Dapagliflozin induces apoptosis by downregulating cFILP$_L$ and increasing cFILP$_S$ instability in Caki-1 cells

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Received June 2, 2022; Accepted September 8, 2022

DOI: 10.3892/ol.2022.13521

Abstract. Dapagliflozin is a sodium/glucose cotransporter 2 inhibitor used recently to treat patients with type 2 diabetes. A recent study has demonstrated that dapagliflozin induces apoptosis in human renal and breast tumor cells. However, to the best of our knowledge, the molecular mechanism underlying dapagliflozin-mediated apoptosis in Caki-1 human renal carcinoma cells has not been elucidated. The present study demonstrated that the dapagliflozin treatment dose-dependently increased cell death in Caki-1 cells. Dapagliflozin treatment also induced apoptosis as confirmed by FITC-conjugated Annexin V/PI staining. Additionally, treatment with dapagliflozin reduced the expression levels of anti-apoptotic proteins, cellular Fas-associated death domain-like interleukin-1-converting enzyme-inhibitory protein (cFLIP)$_L$ and cFLIP$_S$ in Caki-1 cells. Benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl-ketone inhibited dapagliflozin-induced apoptosis, implying that dapagliflozin-induced apoptosis is regulated by a caspase-dependent pathway. By contrast, N-acetylcysteine had no effect on dapagliflozin-induced apoptosis and downregulation of cFLIP$_L$ and cFLIP$_S$ expression. Furthermore, overexpression of cFLIP$_L$, but not cFLIP$_S$, partially inhibited apoptosis induced by dapagliflozin. cFLIP$_L$ and cFLIP$_S$ mRNA levels remained constant in Caki-1 cells after treatment with 0, 20, 40, 60, 80 and 100 μM dapagliflozin. Notably, it was confirmed that cFLIP$_S$ protein levels were reduced due to the increased cFLIP$_S$ instability in dapagliflozin-treated Caki-1 cells. The present study also demonstrated that dapagliflozin had no effect on HK-2 normal human kidney cells. Taken together, the present study revealed that dapagliflozin induced apoptosis via the downregulation of cFLIP$_L$ and an increase in cFLIP$_S$ instability, suggesting that dapagliflozin may be a feasible drug candidate for the treatment of human renal cancer.

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Key words: dapagliflozin, renal carcinoma, apoptosis, cellular Fas-associated death domain-like interleukin-1-converting enzyme-inhibitory protein

Introduction

Renal carcinoma (RC) is responsible for approximately 90% of all kidney cancers in adults (1). RC is classified into several histological cell types based on the genetic, histological and clinical phenotypes; clear cells, granular cells, mixture cells and undifferentiated cells (2-4). The cancer cells are resistant to radiation, chemical and hormone therapies in RC patients and cannot be treated without surgery (5,6). Therefore, it is essential to identify more efficient chemotherapeutic agents for RC treatment.

Dapagliflozin is a new type 2 diabetes drug that decreases blood glucose levels by inhibition of sodium/glucose cotransporter (SGLT2) in the kidney (7-9). It has been reported that empagliflozin, a SGLT2 inhibitor, mediates apoptosis through inhibition of sonic hedgehog signaling molecule expression and migration by activating adenosine monophosphate-activated protein kinase (AMPK) in cervical cancer (10). Previous studies demonstrate that dapagliflozin exerts anti-proliferative and anti-tumor activity in human kidney and breast tumor cells through cell cycle arrest and apoptosis, tumor growth inhibition or AMPK/mTOR signaling pathways (11,12). Moreover, dapagliflozin reduces tumor volume and activates caspase-3, beclin-1 and JNK in solid Ehrlich carcinoma mice (13). Nevertheless, the mechanism underlying dapagliflozin-induced apoptosis has not been presented in human RC.

The cellular Fas-associated death domain-like interleukin-1-converting enzyme-inhibitory protein (cFLIP) is an important apoptosis-regulatory protein associated with apoptosis (14). cFLIP has cFLIP$_L$, cFLIP$_S$ and cFLIP$_R$ isoforms (15). Each of these isoforms has different effects on apoptotic pathways through different mechanisms (16-19). Overexpression of cFLIP suppresses death ligand-mediated cell death and confers resistance to chemotherapeutic agents (20). Constant cFLIP mRNA levels and cFLIP protein stability decrease the sensitivity to anti-cancer drugs in the cFLIP-overexpressing bladder and colorectal cancers (21-23). Hence, modulation of cFLIP, an anti-apoptotic protein, plays a key role in elucidating the mechanism of chemopreventive-mediated apoptosis.

In the present investigation, we showed that dapagliflozin mediated apoptosis in human RC Caki-1 cells by caspase-dependent pathway via the downregulation of cFLIP$_L$ and an increase in cFLIP$_S$ instability.

Materials and methods

Cell culture and materials. Caki-1 cells were purchased from American Type Culture Collection (HTB-46) and maintained in DMEM (LM 001-05; WelGene) with 10% FBS (S001-07; WelGene) and 1% anti-biotic anti-mycotic (AA) solution (LS 203-01; WelGene). HK-2 cells were obtained from the Korean Cell Line Bank (22190) and maintained in RPMI1640 medium (LM 011-01; WelGene) supplemented with 10% FBS and 1% AA solution. The cells were maintained at 37˚C under 5% CO_2 condition. z-VAD-fmk (627610) was obtained from Calbiochem. Dapagliflozin (SC-364481), N-acetylcysteine (NAC; A7250) and cycloheximide (CHX; C1988) were obtained from Sigma-Aldrich.

Cell viability assay. 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assays were analyzed using the Welcount Cell Viability Assay Kit (TR055-01; WelGene). Caki-1 and HK-2 cells were seeded ($0.2x10^5$ cells/well) into three 96-well plates containing DMEM or RPMI1640 with 10% FBS. Cells were expose to 0, 20, 40, 60, 80 and 100 μM of dapagliflozin for 24 h and then cultivated with XTT reagent for 2-3 h at room temperature. The density was measured at 450 nm using a microplate reader (Thermo LabSystems) at 450/690 nm.

FACS analysis. $0.4x10^6$ cells were suspended in 100 μl of cold phosphate-buffered saline (PBS; 70011044; Thermo Fisher Scientific) and 200 μl of 95% ethanol (1.00983.1011; Merck) was mixed, and the sample was while vortexing. The cells were cultivated for 3 h at 4˚C, washed twice with cold PBS, and resuspended in 250 μl of 1.12% sodium citrate buffer (pH 8.4) with 12.5 μg of RNase A (R4875; Sigma-Aldrich; Merck KGaA). The cells were cultivated for 30 min at 37˚C, mixed with 250 μl of 50 μg/ml propidium iodide (PI; P4170; Sigma-Aldrich; Merck KGaA) for 20 min at 37˚C. Cells were analyzed by fluorescence-activated cell sorting (FACS) using CytoFLEX (B53000; Beckman Coulter).

Western blotting. The total cell lysates were prepared by resuspending $0.45x10^6$ cells in 20-50 μl of RIPA lysis buffer (50 mM Tris buffer, 20 mM HEPES, 100 mM NaF; 120 mM NaCl, 0.5% Triton X-100, 100 μM Na_3VO_4, pH 7.6) Total lysates was quantified using a BCA kit (#23225; Thermo Fisher Scientific) according to the manufacturer's protocols. The proteins (30-70 μg) were isolated using 10 or 12% SDS-PAGE gels and electrotransferred onto NC membranes (GE Healthcare). Target proteins were identified using the respective antibodies and Immobilon Western Chemiluminescent HRP Substrate Solution (WBKLS0100; Millipore) and visualized by Davinch-Chemi (CAS-400SM; Davinch-K). Anti-PARP antibody (1:1,000; #9542) and anti-Bax (1:1,000; #2772) were obtain from Cell Signaling Technology. Anti-caspase-3 (1:3,000; ADI-AAP-113) and anti-FLIP (1:700; ALX-804-961-0100) were purchased from Enzo Life Sciences. Anti-Bcl-2 (1:700; sc-7832), anti-Bcl-xL (1:1,000, sc-634), anti-Mcl-1 (1:1,000; sc-12756), anti-cIAP1 (1:1,000; sc-7943), anti-cIAP2 (1:1,000; sc-517317) and anti-β-actin antibody (1:5,000; sc-47778) were supplied by Santa Cruz Biotechnology, and anti-XIAP (1:10,000; 610717) antibody was obtained from BD Biosciences.

Annexin V/PI double staining. Fluorescein isothiocyanate (FITC) Annexin V apoptosis detection kit I (556547; BD Biosciences) was used to determine cell death type. The cells were washed twice with cold PBS, and then cell pellets resuspended in 1X binding buffer. This suspended cells (100 μl) were stained with 5 μl of FITC-conjugated Annexin V and then 5 μl PI. The cells were incubated for 15 min at room temperature in the dark. After adding 400 μl of 1X binding buffer to each tube, the cells were analyzed using CytoFLEX (Beckman Coulter).

RNA isolation and RT-PCR. The levels of $cFLIP_L$ and $cFLIP_S$ mRNA was confirmed using RT-PCR. Total RNA was isolated using Easy-Blue reagent (17061; iNtRON Biotechnology). cDNA was prepared using the M-MLV Reverse Transcriptase (18057018; Thermo Fisher Scientific) according to the manufacturer's protocols. GAPDH was used as an internal control. The primers used to target genes of $cFLIP_L$, $cFLIP_S$ and GAPDH: for $cFLIP_L$, 5'-CGGACTATAGAGTGCTGATGG-3' (forward) and 5'-GATTATCAGGCAGATTCCTAG-3' (reverse); $cFLIP_S$, 5'-TAAGCTGTCTGTCGGGGACT-3' (forward) and 5'-AGA TCAGGACAATGGGCATAG-3' (reverse); GAPDH, 5'-AGG TCGGAGTCAACGGATTTG-3' (forward) and 5'-GTGATG GCATGGACTGTGGT-3' (reverse). The amplified PCR products were separated by electrophoresing on a 1.5% agarose gel with 0.1% ETBR, and the DNA bands were detected by an ultraviolet light gel doc (WGD30; DAIHAN).

Stable transfection. Caki-1 cells were seeded onto 6-well culture plates ($0.25x10^6$ cells/well) and cultivated overnight at 37˚C. Cells were transfected with the pcDNA 3.1-$cFLIP_L$, pcDNA 3.1-$cFLIP_S$ or control pcDNA 3.1 plasmid vectors using LipofectAMINE2000® (11668-019; Thermo Fisher Scientific) in Opti-MEM medium (31985-070; Thermo Fisher Scientific). After 48 h of transfection, the transfected cells were selected using culture medium containing 800 μg/ml G418 (10131-035; Thermo Fisher Scientific). The cells were then exposed to dapagliflozin for 24 h and analyzed for $cFLIP_L$ and $cFLIP_S$ protein expression using western blotting. After 2 or 3 weeks, to rule out the possibility of clonal differences between the generated stable cell lines, pooled Caki-1/pcDNA 3.1, Caki-1/$cFLIP_L$ and Caki-1/$cFLIP_S$ clones were analyzed for $cFLIP_L$ and $cFLIP_S$ protein expression using western blotting.

Statistical analysis. The experiments were performed three independent experiments. One-way ANOVA and post hoc comparisons (Scheffe) and two-way ANOVA followed by post hoc test (Tukey's HSD) were used when comparing the situations. Statistical Package for Social Sciences 27.0 (IBM SPSS Inc.) was utilized for the data analysis. The data were expressed as the mean ± SD, and P-values <0.05 were considered significant.

Results

Dapagliflozin decreases cell viability and induces apoptosis in Caki-1 cells. The anti-cancer effect of dapagliflozin on RC

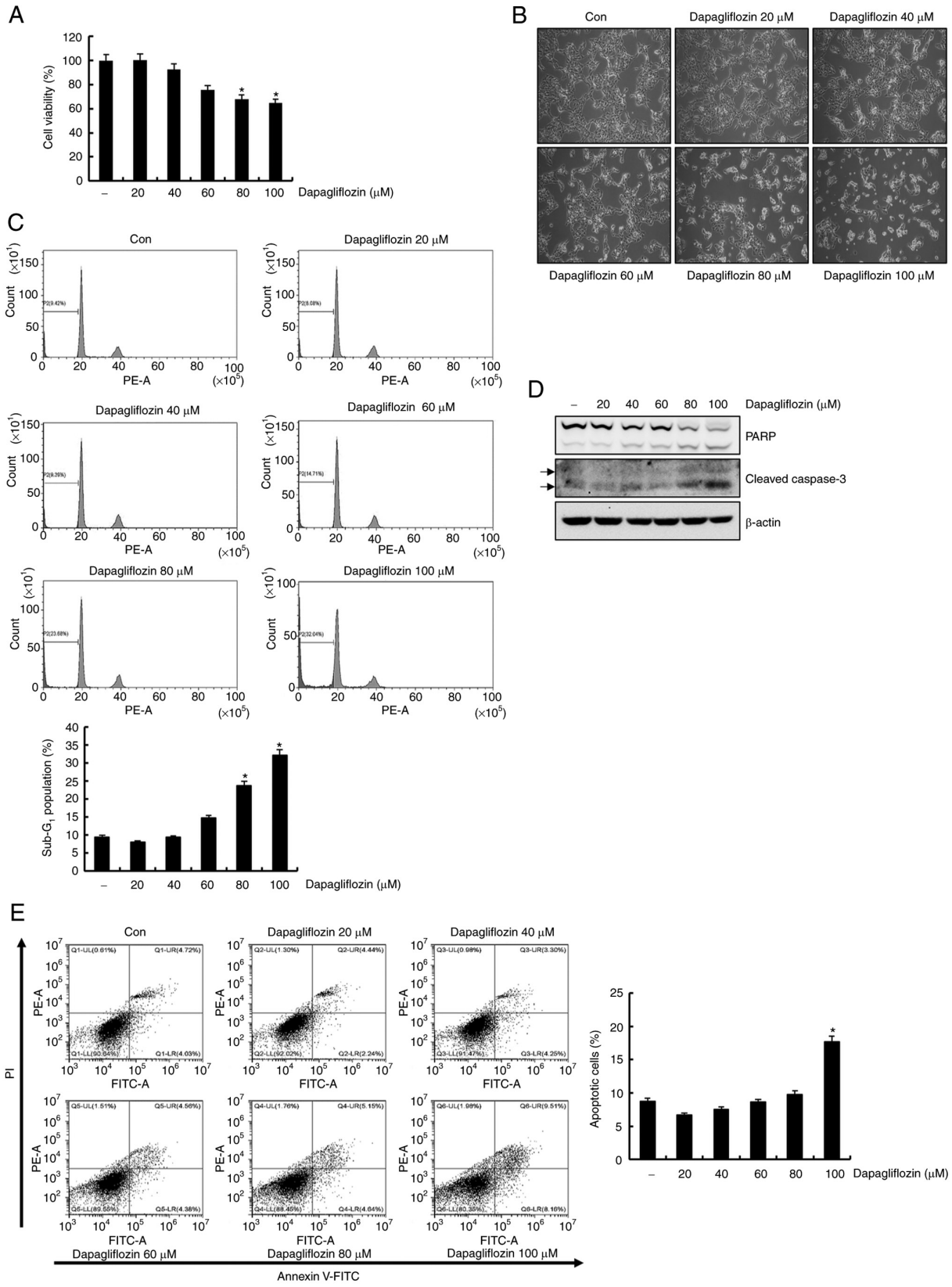

Figure 1. Dapagliflozin induces apoptosis in Caki-1 cells. (A) Cells were treated with dapagliflozin for 24 h. Subsequently, cell viability was analyzed using a 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide assay kit. (B) Caki-1 cells were treated with dapagliflozin for 24 h and morphological findings were observed under a light microscope at a magnification of x200. (C) Caki-1 cells were exposed to dapagliflozin for 24 h. The Sub-G_1 fraction was measured via flow cytometry. The FACS data are indicated in the upper panel. The percentage of the sub-G_1 population is shown in the lower panel. (D) Cells were treated with varying concentrations of dapagliflozin for 24 h. PARP, cleaved caspase-3 and β-actin protein expression was examined by western blotting. β-actin was used as the protein loading control. Cleaved caspase-3 is indicated by arrows. (E) Caki cells were treated with dapagliflozin for 24 h. The type of cell death, which is apoptosis or necrosis, was confirmed via flow cytometry after FITC-conjugated Annexin V/PI staining. The percentage of cells in each quadrant (Q1-UL, necrotic cells; Q1-UR, late apoptotic cells; Q1-LL, live cells; Q1-LR, early apoptotic cells) is indicated (left panel). The percentage of apoptotic cells is shown in the right panel. Data were acquired in three independent experiments and presented as the mean ± SD (n=3). *P<0.05 compared with non-treated cells. Con, dapagliflozin 0 μM; PARP, poly (ADP-ribose) polymerase.

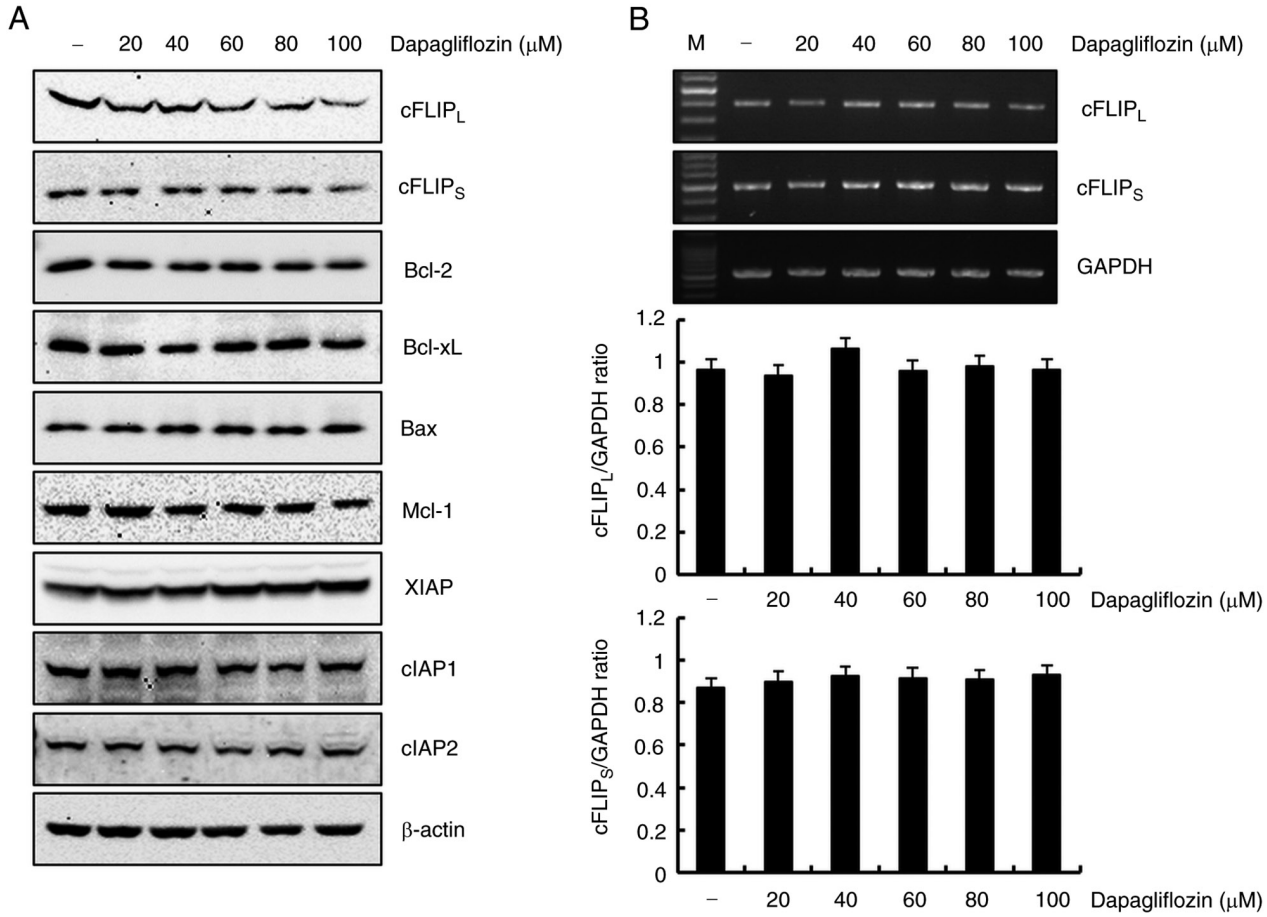

Figure 2. Dapagliflozin inhibits cFLIP$_L$ and cFLIP$_S$ protein expression in Caki-1 cells. (A) Cells were treated with various concentrations of dapagliflozin. At 24 h after treatment, protein expression levels of cFLIP$_L$, cFLIP$_S$, Bcl-2, Bcl-xL, Bax, Mcl-1, XIAP, cIAP1, cIAP2 and β-actin were analyzed using western blotting. β-actin served as the protein loading control. (B) Caki-1 cells were treated with different concentrations dapagliflozin. After 24 h, the levels of cFLIP$_L$, cFLIP$_S$ and GAPDH mRNA (upper panel) were determined using RT-PCR. GAPDH was used as a loading control. The density of cFLIP$_L$, cFLIP$_S$ and GAPDH was analyzed using ImageJ software. Data obtained from RT-PCR of cFLIP$_L$, cFLIP$_S$ and GAPDH were used to evaluate the effect of dapagliflozin on the cFLIP$_L$/GAPDH ratio (middle panel) and cFLIP$_S$/GAPDH ratio (lower panel). Data were acquired in three independent experiments. PARP, poly (ADP-ribose) polymerase; cFLIP, cellular Fas-associated death domain-like interleukin-1-converting enzyme-inhibitory protein Mcl-1, myeloid cell leukemia-1; XIAP, X-linked inhibitor of apoptosis protein; cIAP, cellular inhibitor of apoptosis protein; RT-PCR, reverse transcription PCR; M, marker of RT-PCR.

Caki-1 cells, the cells was investigated by treating the cells with 0, 20, 40, 60, 80 and 100 μM of dapagliflozin. As shown in Fig. 1A, treatment of Caki-1 cells with dapagliflozin showed a dose-dependent reduction in cell viability. High concentrations (80 and 100 μM) dapagliflozin induced the rounded cells of considerable number in Caki-1 cells under light microscopy (Fig. 1B). We then performed flow cytometry analysis of the dapagliflozin-treated Caki-1 cells. Dapagliflozin treatment for 24 h significantly increased the sub-G1 fraction in a dose-dependent manner (Fig. 1C). Exposure to dapagliflozin increased the expression levels of cleavage form of PARP and caspase-3 in Caki-1 cells (Fig. 1D). To determine cell death type induced by dapagliflozin, we analyzed FITC-conjugated Annexin V/PI staining using flow cytometry. Treatment with 100 μM of dapagliflozin increased Annexin V/PI positive cells (Fig. 1E). These observations supported that dapagliflozin induces apoptosis in Caki-1 cells.

Dapagliflozin-induced apoptosis reduces cFLIP$_L$ and cFLIP$_S$ expression levels. The detailed molecular mechanism associated with dapagliflozin-induced apoptosis was studied by treating Caki-1 cells with dapagliflozin and analyzing the expression levels of apoptotic regulatory proteins using western blotting. As shown in Fig. 2A, cFLIP$_L$ and cFLIP$_S$ protein expressions markedly reduced in dapagliflozin-treated Caki-1 cells. However, protein expression levels of Bcl-2, Bcl-xL, Bax, Mcl-1, XIAP, cIAP1 and cIAP2 did not change in response to dapagliflozin treatment. These results demonstrated that dapagliflozin-induced apoptosis suppressed the expression of cFLIP$_L$ and cFLIP$_S$ in Caki-1 cells. To determine whether the dapagliflozin-mediated cFLIP$_L$ and cFLIP$_S$ reduction in protein expression was regulated at the transcriptional level, we confirmed RT-PCR. Exposure Caki-1 cells to dapagliflozin had no effect on cFLIP$_L$ or cFLIP$_S$ expression at the transcriptional level (Fig. 2B). Therefore, dapagliflozin-mediated downregulation of cFLIP$_L$ and cFLIP$_S$ expression levels is modulated at the post-transcriptional level.

Dapagliflozin-mediated apoptosis is inhibited via a caspase signaling pathway. To identify whether activation of the caspase signaling pathway plays an important role in dapagliflozin-mediated apoptosis, Caki-1 cells were

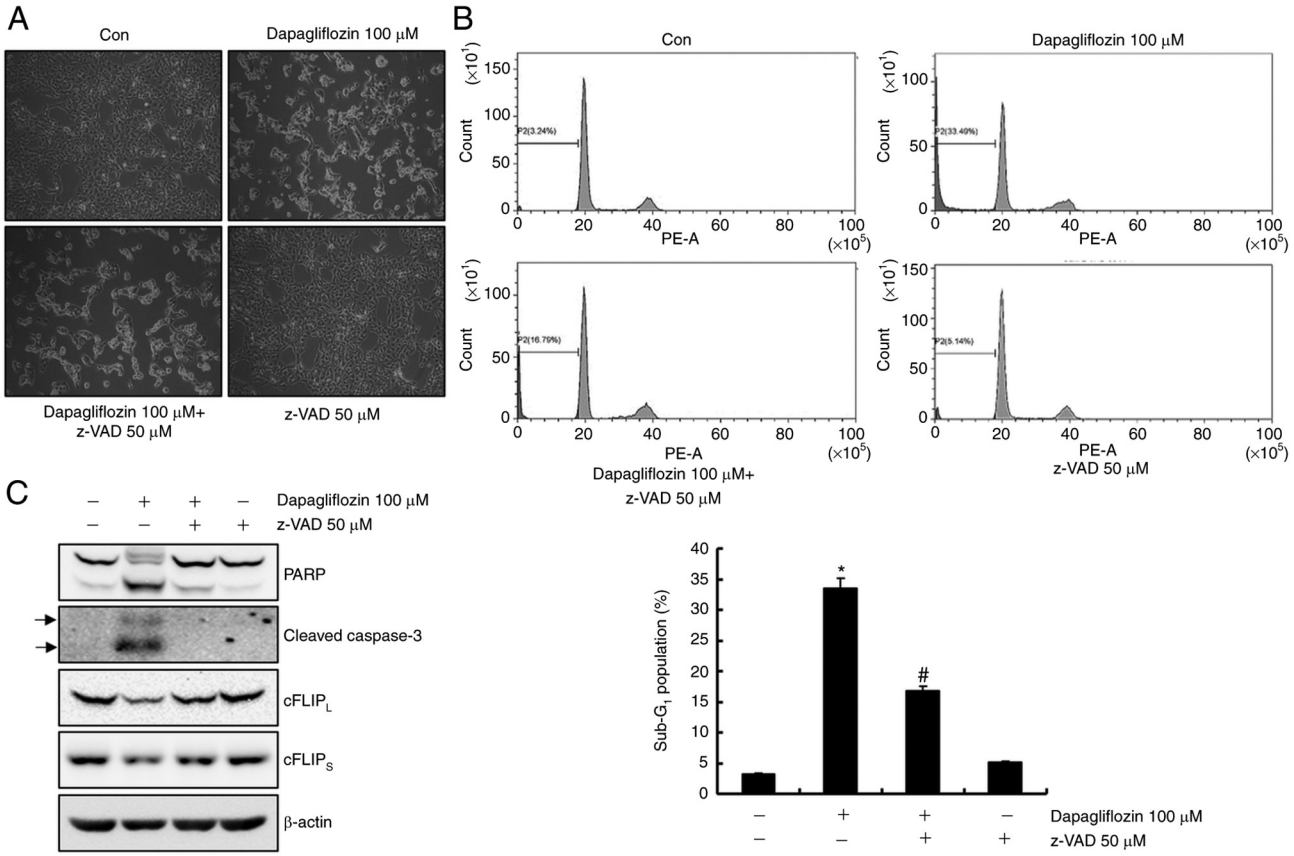

Figure 3. Dapagliflozin-mediated apoptosis is blocked by a caspase signaling pathway in Caki-1 cells. (A) Cells were treated with 100 μM dapagliflozin for 24 h in the presence or absence of z-VAD-fmk. Morphological changes were visualized by light microscopy at a magnification of x200. (B) Caki-1 cells were pretreated with 50 μM z-VAD-fmk or solvent for 30 min and cultivated with dapagliflozin for 24 h. The Sub-G_1 fraction was analyzed by FACS. The FACS data are presented in the upper panel. The percentage of the sub-G_1 population is shown in the lower panel. (C) Caki-1 cells were pretreated with 50 μM z-VAD-fmk or solvent for 30 min and cultivated with 100 μM dapagliflozin for 24 h. Protein expression levels of PARP, cleaved caspase-3, cFLIP$_L$, cFLIP$_S$ and β-actin were examined by western blotting. β-actin was used as a loading control. Cleaved caspase-3 is indicated by arrows. Data were acquired in three independent experiments and are presented as the mean ± SD (n=3). *P<0.05 compared with untreated cells, $^\#$P<0.05 compared with dapagliflozin-treated cells. Con, dapagliflozin 0 μM; z-VAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl-ketone; PARP, poly (ADP-ribose) polymerase; cFLIP, cellular Fas-associated death domain-like interleukin-1-converting enzyme-inhibitory protein.

pretreatment with a pan-caspase inhibitor, z-VAD-fmk. As shown in Fig, 3A and B, pretreatment with z-VAD-fmk inhibited dapagliflozin-mediated apoptosis. Moreover, pretreatment of Caki-1 cells with z-VAD-fmk prevented the cleavage forms of PARP and caspase-3 and restored the cFLIP$_L$ and cFLIP$_S$ expressions levels (Fig. 3C). These findings suggest that dapagliflozin-induced apoptosis is modulated by a caspase signaling pathway through cFLIP$_L$ and cFLIP$_S$ downregulation in Caki-1 cells.

Dapagliflozin-mediated apoptosis is not involved in reactive oxygen species (ROS). ROS causes apoptosis by modulating the expression levels of cFLIP (24). Caki-1 cells were pretreated with the ROS scavenger, NAC, for 1 h and then cultivated with dapagliflozin for 24 h to investigate whether ROS plays a key role in dapagliflozin-induced apoptosis. As shown in Fig. 4A and B, pretreatment with NAC did not prevent dapagliflozin-mediated apoptosis. Furthermore, NAC failed to prevent PARP cleavage and caspase activation and did not restore cFLIP$_L$ and cFLIP$_S$ expression levels in dapagliflozin-treated cells (Fig. 4C). These data suggest that ROS generation is not affected by dapagliflozin-mediated apoptosis.

Dapagliflozin-mediated apoptosis is partially recovered through cFLIP$_L$ downregulation. To determine whether the downregulation of cFLIP$_L$ and cFLIP$_S$ plays an important role in dapagliflozin-induced apoptosis in Caki-1 cells, cFLIP$_L$- and cFLIP$_S$-overexpressing cells were exposed to dapagliflozin. As shown in Fig. 5A, treatment with dapagliflozin considerably caused apoptosis in Caki-1/vector cells, whereas overexpression of cFLIP$_L$ partially inhibited dapagliflozin-mediated apoptosis. In contrast, the overexpression of cFLIP$_S$ did not prevent dapagliflozin-induced apoptosis (Fig. 5B). The expression of cleavage forms of PARP and caspase-3 induced by dapagliflozin treatment was partially blocked by the overexpression of cFLIP$_L$ (Fig. 5C). However, treatment with dapagliflozin in Caki-1/cFLIP$_S$ cells did not affect the cleavage forms of PARP and caspase-3 expression levels (Fig. 5D). These results reveal that the downregulation of cFLIP$_L$ contributes to dapagliflozin-mediated apoptosis. In addition, dapagliflozin may mediate apoptosis in Caki-1/vector cells and even in cFLIP$_S$-overexpressed cells.

Dapagliflozin reduces the expression level of cFLIP$_S$ ascribed by the increase in protein instability. To further investigate

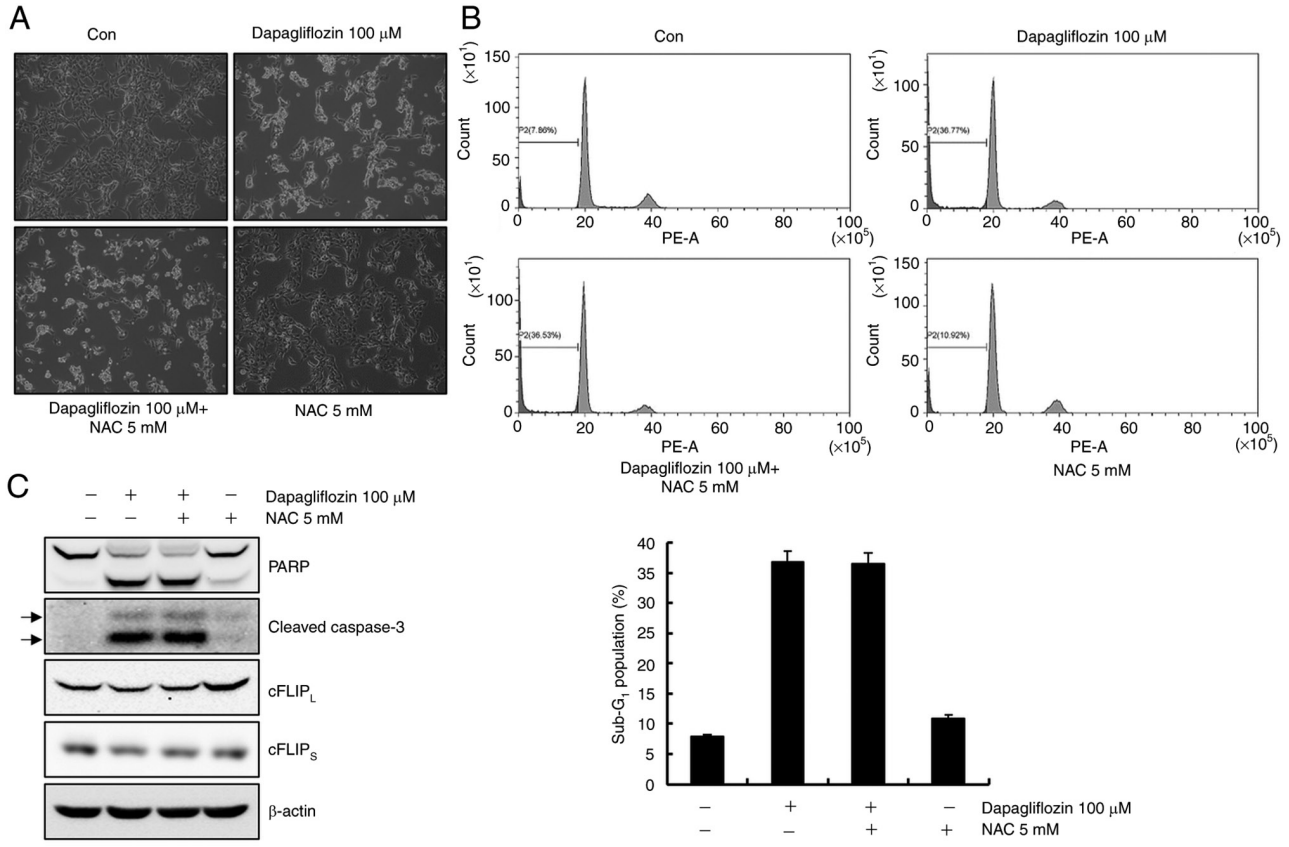

Figure 4. Dapagliflozin-induced apoptosis in Caki-1 cells is not affected by reactive oxygen species. (A) Caki-1 cells were pretreated with 5 mM NAC or vehicle for 1 h and then treated with dapagliflozin for 24 h. Morphological changes were observed by light microscopy at a magnification of x200. (B) Percentages of the Sub-G_1 population were measured by flow cytometry. The FACS data are presented in the upper panel. The percentage of the sub-G_1 population is shown in the lower panel. (C) Caki-1 cells were treated with 5 mM NAC or a vehicle for 1 h before treatment with 100 μM dapagliflozin for 24 h. Protein expression levels of PARP, cleaved caspase-3, cFLIP$_L$, cFLIP$_S$ and β-actin were detected using western blotting. β-actin was used as a loading control. Cleaved caspase-3 is indicated by arrows. Data were acquired in three independent experiments and presented as the mean ± SD (n=3). Con, dapagliflozin 0 μM; NAC, N-acetylcysteine; FACS, fluorescence-activated cell sorting; PARP, poly (ADP-ribose) polymerase; cFLIP, cellular Fas-associated death domain-like interleukin-1-converting enzyme-inhibitory protein.

the molecular mechanism underlying the reduction of cFLIP$_L$ and cFLIP$_S$ expression levels in dapagliflozin-treated cells, we studied protein stability assays of cFLIP$_L$ and cFLIP$_S$. Treatment with dapagliflozin did not affect cFLIP$_L$ or cFLIP$_S$ expression at the transcriptional level (Fig. 2B). After pretreating Caki-1 cells with CHX for 1 h, the cells were treated with dapagliflozin for varying lengths of time; degradation of the cFLIP$_S$ was promoted by dapagliflozin treatment, but not by cFLIP$_L$ (Fig. 6). These findings indicate that the degradation of cFLIP$_S$ protein is facilitated by dapagliflozin treatment and that dapagliflozin treatment induces cFLIP$_S$ protein instability.

Dapagliflozin does not affect cell death in normal human kidney HK-2 cells. We examined the effect of dapagliflozin on normal human kidney HK-2 cells. Dapagliflozin had no effects on cell viability and morphology (Fig. 7A and B). Then, flow cytometry analysis of the dapagliflozin-treated HK-2 cells was conducted. Sub-G1 population was not affected by dapagliflozin treatment (Fig. 7C). Additionally, protein bands of cleavage form of PARP and caspase-3 were not detected in response to dapagliflozin treatment (Fig. 7D). These data indicate that dapagliflozin did not affect cell death in HK-2 cells.

Discussion

In this study, we demonstrated that dapagliflozin exerts potential anti-tumor effects on human RC Caki-1 cells. Dapagliflozin-mediated apoptosis is caused by caspase signaling pathways in Caki-1 cells. Furthermore, the detailed molecular mechanism in dapagliflozin-induced apoptosis is associated with caspase-mediated degradation of cFLIP$_L$ and increase of cFLIP$_S$ instability.

Previous studies have reported that dapagliflozin exerts anti-proliferative and anti-tumor effect (11,12). Consistent with previous studies, our study showed that dapagliflozin treatment significantly increased the sub-G1 fraction in a dose-dependent manner and increased the levels of cleavage forms of PARP and caspase-3. Annexin V/PI positive cells were detected. These findings reveal that dapagliflozin induces apoptosis in Caki-1 cells.

Apoptosis refers to programmed cell death related with caspase activation (25-27). The caspase activation is determined by the regulation of anti- and/or pro-apoptotic proteins (28) Dapagliflozin-mediated downregulation of cFLIP$_L$ and cFLIP$_S$ was caused by their increased degradation, whereas protein expression levels of Bcl-2, Bcl-xL, Bax, Mcl-1, XIAP, cIAP1 and cIAP2 were no changed. These data

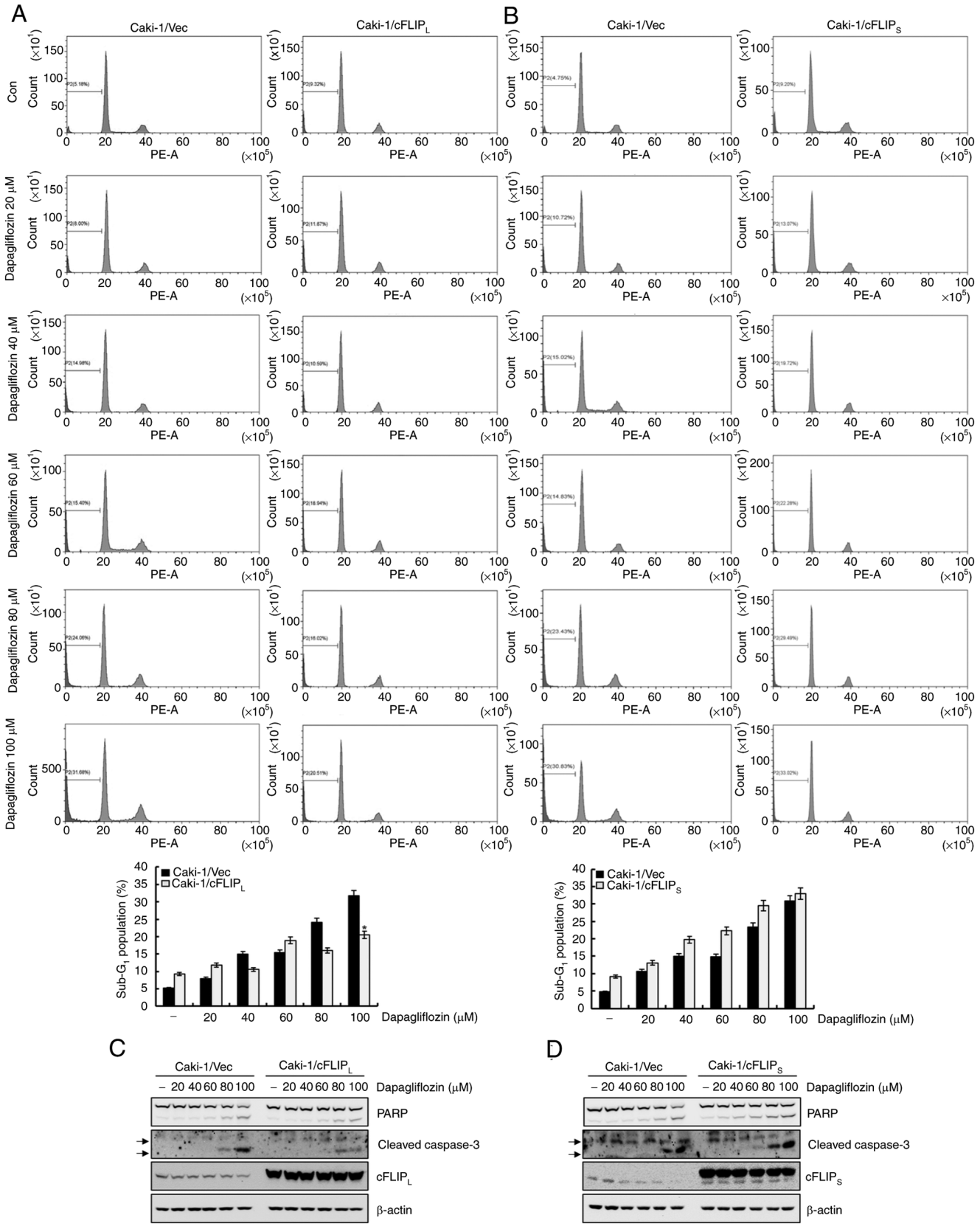

Figure 5. Downregulation of cFLIP$_L$ contributes to dapagliflozin-mediated apoptosis. (A and B) Caki-1/Vec, Caki-1/cFLIP$_L$ and Caki-1/cFLIP$_S$ cells were treated for 24 h with the indicated concentrations of dapagliflozin. The apoptosis levels were determined based on the sub-G$_1$ fraction using flow cytometry. The FACS data are indicated in the upper panels. The percentage of the sub-G$_1$ population is shown in the lower panels. (C) Caki-1/vector and Caki-1/cFLIP$_L$ cells were treated with the indicated concentrations of dapagliflozin. After 24 h, the protein expression levels of PARP, cleaved caspase-3, cFLIP$_L$ and β-actin were analyzed by western blotting. (D) Caki-1/vector and Caki-1/cFLIP$_S$ cells were treated with dapagliflozin for 24 h. Protein expression levels of PARP, cleaved caspase-3, cFLIP$_S$ and β-actin were detected using western blotting. β-actin was used as a loading control. Cleaved caspase-3 is indicated by arrows. Data were acquired in three independent experiments and are presented as the mean ± SD (n=3). *P<0.05 compared with dapagliflozin-treated Caki-1/Vec cells. Vec, vector; Con, dapagliflozin 0 μM; PARP, poly (ADP-ribose) polymerase; cFLIP, cellular Fas-associated death domain-like interleukin-1-converting enzyme-inhibitory protein.

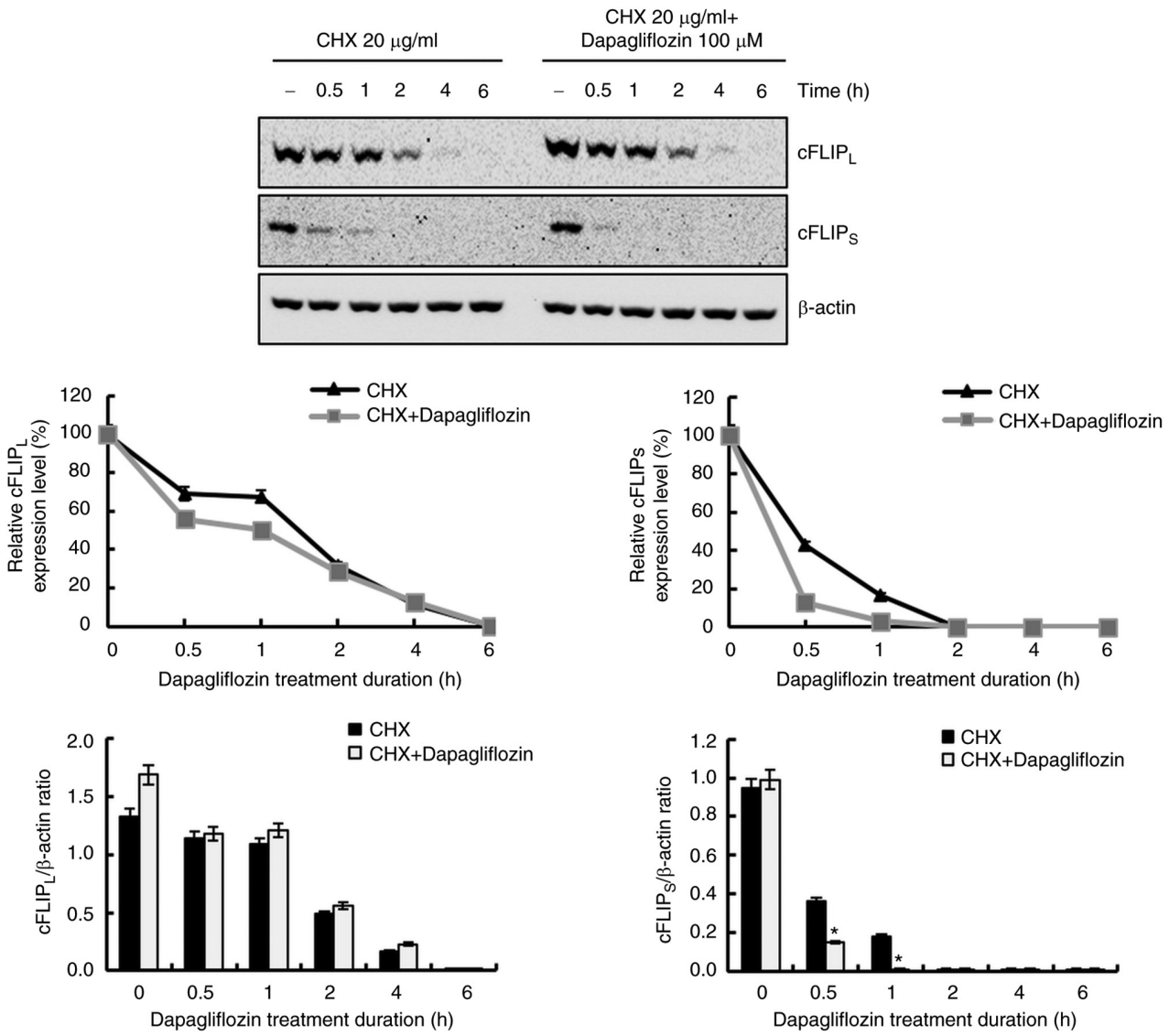

Figure 6. Decrease in cFLIP$_S$ protein levels induced by dapagliflozin treatment occurs due to increased protein instability. Cells were treated with 100 μM dapagliflozin in the presence or absence of 20 μg/ml CHX for the indicated duration. cFLIP$_L$ and cFLIP$_S$ expression levels (upper panel) were detected by western blotting. The density of cFLIP$_L$ and cFLIP$_S$ was analyzed using ImageJ software (middle panel). Data obtained from western blotting of cFLIP$_L$, cFLIP$_S$ and β-actin were used to evaluate the effect of dapagliflozin on the cFLIP$_L$/β-actin ratio and cFLIP$_S$/β-actin ratio (lower panel). β-actin served as a loading control. Data were acquired in three independent experiments and are presented as the mean ± SD (n=3). *P<0.05 compared with CHX only-treated Caki-1 cells. CHX, cycloheximide; cFLIP, cellular Fas-associated death domain-like interleukin-1-converting enzyme-inhibitory protein.

indicate that dapagliflozin-induced apoptosis decreases the expression levels of cFLIP$_L$ and cFLIP$_S$.

cFLIP is a regulator of the apoptotic signaling pathway and is expressed in various cancer cell lines (15,29). Previous studies have demonstrated that cFLIP expression levels are modulated at the proteasome-mediated post-translational level (30,31). In this study, dapagliflozin treatment did not alter cFLIP$_L$ and cFLIP$_S$ mRNA levels. The findings suggest that the dapagliflozin-mediated reduction in the cFLIP$_L$ and cFLIP$_S$ expression levels is modulated at the post-translational level.

Caspase activation regulates apoptosis-regulatory proteins (32,33). Previous studies have shown that dapagliflozin does not affect caspase activation in colon cancer cell lines (34). Contrary to these studies, the present study showed that pretreatment with z-VAD-fmk inhibited sub-G1 cell accumulation, cleavage forms of PARP and caspase-3, and restored the cFLIP$_L$ and cFLIP$_S$ expression levels. These results suggested that dapagliflozin-induced apoptosis might be modulated by the caspase signaling pathway through the downregulation of cFLIP$_L$ and cFLIP$_S$ in Caki-1 cells.

Previous investigations have shown that overexpression of cFLIP modulates apoptosis in several cancer cell lines (35-37). In the present study, overexpression of cFLIP$_L$ partially inhibited dapagliflozin-induced apoptosis, while the overexpression of cFLIP$_S$ failed to inhibit dapagliflozin-induced apoptosis. These data indicate that dapagliflozin-mediated apoptosis is blocked in cFLIP$_L$-overexpressing cells, implying that dapagliflozin-induced apoptosis occurs by the downregulation of cFLIP$_L$.

It has been reported that the downregulation of cFLIP$_S$ is occurred by the increase in protein instability during endoplasmic reticulum (ER) stress-induced apoptosis in human colon tumor cells (38). In the present study, reduction

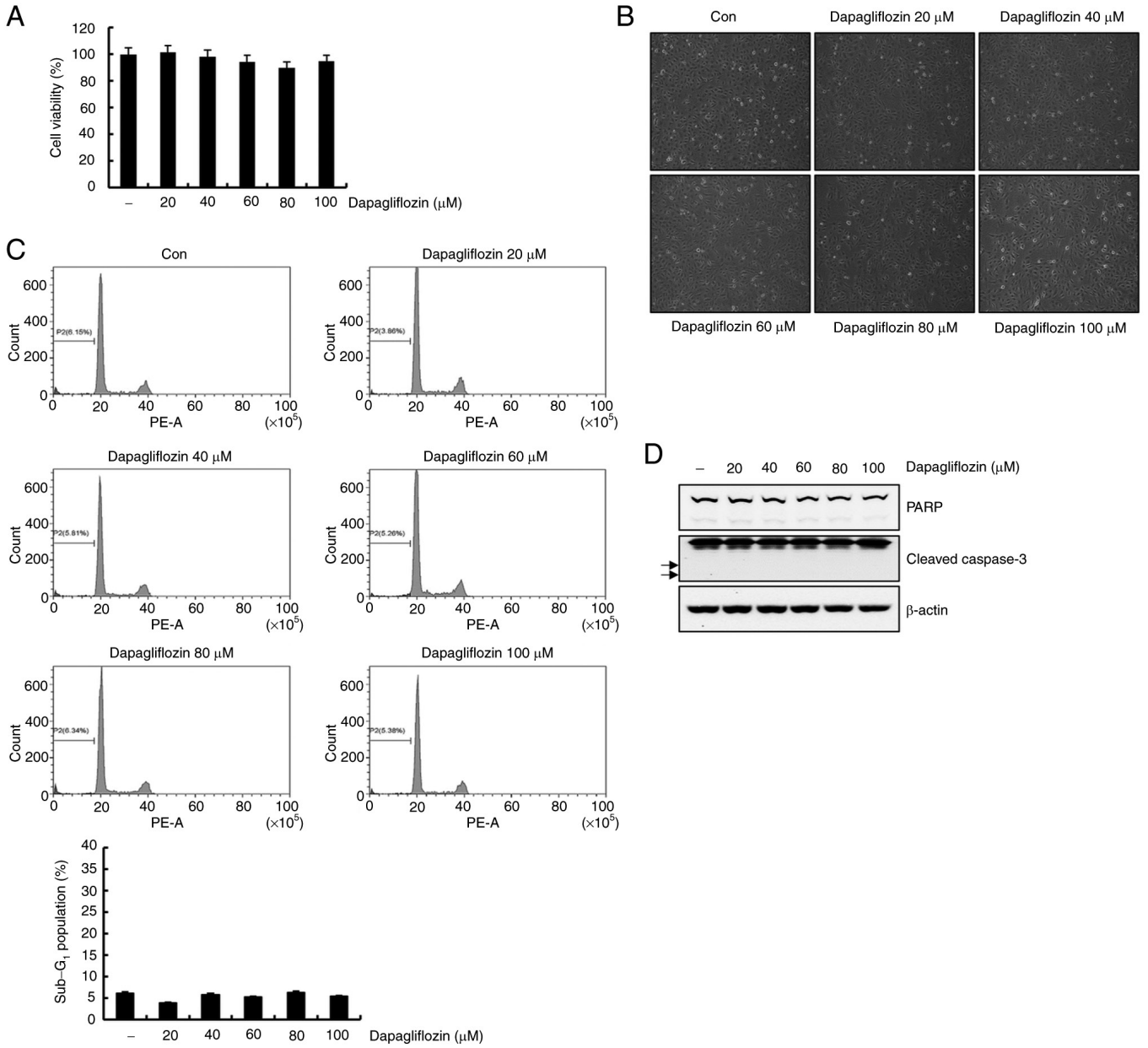

Figure 7. Dapagliflozin does not affect HK-2 cell death. (A) Cells were treated with various concentrations of dapagliflozin. After 24 h, cell viability was measured using a 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide assay kit. (B) HK-2 cells were exposed to dapagliflozin for 24 h. The morphological findings were visualized by light microscope at a magnification of x200. (C) Cells were treated with dapagliflozin for 24 h. The Sub-G_1 population was analyzed by flow cytometry. The FACS data are indicated in the upper panel. The percentage of the sub-G_1 population is shown in the lower panel. (D) HK-2 cells were treated with dapagliflozin for 24 h. PARP, cleaved caspase-3 and β-actin protein expression levels were examined by western blotting. β-actin was used as the protein loading control. Cleaved caspase-3 is indicated by arrows but not detected. Data were acquired in three independent experiments and presented as the mean ± SD (n=3). Con, dapagliflozin 0 μM; PARP, poly (ADP-ribose) polymerase.

of $cFLIP_S$ expression was ascribed by the increased protein instability of $cFLIP_S$ in dapagliflozin-treated cells. This demonstrated that dapagliflozin facilitated the degradation of the $cFLIP_S$, leading to increase instability of $cFLIP_S$.

ROS is an important apoptosis regulator in human cancer cells (39,40). Previous studies reported that ROS regulates cFLIP expression and increases apoptosis (41,42). In the present study, pretreatment with of Caki-1 cells did not inhibit dapagliflozin-mediated apoptosis. These data suggest that dapagliflozin-mediated apoptosis is independent of ROS generation. However, recent studies have shown that dapagliflozin decreases ROS production (43-45). Consistent with the present study, dapagliflozin appears to be a feasible option for reducing ROS production.

Interestingly, dapagliflozin suppresses ER stress-induced apoptosis in normal HK-2 cells (46). In contrast, dapagliflozin did not affect cell death in HK-2 cells in this study.

Taken together, our data indicates that dapagliflozin-induced apoptosis is modulated by caspase signaling pathways through the downregulation of $cFLIP_L$ and an increase in $cFLIP_S$ protein instability in Caki-1 cells. In conclusion, dapagliflozin is a potential chemotherapeutic agent against human renal cancer.

Acknowledgements

Not applicable.

Funding

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (grant no. 2020R 1I1A1A01068857).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JYK conceived and designed the experiments. JHJ performed most of the experiments and analyzed the data. TJL, EGS and IHS conducted data analyses for light microscopy, Annexin V/PI staining and RT-PCR. JHJ drafted and wrote the manuscript. JYK revised the manuscript accordingly. JHJ provided the funding. JHJ and JYK confirmed 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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