Original Research

4′-Chlorodiazepam, a translocator protein (18 kDa) antagonist, improves cardiac functional recovery during postischemia reperfusion in rats

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Abstract

Inhibition of translocator protein (18 kDa) (TSPO) can effectively prevent reperfusion-induced arrhythmias and improve postischemic contractile performance. Mitochondrial permeability transition pore (mPTP) opening, mediated mainly through oxidative stress during ischemia/reperfusion (I/R), is a key event in reperfusion injury. 4′-Chlorodiazepam is a widely used TSPO antagonist. However, whether 4′-chlorodiazepam can improve cardiac functional recovery during postischemia reperfusion by affecting oxidative enzymes, reducing reactive oxygen species (ROS) and thereby inhibiting mPTP opening is still unknown. Cardiac function including heart rate, coronary flow rate, left ventricular developed pressure (LVDP), left ventricular end-diastolic pressure (LVEDP), maximal time derivatives of pressure ($\pm dP/dt$ max) and the severity of ventricular arrhythmias were analyzed in isolated rat hearts during I/R. mPTP opening, ROS and oxidative enzyme activities were measured with fluorometric or spectrophotometric techniques. 4′-Chlorodiazepam did not affect heart rate and coronary flow rate, but abolished the increase in LVEDP, accelerated the recovery of LVDP and $\pm dP/dt$ max, and reduced the severity of ventricular arrhythmias. The mPTP opening probability was reduced by 4′-chlorodiazepam, accompanied by a reduction in ROS level. In addition, the activities of mitochondrial electron transport chain complex I and complex III were increased, while those of xanthine oxidase and NADPH oxidase were reduced. Therefore, 4′-chlorodiazepam may improve cardiac functional recovery during reperfusion, potentially by affecting the activities of oxidative enzymes, reducing ROS and thereby inhibiting mPTP opening. The present study presents evidence that 4′-chlorodiazepam could be a novel adjunct to reperfusion.

Keywords: 4′-chlorodiazepam, translocator protein (18 kDa), postischemia reperfusion, mitochondrial permeability transition pore, reactive oxygen species, oxidative enzyme

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Introduction

Ischemic heart disease is one of the leading causes of cardiac death in Western societies.[1] For recovery, reperfusion using clinical interventions such as coronary artery bypass graft surgery is required for the ischemic heart.[2] Despite significant developments in myocardial protection techniques, reperfusion damage still occurs, and significant morbidity remains a large problem.[2] Novel and reliable approaches to drug development that could enhance recovery upon reperfusion are needed.[2,3]

Mitochondria are cytoplasmic, double-membrane organelles whose main role is to synthesize ATP.[4,5] Mitochondria occupy a large portion of the intracellular space of cardiac myocytes and are located between the myofibrils and just below the sarcolemma.[4] The strategic positioning and abundance of mitochondria ensure a highly efficient localized ATP delivery system to support contraction, metabolism and ion homeostasis.[4,5] Mitochondrial dysfunction has been identified as an important event in ischemia/reperfusion (I/R) damage.[6] Mitochondrial dysfunction affects cell viability through a wide array of events, including loss of ATP synthesis and increased ATP hydrolysis, impairment of ionic homeostasis, formation of reactive oxygen species (ROS) and release of proapoptotic proteins, which are key factors in the generation of irreversible damage.[5,6]

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The translocator protein (18 kDa) (TSPO), formerly known as the mitochondrial benzodiazepine receptor, is a 169 amino acid protein with five transmembrane domains associated with the mitochondrial outer membrane.[7,8] TSPO has a wide spectrum of putative functions, including the regulation of cholesterol transport and the synthesis of steroid hormones, porphyrin transport and heme synthesis, apoptosis, cell proliferation and anion transport.[7–9] Most importantly, TSPO has been proposed to play an important role in mitochondrial regulation, including regulation of mitochondrial membrane potential and the mitochondrial respiratory chain.[7,8]

The mitochondrial permeability transition pore (mPTP) is a voltage-dependent, high-conductance channel located in the inner mitochondrial membrane.[10] Conditions associated with I/R such as ROS accumulation, pH normalization and Ca^{2+} increase are conducive to mPTP opening. mPTP opening is a key event in reperfusion injury.[11] TSPO is an important regulator of mPTP.[12] mPTP opening during I/R has been shown to be mainly mediated through oxidative stress.[11,12] TSPO inhibition can decrease oxidative stress and has been found to be effective in preventing reperfusion-induced arrhythmias.[13–15] In addition, TSPO inhibition has also been shown to markedly improve postischemic contractile performance.[13,15] 4′-Chlorodiazepam is a widely used TSPO antagonist.[14,16] However, whether 4′-chlorodiazepam can improve cardiac functional recovery during postischemia reperfusion by affecting oxidative enzymes, reducing ROS and inhibiting mPTP opening remains unknown.

Materials and methods

This investigation conforms to the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996) and the Policy of Animal Care and Use Committee of Tongji University.

Experimental protocols

The I/R model was established as previously described.[17] Male Sprague–Dawley rats (weighing 250–280 g) were killed by stunning and cervical dislocation. Hearts were rapidly removed into ice-cold Krebs-Henseleit (K–H) buffer containing (mmol/L): NaCl 118.0, KCl 4.7, KH_2PO_4 1.2, $NaHCO_3$ 25, $MgSO_4$ 1.2, $CaCl_2$ 2.5, glucose 11.0. The hearts were then mounted in a Langendorff apparatus and perfused retrogradely at a constant pressure of 75 mmHg with K–H buffer gassed with 95% O_2/5% CO_2 equilibrated at pH 7.35–7.45 and maintained at 37°C.

After a 15-min equilibration period, rat hearts were subjected to the following protocols: (1) control group, 50 min normoxic perfusion ($n = 15$); (2) I/R group, 20 min normoxic perfusion, 15 min ischemia and 15 min reperfusion ($n = 15$); and (3) 4′-chlorodiazepam groups, 20 min normoxic perfusion, 15 min ischemia and 15 min reperfusion with 4′-chlorodiazepam at concentrations of 16 μmol/L ($n = 15$), 32 μmol/L ($n = 15$), or 64 μmol/L ($n = 15$) during the whole perfusion period. 4′-Chlorodiazepam was administered as a pretreatment after the 15 min equilibration period.

Ischemia was established by ligating the left main coronary artery within 2 mm of where it emerges adjacent to the left atrium. The duration of coronary artery ligation was 15 min. The electrocardiogram was monitored using the Data Acquisition System (PowerLab System, ADInstruments, Castle Hill, Australia) and coronary flow was measured by collecting the perfusate over a period of 1 min at regular intervals. Reperfusion was then established by cutting the ligature across a polyvinyl sheath that encompassed the coronary artery.

Analysis of hemodynamic function

When the heart was mounted on the Langendorff apparatus and perfused retrogradely with K–H buffer, a water-filled latex balloon (ADInstruments) was inserted into the left ventricle through the left atrium and the pressure was continuously monitored by a transducer connected to the balloon. The initial value of end-diastolic pressure was set to 5–10 mmHg by adjusting the volume of the balloon. The balloon volume remained constant during the experiment so that changes in the left ventricular end-diastolic pressure (LVEDP) were due to changes in myocardial compliance.

Hemodynamic data were analyzed using a Data Acquisition System (PowerLab System, ADInstruments). The parameters measured included heart rate, coronary flow rate, left ventricular developed pressure (LVDP), LVEDP and maximal time derivatives of pressure measured during contraction ($+dP/dt$ max) and relaxation ($-dP/dt$ max).

Assessment of arrhythmias

Arrhythmias were determined and diagnosed in accordance with the criteria of the Lambeth Conventions.[18] Arrhythmias were categorized as single ventricular premature beats (VPBs), ventricular tachycardia (VT, a run of four or more consecutive VPBs) or ventricular fibrillation (VF, a ventricular rhythm without recognizable QRS complex in which signal morphology changed from cycle to cycle and for which it was impossible to estimate heart rate).

An arrhythmia score was used as previously described.[19] Each heart was given one score representing the most severe type of arrhythmia observed any time during the entire reperfusion period. The arrhythmia was scored as follows: 0, for no arrhythmia; 1, occasional VPBs; 2, frequent VPBs when there were three or more VPBs occurring within 1 min; 3, VT (1 or 2 episodes); 4, VT (3–5 episodes); 5, VT (more than 5 episodes); 6, VF (1–2 episodes); 7, VF (3–5 episodes); and 8, VF (more than 5 episodes).

Isolation of mitochondria and measurement of mPTP opening

Mitochondria were isolated from perfused rat hearts of every group at the end of the protocol. The extraventricular tissue was removed, and the left ventricle was then weighed and parts of the left ventricle were finely minced in ice-cold buffer (160 mmol/L KCl, 10 mmol/L EGTA, 0.5% fatty

acid-free bovine serum albumin, PH 7.4), and brought to a final concentration of 0.1 g/mL of buffer. This tissue suspension was homogenized and centrifuged at 1000 *g* for 10 min at 4°C. The supernatant was then centrifuged at 8000 *g* for 10 min at 4°C to obtain the original mitochondrial pellet, which was resuspended using a homogenizer in suspension buffer (320 mmol/L sucrose, 10 mmol/L Tris-HCl, PH 7.4), and centrifuged again at 8000 *g* for 10 min at 4°C to obtain the final mitochondrial pellet.[16] Two independent methods were used to evaluate the purity of the mitochondrial preparation. First, electron microscopic observations showed very little contamination from broken mitochondria or lysosomes. Second, Western blotting indicated that the mitochondria-marker cytochrome *c* oxidase was at least eight times enriched in the mitochondria preparations while the endoplasmic reticulum-marker calreticulin was markedly reduced to insignificant levels in the purified mitochondria (data not shown). The mitochondrial protein content was determined by using the BCA Protein Assay Kit (Merck, Darmstadt, Germany). Then, using the isolated mitochondria, the mPTP opening was measured fluorometrically using a commercial assay kit (Genmed Scientifics Inc, Arlington, USA) with excitation at 488 nm and emission at 505 nm.

Measurement of ROS and the activities of oxidative enzymes

For the qualitative study of ROS, parts of the left ventricle from every group at the end of the protocol were placed in 20% sucrose for 12 h and embedded in O.C.T. compound. Frozen 10-μmol/L sections were made by cryostat (CM 3050, Leica, Germany) and observed under a fluorescent microscope (BX51, Olympus, Tokyo, Japan) using a commercial assay kit (Genmed Scientifics Inc).

For the quantitative study of ROS, parts of the left ventricle from every group at the end of the protocol were homogenized in phosphate-buffered saline (50 mmol/L sodium phosphate and 150 mmol/L NaCl, pH 7.4) with a PT 10/35 Polytron (Brinkman, Westbury, NY, USA). The protein content was determined using the BCA Protein Assay Kit (Merck). The ROS level was then measured fluorometrically using a commercial assay kit (Genmed Scientifics Inc) with excitation at 488 nm and emission at 520 nm.

Some homogenized solutions made in the quantitative study of ROS were centrifuged at 5000 *g* (4°C) for 10 min, and the supernatant was used for various assays. Protein content was determined by using the BCA Protein Assay Kit (Merck). The enzyme activities of xanthine oxidase, NADPH oxidase and superoxide dismutase (SOD) were evaluated spectrophotometrically using commercial assay kits (Genmed Scientifics Inc).

In addition, the enzyme activities of mitochondrial electron transport chain complex I and complex III were assessed spectrophotometrically using commercial assay kits (Genmed Scientifics Inc) in isolated mitochondria as described above.

Statistical analysis

Data are expressed as mean $\pm$ standard error of the mean (SEM). Statistical analysis of data was performed by applying the χ^2 test or two-way analysis of variance followed by Student–Newman–Keuls *post hoc* test. A P value of less than 0.05 was considered significant.

Results

Effects of 4′-chlorodiazepam on cardiac functional recovery during reperfusion

Addition of 4′-chlorodiazepam to the K–H solution before the occlusion did not change heart rate and coronary flow rate (basal heart rate values for 4′-chlorodiazepam-treated hearts: 280 ± 10 bpm [4′-chlorodiazepam 16 μmol/L group], 290 ± 9 bpm [4′-chlorodiazepam 32 μmol/L group] and 280 ± 8 bpm [4′-chlorodiazepam 64 μmol/L group]; basal coronary flow rate values for 4′-chlorodiazepam-treated hearts: 17.68 ± 0.26 mL/min [4′-chlorodiazepam 16 μmol/L group], 17.87 ± 0.40 mL/min [4′-chlorodiazepam 32 μmol/L group] and 18.02 ± 0.51 mL/min [4′-chlorodiazepam 64 μmol/L group]). During the period of occlusion, the heart rate and coronary flow rate decreased similarly in I/R group and 4′-chlorodiazepam groups by 14% and 48%, respectively. Reperfusion resulted in an increase in coronary flow rate (22.68 ± 0.41 mL/min) to levels above that present prior to ischemia (17.80 ± 0.35 mL/min). However, treatment with 4′-chlorodiazepam did not change coronary flow rate in reperfusion (22.34 ± 0.38 mL/min [4′-chlorodiazepam 16 μmol/L group], 23.07 ± 0.46 mL/min [4′-chlorodiazepam 32 μmol/L group] and 23.76 ± 0.43 mL/min [4′-chlorodiazepam 64 μmol/L group]).

LVDP failed to recover to the baseline even after 15 min of reperfusion, but recovered in 10 min with 4′-chlorodiazepam (Figure 1a). LVEDP was increased during reperfusion by 194% (8.09 ± 0.83 [control group] versus 23.80 ± 1.03 [I/R group], $P < 0.05$) while 4′-chlorodiazepam abolished the increase (Figure 1b). The recovery of $\pm dP/dt$ max in reperfusion was also accelerated by 4′-chlorodiazepam (Figures 1c and d).

Figure 2a shows typical arrhythmias. 4′-Chlorodiazepam effectively reduced the severity of reperfusion-induced ventricular arrhythmias in a dose-dependent manner ($P < 0.05$) (Figure 2b). The anti-arrhythmic effects could be identified from the reduced arrhythmia scores and the decreased incidence of VT and VF (Figures 2c and d). In total, 16, 32 and 64 μmol/L 4′-chlorodiazepam reduced the arrhythmia scores by 30.14%, 60.13% and 80.86% (6.10 ± 0.47 [control group] versus 4.57 ± 0.40 [4′-chlorodiazepam 16 μmol/L group], 3.47 ± 0.36 [4′-chlorodiazepam 32 μmol/L group] and 2.33 ± 0.32 [4′-chlorodiazepam 64 μmol/L group], $P < 0.05$), respectively. The incidence of VT decreased from 80% to 50%, 30% and 10%, respectively ($P < 0.05$) while VF decreased from 100% to 60%, 40% and 10% ($P < 0.05$) (Figures 2c and d).

Effects of 4′-chlorodiazepam on opening probability of mPTP during reperfusion

The opening probability of mPTP was found to be increased by 62.74-fold in reperfusion as compared with the control group (56753 ± 2832 [I/R group] versus 582 ± 50 [control group],

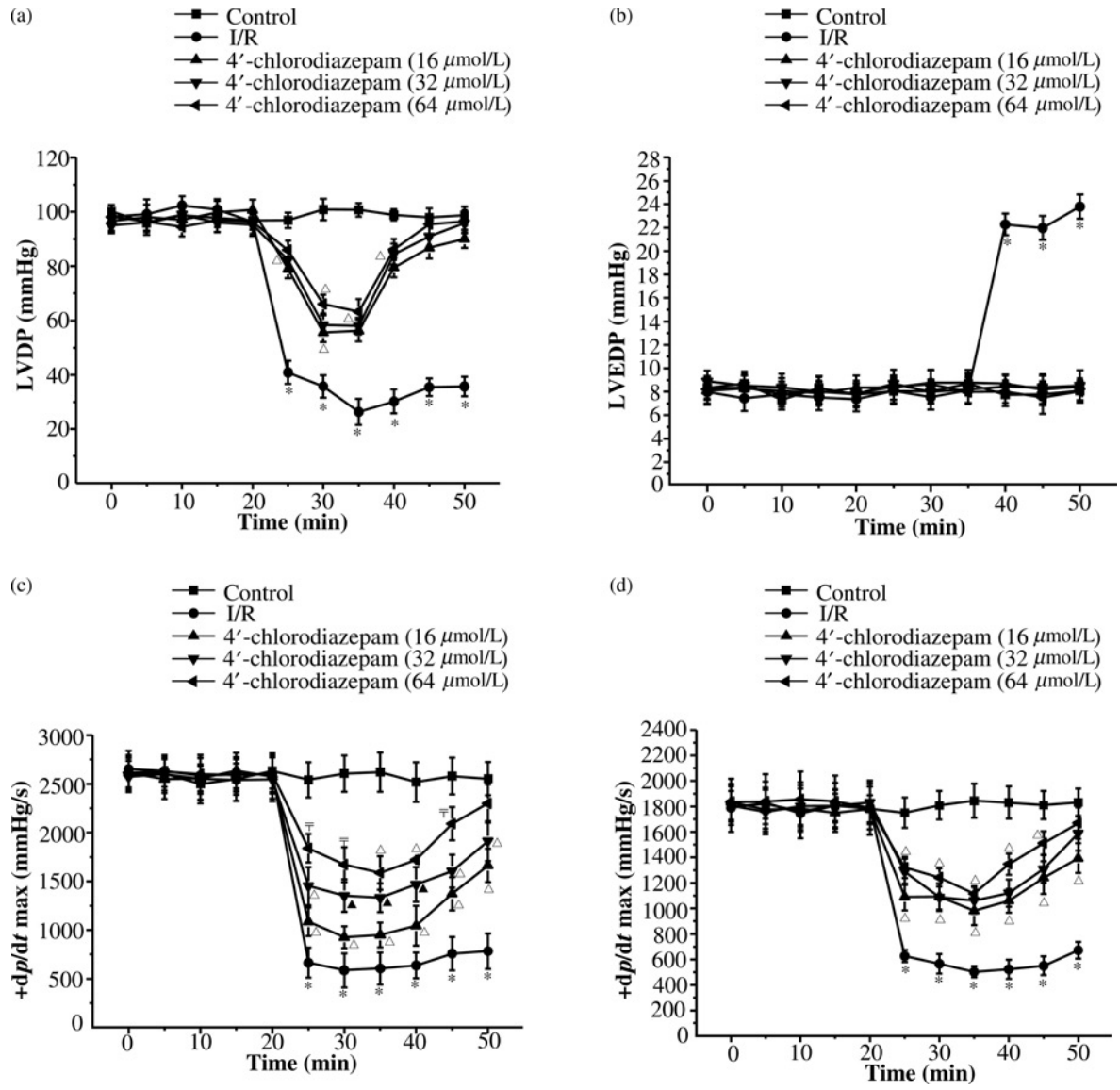


Figure 1 4′-Chlorodiazepam improves cardiac contractile functional recovery during reperfusion. (a) Left ventricular developed pressure (LVDP) changes over time. Values represent mean ± SE ($n = 15$ per group). $^{*}P < 0.05$ versus control group; $^{\Delta}P < 0.05$ versus control group and ischemia/reperfusion (I/R) group. (b) Left ventricular end-diastolic pressure (LVEDP) changes over time. Values represent mean ± SE ($n = 15$ per group). $^{*}P < 0.05$ versus control group. (c) Maximal time derivatives of pressure measured during contraction (+dP/dt max) changes over time. Values represent mean ± SE ($n = 15$ per group). $^{*}P < 0.05$ versus control group; $^{\Delta}P < 0.05$ versus control group and I/R group; $^{\blacktriangle}P < 0.05$ versus control group, I/R group and 4′-chlorodiazepam (16 μmol/L) group; $^{\mp}P < 0.05$ versus control group, I/R group and 4′-chlorodiazepam (16 and 32 μmol/L) groups. (d) Maximal time derivatives of pressure measured during relaxation (−dP/dt max) changes over time. Values represent mean ± SE ($n = 15$ per group). $^{*}P < 0.05$ versus control group; $^{\Delta}P < 0.05$ versus control group and I/R group

$P < 0.05$). The three different doses of 4′-chlorodiazepam used in this study significantly reduced the opening probability of mPTP by 65%, 84% and 93% (56753 ± 2832 [I/R group] versus 20061 ± 563 [4′-chlorodiazepam 16 μmol/L group], 8895 ± 458 [4′-chlorodiazepam 32 μmol/L group] and 4210 ± 439 [4′-chlorodiazepam 64 μmol/L group], $P < 0.05$), respectively (Figure 3).

Effects of 4′-chlorodiazepam on ROS during reperfusion

The ROS level was found to be increased in reperfusion, and 4′-chlorodiazepam reduced the increase in the qualitative assay (Figure 4a). Further study showed that the three different doses of 4′-chlorodiazepam reduced the ROS level by 44%, 56% and 63% (7271 ± 683 [I/R group] versus 4073 ± 429 [4′-chlorodiazepam 16 μmol/L group], 3163 ± 400 [4′-chlorodiazepam 32 μmol/L group] and 2671 ± 378 [4′-chlorodiazepam 64 μmol/L group], $P < 0.05$), respectively (Figure 4b).

Effects of 4′-chlorodiazepam on activities of oxidative enzymes during reperfusion

The activity of mitochondrial electron transport chain complex I and complex III was decreased ($P < 0.05$), while

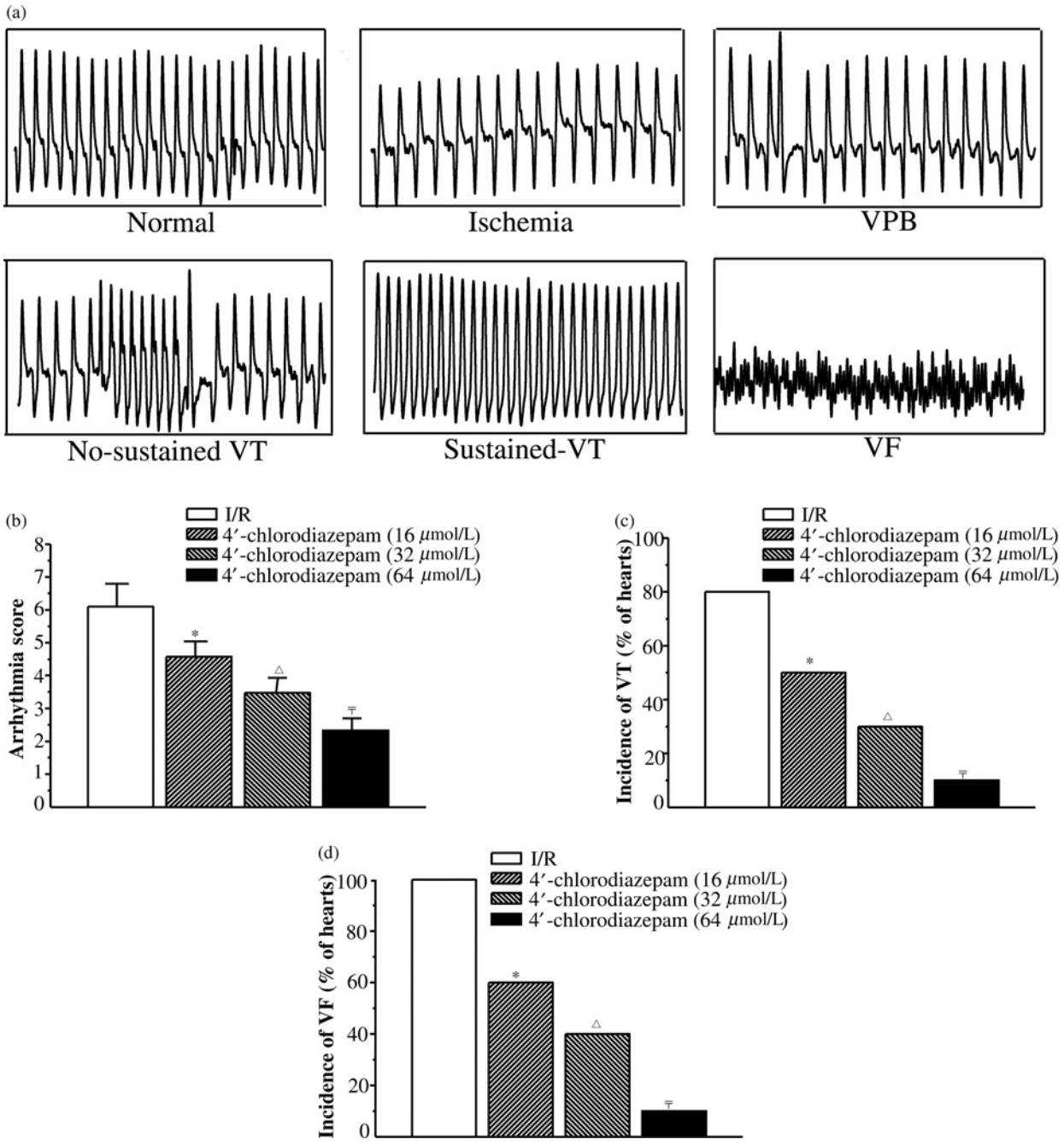


Figure 2 4′-Chlorodiazepam reduces the severity of reperfusion-induced ventricular arrhythmias in rat. (a) Examples of arrhythmias classified according to the Lambeth Conventions. (b) Arrhythmia scores. Values represent mean ± SE ($n = 15$ per group). $^{*}P < 0.05$ versus ischemia/reperfusion (I/R) group; $^{\Delta}P < 0.05$ versus I/R group and 4′-chlorodiazepam (16 μmol/L) group; $^{\mp}P < 0.05$ versus I/R group and 4′-chlorodiazepam (16 and 32 μmol/L) groups. (c) Incidence of ventricular tachycardia. $^{*}P < 0.05$ versus I/R group; $^{\Delta}P < 0.05$ versus I/R group and 4′-chlorodiazepam (16 μmol/L) group; $^{\mp}P < 0.05$ versus I/R group and 4′-chlorodiazepam (16 and 32 μmol/L) groups, $n = 15$ per group. (d) Incidence of VF. $^{*}P < 0.05$ versus I/R group; $^{\Delta}P < 0.05$ versus I/R group and 4′-chlorodiazepam (16 μmol/L) group; $^{\mp}P < 0.05$ versus I/R group and 4′-chlorodiazepam (16 and 32 μmol/L) groups, $n = 15$ per group. VPB, ventricular premature beat; VT, ventricular tachycardia; VF, ventricular fibrillation

that of xanthine oxidase, NADPH oxidase and SOD was increased ($P < 0.05$) in reperfusion. All three different doses of 4′-chlorodiazepam used in this study increased the activity of mitochondrial electron transport chain complex I and complex III and reduced that of xanthine oxidase ($P < 0.05$). In addition, 64 μmol/L 4′-chlorodiazepam reduced the activity of NADPH oxidase ($P < 0.05$) (Table 1).

Discussion

The major findings of the present study are as follows: (1) 4′-chlorodiazepam, a TSPO antagonist, improves cardiac functional recovery during reperfusion in rats. The effects can be identified from the abolition of the increase of LVEDP, the accelerated recovery of LVDP and $\pm dP/dt$ max, and the reduced severity of ventricular arrhythmias; (2) 4′-chlorodiazepam reduces the opening probability of mPTP

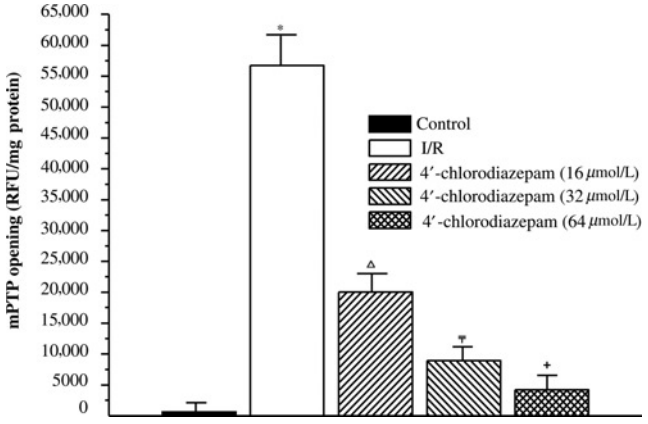


Figure 3 4′-Chlorodiazepam inhibits mitochondrial permeability transition pore opening in reperfusion. Values represent mean ± SE ($n = 15$ per group). $^{*}P < 0.05$ versus control group; $^{\Delta}P < 0.05$ versus control group and ischemia/reperfusion (I/R) group; $^{\mp}P < 0.05$ versus control group, I/R group and 4′-chlorodiazepam (16 μmol/L) group; $^{+}P < 0.05$ versus control group, I/R group and 4′-chlorodiazepam (16 and 32 μmol/L) groups

during reperfusion; (3) 4′-chlorodiazepam decreases the generation of ROS during reperfusion; (4) 4′-chlorodiazepam increases the activity of mitochondrial electron transport chain complex I and complex III, and reduces that of xanthine oxidase and NADPH oxidase.

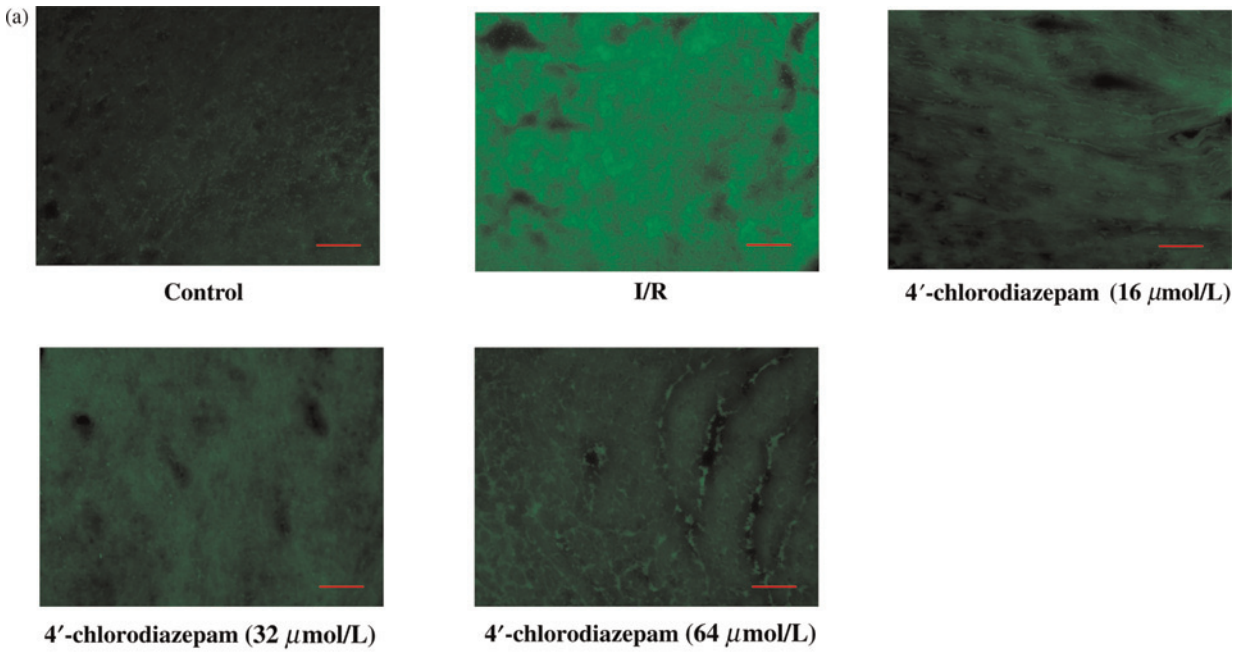
4′-Chlorodiazepam is a widely used TSPO antagonist, and the concentrations we used have been confirmed to be effective in inhibiting TSPO.[14–16] A previous study showed that TSPO inhibition greatly improved postischemic contractile performance in rabbits.[15] In the present study, 4′-chlorodiazepam abolished the increase in LVEDP, and accelerated the recovery of LVDP and $\pm dP/dt$ max in rats. The appearance of arrhythmias is a striking feature in myocardial reperfusion.[20] VF has been regarded as a criterion of potential lethal damage induced by reperfusion injury.[20] TSPO inhibition has been found to be effective in abolishing reperfusion-induced arrhythmias in guinea-pigs and rabbits.[14,15] In this study, it was also found that 4′-chlorodiazepam effectively reduced the severity of ventricular arrhythmias in a dose-dependent fashion in rats. In total, 16, 32 and 64 μmol/L 4′-chlorodiazepam reduced the incidence of VF from a baseline value of 100% to 60%, 40% and 10%, respectively. The anti-arrhythmic effects of 4′-chlorodiazepam, specifically the ability to decrease the incidence of VF and VT, allow the heart to successfully fulfill its role as a pump.[15]

mPTP is located at the contact sites between the outer and the inner mitochondrial membranes and, in its open state, enables free passage of low molecular weight compounds (up to molecular weight 1500) between the mitochondrial inner compartment (matrix) and the cytosol.[10] mPTP has been demonstrated to play a key role in the life and death of cells.[10–12,21] mPTP opens under conditions of elevated mitochondrial calcium, especially when associated with oxidative stress and adenine nucleotide depletion; these are exactly the conditions that occur during reperfusion following a period of ischemia.[11,12,21] mPTP opening is a critical contributing factor in I/R injury.[22–25] A recent study reported that 4′-chlorodiazepam can reduce myocardial infarct size when administered either before or after I/R and that it inhibits mPTP opening.[26] Several reports have indicated that every strategy able to inhibit mPTP opening is potentially cardioprotective in I/R injury.[27–29] In the present study, 4′-chlorodiazepam reduced the opening probability of mPTP during reperfusion, which may be a key to the improvement of cardiac functional recovery.

mPTP is Ca^{2+}-, redox-, voltage- and pH-sensitive. mPTP opening probability is increased by matrix-free Ca^{2+}, ROS, membrane potential depolarization and high pH (>7.0).[30] In physiological settings, Ca^{2+} and ROS are the most important triggers of mPTP opening.[11,12] It has also been found that ROS rather than Ca^{2+} appears to be the more important mPTP trigger in excitable cells such as cardiomyocytes and neurons.[11,12] Additionally, mPTP opening triggered by calcium does not necessarily require an increase in matrix Ca^{2+}, but may be the consequence of an increase in oxidative stress that sensitizes the mPTP to the prevailing Ca^{2+}.[11,12] Therefore, it is suggested that the most critical factor influencing mPTP opening probability is the degree of oxidative stress.[11,12,31] A previous study has shown that TSPO inhibition could counteract H_2O_2-induced impairment of cardiac mitochondrial oxidative phosphorylation as well as cardiomyoblast apoptosis, indicating that TSPO inhibition could prevent the generation of ROS.[13] The present study showed that 4′-chlorodiazepam decreased the generation of ROS during reperfusion, which may be the basis for decreased mPTP opening probability.

Mitochondria are a major source of ROS during reperfusion.[32,33] Two components of the respiratory chain, NADH dehydrogenase in complex I and the ubiquinone–cytochrome *b* area of complex III, are responsible for ROS generation. Decreased oxidative capacity of these complexes leads to increased deleterious production of oxygen radicals.[32,33] In addition, xanthine oxidase and NADPH oxidase are also the primary intracardiac source of ROS during I/R. SOD is the predominant endogenous antioxidant.[34–36] Under I/R, antioxidant defenses are overwhelmed by excess ROS and cellular injury occurs.[32,33] Therefore, the above oxidative enzymes were explored in this study. The activities of mitochondrial electron transport chain complex I and complex III were decreased and that of xanthine oxidase and NADPH oxidase were increased in reperfusion; these findings are consistent with the available literature.[37–40] Moreover, it was also found in this study that the antioxidative effects of 4′-chlorodiazepam were related to the increased activities of mitochondrial electron transport chain complex I and complex III as well as the decreased activities of xanthine oxidase and NADPH oxidase; this may provide a basis for the antioxidative effects.

In conclusion, our results indicate that 4′-chlorodiazepam may improve cardiac functional recovery via alteration of oxidative enzyme activities, reduction of ROS level and thereby inhibition of mPTP opening during reperfusion. In this respect, it would be very interesting to explore the potential of 4′-chlorodiazepam as an alternative adjunct for reperfusion therapy.



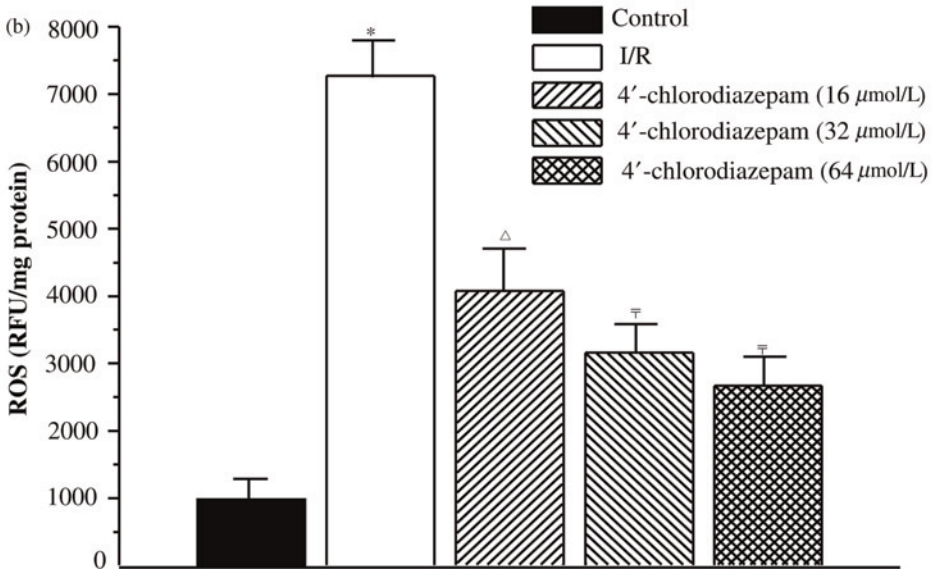


Figure 4 4′-Chlorodiazepam reduces the level of reactive oxygen species (ROS). (a) Representative qualitative assessment images of ROS (×400). Bar, 40 μm. (b) The level of ROS. Values represent mean ± SE ($n = 15$ per group). $^{*}P < 0.05$ versus control group; $^{\triangle}P < 0.05$ versus control group and ischemia/reperfusion (I/R) group; $^{\mp}P < 0.05$ versus control group, I/R group and 4′-chlorodiazepam (16 μmol/L) group

Table 1 Effects of 4′-chlorodiazepam (DZP) on activities of oxidative enzymes during reperfusion ($n = 15$)

Group	Complex I (nmol/L NADH/min/mg)	Complex III (nmol/L ubiquinone/min/mg)	Xanthine oxidase (μmol/L xanthine/min/mg)	NADPH oxidase (μmol/L NADPH/min/mg)	SOD (U/mg)
Control group	121.10 ± 1.52	959.67 ± 13.85	0.14 ± 0.04	0.08 ± 0.01	0.60 ± 0.03
I/R group	60.79 ± 0.99*	614.67 ± 10.16*	0.24 ± 0.03*	0.18 ± 0.02*	1.42 ± 0.10*
DZP (16 μmol/L) group	80.73 ± 1.12*△	879.72 ± 11.02*△	0.15 ± 0.02△	0.16 ± 0.01*	1.36 ± 0.09*
DZP (32 μmol/L) group	85.76 ± 1.34*△	883.89 ± 12.72*△	0.14 ± 0.01△	0.15 ± 0.02*	1.44 ± 0.13*
DZP (64 μmol/L) group	92.89 ± 1.40*△	953.82 ± 13.71△	0.15 ± 0.02△	0.08 ± 0.02△	1.39 ± 0.08*

Complex I, mitochondrial complex I; Complex III, mitochondrial complex III; I/R, ischemia/reperfusion $^{*}P < 0.05$ versus control group; $^{\triangle}P < 0.05$, versus I/R group

Limitations

Hearts were perfused with 4′-chlorodiazepam during the whole experiment in the present study. In order to make this study applicable to clinical practice, future experiments should define the optimal time frame for perfusion with 4′-chlorodiazepam, i.e. duration of 4′-chlorodiazepam perfusion before ischemia and during reperfusion. In addition, future studies should determine whether the effect is reperfusion-specific.

Author contributions: Y-HC designed the experiment and wrote the manuscript. JX and YL established the ischemia reperfusion model and conducted the pharmacological intervention. FL isolated the mitochondria and measured the mPTP opening. DL and HZ measured ROS and the activities of oxidative enzymes and analyzed the data. They have participated sufficiently in the work. JX, DL and HZ contributed equally to this work.

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