Dapagliflozin induces apoptosis by downregulating $cFLIP_L$ and increasing $cFLIP_S$ instability in Caki-1 cells

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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. Benzylxoycarbonyl-Val-Ala-Asp-fluoromethyl-ketone inhibited dapagliflozin-induced apoptosis, implying that dapagliflozin-induced apoptosis is regulated by a caspase-dependent pathway. By contrast, N-acetylcycteine had no effect on dapagliflozin-induced apoptosis and downregulation of cFLIP$_L$ and cFLIP$_S$ expression. Furthermore, overexpression of cFLIP$_S$, but not cFLIP$_L$, 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.

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.

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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% CO₂ 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 (0.2x10⁵ 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 10% FBS. 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 cFLIPₐ and cFLIPₕ mRNA was confirmed using RT-PCR. Total RNA was isolated using Easy-Blue reagent (17061; niRON 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 cFLIPₐ, cFLIPₕ, and GAPDH: for cFLIPₐ, 5'-CGGACTATAGAGTCTGATG-3' (forward) and 5'-GATTATCGGCAGATTTG-3' (reverse); for cFLIPₕ, 5'-TAAGCTGTCTGTCCGGGACT-3' (forward) and 5'-AGA TCAGGACATGGGCATAG-3' (reverse); GAPDH, 5'-AGG TCGGAGTCACGGATTG-3' (forward) and 5'-GTCGAT GCATGGACTGTG-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; DAHAN).

Stable transfection. Caki-1 cells were seeded onto 6-well culture plates (0.25x10⁵ cells/well) and cultivated overnight at 37°C. Cells were transfected with the pcDNA 3.1-cFLIPₕ, pcDNA 3.1-cFLIPₖ, 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 cFLIPₐ and cFLIPₕ 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/cDNA 3.1, Caki-1/cFLIPₕ, and Caki-1/cFLIPₖ clones were analyzed for cFLIPₖ and cFLIPₕ 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-G1 fraction was measured via flow cytometry. The FACS data are indicated in the upper panel. The percentage of the sub-G1 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.
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<sub>L</sub> and cFLIP<sub>S</sub> 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<sub>L</sub> and cFLIP<sub>S</sub> protein expressions markedly reduced in dapagliflozin-treated Caki-1 cells. However, the protein expression levels of Bcl-2, Bcl-xL, Bax, Mcl-1, XIAP, cIAP1, cIAP2 and β-actin did not change in response to dapagliflozin treatment. These results demonstrated that dapagliflozin-induced apoptosis suppressed the expression of cFLIP<sub>L</sub> and cFLIP<sub>S</sub> in Caki-1 cells.

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
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<sub>L</sub> and cFLIP<sub>S</sub> expressions levels (Fig. 3C). These findings suggest that dapagliflozin-induced apoptosis is modulated by a caspase signaling pathway through cFLIP<sub>L</sub> and cFLIP<sub>S</sub> 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<sub>L</sub> and cFLIP<sub>S</sub> 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<sub>L</sub> downregulation. To determine whether the downregulation of cFLIP<sub>L</sub> and cFLIP<sub>S</sub> plays an important role in dapagliflozin-induced apoptosis in Caki-1 cells, cFLIP<sub>L</sub>- and cFLIP<sub>S</sub>-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<sub>L</sub> partially inhibited dapagliflozin-mediated apoptosis. In contrast, the overexpression of cFLIP<sub>S</sub> 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<sub>L</sub> (Fig. 5C). However, treatment with dapagliflozin in Caki-1/cFLIP<sub>S</sub> cells did not affect the cleavage forms of PARP and caspase-3 expression levels (Fig. 5D). These results reveal that the downregulation of cFLIP<sub>L</sub> contributes to dapagliflozin-mediated apoptosis. In addition, dapagliflozin may mediate apoptosis in Caki-1/vector cells and even in cFLIP<sub>S</sub>-overexpressed cells.

Dapagliflozin reduces the expression level of cFLIP<sub>S</sub> ascribed by the increase in protein instability. To further investigate
the molecular mechanism underlying the reduction of cFLIP<sub>L</sub> and cFLIP<sub>S</sub> expression levels in dapagliflozin-treated cells, we studied protein stability assays of cFLIP<sub>L</sub> and cFLIP<sub>S</sub>. Treatment with dapagliflozin did not affect cFLIP<sub>L</sub> or cFLIP<sub>S</sub> 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<sub>S</sub> was promoted by dapagliflozin treatment, but not by cFLIP<sub>L</sub> (Fig. 6). These findings indicate that the degradation of cFLIP<sub>S</sub> protein is facilitated by dapagliflozin treatment and that dapagliflozin treatment induces cFLIP<sub>S</sub> protein instability.

**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<sub>L</sub> and increase of cFLIP<sub>S</sub> 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<sub>L</sub> and cFLIP<sub>S</sub> 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

**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.

**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<sub>1</sub> population were measured by flow cytometry. The FACS data are presented in the upper panel. The percentage of the sub-G<sub>1</sub> 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<sub>L</sub>, cFLIP<sub>S</sub> 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.
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-G1 fraction using flow cytometry. The FACS data are indicated in the upper panels. The percentage of the sub-G1 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.
indicate that dapagliflozin-induced apoptosis decreases the expression levels of cFLIP<sub>L</sub> and cFLIP<sub>S</sub>.

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<sub>L</sub> and cFLIP<sub>S</sub> mRNA levels. The findings suggest that the dapagliflozin-mediated reduction in the cFLIP<sub>L</sub> and cFLIP<sub>S</sub> 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<sub>L</sub> and cFLIP<sub>S</sub> expression levels. These results suggested that dapagliflozin-induced apoptosis might be modulated by the caspase signaling pathway through the downregulation of cFLIP<sub>L</sub> and cFLIP<sub>S</sub> 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<sub>L</sub> partially inhibited dapagliflozin-induced apoptosis, while the overexpression of cFLIP<sub>S</sub> failed to inhibit dapagliflozin-induced apoptosis. These data indicate that dapagliflozin-mediated apoptosis is blocked in cFLIP<sub>L</sub>-over-expressing cells, implying that dapagliflozin-induced apoptosis occurs by the downregulation of cFLIP<sub>L</sub>.

It has been reported that the downregulation of cFLIP<sub>S</sub> 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
of cFLIP<sub>S</sub> expression was ascribed by the increased protein instability of cFLIP<sub>S</sub> in dapagliflozin-treated cells. This demonstrated that dapagliflozin facilitated the degrada-
dition of the cFLIP<sub>S</sub>, leading to increase instability of
cFLIP<sub>S</sub>.

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, dapa-
gliflozin 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<sub>L</sub> and an
increase in cFLIP<sub>S</sub> protein instability in Caki-1 cells. In
conclusion, dapagliflozin is a potential chemotherapeutic
agent against human renal cancer.
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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 JYK conceived and designed the experiments. JHJ performed 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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