

Erythropoietin protects the systolic function of neonatal hearts against ischaemia/reperfusion injury

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Abstract

OBJECTIVES: The effect of erythropoietin (EPO) on neonatal hearts is not well understood. The current hypothesis is that EPO has protective effects against ischaemia-reperfusion when administered prior to ischaemia induction.

METHODS: Systolic and diastolic indices, as well as the Akt and extracellular-regulated kinase (Erk) signalling pathways, were studied *in vivo* using a neonatal pig heart model. Regional ischaemia was induced for 45 min by the ligation of the left anterior descending artery, followed by 90 min of reperfusion. The treatment groups consisted of: (i) untreated controls, (ii) treatment with EPO 3 min prior to ischaemia and (iii) treatment with EPO 24 h before ischaemia. Sophisticated myocardial contractility indices were assessed by pressure/volume loops of the left ventricle. The Akt and Erk pathways were evaluated via a western blot.

RESULTS: Elastance was found to be higher in the group receiving EPO 3 min prior to ischaemia. In addition, preload recruitable stroke work was higher for both groups receiving EPO prior to ischaemia when compared with controls. The time constant of the isovolumic relaxation and end-diastolic pressure-volume relationship did not differ between the three groups after 90 min of reperfusion. Furthermore, EPO treatment enhanced phosphorylation of Akt, but not Erk, and EPO-treated animals showed lower levels of apoptosis-related proteins.

CONCLUSIONS: EPO had a protective effect on neonatal systolic function after ischaemia/reperfusion injury, but no effect on diastolic function. This cardioprotective effect might be mediated by the activation of the Akt pathway.

Keywords: Ischaemia/reperfusion • Cell signalling receptors • Myocardial protection • Molecular biology • Myocardial mechanics

INTRODUCTION

Despite major advances in the treatment of congenital heart defects and improvements in myocardial protection, any additional protection against ischaemia/reperfusion during surgery is valuable. Therefore, the development of new drugs and/or strategies that may lessen the deleterious effects of ischaemia and reperfusion during surgery is necessary [1, 2].

Erythropoietin (EPO) is a member of the cytokine superfamily and was originally described for its critical role in promoting erythrocyte survival and differentiation [3]. More recently, the expression of EPO and its receptor has been described in other organs and tissues, including the brain and heart [4]. Previous studies have shown a direct protective effect of EPO on cardiomyocytes after ischaemia and reperfusion [5], as well as reduced infarct size and attenuated left ventricular dysfunction after permanent coronary ligation [6]. In addition, EPO treatment was found to result in a better left ventricle (LV) pressure recovery

after myocardial ischaemia and reperfusion in isolated hearts of adult rats [7]. The protective effects of EPO in the mature myocardium have been attributed to the activation of survival kinases, including phosphatidylinositol 3-OH kinase-cellular Akt/protein kinase B (PI3K-Akt) and extracellular-regulated kinase (Erk), which have been deemed part of the reperfusion injury salvage kinase (RISK) pathway [8].

Moreover, the favourable effects of EPO have also been demonstrated when it is administered 1 min prior to the injury onset, 24 h after the ischaemia/reperfusion insult and 24 h previous to the insult [9–11].

Despite the demonstrated protective effect of EPO on the adult myocardium after the ischaemia and reperfusion insult [12, 13], the role of EPO and its signalling pathways in the resistance to myocardial ischaemia in the neonatal myocardium is unknown.

Therefore, the aim of our present study was to evaluate the effect of EPO on the RISK pathway and to determine its role in cardioprotection.

MATERIALS AND METHODS

Animal model and heart instrumentation

After approval by the local ethics committee, experiments were carried out using Landrace Large White male piglets weighing 1–2 kg (aged 1–2 days old). All experimental protocols were in accordance with the standards of the Brazilian Council in Animal Experimentation (COBEA), and the 'Guide for Care and Use of Laboratory Animals' published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

The animals were anesthetized with ketamine 20 mg/kg and thiopental 25 mg/kg, mechanically ventilated, and a median sternotomy was performed.

A high-fidelity pressure catheter (Millar Instruments, Houston, TX, USA) was placed in the LV cavity. Four 2-mm piezoelectric crystals (Sonometrics Corporation, Ontario, CA) were placed in the epicardium at the anterior, posterior, base and apex of the LV. The distances between all four crystals on the two axes of the LV were measured by sonomicrometry. A vessel loop was placed around the inferior vena cava and gently snared for 10 s at each time point to simulate changes in ventricular loading to generate pressure/volume loops for a complex myocardial parameter analysis. Animals received 10 ml/kg/h of saline solution for hydration and fluid balance throughout the protocol. Arterial blood was collected for blood gas measurement immediately on gaining arterial access, at the beginning of regional ischaemia, and at 30, 60 and 90 min of regional myocardial reperfusion.

Haemodynamics were continuously recorded using a Sonolab data acquisition system (Sonometrics), and complex myocardial parameters were analysed using Cardiosoft analysis software (Sonometrics).

The pressure/volume loops generated during preload reduction by transient vena cava occlusion allowed the analysis of cardiac contractility indices independent of heart rate and afterload. Ventricular maximum and minimum changes in LV pressure over time ($\pm dP/dt$), preload recruitable stroke work (PRSW), left ventricular time constant of isovolumic relaxation (TAU) and the end-diastolic pressure-volume relationship (EDPVR) were measured.

Myocardial ischaemia/reperfusion and EPO administration

After the initial monitoring, heparin was administered (200 UI/kg) and baseline measurements of cardiac function were taken. Heparin was administered with the intention of avoiding arterial thrombosis or clot embolization during implementation of the protocol. Human recombinant EPO was acquired from Janssen-Cilag (São José dos Campos, SP, Brazil).

All animals were randomly assigned into three groups before the start of the study as described below.

One group received saline solution 3 min prior to myocardial ischaemia (control group; $n = 10$ animals), the second group received 1000 IU/kg of EPO 3 min prior to myocardial ischaemia (EPO3 group; $n = 10$ animals) and the third group received 1000 IU/kg of EPO 24 h prior to myocardial ischaemia (EPO24 group; $n = 10$ animals).

After EPO administration, regional ischaemia was achieved by snaring the left anterior descending artery (LAD) with a 6/0

suture. Ischaemia was confirmed by a visual assessment of cyanosis and dyskinesia of the myocardium supplied by the LAD. The ischaemic time was 45 min, followed by 90 min of reperfusion and observation. Haemodynamic measures were done at 45 min of ischaemia; and at 30, 60 and 90 min of reperfusion.

Western blot analyses and tissue collection

At the end of reperfusion, the piglets were euthanized, hearts were harvested and a segment of the apex of the LV, which was the subject of ischaemia and reperfusion injury, was flash-frozen in liquid nitrogen and stored at -80°C until biochemical analysis. Samples of LV were centrifuged and the soluble fraction was resuspended in Laemmli loading buffer before separation on 8% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. Proteins were transferred from gels to nitrocellulose membranes and incubated with primary antibodies. Secondary antibodies used included alkaline phosphatase-conjugated goat anti-rabbit or anti-mouse immunoglobulin G. Proteins were visualized with a chemiluminescent detection system according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Immunoblots were also incubated with antibodies to β -actin for normalization. The biochemical analyses were done via western blotting with the following antibodies: anti-Erk (SC 93), janus kinase 2 (Jak) (SC 278), signal transducer and activator of transcription 3 (Stat3) (SC 483), endothelial nitric oxide synthase (eNOS) (SC 654), B-cell lymphoma 2 (Bcl-2) (SC 492) Bcl-2-associated death promoter (Bad) (SC 943), Bcl-2-associated X protein (Bax) (SC 493), caspase 8 (SC 5263), phospho-Erk (p-Erk) (SC 7363) and phospho-Stat3 (p-Stat3) (SC 8001) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against Akt (Cell 9272), β -actin (Cell 4967) and phospho-Akt (p-Akt) (Cell 9271) were obtained from Cell Signaling (Danvers, MA, USA). SDS-PAGE reagents were purchased from Bio-Rad (Richmond, CA, USA).

Statistical analyses

All values were expressed as means \pm SD. The functional data were analysed by using the two-way analysis of variance with Bonferroni for multiple comparisons *post hoc* test analyses with GraphPad Prism version 5.0 software for Mac (GraphPad Software, San Diego, CA, USA). Western blot analyses were compared with a control group using the analysis of variance. Data are expressed as means \pm SE. A P -value <0.05 was considered statistically significant.

RESULTS

The myocardial contractility indices were derived from 10 animals from each group and the protein blot analyses were performed on six animals from each group.

Myocardial contractility indices

The EPO effect on myocardial contractility was reflected by higher PRSW values in the EPO3 and EPO24 groups from 45 min of ischaemia up to 90 min of reperfusion when compared with

controls (Fig. 1A). In addition, both EPO treatment regimens resulted in a higher maximum dP/dt values compared with controls (Fig. 1B). Diastolic indices such as minimum dP/dt, EDPVR and TAU were comparable among the three groups (Fig. 1C-E).

Activation of Akt, Erk, Jak and Stat3

At the end of the reperfusion period, we observed an increase in Akt phosphorylation in the EPO3 group compared with the control and EPO24 groups (Fig. 2A). Neither EPO treatment paradigm resulted in increased Erk, Jak or Stat3 phosphorylation (Fig. 2B-D).

Activation of Bad, Bax, Bcl-2 and caspase 8

The administration of EPO 3 min before ischaemia resulted in increased levels of Bax and Bcl-2 proteins compared with control and EPO 24 animals (Fig. 3B and C). Bad levels were not affected by EPO administration. Caspase 8 levels were markedly decreased in the EPO3 and EPO24 groups compared with

controls (Fig. 3A). Finally, eNOS expression levels were higher in the EPO3 group compared with the EPO24 and control groups.

COMMENT

In this study, we investigated the protective effect of EPO in the myocardial function of neonatal hearts after ischaemia and reperfusion injury, as well as the underlying mechanisms of this effect. The neonatal myocardium is more immature compared with the infant and adult myocardia in terms of contractility proteins and Frank-Starling mechanism [14]. Further, often the neonatal heart is exposed, as a result of an underlying congenital heart disease, to variable degrees of cyanosis [10].

We found that EPO had a cardioprotective effect on the preservation of the systolic indices of the LV after ischaemia-reperfusion injury. However, EPO did not have protective effects on the diastolic indices after the reperfusion period. In addition, the Akt pathway was activated when EPO was administered 3 min before ischaemia, but not when EPO was administered 24 h before ischaemia. When we analysed the downstream signalling pathway,

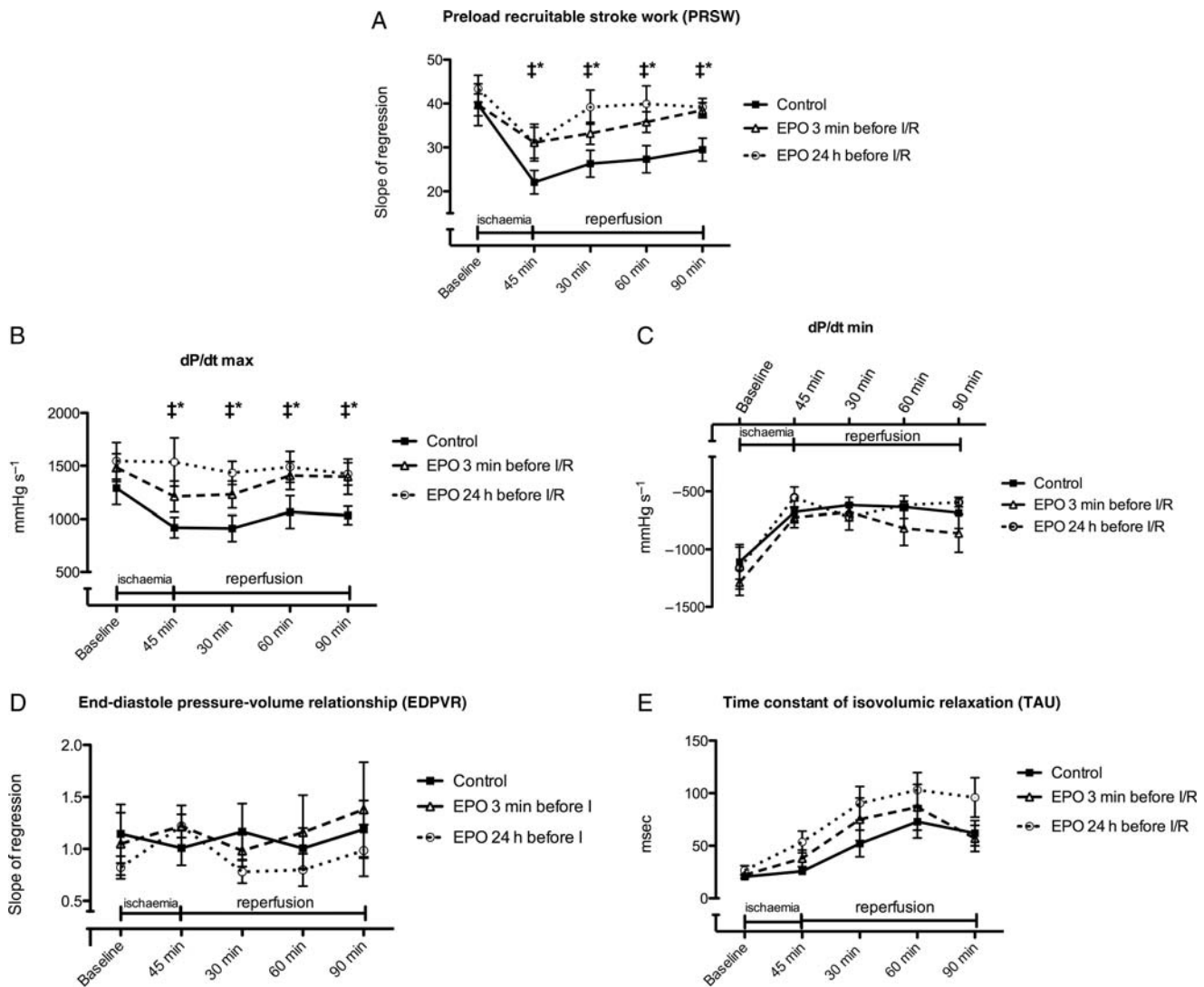


Figure 1: Myocardial contractility indices at baseline, 45 min of ischaemia, and during the reperfusion period. * $P < 0.05$ comparing EPO 24 h before I/R group vs. control group using Bonferroni *post hoc* test. † $P < 0.05$ comparing EPO 3 min before I/R vs. controls using Bonferroni *post hoc* test ($n = 10$ animals per group).

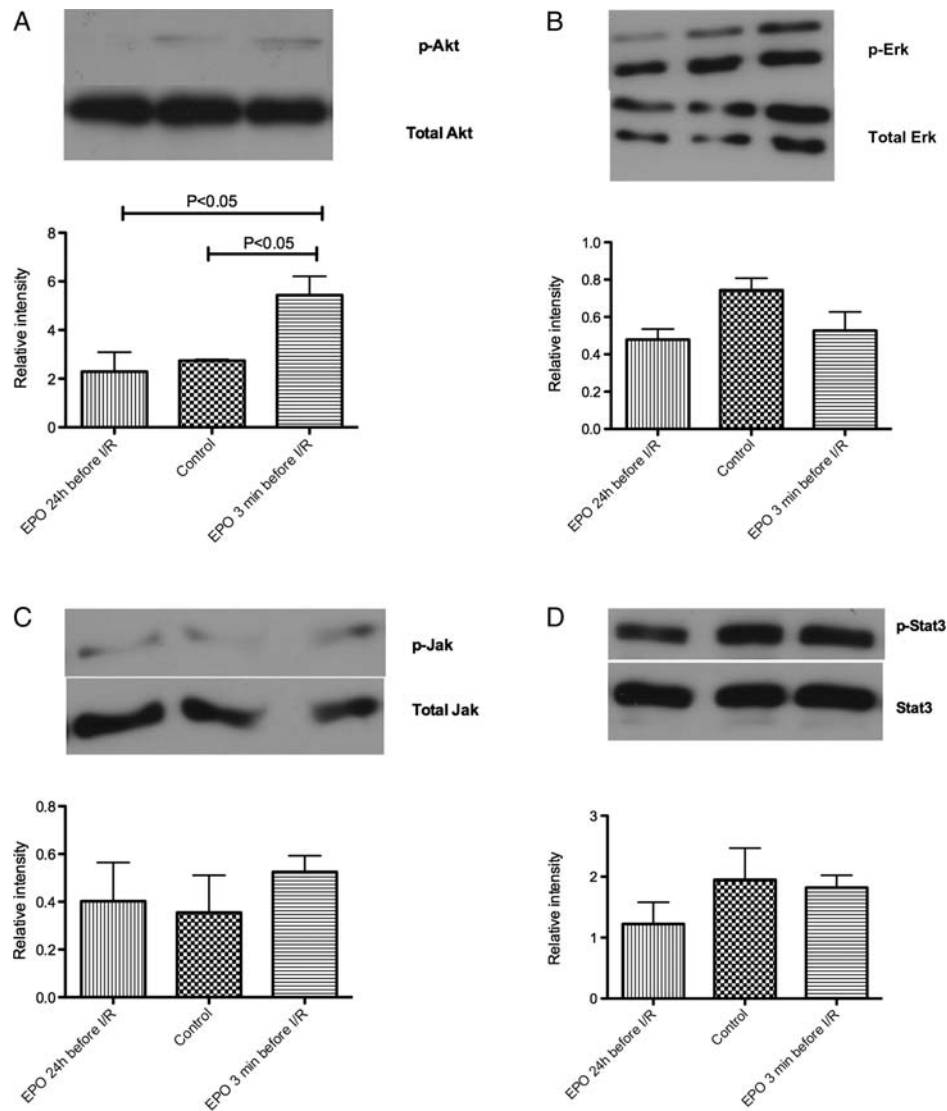


Figure 2: Akt, Erk, Jak and Stat3 western blots after 45 min of regional ischaemia and 90 min of reperfusion. EPO administration 3 min before ischaemia stimulated phosphorylation of Akt, but did not activate Erk, Jak or Stat3. EPO administration 24 h before ischaemia did not activate Akt, Erk, Jak or Stat3. Representative western blots of (A) Akt, (B) Erk, (C) Jak and (D) Stat3 after 90 min of reperfusion ($n = 6$ animals per group).

Bcl-2 and Bax were activated when EPO was administered 3 min prior to ischaemia-reperfusion. This resulted in a reduced expression of caspase 8 in the EPO3 group. When EPO was administered 3 min before the ischaemia-reperfusion injury, eNOS expression was elevated. However, EPO administration 24 h prior to ischaemia-reperfusion had no effect on Bcl-2/Bax expression, though this treatment paradigm did result in a decreased caspase 8 expression. These findings suggest that EPO might be used as an additional protective strategy against neonatal ischaemia and reperfusion during cardiac surgeries or interventional procedures where the myocardium is at risk of ischaemia. Indeed, the protection granted by EPO against ischaemia is comparable to that achieved by ischaemic preconditioning as demonstrated by Nishihara *et al.* [15].

Systolic and diastolic myocardial indices

Several studies have demonstrated the cardioprotective effect of EPO in preserving the systolic function, when it is administered

at the time of ischaemia induction [16, 17]. In addition, EPO has been shown to prevent negative LV remodelling in chronically ischaemic adult animal models [18, 19]. However, previous studies used a mature myocardium, whereas in the present study an immature myocardium was used as a model.

EPO has been described as a potential drug for haemodynamic improvement [10, 20, 21], but to our knowledge few papers have investigated the effect of EPO in infant hearts, and no studies have investigated its effect on neonatal hearts [10, 20]. In addition, the contractility indices used in previous papers, such as maximum dP/dt and recovery of developed LV pressure, were less sensitive compared with those used in the present manuscript. We found that contractility performance was improved with EPO administration 3 min and 24 h prior to ischaemia-reperfusion injury. We observed an increase in the PRSW, which is a very sensitive indicator of contractility function that is not affected by heart rate or preload [22, 23], as well as a higher maximum dP/dt, in the EPO3 and EPO24 groups compared with controls. These two contractility indices were positively affected by EPO after a myocardium reperfusion, with the inotropic

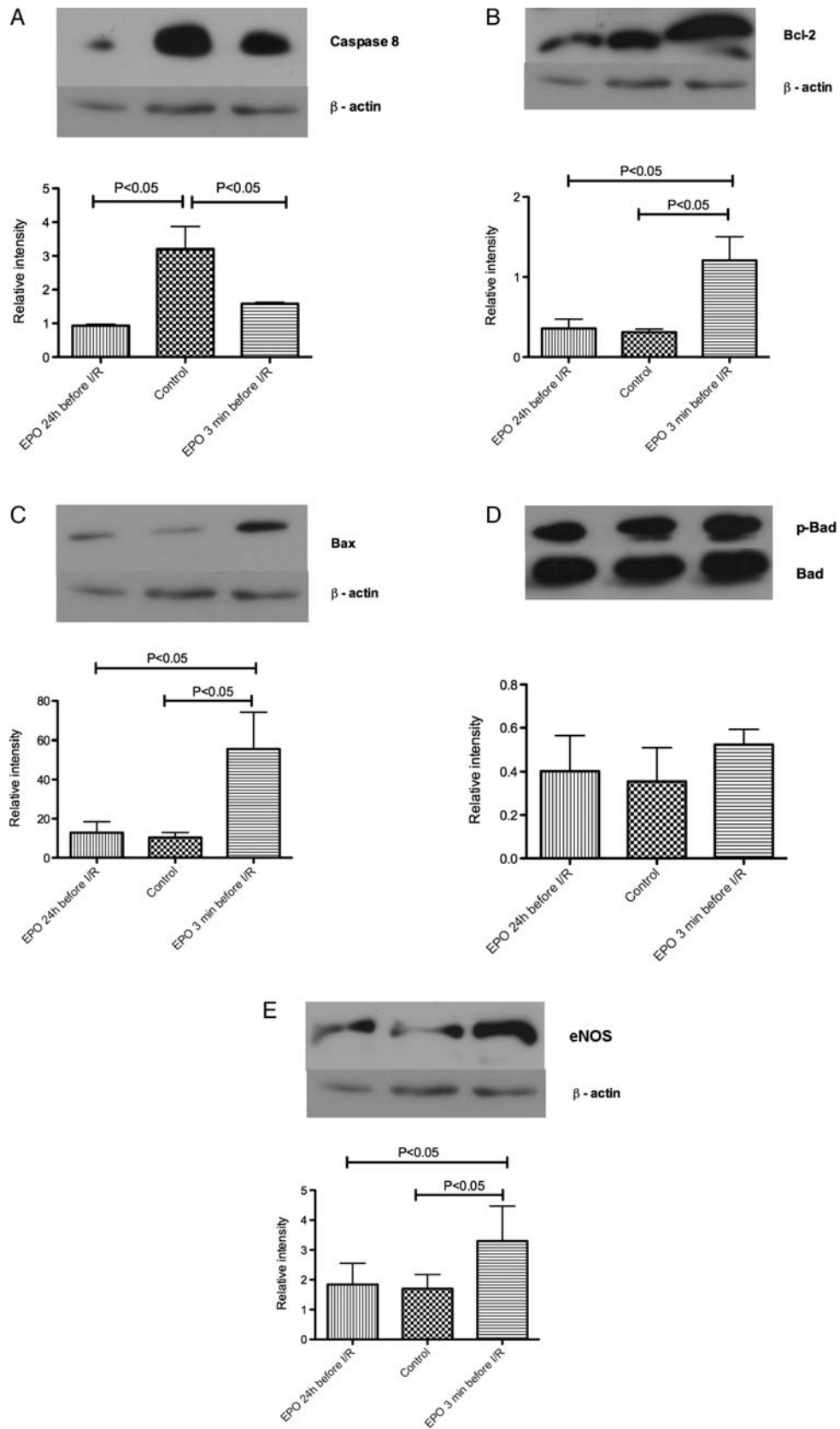


Figure 3: The EPO administration 3 min before myocardial ischaemia results in activation of Bcl-2, Bax and eNOS. The balance of Bcl-2 and Bax activation after EPO administration 3 min before myocardial ischaemia resulted in reduced caspase 8 levels compared with controls. EPO administration 24 h before ischaemia did not activate the Bcl-x protein family, but did result in reduced caspase 8 levels compared with controls. Representative western blots for caspase 8 (A), Bcl-2 (B), Bax (C), Bad (D) and eNOS (E) ($n = 6$ animals per group).

function returning to baseline values in both groups that received EPO in two different regimens of administration.

Previous work has shown an improvement in recovery of LV-developed pressure when EPO was administered 15 or 30 min prior to ischaemia-reperfusion injury in infant rabbit hearts [10]. A separate study observed an increased recovery of the developed pressure of the LV after EPO treatment using Langendorff-perfused adult rat hearts [7]. In the present paper, a relevant model of regional ischaemia-reperfusion using neonatal hearts and sophisticated contractility indices showed systolic function preservation after ischaemia-reperfusion injury with EPO administration similar to previous studies. However, in previous work, the diastolic function after ischaemia and reperfusion injury was not evaluated, and EPO has not been evaluated for cardio-protective effects on the diastolic function of hearts undergoing ischaemia and reperfusion injury. Diastolic function is important for neonatal hearts, as they function near the peak of the Frank-Starling curves. Therefore, any additional increase in arterial pressure or diastolic filling pressure may result in a decrease of stroke work and cardiac output [14]. In the present paper, we used sophisticated diastolic indices such as EDPVR and TAU [17, 20]. TAU is a reliable lusitropism index that is independent of changes in LV loading pressure or the Frank-Starling effect [24]. Both indices were similar in all three groups. To our knowledge, these findings are the first of their kind to be reported.

Cell survival pathways

We investigated the RISK pathway, a term given to describe a group of survival protein kinases including Akt and Erk 1/2 that confer powerful cardioprotection [8]. Downstream activation of the RISK pathway inhibits caspase activation, hence inhibiting cellular apoptosis [8].

In this study, the Akt pathway was activated by EPO, but no activation of the Jak/Stat or Erk pathways was observed. A previous study utilized an isolated infant heart preparation that included perfusion for 30 min prior to perfusion with EPO (1.0 U/ml) for 5 or 15 min, and demonstrated an activation of the PI3K/Akt, Jak/Stat, Erk and protein kinase C pathways [20]. In addition, EPO was used in the perfusate of a Langendorff apparatus for 5 or 15 min in the previous study, whereas EPO was administered intravenously in our experiments. Therefore, the differences between the two studies in the signalling pathways activated may be due to the different ages of heart tissue used and/or the method of EPO delivery.

In addition, the administration of EPO 24 h prior to ischaemia-reperfusion insult has been shown to result in increased Akt activity using an isolated perfused heart model in adult rats [7]. We obtained similar results in the group that received EPO 3 min prior to ischaemia-reperfusion injury, but did not observe the same effect when EPO was administered 24 h prior to the insult. Also, reduced caspase 3 expression was observed previously in hearts that received EPO 24 h prior to the ischaemia-reperfusion injury [7], while we observed lower expression of caspase 8 in both EPO-treated groups. These differences might be due to the immaturity of the neonatal hearts used in the present paper.

We investigated the effect of EPO on the upstream components of the apoptosis pathway and observed increased levels of Bcl-2 and Bax expression in the group that received EPO 3 min prior to ischaemia-reperfusion, but no effect on the expression

of these proteins when EPO was delivered 24 h prior to the insult. The Bcl-2 family of proteins are involved in the response to apoptosis. Some members of this family, such as Bcl-2 and Bcl-XL, are anti-apoptotic, while others such as Bad, Bax and Bid are pro-apoptotic. The sensitivity of cells to apoptotic stimuli can depend on the balance of pro- and anti-apoptotic Bcl-2 proteins. For instance, when there is an excess of pro-apoptotic proteins, cells are more sensitive to apoptosis, but when anti-apoptotic proteins are present in excess, cells tend to be resistant to apoptosis [8]. As we found reduced caspase 8 expression regardless of the timing of EPO administration, but no effect on Bcl-2 and Bax expression with EPO administration 24 h prior to insult, these findings suggest that different pathways act to reduce caspase 8 expression when EPO is administered at 3 min vs. 24 h prior to ischaemia-reperfusion injury.

We observed an increase in eNOS expression in the group that received EPO 3 min before ischaemia-reperfusion, which might be responsible for the protective effect on systolic activity observed in the EPO3 group. eNOS is a downstream target of the RISK pathway, which can potentially inhibit mitochondrial permeability transition pore channel opening to promote cell survival after reperfusion [8]. The upregulation of eNOS by EPO has been shown previously in mature myocardium, but not in neonatal hearts [25].

STUDY LIMITATIONS

The model is relevant to acute ischaemia/reperfusion studies and the findings might not be similar to those on patients who underwent cardioplegic arrest. Regional ischaemia is different from cardioplegia-induced global ischaemia associated with cardiac surgery. Nevertheless, similar cellular protective pathways are activated both in the present model and in hearts under cardioplegic arrest [2, 8, 10].

Serum markers of myocardial damage such as troponin or creatine kinase were not measured for intergroup comparison. However, we dissected the RISK pathway, which is an important cell survival pathway against ischaemia-reperfusion injury. Based on previous papers, we used a dose of 1000 IU/kg [6, 9, 20], which may have been too low to confer protective effects on the diastolic function.

CONCLUSION

In summary, we found that at a dose of 1000 IU/kg EPO treatment improved indices of contractility, but did not improve lusitropism function in the neonatal heart. In the neonatal heart, the RISK pathway is partially activated by Akt, but is not activated by Erk. These results suggest that EPO use prior to an ischaemic cardiac insult, such as neonatal cardiac surgery, could have a protective effect in neonates or infants.

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Conflict of interest: none declared.

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