Metformin-induced apoptosis facilitates degradation of the cellular caspase 8 (FLICE)-like inhibitory protein through a caspase-dependent pathway in human renal cell carcinoma A498 cells

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Abstract. Renal cell carcinoma (RCC) is one of the most common types of cancer in adults. Previous studies have reported that the survival rate was significantly lower for renal cancer patients with diabetes than for those without diabetes. Metformin is a well-known anti-diabetic agent used for the treatment of type 2 diabetes mellitus (T2DM). It also inhibits cell proliferation and angiogenesis and is known to possess antitumor effects. However, the molecular mechanism for metformin-induced apoptosis in renal cell carcinoma is not understood. In the present study, treatment with metformin induced apoptosis in A498 cells in a dose-dependent manner. It was revealed that degradation of cellular caspase 8 (FLICE)-like inhibitory protein (c-FLIP) and activation of procaspase-8 were associated with metformin-mediated apoptosis. By contrast, treatment with metformin did not affect the mRNA level of c-FLIP$_L$ in A498 cells. Treatment with benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (z-VAD-fmk, a pan-caspase inhibitor) almost completely blocked metformin-induced apoptosis and degradation of c-FLIP$_L$ protein. However, N-acetyl-L-cysteine (NAC), a reactive oxygen species (ROS) scavenger, did not inhibit metformin-mediated apoptosis in A498 cells. Taken together, the results of the present study demonstrated that metformin-induced apoptosis involved degradation of the c-FLIP$_L$ protein and activation of caspase-8 in human renal cell carcinoma A498 cells and suggested that metformin could be potentially used for the treatment of renal cancer.

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Introduction

Renal cell carcinoma (RCC), a neoplastic lesion of the kidney in humans, accounts for ~90% of kidney tumors (1). It is difficult to treat with conventional treatments including chemical, hormone and radiation therapy, and cannot be treated without surgery (2,3). A previous report described metformin may improve the incidence of cancer-associated diabetes (4). Thus far, RCC has been treated chemically and immunologically. However, there is an urgent requirement to identify more efficient chemo-preventive agents for treating RCC.

Metformin is the most widely used biguanide drug for treating type 2 diabetes mellitus patients (5). It has been reported that metformin has anti-diabetic and anticancer effects on colorectal and pancreatic cancer cells (6,7). It has also been revealed to exert anti-neoplastic effects in epithelial ovarian cancer (8). Furthermore, metformin has been demonstrated to reduce the risk of cancer prevalence in diabetic patients (9,10). Metformin demonstrated a marked anticancer effect in various cells of different types of human cancer, including breast cancer, renal cancer, glioblastoma, insulinoma and cholangiocarcinoma via cell growth inhibition, cell cycle arrest, apoptosis, adenosine monophosphate-activated protein kinase (AMPK) signaling and tumor growth inhibition (11-15). Although the effect of metformin on A498 cells has been reported (12), the apoptosis-mediated molecular mechanism of action of metformin remains unclear in human renal cell carcinoma A498 cells.

The cellular caspase 8 (FLICE)-like inhibitory protein (c-FLIP) gene makes three isoforms, namely c-FLIP$_L$, c-FLIP$_S$ and c-FLIP$_R$, via alternative splicing in humans. These proteins are well known as anti-apoptotic proteins; each exert this effect via different mechanisms (16). In previous reports, c-FLIP was demonstrated to be an independent negative prognostic factor in ovarian, endometrial and colon cancer cells (17-19). c-FLIP$_L$ is known to be involved in the inhibition of caspase-8 activation-mediated apoptosis (18,20). The activation of caspase-8 leads to death-inducing signaling complex (DISC) and augmented apoptosis via caspase-3 activation. Previous studies have demonstrated that treatment with metformin suppressed the c-FLIP$_L$ protein expression level in human lung adenocarcinoma and bladder cancer (21,22).

In the present study, the mechanism of metformin-mediated apoptosis in human renal cell carcinoma A498 cells was investigated. It was revealed that degradation of c-FLIP$_L$ protein and activation of caspase-8 were associated with metformin-induced apoptosis.

Materials and methods

Cell culture. A498 human renal carcinoma cells were procured from the American Type Culture Collection (ATCC; Manassas, VA, USA). Dulbecco's modified Eagle's medium (DMEM; catalog no. LM 001-05; Welgene, Inc., Kyungsan, Korea) containing 10% fetal bovine serum (FBS; catalog no. S001-07; Welgene, Inc.), 20 mM 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES; catalog no. H0887; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) buffer and 100 μg/ml gentamicin (catalog no. 15710-072; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used as the culture medium. The cells were cultured in an incubator at 37°C with humidified 5% CO$_2$.

Cell morphology. A498 human renal carcinoma cells were treated with an inhibitor in either the absence or presence of metformin (10 mM). Following 24 h incubation, morphological changes were visualized with light microscopy (catalog no. DFC495; Leica Microsystems GmbH, Wetzlar, Germany) at x200 magnification. The images were analyzed using the i-Solution program (IMT i-Solution, Burnaby, BC, Canada).

Flow cytometry analysis. Cell counting was performed using a hemocytometer. Metformin was immediately added to cell cultures at the indicated concentrations. Approximately 0.4x10^6 cells were resuspended in 100 μl PBS (catalog no. 17-517Q; Lonza, Walkersville, MD, USA), and 200 μl of 95% ethanol (catalog no. 1.00983.1011; Merck KGaA) was added during vortexing. The cells were incubated at 4°C for 1 h, washed in PBS, and resuspended in 250 μl 1.12% sodium citrate buffer (pH 8.4) along with 12.5 μl RNase. Incubation was continued for 30 min at 37°C. Cellular DNA was stained with 250 μl (1:1 dilution) propidium iodide (50 μg/ml; catalog no. p4170; Sigma-Aldrich; Merck KGaA) for 30 min at 37°C, and the relative DNA contents of the stained cells were analyzed using fluorescence-activated cell sorting (FACS) on the BD FACS Cato II flow cytometer (BD Biosciences, San Jose, CA, USA).

Western blot analysis. A498 whole-cell lysates were prepared by resuspending 0.4x10^6 cells in 50 μl lysis buffer (137 mM NaCl, 15 mM EGTA, 0.1 mM sodium orthovanadate, 15 mM MgCl$_2$, 0.1% Triton X-100, 25 mM MOPS, 100 μM phenylmethylsulfonyl fluoride and 20 μM leupeptin, adjusted to pH 7.2). The cells were disrupted by sonication and protein extracted at 4°C for 30 min. Protein concentrations were quantified using the BCA assay kit (catalog no. 23225; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The proteins (50 μg) were separated using 10% SDS-PAGE gel and electrotransferred onto nitrocellulose membranes (catalog no. 23225; GE Healthcare, Chicago, IL, USA). The membrane was blocked with 5% skim milk in Tris-buffered saline (TBS) for 30 min at room temperature. The anti c-FLIP$_L$ (dilution, 1:700; catalog no. ALX-804-961) antibody was obtained from Enzo Life Sciences, Inc. (Farmingdale, NY, USA). The anti-PARP (dilution, 1:1,000; catalog no. 9542) antibody was purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). The anti-B-cell lymphoma-2 (Bcl-2); (dilution, 1:700; catalog no. sc-783), anti-B-cell lymphoma-extra-large (Bcl-xL); (dilution, 1:1,000; catalog no. sc-634), anti-myeloid cell leukemia-1 (Mcl-1); (dilution, 1:1,000; catalog no. sc-819), anti-cellular inhibitor of apoptosis 2 (cIAP-2); (dilution, 1:1,000; catalog no. sc-7944) and anti-actin (dilution, 1:2,000; catalog no. sc-1616) antibodies were procured from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). The anti-XIAP (dilution, 1:5,000; catalog no. 610762) antibody was supplied by BD Biosciences (San Jose, CA, USA). Membranes were incubated with the primary antibodies overnight at 4°C. Following six washes with TBS (each for 5 min), the membranes were incubated with the indicated secondary antibody for 1 h at room temperature and washed six times with TBS. The secondary goat anti-rabbit immunoglobulin G (IgG)-horseradish peroxidase (HRP) conjugated (dilution 1:1,000; catalog no. sc-2004) and goat anti-mouse IgG-HRP (dilution 1:1,000; catalog no. sc-2005) antibodies were procured from Santa Cruz Biotechnology, Inc. Specific proteins were detected using an ECL western blotting kit (catalog no. WBKLS0500; Merck KGaA). Proteins were detected using by ImageQuant LAS 4000 Mini Imaging System (GE Healthcare).

Transfection. A498 cells were seeded onto 6-well plates at a concentration of 0.2x10^6 cells/well and incubated overnight at 37°C. The pcDNA 3.1 vector and pcDNA 3.1 c-FLIP$_L$ plasmid were provided by Professor Tae-Jin Lee (Yeungnam University, South Korea). They were then transfected with control plasmid pcDNA 3.1 vector or pcDNA 3.1-c-FLIP$_L$ plasmid for 5 h using lipofectamine 2000 (catalog no. 11668-019; Invitrogen; Thermo Fisher Scientific, Inc.) in Opti-MEM medium (catalog no. 31985-070; Invitrogen; Thermo Fisher Scientific, Inc.). Following transfection, the cells were cultured in DMEM supplemented with 10% FBS for 12 h. Next, the cells were treated with metformin for 24 h. Finally, the cells were analyzed for c-FLIP$_L$ expression using western blotting.

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR). c-FLIP$_L$ mRNA expression was determined by RT-PCR. Total RNA was extracted from A498 cells using the EasyBlue reagent (catalog no. 17061; Thermo Fisher Scientific, Inc.). cDNA was prepared using M-MLV reverse transcriptase (catalog no. 18057018; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. In addition, the total cellular RNA was reverse-transcribed using a random primer and subsequently amplified using PCR. PCR primers were purchased from GenoTech (Daejeon, Korea). GAPDH was used as the internal control. The PCR cycling conditions used were as follows: For c-FLIP$_L$: 95°C for 45 sec, 54°C for 45 sec, 72°C for 45 sec (33 cycles) and for GAPDH: 94°C for 30 sec, 58°C for 45 sec, 72°C for 42 sec (25 cycles). The following primer sequences were used to amplify c-FLIP$_L$ and GAPDH: For c-FLIP$_L$: 5'-CGGACTATAGAGTGCTGA
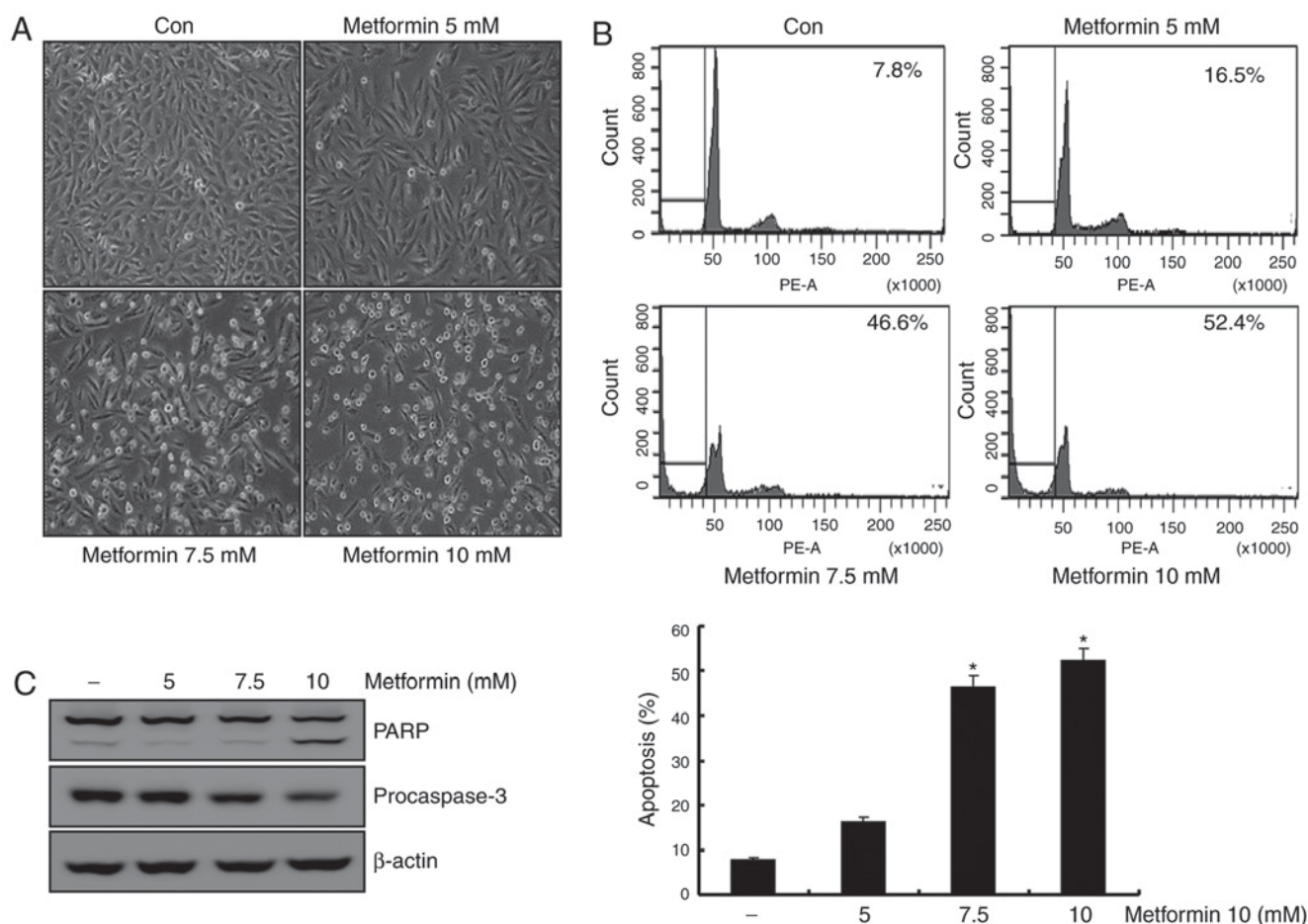
Figure 1. Metformin induces apoptosis in a dose-dependent manner in A498 cells. (A) A498 human renal carcinoma cells were treated with the indicated concentration of metformin. After 24 h, morphological changes were observed using a microscope at x200 magnification. (B) A498 cells were treated with metformin (0, 5, 7.5 and 10 mM) for 24 h. Apoptosis was determined by flow cytometry. Representative FACS histograms are presented in the upper panel, cumulative data are presented in the lower panel. (C) A498 cells were treated with metformin for 24 h, and PARP, procaspase-3 and β-actin expression was analyzed using western blotting. β-actin was used as the loading control. All data are expressed as the mean ± standard deviation of three independent experiments. *P<0.05 compared with untreated cells. FACS, Fluorescence-activated cell sorting; PARP, poly (ADP-ribose) polymerase.

TGG-3' (forward) and 5'-GATTATCAGGCAGATTCCTAG-3' (reverse); and for GAPDH: 5'-AGGTCGGAGTCAACGGAT TTG-3' (forward) and 5'-GTGATGGCATGGACTGTGGT-3' (reverse). PCR products were analyzed by electrophoresis using 1.5% agarose gels and visualized by ethidium bromide using UV light gel (catalog no. WGD30; DAIHAN Scientific, Seoul, Korea).

Measurement of reactive oxygen species. A498 cells were plated in 6 well plates at a density of 0.4×10^6 cells/well and incubated for 24 h. The cells were incubated with metformin for 1 h and loaded with 10 μM 2',7'-dichlorofluorescin diacetate (H$_2$DCFDA; Sigma-Aldrich; Merck KGaA) for 30 min at 37°C. Next, they were washed three times with PBS. Fluorescence was measured using flow cytometry. ROS generation was assessed by the dichlorofluorescence in fluorescence intensity (FL-1, 530 nm) of 10,000 cells using the BD FACS Cato II flow cytometer (BD Biosciences).

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., Chicago, IL, USA). At least three independent experiments were performed. The data were expressed as the mean ± standard deviation and P<0.05 was considered to indicate a statistically significant difference.

Results

Metformin induces apoptosis in human renal cell carcinoma A498 cells. Previous studies reported that metformin induces apoptosis in renal cancer and breast cancer cell lines (12,23). To determine the apoptotic effects of metformin on A498 cells, these cells were treated with various concentrations of metformin (0, 5, 7.5 and 10 mM) for 24 h. In a dose-dependent manner, the metformin-stimulated A498 cells demonstrated features of apoptosis, including cell contraction and rounding and segregation of cells from the well (Fig. 1A). As presented in Fig. 1B, treatment of A498 cells with metformin resulted in a dose-dependent increase in sub-G1 populations. Additionally, treatment of A498 cells with metformin stimulated a reduction in the protein levels of the 32-kDa precursor (procaspase-3), along with the concomitant cleavage of PARP, a protein substrate for caspases (Fig. 1C). These results suggested that metformin-treated A498 cells demonstrated an increase in the Sub-G1 population in a dose-dependent manner.
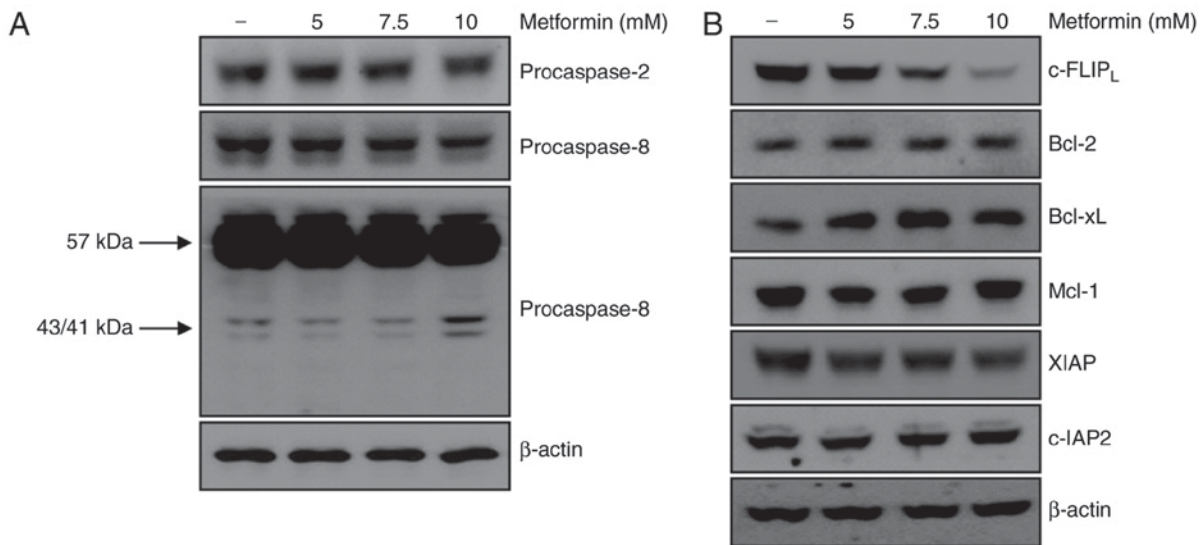
Figure 2. Metformin-induced apoptosis is associated with activation of procaspase-8 and degradation of c-FLIP$_L$. (A) A498 cells were treated with metformin for 24 h, and procaspase-2 and procaspase-8 expression was analyzed using western blotting. β-actin was used as the loading control. (B) A498 cells were treated with various concentrations of metformin for 24 h. c-FLIP$_L$, Bcl-2, Bcl-xL, Mcl-1, XIAP and c-IAP$_2$ expression was determined using western blot analysis. β-actin served as the loading control. 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, baculoviral IAP repeat containing 3.

Metformin-induced apoptosis is modulated through degradation of the c-FLIP$_L$ protein in A498 cells. Caspases are important regulators of apoptotic cell death associated with apoptotic signaling pathways in various cancer cells (24). The present study examined whether activation of the caspase signaling pathway served a key role in metformin-mediated apoptosis. Treatment of A498 cells with metformin did not affect the activation of caspase-2. However, treatment with metformin for 24 h resulted in the appearance of p43/41-kDa fragments of caspase-8 (Fig. 2A). These results demonstrated that caspase-8 activation was involved in metformin-induced apoptosis in A498 cells. The association between metformin-induced apoptosis and regulation of other apoptotic modulators was investigated. As presented in Fig. 2B, protein expression levels of anti-apoptotic molecules including Bcl-2, Bcl-xL, Mcl-1, XIAP and c-IAP$_2$ were not altered by metformin treatment. c-FLIP is a major regulator of the activity of caspase-8 (20). The protein level of c-FLIP$_L$ decreased following metformin treatment in A498 cells in a dose-dependent manner. Taken together, degradation of the c-FLIP$_L$ protein was involved in metformin-induced apoptosis via activation of caspase-8.

Metformin-mediated apoptosis is associated with activation of the caspase signaling pathway. The role of the caspase signaling pathway in metformin-mediated apoptosis was investigated. As presented in Fig. 3A, metformin-induced apoptosis was blocked following pretreatment with a general caspase inhibitor, z-VAD-fmk. Sub-G1 population was markedly decreased by treatment with z-VAD-fmk in the presence of metformin (Fig. 3B). In addition, treatment with z-VAD-fmk prevented the cleavage of PARP and caspase-3 activation (Fig. 3B). These data indicated that metformin-induced apoptosis was mediated by caspase-dependent apoptosis in the presence of z-VAD-fmk.

Metformin-mediated apoptosis is dependent on the degradation of c-FLIP$_L$ protein in A498 cells. The association between caspase-8 activation and metformin-mediated apoptosis was investigated. Treatment with metformin led to c-FLIP$_L$ degradation, which was recovered by z-VAD-fmk (Fig. 4A). To determine whether the mRNA level of c-FLIP$_L$ was associated with protein level in A498 human renal cell carcinoma cells, the c-FLIP$_L$ mRNA level was examined using RT-RCR. As demonstrated in Fig. 4B, c-FLIP$_L$ mRNA level remained constant following metformin treatment in A498 cells at indicated concentrations. A previous study reported that c-FLIP functions as an anti-apoptotic regulator and is overexpressed in various cancer cell lines (25). To determine whether the reduced level of c-FLIP$_L$ was involved in the induction of apoptosis in metformin-treated A498 cells, c-FLIP$_L$ overexpressing cells were established. Overexpression of c-FLIP$_L$ attenuated apoptosis (Fig. 4C) and PARP cleavage (Fig. 4D) induced by metformin. Taken together, metformin-induced apoptosis was associated with the degradation of c-FLIP$_L$ through activation of caspase-8.

ROS is not involved in metformin-mediated apoptosis in A498 cells. ROS is an important regulator of apoptosis (26). A previous report demonstrated that metformin induces ROS production in breast cancer cells (23). Therefore, the capacity of metformin to induce ROS production in A498 human renal carcinoma cells was investigated using flow cytometry. As demonstrated in Fig. 5A, metformin treatment increased ROS production 1 h post-treatment, and pretreatment with the anti-oxidant N-acetyl-L-cysteine (NAC, a ROS scavenger) inhibited metformin-induced ROS production. To confirm whether ROS generation served a key role in metformin-mediated apoptosis, A498 cells were pretreated with NAC for 30 min. Pretreatment with NAC did not inhibit metformin-induced morphological changes and apoptosis in A498 cells (Fig. 5B and C). Furthermore, NAC did not
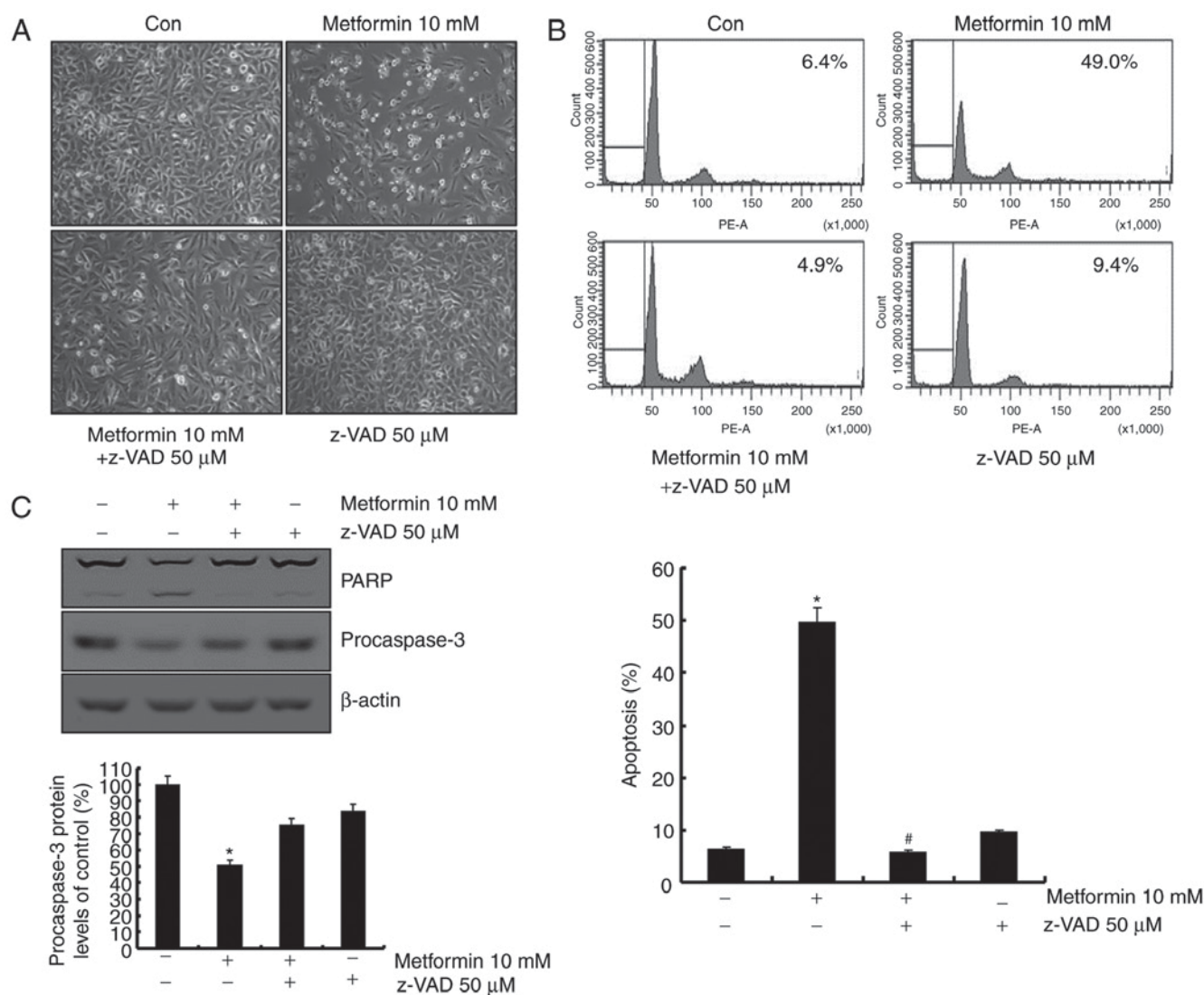
Figure 3. Metformin induces apoptosis by a caspase-dependent signaling pathway in A498 cells. (A) A498 cells were pretreated with 50 μM z-VAD-fmk or a solvent for 30 min and incubated with 10 mM metformin for 24 h. Morphological features were analyzed using light microscopy at x200 magnification. (B) A498 cells were cultured with 50 μM z-VAD-fmk or the vehicle for 30 min prior to treatment with metformin (10 mM) for 24 h. The sub-G1 fraction was determined using flow cytometry. Representative FACS histograms are presented in the upper panel, cumulative data are presented in the lower panel. (C) A498 cells were incubated with 10 mM metformin for 24 h in the presence or absence of 50 μM z-VAD-fmk. PARP, procaspase-3 and β-actin expression was analyzed using western blotting. β-actin was used as the loading control. Band density of procaspase-3 was analyzed using ImageJ software. All data are expressed as the mean ± standard deviation of three independent experiments. *$P<0.05$ compared with untreated cells, #$P<0.05$ compared with metformin-treated cells. z-VAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluorometylhlketone; PARP, poly(ADP-ribose) polymerase.

prevent PARP cleavage, caspase activation and degradation of c-FLIP$_L$ protein in metformin-treated cells (Fig. 5D). These results suggested that ROS generation was not critical for the induction of apoptosis by metformin.

Discussion

Metformin has been used as a therapeutic agent for diabetic patients (27). It was reported to possess various effects, including anti-proliferation, anti-inflammation, anti-angiogenesis and anti-invasion (12,28-30). Metformin also has anti-apoptotic effects on prostate, hepatocellular carcinoma, gall bladder and breast cancer cell lines (31-34). In addition, the insulin receptor may serve a major role in facilitating cancer development via increases in insulin levels (35,36). However, the specific apoptotic mechanism of action of

metformin in human renal cancer cells has not been reported. The present study investigated whether metformin had an anti-cancer effect on human renal cell carcinoma A498 cells. It was revealed that metformin-mediated apoptosis led to the activation of caspase-8 through the downregulation of c-FLIP$_L$ protein expression level in A498 cells.

Apoptosis is the process of programmed cell death that is closely associated with caspase activation. Caspases can be divided into two main groups, namely initiator caspases (caspase-2, -8, -9 and -10) and executioner caspases (caspase-3, -6 and -7) (37). Caspase-8 enhances apoptosis through caspase-3 activation. Caspase-3 is a primary caspase because it is associated with the intrinsic and extrinsic signaling pathways of apoptosis (38). In the present study, the involvement of caspases in metformin-mediated apoptosis was examined. Caspase-8 was activated by metformin,
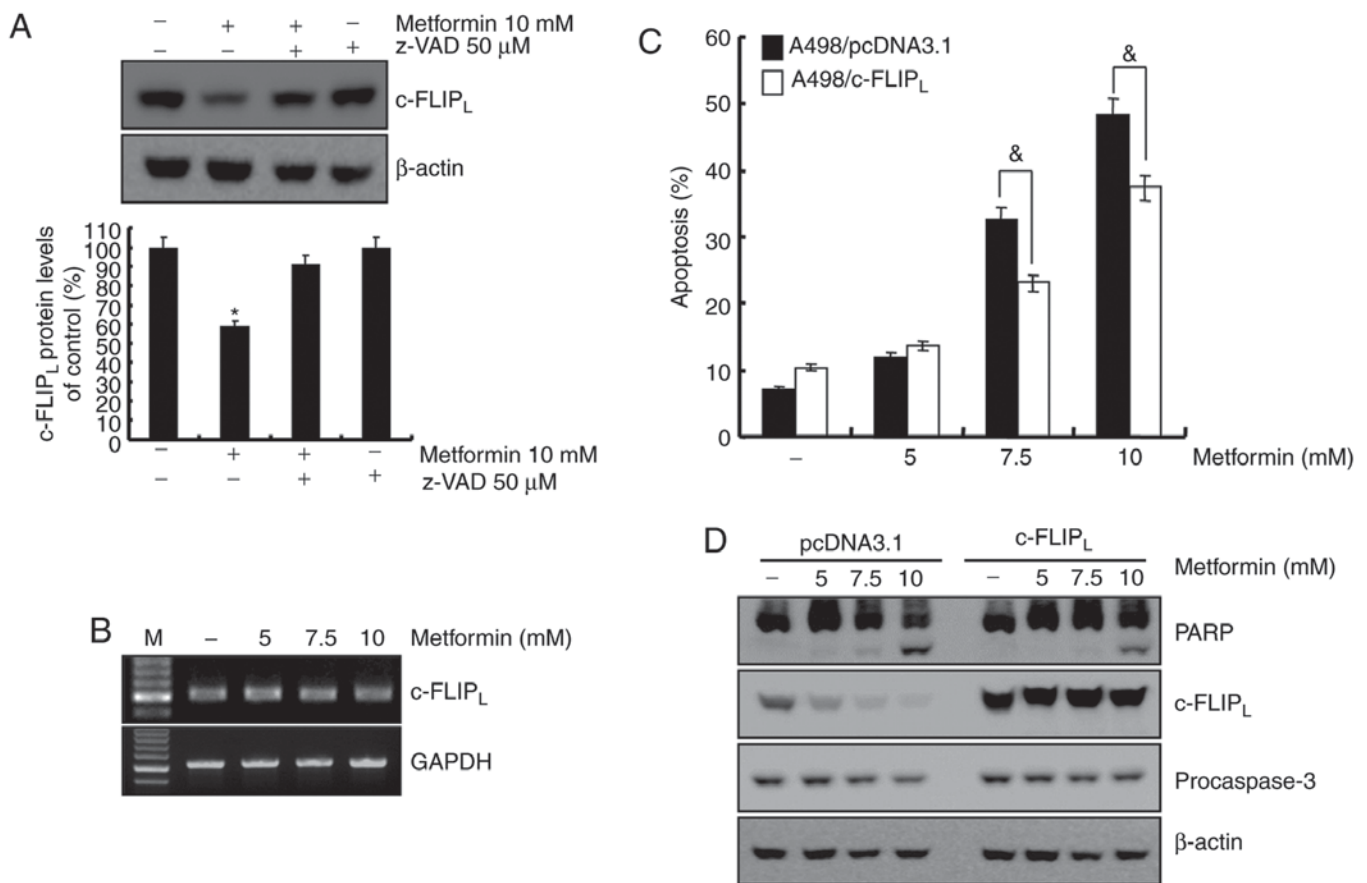
Figure 4. Degradation of c-FLIP$_L$ protein is regulated via a caspase-dependent signaling pathway. (A) A498 cells were treated with 10 mM metformin for 24 h in the presence or absence of 50 mM z-VAD-fmk. c-FLIP$_L$ and β-actin expression was analyzed using western blotting. β-actin was used as the loading control. Band density of the c-FLIPL protein was determined using the ImageJ program. (B) A498 cells were incubated with the indicated concentrations of metformin for 24 h. c-FLIP$_L$ mRNA level was determined using reverse transcription-polymerase chain reaction. (C) A498 cells transfected with pcDNA3.1 or c-FLIP$_L$ plasmid were treated for 24 h with different concentrations of metformin. The sub-G1 cell population was analyzed using flow cytometry. (D) The pcDNA3.1 and c-FLIP$_L$ transfected cells were treated with varying concentrations of metformin. PARP, procaspase-3 and c-FLIP$_L$ expression levels were determined using western blotting. β-actin was used as the loading control. All data are expressed as the mean ± standard deviation of three independent experiments. *P<0.05 compared with untreated cells, &P<0.05 compared with metformin-treated pcDNA3.1 cells. z-VAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoro-me thyl ketone; c-FLIP, cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein; PARP, poly(ADP-ribose) polymerase.

resulting in the emergence of p43/41 kDa fragments (Fig. 2A). Activation of the executioner caspase, caspase-3, was increased via metformin-induced apoptosis (Fig. 1C). In addition, z-VAD-fmk (a pan-caspase inhibitor) completely blocked metformin-induced apoptosis (Fig. 3). These results suggested that metformin-induced apoptosis was associated with caspase activation. Activation of the caspase cascade is modulated via upregulation of pro-apoptotic proteins and/or downregulation of anti-apoptotic proteins (39). In addition, activation of caspase-8 at DISC is blocked via c-FLIP, which inhibits the death receptor-mediated apoptotic pathways (40). In the present study, metformin also decreased c-FLIP$_L$ protein level in A498 cells in a dose-dependent manner (Fig. 2B). However, treatment with metformin did not affect c-FLIP$_L$ mRNA levels (Fig. 4B) and proteasomal degradation (data not shown). Previous reports also indicated that metformin had no effect on c-FLIP$_L$ mRNA level and the proteasomal pathway in non-muscle invasive bladder cancer (NMIBC) (22). It was also revealed that c-FLIP$_L$-over-expressing cells partly prevented metformin-induced apoptosis in A498 cells (Fig. 4). These results suggested that metformin-induced apoptosis occurred via degradation

of c-FLIP$_L$ protein. Metformin has also been reported to suppress the expression levels of anti-apoptotic proteins, including Bcl-2, Bcl-XL, Mcl-1, c-IAP2 and XIAP in primary ovarian cell lines, p53-deficient cells, colorectal and breast cancer cell lines, respectively (41-44). It was demon-strated that Bcl-2, Bcl-XL, Mcl-1, c-IAP2 and XIAP did not affect metformin-treated A498 cells (Fig. 2B). These results demonstrated that suppression of the anti-apoptotic protein, c-FLIP$_L$, was associated with metformin-induced apoptosis in A498 cells.

Reactive oxygen species (ROS) are important mediators of apoptosis in several cancer cell lines (45-48). Previous reports demonstrated that metformin modulated apoptosis through ROS production in hepatoma, ovary and breast cancer cell lines (49,50). Therefore, the association between metformin-mediated apoptosis and ROS generation was investigated. In the present study, metformin was observed to stimulate ROS generation in A498 cells. Pretreatment with N-acetylcysteine did not prevent metformin-induced apoptosis (Fig. 5). These results suggested that metformin-induced ROS generation was not associated with metformin-mediated apoptosis in A498 cells.
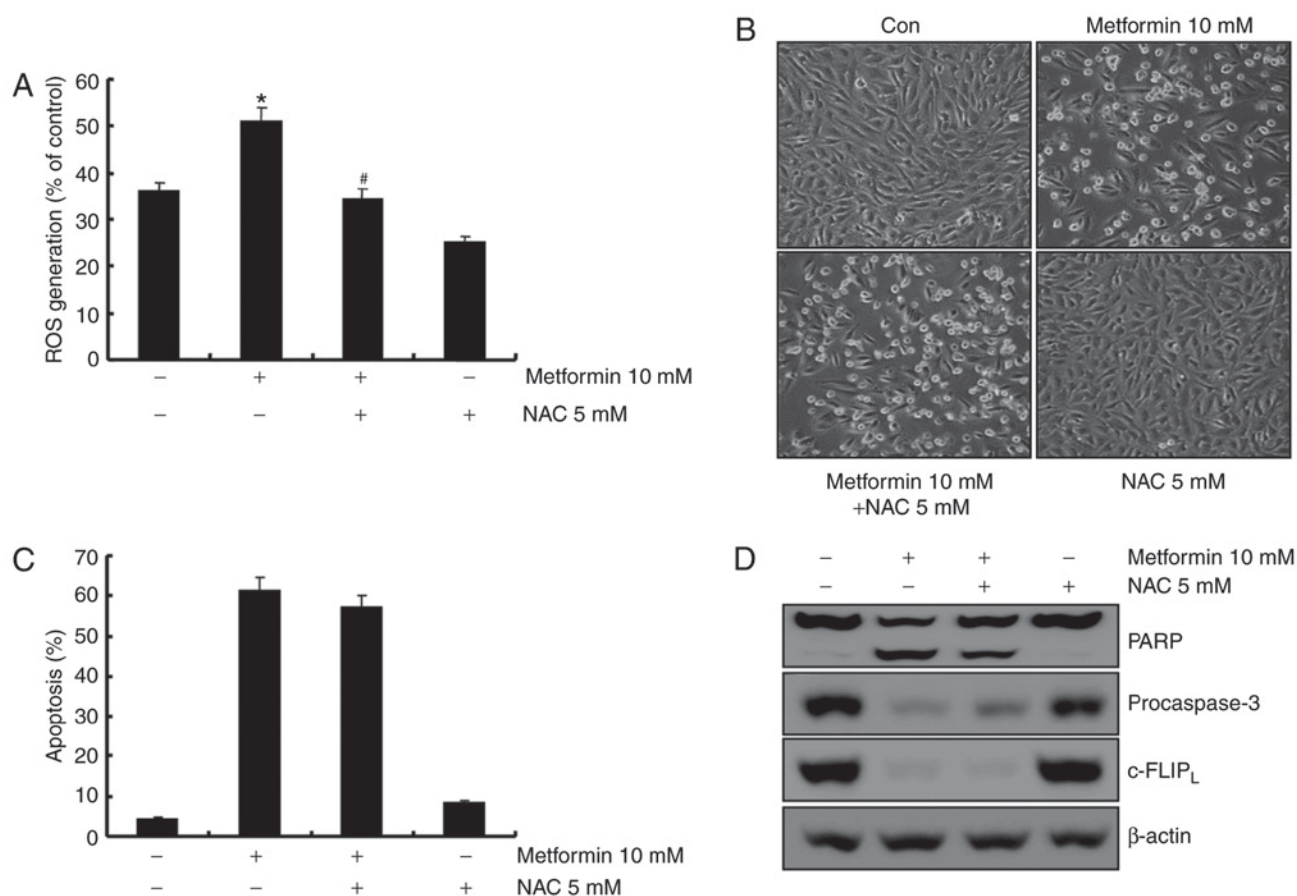
Figure 5. Metformin-mediated apoptosis in A498 cells was not affected by reactive oxygen species generation. (A) Cells were pretreated with 5 mM NAC for 30 min and incubated with 10 mM metformin for 1 h. H_2DCFDA fluorescence was quantified with flow cytometry. (B) A498 cells were incubated with 5 mM NAC or the vehicle for 30 min prior to treatment with 10 mM metformin for 24 h. Morphological changes were analyzed using a light microscope at x200 magnification. (C) A498 cells were pretreated with 5 mM NAC or the solvent for 30 min and incubated in the presence or absence of 10 mM metformin for 24 h. The sub-G1 cell fraction was analyzed using flow cytometry. (D) A498 cells were pretreated with 5 mM NAC or the solvent for 30 min and incubated with 10 mM metformin for 24 h. PARP, procaspase-3, c-FLIP$_L$ and β-actin expression levels were analyzed using western blotting. β-actin was used as the loading control. All data are expressed as the mean ± standard deviation of three independent experiments. $^*P<0.05$ compared with untreated cells, $^#P<0.05$ compared with metformin-treated cells. NAC, N-acetyl-L-cysteine; PARP, poly(ADP-ribose) polymerase; c-FLIP, cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein; ROS, reactive oxygen species.

Taken together, these results demonstrated that metformin-induced apoptosis was mediated by the degradation of c-FLIP$_L$ protein via activation of caspase-8 in A498 human renal cell carcinoma cells. This suggested that metformin can serve the role of a chemotherapeutic agent for diabetes, as well as an anti-cancer agent.

Acknowledgements

Not applicable.

Availability of data and materials

All data generated or analyzed during present study are included within the article.

Authors' contributions

JJ and JK designed the study, collected and analyzed the data. TL, IS and ES advised on the morphological images and performed the data analysis. JJ and JK drafted and wrote the manuscript. TL and JK revised the manuscript critically for intellectual content. All authors gave intellectual input to the study and approved the final version of the manuscript.

Ethics approval and consent to participate

This research was approved by the Ethics Committee of Yeungnam University (Daegu, South Korea). All procedures were performed according to the ethical standards of Ethics Committee of Yeungnam University and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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