Berberine improves insulin resistance in cardiomyocytes via activation of 5′-adenosine monophosphate-activated protein kinase

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ARTICLE INFO
Article history:
Received 12 November 2012
Accepted 19 February 2013

Keywords:
AMPK
Glucose consumption
H9c2
Berberine
Insulin resistance

ABSTRACT

Objective. Insulin resistance plays an important role in the pathogenesis of diabetic cardiomyopathy. Berberine (BBR) is a plant alkaloid which promotes hypoglycemia via increasing insulin sensitivity in peripheral tissues. Little is known of BBR’s role in regulating glucose metabolism in heart.

Materials/methods. We examined the effect and mechanism of BBR on glucose consumption and glucose uptake in insulin sensitive or insulin resistant rat H9c2 cardiomyocyte cells. H9c2 myoblast cells were differentiated into cardiomyocytes and incubated with insulin for 24 h to induce insulin resistance.

Results. BBR-treatment of H9c2 cells increased glucose consumption and glucose uptake compared to controls. In addition, BBR-treatment attenuated the reduction in glucose consumption and glucose uptake in insulin resistant H9c2 cells. Compound C, an inhibitor of AMP-activated protein kinase (AMPK), abolished the enhancement of glucose consumption and glucose uptake mediated by BBR in both insulin sensitive and insulin resistant H9c2 cells compared to controls.

Conclusion. BBR significantly increased AMPK activity, but had little effect on the activity of protein kinase B (AKT) in insulin resistant H9c2 cells, suggesting that berberine improves insulin resistance in H9c2 cardiomyocytes at least in part via stimulation of AMPK activity.

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Keywords: AMPK, Glucose consumption, H9c2, Berberine, Insulin resistance

1. Introduction

The heart is one of the largest energy consumers in the body, and the balance of the energy metabolism in the heart is important for maintaining its physiological function. Under physiological conditions, 70% of the energy supply of the normal heart is provided through oxidation of fatty acid, with 30% derived from oxidation of carbohydrate, including 20%
from glucose oxidation and 10% from glycolysis. However, during the development of cardiovascular diseases, such as cardiomyopathy, myocardial infarction and congestive heart failure, glucose metabolism becomes the main source of energy supply utilized by the heart.

5′-adenosine monophosphate-activated protein kinase (AMPK) is a heterogenous protein trimer composed of three subunits; a functional subunit (α) and regulatory subunits (β, γ). AMPK plays an important role in various intracellular signaling pathways that regulate intracellular energy balance [1]. Activated AMPK triggers glucose uptake, glycolysis, fatty acid oxidation and mitochondria biogenesis, and decreases protein synthesis, glycolysis synthesis and fatty acid synthesis. AMPK has been shown to regulate glucose metabolism through translocation of glucose transporter-4 (GLUT4) to the plasma membrane in 3T3-L1 adipocytes [2]. Activated AMPK results in enhanced glucose metabolism via increasing glycolysis in skeletal muscle [3]. In addition, activation of hepatic AMPK inhibits hepatic glucose production and lipogenesis, as well as cholesterol synthesis [4]. Recently, activation of AMPK was found to phosphorylate cAMP response element-binding protein (CREB)-regulated transcription coactivator 2 (TORC2), which mediates CREB-dependent transcription of peroxisome proliferator-activated receptor gamma coactivator 1 (PGC1) and its subsequent gluconeogenic targets phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6-phosphatase (G6Pase), thus inhibiting the hepatic gluconeogenesis [5]. Furthermore, activation of AMPK has been shown to play a critical role in glucose metabolism of cardiomyocytes [6]. Thus, AMPK might be an important pharmaceutical target for the regulation of energy metabolism in the heart.

Berberine (BBR), is an isoquinoline alkaloid extracted from Coptis chinensis. BBR treatment results in hypoglycemic and insulin-sensitizing activity both in animal models and in patients with type 2 diabetes mellitus [7–11]. Increased glucose consumption mediated by BBR was shown to be a result of activated AMPK. BBR increased glucose uptake via AMP-AMPK-p38 MAPK pathway in L6 rat skeletal muscles [12]. In addition, BBR enhanced glucose metabolism via stimulation of glycolysis due to activation of AMPK. We previously reported that BBR inhibited hepatic gluconeogenesis via activating AMPK [13]. Despite significant advances in our understanding of the hypoglycemic effect of BBR, the role of BBR in regulating glucose metabolism in cardiac cells is unknown. In the present study, we show that BBR stimulates glucose consumption and glucose uptake via activation of AMPK in normal or insulin resistant H9c2 cardiomyocyte cells.

2. Materials and methods

2.1. Materials

DMEM, trypsin-EDTA solution, insulin, 5-amino-1-b-D-ribofuranosyl-1H-imidazole-4-Carb-oxamide (AICAR), Compound C and reagents for western blot analysis were obtained from Sigma-Aldrich (Beijing, China). FCS was obtained from Runsheng Biological Materials (Taiyuan, China). Polyclonal antibodies against phosphorylation of AKT (Ser\(^{172}\)), AKT, phospho-AMPK (Thr\(^{172}\)), AMPKα1/2 and Myosin heavy chain (MF20) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA., USA). Polyvinylidene fluoride (PVDF) membrane was obtained from Millipore (Beijing, China). 2-((N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) Amino)-2-deoxyglucose, 2-NBDG (Invitrogen).

2.2. Cells

H9c2 rat cardiac myoblastic cells were obtained from Cell Resource Center (IBMS, Beijing). Cells were maintained in high glucose Dulbecco’s modification of Eagle’s medium (DMEM) with 10% fetal calf serum (FCS), 2% L-glutamine, 10% sodium bicarbonate, 10% sodium pyruvate, 5% Heps, 1% penicillin/streptomycin and 1% gentamicin in an incubator (37 °C, 5% CO\(_2\)) in 48 well plates. One day before each experiment, cells were incubated in cell culture medium supplemented with 1% FCS to differentiate the cells into cardiomyocytes [14].

2.3. Live fluorescence microscopy of H9c2 Cells labeled with MF20 and Hoechst 33342

After the differentiation process, H9c2 cells were seeded in glass-bottom dishes (Mat-Tek Corporation) and incubated with Myosin heavy chain (MF20, Santa Cruz) antibody (1:200) and Hoechst 33342 (1 μg/ml). The images were obtained using a confocal microscope.

2.4. Glucose consumption

Glucose consumption was measured as previously described [3,15–17]. H9c2 cardiomyocytes were incubated in serum-free, low-glucose (5.5 mM) DMEM overnight. The cells were then treated with BBR and/or other reagents (Compound C 10 μmol/L, AICAR 1 mmol/L) in fresh serum-free low-glucose (5.5 mM) DMEM for 24 h, and 10 μl medium was removed at 3, 6, 12, and 24 h. The glucose concentration in medium was measured with glucose assay reagent (Beijing BHKT Clinical Reagent, Beijing, China) and is based on the glucose oxidase method. The amount of glucose consumption (GC) was calculated by the glucose concentrations of blank wells subtracting the remaining glucose in cell plated wells.

2.5. Glucose uptake in normal and insulin resistant H9c2 cells

In normal cells, H9c2 cardiac myocytes were pre-incubated 3 h in the absence or presence of 10 μmol/L BBR and/or 10 μmol/L Compound C and/or 1 mmol/L AICAR and then 2-NBD-glucose (200 μmol/L) in PBS were added and the cells were incubated for an additional 30 min. For measurement of glucose uptake in insulin resistant cells, H9c2 cells were exposed to insulin (100 nmol/L) treatments for 24 h and then washed 3 times with KRB containing 0.5% BSA at 40-min intervals over 2 h at 37 °C. After 3 h pretreatment of berberine (10 μmol/L) or other reagents, 200 μmol/L 2-NBD-glucose in PBS was added with or without insulin at 100 nmol/L; and the cells were incubated for an additional 30 min. Glucose uptake
was stopped with 3 washes of ice-cold PBS. The fluorescence intensity of cells was recorded by fluorescent microplate.

2.6. Insulin resistance in H9c2 cells

Insulin resistance in H9c2 cells was induced by incubating cells in DMEM with insulin (100 nmol/L) for 24 h [18]. The cells were then left untreated or treated with BBR and/or other reagents (Compound C 10 μmol/L, AICAR 1 mmol/L) and combined with or without insulin (100 nmol/L) for 12 h (for glucose consumption) or 30 min (for glucose uptake) to measure the sensitivity to insulin stimulation.

2.7. Cell viability analysis

Cell viability was determined by MTT assay in 96-well tissue culture plates as described previously [19]. After treatment, the culture medium was removed from the wells, and 200 μl of MTT reagent (Sigma) at a concentration of 1 mg/mL in PBS was added to each well. After 4 h incubation at 37°C, MTT reagent in PBS was removed and then the blue-colored formazan product was solubilized in 0.15 ml of DMSO for 20 min. The absorbance of converted dye was measured at a wavelength of 570 nm.

2.8. Western blot analysis

Quantitative analysis of AKT, P-AKT, AMPK, or p-AMPK(Thr172) expression was performed as previously described [20]. The cells were lysed in ice-cold lysis buffer (50 mmol/L Tris–HCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mmol/L NaCl, 1 mmol/L sodium orthovanadate, 1 mmol/L NaF, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L apro- tinin, 1 mmol/L leupeptin, 1 mmol/L pepstatin A) and centrifuged for 20 min at 14,000 × g. Protein concentration was measured using the bicinchoninic acid (BCA) assay (Pierce BCA protein assay kit). For western blot detection, proteins (80–100 μg) were separated on 12% SDS polyacrylamide gel electrophoresis and electroblotted onto PVDF membranes. Membranes were blocked with 5% (w/v) skim milk or 5% BSA for 2 h at room temperature and then incubated with rabbit polyclonal antibodies (p-AMPK, 1:500, AMPK, 1:1000, p-AKT, 1:1000, AKT, 1:1000, Santa Cruz Biotechnology) with gentle agitation overnight at 4 °C. The membranes were washed 3 times for 10 min each with 15 ml of TBST [10 mmol/L Tris–HCl, 150 mmol/L NaCl and 0.1% (v/v) Tween] and then incubated with secondary antibody (1:1000 goat anti-rabbit IgG horseradish peroxidase conjugate, Santa Cruz Biotechnology) at room temperature for 2 h. Protein was visualized with enhanced chemiluminescence solution and images were generated with GENE Imaging system. The images were quantified using Image Analysis Software (Quantity One). The results were expressed as phosphorylated protein relative to total protein.

2.9. Statistics

Data are expressed in mean ± SE. Differences between two means were analyzed by Student’s t-test. A two-tailed p value <0.05 was considered to be significant.

3. Results

3.1. Differentiation features of H9c2 cells

H9c2 cells are similar to neonatal cardiomyocytes in many ways. For example, the membrane structure, G protein signaling system, electrophysiological properties and reactivity signal stimulation of cardiac hypertrophy are similar between H9c2 cells and neonatal cardiomyocytes. To examine the role of BBR in regulating glucose metabolism in cardiac cells, H9c2 myoblasts were fixed and labeled with myosin heavy chain antibody (MF20) and counterstained with Hoechst as described in Materials and Methods. H9c2 cells grown in medium containing 10% FCS (growth-promoting medium) exhibited a myoblast phenotype, including a spindle-to-stellate shape and were mononucleated (Fig. 1). Differentiated H9c2 cell cultured in medium supplemented with 1% of FCS showed increased fusion and more elongated cells with multiple nuclei. The different morphology was in agreement with previous reports [21,22]. To further characterize differentiation induction, cells were immunolabeled with antibodies against myosin heavy chain (MF20) protein, a marker of muscle differentiation. As shown in Fig. 1, differentiated H9c2 cells exhibited significant expression of myosin heavy chain.

3.2. Low concentrations of BBR did not affect H9c2 cell viability

H9c2 cardiomyoblast cells were incubated in cell culture medium supplemented with 1% FCS to differentiate the cells into cardiomyocytes and the cytotoxicity of BBR was tested in these cells using the MTT assay. BBR was non-cytotoxic concentrations at 50 μmol/L or lower (Fig. 2). In contrast, concentrations of 100 μmol/L or higher resulted in significant

![Fig. 1 – Myosin heavy chain (MF20) content in differentiated H9c2 cells. H9c2 cells cultured in a high-serum media were treated with 10% FCS or 1% FCS overnight and labeled with MF20 (green fluorescence), Hoechst 33342 (nuclei, blue fluorescence). Differentiated H9c2 cell cultures in medium supplemented with 1% of FCS showed increased fusion and more elongated cells with multiple nuclei and cells expressing myosin heavy chain significantly. Images were collected by confocal microscopy with a 400× total magnification.](attachment:image-url)
cytotoxicity. No abnormal features were observed in cells treated with 3.125–50 μmol/L BBR using light microscope analysis (data not shown). A 5–20 μM concentration of BBR was chosen for all subsequent experiments.

3.3. BBR increased glucose consumption and glucose uptake in H9c2 cells through activated AMPK

To investigate the effect of BBR on glucose consumption in H9c2 cells, cells were treated with BBR for 24 h. BBR increased glucose consumption with time in a dose-dependent manner (Fig. 3A). We examined if expression of p-AMPK was altered in H9c2 cells treated with 5–20 μM BBR for 24 h. Total AMPK expression was unaltered between control and BBR treated cells. In contrast, p-AMPK levels and the ratio of p-AMPK to AMPK were significantly increased by BBR treatment in a dose dependent manner (Fig. 3B). We next investigated whether the activated AMPK contributed to the increased glucose consumption in BBR treated cells. AMPK inhibitor Compound C and activator AICAR were added to H9c2 cells treated with or without BBR. Glucose consumption in H9c2 cells was 4-fold higher (p < 0.05) in cells treated with BBR for 24 h compared to controls (Fig. 3C). The presence of Compound C diminished the glucose consumption induced by BBR. In contrast, AICAR enhanced the action of BBR on increasing glucose consumption in H9c2 cells. Moreover, treatment of H9c2 cells with BBR and AICAR for 24 h activated AMPK and increased the ratio of p-AMPK to AMPK. In contrast, the presence of Compound C blocked the activation of AMPK even in the presence of BBR (Fig. 3D). The effect of BBR on glucose uptake was further tested in H9c2 cardiomyocytes. 10 μM concentration of BBR significantly increased basal and insulin simulated-glucose consumption in H9c2 cells (Fig. 4A and 4B). We next examined the effect of BBR on p-AKT in insulin-resistant H9c2 cells. Expression of p-AKT in insulin resistant H9c2 cells was slightly, but not completely blocked by Compound C (Fig. 5C). We next examined the effect of BBR on p-AKT in insulin-resistant H9c2 cells. The presence of Compound C did not affect glucose consumption and glucose uptake in the insulin-resistant cells. BBR treatment at 10 μmol/L also increased the glucose uptake in the insulin-resistant cells.

3.4. BBR increased glucose consumption and glucose uptake in H9c2 cells with insulin resistance

Insulin resistance in H9c2 cells was established by adding insulin (100 nmol/L) to the culture medium for 24 h. As shown in Fig. 4A and 4B, insulin stimulated glucose consumption and glucose uptake in insulin-sensitive H9c2 cells, but cells cultured with insulin (insulin-resistant H9c2 cells) were unresponsive to insulin stimulation. Since Ser473 phosphorylation of AKT is a molecular indicator of insulin resistance, we examined if expression of AKT, a key protein in the insulin signaling pathway, was altered in insulin resistant H9c2 cells. Total AKT expression was unaltered between insulin-sensitive and insulin-resistant H9c2 cells. Treatment of insulin-sensitive H9c2 cells with insulin increased p-AKT levels. In contrast, treatment of insulin-resistant H9c2 cells with insulin did not alter p-AKT levels (Fig. 4C). Thus, adding insulin (100 nmol/L) to the culture medium for 24 h resulted in the establishment of insulin-resistant H9c2 cells. We examined if BBR-treatment altered glucose consumption in H9c2 cells with insulin resistance. BBR-treatment of insulin-resistant H9c2 cells significantly increased basal and insulin stimulated-glucose consumption in insulin-resistant H9c2 cells (Fig. 4D). To examine whether BBR affected the glucose uptake in insulin-resistant cells, BBR at 10 μmol/L was incubated with or without the addition of insulin at 100 nmol/L for 30 min. As shown in Fig. 4E, BBR treatment at 10 μmol/L also increased the glucose uptake in the insulin-resistant cells.

3.5. BBR improved insulin-induced insulin resistance through activation of AMPK

Since the BBR-mediated stimulation in glucose consumption and glucose uptake in insulin-sensitive H9c2 cells was mediated through AMPK, we examined the possibility that BBR improved insulin sensitivity through AMPK activation in insulin resistant H9c2 cells. The effects of BBR on glucose consumption and glucose uptake were examined in the presence of the specific AMPK inhibitor Compound C. As shown in Fig. 5A and 5B, Treatment of insulin resistant H9c2 cells with BBR significantly increased the glucose consumption and glucose uptake in either the absence or presence of insulin. The presence of Compound C did not affect glucose consumption and glucose uptake in insulin-resistant H9c2 cells in either the absence or presence of insulin. In contrast, Compound C attenuated but did not completely block the BBR-mediated stimulation of glucose consumption and glucose uptake in either the absence or presence of insulin. Moreover, BBR significantly stimulated phosphorylation of AMPK in the absence or presence of insulin, this stimulation was suppressed by Compound C (Fig. 5C). We next examined the effect of BBR on p-AKT in insulin-resistant H9c2 cells. Expression of p-AKT in insulin resistant H9c2 cells was slightly, but not significantly increased by BBR treatment in either the absence or presence of insulin (Fig. 5D). Taken together, BBR improves insulin resistance in H9c2 cardiomyocytes at least in part via stimulation of AMPK activity.

4. Discussion

In the present study, the effect and mechanism of BBR on glucose metabolism were examined in both insulin-sensitive and insulin-resistant rat H9c2 cardiomyocytes. The results indicate that BBR stimulates glucose consumption and
glucose uptake in both insulin-sensitive and insulin-resistant H9c2 cardiomyocytes and the mechanism underlying improvement of BBR on insulin resistant in H9c2 cells may be associated with activation of AMPK.

Myocardial insulin resistance and hypoglycemia have been identified as major determinants in the development of diabetic cardiomyopathy. In type 2 diabetes there is a failure to increase glucose disposal into peripheral tissues in
response to insulin leading to chronically elevated levels of glucose in the circulation followed by a compensatory rise in insulin [23,24]. The elevated glucose and insulin levels in turn exacerbate insulin resistance, which results in dysfunction of myocardium and contributes to the pathogenesis of diabetic cardiomyopathy [25].

BBR has been studied as an anti-diabetic drug widely due to its remarkable hypoglycemic effects [9,26]. Recently, the cardiovascular protective effects of BBR have attracted much attention [27-30]. It was initially reported that BBR could significantly improve cardiac function in patients with severe congestive heart failure [31]. The anti-arrhythmic actions of BBR were attributed to its inhibitory effects on IK1, IK, and HERG channel [28]. Recently, it was reported that the BBR attenuation of ischemic arrhythmias maybe related to recovery of depressed I(to) and I(Ca) currents in diabetic rats. In addition, BBR was shown to modulate the sympathetic nervous activity of rats with experimental cardiac hypertrophy.

Fig. 4 – Effect of BBR on glucose consumption and AKT activation in insulin-sensitive and insulin-resistant H9c2 cardiomyocytes treated plus or minus insulin. (A) Insulin-sensitive or insulin-resistant H9c2 cardiomyocytes were incubated in the absence or presence insulin and glucose consumption determined as described in Materials and Methods. (B) 200 μmol/L 2-NBD-glucose was added to H9c2 cells with or without insulin (100 nmol/L) for 30 min to test the ability of glucose uptake; the fluorescence intensity of cells was recorded by fluorescent microplate. (C) Insulin-sensitive or insulin-resistant H9c2 cardiomyocytes were incubated in the absence or presence insulin and AKT (MW: 60 kDa) activation determined by western blot analysis as described in Materials and Methods. A representative blot is depicted. (D) Insulin-resistant H9c2 cardiomyocytes were incubated in the absence or presence of BBR and the absence or presence of insulin and glucose consumption was determined as described in Materials and Methods. (E) H9c2 cells were exposed to insulin (100 nmol/L) for 24 h to induce insulin resistance and then washed 3 times with KRB containing 0.5% BSA at 40-min intervals over 2 h at 37 °C. After 3 h pretreatment of berberine (10 μmol/L), 200 μmol/L 2-NBD-glucose was added to insulin-resistant H9c2 cells with or without insulin (100 nmol/L) for 30 min to test the ability of glucose uptake; the fluorescence intensity of cells was recorded by fluorescent microplate. Data represent the mean ± SE of six experiments. *p < 0.05 compared with control, #p < 0.05 compared with insulin resistant group in presence of insulin.
and improve pressure-load induced left ventricular hypertrophy [27,32]. We previously showed that BBR improved cardiac vascular endothelial function [7] and attenuated loss of cardiac function in acute ischemia reperfusion injury [33]. The present study demonstrates that BBR not only increases glucose consumption and glucose uptake in normal insulin-sensitive H9c2 cells but also enhances both in H9c2 cells with insulin resistance.

Both insulin signaling and AMPK pathways are involved in glucose uptake and consumption in peripheral tissues. Insulin activates insulin receptor substrate-1 (IRS-1) and increases the Glucose transporter type 4 (GLUT-4) translocation to membranes by phosphorylation of AKT [34], while AMPK stimulates GLUT-4 translocation to membranes as well through activation of AS160 [35]. The effects of BBR on the insulin-stimulated glucose uptake pathway are varied and sometimes conflicting which may, in part, be attributed to the variety of cell types and treatment times utilized in these studies. It was reported that BBR exerted its hypoglycemic effect in alloxan-induced diabetic C57BL/6 mice via activated AKT pathway [9]. In contrast, a number of studies indicated that BBR improved glucose metabolism in insulin sensitive cells, including HepG2, C2C12, L6, 3T3-L1 cells, in an insulin independent manner [12,15,36]. BBR stimulated glucose uptake in L6 myotubes through AMPK activation [12]. BBR treatment resulted in increased AMPK activity in 3T3-L1 adipocytes and L6 myotubes, and increased GLUT4 translocation in L6 cells in a PI3K-independent manner [37]. These findings suggest that BBR displays beneficial effects in the treatment of diabetes via stimulation of AMPK activity. In the present study, BBR only weakly, but not significantly stimulated the phosphorylation of AKT, a key molecule in

Fig. 5 – Effect of BBR, Compound C and insulin on glucose consumption and AKT activation in insulin-resistant H9c2 cardiomyocytes. (A) Insulin-resistant H9c2 cardiomyocytes were incubated in the absence or presence of BBR and/or Compound C and/or insulin and glucose consumption was determined as described in Materials and Methods. (B) After 3 h pretreatment of berberine (10 μmol/L) or other reagents in insulin resistant H9c2 cells, 200 μmol/L 2-NBD-glucose in PBS was added with or without insulin at 100 nmol/L and the cells were incubated for an additional 30 min. Glucose uptake was stopped with 3 washes of ice-cold PBS. The fluorescence intensity of cells was recorded by fluorescent microplate. (C) Insulin-resistant H9c2 cardiomyocytes were incubated in the absence or presence of BBR in the absence or presence of insulin and AMPK (MW: 62 kDa) activation was determined by western blot analysis as described in Materials and Methods. A representative blot is depicted. (D) Insulin-resistant H9c2 cardiomyocytes were incubated in the absence or presence of BBR in the absence or presence of insulin and AKT (MW: 60 kDa) activation was determined by western blot analysis as described in Materials and Methods. A representative blot is depicted. Data represent the mean ± SE of six experiments. *p < 0.05 compared with insulin resistant group absence of insulin, #p < 0.05 compared with insulin resistant group in presence of insulin, $, p < 0.05 compared with insulin resistant group treatment with BBR in absence of insulin, ★, p < 0.05 compared with insulin resistant group treatment with BBR in presence of insulin.
the insulin signaling pathway, but strongly promoted the phosphorylation of AMPK. Moreover, BBR-stimulated glucose consumption and glucose uptake were diminished by the AMPK inhibitor Compound C in insulin-resistant H9c2 cells. These data suggest that BBR improves insulin resistance in H9c2 cardiomyocytes, at least in part, by an increase in AMPK activity. We suggest that BBR might provide cardioprotection in insulin resistance and diabetes.

In conclusion, BBR stimulates glucose consumption and glucose uptake in insulin-sensitive and insulin-resistant H9c2 cardiomyocytes. The mechanism underlying improvement of BBR on insulin resistance in H9c2 cells is mediated, at least in part, by an increase in AMPK activity. We suggest that BBR might provide cardioprotection in insulin resistance and diabetes.

Authors’ contributions

Conceived and designed the experiments: Wenguang Chang, Ming Zhang, Li Chen, Hongwei Du. Performed the experiments: Wenguang Chang, Ming Zhang, Jing Li, Zhaojie Meng, Shengnan Wei. Analyzed the data: Wenguang Chang, Ming Zhang. Contributed reagents/materials/analysis tools: Li Chen, Hongwei Du. Wrote the paper: Grant M. Hatch.

Acknowledgment

This work was supported by the National Natural Science Foundation of China (81170745 and 81200598), Jilin Science & Technology Development Plan (201274), Jilin Province Administration of Traditional Chinese medicine science and technology projects (2010-093), and the Heart and Stroke Foundation of Manitoba. G.M.H. is a Canada Research Chair in Molecular Cardiolipin Metabolism.

Conflict of interest

The authors have declared that no competing interests exist.

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