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Action of Metformin on Beta Cell Lipotoxicity

베타세포 지질독성에 대한 메트포르민 작용

2016 년 8 월

서울대학교 대학원

분자의학 및 바이오제약학과

김 홍 일

A thesis of the Degree of Doctor of Medicine

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By

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August 2016

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김 홍 일 의 의학박사 학위논문을 인준함

2016 년 6 월

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ABSTRACT

Action of Metformin on Beta Cell Lipotoxicity

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Introduction: Chronic exposure to elevated levels of free fatty acids contributes to pancreatic beta cell dysfunction. Although it is well known that metformin induces cellular energy depletion and a concomitant activation of adenosine monophosphate-activated protein kinase (AMPK) through inhibition of respiratory chain, previous studies have shown inconsistent results with regard to action of metformin on pancreatic beta cells.

Methods: To examine the effects of metformin on pancreatic beta cells under lipotoxic stress, I measured viability and glucose-stimulated insulin secretion (GSIS) in NIT-1 cells and isolated mouse islets exposed to palmitate and various concentrations of

metformin. To determine the dependence on AMPK, I treated AMPK activator and AMPK antagonist in parallel with metformin and measured levels of AMPK phosphorylation. As markers for cellular metabolism, cellular adenosine diphosphate and triphosphate levels were measured and autofluorescence imaging of the pyridine nucleotides was obtained. I measured messenger RNA levels of endoplasmic reticulum (ER) stress markers, glucose-stimulated calcium influx, intracellular reactive oxygen species (ROS) levels and caspase-3 activity as markers for lipotoxicity.

Results: I find that metformin has protective effects on palmitate-induced beta cell dysfunction. Metformin at concentrations lower than 0.5 mM inhibits palmitate-induced elevations in expression levels of ER stress markers, intracellular ROS level, and caspase-3 activity in a AMPK-independent manner, whereas metformin at the higher concentrations depletes cellular ATP levels, restores calcium influx reduced by palmitate and improves lipotoxic beta cell dysfunction in a AMPK-dependent manner. Cytosolic redox state is increased by metformin at concentrations lower than 0.5 mM at which AMPK activation does not occur.

Conclusions: This study suggests that metformin's action on beta cell lipotoxicity is implemented by different molecular pathways depending on its concentration. Metformin at usual therapeutic dose is supposed to alleviate lipotoxic beta cell dysfunction through inhibition of oxidative stress and ER stress.

Keywords: palmitate-induced beta cell dysfunction, action of metformin, endoplasmic reticulum stress, intracellular reactive oxygen species, AMP-activated protein kinase

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LIST OF ABBREVIATIONS AND SYMBOLS

AMPK: AMP-activated protein kinase

ACC: acetyl-CoA carboxylase

ER: endoplasmic reticulum

T2DM: type 2 diabetes

FFA: free fatty acid

ROS: reactive oxygen species

FBS: fetal bovine serum

BSA: bovine serum albumin

AICAR: 5-aminoimidazole-4-carboxamide ribonucleotide

KRBH: HEPES-balanced Krebs-Ringer-Bicarbonate buffer

HBSS: Hank's buffered salt solution

GSIS: glucose-stimulated insulin secretion

ADP: adenosine diphosphate

ATP: adenosine triphosphate

DCF-DA: 2',7'-dichlorohydro-fluorescein diacetate

NADPH: reduced form of nicotinamide adenine dinucleotide phosphate

PCR: polymerase chain reaction

ATF4: activating transcription factor 4

CHOP: C/EBP homologous protein

GRP94: Glucose-regulated protein 94

FKBP11: FKB506 binding protein 11

SE: standard error

CONTENTS

I. INTRODUCTION

Both insulin resistance and beta cell dysfunction are involved in the pathogenesis of type 2 diabetes (T2DM)[1]. The current evidence indicates that beta cell dysfunction precedes the onset of T2DM and is closely associated with glycemic deterioration in patients with T2DM[2]. For example, beta cell function is believed to be reduced 50% by the time when fasting hyperglycemia is first detected. Thus, preservation of functional beta cell mass is an important therapeutic strategy to prevent progression and treatment failure of T2DM[3].

Dysregulation at the level of the adipocytes in T2DM causes increased lipolysis, resulting in chronic elevation of plasma free fatty acid (FFA) known as lipotoxicity. Lipotoxicity impairs the ability of insulin to stimulate glucose transport and inhibit glucose production, which will decrease glucose utilization in muscle and increase hepatic gluconeogenesis[4]. Prolonged exposure of beta cells to elevated levels of FFA leads to decreased glucose-stimulated insulin secretion (GSIS) and apoptosis[5,6]. Several mechanisms underlying beta cell lipotoxicity have been proposed, such as endoplasmic reticulum (ER) stress, mitochondrial dysfunction, reactive oxygen species (ROS) production, and islet inflammation[7-9].

Elevated levels of FFA combined with chronic hyperglycemia increase demand for insulin biosynthesis, which leads to a heavy load of misfolded proteins in the ER lumen of pancreatic beta cell. Accumulation of misfolded proteins in the ER lumen activates an adaptive system named unfolded protein response[10,11]. The unfolded protein response ameliorates ER stress by inducing a number of downstream signaling pathways which

attenuate protein translation, facilitate protein folding or trigger apoptosis[12].

Mitochondria play a pivotal role in the insulin secretion of pancreatic beta cell by generating ATP and metabolites that can act as factors coupling metabolism to insulin secretion[13]. Decreased mitochondrial mass and transcriptional changes of the proteins from mitochondrial membrane are associated with diabetic state[14]. The production of ROS by mitochondria is important because it leads to oxidative damage that impairs mitochondrial function and activates the cell's apoptotic machinery. In addition, mitochondrial ROS can act as a redox signal affecting a wide range of metabolic functions in the mitochondria, cytosol and nucleus[15]. In pancreatic beta cell, low concentrations of ROS potentiate glucose responsiveness of insulin secretion[16]. On the other hand, chronic elevations of ROS resulting from an imbalance between ROS production and scavenging by antioxidants mediate beta cell failure through several mechanisms including mitochondrial dysfunction, islet inflammation and beta cell apoptosis[17,18]. Thus, antioxidants restore beta cell function under glucolipotoxic condition while in beta cells exposed to low concentrations of ROS, antioxidants can lower insulin secretion[19,20]. Although specific pathways mediating lipotoxic damages have not been fully elucidated, molecular mechanisms triggered by elevated FFAs seem to act interdependently to create a vicious cycle that leads to beta cell failure.

Metformin is commonly used as a first-line drug for the treatment of T2DM. There have been few studies which have investigated the effects of metformin in pancreatic beta cells. Those studies have produced inconsistent and contradictory results as demonstrated in Table 1. Some studies reported metformin restores insulin secretion of cultured islets

Table 1. Summary of results from previous studies on metformin's effect in beta cells

Methods	Results	Reference
Basal and glucose-induced insulin release of cultured rat islet exposed to high FFA or high glucose	Metformin (2.5 μg/ml) restored a normal secretory pattern	Patene G. *et al.* Diabetes (2000)
Insulin secretion of human islet incubated in the presence of 2.0 mmol/L FFA	Metformin (2.4 μg/ml) prevented impairment of insulin release	Lupi R. *et al.* Diabetes (2002)
AMPK activity and insulin secretion of MIN6 cells and human islet	Metformin (1 mM) stimulated AMPK activity and inhibited glucose-stimulated insulin secretion	Leclerc I. *et al.* Am J Physiol Endocrinol Metab (2004)
Apoptosis and insulin release of primary rat beta cells	Metformin (1 mM) reduced glucose responsiveness and resulted in apoptosis	Kefas B.A. *et al.* Biochem Pharmacol (2004)
Apoptosis and cleavage of caspase-3 of MIN6 cells in the absence or presence of palmitate 0.2 mM	Metformin (2 mM) played a dual role in beta cell apoptosis	Jiang Y. *et al.* Int J Biol Sci (2014)

impaired by chronic exposure to elevated FFA[21,22]. On the contrary, others reported that metformin reduces GSIS and viablity of β-cells through AMPK stimulation[23,24]. In one study, metformin was shown to play a dual role in beta cell apoptosis depending on culture condition[25]. The effects of metformin on beta cell function, whether protective or toxic, seem to be partly attributed to the difference in concentration of metformin treated between experiments.

Most of the known molecular targets of metformin have been identified by studies examining drug action on hepatocytes. Molecular pathways mediating the effects of metformin in hepatocytes are shown in Figure 1. It is well known that metformin stimulates AMPK. AMPK phosphorylates and inactivates a downstream effector, acetyl-CoA carboxylase (ACC) which plays an essential role in regulating fatty acid synthesis and oxidation[26]. There are some evidences that AMPK-dependent pathway is required for lipid-lowering and insulin-sensitizing effects of metformin in the liver[27,28]. However, other studies have proposed AMPK-independent pathways for metformin's action in hepatocytes. Metformin is known to directly inhibits complex 1 on the mitochondrial respiratory chain of isolated liver mitochondria at milimolar concentrations[29]. This action of metformin is shown to inhibit hepatic glucose production by decreasing hepatic energy state even in the absence of AMPK activation[30]. A recent study proposed another molecular target of acute metformin treatment in the liver. In this study, glucose-lowering effect of metformin was accompanied by a marked and instant change in cytosolic and mitochondrial redox state of hepatocytes. This action of metformin is attributed to inhibition of mitochondrial redox shuttle, glycerophosphate dehydrogenase, and is

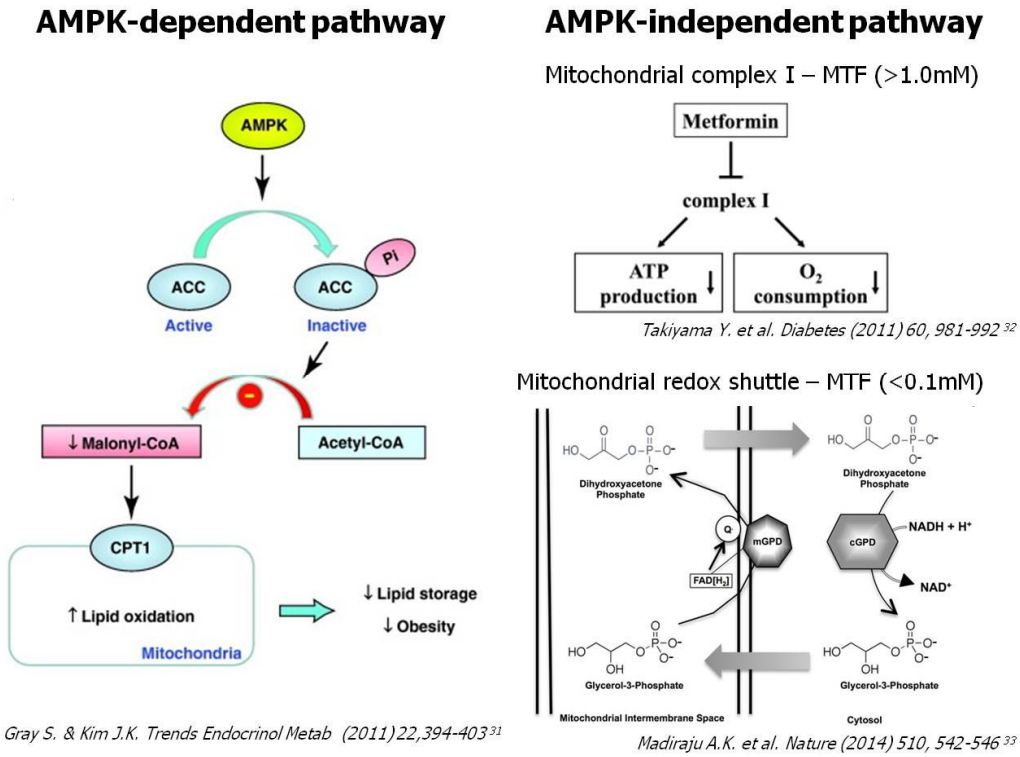


Figure 1. **Molecular pathways mediating action of metformin in hepatocytes**

The effects of metformin on hepatocytes are mediated by AMPK-dependent pathway (left panel) and AMPK-independent pathway (right panels). Mitochondrial complex I (right upper panel) and mitochondrial glycerophosphate dehydrogenase (right lower panel) are proposed as AMPK-independent molecular targets of metformin in hepatocytes.

achieved by relatively low concentration that is comparable to plasma concentration observed in metformin treatment[33].

This study is motivated by the observation of relationship between action of metformin and its concentration and of a considerable gap in metformin concentration between preclinical research and clinical setting. In the present study, I aim to ascertain the effects of metformin on lipotoxicity-induced beta cell dysfunction. In particular, I focus on the relation to dependence on AMPK and to metformin concentration in examining metformin's action on the molecular pathways involved in beta cell lipotoxicity. The results from this study show that metformin restores beta cell dysfunction induced by lipotoxicity through alleviation of ER stress and oxidative stress at concentrations lower than 0.5 mM at which activation of AMPK does not occur, which is in contrast with metformin at the higher concentrations. The findings of this study could be considered clinically relevant as metformin concentration in the pancreas is expected to be low compared to liver concentration and close to plasma concentration observed in the treatment of metformin at usual clinical doses (20 mg/kg) that is known to be in the range of 10 – 40 μM[34].

II. MATERIALS AND METHODS

2.1. Cell culture and reagents

The NIT-1 cell line is derived from NOD/Lt mice. These mice are transgenic for the SV40 large T antigen under the control of a rat insulin promoter, and spontaneously develop beta cell adenoma. At passage 18, most cells are stained positively for insulin, less than 5% are positive for glucagon and none are positive for somatostatin. Insulin secretion is responsive to glucose concentration in the medium[35]. NIT-1 cells were purchased from ATCC (NO. CRL-2055, Manassas, VA, USA) and cultured in RPMI 1640 medium (Gibco Life Technologies, Carlsbad, CA, USA) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco Life Technologies) and 100 U/mL penicillin-100 μg/mL streptomycin. Palmitate was conjugated to fatty acid free bovine serum albumin (BSA) in a 1:3 molar ratio and incubated at 37°C for an hour prior to dilution in culture medium. 1,1-Dimethylbiguanide hydrochloride (metformin), 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) as an AMPK agonist, and compound C as an AMPK antagonist were purchased from Sigma (St. Louis, MO, USA).

2.2. Cell viability assay

Cell viability was assessed by using Cell Counting Kit-8 (Dojindo Molecular Technologies, Rockville, MD, USA)[36]. Fifty microliter of CCK-8 solution was added to 500 μL of culture medium. Cells were incubated for 30min at 37°C and then the

absorbance was measured at 450 nm using a microplate reader. The net absorbance from the plates of cells with the control medium was considered as 100% of cell viability.

2.3. Measurement of glucose-stimulated insulin secretion

NIT-1 cells were seeded in a 6-well plate at 10^4 cells/well in RPMI 1640 containing 10% FBS, after which 200 μM palmitate was added to the medium for 48 h. The cells were starved for 2 h in glucose-free RPMI 1640, washed twice with a glucose-free HEPES-balanced Krebs-Ringer-Bicarbonate buffer (KRBH) containing 0.2% fatty acid-free BSA, and preincubated for 1 h in the same medium. After preincubation, the cells were exposed to KRBH buffer containing 2.5 mM (low glucose) or 16.7 mM (high glucose) glucose for 1 h. Insulin secreted to supernatant was measured by the insulin immunoassay kit (ALPCO, Salem, NH, USA) and was adjusted for total cellular protein of cell lysate. The measurement from the plate of cells with the control medium stimulated by low glucose was considered as 100% of GSIS.

2.4. Isolation of mouse pancreatic islets and measurements of glucose-stimulated insulin secretion and cytosolic calcium level

Female C57BL/6J and BALB/c mice at 24 – 33 weeks of age were used as islet donors. The animal use protocol of this study was approved by the Institutional Animal Care and Use Committee of Seoul National University (IACUC No. SNU-150327-3-2). Isolation of islets from mouse was carried out according to the protocol previously described[37].

After clamping the duodenal opening of the common bile duct and cannulating the bile duct of donor mouse, Hank's buffered salt solution (HBSS) containing 0.8 mg/ml collagenase was injected into the duct. The swollen pancreas was removed and digested in HBSS containing 0.8 mg/ml collagenase at 37°C for 15min. To terminate digestion, HBSS containing 10% FBS and 10mM HEPES was added. Then the solution was filtered with 500 μm mesh and islets were collected from filtered solution by centrifugation at 1500 rpm for 2 min. Islets were purified via Ficoll gradient and the isolated islets were picked selectively with 100-200 μm size into culture plate (15 – 20 per well). Islets were incubated at 37°C in culture medium (RPMI 1640 supplemented with 10% FBS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin) for 3 h. After which, islets were treated with 0.5 mM palmitate and metformin for 24 h. After islets were incubated for 20 min in low glucose (2.5 mM) KRBH buffer, islets medium was replaced with fresh low glucose KRBH. After 40 min exposure to the low glucose, the medium was collected, and islets were incubated with high glucose (16.7 mM) KRBH buffer for 40 min. At the end of incubation, the medium was collected. Insulin was measured by the insulin immunoassay kit (ALPCO, Salem, NH, USA) according to the manufacturer's instructions. Cytosolic calcium levels were detected by using Fluo-4 NW Calcium Assay Kit (Invitrogen, Carlsbad, CA, USA)[38]. Islets were incubated for 40 min at 37°C in low glucose KRBH buffer after 50 μl of 2X dye loading solution was added to each well of a 96-well pate. Then fluorescence was recorded every second for 10 seconds using instrument settings appropriate for excitation at 450 nm and emission at 516 nm. After islets medium was replaced with high glucose KRBH buffer (final glucose concentration

17.5 mM), fluorescence responses were measured every second for 20 seconds.

2.5. RNA extraction and real-time quantitative PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The cDNAs were prepared by reverse transcription with 1 μg of total RNA. Realtime PCR was performed using SYBR Premix Ex Taq reagents (TaKaRa, Shiga, Japan) and a 7500 real-time PCR system (Applied Biosystems, CA, USA). The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA level was used for the internal control. Experiments were performed in duplicate for each sample and repeated four times. Primer sequences of ATF4 were: 5'-CCTGAACAGCGAAGTGTTGG-3' (forward), 5'-TGGAGAACCCATGAGGTTTCAA -3' (reverse), CHOP: 5'-CAACAGAGGTCACACGCACA-3' (forward), 5'-TCTCCT TCATGCGTTGCTTC-3' (reverse), GRP94:5'-AAACGGCAACACTTCGGTCAG-3' (forward), 5'-GCATCCATCTCTTCTCCCTCATC-3' (reverse), FKBP11: 5' –ACACGC TCCACATACACTACACGG-3' (forward), 5'-ATGACTGCTCTT CGCTTCTCTCCC-3' (reverse). Relative mRNA level was expressed as a fold-change relative to vehicle-treated control.

2.6. Measurement of intracellular ROS level

The cells were incubated with 2', 7'-dichlorohydro-fluorescein diacetate (DCF-DA, Invitrogen, Carlsbad, CA, USA) in RPMI 1640 supplemented with 10% FBS for 30 min. Fluorescence was measured with an excitation at 450 nm and emission at 535 nm using a

Victor 3 1420 multilabel counter (PerkinElmer, Boston, MA, USA). The measurement from the plate of cells with the control medium was considered as 100% of intracellular ROS level.

2.7. Caspase-3 activity assay

Caspase-3 activity was measured by using Caspase-3/CPP32 activity Colorimetric Assay Kit (BioVision, Milpitas, CA, USA). Cells were lysed with lysis buffer supplied in the kit, incubated on ice for 10 min, and centrifuged at 10,000 x g for 5 min. The supernatant was collected and protein concentration was determined. 100 µg of protein was diluted to 50 µl with lysis buffer for each assay. The lysate was mixed with 50 µl of 2X reaction buffer containing 10 mM DTT and 5 µl of the 4 mM DEVD-pNA substrate was added. After incubation at 37°C for 2 h, the absorbance from the sample was read at 405 nm using a microplate reader. The net absorbance from the plates of cells with the control medium was considered as 100% of caspase-3 activity.

2.8. Measurement of ADP/ATP ratio

For determination of the ADP/ATP ratio, the ADP/ATP Ratio Assay Kit (Abcam, Cambridge, UK) was used according to manufacturer's instructions. Cells seeded in a 24-well plate and treated were lysed with Nucleotide-releasing Reagent that had been supplied in the kit and 50 µL of the lysate was transferred to a 96-well plate. To measure ATP levels, the luminescence was measured using a Victor 3 1420 multilabel counter (PerkinElmer, Boston, MA, USA) after 100 µL of the substrate solution, also supplied,

was added to each well. To measure ADP levels in the cells, the luminescence was measured again after 10 μL of ADP Converting Enzyme was added to each well.

2.9. 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 sodium dodecyl sulfate—polyacrylamide gel electrophoresis (SDS-PAGE). Proteins on the gel were transferred onto a nitrocellulose membrane. Membranes were incubated with blocking solution (5% skim milk), and then incubated with primary antibodies against phosphorylated AMPK and total AMPK (Cell Signal Technology, Danvers, MA, USA) in 0.1% Tween 20-Tris-buffered saline. Hybridized primary antibodies were detected using a horseradish peroxidase-conjugated IgG antibody (Santa Cruz Biotechnology, Dallas, TX, USA). Bands were detected by using the enhanced chemiluminescence kit (Thermo, Rockford, IL, USA).

2.10. Autofluorescence imaging of the pyridine nucleotides on confocal microscopy

Cells were seeded on chambered coverglass. For the determination of mitochondrial area, cells were loaded with 50 nM MitoTracker Green for 20 minutes. After loading, cells were washed free of probes and further incubated in culture medium. Cells on chambered

coverglass were studied by time-lapse laser confocal microscopy (ZEISS LSM 710, Oberkochen, Germany). Laser excitation was 405 nm for reduced forms of nicotinamide adenine dinucleotide/nicotinamide adenine dinucleotide phosphate (NADH/NADPH), and 488 nm for MitoTracker Green. Fluorescence emission was 420-480 nm for NAD(P)H, and 505-542 nm for MitoTracker Green. The fluorescence of NAD(P)H and MitoTracker Green was imaged simultaneously. Image acquisition conditions were kept constant during each experiment.

2.11. Statistical analysis

Data are expressed as mean ± SE. Statistical significances of the differences were determined by Student's *t* test for paired samples. $P < 0.05$ was considered statistically significant.

III. RESULTS

3.1. Effect of metformin on cell viability of NIT-1 cells

When NIT-1 cells were treated with palmitate (500 μM) for 24 h, cell viability was decreased significantly. Metformin inhibited palmitate-induced cell death of NIT-1 cells over the range of concentration between 0.02 – 1.0 mM. Metformin at the high end of concentration range reduced cell viability in the absence of palmitate and improved viability in the presence of palmitate, but less prominently than metformin at the lower concentrations. AICAR (0.5 – 1.0 mM), an AMPK activator, also inhibited cell death induced by treatment with palmitate (Figure 2).

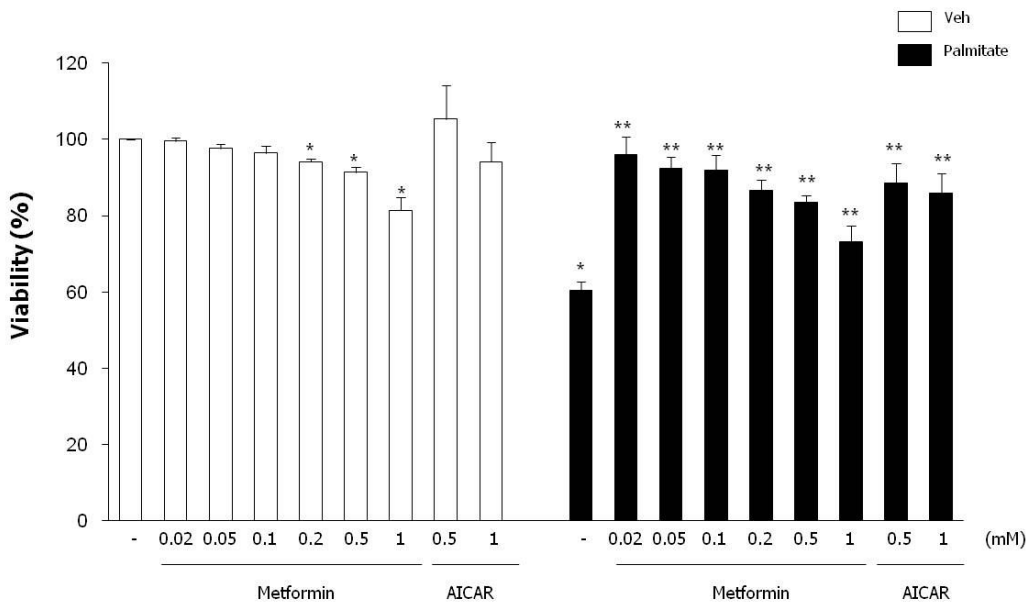


Figure 2. Effect of metformin on cell viability of NIT-1 cells

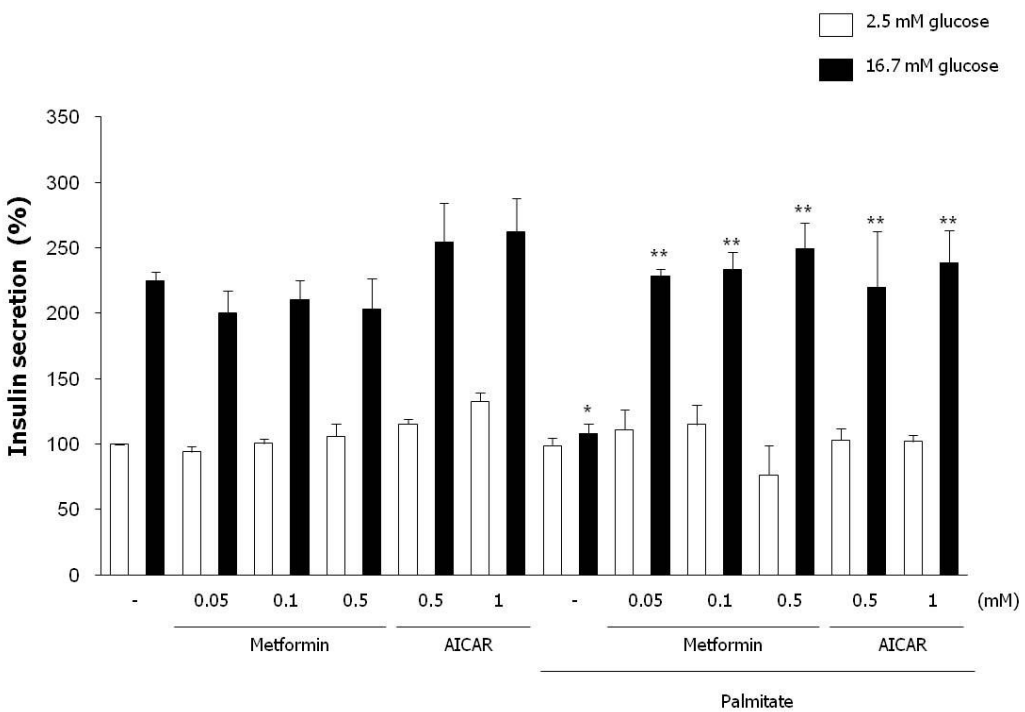
NIT-1 cells were exposed to palmitate (500 μM) and various concentrations of metformin (0.02 mM – 1.0 mM) and AICAR (0.5 mM – 1.0 mM) for 24 h. Cell viability was measured by CCK-8 assay (n=3). *Significantly different (P<0.05) from control. **Significantly different (P<0.05) from palmitate treatment.

3.2. Effect of metformin on glucose-stimulated insulin secretion

In NIT-1 cells, increased insulin secretion responding to high concentration of glucose by about 2.2-fold was nearly abolished by treatment with palmitate (0.2 mM) for 48 h. Metformin restored GSIS impaired by treatment with palmitate over the range of concentration between 0.05 – 0.5 mM. AICAR, an AMPK agonist, exerted a similar effect on GSIS impaired by palmitate (Figure 3A).

Isolated mouse islets exhibited a 4.5-fold increase in insulin secretion responding to high concentration of glucose. Glucose-stimulated insulin secretion was reduced by about 60% in islets exposed to palmitate 0.5 mM for 24 h. Metformin partially restored GSIS impaired by treatment with palmitate at 0.05 mM and 0.5 mM concentrations in mouse islets as in NIT-1 cells (Figure 3B).

A


2.5 mM glucose
16.7 mM glucose
Insulin secretion (%)
350
300
250
200
150
100
50
0
-
0.05
0.1
0.5
0.5
1
-
0.05
0.1
0.5
0.5
1
(mM)
Metformin
AICAR
Metformin
AICAR
Palmitate

B

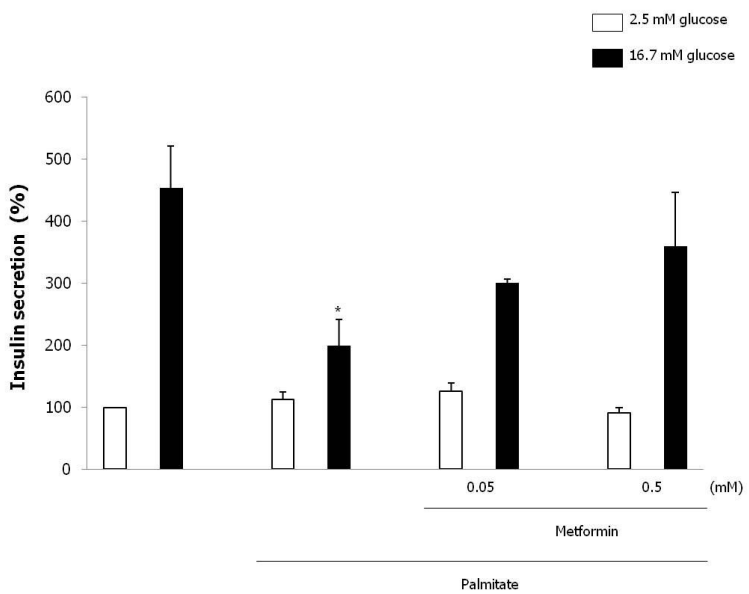

2.5 mM glucose
16.7 mM glucose
Insulin secretion (%)
600
500
400
300
200
100
0
0.05
0.5
(mM)
Metformin
Palmitate

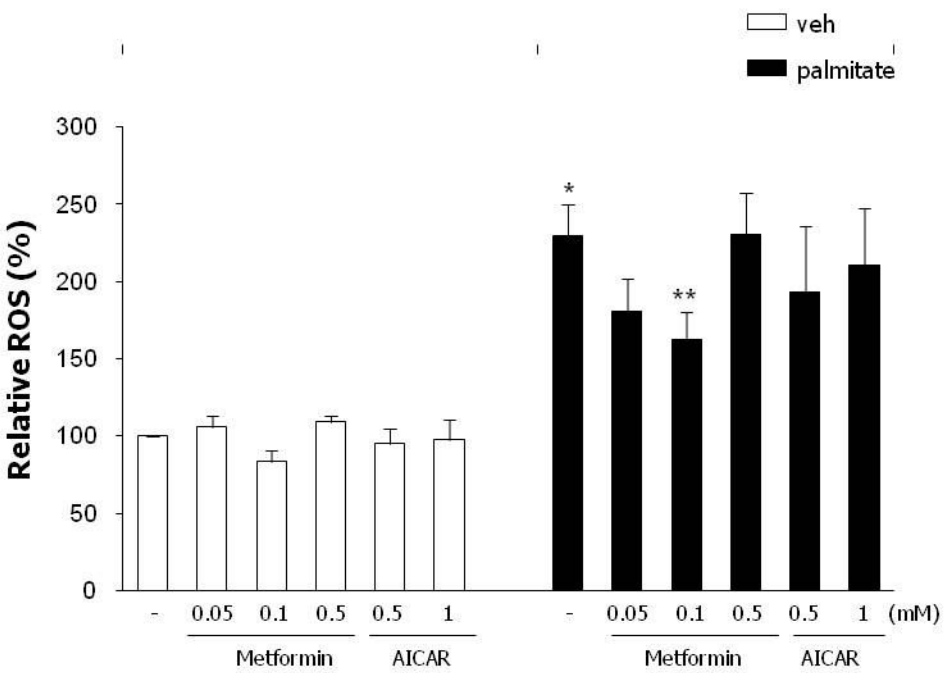
Figure 3. **Effect of metformin on glucose-stimulated insulin secretion**

Glucose-stimulated insulin secretion were measured in NIT-1 cells (n=4) (A) and isolated mouse islets (n=3) (B). NIT-1 cells were treated with palmitate (0.2 mM) for 48 h and mouse islets were treated with palmitate (0.5 mM) for 24 h. Metformin and AICAR treatment were carried out for 24 h. *Significantly different (P<0.05) from control. **Significantly different (P<0.05) from palmitate treatment.

3.3. Action of metformin on intracellular ROS level and caspase-3 activity

Treatment with palmitate (0.5 mM) for 24 h triggered a 2.0 – 2.5 fold increase of intracellular ROS fluorescence in NIT-1 cells. Metformin at 0.5 mM concentration had no effect on intracellular ROS levels increased by treatment with palmitate, whereas metformin at the lower concentrations inhibited ROS increment by palmitate (Figure 4A). In parallel with ROS levels, caspase-3 activity enhanced by treatment with palmitate was inhibited by metformin at 0.05 mM concentration, but not by metformin at the higher concentrations (Figure 4B). AICAR (0.5 – 1.0 mM) had little effect on intracellular ROS level and caspase-3 activity (Figure 4A, 4B).

A


veh
palmitate
Relative ROS (%)
300
250
200
150
100
50
0
*
**
- 0.05 0.1 0.5 0.5 1 - 0.05 0.1 0.5 0.5 1 (mM)
Metformin
AICAR
Metformin
AICAR

B

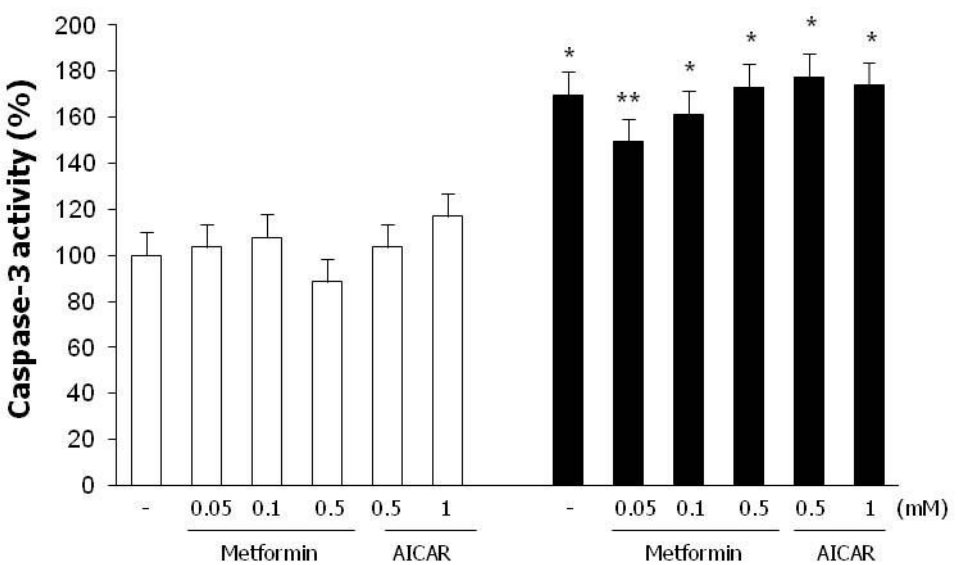

Caspase-3 activity (%)
200
180
160
140
120
100
80
60
40
20
0
*
**
*
*
*
*
- 0.05 0.1 0.5 0.5 1 - 0.05 0.1 0.5 0.5 1 (mM)
Metformin
AICAR
Metformin
AICAR

Figure 4. **Action of metformin on intracellular ROS level and caspase-3 activity**

Intracellular ROS levels were assessed by DCF-DA fluorescence (n=4) (A) and caspase-3 activities were measured by calorimetric assay kit (n=3) (B) in NIT-1 cells incubated in the absence or presence of palmitate (0.5 mM) and treated with various concentrations of metformin and AICAR for 24h. *Significantly different (P<0.05) from control. **Significantly different (P<0.05) from palmitate treatment.

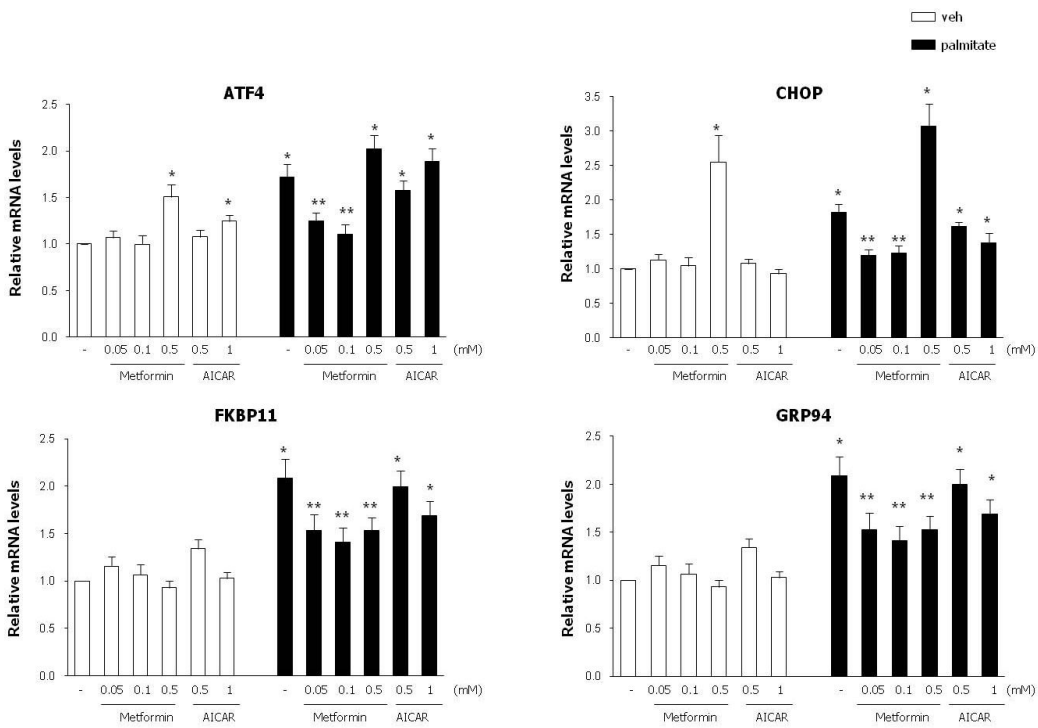
3.4. Action of metformin on mRNA levels of ER stress markers

In response to ER stress, activating transcription factor 4 (ATF4) and C/EBP homologous protein (CHOP) interact to increase protein synthesis leading to oxidative stress and cell death[39]. Glucose-regulated protein 94 (GRP94) and FKB506 binding protein 11 (FKBP11) are well-known genes whose expressions are involved in palmitate-induced ER stress of β cells[40].

In NIT-1 cells, the mRNA levels of *ATF4*, *CHOP*, *FKBP11*, and *GRP94* increased 1.5-2.0 fold over control by treatment with palmitate (0.5 mM) for 24 h. Expression levels of *ATF4* and *CHOP* were elevated by metformin at 0.5 mM concentration even in the absence of palmitate and were inhibited by metformin at the lower concentrations in the presence of palmitate. Metformin suppressed expression levels of *FKBP11* and *GRP94* enhanced by treatment with palmitate over the concentration range between 0.05 mM – 0.5 mM. The effects of AICAR (0.5 – 1.0 mM) on the expression levels of these ER stress markers were not significant (Figure 5A).

We observed similar effects of metformin on expression levels of these ER stress markers in isolated mouse islets exposed to palmitate (Figure 5B).

A


veh
palmitate
ATF4
CHOP
FKBP11
GRP94
Relative mRNA levels
- 0.05 0.1 0.5 0.5 1 - 0.05 0.1 0.5 0.5 1 (mM)
Metformin
AICAR
Metformin
AICAR

B

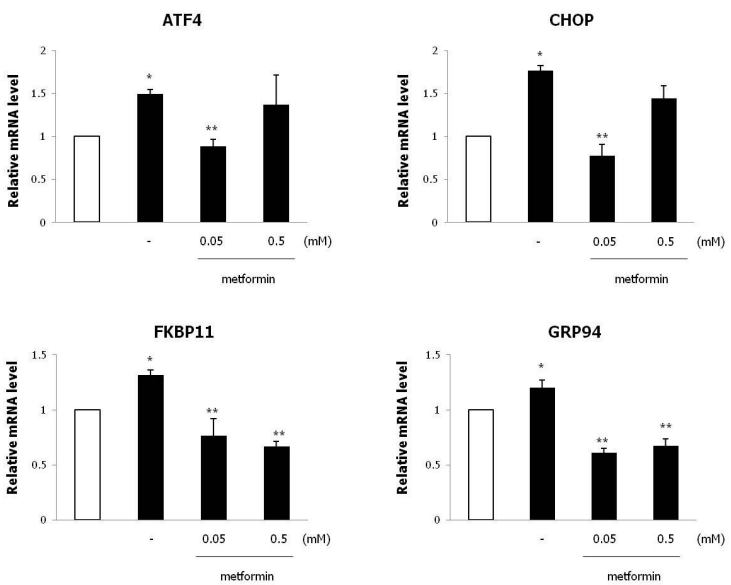

ATF4
CHOP
FKBP11
GRP94
Relative mRNA level
- 0.05 0.5 (mM)
metformin

Figure 5. **Action of metformin on mRNA levels of ER stress markers**

mRNA levels of ER stress markers (ATF4, CHOP, FKBP11, GRP94) were measured by RT-PCR in NIT-1 cells (n=5) (A) and isolated mouse islets (n=3) (B) exposed to palmitate (0.5 mM). Relative mRNA level was expressed as a fold-change relative to vehicle-treated control. Metformin and AICAR treatment were carried out for 24 h. *Significantly different (P<0.05) from control. **Significantly different (P<0.05) from palmitate treatment.

3.5. Action of metformin on glucose-stimulated calcium influx

In isolated mouse islets, cytosolic calcium levels were increased in response to high concentration of glucose (17.5 mM) by about 1.8-fold. Exposure of islets to 0.5 mM palmitate for 24 h significantly reduced glucose-stimulated calcium influx by about 50%. Metformin showed a tendency to restore glucose-stimulated calcium influx suppressed by palmitate as the concentration rises from 0.05 mM to 0.5 mM (Figure 6).

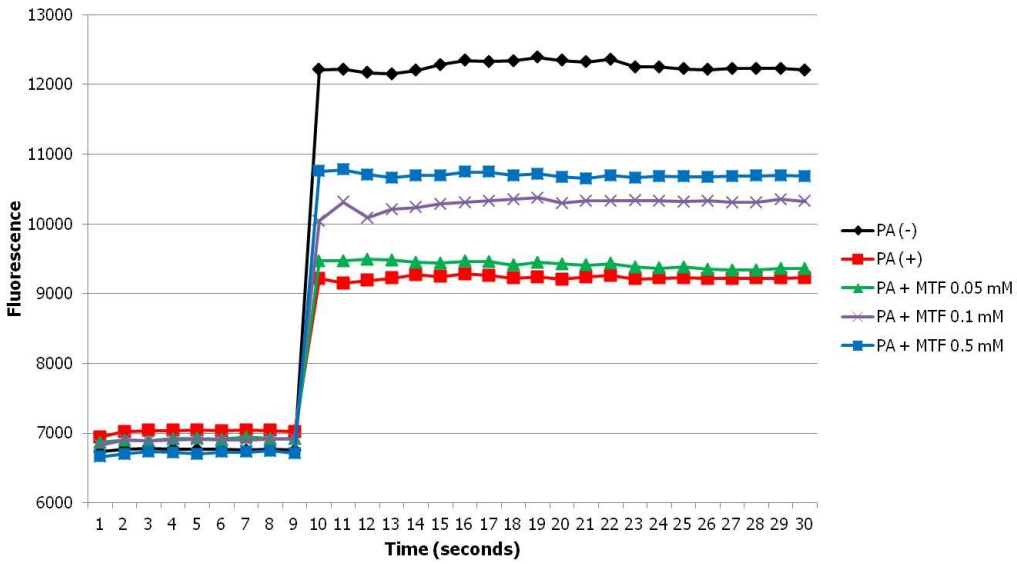


Figure 6. **Action of metformin on glucose-stimulated calcium influx**

Cytosolic calcium levels were measured using the fluo-4 NW fluorescence in isolated mouse islets. The averages of three measurements were plotted. Fluorescence was measured in the presence of 2.5 mM glucose for 10 seconds. Subsequently, glucose concentration was raised to 17.5 mM at t = 10 s for 20 seconds.

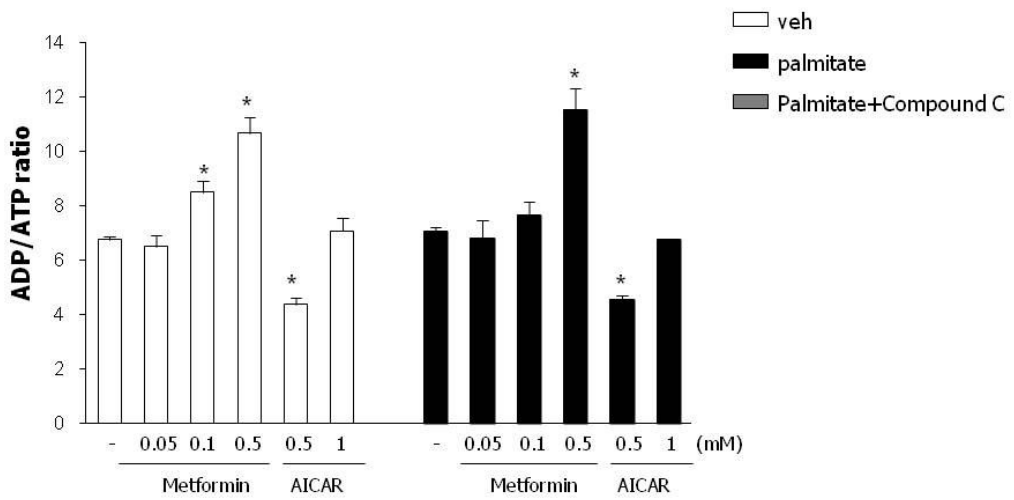
PA: palmitate

MTF: metformin

3.6. Effect of metformin on cellular ADP to ATP ratio and AMPK phosphorylation

Any metabolic stress that tends to deplete cellular ATP levels consequently activates AMPK[41]. Previous work showed a marked reduction of cellular ATP levels in hepatocytes exposed to metformin which indirectly stimulated AMPK[30]. In this study, cellular ADP/ATP ratio was not altered by treatment with palmiate (0.5 mM) for 24 h in NIT-1 cells. Metformin at 0.5 mM concentration increased cellular ADP to ATP ratio (Figure 7A) and levels of AMPK phosphorylation (Figure 5B) in NIT-1 cells regardless of exposure to palmitate. Metformin at the lower concentrations increased neither cellular ADP to ATP ratio nor levels of AMPK phosphorylation in NIT-1 cells exposed to palmitate. Levels of AMPK phosphorylation were nearly nulled by compound C, an AMPK antagonist, when it was added to metformin (Figure 7B).

A


veh
palmitate
Palmitate+Compound C
ADP/ATP ratio
14
12
10
8
6
4
2
0
- 0.05 0.1 0.5 0.5 1 - 0.05 0.1 0.5 0.5 1 (mM)
Metformin
AICAR
Metformin
AICAR

B

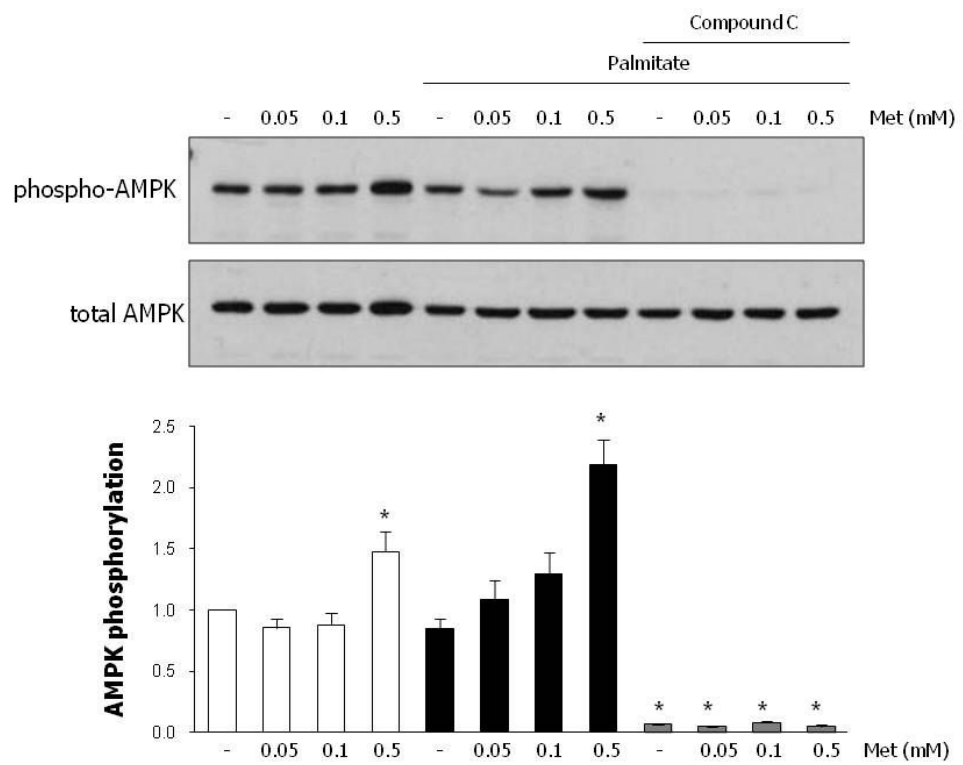

Compound C
Palmitate
- 0.05 0.1 0.5 - 0.05 0.1 0.5 - 0.05 0.1 0.5 Met (mM)
phospho-AMPK
total AMPK
AMPK phosphorylation
2.5
2.0
1.5
1.0
0.5
0.0
- 0.05 0.1 0.5 - 0.05 0.1 0.5 - 0.05 0.1 0.5 Met (mM)

Figure 7. Effect of metformin on cellular ADP to ATP ratio and AMPK phosphorylation

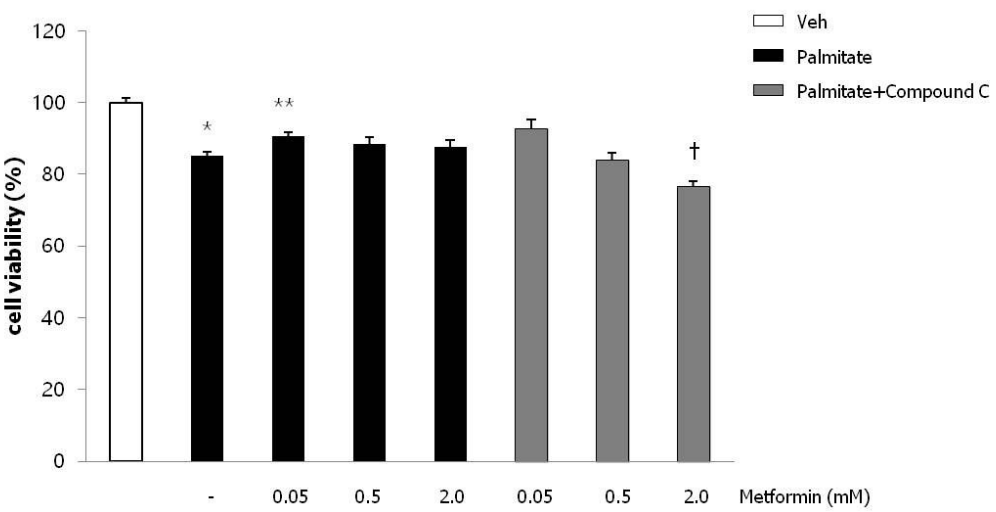
Cellular ADP to ATP ratio (n=4) (A) and levels of AMPK phosphorylations (n=5) (B) were measured in NIT-1 cells incubated in the absence or presence of palmitate (0.5 mM). Metformin and AICAR treatments were carried out for 24 hr and compound C (10 μM) for 1 h. *Significantly different (P<0.05) from vehicle-treated control.

3.7. *Impact of compound C on lipotoxic beta cell dysfunction when added to metformin treatment*

When compound C was added to metformin in NIT-1 cells exposed to palmitate, metformin's effect to improve cell viability at 0.05 mM concentration remained unaffected. On the other hand, viability of NIT-1 cells treated with metformin at 2.0 mM concentration was significantly decreased by addition of compound C (Figure 8A).

Likewise, addition of compound C attenuated improvement of GSIS by metformin at 0.5 mM concentration, but had no effect on GSIS restored by metformin at the lower concentrations (Figure 8B).

A


Veh
Palmitate
Palmitate+Compound C
cell viability (%)
120
100
80
60
40
20
0
*
**
†
-
0.05
0.5
2.0
0.05
0.5
2.0
Metformin (mM)

B

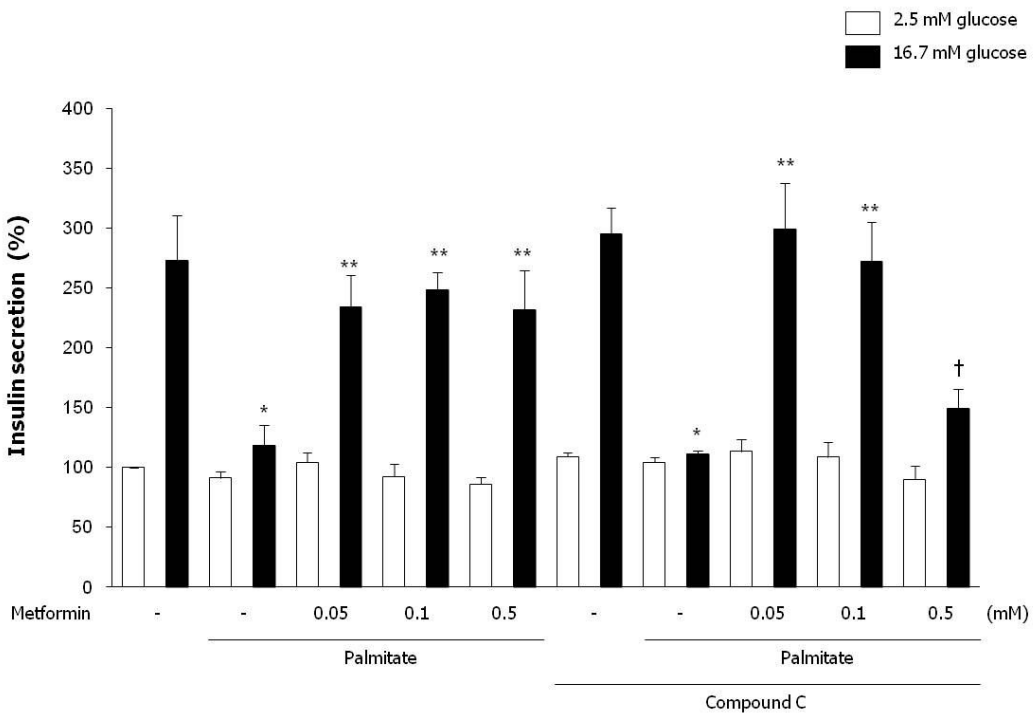

2.5 mM glucose
16.7 mM glucose
Insulin secretion (%)
400
350
300
250
200
150
100
50
0
Metformin
-
-
0.05
0.1
0.5
-
-
0.05
0.1
0.5
(mM)
Palmitate
Palmitate
Compound C

Figure 8. Impact of compound C on lipotoxic beta cell dysfunction when added to metformin treatment

Viability of NIT-1 cell was assayed by trypan blue exclusion method in triplicate and repeated twice (A). Measurements of GSIS of NIT-1 cells were repeated three times (B). Metformin treatments were carried out for 24 h and compound C (5 - 10 μM) was applied to NIT-1 cells exposed to palmitate for 1 hr. *Significantly different (P<0.05) from control. **Significantly different (P<0.05) from palmitate treatment. †Significantly different from palmitate and metformin treatment (P<0.05).

3.8. Impact of compound C on ROS levels induced by palmitate when added to metformin treatment

When compound C (5 μM) was added to metformin treatment in NIT-1 cells exposed to palmitate, concentration-dependent action of metformin on intracellular ROS levels enhanced by treatment with palmitate was not affected by compound C (Figure 9).

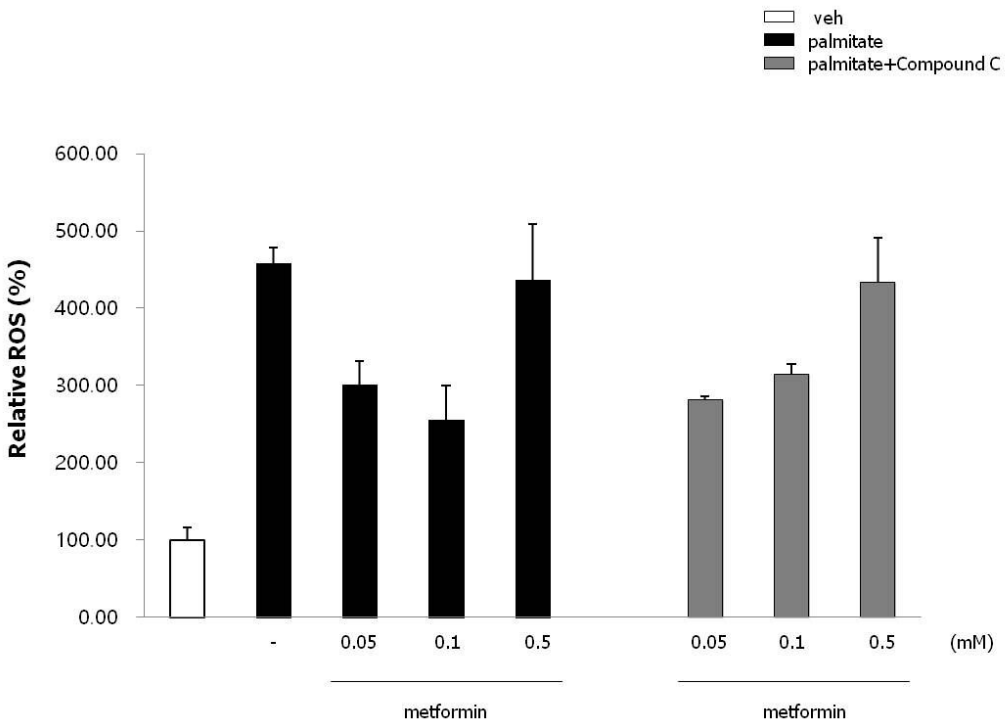


Figure 9. Impact of compound C on ROS levels induced by palmitate when added to metformin treatment

Intracellular ROS levels were measured by DCF-DA fluorescence in duplicate. NIT-1 cells were exposed to palmitate (0.5 mM) and treated with various concentrations of metformin for 24 hr. Compound C (5 μM) was added to metformin treatment for 1 h.

3.9. Impact of compound C on ER stress induced by palmitate when added to metformin treatment

In NIT-1 cells, palmitate-induced elevations in mRNA levels of ER stress markers (ATF4, CHOP, FKBP11, GRP94) were inhibited by metformin in a concentration-dependent manner. Addition of compound C did not alter the effect of metformin on the expression levels of these ER stress markers in NIT-1 cells exposed to palmitate (Figure 10).

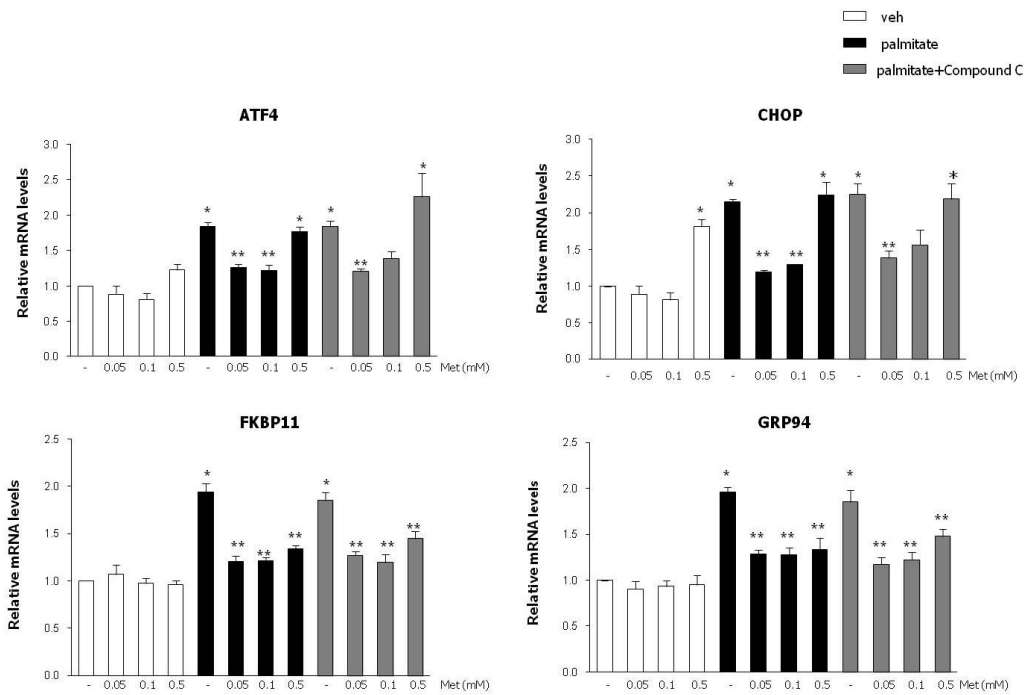


Figure 10. Impact of compound C on ER stress induced by palmitate when added to metformin treatment

mRNA levels of ER stress markers (ATF4, CHOP, FKBP11, GRP94) were measured by RT-PCR in NIT-1 cells treated with various concentrations of metformin for 24 h. Relative mRNA level was expressed as a fold-change relative to vehicle-treated control. Compound C (10 μM) was added to metformin treatment for 1 hr in NIT-1 cells exposed to palmitate (0.5 mM) (n=4). *Significantly different (P<0.05) from control. **Significantly different (P<0.05) from palmitate treatment.

3.10. Effect of metformin on cellular redox state

In NIT-1 cells, cellular NAD(P)H autofluorescence was increased by metformin at 0.05 mM concentration and was unchanged by metformin at 0.5 mM concentration. It made no difference to this action of metformin whether NIT-1 cells were exposed to palmitate or not (Figure 11).

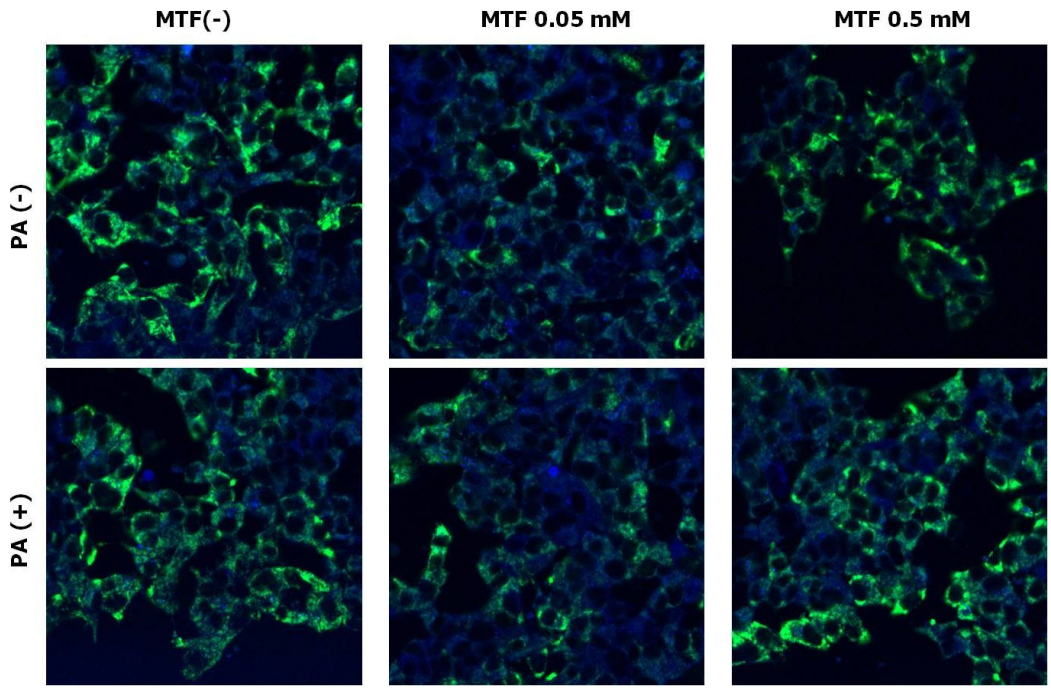


Figure 11. Effect of metformin on cellular redox state

Live cell imaging of excited autofluorescence of cellular NAD(P)H was obtained using laser-scanning confocal microcopy in NIT-1 cells incubated in the absence or presence of palmitate and treated with metformin at 0.05mM and 0.5 mM concentrations. MitoTracker Green was used for staining mitochondria.

PA: palmitate

MTF: metformin

IV. DISCUSSION

Table 2 summarizes the effects of metformin in pancreatic beta cells revealed by the present study. The results show that metformin restores viability and glucose-stimulated insulin secretion of beta cells exposed to palmitate independently of AMPK at lower concentrations (0.05 – 0.1 mM) and dependently of AMPK at higher concentrations (≥0.5 mM). Metformin at lower concentrations attenuates cellular ROS production and ER stress induced by palmitate in a AMPK-independent manner. Cellular ADP to ATP ratio is increased by metformin at higher concentrations, whereas cellular redox state is increased by metformin at lower concentrations regardless of exposure to palmitate. This finding suggests that the effects of metformin on lipotoxic beta cell dysfunction will become dependent on AMPK as metformin concentration goes up to near 0.5 mM. It is thus supposed, that the action of metformin on pancreatic beta cell is implemented by different pathways depending on its concentration (Figure 11). At concentrations lower than 0.5 mM, metformin increases cytosolic redox state, alleviates lipotoxicity-induced oxidative stress and ER stress and consequently improve beta cell function in the presence of lipotoxic stress. This pathway alters neither cellular energy state nor activation of AMPK. At the higher concentrations, metformin stimulates AMPK by inhibiting mitochondrial respiration and depleting cellular energy charge. This AMPK-dependent pathway may account for a dual action of metformin in beta cells, which trigger or alleviate beta cell dysfunction according to the presence of lipotoxic stress.

ER stress signaling is known to be important in regulating beta cell function and

Table 2. Summary of the effects of metformin in beta cell revealed by the present study

	Exposure to Palmitate (-)		Exposure to Palmitate (+)	
Met conc.	Low (0.05 – 0.1 mM)	High (≥0.5 mM)	Low (0.05 – 0.1 mM)	High (≥0.5 mM)
Viability	NC[#]	Dec*	Inc** / AMPK−[†]	Inc / AMPK+[‡]
GSIS	NC	NC	Inc / AMPK−	Inc / AMPK+
ROS level	NC	NC	Dec / AMPK−	NC / AMPK−
ER stress	NC	NC or Inc	Dec / AMPK−	NC or Dec / AMPK−
ADP/ATP ratio	NC	Inc	NC	Inc
Cytosolic redox state	Inc	NC	Inc	NC

Met conc. : Metformin concentration

NC[#] : No change

Dec* : Decreased

Inc** : Increased

AMPK−[†] : AMPK-independent

AMPK+[‡] : AMPK-dependent

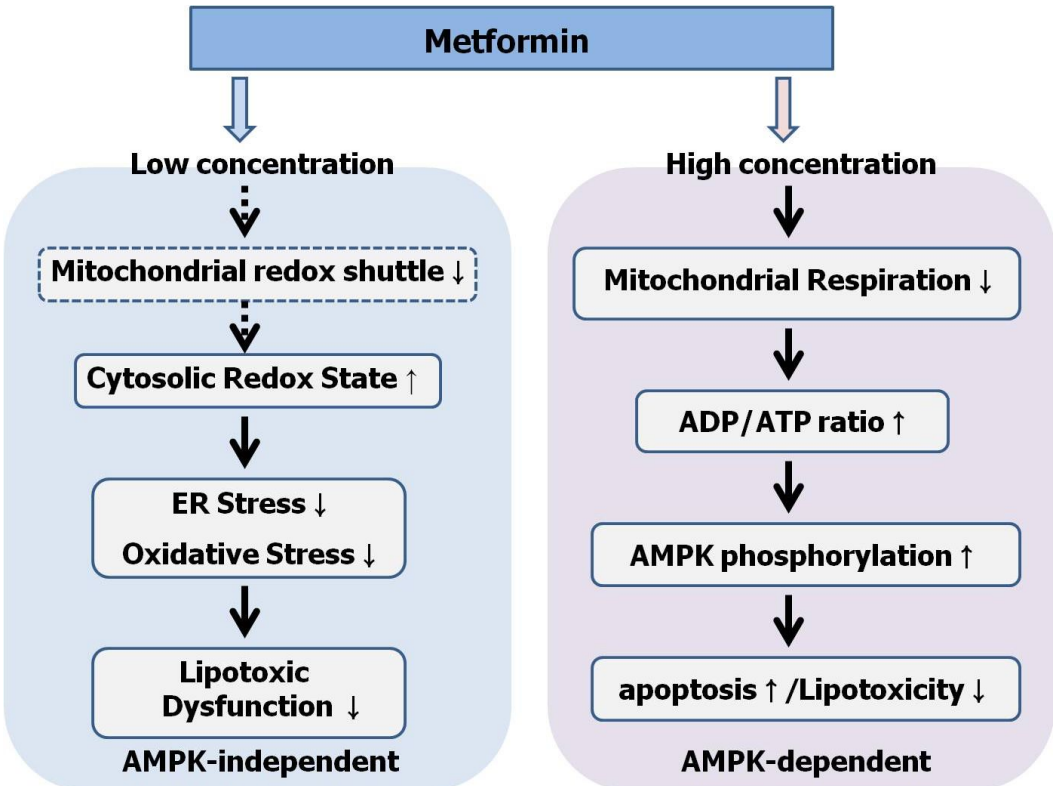


Figure 12. Suggested pathway of metformin's action in beta cell depending on its concentration

Metformin's action on pancreatic beta cell is supposed to be implemented by two pathways depending on its concentration; AMPK-independent pathway at lower concentration and AMPK-dependent pathway at higher concentration

survival and involved at least partially in lipoapoptosis of beta cells[42]. In a study on lipotoxicity of clonal beta cells, metformin showed a protective activity against lipotoxic apoptosis through attenuation of ER stress at concentrations 0.01 – 0.1 mM, which is consistent with the results from this study[43]. On the other hand, in another study, metformin at 5 mM concentration ameliorated thapsigargin-induced dysfunction of clonal beta cells through AMPK pathway, but failed to block ER stress markers[44]. These findings suggest that the action of metformin on ER stress is sensitive to its concentration and a type of ER stress inducer in pancreatic beta cells. In primary rat cardiomyocytes, metformin was shown to induce specific pathways of ER stress signaling leading to strong and persistent induction of CHOP at concentrations higher than 2 mM[45]. In spite of CHOP induction, metformin treatment did not trigger apoptosis, which is also observed in our study in the absence of lipotoxic stress. The authors suggest that cardioprotective effects of metformin might be associated with selective activation of ER stress pathways in the cardiomyocytes. It will be interesting to understand under which circumstances the specific ER stress pathways triggered by metformin treatment mediate protection or apoptosis in different cell types. .

Oxidative stress is another mechanism underlying beta cell lipotoxicity. It was reported that ROS levels are dose- and time-dependently increased by exposure of NIT-1 cells to palmitate and that suppression of NADPH oxidase 2 (NOX2) restores FFA-induced dysfunction of NIT-1 cells[46]. This finding suggests a critical role played by ROS production in beta cell lipotoxicity. Although both ER stress and oxidative stress have been implicated in lipotoxicity-induced beta cell dysfunction, it is difficult to dissect

connection between both mechanisms in mediating the effect of metformin. There is an evidence that a positive feedback cycle exists between oxidative stress and ER stress in beta cell dysfunction and this cycle can be interrupted by superoxide dismutase mimetics and chemical chaperones[47]. In other cell type, it is demonstrated that both protein misfolding in the ER and ROS are required to activate cellular responses causing cell death and antioxidant treatment can reduce ER stress to improve cell survival and protein secretion[48]. There is also evidence that ER stress can induce oxidative damage in pancreatic beta cell though the exact mechanism of ROS generation has not been elucidated[49,50]. In this study, it is likely that metformin, acting as an antioxidant, disrupts the reciprocal link between ER stress and oxidative stress to improve beta cell dysfunction induced by lipotoxicity at concentrations lower than 0.5 mM.

The key finding of this study is that metformin ameliorates lipotoxic beta cell dysfunction through inhibition of oxidative stress and ER stress at lower concentrations independently of AMPK, which is in contrast with metformin at higher concentrations. Although the exact molecular mechanism has not been established by this study, a candidate molecular target is expected to account for the metformin's action that varies according to its concentration. As previously mentioned, metformin exerts its glucose-lowering effect in hepatocytes in an instant manner through alteration of cytosolic and mitochondrial redox state at relatively low concentrations at which AMPK is not activated[33]. At comparable concentrations, metformin is shown to increase cellular redox state of NIT-1 cells regardless of lipotoxic stress in this study, which may result from inhibition of mitochondrial redox shuttle. The cellular redox state influences many

metabolic processes and the availability of reducing equivalents such as NAD(P)H is vital to the cellular oxidative defense systems for counteracting oxidative damage[51]. The acute shift in intracellular redox balance made by metformin at low concentrations at which neither cellular energy state nor AMPK is altered, is supposed to reduce glucose production in hepatocytes and to counteract oxidative stress associated with lipotoxicity in pancreatic beta cells which are considered to be susceptible to oxidative stress due to the lack of an effective antioxidant system[52].

AMPK controls metabolic homeostasis acting as a cellular energy sensor in the target organs for insulin including liver, muscle, and adipose tissue[53]. However, its role in pancreatic beta cell, especially in beta cell dysfunction under lipotoxic condition, remains controversial[54]. In the present study, exposure to palmitate had little effect on cellular ADP/ATP ratio and phosphorylation level of AMPK in spite of definite negative impacts on beta cell function, suggesting that AMPK per se does not play a major role in beta cell lipotoxicity. However, AICAR, a specific activator of AMPK, restored beta cell viability and glucose-induced insulin secretion impaired by lipotoxicty. Metformin also exerted its effects on lipotoxic beta cell dysfunction in a AMPK-dependent manner at concentrations at which activation of AMPK occurs. On the other hand, ER stress and oxidative stress induced by lipotoxicity remained unaffected by activation or inhibition of AMPK. Since AICAR and metformin have been claimed to reduce hepatic lipid accumulation through AMPK[28, 55,56] and accumulation of detrimental lipids is known to be an initiator of beta cell failure[57,58], it is natural to assume that downstream targets of AMPK regulating intracellular lipid would mediate AMPK-dependent effects of

metformin on beta cell lipotoxicity. However, according to previous findings, metformin had no effect on triglyceride overload in beta cell despite its protective effect on lipotoxicity[22,59], further highlighting the difference in AMPK-dependent action of metformin between liver and pancreas. Taken together, AMPK in beta cells mediates the effects of metformin at concentrations equal to or higher than 0.5 mM in the presence of lipotoxic stress via an as-yet-unidentified downstream target, which seems unrelated to known mechanisms underlying beta cell lipotoxicity and hepatic insulin resistance such as oxidative stress, ER stress and intracellular lipid regulation.

Previous studies have demonstrated that calcium influx in islets stimulated with high glucose has been correlated with GSIS profiles and islet cells from diabetic mouse exhibited a poor calcium influx in response to glucose challenge[60,61]. In the present study, chronic exposure of mouse islets to palmitate impaired glucose-stimulated calcium influx, which is in agreement with a previous study[62]. ER calcium depletion through calcium leak channels is a proposed mechanism for deleterious effects of lipotoxicity on metabolism-secretion coupling in beta cell[63,64]. In contrast with cellular redox state and ROS levels, calcium influx response reduced by palmitate was restored by metformin more prominently at 0.5 mM concentration than at 0.05 mM concentration. Metformin at 0.05 mM concentration did not affect glucose-stimulated calcium influx in spite of its action on ER stress, whereas metformin at 0.5 mM concentration partially restored calcium influx response impaired by palmitate. Although it is not clear whether the effect of metformin at 0.5 mM concentration to improve glucose responsiveness of mouse islet is associated with perturbation of ER calcium homeostasis induced by palmitate, this

study demonstrated that the actions on calcium influx altered by lipotoxicity were different between low and high concentration of metformin as cellular redox state and ROS levels were.

It is well known that metformin improves glycemic control by reducing hepatic glucose output[65]. Metformin exerts its effects to inhibit hepatic gluconeogenesis by regulating intrahepatic lipid accumulation through AMPK-dependent pathway and decreasing cellular energy state independently of AMPK[28,30]. It is reported that intracellular ATP content is decreased by metformin at concentrations greater than 0.25 mM with concomitant stimulation of AMPK in primary hepatocytes[26,30]. In contrast, plasma concentrations of metformin at usual clinical doses (20 mg/kg) is known to be in the range of 10 – 40 μM[34]. As the majority of liver blood flow is through the portal vein, which may contain higher concentrations of metformin than those in the general circulation, and metformin accumulates to higher levels in hepatocytes than in plasma due to the high expressions of organic cationic transporter 1 (OCT1) which facilitates cellular uptake of metformin, there will be a difference in tissue metformin concentration between liver and other target organs including pancreas in the treatment of T2DM with metformin. Therefore, it is supposed that metformin at lower concentrations is able to improve beta cell dysfunction induced by lipotoxicity through inhibition of oxidative stress and ER stress while metformin at higher concentrations reduces glucose output and lipogenesis in the liver through activation of AMPK and depletion of cellular energy charge. A study in which plasma and tissue metformin concentrations were measured in rats treated with metformin reported that acute metformin administration led to

metformin concentration in the pancreas of approximately 50 – 60 μM which is lower than in the liver and comparable to lower concentrations of metformin used in this study[31]. Hence, we consider our findings clinically relevant because the action of metformin presented in this study will be a possible explanation for the difference in drug action at therapeutic doses between liver and pancreas regarding the treatment of diabetes.

Several previous studies have investigated the effects of metformin in pancreatic beta cells, but few of them have focused on metformin concentration. This study shows that AMPK-independent action of metformin at concentrations achieved by usual therapeutic dose is mediated by reducing ER stress and cellular ROS levels in beta cell lipotoxicity. Whether metformin is treated acutely or chronically is likely to have influence on the effects of metformin as well as metformin concentration. There is an evidence indicating that both acute and chronic administration of metformin have the same effects on cellular redox state[31]. Although it turns out that acute treatment of metformin administered via intravenous route leads to different tissue metformin concentration between liver and pancreas in rats[33], there is very little evidence from in vivo data to suggest that chronic treatment of metformin leads to the accumulation of metformin in the specific target organ to affect mitochondrial respiration and AMPK activation. In consideration of the very brief plasma half-life of metformin, it is unlikely that chronic treatment of metformin causes significant accumulation in humans. There is also a discrepancy between drug concentration in the lab studies and conventional dose in clinical trials regarding health effects of metformin that recent research has indicated. Further studies are required to clarify which molecular pathway is activated by different concentration of

metformin treated acutely or chronically between major target organs and between experimental and clinical setting.

V. CONCLUSION

Previous findings suggest that AMPK-independent pathway as well as AMPK-dependent pathway mediates the action of metformin in the liver. However, metformin's effect on beta cell function and associated molecular mechanisms in pancreatic beta cell have been rarely studied. Considering the relationships between metformin's action and its concentration, I performed experiments to find out the influence of metformin concentration on molecular mechanisms involved in beta cell lipotoxicity and the roles of AMPK.

In conclusion, I have demonstrated that metformin ameliorates palmitate-induced beta-cell dysfunction through inhibition of ER stress and cellular ROS production at concentrations achieved by usual therapeutic dose in a AMPK-independent manner, which is in contrast with metformin at the higher concentrations. Cytosolic redox state is considered to be a target of this action of metformin in pancreatic beta cell. The findings from this study suggest that there will be a difference in metformin's action at therapeutic doses between target organs including liver and pancreas.

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Abstract in Korean

초 록

서론: 고농도 유리지방산에 장기간 노출되는 것은 췌장베타세포의 사멸과 기능장애를 유발할 수 있다. 메트포르민이 미토콘드리아 호흡과정을 억제함으로써 세포내 에너지 결핍상태를 초래하여 결과적으로 AMP-activated protein kinase (AMPK)를 활성화하는 것은 잘 알려져 있지만 췌장베타세포에 대한 메트포르민의 효과는 기존 연구에서 일관되게 나타나지 않았다.

방법: 베타세포의 지질독성에 대한 메트포르민의 효과를 조사하기 위해 NIT-1 세포주와 마우스에서 분리한 췌장 소도를 팔미트산과 다양한 농도의 메트포르민으로 처리한 후 세포생존능과 인슐린 분비능을 측정하였다. AMPK활성에 대한 의존 여부를 보기 위해 메트포르민과 함께 AMPK 작용제과 길항제를 처리하고 AMPK 인산화를 측정하였다. 세포대사에 대한 표지로 세포내 아데노신 2인산과 3인산 농도를 측정하였고 피리딘 핵산의 자가형광 영상을 촬영하였다. 지질독성에 대한 세포내 표지로 endoplasmic reticulum (ER) stress 관련 유전자의 발현 정도와 포도당 자극에 의한 세포내 칼슘 유입, 활성 산소 농도, caspase-3 활성을 측정하였다.

결과: 메트포르민은 팔미트산에 의해 유발되는 베타세포 기능장애에 대해 보호효과를 보였다. 0.5 mM 농도 미만의 메트포르민은 팔미트산에 의해 상승하는 ER stress 관련 유전자의 발현과 활성산소, caspase-3 활성화를 AMPK 비의존적으로 억제하였다. 반면 그보다 높은 농도의 메트포르민은 세포내 ATP를 낮추고 팔미트산에 의해 감소한 세포내 칼슘 유입을 회복시키며 AMPK 의존적으로 지질독성에 의한 베타세포 기능장애를 호전시켰다. AMPK 활성화를 일으키지 않는 0.5 mM 농도 미만의 메트포르민에 의해 세포질의 산화환원 반응이 증가하였다.

결론: 본 연구는 베타세포지질독성에 대한 메트포르민 작용이 농도에 따라 다른 경로를 통해 이루어질 가능성을 시사하고 있다. 통상적인 치료 용량의 메트포르민은 ER stress와 산화 스트레스 억제를 통해 지질독성이 유발하는 베타세포 기능장애를 완화시키는 것으로 생각된다.

주요어: 메트포르민, 베타세포 지질독성, ER stress, 세포내 활성 산소, AMPK

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