Original Paper

DIDS Reduces Ischemia/Reperfusion-Induced Myocardial Injury in Rats

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Key Words

DIDS • Ischemia/Reperfusion injury • Cardiac function • Apoptosis

Abstract

Background/Aims: Anion channels such as chloride channel are known to participate in the regulation of a wide variety of cellular processes including development, differentiation, proliferation, apoptosis and regeneration. This study was designed to examine the effect of the non-selective anion channel blocker 4,4'-Diisothiocyanostilbene-2, 2'-disulfonic acid (DIDS) on cardiac function and apoptosis using a rat model of ischemia/reperfusion (I/R). **Methods:** Fifty male SD rats were randomly divided into the following groups including sham, I/R and I/R+DIDS (7, 14 or 28 mg/kg). In DIDS group, rats received DIDS treatment (4 ml/kg/hr) at the beginning of reperfusion for 2 hrs using a programmed micro-pump. Cardiac function was evaluated including left ventricular systolic pressure (LVSP), left ventricular end diastolic pressure (LVEDP) as well as positive and negative maximal derivatives of left ventricular pressure ($\pm dp/dt_{max}$). Myocardial infarct size was detected using the double staining with 2, 3, 5-triphenyl-2H-tetra-zolium chloride (TTC) and Evan's blue dye. DNA ladder, TUNEL assay, Bax and Bcl-2 protein levels were evaluated. Levels of ROS and Akt phosphorylation were detected. **Results:** I/R injury compromised cardiac function as manifested by reduced LVSP and $\pm dp/dt_{max}$ as well as pronounced apoptosis. I/R-induced cardiac anomalies were markedly ameliorated by DIDS. DIDS retarded I/R-induced myocardial infarct and apoptosis. In addition, DIDS ameliorated I/R-induced ROS production and Akt dephosphorylation in the heart. **Conclusion:** Taken together, our data revealed that DIDS may protect cardiomyocytes against I/R injury as evidenced by improved cardiac function, Bcl-2, Akt phosphorylation, and reduced myocardial apoptosis, Bax expression, ROS production and myocardial infarct size.

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Introduction

Ischemic heart disease is a leading cause of morbidity and mortality in the developing and developed countries. Despite the advent of therapeutic breakthroughs over the past few decades such as thrombolytic therapy and angioplasty, cardiovascular diseases remain the number one cause of death [1, 2]. Among the various therapeutic strategies to tackle ischemic heart disease, enormous efforts have been undertaken to find therapies for ischemic reperfusion injury occurred when the ischemic myocardium is reperfused with oxygen and substrate-rich blood to paradoxically worsen the infarct size [1-3]. Myocardial ischemia/reperfusion (I/R) is an important pathological and physiological process involved in a variety of common clinical diseases [3]. Accumulating evidence has indicated that myocardial I/R is associated with functional regulation of membrane ion channels in addition to the well-known contribution from oxygen free radicals, Ca^{2+} overload, endothelial cells, nitric oxide and neutrophil [4, 5]. However, the relationship between ion channels and ROS has not been elucidated in the process of I/R. In particular, anion channels such as chloride ion channel are reported to be involved in the generation of myocardial ischemia reperfusion arrhythmia [6, 7]. Early application of anion channel blockers 4,4'-diisothiocyano-2,2'-stilbenedisulfonic acid (DIDS), 4-acetamido-4'-isothiocyanato-stilbene-2,2'-disulphonic acid (SITS) and 5-nitro-2(3-phenylpropylamino) benzoic acid (NPPB) improves the ECG manifestation and reduces the degree of reperfusion arrhythmia [6]. Nonetheless, the possible impact of these anion channel blockers on myocardial I/R injury remains elusive. Furthermore, it remains to be validated for non-selective anion channel blockers as a potential novel therapeutic strategy for the clinical management of myocardial I/R injury-related pathology.

Ample evidence has demonstrated that disruption of intracellular ionic homeostasis is closely associated with cell apoptosis [8-11]. For example, early cell shrinkage or apoptotic volume decrease (AVD), a marked morphological characteristic of apoptosis [12-14], is accompanied with activation of potassium channels, chloride channels and H_2O outflow [14, 15]. Activation of anion channels such as chloride currents has been depicted to play a role in the regulation of cell volume, migration, proliferation, and apoptosis in various types of cells [14, 16-19]. Findings from our group revealed that chloride channels participated in apoptosis while chloride channel blockade alleviated staurosporine-induced apoptosis in cardiomyocytes [20-24]. However, to the best of our knowledge, discrepant reports were seen with regards to the consequence of anion in particular chloride channel inhibition at the levels of organ and whole body [9, 25, 26]. To this end, the present study was designed to examine the effect of DIDS, a non-selective anion channel blocker, on ischemia/reperfusion-induced myocardial injury.

Materials and Methods

Experimental animals

The animal procedures described in this study were approved by the Fourth Military Medical University Institutional Animal Use and Care Committee (Xi'an, China). Adult male Sprague-Dawley (SD) rats were obtained from the animal center at the Fourth Military Medical University. Studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals defined by National Research Council (the National Academies, Eighth Edition, 2011). All rats were maintained at 22 °C with a 12/12-light/dark cycle and received lab chow and water *ad libitum*.

Myocardial ischemia/reperfusion injury model

Adult male rats weighing 250-300 g were anesthetized with pentobarbital sodium (45 mg/kg, i.p.), tracheostomized and ventilated with a small-animal ventilator. Electrodes were attached to 4 limbs to record an electrocardiogram. Myocardial ischemia was produced using a left thoracic incision and placing a 6-0 silk suture around left anterior descending coronary artery (LAD) that was left in place during the period of ischemia (30 min) [27]. Successful creation of myocardial ischemia was verified by the occurrence

of ST-segment elevation on ECG and by color changes in the apex cordis. The sham group underwent thoracotomy and pericardiotomy without coronary artery ligation. After 30 min of ischemia, the slipknot was released and the myocardium was reperused, the chest wall was closed in sutured layers, and ventilation was maintained until the animal regained spontaneous respiration.

Assessment of cardiac function

After rats were anesthetized with sodium pentobarbital, a 1.4 F Millar Mikro-tip catheter transducer (Millar) was passed through the right carotid artery into the left ventricle (LV). LV pressure was digitally processed via the Gould 3P 6600 data acquisition system. Left ventricular systolic pressure (LVSP), Left ventricular end diastolic pressure (LVEDP), positive and negative maximal derivatives of left ventricular pressure ($\pm dP/dt_{\max}$) were monitored or derived by computer algorithm. Cardiac contractile and relaxant function was monitored continuously in sham-operated animals and animals subjected to 30 min of ischemia followed by 4 hrs of reperfusion with or without treatment of DIDS as the dosage of 7, 14 or 28 mg/kg.

Tissue preparation

Rats were anesthetized with pentobarbital sodium (45 mg/kg, i.p.) and were sacrificed using cardiectomy to collect heart tissues. Left ventricular tissues (150-200 mg) were placed in tubes for DNA isolation, and each specimen was immediately frozen in liquid nitrogen prior to storage at -80°C . The remaining ventricular samples were directly immersed in 10% formalin at room temperature for 24 hrs followed by paraffin embedding in 4- μm sections for detection of apoptotic cells using the terminal deoxynucleotide transferase dUTP nick end labeling (TUNEL) method or Bax level using Immunohistochemistry [28].

Measurement of O₂⁻ in the myocardium

Superoxide production in the hearts after 4 hrs of reperfusion with or without treatment of DIDS was measured by dihydroethidium (DHE) staining (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, 10 μm frozen heart sections were fixed with ice acetone for 10 min, and loaded with 5 μM DHE for 1 h at 37°C in a humidified container in the dark. Fluorescence was captured using a laser scanning confocal microscope [29].

Assessment of apoptosis using DNA ladder

Cardiomyocyte apoptosis was assessed using DNA fragmentation (DNA-ladder) as described [30]. In brief, frozen tissue samples (50 mg) were minced in 500 μl of lysis buffer (10 μM Tris-HCl, 0.1 M EDTA and 0.5 % SDS, at pH 8) and were quickly homogenized using 30-50 strokes with a microfuge tube pestle. The tissue was digested with 100 $\mu\text{g}/\text{ml}$ of proteinase K at 55°C for 3 hrs and incubated with RNase A at 37°C for 1 hr. After incubation, tissues were precipitated and centrifuged at $15000\times g$ for 5 min. Supernatants containing DNA were precipitated with isopropanol. After centrifugation at $15000\times g$ for 5 min, the resulting DNA pellets were washed with 75 % ethanol and dissolved in DNA hydration solution at 260 nm by spectrophotometry. Ten μg amount of DNA was loaded onto 1.8 % agarose gels containing 0.5 $\mu\text{g}/\text{ml}$ ethidium bromide. DNA electrophoresis was carried out at 80 V for 1 to 2 hrs. DNA ladders, an indicator of tissue apoptotic nucleosomal DNA fragmentation, were visualized under ultraviolet light and photographed for permanent records.

Detection of apoptosis by TUNEL

In situ DNA fragments were detected using a TUNEL kit (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions. The deparaffinized sections were pretreated with proteinase K (20 mg/ml) for 15 min at room temperature. The sections were incubated with terminal deoxynucleotidyltransferase at 37°C for 1 hr prior to rinsing in PBS. The sections were counterstained with DAPI to permit total nuclei counting. For negative controls the sections were incubated without the enzyme or the nucleotide. Cardiomyocytes with the nucleus-labeled with green fluorescence were deemed as TUNEL positive, all nucleus were labeled with blue fluorescence. For each slide, 5 high magnitude power fields ($\times 400$) were randomly chosen. The index of apoptosis as determined (AI, number of positively stained apoptotic cardiomyocytes/total number of cardiomyocytes counted $\times 100$) calculated. Assays were performed in a blinded manner [28].

Immunohistochemistry analysis of Bax

Immunohistology was performed with a specific antibody against Bax (Cell Signaling Technology Inc., Beverly, MA). Tissue sections were deparaffinized and dehydrated through graded alcohol. Endogenous peroxidase activities were blocked by incubation in 3 % H₂O₂ for 10 min. After three washes with PBS, sections were blocked with 1:50 normal goat serum at 37 °C for 30 min to suppress non-specific background staining. The primary rabbit anti-rat Bax monoclonal antibody was diluted to 1:200. For control sections, PBS was used in place of primary antibody. After incubation at 4°C for 12 hrs, sections were incubated with the secondary goat anti-rabbit IgG antibody (Zhongshan Goldbridge Biotechnology Co, Beijing, China, 1:50) at 37 °C for 30 min, followed by 1:100 diluted in biotin-peroxidase complex at 37 °C for 30 min. The sections were subsequently incubated with 3'3-diaminobenzidine (DAB) 0.5 mg/ml for 10 min, counterstained with hematoxylin and observed under light microscopy. Brown staining in cytoplasm was evaluated as the positive expression. The density of stained myocardium was measured by a computer-assisted image analysis system (Image-Pro Plus 5.0). The program automatically detected the stained cardiomyocytes by their color and the area occupied by cardiomyocyte was calculated. The density was calculated as cardiomyocyte area divided by the total area examined ($\mu\text{m}^2/\text{mm}^2$).

Determination of region of ischemia and infarct size

The region of ischemia and the infarct size were measured by double staining with TTC and Evan's blue dye. At the end of the 24- hr reperfusion period, the ligature around the coronary artery was retied and 2 % Evan's blue dye (1 ml) was injected into left ventricular cavity. The heart was quickly excised and left ventricles were sliced transversely into approximately 1.5-2.0 mm in thickness and incubated for 30 min in a 1 % solution of buffered TTC at 37 °C. Slices were photographed with a digital camera in order to identify normal (stained using Evan's blue dye), infarcted (unstained by TTC and Evan's blue dye) and the non-ischemic (stained brick-red by TTC) myocardium [31]. All measurements in various treatment groups were performed in a blind manner.

Western Blot analysis

Rats were sacrificed following reperfusion with or without DIDS treatment. Left ventricular risk area sections were harvested. Heart tissue was lysed by RIPA containing a protease inhibitor cocktail (Roche). Electrophoresis and immunoblotting were done as described previously [32]. After blocking with 5 % non-fat milk, the membranes were probed overnight at 4°C with Bcl-2 (1:1000, Cell Signaling), Akt (1:1000, Cell signaling), phosphorylated Akt (pAkt, 1:1000, Cell signaling), β -actin (1:1000, abcam). Secondary antibodies conjugated to IRDye TM 800 (1:15 000 dilution Rockland) were detected by using an Odyssey infrared imaging system (LI-COR).

Statistical analysis

Data were presented as mean $\pm$ SEM. The results were subjected to a one-way ANOVA statistical analysis. A Tukey-Grammar *post hoc* test was used to detect any intergroup difference. Both the effect of I/R (e.g., Pre-I, I, R) within a given group (sham, I/R, I/R+DIDS) and the effect between groups (e.g., I/R vs. I/R+DIDS) were compared. A value of $P < 0.05$ was considered statistically significant.

Results

Effect of DIDS on cardiac function

As shown in Table 1, myocardial I/R led to a marked drop in LVSP and $\pm \text{dP/dt}_{\text{max}}$ and an increase in LVEDP compared with the sham group. Treatment with DIDS at the dosage of 7 mg/kg displayed a trend of improved LVSP and $+\text{dP/dt}_{\text{max}}$ ($p > 0.05$ vs. Sham group) as well as significantly recovered LVEDP and $-\text{dP/dt}_{\text{max}}$ ($p < 0.05$ vs. Sham group) in the face of I/R injury. Administration of DIDS at the dosage of 14 mg/kg significantly reversed I/R-induced alterations in LVSP, LVEDP and $\pm \text{dP/dt}_{\text{max}}$ ($p < 0.05$ vs. I/R group). However, the cardioprotective effects elicited at a higher dose of DIDS (28 mg/kg) were comparable to those obtained at 14 mg/kg DIDS ($p > 0.05$ between the two groups). Treatment of DIDS alone (28 mg/kg) had little effect on cardiac contractile function. In addition, I/R but not ischemia

Table 1. Effects of DIDS on various parameters of myocardial function (A: LVSP and LVEDP; B: $+dP/dt_{max}$ and $-dP/dt_{max}$) during both ischemia and reperfusion in rat hearts. Rats were administered DIDS at the dosages of 7, 14 or 28 mg/kg, and were subjected to 30-min ischemia followed by 4-hr reperfusion. LVSP, LVEDP and $\pm dP/dt_{max}$ were determined at 30 min after ischemia and 4 hr after reperfusion. Mean $\pm$ SEM, n = 5, #p < 0.05 vs. Sham operation group; *p < 0.05 vs. I/R group; \$p < 0.05 vs. Pre-ischemia group

A:	N	LVSP (mmHg)			LVEDP (mmHg)		
		Pre-ischemia	Ischemia	Reperfusion	Pre-ischemia	Ischemia	Reperfusion
		10 min	30 min	4 hr	10 min	30 min	4 hr
Sham (S)	5	121.0 $\pm$ 13.3	118.4 $\pm$ 12.8	114.6 $\pm$ 12.8	0.8 $\pm$ 0.3	1.2 $\pm$ 0.5	1.4 $\pm$ 0.6
S+DIDS 28 mg/kg	5	122.5 $\pm$ 10.2	119.1 $\pm$ 8.9	115.4 $\pm$ 9.8	0.9 $\pm$ 0.5	0.8 $\pm$ 0.4	1.1 $\pm$ 0.5
I/R	5	118.6 $\pm$ 16.1	98.4 $\pm$ 9.7#	65.2 $\pm$ 9.3#	0.6 $\pm$ 0.3	3.6 $\pm$ 2.2*	9.4 $\pm$ 3.3#
I/R+DIDS 7 mg/kg	5	123.0 $\pm$ 14.4	101.0 $\pm$ 12.7*	77.4 $\pm$ 16.2#	0.7 $\pm$ 0.4	3.3 $\pm$ 2.1	6.0 $\pm$ 3.3
I/R+DIDS 14 mg/kg	5	119.0 $\pm$ 12.6	100.4 $\pm$ 10.0*	89.4 $\pm$ 9.6**	0.8 $\pm$ 0.3	3.8 $\pm$ 1.9*	4.3 $\pm$ 1.7#
I/R+DIDS 28 mg/kg	5	115.2 $\pm$ 14.3	97.0 $\pm$ 9.4#	83.8 $\pm$ 10.7**	0.9 $\pm$ 0.4	4.1 $\pm$ 2.4*	5.2 $\pm$ 2.0#
B:	N	$+dP/dt_{max}$ (mmHg/sec)			$-dP/dt_{max}$ (mmHg/sec)		
		Pre-ischemia	Ischemia	Reperfusion	Pre-ischemia	Ischemia	Reperfusion
		10 min	30 min	4 hr	10 min	30 min	4 hr
Sham (S)	5	4108 $\pm$ 727	4064 $\pm$ 628	3738 $\pm$ 661	2880 $\pm$ 614	2591 $\pm$ 397	2333 $\pm$ 497
S+DIDS 28 mg/kg	5	4129 $\pm$ 633	4034 $\pm$ 630	4120 $\pm$ 527	2905 $\pm$ 620	2610 $\pm$ 413	2215 $\pm$ 510
I/R	5	3846 $\pm$ 925	2965 $\pm$ 637#	1995 $\pm$ 469#	3023 $\pm$ 582	2028 $\pm$ 388*	1194 $\pm$ 207#
I/R+DIDS 7 mg/kg	5	4131 $\pm$ 916	3058 $\pm$ 633*	2778 $\pm$ 347#	2790 $\pm$ 452	1869 $\pm$ 303	1674 $\pm$ 343
I/R+DIDS 14 mg/kg	5	4057 $\pm$ 694	3023 $\pm$ 514*	3043 $\pm$ 521**	2931 $\pm$ 438	1964 $\pm$ 291*	1878 $\pm$ 208#
I/R+DIDS 28 mg/kg	5	3744 $\pm$ 901	2848 $\pm$ 628#	2861 $\pm$ 573**	2786 $\pm$ 572	1786 $\pm$ 356*	1684 $\pm$ 281#

Fig. 1. DNA gel electrophoresis. Clear DNA ladder formation was observed in myocardial tissues subjected to ischemia-reperfusion (I/R) procedure. Administration of DIDS at various dosages exerted a significant anti-apoptotic effect. Lane 1: Marker; Lane 2: I/R group; Lane 3: I/R+ DIDS 7 mg/kg; Lane 4: I/R+DIDS 14 mg/kg; Lane 5: I/R+DIDS 28 mg/kg; Lane 6: Sham+DIDS 28 mg/kg; Lane 7: Sham group. Data were from three independent experiments each with three mice per group (for a total of nine mice per group).

significantly decreased cardiac contractile capacity as manifested by significantly reduced LVSP, LVEDP and $-dP/dt_{max}$ ($p < 0.05$ vs. pre-ischemia group) along with a trend of decreased $+dP/dt_{max}$ ($p > 0.05$ vs. pre-ischemia group) compared with the pre-ischemia condition, the effect of which was dramatically ameliorated by DIDS at all dosages. These data suggest that DIDS may rescue against I/R-induced decline in cardiac contractile function at the dosages tested.

Fig. 2. TUNEL staining for apoptosis cells: Cells with green nucleomes denote TUNEL positive cells ($\times 400$). A: Representative TUNEL staining images from sham operation, sham operation with DIDS (28 mg/kg), ischemia-reperfusion (I/R), DIDS (7 mg/kg) treatment at the beginning of reperfusion; DIDS (14 mg/kg) at the beginning of reperfusion and DIDS (28 mg/kg) at the beginning of reperfusion; and B: Percentage of TUNEL-positive myocyte nuclei following myocardial I/R in rats. Mean $\pm$ SEM, $n=5$, $\#p < 0.05$ vs. sham group; $*p < 0.05$ vs. I/R group.

Effect of DIDS on I/R injury-induced DNA fragmentation

Myocardial apoptosis was determined using fragmentation of DNA as shown by DNA gel electrophoresis (Fig. 1). DNA ladder formation pattern is characteristic of apoptosis. No apparent DNA 'ladders' were found in the sham group. However, I/R group displayed a typical 'ladder' pattern after the 30 min ischemia and 4 hr reperfusion. DIDS at the dosage of 14 and 28 mg/kg (but not at 7 mg/kg) significantly suppressed the appearance of DNA ladder. Treatment of DIDS alone (28 mg/kg) exhibited no effect on DNA fragmentation.

Effects of DIDS on myocardial apoptotic index after I/R

Myocardial I/R overtly increased the number of TUNEL positive cells (expressed as the percentage of total nuclei in ischemic zone) by $18.2 \pm 4.6\%$ compared with sham hearts ($n=5$, $p < 0.05$ vs. sham group). DIDS treatment (at all three dosages) significantly alleviated I/R-induced myocardial apoptosis as shown by decreased TUNEL-positive cell numbers compared with I/R group ($n=5$, $p < 0.05$ vs. I/R group). These findings are consistent with the results of DNA fragmentation in these rat groups (Fig. 2).

Fig. 3. Effect of DIDS on Bax protein level in a rat model of ischemia-reperfusion (I/R, $\times 400$) injury. A: Representative immunohistochemistry staining in sham operation, sham operation with DIDS (28 mg/kg), ischemia-reperfusion (I/R), DIDS (7 mg/kg) at the beginning of reperfusion; DIDS (14 mg/kg) at the beginning of reperfusion and DIDS (28 mg/kg) at the beginning of reperfusion; B: Pooled data depicting effect of DIDS on Bax protein expression in a rat model of I/R treated with various dosages of DIDS at the beginning of reperfusion or sham operation. Mean $\pm$ SEM, $n=5$, $^{\#}p < 0.05$ vs. sham group; $^{*}p < 0.05$ vs. I/R group.

Fig. 4. Effect of DIDS on Bcl-2 protein level in a rat model of ischemia-reperfusion (I/R) injury. Representative immunoblotting and quantitative assay of Bcl-2 protein expression in sham, sham with DIDS (28 mg/kg), ischemia-reperfusion (I/R), DIDS (7 mg/kg) at the beginning of reperfusion; DIDS (14 mg/kg) at the beginning of reperfusion and DIDS (28 mg/kg) at the beginning of reperfusion. Mean $\pm$ SEM, $n=5$, $^{\#}p < 0.05$ vs. sham group; $^{*}p < 0.05$ vs. I/R group.

Expression of Bax and Bcl-2 proteins after ischemia and reperfusion

Myocardial expression of Bax was significantly upregulated, while Bcl-2 protein was markedly downregulated in I/R group compared with the sham group, the effects of which were significantly ameliorated by DIDS at the dosages of 7, 14 and 28 mg/kg ($n=5$, $p < 0.05$ vs. I/R group) (Fig. 3 and 4).

Effect of DIDS on myocardial ROS after I/R

To test whether the protective effect of DIDS was associated with ROS production after I/R, the level of superoxide anion ($O_2^{\cdot -}$) was detected by DHE staining. Compared with sham

Fig. 5. Effect of DIDS on the ROS levels by dihydroethidine (DHE) staining in a rat model of ischemia-reperfusion (I/R) injury. Representative images and quantitative assay of ROS levels in sham, sham with DIDS (28 mg/kg), ischemia-reperfusion (I/R), DIDS (7 mg/kg) at the beginning of reperfusion; DIDS (14 mg/kg) at the beginning of reperfusion and DIDS (28 mg/kg) at the beginning of reperfusion. Nuclei were labeled with DAPI ($\times 200$). Mean $\pm$ SEM, $n=5$, $^{\#}p < 0.05$ vs. sham group; $^*p < 0.05$ vs. I/R group.

Fig. 6. Effect of DIDS on p-Akt protein levels in a rat model of ischemia-reperfusion (I/R) injury. Representative immunoblotting and quantitative assay of p-Akt and Akt protein expression in sham, sham with DIDS (28 mg/kg), ischemia-reperfusion (I/R), DIDS (7 mg/kg) at the beginning of reperfusion; DIDS (14 mg/kg) at the beginning of reperfusion and DIDS (28 mg/kg) at the beginning of reperfusion. Mean $\pm$ SEM, $n=5$, $^{\#}p < 0.05$ vs. sham group; $^*p < 0.05$ vs. I/R group.

group, I/R dramatically increased myocardial O_2^- accumulation. However, DIDS treatment significantly attenuated I/R-induced ROS (O_2^-) accumulation in the heart (Fig. 5).

Effect of DIDS on myocardial Akt phosphorylation after I/R

Since Akt has been shown to play an important role in cardiomyocyte survival, we detected the level of activated/phosphorylated Akt in the heart after I/R with or without DIDS treatment [33]. Our data revealed that myocardial Akt level was not significantly affected by DIDS treatment alone. However, Akt phosphorylation was significantly decreased in the I/R group, the effect of which was dramatically attenuated by DIDS treatment (Fig. 6).

Fig. 7. Effect of DIDS on myocardial infarct size in a rat model of ischemia-reperfusion (I/R) injury. Pooled data depict effect of DIDS on infarct size in a rat model of I/R treated with various dosages of DIDS (7, 14 and 28 mg/kg) at the beginning of reperfusion or sham operation. Insets: Representative cross-section of heart in sham or I/R group with or without administration of DIDS. Mean $\pm$ SEM, $n=5$, $^{\#}p < 0.05$ vs. sham group; $^*p < 0.05$ vs. I/R group.

Effects of DIDS on myocardial infarct size of myocardial I/R

Following the 30-min ischemia and 4-hr reperfusion, infarction size of the heart was significantly increased in I/R group ($58.4 \pm 11.0\%$) compared with the sham operation group. Sham operation alone did not result in any change in infarct size ($1.0 \pm 1.5\%$). The myocardial infarct size determined by Evan's blue dye-TTC showed that the myocardial infarct size was significantly reduced in DIDS at the dosages of 7 mg/kg ($40 \pm 17\%$), 14 mg/kg ($37 \pm 8\%$) and 28 mg/kg ($41 \pm 7\%$) compared with I/R group ($n=5$, $p < 0.05$ vs. I/R group) (Fig. 7).

Discussion

It is well known that myocardial cell death during ischemia-reperfusion injury usually occurs as a result of both apoptosis and necrosis [34, 35]. In ischemia-reperfusion injury, the irreversible apoptotic damage to myocardium overtly compromises myocardial geometry and contractile function, leading to heart failure [30, 34-36]. Both clinical and experimental evidence have consolidated a beneficial effect of inhibition of apoptotic pathways in the combat against ischemia-reperfusion cardiac injury [23, 26, 37]. The salient findings from this study suggest that DIDS significantly improves cardiac function, lessens TUNEL apoptosis, attenuates DNA fragmentation, and attenuates levels of ROS and pro-apoptotic protein Bax, increases levels of Akt phosphorylation and anti-apoptotic protein Bcl-2, coinciding with reduced myocardial infarct size following ischemia-reperfusion. These findings suggest that DIDS may possess an important protective property against myocardial ischemia-reperfusion injury.

DIDS, a non-selective anion channel blocker, has been reported to rescue against a series of pro-apoptotic stimuli, such as staurosporine, doxorubicin, and ischemia-reperfusion injury [20, 23, 38]. Recent evidence from our group reveals that DIDS significantly attenuates staurosporine-induced cardiomyocyte apoptosis in a PI3K/Akt-dependent manner [20]. DIDS also attenuates ROS accumulation in pancreatic carcinoma cell lines [39]. In addition, DIDS exerts cytoprotective effect via inhibition of pro-apoptotic stimuli-induced apoptotic volume decrease (AVD) by preventing Cl^- fluxes, either by blocking $\text{Cl}^-/\text{HCO}_3^-$ exchanger or chloride channels [16]. Nonetheless, target minimizing the function of $\text{Cl}^-/\text{HCO}_3^-$ exchangers through a bicarbonate-free condition fails to alter the beneficial cytoprotective action of DIDS against staurosporine-induced and ischemia/reperfusion-induced apoptosis in rodent cardiomyocytes [21, 23]. Observation from our current study revealed that DIDS effectively rescued against ischemia-reperfusion-induced cardiac injury in association with lessened

myocardial ROS production and apoptosis. Given the nature of non-selective anion channel for DIDS, inhibition of anion channel such as chloride channel is likely to underscore DIDS-offered beneficial effect against ischemia-reperfusion injury.

Chloride is the most abundant anion capable of passing through cell membranes via anion channels. Anion channels including chloride channel are known to participate in a series of physiological functions in mammalian cells including stabilization of the membrane potential, transfer of chloride for fluid, transport of electrolytes and regulation of pH and cell volume [37, 40]. Numerous subtypes of chloride channels have been identified in the heart including cystic fibrosis transmembrane conductance regulator (CFTR), swelling-activated chloride current ($I_{Cl,swell}$), Ca^{2+} -activated chloride currents ($I_{Cl,Ca}$) and voltage-gated chloride channels (ClC), all of which are subjected to DIDS-induced inhibition [17, 37, 40]. A rather unique role has been reported for anion in particular chloride channels in the development of cardiac hypertrophy, ischemia/reperfusion injury, arrhythmia and congestive heart failure [41-43]. For example, overexpression of human cystic fibrosis transmembrane conductance regulator (CFTR) gene, which encodes a cAMP-regulated chloride channel, led to slowed electrical conduction capacity and sudden cardiac death following isoproterenol challenge, indicating a role of chloride channels in arrhythmogenesis [43]. Our finding that DIDS preserves against myocardial ischemia-reperfusion injury is in line with the earlier notion of activated chloride channel in outward rectification in response to a number of pro-apoptotic stimuli [16, 44]. Enhanced cell volume regulation through chloride and potassium ionic movement across the sarcolemma has been deemed essential for local and remote ischemic preconditioning [8, 19, 45-47]. More evidence suggested that chloride channel blockers are capable of retarding apoptosis via inhibition of outwardly rectifying chloride currents in pathological conditions including ischemia/reperfusion injury, arrhythmia and heart failure [6, 18, 23, 41, 48, 49]. Data from our group revealed that application of DIDS or NPPB may effectively reduce staurosporine- and ischemia-reperfusion-induced cardiomyocyte apoptosis *in vitro* [20-22]. This is strongly supported by our current observations of lessened TUNEL apoptosis, ROS production, DNA fragmentation and late Bax levels, and upregulated Bcl-2 and Akt phosphorylation in response to DIDS treatment in the face of ischemia-reperfusion injury. Our data were also in line with the findings from Mizoguchi and coworkers whereby the non-selective anion channel blockers NPPB and DIDS suppressed myocardial apoptosis, Caspase activation, myocardial infarct size and loss of cardiac function following ischemia and reperfusion upon heterotrophic heart transplantation [25]. Given that necrosis is likely the major contributor to cell death 6 hrs after the onset of the ischemic insult [50, 51] whereas our ischemia-reperfusion occurred within 4.5 hrs, it is plausible to speculate that DIDS may rescue ischemia-reperfusion-induced myocardial dysfunction mainly by way of apoptosis regulation.

Experimental limitation

One major caveat should be considered for our current study in that DIDS is a non-selective anion channel blocker. In addition to suppression of anion channels, DIDS has been reported to possess many other biological effects such as inhibition of Cl^-/HCO_3^- exchanger [20] and ryanodine receptors (RyR) [52]. Our current experimental findings suggested that DIDS rescued against ischemia/reperfusion injury in association with lessened ROS accumulation and Akt dephosphorylation. These DIDS-elicited properties may affect myocardial apoptosis and contractile function independent of anion channel blockade or cell volume regulation. However, it is somewhat difficult to examine the permissive role of these signaling molecules using the *in vivo* murine model of ischemia-reperfusion injury (for example application of the bicarbonate-free condition to assess possible contribution of Cl^-/HCO_3^- exchanger). Although our recent patch clamp study suggested that DIDS-induced anti-apoptotic property is directly associated with inhibition of chloride current in cardiomyocytes [53], further scrutiny is needed to discern the mechanism(s) of action behind DIDS-offered cardioprotection against ischemia-reperfusion injury.

In conclusion, results from our present study indicated that *in vivo* treatment of DIDS rescued against ischemia-reperfusion-induced myocardial contractile anomalies and apoptosis, which was associated with attenuation of ROS accumulation and improvement of Akt signaling. These findings are in line with the earlier observations of DIDS-offered inhibition against cardiomyocyte apoptosis induced by pro-apoptotic stimuli [16, 44, 53]. These findings suggest that the non-selective anion channel blocker leads to inhibition of apoptosis and subsequently reduction of necrosis in cardiomyocytes following ischemia-reperfusion injury. Our finding may offer a novel strategy in the application of non-selective anion channel inhibitors in the clinical management of myocardial ischemia-reperfusion injury [13]. Further investigation is warranted to better elucidate the underlying mechanisms responsible for inhibition of anion channel-elicited pro-survival properties for DIDS and other anion channel blockers.

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Disclosure Statement

None

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