AMPK/mTOR-mediated therapeutic effect of metformin on myocardial ischaemia 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 ischaemia–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 ischaemia 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 1 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 determinate a novel mechanism that DMBG could activate autophagy process to provide a cardio-protective effect against DM induced myocardial IRI.

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Introduction

The presence of diabetes mellitus (DM) patients with higher incidence and fatality on ischaemic 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 ischaemia, but also weakens the efficiency of cardioprotective intervention including ischaemic 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 are used to suggest that DM amplifies heart sensitivity to ischaemia–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 scrutinised, 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 mammalian target of rapamycin (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
Streptozocin (STZ), DMBG and insulin were purchased from Sigma-Aldrich (Billerica, MA). ELISA kit for rat insulin assay was purchased from Elabsience (Wuhan, China). The primary antibodies against ATG5 (Cat. no. 12994, CST, Beverly, MA), BECLIN1 (Cat. no. 3495 T, CST), microtubule-associated protein 1 light chain 3 (LC3) (Cat. no. 4108, CST), 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) 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 Shanghai Animal Care and Use Committee of Zhongshan Hospital, and all animal protocols were executed following the guidelines accordingly. All rats were housed in a controlled environment of 53% humidity, (23 ± 2°C) in a 12/12h 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 levels of glycated haemoglobin a1 (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). The serum insulin was detected using ELISA kit followed by manufacturers’ instructions. Echocardiography was conducted as the described below.

For establishment of DM model rats, 65 mg/kg/day STZ dissolved in saline 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 development of diabetes was verified by measurement of the blood glucose level, which was performed using a monitoring system and test strips (AccuChek Advantage; Roche Diagnostics, Pompano Beach, FL) by sampling of blood with a small scratch on the rat tail. 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.

For myocardial IRI model preparation, the male SD rats were anaesthetised by 30 mg/kg ketamine and conducted the IRI surgery as described previously [11]. Following 1h transitory ligation of the left anterior coronary artery, the hearts were subjected to 3h of reperfusion. The visualisation of the colour return in the formerly pale area was used to indicate the IRI and immediately recorded the electrocardiographic and echocardiographic variations. The SD rats were administered 30 mg/kg/day DMBG intravenously or 1IU/kg/day insulin subcutaneously for 5 days. Five groups of rats (10 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) 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 computerised planimetry (Image J 1.4; National Institutes of Health, Bethesda, MD). 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**

About 10 mg tissues were grinded in liquid nitrogen and added the 500 μl RIPA buffer (ThermoFisher) with protease inhibitor cocktail (Beyotime, Jiangsu, China), and quantified the concentration using bicinchoninic acid (BCA) methods. Aliquots of proteins (40 μg) were added into the lanes of 10% sodium dodecyl sulphate (SDS) polyacrylamide gel, and the proteins were separated through electrophoresis and transferred onto nitrocellulose membranes. Subsequently, the membranes 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) 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**

**Characterisation of the cardiac function of IRI rat treated by DMBG**

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

Moreover, DMBG was used to treat with DM + IRI model. To consider about the impacts derived from DM, insulin combined with DMBG was also examined. The pathological evaluations showed that an obvious myocardial infarction appeared in DM + IRI compared to DM, but compromised upon DMBG treatment (Figure 1(E)). Furthermore, LVEDD and LVESD were remarkably increased while EF and FS were decreased using Amersham Imager 600 system (GE Healthcare, Chicago, IL) whereas the density of the immunoblots was measured with Quantity One 4.62 software (Bio-Rad Laboratories, Inc., Hercules, CA).
in the DM + IRI group by echocardiography (Figure 1(F, G)), and DMBG compromised LVEDD and LVESD as well as enhanced EF and FS level (Figure 1(H, I)), which indicated that DMBG could improve the systolic and diastolic cardiac function, and ameliorate the remodelling of left ventricle after IRI. Additionally, the presence of elevated LDH, CK, CK-MB and AST (Figure 1(J–M)) were consistent with the outcome of echocardiography.

Taken together, DMBG was characterised 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 (Figure 2(A)) and marginal zones (Figure 2(B)) 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 disorganisation, mitochondrial swelling, cellular lyses and a low number of autophagosomes (Figure 2(C)). 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 (Figure 3). It was also found that DMBG stimulated the phosphorylation of AMPK and inhibited the phosphorylation of mTOR in

Figure 1. Characterisation of IRI rat model treated with DMBG. The ratio of heart/body weight (A), insulin secretion (B), levels of plasma glucose (C), HbA1c (D), LVEDD (F), LVESD (G), EF (H), FS (I), LDH (J), CK (K), CK-MB (L) and AST (M) in different groups of rats. Masson’s staining for pathological verification of myocardial infarction with 10 x 20 magnification (E). All data are presented as the mean ± standard error of the mean of five individual experiments. *p < 0.05, versus NC group, †p < 0.05, versus DM + IRI group. NC: negative control; DM: diabetes mellitus; IRI: ischaemia-reperfusion injury; DMBG: metformin; I: insulin.
the diabetic rats (Figure 3) compared with expression in the DM and DM + IRI groups. This result indicated that DMBG can activate autophagy associating with 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 ischaemia, 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 signalling 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. Nevertheless, DMBG has been shown to exert protective effect on the ischaemic heart through not only autophagy but also various other mechanisms, including metabolism effect [31], eNOS activation [32], modulation of cardiac fibroblast activity [33]. The complicated coordination of multiple signalling pathways and regulators triggered by DMBG may also contribute to cardiac function recovery and maintenance.

Overall, the outcomes determinate a novel mechanism that DMBG could activate autophagy process to provide a cardio-protective effect against DM induced myocardial IRI showed that DMBG-induced autophagy observed in the heart of rats with DM after IRI was associated with an activation of AMPK and an inhibition of mTOR. 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.
Geolocation information

The experiments were made in four Central Laboratory of Zhongshan Hospital. The study was approved by the Animal Care and Use Committee of Zhongshan Hospital (Shanghai, China).

Disclosure statement

The authors declare that they have no competing interests.

Author contributions

L. Z. participated in the design of the study, conducted the experiments and drafted the manuscript. X. Z., L. G. and J. G. collected and analysed the data. D. Z. 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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Data availability statement

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

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