Transplant International ISSN 0934-0874

ORIGINAL ARTICLE

Recombinant human erythropoietin protects the liver from hepatic ischemia-reperfusion injury in the rat

Bruno Sepodes,[1] Rui Maio,[2] Rui Pinto,[1] Edward Sharples,[3] Pedro Oliveira,[4] Michelle McDonald,[3] Muhammad Yaqoob,[3] Christoph Thiemermann[3] and Helder Mota-Filipe[1]

1 Faculty of Pharmacy, Unit of Pharmacology and Pharmacotoxicology, University of Lisbon, Av. Forças Armadas, Lisbon, Portugal
2 Unit of Transplantation/Surgery III, Hospital de Santa Maria, Lisbon, Portugal
3 The William Harvey Research Institute, St Bartholomew's and The Royal London School of Medicine and Dentistry, Charterhouse Square, London, UK
4 Unit of Anatomical Pathology, Portuguese Oncology Institute, Lisbon, Portugal

Keywords
apoptosis, erythropoietin, lipid peroxidation, liver, rat, reperfusion injury.

Correspondence
Helder Mota-Filipe PhD, Faculty of Pharmacy, Unit of Pharmacology and Pharmacotoxicology, University of Lisbon, Avenida das Forças Armadas, 1649-019 Lisboa, Portugal. Tel.: +351 21 794 64 22; fax: +351 21 795 74 58; e-mail: hfilipe@ff.ul.pt

Received: 27 December 2005
Revision requested: 24 January 2006
Accepted: 9 June 2006

doi:10.1111/j.1432-2277.2006.00366.x

Summary

Recently, erythropoietin was shown to have both hematopoietic as well as tissue-protective properties. Erythropoietin (EPO) had a protective effect in animal models of cerebral ischemia, mechanical trauma of the nervous system, myocardial infarction, and ischemia-reperfusion (I/R) injury of the kidney. It is not known whether EPO protects the liver against I/R injury. Using a rat model of liver I/R injury, we aimed to determine the effect of the administration of human recombinant erythropoietin (rhEPO) on liver injury. Rats were subjected to 30 min of liver ischemia followed by 2 h of reperfusion. When compared with the sham-operated rats, I/R resulted in significant rises in the serum levels of aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, gamma-glutamyl transferase, tissue lipid peroxidation, caspase-3 activity and altered histology. Administration of rhEPO 5 min before ischemia was able to reduce the biochemical evidence of liver injury; however, this protection was not evident when rhEPO was administered 5 min before reperfusion. Mechanistically, early administration of rhEPO was able to reduce the oxidative stress and caspase-3 activation, suggesting the subsequent reduction of apoptosis. This study provides the first evidence that rhEPO causes a substantial reduction of the liver injury induced by I/R in the rat.

Introduction

The insult to an organ after the onset of reperfusion is a result of the interplay between the different complex mechanisms. Ischemia and reperfusion (I/R) injury is a major cause of morbidity and mortality in patients undergoing the liver surgery and transplantation [1]. Thus, hepatic I/R injury is a complex, multifactorial, pathophysiologic process that includes the action of oxygen-derived free radicals, cytokines, and endothelin [2]. Allied to this is the activation of Kupffer cells and neutrophils [3].

In its classical and originally recognized role, erythropoietin (EPO) and its receptor (EPOR) are essential for the survival, proliferation, and differentiation of erythroid progenitor cells [4]. More recently, it has been recognized that EPO and EPORs are also expressed by other tissues and organs, including the brain and heart [5–7]. Studies have demonstrated a major protective role for EPO in the brain, where locally administered recombinant human EPO (rhEPO) prevented the ischemia-induced neuronal death [6,8]. The same protection was observed after systemic administration of rhEPO, even when administered after the ischemic insult [5]. Also, rhEPO appears to be effective against a wide variety of inflammatory-derived injuries of the nervous system [5,9]. Other benefits found for rhEPO in the setting of I/R of the central nervous system include the positive modulation of tissue perfusion (markedly reducing injury) in critical situations such as

delayed ischemia after sub-arachnoid hemorrhage [10]. In addition, rhEPO significantly improved the survival of rats in a model of shock induced by splanchnic artery occlusion [11], and it also significantly reduced the development of experimental inflammatory bowel disease in the rat [12].

Poststroke myocardial benefits derived from EPO became more evident when rats were administered with rhEPO in a model of myocardial infarction with reperfusion. Administration of rhEPO daily for 7 days reduced the cardiomiocyte loss by approximately 50%, an extent sufficient to normalize hemodynamic function within 1 week after reperfusion [4].

The effect of rhEPO treatment on subsequent I/R injury in the rat kidney was also evaluated. rhEPO had a protective effect on subsequent I/R injury of the kidney, and this effect seems to be associated with induction of heat shock protein 70 (HSP70). In the setting of renal I/R, EPO reduced the DNA fragmentation and prevented the caspase-3 activation, with upregulation of Bcl-X_L and XIAP [15]. Administration of EPO before resuscitation abolished the renal dysfunction and liver injury in hemorrhagic, but not endotoxic shock, in the rat. Hemorrhagic shock (HS) also resulted in significant increases in the kidney of the activities of caspases 3, 8, and 9. This increase in caspase activity was not seen in HS rats treated with EPO [16].

Encouraged by the substantial body of evidence demonstrating the sizeable beneficial effects of EPO in several nonclinical models of I/R injury, we aimed to examine, by using a rat model of I/R of the liver, the effect of rhEPO on liver function after ischemic and subsequent reperfusion, particularly when rhEPO was administered prior to ischemia and when it was administered after ischemia and prior to reperfusion.

Materials and methods

Animals and experimental protocol

Studies were carried out using 42 male Wistar rats (Harlan Ibérica, Barcelona, Spain) weighing 250–320 g. Rats received a standard diet and water *ad libitum* and were cared for in accordance with the Institutional Animal Research Committee *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health (N.I.H. publication No. 85–23, revised 1996), as well as with the EC regulations (O.J. of E.C. L 358/1 18/12/1986). Animals were randomized and allocated into six groups as described: (i) Control Group: data from nonmanipulated animals – rats which were not subjected to any surgical procedure ($n = 6$); (ii) Sham Group: rats which were subjected to the surgical procedures described below except for liver I/R. Rats were administered saline (1 ml/kg i.v.) 5 min prior to liver I/R, and maintained under anesthesia for the duration of the experiment ($n = 9$); (iii) I/R Group: rats which were subjected to the surgical procedures described below and underwent liver ischemia for 30 min followed by reperfusion for 2 h ($n = 7$); (iv) EPO + I/R Group: rats which received rhEPO (1000 IU/kg, i.v.) 5 min prior to liver I/R ($n = 7$); (*v*) I+EPO+R Group: rats which received rhEPO (1000 IU/kg, i.v.) 5 min prior to reperfusion of the organ instead of 5 min prior to ischemia ($n = 9$); (*vi*) EPO Control Group: rats which received rhEPO (1000 IU/kg, i.v.) 5 min prior to liver I/R, and which were subjected to the surgical procedures described below except for liver I/R ($n = 4$). The dose of rhEPO was chosen based on the findings of Parsa *et al.* [17], demonstrating that a single dose of rhEPO (1.000–5.000 IU/kg) could have a profound therapeutic effect [in the setting of myocardial ischemia-reperfusion (I/R)] independent of hematocrit.

Animal surgery

All rats were anesthetized with sodium pentobarbital (Eutasil®, 60 mg/kg i.p; Sanofi Veterinária, Algés, Portugal) and anesthesia was maintained by supplementary i.p. bolii of sodium pentobarbital. As previously described [18], anesthetized rats were placed onto a thermostatically controlled heating mat (Harvard Apparatus Ltd, Kent, UK) and body temperature maintained at 37 ± 0.5 °C by means of a rectal probe attached to a homoeothermic blanket. A tracheotomy was performed to maintain airway patency and to facilitate spontaneous respiration. The right carotid artery was cannulated (PP50, I.D. 0.58 mm, Portex, Kent, UK) and connected to a pressure transducer (Senso-Nor 840, Horten, Norway) for the measurement of mean arterial blood pressure (MAP) from the pulse waveform displayed on a data acquisition system (MacLab 8e; AD Instruments, Hastings, UK). MAP was monitored for the duration of each experiment. The jugular vein was cannulated (PP25, I.D. 0.40 mm; Portex) for the administration of saline or anesthesia as required. A midline laparotomy was performed in order to carefully expose the liver.

Liver I/R

As described previously [19], ligament attachments connecting the liver, diaphragm, abdominal wall and neighboring organs were divided. The liver hilus was exposed to find the common hepatic artery and the portal vein. A vascular microclamp was used to interrupt the portal venous and arterial hepatic blood supply to the cephalad three lobes of the liver during 30 min. The three caudal lobes retained and intact portal inflow and venous outflow, preventing the intestinal venous congestion and possible leakage of bacteria or bacterial products to the

circulation. Reperfusion commenced once the vascular clip was removed, and was allowed to proceed for 2 h. Occlusion was verified visually by change in the color of the liver to a paler shade, and reperfusion by a blush. Other rats were subjected to sham-operation (Sham-operated), which underwent identical surgical procedures to I/R Group rats but did not undergo I/R of the liver clamping and were maintained under anesthesia for the duration of the experiment. At the end of all experiments, rats were sacrificed by an overdose of anesthetic.

Measurement of biochemical parameters

At the end of the reperfusion period, blood was collected into a serum SST® gel and clot activator tube (Becton Dickinson, Le Pont de Claix, France) and was centrifuged (1000 ***g*** for 10 min at room temperature) to separate serum. Serum was analyzed within 24 h by a laboratory for clinical chemistry - Clínica Médica e Diagnóstico Dr Joaquim Chaves (Miraflores, Algés, Portugal). Liver injury was assessed by measuring the rise in the serum levels of alanine aminotransferase (ALT, a specific marker for hepatic parenchymal injury), aspartate aminotransferase (AST, a nonspecific marker for hepatic injury), lactate dehydrogenase (LDH, a marker of nonspecific cellular injury), and gamma-glutamyl transferase (γ-GT, a very sensitive but nonspecific indicator of liver disease with the increased activity found in both the cholestasis and hepatocellular damage). Serum levels of creatinine, urea, and lipase were also determined in order to identify possible remote organ injury (creatine and urea as markers of renal injury, and lipase as a marker for pancreatic injury).

Caspase activity

As described previously [20], 50 μg of cellular protein was incubated with 50 μM substrate in caspase assay buffer (213.5 mM HEPES [pH 7.5], 31.25% sucrose, and 0.3125% CHAPS) for 1 h, and fluorescence was measured on a microplate reader (Fluostar Galaxy; BMG Laboratory Technologies, Aylesbury, UK), with excitation at 380 nm and emission at 460 nm. For each sample, four replicates were assayed with two replicates that contained 50 μM of the caspase-3 inhibitor (Ac-DEVD-CHO) and the remaining pair that contained vehicle (DMSO). Fluorescence readings from wells that contained inhibitor were subtracted from total fluorescence, and results were calculated as nmol AMC/min per milligram protein (Bradford method).

Measurement of liver tissue malondialdehyde

Malondialdehyde (MDA) levels in the liver tissue were determined as an indicator of lipid peroxidation. Briefly, liver tissues, collected at the end of the experiment, were homogenized in 1.15% KCl solution. An aliquot (100 μl) of the homogenate was added to a reaction mixture containing 200 μl of 8.1% (w/v v/v) SDS, 1500 μl of 20% (v/v) acetic acid (pH 3.5), 1500 μl of 0.08% (w/v) thiobarbituric acid, and 700 μl distilled water. Samples were then boiled for 1 h at 95 °C and centrifugated at 3000 *g* for 10 min. The absorbance of the supernatant was measured by spectrophotometry at 650 nm.

Light microscopy

Liver biopsies were taken at the end of reperfusion and were fixed for 1 week in 10% (w/v) PBS-buffered formaldehyde solution at room temperature, dehydrated using graded ethanol and embedded in Paraplast (Sherwood Medical, Mahwah, NJ, USA) and sectioned at 5 μm. Sections were then deparaffinized with xylene, stained with hematoxylin and eosin. All sections were studied by using light microscopy (Dialux 22 Leitz, Esselte Leitz, London, UK).

Materials

Unless otherwise stated, all compounds were obtained from Sigma-Aldrich (Sintra, Portugal). rhEPO was obtained from Janssen-Cilag (Lisbon, Portugal). Pentobarbital sodium (Eutasil®) was obtained from Sanofi Veterinária (Miraflores). All stock solutions were prepared in nonpyrogenic saline (0.9% NaCl; B. Braun, Lisbon, Portugal).

Statistical evaluation

All data are presented as means ± SEM of *n* observations, where *n* represents the number of animals or blood samples studied. For hemodynamics, a two-factorial analysis of variance (ANOVA) was performed. Data without repeated measurements were analyzed by one-factorial ANOVA, followed by a Dunnett's test for multiple comparisons by using a GRAPH PAD PRISM Statistical Package (version 3.0). A P-value <0.05 was considered to be statistically significant.

Results

Effect of rhEPO on hemodynamic parameters

There were no significant baseline differences in hemodynamics between the groups, as assessed by the measurement of MAP (Fig. 1) and heart rates (data not shown). There was a slight fall of the MAP in both the groups subject to I/R, during the ischemic period. This decrease in the MAP was recovered upon reperfusion to mean

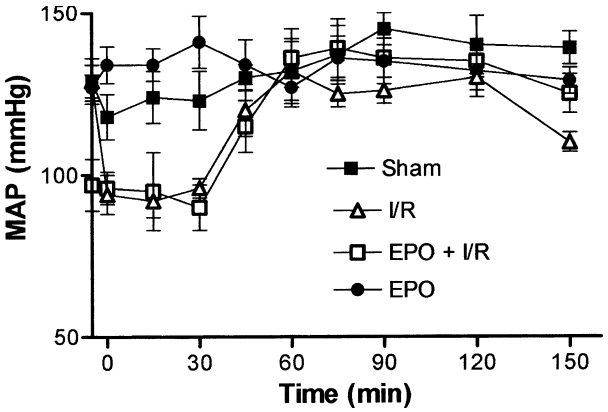

Figure 1 Effect of rhEPO pretreatment on mean arterial pressure (MAP) values. There was a small decline of MAP in both groups subjected to ischemia-reperfusion (I/R), during the ischemic period (I/R group – open triangles, EPO + I/R group – open squares). Upon reperfusion MAP was recovered to mean values not different from those obtained in both sham-group (filled squares) and EPO group (filled circles). Each value is the mean ± SEM for *n* animals (Sham group $n = 9$; I/R group $n = 7$; EPO + I/R group $n = 7$; EPO group $n = 4$).

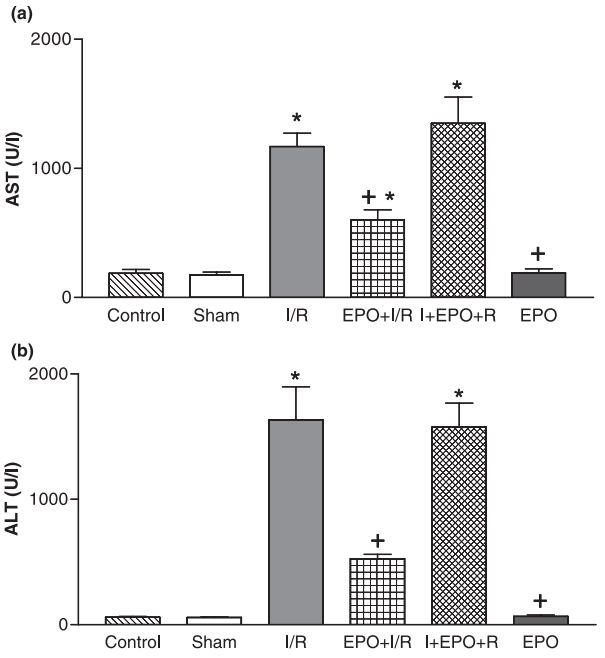

Figure 2 Effect of rhEPO on (a) aspartate aminotransferase (AST) and (b) alanine aminotransferase (ALT) levels in the serum. Within 2 h of reperfusion, the interruption of blood flow to the liver for 30 min led to an increase in the serum levels of AST and ALT. In both cases pretreatment with rhEPO 5 min prior to ischemia significantly reduced this increase. Each value is the mean ± SEM for *n* animals (Control group $n = 6$; Sham group $n = 9$; I/R group $n = 7$; EPO + I/R group $n = 7$; I + EPO + R group $n = 9$; EPO group $n = 4$). (*) $P < 0.05$ *vs.* Sham group; (+) $P < 0.05$ *vs.* I/R group.

values not different from the ones obtained in the sham-group. Again, no significant differences were observed on the heart rate of all groups (data not shown).

Effect of rhEPO on the liver injury caused by I/R injury in the rat

In sham-control rats, surgical procedure did not result in any significant alterations in the serum levels of AST (Fig. 2a), ALT (Fig. 2b), LDH (Fig. 3a), and γ-GT (Fig. 3b). I/R of the liver resulted in significant rises in the serum levels of AST, ALT, LDH and γ-GT, demonstrating the development of hepatocellular injury. In rats subjected to I/R, which had been pretreated with rhEPO before ischemia, no significant rises in the serum levels of ALT, LDH and γ-GT were detected (a significant rise in AST serum levels is still evident after rhEPO pretreatment, although this is still significantly less than the I/R-only group). When rats received rhEPO 5 min prior to reperfusion, significant rises in the serum levels of AST, ALT, LDH, and γ-GT were observed in comparison with the I/R-only group, demonstrating the inexistence of protection over the development of hepatocellular injury. No significant differences were observed for the serum levels of creatinine, urea, and lipase for all the experimental groups (Table 1).

Effect of rhEPO on the rise in the liver tissue levels of MDA caused by I/R injury in the rat

Pretreatment with rhEPO prior to ischemia resulted in a decrease on the liver MDA levels when compared with the liver samples from the I/R group. This indicates that lipid peroxidation of the liver tissue was reduced on the pretreated organs subjected to I/R (Fig. 4).

Effect of rhEPO on caspase activity *in vivo*

Caspase-3 activity was significantly increased in the homogenates of livers at 2 h of reperfusion in the groups subjected to hepatic I/R (Fig. 5), when compared with sham-operated animals. The elevation in caspase-3 activity was significantly reduced by preischemic rhEPO administration (Fig. 5).

Effect of rhEPO on liver histology

Microscopic examination of the liver after 2 h of reperfusion showed injury in the ischemic lobes and centered primarily about the central vein (Fig. 6b). This consisted of hepatocyte necrosis and some neutrophilic infiltration. Many liver cells are swollen and vacuolated. Such alterations were consistent with the raise in the serum levels of ALT, AST, LDH, γ-GT and also MDA and caspase-3 activity in the liver of the experimental group subjected

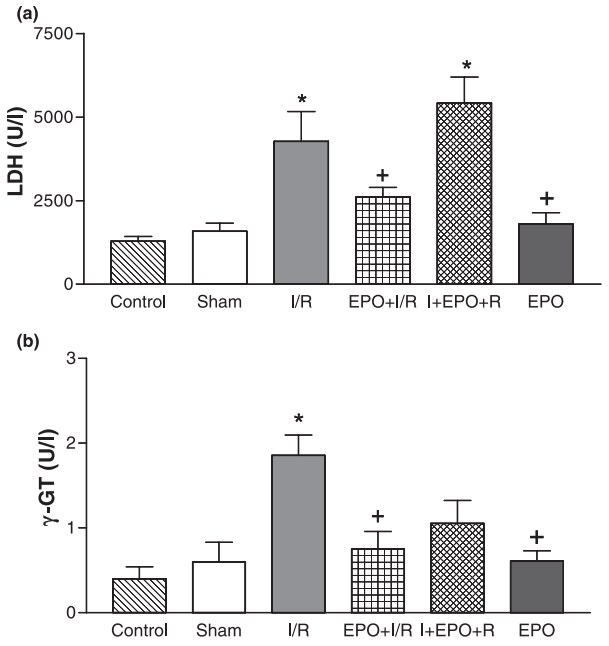

Figure 3 Effect of rhEPO pretreatment on (a) lactate dehydrogenase (LDH) and (b) gamma-glutamyl transferase (γ-GT) serum levels. When compared with sham-operated animals, there was a significant rise in the serum levels of LDH and γ-GT on the group subjected only to liver I/R. Pretreatment of animals with rhEPO 5 min prior to I/R significantly reduced this increase in LDH and γ-GT serum levels. Each value is the mean ± SEM for *n* animals (Control group $n = 6$; Sham group $n = 9$; I/R group $n = 7$; EPO + I/R group $n = 7$; I + EPO + R group $n = 9$; EPO group $n = 4$). (*) $P < 0.05$ *vs*. Sham group; (+) $P < 0.05$ *vs*. I/R group.

Table 1. Effect of human recombinant erythropoietin (rhEPO) pretreatment on urea, creatinine, and lipase serum levels.

	Urea (mg/dl)	Creatinine (mg/dl)	Lipase (U/l)
Sham	48 ± 4	0.4 ± 0.03	8 ± 2
I/R	58 ± 2	0.4 ± 0.06	12 ± 4
EPO + I/R	47 ± 2	0.3 ± 0.05	12 ± 5
EPO	50 ± 4	0.4 ± 0.03	8 ± 2

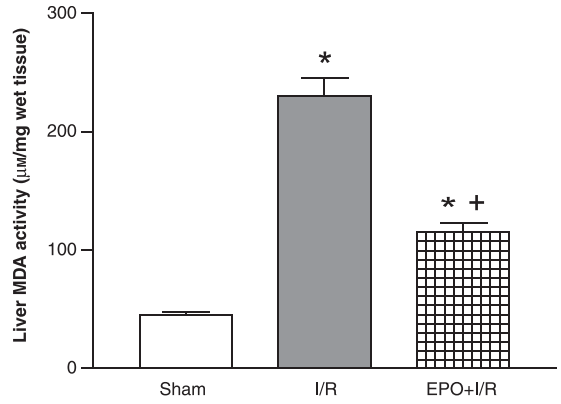

Figure 4 Effect of rhEPO malondialdehyde (MDA) levels in the hepatic tissue. Ischemia followed by reperfusion of the liver led to an increase in lipid peroxidation (as evaluated by MDA) and rhEPO seems to significantly reduce the lipid peroxidation within the tissue. Each value is the mean ± SEM for *n* animals (Sham group $n = 9$; I/R group $n = 7$; EPO + I/R group $n = 7$). (*) $P < 0.05$ *vs*. Sham group; (+) $P < 0.05$ *vs*. I/R group.

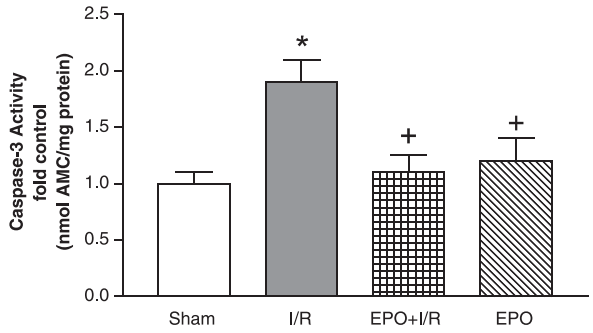

Figure 5 rhEPO-mediated inhibition of caspase-3 activity *in vivo*. In liver homogenates, from rats that were reperfused for 2 h after 30 min of hepatic ischemia, caspase-3 activity was significantly increased compared with sham-operated animals. rhEPO preischemic administration significantly reduced mean caspase-3 activity. (*) $P < 0.05$ *vs*. Sham group; (+) $P < 0.05$ *vs*. I/R group.

only to I/R injury. Animals pretreated with rhEPO (1000 IU/kg, i.v.) before I/R injury (Fig. 6c) showed a preserved tissue architecture when compared with the group only subjected to I/R injury. Liver samples from sham operated animals (Fig. 6a) and animals treated only with rhEPO (Fig. 6d) show no histological signs of injury.

Discussion

A period of ischemia is needed for several surgical procedures on the liver [21,22]. On restoring the blood supply, the liver is subjected to a further insult. Pharmacological modulation of this phenomenon is still not always successful and new therapeutically valid approaches are beginning to be explored. Drugs that are already available and that have a secure safety margin for human use would be of value. Our study brings new insights into the clinical use of rhEPO in the setting of I/R injury of the liver.

We aimed to examine the effect of rhEPO on liver function after ischemic and subsequent reperfusion injury, using a rat model of I/R of the liver. Animals subjected to the I/R procedure alone showed significant rises in the serum levels of AST, ALT, LDH, and γ–GT. The first evidence resulting from the data analysis is the fact that pretreatment with rhEPO prior to ischemia seems to be required in order to obtain the tissue protection against I/R of the organ, as noticed by the reduced serum levels

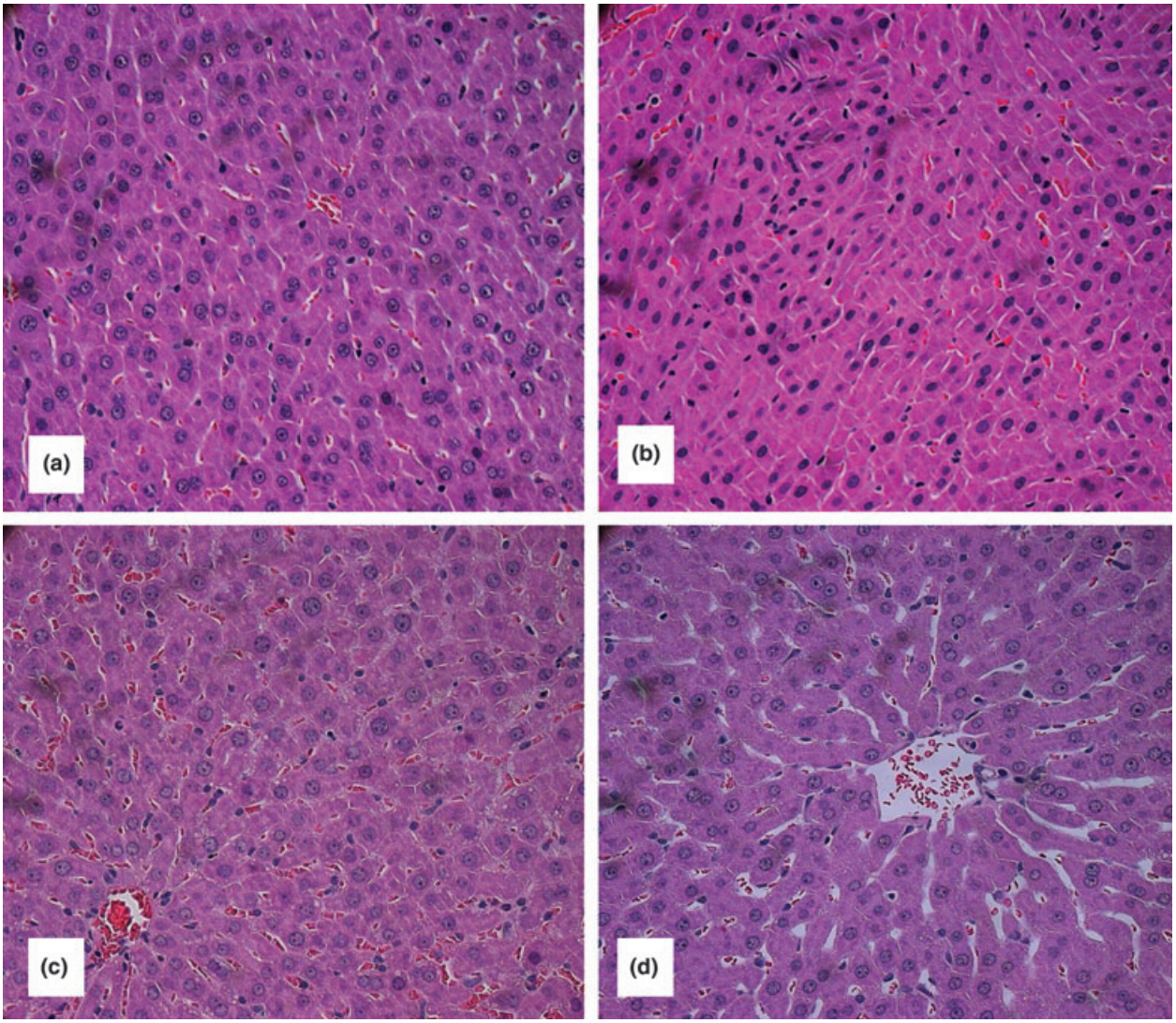

Figure 6 Hepatic histology after I/R. Effect of rhEPO in liver sections. (a) Sham-group rats, (b) rats subjected to I/R alone, (c) rats that received rhEPO prior to I/R injury and (d) represents the tissue from animals only treated with rhEPO. Magnification: 400×. The images are representative of at least four experiments performed on different experimental days.

of AST, ALT, LDH, and γ–GT in the rhEPO + I/R group (versus I/R group). Also, and at least in the liver, this indicates that some short-latency type preconditioning seems to be needed in order to obtain liver protection from I/R injury, as the administration of rhEPO just 5 min prior to reperfusion had no significant protective effect. This provides evidence that, probably, NF-κB – an important mediator of ischemic acute preconditioning – might be a target for rhEPO in the liver. These results are opposite from those obtained by Calvillo *et al.* [4] in the heart, suggesting that there might be some tissue specific issues concerning the treatment with EPO. When looking at the lipid peroxidation (assessed by the MDA tissue levels), pretreatment with rhEPO reduced the MDA levels within the organs collected from animals subjected to I/R, suggesting that rhEPO beneficial effects could also be related to a decreased oxidative injury aimed to cellular targets in the hepatic tissue. Interestingly, pretreatment with rhEPO had no effect on the hematocrit level (data not showed), suggesting that the hepatoprotective effect of this compound is independent of its growth-promoting effects.

The molecular signals by which rhEPO provides its benefit in this model are currently unclear. Previous studies have clearly showed the potential of rhEPO for protecting the tissues under the conditions of hypoxia. The observations obtained from the *in vitro* experiments directly demonstrate that rhEPO prevents apoptosis of

myocardial cells exposed to extreme and prolonged hypoxia. In the model of cardiac injury used by Calvillo *et al.* [4], reduction of medium oxygen content to <1% triggers a majority of cells to enter in apoptosis that can be prevented by rhEPO exposure. In contrast, relatively few cardiomyocytes undergo necrosis, and these do not respond to rhEPO. Similar findings were obtained in the central nervous system, suggesting the involvement of rhEPO only within the apoptosis group [23–26]. Thus, apoptosis and necrosis have been proposed as the mechanisms that produce cellular demise in ischemic rat kidneys [27] and either one may be the target of actions of EPO. Concerning the I/R injury of the kidney, results demonstrated that apoptosis, as well as necrosis, is associated with the cell death in rat kidney with I/R injury [28,29] and that rhEPO exerts its antiapoptotic effect by inducing bcl-2 protein in kidneys and reducing the caspase-3 activity, as it was previously observed in migroglial cells pretreated with EPO [30]. Most recently, it has been demonstrated that in cultured human proximal tubule cells, EPO concentration dependently reduced the cell death and increase in caspase-3 activity caused by either ATP depletion (simulated ischemia) or hydrogen peroxide (oxidative stress) [16]. The same authors also showed that, in the heart, the administration of EPO (300 IU/kg i.v.) before reperfusion also caused a significant reduction in infarct size. In cultured rat cardiac myoblasts (H9C2 cells), EPO also reduced the increase in DNA fragmentation caused by either serum deprivation (simulated ischemia) or hydrogen peroxide (oxidative stress) [16]. In the present work, we confirm that pretreatment with rhEPO reduced the activity of caspase-3, ultimately reducing the apoptosis. The fundamental concept that emerges from the above studies is that rhEPO induces a tolerance of the liver and other organs to a subsequent insult with I/R, and this induction seems to change MAPK expression in ways that promote cell survival.

Finally, a potential role for rhEPO in the recruitment of stem cells into the region of injury has been recently observed for the nervous system [31], which is nevertheless an intriguing but interesting possibility.

Although the effects of rhEPO on the hematocrit (erythrocyte mass) seem to be of minor concern in these acute experiences, this would not be true if rhEPO is used clinically in a chronic dosage regimen. The development of nonerythropoietic analogs of EPO might allow the avoidance of such undesirable effect in the clinical settings stated before. Although tissue-protective cytokines signaling needs to be further clarified, the availability of compounds such as Carbamylated EPO (EPO where all the lysines were transformed to homocitrulline by carbamilation) that do not trigger $(EPOR)_2$ also opens the possibilities to distinguish experimentally between EPO's tissue-protective effects (e.g. antiapoptosis) and its potentially detrimental effects (e.g. pro-coagulant and prothrombotic effects within the microvasculature) and excessive erythropoiesis upon chronic dosing [32]. We will be able to trigger EPO-mediated tissue-protective pathways without cross-talk with the hematopoietic system.

Our results in the model of liver I/R injury strongly support the concept that EPO is a tissue-protective cytokine, and this seems to be true for all the organs expressing the EPO receptor. Proposed mechanisms of protection in models of I/R injury of the kidney, using the same dose of rhEPO (1000 IU/kg), included a reduction in oxidative stress [14], and lower doses (300 IU/kg) of rhEPO reduced the apoptotic cell death [15] and caspase-3 activity [15,16]. Higher doses (ranging from 3000 to 5000 IU/kg) exhibited the ability to increase Bcl2 and HSP70 expression, also reducing the apoptosis [13]. Here, we demonstrate that the mechanisms underlying the hepatic protection from I/R injury might also be the reduction in oxidative stress and caspase-3 activation, and also there is a preserved histology of the organ. The possibility of using rhEPO to induce ischemic tolerance suggests that there are advantages in its clinical application in liver transplantation. Firstly, rhEPO is a safe drug in clinical practice. Secondly, the induction of ischemic tolerance seems to be relatively rapid after a single injection of rhEPO. Thirdly, no additional or special equipment is required for the induction of tolerance in the patient. Clinical studies will be necessary to evaluate the therapeutic properties of rhEPO in preventing an I/R injury not only in the liver, but also when considering the transplantation of other solid organs.

References

1. Taniguchi M, Magata S, Suzuki T, *et al.* Dipyridamole protects the liver against warm ischemia and reperfusion injury. *J Am Coll Surg* 2004; **198**: 758.
2. Serracino-Inglott F, Habib NA, Mathie RT. Hepatic ischemia-reperfusion injury. *Am J Surg* 2001; **181**: 160.
3. Takamatsu Y, Shimada K, Chijiiwa K, Kuroki S, Yamaguchi K, Tanaka M. Role of leukotrienes on hepatic ischemia/reperfusion injury in rats. *J Surg Res* 2004; **119**: 14.
4. Calvillo L, Latini R, Kajstura J, *et al.* Recombinant human erythropoietin protects the myocardium from ischemia-reperfusion injury and promotes beneficial remodeling. *Proc Natl Acad Sci USA* 2003; **100**: 4802.
5. Masuda S, Nagao M, Sasaki R. Erythropoietic, neurotrophic, and angiogenic functions of erythropoietin and regulation of erythropoietin production. *Int J Hematol* 1999; **70**: 1.
6. Morishita E, Narita H, Nishida M, *et al.* Anti-erythropoietin receptor monoclonal antibody: epitope mapping,

quantification of the soluble receptor, and detection of the solubilized transmembrane receptor and the receptor-expressing cells. *Blood* 1996; **88**: 465.
7. Morishita E, Masuda M, Nagao M, Yasuda Y, Sasaki R. Erythropoietin receptor is expressed in rat hippocampal and cerebral cortical neurons, and erythropoietin prevents in vitro glutamate-induced neuronal death. *Neuroscience* 1997; **76**: 105.
8. Digicaylioglu M, Bichet S, Marti H, *et al.* Localization of specific erythropoietin binding sites in defined areas of the mouse brain. *Proc Natl Acad Sci USA* 1995; **92**: 3717.
9. Tan C, Eckardt K, Firth J, Ratcliffe PJ. Feedback modulation of renal and hepatic erythropoietin mRNA in response to graded anemia and hypoxia. *Am J Physiol* 1992; **263**: F474.
10. Grasso G, Buemi M, Alafaci C, *et al.* Beneficial effects of systemic administration of recombinant human erythropoietin in rabbits subjected to subarachnoid hemorrhage. *Proc Natl Acad Sci USA* 2002; **99**: 5627.
11. Squadrito F, Altavilla D, Squadrito G, *et al.* Recombinant human erythropoietin inhibits iNOS activity and reverts vascular dysfunction in splanchnic artery occlusion shock. *Br J Pharmacol* 1999; **127**: 482.
12. Cuzzocrea S, Mazzon E, Di Paola R, *et al.* Erythropoietin reduces the development of experimental, inflammatory bowel disease. *J Pharmacol Exp Ther* 2004; **311**: 1272.
13. Yang C, Li C, Jung J, *et al.* Preconditioning with erythropoietin protects against subsequent ischemia-reperfusion injury in rat kidney. *FASEB J* 2003; **17**: 1754.
14. Patel NS, Sharples EJ, Cuzzocrea S, *et al.* Pretreatment with EPO reduces the injury and dysfunction caused by ischemia/reperfusion in the mouse kidney in vivo. *Kidney Int* 2004; **66**: 983.
15. Sharples EJ, Patel N, Brown P, *et al.* Erythropoietin protects the kidney against the injury and dysfunction caused by ischemia-reperfusion. *J Am Soc Nephrol* 2004; **15**: 2115.
16. Abdelrahman M, Sharples EJ, McDonald MC, *et al.* Erythropoietin attenuates the tissue injury associated with hemorrhagic shock and myocardial ischemia. *Shock* 2004; **22**: 63.
17. Parsa CJ, Matsumoto A, Jihee K, *et al.* A novel protective effect of erythropoietin in the infracted heart. *J Clin Invest* 2003; **112**: 999.
18. Sepodes B, Maio R, Pinto R, *et al.* Tempol, an intracelullar free radical scavenger, reduces liver injury in hepatic ischemia-reperfusion in the rat. *Transplant Proc* 2004; **36**: 849.
19. Mota-Filipe H, Sepodes B, McDonald MC, Cuzzocrea S, Pinto R, Thiemermann C. The novel PARP inhibitor 5-aminoisoquinolinone reduces the liver injury caused by ischemia and reperfusion in the rat. *Med Sci Monit* 2002; **8**: 444.
20. Allen D, Harwood S, Varagunam M, Raftery MJ, Yaqoob MM. High glucose-induced oxidative stress causes apoptosis in proximal tubular epithelial cells and is mediated by multiple caspases. *FASEB J* 2003; **17**: 908.
21. Delva E, Camus Y, Nordlinger B, *et al.* Vascular occlusions for liver resections. Operative management and tolerance to hepatic ischemia: 142 cases. *Ann Surg* 1989; **209**: 211.
22. Huguet C, Addario-Chieco P, Gavelli A, Arrigo E, Harb J, Clement RR. Technique of hepatic vascular exclusion for extensive liver resection. *Am J Surg* 1992; **163**: 602.
23. Juul S, Anderson D, Li Y, Christensen RD. Erythropoietin and erythropoietin receptor in the developing human central nervous system. *Pediatr Res* 1998; **43**: 40.
24. Brines M, Ghezzi P, Keenan S, *et al.* Erythropoietin crosses the blood-brain barrier to protect against experimental brain injury. *Proc Natl Acad Sci USA* 2000; **97**: 10526.
25. Siren A, Fratelli M, Brines M, *et al.* Erythropoietin prevents neuronal apoptosis after cerebral ischemia and metabolic stress. *Proc Natl Acad Sci USA* 2001; **98**: 4044.
26. Celik M, Gokmen N, Erbayraktar S, *et al.* Erythropoietin prevents motor neuron apoptosis and neurologic disability in experimental spinal cord ischemic injury. *Proc Natl Acad Sci USA* 2002; **99**: 2258.
27. Yakamoto K, Wilson D, Brumal R. Outer medullary circulatory defect in ischemic acute renal failure. *Am J Pathol* 1984; **116**: 253.
28. Kaushal G, Singh A, Shah SV. Identification of gene family of caspases in rat kidney and altered expression in ischemia-reperfusion injury. *Am J Physiol* 1998; **274**: 587.
29. Oberbauer R, Scharz C, Regele H, Hansmann C, Meyer TW, Mayer G. Regulation of renal tubular cell apoptosis and proliferation after ischemic injury to a solitary kidney. *J Lab Clin Med* 2001; **138**: 343.
30. Vairano M, Dello Russo C, Pozzoli G, *et al.* Erythropoietin exerts anti-apoptotic effects on rat microglial cells in vitro. *Eur J Neurosci* 2002; **16**: 584.
31. Shringo T, Sorokan S, Shimazaki T, Weiss S. Erythropoietin regulates the in vitro and in vivo production of neuronal progenitors by mammalian forebrain neural stem cells. *J Neurosci* 2001; **21**: 9733.
32. Leist M, Ghezzi P, Grasso G, *et al.* Derivatives of erythropoietin that are tissue protective but not erythropoietic. *Science* 2004; **305**: 239.