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Metformin protects the myocardium against isoproterenol–induced injury in rats through alleviating endoplasmic reticulum stress

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Clinical studies have suggested that metformin, a widely used antidiabetic agent, exerts a direct cardioprotective effect on cardiovascular disease in addition to its blood glucose-lowering activity. This study was designed to identify the role of metformin in rats with isoproterenol (ISO)-induced myocardial injury and to investigate its underlying mechanism. A rat model of myocardial ischemic injury was established by the subcutaneous injection of a high dose of ISO, a β-adrenergic agonist. The results showed that pretreatment of metformin significantly reduced rat mortality induced by ISO, attenuated the increased plasma lactate dehydrogenase activity and myocardium malondialdehyde level, alleviated the hemodynamic disturbance, inhibited the upregulated gene expression of myocardial probrain natriuretic peptide and alleviated the myocardial morphological injury and apoptosis induced by ISO. Furthermore, western blot analysis showed that metformin suppressed the overexpression of the endoplasmic reticulum stress (ERS) markers cleaved caspase-12 and CEBP-homologous protein induced by ISO and increased the phosphorylation of AMP-activated protein kinase (AMPK). In conclusion, these data suggest that metformin might protect the myocardium against acute ischemic injury in rats at least partially by activating AMPK and alleviating aberrant ERS. These findings might provide further experimental evidence for treating patients at risk of ischemic heart disease with metformin.

1. Introduction

Traditionally, the use of metformin, a first-line antidiabetic agent, was debated in treating patients with acute myocardial infarction or heart failure (HF), whereas recent clinical studies have suggested that metformin can benefit the cardiovascular system independent of its blood glucose-lowering activity. In a retrospective cohort study of 16,417 older patients with type 2 diabetes mellitus (T2DM) and HF, the patients treated with metformin had a lower risk of readmission and a reduction in mortality due to HF compared with patients treated with sulfonylureas, insulin or thiazolidinedione (Masoudi et al. 2005). Furthermore, metformin treatment reduced the incidence of nonfatal cardiovascular events (myocardial infarction and stroke) compared with chronic insulin therapy in the second Diabetes and Insulin–Glucose infusion in Acute Myocardial Infarction trial (DIGAMI 2) (Mellbin et al. 2011). These findings suggest that metformin might directly exert protective effects on the heart in addition to its hypoglycemic effect. Therefore, exploring the cardioprotective effect of metformin and its underlying mechanism has become a popular research topic.

Increasing evidence has revealed that aberrant endoplasmic reticulum stress (ERS) is an important mechanism in the pathogenesis of various cardiovascular diseases, including ischemic/reperfusion (I/R) injury (Liu et al. 2008), ischemic heart disease (Azfer et al. 2006; Xin et al. 2011), HF (George et al. 2011), cardiomyopathy (Hamada et al. 2004) and the development of atherosclerosis (Gora et al. 2010). The imbalance of endoplasmic reticulum (ER) homeostasis is known as ERS, which results from various pathophysiological stimuli, such as ischemia, hypoxia, glucose deprivation, cellular redox alteration, Ca^{2+} overload and viral infection (Kaufman 2002). In the early stages of ERS, the unfolded protein response (UPR), specific signaling pathways of ERS, is activated to restore ER homeostasis and promote cell survival. However, prolonged and severe ERS (aberrant ERS) evokes ER-specific apoptotic molecules, including C/EBP homologous protein (CHOP), caspase-12 and c-Jun N-terminal kinase (JNK) (Boyce and Yuan 2006), ultimately leading to cell apoptosis. Our previous work affirmed that the inhibition of ERS could protect rat hearts against I/R injury (Zhang et al. 2009). Therefore, inhibiting or regulating the molecular mechanisms of ERS may provide a therapeutic target for cardiovascular disease.

Dong et al. (2010) reported that AMP-activated protein kinase (AMPK), an important regulator of cellular function in the cardiovascular system, acts as a physiological suppressor of ERS.

Table 1: Effect of metformin on cardiac function in ISO rat hearts

Group	HR (beats/min)	MAP (mmHg)	LVSP (mmHg)	LVEDP (mmHg)	+LV$dp/dt_{max}$ (mmHg/s)	−LV$dp/dt_{max}$ (mmHg/s)
Con (n = 6)	400 ± 10	93 ± 4	123.8 ± 2.7	1.9 ± 0.9	6754 ± 141	4719 ± 175
ISO(n = 6)	447 ± 13*	89 ± 3	118.6 ± 2.6	7.3 ± 1.5**	5497 ± 271**	3563 ± 249**
ISO + Met (n = 6)	428 ± 12	91 ± 4	123.4 ± 4.4	2.0 ± 0.3##	6376 ± 179##	4457 ± 134##

Data are presented as means ± S.E.M. of each group. Con, control; ISO, isoproterenol; Met, metformin; HR, heart rate; MAP, mean arterial blood pressure; LVSP, Left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; +/-LV$dp/dt_{max}$, maximal rate of increase and decrease of left ventricular pressure. * $P < 0.05$ *vs*. Con group; ** $P < 0.01$ *vs*. Con group; ## $P < 0.01$ *vs*. ISO group.

Metformin could activate AMPK in the cardiovascular system (Zou and Wu 2008; Davis et al. 2006). Therefore, we hypothesized that metformin may play a cardioprotective role *via* the inhibition of ERS. We studied the effect of metformin on myocardial ERS in an acute ischemic heart model induced by isoproterenol (ISO) *in vivo*, and found that metformin exerts cardioprotection against ischemic injury induced by ISO, at least partially, by activating AMPK and alleviating aberrant ERS.

2. Investigations and results

2.1. Effect of metformin on the mortality rate in the ISO-treated rats

We first evaluated the effect of metformin on the mortality rate induced by ISO. Our results showed all rats in the Control (Con) group and the ISO plus Met group remained alive, whereas 2 of 9 rats in the ISO group died during the experimental period. The mortality rate was approximately 22.22% in the ISO group compared with the control and ISO plus Met groups ($P < 0.05$). Pretreatment with metformin prevented rat death induced by ISO.

2.2. Effect of metformin on the ISO-induced cardiac dysfunction

As shown in Table 1, no differences were observed in MAP and left ventricular systolic pressure (LVSP) between the 3 groups. However, HR and LVEDP were significantly increased ($P < 0.05$ *vs*. Con; $P < 0.01$ *vs*. Con, respectively), and +/-LV$dp/dt_{max}$ was decreased in the ISO group compared with the Con group ($P < 0.01$ *vs*. Con, Table 1). Interestingly, the metformin pretreatment ameliorated the cardiac dysfunction significantly, with an increased +/-LV$dp/dt_{max}$ ($P < 0.01$ *vs*. ISO) and decreased LVEDP ($P < 0.01$ *vs*. ISO) (Table 1). The level of myocardial proBNP mRNA was elevated in the ISO group compared with that in the Con group ($P < 0.05$ *vs*. Con, Fig. 1). In contrast, metformin significantly depressed the expression of proBNP mRNA induced by ISO compared with that in the ISO group ($P < 0.05$ *vs*. ISO, Fig. 1).

2.3. Effect of metformin on ISO-induced myocardial injury and apoptosis

Our results showed that ISO could greatly increase the plasma LDH activity and the amount of MDA in the myocardium compared with those in the Con group myocardium ($P < 0.01$ *vs*. Con, Table 2). In contrast, metformin reversed the increase in the plasma LDH activity and MDA content in the myocardium induced by ISO ($P < 0.05$ *vs*. ISO; $P < 0.01$ *vs*. ISO, respectively, Table 2). Moreover, the histological sections of ISO-treated hearts showed widespread myocardial structural disorder and subendocardial necrosis, with capillary dilatation and leukocytic infiltration compared with the Con group. In the ISO plus Met group, the ISO-induced subendocardial necrosis, capillary dilatation and leukocytic infiltration were significantly improved (Fig. 2A-C).

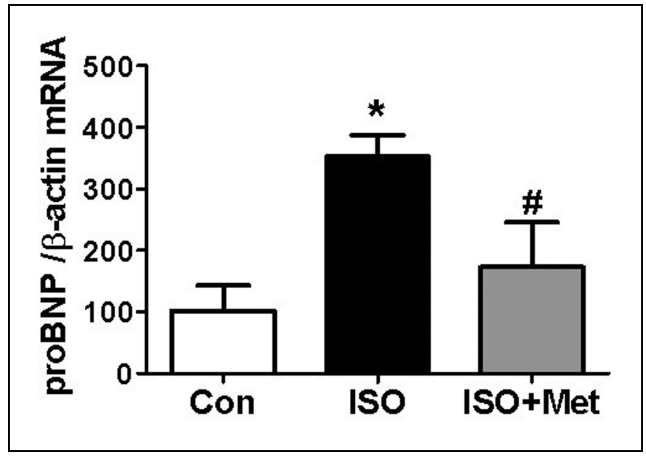

Fig. 1: Metformin decreases the level of proBNP mRNA in myocardium induced by ISO. The level of proBNP mRNA in myocardium of three groups was detected by quantitative real-time PCR and results are relative to β-actin level. Values represent mean ± S.E.M. of four animals in each group. ** $P < 0.01$ *vs*. Con; ## $P < 0.01$ *vs*. ISO (Con, control; ISO, isoproterenol; Met, metformin).

TUNEL staining was used to detect myocardial apoptotic cells in ISO-induced myocardium. The nuclei of the normal cells were stained blue, and TUNEL-positive nuclei were stained brown-yellow (Fig. 3A–C). As illustrated in Fig. 3D, the apoptosis rate of the myocardium was significantly increased in the ISO group compared with the Con group ($P < 0.01$ *vs*. Con). However, in the metformin-pretreated myocardium, the number of apoptotic cells was significantly reduced compared with the myocardium from the rats administered ISO alone ($P < 0.01$ *vs*. ISO, Fig. 3D).

2.4. Effect of metformin on myocardial ERS in ISO-treated hearts

Western blot analysis showed that the ISO treatment dramatically upregulated the cleaved caspase-12 and CHOP protein levels ($P < 0.01$ *vs*. Con, Fig. 4). However, metformin pretreatment significantly decreased the protein levels of cleaved caspase-12 and CHOP compared with the ISO group ($P < 0.01$ *vs*. ISO, Fig. 4).

2.5. Effect of metformin on the expression of phospho-AMPK in ISO-treated hearts

As shown in Fig. 5, metformin pretreatment elevated the expression of p-AMPK protein ($P < 0.01$ *vs*. Con; $P < 0.01$ *vs*. ISO, respectively, Fig. 5), which suggests that AMPK may be involved in the ERS-inhibiting effect of metformin.

3. Discussion

Metformin, a first-line drug in the treatment of T2DM, has been recently considered to provide potential cardiovascular protec-

Table 2: Alleviation of ISO-induced heart injury by metformin

Group	LDH activity in plasma (U/L)	MDA content in myocardium (nmol/mg protein)
Con (n = 6)	355.90 ± 43.67	3.83 ± 0.69
ISO (n = 6)	1039.00 ± 225.20**	10.4 ± 2.13**
ISO + Met (n = 6)	481.00 ± 79.92$^{\#}$	4.53 ± 0.70$^{\#\#}$

Data are presented as means ± S.E.M. of each group. Activity of LDH was measured in plasma, whereas MDA was assayed in myocardial tissues. Con, control; ISO, isoproterenol; Met, metformin; LDH, lactate dehydrogenase; MDA, malondialdehyde. $^{**}P < 0.01$ *vs.* Con group; $^{\#}P < 0.05$ *vs.* ISO group; $^{\#\#}P < 0.01$ *vs.* ISO group.

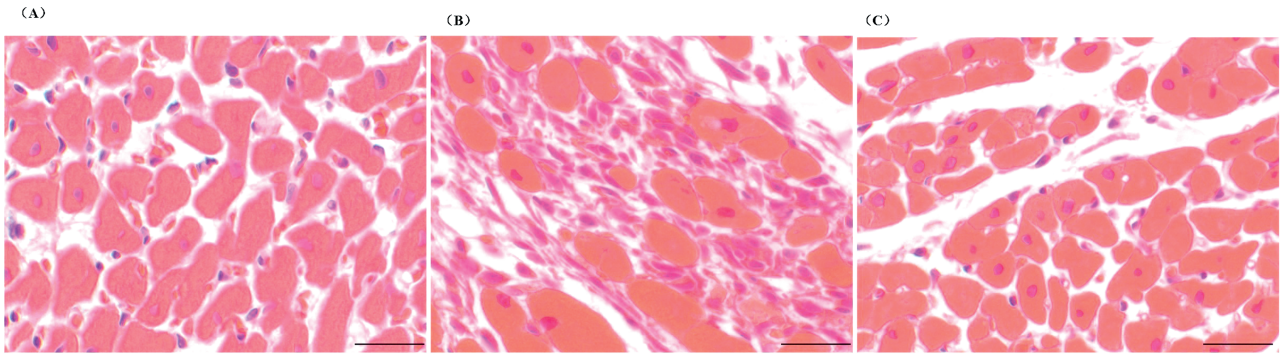

Fig. 2: Metformin ameliorates ISO-induced myocardial injury (hematoxylin–eosin stain, all sections: × 400). (A) myocardium in Con group; (B) myocardium in ISO group; (C) myocardium in ISO + Met group. The bar represents 25 μm (Con, control; ISO, isoproterenol; Met, metformin).

tion independent of its blood glucose-lowering activity. In rodent and canine models of HF, chronic metformin therapy led to significant improvements in cardiac function and remodeling (Gundewar et al. 2009; Yin et al. 2011; Sasaki et al. 2009). In isolated perfused rat hearts, a single dose of metformin 24 h before coronary occlusion significantly reduced the myocardial infarct size (Solskov et al. 2008). However, the underlying mechanism of its cardioprotective effect is unclear and must be further elucidated.

Overdose of ISO, a β-adrenergic agonist and well-known inducer of myocardial injury (Rona 1985), leads to myocardial infarction-like pathological changes and congestive heart failure through catecholamine excess, intracellular Ca^{2+} overload (Singal et al. 1983), inflammatory reactions and oxidative stress (Rona 1985). These mechanisms are important factors related to myocardial injury in ischemic heart disease. We selected this model to confirm the cardiovascular protection of metformin. Consistent with our previous reports (Jiang et al. 2005; Chang

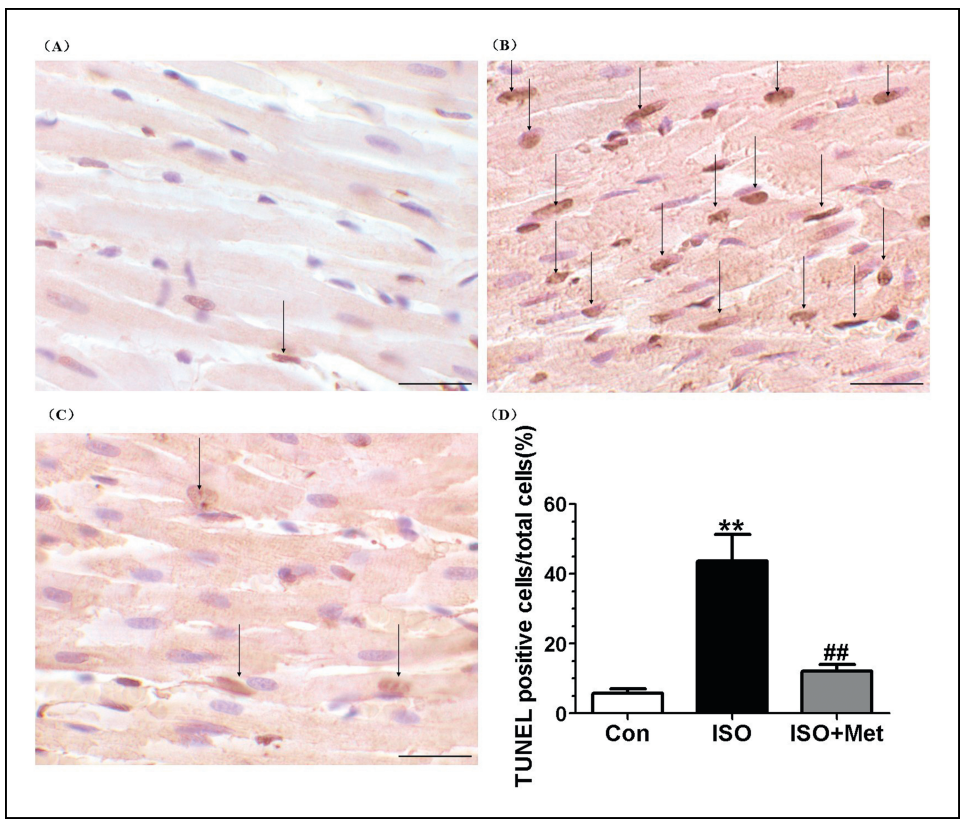

Fig. 3: Metforminin attenuates ISO-induced myocardial apoptosis. (A) myocardium in Con group; (B) myocardium in ISO group; (C) myocardium in ISO+ Met group. Arrows indicate apoptotic nuclei. Original magnification × 400. (D) TUNEL-positive cells were counted in a total of more than 300 myocytes over 3 random fields and expressed as percentage of the total number of nuclei. Data are expressed as mean ± S.E.M. of three independent experiments. $^{**}P < 0.01$ *vs.* Con group; $^{\#\#}P < 0.01$ *vs.* ISO group. The bar represents 25 μm (Con, control; ISO, isoproterenol; Met, metformin).

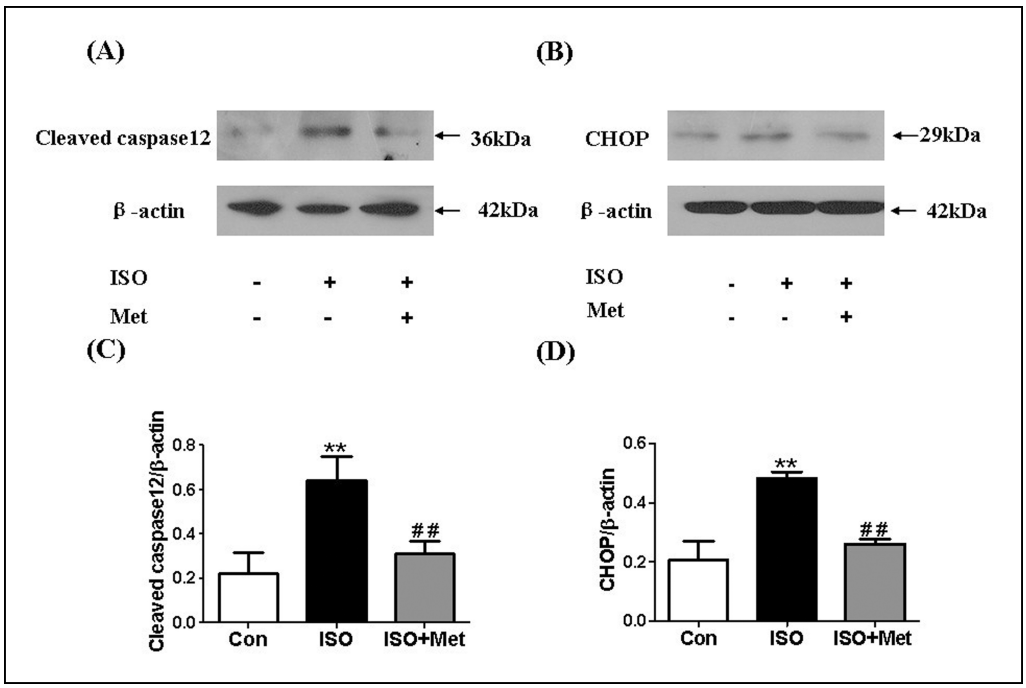

Fig. 4: Metformin inhibites myocardial endoplasmic reticulum stress induced by ISO *in vivo*. Representative protein expression of cleaved caspase-12, CHOP and β-actin(A, B). Quantitative analysis of protein expression of cleaved caspase-12 and CHOP(C, D). Values represent mean ± S.E.M. of three animals in each group. $^{**}P < 0.01$ *vs*. Con; $^{\#\#}P < 0.01$ *vs*. ISO (Con, control; ISO, isoproterenol; Met, metformin).

et al. 2004; Jia et al. 2006), the cardiac function in ISO group was inhibited with LVEDP increasing, and +/-LV$dp/dt_{max}$ declining. As BNP is a powerful marker of ventricular dysfunction (Eindhoven et al. 2012), we detected the level of myocardia proBNP mRNA to assess the severity of HF. We found that the levels of myocardia proBNP mRNA in ISO group significantly increased, which indicated that high doses of ISO induced the severe HF of rat. In the present study, metformin pretreatment obviously improved cardiac function induced by ISO through increasing +/-LV$dp/dt_{max}$, and decreasing LVEDP and the level of myocardia proBNP mRNA. These results suggested that metformin could ameliorate the ISO-induced cardiac dysfunction, which is consistent with previous report by Soraya's lab (Soraya et al. 2012a; Soraya et al. 2012b).

In the ISO group, the increase of LDH activity in plasma indicated the leakage of myocardial intracellular enzymes, and the increase of myocardial MDA content indicated excessive generation of lipid peroxide product. HE staining and TUNEL staining of ISO-treated myocardia showed high doses of ISO induced severe myocardial injury, necrosis and apoptosis. In the present study, metformin pretreatment significantly reduced the level of plasma LDH activity and content of myocardial MDA. Moreover, metformin obviously alleviated myocardial morphological injury and diminished the number of apoptotic cells of the ISO-induced rats. Therefore, the result showed that metformin may protect the heart against ISO-induced ischemic injury *in vivo*, indicating metformin might have potential as a cardioprotective drug.

The endoplasmatic reticulum (ER) is an intracellular organelle that plays an important role in the processing, folding and exporting of newly synthesized proteins, the regulation of Ca^{2+} fluxes and the production of lipids. A variety of insults can interfere with the ER, leading to the accumulation of unfolded and misfolded proteins in the ER. The unfolded protein response (UPR) is activated to restore ER homeostasis by enhancing protein degradation, reducing protein synthesis and expanding the protein folding capacity (Geng et al. 2004; Schröder et al. 2005). An early or initial response of the ERS protects cells against stress or damage, but when ERS is prolonged or overwhelming, ERS-initiated apoptotic signals (including CHOP, JNK and caspase-12) are induced. ERS is an important mechanism in the development of various cardiovascular diseases, such as ischemic heart disease (Azfer et al. 2006; Xin et al. 2011), HF (George et al. 2011). Therefore, inhibiting or regulating ERS has become a target for the prevention and therapy of cardiovascular diseases.

Interestingly, we found that ISO provoked a notable increase in the protein expression of the ERS-mediated apoptosis markers,

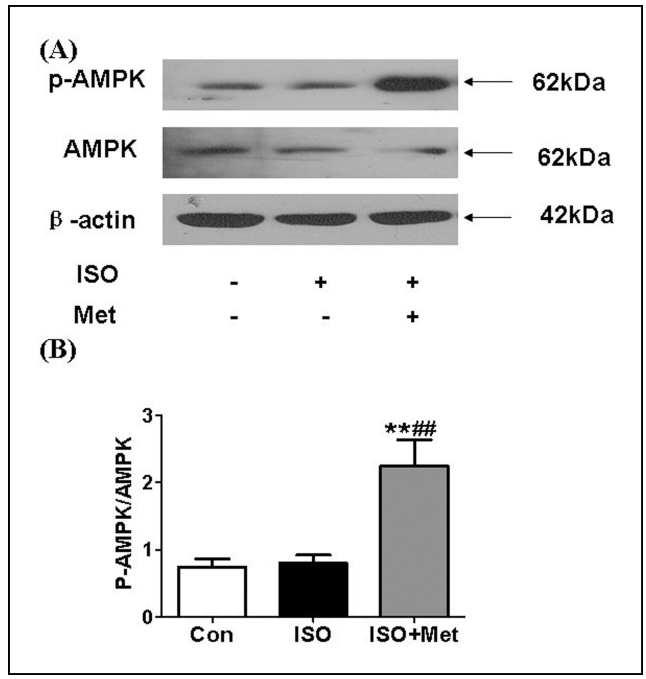

Fig. 5: Metformin increases the phosphorylation of myocardial AMPK in ISO-treated rat hearts. Representative protein expression of p-AMPK, AMPK and β-actin (A) and quantitative analysis of protein expression of p-AMPK (B). Values represent mean ± S.E.M. of three animals in each group. $^{**}P < 0.01$ *vs*. Con; $^{\#\#}P < 0.01$ *vs*. ISO (Con, control; ISO, isoproterenol; Met, metformin).

cleaved caspase-12 and CHOP in the myocardium, indicating that aberrant ERS occurs during the process of ISO-induced myocardial injury. Kim et al. (2010) reported that metformin regulated palmitate-induced apoptosis and ERS by blocking the induction of ERS proteins (GRP78, CHOP, cleaved ATF-6, p-eIF2α and XBP-1) in HepG2 liver cells. In the present study, we found that metformin significantly attenuated the overexpression of cleaved caspase-12 and CHOP in the ISO-treated myocardium. This result demonstrated that metformin could inhibit the aberrant myocardial ERS in ischemic heart injury *in vivo*. Our work suggested that inhibiting the aberrant myocardial ERS contributed to the pleiotropic cardioprotective effect of metformin.

Accumulating studies have indicated that the cardioprotective effect of metformin is partially mediated by the activation of AMPK, an important regulator of energy balance and cellular growth (Gundewar et al. 2009; Yin et al. 2011; Sasaki et al. 2009; Solskov et al. 2008). AMPK is considered as a physiological suppressor of ERS (Dong et al. 2010), and the activation of AMPK protects against cardiomyocyte injury and apoptosis (Yeh et al. 2010; Terai et al. 2005) by elevating eIF-2α and eEF-2 phosphorylation to alleviate the ERS. Moreover, it was reported that the activation of AMPK by metformin inhibited the cardiomyocyte ERS caused by cardioplegia-induced hypoxia/reoxygenation injury *in vitro* (Yeh et al. 2010). Notably, studies from our laboratory have shown that metformin pretreatment significantly increased the phosphorylation of AMPK in ISO-treated hearts *in vivo*, which suggested that the activation of AMPK participates in the metformin-based inhibition of ERS *in vivo*.

In addition to the activation of AMPK, other intracellular signal transduction pathways might be involved in the regulation of aberrant ERS by metformin. In isolated perfused diabetic and non-diabetic rat hearts, the administration of metformin at reperfusion reduced the infarct size by rapidly activating the PI3K/Akt pathway to prevent mitochondrial permeability transition pore opening (Bhamra et al. 2008). Our previous studies have also shown that the PI3K/Akt signaling pathway is involved in inhibiting myocardial ERS (Teng et al. 2011). Therefore, the molecular mechanisms of the metformin-inhibiting effect on ERS require further confirmation.

In summary, this study demonstrated that metformin pretreatment reduced the mortality, attenuated the cardiac dysfunction and ameliorated the tissue damage and myocardial apoptosis caused by ISO, suggesting that metformin protects the myocardium against ISO–induced injury in rats. More importantly, the cardioprotective mechanism of metformin might at least partially be attributed to the activation of AMPK and inhibition of the aberrant ERS. These findings might provide further experimental evidence for treating patients at risk of ischemic heart disease.

4. Experimental

4.1. Materials and reagents

Male Sprague–Dawley (SD) rats (250 ± 10 g) were obtained from the Animal Center, Health Science Center, Peking University (Beijing, China). All animal care and experimental protocols complied with the Animal Management Rule of the Ministry of Health, People's Republic of China (Documentation no. 55, 2001) and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication no. 85–23, revised 1996) and were approved by the Animal Care Committee of Health Science Center, Peking University. Metformin was obtained from Sigma Co. (St. Louis, MO, USA). Antibodies against AMPKα, phosphorylated AMPKα (Thr172) (p-AMPK) were obtained from Cell Signaling Technology (Danvers, MA), antibodies against caspase-12 and CHOP were from Abcam PLC (Cambridge, UK), and the anti-beta-actin antibody and all secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The enhanced chemiluminescence (ECL) kit was from Applygen Technologies (Beijing, China). The nitrocellulose membrane was from Hybond-C (Amersham Life Science, UK). Trizol was purchased from GIBCO (BRL, Rockville, MD, USA), and the dNTPs, M-MuLV RT, oligo$(dT)_{15}$ primer and Taq DNA polymerase were purchased from Promega (Madison, WI, USA). Other chemicals and reagents were of analytical grade.

4.2. Experimental protocol for treating rats with drugs

The ISO-induced myocardial injury model was produced as described previously (Jiang et al. 2005). A total of 27 male SD rats were randomly divided into 3 groups: (1) control (Con, n = 9), in which the rats were subcutaneously (s.c.) injected with saline (2 mL/kg/day, once a day) for 2 days; (2) ISO (n = 9), in which the rats subcutaneously (s.c.) received ISO (20 mg/kg/d dissolved in the same volume of saline as in the control group, once a day) for 2 days; and (3) ISO plus metformin (ISO plus Met, n = 9), in which the rats were pretreated with metformin (250 mg/kg/d, s.c. injection every 12 h) 2 days before the injection of ISO for a total of 4 days, with the administration of ISO being the same as that in the ISO group.

All rats were housed under standard conditions and had free access to water until the end of the experiment. All rats were provided standard rodent chow and fasted overnight after the last injection of the drugs. The rats were anesthetized with urethane (1 g/kg, intraperitoneally), and a catheter filled with heparin saline (500 U/mL) was inserted into the right common carotid artery and then was inserted into the left ventricle to measure mean arterial blood pressure (MAP) and left ventricular pressure. The heart rate (HR), MAP, maximal rates of the increase and decrease of left-ventricular pressure (+/-LV$dp/dt_{max}$), left ventricular systolic pressure (LVSP) and left ventricular end-diastolic pressure (LVEDP) were recorded by a Power Lab system (BL-420F, TaiMeng, Chengdu, China). After the hemodynamic parameters were measured, a blood sample was collected in a heparinized syringe from the left ventricle and transferred to a tube. Plasma was obtained by centrifugation at 3000 revolutions per minute (rpm) for 10 min at 4 °C and stored at − 70 °C to determine lactate dehydrogenase (LDH) activity. All animals were killed by exsanguination, and the hearts were quickly removed.

4.3. Real-time PCR analysis

The total RNA from the left-ventricular myocardium (approximately 50 mg) was extracted with use of the Trizol reagent and reverse transcribed by the use of a reverse transcription system (Promega, Madison, WI, USA). Then, 1 μL of the reaction mixture was used for real-time PCR. The amount of PCR product formed in each cycle was evaluated by SYBR Green I fluorescence. The forward and reverse PCR primers (rat) of probrain natriuretic peptide (proBNP) were proBNP-S, 5′-CTGTGA CGG GCT GAG GTT GT-3′, and proBNP-A, 5′-TGG CAA GTT TGT GCT GGA AG-3′. The control primers were β-actin-S, 5′-GAG ACC TTC AAC ACC CCA GCC-3′, and β-actin-A, 5′-TCG GGG CAT CGG AAC CGC TCA-3′ (AuGCT Biotechnology, Beijing, China). All amplification reactions involved the use of the Mx3000 Multiplex Quantitative PCR System (Stratagene, La Jolla, CA, USA).

4.4. Assay for plasma lactate dehydrogenase (LDH) activity and malondialdehyde (MDA) content

The measurement of the plasma LDH activity was performed as previously described (Song et al. 2009). The content of the lipid peroxidation product MDA in the supernatant extracted from the myocardia was determined by the thiobarbituric acid test (Geng et al. 2004).

4.5. Hematoxylin–eosin staining and apoptosis detection

The hearts were isolated, and cardiac samples were harvested, fixed in 4% paraformaldehyde, and then embedded in paraffin. The embedded hearts were cut into 5-mm sections, stained with hematoxylin–eosin and then examined by light microscopy. Apoptotic cells in the paraffin sections were identified by the TUNEL technique following the manufacturer's instructions (in situ cell death detection kit, Roche Applied Sciences, Shanghai, China). The sections were then counterstained with hematoxylin, and the labeled myocytes were analyzed by light microscopy.

4.6. Western blot analysis

Protein extracts from the myocardia were resuspended in sample buffer containing 2% SDS, 2% β-mercaptoethanol, 50 mmol/L Tris–HCl (pH 6.8), 10% glycerol and 0.05% bromophenol blue. The protein mixture was then placed in boiling water for 10 min and briefly centrifuged at low speed to collect the denatured proteins. The protein samples were resolved on a 10% (for p-AMPKα, AMPKα, cleaved caspase-12, CHOP and β-actin) Tris/glycine SDS–polyacrylamide gel in running buffer containing 25 mmol/L Tris, 192 mmol/L glycine and 0.1% SDS. The proteins were then transferred to a nitrocellulose membrane for 2 h at 4 °C at 200 mA with a transfer buffer

containing 20 mmol/L Tris–HCl (pH 8.0), 150 mmol/L glycine and 20% methanol. Non-specific proteins were blocked by incubating the membrane with 5% non-fat dry milk in TBS-T (20 mmol/L Tris–HCl [pH 7.6], 150 mmol/L NaCl and 0.02% Tween 20) for 1 h at room temperature with agitation. Then, the nitrocellulose membrane was incubated with the primary antibodies anti-beta-actin (1:3000 diluted in TBS-T), anti-AMPKα (1:1000), anti-p-AMPKα (1:1000), anti-CHOP (1:500) or anti-caspase-12 (1:500) overnight at 4–8 °C. After being washed 3 times for 10 min each in TBST, the membrane was incubated with the secondary antibody (horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG) for 1 h at room temperature. The reaction was visualized by ECL, and the films were scanned and analyzed with the NIH imaging software. The protein content was normalized to that of β-actin. All experiments were repeated 3 times.

4.7. Statistical analysis

The data are expressed as the mean ± standard error of the mean (S.E.M.). One-way ANOVA was used to compare more than two groups, and when significant ($P < 0.05$), the Newman-Keuls test was applied to test for differences between individual groups. A $P < 0.05$ was considered statistically significant.

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