Pioglitazone mediates apoptosis in Caki cells via downregulating c-FLIP$_{(L)}$ expression and reducing Bcl-2 protein stability

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Abstract. Pioglitazone is an anti-diabetic agent used in the treatment of type 2 diabetes, which belongs to the thiazolidinediones (TZDs) group. TZDs target peroxisome proliferator-activated receptor γ (PPARγ), which functions as a transcription factor of the nuclear hormone receptor. Pioglitazone has antitumor effects in several cancer types and could be a tool for drug therapy in various cancer treatments. Nevertheless, the molecular basis for pioglitazone-induced anticancer effects in renal cancer (RC) has not yet been elucidated. Thus, the aim of the present study was to investigate the detailed signaling pathway underlying pioglitazone-induced apoptosis in Caki cells derived from human clear cell renal cell carcinoma. As a result, it was demonstrated by flow cytometry analysis and Annexin V-propidium iodide staining that pioglitazone treatment induced apoptotic cell death in a dose-dependent manner in Caki cells. The protein expression levels of cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein (c-FLIP)$_{(L)}$ and Bcl-2, which were determined by western blotting, decreased after pioglitazone treatment in Caki cells. Flow cytometry and western blot analyses demonstrated that pioglitazone-mediated apoptosis was blocked following pretreatment with the pan-caspase inhibitor, z-VAD-fmk, indicating that pioglitazone-induced apoptosis was mediated via a caspase-dependent signaling pathway. However, the reactive oxygen species (ROS) scavenger, N-acetylcysteine (NAC), did not affect pioglitazone-mediated apoptosis and degradation of c-FLIP$_{(L)}$ and Bcl-2 protein. Of note, it was found by western blot analysis that Bcl-2 protein expression was downregulated by the decreased protein stability of Bcl-2 in pioglitazone-treated Caki cells. In conclusion, these findings indicated that pioglitazone-induced apoptosis is regulated through caspase-mediated degradation of FLIP$_{(L)}$ and reduction of Bcl-2 protein stability, suggesting that pioglitazone is a feasible apoptotic agent that could be used in the treatment of human RC.

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Introduction

Pioglitazone is a type 2 anti-diabetic agent included in the thiazolidinedione (TZD) class and is a ligand for synthetic peroxisome proliferator-activated receptor (PPARγ). It is involved in lipid and glucose metabolism and has recently been reported to be associated with the inhibition of numerous cancer cells (1). Pioglitazone is reported to have multiple functions; it is anti-invasive, anti-inflammatory and prevents angiogenesis (2-4). Previous studies have shown that pioglitazone shows marked anti-proliferative and antitumor effects in various types of human cancers, including cancers of bladder, uterus, thyroid, pancreas and breast, via inhibiting the signal transducer and activator of transcription 3 (STAT3), MEK/ERK, p38 mitogen-activated protein kinase (MAPK) and JAK2/STAT3 signaling pathways, and upregulating the expression of AIF and death receptors (DRs) such as DR5 and Fas/CD95 (5-9). Although pioglitazone induces apoptosis in various cancer cell lines, the detailed molecular mechanism underlying pioglitazone-induced apoptosis is not understood in Caki cells derived from human clear cell renal cell carcinoma (ccRCC).

Cellular FADD-like interleukin-1β-converting enzyme inhibitory protein (c-FLIP) is an important anti-apoptotic protein related to cancer cell death. There are three isoforms of c-FLIP, namely, c-FLIP$_{(L)}$, c-FLIP$_{(S)}$ and c-FLIP$_{(R)}$ (10). The c-FLIP$_{(L)}$ shows significant structural similarities with caspase-8 (10) and is associated with TNF-related apoptosis-inducing ligand (TRAIL), Fas, TNF-α and anticancer drug resistance in human malignancies (10-12). The overexpression of c-FLIP inhibits death ligand-induced apoptosis, which may impart resistance to anticancer drugs (13). Moreover, c-FLIP is overexpressed in a wide variety of cancers, including gastric cancer, colorectal cancer (CRC), bladder urothelial cancer and cervical cancer (14-17). Therefore, to specifically regulate the expression and activity of c-FLIP, it is necessary to find a target molecule that does not interfere with caspases-8 and -10; moreover, it is necessary to downregulate c-FLIP mRNA expression or to decrease protein stability via proteasomes.

B-cell lymphoma 2 (Bcl-2) belongs to the Bcl-2 family and can be classified as an anti- or pro-apoptotic protein. Bcl-2

and Bcl-xL are well-known anti-apoptotic proteins, whereas Bax and Bak are widely used as pro-apoptotic proteins (18). Anti-apoptotic Bcl-2 family proteins maintain the mitochondrial membrane, whereas pro-apoptotic Bcl-2 family proteins increase mitochondrial outer membrane permeabilization (MOMP), which is associated with the induction of apoptosis (19-21). Bcl-2 plays a critical role in cancer cell death. Until now, the most effective strategy for targeting the Bcl-2 family has been to use the BH3 mimetic molecules (22). Bcl-2 overexpression is an important mechanism in cancer cells to become resistant to cancer treatment. Overexpression of Bcl-2 is common in many types of human cancers, such as gastric cancer and breast cancer (23-25). Thus, targeting Bcl-2 may be an important strategy to treat cancers.

In the present study, we found that pioglitazone induces apoptosis in human ccRCC Caki cells by activating the caspase-dependent apoptotic signaling pathway via downregulating c-FLIP$_{(L)}$ and reducing Bcl-2 protein stability.

Materials and methods

Cell culture media and reagents. Human ccRCC Caki cells were obtained from the American Type Culture Collection (cat. no. HTB-46; ATCC). Caki cells were maintained in Dulbecco's modified Eagle's medium (DMEM; cat. no. LM 001-05; Welgene) containing 10% fetal bovine serum (FBS; cat. no. S001-07; Welgene) and 1% antibiotic antimycotic (AA) solution (cat. no. LS 203-01, Welgene). Human normal kidney HK-2 cells were purchased from the Korean Cell Line Bank (cat. no. 22190). HK2 cells were maintained in Roswell Park Memorial Institute (RPMI)-1640 (cat. no. LM 011-01; Welgene) medium supplemented with 10% FBS and 1% AA solution. Cells were incubated at 37˚C under 5% CO_2 environment. The compound z-VAD-fmk (cat. no. 627610) was purchased from Calbiochem. Pioglitazone (cat. no. E6910), N-acetylcysteine (NAC; cat. no. A7250) and cycloheximide (CHX; cat. no. C1988) were purchased from Sigma-Aldrich.

Cell viability assay. Cell viability assays were performed using a Welcount Cell Viability Assay Kit (cat. no. TR055-01; WelGene) to determine cell viability. Caki cells were seeded ($0.25x10^5$ cells/well) in two 96-well plates containing DMEM supplemented with 10% FBS. The cells were treated with pioglitazone for 24 h and then incubated with the XTT reagent for 2 h in dark at room temperature. Absorbance was measured at 450 nm using a microplate spectrophotometer (Thermo Labsystems) at 450/690 nm.

Flow cytometry analysis. Approximately $0.4x10^6$ cells were suspended in 100 μl cold PBS (cat. no. 70011044; Thermo Fisher Scientific, Inc.) and 200 μl 95% ethanol (cat. no. 1.00983.1011; Merck) was added while the sample was being vortexed. The cells were incubated at 4˚C for 2 h, washed with PBS and resuspended in 250 μl of 1.12% sodium citrate buffer (pH 8.4) with 10 mg/ml RNase A (cat. no. R4875; Sigma-Aldrich). The cells were further incubated at 37˚C for 40 min. Cellular DNA was stained by incubating the cells with 250 μl propidium iodide (PI) (cat. no. P4170; Sigma) at 37˚C for 20 min. The stained cells were analyzed by fluorescence-activated cell sorting (FACS) using a BD FACSCanto II flow cytometer (BD Biosciences).

Annexin V-PI staining. Annexin V-FITC (cat. no. 556547; BD Biosciences) and PI were used for distinguishing cell death mode. Pioglitazone-treated cells were washed twice in cold PBS and resuspended in binding buffer at a concentration of $2x10^6$/ml. This suspended cells (100 μl) were stained with 5 μl of Annexin V-FITC and 10 μl PI. The cells were incubated for 15 min in the dark at room temperature. After the addition of 400 μl of binding buffer to each tube, the cells were measured by flow cytometry on a FACSCanto II (BD Biosciences).

Western blot analysis. Whole-cell lysates were prepared by suspending $0.45x10^6$ cells in 30-50 μl lysis buffer consisting of 15 mM ethylene glycol tetraacetic acid (EGTA), 137 mM NaCl, 15 mM $MgCl_2$, 0.1 mM sodium orthovanadate, 25 mM MOPS, 100 μM phenylmethanesulfonyl fluoride (PMSF), 0.1% Triton X-100 and 20 μM leupeptin (pH 7.2). The cells were disrupted by sonication, followed by protein extraction by incubating the samples at 4˚C for 30 min. Total protein in the lysates was quantified using the bicinchoninic acid (BCA) assay kit (cat. no. 23225; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The proteins (40-60 μg) were separated using 10-12% SDS PAGE gel and electrotransferred onto nitrocellulose membranes (GE Healthcare). Target proteins were detected using Immobilon Western Chemiluminescent HRP Substrate solution (cat. no. WBULS0100, Millipore). The expressed proteins were visualized using the Image Quant LAS 4000 imaging system (GE Healthcare). Anti-PARP antibody (1:1,000; cat. no. 9542) was purchased from Cell Signaling Technology. Anti-caspase-3 antibody (1:2,000; cat. no. ADI-AAP-113) and anti-c-FLIP (1:700; cat. no. ALX-804-961-0100) antibody were purchased from Enzo Life Sciences. Anti-Bcl-2 antibody (1:700; cat. no. sc-7832), anti-Mcl-1 (1:1,000; cat. no. sc-12756), c-IAP2 (1:1,000; cat. no. sc-517317) and anti-β-actin antibody (1:3,000; cat. no. sc-47778) were supplied by Santa Cruz Biotechnology, Inc. and anti-XIAP (1:5,000; cat. no. 610717) antibody was obtained from BD Biosciences.

RNA isolation and RT-PCR. Bcl-2 mRNA expression was quantified via RT-PCR. Total RNA was extracted from whole cells using EasyBlue reagent (cat. no. 17061; Life Technologies). The cDNA was prepared using M-MLV Reverse Transcriptase (cat. no. 18057018; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Total cellular RNA was reverse-transcribed using random primers and amplified using PCR. GAPDH was used as an mRNA loading control. The sequences of primers used for the amplification of Bcl-2 and GAPDH were as follows: Bcl-2, (forward) 5'-GCCTTCTTT GAGTTCGGTGG-3' and (reverse) 5'-ATCTCCCGGTTG ACGCTCT-3'; GAPDH: (forward) 5'-AGGTCGGAGTCA ACGGATTTG-3' and (reverse) 5'-GTGATGGCATGGACT GTG-GT-3' and the PCR cycling conditions were as follows: for Bcl-2: 95˚C for 5 min, followed by 42 cycles of 95˚C for 45 sec, 53˚C for 30 sec and 72˚C for 30 sec; for GAPDH: 95˚C for 5 min, followed by 25 cycles of 94˚C for 30 sec, 57˚C for 45 sec and 72˚C for 40 sec. The PCR products were analyzed

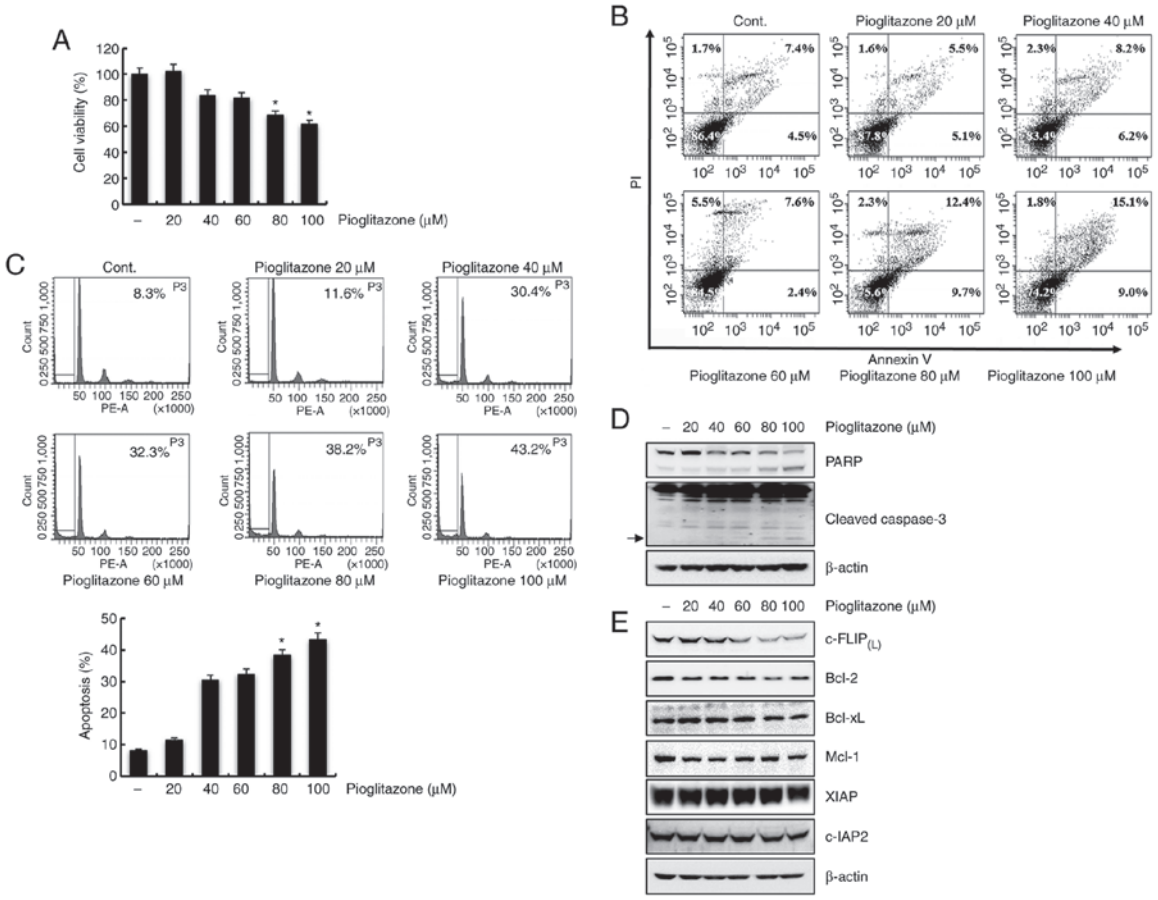

Figure 1. Pioglitazone mediates apoptosis in Caki cells. (A) Caki cells were treated with various concentrations of pioglitazone (0, 20, 40, 60, 80 and 100 μM) for 24 h. Cell viability was measured using the XTT assay kit. (B) Caki cells were treated with pioglitazone for 24 h, collected and stained with Annexin V and PI. Cell death was determined by flow cytometry. Each value corresponds to the percentage of cells in each quadrant (Q1, necrotic cells; Q2, late apoptotic cells; Q3, living cells; Q4, early apoptotic cells). (C) Caki cells were treated with pioglitazone for 24 h. Apoptosis was analyzed by flow cytometry. Representative FACS histograms are presented in the upper panel and cumulative data in the lower panel. (D) The cells were cultured with the indicated concentrations of pioglitazone. PARP, cleaved-caspase-3 and β-actin protein expression levels were determined using western blotting. (E) Caki cells were treated with pioglitazone for 24 h. The expression levels of c-FLIP$_{(L)}$, Bcl-2, Bcl-xL, Mcl-1, XIAP, c-IAP2 and β-actin proteins were detected by western blot analysis. β-actin was used as a control for protein loading. Arrows indicate cleaved forms of caspase-3. The data were obtained from three independent experiments. Data are expressed as the mean ± SD (n=3). *P<0.05 vs. non-treated cells. FACS, Fluorescence-activated cell sorting; PI, propidium iodide; PARP, poly (ADP-ribose) polymerase; c-FLIP, cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein; Bcl-2, B cell lymphoma 2; Bcl-xL, B cell lymphoma-extra large; Mcl-1, myeloid cell leukemia-1; XIAP, X-linked inhibitor of apoptosis protein; c-IAP, cellular inhibitor of apoptosis protein.

by 1.5% agarose gel electrophoresis and visualized with 10% ethidium bromide using a gel system (cat. no. WGD30, Daihan).

Transfection. Caki cells were seeded onto 6-well plates ($0.2x10^6$ cells/well) and incubated overnight at 37˚C. The cells were transfected with pcDNA 3.1 vector and pcDNA 3.1 Bcl-2 plasmid using Lipofectamine 2000 (cat. no. 11668-019; Invitrogen; Thermo Fisher Scientific, Inc.) in Opti-MEM medium (cat. no. 31985-070; Invitrogen; Thermo Fisher Scientific, Inc.). Following transfection, the cells were cultured in DMEM supplemented with 20% FBS for 18 h. Cells were then treated with pioglitazone for 24 h and analyzed for Bcl-2 expression by western blotting.

Statistical analysis. Data were analyzed using one-way ANOVA followed by post hoc comparisons (Student-Newman-Keuls) using the Statistical Package for Social Sciences 8.0 (SPSS Inc.). All experiments were performed in triplicates. The results were expressed as the mean ± SD and result with P<0.05 were considered statistically significant.

Results

Pioglitazone mediates apoptosis in human ccRCC Caki cells. To investigate the anticancer effect of pioglitazone on Caki cells, a series of experiments were performed. Caki cells were treated for 24 h with various concentrations of pioglitazone. As shown in Fig. 1A, treatment with pioglitazone considerably reduced cell viability. To clarify pioglitazone-induced cell death mode, we performed Annexin V-PI double staining using flow cytometry. We confirmed a dose-dependent increase of apoptotic cells in pioglitazone-treated cells (Fig. 1B). The apoptotic effect of pioglitazone was also identified. Pioglitazone treatment for 24 h caused a dose-dependent increase in the sub-G1 cell population (Fig. 1C). Additionally, pioglitazone increased the levels of cleaved PARP and cleaved-caspase-3 in treated cells (Fig. 1D). These results indicate that pioglitazone induces apoptosis in Caki cells. To examine the underlying molecular mechanism involved

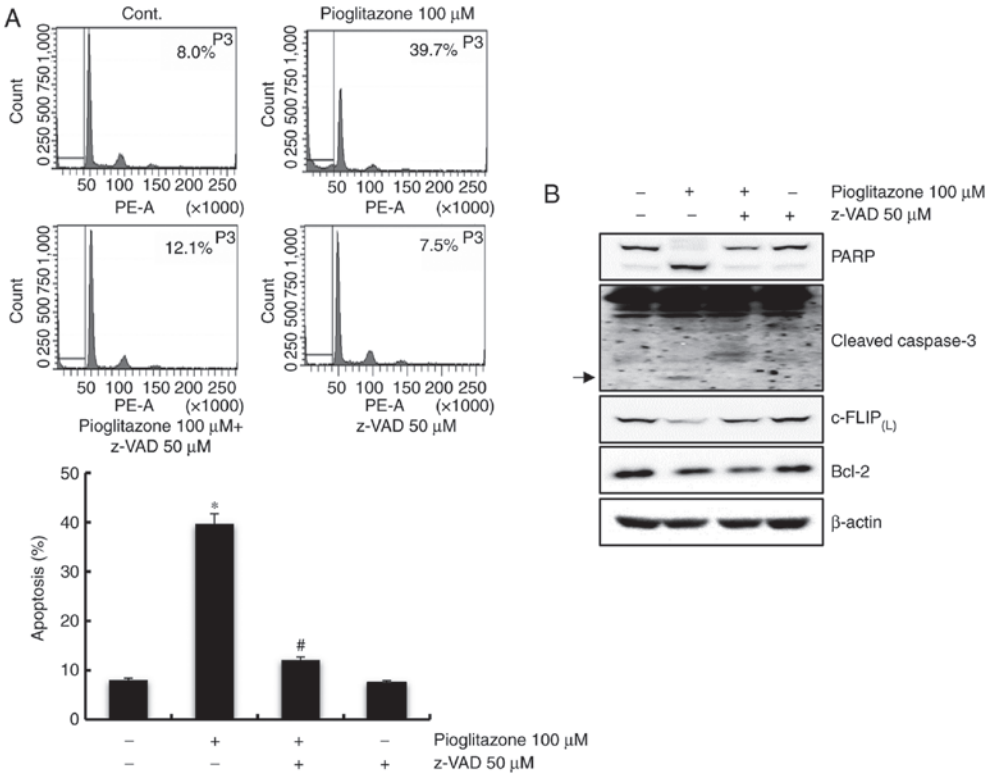

Figure 2. Pioglitazone-induced apoptosis is markedly inhibited via a caspase-dependent pathway. (A) Caki cells were treated with 100 μM pioglitazone for 24 h in the absence or presence of z-VAD-fmk. The sub-G1 cell fraction was measured by flow cytometry. Representative FACS histograms are presented in the upper panel and cumulative data in the lower panel. (B) Cells were pretreated with 50 μM z-VAD-fmk or a vehicle for 30 min and incubated with pioglitazone. After 24 h, the expression levels of PARP, cleaved-caspase-3, c-FLIP$_{(L)}$, Bcl-2 and β-actin proteins were detected by western blotting. β-actin was used as a control for western blot analysis. Arrows indicate cleaved forms of caspase-3. Data was obtained from three independent experiments. Data are expressed as the mean ± SD (n=3). *P<0.05 vs. non-treated cells; $^{\#}$P<0.01 vs. pioglitazone-treated cells. z-VAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone; FACS, Fluorescence-activated cell sorting; PARP, poly(ADP-ribose) polymerase; c-FLIP, cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein; Bcl-2, B cell lymphoma 2.

in pioglitazone-mediated apoptosis, the expression levels of apoptotic-regulatory proteins were confirmed by western blotting. As shown in Fig. 1E, c-FLIP$_{(L)}$ and Bcl-2 expression levels moderately or markedly decreased in pioglitazone-treated Caki cells. However, Bcl-xL, Mcl-1, XIAP and c-IAP2 protein levels were not affected. Taken together, these findings indicate that pioglitazone induces apoptosis and inhibits the expression of c-FLIP$_{(L)}$ and Bcl-2 in Caki cells.

Pioglitazone-induced apoptosis is markedly blocked by a caspase-dependent pathway in Caki cells. To determine whether the caspase-dependent pathway plays a key role in pioglitazone-induced apoptosis, the pan-caspase inhibitor, z-VAD-fmk was used. As shown in Fig. 2A, the pioglitazone-induced apoptosis was significantly blocked by pretreatment with z-VAD-fmk. Furthermore, treatment with z-VAD-fmk inhibited the cleavage of PARP and cleaved-caspase-3, and recovered c-FLIP$_{(L)}$, but not Bcl-2 expression (Fig. 2B). These findings indicate that pioglitazone-mediated apoptosis in Caki cells is regulated by a caspase-dependent pathway via downregulation of c-FLIP$_{(L)}$.

Pioglitazone-mediated apoptosis is not associated with ROS. Studies have shown that ROS can modulate apoptosis by regulating the expression levels of pro-apoptotic proteins, such as caspases or anti-apoptotic proteins such as c-FLIP and Bcl-2 (26). We investigated whether ROS plays a role in pioglitazone-induced apoptosis. Caki cells were pretreated with NAC for 1 h and incubated with pioglitazone for 24 h. Pretreatment with NAC failed to inhibit pioglitazone-mediated apoptosis (Fig. 3A). Additionally, NAC did not affect PARP cleavage, caspase activation, c-FLIP$_{(L)}$ and Bcl-2 expression levels in pioglitazone-treated cells (Fig. 3B). Therefore, these results indicate that pioglitazone-mediated apoptosis is not associated with ROS.

Downregulation of c-FLIP$_{(L)}$ contributes to pioglitazone-mediated apoptosis. We examined whether downregulation of c-FLIP$_{(L)}$ by pioglitazone-induced apoptosis in Caki cells overexpressing c-FLIP$_{(L)}$. Overexpression of c-FLIP$_{(L)}$ significantly decreased pioglitazone-induced apoptosis, whereas treatment with pioglitazone induced significant apoptosis in Caki/vector cells (Fig. 4A). Expression of cleaved PARP and cleaved-caspase-3 induced by pioglitazone treatment was also significantly inhibited by overexpression of c-FLIP$_{(L)}$ (Fig. 4B). Therefore, these findings indicate that the downregulation of c-FLIP$_{(L)}$ contributes to pioglitazone-mediated apoptosis. To confirm the functional role of downregulated Bcl-2 in pioglitazone-treated cells, Caki cells engineered for Bcl-2 overexpression were used. As shown in Fig. 4C, overexpression of Bcl-2 was not associated with pioglitazone-mediated apoptosis. Expression of cleaved PARP and caspase-3 induced

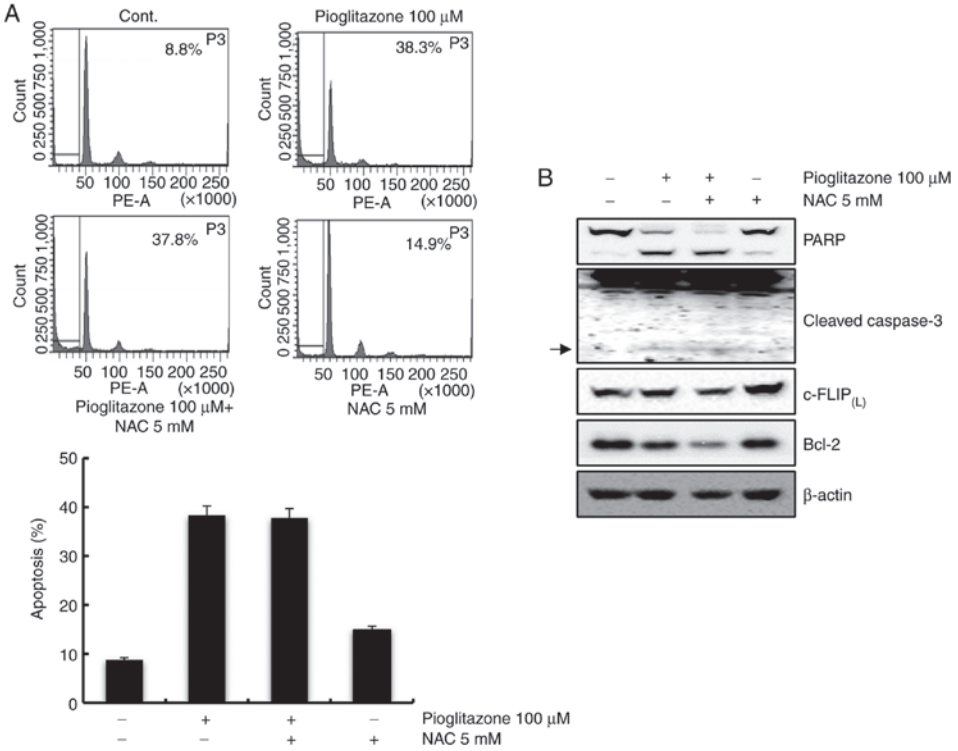

Figure 3. Pioglitazone-induced apoptosis is not mediated by ROS. (A) Caki cells were treated with 5 mM NAC or a vehicle for 1 h before treatment with 100 μM pioglitazone. After 24 h, apoptosis was measured by flow cytometry. Representative FACS histograms are presented in the upper panel and cumulative data in the lower panel. (B) Cells were pretreated with 5 mM NAC or a solvent for 1 h and treated with pioglitazone for 24 h. The expression levels of PARP, cleaved-caspase-3, c-FLIP$_{(L)}$, Bcl-2 and β-actin proteins were detected by western blot analysis. β-actin was used as a loading control protein. Arrows indicate cleaved forms of caspase-3. Data was representative from three independent experiments. Data are expressed as the mean ± SD (n=3). ROS, reactive oxygen species; NAC, N-acetyl-L-cysteine; FACS, Fluorescence-activated cell sorting; PARP, poly(ADP-ribose) polymerase; c-FLIP, cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein; Bcl-2, B cell lymphoma 2.

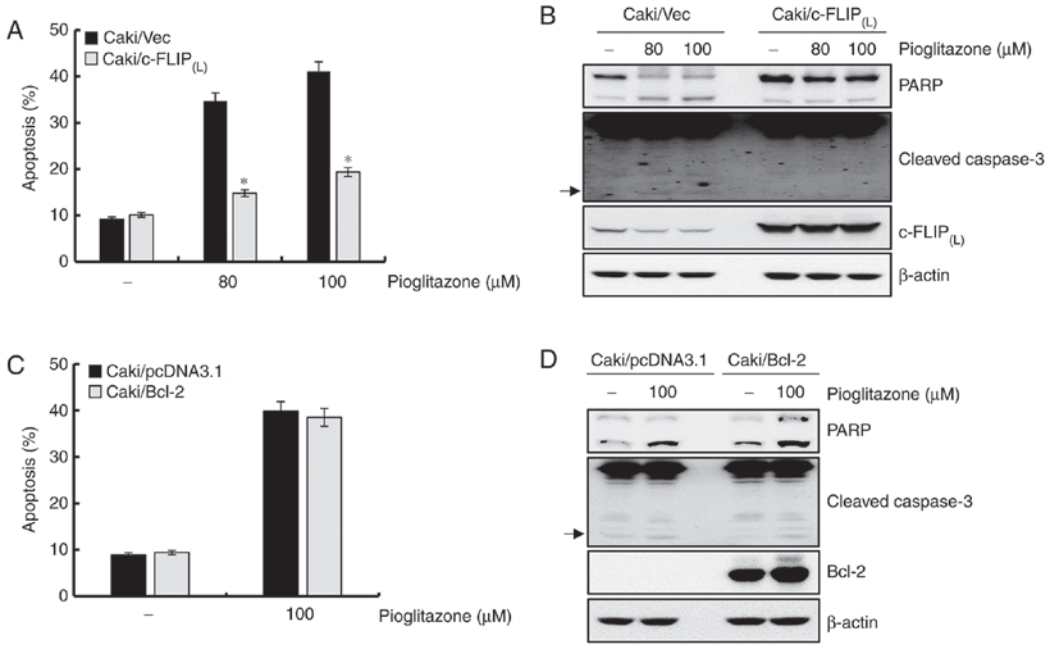

Figure 4. Downregulation of c-FLIP$_{(L)}$ plays a critical role in pioglitazone-mediated apoptosis. (A) Caki/vector and Caki/c-FLIP$_{(L)}$ cells were treated for 24 h with pioglitazone (80 and 100 μM). Apoptosis was analyzed as a sub-G1 cell fraction by FACS. (B) Caki/vector and Caki/c-FLIP$_{(L)}$ cells were treated with 80 and 100 μM pioglitazone for 24 h. Expression levels of PARP, cleaved-caspase-3, c-FLIP$_{(L)}$ and β-actin proteins were detected by western blot analysis. (C) After transient transfection with empty vector or the Bcl-2 expression vector, the cells were treated with 100 μM pioglitazone for 24 h. The sub-G1 cell fraction was measured by flow cytometry. (D) After transient transfection with an empty vector or the Bcl-2 expression vector, the cells were treated with pioglitazone. After 24 h, expression levels of PARP, cleaved-caspase-3, Bcl-2 and β-actin proteins were detected by western blotting. β-actin was used as a control for western blot analysis. Arrows indicate cleaved forms of caspase-3. Data were obtained from three independent experiments. The data are expressed as the mean ± SD (n=3). *P<0.05 vs. pioglitazone-treated Caki/vector cells. c-FLIP, cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein; FACS, Fluorescence-activated cell sorting; PARP, poly(ADP-ribose) polymerase; Bcl-2, B cell lymphoma 2.

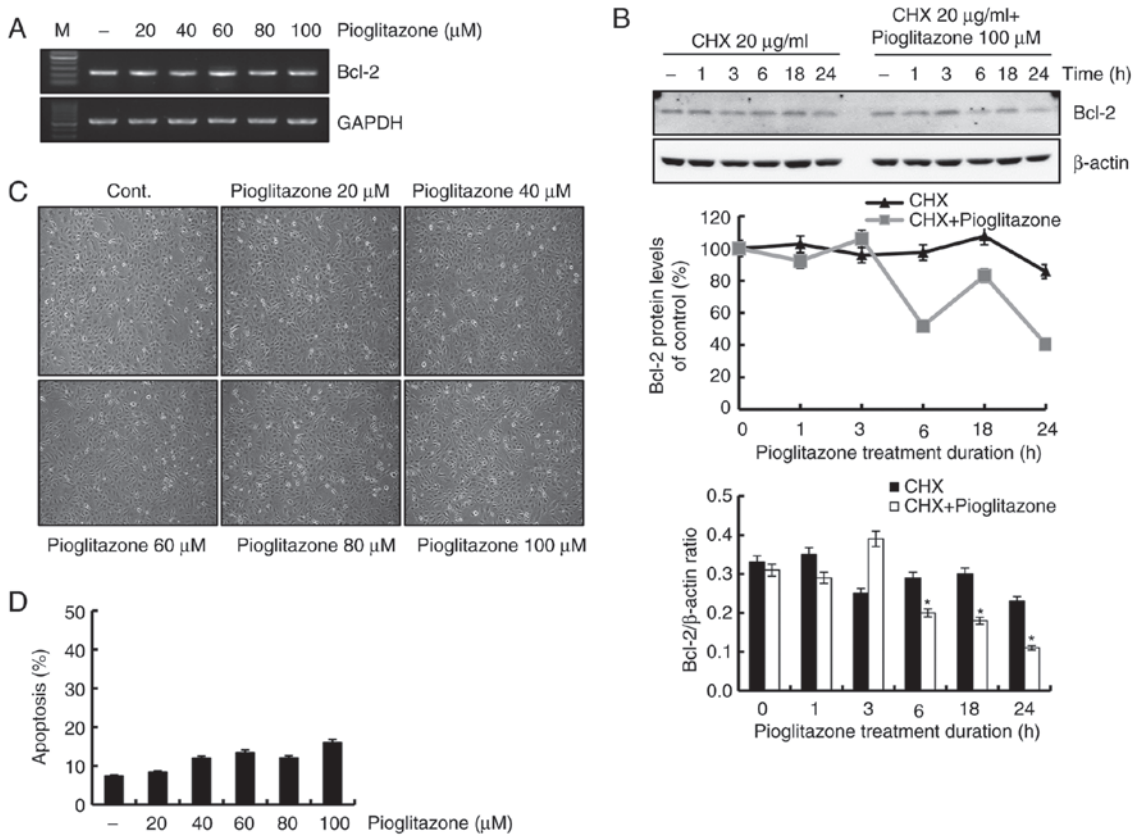

Figure 5. Downregulation of Bcl-2 expression by pioglitazone treatment results from decreased protein stability. (A) Caki cells were treated with pioglitazone. After 24 h, mRNA expression of Bcl-2 was measured via reverse transcription-semi-quantitative PCR. GAPDH was used as a loading control. (B) Cells were treated with or without 100 μM pioglitazone in the presence of 20 μg/ml CHX for the indicated duration. Western blotting was performed to determine Bcl-2 protein expression levels (upper panel). The Bcl-2 density was measured using ImageJ software (middle panel). The data obtained from the western blot analyses of Bcl-2 and β-actin were used to evaluate the effect of pioglitazone on the Bcl-2/β-actin ratio (lower panel). β-actin was used as a loading control protein. (C) HK-2 cells were treated with the indicated concentrations of pioglitazone (0, 20, 40, 60, 80 and 100 μM) for 24 h. The morphological changes were examined under an inverted microscope (magnification, x200). (D) HK-2 cells were treated with pioglitazone (0, 20, 40, 60, 80 and 100 μM) for 24 h. Apoptosis was determined by flow cytometry. Data were obtained from three independent experiments. The data are expressed as the mean ± SD (n=3). *P<0.05 vs. CHX-treated Caki cells. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Bcl-2, B cell lymphoma 2; CHX, cycloheximide.

by pioglitazone-treated cells was not affected by overexpression of Bcl-2. (Fig. 4D), suggesting that the downregulation of Bcl-2 was not related to pioglitazone-induced apoptosis.

Pioglitazone attenuates the expression of Bcl-2 caused by the reduction of protein stability. Whether the pioglitazone-induced decrease in Bcl-2 was regulated at the transcriptional level was evaluated next. As shown in Fig. 5A, the Bcl-2 mRNA level remained constant following treatment with pioglitazone, suggesting that pioglitazone-mediated downregulation of Bcl-2 protein is regulated at the post-transcriptional level. To further clarify the mechanisms underlying the decreased Bcl-2 expression level in pioglitazone-treated cells, a protein stability assay for Bcl-2 was performed. Cells were pretreated with cycloheximide (CHX) for 1 h and then treated with pioglitazone for studying the kinetics. As shown in Fig. 5B, the protein expression level of Bcl-2 reduced more rapidly with the co-treatment of CHX and pioglitazone compared with CHX treatment alone. We also investigated whether treatment with pioglitazone affects the induction of apoptosis in human normal kidney HK-2 cells. However, the sensitivity to apoptosis by pioglitazone was markedly reduced in HK-2 cells, compared with pioglitazone-treated Caki cells (Fig. 5C and D). These results showed that the degradation of Bcl-2 protein was facilitated by pioglitazone treatment and that pioglitazone treatment reduced Bcl-2 protein stability.

Discussion

In this study, it was shown that pioglitazone exerts potent anticancer effects on human ccRCC Caki cells. Pioglitazone-induced apoptosis was mediated by caspase-dependent signaling pathways in treated cells. Moreover, the molecular mechanism of pioglitazone-mediated apoptosis is ascribed to caspase-mediated degradation of c-FLIP$_{(L)}$ protein and reduction of Bcl-2 protein stability.

Pioglitazone, a PPARγ agonist, is used to lower blood glucose levels in patients with type 2 diabetes (27). Studies have shown that pioglitazone may exert antitumor effects in several human cancer cell types, including bladder cancer, acute lymphocytic leukemia and glioma via inducing apoptosis and cell growth inhibition (28-30). Nevertheless, it has been shown that diabetic patients with long-term and high-dose exposure to pioglitazone may increase the risk of bladder cancer (31). However, there is a conflicting study that pioglitazone treatment

does not increase the risk of bladder cancer in diabetic patients and that bladder cancer risk does not correlate with cumulative dose and duration of treatment, indicating that pioglitazone makes it much more effective and safer for glycemic control in diabetic patients (32). Additionally, in our system, pioglitazone treatment did not induce cell proliferation in bladder cancer T24 cells (data not shown). In this study, the antitumor effect of pioglitazone on Caki cells was confirmed. Consistent with previous studies, it was found that increasing concentrations of pioglitazone led to an increased in the sub-G1 cell population.

Pioglitazone and other PPARγ agonists show anticancer activity against several cancer types, such as non-small cell lung carcinoma, acute promyelocytic leukemia, bladder cancer, breast cancer, lung cancer and CRC via enhancing growth arrest, upregulating the expression of DNA damage-inducible 153 gene and PTEN, inactivating the PI3K-Akt pathway, sustaining activated MAPK, modulating DR5 and c-FLIP$_{(L)}$ expression and downregulating Bcl-2 expression (33-38).

Caspase activation regulates apoptotic-regulatory proteins (39). However, there are conflicting reports of caspase involvement in apoptosis induced by PPARγ agonists in cancer cells. Pioglitazone-mediated apoptosis occurs via a caspase-independent pathway in bladder cancer cells (5). In contrast, pretreatment of PC-3 cells with z-VAD-fmk inhibited PPARγ agonist-mediated apoptosis, indicating the involvement of the caspase-dependent pathway in prostate cancer (40). It was also shown that pioglitazone-induced apoptotic cells were remarkably inhibited by pretreatment with z-VAD-fmk. Thus, pioglitazone-mediated apoptosis is regulated by the caspase-dependent apoptotic pathway in Caki cells.

c-FLIP is an important modulator of anti-apoptotic pathway and is expressed in a variety of cancer cell types (41). Previous reports have demonstrated that PPARγ ligands modulate apoptosis via downregulating c-FLIP$_{(L)}$ expression in cervical cancer cell lines (42). To determine whether the downregulation of c-FLIP$_{(L)}$ was involved in pioglitazone-induced apoptosis, c-FLIP$_{(L)}$-overexpressing cells were established in this study. Our results showed that pioglitazone-induced apoptosis was blocked in c-FLIP$_{(L)}$-overexpressing cells, suggesting that pioglitazone-mediated apoptosis occurred via downregulating c-FLIP$_{(L)}$ expression.

Bcl-2 expression is regulated at the transcriptional or post-transcriptional levels (43,44). Studies have indicated that pioglitazone-mediated apoptosis is regulated by suppressed Bcl-2 transcription in hepatocellular carcinoma (45). In contrast, we found that the degradation of Bcl-2 protein was facilitated by pioglitazone treatment without affecting Bcl-2 mRNA expression levels. Thus, our data indicate that pioglitazone-mediated decrease in Bcl-2 protein is regulated at the post-transcriptional level.

ROS are critical regulators of apoptosis in a wide range of human cancer cells (46,47). It has been reported that pioglitazone induces apoptosis by inducing ROS production in lung cancer (48). Thus, we confirmed whether pioglitazone-mediated apoptosis was associated with ROS production. In our study, pretreatment with NAC did not affect pioglitazone-treated cells, thereby providing evidence that pioglitazone-induced apoptosis is independent of ROS production in Caki cells.

Collectively, our results demonstrate that pioglitazone-mediated apoptosis is facilitated by caspase-dependent signaling pathways via downregulating c-FLIP$_{(L)}$ expression and reducing Bcl-2 protein stability in human ccRCC Caki cells. Therefore, based on our study outcomes, we propose that pioglitazone may be a potential therapeutic agent for human RC.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

JYK conceived and designed the study. JHJ and TJL conducted most of the experiments and data analysis, and wrote the manuscript. EGS and IHS conducted data analysis of flow cytometry and Annexin V/PI staining experiments, and wrote and revised the manuscript. JHJ and JYK confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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