Biochemical and Biophysical Research Communications 417 (2012) 147–152

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Metformin prevents endoplasmic reticulum stress-induced apoptosis through AMPK-PI3K-c-Jun NH_2 pathway

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ARTICLE INFO

Article history:
Received 10 November 2011
Available online 23 November 2011

Keywords:
AMPK
JNK
PI3 kinase
NIT-1
Metformin
T2DM
Apoptosis
Thapsigargin

ABSTRACT

Type 2 diabetes mellitus is thought to be partially associated with endoplasmic reticulum (ER) stress toxicity on pancreatic beta cells and the result of decreased insulin synthesis and secretion. In this study, we showed that a well-known insulin sensitizer, metformin, directly protects against dysfunction and death of ER stress-induced NIT-1 cells (a mouse pancreatic beta cell line) via AMP-activated protein kinase (AMPK) and phosphatidylinositol-3 (PI3) kinase activation. We also showed that exposure of NIT-1 cells to metformin (5 mM) increases cellular resistance against ER stress-induced NIT-1 cell dysfunction and death. AMPK and PI3 kinase inhibitors abolished the effect of metformin on cell function and death. Metformin-mediated protective effects on ER stress-induced apoptosis were not a result of an unfolded protein response or the induced inhibitors of apoptotic proteins. In addition, we showed that exposure of ER stressed-induced NIT-1 cells to metformin decreases the phosphorylation of c-Jun NH_2 terminal kinase (JNK). These data suggest that metformin is an important determinant of ER stress-induced apoptosis in NIT-1 cells and may have implications for ER stress-mediated pancreatic beta cell destruction via regulation of the AMPK–PI3 kinase–JNK pathway.

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1. Introduction

Type 2 diabetes mellitus (T2DM) is a metabolic disease that is characterized by high blood sugar levels resulting from defects in insulin sensitivity and glucose utilization [1,2]. Pancreatic beta cells are destroyed by metabolic overload during chronic T2DM conditions [3]. Such destruction is caused by several stresses such as endoplasmic reticulum (ER) stress and reactive oxygen species (ROS) resulting from hyperlipidemic conditions [4–6].

The ER is the site for protein synthesis, folding, modification, and secretion in the cell [7]. Disruption of ER homeostasis by ER stress has been implicated in many kinds of human diseases, including neurodegenerative disease, cystic fibrosis, and diabetes mellitus [8]. Artificially, ER stress can be elicited by chemical components, including tunicamycin, a protein N-glycosylation inhibitor; brefeldin A, which inhibits protein transport from the ER to the Golgi apparatus; and thapsigargin (TG), which blocks ER uptake of calcium by inhibiting the sarcoplasmic/endoplasmic Ca2+-ATPase [9]. ER stress-induced unfolded and misfolded proteins cause the ER stress response [7]. This signal regulates ER chaperone proteins to inhibit protein aggregation and translation and induce the proteasome machinery system for degradation of mis- and un-folded proteins for survival [10]. Otherwise, ER stress activates several pathways leading to cell death, including apoptosis [11]. Non-esterified free fatty acids can be an ER stressor in late T2DM conditions [12]. A recent study [13] has shown a relationship between obesity and ER stress. ER stress has been known as a critical factor for the destruction of pancreatic beta cells because these cells must synthesize and secret abundant insulin to compensate for metabolic needs [14].

Metformin, an AMP-activated protein kinase (AMPK) activator, is widely used for the treatment of T2DM [15,16]. This drug sensitizes insulin responses via hepatic glucose output decrease and glucose uptake and utilization increase [17]. Previously, we have shown protective effects of AMPK against palmitate and ER stress-induced apoptosis in HepG2 cells [18].

In this study, we examined the protective effects of metformin in NIT-1 cells under ER stress. We found that metformin-induced AMPK activation is able to inhibit TG-induced apoptosis via AMPK, PI3 kinase, and JNK inhibition.

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0006-291X/$ - see front matter Published by Elsevier Inc.
doi:10.1016/j.bbrc.2011.11.073

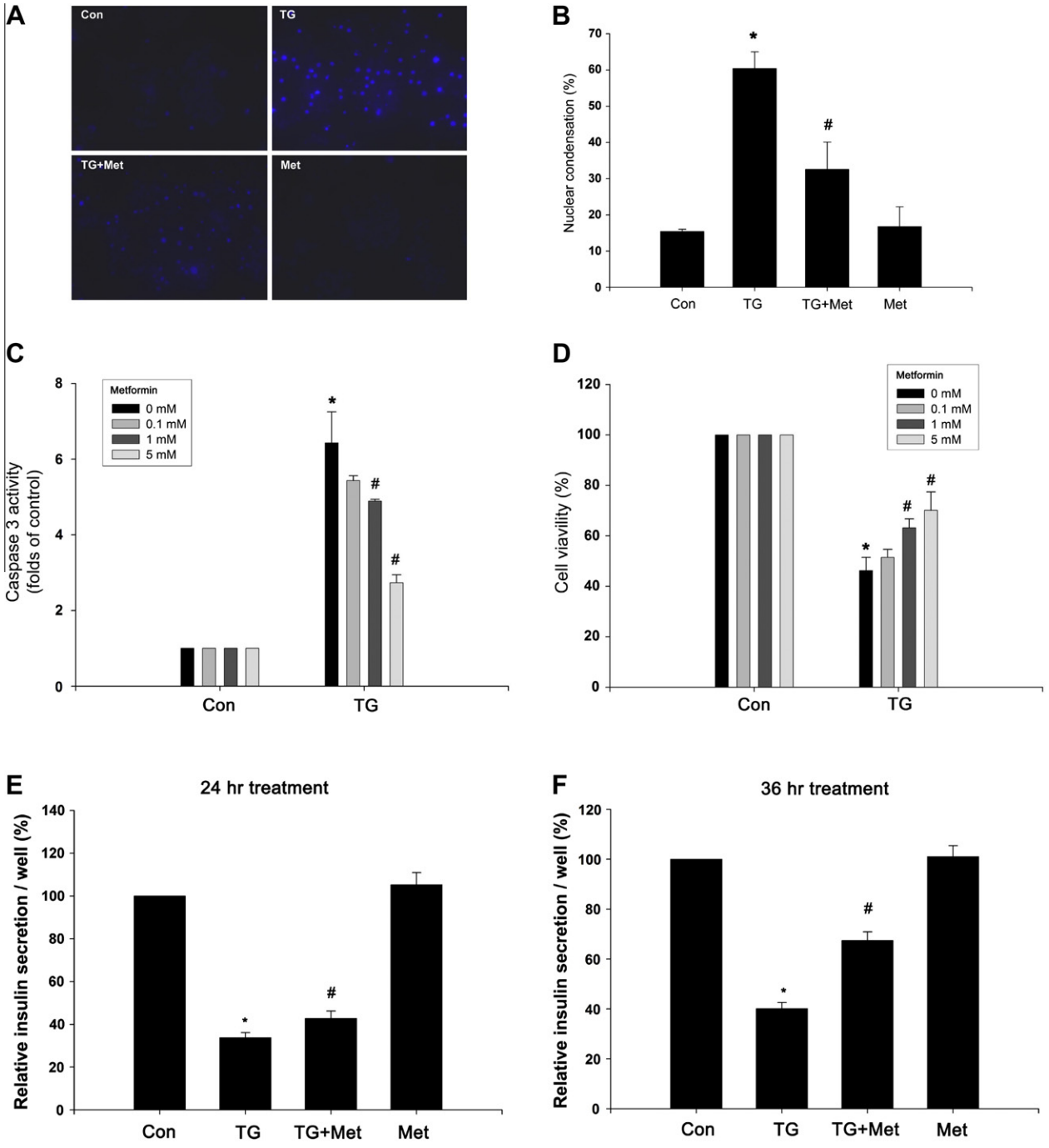

Fig. 1. Metformin blocks thapsigargin-induced apoptosis in a dose-dependent manner. (A) Chromatin condensation assay was performed by Hoechst 33258 staining. (B) Percentage of condensed nuclei as assessed Hoechst 33258 staining of adherent and floating cells. (C) Caspase 3 activity is presented as the mean SD ($n = 4$). (D) Cell viability was measured by MTT assay ($n = 5$). Metformin-induced AMPK was already verified (data not shown). Metformin slightly recovers thapsigargin-induced insulin secretion impairment and induces solely from NIT-1 cells (10^4/well). (E) Twenty six hour treatment. (F) Thirty six hour treatment. Abbreviation used: Con, control; TG, 250 nM thapsigargin; Met, 5 mM metfomin. *Significantly different ($P < 0.05$) from control. #Significantly different ($P < 0.05$) from thapsigargin treatment.

2. Material and methods

2.1. Culture media and reagents

NIT-1 cells were palted at a density of 1×10^5 cells/mL and cultured in DMEM medium supplemented with heat inactivated 10% (v/v) fetal bovine serum (FBS) and 100 U/mL penicillin–100 ug/mL streptomycin. 1,1-Dimethylbiguanide hydrochloride (Metformin) was purchased from Sigma (St. Louis, MO). Thapsigargin as a chemical inducer of the unfolded protein response (UPR) and Wortmannin as a PI3 kinase inhibitor, were purchased from Sigma. Compound c as an AMPK inhibitor, was purchased from calbiochem (San Diego, CA).

2.2. Cell viability assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was dissolved in phosphate buffered saline (PBS) solution at concentration of 5 mg/ml and filtered through a 0.22 μm filter to sterilize and remove insoluble residues then stored in

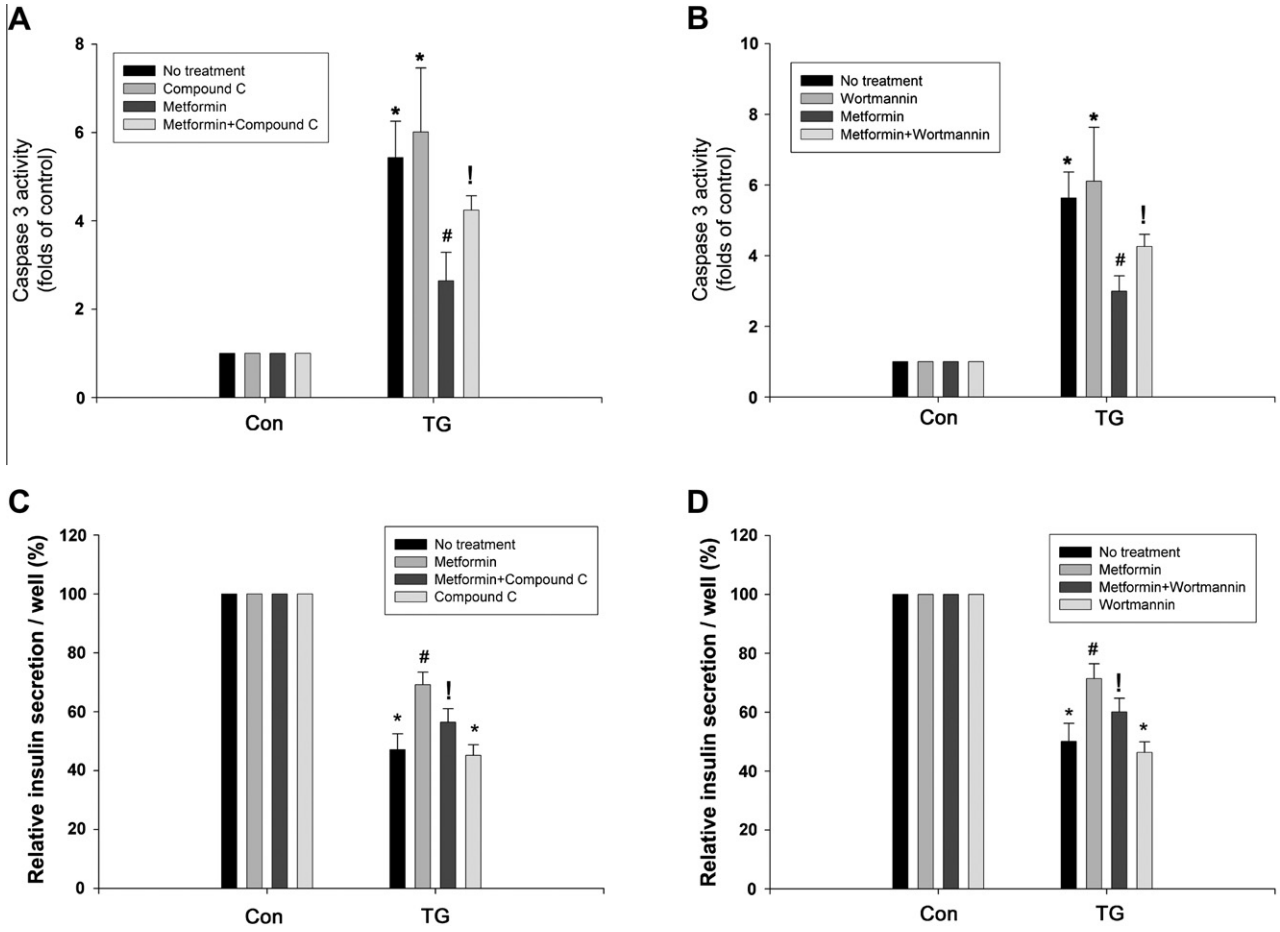

Fig. 2. Metformin inhibits thapsigargin-induced caspase 3 activity and recovers thapsigargin-induced insulin secretion impairment via AMPK and PI3 kinase in NIT-1 cells. (A) The effects of 5 mM metformin and 10 uM compound c, or both, on caspase 3 activity for 24 h. (B) The effects of 5 mM metformin and 1 uM Wortmannin, or both, on caspase 3 activity for 24 h. (C) The effects of 5 mM metformin and 10 uM compound c, or both, on insulin secretion for 36 h. (D) The effects of 5 mM metformin and 1 uM Wortmannin, or both, on insulin secretion for 36 h. Caspase 3 activity and insulin secretion are presented as the mean SD ($n = 3$). Inhibitors-induced inhibition of targets was already verified (data not shown). Abbreviation used: Con, control; TG, 250 nM thapsigargin; Met, 5 mM metfomin. *Significantly different ($P < 0.05$) from control. #Significantly different ($P < 0.05$) from thapsigargin treatment. !Significantly different ($P < 0.05$) from thapsigargin and metformin treatment.

the amber vials at 4 °C for a month. After 24 h incubation, 25 μl of the MTT solution was added to each well of 96-well plates and incubated for 4 h at 37 °C in a humidified atmosphere of 5% CO_2. At end of the incubation period, the media were discarded using a suction pump. The extraction buffer of 20% w/v sodium dodecyl sulfate (SDS) in a solution of 50% of N,N-dimethylformamide (DMF) in demineralized water (50:50, v/v) was prepared at pH 4.7 and filtered through a 0.22 μm filter to remove insoluble residues. The absorbance was determined at 570 nm. The A570 was taken as an index of the cell viability and the activity of mitochondria. The net absorbance from the plates of cells cultured with the control medium (not treated) was considered as 100% of the cell viability and the mitochondrial activity.

2.3. Hoechst 33258 staining

After being treated with thapsigargin and/or metformin for 24 h, the cells incubated with DNA fluorochrome 3 μg/ml of Hoechst 33258 for 20 min. Then, cells were washed with PBS and analyzed by fluorescent microscopy. Cells that exhibited reduced nuclear size, chromatin condensation, intense fluorescence, and nuclear fragmentation were considered as apoptosis.

2.4. RNA extraction and RT PCR analysis

Total RNA was extracted by using TRIzol according to the manufacture's manuals (Invitrogen, Carlsbad, CA). Primer sequences and their respective PCR product lengths were: glucose regulated protein 78, F: 5′-gtcggtgtgttcaagaacgg-3′, R: 5′-ttgcccacctccaatatcaa-3′ (300 bp); CCAAT/enhancer-binding protein homologous protein (CHOP), F: 5′-caacagaggtcacacgcaca-3′, R: 5′-tctccttcatgcgttgcttc-3′ (165 bp); beta-actin, F: 5′-atcactattggcaacgagcg-3′, R: 5′-tcagcaatgcctgggtacat-3′ (200 bp); Bcl-2, F: 5′-ggctggggatgacttctctc-3′, R: 5′-caccccatccctgaagagtt-3′ (138 bp); Bax, F: 5′-ctcaaggccctgtgcactaa-3′, R: 5′-ccagccacaaagatggtcac-3′ (179 bp); cIAP2, F: 5′-ctggtgccaatgacaaggtc-3′, R: 5′-tttgctcggaagttcacagg-3′ (292 bp); spliced XBP-1, F: 5′-gaaccaggagttaagaacacg-3′, R: 5′-aggcaacagtgtcagagtcc-3′ (179 bp), and XIAP, F: 5′-gcggcaatagatagatggca-3′, R: 5′-tcaggggcaaaaggatttct-3′ (197 bp).

2.5. Western blot analysis

Cells were washed with PBS and harvested and incubated in lysis buffer containing 20 mM Hepes (pH 7.4), 1% Triton X-100, 15% glycerol, 2 mM EGTA, 1 mM sodium vanadate, 2 mM dithiothreitol, 10 uM leupeptin, and 5 uM pepstain. Total protein extracts (30 ug) were loaded onto SDS–PAGE, transferred to Hybond-P membranes (Amersham Pharmacia Biotech, Piscataway, NJ). These membranes were binded with antibodies against phosphorylated JNK, total JNK (Cell Signal Technology, Bevery, MA), and beta actin (Sigma). Targetted proteins were detected using horseradish peroxidase conjugated secondary antibodies and reacted with ECL solution (Intron, Seoul, Korea).

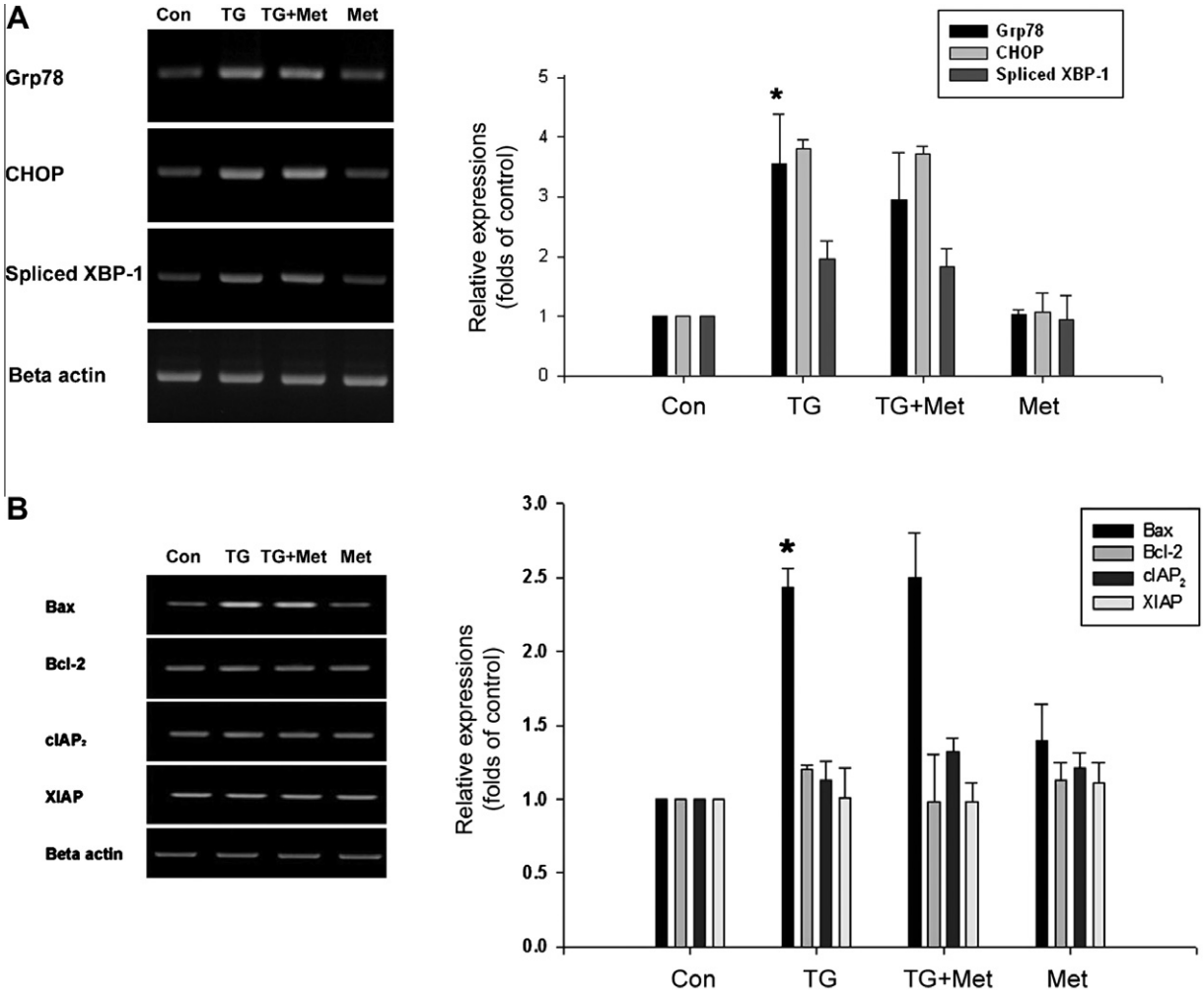

Fig. 3. Metformin is not able to block thapsigargin-induced ER stress indicators (Grp78, CHOP, and XPB-1) in NIT-1 cells. (A) The mRNA expressions of Grp78, CHOP, and spliced XBP-1 were measured by semiquantitative RT-PCR analysis. The treatments were carried out for 24 h. These data are presented as the mean ± SD ($n = 3$). Metformin and thapsigargin are unable to influence the expressions of Bax, Bcl-2, $cIAP_2$, and XIAP in NIT-1 cells. (B) The expressions of these genes were measured by semiquantitative RT-PCR analysis. The treatments were carried out for 24 h. These data are presented as the mean ± SD ($n = 5$). Abbreviation used: Con, control; TG, 250 nM thapsigargin; Met, 5 mM metfomin. *Significantly different ($P < 0.05$) from control.

2.6. Insulin secretion assay from NIT-1 cells

NIT-1 cells were seeded in 1 ml of DMEM containing 25 mM glucose and 10% FBS in a 24 well plate at 10^4 cells/well for 48 h. The cells were washed with HEPES-balanced KRBB containing 2.5 mM glucose and 0.1% fatty acid-free BSA, and preincubated for 1 h at 37 °C in the same medium. After preincubation, the cells were stimulated with 25 mM glucose in HEPES-balanced KRBB at 37 °C for 25 min. Insulin secreted into the supernatant was measured by a radioimmunoassay (Amersham Bioscience, Piscataway, NJ) by using rat insulin as standard.

2.7. Determination of JNK activity

Total cell lysates were assayed for JNK phosphorylation using the Phospho-JNK DuoSet IC ELISA kit (R&D Systems, Minneapolis, MN, USA).

2.8. Determination of caspase-3 activity and cell death

Activity of the caspase-3 class of cysteine protease was determined with the colorimetric activity assay (R&D Systems). Caspase-3 activity was normalized to the total extracted protein concentration. After treatment, culture medium was removed and cells were incubated in PBS of MTT. After 4 h of incubation at 37 °C, NIT-1 cells were solubilized with dimethyl sulfoxide (DMSO).

2.9. Statistical analysis

Statistical comparisons were calculated using analysis of variance. $P < 0.05$ was considered statistically significant. All data are reported as the mean ± SD.

3. Results

3.1. Metformin inhibits ER stress-induced apoptosis

ER stress phosphorylates JNK and impairs insulin sensitivity and beta cell viability [19]. Therefore, we examined a metformin effect in NIT-1 cells under a TG treatment condition. Nuclear condensation (Fig. 1A and B) and caspase-3 activity (Fig. 1C) were elevated, and cell viability (Fig. 1D) was decreased in MTT assay when NIT-1 cells were incubated with TG. Metformin reduced TG-induced chromatin condensation, caspase-3 activity, and cell death in the dose-dependent manner (Fig. 1A–D).

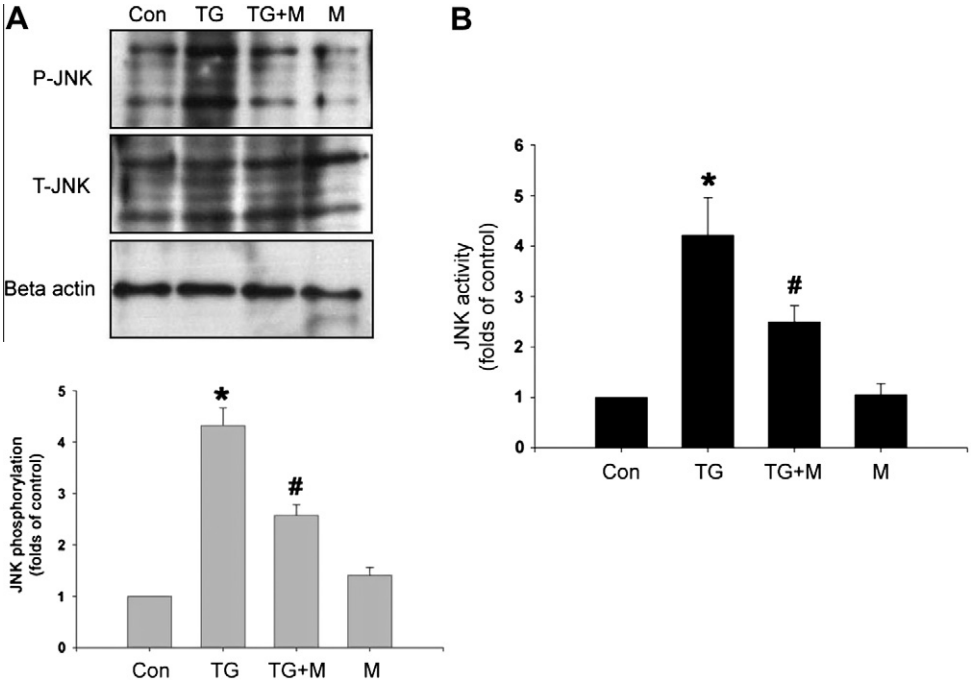

Fig. 4. Metformin inhibits thapsigargin-induced phosphorylation of JNK in NIT-1 cells. (A) The effect of metformin on phosphorylation of JNK was measured by Western blot analysis. (B) JNK activity was measured by using JNK activity assay kit. The treatments were carried out for 24 h. These data are presented as the mean ± SD ($n = 5$). Abbreviation used: Con, control; TG, 250 nM thapsigargin; Met, 5 mM metfomin. *Significantly different ($P < 0.05$) from control. #Significantly different ($P < 0.05$) from thapsigargin treatment.

3.2. Metformin recovers ER stress-induced impaired insulin secretion

A heavy burden on the ER impairs insulin secretion of beta cells [20]. Thus, we verified a metformin effect on impaired insulin secretion by TG. We used 25 mM glucose-induced insulin secretion about 5-folds of basal level (2.5 mM glucose incubation) (data not shown). Glucose-induced insulin secretion was inhibited by TG. Impaired glucose responsiveness was slightly recovered when NIT-1 cells were treated with metformin for 24 h (Fig. 1E); however, the recovery of insulin secretion became more significant and obvious when NIT-1 cells were treated with metformin for 36 h (Fig. 1F).

3.3. AMPK and PI3 kinase inhibitors inhibit metformin-mediated inhibition of TG-induced apoptosis and insulin secretion impairment

Metformin is a well-known AMPK activator [21], and there is a reported linkage between AMPK and the PI3 kinase/Akt pathway [22]. Therefore, we examined the involvement of the AMPK–PI3 kinase/Akt pathway in AMPK–ER stress-induced cell death by using compound C (an AMPK inhibitor) and Wortmannin (a PI3 kinase inhibitor). Caspase-3 activity was induced, and insulin secretion was inhibited by TG treatment in NIT-1 cells as expected (Fig. 2). Compound C (Fig. 2A and C) or Wortmannin (Fig. 2B and D) eliminated the protective and recovery effects of metformin on TG-induced apoptosis and insulin secretion impairment, respectively.

3.4. The protective effects of metformin are not related with the unfolded protein response

UPR is a cellular stress response related to the ER. It reduces the protein load stress in response to the accumulation of mis- and unfolded proteins [23]. The accumulation of ER stress leads to apoptotic cell death [23]. Therefore, we expected that metformin might prevent TG-induced apoptosis via induction of the UPR. TG induced the mRNA expression of several marker genes involved in the UPR in NIT-1 cells expectedly. However, metformin was unable to change the expression of these genes (Fig. 3A).

3.5. Metformin is unable to affect the mRNA expression of Bax, Bcl-2, cIAP2, and XIAP

Bcl-2 plays an important role in caspase-dependent apoptosis, and cIAP2 and X-linked mammalian inhibitor of apoptosis protein (XIAP) play a protective role in ER stress-mediated apoptosis in human breast cancer [24]. Bax, a Bcl-2 associated X protein, also plays an important role in caspase-dependent apoptosis. However, it is able to be localized to the ER to initiate apoptosis, in contrast to Bcl-2, cIAP2, and XIAP [25]. Therefore, we hypothesized that metformin might interrupt TG-mediated effects on the expression of these genes. However, TG induced only Bax, and metformin was unable to inhibit this induction. Bcl-2, cIAP2, and XIAP were not influenced by TG and metformin (Fig. 3B).

3.6. Metformin inhibits TG-induced JNK phosphorylation and activity

TG has been reported to induce JNK-dependent apoptosis in osteoblasts and Jurkat T cells [26], and JNK phosphorylation has been negatively regulated by PI3 kinase/Akt [27]. Therefore, we evaluated the effect of metformin on TG-mediated JNK phosphorylation in NIT-1 cells. TG induced JNK phosphorylation (Fig. 4A) and JNK activity (Fig. 4B) as expected. However, metformin suppressed these inductions (Fig. 4).

4. Discussion

The UPR increases the expression of ER chaperones to adapt to the increased need for protein folding in the ER. The ER is one of the main structures to maintain cell survival. ER stress involves the accumulation of mis- and unfolded proteins [28]. Particularly, abnormal protein synthesis is able to induce UPR and cell death

by ER stress [28]. ER stress also plays a critical role in T2DM [19]. It leads not only to the inhibition of insulin receptor signaling via IRS-1 dephosphorylation but also to the destruction of beta cells [22]. AMPK and PI3 kinase implicate insulin signaling and beta cell survival. In addition, other mechanisms are involved in the recovery effect of metformin. It has been reported that the Bcl-2 family plays an important role in the mitochondrial apoptosis pathway [29]. XIAP and cIAP2 are also related to caspase-dependent apoptosis [30]. Finally, JNK participates in the cellular response to extracellular stress and apoptotic signals [31]. In particular, JNK and caspase have been implicated in mediating apoptotic signals in response to ER stress [32].

This study reports three important observations about the recovery of ER stress-induced apoptosis and insulin secretion impairment in NIT-1 cells by metformin. First, the treatment of compound C and Wortmannin in NIT-1 cells reduced the recovery effect of metformin. Second, metformin was not able to change expressions of ER stress markers and Bax, Bcl-2, cIAP2, and XIAP. Third, metformin potentially inhibited ER stress-induced JNK phosphorylation. These results therefore emphasize an important usage of metformin for treatment of T2DM.

In conclusion, metformin is able to prevent ER stress-induced insulin secretion impairment and apoptosis via the AMPK–PI3 kinase–JNK signal pathway in NIT-1 cells. The results of this study suggest that the modulation of AMPK–PI3 kinase signaling by metformin may reflect a novel pathway in NIT-1 cells that could be controlled to treat T2DM. In addition, metformin may be a good solution for the treatment of metabolic diseases such as T2DM, which is characterized by obesity-induced inappropriate beta cell death.

Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This research did not receive any specific Grant from any funding agency in the public, commercial or not-for-profit sector.

Acknowledgments

We thank Dr. Bong Soo Cha and Dr. Myung Shik Lee for their careful suggestions and providing facilities to manage this research.

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