Recombinant human erythropoietin (rhEPO) preconditioning on nuclear factor-kappa B (NF-kB) activation & proinflammatory cytokines induced by myocardial ischaemia-reperfusion

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Background & objectives: Erythropoietin (EPO), originally identified for its critical hormonal role in promoting erythrocyte survival and differentiation, has shown to have a protective effect in myocardial ischaemia-reperfusion (I-R) injury in animal models. However, the precise mechanisms remain unclear. The objective of this study was to determine the roles of nuclear factor-kappa B (NF-kB) and associated cytokines induced by I-R in the cardioprotection by recombinant human erythropoietin (rhEPO). Morphopathological observations were also made on the ultrastructure of myocardial tissue.

Methods: Myocardial I-R rat model was established by 30 min ligation of left descending coronary and 3 h reperfusion. RhEPO or saline solution was intraperitoneally injected 24 h before I-R insult. The infarct sizes were measured by triphenyltetrazolium chloride (TTC)-Evans blue technique and ultrastructural organizations were observed by a transmission electron microscope. Tumour necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and IL-10 concentrations were analyzed by enzyme-linked immunosorbance assays and NF-kB by electrophoretic mobility shift assay. TNF-α and IL-6 mRNA expression were studied by the reverse-transcription polymerase chain reaction (RT-PCR).

Results: A single bolus injection of 5,000 units/kg of rhEPO 24 h before insult remarkably reduced infarct size and improved ultrastructural organization of I-R myocardium. It greatly suppressed TNF-α and IL-6 expression, but enhanced IL-10 production. It modestly activated NF-kB before I-R insult and markedly attenuated subsequent NF-kB activation during sustained I-R.

Interpretation & conclusion: The suppression of proinflammatory cytokines expression may act by inhibiting NF-kB activation during I-R, but not by induction of IL-10. This might be one of the molecular mechanisms of rhEPO in cardioprotection. In addition, NF-kB was suggested to play a dual role in cardioprotective effects of rhEPO.

Key words: Erythropoietin - IL-10 - ischaemia-reperfusion injury - myocardial - NF-kB
Erythropoietin (EPO) is a hypoxia-induced hormone which is essential for normal erythropoiesis. Recent studies have shown that EPO has broader actions besides the stimulation of erythroid precursors. Many studies have demonstrated the protective effects of EPO or recombinant human erythropoietin (rhEPO) against ischaemia-reperfusion (I-R) injury in a wide variety of tissues including the brain, spinal cord, kidney, vascular smooth muscle cells and heart. In particular, direct cardioprotection of EPO has been well confirmed; it can reduce the myocardial infarction and improve the cardiac function against ischaemia and reperfusion independent of changes in haemoglobin (Hb) concentration, erythrocyte numbers and oxygen-carrying capacity. Several studies have indicated the probable mechanism involved in EPO protection. The action to attenuate apoptosis is likely to be one of the major contributors. However, the precise mechanisms remain unclear.

Nuclear factor-kappa B (NF-κB) is a well-known transcription factor that functions in immune and inflammatory responses. NF-κB plays a pivotal role in myocardial ischaemia-reperfusion injury, and induces many proinflammatory cytokines and chemokines which will greatly contribute to myocardial I-R injury. It has been reported that pretreatment of NF-κB decoy oligodeoxynucleotides, ischaemic preconditioning (IPC), and some forms of pharmacological preconditioning (PPC) can all reduce the extent of myocardial infarction associated with decreased expression of inflammatory cytokines or adhesion molecules.

Recently some researchers reported the anti-inflammatory properties of EPO against brain injury. Agnello et al reported that EPO conferred a strong anti-inflammatory activity on central nervous system (CNS) in a model of experimental autoimmune encephalomyelitis. Similarly, Villa and colleagues demonstrated that rhEPO pretreatment attenuated the production of proinflammatory cytokines and reduced the influx of inflammatory cells into the region of injury in a model of ischaemic brain. Previous studies in the brain indicated that EPO can signal intracellularly through pathways involving NF-κB. Therefore, this study was undertaken to assess whether the cardioprotection by rhEPO was related to attenuation of NF-κB and associated proinflammatory cytokines induced by I-R. As interleukin (IL)-10 has been suggested to play a protective role against I-R injury, in experimental myocardial infarction the effect of EPO on IL-10 was also studied.

Material & Methods

Animals and drugs: Adult male Sprague-Dawley rats (body weight, 400 ± 50 g) were purchased from the Animal Center of the Chinese Academy of Science, Shanghai, China, and divided into experimental groups in a random manner. All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals from the National Institute of Health (NIH Publication No. 85-23, revised 1996). The rats were housed and fed at the Animal Center of Jinling Hospital, Nanjing, at least 10 days before surgery for accustom to the environment. The experimental protocol was approved by the Nanjing University Animal Care and Use Committee. The rhEPO was purchased from Shenyang Sunshine Pharmaceutical CO., LTD. (Shenyang, China). This experiments were performed in Laboratory of Department of Anaesthesiology, Jinling Hospital from May, 2004 to August, 2005.

Experimental protocol: Animals were divided in three experimental groups: (i) the sham operation...
group; (n=36); (ii) the MI-R group (n=42); and (iii) the rhEPO group (n=42). In the sham operation and MI-R groups, 0.9 per cent saline solution (4 ml/kg, ip) was given 24 h prior to the I-R protocol. In rhEPO group, rhEPO (5,000 units/kg diluted in 4 ml/kg saline solution, ip) was injected also 24 h before I-R protocol. This dose of rhEPO has been widely used in analogous animal models of myocardial I-R previously\textsuperscript{5,6}. In each group, hearts were harvested just before I-R, at the end of ischaemia, and at 0.5, 1, 2, and 3 h of reperfusion for myocardial measurements (n=6 for each time point). Additional hearts were harvested at the end of reperfusion for evaluation of myocardial infarct size (n=6 for each group except the sham group).

**Surgical procedure:** The rats were anaesthetized with sodium pentobarbital (50 mg/kg, ip), tracheotomized, intubated and ventilated with room air with a positive pressure respirator (Rodent Respirator, TKR-200C, China) at a stroke volume of 12 ml/kg and a rate of 60 strokes/min. Body temperature, recorded by use of a thermistor (YSI REF 401, Yellow Spring, Ohio, USA) inserted into the rectum, was maintained between 37-37.5°C. Heparin-filled catheters were inserted into the left femoral artery for measurement of blood pressure (BP) and heart rate (HR) with high-sensitivity transducers (Model 42584-05, Abott Critical Care System, North Chicago, IL). Limb lead II of the electrocardiograph was also monitored continuously throughout the experiment. A left thoracotomy was performed via the forth intercostal space with sterile surgical technique and the pericardium was incised. The left descending coronary was ligated for the production of coronary occlusion. After surgical procedure, a 20 min stabilization period was allowed. In all groups except the sham group, coronary artery was occluded for 30 min followed by 3 h reperfusion (removal of the ligature). Myocardial ischaemia was confirmed by the appearance of a regional cyanosis and a progressive marked ST segment elevation in the ECG. Reperfusion was conformed by observing an epicardial hyperemic response. The sham operation group was subjected to thoracotomy and passage of silk ligature around left descending coronary artery without ligation.

**Measurement of infarct size:** Infarct size was evaluated with the triphenyltetrazolium chloride (TTC, Merck, Germany)-Evans blue (Fluka, Switzerland) technique. At the end of each experiment except the sham group, the artery was reoccluded. 2 per cent Evans blue was injected intravenously to separate the left ventricular (LV) area at risk (AAR) for infarction from surrounding normal areas (stained blue). The heart then was harvested and frozen at -20°C for 30 min, cut into 5-6 2 mm thick transverse slices, which were then incubated for 15 min at 37°C in a 1 per cent solution of TTC in phosphate buffer (pH = 7.4) to differentiate the infarcted (pale) from variable (red). Slices were fixed in a 10 per cent formaldehyde solution, and photographed (Nikon 3100, Japan). The corresponding areas were measured by computerized planimetry (ImageJ 1.31v, NIH), and from these measurements infarct size was calculated as a percentage of the AAR.

**Morphopathological observation of ultrastructural organization:** One mm\textsuperscript{3} fresh myocardium tissue, cut from approximately halfway between the apex and the ligation point at the end of reperfusion, was prefixed with 2.5 per cent glutaraldehyde and post-fixed with 1 per cent osmium tetroxide. Then the specimens were immersed in propylene oxide after dehydration with gradient ethanol, embedded with epoxy resin and made into ultrathin sections. The sections were stained subsequently with lead-uranium and the changes of ultrastructural organization were then evaluated by a transmission electron microscope (JEM-1200EX; JEOL Ltd., Tokyo, Japan). In each...
group specimens were taken from six hearts and for each heart four tissue blocks were obtained.

**Nuclear protein extract and electrophoretic mobility shift assay (EMSA):** At every time-point hearts were harvested. Nuclear protein of left ventricle myocardium tissue was extracted and quantified. Briefly, frozen myocardium tissues were homogenized in 0.8 ml ice-cold buffer A composed of 10 mM HEPES pH 7.9, 10 mM KCl, 2 mM MgCl$_2$, 0.1 mM EDTA, 1 mM dithiothreitol (DTT) and 0.5 mM phenylmethysulphonyl fluoride (PMSF) (all from Sigma Chemical Co., USA). Then the homogenates were incubated on ice for 20 min, and vortexed for 30 sec after addition of 50 µl NP-40 (Sigma Chemical Co., USA). The mixture was centrifuged for 10 min (5000 g, 4°C). The pellet was then suspended in 50 µl ice-cold buffer B (50 mM HEPES pH 7.9, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, with 10 per cent (v/v) glycerol) and incubated on ice for 30 min with frequent mixing. After centrifugation for (12000 g, 4°C) 15 min, the supernatants were collected as nuclear extracts and stored at -80°C until use.

EMSA was performed using a commercial kit (Gel Shift Assay System; Promega, Madison, WI, USA). The NF-κB oligonucleotide probe (5’-AGT TGA GGG GAC TTT CCC AGG C-3’) was end-labeled with T4-polynucleotide kinase. Nuclear protein (30 µg) was preincubated in a total volume of 9 µl in a binding buffer, consisting of 10mM Tris-HCl (pH 7.5), 4 per cent glycerol, 1mM MgCl$_2$, 0.5mM EDTA, 0.5mM DTT, 0.5mM NaCl, and 0.05mg/ml poly (di-dc) for 10 min at room temperature. After addition of the $^{32}$P-labeled oligonucleotide probe, the incubation was continued for 20 min at room temperature. Reaction was stopped by adding 1 µl of gel loading buffer and the mixture was subjected to nondenaturing 4 per cent polyacrylamide gel electrophoresis in 0.5xTBE buffer (Tris-borate-EDTA). After electrophoresis was conducted at 390V for 1h, the gel was vacuum-dried and exposed to X-ray film (Fuji Hyperfilm) at -70°C with an intensifying screen.

**Measurement of myocardial cytokines production and expression:** Tumour necrosis factor-alpha (TNF-α), IL-6, and IL-10 concentrations of left ventricle were quantified by enzyme-linked immunosorbance assays (ELISA) (rat TNF-α test kit: Diaclene, Besanson Cedex, France; rat IL-6 and IL-10 test kit: Biosource International, Camarillo, California, USA) according to the manufacturer’s instruction. Values were expressed as pg/mg protein.

TNF-α and IL-6 mRNA expressions were measured by reverse-transcription polymerase chain reaction (RT-PCR). Total RNA was extract with TriPure Isolation Reagent (Roche Molecule Biochemicals, Switzerland) and quantified by absorption at 260 nm. Reverse-transcription (RT) was implemented using Reverse Transcription Systerm (Promega, WI, USA) according to the manufacturer’s protocol. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the normalization control.

The following oligonucleotide pairs were used, each sequence as 5’ to 3’: TNF-α: (sense) CACCACGCTCTTCTGCTACTGAAC, (antisense) CCGGACTCCGTGATGTCTAAGTACT; IL-6: (sense)GACTGATGTTGTGTTGACAGCCACTGC, (antisense)TAGCCACTCTCTGCTGACTCTAACT; GAPDH: (sense) CACGGCAAGTTCAATGGCACA, (antisense) GAATTGTGAGGGAGAGTGCTC.

A total volume of 100 µl reaction contained 2 µl of RT product, 1.5 µmol/l MgCl$_2$, 2.5 U Taq DNA polymerase, 100 µmol/l dNTP, 0.1 µmol/l
primer, and 1× Taq DNA polymerase magnesium-free buffer (Promega, WI, USA). The reaction mixture was overlaid with two drops of mineral oil (Sigma Chemical Co) and incubated in thermocycler (MiniCycle PTC 150, MJ Research Inc, USA) programmed to pre-denature at 95°C for 1 min, anneal at 60°C for 1 min, and extend at 72°C for 2 min, for a total of 30 cycles. The last cycle was followed by incubation at 72°C for 5 min and cooling to 4°C. The polymerase chain reaction products were 546 bp (TNF-α), 509bp (IL-6), and 970 bp (GAPDH), respectively. The products were electrophoresed on a 15 g/l agarose gel and stained with ethidium bromide. The gel was captured as a digital image and analyzed using Scion Image software (Scion Corp, Maryland, USA). Values for each sample were normalized with GAPDH as the control.

Statistical analysis: Statistical analyses were performed by using SPSS 10.0 for windows. Comparisons of infarct size between two groups were performed using Student’s t-test. TNF-α and IL-10 measurements were analyzed by one-way analysis of variance (ANOVA). Significant values were subsequently verified with Student-Newman-Kuels post hoc tests. IL-6 concentrations were analyzed using Kruskal-Wallis test followed by Mann-Whitney test. A \( P < 0.05 \) was considered significant.

Results

Effects on infarct size: The AAR/LV ratio between MI-R group and rhEPO group showed no significant difference. rhEPO significantly reduced the infarct size (28.02 ± 1.34% of AAR) as compared with MI-R group (\( P < 0.001 \), Table I).

<table>
<thead>
<tr>
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<th>AAR/LV (%)</th>
<th>IS/AAR (%)</th>
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<tbody>
<tr>
<td>MI-R group</td>
<td>40.82 ± 2.27</td>
<td>43.28 ± 2.51</td>
</tr>
<tr>
<td>rhEPO group</td>
<td>38.30 ± 1.85</td>
<td>28.02 ± 1.34*</td>
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</table>

Each value represents the mean ± SE of six experiments *\( P < 0.001 \) vs MI-R group

Effects on ultrastructural organization: In sham group, cellular and mitochondrial structures were normal. There was no cellular or tissue swelling and the structure of cytoplasmic part of the sarcomere was normal (Fig. 1A). Mitochondria were close and orderly arranged in the form of round or ellipsoid without swelling. Mitochondrial membranes were intact and cristae were arranged in the form of concentrical ring or vertical line, congested and clear (Fig. 1D). In MI-R group, however, there were apparent cellular and tissue swelling (Fig. 1B). Most myofibrils were either disappeared or disorganized or disarrayed. Mitochondria were swollen obviously and loosely arranged. Mitochondrial membranes were vague or partly ruptured and cristae were obviously loose and dissolved, a lot of vacuoluses were formed (Fig. 1E). In rhEPO group, ultrastructural organization appeared basically close to normal except that cristae were basically congested with a little vacuolation (Fig. 1C, 1F).

Effects on NF-κB activation: NF-κB activation in the nuclear extracts of left ventricle was determined by EMSA at different time-points. NF-κB binding activity was detected at low levels in sham-operated animals throughout the experiment. In the I-R myocardium, I-R greatly induced NF-κB activation which increased at 30 min of reperfusion, declined thereafter, and then rose again at 3 h reperfusion. The administration of rhEPO 24 h prior to I-R modestly activated NF-κB before I-R insult and markedly attenuated subsequent NF-κB activation during sustained I-R compared with MI-R group (Table II, Fig. 2).
Fig. 1. Representative electronmicroscopic views of hearts from the sham group (A, D), MI-R group (B, E) and rhEPO group (C, F) after 30 min of ischaemia and 3 h of reperfusion. The magnification are ×4000 (A-C) and ×20,000 (D-F), respectively. M, mitochondrion; N, nucleus; myo, myofibril.
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**Fig. 2.** Representative electrophoretic mobility shift assay (EMSA) pictures highlighting NF-κB binding activity of the three groups just before I-R and at 3 h of reperfusion, respectively.

**Table II.** Effects of rhEPO on the productions of TNF-α, IL-6, and IL-10 and activities of NF-κB in left ventricle

<table>
<thead>
<tr>
<th></th>
<th>Just before IR</th>
<th>Reperfusion 0.5 h</th>
<th>1 h</th>
<th>2 h</th>
<th>3 h</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NF-κB activity</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>sham group</td>
<td>1.38 ± 0.06</td>
<td>1.41 ± 0.07</td>
<td>1.39 ± 0.06</td>
<td>1.41 ± 0.06</td>
<td>1.43 ± 0.06</td>
</tr>
<tr>
<td>MI-R group</td>
<td>1.39 ± 0.06</td>
<td>1.97 ± 0.10</td>
<td>1.78 ± 0.12</td>
<td>1.69 ± 0.10</td>
<td>2.21 ± 0.14</td>
</tr>
<tr>
<td>rhEPO group</td>
<td>1.63 ± 0.08&quot;</td>
<td>1.63 ± 0.07&quot;</td>
<td>1.58 ± 0.07&quot;</td>
<td>1.55 ± 0.06&quot;</td>
<td>1.53 ± 0.07&quot;</td>
</tr>
<tr>
<td><strong>TNF-α (pg/mg)</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>sham group</td>
<td>ND</td>
<td>11.9 ± 0.5</td>
<td>20.4 ± 1.5</td>
<td>15.6 ± 0.8</td>
<td>9.1 ± 0.4</td>
</tr>
<tr>
<td>MI-R group</td>
<td>ND</td>
<td>28.5 ± 3.2</td>
<td>85.6 ± 3.4</td>
<td>75.7 ± 5.2</td>
<td>26.6 ± 1.9</td>
</tr>
<tr>
<td>rhEPO group</td>
<td>15.2 ± 1.9</td>
<td>27.5 ± 2.1</td>
<td>34.5 ± 2.2&quot;</td>
<td>24.4 ± 1.6&quot;</td>
<td>14.5 ± 2.4&quot;</td>
</tr>
<tr>
<td><strong>IL-6 (pg/mg)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>sham group</td>
<td>ND</td>
<td>307 ± 23</td>
<td>557 ± 39</td>
<td>647 ± 53</td>
<td>574 ± 39</td>
</tr>
<tr>
<td>MI-R group</td>
<td>ND</td>
<td>295 ± 23</td>
<td>1284 ± 125</td>
<td>2565 ± 223</td>
<td>1934 ± 209</td>
</tr>
<tr>
<td>rhEPO group</td>
<td>411 ± 35</td>
<td>494 ± 52&quot;</td>
<td>764±57&quot;</td>
<td>910 ± 72&quot;</td>
<td>712 ± 43&quot;</td>
</tr>
<tr>
<td><strong>IL-10 (pg/mg)</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>sham group</td>
<td>ND</td>
<td>7.65 ± 0.47</td>
<td>24.82 ± 1.95</td>
<td>26.78 ± 2.04</td>
<td>34.28 ± 2.76</td>
</tr>
<tr>
<td>MI-R group</td>
<td>ND</td>
<td>7.52 ± 0.53</td>
<td>25.57 ± 2.07</td>
<td>50.66 ± 4.88</td>
<td>64.11 ± 6.57</td>
</tr>
<tr>
<td>rhEPO group</td>
<td>ND</td>
<td>8.31 ± 0.75</td>
<td>26.40 ± 2.36</td>
<td>57.02 ± 3.91'</td>
<td>78.55 ± 5.58&quot;</td>
</tr>
</tbody>
</table>

The activities of the NF-κB complex were determined by the mean band intensity of electrophoretic mobility shift assay (EMSA). The relative quantitative data to the intensity of background are shown in the Table. ND: not detected by enzyme-linked immunosorbance assays (ELISA) kit.

Values are mean ± SE of six experiments

*P<0.05, **<0.01 compared to MI-R group at the same time point
Effects on myocardial cytokines production and expression: To investigate whether attenuation of activation of NF-κB by rhEPO was associated with reduced inflammatory cytokine production, the levels of myocardial TNF-α and IL-6 were measured. The results showed that both TNF-α and IL-6 were not detectable before I-R in sham group and MI-R group. After 30 min of reperfusion, both increased in MI-R group, and reached a peak at 1 and 2 h reperfusion respectively, and then dropped. Therefore, TNF-α and IL-6 mRNA expression were measured at these peak time-points respectively. rhEPO significantly decreased the production and expression of TNF-α and IL-6 (Table II, Fig. 3).

I-R induced significant IL-10 production after 2 h of reperfusion compared with the sham group, and rhEPO significantly enhanced the release of IL-10 after that time compared to MI-R group.

Discussion

This study demonstrated rhEPO pretreatment of rats prior to experimental induction of I-R reduced the infarct size, protected and preserved the ultrastructural organization of myocardial tissue; and suppressed NF-κB activation and associated proinflammatory cytokines expression during I-R, which might be one of the molecular mechanisms of rhEPO in cardioprotection.

Myocardial infarction develops largely as a result of severe damage to cardiac myocytes and

![Fig. 3. TNF-α and IL-6 mRNA expression in left ventricle at 1 and 2 h reperfusion, respectively. Values were normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the control. Each bar height represents the mean ± SE of six experiments. **P<0.01 vs MI-R group.](image-url)
endothelial cells induced by a complex interaction of multiple cytokines and adhesion molecules activated by reperfusion\textsuperscript{9,10}. TNF-\(\alpha\) is considered as an “upstream” cytokine which is responsible for triggering the cytokine cascade and initiating the inflammatory response in myocardial I-R\textsuperscript{9,10}. Both TNF-\(\alpha\) and IL-6 can regulate the induction of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) in the ischaemic area and may accelerate interactions between transmigrated neutrophils and cardiac myocytes, resulting in promotion of myocardial cell death\textsuperscript{10}. These findings demonstrated the important role of the proinflammatory cytokines as an aggravating factor in the ischaemic myocardium. Our data showed the myocardial TNF-\(\alpha\) and IL-6 expression and production were both significantly reduced in the rhEPO group compared with the MI-R group.

We also found that rhEPO preconditioning attenuated the NF-\(\kappa B\) activation induced by I-R. NF-\(\kappa B\) is a well-known redox-sensitive transcription factor, and is a key regulator of inflammatory response. It was reported to be activated by myocardial I-R and involved in I-R injury, and both cardiac myocytes and interstitial cells were important sources of NF-\(\kappa B\)\textsuperscript{8}. Li et al\textsuperscript{20} found that myocardial ischaemia alone rapidly induced NF-\(\kappa B\) activation and speculated that this early activation might be a signal mechanism for controlling and regulating the immediately-early gene expression during I-R. Maulik et al\textsuperscript{7} also reported elevated binding activities of NF-\(\kappa B\) during different durations of ischaemia or reperfusion. These findings support our results. We observed a biphasic elevation in NF-\(\kappa B\) levels at 30 min and 3 h of reperfusion in MI-R group. The initial rise in NF-\(\kappa B\) may be attributed to the burst of reactive oxygen intermediates (ROI) which peak 2-3 min after the initiation of reperfusion\textsuperscript{21}. The second surge in NF-\(\kappa B\) levels may be the result of the stimulation provided by proinflammatory cytokines which are produced by the initial NF-\(\kappa B\) activation.

Many effector genes, including those encoding TNF-\(\alpha\) and IL-6, are in turn regulated by NF-\(\kappa B\) because the regulatory regions of these genes are responsive to NF-\(\kappa B\). Morishita et al\textsuperscript{11} reported direct evidence for close relationship between NF-\(\kappa B\) and cytokines during myocardial I-R. In vivo transfer of NF-\(\kappa B\) decoy oligodeoxyribonucleotides reduces the extent of myocardial infarction in co-ordination with decreased mRNA of IL-6 and VCAM-1\textsuperscript{11}. In view of these findings, the cardioprotection of rhEPO may be due to reduced proinflammatory cytokines production via downregulation of NF-\(\kappa B\) induced by I-R.

We also showed enhanced production of IL-10 by single bolus injection of rhEPO. IL-10 is a classical anti-inflammatory cytokine, which is known to inhibit the secretion of many proinflammatory mediators such as TNF-\(\alpha\), IL-1, and IL-6 and at the same time upregulates other anti-inflammatory agents\textsuperscript{22}. IL-10 mRNA and protein upregulation was demonstrated in the reperfused infarcted myocardium using a canine model\textsuperscript{17}. Additional investigations indicated that IL-10-deficient mice show an enhanced inflammatory response following experimental myocardial infarction, demonstrated by increased neutrophil recruitment, elevated plasma levels of TNF-\(\alpha\) and high tissue expression of ICAM-1\textsuperscript{18}. IL-10 was therefore suggested to play a protective role in myocardial I-R. Although it was reported that rhEPO augmented ex vivo production of IL-10 in blood cell cultures from haemodialysis patients\textsuperscript{23}, there are no published studies evaluating the effect of rhEPO on IL-10 production in ischaemic-reperfused heart.
Our experiment revealed a significantly increased level of IL-10 in rhEPO group versus MI-R group after 2 h of reperfusion, accompanied by decreased levels of TNF-α and IL-6. One may speculate that rhEPO might inhibit the release of proinflammatory cytokines by enhancement of IL-10 production. However, in the present study, IL-10 did not peak until 2 h of reperfusion, which is subsequent to the peak of TNF-α and IL-6 production. Therefore, the downregulation of TNF-α and IL-6 by rhEPO cannot mainly act through enhancement of IL-10 production. Further studies are needed to determine the exact role of IL-10 in this protection.

Recent studies reported that rhEPO could elicit potent cardioprotection when administered 12-24 h or just 2 h before ischaemia, at the onset of ischaemia, or at the onset of reperfusion, indicating that it can be used in both prevention and treatment of ischaemic episode. Two studies compared the protective effects of rhEPO administered at different time points, but the conclusions are controversial. In our study, the dose chosen for rhEPO preconditioning was 5,000 units/kg, which has been widely used in analogous rat models of myocardial I-R previously. More systematic studies are needed to determine the best regime and time.

It has been well established that NF-κB is activated during preconditioning episode in itself and that inhibition of this NF-κB translocation abolished preconditioning-induced cardioprotection. NF-κB activation during preconditioning can upregulate some downstream anti-apoptotic and cardioprotective genes. In addition, previous studies in the brain indicated that EPO can signal intracellularly through pathways involving NF-κB. Digicaylioglu et al showed that neuronal EPO-receptors activated the neuroprotective signaling pathway via Janus Kinase-2 (JAK2) and NF-κB. In our study, we observed a modest activation of NF-κB before I-R in rhEPO group. Although we did not investigate whether this activation was essential for the cardioprotection of rhEPO, these findings support our hypothesis that modest activation of NF-κB before ischaemic insult and subsequent attenuation of NF-κB activation during sustained I-R contribute to the cardioprotection of rhEPO pretreatment. Therefore, we speculated that NF-κB may serve crucial and complex roles in EPO protection.

In conclusion, we demonstrated that rhEPO preconditioning elicited cardioprotection against I-R injury as demonstrated by reduction of infarct size and preservation of ultrastructural organization of myocardial tissue, and suppressed myocardial TNF-α and IL-6 production and their mRNA expression, and enhanced I-R induced cardiac IL-10 production. The suppressed proinflammatory cytokines expression may act by inhibiting NF-κB activation during I-R, but not by induction of IL-10. This might be one of the mechanisms of rhEPO in cardioprotection. Further studies are needed to throw light on the mechanism of action of rhEPO in cardioprotection against I-R injury. Better understanding of these mechanisms may offer a novel therapeutic strategy in cardioprotection of I-R injury.

Acknowledgment

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