Available online at www.sciencedirect.com
SCIENCE DIRECT®
European Journal of Pharmacology 466 (2003) 181–189
www.elsevier.com/locate/ejphar

A superoxide dismutase mimetic with catalase activity (EUK-8) reduces the organ injury in endotoxic shock

Michelle C. McDonald[a], Roberta d' Emmanuele di Villa Bianca[a,b], Nicole S. Wayman[a], Aldo Pinto[c], Martyn A. Sharpe[d], Salvatore Cuzzocrea[e], Prabal K. Chatterjee[a], Christoph Thiemermann[a,*]

[a] *The William Harvey Research Institute, St. Bartholomew's and The Royal London School of Medicine and Dentistry, Charterhouse Square, London EC1M 6BQ, UK*
[b] *Department of Experimental Pharmacology, Via Domenico Montesano, 49, 80131 Naples, Italy*
[c] *Department of Pharmaceutical Science, Via Ponte don Melillo, 84084 Fisciano (SA), Italy*
[d] *Department of Neurochemistry, Institute of Neurology, University College London, London, UK*
[e] *Istituto di Farmacologia, Università di Messina, Torre Biologica-Policlinico Universatario, Via C. Valeria, 98100 Messina, Italy*

Received 19 February 2003; accepted 25 February 2003

Abstract

Reactive oxygen species contribute to the multiple organ failure in endotoxic shock. Here, we investigate the effects of a salen-manganese complex, which exhibits both superoxide dismutase and catalase activity (EUK-8), on the circulatory failure, renal and liver injury and dysfunction caused by endotoxin in the anaesthetised rat. Endotoxaemia (6 mg/kg i.v., *Escherichia coli* lipopolysaccharide) for 6 h caused hypotension, renal dysfunction and liver injury. Treatment of rats with EUK-8 (0.3 or 1 mg/kg bolus injection followed by an infusion of 0.3 or 1 mg/kg/h) attenuated the renal and liver injury and dysfunction in a dose-related fashion. In addition, the higher dose of EUK-8 attenuated the delayed hypotension caused by endotoxin in the rat. Thus, an enhanced formation of reactive oxygen species importantly contributes to the circulatory failure, as well as the organ injury and dysfunction associated with endotoxic shock. We propose that small molecules, which have the catalytic activity of both superoxide dismutase and catalase, may represent a novel therapeutic approach for the therapy of endotoxic shock.

Keywords: Oxygen radical; Superoxide dismutase; EUK-8; EUK-134; Superoxide anion; Multiple organ failure; Shock

1. Introduction

There is good evidence that septic or endotoxic shock is associated with the generation of reactive oxygen species, such as superoxide anions, hydrogen peroxide and peroxynitrite (Redl et al., 1993). For instance, neutrophils obtained from either rats with endotoxaemia (Simons et al., 1987) or patients with septic shock (Vespasiano et al., 1993) spontaneously generate large quantities of superoxide anions. An enhanced formation of superoxide anions has also been detected by electron spin resonance from heart and liver challenged with endotoxin in vivo (Brackett et al., 1989). In the presence of nitric oxide, superoxide anions generate the potent oxidant peroxynitrite (Beckman et al., 1990). An enhanced formation of peroxynitrite may contribute to the circulatory dysfunction in rats with endotoxic shock (Zingarelli et al., 1997). Interventions that reduce the generation or the effects of reactive oxygen species exert beneficial effects in a variety of models of endotoxic and septic shock. These therapeutic interventions include *N*-acetylcysteine, α-tocopherol, allopurinol, deferoxamine, catalase and superoxide dismutase (Redl et al., 1993; Simon et al., 1996).

The therapeutic efficacy of superoxide dismutase in animals with endotoxic or septic shock is controversial. When given as a pretreatment, superoxide dismutase does improve survival in rodents with endotoxaemia (Warner et al., 1986; Kunimoto et al., 1987; Schineder et al., 1989). In contrast, superoxide dismutase does not reduce or even enhance the respiratory dysfunction caused by endotoxin (or sepsis) in

* Corresponding author. Tel.: +44-207-882-6118; fax: +44-207-251-1685.
E-mail address: c.thiemermann@qmul.ac.uk (C. Thiemermann).

0014-2999/03/$ - see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0014-2999(03)01538-3

sheep, dogs, pigs, rabbits or rats (Traber et al., 1985; Olson et al., 1987; Novotny et al., 1988; Broner et al., 1989; McKechnie et al., 1986; Redl et al., 1990). The following reasons may explain the lack of effect of superoxide dismutase against the organ injury associated with hemorrhagic or septic shock: superoxide dismutase scavenges superoxide, but without efficient removal of the hydrogen peroxide which is produced, levels of hydroxyl radicals may increase (Goode and Webster, 1993). Indeed, superoxide dismutase may function as a pro-oxidant by catalysing the conversion of hydrogen peroxide to hydroxyl radicals (Yim et al., 1990). Neither superoxide dismutase nor superoxide anions easily cross biological membranes. Thus, extracellular superoxide dismutase does not attenuate the effects of superoxide anions generated by intracellular sources (Fridovich, 1995).

Small molecules, which have the catalytic activity of both superoxide dismutase and catalase, such as EUK-8 or EUK-134, have recently been developed (Baker et al., 1998). These agents remove both superoxide anions and hydrogen peroxide and have been shown to exert beneficial effects in a rodent models of ischaemia–reperfusion of the brain (Baker et al., 1998), kidney (Gianello et al., 1996) and heart (Pucheu et al., 1996). These agents also reduce the oxidative stress and mortality in a murine model of amyotrophic lateral sclerosis (Jung et al., 2001). In addition, there is one report that EUK-8 reduces the lung injury caused by endotoxin in the pig (Gonzalez et al., 1995). Here, we investigate the effects of EUK-8 on the circulatory failure and the renal and liver injury/dysfunction caused by endotoxin in the anaesthetised rat.

2. Materials and methods

2.1. Surgical procedure

This study was carried out on 78 male Wistar rats (Tuck, Rayleigh, Essex, UK) weighing 250–320 g receiving a standard diet and water ad libitum. The investigation was performed in accordance with the Home Office *Guidance on the Operation of the Animals (Scientific Procedures) Act 1986*, published by HMSO, London. All animals were anaesthetised with thiopentone sodium (120 mg/kg i.p.) and anaesthesia was maintained by supplementary injections of thiopentone sodium as required. The trachea was cannulated to facilitate respiration and rectal temperature was maintained at 37 °C with a homeothermic blanket. The right carotid artery was catheterised and connected to a pressure transducer (Senso-Nor 840, Senso-Nor, Horten, Norway) for the measurement of phasic and mean arterial blood pressure and heart rate, which were continuously recorded on a data acquisition system (Powerlab® Version 4.0.4, AD Instruments, Hastings, UK) installed on a Dell Dimension 4100 personal computer. The jugular vein was cannulated for the administration of drugs. The bladder was also cannulated to facilitate urine flow and to prevent the possibility of the development of post-renal failure. Upon completion of the surgical procedure, cardiovascular parameters were allowed to stabilise for 15 min. Then, *Escherichia coli* lipopolysaccharide (serotype: 0127:B8, 6 mg/kg i.v.) was injected slowly over 20 min to minimise any acute fall in blood pressure. Thereafter, all animals received an infusion of saline (at 2 ml/kg/h) for a further 6 h, after which blood was taken for further biochemical analysis and the animals were killed by an overdose of anaesthetic.

2.2. Evaluation of the effects of EUK-8 on circulatory failure and multiple organ dysfunction syndrome: experimental design

Initially, five experimental groups were used for the study.

1. LPS Vehicle Group: rats received a bolus injection of vehicle (saline 1 ml/kg i.v.) and 15 min later a slow bolus injection of endotoxin (6 mg/kg i.v.) followed at 30 min later by a continuous infusion of saline (2 ml/kg/h i.v., $n = 15$).
2. LPS EUK-8 0.3 mg/kg Group: rats received a bolus injection of EUK-8 (0.3 mg/kg i.v.) and 15 min later a slow bolus injection of endotoxin (6 mg/kg i.v.) followed at 30 min later by a continuous infusion of EUK-8 (0.3 mg/kg/h at a rate of 2 ml/kg/h i.v., $n = 9$).
3. LPS EUK-8 1 mg/kg Group: rats received a bolus injection of EUK-8 (1 mg/kg i.v.) and 15 min later a slow bolus injection of endotoxin (6 mg/kg i.v.) followed at 30 min later by a continuous infusion of EUK-8 (1 mg/kg/h at a rate of 2 ml/kg/h i.v., $n = 10$).
4. Sham Vehicle Group: rats received a bolus injection of vehicle (saline 1 mg/kg) and 15 min later a bolus injection of saline (instead of endotoxin) followed at 30 min later by a continuous infusion of saline (2 ml/kg/h i.v., $n = 13$).
5. Sham EUK-8 Group: rats received a bolus injection of EUK-8 (1 mg/kg) and 15 min later a bolus injection of saline (instead of endotoxin) followed at 30 min later by a continuous infusion of EUK-8 (1 mg/kg/h at a rate of 2 ml/kg/h i.v. saline, $n = 8$).

After the effects of pretreatment with EUK-8 were determined, additional experiments were carried out to ascertain whether posttreatment (after endotoxin administration) with EUK-8 had any effect on the multiple organ failure and circulatory failure caused by endotoxaemia in the rat. The additional groups include the following.

6. LPS Vehicle POST Group: rats received a slow bolus injection of endotoxin (6 mg/kg i.v.) followed at 120 min later by a continuous infusion of saline ($n = 9$).
7. LPS EUK-8 0.3 mg/kg POST Group: rats received a slow bolus injection of endotoxin (6 mg/kg i.v.) followed at 120 min later by a continuous infusion of EUK-8 (0.3 mg/kg/h i.v., $n = 7$).
8. LPS EUK-8 1 mg/kg POST Group: rats received a slow bolus injection of endotoxin (6 mg/kg i.v.) followed at 120 min later by a continuous infusion of EUK-8 (1 mg/kg/h i.v., $n = 7$).

2.3. Quantification of organ function and injury

Four hours after resuscitation (end of the experiment), 1.5 ml of blood was collected into a serum gel S/1.3 tube (Sarstedt, Germany) from the catheter placed in the right carotid artery. The blood sample was centrifuged (1610 × *g* for 3 min at room temperature) to separate serum. All serum samples were analysed within 24 h by a contract laboratory for veterinary clinical chemistry (Vetlab Services, Sussex, UK). The following marker enzymes were measured in the serum as biochemical indicators of multiple organ injury/dysfunction: Liver injury was assessed by measuring the rise in serum levels of alanine aminotransferase, γ-glutamyl transferase (specific markers for hepatic parenchymal injury) and aspartate aminotransferase (a nonspecific marker for hepatic injury). Renal dysfunction was assessed by measuring the rises in serum levels of creatinine (an indicator of reduced glomerular filtration rate, and hence, renal failure) and urea (an indicator of impaired excretory function of the kidney and/or increased catabolism) (see Baue, 1993; Thiemermann et al., 1995).

2.4. Light microscopy

Organ (lung, liver and kidney) biopsies were taken at the end of the experiment. The biopsies were fixed for 1 week in buffered formaldehyde solution (10% in phosphate-buffered saline, PBS) at room temperature, dehydrated by graded ethanol and embedded in Paraplast (Sherwood Medical, Mahwah, NJ). Sections (thickness: 7 μm) were deparaffinized with xylene, stained with trichromic Van Gieson and studied using light microscopy (Dialux 22 Leitz).

2.5. Immunohistochemical localisation of nitrotyrosine

Tyrosine nitration, an index of the nitrosylation of proteins by peroxynitrite and/or oxygen-derived free radicals, was determined by immunohistochemistry as previously described (Cuzzocrea et al., 1998). At the end of the experiment, the relevant organs were fixed in 10% buffered formaldehyde and 8 μm sections were prepared from paraffin embedded tissues. After deparaffinization, endogenous peroxidase was quenched with 0.3% H_2O_2 in 60% methanol for 30 min. The sections were permeabilized with 0.1% Triton X-100 in PBS for 20 min. Nonspecific adsorption was minimised by incubating the section in 2% normal goat serum in phosphate-buffered saline for 20 min. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 min with avidin and biotin. The sections were then incubated overnight with 1:1000 dilution of primary antinitrotyrosine antibody or with control solutions. Controls included buffer alone or nonspecific purified rabbit IgG. Specific labelling was detected with a biotin-conjugated goat antirabbit IgG and avidin–biotin peroxidase complex.

2.6. Materials

Unless otherwise stated, all compounds were obtained from Sigma-Aldrich (Poole, Dorset, UK). Thiopentone sodium (Intraval Sodium®) was obtained from Rhône Mérieux (Harlow, Essex, UK). Biotin blocking kit, biotin-conjugated goat antirabbit IgG, primary antinitrotyrosine and avidin–biotin peroxidase complex were obtained from

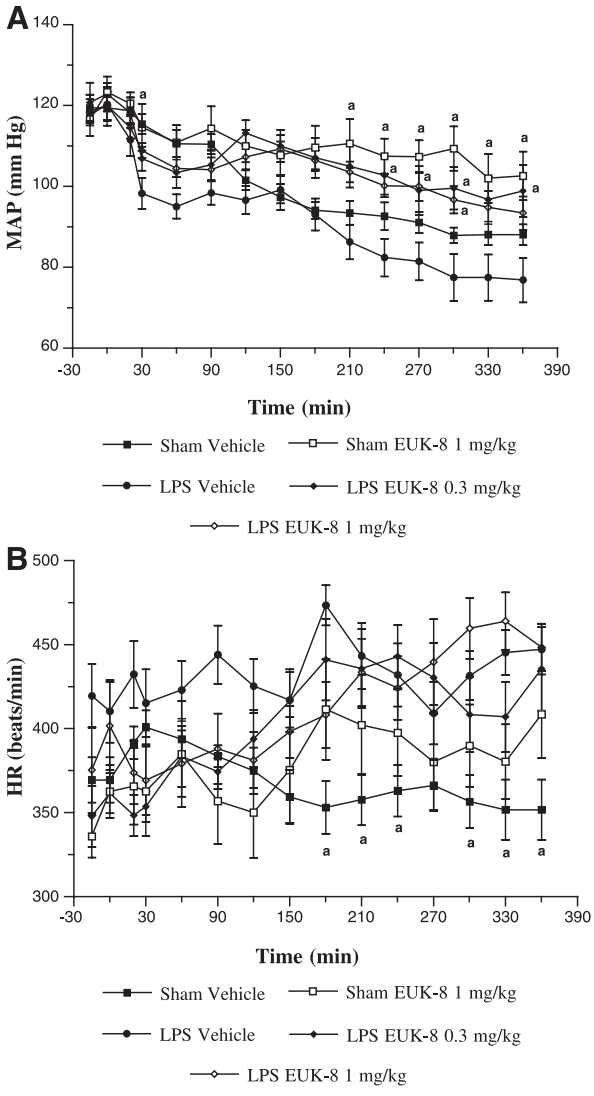

Fig. 1. Alterations in (A) mean arterial blood pressure and (B) heart rate in all experimental groups studied. The following groups were studied: Rats were subjected to the surgical procedure without giving endotoxin and treated with vehicle for EUK-8 (black square, saline, 2 ml/kg i.v. bolus followed by an infusion of 2 ml/kg/h i.v.; Sham Vehicle) or with EUK-8 (open square, 1 mg/kg i.v. bolus injection followed by an infusion of 1 mg/kg i.v.; Sham EUK-8). Alternatively, rats were subjected to 6 h of endotoxaemia and treated with either vehicle (black circle, saline, 2 ml/kg i.v. bolus followed by an infusion of 2 ml/kg/h i.v.; LPS Vehicle), low dose of EUK-8 (black diamond, 0.3 mg/kg i.v. bolus injection followed by an infusion of 0.3 mg/kg i.v.; LPS EUK-8 0.3 mg/kg) or high dose of EUK-8 (open diamond, 1 mg/kg i.v. bolus injection followed by an infusion of 1 mg/kg i.v.; LPS EUK-8 1 mg/kg). [a]$P < 0.05$ when compared to LPS Vehicle Group (black circle).

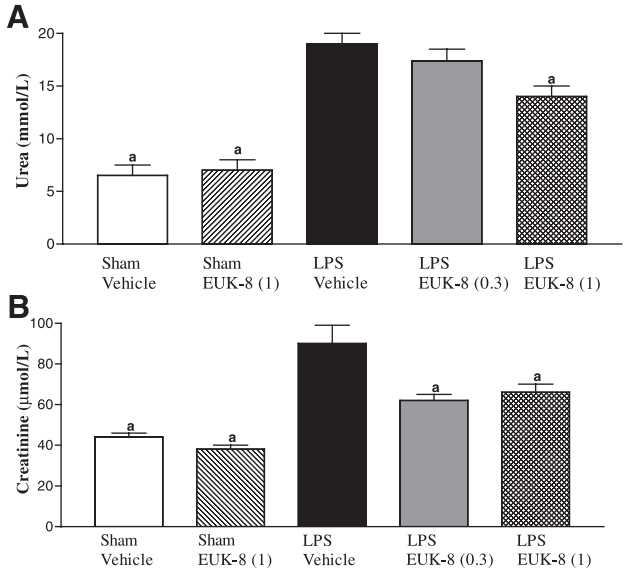

Fig. 2. Alterations in the serum levels of (A) urea and (B) creatinine in rats subjected to (i) the surgical procedure without causing endotoxaemia and treated with vehicle for EUK-8 (open column, saline, 2 ml/kg i.v. bolus followed by an infusion of 2 ml/kg/h i.v.) or with EUK-8 (striped column, 1 mg/kg i.v. bolus injection followed by an infusion of 1 mg/kg/h i.v.), or (ii) endotoxaemia for 6 h and treated with the vehicle for EUK-8 (black column, saline, 2 ml/kg i.v. bolus followed by an infusion of 2 ml/kg/h i.v.), low dose of EUK-8 (grey column, 0.3 mg/kg i.v. bolus injection followed by an infusion of 0.3 mg/kg/h i.v.) or high dose of EUK-8 (hatched column, 1 mg/kg i.v. bolus injection followed by an infusion of 1 mg/kg/h i.v.). [a]$P < 0.05$ when compared to LPS Vehicle Group (black column).

DBA (Milan, Italy). All stock solutions were prepared in nonpyrogenic saline (0.9% NaCl; Baxter Healthcare, Thetford, Norfolk, UK).

2.7. Statistical evaluation

All data are presented as means ± S.E.M. of n observations, where n represents the number of animals or blood samples studied. For repeated measurements (haemodynamics), a two-factorial analysis of variance was performed. Data without repeated measurements (multiple organ injury/failure) was analysed by one-factorial analysis of variance, followed by a Dunnett's test for multiple comparisons. A P-value of less than 0.05 was considered to be statistically significant.

Table 1
The effects of EUK-8 when given as a posttreatment 120 min after the administration of endotoxin

Parameter	LPS vehicle ($n=9$)	LPS EUK-8 0.3 mg/kg ($n=7$)	LPS EUK-8 1 mg/kg ($n=7$)
Urea (mmol/l)	18.6 ± 1.1	16.8 ± 1.4	17.7 ± 1.1
Creatinine (μmol/l)	102 ± 13	67 ± 6	86 ± 10
Aspartate aminotransferase (IU/l)	565 ± 97	599 ± 109	657 ± 91
Alanine aminotransferase (IU/l)	327 ± 89	355 ± 123	453 ± 140
γ-Glutamyl transferase (IU/l)	4.8 ± 0.8	4.7 ± 1.2	4.6 ± 1.2

Rats were treated with either vehicle for EUK-8 (saline, 1 ml/kg i.v. bolus followed by an infusion of 1 ml/kg/h i.v.; LPS vehicle), low dose of EUK-8 (0.3 mg/kg i.v. bolus injection followed by an infusion of 0.3 mg/kg/h i.v.; LPS EUK-8 0.3 mg/kg) or high dose of EUK-8 (1 mg/kg i.v. bolus injection followed by an infusion of 1 mg/kg/h i.v.; LPS EUK-8 1 mg/kg).

3. Results

3.1. Effects of EUK-8 on the delayed fall in blood pressure caused by endotoxin

Baseline values of mean arterial blood pressure in all groups of animals ranged from 113 ± 5 to 119 ± 3 mm Hg and were not significantly different between groups (Fig. 1A). In sham-operated rats (no endotoxin), administration of EUK-8 (at 90 min) did not affect mean arterial blood

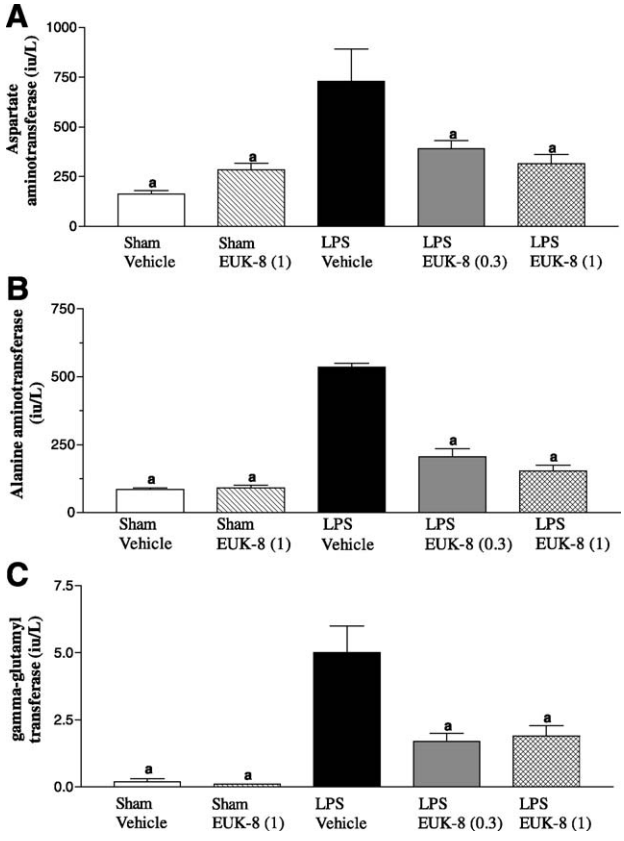

Fig. 3. Alterations in the serum levels of (A) aspartate aminotransferase, (B) alanine aminotransferase and (C) γ-glutamyl transferase in rats subjected to (i) the surgical procedure without causing endotoxaemia and treated with vehicle for EUK-8 (open column, saline, 2 ml/kg i.v. bolus followed by an infusion of 2 ml/kg/h i.v.) or with EUK-8 (striped column, 1 mg/kg i.v. bolus injection followed by an infusion of 1 mg/kg/h i.v.), or (ii) endotoxaemia for 6 h and treated with the vehicle for EUK-8 (black column, saline, 2 ml/kg i.v. bolus followed by an infusion of 2 ml/kg/h i.v.), low dose of EUK-8 (grey column, 0.3 mg/kg i.v. bolus injection followed by an infusion of 0.3 mg/kg/h i.v.) or high dose of EUK-8 (hatched column, 1 mg/kg i.v. bolus injection followed by an infusion of 1 mg/kg/h i.v.). [a]$P < 0.05$ when compared to LPS Vehicle Group (black column).

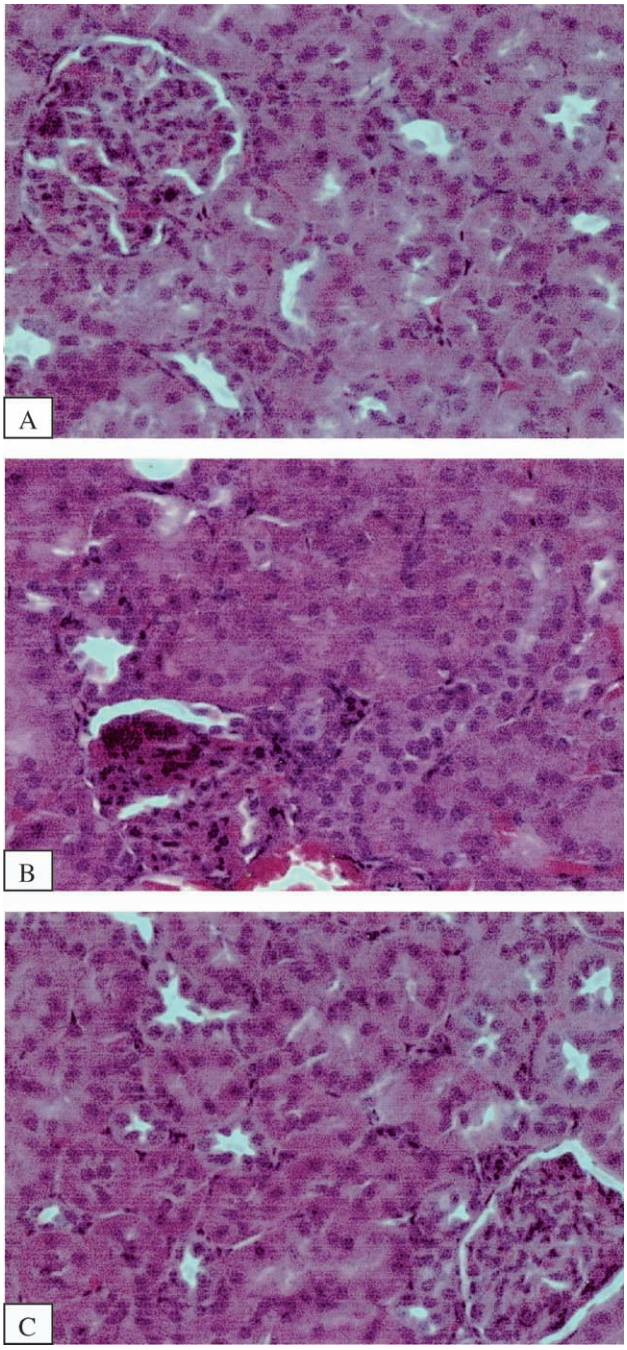

Fig. 4. Representative histological sections of a kidney obtained from (A) rats subjected to the surgical procedure without causing endotoxaemia and treated with vehicle for EUK-8 (saline), (B) rats subjected to endotoxaemia and treated with vehicle for EUK-8 (saline) and (C) rats subjected to endotoxaemia and treated with the high dose of EUK-8.

pressure (Fig. 1A, $P > 0.05$ when compared to sham-operated rats treated with vehicle). In rats subjected to 6 h of endotoxaemia, there was a fall in blood pressure, so that the mean arterial blood pressure at the end of the experiment had fallen to 77 ± 5 mm Hg ($P < 0.05$, Fig. 1A). Treatment of rats with EUK-8 attenuated the fall in mean arterial blood pressure caused by endotoxin (Fig. 1A).

Baseline values of heart rate in all groups of animals ranged from 336 ± 13 to 428 ± 18 beats/min and were not significantly different between groups (Fig. 1B). In control animals, administration of EUK-8 did not result in any

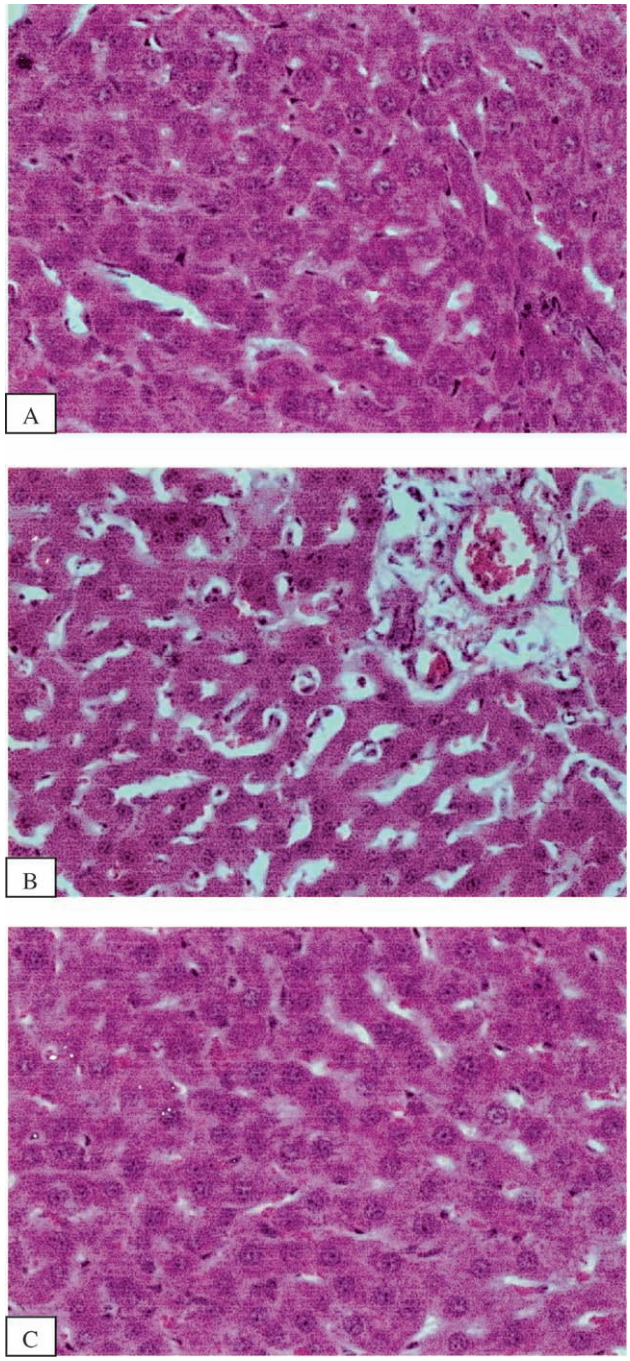

Fig. 5. Representative histological sections of the liver obtained from (A) rats subjected to the surgical procedure without causing endotoxaemia and treated with vehicle for EUK-8 (saline), (B) rats subjected to endotoxaemia and treated with vehicle for EUK-8 (saline) and (C) rats subjected to endotoxaemia and treated with the high dose of EUK-8.

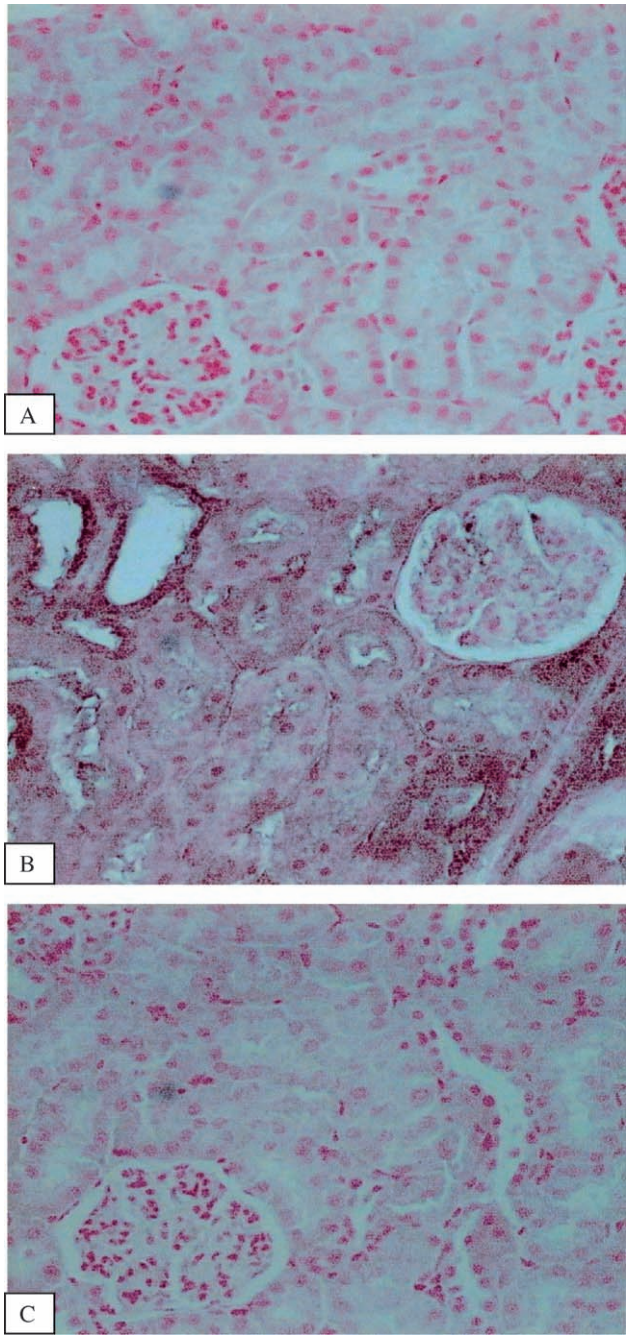

Fig. 6. Representative histological sections of a kidney obtained from (A) rats subjected to the surgical procedure without causing endotoxaemia and treated with vehicle for EUK-8 (saline), (B) rats subjected to endotoxaemia and treated with vehicle for EUK-8 (saline) and (C) rats subjected to endotoxaemia and treated with the high dose of EUK-8. When compared to sham-operated animals (A), endotoxic shock results in substantial renal injury as well as positive (brown) staining for nitrotyrosine (determined by immunohistochemistry), indicating the nitrosylation of proteins. Pretreatment with EUK-8 largely attenuated these pathological alterations associated with endotoxaemia.

significant alterations in heart rate. Endotoxic shock caused a significant increase in heart rate over time (tachycardia) when compared to sham operated animals ($P<0.05$, Fig. 1B). Treatment with EUK-8 had no significant effect on heart rate ($P>0.05$, when compared to rats subjected to endotoxaemia and treated with vehicle; Fig. 1B).

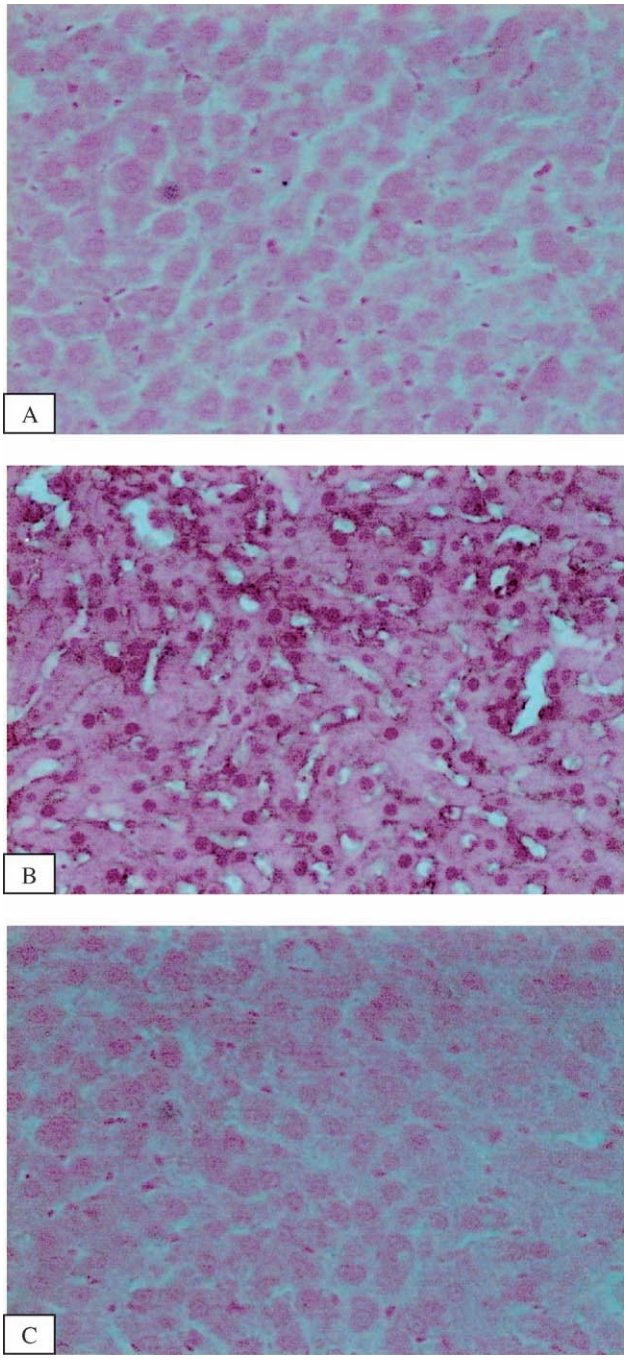

Fig. 7. Representative histological sections of a liver obtained from (A) rats subjected to the surgical procedure without causing endotoxaemia and treated with vehicle for EUK-8 (saline), (B) rats subjected to endotoxaemia and treated with vehicle for EUK-8 (saline) and (C) rats subjected to endotoxaemia and treated with the high dose of EUK-8. When compared to sham-operated animals (A), endotoxic shock results in substantial liver injury as well as positive (brown) staining for nitrotyrosine (determined by immunohistochemistry), indicating the nitrosylation of proteins. Pretreatment with EUK-8 largely attenuated these pathological alterations associated with endotoxaemia.

3.2. Effects of EUK-8 on the multiple organ dysfunction syndrome caused by endotoxaemia in the rat

In sham-operated rats, administration EUK-8 did not result in any significant alterations in the serum levels of urea or creatinine (Fig. 2). When compared with sham-operated rats, endotoxaemia resulted in significant rises in the serum levels of urea and creatinine, demonstrating the development of renal dysfunction. Treatment of rats subjected to endotoxaemia with EUK-8 resulted in a significant attenuation of the renal dysfunction caused by endotoxin (Fig. 2). Although posttreatment with EUK-8, 120 min after the administration of endotoxin, caused a small reduction in the serum levels of creatinine ($P > 0.05$, Table 1), overall, posttreatment with EUK-8 had no significant effect on the renal dysfunction caused by endotoxin (Table 1).

In sham-operated rats, administration of EUK-8 did not result in any significant alterations in the serum levels of aspartate aminotransferase, alanine aminotransferase and γ-glutamyl transferase (Fig. 3). When compared with sham-operated rats, endotoxaemia resulted in significant rises in the serum levels of aspartate aminotransferase, alanine aminotransferase and γ-glutamyl transferase, demonstrating the development of hepatocellular injury. Treatment of rats (prior to endotoxin administration) subjected to endotoxaemia with EUK-8 resulted in an attenuation of the liver injury caused by endotoxin (Fig. 3). However, posttreatment with EUK-8 had no significant effect on the liver injury caused by endotoxin ($P > 0.05$, Table 1).

3.3. Effects of EUK-8 on the formation of nitrotyrosine in the kidney and liver of rats subjected to endotoxaemia

When compared to organs obtained from sham-operated rats, the kidneys and the liver of rats subjected to endotoxaemia showed substantial histological alterations consistent with shock-induced organ injury (Figs. 4 and 5, Panels A and B). The degree of organ injury (as assessed by histology) caused by endotoxaemia was reduced in kidneys (Fig. 4C) and the liver (Fig. 5C) of rats, which were treated with EUK-8 (1 mg/kg i.v.). In addition, the kidneys (Fig. 6B) and liver (Fig. 7B) of rats subjected to endotoxaemia exhibited a marked staining for nitrotyrosine when compared to organs obtained from sham-operated rats (Figs. 6A and 7A). In contrast, the degree of nitrotyrosine staining in these organs (Figs. 6C and 7C) was markedly reduced in rats pretreated with EUK-8 (1 mg/kg i.v.). It should be noted that the administration of EUK-8 in sham-operated rats caused neither any histological alterations nor staining for nitrotyrosine (data not shown).

4. Discussion

Endotoxaemia for 6 h resulted in a substantial increase in the serum levels of urea and creatinine indicating the development of acute renal dysfunction. In addition, endotoxaemia also caused an increase in the serum levels of the aspartate aminotransferase and alanine aminotransferase as well γ-glutamyl transferase, indicating the development of hepatocellular injury. Histological examination confirmed that the rodent model of endotoxic shock used here caused a substantial degree of tissue injury to the kidney and liver. This was associated with tyrosine nitration of proteins in all of these organs, which indicates the formation of peroxynitrite and/or oxygen-derived free radicals (Cuzzocrea et al. 1998). We report here that treatment of rats with EUK-8 (Baker et al., 1998) attenuates both the hepatocellular injury as well as the renal dysfunction (to a lesser extent) caused by endotoxin in the rat. In addition, EUK-8 substantially reduced the nitrosylation of proteins by peroxynitrite/reactive oxygen radicals in the kidney and the liver. Finally, we investigated the effects of EUK-8 when given as a posttreatment (120 min after the administration of LPS). Posttreatment with EUK-8 had no significant effect on the organ injury caused by endotoxaemia, which indicates that (in this rodent model of endotoxaemia) the reactive oxygen species responsible for the development of organ injury are produced within the first 2 h after endotoxin challenge.

There is evidence that EUK-8 also reduces the acute lung injury caused by endotoxin in the anaesthetised pig (Gonzalez et al., 1995). Gonzalez and colleagues provide good evidence that a high dose of EUK-8 (10 mg/kg bolus followed by an infusion of 3 mg/kg/h) significantly reduces many of the features of endotoxin-induced acute lung injury, including arterial hypoxaemia, pulmonary hypertension, decreased dynamic pulmonary compliance and pulmonary oedema.

What then is the mechanism by which EUK-8 protects the kidney and the liver of the rat against injury and dysfunction? EUK-8 is a low-molecular weight, salen-manganese complex that exhibits the catalytic activities of both superoxide dismutase and catalase (Baker et al., 1998). Thus, EUK-8 should remove superoxide anions and hydrogen peroxide and, hence, protect the kidney and the liver against the injury caused by the reactive oxygen species including superoxide anions and hydroxyl radicals. Although it is not known whether EUK-8 scavenges peroxynitrite, EUK-8 should prevent the formation of peroxynitrite by scavenging superoxide anions (Beckman et al., 1990). This study demonstrates that treatment with EUK-8 attenuates the nitrosylation of proteins in the kidney and liver of rats treated subjected to endotoxic shock. This formation of nitrotyrosine moieties is most likely due to the formation of peroxynitrite (Szabo et al., 1998; Zingarelli et al., 1998), although nitrotyrosine formation may also be generated when nitrite interacts with myeloperoxidase (Eiserich et al., 1998). Hence, it is possible that the nitrosylation of proteins observed in organs from rats subjected to shock is the net result of a complex interaction between various oxygen- and nitrogen-derived radicals and oxidants. We, therefore, propose that the beneficial effects of EUK-8

on organ integrity and function are due to the ability of this compound to function as an intracellular superoxide dismutase-mimetic with catalase activity, attenuate the level of superoxide anions in vivo (including tempol and EUK-8) and, hence, reduce the formation of peroxynitrite and the subsequent nitration of proteins.

We have previously reported that tempol reduces the organ injury and dysfunction caused by endotoxic (Leach et al., 1998) and hemorrhagic shock (Mota-Filipe et al., 1999). However, it should be noted that tempol is not very potent and, hence, fairly large quantities (~ 30–100 mg/kg) of this compound are needed to protect tissues in vivo against oxidative stress (Sledzinski et al., 1995; McDonald et al., 1999). In contrast, EUK-8 has the catalytic activity of superoxide dismutase and catalase and, hence, relatively small doses of these compounds exert beneficial effects in the model of endotoxaemia used here.

We also report here that the higher dose of EUK-8 used in our study attenuates the delayed fall in blood pressure caused by endotoxin. The mechanism of this beneficial haemodynamic effect of EUK-8 (or of other superoxide dismutase-mimetics) is not entirely clear. MacArthur and colleagues suggest that deactivation of catecholamines via superoxide anions may account for the vascular hyporeactivity to exogenous catecholamines observed in sepsis, as administration of a superoxide dismutase-mimetic attenuated the hypotension and restored the pressor response to noradrenaline in rats subjected to endotoxaemia (MacArthur et al., 2000).

In conclusion, this study demonstrates for the first time that EUK-8, a water-soluble, cell-permeable salen-manganese complex that exhibits both superoxide dismutase and catalase activity, attenuates the liver injury and (to a lesser extent) the renal dysfunction caused by endotoxin in the rat. These results support the view that the overproduction of reactive oxygen free radicals contributes to the organ injury associated with endotoxic shock. We propose that small molecules such as EUK-8, which permeate biological membranes and function as intracellular superoxide dismutase-mimetic with catalase activity, may be useful in the therapy of conditions associated with endotoxic shock.

Acknowledgements

C.T. is a Senior Fellow of the British Heart Foundation (FS 96/018).

References

Baker, K., Marcus, K.B., Huffman, K., Kruk, H., Malfroy, B., Doctron, S.R., 1998. Synthetic combined superoxide dismutase/catalase mimetics are protective as a delayed treatment in a rat stroke model: a key role for reactive oxygen species in ischemic brain injury. J. Pharmacol Exp. Ther. 284, 215–221.

Baue, A.E., 1993. The multiple organ or system failure syndrome. In: Schlag, G., Redl, H. (Eds.), Pathophysiology of Shock, Sepsis, and Organ Failure. Springer-Verlag, Berlin, pp. 1004–1018.

Beckman, J.S., Beckman, T.W., Chen, J., Marshall, P.A., Freeman, B.A., 1990. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proc. Natl. Acad. Sci. U. S. A. 87, 1620–1624.

Brackett, D.J., Lai, E.K., Lerner, M.R., Wilson, M.F., McCay, P.B., 1989. Spin trapping of free radicals produced "in vivo" in heart and liver during endotoxemia. Free Radic. Res. Commun. 7, 315–324.

Broner, C.W., Shenep, J.L., Stidham, G.L., Stokes, D.C., Fairclough, D., Schonbaum, G.R., Rehg, J.E., Hildener, W.K., 1989. Effect of antioxidants in experimental *Escherichia coli* septicemia. Circ. Shock 29, 77–92.

Cuzzocrea, S., Zingarelli, B., Hake, P., Salzman, A.L., Szabo, C., 1998. Antiinflammatory effects of mercaptoethylguanidine, a combined inhibitor of nitric oxide synthase and peroxynitrite scavenger, in carrageenan-induced models of inflammation. Free Radic. Biol. Med. 24, 450–459.

Eiserich, J.P., Hristova, M., Cross, C.E., Jones, A.D., Freeman, B.A., Halliwell, B., Van Der Vliet, A., 1998. Formation of nitric oxide-derived inflammatory oxidants by myeloperoxidase in neutrophils. Nature 391, 393–397.

Fridovich, I., 1995. Superoxide radical and superoxide dismutases. Annu. Rev. Biochem. 64, 97–112.

Gianello, P., Saliez, A., Bufkens, X., Petinger, R., Misseleyn, D., Hori, B., Malfroy, B., 1996. EUK-134, a synthetic superoxide dismutase and catalase mimetic, protects rat kidneys from ischemia–reperfusion-induced damage. Transplantation 62, 1664–1666.

Gonzalez, P.K., Zhuang, J., Doctrow, S.R., Malfroy, B., Benson, P.F., Menconi, M.J., Fink, M.P., 1995. EUK-8, a synthetic superoxide dismutase and catalase mimetic, ameliorates acute lung injury in endotoxemic swine. J. Pharmacol. Exp. Ther. 275, 798–806.

Goode, H.F., Webster, N.R., 1993. Free radicals and antioxidants in sepsis. Crit. Care Med. 21, 1770–1776.

Jung, C., Rong, Y., Doctrow, S., Baudry, M., Malfroy, B., Xu, Z., 2001. Synthetic superoxide dismutase/catalase mimetics reduce oxidative stress and prolong survival in a mouse amyotrophic lateral sclerosis model. Neurosci. Lett. 304, 157–160.

Kunimoto, F., Morita, T., Ogawa, R., Fujita, T., 1987. Inhibition of lipid peroxidation improves survival rate of endotoxemic rats. Circ. Shock 21, 15–22.

Leach, M., Frank, S., Olbrich, A., Pfeilschifter, J., Thiemermann, C., 1998. Decline in the expression of copper/zinc superoxide dismutase in the kidney of rats with endotoxic shock: effects of the superoxide anion radical scavenger, tempol, on organ injury. Br. J. Pharmacol. 125, 817–825.

MacArthur, H., Westfall, T.C., Riley, D.P., Misko, M.P., Salvemini, D., 2000. Inactivation of catecholamines by superoxide gives new insights on the pathogenesis of septic shock. Proc. Natl. Acad. Sci. U. S. A. 97, 9753–9758.

McDonald, M.C., Zacharowski, K., Bowes, J., Cuzzocrea, S., Thiemermann, C., 1999. Tempol reduces infarct size in rodent models of regional myocardial ischaemia and reperfusion. Free Radic. Biol. Med. 27, 493–503.

McKechnie, K., Furman, B.L., Parratt, J.R., 1986. Modification by oxygen free radical scavengers of the metabolic and cardiovascular effects of endotoxin infusion in conscious rats. Circ. Shock 19, 429–439.

Mota-Filipe, H., McDonald, M.C., Cuzzocrea, S., Thiemermann, C., 1999. A membrane-permeable radical scavenger reduces the organ injury in hemorrhagic shock. Shock 12, 255–261.

Novotny, M.J., Laighlin, M.H., Adams, H.R., 1988. Evidence of lack of importance of oxygen free radicals in *Escherichia coli* endotoxemia in dogs. Am. J. Physiol. 254, H954–H962.

Olson, N.C., Grizzle, M.K., Anderson, D.L., 1987. Effect of polyethylene glycol–superoxide dismutase and catalase on endotoxemia in pigs. J. Appl. Physiol. 63, 1526–1532.

Pucheu, S., Boucher, F., Sulpice, T., Tressallet, N., Bonhomme, Y., Malfroy, B., de Leiris, J., 1996. EUK-8 a synthetic catalytic scavenger of

reactive oxygen species protects isolated, iron-overloaded rat heart from functional and structural damage induced by ischemia–reperfusion. Cardiovasc. Drugs Ther. 10, 331–339.

Redl, H., Lieners, C., Baheartrateami, S., Schlag, G., van Bebber, I.P., Goris, R.J., 1990. SOD in rat models of shock and organ failure. Adv. Exp. Med. Biol. 264, 17–27.

Redl, H., Gasser, H., Schlag, G., Marzi, I., 1993. Involvement of oxygen radicals in shock related cell injury. Br. Med. Bull. 49, 556–565.

Schineder, J., Friderichs, E., Giertz, H., 1989. Protection by recombinant human superoxide dismutase in lethal rat endotoxemia. Prog. Clin. Biol. Res. 308, 913–917.

Simon, H.M., Scalea, T., Paskanik, A., Yang, B., 1996. Superoxide dismutase (SOD) prevents hypotension after hemorrhagic shock and aortic cross clamping. Am. J. Med. Sci. 312, 155–159.

Simons, R.K., Maier, R.V., Lennards, E.S., 1987. Neutrophil function in a rat model of endotoxin-induced lung injury. Arch. Surg. 122, 197–203.

Sledzinski, Z., Wozniak, M., Antosiewicz, J., Lezoche, E., Familiari, M., Bertoli, E., Greci, L., Brunelli, A., Mazera, N., Wajda, Z., 1995. Protective effect of 4-hydroxy-TEMPO, a low molecular weight superoxide dismutase mimic, on free radical toxicity in experimental pancreatitis. Int. J. Pancreatol. 18, 153–160.

Szabo, C., Virag, L., Cuzzocrea, S., Scott, G.S., Hake, P., O'Connor, M.P., Zingarelli, B., Salzman, A., Kun, E., 1998. Protection against peroxynitrite-induced fibroblast injury and artheart rateitis development by inhibition of poly(ADP-ribose) synthase. Proc. Natl. Acad. Sci. U. S. A. 95, 3867–3872.

Thiemermann, C., Ruetten, H., Wu, C.C., Vane, J.R., 1995. The multiple organ dysfunction syndrome caused by endotoxin in the rat: attenuation of liver dysfunction by inhibitors of nitric oxide synthase. Br. J. Pharmacol. 116, 2845–2851.

Traber, D.L., Adams, T., Sziebvert, L., Stein, M., Traber, L., 1985. Potentiation of lung vascular response to endotoxin by superoxide dismutase. J. Appl. Physiol. 58, 1005–1009.

Vespasiano, M.C., Lewandoski, J.R., Zimmerman, J.J., 1993. Longitudinal analysis of neutrophil superoxide anion generation in patients with septic shock. Crit. Care Med. 21, 666–672.

Warner, B.W., Hasselgren, P.O., Fischer, J.E., 1986. Effect of allopurinol and superoxide dismutase in rats with sepsis. Curr. Surg. 43, 292–293.

Yim, M.B., Chock, P.B., Stadtman, E.R., 1990. Copper, zinc superoxide dismutase catalyses hydroxyl radical production from hydrogen peroxide. Proc. Natl. Acad. Sci. U. S. A. 87, 5006–5010.

Zingarelli, B., Day, B.J., Crapo, J.D., Salzman, A.L., Szabo, C., 1997. The potential role of peroxynitrite in the vascular contractile and cellular energetic failure in endotoxic shock. Br. J. Pharmacol. 120, 259–267.

Zingarelli, B., Virag, L., Szabo, A., Cuzzocrea, S., Salzman, A.L., Szabo, C., 1998. Oxidation, tyrosine nitration and cytostasis induction in the absence of inducible nitric oxide synthase. Int. J. Mol. Med. 1, 787–795.