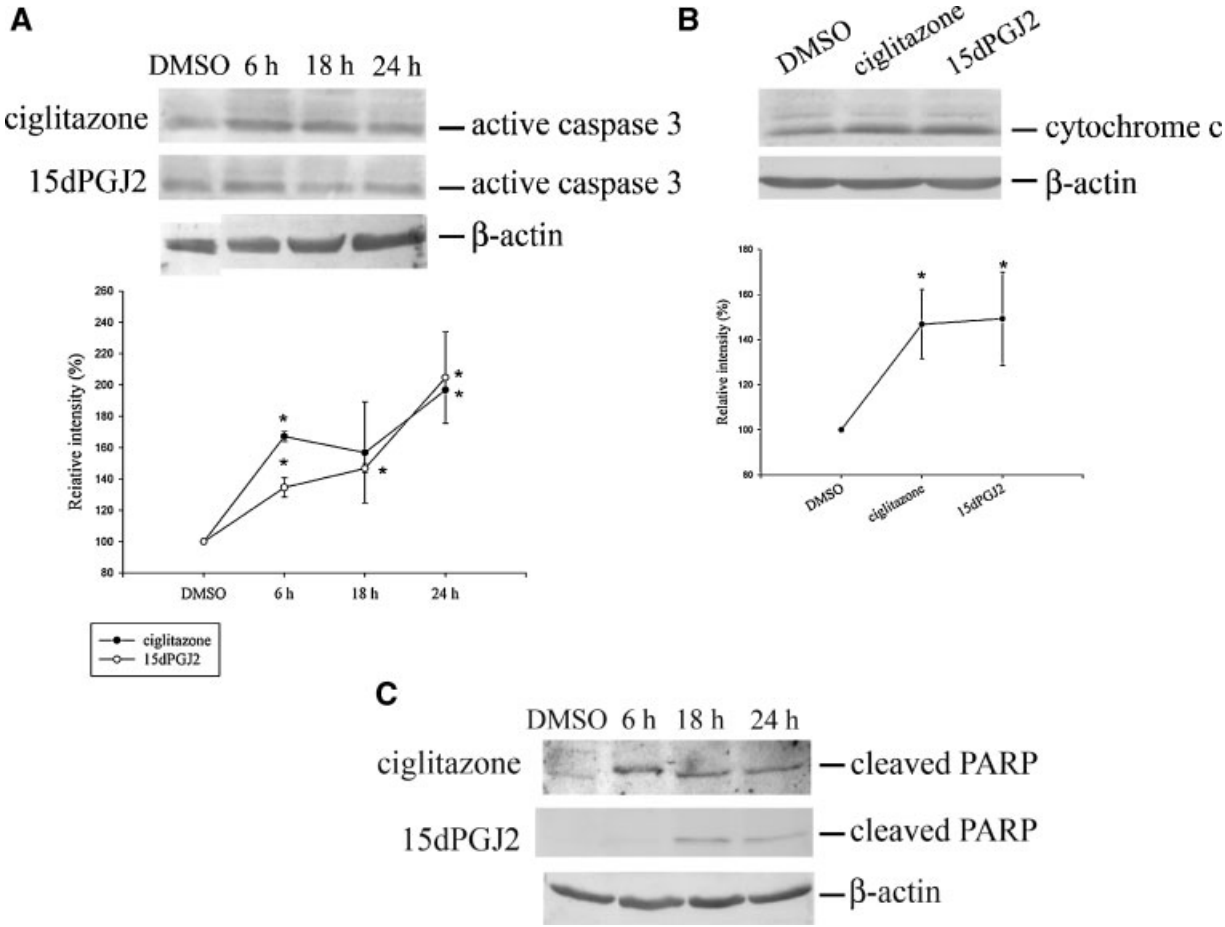

Fig. 3. Western blot analysis of levels of active caspase 3 and cleaved PARP and cytochrome-*c* release in cells treated with ciglitazone or 15dPGJ2. CGTH W-2 cells were treated for different intervals (6, 18, 24 h) with 0.1% DMSO or 10 μM ciglitazone or 15dPGJ2, then cell homogenates were analyzed for active caspase 3 (**A**) or cleaved PARP (**C**) by Western blotting. The cytosolic fractions from cells treated for 6 h were analyzed for cytochrome-*c* (**B**). Immunoblot results are from a representative experiment. Lower panels in A and B; densitometric scans of triplicate blots for active caspase 3 and cytochrome-*c* from three independent experiments, respectively. β-Actin, loading control. *, $P < 0.05$ compared to the DMSO.

Figure 6, the expression of integrin β1 was downregulated with increasing time of treatment. The next experiments supported the involvement of integrin β1 in cell death. Interfering integrin β1 with specific antibodies for 6 h significantly inhibited cell proliferation as compared to those incubated with normal IgG by MTT assay (data not shown). Interestingly, disruption of FA, revealed by diffuse vinculin staining, was only found in apoptotic cells after this treatment (Fig. 7), whereas, vinculin staining in typical FA pattern was observed in cells with normal IgG incubation (Fig. 7).

To provide morphological evidence, structural changes of FA after PPARγ agonist treatment were examined. In control cells, immunofluorescence staining showed a typical patched staining of FA revealed by vinculin staining at the ventral surface of the cells and prominent linear stress fibers by FITC-phalloidin (data not shown). After treatment for 18 h with 10 μM ciglitazone or 15dPGJ2, the vinculin staining at the ventral surface became weak, only that at the cell edges remaining strong, and depolymerization of actin stress fibers occurred (data not shown). Staining for FAK, phosphorylated FAK, paxillin, and phosphorylated paxillin was seen at FA in control cells (Figs. 8 and 9), and became weak and diffuse after treatment with ciglitazone or $15dPGJ_2$ (Figs. 8 and 9).

Since PTP-PEST is able to dephosphorylate paxillin and affect both cell adhesion and cell motility [Shen et al., 2000; Brown and Turner, 2002], we then examined PTP-PEST expression. The total PTP-PEST levels were

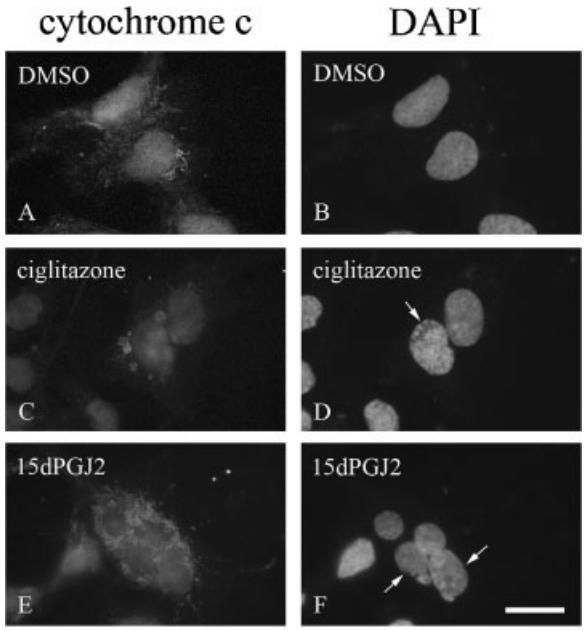

Fig. 4. Cytochrome-*c* immunostaining patterns in PPARγ agonist-treated apoptotic cells. CGTH W-2 cells were treated for 6 h with 0.1% DMSO (**A**, **B**), 10 μM ciglitazone (**C**, **D**), or 10 μM 15dPGJ2 (**E**, **F**), and double-stained with anti-cytochrome-*c* antibodies (A, C, E) and DAPI (B, D, F). Arrows indicate the apoptotic nuclei. Bar = 40 μm.

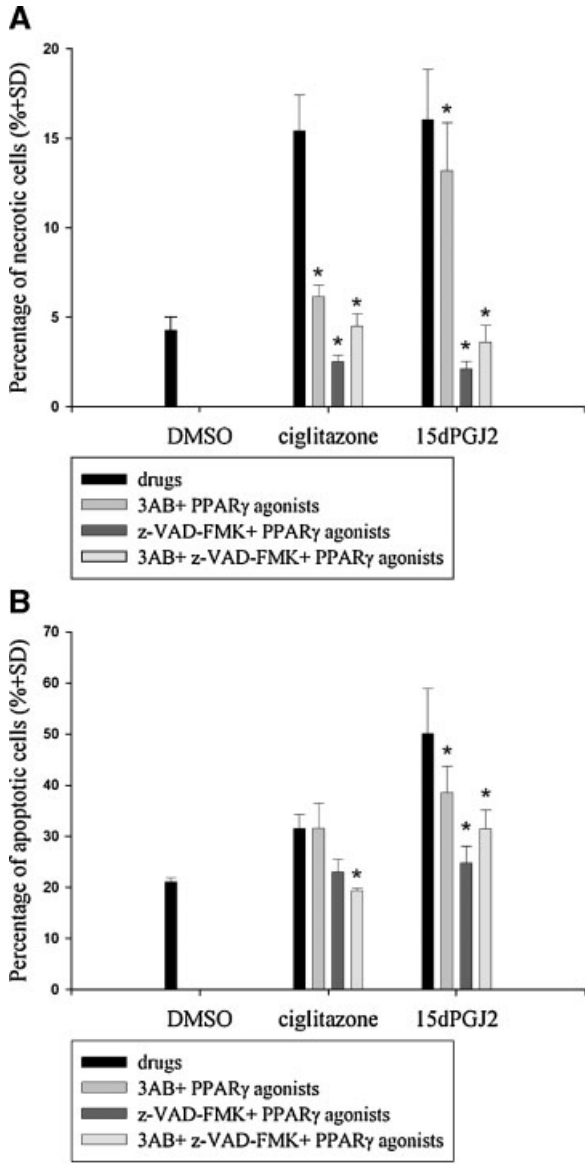

Fig. 5. Effects of the PARP inhibitor 3AB and a broad caspase inhibitor z-VAD-FMK on PPARγ agonist-induced necrosis and apoptosis. CGTH W-2 cells were treated for 6 h with 10 μM ciglitazone, 10 μM 15dPGJ2, 1 mM 3AB plus 10 μM ciglitazone, or 1 mM 3AB plus 10 μM 15dPGJ2, 10 μM ciglitazone with 50 μM z-VAD-FMK and 1 mM 3AB, or 10 μM 15dPGJ2 with 50 μM z-VAD-FMK and 1 mM 3AB, then necrotic (**A**) and apoptotic cells (**B**) were stained and counted. *, $P < 0.05$ compared to the PPARγ agonist-treated groups.

significantly increased after 6 h and reached the peak after 18 h of PPARγ agonist treatment (Fig. 10A). PTEN is reported to be induced by PPARγ agonists [Farrow and Evers, 2003] and can dephosphorylate FAK [Tontonoz et al., 1997] and Akt [Datta et al., 1999]. In this study, PTEN expression was significantly increased 6 h after PPARγ agonist treatment (Fig. 10B). To verify the relation between tyrosine phosphatases and dephosphorylation of paxillin, we examined the effect of orthovanadate pre-treatment on 15dPGJ2-inudced dephosphorylation of FAK and paxillin. Pre-treatment with orthovanadate significantly prevented 15dPGJ2-induced dephosphorylation of FAK and paxillin (Fig. 10C). This observation supported that the tyrosine phosphatases were the upstream regulators for the dephosphorylation of FAK and paxillin. In addition, PTEN was activated as evidenced by the decrease in the levels of phosphorylated PTEN, an inactive form of PTEN, and was accompanied by the dephosphorylation of Akt (Fig. 11).

DISCUSSION

We have demonstrated that two PPARγ agonists, ciglitazone and 15dPGJ2, induced detachment of CGTH W-2 cells from the culture substratum and cell death. These agonists caused downregulation of FA proteins and integrins, and decreased the phosphorylation of FAK and paxillin, the latter being regulated possibly by upregulation of PTEN and PTP-PEST. Antibody perturbation experiment

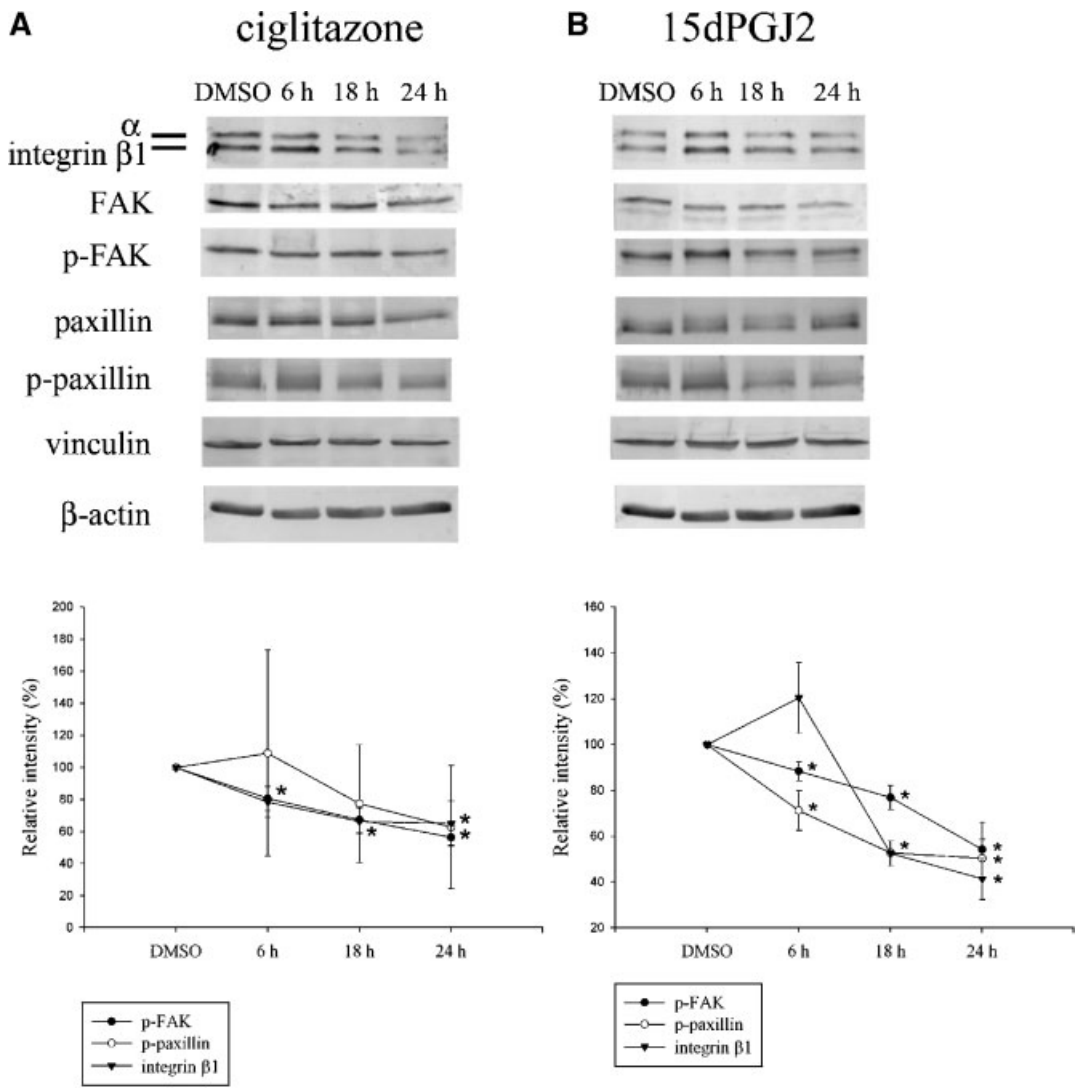

Fig. 6. Time-course study of the effect of ciglitazone or 15dPGJ2 on the expression of FA proteins. CGTH W-2 cells were treated for different intervals with 0.1% DMSO, 10 μM ciglitazone (**A**), or 10 μM 15dPGJ2 (**B**), then the cell homogenates were analyzed for several FA proteins by Western blotting. Immunoblot results are from a representative experiment performed in triplicates with β-actin as loading control. Lower panels in A and B; densitometric scans of triplicate blots for p-FAK, p-paxillin, and intergrin β1. *, $P < 0.05$ compared to DMSO group.

indicated that disrupting integrin-ECM interaction led to the apoptosis. Therefore, disruption of FA accelerated the detachment of CGTH W-2 cells from the ECM and triggered anoikis. Since FAK is important in regulation of anoikis, the reduced levels of total FAK and integrin, and of phosphorylated FAK by PPARγ agonists treatment were correlated with the increased ratios of apoptotic cells. Moreover, cytochrome-*c* caspase 3 and PTEN-Akt pathways were involved in PPARγ agonist-induced apoptosis.

In the present study, staining for F-actin revealed a drastic depolymerization of stress fibers, although total actin levels in CGTH W-2 cells were not affected by PPARγ. Actin is targeted to the FA complex and the organization of actin stress fibers is regulated by the FA complex [Turner, 2000]. PPARγ agonists are reported to inhibit Rho/Rho kinase activity in rat aortic smooth muscle cells [Wakino et al., 2004]. Remodeling of the actin cytoskeleton is regulated by FAK via the Rho family of small GTPases [Carragher and Frame, 2004]. Thus, inhibition of Rho activity by a PPARγ agonist may downregulate FAK expression and affect actin integrity. However, the possibility that

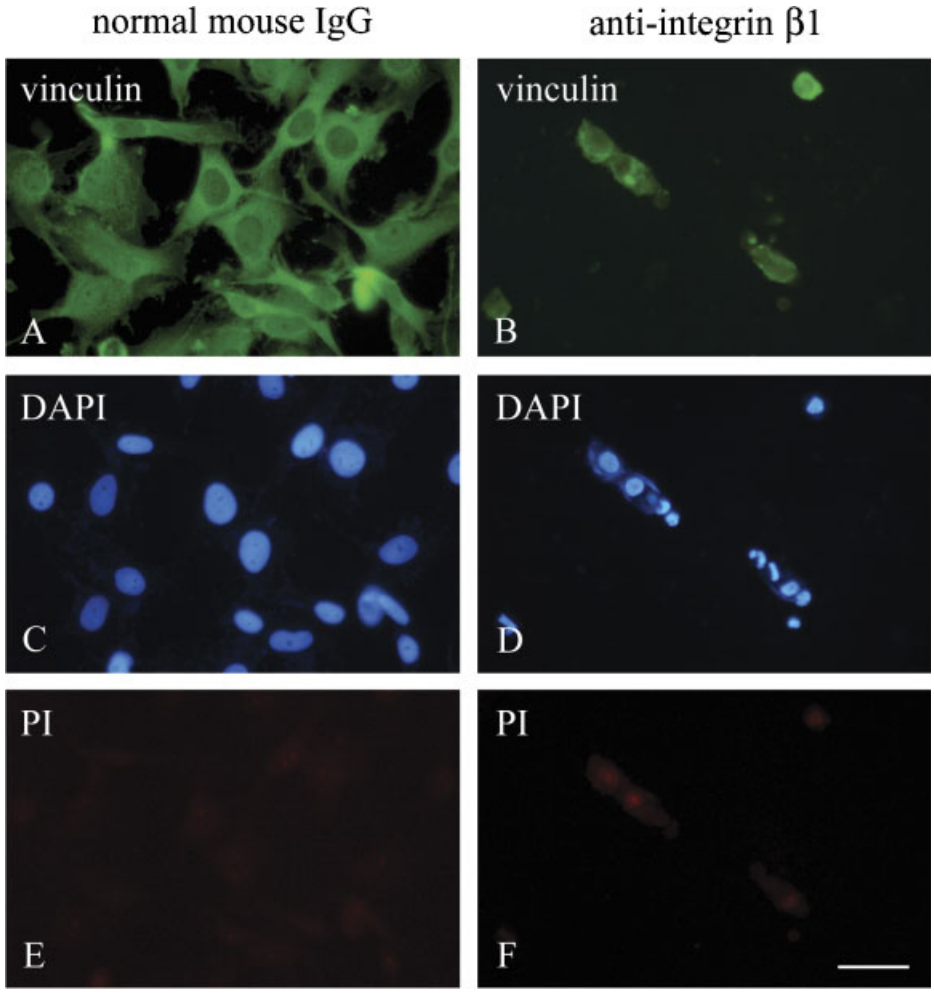

Fig. 7. Effect of anti-integrin β1 antibody perturbation on FA structure, apoptosis, and necrosis. Live CGTH W-2 cells were treated with normal mouse IgG (control; **A**, **C**, **E**) or anti-integrin β1 antibody (**B**, **D**, **F**) for 6 h. The changes of FA were revealed by vinculin immunostaining (A, B) countered stained with DAPI (C, D) and PI (E, F). Bar = 40 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

the present observation of actin disassembly may be due to the collapse of the FA complex cannot be excluded.

Engagement of integrins with their ligands induces the rapid tyrosine phosphorylation of FAK [Burridge and Chrzanowska-Wodnicka, 1996]. The cytoplasmic domain of the integrin β1 tail is bound to FAK and participates in FAK activation [Lee and Juliano, 2004]. The down-regulation of integrin β1 in CGTH W-2 cells may be regulated by the transcriptional effects of PPARγ. A recent study showed that PPARγ ligands inhibit α5 integrin gene transcription in non-small cell lung carcinoma cells [Han et al., 2005]. Moreover, overexpression of death-associated protein kinase, a pro-apoptotic protein, suppresses integrin β1 activity and disrupts matrix survival signals, which in turn decreases the phosphorylation of FAK and paxillin [Wang et al., 2002]. Whether PPARγ agonists affect death-associated protein kinase expression deserves further study. While incubation of CGTH W-2 cells with anti-integrin β1 antibodies to interfere with integrin function, FA disruption was accompanied by apoptosis in CGTH W-2 cells. The present study showed that the reduced expression of integrin β1 induced by PPARγ agonists weakened the cell matrix contacts, which caused cell detachment and finally the cell survival. FA are important in the regulation of anoikis, a type of apoptosis resulted from inadequate or inappropriate cell matrix contacts [Khwaja et al., 1997; Zhan et al., 2004]. After the treatment of ciglitazone or

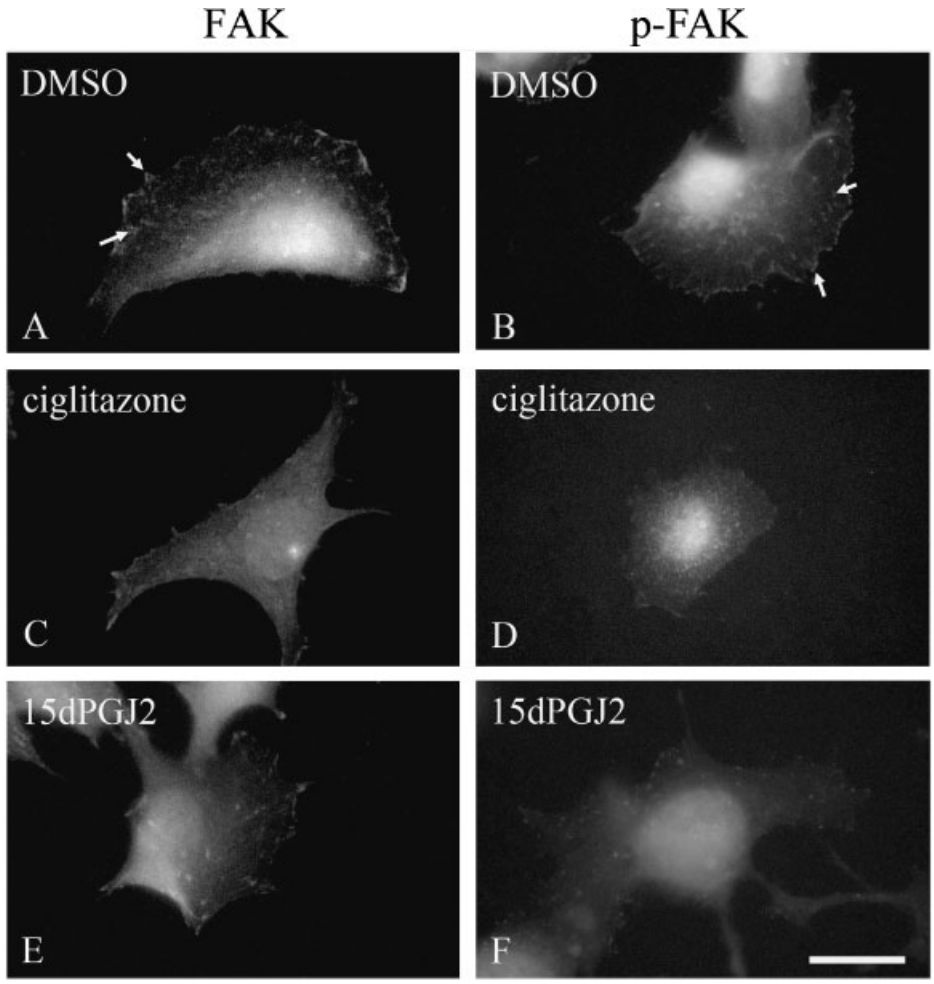

Fig. 8. Effect of ciglitazone or 15dPGJ2 on the distribution of total FAK and phosphorylated FAK. CGTH W-2 cells were treated for 18 h with 0.1% DMSO (**A**, **B**), 10 μM ciglitazone (**C**, **D**), or 10 μM 15dPGJ2 (**E**, **F**) in serum-free medium, then immunostained for FAK (A, C, E) or phosphorylated FAK (B, D, F). Arrows indicate the FA. Bar = 20 μm.

15dPGJ2, the expression levels of integrin β1, phosphorylated levels of FAK and paxillin were decreased, which might lead to anoikis. FAK controls anoikis through PI3K to inactivate Akt, which suppresses cell survival [Khwaja et al., 1997].

Decreased phosphorylation of FAK and paxillin, which may result from either inhibition of tyrosine kinase or activation of PTP, can lead to disassembly of the FA complex [Schaller, 2001; Carragher and Frame, 2004]. Furthermore, Webb et al. [2004] showed that mutations in FA complex proteins, including FAK, Src kinase, p130CAS, and paxillin, could decrease the rate of FA disassembly in murine embryonic fibroblasts [Webb et al., 2004]. In this study, PTEN expression was increased by PPARγ agonists and may be responsible for the subsequent dephosphorylation of FAK. PTEN, a tumor suppressor gene, decreases the phosphorylation of FAK in glioblastoma cells and hepatoma cells [Gu et al., 1999; Haier and Nicolson, 2002; Zhang et al., 2004], and PTEN overexpression in a glioblastoma cell line decreases levels of phosphorylated FAK and leads to inhibition on cell spreading and migration [Tamura et al., 1998]. Moreover, the effect of PTEN overexpression on the decreased adhesion ability appears to be mediated by integrin via binding with fibronectin [Tamura et al., 1998]. Thus, downregulation of integrin by PPARγ agonists in this study may be regulated by the upregulation of PTEN by PPARγ agonists. In addition, PTP-PEST can

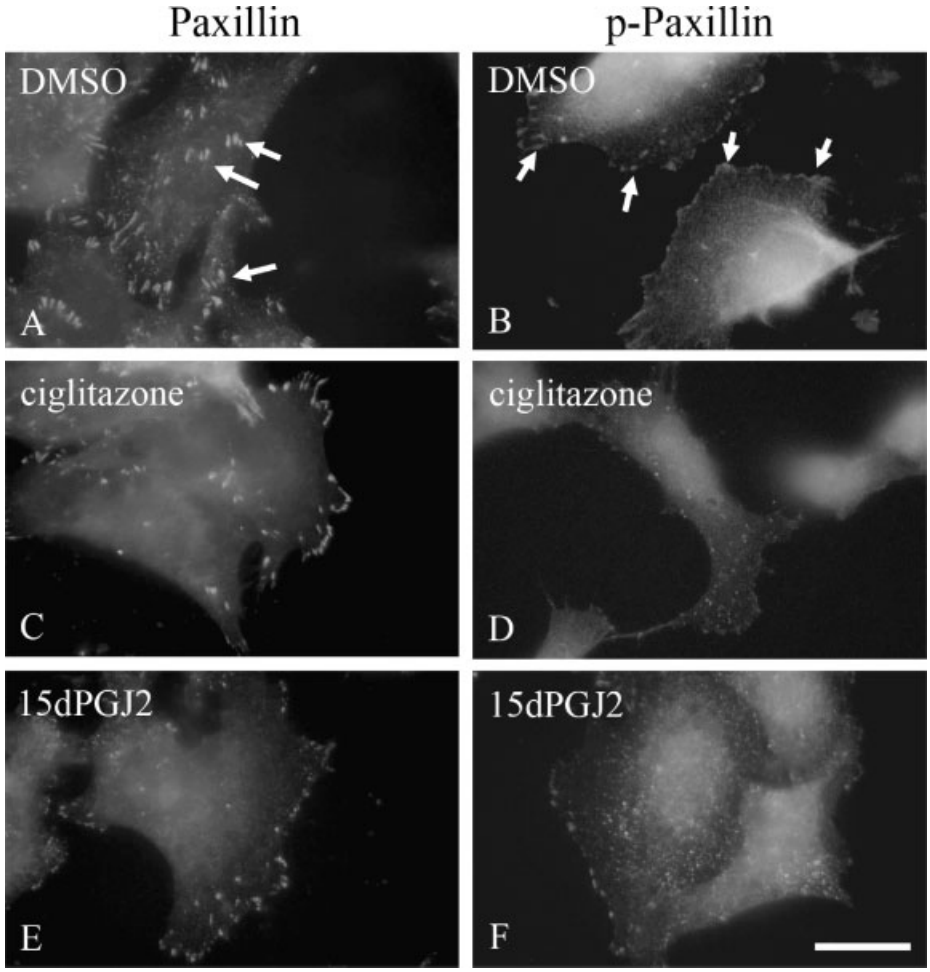

Fig. 9. Effect of ciglitazone or 15dPGJ2 on the distribution of total paxillin and phosphorylated paxillin. CGTH W-2 cells were treated for 18 h with 0.1% DMSO (**A**, **B**), 10 μM ciglitazone (**C**, **D**), or 10 μM 15dPGJ2 (**E**, **F**) in serum-free medium, then immunostained for paxillin (A, C, E) or phosphorylated paxillin (B, D, F). Arrows indicate the FA. Bar = 20 μm.

directly dephosphorylate paxillin or indirectly affect FAK phosphorylation by its action on paxillin [Shen et al., 1998]. The observation that inhibition of PTP activity by orthovanadate completely blocked the dephosphorylation of FAK and paxillin induced by 15dPGJ2 supported the upstream role of PTP in dephosphorylating FAK and paxillin.

Many studies have reported that PPARγ agonists induce apoptosis of cancer cells through the caspase cascade. In human breast cancer cells, 15-dPGJ2 induces cytochrome-*c* release from mitochondria [Pignatelli et al., 2005]. Consistent with previous studies, increased cytosolic levels of cytochrome-*c* in PPARγ agonist-treated cells were associated with the activation of caspase 3. These data provide support for the role of the cytochrome-*c* caspase 3 cascade in the PPARγ agonist-induced apoptosis in this human thyroid cancer cell line. Although both PPARγ agonists caused increases in cytosolic cytochrome-*c* by Western blot analyses, obvious cytosolic staining of cytochrome-*c* was only seen in 15dPGJ2-treated cells. Failure to detect cytochrome-*c* release by immunofluorescence in ciglitazone-treated cells may be explained by different method sensitivity of Western blotting and immunofluorescence. In addition, inactivation of Akt might be resulted from PTEN activation or from inactivation of FAK by PPARγ agonists and may contribute to the apoptosis of CGTH W-2 cells, as reported in pancreatic cancer cells [Farrow and Evers, 2003]. Recent studies on cancer cells indicate that PPARγ agonists inhibit cell growth by cell cycle arrest through

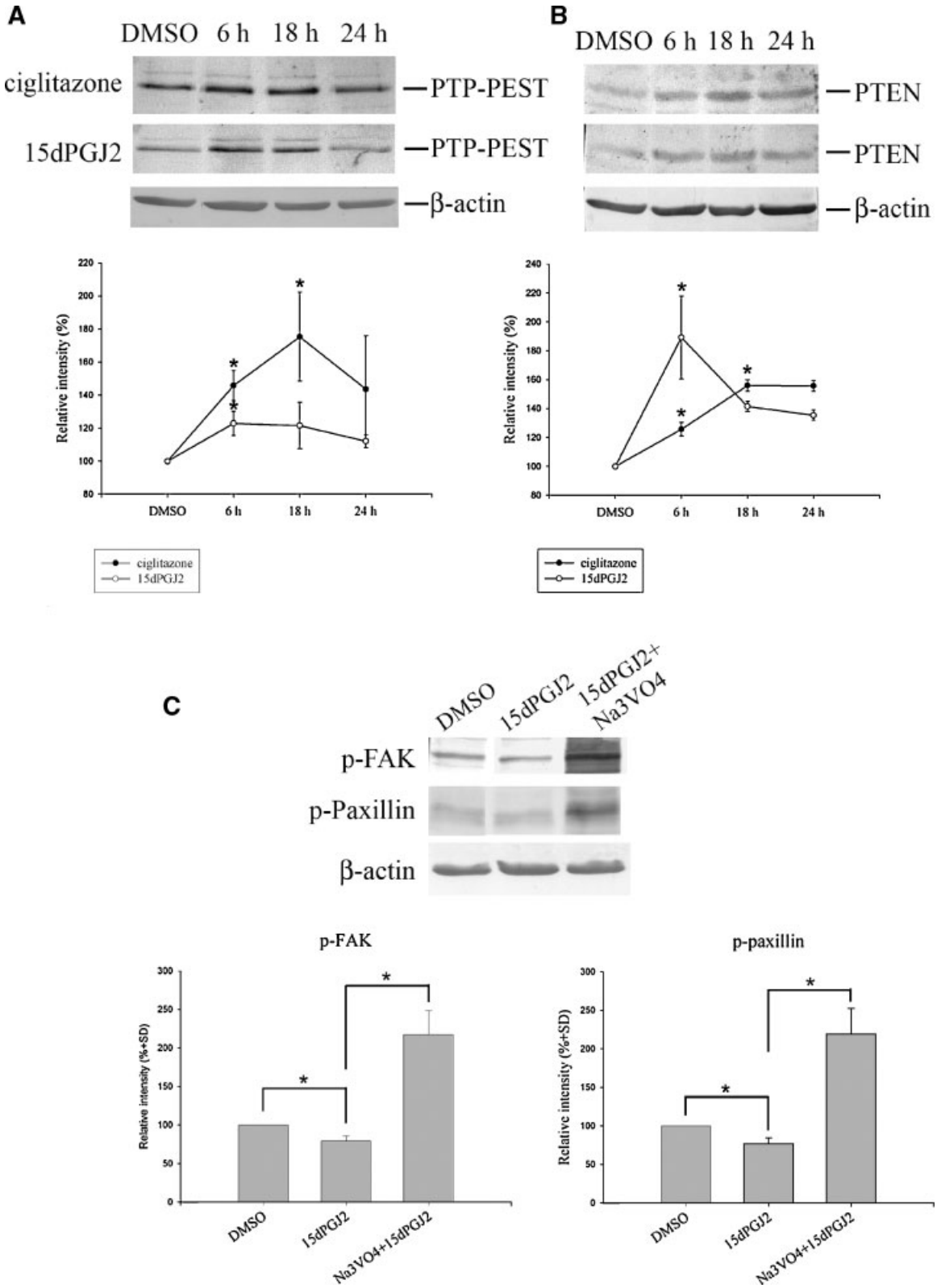

Fig. 10. Time-course studies of the effects of ciglitazone or 15dPGJ2 on the expression of PTP-PEST and PTEN. CGTH W-2 cells were treated for different intervals (6, 18, 24 h) with 0.1% DMSO, 10 μM ciglitazone, or 10 μM 15dPGJ2, then the cell homogenates were analyzed for PTP-PEST (**A**) or PTEN (**B**). The results are from a representative experiment performed in triplicates with β-actin as loading control. Lower panels in A and B: densitometric scans of triplicate blots for PTP-PEST and PTEN, respectively. *, $P < 0.05$ compared to DMSO group. **C**: Effect of orthovanadate on 15dPGJ2-induced dephosphorylation of FAK and paxillin. Cells were treated with 100 μM orthovanadate for 30 min followed by 15dPGJ2 for 6 h. Cell homogenates were analyzed for phosphorylated FAK and phosphorylated paxillin. *, $P < 0.05$ compared to PPARγ agonist-treated groups.

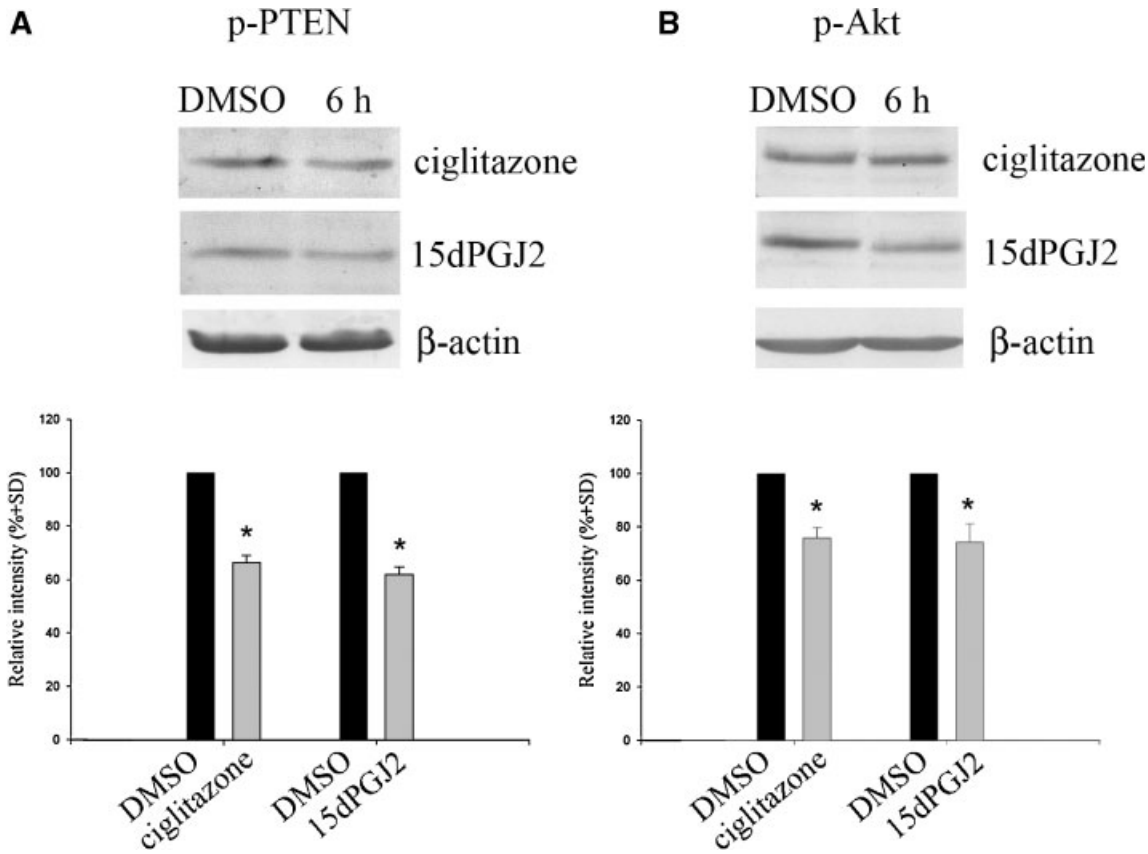

Fig. 11. Effect of ciglitazone or 15dPGJ2 on the expression of phosphorylated PTEN and phosphorylated Akt. CGTH W-2 cells were treated for 6 h with 0.1% DMSO, 10 μM ciglitazone, or 10 μM 15dPGJ2, then the cell homogenates analyzed for phosphorylated PTEN (**A**) and phosphorylated Akt (**B**). Densitometric scans are taken from triplicate blots for phosphorylated PTEN and phosphorylated Akt. *, $P < 0.05$ compared to DMSO group.

induction of p21 and/or p27, or induce apoptosis by increased expression of Bax and caspases 2, 3, 7, and 9 and decreased expression of Bcl-xL or Bcl-2 [Kim et al., 2003; Ray et al., 2004; Strakova et al., 2004; Shen et al., 2005]. In colon cancer and hepatocellular carcinoma, inhibition of NF-κ activity is associated with the apoptosis caused by PPARγ agonists [Okano et al., 2003; Chen and Harrison, 2005]. Our present study did not rule out the possibility of the involvement of other pathways proposed by other investigators.

PARP, a nuclear repair enzyme, is mainly associated with necrosis. It is possible that DNA damage induced by PPARγ agonists results in activation of PARP. In this study, PARP activation may accelerate the cleavage of NAD^+ into ADP-ribose and nicotinamide, and deplete pools of NAD^+ and ATP leading to necrosis. 3AB partially abrogated 15dPGJ2-induced necrosis but completely blocked ciglitazone-induced necrosis, indicating that PARP activation is not the only event leading to 15dPGJ2-induced necrosis. We demonstrated that simultaneous inhibition of PARP and caspase activities completely prevented 15dPGJ2-induced necrosis. The reason why z-VAD-FMK was more effective than 3AB in blocking 15dPGJ2-induced necrosis may be explained by previous findings that caspase 3 is a major mediator of both apoptotic and necrotic cell death [Faubel and Edelstein, 2005], and necrosis can be mediated by part of the apoptotic machinery, such as caspase 8 [Wang et al., 2003].

PARP activation may assist the apoptotic cascade by triggering the translocation of apoptosis inducing factor from the mitochondria to the nucleus, causing DNA condensation and fragmentation, and subsequent apoptosis [Yu et al., 2003; van Wijk and Hageman, 2005]. Inhibition of PARP activity by 3AB had a protective effect on 15dPGJ2-induced apopto-

sis, but not on ciglitazone-induced apoptosis. We further showed that ciglitazone-induced apoptosis was caspase-dependent, while 15dPGJ2-induced apoptosis involved caspase 3 and PARP activation-dependent pathways.

In summary, we demonstrated that, in CGTH W-2 cells, PPARγ agonists induced both apoptosis mediated by the cytochrome-*c* caspase and PTEN-Akt cascades, and necrosis, which was PARP-dependent. While inducing apoptosis, PPARγ agonists also upregulated the expression of PTEN and PTP-PEST, which is related to FA disassembly and cell survival.

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