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European Journal of Cardio-thoracic Surgery 35 (2009) 839–846

EUROPEAN JOURNAL OF CARDIO-THORACIC SURGERY

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Erythropoietin protects from reperfusion-induced myocardial injury by enhancing coronary endothelial nitric oxide production☆,☆☆

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Received 19 September 2008; received in revised form 18 December 2008; accepted 22 December 2008; Available online 23 February 2009

Abstract

Objective: Cardioprotective properties of recombinant human Erythropoietin (rhEpo) have been shown in in vivo regional or ex vivo global models of ischemia–reperfusion (I/R) injury. The aim of this study was to characterize the cardioprotective potential of rhEPO in an in vivo experimental model of global I/R approximating the clinical cardiac surgical setting and to gain insights into the myocardial binding sites of rhEpo and the mechanism involved in its cardioprotective effect. **Methods:** Hearts of donor Lewis rats were arrested with cold crystalloid cardioplegia and after 45 min of cold global ischemia grafted heterotopically into the abdomen of recipient Lewis rats. Recipients were randomly assigned to control non-treated or Epo-treated group receiving 5000 U/kg of rhEpo intravenously 20 min prior to reperfusion. At 5 time points 5–1440 min after reperfusion, the recipients (n = 6–8 at each point) were sacrificed, blood and native and grafted hearts harvested for subsequent analysis. **Results:** Treatment with rhEpo resulted in a significant reduction in myocardial I/R injury (plasma troponin T) in correlation with preservation of the myocardial redox state (reduced glutathione). The extent of apoptosis (activity of caspase 3 and caspase 9, TUNEL test) in our model was very modest and not significantly affected by rhEpo. Immunostaining of the heart tissue with anti-Epo antibodies showed an exclusive binding of rhEpo to the coronary endothelium with no binding of rhEpo to cardiomyocytes. Administration of rhEpo resulted in a significant increase in nitric oxide (NO) production assessed by plasma nitrite levels. Immunostaining of heart tissue with anti-phospho-eNOS antibodies showed that after binding to the coronary endothelium, rhEpo increased the phosphorylation and thus activation of endothelial nitric oxide synthase (eNOS) in coronary vessels. There was no activation of eNOS in cardiomyocytes. **Conclusions:** Intravenous administration of rhEpo protects the heart against cold global I/R. Apoptosis does not seem to play a major role in the process of tissue injury in this model. After binding to the coronary endothelium, rhEpo enhances NO production by phosphorylation and thus activation of eNOS in coronary vessels. Our results suggest that cardioprotective properties of rhEpo are at least partially mediated by NO released by the coronary endothelium.

Keywords: Erythropoietin; Myocardial ischemia–reperfusion injury

1. Introduction

Myocardial protection from ischemia and reperfusion (I/R) injury remains of paramount importance in the setting of global myocardial ischemia associated with open-heart surgical procedures. There has been considerable progress made to date in myocardial protection strategies including the use of blood cardioplegia [1]. Nevertheless, a high risk subset of patients continue to suffer perioperatively from myocardial I/R injury as exhibited by prolonged contractile dysfunction, low-output syndrome, arrhythmias, perioperative myocardial infarction and cardiac failure leading to prolonged intensive care [2]. Thus, there is a compelling necessity to improve myocardial protection from I/R injury during open-heart surgical procedures.

There is a large body of experimental work showing the non-hematopoietic cytoprotective properties of recombinant human Erythropoietin (rhEPO) in a variety of tissues subjected to I/R [3], including the retina [4], brain [5], and cardiovascular system [6]. However, primary targets and down stream mechanisms of the cardioprotective effect of rhEpo remain controversial. The aim of this study was to

☆ Presented at the 22nd Annual Meeting of the European Association for Cardio-thoracic Surgery, Lisbon, Portugal, September 14–17, 2008.

☆☆ This study was funded by the Zurich Centre for Integrative Human Physiology for RT and AB and the Swiss National Science Foundation grants (#112 449 for AB).

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1010-7940/$ – see front matter
doi:10.1016/j.ejcts.2008.12.049

characterize the cardioprotective effect of rhEPO in an in vivo model of cold global I/R injury and to gain insights into the primary targets and down stream mechanisms involved in its cardioprotective effect.

2. Materials and methods

2.1. Animals

Male Lewis rats (Harlan, Holland) weighing 250—300 g were used as donors and recipients. All animals received humane care in compliance with the European Convention on Animal Care. This study was approved by the local institutional ethics committee. The animals were maintained in standard housing conditions with dry diet and water available ad libitum.

2.2. Heterotopic heart transplantation

Donor rats were anesthetized with an i.p. injection of pentobarbital (50 mg/kg of body weight) followed by administration of 500 units of i.v. heparin. The heart was arrested with 10 ml/kg of body weight of cold (4 °C) crystalloid cardioplegic solution and stored in the same cardioplegic solution at 4 °C until implantation.

Recipient rats were anesthetized with an i.p. injection of pentobarbital and the donor heart was transplanted heterotopically according to the technique of Ono and Lindsey [7]. The total duration of global ischemia was 45 min of which the last 15 min were needed for anastomoses.

2.3. Experimental design

Recipient rats were randomized to control non-treated or Epo-treated group receiving 5000 U/kg of rhEpo intravenously 20 min prior to reperfusion. At 5 time points (5, 30, 60, 360, and 1440 min) after reperfusion the recipients ($n = 6$—8 at each point) were sacrificed. Blood, native and grafted hearts were harvested for subsequent analyses.

2.4. Myocardial injury and tissue stress markers

Myocardial injury was assayed by monitoring of the plasma levels of cardiac troponin T isoform (cTnT) using electrochemiluminescent immunoassay (Roche Diagnostics, Switzerland). Plasma levels of atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) were determined by enzymatic immunoassay (Peninsula Laboratories, USA).

2.5. Systemic inflammatory response

Interleukin-6 (IL-6) in blood plasma was assessed using Rat IL-6 Quantikine ELISA Kit (R&D Systems, Minneapolis, MN).

2.6. Measurement of tissue ion and water content

Tissue Na^+ and water content were assessed after drying and burning the tissue sampled in metal-free concentrated HNO_3 using flame photometry and gravimetry, respectively.

2.7. Apoptosis markers and signaling

Caspase 3 and caspase 9 activities were determined in tissue homogenate using colorimetric peptide-based assay kits (R&D Systems). Lysates of rat endothelial cell line (RBE4) treated overnight with 0.5 μM Staurosporine (Sigma—Aldrich, St. Louis, MO) were used as a positive control of apoptosis [8]. The downstream product of active caspase 3, cleaved poly (ADP-ribose) polymerase (PARP) was detected using immuno-blotting with the rat-specific (Asp214) rabbit polyclonal antibody diluted 1:1000 (Cell Signaling). The CardioTACS in situ apoptosis detection kit (R&D Systems) was used to estimate the number of TUNEL-based apoptosis-positive nuclei.

2.8. Red cell, plasma and tissue reduced (GSH)/oxidized Glutathione (GSSG) measurement

Blood and plasma samples were deproteinized and GSH and GSSG were detected using Ellman's reagent as described elsewhere [9]. Tissue samples used for determination of the GSH/GSSH levels were homogenized in equal volumes of solutions containing either 100 mM KCl and 10 mM MOPS-KOH or 5% trichloroacetic acid (TCA) in distilled water. After centrifugation supernatant was used for the GSH/GSSG detection using the same protocol as for the blood/plasma samples (see above).

2.9. Product of nitric oxide (NO) metabolism in plasma

Stable product of NO oxidation, i.e. plasma nitrite level, was detected using chemiluminescence assay by means of CLD-88 (EcoMedica, Switzerland). Plasma samples were collected immediately after blood harvest and concomitant centrifugation (4 min, 8000 g).

2.10. Epo-induced signaling

Binding of rhEpo was assessed in acetone-fixed cryosections of transplanted and native ventricular tissue using antibodies against Epo (R&D Systems).

Rabbit polyclonal antibodies against phosphorylated (Ser 1177) endothelial nitric oxide synthase (eNOS) (Cell Signaling, 1:100) were used for immunohistochemical determination of eNOS phosphorylated eNOS in acetone-fixed frozen sections. Secondary FITC-conjugated antibodies were applied and localization of the specific staining determined using fluorescent microscopy (Zeiss AxioScope 2500).

2.11. K^+ influx measurements in neonatal rat cardiomyocytes

The procedure of cell isolation is described in detail elsewhere [10]. Unidirectional K^+ influx was measured using radioactive tracer technique. The cells (≈400 000 cells per Petri dish, 3 cm ∅) were pre-treated with 1 mM ouabain and/or rhEpo (final activity 10 U/ml) in the presence or absence of 100 μM L-arginine for 30 min. Thereafter flux detection was initiated by adding ^{86}Rb as a tracer for K^+ (final activity 0.5 μCi/ml). Fluxes were assessed over half an hour with tracer accumulation detected 5, 10, 20, and 30 min after the

tracer application. Over this time period the accumulation of tracer was a linear function of the incubation time. Samples (10 μl) of the incubation media were collected and the cells were washed free from extracellular ^{86}Rb with ice-cold incubation medium and finally lysed in 0.1 M NaOH. Cell lysate was then used for detection of the intracellular ^{86}Rb and protein assessment (Bio-Rad protein assay). Unidirectional potassium influx was then calculated from the slope of the ^{86}Rb uptake curve over time and normalized per amount of the ^{86}Rb in the incubation medium and per protein. Active K^+ transport mediated by the Na/K ATPase was calculated as a difference between K^+ fluxes in the presence and in the absence of ouabain. Ouabain-resistant flux component was addressed to as passive K^+ influx.

2.12. Statistical analysis

All data are based on at least six replicates for in vivo experiments and at least five independent experiments when using cultured cardiomyocytes. They are presented as mean ± SEM. The comparison between the experimental groups was performed using ANOVA and two-tailed Student's *t*-test for unpaired samples with normality test and Bonferroni correction when analyzing simple time points (GraphPad Instat.V3.05). The optimal number of experiments per time point and group was chosen in accordance with our previous experience [11]. The level of statistical significance was set at $p < 0.05$.

3. Results

3.1. rhEpo and I/R myocardial damage

Significant reduction of the cardiac TnT plasma levels in the rhEpo-treated group indicated that intravenous application of the rhEpo could efficiently reduce both early and late reperfusion-induced myocardial injury (Fig. 1). In particular, rhEpo abolished acute release of the atrial and brain natriuretic factors into the circulation upon restoration of blood

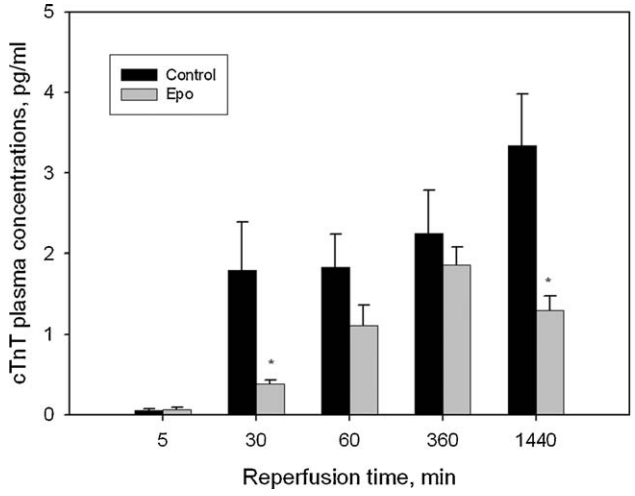

Fig. 1. Plasma troponin T levels. Data are means of 6–8 independent experiments ± SEM ($n = 6–8$). * indicates $p < 0.05$ compared to the corresponding time point in non-treated control group.

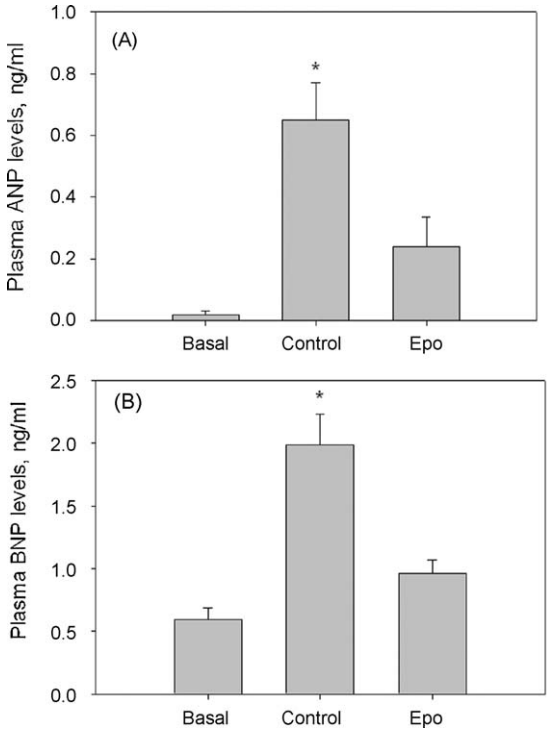

Fig. 2. (A) Plasma levels of atrial natriuretic peptide ANP in non-treated control animals (basal) and 5 min after the onset of reperfusion in non-treated control animals (control) and rhEpo-treated animals (Epo). Data are means of 6–8 independent experiments ± SEM. * indicates $p < 0.05$ compared to the corresponding time point in non-treated control group. (B) Plasma levels of brain natriuretic peptide BNP in non-treated control animals (basal) and 5 min after the onset of reperfusion in non-treated control animals (control) and rhEpo-treated animals (Epo). Data are means of 6–8 independent experiments ± SEM. * indicates $p < 0.05$ compared to the corresponding time point in non-treated control group.

perfusion of the graft (Fig. 2). Systemic inflammatory response assessed as an increase in plasma IL-6 level 6 h after the onset of perfusion was not affected by rhEpo treatment (235 ± 72 pg/ml vs 291 ± 67 pg/ml in control and rhEpo-treated recipients, $p > 0.05$). Thus, cardioprotective effect of Epo was not due to the suppression of the secondary inflammatory response.

The time from the onset of reperfusion to the defibrillation of the grafted heart was shorter in rhEpo-treated than non-treated group (47 ± 2 s vs 66 ± 4 s, respectively, $n = 40$, $p = 00012$).

3.2. Mechanisms of I/R injury

Interaction of rhEpo with its receptor was shown to reduce apoptosis in isolated cardiomyocytes [3]. We have evaluated the degree of apoptosis in transplanted hearts of control and rhEpo-treated animals as well as between the transplanted and native hearts.

To do so we have assessed caspase 9 and caspase 3 activity in ventricular tissue homogenate. The resulting activity was compared between native and transplanted hearts in both control and Epo-treated animal groups and related to the activity of caspases in cell culture where apoptosis was triggered by Staurosporine treatment. The basal activity of caspase 3 and caspase 9 in the native

heart tissue was very low (0.28% of the positive control, Staurosporine-treated vascular endothelial cells for caspase 3 and 0.20% of the positive control for caspase 9). Ischemia—reperfusion resulted in a very modest increase of the activity of both caspases which was only statistically significant between the native and the transplanted hearts 30 min after the onset of perfusion (data not shown). This difference was insignificant when caspases' activity in transplanted hearts of Epo-treated group were compared to the native heart levels. Activation of caspase 3 did not cause detectible PARP cleavage in transplanted hearts at any reperfusion time point. In addition, the number of TUNEL-positive cells ranged between 0 and 4 cells/×20 field and did not differ statistically between non-treated and rhEpo-treated ischemic hearts. Taken together with the data on cardiac TnT release into the circulation these data suggest that most of the ischemia—reperfusion damage represents oncosis rather than apoptosis.

3.3. Cellular ion and water content

Data on tissue water and sodium content shown in Fig. 3A and B provide further confirmation for the preferential oncotic acute reperfusion damage. Reperfusion is followed by a transient increase in tissue sodium and water levels in transplanted hearts of control but not in rhEpo-treated animals. Recovery of the tissue ion/water balance occurred within 30 min of reperfusion in both control grafts and rhEpo-treated grafts.

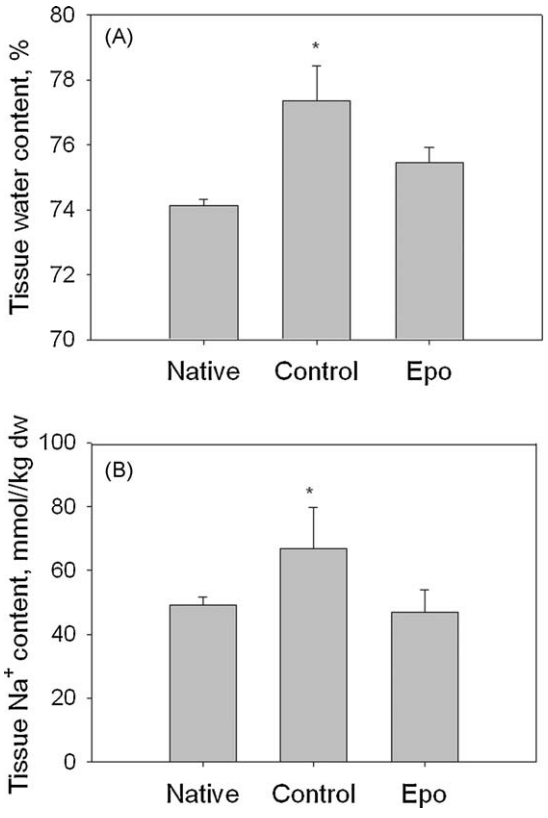

Fig. 3. Effect of I/R and rhEpo on the tissue water and Na^+ content. (A) Water content in ventricular tissue of native non-ischemic hearts of the non-treated control group (native) and in ischemic reperfused hearts of non-treated control group (control) and rhEpo-treated group (Epo) 5 min after the onset of perfusion. (B) Tissue Na^+ content in the same set of samples. Data are means of 6—8 hearts ± SEM. * indicates $p < 0.05$ compared to the value in native hearts.

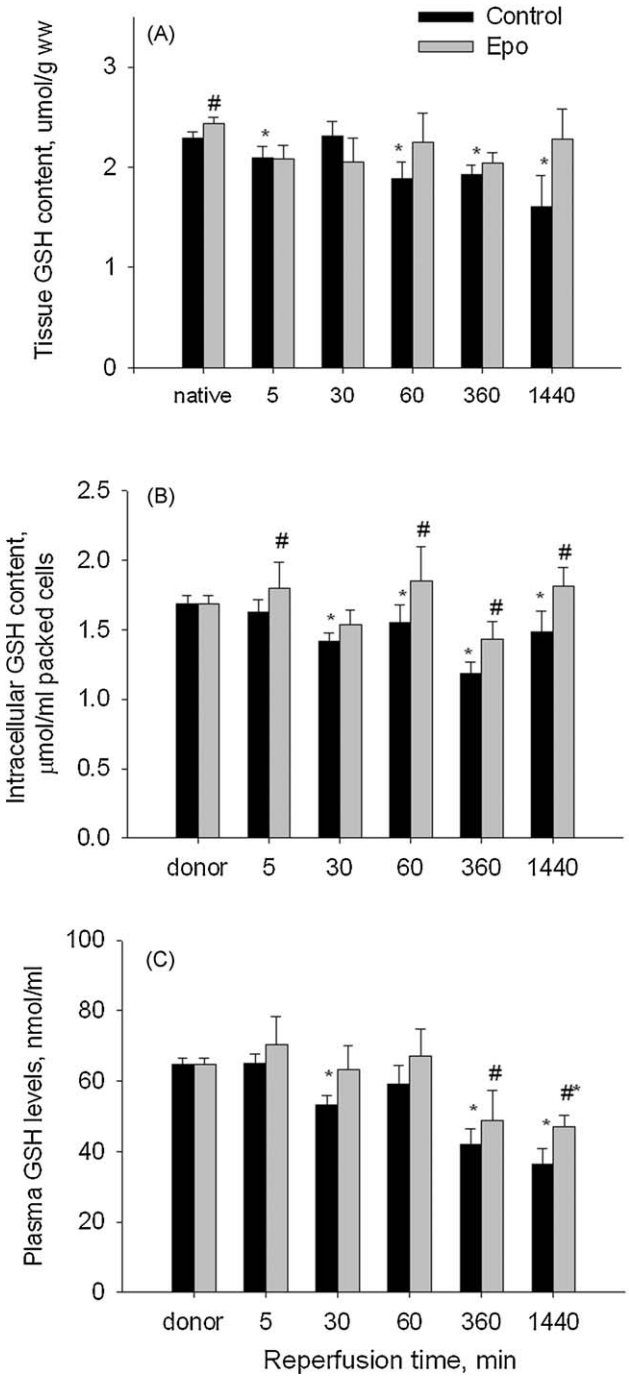

Fig. 4. Reduced glutathione levels in ventricular tissue homogenates of native non-treated (n.control) and native rhEpo-treated (n.Epo) hearts, erythrocytes and plasma. (A) GSH pool of ventricular tissue. Values are means of 6—8 independent heart samples ± SEM. Note that the values for native hearts differ between the control and rhEpo-treated animals (denoted with #, $p < 0.05$). * indicates $p < 0.05$ compared to the native control value. (B) and (C) GSH levels in erythrocytes and plasma. Data are means of 6—8 experiments ± SEM. # indicates $p < 0.05$ compared to the levels in non-treated control animals of the corresponding time point. * denotes $p < 0.05$ compared to the basal level in non-treated animals.

3.4. Reperfusion and oxidative stress

Oxidation is one of the recognized causes of the myocardial damage at reperfusion. Indeed, reperfusion resulted in acute depletion of the GSH pool in ventricular tissue that became even more pronounced with time (Fig. 4A). Administration of rhEpo resulted in an increase in the GSH content in native myocardium ($p < 0.05$). Transplanted hearts of the rhEpo-treated group did not show GSH depletion at any reperfusion time point (Fig. 4A). Intravenous administration of rhEpo also abolished reduction of the GSH levels in erythrocytes and plasma of the recipient animals thus providing systemic defense from the reperfusion-induced oxidative stress (Fig. 4B and C).

3.5. Mechanisms of the cardioprotective action of rhEpo

As shown in Figs. 3 and 4, rhEpo protects transplanted myocardium from reperfusion-induced oxidative stress, edema and Na^+ accumulation. Plasma nitrite levels in recipient animals were significantly upregulated following rhEpo administration indicating an increase in nitric oxide production (Fig. 5). The source of NO production was characterized by using antibodies against phosphorylated (active) forms of endothelial nitric oxide synthase. Interestingly, rhEpo treatment resulted in activation of eNOS selectively in vascular endothelium of coronary vessels but not in the myocardial tissue itself (Fig. 6C and D). Cardiac myocytes are known to express eNOS along with vascular endothelial cells. We therefore investigated localization of rhEpo in both native and transplanted heart tissue. The data presented in Fig. 6A and B indicate that rhEpo does not diffuse from the lumen of the coronary vessels into the myocardium at least for the first 30 min of reperfusion. Both the rhEpo binding and activation of the eNOS are restricted to the endothelial compartment.

In order to address the possible effect of rhEpo on ion and water equilibrium in more details we have monitored the active and passive K^+ transport across the sarcolemmal membrane of primary cultures of cardiac myocytes in the

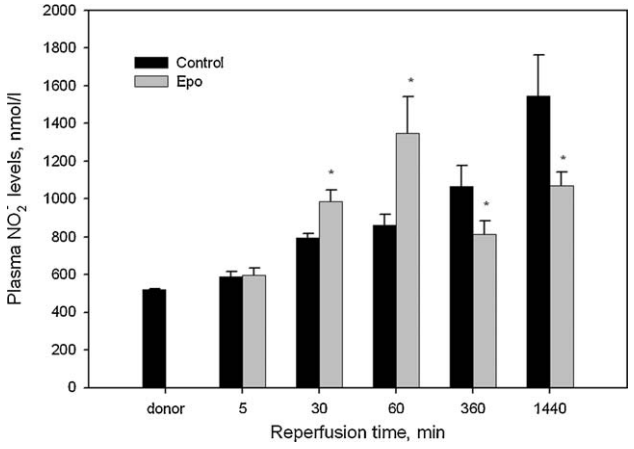

Fig. 5. Nitrite levels in plasma of rhEpo-treated animals (rhEpo) and non-treated control animals (control). Data are means of 6–8 animals ± SEM. * denotes $p < 0.05$ compared to the corresponding time point in non-treated control animals.

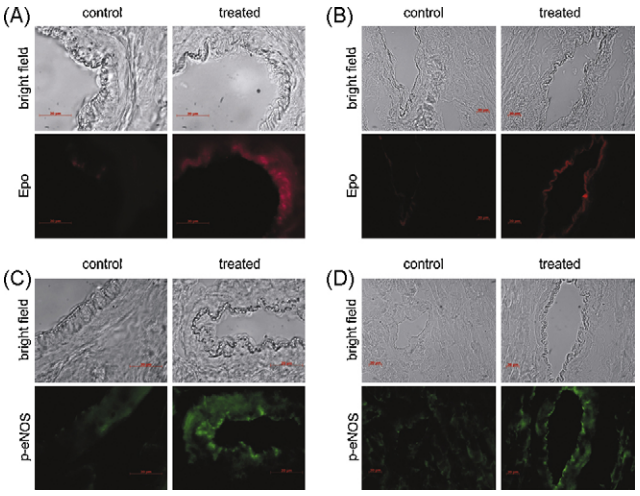

Fig. 6. Localization of the rhEpo binding (stained in red) and eNOS activation (phosphorylated active form stained in green) in the grafts. rhEpo localization in the ventricular tissue of the grafts 5 min after the onset of reperfusion (A) and 30 min after the onset of perfusion (B). Localization of the phospho-eNOS (antibodies against phosphor-Ser 1177) in the transplanted hearts 5 min (C) and 30 min (D) after the onset of perfusion. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

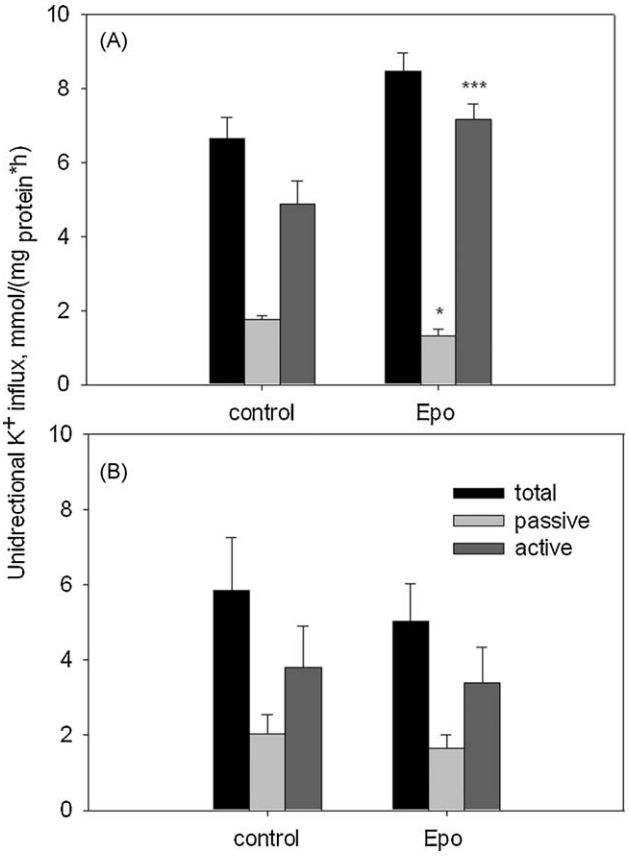

Fig. 7. Total, passive and active K^+ influx components into neonatal rat cardiomyocytes in the presence or in the absence of 10 U/ml rhEpo in the medium containing 100 μM L-arginine (A) or in arginine-free medium (B). Data are means of five independent experiments ± SEM. *** and * indicates $p < 0.001$ and $p < 0.05$ correspondingly when compared to the Epo-free conditions.

presence and in the absence of rhEpo. Administration of rhEpo resulted in stimulation of the active influx of K^+ mediated by the Na/K ATPase along with suppression of passive K^+ movement across the sarcolemma (Fig. 7A). This does not occur when the cell incubation medium is deprived of L-arginine, a substrate of eNOS (Fig. 7B) suggesting the observed effects of rhEpo on both the Na/K ATPase and the passive K^+ flux are not direct but secondary to the Epo-induced stimulation of NO production.

4. Discussion

Choosing an in vivo model of cold global ischemia and warm reperfusion we intended to mimic as closely as possible the clinical setting during open-heart surgical procedures. The narrow time window of cardioprotection for rhEpo makes it almost useless when treating myocardial infarction (first hours after the injury) [3,12]. The preoperative period on the contrary allows precisely timed administration of the drug assuring maximal cardioprotective effect of rhEpo. To our knowledge, the present study is the first one investigating the myocardial action of rhEpo during cold global I/R injury in vivo. Our data indicate that in i.v. administration of rhEpo after the onset of cold global ischemia but prior to warm reperfusion confers an acute cardioprotective effect in rats. This cardioprotection offered by rhEpo was not linked to the suppression of apoptosis but to the reduction of oxidative stress and edema, which was at least partially mediated by the rhEpo-induced stimulation of NO production.

This observation is in line with numerous reports of cardioprotective efficiency of rhEpo obtained using different models and experimental settings including isolated rat cardiomyocytes [6,13—15], in vitro Langendorff model [6,13,16—18] and in vivo coronary artery ligation model [12,14,15,19]. Despite extensive investigations the mechanisms of rhEpo-induced cardioprotection remain a matter of debate. Data generated using primarily isolated cardiomyocytes, but also in some in vivo models suggest that rhEpo protects myocardium from apoptosis [12,15]. However, the role of apoptosis of cardiomyocytes in I/R injury is questionable [1,20]. Our data reveal that, although occurring, apoptosis does not contribute significantly to the cold global I/R injury in our experimental model. Reperfusion induced only marginal activation of caspase 9 and caspase 3 at only one time point (30 min of reperfusion). However, at 30 min of reperfusion rhEpo administration significantly reduced myocardial cell injury reflected by plasma troponin (Fig. 1). Of note, reduction in mechanical overload evidenced by ANP and BNP release into the plasma was detectible even earlier, already 5 min after the onset of blood flow (Fig. 2). We were unable to monitor the changes in downstream targets of caspase activation such as PARP cleavage or DNA fragmentation suggesting that when occurring, apoptotic response was incomplete. Very close to our results, van der Meer and co-workers found only a modest proportion of apoptotic cells positive for active caspase 3 in rat hearts subjected to ischemia—reperfusion with ($1.8 \pm 0.09\%$) or without ($2.1 \pm 0.12\%$) rhEpo treatment [18]. Interestingly, activation of the caspase 3 in their study was mainly restricted to the endothelial cells and fibroblasts. This finding is further supported by Scarabelli and co-workers reporting that apoptosis of endothelial cells precedes cardiomyocyte cell apoptosis in ischemia/reperfusion injury [20]. Parsa and co-workers suggested a potential role of cardiac fibroblasts in modulating cardioprotective effects of Epo in hearts subjected to ischemia—reperfusion [14].

Edema and Na^+ accumulation in the myocardial tissue we have observed (Fig. 3) as well as delayed inflammatory response are also not consistent with the concept of apoptosis as a dominating course of I/R injury. Histological examination revealed extensive myocardial lesions where cell membranes lacked integrity, which became detectible just 30 min after the onset of perfusion when cardiac TnTwas detected in plasma of recipient animals (Fig. 1). Taken together these findings suggest that most of the myocardial damage represented oncotic necrosis.

Our data furthermore indicate that intravenous rhEpo administration would fail to suppress apoptosis in the myocardium even if the latter were occurring for one single reason: rhEpo does not cross the endothelial barrier and hence does not reach the myocardium at least during the first hours of reperfusion (Fig. 6). Thus, data obtained for interaction of the cytokine with isolated cardiomyocytes [15] cannot be compared with those obtained using ex vivo or in vivo models. The target for rhEpo applied intravenously is primarily the endothelium of coronary vessels. Upon binding to the endothelial cells occurring instantaneously (Fig. 6) rhEpo causes activation of the NO production (Fig. 5) and most likely other factors such as endothelin 1 [17] that are mediating cardioprotective effects observed by us and others.

Among the final targets of the rhEpo are reduction of mechanical overload monitored as release of the stress factors ANP and BNP, abrogation of the reperfusion-induced oxidative stress (Fig. 4) and accelerated recovery of the ion and water balance in the myocardial tissue (Fig. 3). It is unlikely that the observed multiple effects are caused by activation of a single signaling pathway. Detailed investigation of involved molecular events in potential signaling pathways is far beyond the scope of this study. Similar suppression of the ANP and BNP release upon reperfusion following ischemia in rhEpo-treated animal models was reported by others [17,19]. Antioxidative effect of rhEpo was also shown in different tissues and experimental settings [3].

We suggest that activation of the NO production in the endothelial cells is at least in part responsible for the lack of GSH depletion in myocardial tissue during reperfusion. There is long-standing debate as to whether NO plays a beneficial or detrimental role in ischemia—reperfusion injury. It seems tempting to extrapolate the results of in vitro experiments using cell cultures to the in vivo pathogenic conditions [21]. Redox environment is the factor decisive for both NO bioavailability and the end products formed which may include both nitrite, nitrate, S- and N-nitrosylation adducts and nitrotyrosine. It has been shown that lower levels of NO production and diffusion into the tissue are associated with its antioxidative properties [22]. The redox environment can in turn modulate the NO function and potentiate its cardioprotective action. This multifaceted action of NO defines its narrow therapeutic safety window for NO in ischemia—reperfusion pathophysiology [22]. In the present

study, administration of rhEpo was followed by increased phosphorylation and activation of eNOS with subsequent enhanced production of NO during the early phase of reperfusion. Upregulation of NO production was associated with reduction of oxidative stress and consequently of myocardial injury. This is in accordance with previous report of Bullard and co-workers who showed that the cardioprotective effect of Epo was associated with a two-fold increase in phosphorylated eNOS [13].

Along with its role as a scavenger of superoxide anions NO has been shown to modulate numerous processes including ion transport [23]. In our study, reduction in myocardial injury was also accompanied by a decrease in tissue edema and Na^+ accumulation. We demonstrated that activation of the NO production triggered by rhEpo treatment of neonatal rat cardiomyocytes (our observations as well as [13,17,21]) modulated Na/K ATPase activity. This is the first report on the role of NOS in regulation of the active K^+ transport in cardiomyocytes. However, our recent study revealed similar coupling between NOS-mediated NO production and activity of the Na/K ATPase in cerebellar granule cells [23]. Along with activation of the Na/K ATPase reduction of the passive K^+ fluxes is observed in the cardiomyocytes where NO generation is induced by rhEpo furthermore facilitating restoration of the transmembrane ion gradients (Fig. 7).

There are limitations in the present study related to its design. Focusing mainly on investigating the mechanisms of Epo-induced cardioprotection, we chose a fixed dose at a fixed time of administration of rhEpo. However, the dose of 5000 U/kg rhEpo used in our study has been shown to confer significant cardioprotection against ischemia—reperfusion [12]. After demonstrating the cardioprotection conferred by rhEpo, we limited our study to the potential role of NO alone. We cannot exclude that other local or systemic factors modulated by rhEpo such as endothelin 1, vascular endothelial growth factor and transforming growth factor may play a significant role in the acute and late cardioprotection induced by rhEpo [3,17]. The involvement of other secondary effectors in the cardioprotection offered by rhEpo warrants further investigations. Moreover, our rodent model does not allow to assess clinical relevance of the obtained results but gives insight for further characterization of mechanisms of potential cardioprotection offered by Epo in human settings.

In summary, intravenous administration of rhEpo after the onset of ischemia but prior to reperfusion protects the heart against cold global I/R. Apoptosis does not seem to play a major role in the process of tissue injury in this model. After binding to the coronary endothelium, rhEpo enhances NO production by phosphorylation and thus activation of endothelial nitric oxide synthase in coronary vessels. Our data suggest that cardioprotective properties of rhEpo are at least partially mediated by nitric oxide released by the coronary endothelium.

Acknowledgment

The authors acknowledge the excellent technical assistance of Mrs Vlasta Strohmeier.

References

[1] Murphy E, Steenbergen C. Mechanisms underlying acute protection from cardiac ischemia—reperfusion injury. Physiol Rev 2008;88:581—609.
[2] Bolli R, Becker L, Gross G, Mentzer Jr RM, Balshaw D, Lathrop DA. Myocardial protection at a crossroads. The need for translation into clinical therapy. Circ Res 2004;95:125—34.
[3] Arcasoy MO. The non-haematopoietic biological effects of erythropoietin. Br J Haematol 2008;141:14—31.
[4] Junk AK, Mammis A, Saviz SI, Singh M, Roth S, Malhotra S, Rosenbaum PS, Cerami A, Brines M, Rosenbaum DM. Erythropoietin administration protects retinal neurons from acute ischemia—reperfusion injury. Proc Natl Acad Sci U S A 2002;99:10659—64.
[5] Wang Y, Zhang ZG, Rhodes K, Renzi M, Zhang RL, Kapke A, Lu M, Pool C, Heavner G, Chopp M. Post-ischemic treatment with erythropoietin or carbamylated erythropoietin reduces infarction and improves neurological outcome in a rat model of focal cerebral ischemia. Br J Pharmacol 2007;151:1377—84.
[6] Wright GL, Hanlon P, Amin K, Steenbergen C, Murphy E, Arcasoy MO. Erythropoietin receptor expression in adult rat cardiomyocytes is associated with an acute cardioprotective effect for recombinant erythropoietin during ischemia—reperfusion injury. FASEB J 2004;18:1031—3.
[7] Ono K, Lindsey ES. Improved technique of heart transplantation in rats. J Thorac Cardiovasc Surg 1969;57:225—9.
[8] Bombeli T, Schwartz BR, Harlan JM. Endothelial cells undergoing apoptosis become proadhesive for nonactivated platelets. Blood 1999;93:3831—8.
[9] Tietze F. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. Anal Biochem 1969;27:502—22.
[10] Schafer BW, Perriard JC. Intracellular targeting of isoproteins in muscle cytoarchitecture. J Cell Biol 1988;106:1161—70.
[11] Tavakoli R, Gazdhar A, Pierog J, Bogdanova A, Gugger M, Pringle IA, Gill DR, Hyde SC, Genoni M, Schmid RA. Electroporation-mediated interleukin-10 overexpression in skeletal muscle reduces acute rejection in rat cardiac allografts. J Gene Med 2006;8:242—8.
[12] Moon C, Krawczyk M, Paik D, Lakatta EG, Talan MI. Cardioprotection by recombinant human erythropoietin following acute experimental myocardial infarction: dose response and therapeutic window. Cardiovasc Drugs Ther 2005;19:243—50.
[13] Bullard AJ, Govewalla P, Yellow DM. Erythropoietin protects the myocardium against reperfusion injury in vitro an in vivo. Basic Res Cardiol 2005;100:397—403.
[14] Parsa CJ, Kim J, Riel RU, Pascal LS, Walton GB, Thompson RB, Petrofski JA, Matsumoto A, Stamler J, Koch WJ. Cardioprotective effects of erythropoietin in the reperfused ischemic heart. A potential role for cardiac fibroblasts. J Biol Chem 2004;279:20655—62.
[15] Tramontano AF, Muniyappa R, Black AD, Blendea MC, Cohen I, Deng L, Sowers JR, Cutaia MV, El-Sherif N. Erythropoietin protects cardiac myocytes from hypoxia-induced apoptosis through an Akt-dependent pathway. Biochem Biophys Res Commun 2003;308:990—4.
[16] Hanlon PR, Fu P, Wright GL, Steenbergen C, Arcasoy MO, Murphy E. Mechanisms of erythropoietin-mediated cardioprotection during ischemia—reperfusion injury: role of protein kinase C and phosphatidylinositol-3-kinase signalling. FASEB J 2005;19:1323—5.
[17] Piuhola J, Kerkela R, Keenan JI, Hampton MB, Richards AM, Pemberton CJ. Direct cardiac actions of erythropoietin (EPO): effects on cardiac contractility. BNP secretion and ischaemia/reperfusion injury. Clin Sci (Lond) 2008;114:293—304.
[18] van der Meer P, Lipsic E, Henning RH, de Boer RA, Suurmeijer AJH, van Veldhuisen DJ, van Gilst WH. Erythropoietin improves left ventricular function and coronary flow in an experimental model of ischemia—reperfusion injury. Eur J Heart Fail 2004;6:853—9.
[19] van der Meer P, Lipsic E, Henning RH, Boddeus K, van der Velden J, Voors AA, van Veldhuisen DJ, van Gilst WH, Schoemaker RG. Erythropoietin induces neovascularization and improves cardiac function in rats with heart failure after myocardial infarction. J Am Coll Cardiol 2005;46:125—33.
[20] Scarabelli T, Stephanou A, Rayment N, Pasini E, Comini L, Curello S, Ferrari R, Knight R, Latchman D. Apoptosis of endothelial cells precedes myocyte cell apoptosis in ischemia/reperfusion injury. Circulation 2001;104:253—6.
[21] Burger D, Lei M, Geoghegan-Morphet N, Lu X, Xenocostas A, Feng Q. Erythropoietin protects cardiomyocytes from apoptosis via up-regulation of endothelial nitric oxide synthase. Cardiovasc Res 2006;72:51—9.

[22] Pacher P, Beckman JS, Liaudet L. Nitric oxide and peroxynitrite in health and disease. Physiol Rev 2007;87:315—424.
[23] Petrushanko IY, Bogdanov NB, Lapina N, Boldyrev AA, Gassmann M, Bogdanova AY. Oxygen-induced regulation of Na/K ATPase in cerebellar granule cells. J Gen Physiol 2007;130:389—98.

Appendix A. Conference discussion

Dr T. Wahlers (*Cologne, Germany*): Yesterday in the Young Investigators Award, the action of Epo on stem cells was reported and they used in rats a dose of 3000 units/kg. You used 5000. Can you give us your rationale using this high dose of Epo and transfer this perhaps to the human side.

Dr Tavakoli: I think one should make a difference between the cell culture model or in vivo model. Besides, the aim of our study was just to prove the cardioprotective potential of Epo and, as I mentioned, get insights into its mechanism. We chose this dose and also the time of administration based on previous studies, again, in an in vivo regional model where the authors have shown that 5000 units/kg is efficient [ref. 19 in the manuscript]. I think in the acute setting of this study the proliferative properties of Epo wouldn't be a major problem.

Dr J. Vaage (*Oslo, Norway*): I think you have a very interesting model by transplanting the hearts back to the animals. I have two questions for you.

You showed that the erythropoietin binds to the endothelium, but you gave it only very briefly before taking out the heart. I think it was 20 min.

Dr Tavakoli: Right.

Dr Vaage: Do you have any data or any information that if you keep it, let's say, 6 h or 24 h, before you could find it located in other places like, for instance, the cardiomyocytes. That's my first question.

My second is more comment. You claim that the effect is partly mediated by endothelial NO. I actually refute that, because you just showed that it is upregulated, which is not in any way an indication that it is the mediator. In order to show that you need either pharmacological blocking or using mice with knockout NO.

Dr Tavakoli: First, your first question about the timing of the application, as I mentioned, this was not a study to investigate the optimal timing of Epo injection. The curve I've shown you indicates that the half-life of Epo is about 2 h. This information was obtained after we started the study, so we did not change the timing and the doses of Epo administration.

Regarding your second input, you know that NO itself is an antioxidative molecule, so this is the reason I suggest that NO is related to the antioxidative and cardioprotective effect of Epo in this model. Therefore, it's one explanation for my assertion that NO is involved in this cardioprotection and antioxidative action of Epo.

Dr Vaage: If I may comment on it. There is, let's say, scientific background for making that suggestion, but I think we must make it very clear at this stage of your investigation it's only a suggestion.

Dr Tavakoli: Correct. It's a suggestion. To be able to say our data indicate that NO is related, we should have put an inhibitor of eNOS and shown that it could abolish the effect of EPO. Hence, it warrants further investigation. I think the best way to say it indicates is that you block the effect of EPO with inhibitors of eNOS.