Scientific Research and Essays Vol. 6(8), pp. 1865-1872, 18 April, 2011
Available online at http://www.academicjournals.org/SRE
DOI: 10.5897/SRE11.087
ISSN 1992-2248 ©2011 Academic Journals

Full Length Research Paper

Long-term diazoxide preconditioning attenuates the cardioprotective effect against isoproterenol induced myocardial injury in a rat model

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Accepted 1 April, 2011

Short-term diazoxide (DZ) preconditioning exerts efficient protective effect on myocardial ischemia injury. This study aimed to investigate the effects of long term DZ preconditioning on isoproterenol (ISO) induced myocardial injury. DZ (20 mg/kg/d) was intraperitoneally injected into rats for different durations (1, 2, 4, 6 and 8 w) and myocardial injury was then introduced through intraperitoneal injection of ISO. The mitochondrial respiratory function and the cardiac troponin (cTnI) level in the blood were measured. In addition, the mRNA and protein expressions of SUR2A were detected. After DZ preconditioning for 1~4 w, the myocardial protective effect was observed in this ISO induced myocardial injury model and was similar to that after 3 days of DZ preconditioning. After DZ preconditioning for 6 to 8 w, the protective effect was markedly attenuated, characterized by impaired mitochondrial respiratory function and increased blood cTnI level. Furthermore, qRT-PCR and western blot assay revealed no significant difference in the mRNA and protein expressions of SUR2A among groups. Long term DZ preconditioning (≥6 w) may attenuate the protective effect against the ISO-induced myocardial injury, which might be associated with the persistent opening of mitochondrial ATP-sensitive potassium channel.

Key words: Diazoxide preconditioning, isoproterenol-induced myocardial injury, mitochondrial adenosine triphosphate -sensitive potassium channel.

INTRODUCTION

Ischemia preconditioning (IP) has been a focus of cardiovascular research since Murry et al. (1986) first reported that short-term ischemia could significantly reduce myocardial infarct size after lethal ischemia (Murry et al., 1986). However, single and transient IP is difficult to implement in clinic for unexpected presentation of myocardial ischemia (MI). Consequently, pharmacological preconditioning using adenosine (Barbagelata et al., 2005; Ross et al., 2005), diazoxide (DZ) (Wang et al., 2004), bradykinin (Liuba et al., 2005; Wei et al., 2004), etc, is considered as a promising strategy in clinical practice for the treatment of MI. Studies have demonstrated that these agents can produce a similar cardioprotective effect to IP. What is the suitable duration for the preconditioning? Some research focused on the relationship between the duration of pharmacological preconditioning and the cardioprotective effect (Hashimi et al., 1998; Loubani and Galinanes, 2001). Actually, prolonged pharmacological preconditioning may result in partial or complete disappearance of protective effect. Hashimi et al. (1998) found that administration of adenosine for 3 to 4 days abolished the cardioprotective effects of IP in rabbits. Loubani and Galinanes (2001) found the myocardium of patients chronically treated with nicorandil, a sensitive mitochondrial ATP-sensitive potassium (mitoK_{ATP}) channel opener, cannot be preconditioned either by ischemia or pharmacologically.

All this findings suggest prolonged pharmacological preconditioning that will fail to protect the heart from ischemia and subsequent reperfusion injury and the loss of protective effects of myocardial preconditioning may be related to the down-regulation of several proteins such as

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receptor A1, PKC and MitoK_{ATP}. DZ is a specific mitoK_{ATP} channel opener and study has revealed the efficient protective effects of short-term DZ preconditioning on MI injury. Whether prolonged DZ preconditioning also attenuates the cardioprotective effects as the aforementioned is unclear. In the present study, a MI rat model was established through injecting isoproterenol (ISO) before which prolonged DZ preconditioning was performed to observe its cardioprotection in MI rats. The potential mechanism of DZ preconditioning was also investigated.

MATERIALS AND METHODS

Animals

Male specific-pathogen-free (SPF) Sprague-Dawley (SD) rats weighing 255±25 g were purchased from the Experimental Animal Center, the Third Military Medical University (Chongqing, China). They were housed under a temperature and light controlled condition. All protocols were conformed with the National Guidelines for the care and use of the laboratory animals.

Reagents

DZ, bovine serum albumin (BSA) and aprotinin were purchased from Sigma Company (Sigma, USA). Rabbit anti-rat SUR2A polyclonal antibody was provided by Santa Cruz biotechnology (Santa Cruz, CA, USA). Peroxidase conjugated goat anti-rabbit IgG was obtained from Zhongshan Golden Bridge Biotechnology Co. Ltd (Beijing, China). QRT-PCR kit was a product of TOYOBO Co. Ltd (Osaka, Japan). The RNA extraction kit was provided by QIAGEN (Chatsworth, CA, USA). Avian myeloblastosis virus (AMV) reverse transciptase was obtained from Promega (Madison, WI, USA). All the primers were synthesized by Bioasia Biotechnology, Co. Ltd (Shanghai, China).

Grouping and treatment

Animals were randomly divided into 8 groups (n = 20 per group). Group 1: control group; Group 2: MI group; Group 3: DZ3d preconditioning (IP) + ISO group; Group 4: DZ1W + MI group; Group 5: DZ2W + MI group; Group 6: DZ4W + MI group; Group 7: DZ6W + MI group and Group 8: DZ8W + MI group. A single subcutaneous injection of ISO (50 mg/kg) was used to establish the MI model (Group 2). Four hours later, animals were killed by decapitation. Rats in Group 3 underwent intraperitoneal injection of DZ (20 mg/kg/d) for 3 consecutive days and ISO administration was carried out 30 min after last DZ injection. Rats in Group 4 to 8 received intraperitoneal injection of DZ (20 mg/kg/d) for 1, 2, 4, 6 and 8 w, respectively and then ISO injection was given 30 min after last DZ injection. Rats in Group 1 were injected with the solvent of equal volume alone for 8 w. The blood and heart samples were collected at the corresponding time points. The whole study was approved by the Animal Ethics Committee.

Isolation of myocardial mitochondria

Rats were sacrificed by decapitation and the heart was obtained. These hearts were washed twice with cold normal saline (NS) and the pericardium, atrial muscle, valves and major blood vessels were removed. The hearts were cut into pieces and homogenated in lysis buffer containing 2 mM phenylmethyl sulfonyfluoride (PMSF). The homogenate was transferred into a 10 ml centrifuge tube followed by centrifugation at 1500 rpm for 3 min. The supernatant was collected and centrifuged at 12000 rpm for 10 min. This process was repeated twice and the supernatant was removed. The final precipitate was myocardial mitochondria. Bradford method was used to determine the concentration of the myocardial mitochondria (protein concentration) and BSA served as a standard (Bradford, 1976). The proteins were stored at -20°C for use. Mitochondrial protein concentration was determined by Lowry method (Stoscheck, 1990) and bovine serum albumin was considered the standard protein. The protein concentration was adjusted to 10 to 20 mg/ml.

Fresh mitochondria suspension was obtained for the measurement of respiratory activity and membrane potential. All procedures were completed at 0 to 4°C.

Assay of mitochondrial respiration function

The mitochondrial respiratory function was measured as previously described (Wang et al., 2004). Briefly, 1.5 ml of reaction buffer was incubated at 28°C for 2 min in Clark oxygen electrode reaction pool. Then 0.1 ml of mitochondrial suspension was added followed by incubation for 1 to 2 min and 10 µl of substrates (0.5 mol/L sodium malate and 0.5 mol/l sodium glutamate) and 5 µl of ADP (0.1 mol/L) were added. The mitochondrial respiratory state was observed, the oxygen consumption curve delineated and the oxygen consumption calculated after addition of ADP (state 3 respiration, ST3) and ADP exhaustion (state 4 respiration, ST4). Mitochondrial respiratory activity was defined as the consumption (nmol O/min/mg) of mitochondrial proteins, while the respiratory control ratio was the ratio of oxygen consumption (ST3/ST4). Double-sandwich antibody immunofluorescence assay was employed to determine the cTnI level in the blood. In brief, 1 to 2 ml of heparinized blood was collected and 0.3 ml of blood was transferred into wells of MI test plate followed by incubation for 5 to 10 min.

The fluorescent signals were determined with a Triage® diagnosis instrument (Biosite, USA) representing the cTnI level. The normal range is 0.05 to 30 ng/ml.

Determination of SUR2A mRNA expression in myocardial tissue by qRT-PCR

Total RNA was extracted from 100 mg of heart tissues according to manufacturer's instructions. After DNase I treatment, 2 µg of RNA was reverse transcribed with AMV reverse transcriptase. A reaction mixture (20 µl) containing 2 µl of cDNA, 0.5 µl of forward primer, 0.5 µl of reverse primer, 10 µl of 2× Mix Tap and 7 µl of diethyl pyrocarbonate (DEPC) treated H_2O was transferred into PCR tubes, and 0.5 µl of corresponding forward and primers for SUR2A gene were added into the PCR tubes. The primers for SUR2A were synthesized in Shanghai Sangon Co Ltd as follows:

5'-GACGAAGATGCCCTGGAAGACC-3' (forward),

5'-GAGGTGACGAGGCAAAGGAAAA-3' (reverse),

and the anticipated length of products was 239 bp.

PCR was performed for 28 cycles. The annealing temperature was maintained at 63°C, and the rest conditions included denaturation at 94°C for 1 min and extension at 72°C for 45 s. At the end of each cycle, fluorescent product was detected by LightCycle system automatically. The non-reverse transcribed template was set as

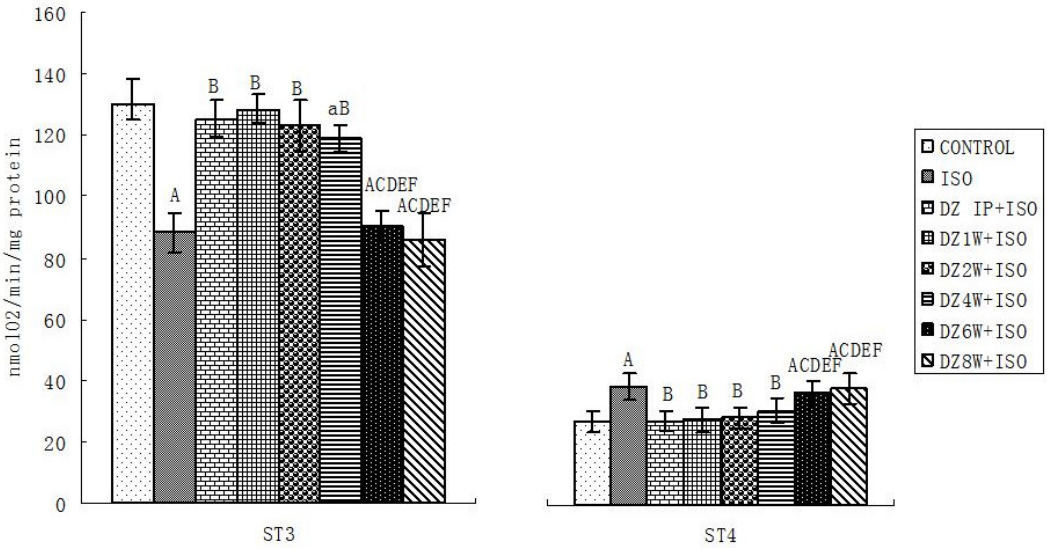

Figure 1. ST3 and ST4 levels in different groups. Diazoxide (DZ) preconditioning for 3 days could efficiently increase the ST3 level and reduce the ST4 level after isoproterenol (ISO) treatment. Meanwhile, DZ preconditioning for 1 to 4 w had similar protective effects. However, DZ preconditioning for 6 to 8 w attenuated these cardioprotective effects. The ST3 level was significantly decreased and the ST4 level markedly increased when compared with other DZ preconditioning groups. A: P<0.05 vs control group; a: P<0.05 vs control group; B: P<0.05vs MI group; C: P<0.05 vs DZ IP group; D: P<0.05 vs DZ1W group; E: P<0.05vs DZ2W group; F: P<0.05 vs DZ4W group.

negative control to exclude contamination of genome DNA. Fluorescence's geometric increase corresponding to exponential increase of the product is used to determine the threshold cycle (Ct) in each reaction. Ct is defined as the fractional PCR cycle number at which the reporter fluorescence is greater than the threshold. The standard curve was delineated with the logarithms of quantitative glyceraldehyde-3-phosphate dehydrogenase (GAPDH) template at various concentrations and the corresponding Ct values.

The Ct value reflects the mRNA expression in each sample; ≥CT value was the difference between Ct_{SUR2A} and Ct_{GAPDH}, which reflects the relative mRNA level of SUR2A in each sample. ≥≥CT is expressed as the difference between ≥CT of DZ untreated rats and logarithm of DZ treated rats, which was used to evaluate the effect of DZ treatment on the expression of SUR2A. $2^{-\geq\geq CT}$ was employed to express the changes in SUR2A gene expression between DZ treatment rats and non-DZ treatment rats.

Detection of SUR2A protein expression by Western blot assay

To detect the protein expression of SUR2A, total protein was extracted from heart tissues. In brief, the hearts were obtained and put into 1 to 2 ml of 95% ethanol followed by boiling for 2 to 3 min. After cooling up, liquid nitrogen was added and tissues were grinded. Then, 1.5 ml of sample were mixed with 0.5 to 1 ml of radioimmunoprecipitation assay (RIPA) lysate in an eppendorf (EP) tube followed by incubation on ice for 20 to 30 min. Centrifugation was performed at 12000 rpm for 15 min at 4°C and supernatant was collected and stored at -70°C. The protein concentration was measured by bicinchoninic acid (BCA) method (Smith et al., 1985). Protein samples (25 µg) were blended with equal volume of 2 × SDS loading buffer followed by boiling for 5 min. Protein separation was performed in 10% separation gel (80 V) and 5% stacking gel (120 V) and proteins were electrically transferred onto polyvinylidene fluoride (PVDF) membrane. After incubation with primary and secondary antibodies, development was performed with an electrogenerated chemiluminescence (ECL) kit. Protein bands then were scanned and the optical density was determined.

Statistical analysis

Statistical analysis was performed with SPSS version 13.0 statistics software and data were expressed as means ± standard deviation ($\bar{x}$ ±SD). One way analysis of variance (ANOVA) was applied for the comparisons among multiple groups and t-test was used for comparisons between two groups. A value of P<0.05 was considered statistically significant.

RESULTS

Prolonged preconditioning attenuated myocardial protective effect of DZ

In MI group, significant changes were found in the myocardial mitochondrial respiratory function. When compared with control group, the mitochondrial state 3 (ST3) was significantly decreased (P<0.05), while state 4(ST4) increased markedly (P<0.05) (Figure 1). In addition, the respiratory control ratio was also significantly reduced when compared with the control group (P<0.05) (Figure 2). After DZ preconditioning (Group 3), the effect of ISO induced impairment on myocardial mitochondrial respiratory function was reduced, the ST3 (P<0.05) (Figure 1) and the respiratory control ratio (P<0.05) (Figure 2) were significantly increased, while

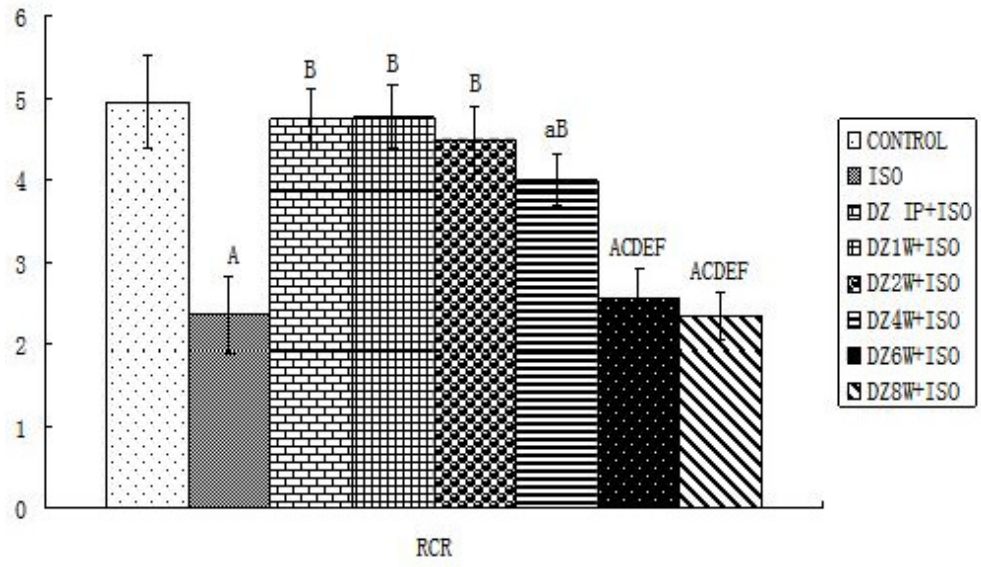

Figure 2. Respiratory control ratio in different groups. Diazoxide (DZ) preconditioning for 3 days and 1~4 w significantly increased the the respiratory control ratio which was decreased by ISO-induced injury. However, the respiratory control ratio in DZ6w+MI group and DZ8w+MI group was lower than that in other DZ preconditioning groups (P<0.05). A: P<0.05 vs control group; a: P<0.05 vs control group; B: P<0.05vs MI group; C: P<0.05 vs DZ IP group; D: P<0.05 vs DZ1W group; E: P<0.05vs DZ2W group; F: P<0.05 vs DZ4W group.

ST4 markedly reduced (P<0.05) (Figure 1) when compared with MI group. After DZ treatment for 1 to 4 w, the myocardial mitochondrial respiratory function was similar to that after DZ preconditioning for 3 days. However, after 6 to 8 w of DZ treatment, the protective effects of DZ preconditioning on ISO induced injury were attenuated, which were characterized by significantly increased myocardial mitochondrial respiratory function, markedly reduced ST3 (P<0.05) (Figure 1), and respiratory control ratio (P<0.05) (Figure 2) and significantly elevated ST4 (P<0.05) (Figure 1).

Prolonged DZ preconditioning increases the cardiac troponin (cTnI) level

The serum cTnI was undetectable in control group (<0.05 ng/ml). ISO-induced myocardial injury significantly elevated the serum cTnI level when compared with control group (P<0.05). In addition, the serum cTnI level was markedly decreased after DZ preconditioning (P<0.05). Meanwhile, the DZ preconditioning also significantly reduced the release of cTnI into the serum (P<0.05). The cTnI level in DZ6W and DZ8W group was higher than that in other DZ preconditioning groups (P<0.05) (Figure 3).

Effect of prolonged DZ preconditioning on SUR2A mRNA expression

Figure 4 shows the melting curve of SUR2A in real-time PCR with a peak at 91.8℃. The melting temperature was uniform and the peak shape was relatively sharp, suggesting high specificity. Analysis showed there was no significant difference in SUR2A expression between different groups (P>0.05), suggesting DZ preconditioning had no effect on the expression of SUR2A in K_{ATP} channel on cardiac membrane (Table 1).

Effect of prolonged DZ preconditioning on protein expression of SUR2A

Western blot assay showed there was no marked difference in SUR2A protein expression between rats treated with DZ for different durations (P>0.05), which suggested various DZ preconditioning had no effect on the protein expression of SUR2A in K_{ATP} channel on cardiac membrane (Figure 5).

DISCUSSION

ISO-induced MI in rats is a classic MI model to evaluate the myocardial ischemic lesion and pharmacological effect (Rona et al., 1959; Tiwari et al., 2009; Keles et al., 2009). Ramesh et al. (1998) found that a single ISO dose was more cardiotoxic than double ISO dose due to ischemic preconditioning. Liu et al. (2003) applied preconditioning through the continuous intraperitoneal injection of DZ (20 mg/kg/d) for 3 d in an ISO-induced MI model and results demonstrated the protective effects of

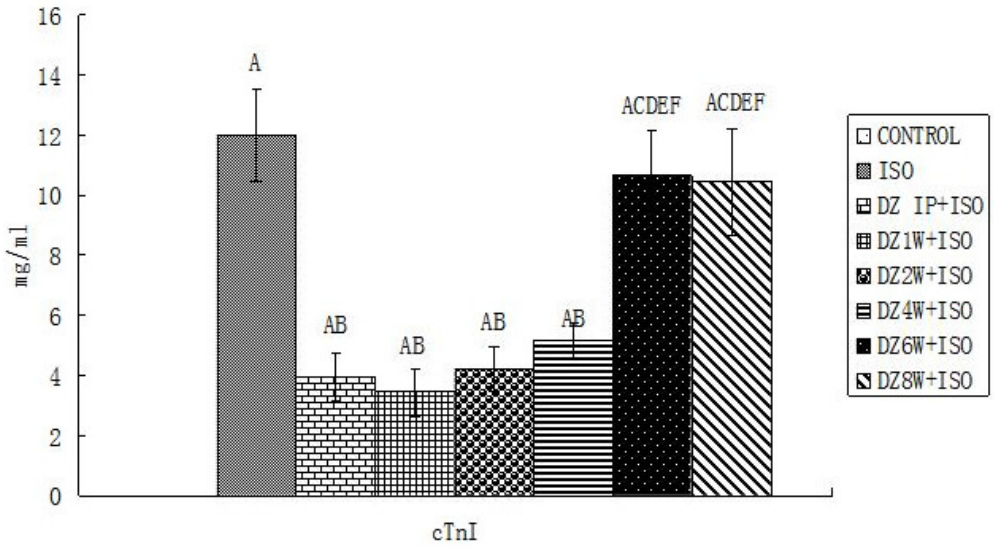

Figure 3. Serum cTnI level in different groups. The cTnI level was rapidly increased after isoproterenol (ISO) injury. Diazoxide (DZ) preconditioning for 3 days, 1~4 w significantly attenuated the cTnI level after ISO injury. However, the cTnI level in DZ6w+MI group and DZ8w+MI group was markedly increased when compared with other DZ preconditioning groups. A: P<0.05 vs control group; B: P<0.05vs MI group; C: P<0.05 vs DZ IP group; D: P<0.05 vs DZ1W group; E: P<0.05vs DZ2W group; F: P<0.05 vs DZ4W group.

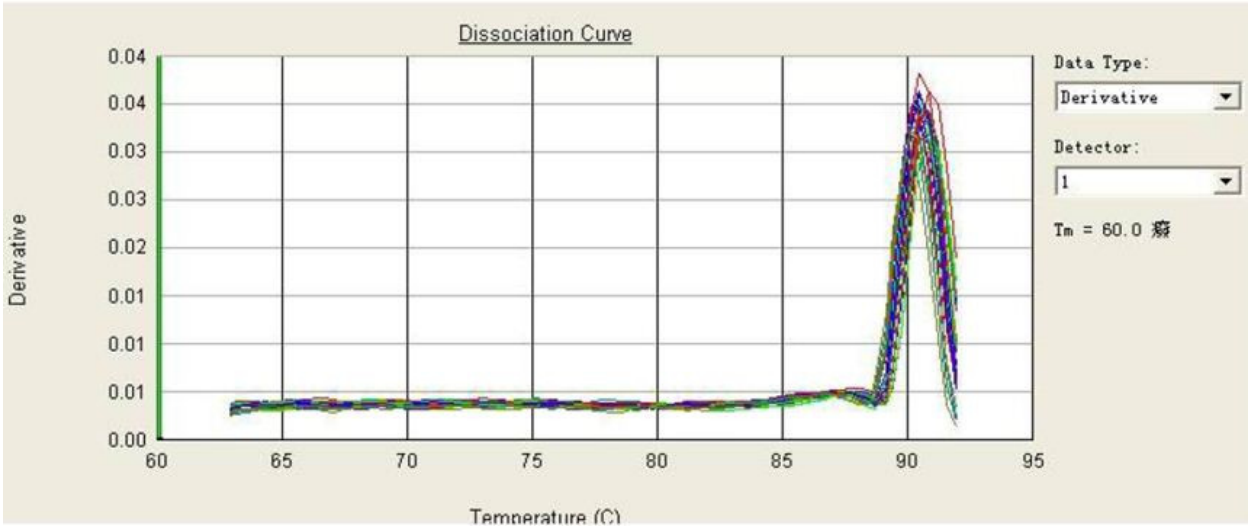

Figure 4. The melting curve of SUR2A in qRT- PCR. The peak of this curve occurred at 91.8℃, the melting temperature was uniform and the peak was sharp showing high specificity.

DZ preconditioning on ISO-induced MI. Thus, a single subcutaneous injection of ISO (50 mg/kg) and intraperitoneal injection of DZ (20 mg/kg/d) for 3 d were employed in the present study. DZ is a specific MitoK_{ATP} channel opener and its myocardial protective effects have been confirmed (Ardehali and O'Rourke, 2005; Costa et al., 2006). In the present study, the protective effect of DZ preconditioning for 3 d was also revealed, which was consistent with the study of Liu et al. (2003). Furthermore, the effects of prolonged DZ preconditioning on MI were also investigated. Results showed intraperitoneal injection of DZ (20 mg/kg/d) for 1 and 2 w effectively protected rats from ISO-induced MI. With further prolongation of preconditioning with DZ, the protective effect on MI began to reduce. The protective effect partially disappeared after DZ preconditioning for 6 and 8 w. A similar phenomenon was also observed by Mayanagi et al. (Garlid et al., 1996). Their study showed

Table 1. SUR2A expression in different groups.

Group	Avg. CT	ΔCT	ΔΔCT	$2^{-\Delta\Delta CT}$
DZ0W	16.88±0.10	0.78±0.10	0.00±0.00	1.00±0.00
DZ1W	16.78±0.07	0.54±0.06	-0.24±0.16	1.18±0.13
DZ2W	17.64±0.16	0.78±0.08	0.00±0.12	1.00±0.08
DZ4W	17.15±0.09	0.93±0.12	0.15±0.12	0.90±0.08
DZ6W	17.60±0.17	0.77±0.21	-0.01±0.16	1.01±0.12
DZ8W	17.59±0.12	0.73±0.20	-0.05±0.14	1.04±0.10

There was no significant difference in the expression of SUR2A among diazoxide (DZ) preconditioning groups.

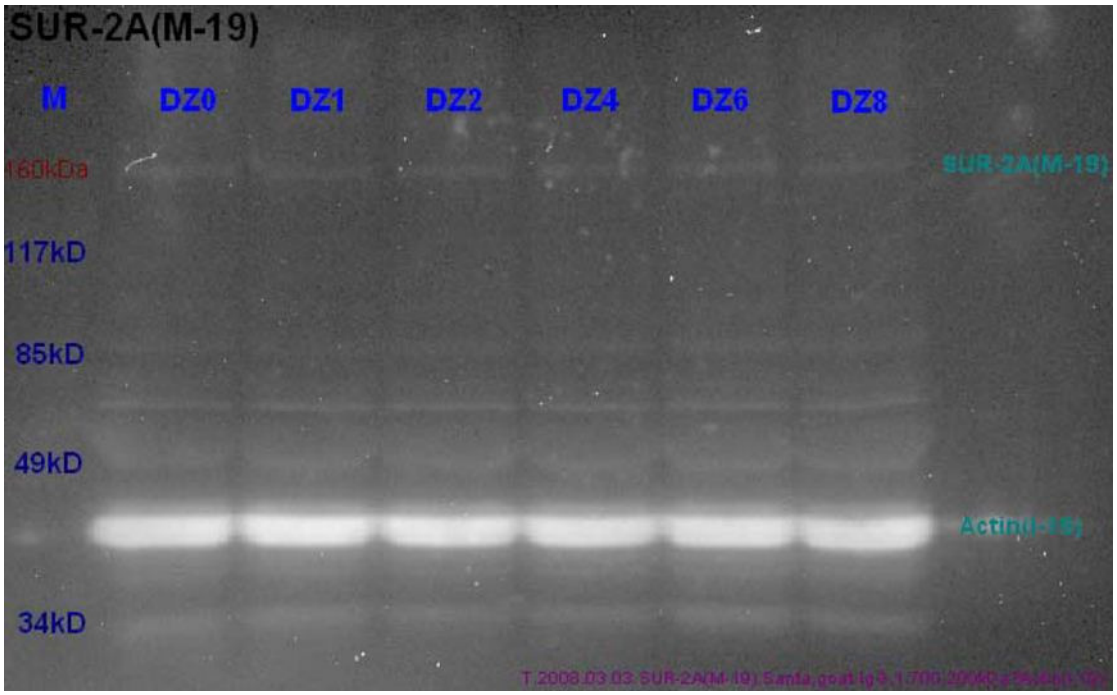

Figure 5. Protein expression of SUR2A in different groups. There was no significant difference in the expression of SUR2A between all diazoxide (DZ) preconditioning groups.

systemic administration of DZ (10 mg/kg) at 24 h before middle cerebral artery occlusion could induce delayed preconditioning against transient focal cerebral ischemia.

After DZ preconditioning for 6 and 8 w, the myocardial mitochondrial respiratory function was compromised and cTnI release was increased after ISO injection when compared with short-term preconditioning. The result suggests that prolonged DZ preconditioning may lead to decreased sensitivity of myocardium to DZ preconditioning, resulting in abolition of myocardial protective effect. The mechanism underlying the absence of myocardial protective effects after long-term DZ preconditioning remains unclear. We speculate that prolonged DZ preconditioning may activate some active sites in the up-stream of K_{ATP} Channel, leading to increased opening frequency of K_{ATP} Channel and subsequent attenuated protective effect (Miki et al., 2002). Adenosine receptors-protein kinase C (PKC)-K_{ATP} channel plays an important role in the ischemic preconditioning. Long-term activation of adenosine receptor, PKC or K_{ATP} channel with specific agonists could reduce the expressions of corresponding receptor, kinase or K_{ATP} channel related proteins (Hashimi et al., 1998; Loubani and Galinanes, 2001). The myocardial protective effects of DZ preconditioning and the signaling pathways following MitoK_{ATP} channel activation such as activation of PKC and p38 cytokinin (mitogen)-activated protein kinase initiated the myocardial protective effects. In addition, patients with diabetes and multiple organ failure may have no response to ischemic or pharmacological preconditioning, because the myocardium undergoes pathological changes which compromise the MitoK_{ATP} channel of these patients and impair the signaling pathways involving in the preconditioning (Ghosh et al., 2001;

Hassouna et al., 2006).

The aforementioned results indicated long-term activetion of some molecules by prolonged preconditioning could down-regulate their expressions, thereby resulting in suppression of their upstream signaling, which led to signal block to the terminal effectors and subsequent compromised myocardial protective effects. K_{ATP} channel plays a central role in the myocardial protection of ischemic preconditioning. It is necessary to investigate the role of K_{ATP} channel in the loss of myocardial protective effect. MitoK_{ATP} channel shares proteins with SarcK_{ATP} channel or is composed of one or two of known K_{ATP} channel subunits. But some studies have the conflict results. These studies show expressions of SUR2A and SUR2B are not observed in mitochondria (Kuniyasu et al., 2003; Lacza et al., 2003; Singh et al., 2003). In addition, the expressions of Kir6.1 and Kir6.2 have no obvious effects on the function of MitoK_{ATP} channel (Seharaseyon et al., 2000) and the function of MitoK_{ATP} channel was not significantly altered after Kir6.1 and Kir6.2 gene deletion (Suzuki et al., 2002, 2001). Thus, MitoK_{ATP} channel may share different genes and proteins with SarcK_{ATP} channel. DZ can open MitoK_{ATP} and SarcK_{ATP} channels. After DZ treatment, the open frequency of MitoK_{ATP} is almost 2000 times as that of SarcK_{ATP} (Garlid et al., 1997).

In the present study, the qRT-PCR and western blot assay showed, after long-term DZ treatment, the mRNA expression of Kir6.2 and protein expression of SarcK_{ATP} were not significantly changed. Maybe the dose of DZ in the preconditioning is not enough to activate SarcK_{ATP} channel in the present study, or the SarcK_{ATP} channel is not involved in the protective effects of DZ preconditioning. We speculate the DZ preconditioning efficiently activates MitoK_{ATP} channel and down-regulates the expressions of related proteins, resulting in complete or partial abolishment of myocardial protective effects. Our results provide evidence to guide the clinical application of pharmacological preconditioning. In the future study, the relationship between the compromised cardioprotection and the down-regulation of MitoK_{ATP} should be elucidated.

ACKNOWLEDGMENTS

This work was supported by National Natural Science Foundation of China (No 30500211). We thank Dr. Jian Chen, Dr. Mingchun Cai and Dr. Kebin Zhang for their technique assistance.

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