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RESEARCH PAPER

β_2-adrenoceptor agonist clenbuterol reduces infarct size and myocardial apoptosis after myocardial ischaemia/reperfusion in anaesthetized rats

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Background and purpose: Considerable evidence indicates that the β_2-adrenoceptor agonist clenbuterol decreases apoptosis in a rodent model of ischaemic cardiomyopathy. In this study, we investigated the effects of clenbuterol on infarct size caused by myocardial ischaemia/reperfusion (I/R) in anaesthetized rats.
Experimental approach: Rats were randomly assigned to the following groups: (i) sham (ii) I/R (iii) clenbuterol + I/R (iv) ICI 118551 + clenbuterol + I/R (v) metoprolol + clenbuterol + I/R (vi) metoprolol + I/R (vii) pertussis toxin + clenbuterol + I/R. Under anaesthesia, left anterior descending coronary artery was occluded for 30 min followed by reperfusion for 2 h.
Key results: Compared with the control I/R group,the clenbuterol (0.5 mg·kg^{-1}, i.p.) group had reduced infarct size, improved diastolic function and sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) activity, increased superoxide dismutase activity, and decreased malondialdehyde (MDA) level and LDH, CK release. Clenbuterol increased the phosphorylation of ERK1/2, which resulted in inhibition of myocardial apoptosis as indicated by the reduction of terminal deoxynucleotidyltransferase end labelling-positive staining, Bax/Bcl-2 mRNA and caspase-3 protein expression. The G_i-protein inhibitor pertussis toxin blocked the clenbuterol-induced improvement in cardiac function and infarct size**.** Pretreatment with ICI 118551(a selective β_2-adrenoceptor antagonist) inhibited the effects of clenbuterol mentioned above. The β_1-adrenoceptor agonist metoprolol had similar effects to clenbuterol but failed to reduce MDA and improve SERCA activity. When administered together, metoprolol and clenbuterol did not induce synergistic effects.
Conclusions and implications: Clenbuterol pretreatment provides significant cardioprotection against ischaemia/reperfusion injury and this is mediated by the β_2-adrenoceptor–G_i-protein signalling. A combination of the β_2-adrenoceptor agonist clenbuterol and the β_1-antagonist metoprolol did not lead to a synergistic anti-apoptotic effect.
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Keywords: β_2-adrenoceptor; myocardial ischaemia/reperfusion; apoptosis

Abbreviations: AAR, area-at-risk; $+dP/dt_{max}$, maximum left ventricular dP/dt; HR, heart rate; LAD, left anterior descending; LV, left ventricular; LVEDP, left ventricular end-diastolic pressure; LVSP, left ventricular systolic pressure; MI/R, myocardial ischaemia/reperfusion; PTX, pertussis toxin; SERCA, sarcoplasmic reticulum calcium-ATPase; TUNEL, terminal deoxynucleotidyltransferase end labelling

Introduction

Ischaemic heart disease is one of the most common causes of death in developed countries. In the ischaemic heart, restoration of the antegrade coronary flow in the infarct-related coronary artery is imperative to limit myocardial ischaemic necrosis (Yellon and Hausenloy, 2007); however, it can also lead to irreversible myocardial damage. This is caused by reperfusion itself which induces the generation of reactive oxygen species, intracellular calcium overload and inflammation, and these changes eventually interact to accelerate myocardial apoptosis (Lin *et al.*, 2005; Timmers *et al.*, 2009).

Clenbuterol is a β_2-adrenoceptor agonist which was first used in the mid-1970s to treat asthma, and has been approved

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for this indication in Europe (Salorinne *et al*., 1975). Recently, clenbuterol has been regarded as a potential treatment for cardiac diseases, especially for the dilated cardiomyopathy of patients who have had a left ventricular assist device implanted (Ahmet *et al*., 2008; Soppa *et al*., 2008).

However, clenbuterol has been reported to induce cardiomyocyte apoptosis in normal healthy animals (Burniston *et al*., 2005a, b; 2006). Whereas, clenbuterol (Xydas *et al*., 2006) and another β_2-adrenoceptor agonist, fenoterol (Ahmet *et al*., 2004), have each been shown to have beneficial effects on the myocardium of animals subjected to coronary artery ligation. Also, clenbuterol decreased apoptosis and improve calcium homeostasis in a rodent model of ischaemic cardiomyopathy (Xydas *et al*., 2006). In addition, clenbuterol elevated Bcl-2 and Bcl-xl protein levels in the rat hippocampus after transient forebrain ischaemia *in vivo* (Zhu *et al*., 1999). We previously reported that the β_2-adrenoceptor agonist clenbuterol reduced myocardial ischaemia/reperfusion (MI/R)-induced cardiomyocyte apoptosis in rat isolated hearts (Liu *et al*., 2008). In rat ventricular cardiomyocytes, there is accumulating evidence showing that β_2-adrenoceptor agonists can activate G_s- and G_i-protein. Coupling to the G_s protein elicits a pro-apoptotic effect, whereas coupling to the G_i protein elicits an anti-apoptotic effect. Thus, the cardioprotection induced by clenbuterol stimulation is likely to be mediated by the β_2-adrenoceptor through coupling to the G_i protein.

The toxic effect induced by stimulation of β-adrenoceptors is mediated primarily by β_1-adrenoceptors, whereas β_2-adrenoceptor stimulation has antiapoptotic and cardioprotective effects. This raises the possibility that combination therapy with a β_2- agonist such as clenbuterol and a β_1-adrenoceptor antagonist such as metoprolol may produce synergistic effects on myocardial ischaemia/reperfusion injury.

Therefore, the aims of the present study were (i) to determine the potential effects of clenbuterol in limiting infarct size and in reducing cardiomyocyte apoptosis after myocardial ischaemia/reperfusion in anaesthetized rats both alone and in combination with metoprolol; (ii) to investigate the role of Gi protein in the cardioprotective effect of clenbuterol; and (iii) to investigate the underlying effects of clenbuterol on haemodynamics and oxidative stress. In addition, we studied the effects of clenbuterol on sarcoplasmic reticulum Ca^{2+}-ATPase activity and the phosphorylation of ERK1/2.

Methods

Experimental animals

Male Sprague-Dawley rats (Grade II, Certificate no. 2004-0007) weighing 250–300 g were purchased from the Experimental Animal Center of Tongji Medical College (Wuhan, China). All animals were treated in accordance with the guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

Experimental protocol

Rats were randomly divided into seven groups, each consisting of eight animals (i) sham group (sham), sham-operated rats in which no tightening of the left anterior descending (LAD) coronary artery sutures was performed; (ii) MI/R group (0.9% saline + MI/R); (iii) clenbuterol group (clenbuterol + MI/R), rats were given clenbuterol (0.5 $mg \cdot kg^{-1}$) by i.p. injection 1 h before ischaemia; (iv) ICI 118551 group (ICI l18551 + clenbuterol + MI/R), rats were pretreated with ICI 118551 (5 $mg \cdot kg^{-1}$) by i.p. injection 30 min before injection of clenbuterol (0.5 $mg \cdot kg^{-1}$); (v) metoprolol + clenbuterol group (metoprolol + clenbuterol + MI/R), rats were given metoprolol (5 $mg \cdot kg^{-1}$) and clenbuterol (0.5 $mg \cdot kg^{-1}$) by i.p. injection 1 h before ischaemia; (vi) metoprolol group (metoprolol + MI/R), rats were given metoprolol (5 $mg \cdot kg^{-1}$) by i.p. injection 1 h before ischaemia; (vii) PTX + clenbuterol group (PTX + clenbuterol + MI/R), rats were given an i.p. injection of pertussis toxin (20 $\mu g \cdot kg^{-1}$) 48 h before the study and clenbuterol (0.5 $mg \cdot kg^{-1}$) was administered as above. All drug concentrations were chosen based on previous publications that investigated β-adrenoceptor-mediated signalling in apoptosis and our preliminary experiments.

Surgical preparation

Rats were anaesthetized with 20% urethane (0.5 $mL \cdot 100g^{-1}$, i.p.). The neck was opened with a ventral midline incision, and a tracheotomy was performed. The rats were ventilated with room air from a positive pressure ventilator (Zhe Jiang university DH-140, 65$strokes \cdot min^{-1}$, 10 $mL \cdot kg^{-1}$). The right carotid artery was advanced with PE-50 tubing (Becton Dickinson) into the left ventricle (LV) to measure LV systolic pressure (LVSP) and end-diastolic pressure (LVEDP), and maximal rate of rise and decline of LV pressure ($\pm$ dP/dt_{max}). A left thoracotomy was performed at the fifth intercostal space and the pericardium was opened to expose the heart. A 5-0 silk suture was passed around the LAD and the ends were pulled through a small vinyl tube to form a snare and then tightened. Coronary artery occlusion was confirmed by ST-segment elevation in the electrocardiogram (ECG) and the presence of regional cyanosis in the myocardium. After 30 min period of ischaemia, the myocardium was reperfused by releasing the snare gently for 2 h (M I/R) (Jin *et al*., 2008). A lead II electrocardiogram and haemodynamic parameters were monitored throughout the duration of the experiment. At the end of the reperfusion period, rats were killed for biochemical studies and other analysis.

Infarct size assessment

Myocardial infarct size was assessed with Evans Blue dye (Sigma) and triphenyl tetrazolium chloride (TTC, Amresco). Briefly, after reperfusion, LAD was re-occluded at the same location, and Evans Blue was infused into the abdominal aorta to determinate the area-at-risk (AAR). After excision of the heart, the LV was cut into 2-mm transverse slices from apex to base. The slices were incubated in 1% TTC (pH 7.4) at 37°C for 20 min and then placed in 4% formaldehyde for 1 day. After TTC staining, infarcted areas are clearly visible as TTC-negative (pale) while viable myocardial areas are stained brick red.

Histopathological analysis

At the end of reperfusion, myocardial tissues were immediately fixed in 10% formalin solution and embedded in paraf-

fin. The paraffin-embedded tissues were sectioned and stained with haematoxylin-eosin and analysed by light microscopy (Olympus BX40, Tokyo, Japan). The following morphological criteria (Mohanty *et al*., 2008) were used to determine the histopathological damage: score 0, no damage; score 1 (mild), interstitial oedema and focal necrosis; score 2 (moderate), diffuse myocardial cell swelling and necrosis; score 3 (severe), confluent areas of inflammation, oedema and necrosis; and score 4 (highly severe), massive area of inflammation, oedema and necrosis.

Measurement of plasma LDH and CK activities and malondialdehyde (MDA), and superoxide dismutase (SOD) in cardiac tissue

The blood samples drawn from the abdominal artery at the end of reperfusion were collected in heparin-treated tubes. These samples were centrifuged at 250× *g* for 15 min. The plasma was used for determination of LDH and CK activities with commercial kits (An *et al*., 2006). The frozen cardiac tissue samples were weighed and homogenized (1:10, w/v) in 50 mmol·L^{-1} phosphate buffer (pH 7.4) and kept in an ice-bath. The MDA level and SOD activity were measured by colourimetric analysis using a spectrophotometer with the associated detection kits (Jiancheng, Nanjing, China) respectively.

Preparation of sarcoplasmic reticulum and measurement of sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) activity

The sarcoplasmic reticulum membrane was prepared according to the methods of Osada *et al*. (1998). Briefly, the LV was homogenized in 10 mL ice-cold lysis buffer (50 mM Na_2HPO_4, 10 mM Na_2EDTA and 25 mM NaF, pH 7.4). The homogenate was centrifuged at 14 000× *g* for 20 min at 4°C, and the supernatant obtained was further centrifuged at 45 000× *g* for 30 min. The pellet obtained was resuspended in storage buffer (30 mM histidine, 0.25 mM sucrose, 10 mM EDTA and 10 mM NaF at pH 7.4), and stored at –80°C. The concentration of protein was determined by a modified Bradford assay.

The activity of Ca^{2+}-ATPase was determined with a kit (Jiancheng, Nanjing, China) by measuring the inorganic phosphate (Pi) liberated from ATP hydrolysis (Henkel *et al*., 1988). Sarcoplasmic reticulum Ca^{2+}-ATPase activity was assayed in a medium (50 mM histidine, 3 mM $MgCl_2$, 100 mM KCl, 5 mM sodium azide, 3 mM ATP and 0.05 mM $CaCl_2$, pH 7.0). Cardiac sarcoplasmic reticulum membranes were added to the reaction mixture at a final concentration of 15–20 μg of protein·mL^{-1}, pre-incubated for 10 min at 37°C. The reaction was initiated by the addition of ATP. Mitochondrial contamination was excluded by determining the activity of azide-sensitive ATPase.

Reverse transcription PCR(RT-PCR) analysis

Total RNA was prepared from rat myocardial tissue samples using Trizol reagent (Gibco BRL, USA) following the manufacturer's protocol. Complementary DNA was generated by SuperScript First-Strand Synthesis System for RT-PCR using oligo(dT) as primer.

The sense and anti-sense primer sequences used were: Bax: 5′-AAGAAGCTGAGCGAGTGTCT -3′; 5′-CAAAGATGGTCAC TGTCTGC-3′; Bcl-2: 5′-CTGGTGGACAACATCGCTCTG-3′; 5′-GGTCTGCTGACCTCACTTGTG-3′; β-actin: 5′-CGTAAAGAC CTCTATGCCAA-3; 5′-AGCCA TGCCAAATGTGTCAT-3. These primer sets yielded PCR products of 360bp, 228bp and 314bp for Bax, Bcl-2 and β-actin respectively. PCR (35 cycles for Bax and Bcl-2, 25cycles for β-actin cDNA) was performed using the GeneAmp PCR System 2400 (PA). The PCR products were electrophoresed through a 1.5% agarose gel and visualized by ethidium bromide staining and UV irradiation. The gel photographs were scanned with a computerized densitometer (SYNGENE, London, UK).

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL) assay

After the reperfusion, the hearts were isolated from each group for the TUNEL assay with a commercial *in situ* apoptosis detection kit (Dead End Colorimetri, TUNEL system, Promega). The TUNEL staining technique labels broken strands of DNA which arise mainly from apoptosis but also from necrosis(Scarabelli *et al*., 1999). The tissues were embedded in paraffin, cut into 4-μm sections. The paraffin was removed from the sections with xylene, they were rehydrated as described previously, and incubated with 20 mg·mL^{-1} proteinase K for 10 min, then rinsed in distilled water. Endogenous peroxidase was blocked by incubating the tissue sections in 3% hydrogen peroxide in methanol for 30 min at room temperature. The sections were then washed several times in PBS and then incubated with equilibration buffer for 10–15 s and TdT-enzyme for 60 min in a humidified atmosphere at 37°C. The sections were subsequently placed in pre-warmed working-dilution stop/wash buffer at room temperature for 10 min and then incubated with streptavidin–peroxidase for 45 min. PBS was used to wash the tissue sections carefully between each step. Diaminobenzidine was used as a chromogen and counterstaining was performed using Mayer's haematoxylin. All steps were performed according to the instructions of the kit. Three sections from each myocardial sample were randomly selected and ten microscopic fields per section were evaluated. In each field, the percentage of TUNEL-positive nuclei was calculated (number of apoptotic myocytes/the total number of myocytes counted × 100). Heart tissue samples for TUNEL analysis were obtained from the margins of noninfarcted but ischaemic areas.

Western blotting

Total protein was extracted with cell lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 3% SDS, 1 mM phenylmethylsulphonyl fluoride and 5 μg·mL^{-1} protease inhibitor cocktail) from area at risk zones of the heart. In total, 50–80 μg of total proteins was loaded onto 10% gradient SDS-polyacrylamide gels. Following electrophoresis, the proteins were transferred to an immobilon-polyvinyliding fluoride (PVDF) membrane. After the non-specific binding sites on the membrane were blocked with 5% non-fat dry milk in TBS-T for 1 h, the membrane was incu-

bated with mouse monoclonal antibody p-ERK1/2 (Santa Cruz Biotechnology), anti-ERK2 antibody (Cell Signaling Technology), or anti-caspase-3 antibody (Santa Cruz Biotechnology), or anti-β-actin antibody (Santa Cruz Biotechnology). Bound antibody was detected by horseradish peroxidase (HRP) conjugated anti-mouse IgG or anti-rabbit IgG. Finally, Immunoreactive bands were visualized with an ECL reagent. The intensity of bands was quantified by densitometry.

Statistical analysis

All data are expressed as means± SEM. Statistical significance of differences between means was analysed by one-way ANOVA followed by Student-Newman-Keuls test. A P-value < 0.05 was considered to be significant.

Materials

Clenbuterol (a β_2-adrenoceptor agonist) and metoprolol (a β_1-adrenoceptor-selective antagonist) were purchased from Sigma (St Louis, MO, USA); ICI 118551 (a β_2-AR-selective antagonist) was from Zeneca Pharmaceuticals (Cheshire, UK); pertussis toxin (PTX, a G_i-protein inhibitor) was from Calbiochem-Novabiochem Co (La Jolla, CA). Nomenclature for receptors and compounds is in accordance with the British Journal of Pharmacology's Guide to Receptors and Channels (Alexander *et al*., 2008).

Results

Effect on myocardial infarct size

To evaluate the effect of clenbuterol on myocardial infarct size, we measured area at risk and infarct size (Figure 1). AAR size was not significantly different among groups. Treatment with clenbuterol, metoprolol + clenbuterol and metoprolol had a smaller infarct size compared with the MI/R group, and there were no differences among the three groups. However, the clenbuterol-induced infarct-limiting protection was completely abolished by the β_2-adrenoceptor blocker, ICI l 18551.

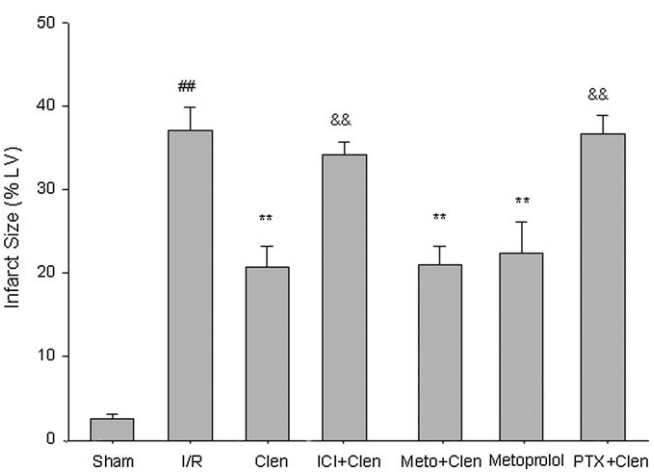


Figure 1 Myocardial infarct size quantified as a percentage of the total left ventricle myocardial infarction. Sham-operated rats or rats with different pretreatments were subjected to 30 min of ischaemia and 2 h of reperfusion. $^{\#\#}P < 0.01$ versus sham; $^{**}P < 0.01$ versus I/R group; $^{\&\&}P < 0.05$ versus clenbuterol. Clen, clenbuterol; ICI, ICI 118551; I/R, ischaemia/reperfusion; meto, metoprolol.

Role of G_i-protein-coupled receptors in clenbuterol-mediated cardioprotection

Rats were treated with PTX 48 h before ischaemia. Figure 2 shows that pretreatment with PTX blocked the clenbuterol-induced improvement in cardiac function ($+dP/dt_{max}$ and LVSP) after ischaemia–reperfusion injury. We also examined the effect of PTX pretreatment on infarct size. As shown in Figure 1, the clenbuterol group had a significantly smaller infarct size than the MI/R group. Pretreatment with PTX blocked the clenbuterol-induced reduction in infarct size.

Haemodynamic data

Pretreatment with clenbuterol decreased the LVEDP compared with the control I/R group during the course of ischaemia and reperfusion ($P < 0.05$), whereas the metoprolol group had lower heart rate (HR) and LVEDP than the control I/R group (Figure 2). Compared with the clenbuterol group, the ICI 118551 + clenbuterol group had a significantly decreased $+dP/dt_{max}$ at 80, 100 and 120 min of reperfusion ($P < 0.05$). There were no differences in LVSP or $+dP/dt_{max}$ among clenbuterol, metoprolol and metoprolol + clenbuterol groups.

Histopathological results

Microscopic histology revealed that the non-infarcted myocardium in the sham group is characterized by an organized pattern and shows normal architecture of the myocardium. In contrast, the myocardium of the control I/R group presented marked oedema, confluent areas of myonecrosis, myofibre loss as compared with those in the sham group. In the clenbuterol, metoprolol and metoprolol + clenbuterol pretreated rats subjected to ischaemia and reperfusion, occasional focal myofibre loss, necrosis and oedema were observed but these were significantly less compared with the control I/R group. The degree of oedema and necrosis was more severe in the ICI 118551 + clenbuterol group than in the clenbuterol group (Figure 3).

Biochemical results

The biochemical indicators of myocardial damage were evaluated after the MI/R injury. A significant increase in LDH, CK levels were observed in the control I/R group as compared with the sham group ($P < 0.01$). In addition, in the control I/R group, a higher level of MDA and lower level of SOD ($P < 0.01$) in comparison with the sham group were observed.

Clenbuterol and metoprolol treatment both alone and in combination led to decreased levels of the LDH and CK activities as compared with the control I/R group ($P < 0.05$). Figure 4 shows that clenbuterol, metoprolol + clenbuterol prevented the increase in I/R-augmented MDA levels. Metoprolol alone tended to reduce the MDA levels but this was not statistically significant. The SOD activities were significantly

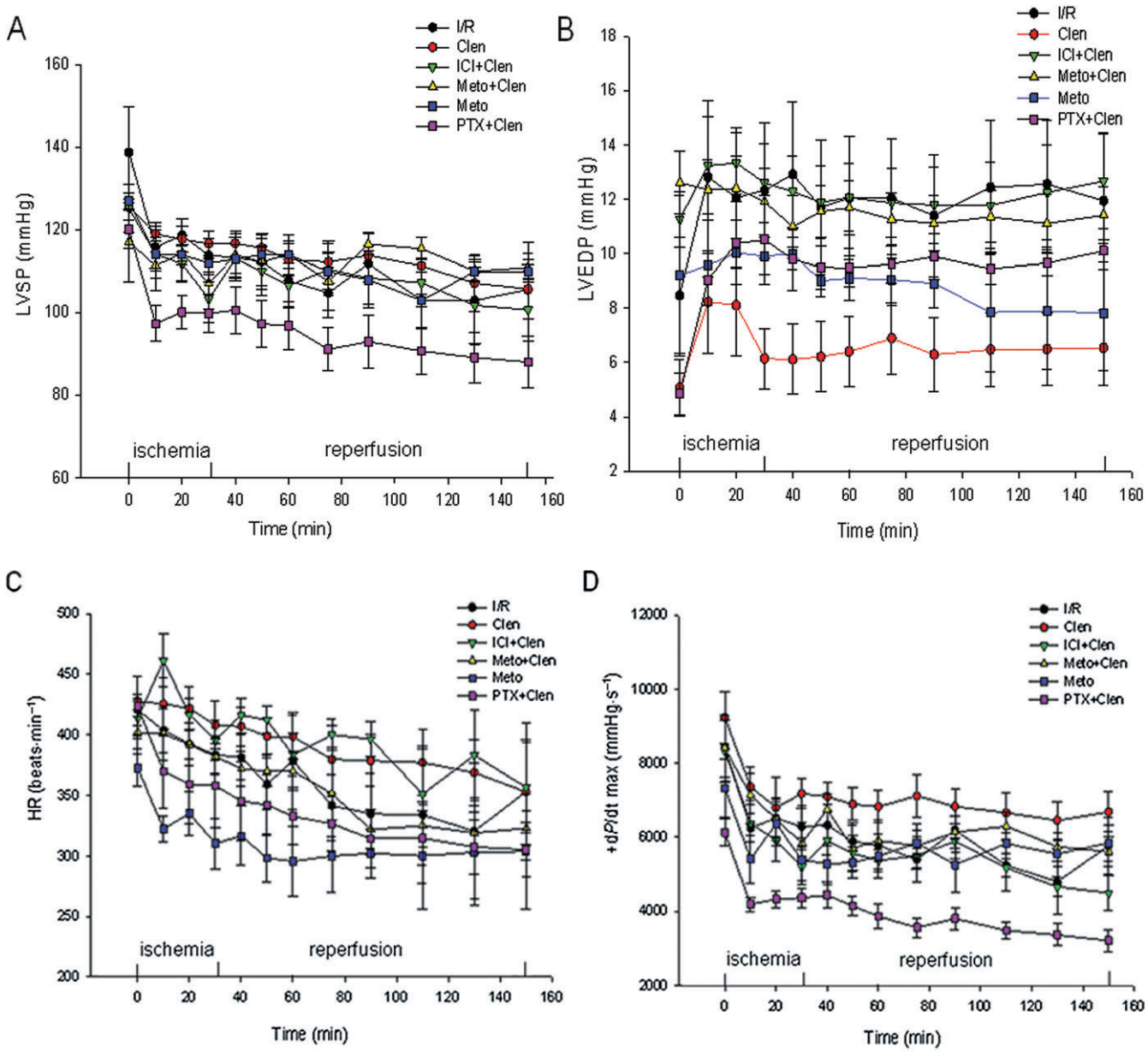


Figure 2 Effect of clenbuterol and metoprolol on haemodynamic variables. Time courses of LVSP(A), LVEDP (B), HR (C) and +dP/dt$_{max}$ (D) were continuously recorded throughout the experiment(0–30 min: ischaemia, 30–150 min: reperfusion). Each point represents the mean ± SEM of eight separate rats. +dP/dt$_{max}$, maximum left ventricular dP/dt; HR, heart rate; LVEDP, left ventricular end-diastolic pressure; LVSP, left ventricular systolic pressure.

increased in the clenbuterol, metoprolol and metoprolol + clenbuterol groups versus the control I/R group, and there were no differences among the three groups ($P > 0.05$). Moreover, pretreatment with ICI 118551 led to an increase in CK, LDH and MDA, and a decrease in SOD compared with the clenbuterol group ($P < 0.05$).

Effect on Ca^{2+}-ATPase activity

The fact that calcium homeostasis plays an important role in the ischaemia/reperfusion-induced myocardial cell injury has been well-documented, and because the re-uptake of Ca^{2+} by the sarcoplasmic reticulum is mainly via sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (>90% Ca^{2+} in cytoplasm), we used an optical assay to examine the activity of Ca^{2+}-ATPase. As depicted in Figure 5, the Ca^{2+}-ATPase activity in control I/R hearts was significantly reduced compared with the sham group (12.47 ± 1.40 μmol $Pi \cdot h^{-1} \cdot mg^{-1}$ protein vs. 50.29 ± 6.42 μmol $Pi \cdot h^{-1} \cdot mg^{-1}$ protein, $P < 0.01$). Interestingly, the activity of Ca^{2+}-ATPase was higher in the clenbuterol, metoprolol and metoprolol + clenbuterol groups (27.86 ± 5.27 μmol $Pi \cdot h^{-1} \cdot mg^{-1}$ protein, 23.01 ± 1.51 μmol $Pi \cdot h^{-1} \cdot mg^{-1}$ protein, 20.15 ± 1.15 μmol $Pi \cdot h^{-1} \cdot mg^{-1}$ protein respectively) compared with the control I/R group. However, the increase reached statistical significance only for clenbuterol ($P < 0.05$, versus the control I/R group) and this

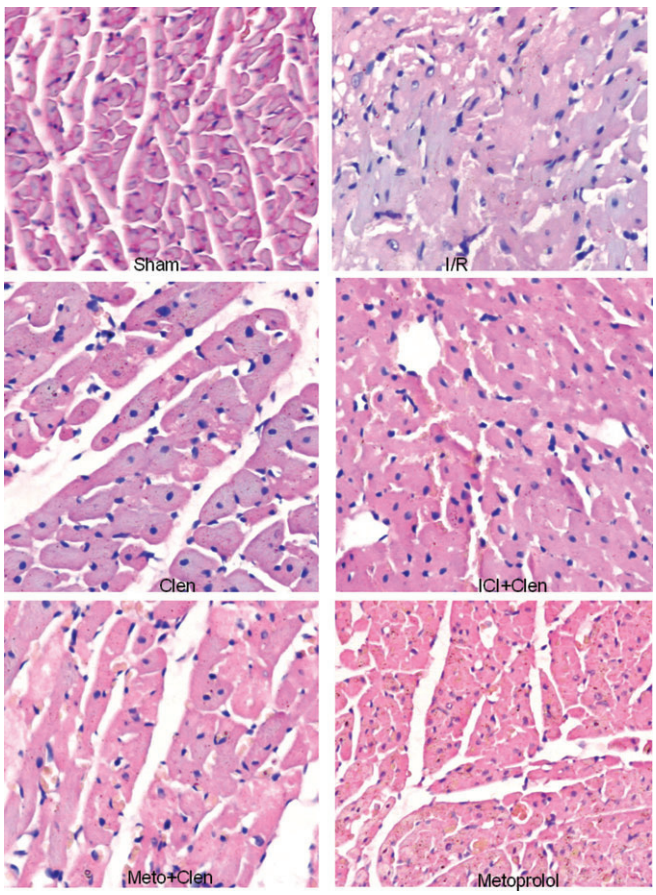


Figure 3 Representative slides of H&E (magnification 200×). A comparison of H&E staining in myocardial tissue from sham-operated rats or rats with different pretreatments after ischaemia 30 min and reperfusion 2 h.

clenbuterol-induced increase in Ca^{2+}-ATPase activity could be prevented by ICI 118551 (12.25 ± 5.68 μmol $Pi \cdot h^{-1} \cdot mg^{-1}$ protein).

Effect on myocardium apoptosis

An increased number of TUNEL-positive cells in the I/R heart indicated that apoptotic death occurred in these animals. The staining of TUNEL was increased in all the LAD ligation groups versus the sham rats, indicating that there was increased apoptosis after surgery in these rats. Clenbuterol and metoprolol treatment both alone and in combination led to decreased levels of TUNEL staining compared with the control I/R group (Figure 6). In contrast, the ICI 118551 + clenbuterol group had an increased number of TUNEL-positive cells compared with the clenbuterol alone group.

Effect of the different treatments on the expression of Bcl-2 and Bax

As shown in Figure 7, clenbuterol and metoprolol pretreatment both alone and in combination significantly increased the expression of Bcl-2 and decreased that of Bax as compared with the control I/R group. The ICI 118551 group revealed a decrease of Bcl-2 and an increase of Bax mRNA levels compared with the clenbuterol group. These results suggested that clenbuterol and metoprolol alone and in combination inhibited myocardial apoptosis, but there was no synergy noted in the effect of metoprolol plus clenbuterol therapy.

Effect of clenbuterol on the expression of caspase-3 protein

We investigated the active p17 subunit of caspase-3, which indicates the proteolytic activation of caspase-3. As illustrated in Figure 8A, an obvious increase of p17 was detectable after myocardial I/R. Clenbuterol and metoprolol pretreatment both alone and in combination reduced the expression of caspase-3 as compared with that in the I/R group, synergy was not seen with the combination therapy. However, ICI 118551 enhanced the activity of caspase-3 compared with the clenbuterol group.

Effect on ERK1/2 activation

Activation of ERK1/2 is associated with cytoprotection in cardiomyocytes and directly mediates the up-regulation of Bcl-2 (Wang *et al*., 2006). ERK1/2 activity was measured using phospho-antibodies. The control I/R group had a decreased level of ERK1/2 activity, the level was up-regulated in the clenbuterol, metoprolol and metoprolol + clenbuterol groups ($P < 0.01$, Figure 8B). ICI 118551 prevented the activation of ERK1/2 induced by clenbuterol.

Discussion

There are four major findings in this study. First, we demonstrated that β_2-adrenoceptor stimulation by clenbuterol leads to a marked reduction of myocardial infarct size in a rat model of myocardial ischaemia/reperfusion. The plausible mechanisms for the reduction of infarct size include improvement of diastolic function, decreased levels of LDH, CK and MDA, increased levels of SOD and Ca^{2+}-ATPase activity, and prevention of cardiomyocyte apoptosis. Second, clenbuterol decreased postischaemic myocardial apoptosis by inhibiting caspase-3 and bax/bcl-2 mRNA expression and increasing the phosphorylation of ERK1/2. Third, we showed that the cardioprotective effect of clenbuterol is mediated by the β_2-adrenoceptor-G_i-protein signalling. Finally, we found no evidence of synergy when the β_2-adrenoceptor agonist was administered combined with a selective β_1-adrenoceptor antagonist, metoprolol.

LDH and CK are important metabolic enzymes in cardiomyocytes, thus the extent of cardiomyocyte CK and LDH release was an important indicator of myocardial I/R injury. In our study, we found that pretreatment with clenbuterol, metoprolol and metoprolol + clenbuterol led to a cardioprotective effect as evidenced by reduced infarct size and the release of LDH and CK. Clenbuterol pretreatment improved recovery of diastolic and systolic function during reperfusion. These results are similar to those of Ahmet *et al*. (2004); they used the β_2-adrenoceptor agonists fenoterol and zinterol in a

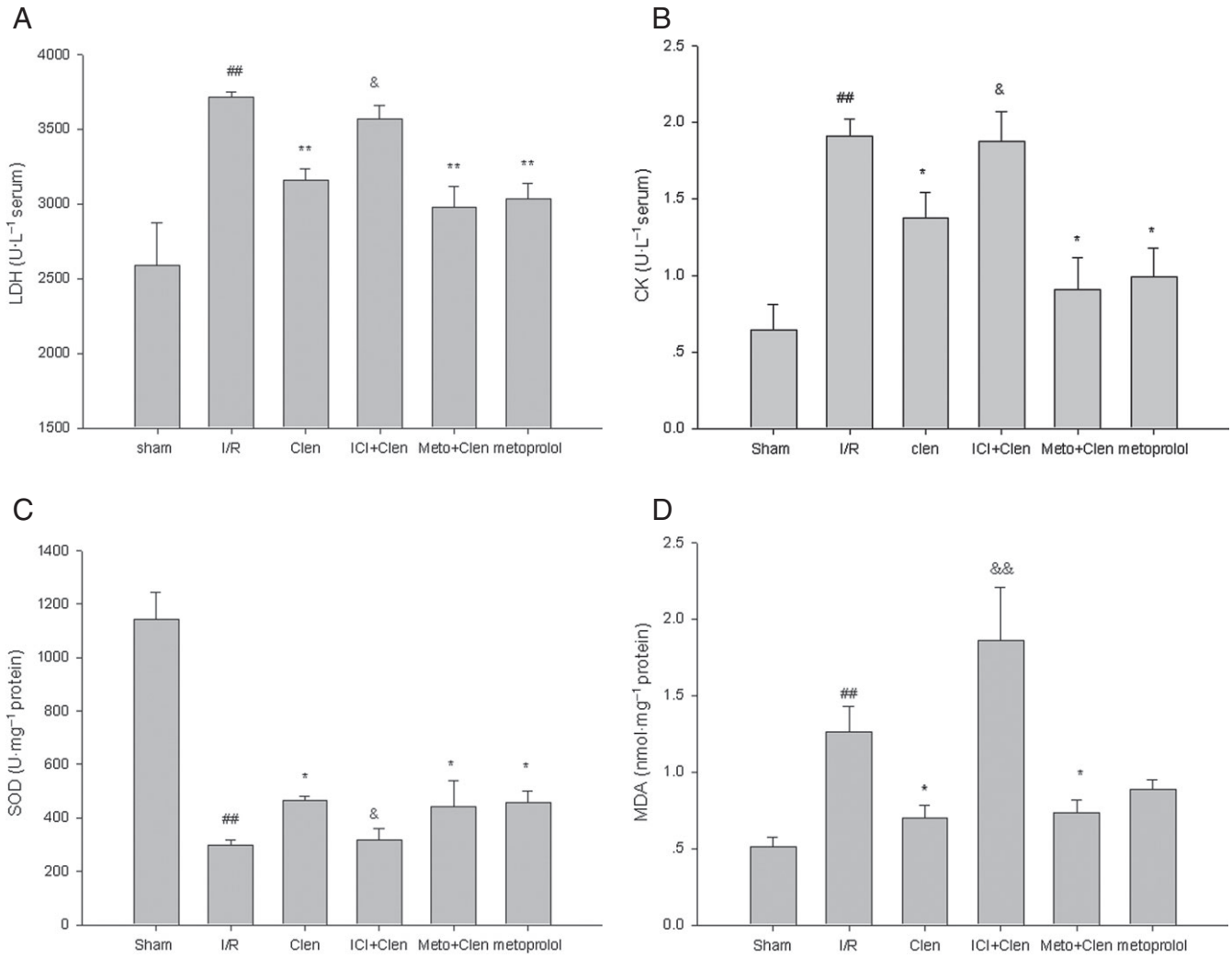


Figure 4 Effect of clenbuterol and metoprolol on serum LDH, CK and myocardial tissues SOD and MDA levels after I/R injury in each group rats. The levels of LDH (A), CK (B), SOD (C) and MDA (D) were measured by using commercial kit supplied from BioAssay Systems. The blood serum samples were collected after 2 h reperfusion to measure the activity of LDH, CK. The myocardial tissue from ischemic zone were taken and measured the activity of SOD and MDA. Results are means ± SEM ($n = 8$ in each groups). $^{\#}P < 0.05$ versus sham, $^{\#\#}P < 0.01$ versus sham, $^{*}P < 0.05$ versus I/R, $^{**}P < 0.01$ versus I/R, $^{\&}P < 0.05$ versus clenbuterol. Clen, clenbuterol; ICI, ICI 118551; I/R, ischaemia/reperfusion; MDA, malondialdehyde; meto, metoprolol; SOD, superoxide dismutase.

rodent LAD-ligation model and found that β_2-adrenoceptor stimulation improved diastolic function.

There is accumulating evidence that oxidative stress induces I/R injury, and reactive oxygen species (ROS) and free radicals have long been recognized to act as major mediators of I/R injury (Nelson *et al*., 1992; Kim *et al*., 2009). MDA, one of the end-products in the lipid peroxidation process, results in cell membrane breakdown and subsequence swelling, which also has been used to assess oxygen-free radical-mediated injury (Ozer *et al*., 2005; Akhlaghi and Bandy, 2009). SOD is an antioxidant enzyme that catalyses the conversion of O_2^- into H_2O_2 and O_2. Reduction of MDA or increase in SOD has been shown to minimize reperfusion injury(Kim *et al*., 2009). In this study, we demonstrated that clenbuterol, metoprolol, and a combination of clenbuterol and metoprolol increased the level of SOD and decreased the content of MDA (except metoprolol alone). However, ICI 118551 could abolish the effect of clenbuterol. The finding that metoprolol was unable to decrease MDA is consistent with the results from previous studies. The β-adrenoceptor antagonist atenolol and metoprolol were found to be ineffective at reducing lipid peroxidation, even at high concentrations *in vitro* (Lysko *et al*., 2000), and metoprolol did not decrease MDA in a model of ischaemia/reperfusion *in vivo* (Zhu *et al*., 2006). These results indicate that metoprolol cannot prevent free-radical-induced lipid oxidation.

It has been demonstrated that ROS decreases the activity of sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), which plays an important role in cardiac calcium handing and myocardial relaxation by coupling the cleavage of ATP to the transport of Ca^{2+} into the sarcoplasmic reticulum lumen. A dysfunction of SERCA has been proposed as a contributing factor to MI/R injury (Tani, 1990; Osada *et al*., 2000). In our study, the I/R rats had a decreased level of Ca^{2+}-ATPase activity compared with the sham-operated rats. The levels of Ca^{2+}-ATPase activity were up-regulated in the clenbuterol, meto-

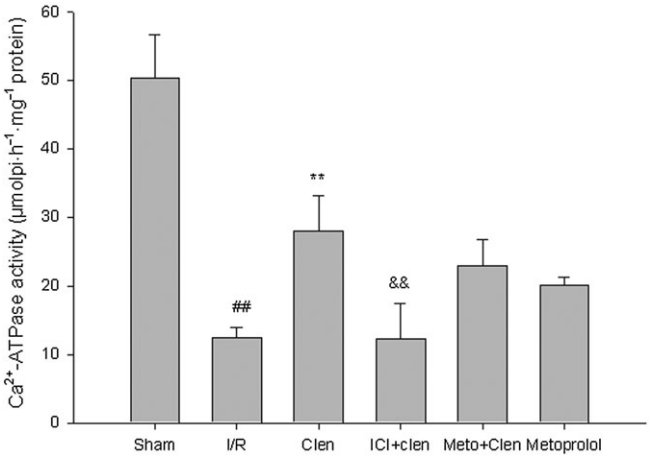


Figure 5 Effect of clenbuterol and metoprolol on sarcoplasmic reticulum Ca^{2+}-ATPase activity in ischaemic/reperfused myocardial tissue. Data are means ± SEM of eight samples. $^{\#\#}P < 0.01$ versus Sham; $^{**}P < 0.01$ versus I/R; $^{\&\&}P < 0.01$ versus clenbuterol. I/R, ischaemia/reperfusion.

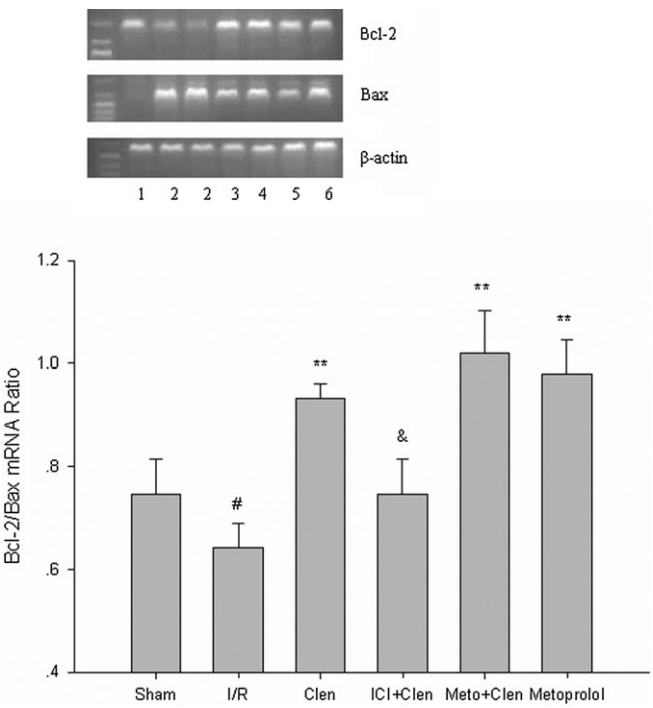
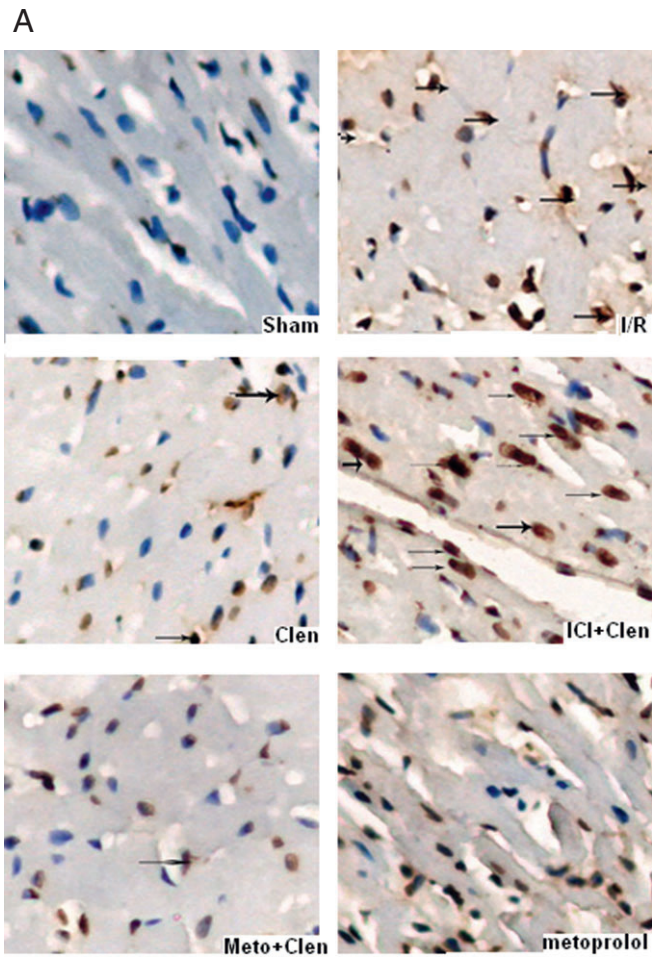


Figure 6 The levels of expression of Bcl-2 and Bax mRNA were detected by RT-PCR in myocardial tissue subjected to 30 min of ischaemia and 2 h of reperfusion. Quantification of the intensities of the Bcl-2 and Bax bands were determined by densitometric scanning of agarose gel and expressed as Bcl-2/Bax ratio. Lane 1: Sham; lane 2: I/R; lane 3: clenbuterol; lane 4: ICI 118551 + clenbuterol; lane 5: metoprolol + clenbuterol; lane 6: metoprolol. Data are means ± SEM of three independent samples. $^{\#}P < 0.05$ versus sham, $^{**}P < 0.01$ versus I/R, $^{\&}P < 0.05$ versus clenbuterol. Clen, clenbuterol; ICI, ICI 118551; I/R, ischaemia/reperfusion; meto, metoprolol.

prolol and metoprol + clenbuterol groups, although the increases reached significance only for clenbuterol. This is consistent with results from a previous study that evaluated the effects of clenbuterol on $SERCA_{2a}$ levels in a rodent model



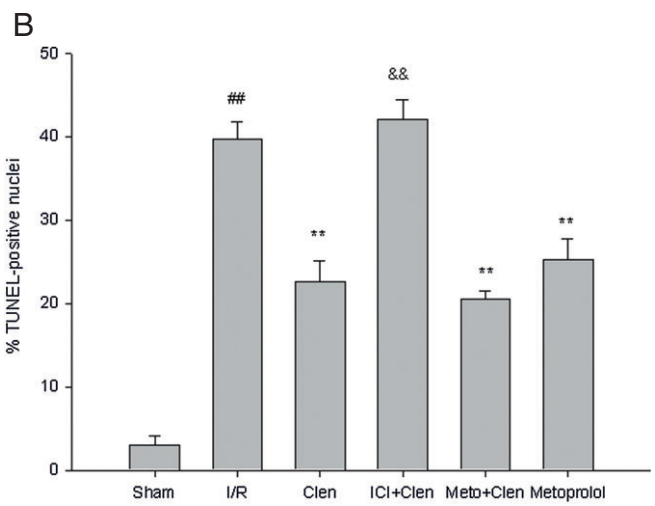


Figure 7 (A) Representative photomicrographs of *in situ* detection of DNA fragment from sham-operated rats or rats with different pretreatments subjected to 30 min of ischaemia followed by 2 h of reperfusion. Arrowheads indicate positive nuclei for TUNEL staining (magnification × 200). (B) Percentage of TUNEL-positive nuclei in sham, I/R and treated groups. $^{\#\#}P < 0.01$ versus sham; $^{**}P < 0.01$ versus I/R; $^{\&\&}P < 0.01$ versus clenbuterol. Clen, clenbuterol; ICI, ICI 118551; I/R, ischaemia/reperfusion; meto, metoprolol; TUNEL, terminal deoxynucleotidyltransferase end labelling.

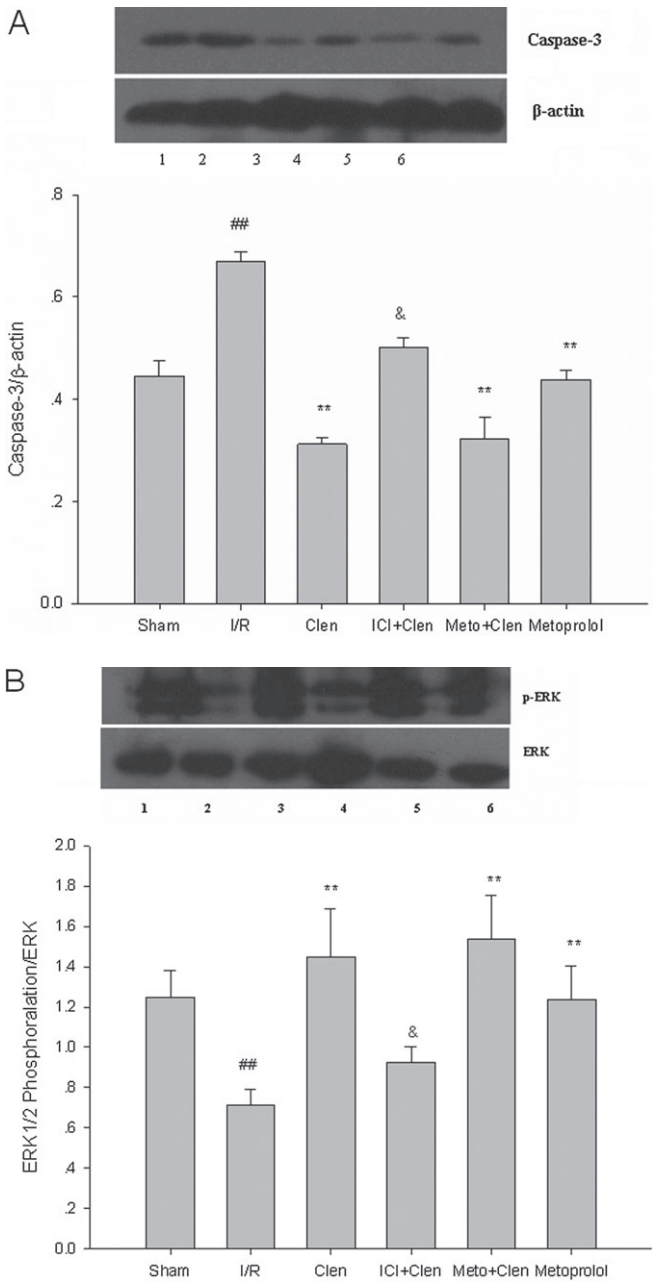


Figure 8 Clenbuterol and metoprolol reduced the expression of cleaved caspase-3 and activated the phosphorylation of ERK1/2 in ischaemic/reperfused myocardial tissue. Rats were pretreated with clenbuterol or/and metoprolol and subjected to 30 min of ischaemia and 2 h of reperfusion. Cleaved caspase-3 (A) and phosphorylated ERK1/2 (B) were detected with Western blot (representative blot above each histogram) analysis by using antibodies specific for caspase p17/β-actin and phosphorylated ERK1/2/total ERK2. The intensity of each band was quantified by densitometry, and data were normalized using the intensity of β-actin or total ERK2. Lane 1: Sham; lane 2: I/R; lane 3: clenbuterol; lane 4: ICI 118551 + clenbuterol; lane 5: metoprolol + clenbuterol; lane 6: metoprolol. $^{\#\#}P < 0.01$ versus sham; $^{**}P < 0.01$ versus I/R; $^{\&}P < 0.05$ versus clenbuterol. I/R, ischaemia/reperfusion.

of ischaemic cardiomyopathy (Xydas *et al*., 2006). It is therefore likely that clenbuterol protects against myocardial ischaemia/reperfusion injury partly by attenuating oxidative myocardial damage and improving calcium handling.

Apoptosis plays a major role in mediating the cell death after ischaemia and reperfusion (Bialik *et al*., 1997; Lv *et al*., 2008). For quantitative analysis of myocardial apoptosis, TUNEL staining is widely used to detect the DNA damage and another of the most widely recognized biochemical features of apoptosis is the activation of a class of cysteine proteases known as caspases (Lv *et al*., 2008). Cells possess multiple caspases, of which caspase-3 activity is required at the step where a protease cascade pathway converges. Under physiological conditions, apoptosis is regulated by the balance of a variety of pro-apoptotic (e.g. bax, Bad) and anti-apoptotic molecules (e.g. Bcl-2, Bcl-x) (Scarabelli *et al*., 1999; Liu *et al*., 2004). Bcl-2 protein, which is an integral membrane protein localized in the outer mitochondrial membrane, nuclear membrane and endoplasmic reticulum (Monaghan *et al*., 1992; Krajewski *et al*., 1993; Zhu *et al*., 1999), can protect against apoptosis induced by ROS producing agents (Nelson *et al*., 1992; Zhu *et al*., 1999; Ozer *et al*., 2005). In contrast, Bax has been shown to actively promote apoptosis, and overexpression of Bax can counteract the anti-apoptotic activity of Bcl-2(Oltvai *et al*., 1993).

ERK1/2, one of the members of MAPK family, is mainly involved in growth factor-induced mitogen signalling and cellular differentiation. ERK1/2 has been demonstrated to protect against ischaemia-reperfusion injury through the inhibition of apoptosis by down-regulation of Bax/Bcl-2 and caspase-3 (Xu *et al*., 2004; Das *et al*., 2009). In this study, we also demonstrated that clenbuterol led to an increase in the ratio of mRNA Bcl-2/Bax and ERK1/2 activation, and reduced the number of TUNEL-positive cells and the expression of caspase-3 protein. The selective β_2-adrenoceptor antagonist ICI 118551 attenuated these effects of clenbuterol. The mechanism for this anti-apoptotic effect may therefore be mediated by stimulation of β_2-adrenoceptors. The results from the present study are consistent with previous reports that clenbuterol and other β_2-adrenoceptor agonists decreased myocardial apoptosis in both *in vivo* (Ahmet *et al*., 2004; Xydas *et al*., 2006) and *in vitro* experimental models (Communal *et al*., 1999; Zhu *et al*., 2001; Liu *et al*., 2008). However, clenbuterol has been reported to cause cardiomyocyte apoptosis in healthy animals (Burniston *et al*., 2005a,b; 2006). When administered to normal healthy animals *in vivo*, β_2-agonists induce cardiomyocyte death by neuromodulation of the sympathetic nervous system and stimulation of the cardiomyocyte β_1-adrenoceptor. In light of this evidence, the β_1-adrenoceptor anatagonist metoprolol should block the cardiotoxity of clenbuterol resulting from its stimulation of the β_1-adrenoceptor. Thus, a combination of the β_1-adrenoceptor antagonist metoprolol and clenbuterol should lead to a synergistic anti-apoptotic effect, based on the theory that stimulation of β_2-adrenoceptors activates pro-apoptotic and anti-apoptotic signals concurrently (Zhu *et al*., 2001). However, synergy was not seen with the combination therapy in our study. This is consistent with prior reports that there was no synergistic anti-apoptotic effect when the two interventions (β_1-adrenoceptor antagonist and β_2-adrenoceptor agonist) were combined in ischaemic heart failure (Xydas *et al*., 2006).

Thus, we speculate that the apparent disparity between the findings of the present work and those of Burniston *et al*. is explained by the different experimental models of rats used

and different doses of clenbuterol. Sichelschmidt *et al*. (2003) reported that ischaemia/reperfusion injury increases PKA, which could phosphorylate the β_2-adrenoceptor and switch coupling of this receptor from G_s to G_i protein (Daaka *et al*., 1997; Zamah *et al*., 2002). The β_2-adrenoceptor is linked to G_i-mediated ERK activation, which is involved in the anti-apoptotic pathway. In our study, the finding that ERK1/2 phosphorylation was increased by clenbuterol is consistent with previous data. We tested the hypothesis that β_2-adrenoceptors protect cardiomyocytes against apoptosis via activation of the G_i protein. In support of this hypothesis, we found that the hearts treated with G_i-protein inhibitor pertussis toxin (PTX) were not protected by clenbuterol, suggesting that the protective effect of clenbuterol is mediated through the G_i protein. Similarly, its has been found that PTX blocks the isoprenaline-induced improvement in postischaemic LVDP and infarct size and that the PKA inhibitors PKI and H-89 limit the protection of isoprenaline, consistent with the hypothesis that PKA-mediated switching of coupling of the β_2-adrenoceptor from G_s to G_i protein is responsible for the protection (Tong *et al*., 2005). Although PKA activity was not measured in the present study, these findings indicate that β_2-adrenoceptors can couple to both G_s and G_i protein, and through a switch in coupling mediated by PKA, β_2-adrenoceptors bind to G_i protein, through which the cardioprotective effect is achieved.

The dosages used for clenbuterol, ICI 118551 and metoprolol administration in the present work were based on previously published reports and our preliminary experiments. Although serum clenbuterol levels were not monitored, the observed changes in infarct size with clenbuterol pharmacotherapy suggest effective and appropriate therapeutic delivery. In addition, metoprolol was also not monitored in our experiments, our finding of a decreased HR is strong evidence of effective drug delivery. In our study, the rats were given a single injection of 0.0016 $mmol \cdot kg^{-1}$ (0.5 $mg \cdot kg^{-1}$) clenbuterol. However, Burniston *et al*. reported that in the heart, a significant incidence of apoptosis was measured after injection of 0.3 $mmol \cdot kg^{-1}$ of either fenoterol or isoprenaline but not clenbuterol. Clenbuterol induced significant cardiomyocyte death at 3 $mmol \cdot kg^{-1}$(Burniston *et al*., 2006). The difference in the doses of clenbuterol might partly contribute to the disparity between the findings of the present work and those of Burniston *et al*.

In our study metoprolol had a similar effect on apoptosis as clenbuterol. This effect of a β_1-adrenoceptor antagonist has also been reported by Zaugg *et al*. (2000); they demonstrated that β-adrenoceptor-mediated apoptosis is largely independent of β_2-adrenoceptor stimulation and mostly mediated via β_1 receptors. The previous study indicated that β_1-adrenoceptor-mediated PKA activation is largely responsible for the apoptosis induced by β-adrenoceptor agonists in adult rat cardiac myocytes. As PKA-mediated switching of coupling of the β_2-adrenoceptor from G_s to G_i protein is responsible for the protection of clenbuterol, metoprolol should not be able to enhance the anti-apoptotic effect of clenbuterol as metoprolol inhibits β_1-adrenoceptor-mediated activation of PKA. Our study and the recent work by Xydas *et al*. (Ahmet *et al*., 2004; Xydas *et al*., 2006) confirmed that a combination of the β_2 -adrenoceptor agonist clenbuterol and the β_1-antagonist metoprolol did not lead to a synergistic anti-apoptotic effect.

In conclusion, our results provide evidence that the β_2-adrenceptor agonist clenbuterol reduces myocardial damage and significantly improves diastolic function, decreases oxidative stress and myocardial apoptosis, and maintains Ca^{2+} handling in the experimental model of I/R injury. Moreover, we demonstrated that the cardioprotective effect of clenbuterol is through the β_2-adrenoceptor-G_i-ERK1/2 signalling. In addition, clenbuterol and metoprolol did not produce a synergistic effect in this model. These results indicate that clenbuterol could have a beneficial effect on cardiac recovery in patients suffering from ischaemic heart disease.

Limitations

The dose-dependence and time-course (including recovery) of clenbuterol on I/R were not investigated. In addition, the mechanisms by which clenbuterol and metoprolol attenuated MI/R injury were not elucidated. As mentioned above, PKA activity and drug-serum levels were also not measured. An improvement in diastolic function, Ca^{2+}-ATPase activity, oxidative stress, and apoptosis and elevated ERK1/2 phosphorylation are only implicated as possible mechanisms. Future studies are needed to characterize more fully the effects and pathways of β_2-adrenoceptor stimulation in I/R injury.

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Conflicts of interest

None.

References

Ahmet I, Krawczyk M, Heller P, Moon C, Lakatta EG, Talan MI (2004). Beneficial effects of chronic pharmacological manipulation of beta-adrenoreceptor subtype signaling in rodent dilated ischemic cardiomyopathy. *Circulation* **110**: 1083–1090.

Ahmet I, Krawczyk M, Zhu W, Woo AY, Morrell C, Poosala S *et al*. (2008). Cardioprotective and survival benefits of long-term combined therapy with beta2 adrenoreceptor (AR) agonist and beta1 AR blocker in dilated cardiomyopathy postmyocardial infarction. *J Pharmacol Exp Ther* **325**: 491–499.

Akhlaghi M, Bandy B (2009). Mechanisms of flavonoid protection against myocardial ischemia-reperfusion injury. *J Mol Cell Cardiol* **46**: 309–317.

Alexander SP, Mathie A, Peters JA (2008). Guide to Receptors and Channels (GRAC). 3rd edn. *Br J Pharmacol* **153** (Suppl. **2**): S1–S209.

An W, Yang J, Ao Y (2006). Metallothionein mediates cardioprotection of isoliquiritigenin against ischemia-reperfusion through JAK2/STAT3 activation. *Acta Pharmacol Sin* **27**: 1431–1437.

Bialik S, Geenen DL, Sasson IE, Cheng R, Horner JW, Evans SM *et al*. (1997). Myocyte apoptosis during acute myocardial infarction in the mouse localizes to hypoxic regions but occurs independently of p53. *J Clin Invest* **100**: 1363–1372.

Burniston JG, Chester N, Clark WA, Tan LB, Goldspink DF (2005a). Dose-dependent apoptotic and necrotic myocyte death induced by the beta2-adrenergic receptor agonist, clenbuterol. *Muscle Nerve* **32**: 767–774.

Burniston JG, Tan LB, Goldspink DF (2005b). beta2-Adrenergic receptor stimulation in vivo induces apoptosis in the rat heart and soleus muscle. *J Appl Physiol* **98**: 1379–1386.

Burniston JG, Tan LB, Goldspink DF (2006). Relative myotoxic and haemodynamic effects of the beta-agonists fenoterol and clenbuterol measured in conscious unrestrained rats. *Exp Physiol* **91**: 1041–1049.

Communal C, Singh K, Sawyer DB, Colucci WS (1999). Opposing effects of beta(1)- and beta(2)-adrenergic receptors on cardiac myocyte apoptosis: role of a pertussis toxin-sensitive G protein. *Circulation* **100**: 2210–2212.

Daaka Y, Luttrell LM, Lefkowitz RJ (1997). Switching of the coupling of the beta2-adrenergic receptor to different G proteins by protein kinase A. *Nature* **390**: 88–91.

Das A, Salloum FN, Xi L, Rao YJ, Kukreja RC (2009). ERK phosphorylation mediates sildenafil-induced myocardial protection against ischemia-reperfusion injury in mice. *Am J Physiol Heart Circ Physiol* **296**: H1236–H1243.

Henkel RD, VandeBerg JL, Walsh RA (1988). A microassay for ATPase. *Anal Biochem* **169**: 312–318.

Jin YC, Kim KJ, Kim YM, Ha YM, Kim HJ, Yun UJ *et al*. (2008). Anti-apoptotic effect of magnolol in myocardial ischemia and reperfusion injury requires extracellular signal-regulated kinase1/2 pathways in rat in vivo. *Exp Biol Med (Maywood)* **233**: 1280–1288.

Kim YM, Ha YM, Jin YC, Shi LY, Lee YS, Kim HJ *et al*. (2009). Palmatine from Coptidis rhizoma reduces ischemia-reperfusion-mediated acute myocardial injury in the rat. *Food Chem Toxicol* **47**: 2097–2102.

Krajewski S, Tanaka S, Takayama S, Schibler MJ, Fenton W, Reed JC (1993). Investigation of the subcellular distribution of the bcl-2 oncoprotein: residence in the nuclear envelope, endoplasmic reticulum, and outer mitochondrial membranes. *Cancer Res* **53**: 4701–4714.

Lin WT, Yang SC, Chen KT, Huang CC, Lee NY (2005). Protective effects of L-arginine on pulmonary oxidative stress and antioxidant defenses during exhaustive exercise in rats. *Acta Pharmacol Sin* **26**: 992–999.

Liu HR, Gao F, Tao L, Yan WL, Gao E, Christopher TA *et al*. (2004). Antiapoptotic mechanisms of benidipine in the ischemic/reperfused heart. *Br J Pharmacol* **142**: 627–634.

Liu P, Xiang JZ, Zhao L, Yang L, Hu BR, Fu Q (2008). Effect of beta2-adrenergic agonist clenbuterol on ischemia/reperfusion injury in isolated rat hearts and cardiomyocyte apoptosis induced by hydrogen peroxide. *Acta Pharmacol Sin* **29**: 661–669.

Lv X, Wan J, Yang J, Cheng H, Li Y, Ao Y *et al*. (2008). Cytochrome P450 omega-hydroxylase inhibition reduces cardiomyocyte apoptosis via activation of ERK1/2 signaling in rat myocardial ischemia-reperfusion. *Eur J Pharmacol* **596**: 118–126.

Lysko PG, Webb CL, Gu JL, Ohlstein EH, Ruffolo RR, Jr, Yue TL (2000). A comparison of carvedilol and metoprolol antioxidant activities in vitro. *J Cardiovasc Pharmacol* **36**: 277–281.

Mohanty IR, Arya DS, Gupta SK (2008). Withania somnifera provides cardioprotection and attenuates ischemia-reperfusion induced apoptosis. *Clin Nutr* **27**: 635–642.

Monaghan P, Robertson D, Amos TA, Dyer MJ, Mason DY, Greaves MF (1992). Ultrastructural localization of bcl-2 protein. *J Histochem Cytochem* **40**: 1819–1825.

Nelson CW, Wei EP, Povlishock JT, Kontos HA, Moskowitz MA (1992). Oxygen radicals in cerebral ischemia. *Am J Physiol* **263**: H1356–H1362.

Oltvai ZN, Milliman CL, Korsmeyer SJ (1993). Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. *Cell* **74**: 609–619.

Osada M, Netticadan T, Tamura K, Dhalla NS (1998). Modification of ischemia-reperfusion-induced changes in cardiac sarcoplasmic reticulum by preconditioning. *Am J Physiol* **274**: H2025–H2034.

Osada M, Netticadan T, Kawabata K, Tamura K, Dhalla NS (2000). Ischemic preconditioning prevents I/R-induced alterations in SR calcium-calmodulin protein kinase II. *Am J Physiol Heart Circ Physiol* **278**: H1791–H1798.

Ozer MK, Parlakpinar H, Cigremis Y, Ucar M, Vardi N, Acet A (2005). Ischemia-reperfusion leads to depletion of glutathione content and augmentation of malondialdehyde production in the rat heart from overproduction of oxidants: can caffeic acid phenethyl ester (CAPE) protect the heart? *Mol Cell Biochem* **273**: 169–175.

Salorinne Y, Stenius B, Tukiainen P, Poppius H (1975). Double-blind cross-over comparison of clenbuterol and salbutamol tablets in asthmatic out-patients. *Eur J Clin Pharmacol* **8**: 189–195.

Scarabelli TM, Knight RA, Rayment NB, Cooper TJ, Stephanou A, Brar BK *et al*. (1999). Quantitative assessment of cardiac myocyte apoptosis in tissue sections using the fluorescence-based tunel technique enhanced with counterstains. *J Immunol Methods* **228**: 23–28.

Sichelschmidt OJ, Hahnefeld C, Hohlfeld T, Herberg FW, Schror K (2003). Trapidil protects ischemic hearts from reperfusion injury by stimulating PKAII activity. *Cardiovasc Res* **58**: 602–610.

Soppa GK, Lee J, Stagg MA, Felkin LE, Barton PJ, Siedlecka U *et al*. (2008). Role and possible mechanisms of clenbuterol in enhancing reverse remodelling during mechanical unloading in murine heart failure. *Cardiovasc Res* **77**: 695–706.

Tani M (1990). Mechanisms of Ca2+ overload in reperfused ischemic myocardium. *Annu Rev Physiol* **52**: 543–559.

Timmers L, Henriques JP, de Kleijn DP, Devries JH, Kemperman H, Steendijk P *et al*. (2009). Exenatide reduces infarct size and improves cardiac function in a porcine model of ischemia and reperfusion injury. *J Am Coll Cardiol* **53**: 501–510.

Tong H, Bernstein D, Murphy E, Steenbergen C (2005). The role of beta-adrenergic receptor signaling in cardioprotection. *FASEB J* **19**: 983–985.

Wang CX, Song JH, Song DK, Yong VW, Shuaib A, Hao C (2006). Cyclin-dependent kinase-5 prevents neuronal apoptosis through ERK-mediated upregulation of Bcl-2. *Cell Death Differ* **13**: 1203–1212.

Xu Z, Ji X, Boysen PG (2004). Exogenous nitric oxide generates ROS and induces cardioprotection: involvement of PKG, mitochondrial KATP channels, and ERK. *Am J Physiol Heart Circ Physiol* **286**: H1433–H1440.

Xydas S, Kherani AR, Chang JS, Klotz S, Hay I, Mutrie CJ *et al*. (2006). beta(2)-Adrenergic stimulation attenuates left ventricular remodeling, decreases apoptosis, and improves calcium homeostasis in a rodent model of ischemic cardiomyopathy. *J Pharmacol Exp Ther* **317**: 553–561.

Yellon DM, Hausenloy DJ (2007). Myocardial reperfusion injury. *N Engl J Med* **357**: 1121–1135.

Zamah AM, Delahunty M, Luttrell LM, Lefkowitz RJ (2002). Protein kinase A-mediated phosphorylation of the beta 2-adrenergic receptor regulates its coupling to Gs and Gi. Demonstration in a reconstituted system. *J Biol Chem* **277**: 31249–31256.

Zaugg M, Xu W, Lucchinetti E, Shafiq SA, Jamali NZ, Siddiqui MA (2000). Beta-adrenergic receptor subtypes differentially affect apoptosis in adult rat ventricular myocytes. *Circulation* **102**: 344–350.

Zhu Y, Prehn JH, Culmsee C, Krieglstein J (1999). The beta2-adrenoceptor agonist clenbuterol modulates Bcl-2, Bcl-xl and Bax

protein expression following transient forebrain ischemia. *Neuroscience* **90**: 1255–1263.

Zhu WZ, Zheng M, Koch WJ, Lefkowitz RJ, Kobilka BK, Xiao RP (2001). Dual modulation of cell survival and cell death by beta(2)-adrenergic signaling in adult mouse cardiac myocytes. *Proc Natl Acad Sci USA* **98**: 1607–1612.

Zhu BQ, Simonis U, Cecchini G, Zhou HZ, Li L, Teerlink JR *et al*. (2006). Comparison of pyrroloquinoline quinone and/or metoprolol on myocardial infarct size and mitochondrial damage in a rat model of ischemia/reperfusion injury. *J Cardiovasc Pharmacol Ther* **11**: 119–128.