

Metformin induces caspase-dependent and caspase-independent apoptosis in human bladder cancer T24 cells

Ji Hoon Jang, Eon-Gi Sung, In-Hwan Song, Tae-Jin Lee* and Joo-Young Kim*

Bladder cancer (BC) is the sixth most common cancer in men. Moreover, chemotherapy for BC leads to various side effects. Metformin is known to induce apoptosis in vitro in many types of cancer. Furthermore, it has feasibility as a drug repositioning used for the treatment of cancer. The molecular mechanism of metformin mediating apoptosis in BC is still unclear. In this study, we showed that metformin stimulated the caspase-dependent apoptotic signaling pathway in T24 cells, a human BC cell line. Moreover, the induced apoptosis was partially inhibited by a general caspase inhibitor, z-VAD-fmk, which suggested that metformin-induced apoptosis in T24 cells is partially caspase-independent. Notably, we observed the nuclear translocation of apoptosis-inducing factors (AIFs) in metformin-promoted apoptosis, which is a typical characteristic of the caspase-independent apoptotic pathway. In addition, we found that metformin-mediated apoptosis occurred via degradation of the cellular FADD-like interleukin-1 β -converting enzyme inhibitory protein (c-FLIP) by facilitating ubiquitin/proteasome-mediated c-FLIP_L degradation. Furthermore, treatment with the reactive oxygen species scavenger N-acetylcysteine, failed to

suppress metformin-induced apoptosis and c-FLIP_L protein degradation in metformin-treated T24 cells. In conclusion, these results indicate that metformin-induced apoptosis was mediated through AIF-promoted caspase-independent pathways as well as caspase-dependent pathways in T24 cells. As such, metformin could be used as a possible apoptotic agent for the treatment of BC. *Anti-Cancer Drugs* 31: 655–662 Copyright © 2020 The Author(s). Published by Wolters Kluwer Health, Inc.

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Introduction

Bladder cancer (BC) is associated with several risk factors, such as smoking, bladder infection, radiation therapy and exposure to specific chemicals [1,2]. Moreover, BC is a common cancer worldwide, with a higher incidence for men than for women [3]. However, current treatments for BC, such as surgery, radiation and chemotherapy are associated with various side effects. Therefore, it is necessary to find a more efficient chemotherapeutic agent for BC.

Metformin has feasibility as a drug repositioning used for the treatment of cancer [4]. Its mechanism involves the degradation of blood glucose via activation of the AMP-activated protein kinase (AMPK). The AMPK regulates ATP and NADPH levels and therefore regulates cell survival and death [5,6]. The activation of AMPK occurs via suppression of mammalian target of rapamycin signaling [7]. Thus, metformin, like other AMPK modulators, is considered an important anti-cancer agent in establishing novel therapeutic strategies for human cancers [8,9]. Previous studies have reported that metformin could

prevent cancer cell growth and proliferation [10,11]. It was also shown to activate apoptotic pathways in several kinds of cancer cells, such as colorectal cancer, cervical cancer, thyroid cancer, bile duct cancer, lung cancer, megakaryoblastic cells and glioma cells via the signal transducer and activator of transcription 3 and transforming growth factor- β /SMAD signaling pathways, cyclin D1 and p53 expression, endoplasmic reticulum stress, insulin-like growth factor (IGF) 1 receptor pathways, invasion, ERK1/2, JNK or PI3K/Akt pathway and migration, respectively [12–18]. Nevertheless, the underlying molecular mechanisms of its anti-cancer effects have not been elucidated in human BC T24 cells. As such, the purpose of this study was to verify the anti-cancer properties of metformin in BC T24 cells and explain its underlying molecular mechanism.

Apoptosis-inducing factor (AIF) is associated with control of the programmed cell death process that is initiated by the mitochondrial signaling pathway also known as the intrinsic pathway [19]. Moreover, the nuclear translocation of AIF is involved in the caspase-independent pathway. Many kinds of cancer cells have mutations in the major apoptotic genes. Furthermore, cancer cells have a resistance to existing drugs, which poses an obstacle in the use of chemotherapeutics for cancer treatment. Thus, the activation of the caspase-independent apoptosis

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signaling pathway has become an aggressive approach to kill cancer cells [20–24].

In the present study, we showed that metformin induces apoptosis in BC T24 cells by activating both caspase-dependent and caspase-independent apoptotic pathways via nuclear translocation of AIF. Additionally, metformin-modulated apoptosis occurs via c-FLIP_L downregulation, by facilitating the ubiquitin/proteasome-mediated pathway.

Materials and methods

Cell culture media and reagents

Human BC T24 cells were obtained from the American Type Culture Collection (Manassas, Virginia, USA). The BC cell line T24 were maintained in Roswell Park Memorial Institute (RPMI)1640 (Welgene, Kyungsan, Korea) medium, containing 10% fetal bovine serum (FBS; Welgene), 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Sigma, St. Louis, Missouri, USA) and 100 µg/ml gentamicin (Invitrogen, South San Francisco, California, USA) was used for the cell culture medium. Cells were cultivated at 37°C in a humidified atmosphere with 5% CO₂. Metformin, N-acetylcysteine (NAC) and cycloheximide (CHX) were purchased from Sigma (Sigma). z-VAD-fmk and MG132 were obtained from Calbiochem (San Diego, California, USA).

Effect on cell morphology

T24 cells were treated with different concentrations of metformin (0, 3, 6, 9, 12 and 15 mM) for 24 h. They were also treated with metformin (12 and 15 mM) with/without inhibitors such as z-VAD-fmk, NAC for 24 h. The morphological changes were examined by an inverted microscope (magnification, ×200) (cat. no. DFC495; Leica, Wetzlar, Germany). The images were taken using the i-Solution program.

Cell viability assay

The XTT assay, performed using the WelCount cell proliferation assay kit (cat. no. TR055-01; WelGene), was used to determine cell viability. T24 cells were seeded at 2.5×10^4 cells/well in two 96-well plates containing RPMI1640 supplemented with 10% FBS. After treatment with metformin for 24 h, cells were incubated with XTT reagent working solution and mix well for 2 h at 37°C CO₂ incubator. The working solution is produced at the rate of 20 µl PMS reagent per 1 ml XTT reagent. Subsequently, the absorbance at 450 nm was quantified using a microplate spectrophotometer (Thermo Labsystems, Santa Rosa, California, USA) at 450/690 nm.

Flow cytometry analysis

0.4×10^6 cells were suspended in 200 µl of PBS (cat. no. 17-517Q; Lonza, Walkersville, Maryland, USA), and 200 µl of 95% ethanol (cat. no. 1.00983.1011; Merck, Darmstadt, Germany) were added while vortexing of the samples.

The cells were incubated at 4°C for 1 h, washed with PBS and resuspended in 250 µl of 1.12% sodium citrate buffer (pH 8.4) added with 12.5 µl of RNase A (cat. no. R4875; Sigma). The cells were further incubated at 37°C for 30 minutes. Cellular DNA was stained by incubating the cells with 250 µl propidium iodide (cat. no. P4170; Sigma) at 37°C for 30 minutes. To determine the relative DNA contents of the stained cells based on fluorescence cells were then performed by fluorescence-activated cell sorting (FACS) using the BD FACS Canto II flow cytometer (BD Biosciences, San Jose, California, USA).

Western blot analysis

T24 cell lysates were prepared by suspending 0.4×10^6 cells in 40 µl of lysis buffer consisting of 15 mM ethylene glycol tetraacetic acid (EGTA), 137 mM NaCl, 15 mM MgCl₂, 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 minutes. The total protein in the lysates was quantified by using the bicinchoninic acid assay kit (Thermo Scientific, Waltham, Massachusetts, USA), according to the manufacturer's instructions. The proteins (40 or 70 µg) were separated using 10 or 12% SDS PAGE gel and electrotransferred onto nitrocellulose membranes (GE Healthcare, Chicago, Illinois, USA). They were specifically detected by Immobilon Western Chemiluminescent HRP Substrate solution (Millipore, Darmstadt, Germany). They were detected using the Image Quant LAS 4000 Imaging System (GE Healthcare). The anti-PARP (1:1000) antibody was supplied by Cell Signaling Technology. The anti-caspase-3 (1:3000) and anti-c-FLIP (1:1000) antibodies were obtained from Enzo Life Sciences (Farmingdale, New York, USA). The anti-Bcl-2 (1:1000), anti-Mcl-1 (1:1000) and anti-actin (1:3000) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and anti-XIAP (1:5000) antibody was obtained from BD Biosciences.

RNA isolation and RT-PCR

c-FLIP_L mRNA expression level was quantified via RT-PCR. Total RNA was extracted from T24 cells by the EasyBlue reagent (cat. no. 17061; Life Technologies, Korea). The cDNA was prepared using the M-MLV reverse transcriptase (cat. no. 18057018; Thermo Scientific), according to the manufacturer's instructions. PCR primers were procured from GenoTech (Daejeon, Korea). GAPDH primer was used as a loading control. The primers used for the amplification on c-FLIP_L and GAPDH were the following: c-FLIP_L: 5'-CGGACTATAGAGTGCTGATGG-3' and 5'-GACT5'-GATTATCAGGCAGATTCCTAG-3'; GAPDH: 5'-AGGTCGGAGTCAACGGATTTG-3' and 5'-GTGATGGCATGGACTGTG-GT-3'. PCR reaction was carried out using the following cycling conditions:

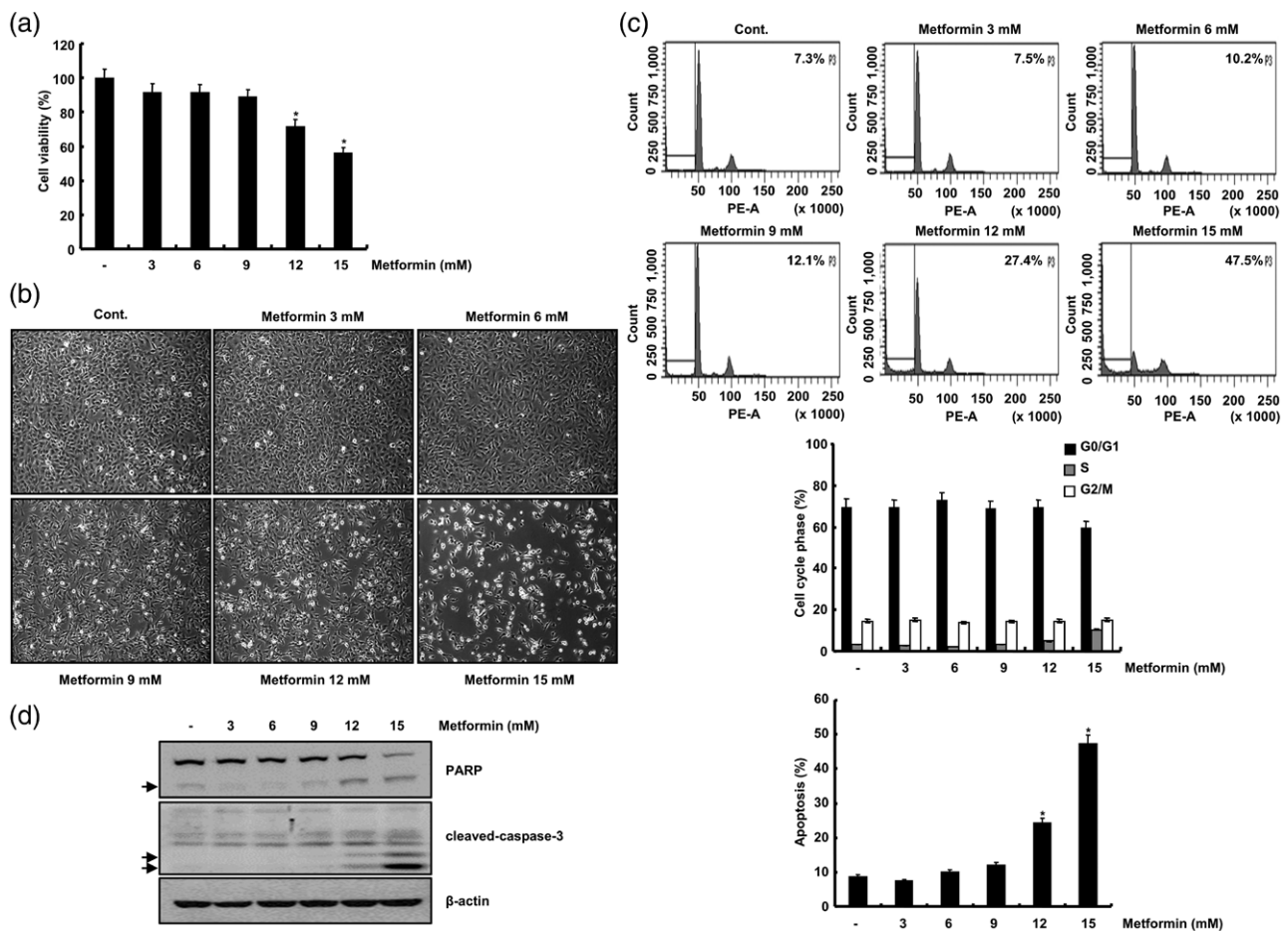
c-FLIP_L: 94°C for 5 min, followed by 34 cycles of 94°C for 40 sec, 54°C for 45 sec and 72°C for 40 sec; GAPDH: 95°C for 5 min, followed by 24 cycles of 94°C for 30 sec, 58°C for 45 sec and 72°C for 40 sec and with last expansion at 72°C for 5 min. The PCR products were performed by 1.5% agarose gel electrophoresis and visualized with 10% ethidium bromide using a gel documentation system (cat. no. WGD30; DAIHAN, Korea).

Measurement of apoptosis-inducing factor nuclear translocation

The cells were harvested and washed three times with cold PBS, and then incubated with an extraction buffer consisting of HEPES 10 mM, sucrose 250 mM, KCl 10 mM, MgCl₂ 1.5 mM, EDTA 1 mM, EGTA 1 mM, digitonin 0.05 % and PMSF 1 mM at 4°C for 10 min, then centrifuged at 10,000 g at 4°C for 10 min. The supernatant

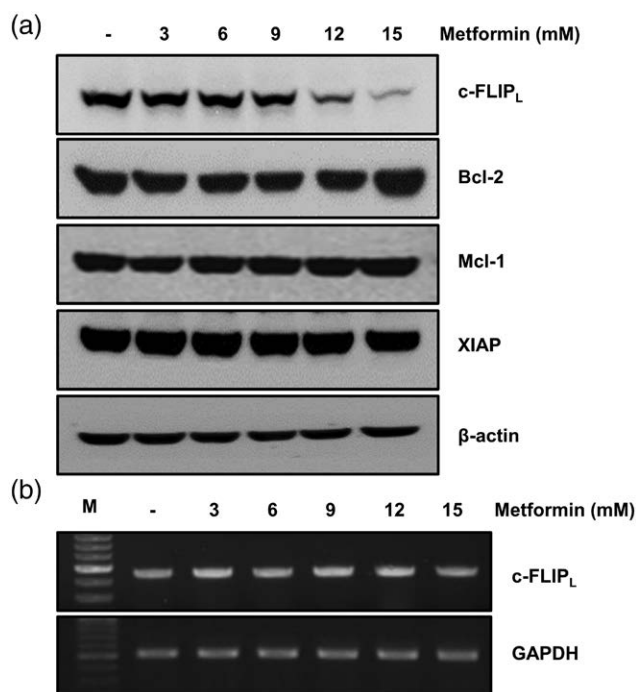
containing cytosolic proteins was removed and the pellet was incubated in a nuclear extraction buffer consisting of NaCl 350 mM, EDTA 1 mM, EGTA 1 mM, Tris-HCl 10 mM, pH 7.4 and protease inhibitors at 4°C for 20 min, and then centrifuged at 10,000 g at 4°C for 10 min. The proteins were loaded onto a 12% SDS-polyacrylamide gels and transferred on nitrocellulose membranes. After blocking in 5% skim milk at room temperature for 30 min, the membranes were probed with anti-AIF (1:1000), anti- α -tubulin (1:2000), and anti-lamin B (1:2000) antibodies, followed by incubation with a horseradish peroxidase-conjugated secondary antibody. Antibodies were procured from Santa Cruz Biotechnology (Santa Cruz, California, USA). The bands were detected by Immobilon Western Chemiluminescent HRP Substrate solution (Millipore Corporation, Bedford, Massachusetts, USA).

Fig. 1



Metformin induces apoptosis in T24 cells dose-dependently. (a) T24 cells were treated with the indicated concentration of metformin. After 24 h, cell viability was assessed using the XTT assay. (b) The morphological changes were examined by inverted microscope (magnification, $\times 200$). (c) T24 cells were treated with metformin (0, 3, 6, 9, 12 and 15 mM) for 24 h. FACS analysis is shown in the upper panel. Apoptosis confirmed by flow cytometry is shown in the middle panel. Cell cycle phase is shown in the lower panel. (d) T24 cells were treated with metformin. PARP, cleaved-caspase-3 and β -actin expression levels were detected by western blot. β -actin was used as a control. Arrows indicate PARP and caspase-3 cleavage form. Data are representative from three independent experiments. The data are expressed as mean \pm SD ($n = 3$). * $P < 0.05$ compared to non-treated cells. FACS, fluorescence-activated cell sorting.

Fig. 2



Metformin suppresses the expression of c-FLIP_L proteins in T24 cells. (a) T24 cells were treated with various concentrations of metformin for 24 h. c-FLIP_L, Bcl-2, Mcl-1, XIAP and β-actin expression levels were detected by western blot. β-actin was used as a control. (b) T24 cells were cultured with the concentrations of metformin. After 24 h, the c-FLIP_L mRNA level was analyzed by RT-PCR. Data are representative from three independent experiments.

Statistical analysis

Data were analyzed using one-way analysis of variance followed by posthoc comparisons using the Statistical Package for Social Sciences 8.0 (SPSS Inc., Chicago, Illinois, USA). All experiments were performed three independent times. The results were expressed as the mean ± SD and *P* values of less than 0.05 were considered to be statistically significant.

Results

Metformin induces apoptosis in human bladder cancer T24 cells

In our previous study, we reported that metformin mediated apoptosis in human renal cancer A498 cells [25]. To determine the apoptotic effects of metformin on the cell growth of human BC T24 cells, the cells were treated with different concentrations of metformin for 24 h. Our results showed that cell viability decreased in a dose-dependent manner in metformin-treated T24 cells (Fig. 1a). Next, we verified the apoptotic effects of metformin in T24 cells. As shown in Fig. 1b, treatment of T24 cells increased the typical characteristics of apoptosis, including cell shrinkage

and detachment of cells from the culture vessel. The metformin-treated T24 cells caused a distinguishable and dose-dependent increase in sub-G1 phase cells (Fig. 1c). Additionally, treatment of T24 cells with metformin increased cleavage of PARP and cleaved-caspase3 (Fig. 1d). These results indicate that metformin induces a significant increase of apoptotic cells in the T24 cells dose-dependently.

Metformin suppresses the expression of c-FLIP_L proteins in T24 cells

To examine the underlying mechanism involved in metformin-induced apoptosis, we investigated the expression levels of various apoptotic-regulatory proteins by western blot. As shown in Fig. 2a, the c-FLIP_L protein levels were decreased in a dose-dependent manner in metformin-treated T24 cells. However, Bcl-2, Mcl-1 and XIAP protein levels were not altered in metformin-treated cells. Next, we analyzed whether the metformin-induced c-FLIP_L reduction was controlled at the transcriptional level. As shown in Fig. 2b, the c-FLIP_L mRNA level remained constant after treatment of T24 cells with metformin at various concentrations. Therefore, our results suggest that the metformin-induced reduction of c-FLIP_L protein levels is regulated at the post-transcriptional level.

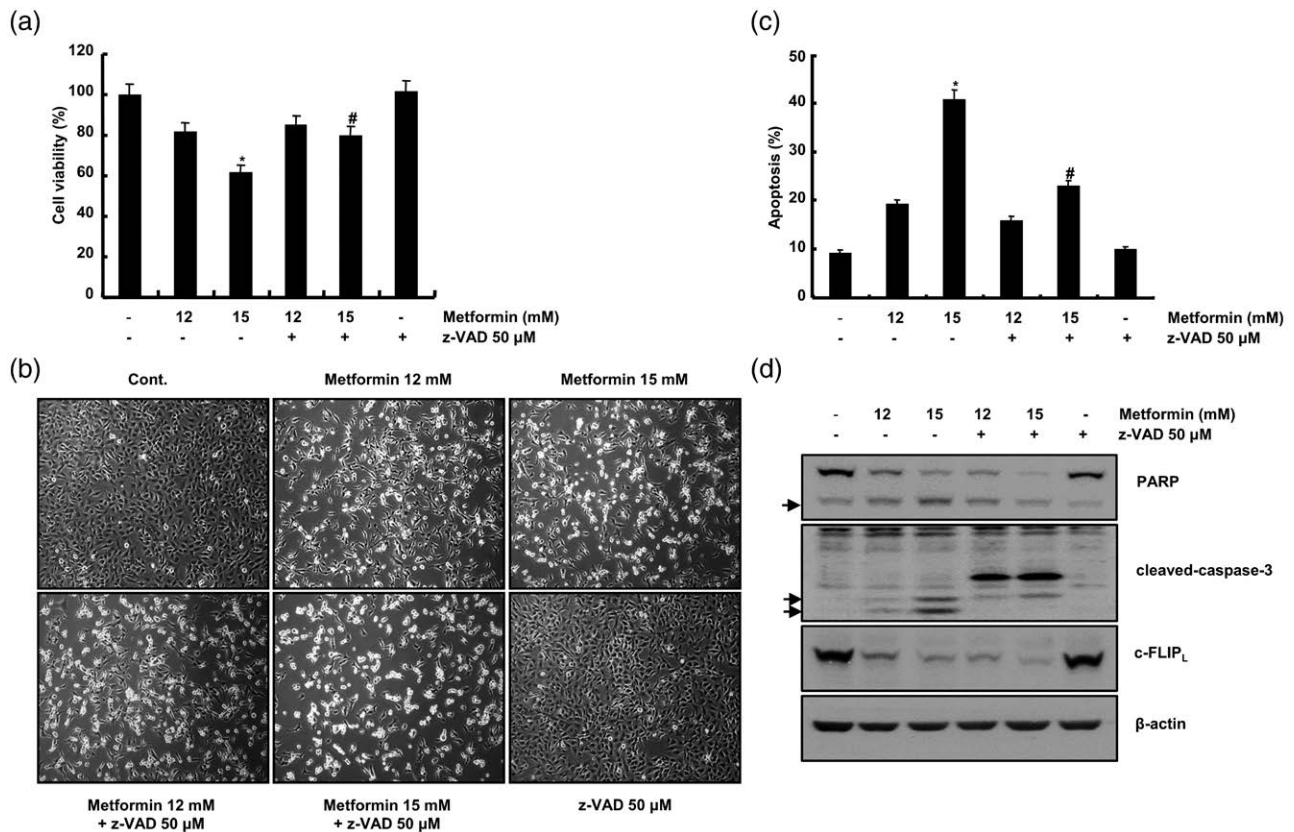
Metformin-mediated apoptosis in T24 cells is partially blocked by inhibition of the caspase-dependent signaling pathway

We investigated whether the caspase signaling pathway played a role in metformin-induced apoptosis. As shown in Fig. 3a and b, metformin mediating cell viability, morphological changes apoptosis was partially inhibited by pretreatment with z-VAD-fmk, general caspase inhibitor, as revealed by XTT assay and microscopy. Sub-G1 population was partially prevented by treatment with z-VAD-fmk in the presence of metformin (Fig. 3c). In addition, treatment with z-VAD-fmk partially prevented the cleavage of caspase-3 and PARP, but not c-FLIP_L (Fig. 3d). These findings indicate that metformin-mediated apoptosis in BC cells is partially regulated by the caspase-dependent pathway.

Metformin-induced apoptosis in T24 cells is independent of reactive oxygen species levels

Reactive oxygen species (ROS) are a known key regulator of apoptosis [26]. To examine whether ROS played a function in metformin-induced apoptosis, T24 cells were pretreated with ROS scavenger, NAC for 30 minutes, and then treated with metformin for 24 h. NAC did not prevent metformin mediating cell viability, morphological changes and apoptosis in T24 cells (Fig. 4a–c). Moreover, NAC treatment failed to block the PARP cleavage, as well as activation of caspase and c-FLIP_L degradation in metformin-treated cells (Fig. 4d). The results showed

Fig. 3



Metformin-mediated apoptosis in T24 cells is partially blocked by inhibition of caspase-dependent signaling pathway. (a) T24 cells were pretreated with 50 μ M z-VAD-fmk or a solvent for 30 minutes and incubated with 12 and 15 mM metformin for 24 h. Cell viability was assessed using the XTT assay. (b) T24 cells were incubated with 50 μ M z-VAD-fmk or a vehicle for 30 min before treatment with metformin (12 and 15 mM). After 24 h, the morphological changes were examined using an inverted microscope (magnification, $\times 200$). (c) Cells were pretreated with 50 μ M z-VAD-fmk or a solvent for 30 minutes and incubated with 12 and 15 mM metformin for 24 h. The sub-G1 cell fraction was confirmed by flow cytometry. (d) T24 cells were treated with 50 μ M z-VAD-fmk or a vehicle for 30 minutes before treatment with metformin for 24 h. PARP, cleaved-caspase-3, c-FLIP_L and β -actin expression levels were detected by western blot. β -actin was used as a control. Arrows indicate PARP and caspase-3 cleavage form. Data are representative from three independent experiments. The data are expressed as mean \pm SD ($n = 3$). * $P < 0.05$ compared to non-treated cells, # $P < 0.01$ compared with metformin-treated cells.

that ROS was not affected by metformin-induced apoptosis in T24 cells.

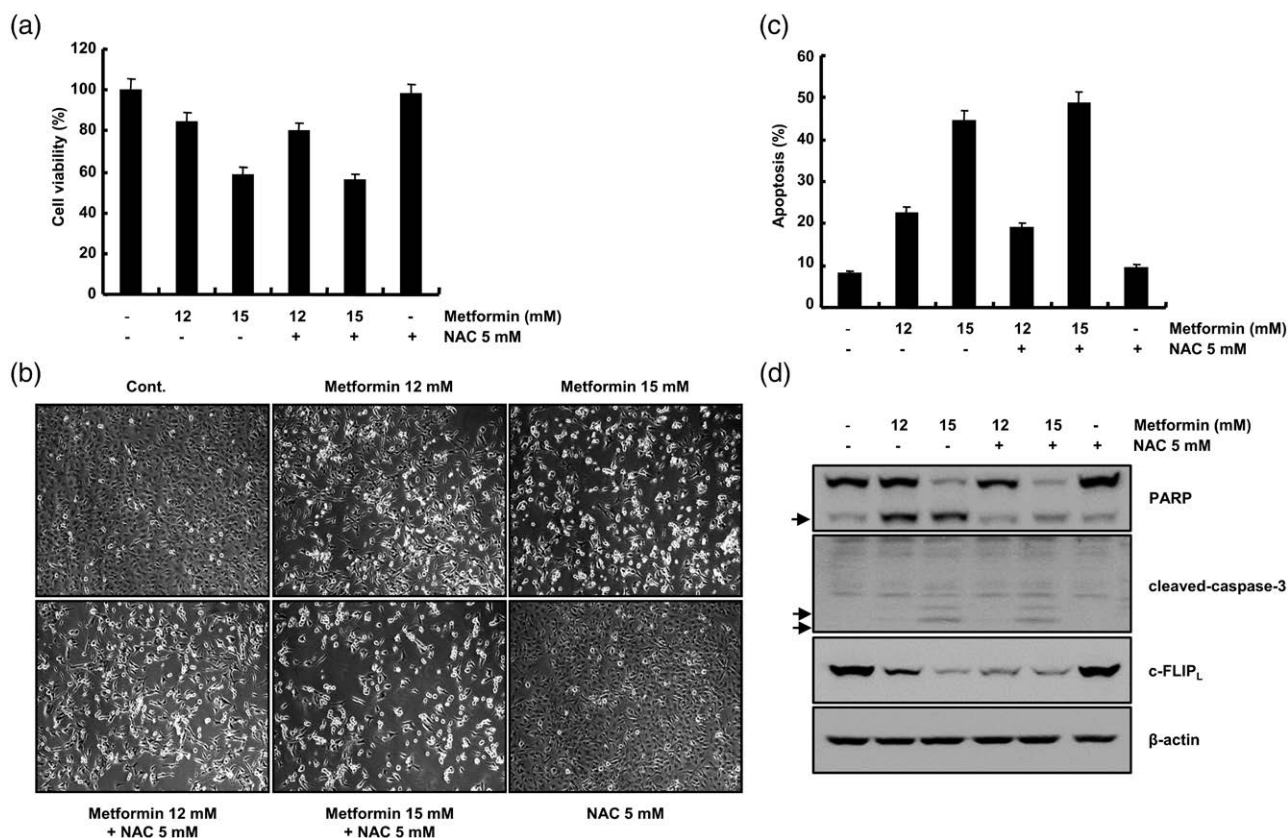
The metformin-mediated caspase-independent pathway is modulated by apoptosis-inducing factor translocation in T24 cells

To investigate the molecular mechanism of caspase-independent apoptosis, we examined the role of AIF in metformin-induced apoptosis. We detected AIF localization via western blot with fractionated cellular components. As shown in Fig. 5, metformin treatment induces AIF release in the cytosol and translocation to the nuclei. Moreover, AIF was found to be decreased in the mitochondria (Fig. 5). These findings suggest that metformin-mediated apoptosis in T24 cells is modulated by translocation of AIF from mitochondria to nucleus through the caspase-independent pathway.

Metformin-mediated c-FLIP_L protein degradation is regulated by the activation of the proteasomal signaling pathway in T24 cells

To further examine the underlying molecular mechanism of the decreased c-FLIP_L protein expression levels in metformin-treated cells, we performed a protein stability assay of c-FLIP_L. T24 cells were treated metformin in the absence or presence of CHX in order to obtain protein degradation kinetics. Our results revealed that c-FLIP_L protein degradation was facilitated by metformin treatment (Fig. 6a), implying that metformin treatment resulted in a decrease in the stability of the c-FLIP_L protein. Thus, we used a cell-permeable proteasome inhibitor, MG132, to identify the connection between c-FLIP_L protein levels and ubiquitin proteasome-mediated post-transcriptional regulation. The cells were pretreated MG132 for 1 h and then treated with metformin for 24 h. Our results showed that c-FLIP_L partially recovered metformin-induced

Fig. 4



ROS are not involved in metformin-induced apoptosis. (a) Cells were cultured with 5 mM NAC or a vehicle for 30 minutes before treatment with metformin (12 and 15 mM). After 24 h, cell viability was determined by XTT assay. (b) T24 cells were pretreated with 5 mM NAC or a solvent for 30 minutes and then incubated with metformin for 24 h. Morphological features were examined by inverted microscope (magnification, $\times 200$). (c) Cells were cultured with 5 mM NAC or a vehicle for 30 minutes before treatment with metformin (12 and 15 mM). After 24 h, the sub-G1 fraction was confirmed by flow cytometry. (d) T24 cells were incubated with different concentrations of metformin for 24 h in the presence or absence of 5 mM NAC. PARP, cleaved-caspase-3 and β -actin expression levels were detected by western blot. β -actin was used as a control. Arrows indicate PARP and caspase-3 cleavage form. Data are representative from three independent experiments. The data are expressed as mean \pm SD ($n = 3$). * $P < 0.05$ compared to non-treated cells. NAC, N-acetylcysteine; ROS, reactive oxygen species.

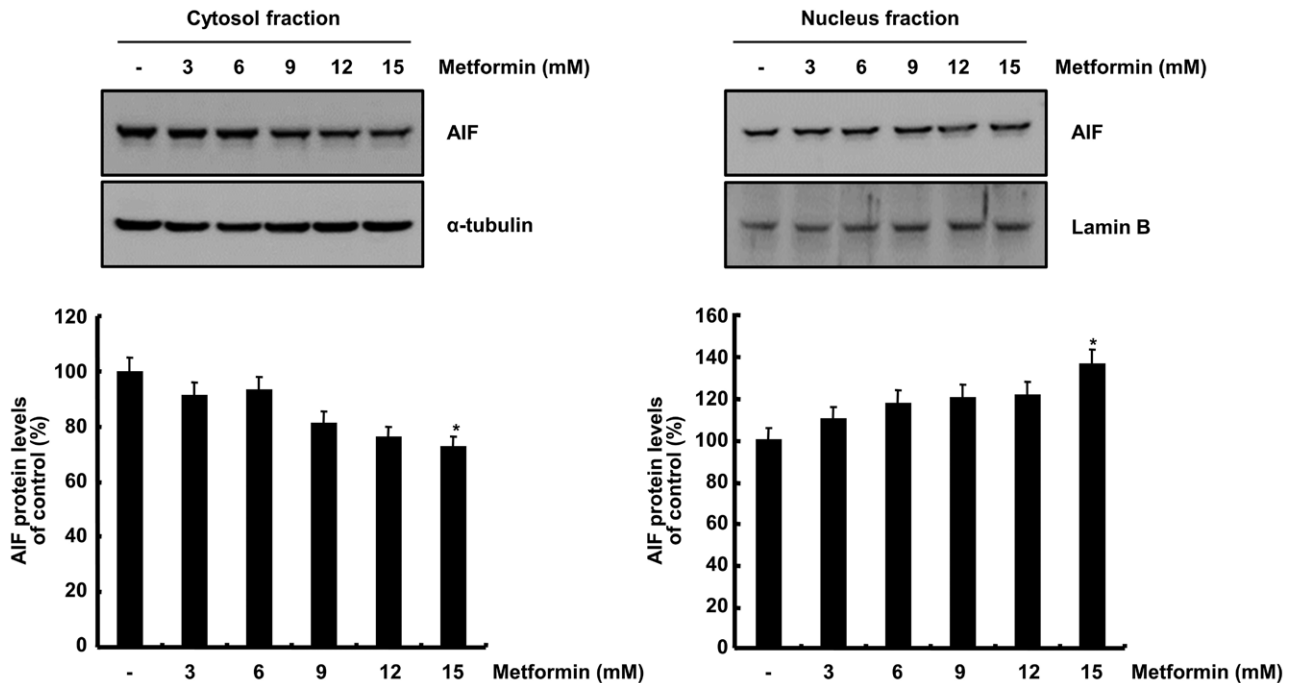
apoptosis in T24 cells treated with MG132 (Fig. 6b). These data suggest that metformin mediating apoptosis is modulated by downregulation of c-FLIP_L expression at the post-transcriptional level.

Discussion

In the present study, we showed that metformin exhibits meaningful anti-cancer activity against human BC cells. Moreover, we showed that metformin-induced apoptosis was regulated by both caspase-dependent and caspase-independent signaling pathways in human BC T24 cells. To our knowledge, this is the first study demonstrating that metformin mediated caspase-independent apoptosis occurs via nuclear translocation of AIF in T24 cells. Furthermore, we showed that metformin downregulated c-FLIP_L expression at the post-transcriptional level and that c-FLIP_L protein expression levels were downregulated by promoting protein degradation in metformin-treated cells.

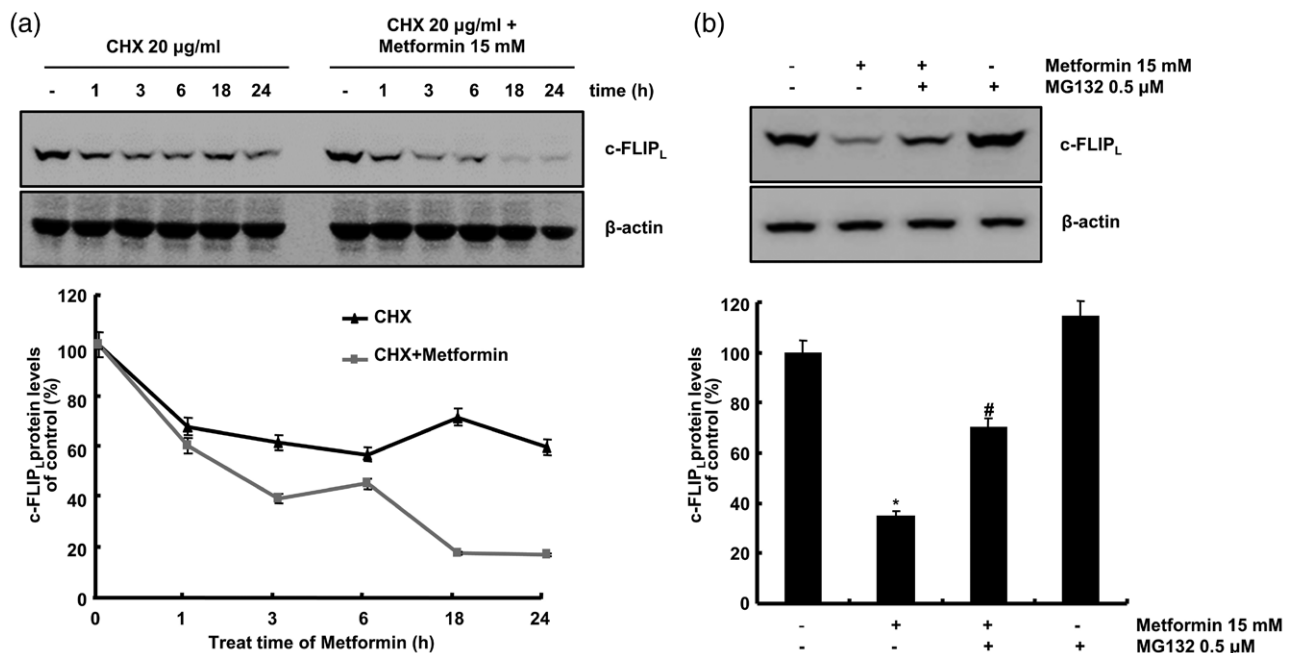
Metformin is currently used to treat patients with type 2 diabetes [27]. Previous studies have reported that metformin shows a marked anticancer effect in several human cancer cell types, including cervical cancer, ovarian cancer and bile duct cancer through inhibition of cell proliferation, apoptosis, AMPK signaling and suppression of tumor growth [11,15,28]. However, the molecular mechanism of metformin in human BC cells has not yet been elucidated. Thus, we studied whether metformin had an anti-tumor effect on BC T24 cells. In our previous study, we proved that metformin-induced apoptosis caused the activation of caspase-3 and cleavage of PARP in the human renal cancer A498 cells [25]. Similarly, metformin stimulated the caspase-dependent apoptotic pathway in T24 cells dose-dependently, which was partially inhibited by treatment with the general caspase inhibitor, z-VAD-fmk. These results indicate that metformin-induced apoptosis is regulated by caspase-dependent and caspase-independent apoptotic signaling pathways.

Fig. 5



AIF translocation is required for metformin-induced apoptosis in T24 cells. Cells were treated with metformin for 24 h. Next, cytoplasmic and nucleus fractions were prepared and detected by western blot using AIF, α -tubulin (cytosolic loading control) and lamin B (nucleus loading control) antibodies. Data are representative from three independent experiments. The data are expressed as mean \pm SD ($n = 3$). * $P < 0.05$ compared to non-treated cells. AIF density was performed by the ImageJ software.

Fig. 6



Metformin-mediated apoptosis occurs via proteasomal signaling pathways in T24 cells. (a) Cells were treated with or without metformin 15 mM in the presence or absence of 20 μ g/ml cycloheximide (CHX) for the indicated times. c-FLIP_L and β -actin expression levels were detected by western blot. β -actin was used as a control for western blot. (b) T24 cells were incubated with 0.5 μ M MG132 or a vehicle for 1 h before treatment with metformin for 24 h. Data are representative from three independent experiments. The data are expressed as mean \pm SD ($n = 3$). * $P < 0.05$ compared to non-treated cells, # $P < 0.01$ compared to metformin-treated cells. The c-FLIP_L density was analyzed by the ImageJ software.

The AIF protein is normally located in the outer membrane of the mitochondria and is translocated to the nucleus after activation of apoptotic pathways, where it involves in caspase-independent apoptotic events, such as chromatin condensation and DNA fragmentation [29,30]. In this study, we found that metformin treatment caused AIF release from the mitochondria followed by nuclear translocation in T24 cells, which indicates that metformin-mediated caspase-independent apoptosis is modulated by AIF translocation.

Previous studies have proved that c-FLIP_L expression is regulated at the ubiquitin proteasome-mediated post-transcriptional level or the transcriptional level [31,32]. Our results showed that metformin treatment did not alter c-FLIP_L mRNA expression. However, MG132 partially blocked metformin-mediated c-FLIP_L down-regulation. In addition, metformin promoted the ubiquitin/proteasome-mediated degradation of the c-FLIP_L protein, resulting in downregulation of c-FLIP_L. These findings suggest that the metformin-mediated decrease in c-FLIP_L expression was regulated at the post-transcriptional level.

In conclusion, this study is the first to demonstrate that metformin-induced apoptosis is mediated by caspase-dependent and caspase-independent signaling pathways through the activation of the AIF signaling pathway and increasing c-FLIP_L protein instability in human BC T24 cells. As such, we propose that metformin may be a potential candidate for novel anti-cancer drugs for BC.

Acknowledgements

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Conflicts of interest

There are no conflicts of interest.

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