Erythropoietin in cardiac ischemia

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Chapter 2

Erythropoietin improves left ventricular function and coronary flow in an experimental model of ischemia-reperfusion injury

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Abstract

Background: Recent studies show that erythropoietin (EPO) plays a protective role in brain ischemia. In this condition, administration of EPO protects neurons from ischemic damage. Recently, it has been shown that in patients with chronic heart failure (CHF), EPO treatment improved cardiac function. In the present study we assessed the role of EPO and EPO-receptor (EPO-R) in the heart.

Methods and Results: We studied the presence and functionality of the EPO-R in isolated rat hearts in the Langendorff set-up. Hearts were perfused for 20 minutes with 10 U/ml EPO or vehicle. Immunohistochemistry revealed the presence of the EPO-R on endothelial cells, fibroblasts and to a lesser extent cardiomyocytes. Furthermore, perfusion with EPO resulted in a 50% increase in the phosphorylated MAP kinases p42/p44. To evaluate the protective role of EPO in cardiac ischemia, we performed low-flow (0.6 ml/min) ischemia/reperfusion experiments in isolated rat hearts. Administration of EPO (10 U/ml) reduced the cellular damage by 56% ($p<0.05$) during reperfusion, diminished apoptosis by 15% ($p<0.05$) and resulted in a significantly improved recovery of left ventricular pressure ($p=0.02$) and coronary flow ($p=0.01$).

Conclusion: The present data suggest that a functional EPO-R is present in rat adult cardiac tissue and that exogenous EPO administration improves cardiac function after ischemia/reperfusion injury.

Introduction

In response to ischemia, mammalian cells express a variety of proteins, including erythropoietin (EPO) and vascular endothelial growth factor (VEGF) ([1]). The regulation of these two proteins is mediated by hypoxia inducible factor 1 (HIF-1). Expression of HIF-1 increases exponentially, as cellular O_2 concentrations decrease ([1;2]). Erythropoietin (EPO) is a glycoprotein hormone, primarily produced in the kidney. It mediates the physiological response to hypoxia by increasing red blood cell production. However, expanding evidence suggests that EPO also plays a major role in non-erythropoietic processes.

Several reports showed its efficacy in brain and retinal diseases ([3-5]). A study in rats subjected to cerebral ischemia showed a significant reduction in brain infarct size ([5;6]). Specificity and biological relevance of these changes were demonstrated by the observation that neutralization of endogenous EPO with soluble EPO-R augments ischemic brain damage ([7]). During ischemia, the EPO-receptor (EPO-R) is locally upregulated in brain tissue ([8]). After binding with its receptor, EPO signals through various intracellular pathways, including the MAP p42/p44 and JAK2-STAT5 tyrosine kinases ([9]). It was recently shown that activation of these pathways by EPO resulted in anti-apoptotic effect in various tissues including brain, retinal cells and erythroid precursor cells ([3;10;11]).

Little is known about the presence and protective role of EPO and its receptor in the heart. Juul *et al,* have described the presence of EPO and EPO-R in human fetal cardiac tissue ([12]). Experiments with knock-out mice, deficient for the genes expressing EPO and EPO-R, provide more evidence for its role in cardiac tissue, as both $EPO^{-/-}$ and $EPO\text{-}R^{-/-}$ mice suffer from ventricular hypoplasia and abnormalities in the vascular network ([13]). Silverberg *et al,* have shown that EPO treatment, in patients with CHF, results in an increased left ventricular ejec-

tion fraction, as compared with the placebo control group and there is a growing interest in this subject in the last few years ([14;15]).
The present study was designed to examine the presence and functionality of the EPO-R in adult cardiac tissue. In addition, we evaluated the protective effects of exogenous EPO administration in ischemia/reperfusion injury in the isolated rat heart.

Methods

Study design

Langendorff experiments were performed in isolated rat hearts that were perfused with EPO (10 U/ml) or vehicle for 20 minutes. These hearts were used to determine the expression and localization of EPO-R, and common signaling pathways were explored. To determine the protective effects of EPO treatment on ischemia/reperfusion injury, we studied 2 experimental groups (each consisting of 6 rats): ischemia/reperfusion without EPO and ischemia/reperfusion with EPO.

Langendorff perfusion

This well established experimental set-up has been described earlier ([11;16-19]). In short, rats were anaesthetised with isoflurane in O_2/N_2 and 500 U of heparin were injected in the tail vein. The heart was rapidly excised and the aorta was immediately perfused retrogradely by a modified Tyrode solution (glucose 10, NaCl 128.3, KCl 4.7, $NaHCO_3$ 20.2, $CaCl_2$ 1.35, NaH_2PO_4 0.42, $MgCl_2$ 1.05; all mmol/liter) and was equilibrated with 95% O_2 and 5% CO_2. Perfusion pressure was maintained at 60 mmHg. Coronary flow (CF) was measured by a microprocessor, which controlled the perfusion pressure by adjusting a peristaltic perfusion pump. CF and left ventricular pressure (LVP) were monitored continuously. After equilibrating for 15 minutes, hearts were subjected to low flow ischemia (0.6 ml/min) for 40 minutes, followed by a 2 hours reperfusion period at a constant 60 mmHg perfusion pressure. EPO (10 U/ml) or vehicle was administered from stabilization throughout the protocol. All the experiments conform with the Guide for the Care and Use of Laboratory Animals published by the US National Institutions of Health.

Analysis of coronary effluent

During stabilization (t=5 minutes), ischemia (t=20, t=30 and t=54 minutes) and reperfusion (t=55, t=56, t=57, t=60, t=70, t=90, t=120 and t=150 minutes), coronary perfusate samples were collected. Purines, a sensitive indicator of myocardial ischemia, were determined by high-performance liquid chromatography (HPLC) as previously described ([20;21]). The total amount of purines released during ischemia and reperfusion, corrected for coronary flow and left ventricular weight, was calculated (area under the curve).

RT-PCR

Snap-frozen LV tissues were used to extract total RNA. Total RNA was isolated using the method of acid guanidium thiocyanate lysis ([22]). RNA was quantified using a GeneQuant II (Pharmacia Biotechnology). First strand cDNA was synthesized from 1 μg RNA using the RT-PCR Core kit (Perkin-Elmer). Reverse transcriptase (RT) PCR for EPO-R was performed using a forward (5'- AGGACACCTACCTGGTATTGGA-3') and reverse primer (5'-CAGGCCCAGAGA-

GGTTCTCA-3'), yielding a product of 73 bp. To determine the specificity of the PCR reaction the amplicon was digested with Nci I to obtain the expected 39 bp and 34 bp fragments.

Western blotting

Snap frozen LV tissues were homogenized in Radio-Immuno-Precipitation-Assay (RIPA) buffer (1% NP40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 10 mM -mercaptoethanol, 10 mg/ml PMSF, 5 µl/ml aprotinin, 100 mM sodium orthovanadate, 5 µl/ml benzamidine, 5 µl/ml pepstatine A, 5 µl/ml leupeptine in 1× PBS). Protein concentrations were determined using the DC assay (Bio-Rad) with a bovine albumin standard. Protein levels of EPO-R, phosphorylated MAP kinases p42/p44 and phosphorylated STAT5 were determined by Western blot. Protein samples (50 µg) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to nitrocellulose membranes, followed by staining with Ponceau S solution (Sigma). Membranes were incubated with primary antibody against phosphorylated MAP kinases p42/p44 (1:1000 dilution, New England Biolabs), phosphorylated STAT5 (1:1000 dilution, Upstate biotech) and EPO-R (1:250 dilution; C-20 and M-20, Santa Cruz Biotechnology). Horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (1:2000, Santa Cruz Biotechnology) was used as secondary antibody. Signals were detected by the ECL-detection method (Amersham) and quantified by densitometry.

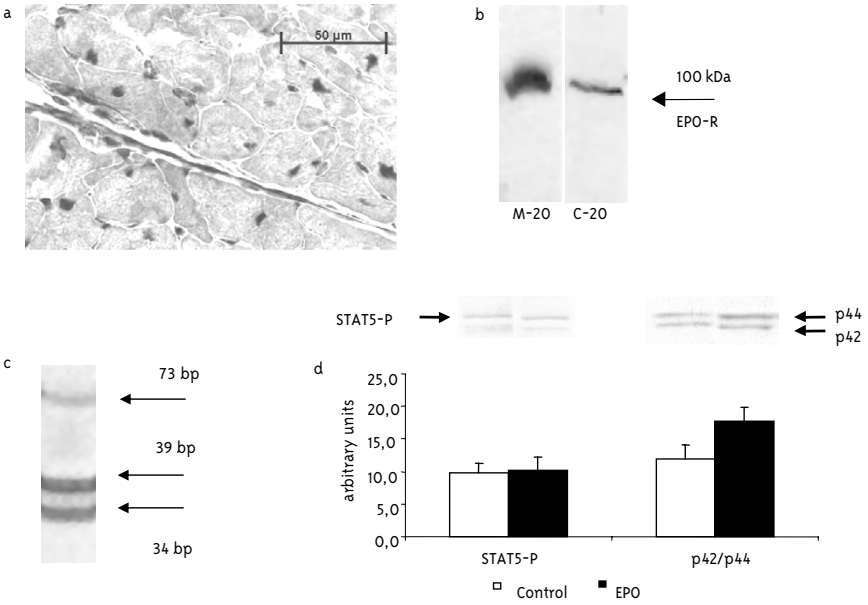
Immunohistochemistry

For immunohistochemistry, cryosections (4 µm) from a midpapillary slice of the left ventricle were fixed in acetone. Hereafter, sections were incubated with two different polyclonal anti-EPO-R antibodies (1:50) (M-20, C-20; Santa Cruz). A two-step indirect peroxidase detection system was employed to visualize the expression pattern of the EPO-R. Sections of placenta and breast carcinoma were used as a positive control ([23;24]). Slides omitting the primary antibody and preincubation with blocking peptide (10:1) were used as negative controls. For apoptosis detection, sections were incubated with an antibody that specifically recognizes the active form of caspase-3 (1:50; New England Biolabs), as previously reported ([25]). For quantitative analysis, active caspase-3 positive cells in thirty random fields per section (80-120 cells per field) were counted at high-power magnification. Tissue sections of colonic adenocarcinoma served as a positive control ([26;27]).

Statistical Analysis

Values shown are mean ± SEM. We used a linear regression model with repeated measures to compare the functional responses to EPO treatment. Data regarding the purine overflow and caspase-3 immunohistochemistry were analyzed by Student's t-test. Statistical significance was defined as $p<0.05$.

Figure 1a) Immunostaining for EPO-R in normal cardiac tissue. Staining is predominantly observed in interstitial cells, including endothelial cells and fibroblasts. Weak staining is observed in cardiomyocytes. b) Western blot analysis of EPO-R expression in non-ischemic tissue with two different antibodies (M-20, C-20). c) RT-PCR analysis of EPO-R mRNA transcripts (73 bp), after partial digestion with restriction enzyme Nci I, two specific products (39 bp and 34 bp) are obtained. d) Effects of EPO (10 U/ml) on phosphorylated STAT5 (92 kDa) and MAP kinase p42/p44 (42 and 44 kDa), assessed by Western blot analysis (n=6).

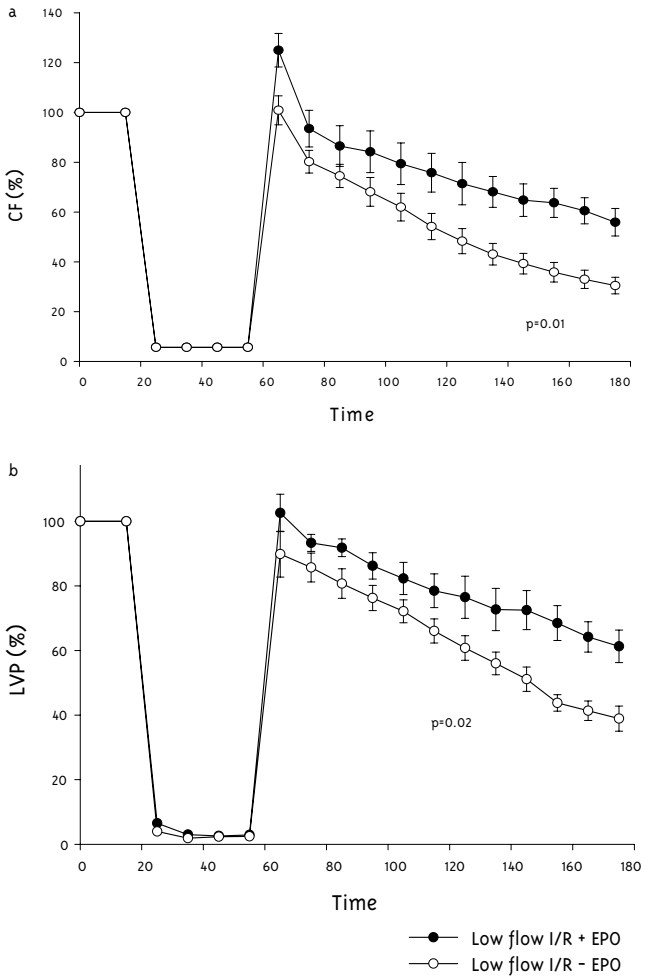


Results

Expression pattern and functionality of EPO-R in heart

We determined the expression of EPO-R in normal cardiac tissue by immunohistochemistry. Immunostaining for EPO-R was predominantly observed in interstitial cells, including endothelial cells and fibroblasts. Cardiomyocytes showed weak expression of the EPO-R (Fig1a). We found similar expression patterns with both antibodies, while incubation with 10x excess of blocking peptide completely abolished the signal (data not shown). Western blotting revealed a specific signal for the EPO-R with both antibodies at the expected size of 100 kDa (Fig 1b). Further, we studied the expression levels of the EPO-R after ischemia-reperfusion injury. In the group without EPO perfusion, we did not observe a change in the expression level of the EPO-R compared to non-ischemic tissue. However, EPO treatment during ischemia-reperfusion induced a 26 ± 8.3% downregulation of the EPO-R. Further, RT-PCR revealed EPO-R gene transcription in the rat heart. Specificity of the product was confirmed by restriction fragment length analysis (Fig 1c). We further explored potential signal transduction pathways of the EPO-R, by infusing 10 U/ml EPO in Langendorff-perfused hearts for twenty minutes. This resulted in a 50% increase in levels of phosphorylated MAP kinase p42/44 in the left ventricles of EPO-perfused hearts compared with vehicle perfused hearts (Fig 1d). No increase in the levels of phosphorylated STAT5 were detected.

Figure 2. Effects of EPO treatment on CF (2A) and LVP (2B). Values are shown as a percentage from baseline (n=12).



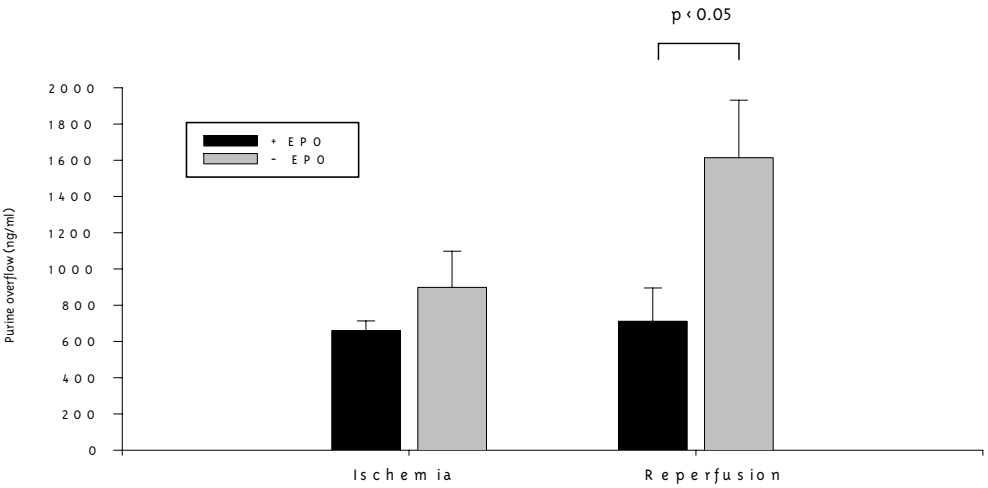
Effects of EPO on Cardiac Function

To test the potential protective effects of EPO in the heart, we performed low-flow ischemia/reperfusion experiments in isolated rat hearts. Baseline characteristics, body weight, heart weight, CF and LVP did not differ between both groups (data not shown). During low-flow ischemia the cardiac function decreased to a similar extent in the two groups, irrespective of EPO treatment. During reperfusion, post-ischemic hyperemia occurred in both groups, but CF was restored to a significantly higher level during the two hours reperfusion period in the EPO treated group ($p=0.02$, Fig 2a). Furthermore, LVP was significantly increased throughout the reperfusion period in the EPO treated group compared with the vehicle treated group ($p=0.01$, Fig 2b).

Effects of EPO on Cellular Damage

To determine the effect of EPO perfusion on cellular damage, we measured purine overflow at different time points during stabilization, ischemia and reperfusion period. Total overflow

Figure 3. Effect of EPO perfusion on total purine overflow during ischemia and reperfusion (area under the curve). Purines are a marker for ATP breakdown and therefore an indicator of reversible and irreversible damage to the myocardium (n=12).



of purines during reperfusion showed a 56% decrease (711 ± 183 nmol/g versus 1614 ± 317 nmol/g) ($p<0.05$) in the EPO treated group, compared with the vehicle treated hearts. A smaller difference was observed during the ischemic period between the EPO perfused hearts and the control group, 660 ± 53 nmol/g versus 898 ± 200 nmol/g, respectively (p=NS; Fig 3). No purines were detected at baseline.

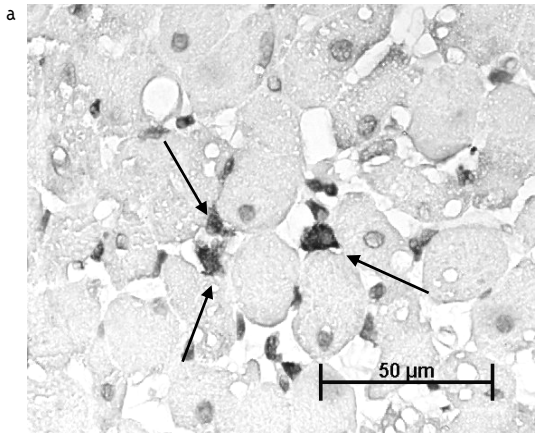
Furthermore, we studied the anti-apoptotic effects of EPO perfusion on the heart. Staining with anti-active caspase-3 was mostly restricted to endothelial cells and fibroblasts (Fig 4a). The hearts perfused with EPO demonstrated a 15% reduction in apoptotic cells (2.1% ± 0.12 versus 1.8% ± 0.09) ($p<0.05$, Fig 4b).

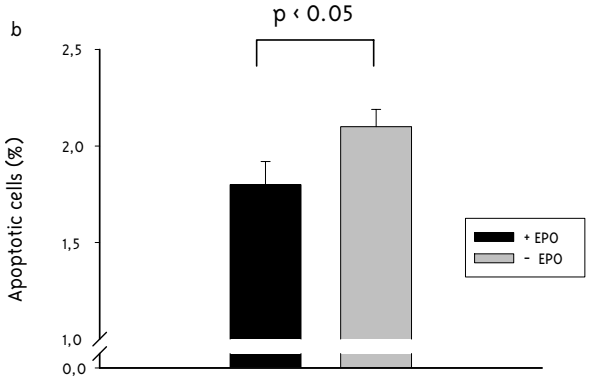
Discussion

In the present study, we demonstrated the presence of a functional EPO-R in adult cardiac tissue and we showed that EPO administration limited cardiac damage and preserved cardiac function after ischemia/reperfusion injury. However, the mechanism by which EPO preserves cardiac function is currently unknown.

We found that EPO stimulation increases the levels of phosphorylated MAP kinases p42/p44 in normal rat heart. This pathway has already been implicated as a survival pathway in cardiac cells after ischemia/reperfusion injury, by inhibiting apoptosis ([28-30]). A study by Yue *et al.* provided more evidence for the role of MAP kinases in ischemia/reperfusion injury by demonstrating that inhibition of the MAP kinases p42/p44 pathway exacerbated cardiac injury and showed a diminished functional recovery compared with control hearts ([31]). Thus, activation of this pathway seems to be important for survival of cardiac cells by protecting them from programmed cell death. With respect to STAT5, we did not observe difference in the amount of phosphorylated STAT5 after EPO perfusion for twenty minutes. This might be related to

Figure 4a) Immunostaining for active caspase-3 in ischemic cardiac tissue without EPO treatment. b) Percentage of active caspase-3 positive cells at the end of reperfusion in isolated rat hearts perfused with or without EPO and subjected to 40 min. of low-flow ischemia and 2 hours of reperfusion (n=12).





different time-points of STAT5 phosphorylation after perfusion with EPO. Furthermore, this pathway could play a minor role in the cardiac EPO signaling, as shown in vascular smooth muscle cells by Ammarguellat *et al* ([32]). Future experiments will be needed to precisely explore the EPO signaling pathways in the heart.

Both ischemic and reperfused myocardium can undergo apoptosis, however, during reperfusion, accelerated apoptosis occurs in cardiac cells ([33]). We observed that EPO limits cardiac damage by 56% during reperfusion. A recent paper from Scarabelli *et al.* showed that in the early stages of reperfusion, apoptosis is first seen in endothelial cells and is spreading to surrounding cardiac myocytes, suggesting that reperfusion induces the release of pro-apoptotic mediators from endothelial cells ([34]). We found that the EPO-R was predominantly localized to endothelial cells and fibroblasts. Interestingly, we observed in these cells a reduction in apoptosis of 15%, when the hearts were perfused with EPO. By preventing apoptosis in these cells, it is tempting to speculate that EPO can preserve vascular flow and ultimately protect the

myocardium. Although a reduction of 15% in apoptotic cells seems modest, recent investigations suggest that apoptosis after myocardial infarction is progressive, and therefore small amounts of apoptotic cells may result in more extensive cell loss ([35]). Recent data reported on the beneficial effects of preconditioning in the rodent heart in which exposure of wild-type mice to intermittent hypoxia resulted in protection from ischemia-reperfusion injury ([36]). Ischemic preconditioning was absent in mice heterozygous for a knockout in the HIF-1α gene. Further, in wild-type mice, EPO administration at 24 hours prior to *ex vivo* ischemia-reperfusion resulted in a reduction in apoptosis and an increased cardiac recovery. While these findings are in accordance with our results the present study suggests that there is no need for an extended period of pretreatment with EPO to exert its protective effects.

In addition to its anti-apoptotic effects, EPO may protect the myocardium through other mechanisms that have not been assessed in this work. Oxidative stress plays an important role in the reperfusion damage observed in the myocardium ([37]). Recent research suggests that EPO can also directly protect tissue against the effects of free radicals ([38]). Furthermore, it has been shown that EPO may increase the nitric oxide (NO) production when EPO-induced erythrocytosis occurs, reviewed by Smith *et al.*([39]). Transgenic mice overexpressing human erythropoietin showed higher NO synthase levels and an increased NO-mediated endothelium derived relaxation ([40]). On the other hand, Noguchi *et al* showed that one-week of erythropoietin treatment in rabbits resulted in a decreased response to endothelium dependent vasodilators ([41]). EPO has also been shown to act as a cardioprotective agent, by modulating the cardiac Na^+-K^+-pump ([42]).

EPO has been widely used in clinical practice for more than a decade. A recent study of Silverberg *et al*. showed the beneficial effects of rh-EPO therapy in CHF patients ([14]). They conducted a placebo controlled study in 32 mild anemic patients with severe CHF (NYHA ≥III) and treated them with rh-EPO. Over a mean of 8.2 +/- 2.6 months, left ventricular ejection fraction increased by 5.5% in the treatment group, compared to a decrease of 5.4% in the control group. These results strongly suggest an important role for rh-EPO in patients with CHF. Although correction of anemia has beneficial effects on cardiac function, non-erythropoietic effects are also likely to play a role. More evidence for non-erythropoietic effects of EPO in human was provided by Ehrenreich *et al.* ([43]). They recently conducted a pilot double blind randomized clinical trial to investigate the acute effects of EPO treatment in patients with ischemic stroke. Administration of EPO within 8 hrs after stroke reduced brain infarct size and improved the clinical outcome. As there are many similarities between brain and heart ischemia, EPO administration may become an adjunctive therapy for the treatment of acute coronary syndromes. Further work is needed to determine the mechanisms by which EPO reduces cardiac damage and preserves cardiac function.

In conclusion, this study suggests that EPO treatment is effective in reducing myocardial damage and preserving cardiac function after ischemia/reperfusion injury. This implies an organ protective role of EPO beyond erythropoiesis and warrants the search for organ specific EPO analogues.

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