AMPK/mTOR-mediated therapeutic effect of metformin on myocardial ischemia reperfusion injury in diabetic rat

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Abstract

BACKGROUND: The autophagy associated signalling pathways such as AMPK/mTOR previously were suggested to play a crucial role in protecting from ischemia-reperfusion injury (IRI). The objective of this study was to evaluate the effect of metformin (DMBG) on autophagy during myocardial IRI with diabetes mellitus (DM). **METHODS:** The DM rat model was established using streptozocin, and further induced ischemia model via transitory ligation of the left anterior coronary artery and following reperfusion. The model rats were treated with 400 mg/kg/day DMBG for one week. Autophagosomes were investigated using transmission electron microscopy. Autophagy-associated signalling pathways were detected by western blot. **RESULTS:** The myocardial infarct size was shown to significantly increase in the DM rats exposed to IRI compared to negative control, but decrease in DMBG treated. The mature autophagosomes were elevated in infarction and marginal zones of DM+IRI+DMBG compared to DM+IRI. Furthermore, the increasing protein levels of LC3-II, BECLIN 1, autophagy related 5 (ATG5) and AMP-activated protein kinase suggested activated autophagy-associated intracellular signalling AMPK and mTOR pathways upon DMBG treated. **CONCLUSIONS:** Taken together, the outcomes demonstrated that DMBG could activate autophagy process to provide a cardio-protective effect against DM induced myocardial IRI.

Background

The presence of diabetes mellitus (DM) patients with higher incidence and fatality on ischemic heart disease (IHD) cause the increasing concern even though the current clinical intervention and treatment score remarkable progress [1]. Clinical findings have shown that diabetes is not only apt to induce the insult of cardiogenic ischemia, but also weakens the efficiency of cardioprotective intervention including ischemic pre- and post-conditioning. The patients with diabetes show poorer recovery following acute myocardial infarction (AMI), demonstrated as poorer prognosis following AMI. The common clinical indications of myocardial infarct size is used to suggest that DM amplifies heart sensitivity to ischemia-reperfusion injury (IRI). Increased infarct size and higher rates of acute congestive heart failure onset subsequent to reperfusion treatment were detected in patients with DM, compared with those in control individuals.

Metformin (DMBG) has been developed as a prospective agent with a good safety profile that is widely used as a first-line treatment for type II diabetes and steatohepatitis [2] by controlling insulin [3] and regulating people's appetite [4]. The therapeutic effects of DMBG are due to improving the insulin resistance of liver and muscles, as well as enhancing the translocation of glucose transporters 4 (GLUT4) [5]. Additionally, metformin also exerts as an AMP-activated protein kinase (AMPK) activator to induce AMPK-mediated autophagy process in hepatic steatosis [6] and hepatocellular carcinoma [7]. Previous clinical studies have determined that metformin is beneficial to protect from AMI [8] and reduce the cardiovascular death in people with DM [9]. Nevertheless, the mechanisms underlying the pharmacological actions of DMBG in AMI with DM, although already scrutinized, still remain to be fully elucidated.

No direct evidence between DMBG and autophagy in cardiac IRI has been revealed so far, and we assume that DMBG may also govern the associated signalling pathways to induce autophagy in the infarct zone to alleviate AMI. The objective of the present study was to evaluate the effect of DMBG on autophagy during cardiac IRI in rats with DM, with a focus on autophagy-associated factors and pathways, including microtubule-associated protein 1 light chain 3 II (LC3-II), BECLIN 1, Autophagy related 5 (ATG5) and AMPK and mTOR. The results revealed that DMBG improved the function of autophagy and decreased infarct size in diabetes-induced IRI by increasing the expression of ATG5 and BECLIN-1 in a streptozotocin (STZ)-induced diabetes rat model.

Methods

Reagents

Streptozotocin (STZ), DMBG and insulin were purchased from Sigma-Aldrich (St. Louis, MO, USA). The primary antibodies against ATG5 (Cat. No. 12994, CST, Beverly, MA, USA), BECLIN1 (Cat. No. 3495T, CST), microtubule-associated protein 1 light chain 3 (LC3) (Cat. No. 4108, CST), mammalian target of rapamycin (mTOR) (Cat. No. 2983, CST), phosphorylated (p-) mTOR (Cat. No. 5536, CST), AMPK (Cat. No. 5831, CST), p-AMPK (Cat. No. 2535, CST) and GAPDH (Cat. No. AF1186, Beyotime Biotechnology, Shanghai, China), as well as the secondary antibodies rabbit anti-mouse (A27025, ThermoFisher Scientific, Waltham, MA, USA) and donkey anti-rabbit (SA1-200, ThermoFisher) were purchased.

Experimental animals

Total seventy male Sprague-Dawley (SD) rats with of 12-week-old (160–200 g) acquired from Shanghai SLAC Laboratory Animal Co. Ltd. (Shanghai, China) were enrolled in this study. The study was approved by the Animal Care and Use Committee of Zhongshan Hospital (Shanghai, China), and all animal protocols were executed following the guidelines accordingly. All rats were housed in a controlled environment of 53% humidity, ($23 \pm 2^{\circ}\text{C}$) in a 12/12 h dark/light cycle with free access to high calorie diet (60% normal fodder plus 15% animal fat, 20% sucrose and 5% cholesterol) just as previously described [10]. The rats were anaesthetised using 50 mg/kg pentobarbital, and the blood was sampled from caudal vein. The levels of glycated hemoglobin $\alpha 1$ (HbA1c), plasma glucose, lactate dehydrogenase (LDH), creatine phosphokinase (CK), CK isoenzyme MB (CK-MB) and aspartate aminotransferase (AST) were detected biochemical analyser (BK-400, BIOBASE, Jinan, Shandong, China). Echocardiography was conducted as the described below.

For establishment of DM model rats, 65 mg/kg/day STZ dissolved in 0.1 M citrate buffer (pH 4.1) was intraperitoneally injected in the male SD rats for one week prior to surgery. Control animals were administered with an equivalent volume of saline solution. The rat with the level of blood glucose reaching more than 300 mg/dl 10 successive days following STZ treatment was considered as type II diabetes, whereas those with blood glucose levels lower than that were excluded from the experiment. Diabetic and metabolic parameters were measured to ensure that the experimental groups were well-

prepared. Rats were sacrificed using cervical dislocation, following with the heart tissues harvest for the next experiment, and the body was transferred to the Animal Department of the school after experiment.

For myocardial IRI model preparation, the male SD rats were anesthetized by 30 mg/kg ketamine and conducted the IRI surgery as described previously [11]. Following 1 h transitory ligation of the left anterior coronary artery, the hearts were subjected to 3 h of reperfusion. The visualization of the colour return in the formerly pale area was used to indicate the IRI and immediately recorded the electrocardiographic variations. The SD rats were administered 30 mg/kg/day DMBG intravenously or 1 IU/kg/day insulin subcutaneously for five days. Five groups of rats (ten rats in each group) were included: (1) negative control (NC); (2) diabetic (DM); (3) diabetic with IRI (DM + IRI); (4) diabetic with IRI and treated with DMBG (DM + IRI + DMBG); (5) diabetic with IRI treated with insulin and DMBG (DM + IRI + I + DMBG).

Echocardiography

Rats were sedated and exposed shaved chests. Rats were placed in a left lateral decubitus position and scanned by a Vivid-i™ ultrasound system (GE Healthcare Life Sciences, Pittsburgh, PA, USA) using a 10S transducer and a cardiac application with 10 MHz, 2.5 cm depth and 225–350 fps of transmission frequency as previously described [12]. The measurements included short-axis cross sections of the apex and papillary muscles levels. The heart rate was calculated from the R-R interval of the electrocardiogram (ECG) signal. The end-systolic and end-diastolic LV areas (ESA and EDA, respectively) were measured from the ultrasound B-scan at the PM level. Enddiastole was defined by the ECG R-wave, and the end-systole defined as the image with the smallest LV area. The fractional area change (FAC) was defined as the ratio of the difference between end-diastolic area and end-systolic area, divided by the end-diastolic area. Left ventricular end-diastolic dimension (LVEDD), left ventricular end-systolic dimension (LVESD), interventricular septal depth (IVSD), ejection fraction (EF) and fractional shortening (FS) were calculated as the average values of three cardiac cycles.

Masson's Trichrome Staining

Myocardial infarct size was measured in order to determine the extent of IRI. 4 µm paraffin slides were conducted Masson's staining following the manufacturer's instructions (Solarbio, Beijing, China) to assess the myocardial infarct size. The extent of the necrotic area was imaged by an Olympus BX-51 microscope and measured by computerized planimetry (Image J 1.4; National Institutes of Health, Bethesda, MD, USA). Infarct size was expressed as the percentage of the total weight of the area at risk of left ventricle. The silk-like fibres indicated the early phase while the appearance of collagen deposition indicated the late phase of myocardial infarction. The muscle fibres appeared red and the collagen fibres appeared blue.

Western blotting

10 mg tissues were grinded in liquid nitrogen and added the 500 µl RIPA buffer (ThermoFisher) with protease inhibitor cocktail (Beyotime), and quantified the concentration using BCA methods. Aliquots of proteins (40 µg) were added into the lanes of 10% SDS polyacrylamide gel, and the proteins were

separated through electrophoresis and transferred onto nitrocellulose membranes. Subsequently, the membranes were congested with 5% nonfat dry milk in 0.01 M PBS buffer (pH 7.4) and 0.05% Tween-20 for 1 h at room temperature (RT). The blocked membranes were then incubated with primary antibodies of LC3 (1:2000), BECLIN1 (1:2000), ATG5 (1:2000), AMPK (1:2000), p-AMPK (1:500), mTOR (1:1000), p-mTOR (1:500) and GAPDH (1:10000) overnight at 4°C, followed by incubation with the appropriate secondary antibodies (horseradish peroxidase-conjugated rabbit anti-mouse diluted with 1: 10000 and donkey anti-rabbit diluted with 1: 5000) for 30 min at RT. The expression was determined by enhanced chemiluminescence method using Amersham Imager 600 system (GE Healthcare) whereas the density of the immunoblots was measured with Quantity One 4.62 software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Transmission electron microscopy

Immunoelectron microscopy was performed according to a previous protocol [13]. In brief, 6 µm sections were crosslinked within the following solution in order: 2.5% glutaraldehyde for 2 h, 2% agarose II for 1 h and 1% osmium tetroxide for 1 h all at RT. Following fixation, dehydration using an ethanol dilution series and infiltration in resin mixture of propylene oxide/Epon812, the sections were stained with lead citrate and uranyl acetate. Autophagosomes were identified by microscopic examination, which was performed using JEOL JEM1230 electron microscope as previously reported [14].

Statistical analysis

GraphPad Prism software 5.1 (GraphPad Software, Inc., La Jolla, CA, USA) was used to perform statistical analysis. The results of experimental data are expressed as the mean ± standard error of the mean. Student's t-test and One Way Anova were used for evaluating differences between two and multiple groups, respectively. For significant differences, Student-Newman-Keuls specific post hoc tests were performed for comparisons between treatment groups. P value less than 0.05 was considered to indicate a statistically significant difference.

Results

Characterization of the cardiac function of IRI rat treated by DMBG

Initially, STZ treatment significantly reduced body weight ratio (Fig. 1A), and inhibited insulin secretion (Fig. 1B), and increased diabetic parameters including the plasma glucose (Fig. 1C), HbA1c (Fig. 1D) in DM compared to NC group, which indicated a successful DM rat model.

Moreover, pathological evaluations showed that an obvious myocardial infarction appeared in DM + IRI compared to DM, but compromised upon DMBG treatment (Fig. 1E). Furthermore, the ratio of heart/body weights (Fig. 1F, G) were reduced in the DM + IRI group compared to DMBG treated. LVEDD and LVESD were remarkably increased while EF and FS were decreased in the DM + IRI group by echocardiography,

and DMBG compromised LVEDD and LVESD as well as enhanced EF and FS level, which indicated that DMBG could improve the systolic and diastolic cardiac function, and ameliorate the remodelling of left ventricle after IRI (Fig. 1H-K). Additionally, the presence of elevated LDH, CK, CK-MB and AST (Fig. 1L-O) were consistent with the outcome of echocardiography.

Taken together, DMBG was characterized as a phenotype that could efficiently improve the cardiac function of IRI.

The activated autophagy in the infarction and marginal zones upon DMBG treatment

Transmission electron microscopy was used to investigate the effects of DMBG on autophagic flux, which are generally formed in cells undergoing the autophagic process and constitute an indicator for analysis of the extent of autophagic induction. Quantitative analysis showed that the counting of mature autophagosomes in infarction (Fig. 2A) and marginal zones (Fig. 2B) in the DM, DM + IRI + DMBG and DM + IRI + I + DMBG groups were all higher than those in the DM + IRI group, which suggested that induction of IRI caused changes in the myocardium with myofibril disorganization, mitochondrial swelling, cellular lyses and a low number of autophagosomes (Fig. 2C). The structural changes observed in the IRI group were reversed in the DM + IRI + DMBG and DM + IRI + I + DMBG groups. These results indicated that DMBG treatment activates autophagy in the heart of diabetic rats.

The activated autophagy associated signalling pathway upon DMBG treatment

In order to confirm the effect of DMBG on autophagic flux, western blot analysis was performed to detect the protein expression of autophagy associated signalling pathways including AMPK and mTOR. The results showed that the expression levels of the autophagosome membrane-associated form LC3-II, ATG5 and BECLIN-1 were downregulated in the DM + IRI group compared with those in the pure type II DM rats. In contrast, the protein expression levels of LC3-II, ATG5 and BECLIN-1 were significantly increased following DMBG treatment ($p < 0.05$) compared with those in the DM and DM + IRI groups (Fig. 3). It was also found that DMBG activated the expression of p-AMPK and inhibited the expression of p-mTOR in the diabetic rats (Fig. 3) compared with expression in the DM and DM + IRI groups. This result indicated that DMBG can activate autophagy by regulating the mTOR/AMPK pathway, partially inhibiting IRI to subsequently decrease infarct size and protect heart cells in rats with DM.

Discussion

DMBG, as a kind of synthetic biguanide, is currently one of the most frequently recommended medications for type II diabetes treatment around the world via decreasing plasma triglycerides (TG) and low density lipoprotein (LDL) cholesterol levels as well as declining the systolic and diastolic blood pressure, and vasoprotective effects [2]. Numerous clinical experiments have been piloted to investigate

the effects of DMBG metabolism in diabetics, although these experiments have used dissimilar groups of individuals [15]. In order to investigate the hypothesis that DMBG can improve autophagic function and decrease infarct size in diabetes-induced IRI, the present study used a model of STZ-induced diabetes in rats. Infarct size was used to assess IRI, which increased significantly in DM rats. DMBG treatment decreased the infarct size in the heart of DM rats treated with IRI. Therefore, the results showed that treatment with DMBG could alleviate IRI in the STZ-induced diabetic rats. In order to further understand the mechanisms by which DMBG alleviates IRI, the present study examined the autophagic profile in different experimental subjects as previous studies have shown that DMBG possesses an autophagy-promoting function in other diseases, such as cystic echinococcosis [16], prion infection [17] and colon cancer [18]. These findings imply that autophagic dysfunction may be involved in the process of myocardial ischemia, which can be reversed by DMBG treatment.

To investigate the effect of DMBG on autophagy at the molecular level, we examined the expression of autophagy-related factors using western blot analysis. It was found that ATG5, BECLIN-1 and AMPK were upregulated, whereas the expression of mTOR was inhibited in the DM + IRI model. ATG5 has been previously reported to be closely related with the induction of autophagy in multiple types of cancer and cardiac diseases [19–21], which is consistent with our data. In addition, LC3, a particular indicator of autophagy in mammalian cells, has an autophagosome membrane-associated form LC3-II and cytoplasmic form (LC3-I). The ratio of LC3-II to LC3-I is a key biomarker of the formation of autophagosomes and activation of autophagy [22]. The digestion of sequestered substances in autophagosomes is initiated by the fusion of lysosomes with the outer membrane of the autophagosome [23]. Studies have shown that the recruitment of membranes for the formation of autophagosomes is investigated by BECLIN1, a downstream effector in the process of autophagy [24, 25]. Similarly, it has been found that DMBG can induce the phosphorylation of AMPK and inhibit the phosphorylation of mTOR [26], and mTOR kinase has been found to play an important role in several cancer- and metabolic disease-related pathways upon DMBG [27]. It has also been suggested that modulation of the mTOR signaling pathway can have a significant function in mediating the beneficial effects of DMBG.

Previous studies have revealed that DMBG can protect neurons [28], the kidneys [29] and liver [30] from IRI, although the mechanism has not been fully elucidated. The present study mainly focused on the effect of DMBG on diabetes combined with cardiac IRI, and investigated the underlying mechanism on the mTOR/AMPK pathway. The results revealed the role of autophagy in IRI and demonstrated the connection between DMBG and autophagy for a potential therapeutic strategy in clinical application.

Conclusions

The present showed that DMBG treatment induced the activation of autophagy function by activating AMPK, LC3-I, LC3-II, ATG5 and BECLIN-1, and inhibiting mTOR in the heart of rats with DM. Therefore, DMBG activates autophagy and decreases infarct size in the heart by regulating the mTOR/AMPK pathway and may offer a promising strategy for treating IRI in diabetes. Additionally, based on the

present study, the glucose-dependent therapeutic strategy may be introduced to investigate the myoprotective effects on IRI in patients with diabetes in the future.

Abbreviations

IRI	ischemia-reperfusion injury
DM	diabetes mellitus
ATG5	autophagy related 5
IHD	ischemic heart disease
AMI	acute myocardial infarction
GLUT4	glucose transporters 4
AMPK	AMP-activated protein kinase
STZ	Streptozocin

Declarations

Acknowledgement

Not applicable.

Authors’ contributions

LZ participated in the design of the study, conducted the experiments and drafted the manuscript. XZ, LG and JG collected and analyzed the data. DZ designed the study, revised the manuscript and is responsible for authenticity of data. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The study was approved by the Animal Care and Use Committee of Zhongshan Hospital (Shanghai, China).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Characterization of IRI rat model treated with DMBG. The heart weight (A), insulin secretion (B), levels of plasma glucose (C), HbA1c (D), body weight (F), ratio of heart/body weight (G), LVEDD (H), LVESD (I), EF (J), FS (K), LDH (L), CK (M), CK-MB (N) and AST (O) in different groups of rats. Masson's staining for pathological verification of myocardial infarction with 10 × 20 magnification (E). All data are presented as the mean ± standard error of the mean of five individual experiments. *p<0.05, vs. DM group, #p<0.05, vs. DM + IRI group.

vs. DM + IRI group. NC, negative control; DM, diabetes mellitus; IRI, ischemia-reperfusion injury; DMBG, metformin; I, insulin.

Figure 2

The distribution of autophagosomes in infarction and marginal zones. The profiles and the analysis (C) of autophagosomes in infarction (A) and marginal zones (B) of IRI rat model treated with DMBG. Scale bar=0.5 μ m. Regions of infarction are outlined by a red frame. Autophagosomes are indicated by red arrows, which were counted in at least ten different fields. * $p<0.05$, vs. DM group, # $p<0.05$, vs. DM + IRI group. NC, negative control; DM, diabetes mellitus; IRI, ischemia-reperfusion injury; DMBG, metformin; I, insulin.

Figure 3

The activation of mTOR and AMPK signalling pathways upon DMBG. Protein levels of LC3-I, LC3-II, BECLIN-1 and ATG5, and the ratios of p-mTOR/mTOR and p-AMPK/AMPK (top) and the statistical analysis (below). All data are presented as the mean $\pm$ standard error of the mean of three individual experiments. * $p < 0.05$, vs. DM group, # $p < 0.05$, vs. DM + IRI group. NC, negative control; DM, diabetes mellitus; IRI, ischemia-reperfusion injury; DMBG, metformin; I, insulin.

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