



Long-term *in vitro* Treatment of Metformin Altered Biological and Biochemical Properties of Cardiac Cells

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Abstract

A cardiovascular complication from diabetes, especially ischemic heart disease (IHD), is a major cause of death in diabetic patients. Metformin is an effective anti-diabetic drug that has been used worldwide. Several studies have reported the cardioprotective effect of Metformin in an *in vivo* and *ex vivo* model. However, information on the biological alterations of long-duration treatment with Metformin at the cellular and molecular levels is still unclear. The purpose of this study was to demonstrate the effects of long-duration cardioprotective doses of Metformin treatment on the biological, biochemical and proteomes characteristics of cardiac cells. Rat cardiac myoblast cell lines (H9c2) were cultured for 30 days in Dulbecco's modified Eagle's medium (DMEM) in both the presence and absence of 3mM Metformin, which was optimized as a cardioprotective dose, in a simulated ischemia/reperfusion model. The changes in cell proliferation were determined by the characteristic of the growth curve and calculated populations doubling time (PDT). The cellular morphology was measured by F-actin cytoskeleton staining with phalloidin conjugated with TRITC and visualized under a fluorescence microscope. The phosphorylation of p38 MAPK was demonstrated by western blotting. The alteration of the expressed cellular protein was determined by a proteomics technique. The results showed that the long term effects of 3 mM Metformin was an increase of cell proliferation, decreased populations' doubling time, slight disorganized actin, and decreased phosphorylation of the p38 MAPK level. In conclusion, the data suggested that the *in vitro* effect of long-duration Metformin treatment altered the biological, biochemical and proteome characteristics of the cardiac cells.

Keywords: diabetes, metformin, longterm effects of metformin, cardiac cell, proteomics

Introduction

Diabetes Mellitus (DM) is a group of metabolic disorders caused by the malfunctioning of the pancreas not producing sufficient insulin or ineffective insulin signaling and responses (Rossi, 2010). World Health Organization (WHO) has predicted that the number of DM patients around the world will increase up to a total of 438 million people in 2026 (Hu, 2011). It has been reported that DM patients have a two to four times higher risk of cardiovascular

disease than non-diabetic patients (Morrish, Wang, Stevens, Fuller, & Keen, 2001). In particular, ischemic heart disease is a major cardiovascular complication causing mortality in diabetic patients.

Glycemic control is the primary approach to preventing the progression and complications of diabetes. The American Diabetes Association (ADA) has recommended that Metformin is the first drug of choice which has been used worldwide for decades (Hauton, 2011). In addition, a previous study reported that Metformin could activate p38 Mitogen-activated



Protein Kinase (p38-MAPK) in cardiomyocytes (Capano & Crompton, 2006). Activation of p38-MAPK induces translocation of Bax protein into the mitochondria (Kravchuk, Grineva, Bairamov, Galagudza, & Vlasov, 2011). Bax protein plays an important role in cellular apoptosis. Stimulated Bax protein results in an abundance of cellular apoptosis (Kravchuk et al., 2011). However, several studies have reported the beneficial effects of Metformin as a cardioprotectant such as reducing infarct size, decreased risk of heart failure and improved ventricular function (ejection fraction) in an Ischemia/Reperfusion (I/R) model (Yin et al., 2011, Wang, Zhang, Li, & Zhao, 2011). In addition, Metformin has shown the ability to prevent cellular apoptosis in primary cardiomyocytes (Wang et al., 2011). It has also been reported that the mechanism of action of Metformin can be possibly due to the attenuation of AGEs production (Rahbar et al., 2000), which could cause adverse effects in the diabetic heart (Rahbar et al., 2000).

Normally, diabetic patients chronically receive anti-diabetic drugs from the first time that they have been diagnosed which continues over the long term. However, the effect of long term Metformin exposure on cardiac cell biology has not been investigated. The purpose of this study was therefore to demonstrate the effects of long exposure duration of Metformin on biological alterations as well as the changing of proteins expression, by proteomic technique, in cardiac cells. We hypothesized that Metformin could possibly cause the biological, biochemical alterations and protein expression in cardiac cells treated with long-duration exposure.

Methods and Materials

Cell line and culture

Cardiac myoblast cell line (H9c2) was purchased from American Type Cell Culture (ATCC-

CRL1446) and was cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 5,000 units of penicillin and streptomycin. Cells were cultured at 37°C, 5% CO₂ + 95% O₂ throughout the experiments.

Simulated Ischemia and Reperfusion (sI/R)

Simulated ischemia was induced by incubating H9c2 cell with specific modified Krebs-Henseleit buffer (137 mM NaCl, 3.8 mM KCl, 0.49 mM MgCl₂, 0.9 mM CaCl₂, and 4.0 mM HEPES) with 20 mM 2-deoxyglucose, 20 mM sodium lactate, and 1 mM sodium dithionite at pH 6.5. After simulated ischemia was achieved, the ischemic buffer was removed and the cells were subjected to reperfusion by the addition of 2 ml of complete medium before further incubating at 37°C, 5% CO₂ for 24 hours (Kumphune, Jernsri, & Paiyabhroma, 2012).

Measurement of cellular injury

The activity of LDH activity (U/L) was measured from collected supernatant of culture medium, after simulated ischemia/reperfusion, using a specific commercially available kit (Human, Germany). The absorbance was measured at λ 340 nm exactly after 1, 2, and 3 minutes. The mean absorbance change per minute ($\Delta A/\text{min}$) was used to calculate LDH activity.

Measurement of cell viability assay

The measurement of cell viability was performed by MTT cell survival assay based on the reduction of MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) in the presence of mitochondrial reductases. At the end of reperfusion period, cells were incubated with 0.01g/ml MTT solution for 2 hours at 37°C. After that, dimethyl sulfoxide (DMSO) was used for dissolving the converted dye. The optical density was determined spectrophotometrically at λ 490 nm.



The percentage of cell viability was calculated by comparing the optical density of treated samples with untreated control group (100% viability).

Optimization of cardioprotective dose of Metformin in cardiac cell subjected to simulated ischemia and toxicity

H9c2 (1×10^5 cells/ml) was cultured in 24 well-plate until reach 80% confluence. Then, cells were cultured in DMEM medium that supplemented with 10% FBS in the presence of 0, 1 μ M, 10 μ M, 100 μ M, 1 mM, 2 mM, 3 mM, 4 mM, 5 mM, and 10 mM of Metformin (Glucophage®, MERCK) for 24 hrs. On the next day, the culture medium was collected for measuring lactate dehydrogenase (LDH) activity and cell viability was measured by MTT cell survival assay. Similar set of experimental design was performed, after 24 hrs of Metformin exposure, cells were subjected to 40 mins of sI and 24 hrs of R for assessing the cardioprotective effect of anti-diabetic drugs. After that, the cardioprotective dose of Metformin was manifested and applied in others experiments.

Culture of cardiac cell in the presence of cardioprotective dose of Metformin

H9c2 was cultured in the presence and absence of cardioprotective dose of Metformin for 30 days. At the end of long term exposure of Metformin, for 30 days, cells were then subcultured and for measuring growth curve and population doubling time, cytoskeletal organization, level of p38 MAPK, and protein extraction for further proteomics assay.

Growth curve and population doubling time (PDT)

H9c2 cells that chronically treated with cardioprotective dosed of Metformin and control untreated cells at density at 1×10^5 cells/ml was seeded into 6 well-plates and was cultured for 7 days in the presence and absence of the optimized concentration of Metformin. Cells were harvested and counted every day for 7 days. The growth curve was

plotted between times (days) and cell concentration by using MTT assay. In addition, the cell number was used to calculate for population doubling time (PDT).

Staining and visualizing the actin cytoskeleton organization

H9c2 cells that chronically treated with cardioprotective dosed of Metformin and control untreated cells were grown on steriled-cell culture slide and were cultured in DMEM medium at sub-confluent density for 2 days. Cells on culture slides were washed twice with phosphate buffer saline (PBS) before fixing with fixative agent (2% (v/v) formaldehyde, 0.05% (v/v) glutaraldehyde). The cells on culture slides were permeabilized with 0.5% Triton-X 100 in PBS and were stained with 50 μ g/ml of phalloidin conjugated (TRITC) (Amresco, USA) for 40 mins at room temperature in the dark moist chamber. Subsequently, the culture slides were washed with PBS, before nuclear staining with 0.01 μ g/ml 4', 6-diamidino-2-phenylindole (DAPI) for 20 mins and then mounted by adding 20 μ l of 50% (v/v) glycerol and sealed the edges with nail varnish (Jemsri & Kumphune, 2012). The actin cytoskeleton was visualized under fluorescence microscope (Zeiss, Germany).

Immunoblot Analysis

Extracted proteins from H9c2 cell were separated on 12 % SDS- polyacrylamide gel and immunoblot with anti-phosphorylated p38 MAPK. The intensity of each band was measured by Chemidoc™ XRS (BIORAD, USA). Comparison of the band intensity between lanes provided information on relative abundance of the protein (Kumphune et al., 2012).

Analysis of proteins by shotgun proteomic technique by GeLC/MS

Proteins were extracted by lysis buffer (0.5% SDS). The concentration of extracted protein was estimated by using the method of Lowry (Lowry, Rosenbrough, Farr, & Randall, 1951). Twenty five



micrograms of cardiac cell lysate proteins were subjected to 2D SDS-PAGE and stained with Coomassie brilliant blue dye. Then the protein bands were cut into 13 fractions and consequently destained with 200 μ l of 50% methanol in 25 mM ammonium bicarbonate solution. The small pieces of gels were shaken for 10 mins for 3 times at 37° and dehydration by using 100 % acetonitrile and were shaken for further 5 mins. After that, the gels were dried and reduced disulfide bond with 10 mM dithiothreitol in 10 mM ammonium bicarbonate at 56°C for 1 hr and gels were then alkylated at room temperature for 1 hr in the dark and in the presence of 100 mM iodoacetamide in 10 mM ammonium bicarbonate. Then, gels were rehydrated twice with 100% of acetonitrile for 5 mins and tryptic digested with 10 μ l of trypsin solution (10 ng/ μ l trypsin in 50% acetonitrile/10 mM ammonium bicarbonate) at 37°C overnight. Protein was extracted by using 30 μ l of 50% acetonitrile in 0.1% formic acid. Extracted peptides were subjected to peptide separation and analysis by Nano LC-MS for Synapt HDMS system, NanoAquity system (Water Corp., Milford, MA). The mass spectrum was normalized with an internal BSA and determined by Decyder™ MS database.

Statistical analysis

Data was presented in Mean \pm Standard Error of Mean (S.E.M) and was analyzed by Graph Pad Prism 5.0. The different of coefficient of variance (CV) in each group was analyzed by ANOVA or t-test. P value < 0.05 was considered as statistically significant.

Results

Determination of cardioprotective dose of Metformin from simulated ischemia and reperfusion injury in cardiac H9c2 cell line

H9c2 cells were cultured in DMEM medium supplemented with a various concentrations of Metformin for 24 hrs prior to sI/R. The results showed that sI/R for 40 minutes caused cardiac H9c2 cell death and decreased the percentage of cell viability to 33.28 ± 4.37 %, when compared to untreated control groups. All concentration of Metformin, excepted 3 mM and 4 mM, could not increase the cell viability of cardiac cell after sI/R. However, treatment of Metformin at 3 mM and 4 mM could significantly increase the cell viability of cardiac cell, after sI/R, when compared to sI/R group (60.25 ± 7.03 % and 77.43 ± 7.57 %, respectively, versus 33.28 ± 4.37 %, p value < 0.05.) (Figure 1A). Moreover, sI/R caused the released LDH activity increased up to 184.70 ± 13.96 U/ml whereas the released LDH activity of 3 mM, 4 mM, and 5 mM groups was significantly reduced than that of sI/R group (86.30 ± 5.08 U/ml, 83.07 ± 4.11 U/ml, and 119.4 ± 9.81 U/ml, respectively, versus 184.70 ± 13.96 U/ml, p value < 0.05.) (Figure 1B). To ensure that treatment of Metformin did not cause any harmful effect to H9c2 cells. The measurement of toxicity was performed. The results showed that treatment of Metformin at 5 mM and 10 mM resulted in significantly reduced cell viability, when compared to untreated control group (63.52 ± 7.55 % and 32.92 ± 4.11 % respectively, p value < 0.05) (Figure 1C). In addition, the results showed a significant higher in released LDH activity in cells treated with 5 mM and 10 mM of Metformin than that of untreated control group (85.92 ± 5.90 U/ml and 116.3 ± 4.59 U/ml, respectively, versus 58.21 ± 4.53 U/ml, p value < 0.05) (Figure 1D). Therefore, the results suggested that 3 mM of Metformin was the lowest concentration with cardioprotective effect without producing cellular toxicity. This concentration was used in experiments throughout this study.

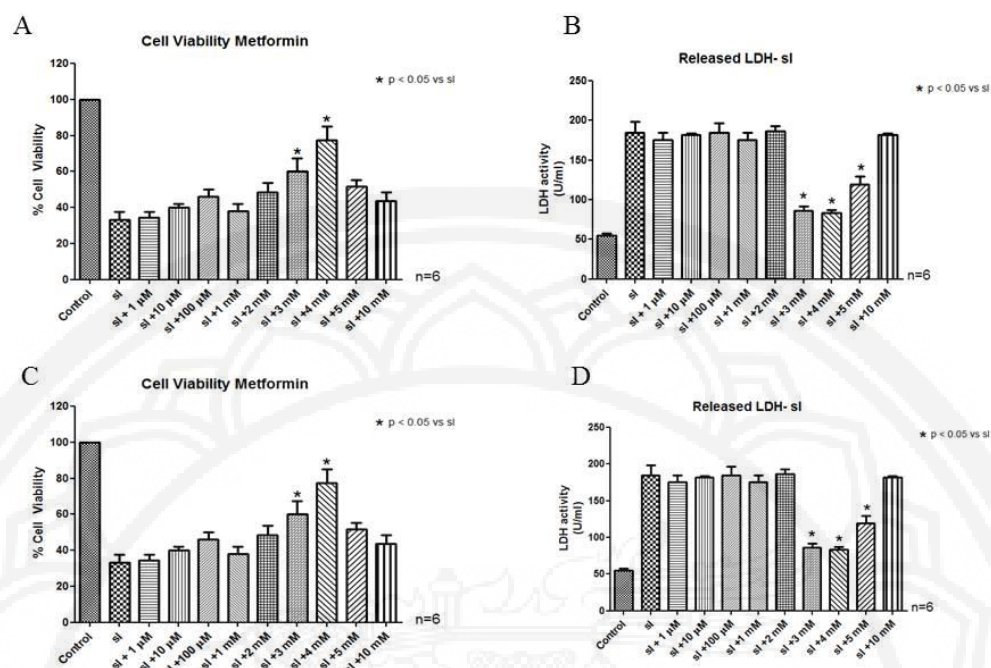


Figure 1 Optimization of cardioprotective dose of Metformin in cardiac cell

H9c2 cells were exposed to 1 μ M–10 mM Metformin for 24 hrs followed by si/R (1A, 1B). Toxicity of Metformin on cardiac cell presents in 1C and 1D. The MTT assay (1A, 1C) was used for determining the cell viability and released LDH activity (1B, 1D) was measured from collected culture medium. $p < 0.05$ compared to control group was considered as statistically significance

Long-term treatment of cardioprotective dose of Metformin increased cardiac cell proliferation

Treatment of 3 mM Metformin for 30 days could reduce the population doubling when compared with untreated control group (22.24 ± 2.19 hrs vs 27.36 ± 1.375 , p value < 0.05). (Table 1). Moreover,

the growth curved showed that treatment of 3 mM of Metformin for 30 days caused dramatically increase in cell number, when compared with untreated control groups (Figure 2A). The linear regression equation of growth curve in Metformin treated group and control group was derived as $y = 0.0142X + 0.8492$ and $y = 0.0092X + 0.6117$, respectively (Figure 2B). The linear regression of growth curve in Metformin treated group showed greater slope than that of control group suggesting the higher growth rate of cardiac cell with long-term exposure to Metformin.

Table 1 Effect of long-duration Metformin treatment on population doubling time (PDT)

	Control	Metformin	P value
Mean PDT (hrs)	27.36	22.24	0.014
S.E.M	1.375	2.195	

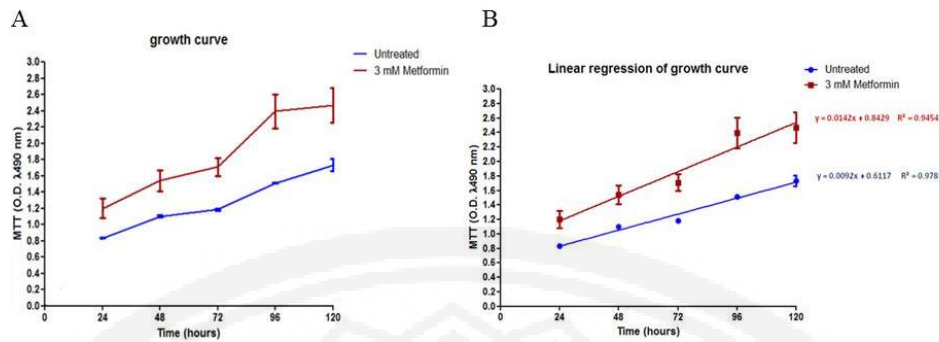


Figure 2 Increased cell proliferation by long-term treatment of cardioprotective dose of Metformin

H9c2 cells were cultured for 30 days in the presence (red line) and absence (blue line) of 3 mM of Metformin. After that, cells were counted for 7 days. The growth curve (2A) and linear regression of growth curve (2B)

Long-term treatment of cardioprotective dose of Metformin change in actin cytoskeleton organization

Stained actin of H9c2 cell with phalloidin conjugated-TRITC followed by nuclear staining with

DAPI showed that there were organized of actin filament and intact of stress fiber in untreated control cells. However, treatment with 3 mM Metformin for 30 days caused changes of actin organization by reducing actin accumulation at the cell border (Figure 3, arrow), but did not effect on shape and size of the cardiac cell when compared with untreated control group (Figure 3).

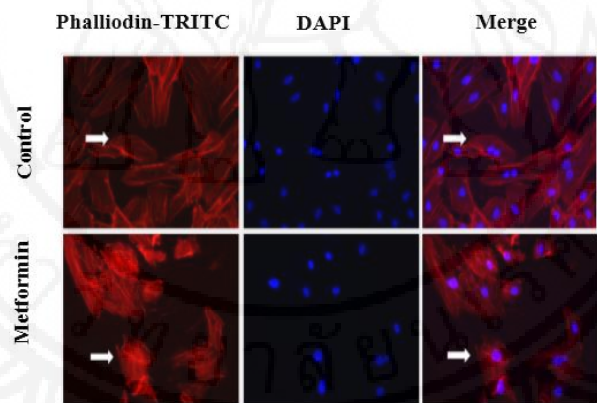


Figure 3 Effect of long-duration metformin treatment on cellular morphology and actin organization

H9c2 cells were cultured for 30 days in the presence and absence of 3 mM of metformin. The actin cytoskeleton were stained with phalloidin conjugated (TRITC) represent in red and nuclear stained with 4', 6-diamidino-2-phenylindole (DAPI) represent in blue

p38 MAPK phosphorylation of cardiac H9c2 cell treated with cardioprotective dose of Metformin.

The basal level of p38 MAPK was determined in both H9c2 cells that chronically treated with

cardioprotective dosed of Metformin and control untreated cells were determined. The resulted showed that treatment of 3 mM of Metformin for 30 days on cardiac H9c2 cell significantly decreased phosphorylation of p38 MAPK when compared with untreated control group, whereas the basal level of p38 MAPK was not altered. (p value < 0.05) (Figure 4).

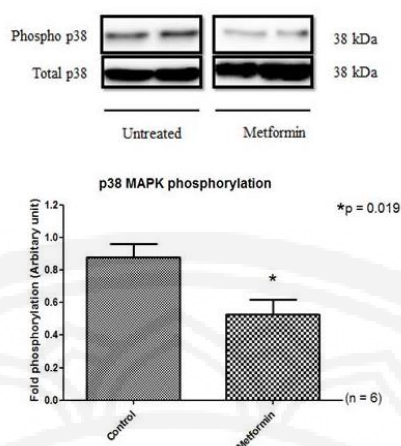


Figure 4 Effect of long-duration metformin treatment on the phosphorylation of p38 MAPK. H9c2

Cells were cultured for 30 days in the presence and absence of 3 mM of metformin. After that, the phosphorylation of p38 MAPK was compared. Treatment of 3 mM metformin decreased the activation of p38 MAPK when compared with untreated control group. Immunoblots (top) and quantitation (bottom) of p38 MAPK phosphorylation normalized to total protein

Cardiac cellular protein changing in long-term exposure to cardioprotective dose of Metformin.

The protein expression profile were determined in H9c2 cells that chronically treated with cardioprotective dosed of Metformin and control untreated cells by 2D SDS-PAGE and Nano LC-MS/MS (ESI-TOF). The results showed the difference of protein expression patterns (Figure 5A). There are 1,018 differentially expressed proteins were identified. The different of protein level in each group was analyzed by ANOVA or t-test (p value <

0.05). There were 35 proteins found to be up-regulated in cardiac cell treated with 3 mM Metformin for 30 days. These protein are involved in several mechanisms, including signal transduction, stress response, transportation, metabolic process, transcription, cell division, translation, DNA repair, cell structure, and unknown function (Figure 5B). The biological function of candidates proteins that related to the previously resulted were selected for predicting the effect of Metformin and treated cardiac H9c2 cell summarized (Table 2). For example, synaptonemal complex protein 2 (SYCP2), Rho guanine nucleotide exchange factor (rho-GEF), and cell cycle-related kinase (CCRK), which plays important role in cell proliferation signaling were significantly increased in treated Metformin group. Moreover, the down-regulation of actin gamma, which maintenance of the cytoskeleton was also found in Metformin and treatment.

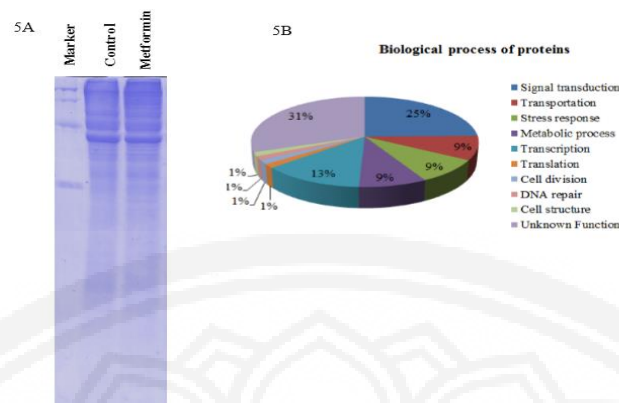


Figure 5 Twenty-five micrograms of total protein were separated by SDS-PAGE and stained with Coomassie blue (5A)

The biological process of candidate proteins in (5B). Long-term treatment of 3 mM of Metformin H9c2 cells after treated with long duration anti-diabetic drugs derived from UniprotKB database on cardiac H9c2 cells caused changed the protein expressions in several biological processes.

Table 2 Relative ratio of biological process proteins altered after anti-diabetic drugs

Proteins Name	Accession Number	Biological process	Relative intensity ratio	
			Control	Metformin
Signal Transduction				
Rho guanine nucleotide exchange factor (GEF) 17 (predicted)	gi 149068757	actin cytoskeleton organization,	2.68	6.91
	gi 210032529	cell proliferation	6.49	5.03
rasGTPase-activating-like protein IQGAP1	gi 149044090	Ras GTPase activator activity	0	3.41
Thousand and one amino acid protein kinase 2 beta	gi 157821543	Serine/threonine-protein kinase	0	7.37
NT-3 growth factor receptor isoform 1 precursor		tyrosine-protein kinase, actin cytoskeleton reorganization		
cell cycle-related kinase (CCRK)	gi 397174822	cell proliferation	0	9.61
Stress Response				
heat shock cognate 71 kDa protein-like isoform 1	gi 158081745	promote lysosomal degradation	0	12.22
heat shock-related 70	gi 109492762	protein folding	5.48	6.95
heat shock protein HSP 90-alpha	gi 11177910		5.99	10.08
Transportation				
organic anion transporter K8	gi 19071449	Na+-independent transport of organic anions and drugs	0	4.68
Metabolic Process				
PKLR pyruvate kinase	gi 185134818	phosphoenolpyruvate into pyruvate	3.70	8.88
mitochondrial ATP synthase	gi 89574017	and ATP	8.84	10.01
cytochrome c oxidase subunit IV isoform 2	gi 149031018	produces ATP from ADP	6.31	8.61
		mitochondrial respiratory chain		
Cell Division				
synaptonemal complex protein 2, isoform	gi 149034074	meiotic nuclear division	0	11.26
Structure				
actin, gamma 2	gi 149036532	cell motility and in the maintenance of the cytoskeleton	3.16	2.50



Discussion

The major findings of this study are the cardioprotective effects in pretreatment of 3 mM of Metformin in an *in vitro* ischemia/reperfusion (I/R) injury. Long-term treatment of 3 mM Metformin activated cell proliferation, disorganized of actin cytoskeletal, and decreases the level of phosphorylated p38 MAPK. Moreover, treatment of Metformin caused cardiac cell proteins alteration such as proteins that involve in signal transduction, stress response, transportation process, metabolic process, transcription machinery, cell division, translation process, and DNA repair.

This study showed the cardioprotective of Metformin pre-treatment of *in vitro* sI/R, increased the H9c2 cells viability of without causing cellular toxicity. The proteomics data showed that Thousand and one amino acid protein kinase 2 (TOAK2) was involved in cellular osmotic stress response (Chen et al., 2003), was significantly increased by Metformin treatment. Chen Z. *et al* reported that activated TOAK2 could phosphorylate MKK3/6 results in phosphorylation of p38 MAPK. The information from proteomics study could be explained the effect of Metformin on cell proliferation and decreasing of population doubling time, by the Metformin resulted in up-regulation of synaptonemal complex protein 2 (SYCP2), Rho guanine nucleotide exchange factor (rho-GEF), ras GTPase-activating-like protein, and cell cycle-related kinase (CCRK) which plays important role in meiotic nuclear division during cell division and cell proliferation (Costa et al., 2005; Alberts et al., 2002). CCRK was reported that associated with glioblastoma tumorigenesis and plays important roles in cancer cell proliferation (Ng et al., 2007). It is interesting that Metformin could up-regulate proteins related to cancer. It is noteworthy that the effect of Metformin

on CCRK in cardiac cell need to be further investigated in attempt to clarify the safety of using this drug.

Osmotic stress causes the electrolyte and water transport dysfunction. In this case, cell responded to this insults by activating the membrane transporter protein for stabilizing the electrolyte and water (Mager, Boer, Siderius, & Voss, 2005). Organic anion transporter K8 (OAT-K8), was also found to increase in long term Metformin treatment in cardiac cell. The results suggest that treatment of Metformin may cause osmotic stress leading to activation of OAT-K8 protein.

Pyruvate kinase, liver and red blood cell (PKLR) pyruvate kinase, mitochondrial ATP synthase, and cytochrome c oxidase significantly increased indicate that the function of cardiac mitochondrial may increase up to response to the growth rate of cardiac cell or treatment of Metformin may improve the cellular respiration of cardiac cell and subsequently results in increasing of intracellular ATP, which is the key of several cellular metabolisms (Kucharczyk et al., 2009).

Moreover, Heat shock-related 70, and heat shock protein HSP 90-alpha also increased in long term Metformin treatment. Metformin treatment may associate with the protein folding which leads to improve the protein structure and function (Neckers, 2007).

The possible explanation of how treatment of Metformin causes biological and biochemical alteration is summarized in figure 6.

However, there are limitations found in this study. The concentration of Metformin used in this study was higher than the real therapeutic concentration of the drugs that have been using for treatment the diabetic patients. Moreover, the environment in an *in vitro* model was not found in the real physiology. The human physiology has excretory process via renal system which could excrete some of drugs and toxicants from blood stream result in

the reduction of the harmful effect. In addition, simulated ischemia/reperfusion used in this study might not well reflect the real physiological condition of I/R injury.

Therefore, the *in vivo* experiments of long-term treatment of Metformin in whole protein expression

need to be further investigated and will provide some mechanistic insight concerning the effect of Metformin on cardiovascular responses and the possibility of adverse effects of using Metformin, which certainly provide promising drug safety issue for diabetes patients in the future.

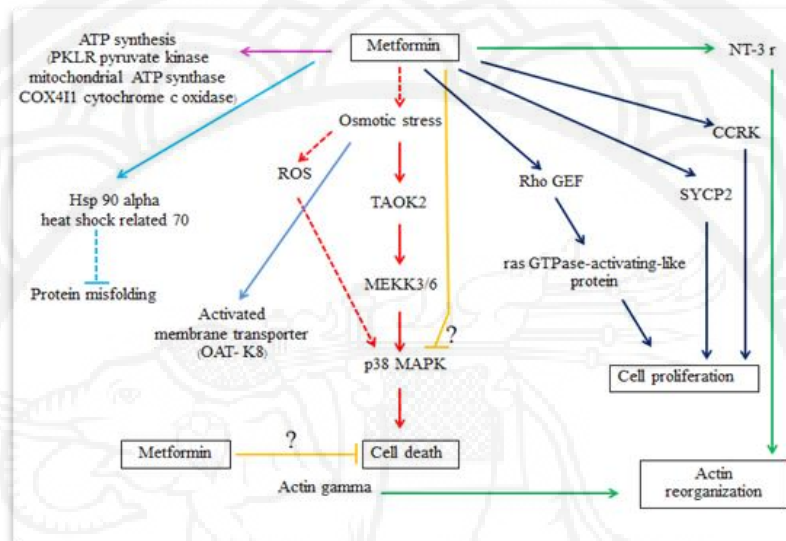


Figure 6 The proposed mechanism of long duration exposure of cardioprotective dose of Metformin on cardiac H9c2 cell

Conclusion and Suggestion

In summary, the present study demonstrates the long term effects of Metformin treatment caused enhanced cell proliferation, reduced the phosphorylation of p38 MAPK, and changes in cardiac cell proteins expression.

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