Erythropoietin Protects the Intestine Against Ischemia/Reperfusion Injury in Rats

Ensari Guneli,[1] Zahide Cavdar,[2] Huray Islekel,[2] Sulen Sarioglu,[3] Serhat Erbayraktar,[4] Muge Kiray,[5] Selman Sokmen,[6] Osman Yilmaz,[1] and Necati Gokmen[7]

[1]Department of Laboratory Animal Sciences, Health Sciences Institute, Dokuz Eylul, University, Izmir, Turkey; [2]Department of Biochemistry, Health Sciences Institute, Dokuz Eylül University, Izmir, Turkey; [3]Department of Pathology, Faculty of Medicine, Dokuz Eylul University, Izmir, Turkey; [4]Department of Neurosurgery, Faculty of Medicine, Dokuz Eylul University, Izmir, Turkey; [5]Department of Histology and Embryology, Faculty of Medicine, Dokuz Eylul University, Izmir, Turkey; [6]Department of Surgery, Faculty of Medicine, Dokuz Eylul University, Izmir, Turkey; [7]Department of Anesthesiology and Reanimation, Faculty of Medicine, Dokuz Eylul, University, Izmir, Turkey

Previous studies have shown that erythropoietin (EPO) has protective effects against ischemia/reperfusion (I/R) injury in several tissues. The aim of this study was to determine whether EPO could prevent intestinal tissue injury induced by I/R. Wistar rats were subjected to intestinal ischemia (30 min) and reperfusion (60 min). A single dose of EPO (5000 U/kg) was administered intraperitoneally at two different time points: either at five minutes before the onset of ischemia or at the onset of reperfusion. At the end of the reperfusion period, jejunum was removed for examinations. Myeloperoxidase (MPO), malondialdehyde (MDA), and antioxidant defense system were assessed by biochemical analyses. Histological evaluation was performed according to the Chiu scoring method. Endothelial nitric oxide synthase (eNOS) was demonstrated by immunohistochemistry. Apoptotic cells were determined by TUNEL staining. Compared with the sham, I/R caused intestinal tissue injury (Chiu score, 3 ± 0.36 vs 0.4 ± 0.24, $P < 0.01$) and was accompanied by increases in MDA levels (0.747 ± 0.076 vs 0.492 ± 0.033, $P < 0.05$), MPO activity (10.51 ± 1.87 vs 4.3 ± 0.45, $P < 0.05$), intensity of eNOS immunolabelling (3 ± 0.4 vs 1.3 ± 0.33, $P < 0.05$), the number of TUNEL-positive cells (20.4 ± 2.6 vs 4.6 ± 1.2, $P < 0.001$), and a decrease in catalase activity (16.83 ± 2.6 vs 43.15 ± 4.7, $P < 0.01$). Compared with the vehicle-treated I/R, EPO improved tissue injury; decreased the intensity of eNOS immunolabelling (1.6 ± 0.24 vs 3 ± 0.4, $P < 0.05$), the number of TUNEL-positive cells (9.2 ± 2.7 vs 20.4 ± 2.6, $P < 0.01$), and the high histological scores (1 ± 0.51 vs 3 ± 0.36, $P < 0.01$), and increased catalase activity (42.85 ± 6 vs 16.83 ± 2.6, $P < 0.01$) when given before ischemia, while it was found to have decreased the levels of MDA (0.483 ± 0.025 vs 0.747 ± 0.076, $P < 0.05$) and MPO activity (3.86 ± 0.76 vs 10.51 ± 1.87, $P < 0.05$), intensity of eNOS immunolabelling (1.4 ± 0.24 vs 3 ± 0.4, $P < 0.01$), the number of TUNEL-positive cells (9.1 ± 3 vs 20.4 ± 2.6, $P < 0.01$), and the number of high histological scores (1.16 ± 0.4 vs 3 ± 0.36, $P < 0.05$) when given at the onset of reperfusion. These results demonstrate that EPO protects against intestinal I/R injury in rats by reducing oxidative stress and apoptosis. We attributed this beneficial effect to the antioxidative properties of EPO.

Online address: http://www.molmed.org
doi: 10.2119/2007–00032.Guneli

INTRODUCTION

The restoration of blood flow to an ischemic region leads to tissue injury at a greater rate than the original ischemic insult, an event called reperfusion injury. Among the abdominal organs, the small intestine is probably the most sensitive to ischemia/reperfusion (I/R) induced injury (1). It occurs frequently in a variety of clinical conditions, including mesenteric artery occlusion, abdominal aneurism surgery, trauma, shock, and small intestinal transplantation, and is associated with high morbidity and mortality (2). Although the exact mechanisms involved in the pathogenesis of intestinal I/R injury have not been fully elucidated, it is generally believed that oxidative stress mediators such as reactive oxygen species (ROS), polymorphonuclear neutrophils, and nitric oxide (NO) play an important role (3).

Erythropoietin (EPO) is a glycoprotein cytokine produced primarily by the kidney in the regulation of red blood cell production. In addition to this well-known and widely recognized effect, many studies have shown that EPO also acts as a tissue protecting factor (4–7). The favorable effects of the EPO-related changes are not fully understood, although its antiapoptotic, antioxidative, and antiinflammatory properties as well as its angiogenic potential seem to be related to the EPO-mediated protective effect (5,8,9). The protective effects of EPO are mediated through the tissue-protective EPO receptor, consisting of a heteromeric complex containing an EPO receptor and a β common receptor subunit (10). However, little is known about the presence and protective role of EPO and its recep-

Address correspondence and reprint requests to Serhat Erbayraktar, Department of Neurosurgery, Faculty of Medicine, Dokuz Eylul University, Inciralti, Izmir 35340, Turkey. Phone: + 90 232 4123333; Fax: + 90 232 2590541; E-mail: *s.erbayraktar@deu.edu.tr.*
Submitted April 20, 2007; Accepted for publication August 17, 2007.

tor in the intestine. Juul SE et al (1999) have demonstrated that EPO receptors are expressed on the intestinal tissue in rodents and humans (11). EPO was found to have a beneficial effect on intestinal damage such as colitis (12), necrotizing enterocolitis, (13) and anastomosis (14) in animal models. Previous studies have shown that EPO exhibits protective effects on tissue injury associated with I/R in many tissues such as cardiac (15), kidney (16), liver (17), lung (18), brain (19), and retina (20). However, it is not known whether EPO has a protective effect in the intestinal injury associated with I/R. Under the light of these data, the aim of the present study therefore was to determine whether recombinant human erythropoietin (rHuEPO) has a protective effect on intestinal I/R injury in rats.

MATERIALS AND METHODS

Animals

Male albino Wistar rats (200–250 g) were used in the present study. All the animals were kept under optimum conditions (21 ± 1° C, 40 to 70 percent humidity, 12/12 darkness-lightness cycle) at Dokuz Eylül University's Laboratory Animal Unit and were fed ad libitum with standard pellet diet and water. The experimental protocol was approved by Dokuz Eylül University's Ethic Committee for Animal Research.

Experimental Groups

The rats were randomly divided into four groups as described: (a) Sham group: All the surgical steps were performed, except that intestinal I/R was not induced. Animals were kept under anesthesia for the duration of the intestinal I/R method. The sham group served as control of I/R group; (b) I/R group: Intestinal I/R was performed and served as control of rHuEPO-administered groups; (c) EPO + I/R group: Intestinal I/R was performed and rHuEPO was administered five minutes before the onset of ischemia; (d) I + EPO + R group: Intestinal I/R was performed and rHuEPO was administered at the onset of reperfusion.

A single dose of rHuEPO (5000 U/kg) was injected via i.p. route. rHuEPO (NeoRecormon, Roche, Germany) were purchased commercially.

Technique of Intestinal I/R

Feeding of the animals was stopped 12 h prior to the start of the intestinal I/R procedure and they received only water. The rats were anesthetized with urethane (1500 mg/kg, i.p.) and their temperature was regulated by means of a lamp light bulb during the test. Intestinal I/R was induced as follows: The rats were placed in the supine position and secured in the dissection tray. The abdominal region was shaved and cleaned with antiseptic solutions. The intestinal region was reached by means of midline laparotomy. Superior mesenteric artery (SMA) was subjected with care and occluded with an atraumatic microvascular clamp, thus intestinal ischemia was created in 30 min. Ischemia was recognized by the existence of pulseless or pale color of the intestine. The abdominal region was then closed. Following ischemia, the clamp was removed and 60 min reperfusion was induced. The return of the pulses and the reestablishment of the pink color were assumed to be the reperfusion of the intestine. At the end of reperfusion, the jejunal segment was taken out, and the animals were killed by exsanguination.

Tissue Preparation for Biochemical Analysis

All tissues were washed two times with cold saline solution and homogenized using a glass Teflon homogenizer (B. Brawn, Germany) in buffer at a ratio of 1/10 (50 mM potassium phosphate buffer pH: 7.8, containing 0.5 mmol/L PMSF, 10 μg/mL aprotinin) after cutting the tissue into small pieces with scissors and centrifuged at 2500*g*. Malondialdehyde (MDA) analyses were measured at this homogenate stage. The homogenate was then centrifuged at 45 000*g* for 30 min. The supernatant was used for colorimetric determination of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) enzyme activities. For glutathione (GSH) and myeloperoxidase (MPO) assay, tissue preparation details were mentioned in the analysis section. All preparation procedures were performed at +4° C. All homogenates were stored at –80° C prior to testing.

MDA Assay

The MDA assay was based on the condensation of one molecule of malondialdehyde with two molecules of thiobarbituric acid (TBA) in the presence of reduced agents. The TBA + MDA complex was analyzed by HPLC system as described by Tatum et al. (21). Briefly, the HPLC system (Shimadzu VP Class, Shimadzu Corporation, Japan) consisted of a LC-10 ADVP pump system (Shimadzu VP) equipped with an automatic injector (SIL-10 ADVP), RF-10XL fluorescence detector and a personal computer using Class VP 6.1 Software. Aliquots of TBA + MDA samples were injected on a C18 column (Nucleosil 100-5, 150-4.6 mm; Macherey–Nagel Incorporation, Bethlehem, PA, USA) maintained at 30° C, followed by fluorimetric detection at 550 nm after excitation at 340 nm. Serial concentrations (0.75 μM – 50 μM) of 1, 1, 3, 3-tetraethoxypropane (TEP) were used as standard. Measurements were expressed in terms of MDA normalized to the tissue protein content.

Thiobarbituric acid (TBA), 1,1,3,3-Tetraethoxypropane (TEP), butylated hydroxytoluen (BHT), potassium monobasic phosphate (KH_2PO_4), potassium dibasic phosphate (K_2HPO_4), sodium hydroxide (NaOH), sodium dodecylsulphate (SDS), ethanol, pyridine, n-butanol, and HPLC grade methanol were obtained from Sigma Chemicals, Germany.

Determination of MPO Activity

MPO activity was measured in tissues with commercially available ELISA kit (Bioxytech MPO-EIA, Oxis Research, Portland, OR, USA). Briefly, tissue samples were homogenized in 50 mM potassium phosphate buffer, pH: 7.8, containing 0.5 mmol/L PMSF, 10 μg/mL aprotinin, five percent 0.5 percent hexadecyltrimethylammonium bromide

(HETAB), and centrifuged at 40,000*g* for 15 min at +4° C. Then, the supernatant was assayed according to the manufacturer's instructions. The absorbance was read at 405 nm using Multi-Detection MicroPlate Reader (Synergy HT, BioTek Instruments, Inc., Winooski, VT, USA). Quantifications were achieved by the construction of standard curve using known concentrations of MPO. Results were expressed as ng/mg protein.

Determination of SOD Activity

For determination of SOD activity, the colorimetric assay (Bioxytech SOD-525, Oxis Research) was used. This method is based on the SOD-mediated increase in the rate of autooxidation of tetrahydrobenzofluorene in an aqueous alkaline solution to yield a chromophore with maximum absorbance at 525 nm. This absorbance was measured by spectrophotometer (Varian, Carry 50 UV-Visible, Australia). Results were expressed as U/mg protein.

Determination of CAT Activity

CAT activity was determined by means of commercially available colorimetric assay (Bioxytech, Catalase 520, Oxis Research) and performed according to the manufacturer's instructions. One unit of enzyme activity was defined as the amount of catalase that is available causing a change in absorbance at 520 nm for ten minutes. CAT activity was expressed as U/mg protein.

Measurement of Glutathione Peroxidase (GSH-Px) Activity

GSH-Px activity was measured by automated spectrophotometric method (Hitachi Modular Analytics, Roche Diagnostics Inc., Tokyo, Japan). The enzymatic reaction was initiated by the addition of cumene hydroperoxide (CuOOH) to the reaction mixture containing GSH, NADPH, EDTA, $NaNO_3$, and glutathione reductase. The change in the absorbance at 340 nm was monitored.

Measurement of GSH Levels

Colorimetric assay for assessment of reduced glutathione concentration (Bioxytech, GSH-400, Oxis Research) was used. Firstly, the tissue was homogenized in precipitation reagent (Bioxytech GSH-420, Oxis Research) and homogenate was centrifuged at 3000*g* for ten minutes at +4° C and upper aqueous layer was used for assay. Then, the level of reduced glutathione was measured at 412 nm by spectrophotometer (Varian, Carry 50 UV-Visible, Australia). Results were expressed as μmole/mg protein.

Protein Determination

The protein content in each tissue sample was determined using the bicinchoninic acid protein assay (BCA) (Sigma Chemicals, Germany). Bovine serum albumin was used as a standard (22).

Histological Analysis

Serial sections were taken from ten percent formalin fixed paraffin embedded tissue blocks of intestinal tissues and stained with Hematoxilen & Eosin (H&E). Tissue injury in the intestinal mucosa was evaluated using light microscopy according to the criteria described by Chiu et al (1970) and graded from 0 to 5 (23). The grades are: *Grade 0*: Normal mucosa; *Grade 1*: Formation of subepithelial detachments at the tip of the villi with capillary congestion; *Grade 2*: Subepithelial detachments exerted a moderate amount of upward push on the mucosa epithelium; *Grade 3*: Large subepithelial detachments exerted a massive amount of upward push on the mucosa epithelium along the villi and few denuded villus tips were observed; *Grade 4*: The villi were denuded to the level of lamina propria and dilated capillaries; *Grade 5*: Presence of ulceration, disintegration of lamina propria, and hemorrhage.

Detection of Apoptosis by TUNEL Method

Four-micrometer-thick sections were collected on poly-L-lysine-coated glass slides. The nuclear DNA fragmentation of the apoptotic cell was labeled in situ by the terminal deoxynucleotidyl treatments with xylene and rehydration with progressively decreasing alcohol concentrations followed by phosphate-buffered saline (PBS), each section was treated with 20 μg/mL proteinase K (Sigma) in 0.1 mol/L Tris/HCL buffer (pH 7.4) for 15 min. After rinsing with PBS, endogenous peroxidase activity was blocked with three percent hydrogen peroxide (H_2O_2) for five minutes. After rinsing with PBS, they were incubated with 0.5 U/μL terminal deoxynucleotidyl transferase (Boerhinger Mannheim, Germany) and 0.05 nmol/μL biotinylated deoxyuridine triphosphate in terminal deoxynucleotidyl transferase buffer (Boerhinger Mannheim) for 60 min in a humidified chamber at 37° C. Each slide was then observed with a microscope to check the staining quality before image acquisition. For each animal, five sections were analyzed by counting apoptotic bodies in five randomly chosen fields.

Immunohistochemistry

Immunohistochemical staining was used to locate eNOS expression in jejunal tissue. Sections were incubated at 60° C overnight, and then deparaffinized in xylene for 30 min. After rehydrating through a graded ethanol series, sections were treated with two percent trypsin at 37° C for 15 min. Sections were then incubated in a solution of three percent H_2O_2 for 15 min to inhibit endogenous peroxidase activity. Next, the sections were incubated overnight with anti-eNOS antibody (GeneTex, Inc, San Antonio, TX, USA) and then for another 30 min with the biotinylated mouse secondary antibody. The bound secondary antibody was then amplified with Vector Elite ABC kit (Vectastain, Vector Laboratories, Burlingame, CA, USA). The antibody-biotin-avidin-peroxidase complexes were visualized using 0.02 percent DAB and nuclei were counterstained with Harris hematoxylin. The sections were finally mounted onto lysine-coated slides. The images were analyzed using a computer-assisted image analyzer system consisting of a microscope (Olympus BX-50, Tokyo, Japan) equipped with a

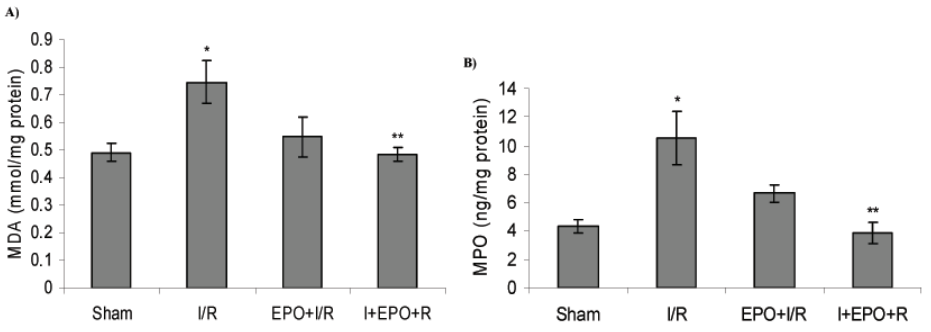

Figure 1. Jejunal tissue levels of malondialdehyde (MDA) and myeloperoxidase (MPO) in sham-operated animals, vehicle-treated I/R animals, and I/R animals treated with EPO five minutes before ischemia and at the onset of reperfusion. Data are mean ± S.E.M. (n = 5–7 per group). *$P < 0.05$ vs sham; **$P < 0.05$ vs vehicle-treated I/R animals (one-way ANOVA followed by Tukey's post-test). Note that there is a significant decrease in the MDA and MPO levels in the I + EPO + R group compared with the I/R group. Sham; intestinal I/R was not induced, I/R; intestinal I/R, EPO + I/R; rHuEPO administered five minutes before ischemia, I + EPO + R; rHuEPO administered onset of reperfusion.

high-resolution video camera (JVC TK-890E, Japan). This analysis was performed in at least ten areas per jejunal section, in two sections from each animal at ×40 magnification. The immunolabelling scores were evaluated blindly. Immunolabelling intensity was graded as mild (1), moderate (2), strong (3), and very strong (4).

Statistical Analysis

SPSS statistical package was used for data analysis (version 11.0, SPSS Inc., Chicago, IL, USA). The difference among groups was assessed with one-way ANOVA and Tukey HSD test was used to examine the difference between two groups. Statistical significance was accepted for the P values lower than 0.05; the arithmetic mean ± S.E.M. was used to define distribution.

RESULTS

Effects of rHuEPO on MDA Levels and MPO Activity in the Intestinal Tissue Subjected to I/R in Rats

MDA levels examined as an indicator of lipid peroxidation are shown in Figure 1A. Compared with the sham group, MDA levels in the jejunal tissue in the I/R group were found to have increased (0.747 ± 0.076 vs 0.492 ± 0.033 mmol/mg protein, $P < 0.05$). As for the rHuEPO-administered groups, a significant decrease in the MDA levels was observed in the I + EPO + R group (0.483 ± 0.025 vs 0.747 ± 0.076 mmol/mg protein, $P < 0.05$), while there was a decrease in the EPO + I/R group but the difference was not significant when compared with I/R group ($P > 0.05$).

MPO activity examined as an indicator of neutrophil accumulation is shown in Figure 1B. Compared with the sham group, MPO activity in the jejunal tissue in the I/R group were found to have increased (10.51 ± 1.87 vs 4.3 ± 0.45 ng/mg protein, $P < 0.05$). As for the rHuEPO-administered groups, a significant decrease in the MPO activities was observed in the I + EPO + R group (3.86 ± 0.76 vs 10.51 ± 1.87 ng/mg protein, $P < 0.05$) while there was a decrease in the EPO + I/R group, but the difference was not significant when compared with the I/R group ($P > 0.05$).

Effects of rHuEPO on Antioxidant Activity in the Intestinal Tissue Subjected to I/R in Rats

The levels of enzymatic activity (SOD, CAT, and GSH-Px) and non-enzymatic levels (GSH) in the jejunal tissue are shown in Table 1. Compared with the sham group, the I/R group exhibited slight changes in the enzymatic activity of SOD and GSH-Px and also in the levels of GSH, but these differences were not significant ($P > 0.05$); however, a significant decrease was determined in the level of CAT activity (16.83 ± 2.6 vs 43.15 ± 4.7 U/mg protein, $P < 0.01$). As for the rHuEPO-administered groups, only the EPO + I/R group was found to have an increased CAT activity (42.85 ± 6 vs 16.83 ± 2.6 U/mg protein, $P < 0.01$) when compared with the I/R group.

Table 1. Jejunal tissue levels of antioxidant system elements (superoxide dismutase (SOD, catalase (CAT) and gluthatione peroxidase (GSH-Px)) and glutathione (GSH)) in sham-operated animals, vehicle-treated I/R animals, and I/R animals treated with EPO five minutes before ischemia and at the onset of reperfusion.

	Enzymatic antioxidants		Non-enzymatic antioxidant	
	SOD (U/mg/protein)	CAT (U/mg/protein)	GSH-Px (U/mg/protein)	GSH (µmol/mg/protein)
Sham	4.94 ± 1.24	43.15 ± 4.7	0.036 ± 0.007	0.381 ± 0.079
I/R	6.18 ± 0.43	16.83 ± 2.6[a]	0.035 ± 0.010	0.275 ± 0.025
EPO + I/R	5.34 ± 0.66	42.85 ± 6[b]	0.034 ± 0.013	0.262 ± 0.022
I + EPO + R	5.15 ± 0.88	29.31 ± 4.6	0.032 ± 0.008	0.280 ± 0.028

[a]$P < 0.01$ vs sham.

[b]$P < 0.01$ vs vehicle-treated I/R animals.

Data are mean ± S.E.M. (n = 5-7 per group). Note that there is a significant increase in the CAT levels in the EPO + I/R group compared with the I/R group. Sham; intestinal I/R was not induced, I/R; intestinal I/R, EPO + I/R; rHuEPO administered five minutes before ischemia, I + EPO + R; rHuEPO administered onset of reperfusion.

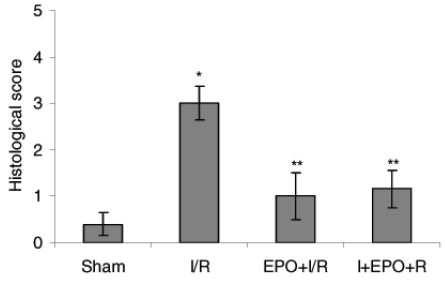

Figure 2. Intestinal mucosal injury evaluated by Chiu scoring system in sham-operated animals, vehicle-treated I/R animals, and I/R animals treated with EPO five minutes before ischemia and at the onset of reperfusion. Grading as (0 = normal mucosa, 1 = slight-, 2 = moderate-, 3 = massive subepithelial detachments, 4 = denudes villi, 5 = ulceration). Data are mean ± S.E.M. (n = 5–7 per group). *$P < 0.01$ vs sham; **$P < 0.05$ vs vehicle-treated I/R animals (one-way ANOVA followed by Tukey's post-test). Note that there is a significant decrease in the intestinal injury score after treatment with EPO compared with the vehicle treated I/R animals. Sham; intestinal I/R was not induced, I/R; intestinal I/R, EPO + I/R; rHuEPO administered five minutes before ischemia, I + EPO + R; rHuEPO administered onset of reperfusion.

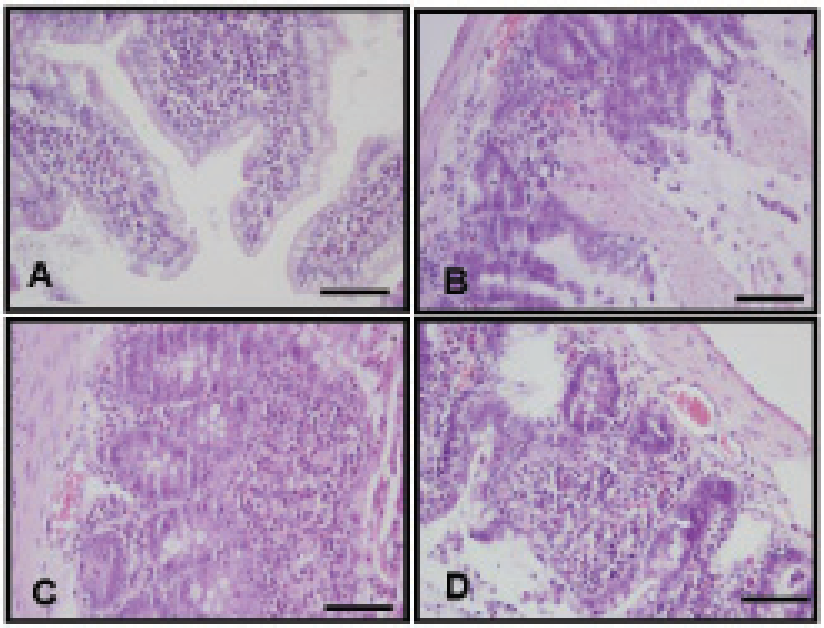

Figure 3. Photomicrographs of the jejunal tissue stained by the hematoxylin and eosin in sham-operated animals, vehicle-treated I/R animals, and I/R animals treated with EPO five minutes before ischemia and at the onset of reperfusion. (A) Sham: Histological features of normal jejunal tissue were observed, (B) I/R: The villi are denuded to the level of the lamina propria and dilated capillaries, (C) EPO + I/R: The villi are preserved (D) I + EPO + R: Erosion of the surface epithelium while the architecture of the villi are preserved. Sham; intestinal I/R was not induced, I/R; intestinal I/R, EPO + I/R; rHuEPO administered five minutes before ischemia, I + EPO + R; rHuEPO administered onset of reperfusion (40×).

Effects of rHuEPO on Histological Changes in the Intestinal Tissue Subjected to I/R in Rats

H&E staining was carried out to determine the histological changes in the jejunal tissue. Histological evaluation was performed according to the Chiu scoring method. Data related to scoring obtained by means of H&E staining as well as the microphotographs are shown in Figure 2 and Figure 3. As expected, no mucosal injury was observed in the sham group. According to the Chiu scoring system, the injury in the I/R group was found to have increased compared with the sham group (3 ± 0.36 vs 0.4 ± 0.24, $P < 0.01$). It was determined that rHuEPO administered both before ischemia and at the onset of reperfusion significantly prevented the mucosal injury caused by I/R (EPO + I/R 1 ± 0.51, $P < 0.01$ and I + EPO + R 1.16 ± 0.4 vs I/R 3 ± 0.36, $P < 0.05$, respectively).

Effects of rHuEPO on the Apoptotic Changes in the Intestinal Tissue Subjected to I/R in Rats

Localization of apoptotic cells in the jejunal tissue was made using the TUNEL staining method. The number of apoptotic cells (TUNEL-positive cells) is shown in Figure 4. Microphotographs of apoptotic cells stained by the TUNEL method are shown in Figure 5. Fewer TUNEL-positive cells were observed in the sham group (4.6 ± 1.2). Compared with the sham, the numbers of TUNEL-positive cell were found to have increased in the I/R group (20.4 ± 2.6 vs 4.6 ± 1.2, $P < 0.001$). However, a decrease in the numbers of TUNEL-positive cell was observed in rHuEPO administered in groups both before ischemia and at the onset of reperfusion groups (EPO + I/R 9.2 ± 2.7 and I + EPO + R 9.1 ± 3 vs I/R 20.4 ± 2.6, $P < 0.01$).

Effects of rHuEPO on the eNOS Expression in the Intestinal Tissue Subjected to I/R in Rats

We used immunohistochemical staining to localize eNOS expression. Immunohistochemical staining was scored in a semiquantitative manner to determine the differences between the groups in the distribution patterns of intensity of eNOS immunolabelling of the intestinal tissue (Figure 6). The intensity of the staining was recorded as mild (1), moderate (2), strong (3), and very strong (4). Microphotographs of eNOS immunoreactivity in the jejunal tissue are shown in Figure 7. According to the scoring system, the intensity of eNOS immunolabelling in the I/R group was also found to have increased compared with the sham group (3 ± 0.4 vs 1.3 ± 0.33, $P < 0.05$). However, it was determined that rHuEPO administered both before ischemia and at the onset of reperfusion sig-

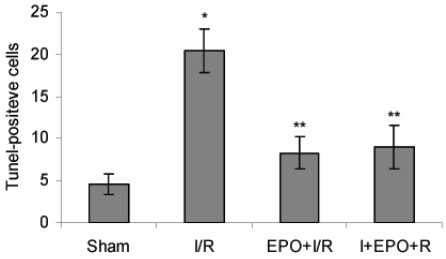

Figure 4. The number of apoptotic cells in the jejunal tissue determined by TUNEL staining in sham-operated animals, vehicle-treated I/R animals, and I/R animals treated with EPO five minutes before ischemia and at the onset of reperfusion. Results are expressed as mean TUNEL positive nuclei/observed field ± S.E.M. (n = 5–7 per group). *$P < 0.001$ vs sham; **$P < 0.01$ vs vehicle-treated I/R animals (one-way ANOVA followed by Tukey's post-test). Note that there is a significant decrease in the TUNEL-positive cells in EPO treated I/R animals compared with the vehicle treated I/R animals. Sham; intestinal I/R was not induced, I/R; intestinal I/R, EPO + I/R; rHuEPO administered five minutes before ischemia, I + EPO + R; rHuEPO administered onset of reperfusion.

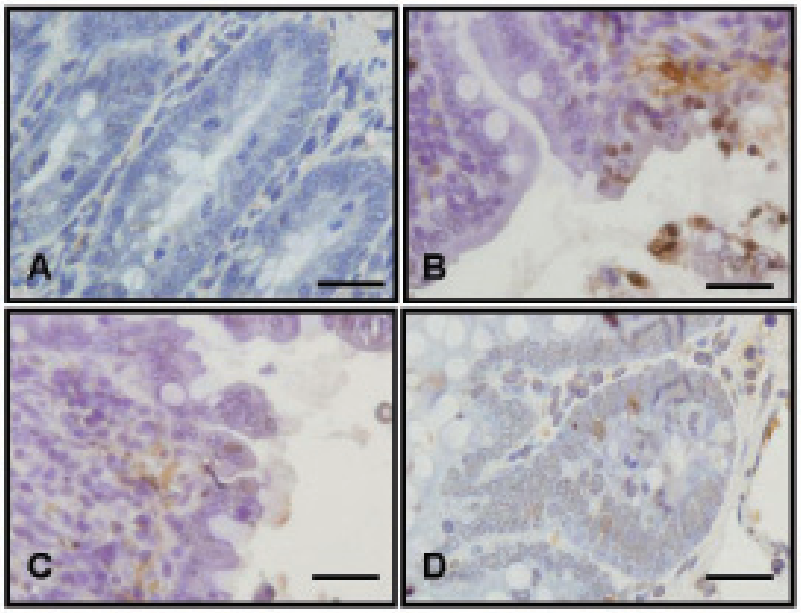

Figure 5. Photomicrographs of apoptotic cells in the jejunal tissue stained by the TUNEL method in sham-operated animals, vehicle-treated I/R animals, and I/R animals treated with EPO five minutes before ischemia and at the onset of reperfusion. The dark brown dots correspond to representative TUNEL-positive nuclei. (A) Sham: Intestinal I/R was not induced, (B) I/R: Intestinal I/R, (C) EPO + I/R: rHuEPO administered five minutes before ischemia, (D) I + EPO + R: rHuEPO administered onset of reperfusion (200×).

nificantly decreased (EPO + I/R 1.6 ± 0.24, $P < 0.05$ and I + EPO + R 1.4 ± 0.24 vs I/R 3 ± 0.4, $P < 0.01$, respectively).

DISCUSSION

Given the fact that histological assessment made using a microscopic scoring system has been accepted as a good standard in the evaluation of I/R injury in the intestinal tissue (23,24), the present study has established that a high single dose of rHuEPO administered both before ischemia and at the onset of reperfusion protected the intestinal tissue against I/R injury in rats. Data from the present study demonstrate that antiapoptotic, antioxidative, and antiinflammatory properties seem to be related to the EPO-mediated protective effect against intestinal I/R injury. The present study, to the best of our information, is the first study demonstrating the effects of rHuEPO in preventing the I/R-induced intestinal injury.

Oxidative stress plays an important role in the intestinal I/R injury. In the intestinal tissue subjected to I/R, activated neutrophils induce tissue injury through the production and release of ROS and cytotoxic proteins (for example, proteases, MPO, lactoferrin) into the extracellular fluid, constituting the inflammatory cascades that trigger the radical-induced I/R injury (3,25,26). MPO activity is commonly used to measure the extent of inflammation in intestinal tissues subjected to I/R injury (3). In the present study, intestinal I/R caused an elevation in tissue MPO activity, indicating the presence of enhanced leukocyte recruitment in the inflamed tissue, while the increased intestinal MDA level, an indicator of lipid peroxidation, verified the oxidative damage in the intestinal tissue.

Some researchers reported the antiinflammatory properties of EPO against I/R induced tissue injury (9,19,27). The present study determined that the MPO activity decreased with the application of rHuEPO, but a significant decrease was observed in the group in which rHuEPO was administered at the onset of reperfusion. The inhibition of neutrophil recruitment into the tissue is reflected by the partial capacity of rHuEPO to reverse the neutropoenia observed during reperfusion.

ROS are potent oxidizing agents, the damage of cellular membranes by lipid peroxidation being a major consequence. Previous studies established that EPO inhibits lipid peroxidation in the oxidative damage induced by in vitro (28,29) and in vivo (17,30,31) models. These studies also established that EPO inhibited lipid peroxidation induced by strong hydroxyl radicals (·OH) formed by iron-mediated Fenton reaction. In the present study, it was found that rHuEPO administered at the onset of both ischemia and reperfusion caused a decrease in MDA levels, but a really significant decrease was observed in the group in which rHuEPO was administered at the onset of reperfusion. The present study as well as a number of other previously conducted

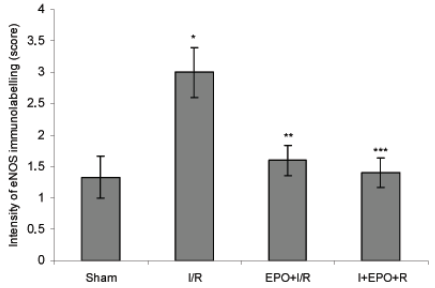

Figure 6. The intensity of eNOS immunolabelling evaluated by scoring system in sham-operated animals, vehicle-treated I/R animals, and I/R animals treated with EPO five minutes before ischemia and at the onset of reperfusion. Grades of score: mild (1), moderate (2), strong (3), and very strong (4). Data are mean ± S.E.M. (n = 3–5 per group). *$P < 0.05$ vs sham; **$P < 0.05$ vs vehicle-treated I/R animals; ***$P < 0.01$ vs vehicle-treated I/R animals (one-way ANOVA followed by Tukey's post-test). Note that there is a significant decrease in EPO groups compared with the I/R group. Sham; intestinal I/R was not induced, I/R; intestinal I/R, EPO + I/R; rHuEPO administered five minutes before ischemia, I + EPO + R; rHuEPO administered onset of reperfusion.

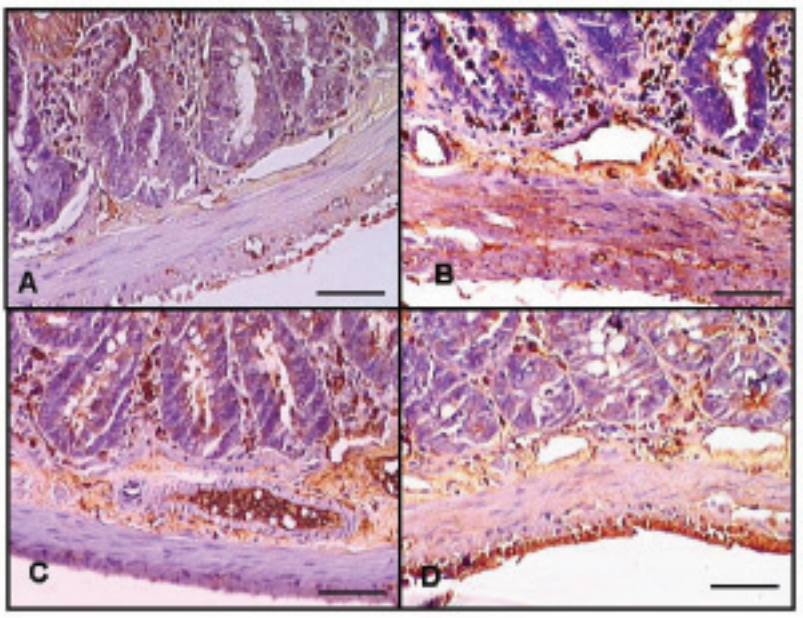

Figure 7. Immunolabelling of endothelial nitric oxide synthase (eNOS) in the jejunal tissue in sham-operated animals, vehicle-treated I/R animals, and I/R animals treated with EPO five minutes before ischemia and at the onset of reperfusion. Note that mild immunolabelling of eNOS is detected in jejunal specimens from sham animals, strong immunoreactivity is detected in I/R animals and moderate immunoreactivity is detected in rHuEPO administered groups. (A) Sham: Intestinal I/R was not induced, (B) I/R: Intestinal I/R, (C) EPO + I/R: rHuEPO administered five minutes before ischemia, (D) I + EPO + R: rHuEPO administered onset of reperfusion (40×).

researches demonstrated that EPO had a direct antioxidant function by scavenging ROS from the environment. EPO is a glycoprotein hormone containing approximately 30 percent carbohydrate, 11 percent sialic acid, 11 percent total hexose, and 8 percent *N*-acetylglucosamine. Cross et al (1984) has shown that small glycopolypeptides are powerful ·OH scavengers (32). Such scavenging action, as they have pointed out, is to be expected from the high sugar content of the glycopolypeptides. Thus, protection by EPO may be mediated through the scavenging action of its sugar moiety. Mechanistic studies suggest that Bcl-2 might be mediated in the antioxidant activity of EPO (33).

There are many studies revealing the association of EPO with the antioxidant system. These studies showed that EPO increased the activity of antioxidant enzymes, such as SOD, CAT, and GSH-Px (28,29,34–37). In the present study, it also was found that rHuEPO administered prior to ischemia significantly elevated the level of CAT activity when compared with the I/R group. In conformity with the results obtained from previous studies on the antioxidant system, we found that EPO might be capable of increasing the activity of CAT or restoring this enzyme, which decreases due to I/R in the intestinal tissue. Findings obtained from the present study and a handful of previously conducted studies have verified that EPO might be capable of acting as a direct antioxidant as well by activating antioxidant defense mechanisms.

Apoptosis, known as programmed cell death, is a form of cell death that serves to eliminate dying cells in proliferating or differentiating cell populations (38). However, activation of apoptosis in pathologic states results in rapid and extensive cell death with consequent tissue dysfunction. Previous studies reported that apoptosis is a major mode of cell death in the intestinal damage induced by I/R (39,40). In the present study, the number of TUNEL-positive cells as an indicator of apoptosis increased significantly after 30 min ischemia followed by 60 min reperfusion in the jejunal tissue. In previous studies dealing with I/R injury, EPO enhanced functional and morphologic tissue recovery, mainly through its antiapoptotic action. For example, rHuEPO was found to be effective in reducing the number of TUNEL-positive cells in I/R-induced injury in the heart (41). Sharples et al (2004) determined that rHuEPO reduced the number of TUNEL-positive cells prior to both ischemia and reperfusion in renal I/R injury (16). In the present study, an increased number of TUNEL-positive cells in the jejunal tissue subjected to I/R was also reduced by rHuEPO. Despite well-characterized reductions in apoptosis after EPO treatment, the mechanisms mediating the effects of EPO are not yet fully understood. It was reported that

Jak2-STAT-Bcl-2 pathway are involved in mediating the antiapoptotic effect of EPO (42).

Oxidative stress is known to induce apoptosis by damaging DNA, oxidizing membrane lipids, and/or directly activating the expression of the genes/proteins responsible for apoptosis (43–45). Kojima et al (2003) have reported that oxidative stress after I/R plays an important role in induction of apoptosis in the intestinal mucosa (46). In the present study, increased oxidative stress in intestinal I/R may be responsible for apoptosis. Therefore, the inhibitory effect of EPO on the I/R-induced ROS production may be the underlying mechanism for its protective effect against apoptosis.

Although NO is an important signaling molecule in physiological processes, its protective or detrimental role in intestinal I/R injury is still controversial. It has been reported that endothelial nitric oxide synthase (eNOS)-derived NO may have a protective role at the onset of I/R of the small intestine (47). However, evidence also suggests that eNOS can become "dysfunctional" during oxidative stress. It has been demonstrated that superoxide anions may react with NO released by eNOS and thereby turn into cytotoxic oxidant peroxynitrite (48). Therefore, an altered function of eNOS may play a role in intestinal I/R. In the present study, the 30 min ischemia followed by 60 min reperfusion increased eNOS expression in the jejunal tissue. These results show that intestinal I/R injury may be related to increased NO production associated with eNOS, producing peroxynitrite. However, our study showed that increased eNOS expression induced by I/R decreased with rHuEPO treatment, thus it might be reducing peroxynitrite, and causing enhanced intestinal I/R injury. These results demonstrate that inhibitory effect of EPO on the eNOS-mediated NO overproduction may be the underlying mechanism for its protective effect against intestinal I/R injury. However, these results contradict a recent study which has demonstrated that EPO increases eNOS expression in cardiomyocyte in both in vitro and in vivo models of I/R (49). On the other hand, previous studies in cultured endothelial cells indicated that EPO had little or no effect on increasing eNOS activity (50,51). Wang and Waziri (1999) reported that 24 h incubation of human coronary artery endothelial cells with EPO-inhibited NO production and eNOS expression (52). Calapai et al (2000) showed that increase of NO production in the hippocampus, as observed after ischemia, was reduced in animals treated with rHuEPO (53). Briefly, as reviewed by Li et al (2002), effects of EPO on eNOS expression are still controversial (54). Nevertheless, elucidation of the role of NO pathway in intestinal protection of EPO will need further investigations.

In conclusion, a single dose of EPO protects the intestinal tissue against I/R injury in rats, demonstrating antioxidant, anti-inflammatory, and antiapoptotic effects. Future experiments will be needed to precisely explore the EPO signaling pathways in the intestine to delineate the benefits of EPO therapy and incorporate its potential use into clinical practice in the future.

ACKNOWLEDGMENTS

This research was supported by grants from Dokuz Eylul University Research Foundation (Project 2005. KB. SAG.031).

REFERENCES

1. Granger DN, Korthuis RJ. (1995) Physiologic mechanisms of postischemic tissue injury. *Annu. Rev. Physiol*. 57:311–32.
2. Brandt, JL. (2003) Mesenteric vascular disease. Current Diagnosis and Treatment in Gastroenterology. New York: Section 1.9.
3. Mallick IH, Yang W, Winslet WC, Seifalian AM. (2004) Ischemia–Reperfusion Injury of the Intestine and Protective Strategies Against Injury. *Dig. Dis. Sci*. 49:1359–77.
4. Erbayraktar S et al. (2006) Carbamylated erythropoietin reduces radiosurgically-induced brain injury. *Mol. Med*. 12:74–80.
5. Siren AL, Ehrenreich H. (2001) Erythropoietin—a novel concept for neuroprotection. *Eur. Arch. Psychiatry Clin. Neurosci*. 25:179–84.
6. Celik M et al. (2002) Erythropoietin prevents motor neuron apoptosis and neurologic disability in experimental spinal cord ischemic injury. *Proc. Natl. Acad. Sci*. 99:2258–63.
7. Erbayraktar S et al. (2003) Asialoerythropoietin is a nonerythropoietic cytokine with broad neuroprotective activity in vivo. *Proc. Natl. Acad. Sci*. 100:6741-6746.
8. Erbayraktar S, Yilmaz O, Gokmen N, Brines M. (2003) Erythropoietin is a multifunctional tissue-protective cytokine. *Curr. Hematol. Rep*. 2:465-470.
9. Contaldo C et al. (2007) Human recombinant erythropoietin protects the striated muscle microcirculation of the dorsal skinfold from postischemic injury in mice. *Am. J. Physiol. Heart. Circ. Physiol*. 293:274-283.
10. Brines M et al. (2004) Erythropoietin mediates tissue protection through an erythropoietin and common beta-subunit heteroreceptor. *Proc. Natl. Acad. Sci*. 101:14907–12.
11. Juul SE et al. (1999) Why is erythropoietin present in human milk? Studies of erythropoietin receptors on enterocytes of human and rat neonates. *Pediatr. Res*. 46: 263–8.
12. Cuzzocrea S et al. (2004) Erythropoietin reduces the development of experimental inflammatory bowel disease. *J. Pharmacol. Exp. Ther*. 311:1272–80.
13. Ledbetter DJ, Juul SE. (2000) Erythropoietin and the incidence of necrotizing enterocolitis in infants with very low birth weight. *J. Pediatr. Surg*. 35:178–82.
14. Fatouros M et al. (1999) Alterations in body weight, breaking strength, and wound healing in wistar rats treated pre- and postoperatively with erythropoietin or granulocyte macrophage-colony stimulating factor: Evidence of a previously unknown anabolic effect of erythropoietin? *J. Lab. Clin. Med*. 133:253–9.
15. Lipsic E et al. (2004) Timing of erythropoietin treatment for cardioprotection in ischemia-reperfusion. *J. Cardiovasc. Pharmacol*. 44:473–9.
16. Sharples EL et al. (2004) Erythropoietin protects the kidney against the injury and dysfunction caused by ischemia-reperfusion. *J. Am. Soc. Nephrol*. 15:2115–24.
17. Solaroglu A, Dede FS, Okutan E, Bayrak A, Haberal A, Kilinc K. (2004) A single dose of erythropoietin attenuates lipid peroxidation in experimental liver ischemia–reperfusion injury in the rat fetus. *J. Matern. Fetal. Neonatal. Med*. 16:231–4.
18. Wu H et al. (2006) Pretreatment with recombined human erythropoietin attenuates ischemia—reperfusion-induced lung injury in rats. *Eur. J. Cardiothorac. Surg*. 29:902–7.
19. Villa P et al. (2003) Erythropoietin selectively attenuates cytokine production and inflammation in cerebral ischemia by targeting neuronal apoptosis. *J. Exp. Med*. 198:971–5.
20. Junk AK et al. (2002) Erythropoietin administration protects retinal neurons from acute ischemia-reperfusion injury. *Proc. Natl. Acad. Sci*. 99:10659–64.
21. Tatum VL, Changchit C, Chow CK. (1990) Measurement of malonaldehyde by HPLC with fluorescence detection. *Lipids* 25:226–9.
22. Wiechelman KJ, Braun RD, Fitzpatrick JD. (1988) Investigation of the bicinchoninic acid protein

assay: identification of the groups responsible for color formation. *Anal. Biochem.* 175:231–7.
23. Chiu CJ, McArdle AH, Brown R, Scott HJ, Gurd FN. (1970) Intestinal mucosal lesion in low-flow states. I. A morphologic, hemodynamic and metabolic reappraisal. *Arch. Surg.* 101:478.
24. Park PO, Haglund U, Bulkley GB, Falt K. (1990) The sequence of development of intestinal tissue injury following strangulation ischemia and reperfusion. *Surgery.* 107:574.
25. Zimmerman BJ, Granger DN. (1994) Mechanisms of reperfusion injury. *Am. J. Med. Sci.* 307:284–92.
26. Kettle AJ, Winterbourn CC. (1997) Myeloperoxidase: a key regulator of neutrophil oxidant production. *Redox. Rep.* 3:3–15.
27. Liu X et al. (2006) Mechanism of the cardioprotection of rhEPO pretreatment on suppressing the inflammatory response in ischemia–reperfusion. *Life Sci.* 78:2255–64.
28. Chattonadhyay T, Das C, Bandyopadhyay D, Datta AG. (2000) Protective effect of erythropoietin on the oxidative damage of erythrocyte membrane by hydroxyl radical. *Biochem. Pharmacol.* 59:419–25.
29. Chakraborty M, Ghosal J, Biswas T, Data AG. (1988) Effect of erythropoietin on membrane lipid peroxidation, superoxide dismutase, catalase, and glutathione peroxidase of rat RBC. *Biochem. Med. Metab. Biol.* 40:8–18.
30. Akisu M, Kullahcioglu GF, Baka M, Husseyinov A, Kultursay N. (2001a) The role of recombinant human erythropoietin in lipid peroxidation and platelet-activating factor generation in a rat model of necrotizing enterocolitis. *Eur. J. Pediatr. Surg.* 11:167–72.
31. Akisu M, Tuzun S, Arslanoglu S, Yalaz M, Kultursay N. (2001b) Effect of recombinant human erythropoietin administration on lipid peroxidation and antioxidant enzyme(s) activities in preterm infants. *Acta. Med. Okayama.* 55:357–62.
32. Cross CE, Halliwell B, Allen A. (1984) Antioxidant protection: A function of tracheobronchial and gastrointestinal mucus. *Lancet.* 1:1328–30.
33. Digicaylioglu M, Lipton SA. (2001) Erythropoietin-mediated neuroprotection involves cross-talk between Jak2 and NF-κB signaling cascades. *Nature.* 412:641–7.
34. Genc S, Akhisaroglu M, Kuralay F, Genc K. (2002) Erythropoietin restores glutathione peroxidase activity in 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine-induced neurotoxicity in C57BL mice and stimulates murine astroglial glutathione peroxidase production in vitro. *Neurosci. Lett.* 321:73–76.
35. Kumral A et al. (2005) Protective effects of erythropoietin against ethanol-induced apoptotic neurodegeneration and oxidative stress in the developing C57BL/6 mouse brain. *Brain Res. Dev. Brain Res.* 160:146–56.
36. Sakanaka M et al. (1998) In vivo evidence that erythropoietin protects neurons from ischemic damage. *Proc. Natl. Acad. Sci.* 95:4635–40.
37. Turi S, Nemeth I, Varga I, Bodrogi T, Matkovics B. (1992) The effect of erythropoietin on the cellular defense mechanism of red blood cells in children with chronic renal failure. *Pediatr. Nephrol.* 6:536–41.
38. Kerr JF, Wyllie AH, Currie AR. (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer.* 26: 239–57.
39. Ikeda H et al. (1998) Apoptosis is a major mode of cell death caused by ischaemia and ischaemia/reperfusion injury to the rat intestinal epithelium. *Gut.* 42:530–7.
40. Noda T, Iwakiri R, Fujimoto K, Matsuo S, Aw TY. (1998) Programmed cell death induced by ischemia–reperfusion in rat intestinal mucosa. *Am. J. Physiol.* 274:270–6.
41. Parsa CJ et al. (2004) Cardioprotective effects of erythropoietin in the reperfused ischemic heart: a potential role for cardiac fibroblasts. *J. Biol. Chem.* 279:20655–62.
42. Brines M and Cerami A. (2006) Discovering erythropoietin's extra-hematopoietic functions: biology and clinical promise. *Kidney Int.* 70:246–50.
43. Bai J, Cederbaum AI. (2001) Mitochondrial catalase and oxidative injury. *Biol. Signals Recept.* 10: 189–99.
44. Buttke TM, Sandstrom PA. (1994) Oxidative stress as a mediator of apoptosis. *Immunol. Today.* 15:7–10.
45. Curtin JF, Donovan M, Cotter TG. (2002) Regulation and measurement of oxidative stress in apoptosis. *J. Immunol. Methods.* 265:49–72.
46. Kojima M et al. (2003) Effects of antioxidative agents on apoptosis induced by ischaemia-reperfusion in rat intestinal mucosa. *Aliment Pharmacol. Ther.* 1:139–45.
47. Roviezzo F et al. (2007) Protective role of PI3-kinase-Akt-eNOS signaling pathway in intestinal injury associated with splanchnic artery occlusion shock. *Br. J. Pharmacol.* 151:377–83.
48. Munzel T, Daiber A, Ullrich V, Mulsch A. (2005) Vascular consequences of endothelial nitric oxide synthase uncoupling for the activity and expression of the soluble guanylyl cyclase and the cGMP-dependent protein kinase. *Arterioscl. Thromb. Vasc. Biol.* 25:1551–7.
49. Rui T et al. (2005) Erythropoietin prevents the acute myocardial inflammatory response induced by ischemia/reperfusion via induction of AP-1. *Cardiovasc. Res.* 65:719–27.
50. Lopez Ongil SL, Saura M, Lamas S, Rodriguez Puyol M, Rodriguez Puyol D. (1996) Recombinant human erythropoietin does not regulate the expression of endothelin-1 and constitutive nitric oxide synthase in vascular endothelial cells. *Exp. Nephrol.* 4:37–42.
51. Banerjee D, Rodriguez M, Nag M, Adamson JW. (2000) Exposure of endothelial cells to recombinant human erythropoietin induces nitric oxide synthase activity. *Kidney Int.* 57:1895–904
52. Wang XQ, Vaziri ND. (1999) Erythropoietin depresses nitric oxide synthase expression by human endothelial cells. *Hypertension.* 33:894–9
53. Calapai MC et al. (2000) Erythropoietin protects against brain ischemia injury by inhibition of nitric oxide formation, *Eur. J. Pharmacol.* 401:349–356.
54. Li H, Wallerath T, Münzel T, Förestermann U. (2002) Regulation of endothelial-type NO synthase expression in pathophysiology and in response to drugs, *Nitric Oxide.* 7:149–64.