Attenuation of Inflammation and Apoptosis by Pre- and Posttreatment of Darbepoetin- α in Acute Liver Failure of Mice

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In many liver disorders inflammation and apoptosis are important pathogenic components, finally leading to acute liver failure. Erythropoietin and its analogues are known to affect the interaction between apoptosis and inflammation in brain, kidney, and myocardium. The present study aimed to determine whether these pleiotropic actions also exert hepatoprotection in a model of acute liver injury. C57BL/6J mice were challenged with D-galactosamine (Gal) and *Escherichia coli* lipopolysaccharide (LPS) and studied 6 hours thereafter. Animals were either pretreated (24 hours before Gal-LPS exposure) or posttreated (30 minutes after Gal-LPS exposure) with darbepoetin- α (DPO, 10 μ g/kg i.v.). Control mice received physiological saline. Administration of Gal-LPS caused systemic cytokine release and provoked marked hepatic damage, characterized by leukocyte recruitment and microvascular perfusion failure, caspase-3 activation, and hepatocellular apoptosis as well as enzyme release and necrotic cell death. DPO-pretreated and -posttreated mice showed diminished systemic cytokine concentrations, intrahepatic leukocyte accumulation, and hepatic perfusion failure. Hepatocellular apoptosis was significantly reduced by 50 to 75% after DPO pretreatment as well as posttreatment. In addition, treatment with DPO also significantly abrogated necrotic cell death and liver enzyme release. In conclusion, these observations may stimulate the evaluation of DPO as hepatoprotective therapy in patients with acute liver injury. (*Am J Pathol* 2007, 170:1954–1963; DOI: 10.2353/ajpath.2007.061056)

lenge.¹ This illness can rapidly progress to coma and death from cerebral edema and multiorgan dysfunction.² Prognosis of ALF depends not only on primary hepatic injury but also on complications that occur during the course of disease. This also includes major liver surgery and liver transplantation, which are prone to complications by endotoxemia and sepsis.^{3,4} Infection receives particular attention because it has been established that patients with ALF are abnormally susceptible to infectious pathogens as a result of multiple immunological defects.⁵ In line with this, established bacterial infections are reported to occur in ~80% of cases.⁶ Within the extremely complex cascade of pathophysiological events, representing the systemic inflammatory response, great attention has recently been brought to the contribution of apoptosis in organ dysfunction and failure.^{7,8} Hepatocellular apoptosis represents not only a crucial step in ALF but functions also as a signal for leukocyte migration and attack on parenchymal cells,^{9,10} thereby establishing a vicious circle with aggravation of leukocytic inflammation and final cell death. It has been shown that preventing apoptosis in hepatocytes by caspase inhibition suppresses leukocyte transmigration and leukocyte-dependent liver cell necrosis.¹⁰ Although controversies still exist regarding which mode of cell death, apoptosis or necrosis, predominates in various forms of acute liver injury, it becomes evident that treatment strategies should target downstream consequences of inflammatory activation.¹¹ Thereby, pleiotropic agents, exerting multiple functions, may be of particular interest because of their broad potential to interfere with relevant pathways of disease.

Erythropoietin (EPO), initially known as renal glycoprotein hormone and which promotes the survival, proliferation, and differentiation of erythrocytic progenitors in hematopoietic tissues, has meanwhile been recognized

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Acute liver failure (ALF) is a gastrohepatointestinal emergency that continues to be a huge therapeutic chal-

as an anti-apoptotic and tissue-protective pleiotropic cytokine.^{12–14} Recent studies have identified multiple paracrine/autocrine functions of EPO and its long-acting analogue darbepoetin- α (DPO) that coordinate local responses to injury by attenuating both apoptotic and inflammatory causes of cell death in brain, kidney, and myocardium.^{15–17} Encouraged by these reports, we determined whether DPO is protective in ALF by pre- and posttreating mice exposed to D-galactosamine (Gal) and *Escherichia coli* lipopolysaccharide (LPS).

Materials and Methods

Animal Model and Experimental Groups

Male C57BL/6J mice (Charles River Laboratories, Sulzfeld, Germany) were used at 8 to 10 weeks of age with a body weight of ~20 g. Animals were kept on water and standard laboratory chow *ad libitum*. All animals received humane care according to the German legislation on protection of animals and the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication 86-23, revised 1985).

For induction of acute liver injury, animals were injected with Gal (720 mg/kg body weight i.p.; Sigma-Aldrich, Taufkirchen, Germany) and LPS (10 μ g/kg body weight i.p., serotype 0128:B12; Sigma-Aldrich). Concentrations of Gal and LPS were used in accordance with previously published work.^{18–20} Via retro-orbital vein puncture, animals were treated with DPO (10 μ g/kg body weight, Aranesp; Amgen Europe, Breda, The Netherlands) at either 24 hours before induction of acute liver injury ($n = 7$, Gal-LPS/DPO_{pre}) or 30 minutes after induction of liver injury ($n = 7$, Gal-LPS/DPO_{post}). Physiological saline-treated animals with liver injury served as Gal-LPS controls ($n = 7$, Gal-LPS). Sham-treated animals, without induction of acute liver injury and receiving only isotonic saline, served as sham controls ($n = 7$, control). Six hours after Gal-LPS exposure, *in vivo* analysis of the hepatic microcirculation as well as blood and tissue sampling were performed in the four groups mentioned above.

To achieve specific information on the relation between necrosis, apoptosis, inflammation, and tissue perfusion, we studied the time course of events by means of *in vivo* fluorescence microscopy in additional sets of saline-treated animals and animals with posttreatment of DPO at 2 and 4 hours after Gal-LPS exposure ($n = 5$ animals per time point and group each). These animals further served for sampling of blood and liver tissue for subsequent analysis (see below).

Intravital Fluorescence Microscopy

For *in vivo* analysis of the hepatic microcirculation 2, 4, and 6 hours after Gal-LPS exposure, ketamine/xylazine-anesthetized animals (75/25 mg/kg body weight i.p.) were placed in supine position on a heating pad for maintenance of body temperature at 36 to 37°C. Polyethylene catheters (PE 50, ID 0.28 mm; Smiths Medical

International Ltd., Kent, UK) in the left carotid artery and jugular vein served for continuous monitoring of hemodynamics and injection of fluorescent dyes. After transverse laparotomy, the animals were positioned on their left side, and the left liver lobe was exteriorized and covered with a glass slide for intravital fluorescence microscopy. Using a Zeiss fluorescence microscope equipped with a 100 W mercury lamp and different filter sets for blue, green, and UV epi-illumination (Axiotech Vario; Zeiss, Jena, Germany), microscopic images were taken by water immersion objectives ($\times 20/0.50W$, $\times 40/0.8W$; Zeiss), televised using a charge-coupled device video camera (FK 6990A-IQ; Pieper, Berlin, Germany), and recorded on videotape for subsequent off-line evaluation.⁹

Blood perfusion within individual sinusoids was studied after tissue contrast enhancement by sodium fluorescein (2 μ mol/kg body weight i.v.; Merck, Darmstadt, Germany) and blue light epi-illumination (450 to 490/ >520 nm, excitation/emission wavelength).⁹ *In vivo* labeling of leukocytes with rhodamine-6G (1 μ mol/kg body weight i.v.; Merck) and green light epi-illumination (530 to 560/ >580 nm) enabled quantitative analysis of intrahepatic leukocyte flow behavior.⁹ For analysis of apoptotic cell death, *in vivo* staining of hepatocellular nuclei was achieved by intravenous injection of bisbenzimidazole (Hoechst 33342, 10 μ mol/kg; Sigma-Aldrich) and UV epi-illumination (330 to 380/ >415 nm).^{9,21}

Assessment of hepatic microcirculatory parameters was performed off-line by frame-to-frame analysis of the videotaped images at magnifications of 424- and 823-fold, using a computer-assisted image analysis system with a 19-inch monitor (CapImage; Zeintl, Heidelberg, Germany). Within 10 acini and postsinusoidal venules per animal, microcirculatory analysis included the determination of sinusoidal perfusion, representing the number of perfused sinusoids in percentage of all sinusoids visible as well as the number of adherent leukocytes, located within postsinusoidal venules (given as cells/mm² endothelial surface, calculated from diameter and length of vessel segment studied, assuming cylindrical geometry) and not moving during an observation period of 20 seconds.⁹ Apoptotic cell death was analyzed within 10 lobules per animal by counting the number of cells that showed apoptosis-associated condensation, fragmentation, and crescent-shaped formation of chromatin (given as cells/mm² observation field).^{9,21}

Sampling and Assays

After *in vivo* microscopy, animals were exsanguinated by puncture of the vena cava inferior for immediate separation of ethylenediaminetetraacetic acid plasma. Aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, and glutamate dehydrogenase activities were measured spectrophotometrically as indicators for hepatocellular disintegration and necrosis. Ethylenediaminetetraacetic acid plasma further served for analysis of tumor necrosis factor (TNF)- α and interleukin (IL)-6, using commercially available enzyme-linked immunosorbent assay kits in accordance with the manufacturer's instruc-

tions (Pierce Biotechnology, Rockford, IL). Liver tissue was sampled for subsequent Western blot protein analysis, histology, and immunohistochemistry.

Histology and Immunohistochemistry

Liver tissue was fixed in 4% phosphate-buffered formalin for 2 to 3 days and then embedded in paraffin. From the paraffin-embedded tissue blocks, 4- μ m sections were cut and stained with hematoxylin and eosin (H&E). Necrosis was assessed by morphological features, such as vacuolization, swollen cytoplasm with disrupted cell and organelle membranes, as well as lytic nuclear changes.²² The percentage of necrosis was estimated by evaluating the number of microscopic fields with necrosis compared with the entire histological section.²³ To evaluate hepatocyte replication, mitotic figures were counted in 1000 hepatocytes (400-fold magnification) and given as mitotic index (number of mitotic figures per 1000 hepatocytes).²⁴

For the immunohistochemical study of cleaved caspase-3, 4- μ m sections of paraffin-embedded specimens were incubated overnight at 4°C with a rabbit polyclonal cleaved caspase-3 antibody (1:500, 9661 lot 15; Cell Signaling Technology, Frankfurt, Germany). This antibody detects endogenous levels of the large fragment (17/19 kd) of activated caspase-3 but not full-length caspase-3.²⁵ For the development of cleaved caspase-3, a biotinylated anti-rabbit immunoglobulin antibody was used as a secondary antibody for streptavidin-biotin complex peroxidase staining (1:20, horseradish peroxidase, P 0448; DakoCytomation, Hamburg, Germany). 3,3'-Diaminobenzidine (S 3000; DakoCytomation) was used as chromogen. The sections were counterstained with hemalaun. Cleaved caspase-3-positive hepatocytes were counted within 30 consecutive fields ($\times 40$ objective/0.65 numeric aperture) and are given as cells per mm².

Western Blot Analysis

For Western blot analysis of protein levels of cleaved caspase-3, Bcl-X_L, Bax, proliferating cell nuclear antigen (PCNA), and phosphorylated endothelial nitric-oxide synthase (phospho-eNOS^{Ser1177}), liver tissue was homogenized in lysis buffer (10 mmol/L Tris, pH 7.5, 10 mmol/L NaCl, 0.1 mmol/L ethylenediaminetetraacetic acid, 0.5% Triton X-100, 0.02% NaN₃, and 0.2 mmol/L phenylmethyl sulfonyl fluoride), incubated for 30 minutes on ice, and centrifuged for 15 minutes at 10,000 $\times g$. The supernatant was saved as whole protein fraction. Before use, the buffer received a protease inhibitor cocktail (1:100 v/v; Sigma-Aldrich). Protein concentrations were determined using the bicinchoninic acid protein assay (Sigma-Aldrich) with bovine serum albumin as standard. Sixty μ g of protein/lane (cleaved caspase-3 and phospho-eNOS) or 20 μ g of protein/lane (Bcl-X_L, Bax, PCNA) were separated discontinuously on sodium dodecyl sulfate polyacrylamide gels (12%) and transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore,

Eschborn, Germany). After blockade of nonspecific binding sites, membranes were incubated for 2 hours at room temperature with a rabbit polyclonal anti-cleaved caspase 3 (Asp 175, 1:1000; Cell Signaling Technology), a mouse monoclonal anti-Bcl-X_L (1:1000; BD PharMingen, Heidelberg, Germany), a mouse monoclonal anti-Bax (1:250; BD PharMingen), a rabbit polyclonal anti-PCNA (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA), or a rabbit polyclonal anti-phospho-eNOS (1:500; Cell Signaling Technology), followed by secondary peroxidase-linked goat anti-rabbit or anti-mouse antibodies (cleaved caspase-3, 1:2000; PCNA, 1:8000; and phospho-eNOS, 1:2500; Cell Signaling Technology; Bcl-X_L, 1:10,000; and Bax, 1:20,000; Sigma-Aldrich). Protein expression was visualized by means of luminol-enhanced chemiluminescence (ECL Plus; Amersham Pharmacia Biotech, Freiburg, Germany) and exposure of the membrane to a blue light-sensitive autoradiography film (Kodak BioMax Light Film; Kodak-Industrie, Chalon-sur-Saone, France). Signals were densitometrically assessed (Quantity One, Gel Doc XR; Bio-Rad Laboratories GmbH, Munich, Germany) and normalized to the β -actin or β -tubulin (for phospho-eNOS only) signals (mouse monoclonal anti- β -actin antibody, 1:20,000; Sigma-Aldrich; or rabbit polyclonal anti- β -tubulin antibody, 1:500; Santa Cruz Biotechnology).

Statistical Analysis

All data are expressed as means $\pm$ SEM. After proving the assumption of normality and equal variance across groups, differences between groups were assessed using analysis of variance followed by the appropriate post hoc comparison test. Statistical significance was set at $P < 0.05$. Statistics were performed using the software package SigmaStat (Jandel Corp., San Rafael, CA).

Results

DPO Attenuates Intrahepatic Inflammation and Systemic Cytokine Concentrations after Gal-LPS Challenge

Analysis of hepatic microcirculation by fluorescence microscopy is given in Figure 1. In the Gal-LPS-treated mice, *in vivo* microscopy of the livers revealed characteristic features of acute injury, including severe sinusoidal perfusion failure of up to 40% and increased white blood cell accumulation with ~ 250 adherent leukocytes per mm² endothelial surface of postsinusoidal venules. DPO-pretreated and, in particular, DPO-posttreated animals exhibited an improvement of hepatic microvascular perfusion by $\sim 17\%$ and a 50% reduction of inflammatory leukocyte adherence, respectively (Figure 1).

Gal-LPS exposure was characterized by high concentrations of IL-6 when compared with control animals without liver injury. Pretreatment and, in particular, posttreatment with DPO was capable of significantly reducing systemic IL-6 concentrations (Table 1). At 6 hours after

Figure 1. Hepatic sinusoidal perfusion (left) and venular leukocyte adherence (right) in animals that were injected with Gal (720 mg/kg body weight i.p.) and LPS (10 μ g/kg body weight i.p.) for induction of acute liver injury and either pretreated (24 hours before Gal-LPS exposure) or posttreated (30 minutes after Gal-LPS exposure) with DPO (DPO_{pre} and DPO_{post}; 10 μ g/kg i.v.). Endotoxemic controls were treated with saline only (Gal-LPS). Sham-operated animals without liver injury served as controls (control). Values are given as means $\pm$ SEM; analysis of variance and post hoc comparison test; * P < 0.05 versus control; # P < 0.05 versus Gal-LPS.

Gal-LPS exposure, TNF- α levels were already well less than 100 pg/ml and did not differ between the four groups studied (Table 1).

DPO Ameliorates Apoptotic Cell Death and Caspase-3 Cleavage after Gal-LPS Challenge

Analysis of apoptotic cell death is given in Figures 2 and 3, including *in vivo* microscopy of hepatocellular apoptosis as well as immunohistochemistry and Western immunoblot for cleaved caspase-3. Compared with sham control animals, Gal-LPS induced a notable increase of hepatocellular apoptosis, as indicated by the $\sim$ 12-fold rise in the number of bisbenzimid-stained hepatocytes with nuclear chromatin condensation and fragmentation (Figure 2, left). In support of these *in vivo* data, immunohistochemistry (Figure 2, right) and Western immunoblot (Figure 3) revealed an intense activation of the effector caspase-3 in Gal-LPS-exposed liver tissue. Not only pre-

treatment but also posttreatment of DPO remarkably ameliorated the extent of apoptotic cell death to almost sham control values (Figures 2 and 3).

DPO Reduces Hepatic Morphological Damage and Liver Enzyme Release after Gal-LPS Challenge

H&E histopathology of Gal-LPS-exposed liver exhibited disruption of the general architecture, microvascular disintegration, as well as tissue apoptosis and necrosis (Figure 4). Morphological criteria, such as vacuolization, swollen cytoplasm with disrupted cell and organelle membranes, as well as lytic nuclear changes served to determine necrosis and revealed a substantial amount of necrosis with a fraction of 50% positive fields on Gal-LPS exposure (Table 1). In line with histopathology, liver injury caused a threefold to eightfold rise in transaminase levels when compared with saline exposed controls (Table 1). Sections obtained from DPO pre- and posttreated animals appeared different, often exhibiting grossly retained general architecture and lacking evidence of major morphological injury (Figure 4). Occasional sections mostly of the posttreated animals exhibited minor to moderate injury (Table 1). Accordingly, the fraction of observation fields encompassing necrotic tissue amounted to only 10 and 18% on DPO pre- and posttreatment, respectively. Concomitantly, transaminase levels were found significantly reduced in DPO pre- and posttreated animals but still exceeded control values (Table 1). Analysis of H&E-stained liver tissue sections for mitotic figures revealed no differences between groups exhibiting very low numbers of proliferating cells (<0.1% mitotic figures).

Time Course of Events after Gal-LPS Challenge and Effect of Posttreatment with DPO

To assess specific information on the relation between necrosis, apoptosis, inflammation, and tissue perfusion, we studied the time course of events at 2 and 4 hours after Gal-LPS exposure with and without DPO posttreat-

Table 1. Systemic Concentrations of TNF- α and IL-6 (pg/ml), Tissue Necrosis (%), and Transaminase Activities (U/L) in Animals That Were Injected with Gal (720 mg/kg Body Weight i.p.) and LPS (10 μ g/kg Body Weight i.p.) for Induction of Acute Liver Injury and Either Pretreated (24 Hours before Gal-LPS Exposure) or Posttreated (30 Minutes after Gal-LPS Exposure) with Darbepoetin (DPO_{pre} and DPO_{post}; 10 μ g/kg i.v.)

	Control	Gal-LPS		
			DPO _{pre}	DPO _{post}
TNF- α	40 $\pm$ 3	58 $\pm$ 4	46 $\pm$ 3	41 $\pm$ 9
IL-6	302 $\pm$ 60	4869 $\pm$ 228*	2734 $\pm$ 630*	1648 $\pm$ 934 [†]
Necrosis	0.7 $\pm$ 0.5	50 $\pm$ 10*	11 $\pm$ 7* [†]	18 $\pm$ 7* [†]
ALT	33 $\pm$ 4	267 $\pm$ 51*	92 $\pm$ 24* [†]	145 $\pm$ 76* [†]
AST	140 $\pm$ 13	453 $\pm$ 13*	249 $\pm$ 51* [†]	267 $\pm$ 91* [†]
LDH	1249 $\pm$ 266	3468 $\pm$ 295*	1627 $\pm$ 329 [†]	1439 $\pm$ 667 [†]
GLDH	15 $\pm$ 2	59 $\pm$ 11*	33 $\pm$ 6 [†]	33 $\pm$ 12 [†]

Control animals received isotonic saline only (control). For further information, please see Materials and Methods. Values are given as means $\pm$ SEM; analysis of variance and post hoc comparison test.

* P < 0.05 versus control.

[†] P < 0.05 versus Gal-LPS.

ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase; GLDH, glutamate dehydrogenase.

Figure 2. Representative images with quantitative analysis of apoptotic hepatocytes in animals that were injected with Gal (720 mg/kg body weight i.p.) and LPS (10 μ g/kg body weight i.p.) for induction of acute liver injury and either pretreated (24 hours before Gal-LPS exposure) or posttreated (30 minutes after Gal-LPS exposure) with DPO (DPO_{pre} and DPO_{post}; 10 μ g/kg i.v.). Endotoxic controls were treated with saline only (Gal-LPS). Sham-operated animals without liver injury served as controls (control). Hepatocellular apoptosis was assessed by either fluorescence microscopy and bis-benzimide staining (left) or immunohistochemistry for cleaved caspase-3 (right). Note the individual hepatocytes with condensation, fragmentation, and/or margination of nuclear chromatin as characteristic signs of apoptotic cell death on Gal-LPS exposure and the marked reduction of cell apoptosis in the images of Gal-LPS/DPO_{pre} animals, being also representative for those found in Gal-LPS/DPO_{post} animals. Values are given as means $\pm$ SEM; analysis of variance and post hoc comparison test; * P < 0.05 versus control; # P < 0.05 versus Gal-LPS. Original magnifications: $\times 823$ (left); $\times 1600$ (right).

ment. At the early time point of 2 hours, Gal-LPS exposure is characterized by a massive hepatic inflammatory response, as indicated by the huge number of leukocytes firmly interacting with the endothelium of postsinusoidal venules (Figure 5A), a remarkable impairment of hepatic perfusion with $\sim 20\%$ of nonperfused sinusoids (Figure 5B) and high levels of TNF- α and IL-6 (Table 2). Although very limited, apoptotic cell death was found already present (Figure 5C). At 4 hours, perfusion failure persisted and became even more pronounced at 6 hours with a concomitant rise of apoptotic and necrotic tissue injury (Figure 5, C and D).

Posttreatment with DPO was found to be capable of dampening each of the individual features of acute liver injury on Gal-LPS exposure (Figure 5, A–D; Table 2). Notably, DPO most effectively inhibited the early microcirculatory deterioration of the liver (Figure 5, A and B), most supposedly resulting in subsequent amelioration of tissue damage, as given by the significantly lower extent of tissue apoptosis and necrosis (Figure 5, C and D). In

Figure 3. Representative Western blot and densitometric analysis of cleaved caspase-3 protein levels in livers of animals that were injected with Gal (720 mg/kg body weight i.p.) and LPS (10 μ g/kg body weight i.p.) for induction of acute liver injury and either pretreated (24 hours before Gal-LPS exposure) or posttreated (30 minutes after Gal-LPS exposure) with DPO (DPO_{pre} and DPO_{post}; 10 μ g/kg i.v.). Endotoxic controls were treated with saline only (Gal-LPS). Sham-operated animals without liver injury served as controls (control). β -Actin served as loading control. Values are given as means $\pm$ SEM; analysis of variance and post hoc comparison test; * P < 0.05 versus control; # P < 0.05 versus Gal-LPS.

line with the above-mentioned data on liver cell apoptosis and necrosis, transaminase levels, as represented by alanine aminotransferase in Table 2, reflect the kinetics of liver tissue injury, being most and almost exclusively apparent at 6 hours after the Gal-LPS exposure.

Bcl-X_L, Bax, and PCNA Protein Levels after Gal-LPS Challenge

Using Western blot analysis (Figure 6), we show that expression of anti-apoptotic Bcl-X_L did not markedly dif-

fer among saline-treated control and Gal-LPS livers with and without DPO. Protein level of proapoptotic Bax was found slightly increased in Gal-LPS livers. DPO posttreatment caused some reduction of Bax protein levels compared with Gal-LPS livers alone (Figure 6). In line with H&E histopathology with assessment of mitotic figures, proliferative capacity of the liver, as assessed by PCNA protein levels, did not differ between the groups studied (Figure 6). To address a possible action of DPO on the hepatic microvasculature, we analyzed the phospho-eNOS protein expression in liver tissue at 2, 4, and 6 hours after Gal-LPS exposure and posttreatment with DPO (Table 2). During the whole time course, DPO-treated animals revealed higher levels of phospho-eNOS protein in liver tissue when compared with the saline-treated animals (Table 2).

Discussion

Herein, we communicate the following major findings: administration of DPO at 24 hours before induction of acute liver injury significantly reduced microcirculatory deterioration, inhibited apoptotic cell death, and attenuated final tissue damage. A key observation was that delaying the administration of DPO for 30 minutes after the induction of liver injury not only maintained hepatoprotection but also was more effective with respect to the anti-inflammatory property. Analyzing the time course of events, we could further demonstrate that posttreatment with DPO was capable of effectively dampening the early microcirculatory deterioration of the liver, with most supposedly subsequent amelioration of tissue damage. These findings might open the possibility to treat a patient successfully not only at risk of liver failure but also within a certain time period after suffering from acute liver dysfunction.

The EPO analogue DPO is an erythropoiesis-stimulating protein that exerts similar physiological responses as EPO. Hyperglycosylation of DPO is thought to cause a marked reduction in EPO receptor binding affinity with, thus, reduced receptor binding and internalization, leading to the prolonged *in vivo* half-life of ~24 to 26 hours.²⁶ Convincing evidence is available that EPO as well as DPO act as neurotrophic and neuroprotective factors in the brain. In animal studies both agents have been reported to be beneficial in treating global and focal cerebral ischemia, reducing nervous system inflammation and improving neurological outcome.^{16,27–30} Clinical experience in stroke patients has further underlined the value of EPO for use as a neuroprotective drug.³¹ Besides the brain, heart and kidney are protected by EPO or

Figure 4. Representative H&E-stained liver tissue sections of animals that were injected with Gal (720 mg/kg body weight i.p.) and LPS (10 μ g/kg body weight i.p.) for induction of acute liver injury and either pretreated (24 hours before Gal-LPS exposure) or posttreated (30 minutes after Gal-LPS exposure) with DPO (DPO_{pre} and DPO_{post}; 10 μ g/kg i.v.). Endotoxic controls were treated with saline only (Gal-LPS). Sham-operated animals without liver injury served as controls (control). Note maintenance of the general architecture and morphology of the DPO-treated livers in contrast to the deterioration of liver integrity in the untreated Gal-LPS-exposed liver. Original magnifications, $\times 200$.

Figure 5. Hepatic venular leukocyte adherence (**A**), sinusoidal perfusion (**B**), hepatocellular apoptosis (**C**), and tissue necrosis (**D**) in animals which were injected with Gal (720 mg/kg body weight i.p.) and LPS (10 μ g/kg body weight i.p.) for induction of acute liver injury and either posttreated (30 minutes after Gal-LPS exposure) with saline (Gal-LPS) or with DPO (DPO_{post}; 10 μ g/kg i.v.). Analyses were performed at 2, 4, and 6 hours after the Gal-LPS-challenge. Values are given as means $\pm$ SEM; analysis of variance and post hoc comparison test; [#] $P < 0.05$ versus Gal-LPS at the respective time points; [†] $P < 0.05$ versus 2 hours; [‡] $P < 0.05$ versus 2 and 4 hours.

DPO in the setting of ischemia/reperfusion.^{15,17,32} This protection by EPO appears to depend strongly on, among others, the anti-apoptotic effect of EPO on neurons and motoneurons, cardiac myocytes and fibroblasts, as well as tubular epithelial cells^{12,13} and endothelial cells.³³ Most recently, EPO has been reported to reduce the oxidative stress and caspase activation in the postischemic liver, if administered before ischemia.³⁴ Thus, it is tempting to speculate that DPO would also confer protection in acute liver injury.

Gal-LPS exposure caused both systemic inflammatory response and local tissue injury. Levels of IL-6 were found highest at 2 hours but still elevated at 4 and 6 hours after Gal-LPS exposure.³⁵ In contrast, TNF- α concentrations were already below 100 pg/ml at 4 hours, corresponding to the immediate but very transient rise of TNF- α with a maximum at 1.5 to 2 hours on Gal-LPS exposure.^{35,36} In line with other reports,^{37,38} Gal-LPS-exposed livers present with leukocyte accumulation, perfusion failure, as well as apoptotic and necrotic cell death. Hepatotoxicity of leukocytes has mainly been ascribed to the adherence-dependent oxidant stress and

degranulation,³⁹ leading to oncotic necrotic cell death. However, hepatocytes undergoing apoptosis trigger transmigration and attack of leukocytes with expansion and aggravation of the injury beyond the original apoptotic cells.^{10,40} Along with the report of Faouzi and coworkers⁴¹ demonstrating that chemokines are the cause of hepatic inflammation in a model of Fas receptor-mediated hepatocellular apoptosis, mediators generated during apoptosis may be involved in the amplification of the inflammatory response.⁴² Similarly to observations in septic liver models with prevention of apoptosis as well as leukocyte adherence and leukocyte-dependent injury by treatment with a pan-caspase inhibitor,^{9,10} the anti-inflammatory effect of DPO in the present study may not arise from a direct antagonism of leukocytes, but rather by the anti-apoptotic effect on hepatocytes. This view is supported by a recent study on ischemic brain injury, demonstrating that rhEPO attenuates ischemia-induced cytokine production and inflammation by reducing neuronal apoptosis.⁴³ Thus, the present observation that DPO reduced the Gal-LPS-associated rise in IL-6 might

Table 2. Systemic Concentrations of TNF- α and IL-6 (pg/ml), ALT Activities (U/L), and Hepatic Phospho-eNOS Protein Expression (Relative Density of Phospho-eNOS/ β -Tubulin) in Animals That Were Injected with Gal (720 mg/kg Body Weight i.p.) and LPS (10 μ g/kg Body Weight i.p.) for Induction of Acute Liver Injury and Either Posttreated (30 Minutes after Gal-LPS Exposure) with Saline (Gal-LPS) or with Darbepoetin (DPO_{post}; 10 μ g/kg i.v.)

		2 Hours	4 Hours	6 Hours
TNF- α	Gal-LPS	405 $\pm$ 241	53 $\pm$ 12	58 $\pm$ 4
	Gal-LPS/DPO _{post}	29 $\pm$ 6*	34 $\pm$ 5	41 $\pm$ 9
IL-6	Gal-LPS	8495 $\pm$ 1166	4069 $\pm$ 666 [†]	4869 $\pm$ 228 [†]
	Gal-LPS/DPO _{post}	2198 $\pm$ 1319*	1758 $\pm$ 727*	1648 $\pm$ 934*
ALT	Gal-LPS	61 $\pm$ 11	69 $\pm$ 12	267 $\pm$ 51 [‡]
	Gal-LPS/DPO _{post}	43 $\pm$ 3	51 $\pm$ 9	145 $\pm$ 76*
Phospho-eNOS	Gal-LPS	2.3 $\pm$ 0.8	3.4 $\pm$ 0.0	1.2 $\pm$ 0.4
	Gal-LPS/DPO _{post}	4.0 $\pm$ 1.6	4.8 $\pm$ 2.1	5.6 $\pm$ 3.6*

Analyses were performed at 2, 4, and 6 hours after the Gal-LPS challenge. For further information, please see Materials and Methods. Values are given as means $\pm$ SEM; analysis of variance and post hoc comparison test.

* $P < 0.05$ versus Gal-LPS at the respective time points.

[†] $P < 0.05$ versus 2 hours.

[‡] $P < 0.05$ versus 2 and 4 hours.

Figure 6. Representative Western blot analysis of Bcl-X_L, Bax, and PCNA protein levels in livers of animals that were injected with Gal (720 mg/kg body weight i.p.) and LPS (10 μg/kg body weight i.p.) for induction of acute liver injury and either pretreated (24 hours before Gal-LPS exposure) or posttreated (30 minutes after Gal-LPS exposure) with DPO (DPO_{pre} and DPO_{post}; 10 μg/kg i.v.). Endotoxic controls were treated with saline only (Gal-LPS). Sham-operated animals without liver injury served as controls (control). β-Actin served as loading control.

just be interpreted as the consequence of reduced hepatocellular apoptosis on DPO treatment.

One potential mechanism for the hepatoprotection by DPO could be the modulation of apoptosis-regulating genes of the Bcl-2 family. It has been described that Bax protein was found to be increased in response to Gal/TNF-α exposure and related to increased hepatocellular apoptosis.⁴⁴ Moreover, sequential analysis of proapoptotic and anti-apoptotic gene expression in the Gal-LPS-exposed liver by Liu and coworkers⁴⁵ indicated that there is an early block in transcription of anti-apoptotic Bcl-2 and Bcl-X_L at ~2 hours after Gal-LPS exposure, before the proapoptotic gene Bax is transcriptionally activated. At 6 hours after challenge with Gal and LPS, increased Bax expression then coincides with massive hepatocellular apoptosis.⁴⁵ This time-dependent alteration in the balance between gene expression of anti-apoptotic and proapoptotic proteins induced by Gal-LPS fits in with the present observation of increased Bax but unchanged Bcl-X_L protein levels in livers at 6 hours after Gal-LPS exposure. The early transcriptional block of Bcl-X_L on Gal-LPS exposure of livers might be the reason why we could not observe an effect of DPO on this anti-apoptotic protein, although it has often been described that EPO maintains cell viability via up-regulation of Bcl-X_L.¹² In contrast to Bcl-X_L, we could observe a decrease of the proapoptotic Bax protein in Gal-LPS-exposed mice on DPO treatment, which could, at least in part, mediate the hepatoprotective effects. However, the only slight reduction in the Bax protein levels implies that additional mechanisms that are activated on receptor signaling¹⁴ might have contributed to the amelioration of hepatic tissue injury. This view is in line with extensive work of the group of Cerami and colleagues,^{46,47} demonstrating that EPO analogs that do not bind the classical EPO receptor retain its anti-inflammatory and anti-apoptotic actions, implying an action through other receptors, such as the common β receptor (βcR), or even receptor-independent actions.⁴⁷

Decreased intrahepatic leukocyte accumulation will in turn reduce flow resistance with subsequent alleviation of microvascular perfusion. The question to which extent the

reduced perfusion failure is just a consequence of hepatic tissue protection by DPO or a result of DPO-specific actions on the vasculature, including up-regulation of vasoactive mediator systems in endothelial and vascular smooth muscle cells (for references, see Ref. 13), cannot be completely answered so far. However, the present observation of DPO-associated up-regulation of phospho-eNOS could at least be partly responsible for the beneficial effects of DPO on the hepatic microvasculature. This view is supported by *in vitro* results demonstrating that EPO stimulates eNOS expression with the consequence of raised NO production.⁴⁸ With the fact that tissue necrosis concomitantly occurring with massive apoptosis in Gal-LPS exposed livers has been interpreted as the consequence of microvascular injury and inflammatory breakdown of endothelial integrity,⁴⁵ we would like to state that DPO may mainly exert its protection by interfering with these interwoven mechanisms of apoptosis, inflammation, and microvascular injury. This is further underscored by reports demonstrating that EPO prevents LPS-induced apoptosis in endothelial cells.³³ Despite this very convincing potential of DPO to reduce Gal-LPS-induced liver injury, the time frame and the high mortality rate of the herein used model³⁶ does not allow appropriately judgment whether treatment of DPO would also promote liver regeneration. For this, models other than the Gal-LPS induced ALF would be more suited.

Being aware of the complicity to distinguish unequivocally between apoptosis and oncotic necrosis, we used multiple assays to assess apoptotic cell death. The uniform results from these assays allowed us to identify—apart from necrotic injury—apoptotic cell death as a dominant mode of death on Gal-LPS exposure, which could almost be completely prevented by pre- and post-treatment with DPO. Of interest, DPO significantly augmented global morphological recovery, including tissue necrosis. This is in contrast to other reports studying isolated cell cultures, demonstrating that EPO exposure to cardiomyocytes does not affect the number of hypoxia-induced necrotic cells but reduced apoptotic cells by half.¹⁵ Accordingly, the anti-necrotic property of DPO, observed in the present study, is most likely an indirect effect that can be attributed to DPO interfering with the vicious circle between apoptosis, inflammation, and necrotic cell death in the *in vivo* system.

Taken together, pre- and posttreatment with DPO significantly lowers acute liver injury and augments morphological recovery. These observations may stimulate the evaluation of DPO, a compound established as clinically safe, as a hepatoprotective therapy in patients with acute liver injury.

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