Metformin induces TPC-1 cell apoptosis through endoplasmic reticulum stress-associated pathways *in vitro* and *in vivo*

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Abstract. Thyroid cancer is among the most common types of malignant tumor of the endocrine system. The role of metformin in the inhibition of cancer cell proliferation and induction of apoptosis is widely accepted. The present study explored the effect and the underlying mechanisms of metformin on human thyroid cancer TPC-1 cells. Following treatment of TPC-1 cells with different concentrations of metformin, cell proliferation and apoptosis were analyzed by cell counting kit-8 (CCK-8) assay and flow cytometry, respectively. Reverse transcription-quantitative PCR and western blotting were used to detect alterations in the mRNA and protein expression levels, respectively, for heat shock protein family A member 5 (HSPA5, also known as Bip), DNA damage-inducible transcript 3 (DDIT3, also known as CHOP) and caspase-12. The results demonstrated that treatment with metformin inhibited proliferation and induced apoptosis in a concentration and time-dependent manner. In addition, treatment with metformin increased the expression of Bip, CHOP and caspase-12 *in vitro*, activating endoplasmic reticulum (ER) stress. Thapsigargin treatment enhanced the apoptosis induced by metformin. Inhibition of ER stress by 4-phenylbutyrate reversed the metformin-induced apoptosis. Finally, treatment with metformin inhibited thyroid cancer growth and increased the expression of Bip and CHOP in a TPC-1 cell xenograft model. These results indicated that metformin increased the apoptotic rate of thyroid cancer cells via ER stress-associated mechanisms.

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Introduction

Thyroid cancer is among the most common types of endocrine cancer worldwide, and its incidence is increasing (1). It has been reported that thyroid cancer has the most rapidly increasing rate of incidence of all cancer types among women, and the second among men (2). Thyroid cancer can be characterized into papillary (PTC), follicular (FTC), poorly differentiated (PDTC) and anaplastic thyroid cancer (ATC) by histopathological characteristics. PTC is the most common type of thyroid cancer and accounts for >90% of all thyroid cancer cases (3). Although multiple studies have investigated the molecular mechanisms of PTC, it remains to be fully characterized. Recent studies suggest that, as an endocrine cancer, the pathogenesis of PTC may be associated with cell metabolism, and that targeting of cancer cell metabolism may be a novel approach for the prevention or treatment of PTC (4).

Metformin, a first-line antidiabetic agent, is known to activate AMP-activated protein kinase (AMPK) and has been demonstrated to be a potential anticancer agent (5,6). Epidemiologists have reported that diabetic patients who receive metformin demonstrate a lower risk and incidence of multiple types of cancer (7,8). Furthermore, a number of pharmacological studies have revealed the antiproliferative and antimetastatic effects of metformin in multiple types of cancer, including thyroid cancer (9-11). Although the use of metformin decreases the incidence of cancer and induces cell death in cancer cells, its mechanism of action has not been fully investigated in thyroid cancer.

Endoplasmic reticulum (ER) stress-associated apoptosis is a major pathway in the regulation of cell apoptosis. The ER is an essential organelle for protein synthesis, folding and trafficking. A number of cellular stress conditions, such as hypoxia and metabolic stress, lead to the accumulation of nascent or unfolded proteins in the ER lumen. ER stress-induced unfolded or misfolded proteins regulate ER chaperone proteins to inhibit protein aggregation and translation and induce degradation by the proteasome; this process is termed the unfolded protein response (UPR) (12). If ER stress is persistent and unresolved, UPR triggers several signaling pathways

leading to apoptosis. Recent studies have suggested that ER stress induces apoptosis via increasing the expression of DNA damage-inducible transcript 3 (DDIT3, also known as CHOP), JNK and caspase-12 (13). Granato *et al* (14) revealed that metformin enhances the cytotoxic effect of bortezomib against PEL cells by altering UPR activation and upregulating the expression of CHOP.

The present study investigated the effect of metformin on the proliferation and apoptosis of thyroid cancer TPC-1 cells, and explored the underlying molecular mechanism *in vitro* and *in vivo*. The results demonstrated that metformin inhibited cell proliferation and induced apoptosis via an ER stress-associated mechanism. The present findings suggested that metformin treatment may have therapeutic potential for patients with papillary thyroid cancer.

Materials and methods

Cell culture. The human thyroid cancer cell line, TPC-1, was obtained from the Type Culture Collection of the Chinese Academy of Sciences. The cells were cultured in RPMI-1640 (HyClone; GE Healthcare Life Sciences), supplemented with 10% fetal bovine serum (Gemini Bio Products) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin), at 37˚C in a humidified atmosphere with 5% CO_2.

Cell viability assay. Metformin, thapsigargin and 4-phenylbutyrate (4-PBA) were purchased from Sigma-Aldrich (Merck KGaA) and dissolved in dimethyl sulfoxide (DMSO). Cell viability was detected by Cell Counting kit-8 (CCK-8; Dojindo Molecular Technologies, Inc.) assay. TPC-1 cells were seeded into 96-well plates at a density of $5x10^3$ cells/well. The medium was removed and replaced with medium containing metformin at 1.25, 2.5, 5, 10 or 20 mmol/l for 24 or 48 h. Cells treated with 20 mmol/l metformin for 24 h were pretreated with 1 μmol/l thapsigargin and/or 1 mmol/l 4-phenylbutyrate for 1 h. Then, the cells were incubated with 10 μl CCK-8 solution for 1 h. The OD values were measured by absorbance using a microplate reader (Thermo Fisher Scientific, Inc.) at a wavelength of 450 nm, and cell viability was quantified as a percentage of the control using the OD values.

Apoptosis analysis. For cell apoptosis analysis, TPC-1 cells were analyzed using an Annexin V-FITC Apoptosis Detection kit (BD Biosciences), according to the manufacturer's instructions. Briefly, cells were collected after treatment with various concentrations of metformin for 24 h. The cell suspension was transferred to a 5 ml tube prior to the addition of 5 μl Annexin V and 5 μl propidium iodide (PI). After incubation in the dark at room temperature for 15 min, 400 μl binding buffer was added. Apoptosis was detected with a Epics XL-MCL ADC flow cytometer (Beckman Coulter, Inc.), according to the manufacturer's instructions. Data were analyzed using the EXP032ADC operation system.

Western blot analysis. TPC-1 cells were harvested and total protein was extracted on ice using RIPA lysis buffer (Beyotime Institute of Biotechnology) with a protease inhibitor cocktail (104 mM AEBSF, 80 μM aprotinin, 5 mM bestatin, 1.5 mM E-64, 2 mM leupeptin, and 1.5 mM pepstatin A; MedChemExpress LLC) for 20 min. The protein concentration was determined using a bicinchoninic acid kit (Beyotime Institute of Biotechnology). Equal amounts of protein ~30 μg were separated by 12% SDS-PAGE and transferred into PVDF membranes (Roche Diagnostics). The membranes were immunoblotted at 4˚C overnight, with the following primary antibodies: heat shock protein family A member 5 (HSPA5, also known as Bip; 1:800 dilution; cat. no. 11587-1-AP), CHOP (1:300 dilution; cat. no. 15204-1-AP), caspase-12 (1:500 dilution; cat. no. 55238-1-AP) and β-actin (1:5,000 dilution; cat. no. 60008-1-Ig), all from ProteinTech Group, Inc. This was followed by six 5-min washes with PBS. Then the membranes were incubated with a horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (1:5,000 dilution; cat. no. JH-0011; DingGuo BioTech Co., Ltd.) for 1 h at room temperature, and washed six more times with PBS. The proteins were visualized using an enhanced chemiluminescence western blotting detection kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's instruction. β-actin served as internal control.

Total RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated from TPC-1 cells using TRIzol (Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. cDNA was synthesized using a Reverse script RT reagent kit (Takara Biotechnology Co., Ltd.). SYBR-Green (Takara Biotechnology Co., Ltd.) was used for the qPCR to quantify the expression of Bip, caspase-12 and CHOP on the real-time PCR detection system Mx-3005P (Agilent Technologies, Inc.), according to the manufacturer's instructions. β-actin was used as a housekeeping control gene. The primer sequences used were as follows: Bip, forward, 5'-GAACGTCTG ATTGGCGATGC-3' and reverse, 5'-ACCACCTTGAACGG CAAGAA-3'; caspase-12, forward, 5'-GCTCAGGAAATGGA AACAGC-3' and reverse, 5'-AGTGCTTGGTCCCACAGATT-3'; CHOP, forward, 5'-TGGAAGCCTGGTATGAGGAC-3' and reverse, 5'-AAGCAGGGTCAAGAGTGGTG-3'; and β-actin, forward, 5'-CCTGGCACCCAGCACAAT-3' and reverse, 5'-GGGCCGGACTCGTCATAC-3'. The thermocycling conditions were: 95˚C for 60 sec, and 40 cycles of 95˚C for 15 sec, 60˚C for 15 sec, and 72˚C for 45 sec. Relative fold changes in mRNA expression were calculated using the formula $2^{-\Delta\Delta Cq}$ (15).

Tumor xenograft mouse models. Ten male immunodeficient BALB/c nude mice (age, 4-6 weeks; weight, 16±2 g) were purchased from Vital River Laboratories Co., Ltd. The animals were housed under pathogen-free conditions at the Institute of Medicine Zhengzhou University at 25±2˚C and 70±5% humidity, under a 12-h light/dark cycle and access to food and water *ad libitum*. TPC-1 cells in the log-phase were suspended in serum-free culture medium with 50% Matrigel (BD Biosciences) at a density of $1x10^7$ TPC-1 cells in 200 μl. Tumor xenografts were established by subcutaneous inoculation into the right flank of nude mice. The mice were randomly divided into two groups of 5 mice: the control group, administered with PBS containing 10% DMSO; and the metformin group, administered with 350 mg/kg intragastric metformin infusion, daily (16-18). The volume of the tumors was measured using calipers every week. Tumor volume was calculated according to the following formula: Tumor volume (mm^3) = (length x $width^2$)/2. The maximum tumor diameter in the present study was 15.67 mm,

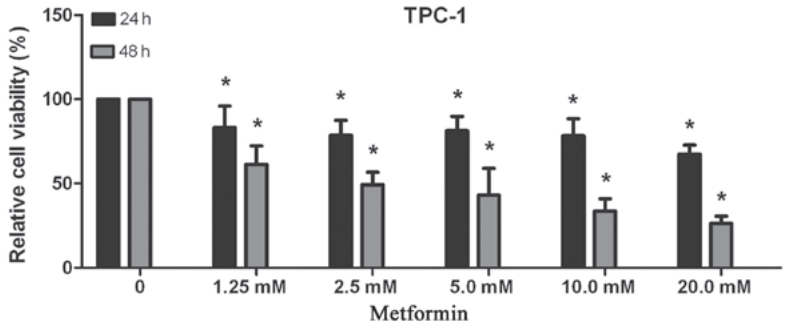

Figure 1. Metformin inhibits proliferation in TPC-1 cells. Cells were treated with the indicated concentrations of metformin for 24 and 48 h. Cell viability was examined by Cell Counting Kit-8 assay. *P<0.05 vs. 0 mM.

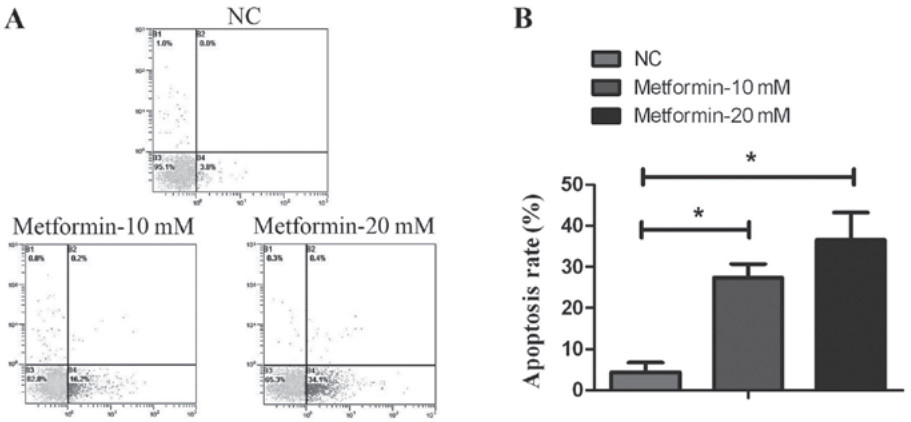

Figure 2. Metformin induces apoptosis in TPC-1 cells. TPC-1 cells were treated with 10 or 20 mmol/l metformin for 24 h, stained with Annexin V-FITC and propidium iodide, then analyzed by detection by flow cytometry. (A) Representative plots. (B) Quantification of apoptosis rate. *P<0.05 vs. NC. NC, negative control

which was acceptable under the ethical guidelines. After 6 weeks, the animals were sacrificed by cervical dislocation following anesthesia by 1% pentobarbital (50 mg/kg) injection (19), and the tumor tissues were removed and measured. Xenograft tumors were harvested, fixed in 10% formalin for 24 h at room temperature, embedded in paraffin, and cut into 4 μm sections for immunohistochemical analysis.

Immunohistochemistry. The slides were immersed in heated antigen retrieval solution (10 mmol/l citrate buffer, pH 6.0), and subsequently treated with 3% hydrogen peroxide for 10 min. After washing with PBS, the slides were incubated with primary antibody (Bip, cat. no. 11587-1-AP, 1:50 dilution; CHOP, cat. no. 15204-1-AP, 1:50 dilution; both from ProteinTech Group, Inc.) at 4˚C overnight, and then with secondary antibody (cat. no. PV9000; 1:500 dilution; OriGene Technologies, Inc.) for 20 min at room temperature. The reaction was developed using a 3,3'-diaminobenzidine kit (1:50 dilution in buffer; OriGene Technologies, Inc.). Finally, the slides were counterstained in hematoxylin prior to dehydration and mounting. Staining results were observed under a light microscope (CX31; Olympus Corporation; original magnification, x200). Sections were scored semi-quantitatively for the extent of immunoreaction as follows: 0, 0% immunoreactive cells; 1, <5% cells; 2, 5-50% immunoreactive cells; and 3, >50% immunoreactive cells. In addition, the intensity of staining was scored semi-quantitatively as: 0, negative; 1, weak; 2, intermediate; and 3, strong. The final immunoreaction score was calculated as the sum of both parameters (extent and intensity) (20).

Statistical analysis. All data are presented as the mean ± standard deviation from at least three independent experiments. Differences between groups were assessed using Student's t-test followed by Shapiro-Wilk W test, or one-way ANOVA followed by Bonferroni test. P<0.05 was considered to indicate a statistically significant difference, and analyses were performed using SPSS 17.0 software (SPSS Inc.).

Results

Metformin inhibits cell viability in TPC-1 cells. TPC-1 cells were seeded into 96-well plates. Following treatment with metformin at different concentrations (1.25, 2.5, 5, 10 or 20 mmol/l) for 24 or 48 h, cell viability was detected by CCK-8 assay. As presented in Fig. 1, the inhibitory effect of metformin on proliferation increased with the increase in concentration and duration of treatment. The IC_{50} values of metformin for 24 and 48 h were 324.865 and 2.684 mmol/l, respectively. These data indicated that metformin inhibited cell viability in a concentration- and time-dependent manner.

Metformin induces cell apoptosis in TPC-1 cells. To investigate whether the inhibition of cell proliferation was due to an increased rate of apoptosis, the proportion of apoptotic cells was detected by flow cytometry using

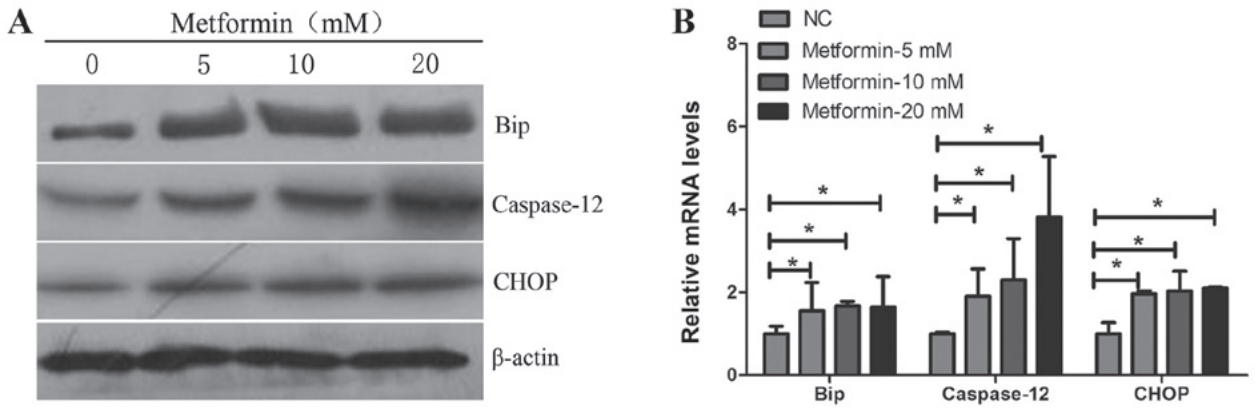

Figure 3. Metformin activates ER stress and ER stress-associated apoptosis. TPC-1 cells were treated with 5, 10 or 20 mmol/l metformin for 24 h. (A) Protein expression levels of Bip, caspase-12 and CHOP were detected by western blotting. β-actin was used as a loading control. (B) mRNA expression levels of Bip, caspase-12 and CHOP were detected by reverse transcription-quantitative PCR. β-actin was used as an internal control. *P<0.05 vs. NC. ER, endoplasmic reticulum; Bip, heat shock protein family A member 5; CHOP, DNA damage-inducible transcript 3; NC, negative control.

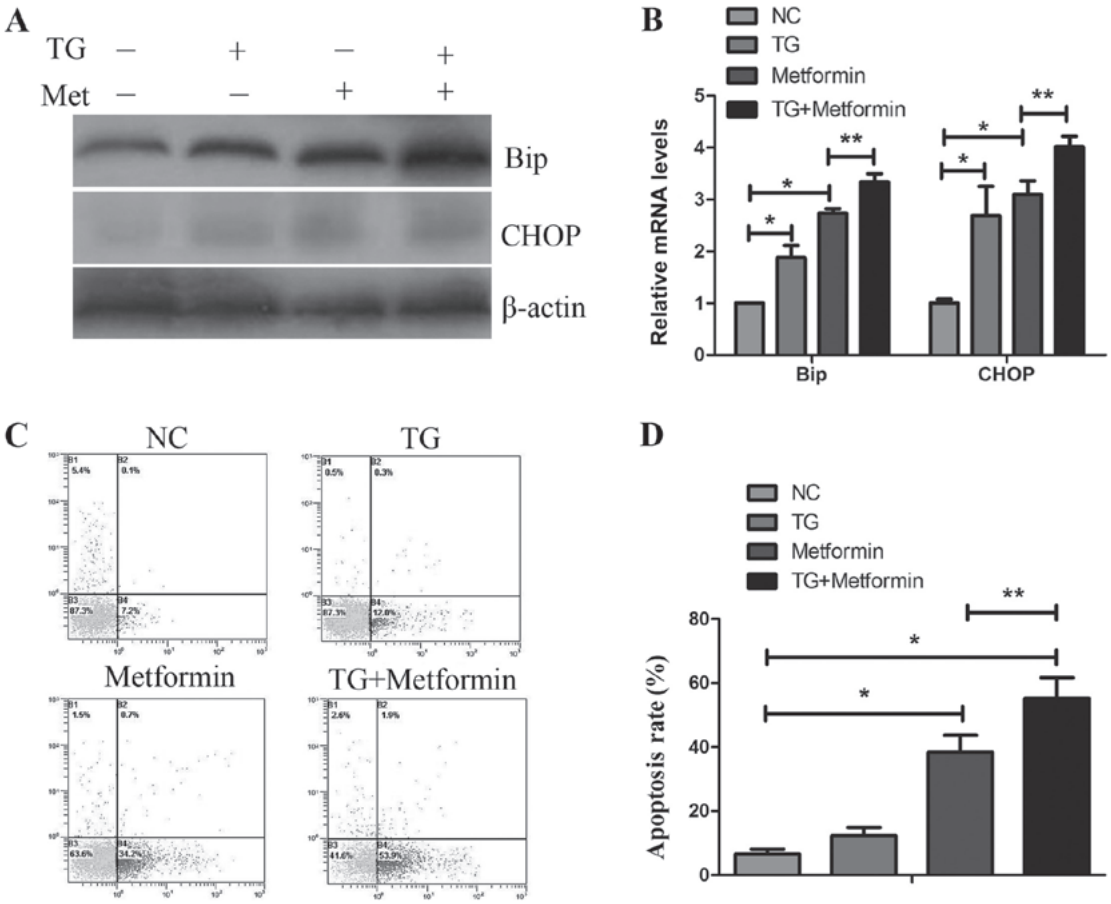

Figure 4. TG enhances metformin-mediated apoptosis in TPC-1 cells. TPC-1 cells were pretreated with TG for 1 h, then treated with or without metformin for 24 h. (A) The protein expression levels of Bip and CHOP were detected by western blotting, using β-actin as a loading control. (B) mRNA expression levels of Bip and CHOP following treatment with TG and/or metformin in TPC-1 cells. (C) TPC-1 cells were stained with Annexin V-FITC and propidium iodide, and analyzed flow cytometry. Representative plots are shown (D). Quantification of apoptosis rate. *P<0.05, with comparisons indicated by brackets. TG, thapsigargin; Bip, heat shock protein family A member 5; CHOP, DNA damage-inducible transcript 3; NC, negative control; Met, metformin.

Annexin V-FITC/PI staining, following treatment with different concentrations of metformin. As presented in Fig. 2, the apoptosis rate following metformin treatment was significantly increased compared with the control group. These findings indicated that metformin induced apoptosis of thyroid cancer TPC-1 cells.

Metformin induces ER stress and ER stress-associated apoptosis in TPC-1 cells. The ER stress-associated apoptotic pathway serves an important role in apoptosis induced by anticancer agents (22). Following treatment with metformin at different concentrations for 24 h, the mRNA and protein expression levels of the ER molecular chaperone Bip, and the

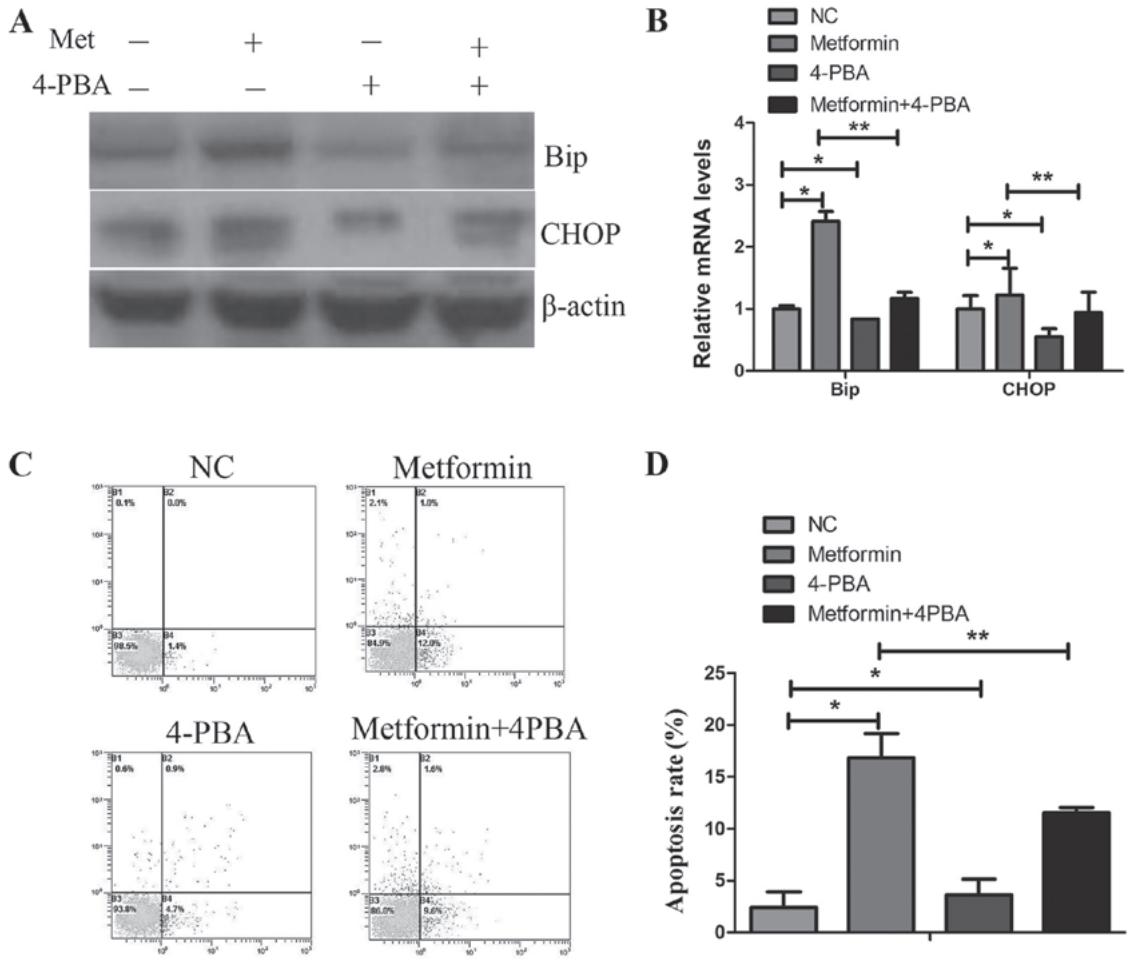

Figure 5. 4-PBA reverses the metformin-mediated apoptosis in TPC-1 cells. TPC-1 cells were pretreated with 4-PBA for 1 h, then treated with or without metformin for 24 h. (A) The protein expression levels of Bip and CHOP were detected by western blotting, using β-actin as a loading control. (B) mRNA expression levels of Bip and CHOP following treatment with 4-PBA and/or metformin in TPC-1 cells. (C) TPC-1 cells were stained with Annexin V-FITC and propidium iodide, and analyzed flow cytometry. Representative plots are shown (D). Quantification of apoptosis rate. *P<0.05, with comparisons indicated by brackets. 4-PBA, 4-phenylbutyrate; Bip, heat shock protein family A member 5; CHOP, DNA damage-inducible transcript 3; NC, negative control; Met, metformin.

ER-associated apoptosis genes CHOP and caspase-12, were detected in TPC-1 cells by RT-qPCR and western blotting. As presented in Fig. 3, the mRNA and protein expression levels of Bip, CHOP and caspase-12 were significantly increased with increased concentrations of metformin for 24 h. These data suggested that metformin may induce ER stress-mediated apoptosis.

Activation of ER stress by thapsigargin enhances metformin-mediated apoptosis in TPC-1 cells. To detect whether ER stress serves an important role in apoptosis mediated by metformin, TPC-1 cells were pretreated with the ER stress activator thapsigargin (1 μmol/l) for 1 h, followed by treatment with metformin for 24 h. As presented in Fig. 4C and D, treatment with thapsigargin enhanced the metformin-mediated apoptosis. Furthermore, treatment with thapsigargin and metformin further increased the expression levels of Bip and CHOP compared with either treatment alone (Fig. 4A and B). These data indicated that activation of ER stress enhanced the anticancer effect of metformin in TPC-1 cells.

Inhibition of ER stress by 4-PBA reverses metformin-induced apoptosis in TPC-1 cells. To further confirm whether ER stress has an important role in apoptosis induced by metformin, TPC-1 cells were pretreated with the ER stress inhibitor 4-PBA (1 mmol/l) for 1 h, followed by treatment with metformin for 24 h. As presented in Fig. 5C and D, treatment with 4-PBA decreased the metformin-mediated growth apoptosis. Furthermore, 4-PBA and metformin co-treatment decreased the Bip and CHOP mRNA and protein expression levels, compared with metformin treatment alone. These results further confirmed that the ER stress process was required for metformin-induced apoptosis in TPC-1 cells.

Metformin inhibits TPC-1 tumor cell growth and increases the expression of Bip and CHOP in vivo. Next, the effects of metformin on TPC-1 cells were investigated *in vivo* using a xenograft mouse model (Fig. 6A). The results demonstrated that the tumor volume (Fig. 6B and C) and the tumor weight (Fig. 6D) in the control group at the end of the treatments were 391.0±157.6 mm^3 and 1.08±0.25 g, respectively, whereas those in the metformin group were 153.1±66.5 mm^3 and 0.55±0.13 g, respectively. These data indicated that treatment with metformin significantly decreased the tumor growth rate and tumor weight compared with the control group (Fig. 6B-D). The protein expression levels of Bip and CHOP in the tumor

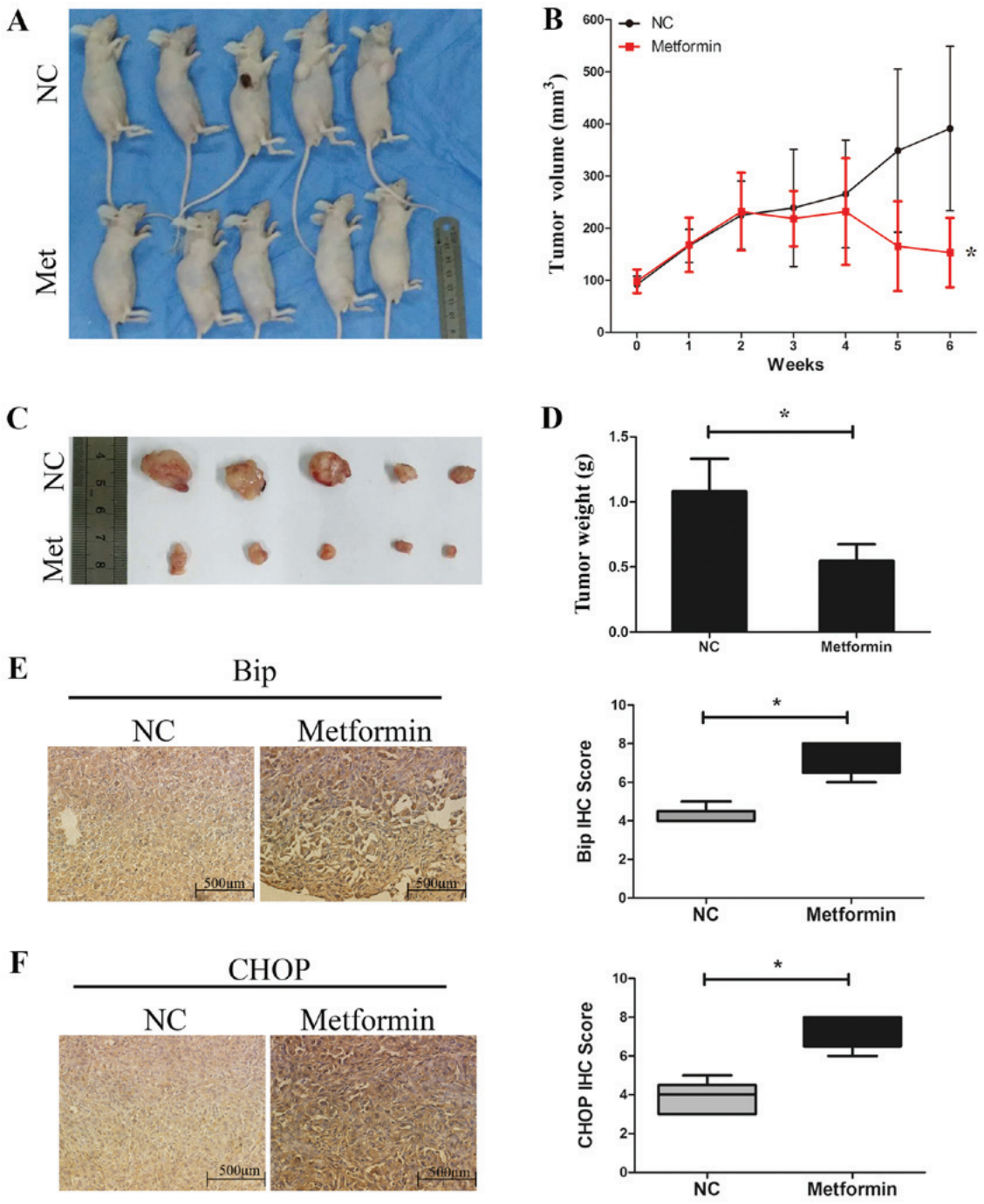

Figure 6. Metformin inhibits tumor growth in TPC-1 xenografts. (A) Tumor-bearing xenograft mice. (B) Tumor volumes over time for BALB/c nude mice following treatment with PBS or metformin. (C) Photographs of the tumors and (D) tumor weights measured in each group at the endpoint of the experiment. (E) Immunohistochemistry analysis for Bip and (F) CHOP expression in the xenograft tumor tissues. Original magnification, x200. *P<0.05. NC, negative control; Met, metformin; Bip, heat shock protein family A member 5; CHOP, DNA damage-inducible transcript 3.

xenograft tissues were detected by immunohistochemistry. As presented in Fig. 6E and F, the expression levels of Bip and CHOP were significantly increased in the tumor tissues from the metformin group compared with the control group, which is consistent with the aforementioned *in vitro* results of the present study.

Discussion

Metformin, commonly used for the treatment of diabetes mellitus through increasing insulin sensitivity and improving glycemic control, has been demonstrated to inhibit cancer cell proliferation and induce cancer cell death (23). Previous studies have reported that metformin inhibits the carcinogenesis of endocrine tumors through activating AMPK, leading to the downregulation of mTOR activation, which serves an important role in cell metabolism (24-26). Evidence also suggests that metformin reduces chemoresistance in ovarian cancer (27), bladder cancer (28), breast cancer (6) and esophageal squamous carcinoma (29). The present results suggested that cellular proliferation was suppressed following treatment with metformin in TPC-1

cells. Flow cytometry analysis revealed that metformin significantly increased the rate of apoptosis in TPC-1 cells, in a concentration and time-dependent manner. Mechanistically, metformin may induce TPC-1-cell apoptosis through its regulation of ER-stress associated pathways.

In the present study, the metformin dosing range used *in vitro* was 1.25-20 mmol/l, while the serum levels of metformin in patients with type 2 diabetes (average 1,500 mg oral daily dose) have been reported at 2-6 μmol/l, with peak levels of 38 μmol/l and steady state ranges of 15.5 μmol/l (21). A recent study has indeed noted that the dosing of metformin *in vitro* is a contentious issue, with concerns that supra-physiological doses result in off-target effects and are not reflective of *in vivo* events (21). However, it can be speculated that the purpose of using metformin for diabetes treatment is the regulation of insulin sensitivity and improvement of glycemic control, while the use of metformin for cancer treatment would aim at inducing apoptosis. In the present study, the anticancer effect of metformin was explored in thyroid cancer cells, instead of a diabetic model. Our previous study also indicated that metformin at 20 mM exhibits anticancer effects in gallbladder cancer (18). Similar *in vitro* concentrations have been used in other studies (16,30,31).

Deregulation of apoptosis can lead to the development or progression of tumorigenesis. To investigate the mechanism of metformin-induced cell death, the ER stress pathway-mediated apoptosis was examined. Increasing evidence suggests that ER stress-associated apoptosis is involved in the apoptosis induced by anticancer agents (32). The ER triggers adaptive protective processes with the accumulation of unfolded or misfolded proteins (33). This homeostatic mechanism is mediated by UPR via three ER transmembrane receptors: Activating transcription factor 6 (ATF6), protein kinase dsRNA-like ER kinase (PERK) and inositol-requiring enzyme 1α (IRE1α) (34). Increasing evidence suggests that ER stress serves a protective role against tumor invasion, metastasis and chemoresistance (35,36). However, when ER stress is severe or prolonged, the ATF6, PERK and IRE1α signaling pathways are activated, resulting in increased expression of CHOP, caspase-12 and JNK (37), and subsequently leading to apoptosis.

Recent studies have suggested that ER stress serves a role in chemoresistance in colorectal cancer (38). A previous study has reported that ER stress induces apoptosis via the JNK/p38 pathway following treatment with protodioscin (39). However, the role of metformin in thyroid cancer remained unclear. The present study demonstrated that the mRNA and protein expression levels of Bip increased with metformin treatment in TPC-1 cells. Bip is an abundant and key ER chaperone. It has been proposed that under conditions of ER stress, the expression of Bip is increased and the three ER stress sensors IRE1α, PERK and ATF6, are activated to alter transcriptional and translational programs, indicating that the expression and activation of Bip has a key role in ER stress (40). The present study also observed that the expression of the ER stress-associated apoptosis genes CHOP and caspase-12 increased when TPC-1 cells were treated with metformin. CHOP, a key transcription factor, is the most well-characterized proapoptotic pathway in the ER (41). Investigating the relationship between ER stress and apoptosis, it was demonstrated that activation of ER stress by thapsigargin enhanced the sensitivity of TPC-1 cells to metformin, while inhibition of ER stress by 4-PBA reversed metformin-induced apoptosis in TPC-1 cells. Furthermore, a xenograft mouse model was used to investigate the role of metformin *in vivo*. Treatment with metformin significantly decreased tumor growth compared with the control group. Furthermore, the protein expression levels of Bip and CHOP were increased in tumor tissues treated with metformin compared with the control group, which was consistent with the *in vitro* results. These data indicated that metformin may suppress cell proliferation via induction of ER stress-associated apoptosis. Cho *et al* (42) revealed that treatment with 100 mg/ml metformin decreases tumor growth by 47-60% compared with the control group in a thyroid cancer BPH10-3SC xenograft mouse model. Thakur *et al* (43) reported that treatment with metformin leads to a significant reduction in thyroid cancer metastatic growth in the FTC133 model, but not in the BCPAP model. A recent study also reported that metformin is a potential anticancer agent in multiple types of cancer (44). Based on these studies, metformin may possess anticancer effect in other thyroid cancer cell models. In the present study, treatment with metformin in the TPC-1 xenograft model decreased tumor growth by ~60% compared with the control group. However, there are several limitations to the present results: the use of only one thyroid cancer cell line; the lack of a positive control (for example sorafenib, a known anticancer drug used to treat thyroid cancer); and the lack of other mechanistic controls to compare the effects of metformin treatment. Therefore, further studies are required in order to fully investigate the anticancer effect of metformin in ER-induced apoptosis in thyroid cancer.

Recently, Yang *et al* (45) reported that metformin induced ER stress-dependent apoptosis, suggesting a promising therapeutic strategy for prostate cancer. In the present study, the results demonstrated that metformin could also inhibit proliferation and induce apoptosis in thyroid cancer TPC-1 cells, by targeting the ER stress-associated apoptotic pathway. These findings indicated that metformin may have therapeutic application in the treatment of thyroid cancer. Multiple studies have recently focused on the relationship between apoptosis and autophagy. Autophagy and apoptosis are both important biological processes and their relationship is complex. Wang *et al* (46) revealed that autophagy activated by metformin reversed hyperglycemia-induced cardiomyocyte apoptosis in H9c2 cells. Similar results were observed in the Xiao *et al* and Li *et al* studies (47,48), indicating that autophagy may have a protective role in metformin-induced apoptosis. The role and mechanism of autophagy in metformin-induced apoptosis in thyroid cancer remains unclear, and it will be investigated further in our future study.

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Availability of data and materials

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

Authors' contributions

JY, LQ, KC, SS and RL performed the experiments, WZ and CZ designed the study, JY and LQ prepared and wrote the study. All authors read and approved the final manuscript and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The animal protocol was approved by the Institutional Animal Care and Use Committee of the First Affiliated Hospital of Zhengzhou University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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