ORIGINAL PAPER

Activation of Akt/GSK-3β signaling pathway is involved in intermedin$_{1\text{-}53}$ protection against myocardial apoptosis induced by ischemia/reperfusion

Jun-Qiu Song · Xu Teng · Yan Cai · Chao-Shu Tang · Yong-Fen Qi

Abstract Intermedin (IMD) is a novel member of the calcitonin/calcitonin gene-related peptide family. We investigated the cardioprotective mechanism of IMD$_{1\text{-}53}$ in the in vivo rat model of myocardial ischemia/reperfusion (I/R) injury and in vitro primary neonatal cardiomyocyte model of hypoxia/reoxygenation (H/R). Myocardial infarct size was measured by 2,3,5-triphenyl tetrazolium chloride staining. Cardiomyocyte viability was determined by trypan blue staining, cell injury by lactate dehydrogenase (LDH) leakage, and cardiomyocyte apoptosis by terminal deoxyribonucleotidyl transferase-mediated dUTP nick-end labeling assay, Hoechst staining, gel electrophoresis and caspase 3 activity. The translocation of mitochondrial cytochrome c of myocardia and expression of apoptosis-related factors Bcl-2 and Bax, phosphorylated Akt and phosphorylated GSK-3β were determined by western blot analysis. IMD$_{1\text{-}53}$ (20 nmol/kg) limited the myocardial infarct size in rats with I/R; the infarct size was decreased by 54%, the apoptotic index by 30%, and caspase 3 activity by 32%; and the translocation of cytochrome c from mitochondria to cytosol was attenuated. IMD$_{1\text{-}53}$ increased the mRNA and protein expression of Bcl-2 and ratio of Bcl-2 to Bax by 81 and 261%, respectively. IMD$_{1\text{-}53}$ (1×10^{-7} mol/L) inhibited the H/R effect in cardiomyocytes by reducing cell death by 43% and LDH leakage by 16%; diminishing cellular apoptosis; decreasing caspase 3 activity by 50%; and increasing the phosphorylated Akt and GSK-3β by 41 and 90%, respectively. The cytoprotection of IMD$_{1\text{-}53}$ was abolished with LY294002, a PI3K inhibitor. In conclusion, IMD$_{1\text{-}53}$ exerts cardioprotective effect against myocardial I/R injury through the activation of the Akt/GSK-3β signaling pathway to inhibit mitochondria-mediated myocardial apoptosis.

Keywords Intermedin · Myocardium · Ischemia/reperfusion · Apoptosis · Akt/GSK-3β

J.-Q. Song · X. Teng · Y. Cai · C.-S. Tang · Y.-F. Qi
Department of Physiology and Pathophysiology, Peking University Health Science Center, 100191 Beijing, China

C.-S. Tang · Y.-F. Qi
Key Laboratory of Molecular Cardiovascular Science, Ministry of Education, 100191 Beijing, China

C.-S. Tang · Y.-F. Qi (✉)
Institute of Cardiovascular Diseases, Peking University First Hospital, 100034 Beijing, China
e-mail: yongfenqi@yahoo.com

Introduction

Myocardial ischemia/reperfusion (I/R) injury is defined as exacerbated tissue injury along with inhibition of cardiac function during reperfusion after prolonged myocardial ischemia. It is an important clinical problem associated with procedures such as thrombolysis, heart transplantation and coronary bypass surgery. Recent studies showed that myocardial I/R can activate endogenous protective mechanisms and cause the release of some cardiovascular system-derived bioactive substances, such as adenosine, nitric oxide, adrenomedullin (ADM) and calcitonin gene-related peptide (CGRP). Therefore, seeking new endogenous cardioprotective substances and shedding light on their effects and mechanisms is a promising strategy for therapy in ischemic heart disease.

Intermedin (IMD), also known as adrenomedullin 2 (ADM 2), is a novel member of the calcitonin/CGRP family [1, 2]. Human IMD gene encodes a prepropeptide of 148 amino acids with a signal peptide for secretion at the N terminus. IMD_{1-53} can be generated from prepro-IMD by proteolytic cleavage. Previous studies indicated that IMD exists extensively in the heart, kidney, blood vessels, hypothalamus, gastrointestinal tract, lung, spleen, pancreas, skin, thymus and ovary. Intraperitoneal administration of IMD dose-dependently reduced blood pressure in normal and spontaneously hypertensive rats [1]. Our previous investigations have shown that IMD_{1-53} ameliorates impaired cardiac function in an ex vivo model of myocardial I/R injury and isoproterenol-induced myocardial ischemia injury [3, 4]. The biological action of IMD is mediated by calcitonin receptor-like receptor (CRLR) and receptor activity-modifying proteins (RAMPs) acting as molecular chaperones for trafficking CRLR from the endoplasmic reticulum and Golgi apparatus to the cell surface. IMD can produce some cardiovascular effects through activation of the cAMP/protein kinase A (PKA) pathway and induction of nitric oxide synthesis [5], but the cellular and molecular mechanisms of IMD protection against myocardial I/R injury remain unclear.

A growing body of evidence suggests that apoptosis of cardiomyocytes is one of the major contributors to myocardial I/R injury. Apoptosis could be mediated by the pathways of cell-surface death receptor, mitochondria or endoplasmic reticulum. Cardiomyocytes are rich in mitochondria, and mitochondria-mediated apoptosis plays a crucial role in the pathogenesis of myocardial I/R injury. The release of cytochrome c from mitochondria to cytosol leads to the activation cascades of caspase 9 and caspase 3, which function to execute the final apoptotic event. The Bcl-2 family members are important regulators of mitochondria-mediated apoptosis, which are divided into anti-apoptotic proteins such as Bcl-2 and pro-apoptotic proteins such as Bax. Bcl-2/Bax ratio is often adopted to represent the extent of apoptosis.

Akt, a serine/threonine kinase, is a primary mediator of the downstream effects of phosphatidylinositol-3 kinase (PI3K), coordinating a variety of intracellular signals and controlling cell responses to extrinsic stimuli and regulating cell proliferation and survival. Under normal physiological conditions, Akt is activated by growth factors such as insulin or insulin-like growth factors. Once activated, Akt is translocated from the cell membrane to various subcellular compartments, including the Golgi, endoplasmic reticulum, mitochondria and nucleus, where it phosphorylates substrates or interacts with other molecules [6]. In response to myocardial I/R injury, activated Akt protects against myocyte apoptosis and functions to promote cellular survival [7]. Glycogen synthase kinase 3 (GSK-3) contributes to

many cellular processes such as gene expression, cell division, glycogen metabolism, survival and apoptosis. Its two isoforms, GSK-3α (51 kDa) and GSK-3β (47 kDa), with a high degree of sequence similarity in their catalytic domains, are regulated by phosphorylation at Ser^{21} and Tyr^{279} for GSK-3α and Ser^9 and Tyr^{216} for GSK-3β. Both the $Ser^{21/9}$ and $Tyr^{279/216}$ sites on GSK-3 modulate functioning in opposing directions, with phosphorylation of $Ser^{21/9}$ decreasing GSK-3 activity and phosphorylation of $Tyr^{279/216}$ increasing GSK-3 activity [8]. Akt can phosphorylate GSK-3β at Ser^9, and the phosphorylated GSK-3β with decreased activity is involved in the cardioprotective effect of ADM and bradykinin against myocardial I/R injury [9, 10].

In the present study, we investigated the protective effects of IMD_{1-53} on cardiac myocyte apoptosis induced by myocardial I/R in vivo or cardiomyocyte hypoxia/reoxygenation (H/R) in vitro and its underlying signaling pathway. We found that IMD_{1-53} exerts cardioprotection against myocardial I/R injury or cardiomyocyte H/R injury and the activation of Akt/GSK-3β signaling pathway plays an important role in mediating the anti-apoptotic effect of IMD_{1-53}.

Materials and methods

Materials

Male Sprague-Dawley (SD) rats (211 ± 8 g) and 1- to 2-day-old SD rats were obtained from the Animal Center, Health Science Center, Peking University (Beijing, China). All animal care and experimental protocols complied with the Animal Management Rule of the Ministry of Health, People's Republic of China (Documentation 55, 2001). Synthetic rat IMD_{1-53} peptide was from Phoenix Pharmaceutical Inc. (Belmont, CA, USA). Rabbit polyclonal anti-Bcl-2 (sc422), anti-Bax (sc493), anti-cytochrome c (sc7159) and goat polyclonal anti-β-actin (sc1616) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal anti-Akt (ab9272) and anti-phospho-Akt (ab9271) antibodies were from Abcam Inc. (Cambridge, MA, USA). Rabbit polyclonal anti-GSK-3β (9315) and anti-phospho-GSK-3β (9336s) antibodies were from Cell Signaling Technology (Beverly, MA, USA). N,N-dimethyl-p-phenylenediamine sulphate, sodium dodecyl sulfate (SDS) and PI3K inhibitor LY294002 were from Sigma Co. (St. Louis, MO, USA). Trizol was from GIBCOL (BRL, Rockville, MD, USA) and dNTP, M-MuLV RT, Oligo (dT) 15 primer and Taq DNA polymerase were from Promega (Madison, WI, USA). Sequences of oligonucleotide primers were Bax-S, 5′-AGGATGATTGCTGACGTGGAC-3′, and Bax-A, 5′-TCAGCCCATCTTCTTCCAGA-3′; Bcl-2-S,

5′-CACCCCTGGTGGACAACATC-3′, and Bcl-2-A, 5′-C
CAGGTATGCACCCAGAGTGA-3′; β-actin-S, 5′-ATC
TGGCACCACACCTTC-3′, and β-actin-A, 5′-AGCCA
GGTCCAGACGCA-3′ (AuGCT Biotechnology, Beijing,
China). Other chemicals and reagents were of analytical
grade.

Myocardial I/R model in rats

Male SD rats were anaesthetized by use of urethane (1 g/kg)
through intraperitoneal injection. The trachea was
intubated for artificial ventilation with room air. A cannula
was inserted into the right carotid artery for measurement
of blood pressure. Thoracotomy was performed between
the sternum and left costa. The pericardium was opened
and the heart exposed. A 3/0 silk suture was passed around
the left anterior descending coronary artery, and the coro-
nary artery was occluded by pulling on the suture tightly.
After 30 min of myocardial ischemia, the suture was
loosened for 120 min for reperfusion. The sham-operated
animals underwent the same surgical procedures except
that the suture around the left anterior descending coronary
artery was not fastened. Normal saline or IMD_{1-53}
(20 nmol/kg) was infused from the left femoral vein
20 min after the onset of ischemia for 10 min. A standard
limb lead II was used for electrocardiography monitoring
during I/R.

Preparation of primary neonatal cardiomyocytes and H/R treatment

Neonatal rat cardiac myocytes were isolated from 1- to
2-day-old SD rats. Briefly, the excised hearts were washed
in Hanks balanced salt solution (HBSS; Ca^{2+}-Mg^{2+} free)
and the ventricular tissues were minced by use of fine
scissors in HBSS containing trypsin (0.05%) and collage-
nase (0.055%), and digested at 37°C. Cells were isolated by
multiple 10-min rounds of tissue digestion. After each
incubation, the supernatant was added to an equal volume of
DMEM containing 20% fetal bovine serum. The total cell
suspensions were centrifuged at 1,000 rpm for 10 min.
Supernatants were discarded and the cell pellets were
resuspended in DMEM containing 10% fetal bovine serum.
The cells were plated onto plastic culture dishes for 90 min
so that most of the non-myocytes attached to the dish, and
the myocytes remained in the suspension. The myocytes
were harvested and seeded onto 60-mm culture dishes at 10^5
cells per cm^2. 5-Bromo-2′-deoxyuridine (100 μmol/L) was
added during the first 48 h to inhibit proliferation of non-
myocytes. For simulation of ischemia, the cell medium was
replaced with modified Esumi's ischemic buffer (in mmol/L:
137 NaCl, 12 KCl, 0.49 $MgCl_2$, 0.9 $CaCl_2$, 4 HEPES, 10
deoxyglucose, and 20 sodium lactate, pH 6.2). This buffer

has high potassium content and a low acidic pH and hence
mimics the condition of cells within the heart on oxygen
deprivation [11]. The cells were incubated in a hypoxic
chamber with humidified atmosphere of 5% CO_2/0% O_2
balanced with N_2 at 37°C for 6 h. For control conditions,
cells were cultured with Esumi's control buffer (in mmol/L:
137 NaCl, 3.8 KCl, 0.49 $MgCl_2$, 0.9 $CaCl_2$, 4.0 HEPES, 10
deoxyglucose, pH 7.4) in a humidified atmosphere of 5%
CO_2/21% O_2 balanced with N_2 at 37°C for 6 h [11]. The
cells were then returned to the standard incubator for a
further 4 h of reoxygenation. To investigate the protective
effect of IMD_{1-53} against H/R injury, cardiomyocytes were
incubated with IMD_{1-53} (1×10^{-7} mol/L) for 4 h at the
point of reoxygenation, in the presence and absence of
LY294002 (10 μmol/L). Following dose-response experi-
ments (see supplementary material), the dose of
1×10^{-7} mol/L was chosen to evaluate the role of IMD_{1-53}
in protection.

Assessment of myocardial infarct size

At the end of reperfusion, the suture around the left anterior
descending coronary artery was retightened, and 1.5 mL of
2% Evans blue dye was injected via the left femoral vein.
The area at risk (AAR) was identified by negative staining
with Evans blue, and the infarct area (IA) was confirmed as
the unstained part in the risk area following 1% triphenyl
tetrazolium chloride (TTC) staining. The tissue was sliced
and the weights of the AAR and IA were determined.
Myocardial infarct size was expressed as a weight ratio of
IA to AAR (IA/AAR).

Terminal deoxyribonucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay and Hoechst staining

At the end of reperfusion, cardiac samples were harvested
and fixed in 4% paraformaldehyde. After being frozen in
embedded medium, samples were cut into 5-μm sections
and TUNEL assay was performed according to the manu-
facturer's instructions (Roche Applied Science, Penzberg,
Germany). Under light microscope, the TUNEL-positive
nuclei of cardiomyocytes were stained dark purple. For
each slide, 10 fields were randomly chosen, and a total of
100 cells per field were counted. The apoptotic index was
determined (number of apoptotic cardiomyocytes/total
number of cardiomyocytes counted) from a total of 50
fields per heart, and the assay was performed in a blinded
manner.

Hoechst staining was performed according to the man-
ufacturer's instructions (Applygen, Beijing, China) and the
condensed or fragmented apoptotic nuclei were observed
under fluorescence microscope.

RT-PCR assay for determining Bcl-2 and Bax mRNA level

Total myocardial RNA was prepared by in situ lysis with Trizol reagent. One microgram of total tissue RNA was reverse transcribed into single-strand cDNA with use of M-MuLV reverse transcriptase and oligo (dT) 15 primers. PCR was performed in a 0.2-mL tube containing 2 μL tissue cDNA, 5 μmol/L each of Bcl-2-S and Bcl-2-A primer mixture in 1 μL, 2.5 mmol/L dNTP mixture in 1 μL, 1.5 mmol/L $MgCl_2$ in 1.5 μL, 10× PCR buffer in 2.5 μL, and 1.25 U Taq DNA polymerase, in a total volume of 25 μL. After being denatured at 95°C for 5 min, the solution underwent PCR at 94°C for 30 s, 60°C for 30 s, and 72°C for 40 s for 30 cycles, then 72°C for 5 min. An amount of 6 μL of PCR product was separated in 1.5% agarose gel and stained with ethidium bromide. The optical density of the 431-bp band was measured by use of the Gel Documentation System (Bio-Rad, Hercules, CA, USA). The relative amount of Bax and β-actin mRNA was also determined by the above method with 407- and 532-bp products, respectively; all results were repeated three times.

Western blot analysis

Protein extracts from myocardia or cardiomyocytes were resuspended in sample buffer containing 2% SDS, 2% β-mercaptoethanol, 50 mmol/L Tris–HCl (pH 6.8), 10% glycerol and 0.05% bromophenol blue. The protein mixture was then placed in boiling water for 10 min and briefly centrifuged at low speed to collect the denatured proteins. Protein samples were resolved on a 12% (for Bax, Bcl-2 and cytochrome c) or 10% (for Akt, phospho-Akt, GSK-3β, phospho-GSK-3β and β-actin) Tris/glycine SDS–polyacrylamide gel in running buffer containing 25 mmol/L Tris, 192 mmol/L glycine, and 0.1% SDS. The proteins were then transferred to a nitrocellulose membrane for 2 h at 4°C at 100 mA with a transfer buffer containing 20 mmol/L Tris–HCl (pH 8.0), 150 mmol/L glycine, and 20% methanol. Non-specific proteins were blocked by incubating the membrane with 5% non-fat dry milk in TBS-T (20 mmol/L Tris–HCl [pH7.6], 150 mmol/L NaCl and 0.02% Tween 20) for 1 h at room temperature with agitation. The primary antibody was added to the membrane at 1:1,000 dilution in TBS-T and incubated at room temperature overnight with agitation. After being washed three times for 10 min each in TBS-T, the fluorescein-linked secondary antibody at 1:3,000 dilution in TBS-T was added to the membrane and incubated at room temperature for 1 h with agitation. The membrane was then washed three times for 10 min each in TBS-T, and underwent enhanced chemiluminescence detection. Autoradiograph was scanned and relative density quantified.

Extraction and electrophoresis of DNA

Myocytes cultured on dishes were scraped off and centrifuged at 8,000 rpm for 5 min in PBS. The pellet resuspended in 0.5 mL of lysis buffer containing 75 mmol/L NaCl, 10 mmol/L Tris–HCl, 1% SDS, 10 mmol/L EDTA, pH 8.0, and 0.2 mg/mL proteinase K was digested at 55°C for 3 h, and the lysate was incubated with RNase A at 37°C for 1 h. After extraction with a mixture of phenol, chloroform and isoamyl alcohol, DNA was precipitated by sodium acetate and water-free ethanol and centrifuged at 12,000 rpm for 20 min. The pellet was washed with 75% ethanol twice and dissolved in TE buffer containing 10 mmol/L Tris–HCl, 1 mmol/L EDTA, pH 8.0. After quantification with use of a spectrophotometer, 10 μg of DNA was loaded and electrophoresed on a 1.8% agarose gel containing ethidium bromide (0.5 μg/mL). As a hallmark of apoptosis, DNA laddering was visualized under ultraviolet light and photographed.

Measurement of caspase 3 activity

Myocardial caspase 3 activity was determined by use of colorimetric assay kits (Beyotime Institute of Biotechnology, Jiangsu, China) according to the manufacturer's instructions. Results were expressed as nmol/h/mg protein.

Statistical analysis

Data are expressed as mean ± SEM. One-way ANOVA was used to compare more than two groups, and when significant ($P < 0.05$), the Tukey HSD test was applied to test for differences between individual groups. A $P < 0.05$ was considered statistically significant.

Results

IMD$_{1-53}$ treatment attenuated I/R-induced myocardial necrosis and apoptosis

I/R treatment produced obvious myocardial infarction, and TUNEL staining showed many apoptotic myocytes in the ischemic area. The nuclei of apoptotic cells were stained dark purple (Fig. 1c). Compared with I/R alone, with I/R + IMD treatment, the IA/AAR ratio was decreased (0.276 ± 0.027 vs. 0.599 ± 0.041, $P < 0.01$; Fig. 1b), and the number of TUNEL-positive myocytes was decreased as indicated by the apoptotic index (0.347 ± 0.040 vs. 0.497 ± 0.031, $P < 0.01$; Fig. 1c). Caspase 3 was activated with I/R alone, but with I/R + IMD, the activity was lower than that with I/R alone (58.49 ± 5.25 vs. 86.52 ± 7.83 nmol/h/mg, $P < 0.01$; Fig. 1d).

Fig. 1 IMD attenuates ischemia-reperfusion (I/R)-induced myocardial necrosis and apoptosis. **a** Images of cardiac TTC staining (surviving tissues *red*, infarcted areas *white*). **b** Infarct size was expressed as IA/AAR. Values are means ± SEM. ** $P < 0.01$ vs. I/R. **c** Representative image and quantitative results of myocardial TUNEL staining. *Arrows* indicate apoptotic nuclei. Original magnification ×200. Values are means ± SEM. ** $P < 0.01$ vs. Sham treatment; ## $P < 0.01$ vs. I/R. **d** Myocardial caspase 3 activity. Values are means ± SEM. $n = 5$. ** $P < 0.01$ vs. Sham treatment; ## $P < 0.01$ vs. I/R. (Color figure online)
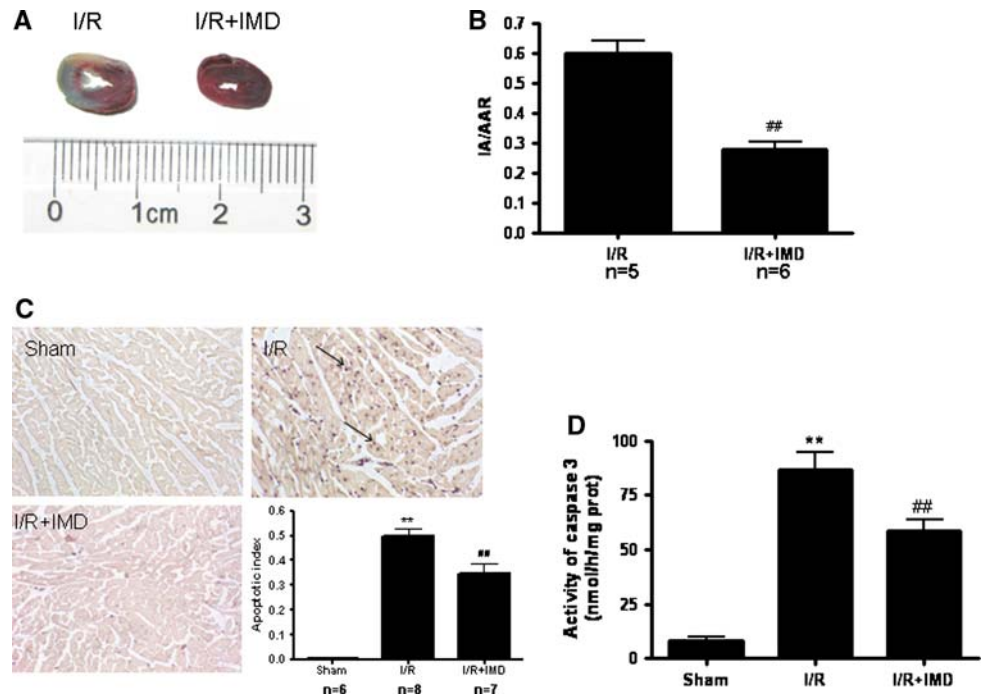
IMD_{1-53} administration inhibited the mitochondria-mediated myocardial apoptosis induced by I/R

Western blot analysis revealed that cytochrome c mainly located in mitochondria and could not be found in cytosol of myocytes with sham treatment. The level of cytochrome c expression in mitochondria among various groups was similar. The level of cytosolic cytochrome c with I/R + IMD treatment was lower than that with I/R alone (Fig. 2a). Thus, myocardial I/R led to translocation of cytochrome c from mitochondria to cytosol and IMD_{1-53} treatment could inhibit this process.

RT-PCR revealed that compared with sham treatment, I/R treatment produced decreased Bcl-2/Bax ratio by 45% ($P < 0.05$). Compared with I/R treatment alone, I/R + IMD treatment increased the ratio by 81% ($P < 0.05$; Fig. 2b). Western blot analysis revealed that the Bcl-2/Bax ratio with I/R treatment was not significantly different from that with sham treatment, but compared with I/R treatment, I/R + IMD treatment increased the ratio by 261% ($P < 0.01$, Fig. 2c).

IMD_{1-53} administration increased the phosphorylation of myocardial Akt and GSK-3β with I/R

Western blot analysis revealed that compared with sham treatment, I/R treatment increased the ratios of P-Akt/Akt and P-GSK-3β/GSK-3β by 144 and 93% (both $P < 0.05$), respectively, and compared with I/R alone treatment, I/R + IMD treatment increased the ratios by 82% ($P < 0.05$) and 67% ($P < 0.01$), respectively (Fig. 3).
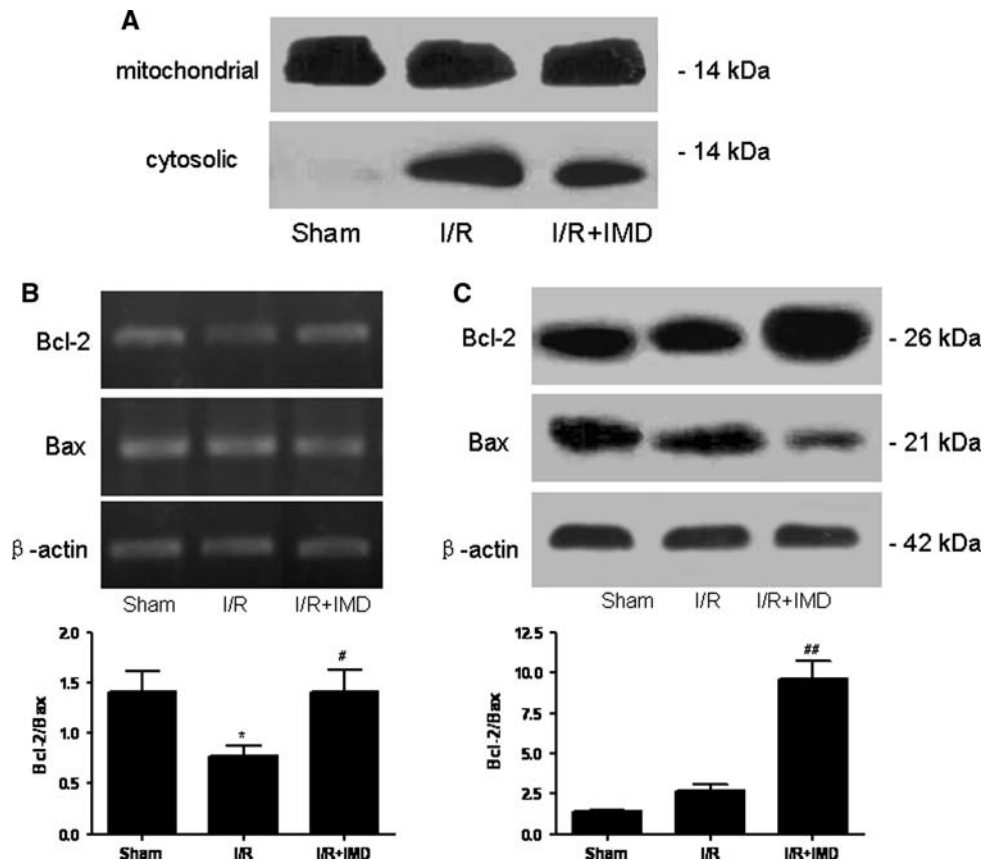
The protective effect of IMD_{1-53} on H/R-induced apoptosis was abolished by preincubation with LY294002, a PI3K inhibitor

Hoechst staining and gel electrophoresis revealed condensed or fragmented apoptotic nuclei with H/R treatment. Incubation with 1×10^{-7} mol/L of IMD_{1-53} attenuated the extent of apoptosis, and preincubation with LY294002, a PI3K inhibitor, abolished the effects of IMD_{1-53} (Fig. 4a, b). Activity of caspase 3 was increased with H/R treatment but was lower with H/R + IMD treatment than with H/R alone (35.91 ± 3.64 vs. 71.40 ± 6.02 nmol/h/mg, $P < 0.01$). LY294002 treatment (10 μmol/L) effectively attenuated the IMD_{1-53} effect (56.70 ± 4.15 vs. 35.91 ± 3.64 nmol/h/mg for H/R + IMD treatment, $P < 0.05$; Fig. 4c).
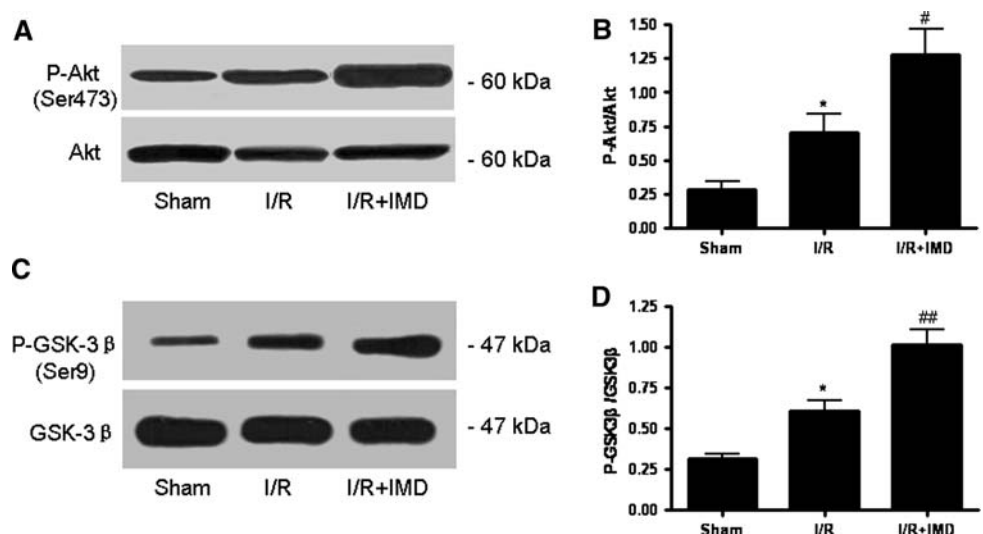
IMD_{1-53} increases the phosphorylation of cardiomyocyte Akt and GSK-3β with H/R in vitro

On western blot analysis, compared with control treatment, H/R treatment increased the ratios of P-Akt/Akt and P-GSK-3β/GSK-3β by 205% ($P < 0.01$) and 57% ($P < 0.05$), respectively. Compared with H/R alone treatment, H/R + IMD increased the ratios of P-Akt/Akt and P-GSK-3β/GSK-3β by 41% ($P < 0.05$) and 90% ($P < 0.01$), respectively. Compared with H/R + IMD treatment, that with the LY294002 decreased the ratios of P-Akt/Akt and P-GSK-3β/GSK-3β by 86 and 91% (both $P < 0.01$), respectively (Fig. 5).

Discussion

The major finding in this study is that IMD_{1-53} protected against I/R induced cardiomyocyte apoptosis in the in vivo rat model and in in vitro-cultured cardiomyocytes. We also demonstrated that the mechanism of cardioprotection produced by IMD_{1-53} involved the activation of Akt/GSK-3β signaling pathway by using the PI3K inhibitor (LY294002), which could abrogate the protective effect of IMD_{1-53}. To the best of our knowledge, this is the first report that links IMD_{1-53}-induced cardioprotection and the Akt/GSK-3β signaling pathway.

Roh et al. [1], using a phylogenetic profiling approach, identified a novel calcitonin/CGRP family peptide, intermedin, from the genomes of humans and other vertebrates. Recently Takei et al. [2] identified adrenomedullin 2 in

Fig. 4 IMD inhibits the cardiomyocyte apoptosis induced by H/R, which could be abolished by the PI3K inhibitor LY294002. **a** Hoechst staining of myocyte nuclei among groups. The *arrows* indicate condensed nuclei adjacent to the normal. **b** Representative photograph of DNA fragmentation in cardiomyocytes. *0*: DNA size marker; *1*: control; *2*: H/R; *3*: H/R + IMD; *4*: H/R + IMD + LY294002. **c** Caspase 3 activity in cardiomyocytes. Values are means ± SEM ($n = 5$). ** $P < 0.01$ vs. control; ## $P < 0.01$ vs. H/R; & $P < 0.05$ vs. H/R + IMD
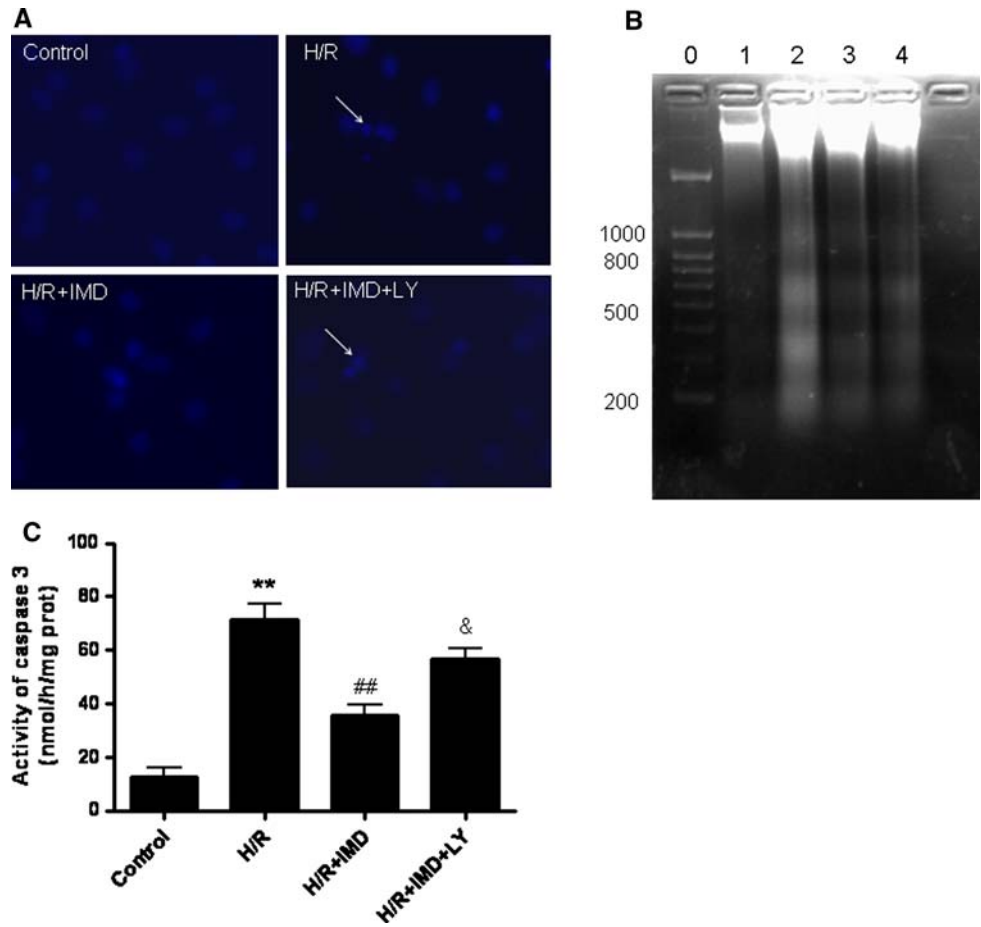
Fig. 5 IMD increases the phosphorylation of cardiomyocyte Akt and GSK-3β under H/R as indicated by the ratio of P-Akt to Akt and P-GSK-3β to GSK-3β as quantitative analysis. Values are means ± SEM ($n = 6$). * $P < 0.05$, ** $P < 0.01$ vs. control; # $P < 0.05$, ## $P < 0.01$ vs. H/R; && $P < 0.01$ vs. H/R + IMD
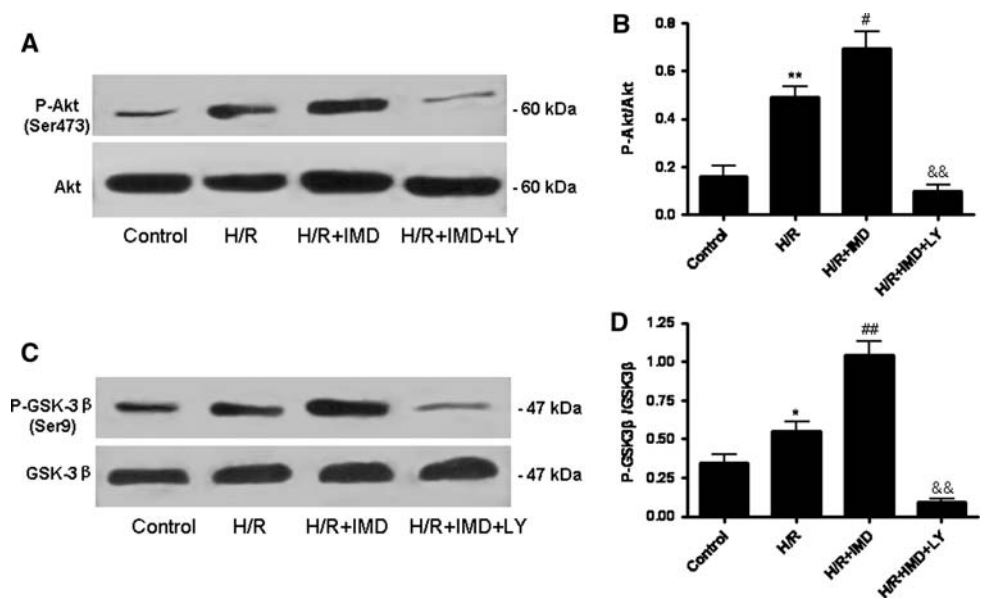
mammals (mice, rats and humans) with the same nucleotide and amino acid sequence as intermedin. Therefore, adrenomedullin 2 and intermedin were considered the same peptide. Amino acid sequence analysis showed that the cleavage sites of the precursor of prepro-intermedin exist between two basic amino acids at Arg93-Arg94 and Arg147-Gly148, resulting in the production of prepro-intermedin$_{95-147}$ (IMD$_{1-53}$). Previously, we reported that IMD$_{1-53}$ is an endogenously degraded bioactive fragment of prepro-intermedin and plays a role in the regulation of cardiovascular

homeostasis [3]. Calcitonin/CGRP family members can bind to the CRLR/RAMP complex on the cell plasma membrane [15]. The ligand-binding selectivity of CRLR is regulated by RAMPs (RAMP1, 2 and 3). IMD represents a nonselective agonist for the CRLR/RAMP complex and exerts its biological effects through activating the CRLR/RAMP system and elevating the intracellular cAMP level [1, 15].

The potential mechanisms of myocardial I/R injury have not been well explained. Calcium overload, oxidative stress, disturbance of energy metabolism and inflammation are all involved. In this study, I/R in rats led to myocardial infarction. Moreover, cultured cardiomyocytes subjected to H/R showed large-scale cell death and leakage of LDH (see supplementary material). Previously, we reported that the cardioprotection of IMD_{1-53} against myocardial I/R injury ex vivo could improve cardiac function after I/R and attenuate myocardial lipid peroxidation [3, 4]. The present study demonstrated that IMD_{1-53} limited the infarct size in the rat model of myocardial I/R injury. Trypan blue staining and determination of LDH activity further confirmed that IMD_{1-53} suppressed cell death of in vitro-cultured cardiomyocytes induced by H/R (see supplementary material).

Cardiomyocyte apoptosis is one of the major pathogenic mechanisms underlying myocardial I/R injury. Inhibition of cardiomyocyte apoptosis could prevent the loss of contractile cells and minimize cardiac injury induced by myocardial I/R. In this study, myocardial I/R injury in vivo and H/R injury in in vitro-cultured cardiomyocytes led to caspase 3 activation and apoptosis, as was found previously [9]. We demonstrated for the first time that IMD_{1-53} decreased apoptosis in the rat model of myocardial I/R injury and in cultured cardiomyocytes.

Cardiomyocytes are rich in mitochondria, and mitochondria-mediated apoptosis plays an important role in I/R injury pathogenesis. Under normal conditions, cytochrome c locates in mitochondria. Once released cytochrome c binds to the C-terminal domain of the apoptotic protease activating factor-1 (Apaf-1), it induces a conformational change that permits its activation. In the presence of the nucleotide dATP, the Apaf-1/cytochrome c complex oligomerizes into a heptameric structure, which allows interaction of procaspase 9 with Apaf-1, thus placing individual procaspase 9 molecules in close proximity with each other and promoting their activation [16]. Active caspase 9 can then cleave and activate procaspases 3 and 7 to execute the cell death program. We found that myocardial I/R led to translocation of cytochrome c from mitochondria to cytosol, and IMD_{1-53} treatment could inhibit this process.

The Bcl-2 protein family members are potent regulators of mitochondrial changes during apoptosis. In terms of function, they are classified into two types: anti-apoptotic members such as Bcl-2 and Bcl-X_L or pro-apoptotic members such as Bax, Bak and Bad. Activation of Bax and Bak upon induction of apoptosis involves their oligomerization, integration into the mitochondrial membrane and formation of non-selective channels/lipidic pores [17]. The anti-apoptotic family members bind to the multi-domain pro-apoptotic members and prevent them from mediating the release of cytochrome c. Bcl-2/Bax ratio is often adopted to represent the extent of apoptosis. In this study, we determined the expression of Bcl-2 and Bax at the mRNA and protein levels. The expression levels of mRNA and protein do not correlate necessarily. The process of expression is complicated which includes transcription, translation and post-translational modification. So the difference of Bcl-2/Bax ratio between mRNA and protein is possible. Fortunately, the ratios at both mRNA and protein levels with I/R + IMD treatment were statistically increased compared with I/R alone, which indicated the anti-apoptotic effect of IMD_{1-53}.

At the early stage of myocardial reperfusion, some endogenous protein kinases that confer powerful cardioprotection, including Akt and ERK 1/2 [18], are activated. Previous studies revealed that activation of the PI3K/Akt pathway attenuates mitochondria-mediated apoptosis. Insulin administered early during reperfusion in the perfused heart limited the infarct size through activation of the PI3K/Akt pathway and phosphorylation of pro-apoptotic protein Bad [19]. Overexpression of Akt in cultured cardiomyocytes lessened H/R induced cell death and preserved Bcl-2 level in mitochondria. These effects were similar to those of hypoxic preconditioning [20]. As a downstream effector of Akt, GSK-3β is phosphorylated at Ser^9 by Akt, and the phosphorylated GSK-3β provides cardioprotection against myocardial I/R injury [21]. Bradykinin administered before reperfusion produced cardioprotection involving GSK-3β phosphorylation and later inhibition of the opening of mitochondrial permeability transition pore (mPTP), which was thought to be one of the critical factors mediating cytochrome c release from mitochondria to cytosol [10]. Ischemic preconditioning and erythropoietin offered cardioprotection against myocardial I/R injury and allowed for translocation of phosphorylated GSK-3β from cytosol to mitochondria. The phosphorylated GSK-3β interacted with adenine nucleotide translocase (ANT), one of the mPTP components, and reduced the interaction of ANT and cyclophilin D. The opening of mPTP was then inhibited [22]. In the present study, I/R led to increased Akt and GSK-3β phosphorylation. Infusion of IMD_{1-53} before reperfusion further increased Akt and GSK-3β phosphorylation. In cultured cardiomyocytes, H/R also increased Akt and GSK-3β phosphorylation. Incubation with IMD_{1-53} at reoxygenation further increased Akt and GSK-3β phosphorylation and attenuated myocyte apoptosis. The protective effect of IMD_{1-53} against H/R injury could be abolished in part by the PI3K inhibitor

LY294002, so IMD$_{1-53}$ exerted cardioprotection through activating an endogenous Akt/GSK-3β signaling pathway.

In summary, our results demonstrate that IMD$_{1-53}$ exerts cardioprotection against myocardial I/R injury by reducing mitochondria-mediated apoptosis, and the activation of the Akt/GSK-3β signaling pathway is involved in the cardioprotective effect of IMD$_{1-53}$.

References

1. Roh J, Chang CL, Bhalla A, Klein C, Hsu SY (2004) Intermedin is a calcitonin/calcitonin gene-related peptide family peptide acting through the calcitonin receptor-like receptor/receptor activity-modifying protein receptor complexes. J Biol Chem 279:7264–7274

2. Takei Y, Inoue K, Ogoshi M, Kawahara T, Bannai H, Miyano S (2004) Identification of novel adrenomedullin in mammals: a potent cardiovascular and renal regulator. FEBS Lett 556:53–58

3. Yang JH, Jia YX, Pan CS, Zhao J, Ouyang M, Yang J et al (2005) Effects of intermedin (1–53) on cardiac function and ischemia/reperfusion injury in isolated rat hearts. Biochem Biophys Res Commun 327:713–719

4. Jia YX, Yang JH, Pan CS, Geng B, Zhang J, Xiao Y et al (2006) Intermedin1-53 protects the heart against isoproterenol-induced ischemic injury in rats. Eur J Pharmacol 549:117–123

5. Yang JH, Pan CS, Jia YX, Zhang J, Zhao J, Pang YZ et al (2006) Intermedin 1–53 activates L-arginine/nitric oxide synthase/nitric oxide pathway in rat aortas. Biochem Biophys Res Commun 341:567–572

6. Parcellier A, Tintignac LA, Zhuravleva E, Hemmings BA (2008) PKB and the mitochondria: AKTing on apoptosis. Cell Signal 20:21–30

7. Fujio Y, Nguyen T, Wencker D, Kitsis RN, Walsh K (2000) Akt promotes survival of cardiomyocytes in vitro and protects against ischemia-reperfusion injury in mouse heart. Circulation 101:660–667

8. Forde JE, Dale TC (2007) Glycogen synthase kinase 3: a key regulator of cellular fate. Cell Mol Life Sci 64:1930–1944

9. Yin H, Chao L, Chao J (2004) Adrenomedullin protects against myocardial apoptosis after ischemia/reperfusion through activation of Akt-GSK signaling. Hypertension 43:109–116

10. Park SS, Zhao H, Mueller RA, Xu Z (2006) Bradykinin prevents reperfusion injury by targeting mitochondrial permeability transition pore through glycogen synthase kinase 3beta. J Mol Cell Cardiol 40:708–716

11. Esumi K, Nishida M, Shaw D, Smith TW, Marsh JD (1991) NADH measurements in adult rat myocytes during simulated ischemia. Am J Physiol 260:H1743–H1752

12. Lee YS, Kang YJ, Kim HJ, Park MK, Seo HG, Lee JH et al (2006) Higenamine reduces apoptotic cell death by induction of heme oxygenase-1 in rat myocardial ischemia-reperfusion injury. Apoptosis 11:1091–1100

13. Chang L, Xu J, Yu F, Zhao J, Tang X, Tang C (2004) Taurine protected myocardial mitochondria injury induced by hyperrhomocysteinemia in rats. Amino Acids 27:37–48

14. Baxter GF, Mocanu MM, Brar BK, Latchman DS, Yellon DM (2001) Cardioprotective effects of transforming growth factor-beta1 during early reoxygenation or reperfusion are mediated by p42/p44 MAPK. J Cardiovasc Pharmacol 38:930–939

15. Kuwasako K, Cao YN, Nagoshi Y, Tsuruda T, Kitamura K, Eto T (2004) Characterization of the human calcitonin gene-related peptide receptor subtypes associated with receptor activity-modifying proteins. Mol Pharmacol 65:207–213

16. Boatright KM, Renatus M, Scott FL, Sperandio S, Shin H, Pedersen IM et al (2003) A unified model for apical caspase activation. Mol Cell 11:529–541

17. Lassus P, Opitz-Araya X, Lazebnik Y (2002) Requirement for caspase-2 in stress-induced apoptosis before mitochondrial permeabilization. Science 297:1352–1354

18. Hausenloy DJ, Yellon DM (2007) Reperfusion injury salvage kinase signalling: taking a RISK for cardioprotection. Heart Fail Rev 12:217–234

19. Jonassen AK, Sack MN, Mjøs OD, Yellon DM (2001) Myocardial protection by insulin at reperfusion requires early administration and is mediated via Akt and p70s6 kinase cell-survival signaling. Circ Res 89:1191–1198

20. Uchiyama T, Engelman RM, Maulik N, Das DK (2004) Role of Akt signaling in mitochondrial survival pathway triggered by hypoxic preconditioning. Circulation 109:3042–3049

21. Gross ER, Hsu AK, Gross GJ (2004) Opioid-induced cardioprotection occurs via glycogen synthase kinase beta inhibition during reperfusion in intact rat hearts. Circ Res 94:960–966

22. Nishihara M, Miura T, Miki T, Tanno M, Yano T, Naitoh K et al (2007) Modulation of the mitochondrial permeability transition pore complex in GSK-3beta-mediated myocardial protection. J Mol Cell Cardiol 43:564–570