Protective effects of a superoxide dismutase mimetic and peroxynitrite decomposition catalysts in endotoxin-induced intestinal damage

*,[1]Daniela Salvemini, [2]Dennis P. Riley, [2]Patrick J. Lennon, [1]Zhi-Qiang Wang, [1]Mark G. Currie, [3]Heather Macarthur & [1]Thomas P. Misko

[1]Discovery Pharmacology, G.D. Searle, Monsanto Co, 800 N. Lindbergh Blvd, St. Louis, MO 63167, U.S.A.; [2]Chemical Sciences, Monsanto Co, 800 N. Lindbergh Blvd, St. Louis, Missouri MO 63167, U.S.A. and [3]Department of Pharmacological and Physiological Sciences, Saint Louis University School of Medicine, 1402 South Grand Blvd, St. Louis, Missori MO 63104, U.S.A.

1   The relative contributions of superoxide anion (O_2^-) and peroxynitrite (PN) were evaluated in the pathogenesis of intestinal microvascular damage caused by the intravenous injection of *E. coli* lipopolysaccharide (LPS) in rats. The superoxide dismutase mimetic (SODm) SC-55858 and the active peroxynitrite decomposition catalysts 5,10,15,20-tetrakis(2,4,6-trimethyl-3,5-disulphonatophenyl)-porphyrinato iron (III) and 5,10,15,20-tetrakis(N-methyl-4′-pyridyl)-porphyrinato iron (III) (FeTMPS, FeTMPyP respectively) were used to assess the roles of O_2^- and PN respectively.

2   The intravenous injection of LPS elicited an inflammatory response that was characterized by a time-dependent infiltration of neutrophils, lipid peroxidation, microvascular leakage (indicative of microvascular damage), and epithelial cell injury in both the duodenum and jejunum.

3   Administration of the SODm SC-55858, FeTMPS or FeTMPyP at 3 h post LPS reduced the subsequent increase in microvascular leakage, lipid peroxidation and epithelial cell injury. Inactive peroxynitrite decomposition catalysts exhibited no protective effects. Only, SC-55858 inhibited neutrophil infiltration.

4   Our results suggest that O_2^- and peroxynitrite play a significant role in the pathogenesis of duodenal and intestinal injury during endotoxaemia and that their removal by SODm and peroxynitrite decomposition catalysts offers a novel approach to the treatment of septic shock or clinical conditions of gastrointestinal inflammation. Furthermore, the remarkable protection of the intestinal epithelium by these agents suggests their use during chemo- and radiation therapy, cancer treatments characterized by gastrointestinal damage. Potential mechanisms through which these radicals evoke damage are discussed.

Introduction

Peroxynitrite (PN) is a highly reactive oxidant produced by the combination of nitric oxide (NO) with superoxide anion (O_2^-) at rates approaching the diffusion limit (Beckman *et al.*, 1990; Ischiropoulos *et al.*, 1992; Beckman & Crow, 1993; Huie & Padmaja, 1993). Under physiological conditions the removal of O_2^- occurs *via* endogenous superoxide dismutases (SOD). Under pathophysiological conditions such as endotoxic shock, the increase in the amount of both NO and O_2^- is such that the reaction between these two free radicals is favoured over that between O_2^- and SOD (Ischiropoulos *et al.*, 1992; Beckman, 1994; Taylor *et al.*, 1995). The overproduction of NO by the inducible nitric oxide synthase enzyme (iNOS) may play a role in the damage that is observed in the microvasculature and mucosa of the gut following the administration of LPS to rats (Salter *et al.*, 1991; Boughton-Smith *et al.*, 1993). Recent indirect evidence, however, points to PN as the culprit in such

damage and raises the interesting possibility that the formation of peroxynitrite from NO and O_2^-, rather than NO itself, is the major cause of gastric injury observed in the gut during sepsis (Lamarque & Whittle, 1995a, b). To date, the data that has been generated to support the role of peroxynitrite *in vivo* is indirect and has relied mainly on pharmacological (presumed inhibition of PN through reduction of NO by NOS inhibitors) and biochemical (measurement of nitrotyrosine, a marker for peroxynitrite mediated reactions) approaches. In order to explore directly the role(s) of peroxynitrite in disease, we have developed a class of porphyrin-containing compounds which catalytically decompose peroxynitrite to nitrate (Stern *et al.*, 1996). These catalysts do not directly react with either O_2^- or NO (Stern *et al.*, 1996) and therefore can be utilized to assess the direct contributions of peroxynitrite (Stern *et al.*, 1996; Misko *et al.*, 1998; Salvemini *et al.*, 1998). Two of these catalysts, FeTMPS and FeTMPyP, were found to be effective both *in vitro* and *in vivo* at removing peroxynitrite and preventing its cytotoxic effects (Salvemini *et al.*, 1998; Misko *et al.*, 1998). More importantly, pharmacological use of these catalysts allowed us to demonstrate for the first time that,

*Author for correspondence.

indeed, peroxynitrite plays a key role *in vivo* in the development of the inflammatory response since its removal resulted in potent anti-inflammatory effects (Salvemini *et al.*, 1998).

In addition to its role in the formation of PN, little is known about the participation of O_2^- in the sequence of events underlying endotoxin-induced intestinal injury. This was examined in the present study by using a superoxide dismutase mimetic (SODm) which catalytically removes O_2^- without interfering with NO, $ONOO^-$ or other radicals such as the hydroxyl radical or hydrogen peroxide (Riley *et al.*, 1996). These are low molecular weight, manganese containing, non-peptide molecules possessing the function and catalytic rates of native SOD enzymes, but having the advantage of being much smaller molecules (MW 400-500 *vs* MW 30,000 for the mimetic and native enzyme, respectively). In the present study we have used, SC-55858 a water soluble pentaaza macrocyclic Mn(II) complex containing the *2R, 3R, 8R, 9R*-all-*trans*-fused cyclohexano substituents on the carbon atoms of the macrocycle (Figure 1) (Riley *et al.*, 1997). SC-55858 is an excellent SOD catalyst ($Mn(C_{18},H_{37}N_5)Cl_2$; Mo. Wt. = 449.4) with a second-order catalytic rate constant at pH = 7.4 of $1.2 \times 10^{+8}$ M^{-1} s^{-1} rivaling that of the native Mn SOD enzyme (Riley *et al.*, 1997). These mimetics potentially differ from other so-called metal based SODm such as the manganese complex with beta-octabromo-meso-tetrakis-(N-methylpyridinium-4-yl) porphyrin or manganese [III] tetrakis 4-benzoic acid porphyrin (Batinic-Haberle *et al.*, 1997) or the manganese [III] tetrakis(benzoic acid) porphyrin (Day *et al.*, 1995; Gardner *et al.*, 1996) in that they are more chemically stable and they possess much faster rates for the dismutation of O_2^-. Additionally, the porphyrin complexes should not necessarily be regarded as SOD mimetic since they have not been shown to perform an actual catalytic dismutation (Weiss *et al.*, 1993), but rather have been demonstrated to perform the stoichiometric dismutation of superoxide to hydrogen peroxide.

SC-55858, FeTMPS and FeTMPyP were used to dissect pharmacologically the contributions of O_2^- and peroxynitrite to the intestinal injury resulting from systemic exposure to endotoxin.

Methods

Surgical procedure

Male Sprague Dawley rats (200 – 230 g) were anaesthetized with inactin (100 mg kg^{-1} intraperitoneally). The trachea was cannulated to facilitate respiration and body temperature maintained at 37°C by means of a heating pad. The left
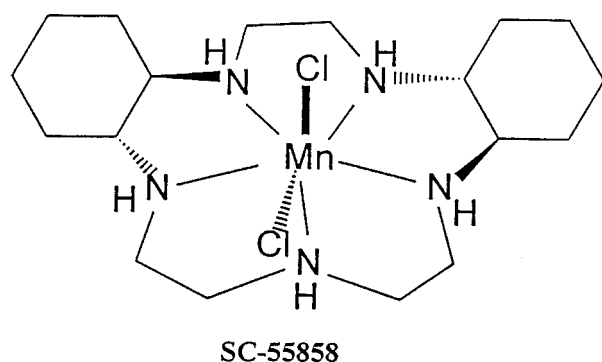
SC-55858

Figure 1 Structure of the superoxide dismutase mimetic SC55858.

femoral vein was cannulated for the administration of drugs. Lipopolysaccharide from *E. coli* (LPS; 3 mg kg^{-1}, serotype O111 : B4) was administered as a bolus intravenous (i.v) injection at a volume of 0.3 ml. Control animals received isotonic saline at the same volume and by the same route.

Drug administration

Drugs were dissolved in isotonic saline. All drugs were given by i.v. injection at a volume of 0.3 ml at 3 h (therapeutic protocol) after the administration of saline or LPS (a time point when damage was seen) and the animals sacrificed 2 h later (in order to determine effects of drugs on further damage). Control animals received isotonic saline at the same volume (0.3 ml) and by the same route (i.v.). In some experiments, animals received a single dose of colchicine (1.5 mg kg^{-1}, Salvemini *et al.*, 1995) 15 min before the injection of LPS. Animals were sacrificed at 3 and 5 h after LPS. Experiments were also performed with SODm and PN decomposition catalysts in the colchicine treated rats.

Myeloperoxidase activity

Myeloperoxidase (MPO), a haemoprotein located in azurophil granules of neutrophils, has been used as a biochemical marker for neutrophil infiltration into tissues (Bradley *et al.*, 1982). In the present study, MPO was measured spectrophotometrically by a method similar to that described previously (Laight *et al.*, 1994). At the specified times following the injection of LPS, segments of duodenums and jejunums were removed and homogenized in ice-cold phosphate buffer (50 mM, pH 6) containing 0.5% hexadecyltrimethylammonium bromide (HTAB), freeze-thawed three times and centrifuged (10,000 × g, 15 min, 4°C). Following centrifugation, 10 μl aliquots of each of the supernatants were mixed with 200 μl of assay buffer (50 mM phosphate buffer, pH 6, containing 0.5% HTAB, 0.167 mg ml^{-1} *O*-dianisidine hydrochloride and 0.0005% hydrogen peroxide). Changes in absorbance at 460 nm were measured spectrophotometrically over 5 min and MPO activities were expressed as mU g^{-1} wet tissue).

Lipid peroxidation

Malondialdehyde (MDA) has been used as a biochemical marker for lipid peroxidation and was measured by a method similar to that described previously (Okhawa *et al.*, 1979). Levels of MDA were measured at 0, 3 and 5 h post-injection of LPS. Segments of duodenum and jejunum were removed and immediately frozen in liquid nitrogen, and homogenized in potassium chloride (1.15%). 2 ml of chloroform was added to each homogenate and the samples were then spun for 30 min. The organic layer of the sample was removed and dried under nitrogen gas and reconstituted with 100 μl of saline. MDA production was estimated by measuring thiobarbituric acid (TBA)-reacting compounds. Briefly, to each sample was added the following reaction mixture: 20 μl of 8.1% sodium dodecyl sulphate (SDS), 150 μl of 20% acetic acid solution (pH 3.5), 150 μl of 0.8% TBA and 400 μl of distilled water. The mixture was heated at 95°C for 15 min and allowed to cool. To extract the chromogenic product, 100 μl distilled water and 500 μl of n-butanol and pyridine (15:1, v v^{-1}, containing 0.05% butylated hydroxytoluene) was added to the samples which were centrifuged (4000 r.p.m., 10 min) to facilitate phase separation. The organic layer was removed and MDA measured by reading the absorbance at 532 nm. Results are expressed as nmol MDA g^{-1} tissue.

Intravascular volume

Changes in intravascular volume in the intestinal tissue were determined in an additional group of rats by administering ^{125}I-labelled bovine serum albumin ($[^{125}I]$-BSA) intravenously (0.5 ml; 0.5 μCi) 2 min before surgical removal of the jejunum. The tissue and plasma content of radiolabel was determined and intravascular volume expressed as μl g^{-1} tissue. This value was subtracted from that obtained in the plasma leakage studies to obtain a measure of the intestinal plasma albumin leakage.

Plasma leakage

Intestinal vascular permeability was determined as the leakage into the jejunal tissue of $[^{125}I]$ -BSA administered intravenously (0.5 ml; 0.5 μCi) together with either LPS (3 mg kg^{-1}) or isotonic saline. At various times (1–6 h) after LPS or saline administration, segments (3–5 cm) of duodenal or jejunal tissues were ligated and removed. The intestinal tissues were rapidly washed, blotted dry and weighed. Blood (0.5 ml) was collected into tubes containing tri-sodium citrate (0.318% final concentration) and plasma prepared by centrifugation (10,000 $\times$ g for 10 min). The $[^{125}I]$-BSA content in segments of whole tissue and in aliquots of plasma (100 μl) was determined in a gamma counter. The total content of plasma in the intestinal tissues was expressed as μl g^{-1} tissue.

Intestinal epithelial cell isolation

Intestinal epithelial cells were isolated from segments of the intestine as described previously (Lentze *et al.*, 1985). A segment of proximal small intestine was obtained and slowly flushed with 50 ml of a solution containing 0.15 M NaCl dithiothreitol (DTT). The segment was then filled with 5 ml of a solution containing (in mM): KCl 1.5, NaCl 96, sodium citrate 27, KH$_2$PO$_4$ 8 and Na$_2$HPO$_4$ 5.6 (pH 7.3); the proximal and the distal ends were ligated. The segments were then immersed for a period of 15 min

in phosphate-buffered saline (PBS) kept at 37°C which was bubbled with 95% O$_2$-5% CO$_2$. The solution from inside the segments was removed and another containing 1.5 mM EDTA and 0.5 mM DTT was added for 5 min, after which epithelial cells were collected (Tepperman *et al.*, 1993). The preparation was subsequently washed twice with PBS (pH 7.4), centrifuged for 5 min (800 $\times$ g) and resuspended in a buffer containing N-2-hydroxyethylpiperazipine-N-2-ethane-sulphonic acid (HEPES, 10 mM), sucrose (320 mM), DTT (1 mM), soybean trypsin inhibitor (10 μg ml^{-1}), leupeptin (10 μg ml^{-1}) and aprotinin (2 μg ml^{-1}). This technique yielded a preparation of epithelial cells at 99% purity (Tepperman *et al.*, 1993), which was confirmed by formaldehyde fixation and staining with hematoxylin-eosin-safran (HES). In all experiments an aliquot of cells isolated from control, and from the experimental groups under investigation was examined for viability as determined by trypan blue dye exclusion (0.5% trypan blue in PBS). This technique has been shown to be a reliable index of epithelial cell injury (Tepperman *et al.*, 1993). Results are expressed as per cent of damaged cells.

Materials

$[^{125}I]$-bovine bovine serum albumin was obtained from Amersham International (U.S.A.). Human polymorpho-nuclear leukocyte myeloperoxidase was obtained from Calbiochem (La Jolla, CA, U.S.A.). All other chemicals and reagents were obtained from Sigma (St. Louis, MO, U.S.A.). SC-55858 and the PN decomposition catalysts were synthesized as described previously (Stern *et al.*, 1996; Riley *et al.*, 1996).

Statistical analysis

Results are expressed as mean $\pm$ s.e.mean for (*n*) rats. The results were analysed by Student's unpaired *t*-test to determine the significant differences between means, or by a two-way ANOVA followed by a least significant procedure to determine
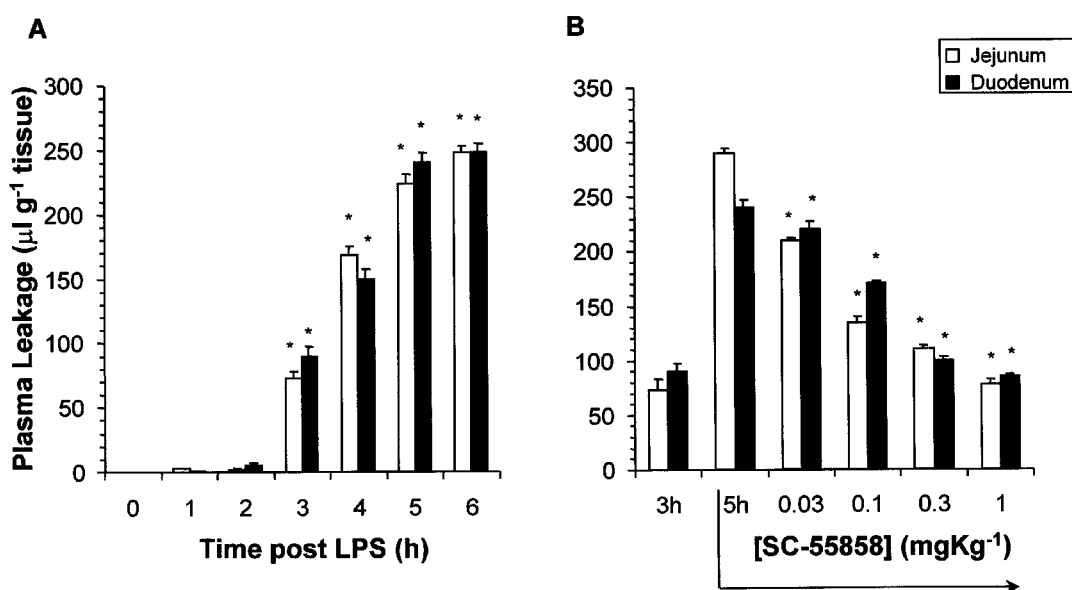
Figure 2 (A) Time-dependent induction of plasma leakage in the jejunum and duodenum following LPS (3 mg kg^{-1}). (B) SC-55858 (0.03–1 mg kg^{-1}) administered at 3 h post LPS, inhibited in a dose-dependent manner, the further increase in plasma leakage observed in jejunum and duodenum at the 5 h time-point. Each bar represents the mean $\pm$ s.e.mean of five experiments. *$P < 0.05$.

the nature of this response. A *P* value of <0.05 was considered to be statistically significant.

Results

Selectivity of SODm and PN-decomposition catalysts

In a previous series of papers (Riley *et al.*, 1996; 1997), we have shown that the pentaaza macrocyclic ligand complexes of Mn(II) can be not only highly active catalysts for the dismutation of O_2^- but that they are also highly selective. The complex SC-55858 has, for example, been found to catalytically dismute O_2^- at a rate exceeding 10^{+8} molecules of O_2^- per molecule of complex per second at pH = 7.4 and 21°C, at a rate comparable to the native Mn SOD enzyme. Remarkably, this complex does not react with hydrogen peroxide under the same conditions (Riley *et al.*, 1996; 1997), nor does it react with other biologically relevant oxidants such as PN or nitric oxide. Thus, in our assays to monitor catalytic catalase activity using oxygen electrodes (Marshall & Worsfold, 1978; Pasternack & Pysnik, 1983), in which total oxygen concentration evolved from the reaction of hydrogen peroxide with catalase (or any putative catalase mimic) is quantitatively monitored, no catalytic activity is observed between SC-55858 and H_2O_2, further no stoichiometric reaction is observed to occur between SC-55858 and H_2O_2 as monitored *via* spectrophotometric or electrochemical (cyclic voltammetric) techniques. The stopped flow assay developed for monitoring peroxynitrite catalytic activity (Stern *et al.*, 1996) was utilized to assess the PN activity of SC-55858. No catalytic PN activity is observed with SC-55858, nor is there any stoichiometric reactivity with PN observed with SC-55858, as monitored spectrophotometrically. The PN catalysts utilized in this study were shown to possess no catalase nor any direct catalytic SOD activity (Stern *et al.*, 1996). These substrate specificities allow us to probe directly the physiological roles that the small molecules, O_2^- and PN, play by studying the effects that such selective catalysts exhibit *in vivo*.

### SODm and PN-decomposition catalysts reduce LPS-induced plasma leakage

In control rats or in rats that received an intravenous injection of LPS, the intravascular blood volume in the duodenum or jejunum as determined by the tissue level [^{125}I]-BSA injected 2 min before the removal of these tissues did not change over the entire period of the experiment (6 h). LPS elicited a pronounced increase in plasma leakage in the jejunum and duodenum by 3 h (Figure 2A). The therapeutic injection of the SODm, SC-55858 (0.03 – 1 mg kg^{-1}, *n* = 5) (Figure 2B) or the PN decomposition catalysts, FeTMPS and FeTMPyP (1 – 30 mg kg^{-1}, *n* = 5) at 3 h post LPS (time point when significant damage was already seen) caused a dose-dependent inhibition of the leakage observed at the 5 h time point (Figure 3A and B). On the other hand, the inactive PN decomposition catalysts, H_2TMPS or ZnTMPyP (30 mg kg^{-1}, *n* = 5) had no effect (Figure 3A and B).

Differential effects of SODm and PN-decomposition catalysts on LPS-induced neutrophil infiltration

The intravenous injection of LPS elicited a time-dependent neutrophil infiltration into the duodenum and jejunum as evidenced by the presence of MPO (an index of neutrophil influx) (Figure 4A). At the highest doses tested, the therapeutic injection (3 h post LPS) of the SODm, SC-55858 (1 mg kg^{-1}, i.v, *n* = 5), Figure 4B) prevented the subsequent increase in neutrophil infiltration observed at the 5 h time point. In contrast, FeTMPS and FeTMPyP (30 mg kg^{-1}, i.v, *n* = 5) had no effect (Figure 4B).

SODm and PN-decomposition catalysts reduce LPS-induced lipid peroxidation

Lipids of biological membranes are often important targets for modification by O_2^- and peroxynitrite and MDA is a product that is formed as a consequence of free radical mediated lipid peroxidation. Consistent with the increase in vascular
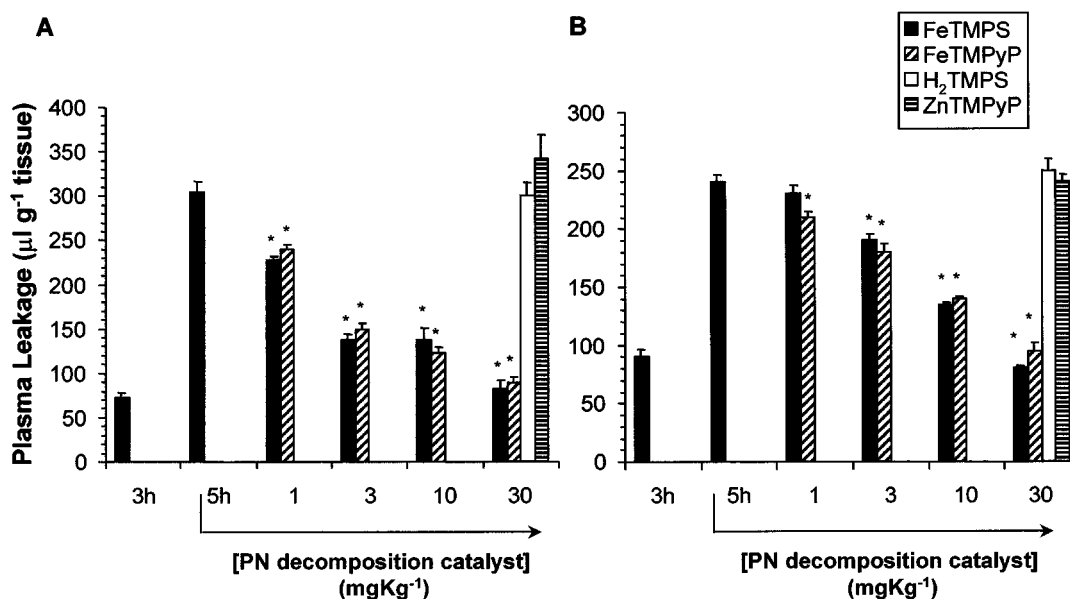
Figure 3 Effects of peroxynitrite decomposition catalysts on plasma leakage. FeTMPS (1 – 30 mg kg^{-1}) or FeTMPyP (3 – 30 mg kg^{-1}), administered at 3 h post LPS, inhibited in a dose-dependent manner, the increase in plasma leakage observed in jejunum (A) and duodenum (B) at the 5 h time-point. H_2TMPS or ZnTMPyP (both at 30 mg kg^{-1}) had no effect (A and B). Each bar represents the mean ± s.e.mean of five experiments. *P < 0.05.

A

B
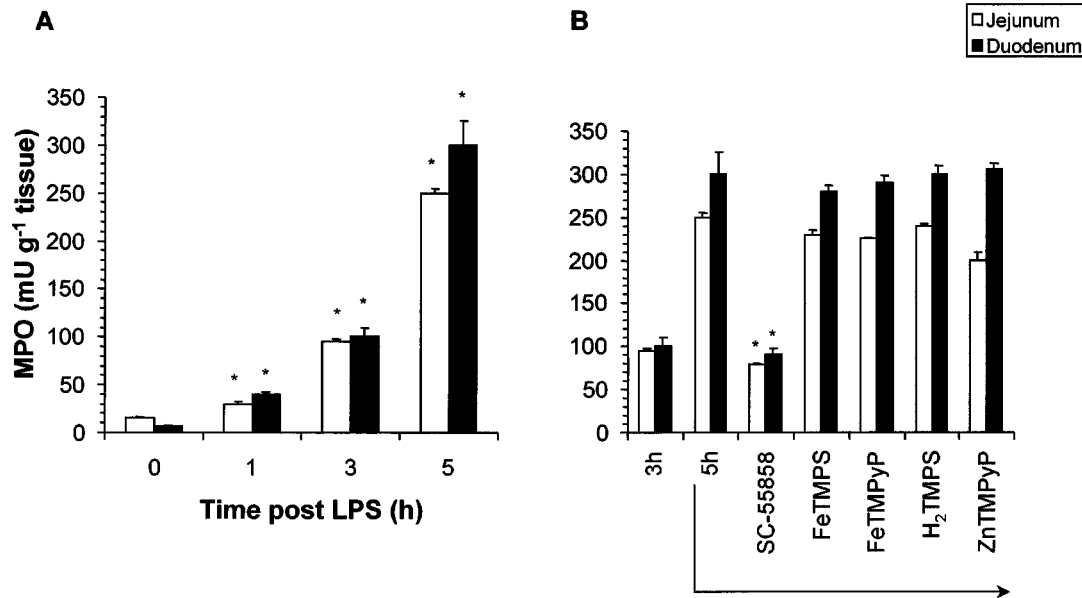
☐ Jejunum
■ Duodenum

Figure 4 LPS caused a time-dependent infiltration of neutrophils into the jejunum and duodenum (A). The SODm, SC-55858 (1 mg kg^{-1}) when given at 3 h post LPS attenuated further neutrophil accumulation observed at 5 h (B). No effect was seen with active (FeTMPS, FeTMPyP) or inactive (H$_2$TMPS, ZnTMPyP) PN decomposition catalysts (30 mg kg^{-1}) (B). Each bar represents the mean±s.e.mean of five experiments. *$P < 0.05$.

A

B

☐ Jejunum
■ Duodenum
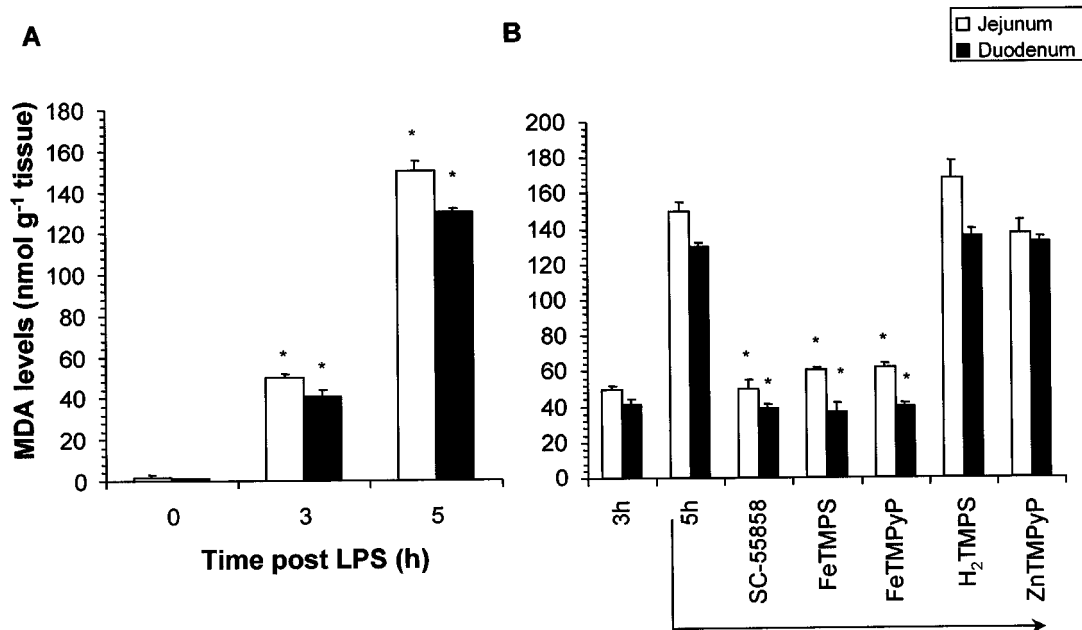
Figure 5 LPS caused a time-dependent increase in lipid peroxidation of jejunal and duodenal segments (A). SC-55858 (1 mg kg^{-1}), FeTMPS or FeTMPyP (30 mg kg^{-1}) when given at 3 h post LPS attenuated the lipid peroxidation observed at 5 h (B). The inactive PN decomposition catalysts (H$_2$TMPS, ZnTMPyP; 30 mg kg^{-1}) had no effect (B). Each bar represents the mean±s.e.mean of five experiments. *$P < 0.05$.

permeability, the intravenous injection of LPS elicited lipid peroxidation of the intestinal membranes as evidenced by the time-dependent increase in measurable MDA in both duodenal and jejunal preparations (Figure 5A). No lipid peroxidation products were observed at time 0 or 1 h after injection but significant peroxidation was observed at the 3 and 5 h time points (Figure 5A). At the highest doses tested, the therapeutic injection (3 h post LPS) of the SODm, SC-55858 (1 mg kg^{-1}, i.v, $n = 5$, Figure 5B) or FeTMPS and FeTMPyP (30 mg kg^{-1}, i.v, $n = 5$, Figure 5B) prevented the subsequent increase in

intestinal epithelial cell damage observed at the 5 h time point. As expected, H$_2$TMPS or ZnTMPyP (30 mg kg^{-1}, i.v, $n = 5$) had no effect (Figure 5B).

LPS-induced epithelial cell damage and effects of SODm and PN-decomposition catalysts

At 3 and 5 h after the intravenous injection of LPS, the percentage of damaged intestinal epithelial cells was 26±2 and 74±3%, respectively (Figure 6A). At the highest doses tested,
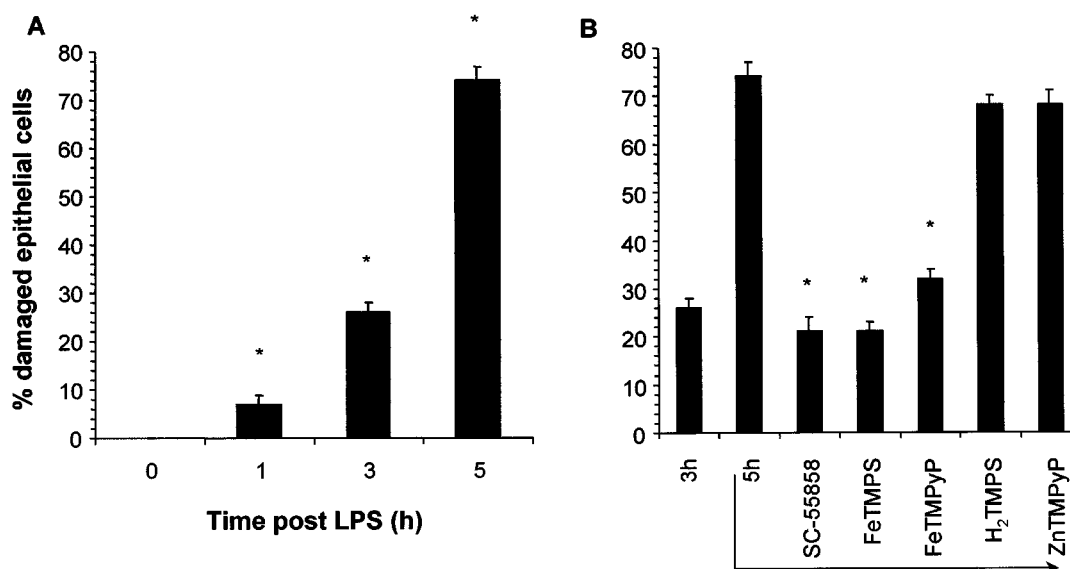
Figure 6 LPS caused a time-dependent damage of intestinal epithelial cells (A). SC-55858 (1 mg kg^{-1}), FeTMPS or FeTMPyP (30 mg kg^{-1}) when given at 3 h post LPS attenuated further epithelial cell damage observed at 5 h (B). The inactive peroxynitrite decomposition catalysts H$_2$TMPS or ZnTMPyP had no protective effect (B). Each bar represents the mean±s.e.mean of five experiments. *$P < 0.05$.

the therapeutic injection of the SODm, SC-55858 (1 mg kg^{-1}, $n = 5$, Figure 6B) or FeTMPS and FeTMPyP (30 mg kg^{-1}, $n = 5$, Figure 6B) at 3 h post LPS prevented the subsequent increase in intestinal epithelial cell damage observed at the 5 h time point (Figure 6B. H$_2$TMPS or ZnTMPyP (30 mg kg^{-1}, $n = 5$) were ineffective (Figure 6B).

Neutrophil contribution to LPS-mediated intestinal damage: effects of colchicine treatment

To determine the relative contribution of neutrophils to endotoxin-induced intestinal damage, animals were made neutropenic by pre-treatment with colchicine as described in the Methods section: all parameters of inflammation were subsequently measured at 3 and 5 h after LPS. Colchicine inhibited neutrophil infiltration (from 95 ± 3 and 250 ± 5 to 15 ± 7 and 20 ± 3 mU MPO g^{-1} tissue at 3 and 5 h for LPS and LPS + colchicine respectively, $n = 5$), lipid peroxidation (from 50 ± 3 and 150 ± 3 to 10 ± 3 and 100 ± 3 nmol MDA g^{-1} tissue at 3 and 5 h for LPS and LPS + colchicine respectively, $n = 5$), plasma leakage (from 73 ± 5 and 233 ± 8 to 25 ± 2 and 156 ± 5 μl g^{-1} tissue at 3 and 5 h for LPS and LPS + colchicine respectively, $n = 5$) and epithelial cell damage (from 26 ± 2 and $74 \pm 3\%$ to 3 ± 2 and $45 \pm 5\%$ damaged cells at 3 and 5 h for LPS and LPS + colchicine respectively, $n = 5$). Interestingly, although colchicine was as effective in inhibiting neutrophil infiltration at 3 and 5 h, the degree of protection towards lipid peroxidation, plasma leakage and epithelial cell damage was greater at the 3 h time point. We next assessed the effects of the SODm and PN decomposition catalysts in colchicine-treated rats. For this purpose, SC-55858 (1 mg kg^{-1}), FeTMPS or FeTMPyP (30 mg kg^{-1}) were given 3 h after endotoxin. All drugs prevented damage seen at the 5 h time point. Levels of plasma leakage went from: 25 ± 2 and 156 ± 5 μl g^{-1} tissue for colchicine alone at 3 and 5 h to 20 ± 3, 25 ± 7 and 25 ± 4 μl g^{-1} tissue for colchine in the presence of SC-55858, FeTMPS and FeTMPyP respectively at 5 h, $n = 5$, levels of MDA went from: 10 ± 3 and 100 ± 3 nmol MDA g^{-1} tissue for colchicine alone at 3 and 5 h to 13 ± 2, 12 ± 1 and 10 ± 2 nmol MDA g^{-1} tissue for colchine in the presence of SC-55858, FeTMPS and

FeTMPyP respectively at 5 h, $n = 5$, and percentage of damaged epithelial cells went from: 3 ± 2 and $45 \pm 5\%$ damaged cells for colchicine alone at 3 and 5 h to 2 ± 0.3, 3 ± 1 and $2 \pm 1\%$ damaged cells for colchicine in the presence of SC-55858, FeTMPS and FeTMPyP respectively at 5 h, $n = 5$.

Discussion

Some of the characteristics of endotoxin shock include hypotension, intravascular coagulation, increases in micro-vascular permeability and multi-organ damage with the lung and gastro-intestinal tract being prime targets (Schlag *et al.*, 1991). Peroxynitrite is a powerful oxidant which is highly reactive towards biological molecules including protein and non-protein sulfhydryls, DNA, and membrane phospholipids (Beckman *et al.*, 1990; Radi *et al.*, 1991a, b; Graham *et al.*, 1993). Peroxynitrite is also stable enough to cross several cell diameters to reach targets before becoming protonated and decomposing (Crow & Beckman, 1995). It is, therefore, not surprising that evidence is increasing for a major role for peroxynitrite in the development of tissue damage during inflammation (Lamarque & Whittle, 1995a, b; Dawson, 1995; Shigenawa *et al.*, 1997) as well as in human subjects during sepsis (Fukuyama *et al.*, 1997). Nevertheless, as indicated earlier, implications for the participation of peroxynitrite in diseases has relied on indirect evidence, mainly through the measurement of nitrotyrosine. The presence of nitrotyrosine at the site of injury does not however prove that peroxynitrite caused the damage, simply that it was formed. By using pharmacological agents specific for peroxynitrite decomposition (Salvemini *et al.*, 1998; Misko *et al.*, 1998), our results clearly indicate that peroxynitrite is the major cause of the intestinal damage following endotoxin administration. Thus, FeTMPS and FeTMPyP, which we have demonstrated to be active peroxynitrite decomposition catalyst (Stern *et al.*, 1996; Misko *et al.*, 1998; Salvemini *et al.*, 1998) protected against microvascular injury, lipid peroxidation and epithelial cell injury.

The fact that the SOD mimetic, SC-55858, was also protective indicates that superoxide anions also play an important role in the damage evoked by endotoxin. Numerous cell types release superoxide anions during the inflammatory response and these include endothelial cells, epithelial cells, macrophages and neutrophils (Broner *et al.*, 1988; Henson & Johnston 1988). As seen here, neutrophils do infiltrate into the intestine and their activation at the site of injury may contribute to O_2^- production and subsequent damage. Evidence does exist to support a damaging role of superoxide in the gastro-intestinal tract. For instance, the native enzyme SOD inhibits microvascular injury following ischaemia-reperfusion of the intestine and stomach (Droy-Lefaix *et al.*, 1991; Haglind *et al.*, 1994; Xia *et al.*, 1995) and direct generation of superoxide anions from xanthine-xanthine oxidase infused intra-arterially provokes gastric mucosal injury (Espluges & Whittle, 1989; Lamarque & Whittle, 1995a, b). Although neutrophils are probably the main source for the release of superoxide, and presumably playing a major role in the generation of peroxynitrite, a neutrophil involvement is not an obligatory requirement for the damage observed after endotoxin. Indeed, the protective effects of the peroxynitrite-decomposition catalysts were obtained despite the lack of effect by the catalysts on neutrophil infiltration. In contrast to peroxynitrite decomposition catalysts, SODm did inhibit neutrophil infiltration. Our findings with the SOD mimetic SC-55858 are consistent with a role for O_2^- in mediating neutrophil adhesion and infiltration (Scraufstatter *et al.*, 1987; Warren *et al.*, 1990) and with numerous reports indicating an inhibition of neutrophil infiltration at the site of inflammation by the native SOD enzyme (Hirschelmann & Bekemeier 1981; Boughton-Smith *et al.*, 1993; Salvemini *et al.*, 1996). Thus, besides inhibiting the formation of peroxynitrite, part of the protective effect of the SODm could be attributable to inhibition of neutrophil recruitment. Evidence does exist to suggest that the damaging effect of superoxide anions in the gastro-intestinal tract is neutrophil-independent since depletion of circulating neutrophils did not affect xanthine-xanthine oxidase induced mucosal damage (Deitch *et al.*, 1990). Because neutrophils are only a part of the inflammatory cell infiltrate, we investigated the response of animals made neutropenic by colchicine treatment. Neutrophils appear to contribute to the damage observed at the earlier (3 h) but not later (5 h) time point since in neutropenic rats, plasma leakage, lipid peroxidation and epithelial cell injury were markedly attenuated at the 3 h time point, and only slightly attenuated at the later time point. This suggests that cells other than neutrophils are responsible for the damage during the later stages of the inflammatory response. SC-55858, FeTMPS and FeTMPyP did not lose their inhibitory activity in the colchicine treated animals suggesting that both O_2^- and peroxynitrite are still involved in the damage. Macrophages and epithelial cells are a likely source. These cells are able to generate large amounts of O_2^-, are induced by endotoxin to express iNOS and have as a consequence, the potential to generate peroxynitrite (Schlag *et al.*, 1991; Gow *et al.*, 1998).

There is no doubt that large amounts of NO are released from iNOS in animal models of endotoxaemia as well as in humans with sepsis. However, the use of selective inhibitors of iNOS for sepsis is problematic. This assertion stems from the fact that although iNOS inhibitors prevent the hyporeactivity and the hypotension following endotoxin administration it is very difficult to improve survival. It is therefore tempting to speculate that in the presence of a failing eNOS system (Forstermann & Leinert, 1995) optimal organ-perfusion and function may have to rely on some of the NO formed from iNOS. Thus, when the iNOS inhibitors prevent the release of NO from iNOS and eliminate part of its damaging effects (presumably through the formation of peroxynitrite) these same inhibitors will also remove a critical portion of NO required to maintain normal physiology. It is therefore possible that since neither SODm or peroxynitrite decomposition catalysts interact with NO (Misko *et al.*, 1998), they are able to remove the harmful effects due to the overproduction of NO without compromising its ability to help maintain vital organ perfusion. This hypothesis is under current investigation. The pharmacological separation of the positive effects of NO from its negative effects in shock and other types of inflammatory disorders has, until now, been almost impossible.

In conclusion we have demonstrated that superoxide and peroxynitrite are associated with the intestinal damage evoked by endotoxin. The PN decomposition catalysts and SODm catalysts may offer a novel approach for manipulating the sequence of events that are associated with shock. The further use of these catalysts as pharmacological tools in animal models of human disease may lead us to a better understanding of when and where superoxide and peroxynitrite play key roles in the development of inflammatory diseases such as sepsis. This in turn should provide us with more effective treatment strategies for diseases in the clinic. In addition to inflammation, the future clinical utility for agents such as these may be as adjunct therapy for the gastro-intestinal damage associated with chemo- and radiation therapy for cancer.

In conclusion, SODm and peroxynitrite decomposition catalysts are not only useful tools for the pharmacological dissection of free radical-mediated pathology, but also offer promise as disease-modifying therapeutic agents capable of preserving the positive aspect of the double-edge sword of nitric oxide action.

References

BATINIC-HABERLE, I., LIOCHEV, S.I., SPASOJEVIC, I. & FRIDO-VICH, I. (1997). A potent superoxide dismutase mimic: manganese beta-octabromo-meso-tetrakis-(N-methylpyridinium-4-yl)porphyrin. *Arch. Biochem. Biophys.*, **343**, 225–233.

BECKMAN, J.S. (1994). Peroxynitrite versus hydroxyl radical: The role of nitric oxide in superoxide-dependent cerebral injury. *Ann. NY Acad. Sci.*, **738**, 69–75.

BECKMAN, J.S., BECKMAN, T.W., CHEN, J., MARSHALL, P.M. & 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.

BECKMAN, J.S. & CROW, J.P. (1993). Pathological implications of nitric oxide, superoxide and peroxynitrite formation. *Biochem. Soc. Trans.*, **21**, 330–334.

BOUGHTON-SMITH, N.K., EVANS, S.M., LASZLO, F., WHITTLE, B.J. & MONCADA, S. (1993). The induction of nitric oxide synthase and intestinal vascular permeability by endotoxin in the rat. *Br. J. Pharmacol.*, **110**, 1189–1195.

BRADLEY, P.P., PRIEBAT, D.A., CHRISTENSEN, R.D. & ROTHSTEIN, G. (1982). Measurement of cutaneous inflammation. Estimation of neutrophil content with an enzyme marker. *J. Clin. Invest. Dermatol.*, **78**, 206–209.

BRONER, C.W., SHENEP, J.L., STIDHAM, G.L., STOKES, D.C. & HILDNER, B.S. (1988). Effects of scavengers of oxygen-derived free radicals on mortality in endotoxin-challenged mice. *Crit. Care Med.,* **16,** 848 – 851.

CROW, J.P. & BECKMAN, J.S. (1995). The role of peroxynitrite in nitric oxide mediated toxicity. *Current Topics in Microbiology and Immunology.,* **196,** 51 – 73.

DAWSON, V.L. (1995). Nitric oxide: role in neurotoxicity. *Clin. Exp. Pharm. Physiol.,* **22,** 305 – 308.

DAY, B.J., SHAWEN, S., LIOCHEV, S.I. & CRAPO, J.D. (1995). A metalloprotin superoxide dismutase mimic protects against paraquat-induced endothelial cell injury, in vitro. *J. Pharmacol. Exp. Ther.,* **275,** 1227 – 1232.

DEITCH, E.A., BRIDGES, W., BERG, R., SPECIAN, R.D. & GRANGER, N. (1990). Hemorrhagic shock-induced bacterial translocation: the role of neutrophils and hydroxyl radicals. *J. Trauma,* **30,** 942 – 951.

DROY-LEFAIX, M.T., DROUET, Y., GERAUD, G., HOSFOD, D. & BRAQUET, P. (1991). Superoxide dismutase (SOD) and the PAF-antagonist (BN 52021) reduce small intestinal damage induced by ischemia-reperfusion. *Free Rad. Res. Commun.,* **12 – 13,** 725 – 735.

ESPLUGUES, J.V. & WHITTLE, B.J. (1989). Gastric damage following local intra-arterial administration of reactive oxygen metabolites in the rat. *Br. J. Pharmacol.,* **97,** 1085 – 1092.

FÖRSTERMANN, U. & LEINERT, H. (1995). Nitric oxide synthase: expression and expressional control of the three isoforms. *Naunyn-Schmiedebergs Arch. Pharmacol.,* **352,** 351 – 364.

FUKUYAMA, N., TAKEBAYASHI, Y., HIDA, M., ISHIDA, H., ICHIMORI, K. & NAKAZAWA, H. (1997). Clinical evidence of peroxynitrite formation in chronic renal failure patients with septic shock. *Free Radical Biol. Med.,* **22,** 771 – 774.

GARDNER, P.R., NGUYEN, D.D.-H. & WHITE, C.W. (1996). Superoxide scavenging by Mn (II/III) tetrakis (1-methyl-4-pyridyl) porphyrin in mammalian cells. *Arch. Biochem. Biophys.,* **325,** 20 – 28.

GOW, A.J., THOM, S.R. & ISCHIROPOULUS, H. (1998). Nitric oxide and peroxynitrite mediated pulmonary cell death. *Am. J. Physiol.,* **274,** L112 – L118.

GRAHAM, A., HOGG, N., KALYANARAMAN, B., O'LEARY, V., DARLEY-USMAR, V. & MONCADA, S. (1993). Peroxynitrite modification of low-density lipoprotein leads to recognition by the macrophage scavenger receptor. *FEBS Lett.,* **330,** 181 – 185.

HAGLIND, E., XIA, G. & RYLANDER, R. (1994). Effects of antioxidants and PAF receptor antagonist in intestinal shock in the rat. *Circ. Shock,* **42,** 83 – 91.

HENSON, P.M. & JOHNSTON, R.B. (1987). Tissue injury and inflammation. Oxidants, proteinases and cationic proteins. *J. Clin. Invest.,* **79,** 669 – 674.

HIRSCHELMANN, R. & BEKEMEIER, H. (1981). Effects of catalase, peroxidase, superoxide dismutase and 10 scavengers of oxygen radicals in carrageenan edema and in adjuvant arthritis of rats. *Experientia,* **37,** 1313 – 1314.

HUIE, R.E. & PADMAJA, S. (1993). The reaction of NO with superoxide. *Free Rad. Res. Commun.,* **18,** 195 – 199.

ISCHIROPOULOS, H., ZHU, L. & BECKMAN, J.S. (1992). Peroxynitrite formation from macrophage-derived nitric oxide. *Arch. Biochem. Biophys.,* **298,** 446 – 451.

LAIGHT, D.W., LAD, N., WOODWARD, B. & WATERFALL, J.F. (1994). Assessment of myeloperoxidase activity in renal tissue after ischemia/reperfusion. *Eur. J. Pharmacol..,* **292,** 81 – 88.

LAMARQUE, D. & WHITTLE, B.J.R. (1995a). Involvement of superoxide and xanthine oxidase in neutrophil-independent rat gastric damage induced by NO donors. *Br. J. Pharmacol.,* **116,** 1843 – 1848.

LAMARQUE, D. & WHITTLE, B.J.R. (1995b). Role of oxygen-derived metabolites in the rat gastric mucosal injury induced by nitric oxide donors. *Eur. J. Pharmacol.,* **277,** 187 – 194.

LENTZE, M.J., COLONY, P.C. & TRIER, J.S. (1985). Glucocorticoid receptors in isolated intestinal epithelial cells in rats. *Am. J. Physiol.,* **249,** G58 – G65.

MARSHALL, M.J. & WORSFOLD, W. (1978). Superoxide dismutase: a direct, continuous assay using the oxygen electrode. *Anal. Biochem.,* **86,** 561 – 567.

MISKO, T.P., HIGHKIN, M.K., VEENHUIZEN, A.W., MANNING, P.T., STERN, M.K., CURRIE, M.G & SALVEMINI, D. (1998). Characterization of the cytoprotective action of peroxynitrite decomposition catalysts. *J. Biol. Chem.,* **273,** 15646 – 15653.

OHKAWA, H., OHISHI, H. & YAGI, K. (1979). Assay for lipid peroxides in animal tissue by thiobarbituric acid reaction. *Anal. Biochem.,* **95,** 351 – 358.

PASTERNACK, R.F. & PYSNIK, D. (1983). Catalysis of the Dispropostionation of Superoxide by Metalloporphyrins. In *Oxy Radicals and Their Scavenger Systems.* eds. Cohen G & Greenwald R. p. 151, The Netherlands: Elsevier.

RADI, R., BECKMAN, J.S., BUSH, K.M. & FREEMAN, B.A. (1991a). Peroxynitrite-induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitric oxide. *Arch. Biochem. Biophys.,* **288,** 481 – 487.

RADI, R., BECKMAN, J.S., BUSH, K.M. & FREEMAN, B.A. (1991b). Peroxynitrite oxidation of sulfhydryls. The cytotoxic potential of superoxide and nitric oxide. *J. Biol. Chem.,* **266,** 4244 – 4250.

RILEY, D.P., HENKE, S.L., LENNON, P.J., WEISS, R.H., NEUMANN, W.L., RIVERS, W.J., ASTON, K.W., SAMPLE, K.R., RAHMAN, H., LING, C.-S., SHIEH, J.-J., BUSCH, D.H. & SZULBINSKI, W. (1996). Synthesis, characterization and stability of manganese (II) C-substituted 1,4,7,10,13-pentaazacyclopentadecane complexes exhibiting superoxide dismutase activity. *Inorg. Chem.,* **35,** 5213 – 5231.

RILEY, D.P., LENNON, P.J., NEUMANN, W.L. & WEISS, R.H. (1997). Toward the rational design of superoxide dismutase mimics: Mechanistic studies for the elucidation of substituent effects on the catalytic activity of macrocyclic manganese(II) complexes. *J. Am. Chem. Soc.,* **119,** 6522.

SALTER, M., KNOWLES, R.G. & MONCADA, S. (1991). Widespread tissue distribution, species distribution and changes in activity of Ca^{2+}-dependent and Ca^{2+}-independent nitric oxide synthases. *FEBS Lett.,* **291,** 145 – 149.

SALVEMINI, D., MANNING, P.T., ZWEIFEL, B.S., SEIBERT, K., CONNOR, J., CURRIE, M.G., NEEDLEMAN, P. & MASFERRER, J.L. (1995). Dual inhibition of nitric oxide and prostaglandin production contributes to the antiinflammatory properties of nitric oxide synthase inhibitors. *J. Clin. Invest.,* **96,** 301 – 308.

SALVEMINI, D., WANG, Z.-Q., STERN, M.K., CURRIE, M.G. & MISKO, T.P. (1998). Peroxynitrite decomposition catalysts: novel therapeutics for peroxynitrite-mediated pathology. *Proc. Natl. Acad. Sci. U.S.A.,* **95,** 2659 – 2663.

SALVEMINI, D., WANG, Z.Q., WYATT, P.S., BOURDON, D.M., MARINO, M.H., MANNING, P.Y. & CURRIE, M.G. (1996). Nitric oxide: a key mediator in the early and late phase of carrageenan-induced rat paw inflammation. *Br. J. Pharmacol.,* **118,** 829 – 838.

SCHLAG, G., REDL, H. & HALLSTRÖM, S. (1991). The cell in shock: the origin of multiple organ failure. *Resuscitation,* **21,** 137 – 180.

SCRAUFSTATTER, I.U., HYSLOP, P.A., JACKSON, J. & COCHRANE, C.C. (1987). Oxidant injury of cells. *Int. J. Tissue. React.,* **9,** 317 – 324.

SHIGENAGA, M.K., LEE, H.H., BLOUNT, B.C., CHRISTEN, S., SHIGENO, E.T., YIP, H. & AMES, B.N. (1997). Inflammation and NO(X)-induced nitration: assay for 3-nitrotyrosine by HPLC with electrochemical detection. *Proc. Natl. Acad. Sci. U.S.A.,* **94,** 3211 – 3216.

STERN, M.K., JENSEN, M.P. & KRAMER, K. (1996). Peroxynitrite Decomposition Catalysts. *J. Am. Chem. Soc.,* **118,** 8735 – 8736.

TAYLOR, D.E., GHIO, A.J. & PIANTADOSI, C.A. (1995). Reactive oxygen species produced by liver mitochondria of rats in sepsis. *Arch. Biochem. Biophys.,* **316,** 70 – 76.

TEPPERMAN, B.L., BROWN, J.F. & WHITTLE, B.J.R. (1993). Nitric oxide synthase induction and intestinal epithelial cell viability in rats. *Am. J. Physiol.,* **265,** G214 – G218.

WARREN, J.S., YABROFF, K.R., MANDEL, D.M., JOHNSON, K.J. & WARD, P.A. (1990). Role of O_2^- in neutrophil recruitment into sites of dermal and pulmonary vasculitis. *Free. Rad. Biol. Med.,* **8,** 163 – 172.

WEISS, R.H., FLICKINGER, A.G., RIVERS, W.J., HARDY, M.D., ASTON, K.W., RYAN, U.S. & RILEY, D.P. (1993). Evaluation of putative superoxide dismutase mimics. *J. Biol. Chem.,* **268,** 23049.

XIA, Z.F., HOLLYOAK, M., BARROW, R.E., HE, F., MULLER, M.J. & HERNDON, D.N. (1995). Superoxide dismutase and leupeptin prevent delayed reperfusion injury in the rat small intestine during burn shock. *J. Burn Care Rehab.,* **16,** 111 – 117.