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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

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1 Endotoxaemia causes an enhanced formation of reactive oxygen species (ROS) which contribute to the multiple organ dysfunction syndrome (MODS) in septic shock. Here we investigate (i) the effects of endotoxin on the expression of two isoforms of superoxide dismutase (SOD), namely Cu/Zn-SOD (cytosol) and Mn-SOD (mitochondria) in the rat kidney, and (ii) the effects of the radical scavenger tempol on the MODS caused by lipopolysaccharide (LPS, *E. coli*, 6 mg kg^{-1} i.v.) in the rat.

2 Endotoxaemia resulted in a rapid, but transient, decline in the expression of both mRNA and protein of Cu/Zn-SOD as well as an increase in the expression of the mRNA of Mn-SOD in the kidney. Endotoxaemia for 6 h also caused hypotension, acute renal dysfunction, hepatocellular injury, pancreatic injury and an increase in the plasma levels of nitrite/nitrate.

3 Pretreatment of rats with tempol (100 mg kg^{-1} i.v. bolus injection, 15 min prior to LPS followed by an infusion of 30 mg kg^{-1} i.v., $n=9$) did not affect the circulatory failure, but attenuated the renal dysfunction and the hepatocellular injury/dysfunction caused by LPS. Tempol did not affect the rise in nitrite/nitrate caused by endotoxin.

4 These results imply that an enhanced formation of ROS (including superoxide anions) in conjunction with inadequate defences against such ROS contributes to the injury and dysfunction of the kidney and the liver in endotoxic shock.

Keywords: Endotoxin shock; oxygen radicals; superoxide dismutase; superoxide anions; tempol

Introduction

There is good evidence that endotoxaemia, sepsis and septic shock are associated with the generation of reactive oxygen species (ROS) such as superoxide anions, hydrogen peroxide and peroxynitrite. For instance, elevated levels of hydrogen peroxide have been documented in the expired breath of patients with adult respiratory distress syndrome (ARDS) and/or hypoxaemic respiratory failure (Baldwin *et al*., 1986; Sznajder *et al*., 1989). 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 murine macrophages challenged with endotoxin *in vitro* (Brackett *et al*., 1989). In the presence of nitric oxide (NO), 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). The overproduction of ROS in shock leads to a considerable oxidant stress as indicated by lipid peroxidation, high blood levels of malondialdehyde and conjugated dienes as well as the consumption of the endogenous antioxidants vitamin C and E (Novelli, 1997). Interventions which reduce the generation, or the effects, of ROS exert beneficial effects in a variety of models of endotoxic and septic shock. These therapeutic interventions include N-acetylcystein, α-tocopherol, allopurinol, deferoxamine, catalase and superoxide dismutase (SOD) (see Redl *et al*., 1993).

The therapeutic efficacy of SOD in animals with endotoxic or septic shock is controversial. When given as a pretreatment, SOD does improve survival in rodents with endotoxaemia (Warner *et al*., 1986; Kunimoto *et al*., 1987; Schneider *et al*., 1989). In contrast, SOD does not reduce or even enhances the respiratory dysfunction caused by endotoxin (or sepsis) in sheep, dogs, pigs, rabbits or rats (Traber *et al*., 1985; Olsen *et al*., 1987; Novotny *et al*., 1988; Broner *et al*., 1989; McKechnie *et al*., 1986; Redl *et al*., 1990a). Moreover, recombinant human SOD does not attenuate the multiple organ injury caused by hypovolaemic-traumatic shock in baboons (Redl *et al*., 1990b). The following reasons may explain the lack of effect of SOD against the organ injury associated with endotoxic or septic shock: (1) SOD scavenges superoxide anions, but without efficient removal of the hydrogen peroxide which is produced, levels of hydroxyl radicals may increase (Goode & Webster, 1993). Indeed, SOD may function as a pro-oxidant by catalysing the conversion of hydrogen peroxide to hydroxyl radicals (Yim *et al*., 1990). (2) Neither SOD nor superoxide anions easily cross biological membranes. Thus, an increase in the amounts of extracellular SOD does not attenuate the effects of superoxide anions generated by intracellular sources (Fridovich, 1995). In contrast to SOD, spin trapping nitrones, such as N-*tert*-butyl nitrone (PBN), consistently improve outcome in rat models of endotoxic (McKechnie *et al*., 1986; Hamburger & McCay, 1989) and traumatic shock (Novelli *et al*., 1985; Novelli, 1992). Similarly, pretreatment of rats with a cyclic analogue of PBN reduces mortality in rat models of endotoxaemia and bacteraemia (French *et al*., 1994).

The detrimental effects of free radicals generated either under physiological or pathophysiological conditions is limited

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by endogenous antioxidants and tissue damage only occurs when the generation of these radicals is dramatically enhanced and/or when the antioxidant defences are decreased (Goode & Webster, 1993). Interestingly, the levels of glutathione in the bronchoalveolar lavage fluid of patients with sepsis and acute lung injury are reduced (Pacht *et al*., 1991). The plasma levels of vitamin E (Novelli, 1997) and vitamin C (Borrelli *et al*., 1996) are reduced in patients with sepsis. Unfortunately, there are no data regarding the alterations in the plasma or tissue levels of SOD in animals or man with endotoxaemia.

Here we investigate the effects of endotoxaemia on the expression of the isoforms of superoxide dismutase (SOD) located in the cytosol (Cu/Zn-SOD) and in the mitochondria (Mn-SOD) of the rat kidney. Having demonstrated that endotoxaemia leads to a transient, but profound reduction in the cytosolic levels of Cu/Zn-SOD, we have subsequently investigated the effects of the novel superoxide anion scavenger, tempol, on the circulatory failure and MODS (renal dysfunction, liver injury and dysfunction, pancreatic injury) caused by endotoxin in the rat. Tempol (4-hydroxy-tempo) is a water-soluble analogue of the spin label tempo, which is widely employed in electron spin resonance spectroscopy (Laight *et al*., 1997). Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl) is a stable piperidine nitroxide (stabe free radical) of low molecular weight, which permeates biological membranes and scavenges superoxide anions *in vitro* (Laight *et al*., 1997).

Methods

Surgical procedure

This study was carried out on 62 male rats (Tuck, Rayleigh, Essex, U.K.) weighing 240–320 g receiving a standard diet and water *ad libitum*. All animals were anaesthetized with thiopentone sodium (Intraval Sodium®, 120 mg kg^{-1} 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 (BioScience, Sheerness, Kent, U.K.). The left carotid artery was cannulated and connected to a pressure transducer (Senso-Nor 840, Senso-Nor, Horten, Norway) for the measurement of phasic and mean arterial blood pressure (MAP) and heart rate (HR) which were displayed on a data acquisition system (MacLab 8e, ADI Instruments, Hastings, U.K.). The femoral 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 stabilize for 15 min. It should be noted that all animals received a total fluid replacement of 4 ml kg^{-1} h^{-1} (physiological saline solution, as an in i.v. infusion into the femoral vein) throughout the experiment.

Effects of endotoxin on the expression of Cu/Zn-SOD and Mn-SOD in the kidney of the rat

At 15 min after the end of the surgical procedure, all rats received either vehicle ($n=15$) or *E. coli* lipopolysaccharide (LPS, 10 mg kg^{-1} i.v., $n=15$) as a slow injection over 15–20 min. At 0.5, 1, 2, 4 or 6 h after injection of LPS or vehicle ($n=3$ per time point), groups of animals were killed by an overdose of anaesthetic. Subsequently, the right kidney was removed, snap frozen in liquid nitrogen and stored at −80°C.

Evaluation of the effects of tempol on circulatory failure and MODS: Experimental design

After recording baseline haemodynamic parameters and 15 min prior to the injection of LPS, animals were pretreated with either tempol (100 mg kg^{-1} i.v. bolus injection followed by an infusion of 30 mg kg^{-1} i.v., $n=9$) or vehicle (saline, $n=11$). This dose was chosen as a similar dose of Tempoe prevented the pancreatic injury caused by cerulein in the rat (Sledzinski *et al*., 1995). At time 0 (e.g. 15 min after administration of tempol), all animals received *E. coli* lipopolysaccharide (LPS, 6 mg kg^{-1} i.v.) as a slow injection over 15–20 min. It should be noted that all animals received a total fluid replacement of 4 ml kg^{-1} h^{-1} (as an i.v. infusion into the femoral vein) throughout the experiment. To elucidate the effects of the above interventions on any of the parameters measured in rats injected with vehicle rather than LPS, these animals received bolus injections of either saline ($n=9$) or tempol (100 mg kg^{-1} i.v. bolus injection, 15 min prior to LPS followed by an infusion of 30 mg kg^{-1} i.v., $n=4$).

Quantification of organ function and injury

Six hours after the injection of LPS, 1.5 ml of blood was collected into a serum gel S/1.3 tube (Sarstedt, Germany) from a catheter placed in the carotid artery. The blood sample was centrifuged ($1610\times g$ 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, U.K.). The following marker enzymes were measured in the serum as biochemical indicators of multiple organ injury/dysfunction: (1) Liver injury was assessed by measuring the rise in serum levels of alanine aminotransferase (ALT, a specific marker for hepatic parenchymal injury) and aspartate aminotransferase (AST, a non-specific marker for hepatic injury), γ-glutamyl-transferase (γ-GT) and bilirubin (a specific indicator of liver dysfunction) (Baue, 1993; Hewett *et al*., 1993); (2) 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); (3) Pancreatic injury was assessed by measuring the rise in serum lipase (see Thiemermann *et al*., 1995; Ruetten & Thiemermann, 1997).

Measurement of serum nitrate and nitrite

Nitrite and nitrate are the primary oxidation products formed when NO reacts with oxygen and, therefore, the nitrite/nitrate concentration in plasma can be used as an indicator of NO synthesis. Blood was collected into heparinized capillary tubes and centrifuged ($1610\times g$ for 5 min at room temperature) to separate cells and plasma. The nitrate in the sample was enzymatically converted to nitrite according to the method of Schmidt *et al*. (1992). Briefly, nitrate was stoichiometrically reduced to nitrite by incubation of sample aliquots (50 μl) for 15 min at 37°C, in the presence of nitrate reductase (1 u ml^{-1}, E.C. 1.6.6.2), NADPH (500 μM) in a final volume of 80 μl. When nitrate reduction was complete, the unused NADPH which interferes with the subsequent nitrite determination, was oxidised by lactate dehydrogenase (100 iu ml^{-1}) and sodium pyruvate (100 mM), in a final reaction volume of 100 μl and incubated for 5 min at 37°C. Subsequently, total nitrite in plasma was assayed by adding 100 μl of Griess reagent (4% sulphanilamide and 0.2% naphtylethylenediamide in 10%

phosphoric acid) to 100 μl samples of serum (Green *et al*., 1981). The increase in optical density was measured at 550 nm (reference filter: 650 nm) with a Molecular Devices microplate reader (Richmond, CA, U.S.A.). Total nitrite/nitrate concentrations (μM) were calculated by comparison with the optical density of standard solutions (0–200 μM) of sodium nitrate (also stoichiometrically converted to nitrite) prepared in plasma.

Preparation of tissue homogenates (kidney)

Kidneys from control rats and LPS-treated rats were frozen in liquid nitrogen. Whole kidneys were homogenized in a buffer comprising of: Tris/HCl (20 mM, pH 8.0), NaCl (137 mM), glycerol (10% w v^{-1}), EDTA (5 mM), phenylmethylsulphonyl fluoride (1 mM) and leupeptin (15 μg ml^{-1}). The tissue extract was centrifuged at low speed to remove cell debris and the supernatant was diluted 1 : 1 with water. The cytoplasmic and particulate fractions of total kidney homogenate were prepared by a single centrifugation step (100,000 $\times g$ for 60 min, 4°C) using an Optima TLX Ultracentrifuge (Beckman, Germany). The amount of protein in the cytoplasmic fractions of the homogenates was determined using the Bradford method (Bio-Rad Imaging Densitometer GS-700, Software: Molecular Analyst).

RNA isolation and RNase protection analysis

RNA was isolated according to the procedure described by Chomczynski & Sacchi (1987). Briefly, 20 μg of total RNA extracted from the kidneys (obtained at different time points before or after LPS) were used for RNase protection assays as previously described (Werner *et al*., 1992). The DNA probes were cloned into the transcription vector pBluescript II KS (+) and linearised. An antisense transcript was synthesized *in vitro* using T3 or T7 RNA polymerase and [^{32}P]UTP (800 Ci $mmol^{-1}$). RNA samples were hybridized at 42°C overnight with 100,000 c.p.m. (Cerenkov counting, counting efficiency: ~30%) of the labelled antisense transcript. Hybrids were digested with RNase A (36 μl ml^{-1}) and T1 (720 μl ml^{-1}) for 1 h at 30°C. Under these conditions, every single mismatch is recognized by the RNases. Protected fragments were separated on 5% w v^{-1} acrylamide/8M urea gels and analysed using a PhosphoImager (Fuji, Japan). All protection assays were carried out with at least two different sets of RNA from independent cell culture experiments.

Probe DNAs

The rat Cu/Zn-SOD cDNA probe was cloned by polymerase chain reaction using 5′-GCT GAA GGG CGA CGG TCC GGT-3′ as a 5′-primer and 5′-TCT TGT TTC TCG TGG ACC ACC-3′ as a 3′-primer. The amplified cDNA fragment corresponds to nucleotides 20–370 of the published sequence (Puga & Oates, 1987). Furthermore, we cloned a cDNA fragment of rat Mn-SOD by polymerase chain reaction using 5′-GTC GCT TAC AGA TTG CCG CCT GC-3′ as a 5′-primer and 5′-CTA CTA CAA AAC ACC CAC CAC GG-3′ as a 3′-primer. The cDNA fragment corresponds to nucleotides 481–731 of the published sequence (Ho & Crapo, 1987).

Western blot analysis

Fifty μg of protein from cytoplasmic fractions of kidneys obtained from rats which had either not received LPS (control) or which had received LPS for 30 min, 1, 2, 4 or 6 h was separated using SDS-gel-electrophoresis. After transfer to a polyvinylidene fluoride (PVDF) membrane, Cu/Zn-SOD protein was detected using a polyclonal antiserum directed against human Cu/Zn-SOD (Upstate Biotechnology, Lake Placid, NY, U.S.A.). A secondary antibody coupled to horseradish peroxidase and the enhanced chemiluminescence (ECL) detection system were used to visualize Cu/Zn-SOD protein.

Materials

Tempol, bacterial lipopolysaccharide (*E. coli* serotype 0.127:B8), trifluoroacetic acid, hydrochloric acid, sulphuric acid, sulphanilamide, naphthylethylenediamide and phosphoric acid were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). Sodium thiopentone (Intraval®) was obtained from Rhone Mérieux Ltd. (Harlow, Essex, U.K.). Phenylmethylsulphonyl fluoride and leupeptin were supplied by Sigma Biochemicals (Deisenhofen, Germany), the antibody coupled to horseradish peroxidase was from Biomol (Hamburg, Germany) and the ECL detection system was from Amersham (Braunschweig, Germany).

Statistical evaluation

All values in figures and text are expressed as means ± s.e.mean of n observations, where n represents the number of animals or blood samples studied. The data were analysed using ANOVA followed by Bonferroni's test for multiple comparisons. A P-value of less than 0.05 was considered to be statistically significant.

Results

Effects of endotoxaemia on the expression of Cu/Zn-SOD (mRNA and protein) and Mn-SOD (mRNA) in the kidney of the rat

Figure 1A depicts the kinetics of the expression of Cu/Zn-SOD mRNA and protein in homogenates of kidneys obtained from two separate series of experiments. Treatment of rats with LPS led within 30 min to a very substantial decrease (12.5 fold) in the mRNA for Cu/Zn-SOD (Figure 1A). This decrease in Cu/Zn-SOD mRNA expression was followed by a substantial decrease (5–10 fold) in the expression of Cu/Zn-SOD protein in the kidney (whole kidney extracts) of these animals (Figure 1A). The reduction in the expression of Cu/Zn-SOD protein was only transient, as high levels of Cu/Zn protein were restored within 2 h after administration of LPS (Figure 1A). The kinetics of the expression of the mRNA of the mitochondrial isoform of SOD, Mn-SOD, are depicted in Figure 1B. Injection of LPS resulted in a time-dependent, 9 fold (at 6 h) increase in Mn-SOD mRNA in the kidney of these animals (Figure 1B). As the necessary antibodies to this protein are currently not commercially available, the kinetics of the expression of Mn-SOD protein could not be determined.

Effects of tempol on the circulatory failure caused by endotoxaemia

Baseline values of MAP in all groups of animals ranged from 122 ± 3 to 138 ± 3 mmHg, and were not significantly different between groups (Table 1). Administration of tempol at time −15 min (e.g. prior to administration of LPS), caused a significant fall in MAP. Infusion of LPS (6 mg kg^{-1} i.v.)

produced a progressive fall in MAP from 131±3 mmHg (baseline, prior to infusion of LPS) to 82±5 mmHg at 6 h ($P<0.05$, $n=11$). Treatment of rats which had received LPS with tempol did not significantly affect the hypotension caused by LPS (Table 1).

Baseline values of heart rate in all groups of animals ranged from 365±13 to 383±11 beats min^{-1} (b.p.m.) and were not significantly different between groups (Table 1). In animals which received an injection of vehicle for LPS rather than LPS, administration of tempol did not result in any significant alterations in heart rate. When compared to rats which had received vehicle rather than LPS, endotoxaemia for 6 h did not result in a significant change in heart rate. In rats treated with LPS, tempol had no significant effect on heart rate.

Effects of tempol on the multiple organ dysfunction syndrome caused by endotoxaemia

In animals which received an injection of vehicle for LPS rather than LPS, administration of saline or tempol did not result in any significant alterations in the serum levels of urea or creatinine (Figure 2A and B). When compared to rats which had received vehicle (saline) rather than LPS, endotoxaemia for 6 h resulted in significant rises in the serum levels of urea and creatinine (Figure 2A and B). Treatment of rats which had received LPS with tempol attenuated the renal dysfunction caused by LPS ($P<0.05$, Figure 2A and B).

In animals which received an injection of vehicle for LPS rather than LPS, administration of saline or tempol did not result in any significant alterations in the serum levels of bilirubin, γ-GT, AST and ALT (Figure 3A–D). When compared to rats which had received vehicle (saline) rather than LPS, endotoxaemia for 6 h resulted in significant rises in the serum levels of bilirubin, γ-GT, AST and ALT (Figure 3A–D). Treatment of rats which had received LPS with tempol significantly attenuated the rise in bilurubin, γ-GT and AST caused by LPS ($P<0.05$, Figure 3A–C). Although tempol also caused a 37% reduction in the serum levels of ALT (when compared to LPS-rats), this effect was not significant (Figure 3D).

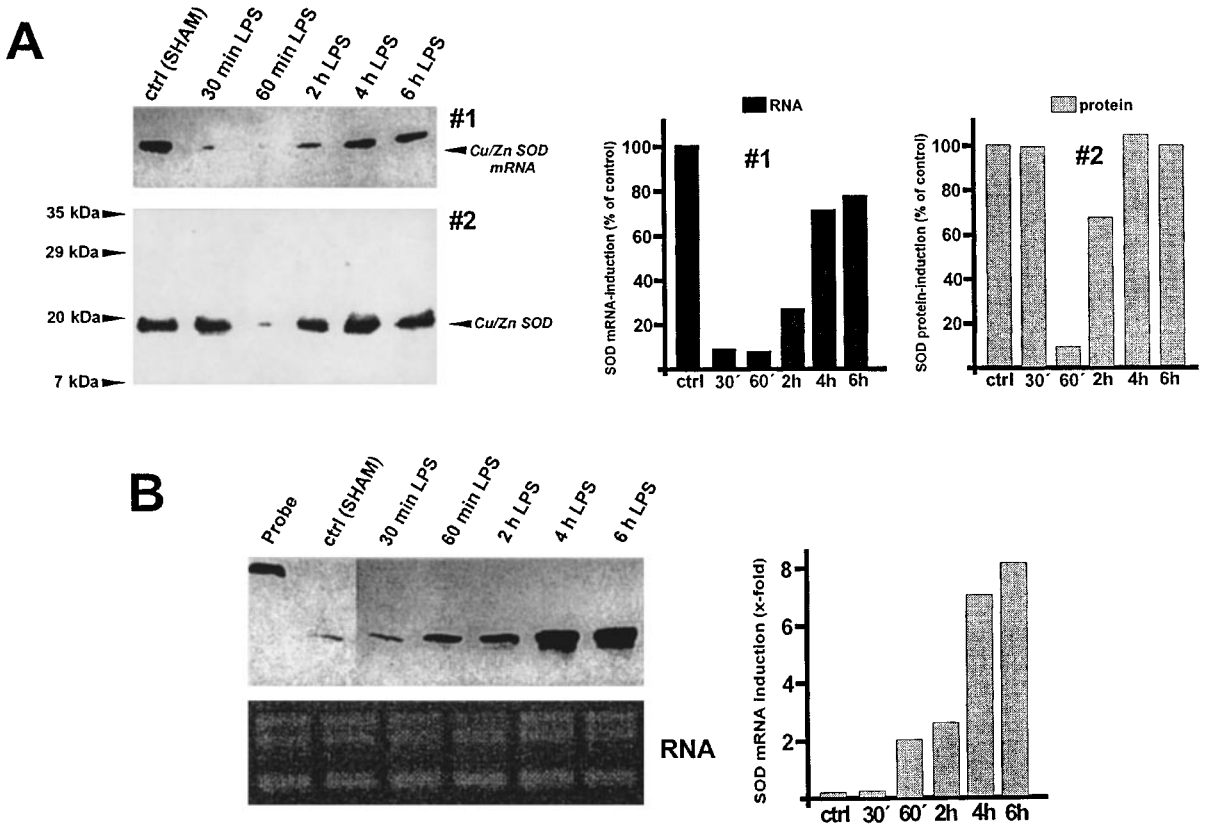

Figure 1 Effects of endotoxaemia on the kinetics of the expression of (A) mRNA and protein of Cu/Zn-SOD and (B) mRNA of Mn-SOD in the kidney (whole kidney extracts) of the rat. (A), Upper panel: Thirty μg of total cellular RNA from kidneys obtained from rats treated with either vehicle (for LPS) or lipopolysaccharide (LPS) at 30 min, 60 min, 2, 4 or 6 h after injection of LPS was analysed for Cu/Zn-SOD mRNA by RNase protection analysis. (A), Lower panel: Fifty μg of protein from the isolated cytoplasmatic fraction of total kidney homogenates obtained from rats treated with either vehicle (for LPS) or lipopolysaccharide (LPS) at 30 min, 60 min, 2, 4 or 6 h after injection of LPS was analysed by immunoblotting for the presence of Cu/Zn-SOD protein. Cu/Zn-SOD with an estimated molecular weight of 15 kDa was detected as indicated by the arrow. Two independent series of control rats and LPS-treated rats ($n=3$ for every data point) were used for analysis as indicated (by #1 and #2). 100% refers to the expression level of Cu/Zn-SOD in sham-operated animals which were not treated with LPS, as Cu/Zn is constitutively expressed in the kidney. (B) Thirty μg of total cellular RNA from kidneys obtained from rats treated with either vehicle (for LPS) or lipopolysaccharide (LPS) at 30 min, 60 min, 2, 4 or 6 h after injection of LPS was analysed for the levels of Mn-SOD mRNA by RNase protection analysis. Please note that Mn-SOD is constitutively expressed in the kidney, but is substantially induced by LPS. The kinetic alterations in the expression of mRNA and protein of Cu/Zn-SOD (A) and in the expression of mRNA of Mn-SOD were assessed by image analysis and scanning densitometry and expressed as % of control (pre-LPS value). Every data point depicted in this figure is a representative example of three individual experiments (animals). None of the results were pooled and the variability between experiments was relatively small. For instance, the maximum inhibition of the expression of Cu/Zn-SOD ranged between 80 and 95% (when compared to the respective control values obtained for time point zero, that is prior to administration of LPS).

Table 1

Group	*Baseline*	*0 min*	*120 min*	*240 min*	*360 min*
LPS + saline					
HR	383 ± 11	384 ± 9	400 ± 8	408 ± 14	400 ± 10
MAP	131 ± 3	1287 ± 4	110 ± 2	99 ± 4	82 ± 5
LPS + tempol					
HR	366 ± 14	355 ± 14	360 ± 13	371 ± 11	385 ± 11
MAP	122 ± 3	108 ± 2*	103 ± 2	104 ± 3	94 ± 4
Control + saline					
HR	368 ± 8	357 ± 11	371 ± 8	370 ± 6	368 ± 8
MAP	129 ± 3	128 ± 1	117 ± 4	108 ± 3	107 ± 5*
Control + tempol					
HR	365 ± 13	361 ± 14	369 ± 12	367 ± 14	373 ± 18
MAP	138 ± 2	121 ± 2	122 ± 2	115 ± 2	115 ± 2*

Alterations in heart rate (HR) and mean arterial blood pressure (MAP) in all animal groups studied at baseline (−15 min), time 0, 120, 240 and 360 min. Rats were treated with vehicle (saline) rather than LPS and subsequently received either vehicle for tempol (saline, 1 ml kg^{-1} i.v., $n=9$) or tempol (100 mg kg^{-1} i.v. bolus injection, followed by an infusion of 30 mg kg^{-1} i.v., $n=4$). Two separate groups of animals were pretreated with either vehicle for tempol (saline, LPS-control, 1 ml kg^{-1} i.v., $n=11$) or tempol (100 mg kg^{-1} i.v. bolus injection, followed by an infusion of 30 mg kg^{-1} i.v., $n=9$) and subsequently received (15 min later) an injection of lipopolysaccharide (LPS, *E. coli*, 6 mg kg^{-1} i.v.).

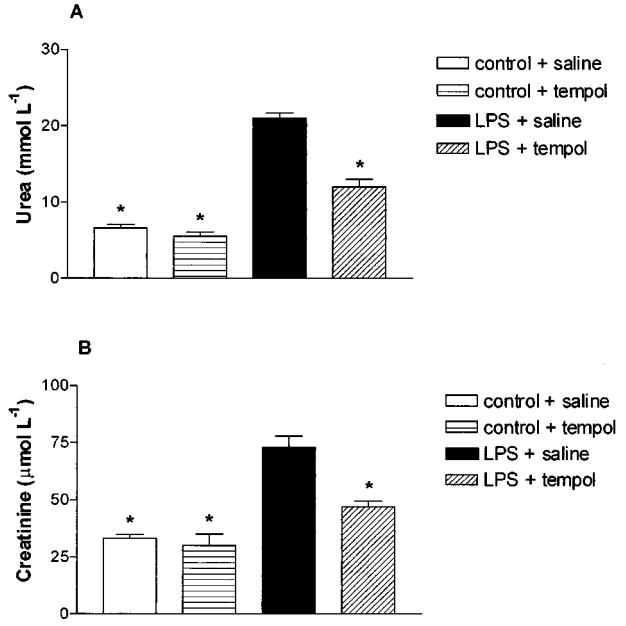

Figure 2 Effects of tempol on the increase in the serum levels of (A) urea and (B) creatinine (indicators of renal dysfunction) caused by LPS (6 mg kg^{-1} i.v.) in the anaesthetized rat. Rats were treated with vehicle (saline) rather than LPS and subsequently received either vehicle for tempol (saline, 1 ml kg^{-1} i.v., $n=9$) or tempol (100 mg kg^{-1} i.v. bolus injection, followed by an infusion of 30 mg kg^{-1} i.v., $n=4$). Two separate groups of animals were pretreated with either vehicle for tempol (saline, LPS-control, 1 ml kg^{-1} i.v., $n=11$) or tempol (100 mg kg^{-1} i.v. bolus injection, followed by an infusion of 30 mg kg^{-1} i.v., $n=9$) and subsequently received (15 min later) an injection of lipopolysaccharide (LPS, *E. coli*, 6 mg kg^{-1} i.v.). *$P<0.05$ represents significant difference when compared to LPS-control.

In animals which received an injection of vehicle for LPS rather than LPS, administration of saline or tempol did not result in any significant alterations in the serum levels of lipase (Figure 4). When compared to rats which had received vehicle (saline) rather than LPS, endotoxaemia for 6 h resulted in a significant rise in the serum levels of lipase (Figure 4). Treatment of rats which had received LPS with tempol did not affect the pancreatic injury caused by LPS ($P<0.05$, Figure 4).

Effects of tempol on the rise in the plasma levels of nitrite/nitrate caused by endotoxaemia

In animals which received an injection of vehicle for LPS rather than LPS, administration of saline or tempol did not result in any significant alterations in the plasma levels of nitrite/nitrate (Figure 5). When compared to rats which had received vehicle (saline) rather than LPS, endotoxaemia for 6 h resulted in significant rises in the plasma levels of nitrite and nitrate (Figure 5). Treatment of rats which had received LPS with tempol did not affect the rise in the serum levels of nitrite/nitrate caused by LPS ($P>0.05$, Figure 5).

Discussion

In the rat model of endotoxic shock used here, 6 h of endotoxaemia resulted in a substantial increase in the plasma levels of urea and creatinine indicating the development of acute renal dysfunction. Pretreatment of rats with the stable piperidine nitroxide, tempol, which permeates biological membranes and scavenges superoxide anions (Laight *et al*., 1997), attenuated the renal dysfunction caused by endotoxin in the rat. Endotoxaemia also resulted in an increase in the serum levels of bilirubin, AST and γ-GT indicating the development of liver injury and dysfunction. Pretreatment of rats with tempol also attenuated this liver injury and dysfunction caused by endotoxin. Tempol however, did not affect the rise in the serum levels of lipase and hence, the pancreatic injury caused by endotoxin in the rat.

Most notably, this study also demonstrates that endotoxaemia results in a substantial, transient decline in the expression of the cytosolic isoform of SOD (Cu/Zn-SOD) in the kidney of rats with endotoxaemia. Within 30 min after injection of endotoxin, the expression of mRNA and protein of Cu/Zn-SOD declined to less than 10% of baseline. Interestingly, this rapid and substantial decline in the expression Cu/Zn-SOD was transient, as the expression of mRNA and protein of this endogenous antioxidant enzyme returned towards baseline at 2–4 h after injection of endotoxin. The mechanism(s) underlying this downregulation of the expression of Cu/Zn-SOD are unclear. In fact, there is little

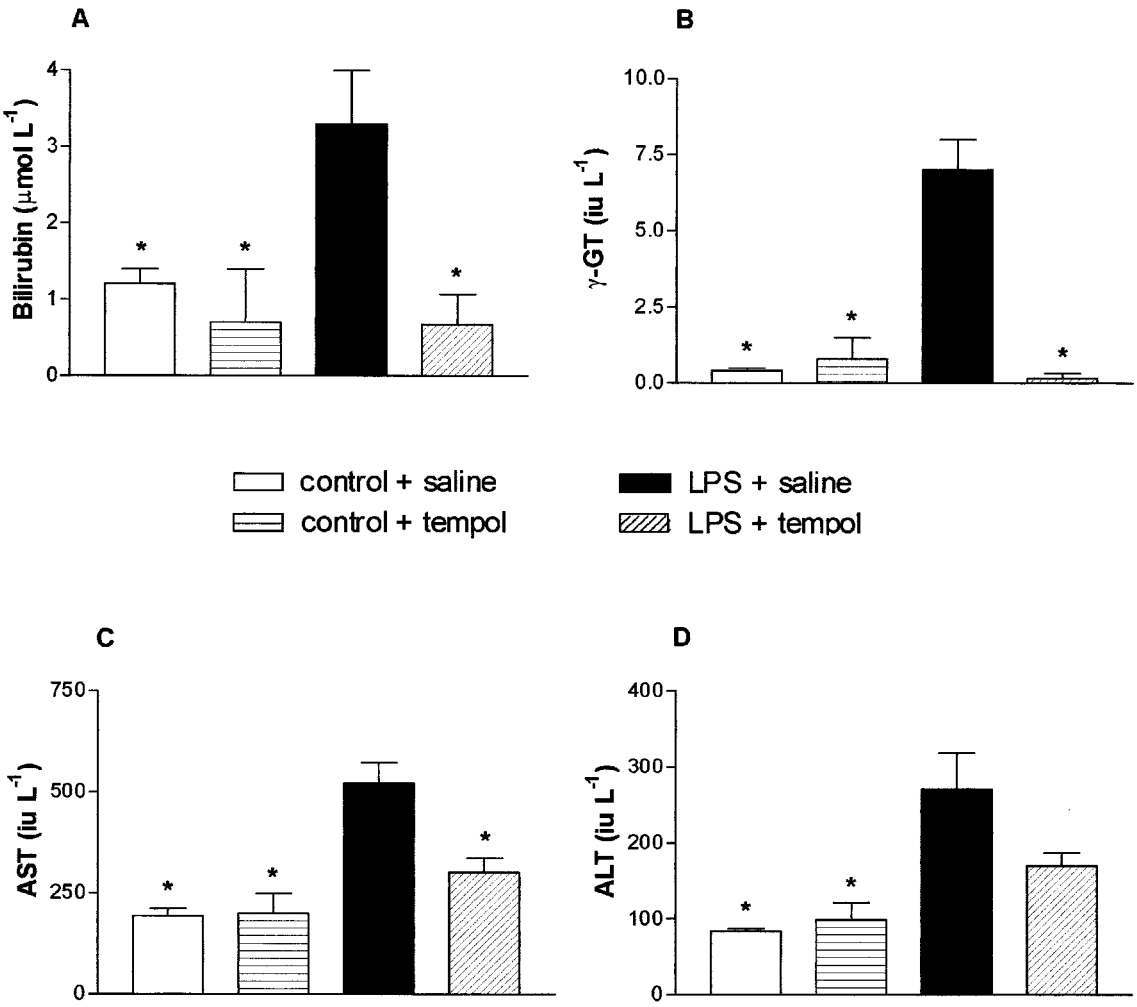

Figure 3 Effects of tempol on the increase in the serum levels of (A) bilirubin, (B) γ-GT, (C) AST and (D) ALT (indicators of hepatic dysfunction and injury) caused by LPS (6 mg kg^{-1} i.v.) in the anaesthetized rat. Rats were treated with vehicle (saline) rather than LPS and subsequently received either vehicle for tempol (saline, 1 ml kg^{-1} i.v., $n=9$) or tempol (100 mg kg^{-1} i.v. bolus injection, followed by an infusion of 30 mg kg^{-1} i.v., $n=4$). Two separate groups of animals were pretreated with either vehicle for tempol (saline, LPS-control, 1 ml kg^{-1} i.v., $n=11$) or tempol (100 mg kg^{-1} i.v. bolus injection, followed by an infusion of 30 mg kg^{-1} i.v., $n=9$) and subsequently received (15 min later) an injection of lipopolysaccharide (LPS, *E. coli*, 6 mg kg^{-1} i.v.). *$P<0.05$ represents significant difference when compared to LPS-control.

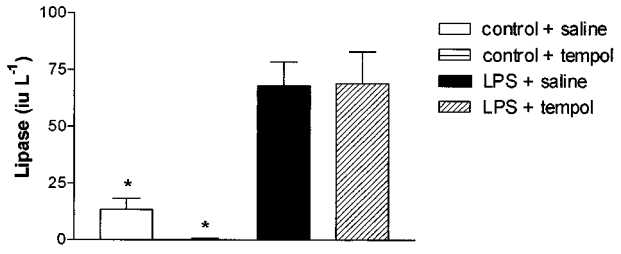

Figure 4 Effects of tempol on the increase in the serum levels of lipase caused by LPS (6 mg kg^{-1} i.v.) in the anaesthetized rat. Rats were treated with vehicle (saline) rather than LPS and subsequently received either vehicle for tempol (saline, 1 ml kg^{-1} i.v., $n=9$) or tempol (100 mg kg^{-1} i.v. bolus injection, followed by an infusion of 30 mg kg^{-1} i.v., $n=4$). Two separate groups of animals were pretreated with either vehicle for tempol (saline, LPS-control, 1 ml kg^{-1} i.v., $n=11$) or tempol (100 mg kg^{-1} i.v. bolus injection, followed by an infusion of 30 mg kg^{-1} i.v., $n=9$) and subsequently received (15 min later) an injection of lipopolysaccharide (LPS, *E. coli*, 6 mg kg^{-1} i.v.). *$P<0.05$ represents significant difference when compared to LPS-control.

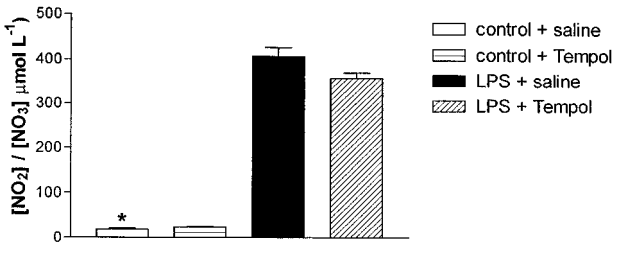

Figure 5 Effects of tempol on the increase in the plasma serum levels of nitrite/nitrate (indicators of NO formation) caused by LPS (6 mg kg^{-1} i.v.) in the anaesthetized rat. Rats were treated with vehicle (saline) rather than LPS and subsequently received either vehicle for tempol (saline, 1 ml kg^{-1} i.v., $n=9$) or tempol (100 mg kg^{-1} i.v. bolus injection, followed by an infusion of 30 mg kg^{-1} i.v., $n=4$). Two separate groups of animals were pretreated with either vehicle for tempol (saline, LPS-control, 1 ml kg^{-1} i.v., $n=11$) or tempol (100 mg kg^{-1} i.v. bolus injection, followed by an infusion of 30 mg kg^{-1} i.v., $n=9$) and subsequently received (15 min later) an injection of lipopolysaccharide (LPS, *E. coli*, 6 mg kg^{-1} i.v.). *$P<0.05$ represents significant difference when compared to LPS-control.

information regarding the factors which regulate the transcription of this isoform of SOD, as Cu/Zn-SOD is constitutively expressed in glomerular mesangial cells and not regulated by cytokines and growth factors (Yoshioka *et al*., 1994; Stephanz *et al*., 1996). Nevertheless, our results indicate that the ability of tissues (such as the kidney) to counteract the consequences of the well-documented overproduction of ROS in endotoxic shock, is substantially reduced in the primary stages (e.g. in the first 2 h) of endotoxaemia.

We also demonstrate that endotoxaemia results in a progressive increase in the expression (of the mRNA) of mitochondrial isoform of SOD, Mn-SOD, in the kidney. This was not entirely surprising as LPS as well as pro-inflammatory cytokines including tumour necrosis factor-α (TNFα) as well as the interleukins (IL) IL-1α and IL-1β induce the expression of Mn-SOD in glomerular mesangial cells and epithelial cells of the rat (Gwinner *et al*., 1995; Stephanz *et al*., 1996). The transcriptional induction of the Mn-SOD gene in certain lung-derived cell lines is secondary to the activation by ROS of the transcription factor NF-κB (Das *et al*., 1995; Warner *et al*., 1996). Interestingly, exposure of rat mesangial cells *in vitro* to hydrogen peroxide also enhanced the expression of Mn-SOD (Yoshioka *et al*., 1994). Taken together, these findings support the view that endotoxin (and/or pro-inflammatory cytokines) enhance the expression of Mn-SOD, but cause a transient (but substantial) reduction in the expression of Cu/Zn-SOD. Clearly, these results demonstrate that the genes for these two isoforms of SOD are differentially regulated.

What then, is the mechanism by which tempol protects the kidney or the liver of the rat against injury and dysfunction? Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl) is a stable piperidine nitroxide (stable free radical) of low molecular weight, which permeates biological membranes and scavenges superoxide anions *in vitro* (Laight *et al*., 1997). In addition, tempol inhibits the catalytic action of transition metal irons and, hence, attenuates the formation of hydroxyl radicals (Monti *et al*., 1996). Similarly, tempol protects cultured rabbit epithelial cells against the injury caused by hydrogen peroxide (Reddan *et al*., 1992). Thus, tempol scavenges intracellular superoxide anions and prevents the formation of hydroxyl radicals. Unlike recombinant SOD, which is not able to cross biological membranes (Fridovich, 1995), tempol is a water-soluble, small molecule which permeates biological membranes and, hence, will function as an intracellular scavenger of superoxide anions. We therefore propose that the beneficial effect of tempol on renal and hepatic integrity and function are due to the ability of this stable free nitroxide radical to function as an intracellular scavenger of superoxide anions and other free radical species. Indeed, there is evidence that stable nitroxide radicals, such as tempol or Tempo, also exert beneficial effects in other conditions associated with an overproduction of ROS. Tempol also attenuates the cardiotoxic effects of adriamycin, which are secondary to the generation of superoxide anions and hydroxyl radicals (Monti *et al*., 1996). Similarly, Tempo reduces the degree of tissue injury caused by regional ischaemia and reperfusion of the rat heart (Gelvan *et al*., 1991).

Surprisingly, tempol did not affect the pancreatic injury caused by endotoxaemia. There is evidence that tempol can cross the membrane of pancreatic cells and is excreted in pancreatic juice. Moreover, infusion of tempol (23 mg kg^{-1} h^{-1} i.v. for 3 h) attenuates the oedema formation and the pancreatic injury (determined by light microscopy) caused by cerulein in the rat (Sledzinski *et al*., 1995). There is little information regarding the mechanism(s) underlying the pancreatic injury caused by endotoxin. The results of our study suggest that the pancreatic injury caused by LPS in the rat is not primarily mediated by superoxide anions.

In rats which had received vehicle rather than LPS, administration of tempol caused a fall in blood pressure of approximately 20 mmHg, which was maintained throughout the infusion of this nitroxide radical. The mechanism(s) underlying this vasodilator effect of tempol are not entirely clear, but may be due to the ability of tempol to enhance the bioavailability of nitric oxide generated by the vascular endothelium (Zöllner *et al*., 1997). Although tempol also caused a fall in blood pressure prior to administration of LPS, it did not enhance the hypotension associated with prolonged periods of endotoxaemia. In fact, the fall in blood pressure caused by endotoxin in rats pretreated with tempol appeared to be less than in the respective control group.

The increase in nitrite/nitrate observed in this study are secondary to an overproduction of NO by iNOS, which contributes to the circulatory failure, but not to the multiple organ dysfunction caused by endotoxin in the rat (Wray *et al*., 1998). There is limited evidence that certain nitrones (e.g. phenyl N-tert-butyl nitrone, PBN), which also scavenge superoxide anions, also attenuate the formation of NO in rats with endotoxaemia (Miyajima & Kotake, 1997). It is, however, unlikely, that any of the observed effects of tempol are due to the ability of this stable free radical to prevent the formation of NO by iNOS, as tempol did not affect the increase in the plasma levels of nitrite/nitrate caused by endotoxin.

In conclusion, this study demonstrates that the stable nitroxide radical tempol attenuates the renal and liver injury/dysfunction (but not the pancreatic injury) caused by endotoxin in the rat. These results support the view that the overproduction of reactive oxygen (or nitrogen) free radicals contributes to the organ injury associated with endotoxaemia. We also demonstrate that endotoxaemia results (i) within 30 min in a transient downregulation of the expression of Cu/Zn-SOD (mRNA and protein), and (ii) in a progressive increase in the expression of Mn-SOD (mRNA). We propose that the downregulation of the cytosolic isoform of SOD sensitizes tissues and organs (including the kidney and liver) to superoxide-mediated tissue injury. Our results also support the notion that ROS (including superoxide anions) contribute to the injury and dysfunction of the kidney and liver. Finally, we propose that small molecules, such as tempol, which permeate biological membranes and function as intracellular radical scavengers, may be useful in the therapy of conditions associated with local or systemic inflammation.

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