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Erythropoietin in cardiac ischemia

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Chapter 3

Timing of erythropoietin treatment for cardioprotection in ischemia/reperfusion

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Abstract

Erythropoietin (EPO) is a hormone known to stimulate hematopoiesis. However, recent research suggests additional properties of EPO, such as protection against ischemia/reperfusion (I/R) injury in various tissues. We studied the effect of timing of EPO administration on cardioprotection during I/R in the heart. Male Sprague-Dawley rats were subjected to 45 minutes of coronary occlusion, followed by 24-hours of reperfusion. Animals were randomized to receive saline or single dose of EPO (5,000 IU/kg) either 2 hours before I/R, at the start of ischemia or after the onset of reperfusion. The ratio of infarct area/area at risk (planimetry), left ventricular (LV) function (pressure development) and apoptosis (number of active caspase-3 positive cells) were determined after 24-hour reperfusion. Administration of EPO during different time points resulted in a 19-23 % ($p < 0.05$) reduction in the infarct area/area at risk, which was accompanied by a trend towards better LV hemodynamic parameters. Apoptosis was significantly attenuated in groups treated with EPO at the start of ischemia (29% reduction) and after the onset of reperfusion (38%), and to a lesser extent (16%) in the group pre-treated with EPO. Thus, *in vivo* administration of EPO at different time points protects the myocardial structure and preserves cardiac function during I/R. Cardioprotective effect of EPO is associated with inhibition of apoptosis.

Introduction

Erythropoietin (EPO) is an endogenous hematopoietic hormone produced by the kidney in response to hypoxia. EPO targets erythroid progenitor cells in bone marrow to increase the number of mature red blood cells ⁽¹⁾. Rather than directly stimulating the proliferation, EPO inhibits the apoptosis of erythroid precursor cells ⁽²⁾. Independent of its hematopoietic effect, EPO was recently shown to be protective in vascular disease ⁽³⁾. Furthermore, systemic administration of EPO to rats subjected to cerebral ischemia/reperfusion (I/R) resulted in a significant reduction in brain infarct size ⁽⁴⁾. Application of EPO was beneficial also in the setting of hypoxic retinal disease ⁽⁵⁾ and renal ischemic injury ⁽⁶⁾. Protection against apoptosis was implicated as a possible mechanism of the observed EPO effects ^(7,8), suggesting the extension of EPO anti-apoptotic property to other tissues. Both *in vitro* and *in vivo*, EPO has been shown to activate a number of signaling kinases (Akt, MAP-kinase, STAT-5) associated with the prevention of apoptosis ^(9,10). Accordingly, activation of these pathways during cardiac ischemia was reported to have cytoprotective effects ⁽¹¹⁾.

Recently, evidence is accumulating for a protective role of EPO during ischemia in the heart. In isolated cardiomyocytes, EPO was shown to protect against hypoxia-induced apoptosis, through an Akt-dependent pathway ⁽⁹⁾. Pre-treatment with EPO increased functional recovery and decreased apoptosis in isolated rat hearts subjected to I/R 24-hours later ⁽¹²⁾. In a study performed by our group, perfusion with EPO during *ex vivo* (Langendorff) ischemia/reperfusion improved left ventricular (LV) function and limited cellular damage ⁽¹³⁾. Repeated administration of EPO in a rat coronary I/R model reduced cardiomyocyte loss and normalized diastolic hemodynamic dysfunction within 1 week after reperfusion ⁽¹⁴⁾. Most recently, Parsa et al. ⁽¹⁵⁾ showed cardioprotective and anti-apoptotic effects of EPO administered to rabbits at the time of myocardial infarction.

In-vitro, EPO also exhibits angiogenic potential in myocardial tissue, which could also

account for its cardioprotective effect (¹⁶).

However, the optimal timing of EPO administration during I/R in the heart remains unknown. The aim of our study was to provide a clinically relevant “window of opportunity” for EPO treatment. Applied into the clinical situation, EPO would be administered to patients presenting with chest pain- without irreversible ischemic damage (unstable angina), with already evolving myocardial infarction, or undergoing revascularization procedures. Accordingly, we investigated the effects of EPO treatment at 3 different time points during I/R procedure (before and at the onset of ischemia, and after the start of reperfusion).

Methods

Animal model

The experiments were conducted in accordance with the international Guide for the Care and Use of Laboratory Animals. The experimental protocol was approved by the Animal Research Committee of the University of Groningen.

Male Sprague-Dawley rats (270-320 g) were obtained from Harlan (Zeist, The Netherlands). At the time of operation anesthesia was induced and maintained with 2.0-2.5% isoflurane (Isofluraan, Rhodia Organique Fine Ltd., UK). The trachea was intubated and the rats were mechanically ventilated (Amsterdam Infant Ventilator, Hoek/Loos, Schiedam, The Netherlands) using room air enriched with 1.0 l/min oxygen.

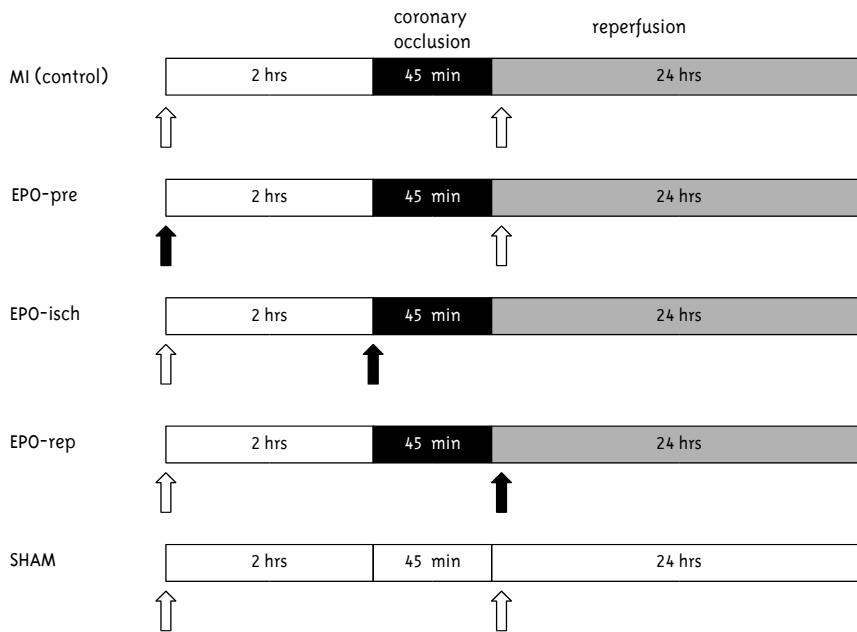
Left thoracotomy was performed and the heart exposed through the fifth intercostal space. The pericardium was incised and a 6-0 silk suture (Perma-Hand seide, Johnson& Johnson, Belgium) was placed around the proximal portion of the left coronary artery, beneath the left atrial appendage. The ligature ends were passed through a small length of plastic tube to form a snare. For coronary artery occlusion, the snare was pressed onto the surface of the heart directly above the coronary artery and hemostat was applied to the snare. Ischemia was confirmed by the blanching of the myocardium and dyskinesis of the ischemic region.

After 45 min. of occlusion, the hemostat was removed and snare released for reperfusion, with the ligature left loose on the surface of the heart (¹⁷). Successful reperfusion was indicated by the restoration of normal rubor. The wounds were sutured and the thorax was closed under negative pressure. The rats were weaned from mechanical ventilation and returned to cages to recover. In sham-operated rats, the same procedure was executed, without tightening the snare. Body core temperature was monitored during the surgical procedure with a rectal thermometer and maintained between 37⁰- 38⁰C by heating pads.

Experimental protocol

Animals were randomly allocated to 5 groups. To determine the effect of timing on the cardioprotective effect of EPO during ischemia/reperfusion, 3 groups of rats received recombinant human EPO (5.000 IU/kg in 0.5 ml of saline, i.p.) at 3 different time points: 2 hours before I/R (EPO-pre group; n=25), at the start of ischemia (EPO-isch group; n=16) and 5 min. after the onset of reperfusion (EPO-rep group; n=20). Control (MI group; n=20) and sham-operated (SHAM group; n=6) groups received corresponding injections of saline (0.5 ml, i.p.; Fig. 1).

Figure 1. Experimental protocol. Coronary artery occlusion was maintained for 45 min., followed by 24-hour reperfusion. Solid arrows indicate the time of EPO (5.000 IU/kg, i.p.) administration. Open arrows indicate the time of corresponding saline injections.



Hemodynamic measurements

Twenty-four hours after the coronary reperfusion, rats were reanesthetized. Microtip pressure transducer (Millar Instr. Inc., Houston, TX) was inserted into the left ventricular cavity via the right carotid artery. Left ventricular systolic pressure (LVSP) and its first derivatives (dP/dt_{max} and dP/dt_{min}), left ventricular end-diastolic pressure (LVEDP), and heart rate were measured. The catheter was retracted into the aortic arch and arterial systolic and diastolic blood pressures were recorded.

Measurement of Infarct Size

At the end of hemodynamic measurements, the chest was reopened, the heart rapidly excised and retrogradely perfused with 5 ml saline to remove any blood. The coronary artery was reoccluded by tightening the ligature that had remained at the site of the previous occlusion, and the heart was injected through the aorta with trypan blue (0.4%, Sigma Chemical, St. Louis, MO), to stain the perfused myocardium blue, whereas the non-perfused area at risk (AR), remained unstained. The heart was trimmed of the right ventricle and both atria and sliced transversely into 2-mm thick sections, which were incubated for 10 min in 37°C nitro blue tetrazolium (Sigma Chemical, St. Louis, MO; 1 mg/ml Sørensen buffer, pH 7.4) to delineate the viable area (stained) and the infarcted area (IA- unstained) inside the AR, as described before (¹⁷). The sections were weighed and different regions were then measured by computed planimetry. Total weight of AR was calculated and expressed as percentage of total LV weight, IA was expressed as percentage of AR. Afterwards the sections were fixed in 10% formalin and embedded in paraffin.

Immunohistochemistry

Paraffin embedded sections were dewaxed in xylene and dehydrated through graded alcohols.

For the assessment of apoptosis the antigen retrieval was carried out by microwave treatment in a citrate buffer (10mM, pH 6.0). The endogenous peroxidase activity was blocked by 3% H₂O₂. The sections were then incubated for 1 hour at room temperature with rabbit polyclonal active caspase-3 antibody (1:50; New England Biolabs, Beverly, MA) ⁽¹⁸⁾, followed by incubation with peroxidase conjugated goat anti-rabbit and rabbit anti-goat IgG for 30 minutes at room temperature.

To determine the proliferative activity, sections were immersed overnight at 80°C in Tris-HCl solution (0.1 M, pH 9.0) and incubated for 1 hour with monoclonal mouse anti-Ki67-antigen antibody (1:25; Immunotech Marseille, France) ⁽¹⁹⁾, followed by incubation with peroxidase conjugated rabbit anti-mouse and goat anti-rabbit IgG for 30 minutes at room temperature.

In both stainings 3,3' diaminobenzidine (DAB) was used as chromogen and the sections were counterstained with hematoxylin.

Positively stained cells were counted in ten randomly selected fields in the AR at 400x amplification. Anti-Ki67 staining was considered positive only when localization was nuclear. Blinded for treatment, the results from each heart sample were quantified and expressed as a percentage of total number of cells/nuclei. Tissue sections of colonic adenocarcinoma served as a positive control for both stainings ⁽²⁰⁾.

Serum EPO analysis

Serum human EPO levels were measured 24 hours after the surgery using the IMMULITE® EPO assay (DPC, Los Angeles, CA), which has been described before ⁽²¹⁾.

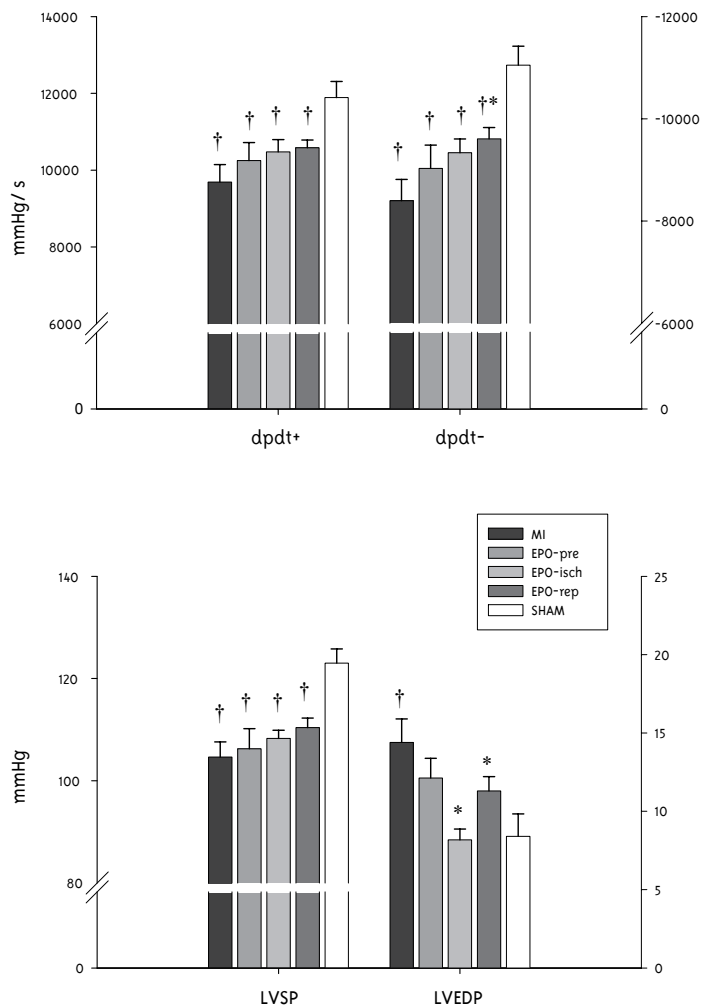
Materials

All experiments were performed using recombinant human Erythropoietin (rhEPO) alfa (EPREX, Janssen-Cilag, Tilburg, The Netherlands; 10.000 IU/ml). The rhEPO contains the same amino acid sequence as natural human EPO and possesses the same biological activity. It is ~ 80% homologous to rodent EPO, and it has been shown to be biologically active in rodents ⁽²²⁾.

Statistical analysis

Data are presented as mean ± SEM. Statistical analysis between groups was performed by one-way ANOVA followed by LSD post-hoc analysis. Pearson's correlation coefficients were calculated to determine the relationship between infarct size and LV function. Differences were considered significant at p<0.05. Animals with risk area, which exceeded 2SD of the average of all animals were excluded from the further analysis (MI: n=1; EPO-pre: n=2; EPO-isch: n=1; EPO-rep: n=1).

Figure 2. Left ventricle (LV) hemodynamic parameters, 24-hours after reperfusion. Results are expressed as mean \pm SEM. LVSP indicates left ventricular systolic pressure; LVEDP-left ventricular end-diastolic pressure; dP/dtmax and dP/dtmin - maximal rate of increase and decrease of ventricular pressure; * $p < 0.05$ vs. MI (control), † $p < 0.05$ vs. SHAM.



Results

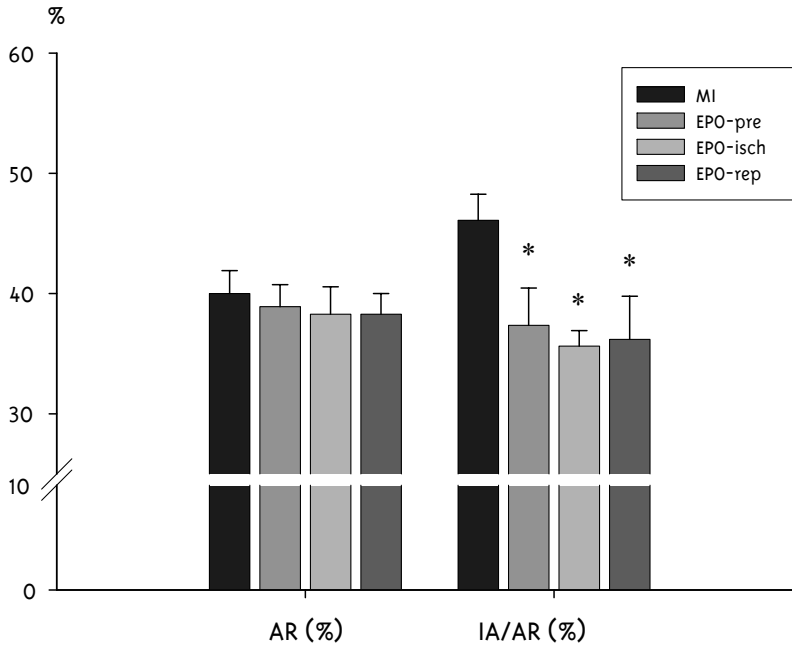
Mortality

Overall 24-hour mortality in rats subjected to I/R was 24.7%. There was no significant difference in mortality rate between the non-treated MI group and groups treated with EPO (data not shown).

Hemodynamic measurements

In vivo cardiac function was measured 24 hours after reperfusion by Millar catheterization. The data are presented in Figure 2. I/R in all groups resulted in a significant reduction

Figure 3. Effects of EPO on infarct size. Area at risk (AR) is expressed as a percentage of total LV weight. Infarct area (IA) is expressed as a percentage of area at risk (IA/AR), * $p < 0.05$ vs. MI (control).



of LVSP, dP/dt_{\max} , dP/dt_{\min} when compared to sham-operated animals. Between the groups subjected to I/R, there was no significant difference in LVSP and LV dP/dt_{\max} , although a trend towards better systolic hemodynamic parameters was present in all three groups treated with EPO. LVEDP was significantly lower in EPO-isch and EPO-rep groups when compared to MI group. LV dP/dt_{\min} was significantly enhanced in EPO-rep group compared to MI group. LV function (dP/dt_{\max} and dP/dt_{\min}) correlated with the infarct size ($r = -0.35$, $p = 0.02$ and $r = 0.38$, $p = 0.01$, respectively). Mean arterial pressure was not significantly different between treated and non-treated groups subjected to I/R (data not shown).

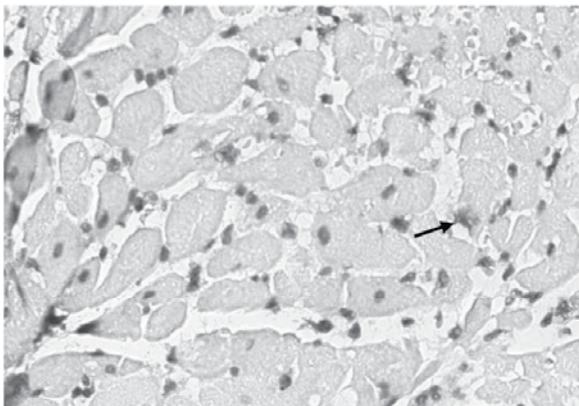
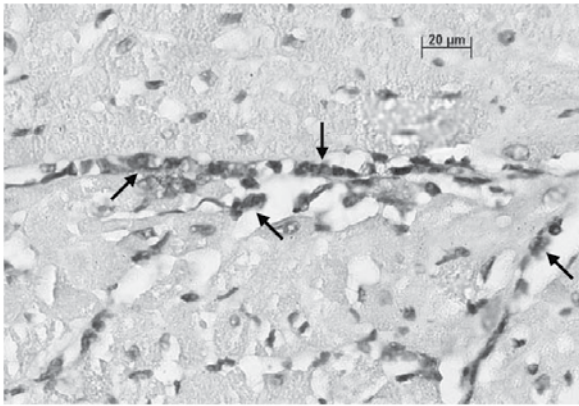
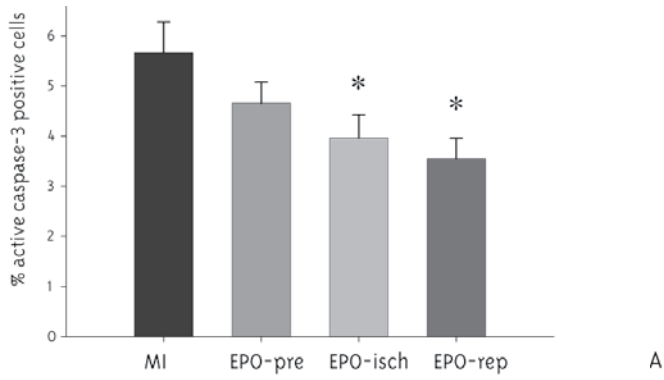
Infarct size

Infarct sizes in the I/R groups are shown in Figure 3. There were no significant differences in the potential ischemic zone- area at risk (AR) between the control (MI) group and the three treatment groups. Similar areas of LV were thus exposed to I/R injury. In all groups treated with EPO, a significant reduction of the infarct area within AR was obtained compared with MI group (EPO-pre: 18.9%; EPO-isch: 22.8%; EPO-rep: 21.5% infarct size reduction), with no differences between the EPO treated groups.

Assessment of Apoptosis

Administration of EPO resulted in reduction in active caspase-3 positive cells, which was statistically significant in EPO-isch and EPO-rep groups (EPO-isch: 28.6%; EPO-rep: 37.5%

Figure 4. Effects of EPO on apoptosis. A) Graphic representation of active caspase-3 positive cells. Data are shown as a percentage of total number of cells, * $p < 0.05$ vs. MI (control). B) Representative active caspase-3 stained LV section from a MI group (control). C) Representative active caspase-3 stained LV section from an EPO-rep group. Arrows indicate active caspase-3 positive cells.



reduction compared to MI group; Fig. 4). A smaller difference, statistically non-significant, was observed in EPO-pre group (16.1% reduction).

Active caspase-3 staining was mostly limited to the non-infarcted part of the area at risk, mainly to subendocardial and subepicardial regions. There were few active caspase-3 positive cells found outside the area at risk. No apoptotic cells were observed in the sham-operated group. Predominantly endothelial cells and fibroblasts were positive for active caspase-3 staining.

Determination of Proliferative Activity

Overall, the percentage of Ki67-antigen positive nuclei was very low (0.78% on average). We did not observe significant difference in proliferative activity as measured by the number of Ki67-antigen positive cells among the groups subjected to I/R (MI group: $0.75 \pm 0.25\%$; EPO-pre: $0.99 \pm 0.18\%$; EPO-isch: $0.72 \pm 0.14\%$; EPO-rep: $0.68 \pm 0.23\%$ of total number of nuclei). In this respect mainly capillary wall cells appeared positive for the staining.

Serum EPO levels

In rats, rhEPO is promptly absorbed after i.p. injection, with a plasma half-life of 7 hours⁽²³⁾. Twenty-four hours after reperfusion elevated levels of serum EPO were detected in the actively treated groups (EPO-pre: $4,236 \pm 145$ mIU/ml; EPO-isch: $5,213 \pm 360$ mIU/ml; EPO-rep: $5,597 \pm 209$ mIU/ml). Recombinant human EPO was undetectable in MI and sham-operated rats.

Discussion

In the present study, we show that a single dose of EPO at different time points during ischemia/reperfusion reduces the infarct size, which is accompanied by an improved LV function. To this point, these data confirm the previously reported cardioprotection by EPO in an I/R model^(14;15). However, we take this concept one step further, by comparing the cardioprotective effects of EPO treatment over a relevant time frame. We demonstrate that the observed beneficial effects are independent of timing of EPO administration, from pre-ischemic until after the start of reperfusion.

In our study, administration of EPO in all groups, including the group receiving EPO after the onset of reperfusion, markedly reduced the infarct size by limiting the area of irreversibly damaged tissue after ischemia/reperfusion injury and increasing the extent of viable myocardium. Since infarct size was significantly correlated with LV function (dP/dt_{max} and dP/dt_{min}), reducing the mass of infarcted tissue led also to improved LV hemodynamic parameters in groups treated with EPO (statistically significant LVEDP reduction in EPO-isch and EPO-rep groups and LV dP/dt_{min} in EPO-rep group).

Two forms of cell death are implicated during ischemia/reperfusion injury, namely apoptosis and necrosis⁽²⁴⁾. Although the exact contribution of these two forms of death is unclear, apoptosis progressively develops and accelerates during the reperfusion^(18;25). Since EPO is known to have anti-apoptotic properties, we investigated the level of apoptosis by measuring the percentage of active caspase-3 positive cells. In all actively treated groups, EPO administration reduced the rate of apoptosis within the risk area. Surprisingly, apoptosis was most attenuated in the group treated with EPO after the start of reperfusion. The “window of

protection” may thus be explained by the ability of the exogenous EPO to protect the cells in the risk area against the reperfusion-induced programmed cell death. Primarily protection against reperfusion damage confirms our previous results, in which the protective effects of EPO were mainly observed during the reperfusion of isolated rat hearts subjected to I/R⁽¹³⁾. Scarabelli et al.⁽¹⁸⁾ showed that in the early stages of reperfusion, apoptosis is firstly seen only in endothelial cells and is gradually spreading to surrounding cells. In our experiment we observed apoptosis specifically in endothelial and interstitial cells. We may hence hypothesize that EPO cardioprotection may be explained by preservation of the endothelial function and vascular flow in coronary vessels.

Furthermore, early apoptosis after myocardial infarction is associated with later expansion of the infarct size and LV remodeling⁽²⁶⁾. This is supported by a study by Moon et al., where a single dose of EPO at the time of myocardial infarction in rats led to a 50% reduction in early apoptosis, with subsequent prevention of LV dysfunction over a period of 8 weeks⁽²⁷⁾.

In addition to its anti-apoptotic effects, erythropoietin was recently shown to mobilize progenitor cells from the bone marrow⁽²⁸⁾ and stimulate neovascularization⁽¹⁶⁾, which was associated with the regeneration of the myocardium⁽²⁹⁾. Although we did not find any increase in proliferative activity in groups treated with EPO, this mechanism may play a role in a long-term EPO effect, positively shifting the balance between the cell death and regeneration in the infarcted myocardium⁽³⁰⁾.

Since we assessed the short-term action of EPO, its effect on hemoglobin levels would be largely limited and can not explain the described results. Previously it has been shown that a single high-dose of EPO does not increase hemoglobin levels during the first 2-3 days^(15;27).

The molecular signals by which EPO provides its benefit in this study remain largely unresolved and their analysis goes beyond the scope of this article. In neuronal and hematopoietic cells EPO activates various protein kinase cascades^(31;32). The primary kinase signaling pathway is the stress responsive Jak-2, activation of which leads to downstream phosphorylation of STAT-5, Akt-1 and MAPK. In the myocardium, the activation of these pro-survival kinase cascades during the first minutes of reperfusion has been shown to attenuate reperfusion-induced apoptosis⁽²⁴⁾.

In previous experiments we found that EPO increases the levels of phosphorylated MAPK p42/p44 in isolated rat hearts⁽³³⁾. However, the level of STAT-5 and MAPK activation peaks at 5-30 minutes after EPO exposure, with returning to baseline values within 1 hour⁽³⁴⁻³⁶⁾. Accordingly, in the present study we did not detect any difference in STAT-5 and MAPK phosphorylation between MI and EPO groups 24 hours after the reperfusion (data not shown). This may also explain the somewhat smaller anti-apoptotic effect found in the group pre-treated with EPO 2 hours before I/R, as it would result in insufficient activation of STAT-5/ MAPK pathways at the time of ischemia/reperfusion. However, further studies are needed to determine the precise mechanism of EPO induced cardioprotection.

Additional mechanisms have been implicated in the protective role of EPO in vascular diseases. EPO was shown to modulate NO activity and thus could account for restored vascular homeostasis^(37;38). Furthermore, antioxidative role of EPO could also play a role in cardioprotection during I/R⁽³⁹⁾.

The clinical benefit of non-erythropoietic effects of EPO has been implicated by Ehrenreich et al.⁽⁴⁰⁾ in a pilot, double-blind, randomized clinical trial investigating the acute effects of EPO treatment in patients with ischemic stroke. There, administration of EPO within 8 hrs

after stroke reduced brain infarct size and improved the clinical outcome. This short-term therapy with high-dose of EPO proved to be both safe and well tolerated. The serum EPO levels achieved in these patients (4.000-6.000 mIU/ml) were well comparable with those measured in our study.

As there are many similarities between brain and heart ischemia, EPO administration may provide an adjunctive therapy for the treatment of acute coronary syndromes. So far the therapeutic strategies are more directed to shortening the time of ischemia (“open artery” theory) and to lesser extend to approaches salvaging the cardiac tissue during reperfusion. Prospective EPO therapy may thus protect the “area at risk” during ischemia and (particularly) reperfusion.

Conclusion

In our study, we have shown that in vivo administration of EPO at different time points protects the myocardial structure and preserves cardiac function during I/R. Cardioprotective effect of EPO extends beyond the start of reperfusion, providing a broad “window of opportunity” for the potential treatment of acute coronary syndromes.

The present results and those of other groups, including the data on the safety of EPO administration in the clinical practice, warrant a pilot study with EPO treatment in patients with acute coronary syndromes.

Acknowledgments

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