

Bakuchiol attenuates myocardial ischemia reperfusion injury by maintaining mitochondrial function: the role of silent information regulator 1

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Abstract Ischemia reperfusion (IR) injury (IRI) is associated with poor prognoses in the settings of both cardiac surgery and ischemic heart disease and causes mitochondrial oxidative stress and cell death. Silent information regulator 1 (SIRT1), a member of the histone deacetylase family, exerts anti-IRI effects. Bakuchiol (BAK), an analog of resveratrol and a monoterpene phenol isolated from the seeds of *Psoralea corylifolia* (Leguminosae), protects tissues from injury. This study was designed to investigate the protective effects of BAK treatment in the setting of myocardial IRI and to elucidate the potential mechanism of those effects. Prior to induction of IR, isolated rat hearts or cardiomyocytes were exposed to BAK in either the absence or presence of the SIRT1 inhibitors Sirtinol and SIRT1 siRNA. BAK exerted cardioprotective effects, as evidenced by the improvements

noted in cardiac function following ischemia, attenuated myocardial apoptosis, and changes in several biochemical parameters (including increases in the level of the anti-apoptotic protein Bcl2, decreases in the level of the pro-apoptotic protein Bax, and decreases in the cleaved Caspase 3 level). However, Sirtinol and SIRT1 siRNA each blocked BAK-induced cardioprotection by inhibiting SIRT1 signaling. Additionally, BAK significantly increased the activities of mitochondrial succinate dehydrogenase, cytochrome c oxidase, and mitochondrial superoxide dismutase and decreased the production of malondialdehyde. These findings suggested that BAK significantly attenuated IR-induced mitochondrial oxidative damage. However, Sirtinol and SIRT1 siRNA abolished BAK-dependent mitochondrial function. In summary, our results demonstrate that BAK treatment attenuates IRI by attenuating IR-induced mitochondrial oxidative damage via the activation of SIRT1/PGC-1 α signaling.

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Keywords Ischemia reperfusion injury · Bakuchiol · Silent information regulator 1 · Mitochondrial oxidative damage

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Introduction

Ischemic heart disease, also known as coronary atherosclerotic heart disease, has become a leading cause of death worldwide, accounting for 7.4 million deaths in 2012 (<http://www.who.int/mediacentre/factsheets/fs310/en/>); its prevalence continues to increase in developing countries [1]. Previous evidence has indicated that not only ischemia itself but also reperfusion contributes to the tissue injury sustained following myocardial ischemia, a phenomenon known as ischemia/reperfusion (IR) injury (IRI) [2]. Mitochondria play an important role in IRI [3]. The calcium overload and increased levels of oxidative stress that occur in the setting of IRI result in damage to the mitochondrial membrane and electron transport chain, the opening of mitochondrial permeability transition pores and the uncoupling of mitochondrial oxidative phosphorylation; these changes subsequently trigger the deterioration of mitochondrial structure and function, resulting in diminished cellular metabolism. The consequent low ATP levels and altered ion homeostasis result in the rupture of the plasma membrane and cell death in the myocardium. Therefore, mitochondria appear to be a key factor in cardiomyocyte death, and protecting mitochondria from oxidative stress and maintaining mitochondrial function may represent a means of ameliorating myocardial IRI [3]. Preconditioning the heart with multiple brief episodes of nonlethal ischemia episodes in combination with alternating periods of reflow has been shown to reduce the size of infarcts caused by subsequent ischemic insults, a phenomenon known as myocardial ischemic preconditioning (IPC) [4]. However, the classic IPC procedure requires that interventions be applied directly to the heart, which may potentiate additional injuries to target tissues and arteries and limit its clinical applicability; alternative methods of mimicking the cardioprotective effects of classic ischemic conditioning have been investigated, including both pharmacologic conditioning and remote conditioning [5].

Bakuchiol (BAK), a monoterpenic phenol isolated from the seeds of *Psoralea corylifolia* (Leguminosae) [6], exerts organic protective effects in both the liver [7, 8] and the skin [9]. Recent studies have demonstrated that BAK exerts a wide spectrum of pharmacologic effects, including anti-oxidative [10, 11], anti-inflammatory [12], anti-aging [9, 10], and anti-diabetic [13] effects. However, oxidative stress, inflammation and diabetes are the primary elements of myocardial IRI [14–16], which suggests that BAK may play a promising role in attenuating IRI.

Silent information regulator 1 (SIRT1) is a histone deacetylase that depends on nicotinamide adenine dinucleotide (NAD⁺) [17] and plays a key role in the longevity effects of calorie restriction; its cardioprotective effects are

also well known [17, 18]. The activation of SIRT1 by IPC and resveratrol, a natural phenolic phytoalexin [19], reportedly attenuates myocardial IRI [20, 21]. Previous studies have demonstrated that resveratrol exerts cardioprotective effects by protecting cardiomyocytes from oxidative stress and improving mitochondrial function via the SIRT1 signaling pathway [22, 23]. Additionally, SIRT1 activates peroxisome proliferator-activated receptor- γ co-activator-1 α (PGC-1 α), which attenuates oxidative damage. Previous studies have indicated that the up-regulation of PGC-1 α depends on SIRT1 and protects tissues in both cerebral ischemia [24, 25] and renal IRI [26]. Mitochondrial dysfunction is a pathological consequence of IRI; maintaining mitochondrial function following ischemia may preserve organ function; SIRT1/PGC-1 α activation following IRI accelerates mitochondrial biogenesis and dynamics and ameliorates mitochondrial damage, which contributes to the recovery of mitochondrial protein expression and function [26, 27]. BAK is an analogue of resveratrol and has a resveratrol-like structure [28]. Thus, BAK likely attenuates myocardial IRI by maintaining mitochondrial function via SIRT1. However, the definitive role of SIRT1/PGC-1 α signaling and its relationship with the cardioprotective effects of BAK in the setting of IRI have not been investigated.

Therefore, this study was designed to evaluate the protective effects of BAK in the setting of myocardial IRI and to investigate its potential effects on both myocardial mitochondrial redox states and mitochondrial function. The involvement of SIRT1/PGC-1 α signaling in the maintenance of mitochondrial function was also evaluated.

Materials and methods

Materials

BAK, 4',6-diamino-2-phenylindole (DAPI), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethylsulfoxide (DMSO), and a protease inhibitor cocktail were purchased from Sigma-Aldrich (St. Louis, MO, USA). The SIRT1 siRNA and antibodies against PGC-1 α , Bcl2, Bax, and β -actin were purchased from Cell Signaling Technology (Beverly, MA, USA). Sirtinol and the antibodies against Caspase 3 and SIRT1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The kits used to measure the hydrogen peroxide (H₂O₂), glutathione (GSH), oxidized glutathione (GSSG), lactate dehydrogenase (LDH), creatine kinase-MB (CK-MB), superoxide dismutase (SOD), and malondialdehyde (MDA) levels were purchased from the Institute of Jiancheng Bioengineering (Nanjing, Jiangsu, China). The terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) kits

were purchased from Roche (Mannheim, Germany). The rabbit anti-goat, goat anti-rabbit, and goat anti-mouse secondary antibodies were purchased from the Zhongshan Company (Beijing, China).

Preparation of the perfused isolated rat hearts and BAK treatment

The present study was performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (National Institutes of Health Publication No. 85-23, revised in 1996) and approved by the Ethics Committee of the Fourth Military Medical University. Healthy adult male Sprague–Dawley (SD) rats (weighing between 220 and 250 g) and newborn SD rats (1–2 days old) were obtained from the animal center of the Fourth Military Medical University. As described in a previous study [29], the SD rats were intraperitoneally anesthetized with sodium pentobarbital (50 mg/kg). Twenty minutes following an intraperitoneal injection of 500 U/kg of heparin sodium, the chest was opened. The heart was quickly removed and retrogradely perfused through the aorta with a non-circulating Langendorff apparatus (Radnoti Glass Technology Inc., USA) at a constant pressure of 80 mmHg. The perfusate was Krebs–Henseleit buffer (KHB) containing (in mM) 118 NaCl, 4.7 KCl, 1.2 MgSO₄, 1.2 KH₂PO₄, 1.25 CaCl₂, 25 NaHCO₃, and 11 glucose (pH 7.4, 37 °C), which was continuously gassed with 95 % O₂ and 5 % CO₂. The left ventricular developed pressure (LVDP) and heart rate (HR) were each monitored using a transducer (Model 100 BP-Biopac System Inc., USA) connected to a water-filled latex balloon inserted into the left ventricle via the left atrium. The BAK was dissolved in DMSO as stock solution and used in 3 days. At the beginning of the experiment, the left ventricular end-diastolic pressure (LVEDP) was adjusted to approximately 5 mmHg by inflating the balloon. The isolated heart was surrounded by a homoeothermic glass cover (37 °C) to maintain a constant temperature. All data were recorded and stored using the AcqKnowledge 3.8.1 software package and a Biopac Data Acquisition System (Model 100 BP-Biopac System Inc., Goleta, CA, USA). To establish the IRI model, the isolated perfused rat hearts were subjected to 45 min of ischemia, followed by 60 min of reperfusion. The various concentrations of BAK (0.25 μM, 0.5 μM, and 1 μM) were administrated for 5 min before the IR injury. After different treatments, the heart rate (HR), left ventricular developed pressure (LVDP) and rate of change of pressure development (+dP/dt max) were monitored. The coronary flow (CF) was calculated and collected during reperfusion for biochemical assays. The experimental protocols are included in Fig. 1a–c.

Cell culture, ischemia reperfusion (IR) treatment, and siRNA transfection

The primary cultures of neonatal rat cardiomyocytes from 1- to 2-day-old Sprague–Dawley rats were prepared using a previously reported method [30]. The cells were cultured in Dulbecco's modified Eagle's medium from Gibco (Invitrogen, Life Technologies, Ghent, Belgium), which was supplemented with 10 % fetal bovine serum (FBS) (Biobrom GmbH, Germany), 2 mM L-glutamine, 30 mg/l endothelial cell growth factor supplement (EGCS), 100 IU/ml penicillin, and 100 mg/ml streptomycin at 37 °C and 5 % CO₂. The cells were transiently transfected with control siRNA or SIRT1 siRNA at 50 pM for 24 h using Lipofectamine RNAiMAX reagent (Invitrogen, Carlsbad, CA, USA) in DMEM [31]. After transfection with SIRT1 siRNA for 24 h, IR was then induced in the cardiomyocytes pre-treated with or without BAK for additional 2 h [32]. Then, the cells were harvested for further analysis.

Myocardial apoptosis analyses

A portion of the myocardium from the mid-left ventricle and treated cardiomyocytes were fixed in 4 % formalin. The level of cardiomyocyte apoptosis was analyzed via TUNEL staining. The TUNEL-positive cells that exhibited green nuclear staining, as well as the cells exhibiting blue nuclear DAPI staining, were counted within 5 randomly chosen fields under high-power magnification. The index of apoptosis was expressed as the ratio of positively stained apoptotic myocytes or cardiomyocytes to the total number of myocytes or cardiomyocytes counted × 100 %.

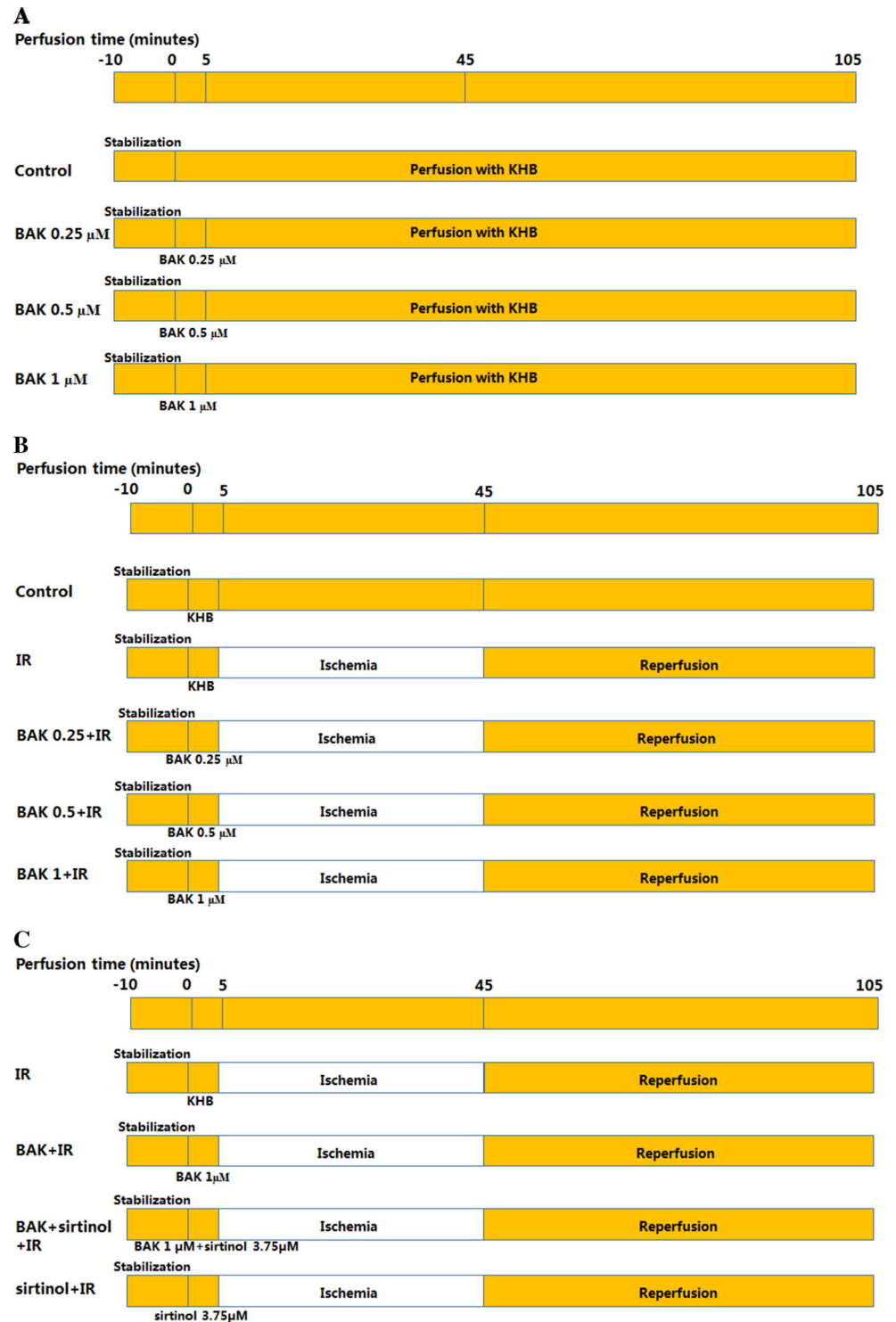
Myocardial necrosis analyses

Myocardial necrosis was estimated based on the levels of both LDH and CK-MB in the coronary effluent and cell lysis buffer. Respectively, the samples were added the substrate buffer and nicotinamide adenine dinucleotide to analyze the LDH. After incubation in 37 °C for 15 min, 2,4-dinitrophenylhydrazine (DNPH) was mixed and incubated for additional 15 min. At last, the 0.4 M NaOH was added at room temperature; the mixture was measured using an FLX 800 fluorescence microplate reader (Biotech Instruments, Inc., USA) at 440 nm. Moreover, the contents of CK-MB were analyzed using creatine kinase MB isoenzyme colorimetric assay kit in accordance with the manufacturer's instructions.

Mitochondria isolation and oxidative damage analyses

The myocardial mitochondrial and cytosolic samples were prepared via differential centrifugation [33]. Briefly, frozen

Fig. 1 Experimental protocols. **a** The effects of BAK treatment on the normal isolated rat hearts. **b** The effects of BAK treatment on the IR-injured isolated hearts. **c** The effects of BAK and Sirtinol pre-treatment on the IR-injured isolated hearts. *BAK* bakuchiol, *KHB* Krebs–Henseleit buffer



myocardial samples and cell lysis buffer were homogenized in 10 volumes of buffer A (250 mM sucrose; 10 mM Tris–HCl, pH 7.4; 1 mM EDTA, pH 7–8; 1 mM orthovanadate; 1 mM NaF; 0.3 mM phenylmethylsulfonyl fluoride (PMSF); and 5 μg/ml each of leupeptin, aprotinin and pepstatin A) and subjected to serial centrifugations at 1000,

10,000 and 100,000×g. The 1000 g pellet (nuclear fraction) was discarded, whereas the 10,000 g pellet (mitochondrial fraction) was washed in buffer A and re-centrifuged. The resulting pellet was re-suspended in buffer B (150 mM NaCl; 20 mM Tris–HCl, pH 7.4; 10 mM EDTA, pH 7–8; 1 mM orthovanadate; 1 mM NaF; 0.3 mM

PMSF; 0.5 µg/ml pepstatin A; 5 µg/ml each of leupeptin and aprotinin; and 1 % NP-40) and subjected to centrifugation at $21,000\times g$ for 10 min. The resulting supernatant was defined as the mitochondrial fraction. The 100,000 g supernatant was defined as the cytosolic fraction. All isolated mitochondrial and cytosolic samples were kept on ice and used within 3 h of isolation. As indicators of mitochondrial oxidative stress, the activity level of SOD and the levels of H_2O_2 , GSH, GSSG, and MDA in the mitochondrial fraction were determined using commercially available kits. After mixed the different reagents with mitochondrial fraction in each kit, the SOD level was measured using an FLX 800 fluorescence microplate reader at 550 nm; while, H_2O_2 level was analyzed at 405 nm. The values of GSH and GSSG were calculated based on the mitochondrial redox potential (E_h) using the Nernst equation, as described previously [33]. Furthermore, the activity levels of succinate dehydrogenase (SDH, complex II) and cytochrome c oxidase (COX, complex IV) were also spectrophotometrically measured in the left ventricle tissue homogenates as previously described [34].

Western blot analyses

The myocardial and cardiomyocyte samples were homogenized in lysis buffer (Beyotime Biotechnology, Haimeng, Jiangsu, China) using a 1 % protease inhibitor cocktail. The lysates were centrifuged for 15 min at $12,000\times g$, and the resulting supernatant was transferred to a new tube and stored at $-70^\circ C$. The protein concentrations were determined using a Bradford protein assay kit, and the proteins were separated via electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked for 1 h in Tris-buffered saline and Tween 20 (TBST, pH 7.6) containing 5 % non-fat dry milk before being incubated overnight at $4^\circ C$ with antibodies against SIRT1, PGC-1 α (1:500 dilutions), Bcl2, Bax, Caspase 3, and β -actin (1:1000 dilution). The membranes were then washed with TBST and subsequently probed with the appropriate secondary antibodies (1:5000 dilution) at room temperature for 90 min. The membranes were washed again in TBST, and the protein bands on the membranes were detected using a Bio-Rad imaging system (Bio-Rad, Hercules, CA, USA) and quantified using the Quantity One software package (West Berkeley, CA, USA).

Statistical analysis

The results are expressed as the means \pm standard deviations (SDs). Significance was determined using a two-tailed Student's *t* test and one-way ANOVA, followed by the Tukey post hoc test, using statistical software (Prism 5.0 GraphPad Software, Inc., La Jolla, CA, USA); $P < 0.05$ was considered to indicate significant differences.

Results

The effects of BAK on LVDP, LDH release, CK-MB release, and SIRT1 expression in normal rat hearts

Following 110 min of perfusion, BAK (0.25, 0.5, or 1 µM) treatment did not affect the LVDP ($P > 0.05$ vs. the control group, Fig. 2a). Additionally, BAK treatment did not affect the LDH (Fig. 2b) or CK-MB (Fig. 2c) release in the coronary effluent ($P > 0.05$ vs. the control group). Interestingly, BAK significantly increased the levels of SIRT1 and PGC-1 α expression in the normal hearts in a dose-dependent manner (Fig. 2d).

The effects of BAK on cardiac function, apoptotic index, LDH release, and CK-MB release in IR-injured hearts

Following the IR procedure, the absolute values of LVDP, $+dP/dt$ max, CF, and HR decreased significantly compared with their baseline values ($P < 0.05$). BAK treatment (0.25, 0.5, or 1 µM) significantly increased the functional recovery of post-ischemic hearts, as demonstrated by dose-dependent increases in the LVDP, $+dP/dt$ max, CF, and HR values throughout the reperfusion period ($P < 0.05$ vs. the IR group, Fig. 3a–d). The apoptotic index in IR hearts was 59.80 ± 4.73 %, and BAK treatment (0.25, 0.5, or 1 µM) facilitated significant dose-dependent reductions in the apoptotic index to 39.10 ± 4.26 , 29.55 ± 3.41 , and 16.48 ± 3.49 % ($P < 0.05$ vs. the IR group), respectively (Fig. 4a). The total amounts of released LDH and CK-MB in the IR group were 131.79 ± 7.50 (Fig. 4b) and 62.44 ± 4.59 IU/L/g of wet weight, respectively (Fig. 4c). BAK treatment (0.25, 0.5, or 1 µM) also significantly reduced LDH release to 99.17 ± 5.42 , 82.29 ± 4.66 , and 69.17 ± 4.40 IU/L/g of wet weight ($P < 0.05$ vs. the IR group), respectively, and reduced CK-MB release to 55.18 ± 4.18 , 40.03 ± 3.94 , and 33.15 ± 3.50 IU/L/g of wet weight ($P < 0.05$ vs. the IR group), respectively. These changes were most significant in response to 1 µM BAK; therefore, this value was selected for pre-treatment in subsequent experiments.

The effects of BAK on the mitochondrial oxidative damage indicators of IR-injured hearts

The mitochondrial oxidative damage indicators underwent additional measurements. As expected, the mitochondrial SDH (Fig. 5a), COX (Fig. 5b), and SOD activities (Fig. 5c) and mitochondrial E_h (Fig. 5f) were significantly decreased, whereas the MDA (Fig. 5d) and H_2O_2 (Fig. 5e) content was significantly increased, in the IR group ($P < 0.05$ vs. the control group). BAK treatment

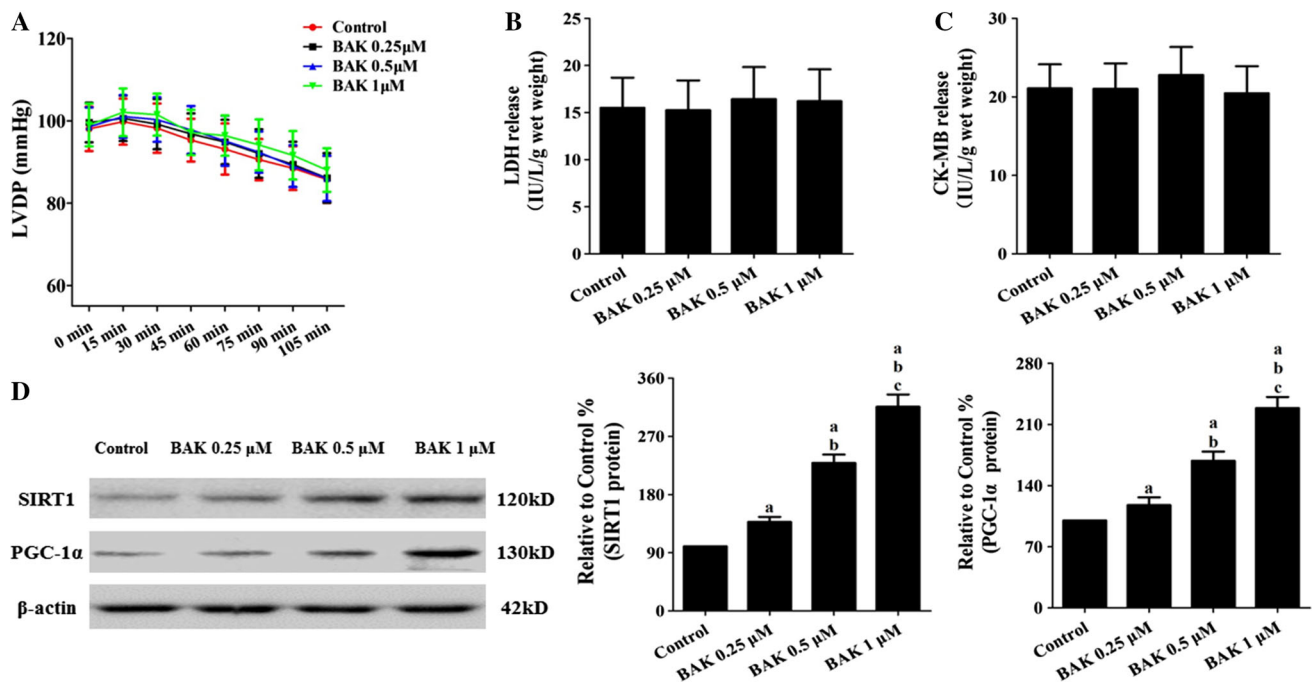


Fig. 2 The effects of BAK on LVDP, LDH release, CK-MB release, and SIRT1 and PGC-1 α expression in the normal rat hearts. **a** A representative *line graph* of the LVDP curves. **b** The level of LDH was normalized to the net heart weight and is expressed in IU/g. **c** The level of CK-MB was normalized to the net heart weight and is expressed in IU/g. **d** A representative image of the SIRT1 and PGC-

1 α Western blot results is depicted. The results are expressed as the means \pm SDs, $n = 8$, ^a $P < 0.05$ versus the control group, ^b $P < 0.05$ versus the IR group, ^c $P < 0.05$ versus the 0.25 μ M BAK + IR group, ^d $P < 0.05$ versus the 0.5 μ M BAK + IR group. BAK bakuchiol, LVDP left ventricular peak developing pressure

significantly increased the SDH, COX, SOD activities and mitochondrial E_h , as well as decreased the MDA content and H_2O_2 formation ($P < 0.05$ vs. the IR group). These effects were most significant with 1 μ M BAK, which increased the SDH, COX, SOD activities and mitochondrial E_h to 6.30 ± 0.49 U/mg of protein, 67.08 ± 4.31 mM/min/mg of protein, 19.40 ± 1.53 U/mg of protein, and 191.24 ± 16.88 mV, respectively, and decreased the MDA content and H_2O_2 formation to 1.14 ± 0.19 nM/mg of protein and 91.32 ± 5.11 μ M/mg protein, respectively.

The effects of BAK on SIRT1, PGC-1 α , Bcl2, Bax, and cleaved Caspase 3 expression in IR-injured hearts

As depicted in Fig. 6, the levels of SIRT1, PGC-1 α , and Bcl2 expression significantly decreased, whereas Bax and cleaved Caspase 3 expression significantly increased, in the IR group ($P < 0.05$ vs. the control group). BAK treatment significantly increased the levels of SIRT1, PGC-1 α , and Bcl2 expression and decreased the level of Bax and cleaved Caspase 3 expression ($P < 0.05$ vs. the IR group). These effects were most significant with 1 μ M BAK, which increased the levels of SIRT1, PGC-1 α , and Bcl2

expression to 61.08 ± 4.20 , 67.30 ± 4.77 , and 74.69 ± 4.46 %, respectively, and decreased the levels of Bax and cleaved Caspase 3 expression to 96.16 ± 4.75 and 166.91 ± 15.88 %, respectively.

The effects of BAK and Sirtinol on LVDP, LDH release, and CK-MB release in IR-injured isolated hearts

Additional experiments were performed to determine the role of SIRT1/PGC-1 α signaling in the myocardial protective effects of BAK. Sirtinol was used as an SIRT1 inhibitor. As depicted in Fig. 7a–c, BAK (1 μ M) treatment significantly increased LVDP to 64.98 ± 7.25 mmHg following 60 min of reperfusion and decreased LDH and CK-MB release to 65.04 ± 4.37 and 35.69 ± 3.64 IU/L/g of wet weight ($P < 0.05$ vs. IR group), respectively. Sirtinol (3.75 μ M), a specific inhibitor of SIRT1 based on our previous research [32], reversed the protective effects of BAK, decreased LVDP to 50.4 ± 6.6 mmHg, and increased LDH and CK-MB release to 107.12 ± 6.59 and 55.90 ± 4.50 IU/L/g of wet weight ($P < 0.05$ vs. BAK + IR group), respectively. Compared with the IR group, Sirtinol + IR treatment did not affect the LVDP, LDH release, and CK-MB release ($P > 0.05$). However,

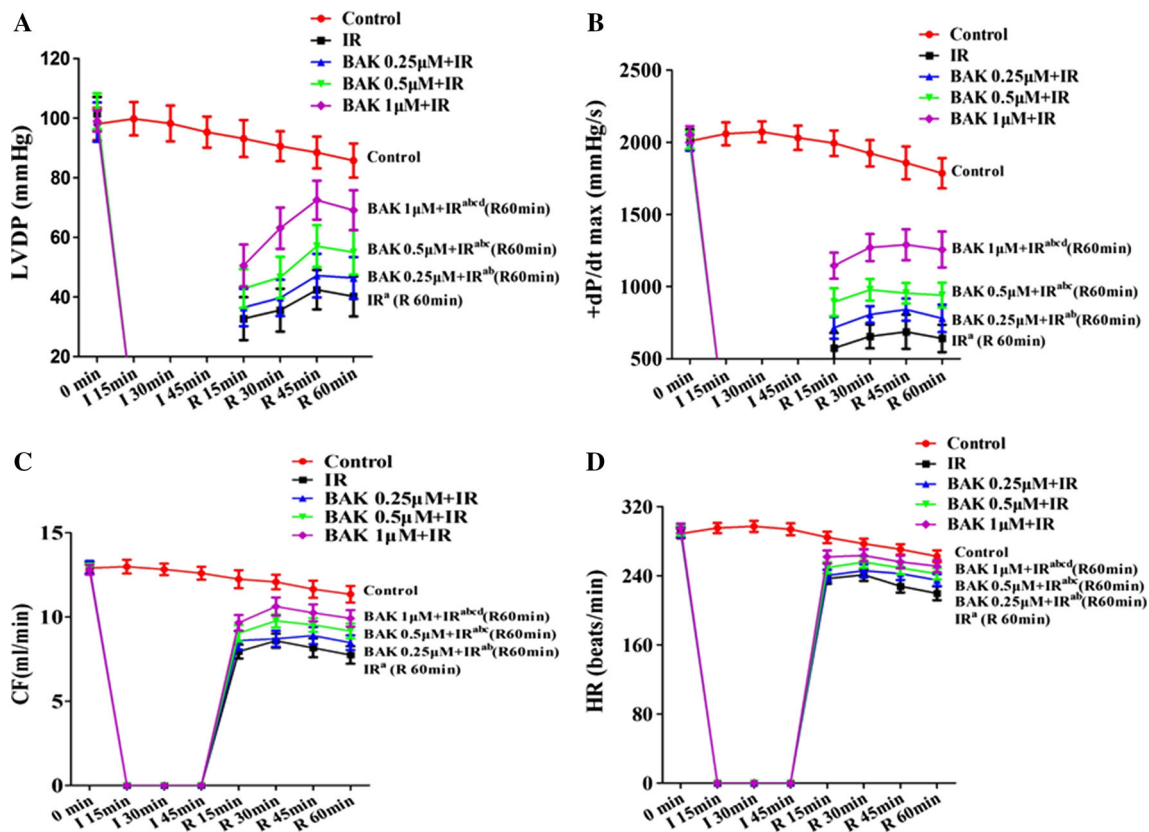


Fig. 3 The effects of BAK on cardiac function in the IR-injured hearts. **a** A representative line graph of the LVDP curves. **b** A representative line graph of the +dP/dt curves. **c** A representative line graph of the CF curves. **d** A representative line graph of the HR curves. The results are expressed as the means \pm SDs, $n = 8$,

^a $P < 0.05$ versus the control group, ^b $P < 0.05$ versus the IR group, ^c $P < 0.05$ versus the 0.25 μM BAK + IR group, ^d $P < 0.05$ versus the 0.5 μM BAK + IR group. BAK bakuchiol, LVDP left ventricular peak developing pressure, +dP/dt max the maximum rate of pressure change in the ventricle, CF coronary flow, HR heart rate

Sirtinol treatment alone did not affect the expressions of these targets.

The effects of BAK and Sirtinol on the mitochondrial oxidative damage indicators of IR-injured hearts

As depicted in Fig. 7d–i, BAK treatment significantly increased the SDH, COX, and SOD activities and mitochondrial E_h to 6.37 ± 0.48 U/mg of protein, 69.18 ± 4.60 mM/min/mg of protein, 18.93 ± 1.69 U/mg of protein, and 186.93 ± 15.48 mV, respectively, and decreased the MDA content and H_2O_2 formation to 1.09 ± 0.17 nM/mg of protein and 89.55 ± 5.01 μM /mg protein, respectively ($P < 0.05$ vs. IR group). Sirtinol reversed the mitochondrial protective effects of BAK and decreased the SDH, COX, and SOD activities and mitochondrial E_h to 5.29 ± 0.41 U/mg of protein, 51.65 ± 3.77 mM/min/mg of protein, 13.17 ± 1.27 U/mg of protein, and 168.21 ± 16.42 mV, respectively. It also decreased MDA content and H_2O_2 formation to 1.78 ± 0.19 nM/mg of protein and 116.57 ± 5.98 μM /mg protein,

respectively ($P < 0.05$ vs. BAK + IR group). Compared with the IR group, Sirtinol + IR treatment did not affect the SDH, COX, or SOD activities, mitochondrial E_h , MDA content, or H_2O_2 formation ($P > 0.05$).

The effects of BAK and Sirtinol on SIRT1, PGC-1 α , Bcl2, Bax, and cleaved Caspase 3 expression in IR-injured hearts

As depicted in Fig. 8, BAK treatment significantly increased the levels of SIRT1, PGC-1 α , and Bcl2 expression to 294.03 ± 17.52 , 279.20 ± 16.28 , and 336.37 ± 19.29 %, respectively, and decreased the level of Bax and cleaved Caspase 3 expression to 19.78 ± 2.14 and 146.22 ± 14.61 %, respectively ($P < 0.05$ vs. IR group). Sirtinol not only effectively inhibited SIRT and PGC-1 α expression ($P < 0.05$, shown in Supplementary Fig. 1) but also reversed the effect of BAK and decreased the levels of SIRT1, PGC-1 α , and Bcl2 expression to 86.48 ± 4.61 , 188.09 ± 10.64 , and 77.16 ± 4.71 %, respectively, and increased the levels of Bax and cleaved Caspase 3

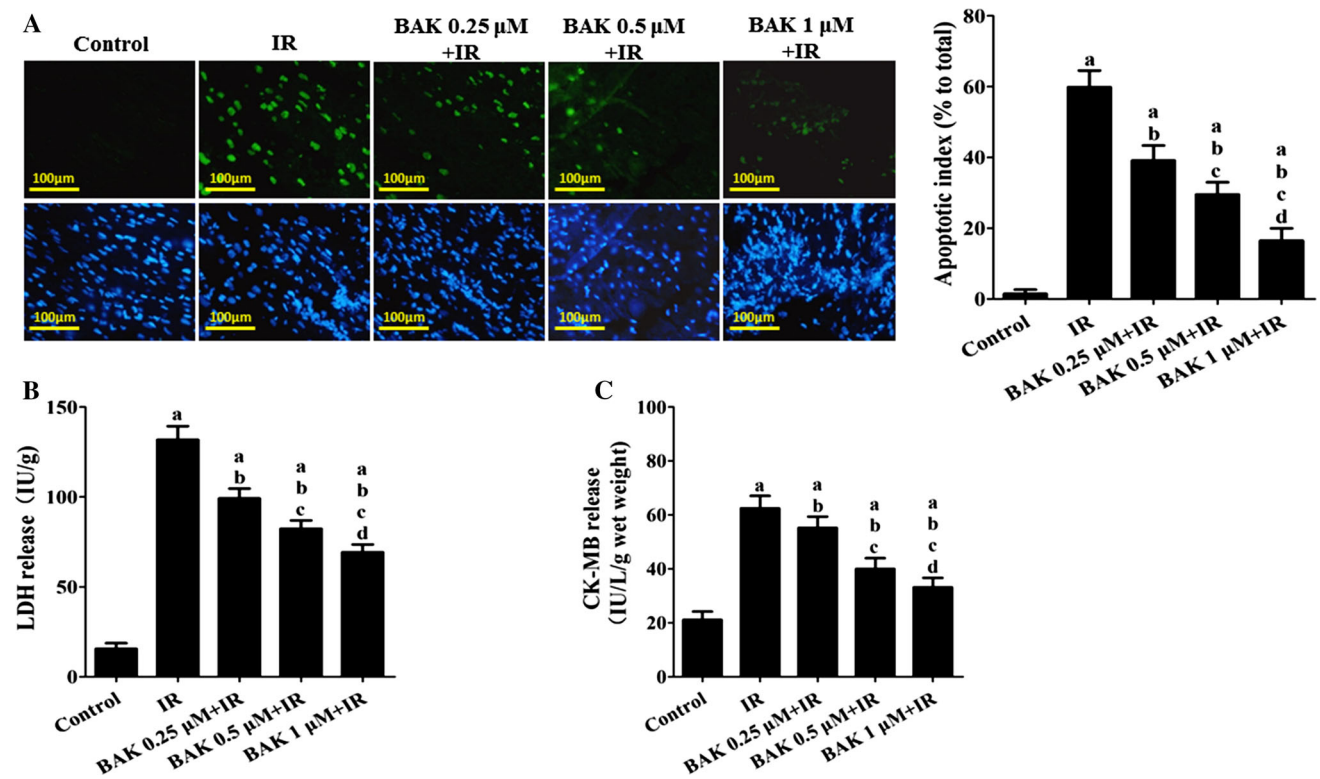


Fig. 4 The effects of BAK on apoptotic index, LDH release, and CK-MB release in the IR-injured hearts. **a** Representative images of apoptotic cardiomyocytes are presented ($\times 400$). The apoptotic cells were detected using immunofluorescence staining with TUNEL (green), and the nuclei were labeled with DAPI (blue). **b** The level of LDH was normalized to the net heart weight and is expressed in

IU/g. **c** The level of CK-MB was normalized to the net heart weight and is expressed in IU/g. The results are expressed as the means \pm SDs, $n = 8$, ^a $P < 0.05$ versus the control group, ^b $P < 0.05$ versus the IR group, ^c $P < 0.05$ versus the 0.25 μ M BAK + IR group, ^d $P < 0.05$ versus the 0.5 μ M BAK + IR group. BAK bakuchiol (Color figure online)

expression to 80.05 ± 4.83 and 206.21 ± 20.55 %, respectively ($P < 0.05$ vs. BAK + IR group). Compared with the IR group, Sirtinol + IR treatment effectively decreased the levels of SIRT1 and PGC-1 α expression to 19.09 ± 2.35 and 25.64 ± 3.26 %, respectively ($P < 0.05$).

The effects of BAK and SIRT1 siRNA pretreatment on the cell viability, apoptotic index, and mitochondrial oxidative damage indicators of IR-injured cardiomyocytes

The cardiomyocyte results were consistent with those that were obtained from the isolated rat. The cell viability was analyzed by the MTT assay. Pretreatment with BAK markedly increased cell viability following IR (vs. control siRNA + IR group, $P < 0.05$, Supplementary Fig. 2A). In addition, BAK pretreatment significantly decreased the apoptotic index (vs. control siRNA + IR group, $P < 0.05$, Supplementary Fig. 2B). However, the protective effect of BAK pretreatment was abolished by SIRT1 siRNA (vs. control siRNA + BAK + IR group, $P < 0.05$). Significantly

reduced levels of LDH (Supplementary Fig. 2C) and MDA (Supplementary Fig. 2E) were observed in the control siRNA + BAK + IR group compared with the control siRNA + IR group ($P < 0.05$). In addition, BAK pretreatment significantly increased mitochondrial SOD (vs. control siRNA + IR group, $P < 0.05$, Supplementary Fig. 2D). However, SIRT1 siRNA pretreatment abolished most of the protective effect afforded by BAK pretreatment ($P < 0.05$). Compared with the control group, control siRNA had no effect on the cell viability, apoptotic index, and LDH, SOD, and MDA release.

The effects of BAK and SIRT1 siRNA pretreatment on SIRT1, PGC-1 α , Bcl2, Bax, and cleaved Caspase 3 expression in IR-injured cardiomyocytes

As depicted in Supplementary Fig. 3, BAK pretreatment significantly increased SIRT1, PGC-1 α , and Bcl2 and decreased Bax and cleaved Caspase 3 expression (vs. the control siRNA + IR group, $P < 0.05$). However, the effect of BAK pretreatment on the expression of these proteins was abolished by SIRT1 siRNA (vs. control siRNA + BAK + IR

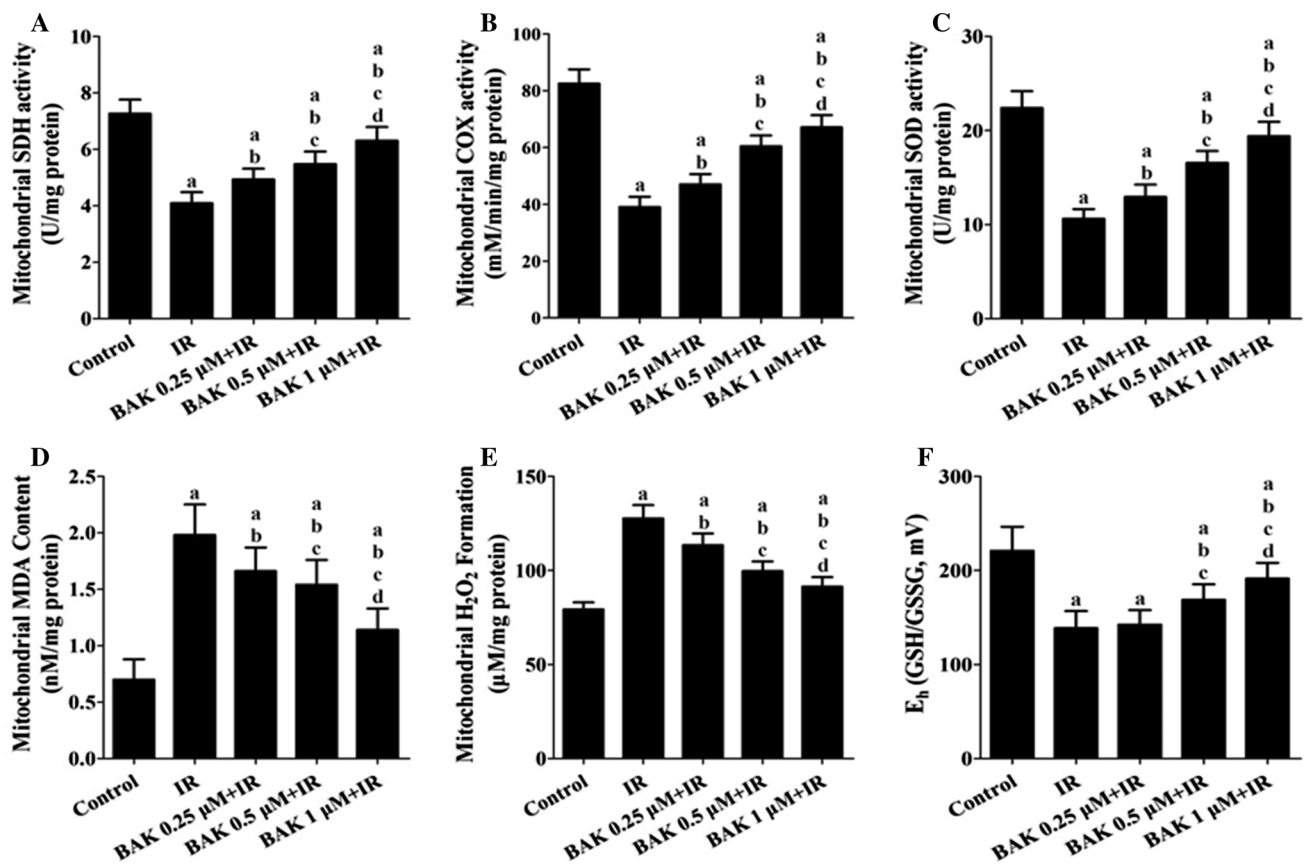


Fig. 5 The effects of BAK on the mitochondrial oxidative damage indicators of the IR-injured hearts. **a** SDH activity in the mitochondria. **b** COX activity in the mitochondria. **c** SOD activity in the mitochondria. **d** MDA production in the mitochondria. **e** H₂O₂ production in the mitochondria. **f** GSH and GSSG in mitochondria were calculated based on the redox potential (E_h). The results are

expressed as the means \pm SDs, $n = 8$, ^a $P < 0.05$ versus the control group, ^b $P < 0.05$ versus the IR group, ^c $P < 0.05$ versus the 0.25 μ M BAK + IR group, ^d $P < 0.05$ versus the 0.5 μ M BAK + IR group. BAK bakuchiol, SDH succinate dehydrogenase, COX cytochrome c oxidase, SOD superoxide dismutase, MDA malondialdehyde, E_h mitochondrial redox potential

group, $P < 0.05$). As expected, SIRT1 siRNA + IR treatment significantly decreased SIRT1 expression (vs. IR group, $P < 0.05$).

Discussion

BAK, a monoterpene phenol isolated from the seeds of *P. corylifolia* (Leguminosae) [6], exerts many biological and pharmacological effects. Park et al. observed that BAK protects against the hepatocyte toxicants tert-butyl hydroperoxide and carbon tetrachloride and attenuates D-GalN-induced toxicity in the liver [8]. In another study, Park et al. suggested that BAK protects the liver from fibrosis and cirrhosis via caspase-3-dependent apoptosis by preventing the c-Jun NH2-terminal kinase-mediated mitochondrial translocation of Bax [7]. Additionally, Chaudhuri and Bojanowski observed that BAK, a retinol-like functional compound, significantly improves the lines and wrinkles, pigmentation, elasticity, firmness, and photo-

damage of the skin [9]. Importantly, although the direct relationship between BAK and myocardial IRI has not been elucidated, BAK has been indicated to inhibit the pathogenesis of myocardial IRI, including oxidative stress, inflammation, and diabetes [14–16]. Seo et al. confirmed that BAK inhibits oxidative stress-induced ROS production and mitochondrial dysfunction [10]. Moreover, Choi et al. suggested that BAK is a potent anti-inflammatory compound [12], and Krenisky et al. observed the protective effects of BAK in the setting of diabetes [13]. Therefore, BAK likely plays a protective role in myocardial IRI. In this study, BAK pre-treatment protected isolated rat hearts, as demonstrated by improvements in the physiological, enzymologic, and cell survival indices, including improvements in the LDVP, dP/dt max, CF, heart rate, and LDH and CK-MB release, as well as decreased cardiomyocyte apoptosis.

SIRT1 is a member of the class III group of histone deacetylases, which deacetylate many transcription factors and nuclear co-activators and are involved in the protection

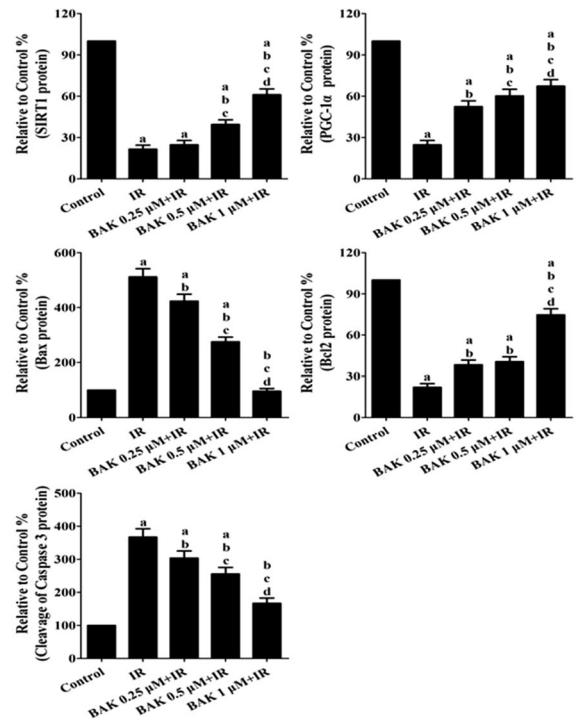
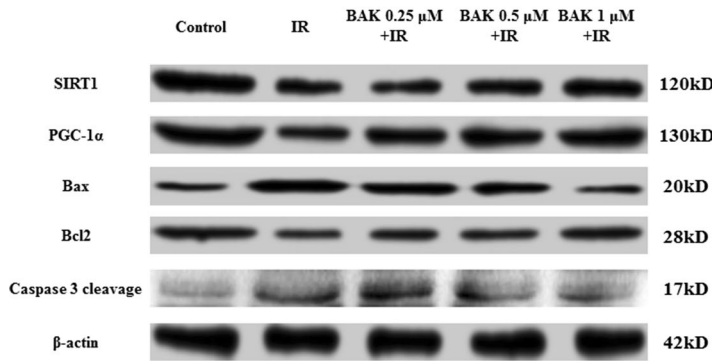


Fig. 6 The effects of BAK on SIRT1, PGC-1α, cleaved Caspase 3, Bcl2 and Bax expression in the IR-injured hearts. Representative images of the Western blot results are provided. The results are expressed as the means ± SDs, n = 8, ^aP < 0.05 versus the control

group, ^bP < 0.05 versus the IR group, ^cP < 0.05 versus the 0.25 μM BAK + IR group, ^dP < 0.0 versus the 0.5 μM BAK + IR group. *BAK* bakuchiol

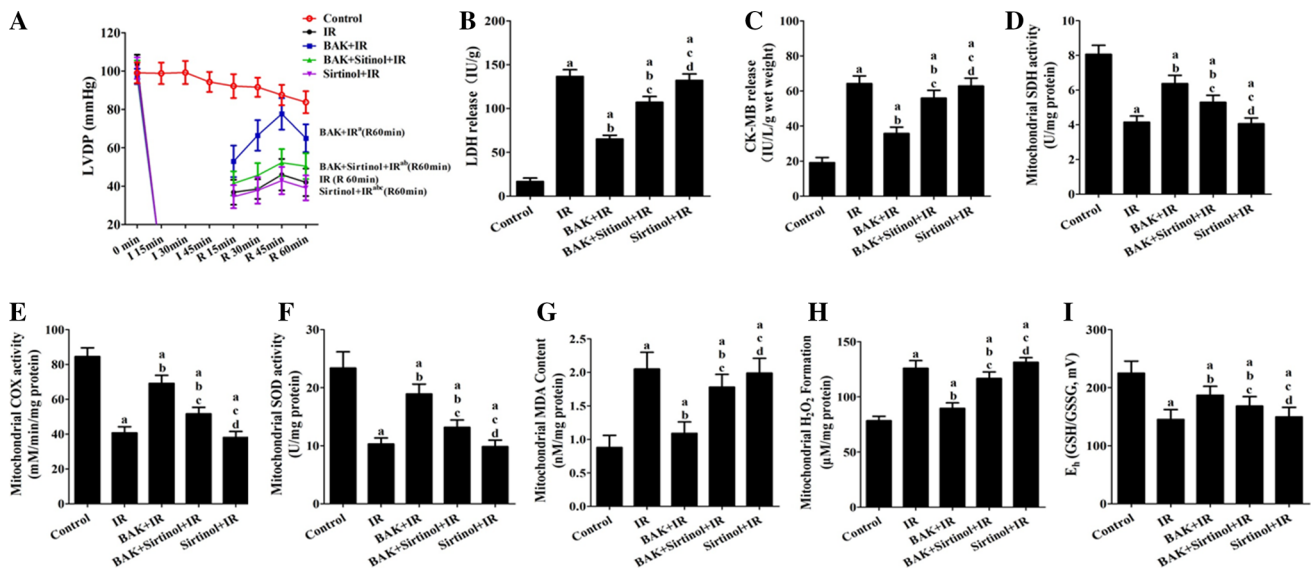


Fig. 7 The effects of BAK and Sirtinol on LVDP, LDH release, CK-MB release, and the mitochondrial oxidative damage indicators of the IR-injured isolated hearts. **a** A representative line graph of the LVDP curves. **b** The level of LDH was normalized to the net heart weight and is expressed in IU/g. **c** The level of CK-MB was normalized to the net heart weight and is expressed in IU/g. **d** SDH activity in the mitochondria. **e** COX activity in the mitochondria. **f** SOD activity in the mitochondria. **g** MDA production in the mitochondria. **h** H₂O₂

production in the mitochondria. **i** GSH and GSSG in the mitochondria were calculated based on the redox potential (E_h). The results are expressed as the means ± SDs, n = 8, ^aP < 0.05 versus the IR group, ^bP < 0.05 versus the BAK + IR group, ^cP < 0.05 versus the BAK + Sirtinol + IR group. *BAK* bakuchiol, *LVDP* left ventricular peak developing pressure, *SDH* succinate dehydrogenase, *COX* cytochrome c oxidase, *SOD* superoxide dismutase, *MDA* malondialdehyde, E_h mitochondrial redox potential

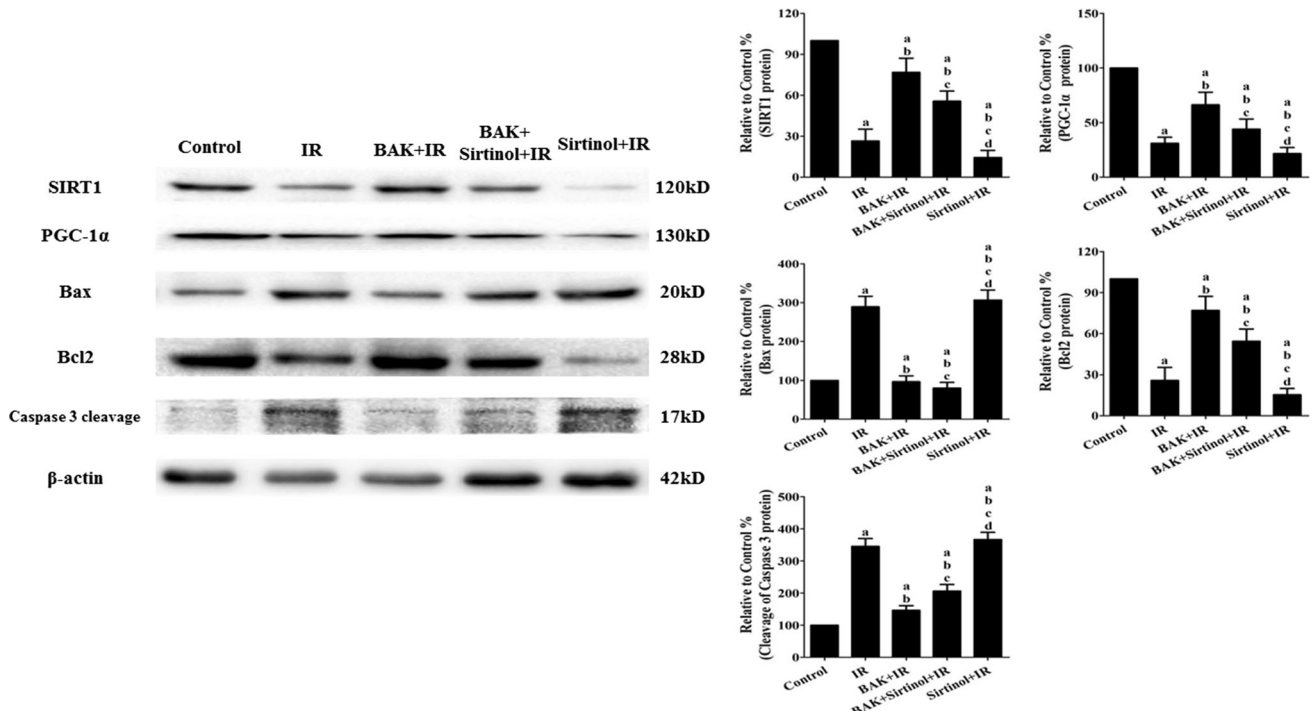


Fig. 8 The effects of BAK and Sirtinol on SIRT1, PGC-1α, cleaved Caspase 3, Bcl2 and Bax expression in the IR-injured hearts. Representative images of the Western blot results are provided. The results are expressed as the means ± SDs, n = 8, ^aP < 0.05 versus

the control group, ^bP < 0.05 versus the IR group, ^cP < 0.05 versus the BAK + IR group, ^dP < 0.05 versus the BAK + Sirtinol + IR group. *BAK* bakuchiol

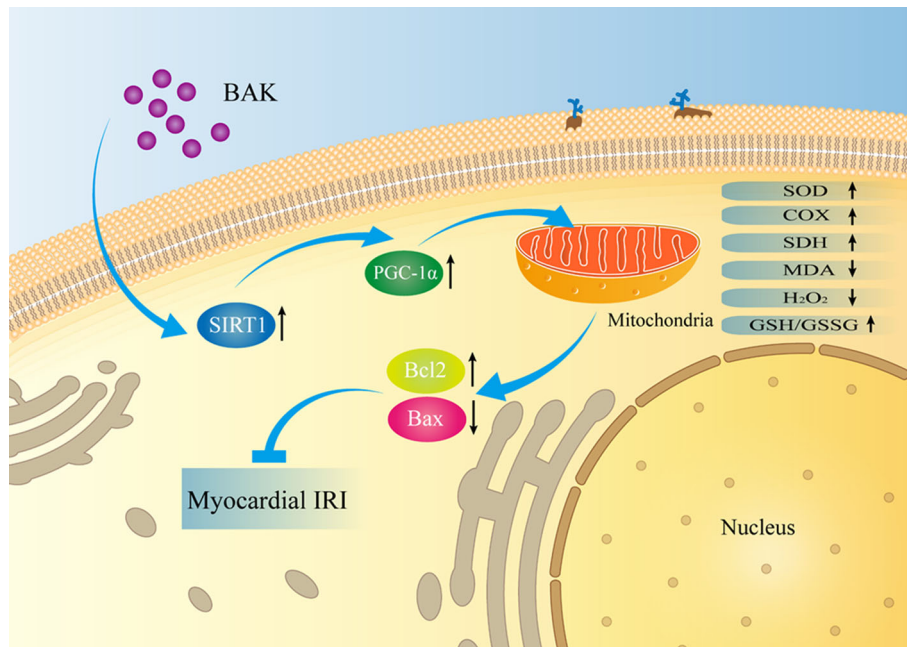


Fig. 9 The SIRT1-dependent mechanism of the cardioprotective effects against IRI afforded by BAK. BAK upregulated SIRT1, which subsequently triggered PGC-1α expression. The downstream effects included improvements in SDH, COX, and SOD activities; improvements in mitochondrial redox potential (E_h); and decreased MDA and H₂O₂ contents in the mitochondria. These changes resulted in an

increase in the level of the anti-apoptotic factor Bcl2 and a decrease in the level of the pro-apoptotic factor cleaved Caspase 3 and Bax, resulting in cardioprotective effects against myocardial IRI. *SIRT1* silent information regulator 1, *IRI* ischemia reperfusion injury, *BAK* bakuchiol, *SDH* succinate dehydrogenase, *COX* cyclooxygenase, *SOD* superoxide dismutase, *MDA* malondialdehyde

of many organs and tissues, including the heart and brain [17]. Zhang et al. observed that interferon regulatory factor 9 mediated myocardial IRI by negatively regulating the SIRT1-p53 axis in the setting of IR, both in vivo and in vitro. SIRT1 protects cardiomyocytes from myocardial IRI via deacetylase activity, which promotes the deacetylation of acetyl-p53 and downregulates a downstream apoptosis-related signaling cascade [35]. Lysine deacetylation occurs during IPC, and Nadtochiy et al. suggested that increased SIRT1 activity plays a role in this phenomenon. The inhibition of SIRT1, either directly or by restricting the availability of its substrate, NAD⁺, inhibits IPC. These data suggest a role for SIRT1-mediated lysine deacetylation in the mechanism underlying acute IPC [20]. Moreover, Hong et al. observed that resveratrol downregulates c-Myc and β -catenin via SIRT1 activation, mimicking the effects of IPC [21]. Additionally, Paschalaki et al. observed that SIRT1 upregulation attenuated DNA damage and the senescence of endothelial progenitor cells in smokers and patients with chronic obstructive pulmonary disease, exerting cardiovascular protective effects [36]. Furthermore, Li et al. demonstrated that resveratrol protects cardiomyocytes from oxidative stress via SIRT1 and mitochondrial biogenesis signaling pathways [22]. Similarly, Shalwala et al. suggested that SIRT1 activation mediates sildenafil- and resveratrol-induced cardioprotection against IRI in mice [37], and Sun et al. observed that resveratrol protects hypothermic preserved isolated rat hearts from injury via the upregulation of SIRT1 expression [38]. Importantly, previous studies have demonstrated that SIRT1 activates PGC-1 α and plays a role in the attenuation of IRI. Zhu et al. suggested that icariin protects against IRI by enhancing SIRT1-dependent PGC-1 α expression in the setting of cerebral ischemic stroke [25], and Fu et al. demonstrated that α -lipoic acid upregulates SIRT1 expression [24]. The overexpression of SIRT1 activates the transcriptional activity of PGC-1 α and protects the brain against focal ischemia in mice [24]. BAK is an analog of resveratrol, which inhibits ROS generation in cardiomyocytes via SIRT1 [22]. Therefore, we hypothesized that BAK protected tissues from myocardial IRI by attenuating oxidative stress via the SIRT1/PGC-1 α pathway. In this study, treating normal hearts with BAK induced a dose-dependent increase in SIRT1 and PGC-1 α expression in the absence of significant differences in the LDVP and LDH levels and CK-MB release. IR significantly decreased both SIRT1 and PGC-1 α expression in isolated hearts, whereas BAK pre-treatment significantly restored SIRT1 and PGC-1 α expression in a dose-dependent manner. However, inhibiting SIRT1 with Sirtinol and SIRT1 siRNA abolished the BAK-mediated increases in the expression of SIRT1 and PGC-1 α . Moreover, the SIRT1 upregulation induced by BAK treatment was also

related to an increase in the level of the apoptosis-related factor cleaved Caspase 3 and anti-apoptotic factor Bcl2, as well as a reduction in the level of the pro-apoptotic factor Bax. Additionally, previous studies have demonstrated that SIRT1 inhibits apoptotic signals by inducing Bcl2 expression and reducing Bax expression [39]. Therefore, BAK pre-treatment protects cardiac tissue via SIRT1-dependent PGC-1 α activation and the induction of downstream signals associated with anti-apoptotic effects in the heart.

Resveratrol treatment has been shown to improve mitochondrial biogenesis and function in the setting of IRI, while the inhibition of SIRT1 reversed these benefits [20, 23], indicating that SIRT1 activity reduces mitochondrial oxidative stress and maintains mitochondrial function. Additionally, Della-Morte et al. suggested that the resveratrol-mediated upregulation of SIRT1 inhibits mitochondrial uncoupling protein 2 (UCP2), which has been linked to mitochondrial dysfunction [40]. Das et al. also determined that the resveratrol-mediated upregulation of SIRT1 protects cardiac tissue by activating the Forkhead box, subgroup O (Foxo3a)/PTEN-induced kinase 1 (PINK1)/PARKIN signaling pathway, resulting in mitochondrial fission and mitophagy, as well as the attenuation of oxidative injury [41]. Mitochondrial oxidative stress is a significant pathological consequence of IRI and increases mitochondrial oxidative damage, which subsequently accelerates both the development and progression of oxidative stress. In this study, we investigated the redox status of mitochondria following ischemia in isolated hearts. The results of these experiments suggested that BAK pre-treatment maintained mitochondrial function, as evidenced by significant improvements in the mitochondrial SDH, COX, and SOD activities. Moreover, increased mitochondrial E_h and decreased mitochondrial MDA content and H₂O₂ formation were also observed, which indicates that the mitochondrial oxidative damage caused by IRI was attenuated. However, Sirtinol and SIRT1 siRNA abolished the improvements in mitochondrial function secondary to BAK pre-treatment. Together, these findings indicate that BAK attenuates myocardial IRI-induced mitochondrial oxidative damage via SIRT1 activation.

Our findings suggest that BAK treatment exerts promising cardioprotective effects in the setting of IRI. These effects were primarily attributed to the activation of SIRT1/PGC-1 α signaling and the attenuation of mitochondrial oxidative injury, as SIRT1/PGC-1 α signaling attenuates the myocardial IRI-induced mitochondrial oxidative damage (Fig. 9). These results indicate that BAK may play a promising role in the treatment of myocardial IRI in the setting of cardiac surgery and ischemic heart disease.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest in the studies described.

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