Itraconazole attenuates hepatic gluconeogenesis and promotes glucose uptake by regulating AMPK pathway

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Abstract. The primarily metabolic abnormality in type 2 diabetes mellitus (T2DM) is the defect in gluconeogenesis and glucose uptake. Itraconazole (ITCZ) is a traditional azole drug with anti-fungal and anticancer properties. However, limited attention has been directed towards the contribution of ITCZ to hepatic gluconeogenesis and glucose uptake in T2DM. The present study aimed to investigate the potential effects of ITCZ on hepatic gluconeogenesis and glucose uptake as well as the underlying mechanisms. No obvious change in cell viability was detected by MTT assay in HepG2 cells with ITCZ treatment at gradually increasing concentrations. Western blot analysis demonstrated that the phosphorylation level of 5' adenosine monophosphate-activated protein kinase (AMPK) was significantly elevated by ITCZ treatment at ≥5 μg/ml (P<0.05). Moreover, ITCZ repressed the gluconeogenesis of HepG2 cells, as evidenced by the dose-dependently increased glycogen synthase kinase 3β phosphorylation level and a notably decreased glucose production rate (P<0.05). Simultaneously, the expression of peroxisome proliferator-activated receptor γ co-activator 1α, phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) in HepG2 cells was reduced by ITCZ in a dose-dependent manner (P<0.001). Furthermore, a 2-deoxyglucose uptake assay revealed that the glucose uptake of HepG2 cells was notably enhanced, accompanied by the ITCZ dose-dependent upregulation of glucose transporter-4 (GLUT-4) (P<0.05). Conversely, silencing of AMPK by small interfering RNA resulted in an increase of ITCZ-reduced gluconeogenesis and inhibition of ITCZ-induced glucose uptake with relative upregulation of PEPCK and G6Pase and downregulation of GLUT4 in the presence of 50 μg/ml ITCZ (P<0.05). Overall, the results indicated that AMPK has an important role in regulating ITCZ-induced glucose uptake by stimulating GLUT4 in HepG2 cells. Therefore, ITCZ may become a promising candidate for T2DM therapy.

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Introduction

Diabetes is a multifactorial disease with ~366 million individuals affected worldwide in 2013. An estimated 36% of these patients come from the Western Pacific region, particularly China and India (1). It is majorly classified into type 1 diabetes mellitus (T1DM) and T2DM. T2DM accounts for 90-95% of all diabetes cases and is a chronic metabolic disease characterized by insulin resistance and impaired glucose and lipid metabolism (2-4). Uncontrolled glucose production and reduced glucose uptake are considered to be the main driver of impaired glucose metabolism (5,6). The liver is the major organ for maintaining glucose homeostasis by releasing glucose into the circulation when fuel is limited and converting glucose into lipid when fuel is in excess (7). In the diabetic state, hepatic gluconeogenesis contributes to 60% of the continuous glucose production (8). Hence, inhibiting gluconeogenesis and improving glucose uptake are considered to be effective approaches for the treatment and prevention of T2DM.

Itraconazole (ITCZ) is an orally bioavailable azole drug approved by the US Food and Drug Administration for the treatment and prevention of fungal infections by targeting lanosterol 14α-demethylase to repress the conversion of lanosterol to ergosterol in fungi (9). Moreover, ITCZ is the only azole anti-fungal that is known to have potent anti-angiogenic and anti-proliferative activities in non-small cell lung cancer and glioblastoma cells (10,11), and has been shown to prolong the overall survival of patients with metastatic pancreatic cancer, ovarian cancer and breast cancer (12-15). Sano *et al* (16) reported that ITCZ was capable of reducing the inflammatory degree, mucosal hyperplasia and *Candida* infection in alloxan-induced diabetic rats (16), while Verspeelt *et al* (17) reported that ITCZ was safe and efficient against dermatological disorders in diabetic patients. Apart from that, Head *et al* (18) reported that ITCZ activated the adenosine monosphosphate-activated protein kinase (AMPK) signaling pathway in human umbilical vein cells. Activation of AMPK suppressed glucose production and stimulated glucose uptake in the liver, which is recognized as an attractive therapeutic strategy for the treatment of T2DM and associated metabolic disorders (19). It was therefore speculated that ITCZ may be promising for adjusting the abnormal of hepatic glucose

metabolism. However, these effects and the underlying mechanisms have remained to be fully elucidated.

In the present study, HepG2 cells were subjected to various concentrations of ITCZ for various durations to detect the cytotoxicity and explore the effect of ITCZ on the phosphorylation level of AMPK and subsequent hepatic gluconeogenesis and glucose uptake. The results showed that there was no cytotoxicity of ITCZ to HepG2 cells, and activation of AMPK decreased the expression of peroxisome proliferator-activated receptor γ co-activator 1α (PGC-1α), phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G-6-Pase) to block gluconeogenesis and elevated the expression level of glucose transporter-4 (GLUT4) to promote glucose uptake in HepG2 cells.

Materials and methods

Cell culture. The HepG2 cell line was obtained from the cell bank of the Shanghai Institutes for Biological Sciences (Chinese Academy of Sciences, Shanghai, China). HepG2 cells were grown in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with streptomycin/penicillin (100 U/ml) and 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA) at 37˚C in a 5% CO_2 incubator. Upon reaching 80-90% confluency, cells were digested for subsequent experiments.

Drug treatment. ITCZ (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was dissolved in dimethyl sulfoxide (DMSO). Cells were exposed to ITCZ at an ascending range of concentrations (0, 0.5, 2, 5, 10, 20 or 50 μg/ml) for 24 or 6 h for MTT assay or AMPK expression detection, respectively. For the AMPK interference assay, cells were cultured with 50 μg/ml ITCZ for 24 h. Increased concentrations of ITCZ (0, 5, 10, 50 μg/ml) were employed for the other experiments. Untreated cells (0 μg/ml ITCZ) were used as a control.

MTT assay. Cells were seeded in 96-well plates at a density of $2x10^3$ cells per well and cultured to 80% confluence. Cells were subsequently incubated with the indicated concentrations of ITCZ for 24 h with five replicates for each condition, including blank wells and untreated controls. Thereafter, cells were exposed to 8 μl MTT (0.2 mg/ml; Sigma-Aldrich; Merck KGaA) for 4 h prior to incubation with 200 μl DMSO (Sigma-Aldrich; Merck KGaA) to dissolve the crystals. Optical density (OD) values were assessed at 490 nm using a microplate reader (BioTek, Winooski, VT, USA).

Glucose production assay. A glucose production assay was performed according to previously published methods (20). In brief, HepG2 cells were seeded in 6-well plates at a density of $5x10^5$ cells per well and exposed to indicated concentrations of ITCZ for 6 h, followed by washing twice with PBS to remove the remaining glucose. The cells were then incubated with 100 μl glucose production medium containing 2 mM of sodium pyruvate and 20 mM of sodium lactate (Sigma-Aldrich; Merck KGaA) for 4 h. 50 μl supernatant was collected and the glucose concentration was measured by a Glucose (HK) Assay kit (Sigma-Aldrich; Merck KGaA) following the manufacturer's instructions.

2-Deoxyglucose uptake assay. Glucose uptake was analyzed as described in a previous study (21). In brief, HepG2 cells were seeded in 24-well plates and subjected to serum-free DMEM supplemented with 5.5% glucose for 24 h, followed by incubation in sequence with ITCZ for 6 h and [^{14}C]-2-deoxy-d-glucose (2-DG) transport buffer (Seebio Biotech, Inc., Shanghai, China) for 30 min. Thereafter, the cells were washed twice with PBS and lysed with 200 mg/ml NaOH. The glucose uptake was determined by the radioactivity of [^{14}C]-2-DG using a TopCount scintillation counter (Packard, Meriden, CT, USA).

RNA interference of AMPK. The small interfering (si)RNA for AMPK-α1 and control siRNA were synthesized by GenePharma Co., Ltd. (Shanghai, China). Sequences were as follows: AMPK-siRNA, 5'-AATTACTTCTGGTGCAGC ATAGCGG-3' and control siRNA, 5'-TTCTCCGAACGT GTCACGT-3'. 75 pmol AMPK-siRNA and control siRNA were transfected into HepG2 cells, respectively, by using Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Cells were harvested after 24 h of transfection for subsequent experiments.

Reverse-transcription quantitative polymerase chain reaction analysis (RT-qPCR). Total RNA in each group was extracted by an RNA extraction kit (Tiangen Biotech, Beijing, China) strictly following the manufacturer's instructions and reverse-transcribed into complementary DNA. A High Capacity cDNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used to convert RNA into cDNA. Briefly, total RNA (0.5 μg/μl) 3 μg, Oligo dT Primer 1 μl (Takara Biotechnology Co., Ltd., Dalian, China), dNTP Mixture 1 μl (Takara Biotechnology Co., Ltd.) and Rnase-free ddH_2O (Solarbio Science and Technology Ltd., Beijing, China) were mixed with a total mixture of 10 μl. The mixture was stirred at 70˚C for 5 min and put into ice immediately. Then 10 μl mixture of template RNA and primer, 4 μl 5X PrimeScript II buffer, 0.5 μl RNase inhibitor, 1 μl PrimeScript II RTase and 4.5 μl RNase-free ddH_2O were mixed and then reacted at 45˚C for 45 min and at 95˚C for 5 min. PCR reactions were performed with using SYBR-Green mastermix (Solarbio, Beijing, China) in an Exicycler™ 96 (Bioneer, Daejeon, Korea) with the following composition and cycling profile: PrimeSTAR buffer 5.0 μl (Takara Biotechnology Co., Ltd.); dNTP Mixture (2.5 mM) 2.5 μl (Takara Biotechnology Co., Ltd.); forward primer (10 mM); 1.0 μl, reverse primer (10 mM) 1.0 μl; genomic DNA 1.0 μl (Takara Biotechnology Co., Ltd.); PrimeSTAR HS DNA Polymerase (2.5 U/μl) 0.5 μl (Takara Biotechnology Co., Ltd.); DEPC-treated water (Solarbio) 14 μl; initial denaturation at 95˚C for 5 min, followed by 30 cycles of 95˚C for 20 sec, 60˚C for 20 sec and 72˚C for 30 sec. Primer sequences (synthesized by Sangon Biotech Co., Ltd. Shanghai, China) were as follows: Glycogen synthase kinase (GSK)3β forward, 5'-CCT TAACCTGGTGCTGGACT-3' and reverse, 5'-AGCTCTGGT GCCCTGTAGTA-3'; PGC-1 forward, 5'-ATGCACTGACAG ATGGAGACGTGAC-3' and reverse, 5'-GTTCCTATACCA TAGTCATGCATTG-3'; PEPCK forward, 5'-GCTCTGAGG AGGAGAATGG-3' and reverse, 5'-TGCTCTTGGGTGACG ATAAC-3'; G6Pase forward, 5'-GTGAATTACCAAGAC

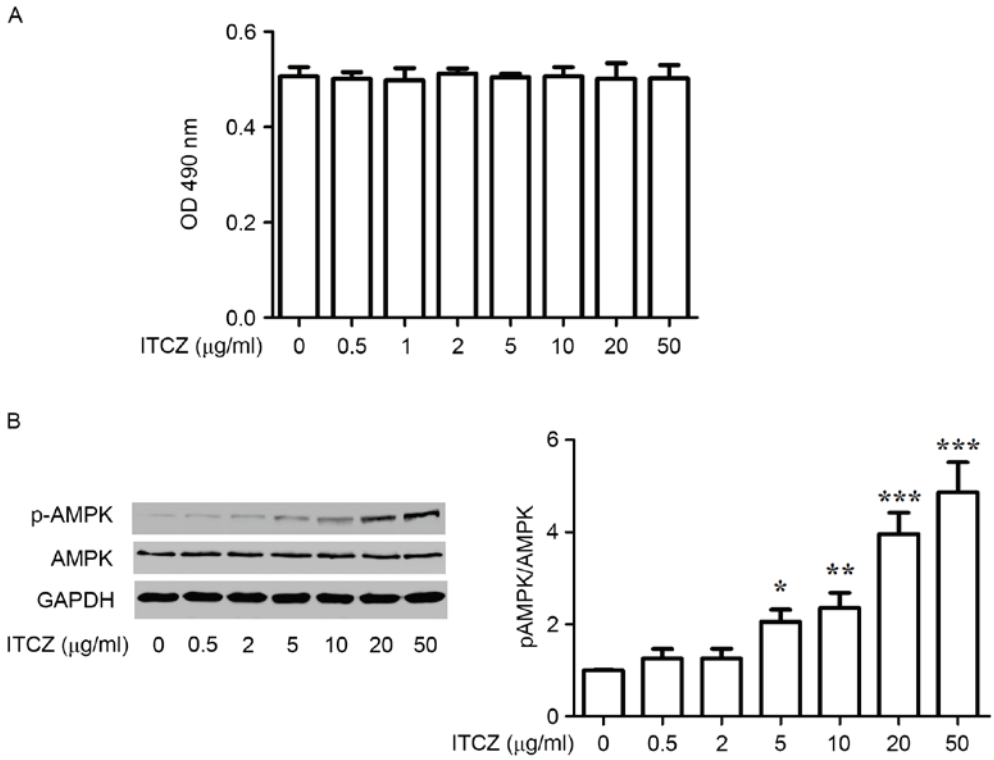

Figure 1. ITCZ activates the AMPK pathway without significant cytotoxicity in HepG2 cells. (A) HepG2 cells were seeded in 96-well plates with 5 replicates in each testing point and exposed to increased concentrations of ITCZ (0.5, 1, 3, 2, 5, 10, 20 or 50 μM) for 24 h, followed by MTT assay. The absorbance was read at 490 nm. (B) HepG2 cells were incubated with indicated concentrations of ITCZ for 6 h. Representative images of western blots showing p-AMPK and AMPK protein expression levels are shown. GAPDH was used as an internal control. The p-AMPK/AMPK ratio is shown in the bar graph. All values are expressed as the mean ± standard deviation from three independent experiments. *P<0.05, **P<0.01, ***P<0.001 vs. 0 μg/ml ITCZ. p-AMPK, phosphorylated 5' adenosine monophosphate-activated protein kinase; ITCZ, itraconazole; OD, optical density.

TCCCAG-3' and reverse, 5'-GCCCATGGCATGGCCAGAGGG-3'; GLUT4 forward, 5'-GCCAGCCTACGCCACCATA-3' and reverse, 5'-ACCCATAGCATCCGCAAC-3'; GAPDH forward, 5'-CACCCACTCCTCCACCTTTG-3' and reverse, 5'-CCACCACCCTGTTGCTGTAG-3'. Relative mRNA levels were calculated by the $2^{-\Delta\Delta Cq}$ method (22). GAPDH served as an internal control.

Western blot analysis. Total protein was extracted by lysing HepG2 cells with radioimmunoprecipitation assay buffer containing 1% phenylmethanesulfonyl fluoride (Beyotime Institute of Biotechnology, Inc., Haimen, China). The protein concentration was quantitated with a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology, Inc.). Equal amounts of protein from each sample were loaded and separated by 10% SDS-PAGE followed by electrotransfer onto polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). After blocking with 5% non-fat milk for 1 h, the membranes were probed with primary antibodies against AMPK-α1 (ab32047), phosphorylated (p)-AMPK (phospho S487) (ab131357), GSK3β (ab32391) (all diluted at 1:5,000; Abcam, Cambridge, MA, USA), p-GSK3β (ab75745), PGC-1α (ab54481) (both diluted at 1:1,000; Abcam), PEPCK (sc-377027) (diluted at 1:100; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), G-6-Pase (ab83690) (diluted at 1:250; Abcam) and GLUT4 (ab654) (diluted at 1:2,500; Abcam) at 4˚C overnight, and subsequently incubated with the corresponding secondary horseradish peroxidase-conjugated anti-rabbit (ab6721) or anti-mouse (ab6785) immunoglobulin G antibodies (1:5,000 dilution; Beyotime Institute of Biotechnology, Inc.) at 37˚C for 45 min. Unbound antibodies in each step were washed with Tris-buffered saline containing Tween-20 four times. The target bands were visualized by enhanced chemiluminescence solution (Qihai Biotec, Shanghai, China) and relative band intensities were analyzed using Gel-Pro-Analyzer software (Media Cybernetics, Bethesda, MD, USA). GAPDH was served as an internal control.

Statistical analysis. Statistical analysis was performed using GraphPad Prism 5.0 software (GraphPad Inc., La Jolla, CA, USA). Values are expressed as the mean ± standard deviation. Differences between groups were analyzed using one-way analysis of variance. P<0.05 was considered to indicate a statistically significant difference.

Results

ITCZ activates AMPK pathway without significant cytotoxicity in HepG2 cells. An MTT assay was performed to assess the cytotoxicity of ITCZ. There were no significant differences in HepG2 cell viability between the control group and ITCZ at the concentrations applied (Fig. 1A).

Next, western blot analysis was used to evaluate the phosphorylation of AMPK. The results showed that after 6 h, the phosphorylation level of AMPK in HepG2 cells was significantly and dose-dependently elevated by ITCZ at ≥5 μg/ml

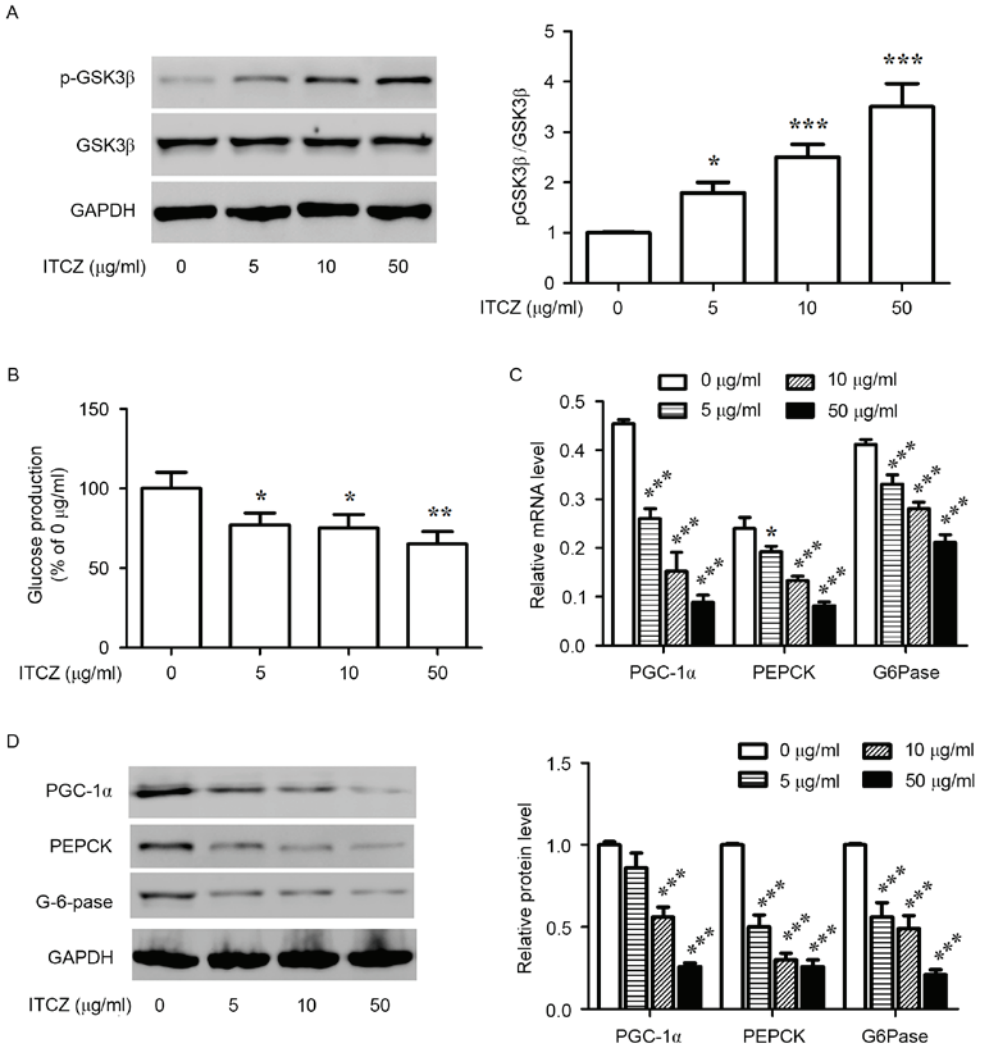

Figure 2. ITCZ attenuates hepatic gluconeogenesis. HepG2 cells were treated with different concentrations of ITCZ (0, 5, 10 or 50 μg/ml) for 6 h in flasks or 6-well plates. (A) Western blot analysis was performed to detect the expression and phosphorylation level of GSK3β. Representative blots and corresponding densitometric analysis are shown. (B) HepG2 cells were seeded in 6-well plates, incubated with ITCZ at the indicated concentrations, and glucose production was evaluated by a Glucose Assay kit. (C) The mRNA expression levels of PGC-1α, PEPCK and G6Pase were analyzed by reverse-transcription quantitative polymerase chain reaction analysis. (D) The protein expression levels of PGC-1α, PEPCK and G6Pase were analyzed by western blot. A series of representative images are shown. GAPDH served as an internal control. The above experiments were performed in triplicate and values are expressed as the mean ± standard deviation. *P<0.05, **P<0.01, ***P<0.001 vs. 0 μg/ml ITCZ. p-GSK, phosphorylated glycogen synthase kinase; ITCZ, itraconazole; PGC-1α, peroxisome proliferator-activated receptor γ co-activator 1α; PEPCK, phosphoenolpyruvate carboxykinase; G6Pase, glucose-6-phosphatase.

compared with that in the ITCZ un-treated control (P<0.05; Fig. 1B). Therefore, 5, 10 and 50 μg/ml ITCZ was used for subsequent experiments. The above results suggested that ITCZ was safe for HepG2 cells and activated the AMPK pathway in HepG2 cells.

ITCZ attenuates hepatic gluconeogenesis. To explore the effect of ITCZ on hepatic gluconeogenesis, the glucose oxidase reagent, RT-qPCR and western blot analysis were used to analyze glucose production and the expression of associated genes of the gluconeogenesis pathway. It was discovered that after ITCZ treatment for 24 h, the phosphorylation level of GSK3β was notably increased in a dose-dependent manner (P<0.05; Fig. 2A), indicating an inhibitory effect of ITCZ on hepatic gluconeogenesis. In line with this, the glucose levels in HepG2 cells were obviously and dose-dependently decreased by ITCZ treatment compared with those in the ITCZ un-treated control (P<0.05; Fig. 2B). Furthermore, a significant and dose-dependent decline of PGC-1α, PEPCK and G6Pase expression at the mRNA and protein level compared with the 0 μg/ml ITCZ group was noted (P<0.05; Fig. 2C and D). The above results indicated that ITCZ attenuated hepatic gluconeogenesis by downregulating PGC-1α, PEPCK and G6Pase.

ITCZ promotes glucose uptake. To address the effect of ITCZ on hepatic glucose uptake, a glucose uptake assay, RT-qPCR and western blot analysis were performed to detect the status of glucose uptake and the expression of associated genes. The results showed that 10 μg/ml ITCZ significantly enhanced the level of glucose uptake by 1.35-fold of that of the 0 μg/ml ITCZ group (P<0.05; Fig. 3A), and an even greater elevation was observed with 50 μg/ml ITCZ treatment with increasing level of glucose uptake by 1.5-fold of that of the 0 μg/ml ITCZ group (P<0.01). In parallel with this, the mRNA and protein expression of GLUT4 was significantly elevated in an ITCZ dose-dependent manner in comparison with the 0 μg/ml ITCZ

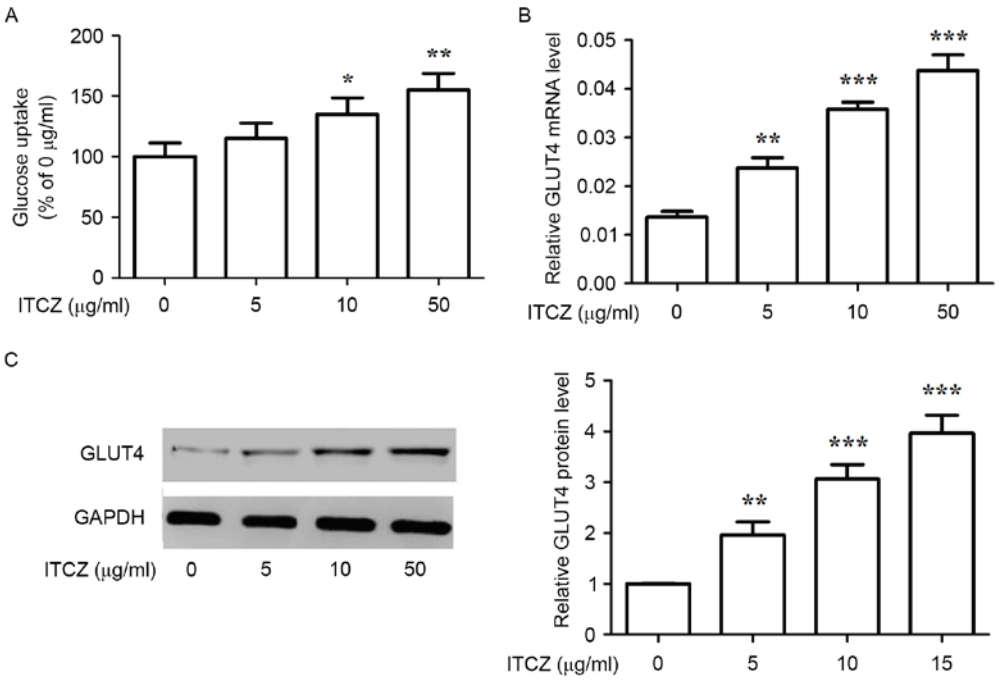

Figure 3. ITCZ promotes glucose uptake. HepG2 cells were incubated with different concentrations of ITCZ (0, 5, 10 and 50 μg/ml) for 6 h in flasks or 24-well plates. (A) HepG2 cells were seeded in 24-well plates, incubated with ITCZ at the indicated concentrations and the glucose uptake was determined via a [^{14}C]-2-deoxy-D-glucose radioactivity assay. (B) Reverse-transcription quantitative polymerase chain reaction analysis of GLUT4 mRNA expression. (C) Western blot analysis of GLUT4 protein expression levels. Representative blot images are shown on the left. GAPDH was used as an internal control. The above experiments were performed triplicate and values are expressed as the mean ± standard deviation. *P<0.05, **P<0.01, ***P<0.001 vs. 0 μg/ml ITCZ. GLUT4, glucose transporter-4; ITCZ, itraconazole.

group (P<0.01; Fig. 3B and C). The above results confirmed that ITCZ promoted hepatic glucose uptake.

Silencing of AMPK reverses ITCZ-induced anti-gluconeogenetic and pro-glucose uptake effects in HepG2 cells. To further examine whether the anti-gluconeogenic and pro-glucose uptake effects of ITCZ were mediated by the AMPK pathway, AMPK was silenced by siRNA interference. As shown in Fig. 4A, AMPK knockdown completely inhibited the anti-gluconeogenetic effects of ITCZ (P<0.05). Simultaneously, it also significantly inhibited the pro-glucose uptake effects of ITCZ (P<0.05; Fig. 4B). Similarly, the ITCZ-induced changes in these proteins were completely inhibited by AMPK siRNA, resulting in basal levels (Fig. 4C). Taken together, while the exact role of the AMPK pathway in the anti-gluconeogenic and pro-glucose uptake effects of ITCZ in HepG2 cells remains to be fully elucidated, the results indicated that the anti-gluconeogenic and pro-glucose uptake effects of ITCZ were mediated via the AMPK pathway, as suppression of AMPK apparently inhibited these processes.

Discussion

ITCZ is a triazole anti-fungal agent with a cytostatic effect in various malignancies, and has been shown to suppress inflammation and infection in diabetic rats (23). However, the effect of ITCZ on hepatic gluconeogenesis and glucose uptake in T2DM and the underlying mechanisms have remained to be fully elucidated. The present study confirmed that ITCZ was able to activate the AMPK pathway to suppress hepatic gluconeogenesis by repressing the expression of PGC-1α, PEPCK and G-6-Pase and promote glucose uptake by increasing the expression of GLUT4 in HepG2 cells. The present study observed that ITCZ was non-cytotoxic to hepatocytes, which was consistent with the results by Verspeelt *et al* (17). AMPK is a heterotrimeric serine/threonine kinase ubiquitously expressed in mammalian organs, including the liver, and acts as a major cellular energy sensor and a master regulator of metabolic homeostasis. Activation of AMPK induced inhibition of hepatic gluconeogenesis and promotion of glucose uptake is the main focus of T2DM drugs, such as methotrexate, ginsenoside Rg, cocoa flavonoids and metformin (24-26). Accumulating evidence has proved that activated AMPK phosphorylated CRE-binding protein (CREB)-regulated transcription co-activator 2 (CRTC2) and prevented the transportation of CRTC2 from cytoplasm to the nucleus, ultimately leading to the downregulation of gluconeogenic genes in the liver (27). AMPK also regulates the expression of GLUT4 to participate in the glucose uptake process (28). The present study found that the AMPK pathway was dose-dependently activated by ITZC treatment in HepG2 cells, suggesting an anti-diabetic effect of ITZC.

GSK3β is an isoform of GSK3 that negatively regulates insulin-mediated glucose homeostasis and glycogen synthesis. Phosphorylation of the N-terminal Ser 9 in GSK3β has been reported to exhibit anti-diabetic effects in animal models and *in vitro* (29). Overexpression and activation of GSK3β are considered to be potential reference indexes for evaluation of patients with T2DM and obese animal models (29). The present study observed an ITCZ dose-dependently increased activation of GSK3β and the corresponding reduction of glucose production in HepG2 cells, indicating that ITCZ

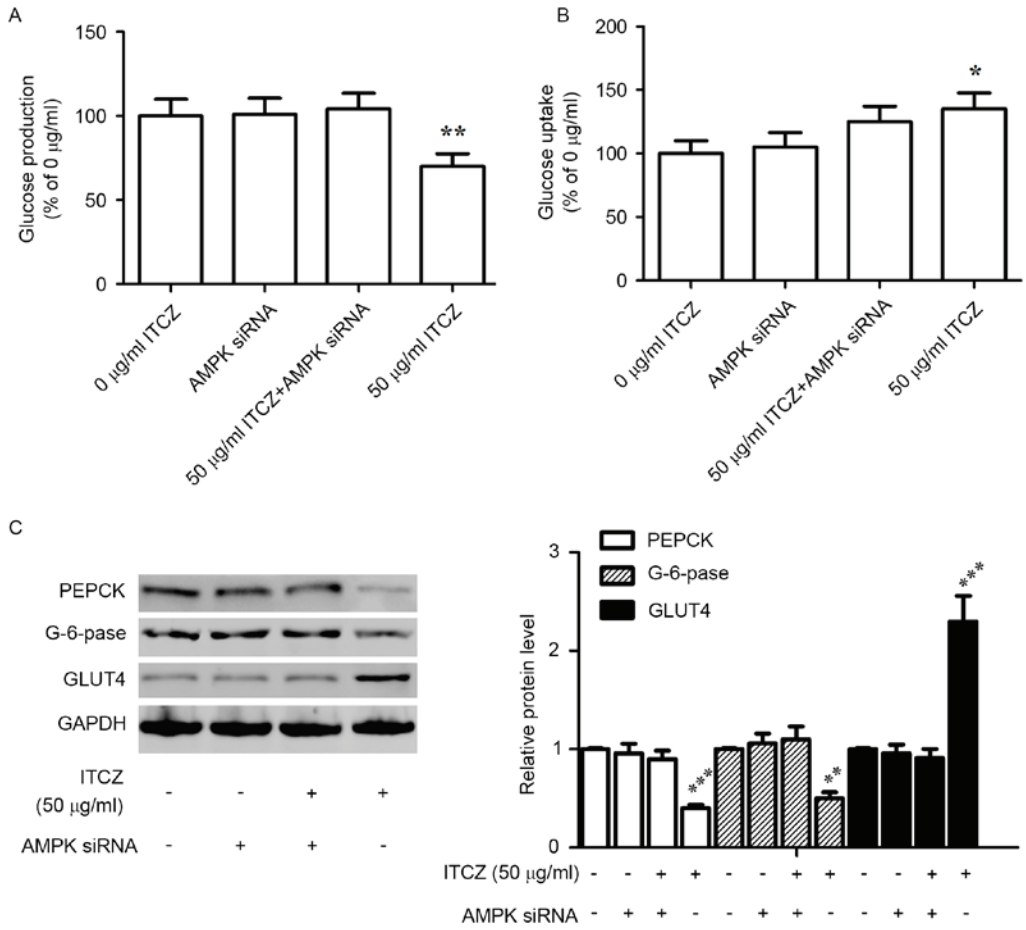

Figure 4. Silencing of AMPK reverses ITCZ-induced anti-gluconeogenic and pro-glucose uptake effects in HepG2 cells. Cells with or without AMPK siRNA interference were cultured in flasks or plates with 50 μg/ml ITCZ for 24 h. (A) Cell gluconeogenic capacity was measured by a glucose production assay. (B) Cell glucose uptake was determined via the radioactivity of [^{14}C]-2-deoxy-D-glucose. (C) Representative images of western blots showing the expression of PEPCK, G6Pase and GLUT4 are shown, and expression levels were quantified in a bar graph. GAPDH was used as internal control. The above experiments were performed in triplicate and values are expressed as the mean ± standard deviation. *P<0.05, **P<0.01, ***P<0.001 vs. 50 μg/ml ITCZ + AMPK siRNA. GLUT4, glucose transporter-4; ITCZ, itraconazole; AMPK, small interfering RNA targeting 5' adenosine monophosphate-activated protein kinase; PEPCK, phosphoenolpyruvate carboxykinase; G6Pase, glucose-6-phosphatase.

attenuated hepatic gluconeogenesis in a dose-dependent manner.

The rate of gluconeogenesis is controlled by PEPCK and G-6-Pase. Activated PEPCK catalyzes the reaction from phosphoenolpyruvate to oxaloacetic acid, which is a rate-limiting step in gluconeogenesis (30). Activated G-6-Pase catalyzes the production of free glucose from glucose 6-phosphate, which is the final step of gluconeogenesis (31). Thus, pharmacological intervention of the expression of PEPCK and G-6-Pase is regarded as a potential approach for the treatment of metabolic aberrations. In addition, CRTC2-stimulated PGC-1α is a co-activator protein capable of increasing the transcriptional activation of the G-6-Pase and PEPCK promoters by directly interacting with forkhead box O1 and hepatocyte nuclear factor 4α (27,31,32). Knockout or knockdown of PGC-1α in mice led to lower blood glucose levels, reduction of gluconeogenic gene expression and activation of AMPK (33,34). The present study confirmed that ITCZ caused a dose-dependent inhibition of PGC-1α, PEPCK and G-6-Pase in HepG2 cells at the transcriptional level, accompanied by a significant decrease in glucose production. However, these effects were completely abrogated after silencing of AMPK in ITCZ-treated HepG2 cells. These results indicated that ITCZ decrease the glucose synthesis rate to reduce gluconeogenesis through AMPK-mediated downregulation of PGC-1α, PEPCK and G-6-Pase in HepG2 cells. The GLUT protein family consists of a series of integral membrane proteins involved in the eukaryotic transportation of monosaccharides, polyols and other small carbon molecules across cell membranes. Among all fourteen members in humans, GLUT4, encoded by the SLC2a4 gene, is well known as a determinant of the cell glucose uptake rate, which is closely associated with metabolic abnormalities in insulin-resistant T2DM (35). Numerous flavonoids and phenolic compound increase the glucose uptake by promoting the translocation of GLUT4 to the plasma membrane in cells to relieve T2DM. It is a well-established fact that activated AMPK phosphorylates GLUT4 enhancer factor to induce GLUT4 transcription (36). The present study identified a significantly elevated glucose uptake of HepG2 cells after treatment with various concentrations of ITCZ, along with a dose-dependent enhancement of GLUT4 expression. Furthermore, silencing of hepatic AMPK resulted in inhibition of ITCZ-induced downregulation of GLUT4 and increased glucose uptake by HepG2 cells. It is

therefore concluded that ITCZ-induced glucose uptake and inhibition of glucose production via suppression of GLUT4 in HepG2 cells is mediated by AMPK.

In conclusion, the present study revealed that ITCZ attenuate hepatic gluconeogenesis and promoted glucose uptake without cytotoxicity to HepG2 cells, and that these effects were mediated via the AMPK pathway. These preliminarily results identified the anti-diabetic roles of ITCZ in HepG2 cells, indicating that ITCZ may serve as a potential drug for the treatment of T2DM.

Acknowledgements

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