Abstract. Endomorphins (EMs) have important roles in the body with regards to analgesia, feeding behavior, gastrointestinal movement and inflammatory reaction. Recent studies have reported that EMs may also participate in chronic hypoxia in the protection of rat myocardial ischemia/reperfusion; however, the mediator and underlying mechanisms remain to be elucidated. The aim of the present study was to investigate the effects of EM-1 postconditioning on myocardial ischemia/reperfusion injury (MIRI) and myocardial cell apoptosis in a rat model, and to assess its likely mechanisms. A total of 48 male Sprague Dawley rats were randomly divided into four groups: Sham group, ischemia/reperfusion group (IR group), ischemic postconditioning group (IPO group) and EM-1 postconditioning group (EM50 group). A MIRI model was established via occlusion of the left anterior descending branch of the coronary artery for 30 min, followed by reperfusion for 120 min in vivo. Hemodynamic indexes were recorded and analyzed. Following reperfusion, plasma lactate dehydrogenase (LDH), creatine kinase-MB (CK-MB), malondialdehyde (MDA), superoxide dismutase (SOD), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) contents or activities were measured, infarct size was determined, and the expression levels of B-cell lymphoma 2 (Bcl-2) and Bcl-2-associated X protein (Bax) mRNA and cleaved caspase-3 protein were assessed. In the IR group, mean arterial pressure (MAP) and heart rate (HR) were decreased compared with in the sham group. In addition, LDH and CK-MB levels were increased; IL-6, TNF-α and MDA content was increased; SOD activity was decreased; the Bcl-2/Bax ratio was decreased; and cleaved caspase-3 protein expression levels were increased in the IR group. Compared with in the IR group, in the IPO and EM50 groups, MAP and heart rate (HR) were recovered to various extents post-reperfusion: LDH and CK-MB levels were decreased; IL-6, TNF-α and MDA content was decreased; SOD activity was increased; infarct size was reduced; the Bcl-2/Bax ratio was increased; and cleaved caspase-3 protein expression levels were decreased. In conclusion, EM-1 postconditioning was revealed to reduce I/R injury and inhibit myocardial cell apoptosis, which may be associated with reductions in oxidative stress and inflammatory reactions.

Introduction

Endomorphins (EMs) are a type of opioid peptide, which were initially identified in 1997 by Zadina et al (1); their identification prompted research into endogenous opioid peptides. EMs have important roles in the body with regards to analgesia (2), feeding behavior (3), gastrointestinal movement (4) and inflammation (5). In recent years, EMs have been reported to have an important role in cardiovascular biology (6), and have been shown to protect endothelial cells by slowing down the process of apoptosis and promoting proliferation under physiological concentrations (7,8).

In recent years, the incidence of acute myocardial infarction has increased, and myocardial ischemia/reperfusion injury (MIRI) is considered a complex problem. Due to the time limit of ischemic preconditioning (IPC) and the invasive operation associated with ischemic postconditioning (IPO), pharmacological postconditioning (9) is considered a more feasible measure to reduce MIRI. Previous studies have suggested that exogenous opioids, including morphine, fentanyl and remifentanil, can resist MIRI and exert myocardial protection; however, the long-standing or excessive use of exogenous drugs may produce toxicity to the organism (10,11). Therefore, the use of EMs, which are endogenous opioids, may be considered a promising novel therapeutic strategy. Numerous studies have confirmed that MIRI can induce oxidative stress, mito-
chondrial dysfunction and inflammation (12, 13). Furthermore, previous studies have reported that EMs have an important role in clearing oxygen free radicals (14) and inhibiting mitochondrial dysfunction (15). It has also been demonstrated that EMs may participate in chronic hypoxia in the protection of rat MIRI (16). There are two types of EM: EM-1 and EM-2. EM-1 is widely distributed in the brain (17) and may have a certain correlation with cardiovascular regulatory function.

Cell apoptosis has a substantial role in MIRI; it is an important process in the occurrence and development of MIRI (18), and a relevant mechanism underlying myocardial damage and myocardial cell loss (19-21). Therefore, the present study aimed to determine the effects of EM-1 postconditioning on MIRI and myocardial cell apoptosis in a rat model, and to analyze the underlying mechanisms.

Materials and methods

Animals. Male clean-grade Sprague Dawley rats (weight, 250-350 g; age, 3 months) were obtained from the Animal Center of Bengbu Medical College (Bengbu, China). The rats were fed a normal diet and had ad libitum access to water. All rats were housed in cages at 25±1°C with a fixed 12-h light/dark cycle. All animal procedures were conducted in accordance with the United States National Institutes of Health Guide, and were approved by the Animal Use and Care Committee of Bengbu Medical College.

Materials and primary reagents. EM-1 was purchased from Sigma-Aldrich (Merck Millipore, Darmstadt, Germany). Lactate dehydrogenase (LDH), malondialdehyde (MDA) and superoxide dismutase (SOD) activity kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The creatine kinase MB (CK-MB) isoenzyme enzyme-linked immunosorbent assay (ELISA) kit, interleukin-6 (IL-6) ELISA kit and tumor necrosis factor-α (TNF-α) ELISA kit were purchased from Biosalvin Co., Ltd. (Jiangsu, China). cDNA (#K1622) and polymerase chain reaction (PCR) kits were purchased from Fermentas (Thermo Fisher Scientific, Inc., Waltham, MA, USA #K0171). B-cell lymphoma 2 (Bcl-2), Bcl-2-associated X protein (Bax) and β-actin primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai China). Mouse β-actin antibody was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA).

Experimental method

MIRI rat model generation in vivo. All rats were fasted for 12 h and were given ad libitum access to water prior to the trial. The rats were injected with 4% chloral hydrate (1 ml/100 g) intraperitoneally prior to the operation. Anaesthetized rats were fixed on the operating table in a supine position. A tracheal cannula was inserted into the rats, which was connected to a breathing machine (tidal volume, 2-3 ml/100 g; respiratory rate, 70-80 times/min). The right carotid artery of each rat was separated and an arterial cannula was inserted, which was connected to the Med-Lab system (Nanjing Medease Science and Technology Co., Ltd., Nanjing China), in order to record changes to heart rate (HR) and mean arterial pressure (MAP). The chest of each rat was sheared and an incision was made along the left sternal border, separating the pericardium and exposing the heart. Subsequently, 5-0 fine silk was threaded through the bottom of the left anterior descending coronary artery (LAD). Following LAD ligation, ST-T elevation shown in the electrocardiogram indicated the success of ischemia. The LAD was ligated for 30 min (ischemia) followed by 120 min reperfusion in vivo, following 20 min stabilization. The MIRI model was thus accomplished (22).

Animal experimental groups. Male Sprague Dawley rats (n=48) were randomly divided into four groups (n=12/group): Sham group, ischemia/reperfusion group (IR), IPO group and EM-1 postconditioning (50 µg/kg) group (EM50). The groups underwent the following procedures: i) Sham group, LAD ligation with no other intervention for 150 min; ii) IR group: LAD was ligated for 30 min (ischemia), and was reperfused for 120 min in vivo; iii) IPO group, after 30 min ischemia, three cycles of LAD clamping for 15 sec and declamping for 15 sec were performed prior to reperfusion; iv) EM50 group: EM-1 (50 µg/kg) was administered intravenously following LAD ligation for 25 min, subsequently the LAD was reperfused for 120 min in vivo.

Determination of hemodynamic characteristics. MAP and HR were continuously monitored and recorded using the Med-Lab hemodynamic system throughout the whole process of the experiment. Rate pressure product (RPP) was calculated using the following equation: RPP = MAP x HR.

Plasma IL-6, TNF-α and MDA content, and LDH, CK-MB and SOD activity. Following reperfusion, arterial blood samples were collected and placed in heparinized centrifuge tubes, and were centrifuged at 1,509 x g for 20 min at 4°C. The supernatant was collected and stored at -80°C. MDA content, and LDH and SOD activities were measured using colorimetric assays. IL-6 and TNF-α content, and CK-MB activity were measured by enzyme-linked immunosorbent assay using commercially available kits according to the manufacturers' protocols.

Determination of myocardial infarct size. The rats were injected with 4% chloral hydrate (1 ml/100 g) intraperitoneally and sacrificed by beheading prior to heart removal. The heart was removed alongside the LAD at the end of reperfusion, and 1% Evans blue was injected into the heart through the aorta. The heart was subsequently cut into 2 mm sections vertical to the longitudinal axis after freezing, and the sections were dyed with 1% triphenyl tetrazolium chloride in a 37°C water bath for 10-15 min. Subsequently, the sections were fixed in 10% formalin buffer and were divided into various regions according to color; the blue zone is considered the non-infarcted zone, the red zone is the area at risk (AAR), and the white zone is used to determine infarct size (IS). The relative area was measured using Image-Pro Plus software 6.0 (Media Cybernetics, Inc., Rockford, MD, USA) following image acquisition. The myocardial IS was expressed as a percentage of the AAR.

Detection of Bcl-2 and Bax mRNA levels by reverse transcription (RT)-PCR. Following the operation, left anterior myocardial tissues (0.1 g) were collected from each group and
homogenized, and total RNA was extracted using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol.

Total RNA (2 µg) was reverse transcribed to cDNA in a 25 µl PCR reaction volume, containing 1 µl forward primer, 1 µl reverse primer, 9 µl nuclease-free water, 12.5 µl PCR Master mix and 1.5 µl cDNA (all reagents were obtained from Fermentas; Thermo Fisher Scientific, Inc.) Subsequently RNA concentration was detected, and RT and PCR amplification were conducted. The primer sequences are shown in Table I. The PCR cycling conditions were as follows: Initial denaturation at 95˚C for 3 min; followed by 30 cycles of denaturation at 95˚C for 50 sec, annealing at 59.4˚C (β-actin), 64.5˚C (Bax), 61.5˚C (Bcl-2) for 50 sec, and extension at 72˚C for 60 sec; and final extension at 72˚C for 10 min; PCR products were then maintained at 4˚C. The PCR products were analyzed by 1% agarose gel electrophoresis and were stained with ethidium bromide. The densitometric results for Bcl-2 and Bax were compared with corresponding β-actin levels to account for loading differences.

Tanon Dots 3.1.2 software (Tanon Science & Technology Co., Ltd., Shanghai, China) was used for analysis.

Detection of cleaved caspase-3 protein by western blotting. Following the operation, rat ventricular tissues (0.1 g) were collected from each group and were homogenized in 1 ml protein extraction buffer (10 µl phenylmethylsulfonyl fluoride, 990 µl lysis buffer). The supernatant was collected following centrifugation (12,000 x g for 30 min at 4˚C) and protein content was determined using the bicinchoninic acid method. The quantity of the total protein samples was 20 µg, which was diluted in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer. Following denaturation, the samples were separated by 12% SDS-PAGE and were transferred to polyvinylidene difluoride membranes. The

<table>
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<th>Variable</th>
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Data are presented as the mean ± standard deviation. *P<0.01 vs. Sham group; *P<0.05 vs. IR group. Sham, left anterior descending coronary artery ligation with no other intervention; IR, ischemia/reperfusion; IPO, ischemic postconditioning; EM50, endomorphin-1 (50 µg/kg) postconditioning; MAP, mean arterial pressure; HR, heart rate; RPP, rate pressure product.
membranes were blocked with 5% skimmed milk at 37˚C for 120 min. The membranes were then incubated at 4˚C overnight with rabbit cleaved caspase-3 antibody (1:1,000; cat. no. #9664) and mouse β-actin antibody (1:500; cat. no. #BM0627). Subsequently, membranes were incubated for 60 min at 37˚C with horseradish peroxidase (HRP)-conjugated anti-mouse immunoglobulin (Ig)G (1:10,000; cat. no. #BA1050) or HRP-conjugated anti-rabbit IgG (1:10,000; cat. no. #BA1054) secondary antibodies. The membranes were visualized using a chemiluminescent HRP substrate. The band densities were determined and analyzed with an automatic digital gel image analysis system Tanon 3500 (Tanon Science & Technology Co., Ltd., Shanghai, China).

Statistical analysis. Data are presented as the mean ± standard deviation (n=12). One-way analysis of variance followed by least significance difference test was used for multiple comparisons. All data were analyzed using GraphPad Prism version 4.0 software (GraphPad Software, Inc., San Diego, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Alterations to hemodynamic characteristics. Basal HR, MAP and RPP were not identified to be significantly different between the groups at baseline (P>0.05). During the period of ischemia, HR, MAP and RPP were significantly decreased in the IR group (P<0.01) compared with in the sham group. Compared with in the IR group, HR, MAP and RPP were increased in the IPO and EM50 groups (P<0.05, P<0.01). During the period of reperfusion, HR, MAP and RPP were significantly decreased in the IR group (P<0.01) compared with the sham group. Compared with in the IR group, HR, MAP and RPP were increased in the IPO and EM50 groups (P<0.05, P<0.01) (Table II).

Alterations in LDH and CK-MB plasma activities. In the IR, IPO and EM50 groups, LDH and CK-MB activities were significantly higher compared with in the sham group (P<0.01). Compared with in the IR group, LDH and CK-MB activities were significantly decreased in the IPO and EM50 groups (P<0.01) compared with the sham group. Compared with in the IR group, HR, MAP and RPP were increased in the IPO and EM50 groups (P<0.05, P<0.01) (Fig. 1; P<0.01).

Alterations in MDA content and SOD activity in the plasma. Compared with Sham group, in IR, IPO, and EM50 groups, SOD activity was significantly reduced and MDA content was increased. Compared with in the IR group, SOD activity was significantly increased and MDA content was significantly decreased in the IPO and EM50 groups (Fig. 2; P<0.01).

Alterations in IL-6 and TNF-α plasma content. In the IR group, IL-6 and TNF-α levels were significantly increased...
Comparisons between the sham and IR groups revealed significant decreases in IL-6 and TNF-α levels in the IPO and EM50 groups (P<0.01; Fig. 3). Compared with the IR group, IL-6 and TNF-α levels were significantly decreased in the IPO and EM50 groups (P<0.01; Fig. 3).

**Alterations to myocardial infarct size in rats.** Myocardial infarct size (% IS/AAR) was significantly decreased in the IPO and EM50 groups (P<0.01; Fig. 4).

**Alterations in the mRNA expression levels of myocardial Bcl-2 and Bax.** The results of the RT-PCR revealed that, compared with the sham group, the mRNA expression levels of Bcl-2 and the ratio of Bcl-2/Bax were significantly reduced (P<0.01), whereas the expression levels of Bax were significantly increased (P<0.01) in the IR group. Compared with the IR group, the mRNA expression levels of Bcl-2 and the ratio of Bcl-2/Bax were significantly increased (P<0.01), whereas the mRNA expression levels of Bax were significantly reduced (P<0.01) in the IPO and EM50 groups (Fig. 5).

**Alterations in the protein expression levels of cleaved caspase-3.** The protein expression levels of cleaved caspase-3 were higher in the IR, IPO and EM50 groups compared with the sham group (P<0.05, P<0.01). Compared with the IR group, the ratio of cleaved caspase-3/β-actin was significantly reduced in the IPO and EM50 groups (P<0.01; Fig. 6).
response, the development of which is complex. TNF-α is a cytokine that is predominantly produced by macrophages, which is involved in the formation and development of MIRI. It has previously been reported that excessive activation of TNF-α can significantly damage myocardial function and prompt myocardial cell apoptosis, thus increasing myocardial damage (30). IL-6 is considered a marker of inflammation, which is responsible for inflammatory regulation and is also closely associated with the occurrence and development of MIRI (31). The present study indicated that in the IPO and EM50 groups the plasma levels of IL-6 and TNF-α were reduced compared with in the IR group. These findings suggested that EM-1 postconditioning may alleviate MIRI by inhibiting the inflammatory response.

Cell apoptosis is the primary mechanism underlying MIRI. Cell apoptosis is controlled by several genes and enzymatic reactions, the molecular regulatory mechanism of which is complex. The Bcl-2 family has an important role in cell apoptosis, and the Bcl-2 gene is the most representative anti-apoptotic gene of the Bcl-2 family. The Bax gene shares 21% amino acid sequence homology with the Bcl-2 gene, and is able to suppress the anti-apoptotic function of Bcl-2; the Bcl-2/Bax ratio (32,33) determines the occurrence of cell apoptosis. The cysteine aspartic acid specific protease, or caspase, family also has an important role in mediating apoptosis. Caspase-3 is an important caspase family enzyme that induces the execution of apoptosis, and is involved in the process of cell apoptosis after activation by enzyme digestion. In addition, caspase-3 is an important proteinase of the caspase enzyme cascade reaction; therefore, caspase-3 is considered an enzymatic marker of apoptosis. The present study demonstrated that in the IPO and EM50 groups the Bcl-2/Bax ratio was increased, whereas the protein expression levels of cleaved caspase-3 were reduced compared with in the IR group; therefore, EM-1 postconditioning may produce anti-apoptotic effects in MIRI.

There are several mechanisms underlying MIRI and their relationship is complex. Causal relationships may exist between the mechanisms, and they may interact with each other leading to myocardial injury. The number of neutrophils is markedly increased during reperefusion, and neutrophils produce an excess of oxygen free radicals, which can in turn induce oxidative stress, lipid peroxidation and inflammation, thus promoting cell apoptosis leading to further injury of myocardial tissue. It has previously been reported that the production of oxygen free radicals, accumulation of neutrophils and complement activation may be associated with the inflammatory response (34). Furthermore, the excessive activation of TNF-α can prompt myocardial cell apoptosis and increase myocardial damage (30). The present study demonstrated that EMs may produce anti-apoptotic effects by resisting oxidative stress, lipid peroxidation and inhibiting the inflammatory response; it may be hypothesized that EMs also produce an anti-apoptotic effect via certain signaling pathways, such as the phosphoinositide 3-kinase/Akt and extracellular signal-regulated kinases /2 signaling pathways; the related mechanisms require further study.

In conclusion, the present study demonstrated that EM-1 postconditioning may exert myocardial protection and anti-apoptotic effects by resisting oxidative stress, lipid peroxidation and inhibiting the inflammatory response.

Discussion

The myocardial restoration of blood flow following ischemia may induce significant pathological and physiological reperfusion-associated alterations to myocardial cells and the local vascular network, further aggravating myocardial damage; this process is known as MIRI. In the present study, a successful rat model of MIRI was generated in vivo. Hemodynamic indexes, myocardial enzymes and myocardial infarct size all reflect the degree of I/R damage. Previous studies have demonstrated that the primary mechanisms underlying MIRI may include excessive production of free radicals, infiltration of inflammatory cells, mitochondrial dysfunction and apoptosis of myocardial cells (19,23-25). Therefore, reducing the generation of free radicals (26), inhibiting the inflammatory response and suppressing apoptosis (27) may markedly reduce I/R injury.

The myocardial restoration of blood flow following ischemia may produce a large number of free radicals, which can cause oxidative stress, lipid peroxidation and lead to further injury of the myocardial tissue. SOD is one of the most important radical scavenging enzymes, whereas MDA is a metabolite of lipid peroxidation; therefore, measuring plasma SOD activity and MDA content can reflect the production of oxygen free radicals and the degree of myocardial cell damage (28). The results of the present study indicated that SOD activity was increased and MDA content was reduced in the plasma from IPO and EM50 groups compared with in the IR group. These results suggested that EM-1 postconditioning may alleviate MIRI by reducing the production of free radicals.

Inflammation is an important mechanism that is closely associated with the occurrence and development of MIRI (29). MIRI is characterized by a local or systemic inflammatory response, the development of which is complex. TNF-α is a cytokine that is predominantly produced by macrophages, which is involved in the formation and development of MIRI. It has previously been reported that excessive activation of TNF-α can significantly damage myocardial function and prompt myocardial cell apoptosis, thus increasing myocardial damage (30). IL-6 is considered a marker of inflammation, which is responsible for inflammatory regulation and is also closely associated with the occurrence and development of MIRI (31). The present study indicated that in the IPO and EM50 groups the plasma levels of IL-6 and TNF-α were reduced compared with in the IR group. These findings suggested that EM-1 postconditioning may alleviate MIRI by inhibiting the inflammatory response.

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In conclusion, the present study demonstrated that EM-1 postconditioning may exert myocardial protection and anti-apoptotic effects by resisting oxidative stress, lipid peroxidation and inhibiting the inflammatory response.
Although EMs only have four amino acid residues, they contain abundant information. Therefore, in the future, EMs may have broad application prospects, not only in analgesia, but also in the treatment of cardiovascular conditions.

Acknowledgements

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