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Role of mast cells, neutrophils and nitric oxide in endotoxin-induced damage to the neonatal rat colon

[1]J.F. Brown, K.A. Chafee & B.L. Tepperman

Department of Physiology, Faculty of Medicine, University of Western Ontario, London, Ontario, Canada, N6A 5C1

1 The mechanisms involved in mediating bacterial endotoxin lipopolysaccharide (LPS)-induced injury in the colon of neonatal rat pups aged 10–12 days was examined.

2 Administration of LPS (3 mg kg^{-1}, i.p.) caused a time-related increase in the plasma concentration of rat mast cell protease-II (RMCP-II) which was attenuated dose-dependently, by the non-selective mast cell stabilizer doxantrazole (0.05–5 mg kg^{-1}, i.p.). The selective connective tissue mast cell stabilizer ketotifen (5–25 mg kg^{-1}, i.p.) was without effect at the lower dose and had only a limited inhibitory effect at the higher dose.

3 In addition, doxantrazole (5 mg kg^{-1}, i.p.) inhibited mast cell degranulation in response to LPS in sections of neonatal rat colon, but ketotifen (5 mg kg^{-1}, i.p.) was without effect.

4 The increase in plasma RMCP-II concentration in response to LPS treatment preceded increases in tissue myeloperoxidase (MPO) activity, inducible nitric oxide synthase (iNOS) activity and tissue lipid peroxidation. These events were all attenuated by pretreatment with doxantrazole (5 mg kg^{-1}, i.p.), antineutrophil serum (100 μl kg^{-1}, i.p.), dexamethasone (2 mg kg^{-1}, i.p.) and the selective iNOS inhibitor, aminoguanidine (25 mg kg^{-1}, i.p.).

5 In addition, lipid peroxidation was inhibited by pre-administration of the antioxidant enzymes superoxide dismutase (2000 u kg^{-1}, i.p.) and catalase (2000 u kg^{-1}, i.p.), the xanthine oxidase inhibitor allopurinol (100 mg kg^{-1}, i.p.) and the peroxyl scavenger deferoxamine (10 mg kg^{-1}, i.p.), suggesting the involvement of reactive oxygen metabolites in the colonic injury.

6 These findings suggest that the sequence of events resulting in colonic damage in the neonatal rat following administration of LPS include mast cell degranulation, neutrophil infiltration, elevation in iNOS activity and subsequent lipid peroxidation.

Keywords: Lipopolysaccharide; rat mast cell protease II; myeloperoxidase; inducible nitric oxide synthase; aminoguanidine; doxantrazole; colonic injury; neonatal rats

Introduction

Administration of bacterial endotoxin lipopolysaccharide (LPS) to adult animals is associated with a widespread increase in the activity of the inducible isoform of nitric oxide synthase (iNOS; Knowles *et al*., 1990). This increase in iNOS activity is associated with inflammation affecting numerous organs including the gastrointestinal tract (Boughton-Smith *et al*., 1993). It has been shown that following infection or administration of bacterial endotoxin lipopolysaccharide (LPS) to adult animals, the iNOS enzyme is expressed and generates large quantities of NO which is associated with colonic epithelial cell injury (Tepperman *et al*., 1993). It is proposed that NO in conjunction with other reactive oxygen metabolites mediates this damage (Darley-Usmar *et al*., 1992). Recently we have demonstrated that LPS administration also results in an increase in iNOS activity in the colon of neonatal rats aged between 10–25 days, with maximal activity occurring between 10 and 15 days of age (Brown & Tepperman, 1997a). This increase in colonic iNOS activity coincided with the age in which the greatest degree of colonic damage was evident. These findings suggest that the neonatal rat colon is highly susceptible to the detrimental effects of LPS-induced iNOS activity when compared to more mature animals.

The large amounts of NO produced by the inducible isozyme can interact with reactive oxygen metabolites such as superoxide resulting in the propagation of the highly reactive species peroxynitrite. This potent oxidizing agent can initiate lipid peroxidation and can produce extreme membrane damage (Beckman *et al*., 1990). Lipid peroxidation has been implicated in the pathophysiology of many disease processes (Balasubramanian *et al*., 1992) and has been proposed as a mechanism whereby ischaemia and bacterial infection cause damage. The peroxidation of membrane phospholipid polyunsaturated fatty acids causes severe damage to cellular membranes, organelles and their associated enzymes which results in structural and functional damage to the membrane (Perret *et al*., 1994). Recently we have demonstrated that activation of colonic iNOS activity in the neonate was associated with an increase in lipid peroxidation. Furthermore, the increase in lipid peroxidation correlated with an increase in the extent of histological damage (Brown & Tepperman, 1997a).

LPS also causes degranulation of inflammatory mast cells (Morrison & Betz, 1977) residing in the gastric mucosa, resulting in the release of numerous inflammatory mediators including histamine, platelet activating factor and oxidants, which have been shown to increase epithelial permeability (Kanwar & Kubes, 1994). Thus, colonic tissue lipid peroxidation may be mediated by the production of NO and other oxygen metabolites by activated neutrophils and macrophages (Wallace *et al*., 1990) or could occur through mast cell degranulation (Kanwar *et al*., 1994).

It is therefore proposed that the colonic injury in the neonate following exposure to LPS may be a result of lipid peroxidation mediated by NO and other reactive oxygen metabolites or may be in response to the proinflammatory mediators released by mast cells, with potent oxidizing

[1] Author for correspondence.

capabilities. In the present study the effects of LPS administration on lipid peroxidation in the colon of neonatal rats have been examined. The potential contribution of NO and mast cell degranulation in this process has also been studied.

Methods

Animals

Pregnant female Sprague Dawley dams in late gestation were purchased from Canada Breeding Labs (St Constant, Quebec) and were maintained in a temperature controlled environment ($22 \pm 1^{\circ}C$) on a 12 h light and dark cycle with chow and water available *ad libitum*. The day of parturition was termed day zero and all pups were maintained with their natural mother. All studies were approved by the University of Western Ontario Animal Care Committee, and all animals were treated according to the guidelines set out by the Canadian Council on Animal Care.

Treatments

Bacterial endotoxin-induced injury At 10–12 days *post partum* pups of either sex were placed randomly into experimental groups treated with either saline vehicle (control) or *Escherichia coli* lipopolysaccharide (LPS; Serotype 0111:B4, Sigma; 3 mg kg^{-1}, i.p. in sterile saline). After treatment, all pups were returned to their mother to suckle.

Drug treatments Animals were further subdivided to receive the following treatments before administration of LPS: (1) anti-neutrophil serum (ANS; Accurate Chemical and Scientific Corporation, NY., 10 μl of antiserum, i.p., 2 h prior to LPS), which at this dose and with the same treatment regime has previously been shown to reduce the number of circulating neutrophils to less than 5% of control numbers (Brown & Tepperman, 1997b) and the animals remain neutropenic for up to 48 h (Buell & Berin, 1994); (2) the steroidal anti-inflammatory dexamethasone (2 mg kg^{-1}, i.p.; 1 h before LPS), which at this dose inhibits both the induction of Ca^{2+}-independent NO synthase activity (Tepperman *et al*., 1994) and the adherence of circulating leukocytes to vascular walls (McCafferty *et al*., 1995); (3) the selective iNOS inhibitor aminoguanidine (0.05–25 mg kg^{-1}, i.p.) or the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME; 2 mg kg^{-1}, i.p.), both inhibitors were administered immediately after LPS, or the inactive stereoisomer D-NAME; (4) the selective connective tissue mast cell stabilizer ketotifen or the non-selective mast cell stabilizer doxantrazole (0.05–25 mg kg^{-1}, i.p.) administered 1 h before LPS or (5) the antioxidant enzymes superoxide dismutase (SOD; 2000 u kg^{-1}, i.p.) administered 15 min before LPS or (6) the xanthine oxidase inhibitor allopurinol (100 mg kg^{-1}, i.p.) and the peroxyl scavenger deferoxamine (10 mg kg^{-1}, i.p.) administered 1 h and 15 min before LPS, respectively. These doses of SOD, allopurinol and deferoxamine have previously been shown to inhibit sodium nitroprusside-induced gastric mucosal damage (Lamarque & Whittle, 1995a, b).

Measurement of nitric oxide synthase activity

At various times following administration of LPS rat pups were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, i.p.). A mid-line abdominal incision was made and samples of distal colon were rapidly removed, washed in ice-cold saline, blotted dry and placed on ice. Ice-cold homogenization buffer (pH 7.4), consisting of HEPES 10 mM, sucrose 0.32 M, EDTA 0.1 mM, dithiothreitol 1 mM, soyabean trypsin inhibitor 10 μg ml^{-1}, leupeptin 10 μg ml^{-1} and aprotinin 2 μg ml^{-1}, was quickly added to produce a final tissue content of 33% w/v. Enzyme activity was released by homogenization (Ultra-Turrax) on ice for 15 s at full speed and cell debris was removed by centrifugation at 10,000 *g* for 20 min at 4°C. Conversion of L-[^{14}C]-arginine to L-[^{14}C]-citrulline was measured as an index of NO synthase activity and was performed as described previously (Brown *et al*., 1992). Briefly, 20 μl of tissue homogenate supernatant was incubated for 10 min at 37°C with 50 μl of substrate buffer (pH 7.4) consisting of (final concentrations) 30 mM potassium phosphate, 150 μM $CaCl_2$ 0.7 mM $MgCl_2$, 15 μM L-[^{14}C]-arginine (800,000 d.p.m. ml^{-1}; ICN, Mississauga, Ontario, Canada), 0.1 mM NADPH and 10 mM L-valine. Unconverted L-[^{14}C]-arginine was removed by addition of 0.5 ml of a 1 : 1 suspension of DOWEX (AG 50 W-8; Sigma Chemical Co., St. Louis, MO) in water. Distilled water (1 ml) was added to each tube and the resin was allowed to settle for 20 min before removal of 975 μl of the liquid phase for estimation of product formation by liquid scintillation counting. Product formation that was inhibited by removal by Ca^{2+} from the substrate buffer by addition of EGTA (1 mM) and by the incubation with the NO synthase inhibitor L-NAME (300 μM; Sigma Chemical Co., St. Louis, MO), was used as an index of constitutive Ca^{2+}-dependent NO synthase activity. Inducible Ca^{2+}-independent NO synthase activity was measured as that activity which was inhibited by *in vitro* incubation with L-NAME but not by EGTA.

Measurement of plasma nitrite concentrations

Four hours after administration of LPS (3 mg kg^{-1}, i.p.), animals were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, i.p.) and 0.2 ml of blood was removed by cardiac puncture, transferred to tubes containing sodium citrate (0.32% final concentration) and plasma was prepared by centrifugation (10,000 *g* for 2 min). Nitrite (NO_2) content was determined spectrophotometrically by a procedure based on the Griess reaction (Green *et al*., 1982). Plasma was deproteinized with 35% sulphosalicyclic acid and centrifuged at $10{,}000 \times g$ for 15 min at 4°C, and the supernatant was combined with NH_4Cl buffer and 5% NaOH before analysis. Sample aliquots were mixed, with the Griess reagent that contained 1 part 0.1% naphthylethylenediamine dihydrochloride in distilled H_2O plus 1 part 1% sulphanilamide in 5% concentrated H_3PO_4 and absorbance was determined at 546 nm.

Measurement of myeloperoxidase activity

Myeloperoxidase (MPO) levels were measured to provide an index of polymorphonuclear leukocyte infiltration (Krawisz *et al*., 1984). MPO activity was determined as described by Wallace (1987). Briefly, frozen samples of whole colon were suspended in 50 mM phosphate buffer containing 0.5% hexadecyltrimethylammonium bromide (HTAB; pH 6.0; Sigma) at a tissue concentration of 50 mg ml^{-1}. Samples were homogenized three times for 30 s each, freeze-thawed three times in an acetone dry-ice bath and centrifuged at 40,000 *g* for 15 min at 4°C. MPO activity in the supernatant was determined by adding 100 μl of the supernatant to 2.9 ml of 50 mM phosphate buffer (pH 6.0) containing 0.167 mg ml^{-1}

O-dianisidine hydrochloride (Sigma) and 0.0005% w/v hydrogen peroxide. The change in absorbance at 460 nm over a 3 min period was measured. MPO activity was defined as that which would convert 1 mol of hydrogen peroxide to water in 1 min at 22°C (Bradley *et al*., 1982).

Measurement of lipid peroxidation

At various times following administration of LPS, lipid peroxidation of colonic tissue was measured according to a method previously described byYagi (1976). Briefly, 'ice-cold' Earle's balanced salt solution was added to samples of colonic tissue to produce a final tissue content of 100 mg ml^{-1}. Tissue was homogenized (Ultra-Turrax) on ice for 15 s at full speed before sonication for 5 s also on ice. Trichloroacetic acid (5%, 1.8 ml) was added to 200 μl of tissue homogenate which was centrifuged at 3000 *g*, for 20 min at 4°C. An equal volume of supernatant was added to freshly prepared 0.6% thiobarbituric acid. The mixture was then vortexed and boiled for 10 min before extraction of the coloured product by addition of *n*-butanol and centrifugation at 3000 *g* for 15 min at 4°C. A 1 ml aliquot of the upper phase was removed and an additional 1 ml of *n*-butanol was added before the fluorescence was measured at 515 nm excitation and 553 nm emission. Data are presented against a standard curve of malondialdehyde (MDA) in methanol (1 : 1000). The solution was mixed 1 : 1 with 0.2 N HCl and left overnight at room temperature. The solution was further diluted in methanol to achieve a final concentration of 50 nmol of MDA in 1 ml. Aliquots of this MDA solution were added to 1.5 ml of Earle's balanced salt solution without phenol red (GIBCO) to produce standards containing 0.1 to 2.5 nmol of MDA. Thiobarbituric acid (1.5 ml, 0.6%) was added to each tube before the contents were boiled and the standards were then treated exactly as the samples.

Mucosal mast cell degranulation

At various times after administration of LPS (2 mg kg^{-1}, i.p.), animals were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, i.p.) and 0.2 ml of blood was removed by cardiac puncture, transferred to tubes containing sodium citrate (0.32% final concentration) and plasma was prepared by centrifugation (10,000 *g* for 2 min). The plasma concentration of rat mast cell protease II (RMCP-II) was measured as an index of mucosal mast cell degranulation (Kanwar *et al*., 1994) with a commercially available enzyme-linked immunosorbent assay (Morendun Animal Health, Scotland). The number of mucosal mast cells in sections of distal colon were determined as described previously (Appleyard & Wallace, 1995). Briefly, samples of the distal colon were fixed in Carnoy's fixative, processed by routine techniques, and mounted on glass slides. The sections were stained with alcian blue and counterstained with safranine O to permit visualization of mucosal mast cells. The number of mucosal mast cells in five randomly selected medium-power fields was counted, and the mean number of mast cells per field was calculated. The observer was unaware of the treatments the rats had received.

Statistical analysis

Results are presented as mean $\pm$ s.e.mean with *n* equal to the number of animals. Data were analysed by analysis of variance and Dunnett's or Newman-Keuls test for multiple comparisons, where $P<0.05$ was the minimum accepted level of significance for all groups.

Drugs

Aminoguanidine, dexamethasone, deferoxamine, ketotifen, allopurinol and *Escherichia coli* lipopolysaccharide (serotype 0111:B4) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Superoxide dismutase (5,000 u mg^{-1}) was purchased from Boehringer Mannheim (Germany). Doxantrazole was purchased from Aldrich Chemical Co. Inc. (Milwaukee, WI, U.S.A.). Aminoguanidine and allopurinol were dissolved in equimolar solutions of sodium hydroxide and then diluted in sterile saline. All other drugs were dissolved in sterile saline immediately before administration.

Results

Effect of LPS administration

In response to administration of LPS (3 mg kg^{-1}, i.p.) there was a time-related increase in colonic Ca^{2+}-independent iNOS activity. The increase in iNOS activity was significantly different ($P<0.05$) from control animals, 2 h after administration of LPS (Figure 1a). In addition, the pattern of lipid peroxidation in response to LPS treatment followed a similar course of events as the alterations in iNOS activity, being elevated 2 h after LPS administration and remaining elevated at 6 h (Figure 1b).

Administration of LPS also resulted in a rapid and significant ($P<0.05$) increase in the plasma concentration of rat mast cell protease-II (RMCP-II) with a significant increase being observed as early as 30 min post LPS administration (Figure 2a). Furthermore, LPS administration was also associated with an increase in tissue myeloperoxidase (MPO) activity which commenced 2 h after administration of LPS and remained elevated at 6 h (Figure 2b).

Effect of mast cell stabilizers

Administration of the non-selective mast cell stabilizer doxantrazole (0.05 – 5 mg kg^{-1}, i.p.) 2 h before the administration of LPS, caused a dose-dependent reduction in the concentration of plasma RMCP-II measured 1 h after LPS administration (Figure 3). However, the selective connective tissue mast cell stabiliser, ketotifen, was without effect at the standard dose (5 mg kg^{-1}, i.p.), but at a higher dose (25 mg kg^{-1}, i.p.) a small, but nevertheless significant reduction in the plasma concentration RMCP-II was observed (Figure 3).

In addition, doxantrazole (5 mg kg^{-1}, i.p.) inhibited the LPS-associated reduction in the number of mucosal mast cells observed with light microscopy, whereas ketotifen (5 mg kg^{-1}, i.p.) was unable to inhibit the LPS-associated decrease in mast cell number (Figure 4). Furthermore, pretreatment with doxantrazole (5 mg kg^{-1}) also attenuated the LPS-associated increase in iNOS activity (Figure 5a), MPO activity (Figure 5b) and lipid peroxidation (Figure 5c) measured 4 h after the administration of LPS.

Effect of superoxide dismutase

Administration of the antioxidant enzyme, superoxide dismutase (2000 u kg^{-1}, i.p.) 15 min before the administration of LPS (3 mg kg^{-1}, i.p.) inhibited the LPS-associated increase in

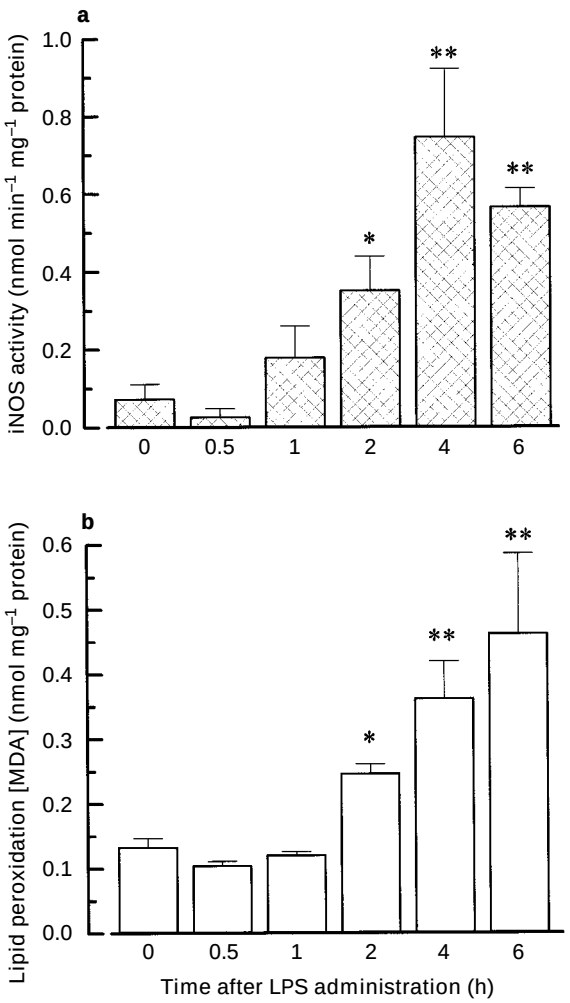


Figure 1 Effect of LPS (3 mg kg^{-1}, i.p.) administration upon colonic tissue: (a) iNOS activity, measured as the Ca^{2+}-independent conversion of [^{14}C]-L-arginine to [^{14}C]-L-citrulline, and (b) lipid peroxidation, measured as the amount of thiobarbituric acid reactive lipid. All values are mean ± s.e.mean ($n = 6-8$), where $^{**}P < 0.01$ and $^{*}P < 0.05$ for difference from values at time zero by analysis of variance and Dunnett's test for multiple comparisons.

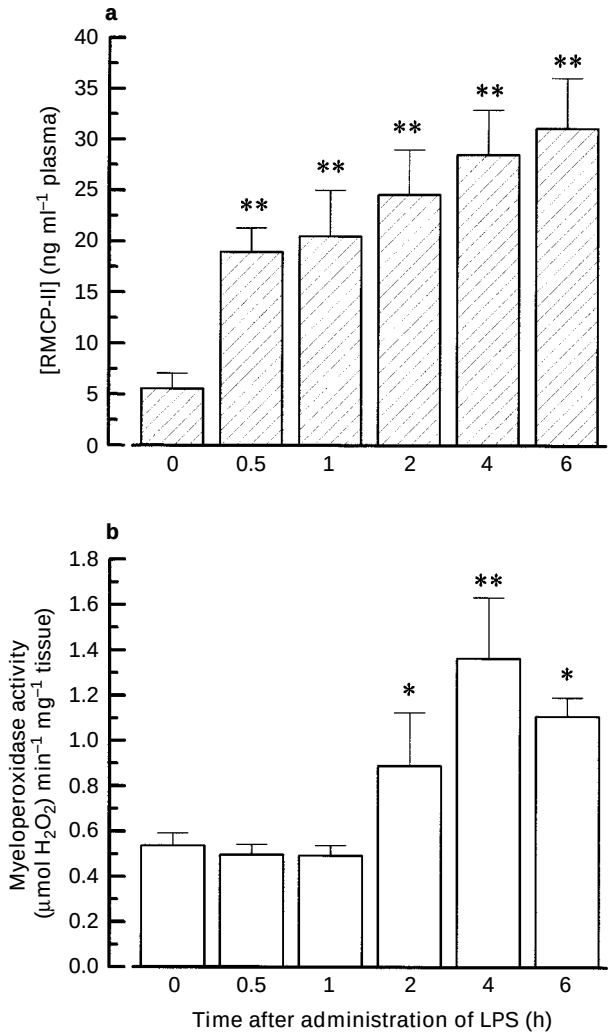


Figure 2 Effect of LPS (3 mg kg^{-1}, i.p.) administration on (a) mucosal mast cell degranulation measured as the plasma concentration of rat mast cell protease II (RMCP-II) and (b) myeloperoxidase activity measured as μmol of hydrogen peroxide min^{-1} mg^{-1} protein. Data are presented as mean ± s.e.mean ($n = 7-8$), where $^{**}P < 0.01$ for difference from animals at time t = 0, by analysis of variance and Dunnett's test.

mast cell degranulation (Figure 6), measured 1 h after administration of LPS. In addition, superoxide dismutase inhibited lipid peroxidation measured 4 h after administration of LPS (Figure 7).

Effect of allopurinol and deferoxamine

Both allopurinol and deferoxamine inhibited the LPS-associated increase in mucosal mast cell degranulation measured 1 h after administration of LPS (Figure 6). Furthermore, the increase in lipid peroxidation in response to LPS was inhibited by pre-administration of the xanthine oxidase inhibitor allopurinol (100 mg kg^{-1}, i.p.) and the peroxyl scavenger deferoxamine (100 mg kg^{-1}, i.p.) administered 1 h and 15 min before LPS (3 mg kg^{-1}, i.p.) treatment, respectively (Figure 5).

Effect of antineutrophil serum, aminoguanidine and dexamethasone

Pretreating animals with antineutrophil serum (ANS; 100 μl kg^{-1}, i.p.) 2 h before the administration of LPS (3 mg kg^{-1}, i.p.) abolished the LPS-associated increase in iNOS activity (Figure 5a), MPO activity (Figure 5b) and lipid peroxidation (Figure 5c). In addition, pretreating animals with dexamethasone (2 mg kg^{-1}, i.p.) 1 h before the administration of LPS attenuated the LPS associated increase in iNOS activity (Figure 5a), MPO activity (Figure 5b) and lipid peroxidation (Figure 5c) measured 4 h after the administration of LPS. Furthermore, the selective iNOS inhibitor aminoguanidine (25 mg kg^{-1}, i.p.) administered immediately after LPS also attenuated the increase in iNOS activity, MPO activity and lipid peroxidation (Figure 5a, b and c, respectively). Aminoguanidine (0.05–25 mg kg^{-1}, i.p.) also dose-dependently inhibited the LPS-associated increase in plasma nitrite concentrations (Table 1). However, at the highest dose (25 mg kg^{-1}, i.p.), aminoguanidine did not affect the basal concentration of plasma nitrite (Table 1).

Discussion

The findings of this present study indicate that administration of LPS to neonatal rat pups of between 10 and 12 days of age, results in a time-related increase in colonic iNOS activity.

These data conform and extend our previous findings in adult rats which demonstrated that colonic epithelial cells were able to synthesize NO via the inducible isoform of NOS 4 h after administration of LPS (Tepperman *et al*., 1994). Furthermore, we have also demonstrated that the increase in iNOS activity is associated with an increase in tissue thiobarbituric acid reactive lipids. Thiobarbituric acid reactive lipids are considered to be valid markers of membrane lipid peroxidation (Janero, 1990) and an increase in these lipids is commonly associated with inflammation of numerous tissues including the colon. Indeed, an increase in lipid peroxidation has been

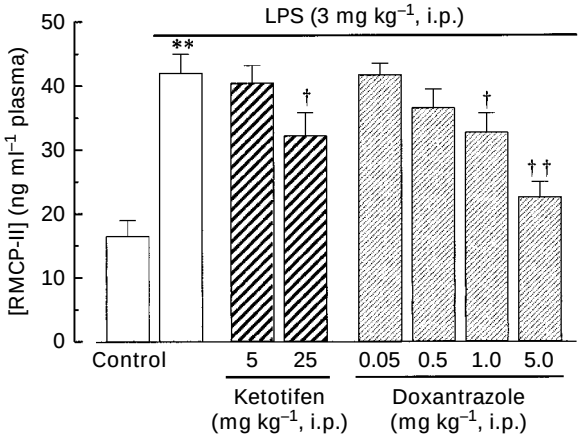


Figure 3 Effect of the connective tissue selective mast cell stabilizer ketotifen (5 and 25 mg kg^{-1}, i.p.) and the non-selective mast cell stabilizer doxantrazole (0.05–5 mg kg^{-1}, i.p.) on mucosal mast cell degranulation. Both agents were administered 1 h before the administration of LPS (3 mg kg^{-1}, i.p.) and mucosal mast cell degranulation was measured 1 h after administration of LPS as the plasma concentration of RMCP-II. Data are presented as mean ± s.e. mean ($n=6$), where $^{**}P<0.01$ for difference from control animals; $\dagger\dagger P<0.01$ and $\dagger P<0.05$ for difference from LPS-treated animals by analysis of variance and Newman-Keuls test.

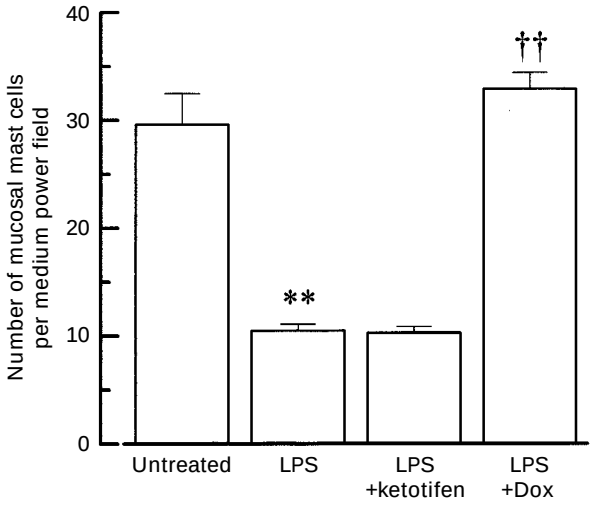
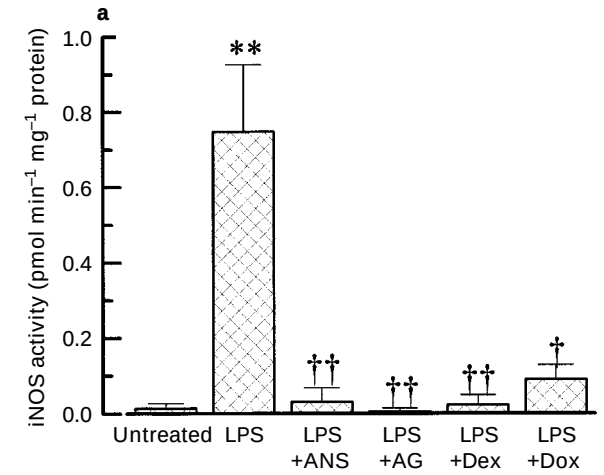
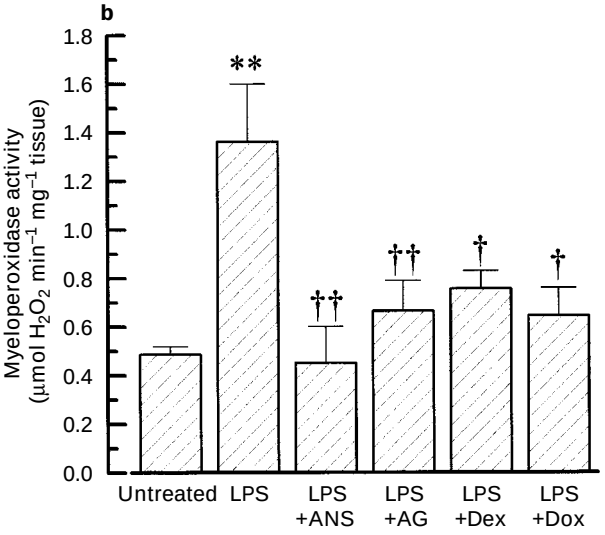


Figure 4 Effect of the selective connective tissue mast cell stabilizer ketotifen (5 mg kg^{-1}, i.p.) and the non-selective mast cell stabilizer doxantrazole (Dox; 5 mg kg^{-1}, i.p.) on the number of microscopically visible mucosal mast cells in whole thickness sections of colonic mucosa stained with alcian blue and safranine O. Both mast cell stabilizers were administered 1 h before the administration of LPS (3 mg kg^{-1}, i.p.) and tissue samples were removed 4 h after administration of LPS. Data are presented as mean ± s.e.mean ($n=6$), where $^{**}P<0.01$ for difference from control animals; $\dagger\dagger P<0.01$ for difference from LPS-treated animals, by analysis of variance and Newman-Keuls test for multiple comparisons.





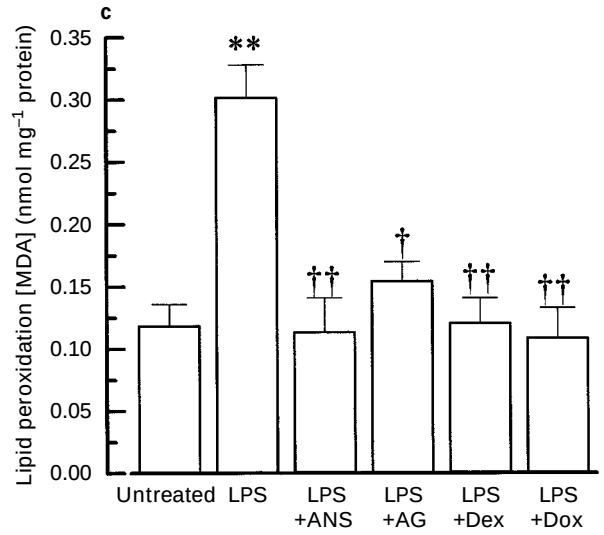


Figure 5 Effect of pretreatment with antineutrophil serum (ANS; 100 μl kg^{-1}, i.p.) the steroidal anti-inflammatory agent dexamethasone (Dex; 2 mg kg^{-1}, i.p.), the mast cell stabilizer doxantrazole (Dox; 5 mg kg^{-1}, i.p.) and the selective iNOS inhibitor aminoguanidine (AG; 25 mg kg^{-1}, i.p.) on (a) iNOS activity, (b) myeloperoxidase activity and (c) lipid peroxidation in response to LPS treatment (3 mg kg^{-1}, i.p.) All measurements were made 4 h after administration of LPS and data are presented as mean ± s.e.mean ($n=8$), where $^{**}P<0.01$ for difference from control; $\dagger\dagger P<0.01$ and $\dagger P<0.05$ for difference from LPS treated by analysis of variance and Newman Keuls test for multiple comparisons.

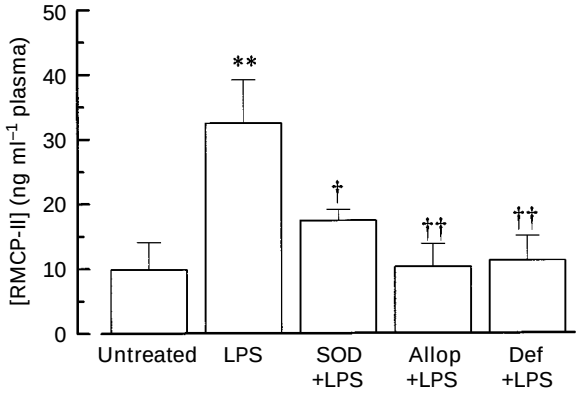


Figure 6 Effect of pretreatment with the antioxidant enzyme superoxide dismutase (SOD; 2000 u kg^{-1}, i.p.; 15 min before the administration of LPS), the xanthine oxidase inhibitor allopurinol (Allop; 100 mg kg^{-1}, i,p.; 1 h before the administration of LPS) and the peroxyl scavenger deferoxamine (Def; 10 mg kg^{-1}, i.p.; 15 min before the administration of LPS), on plasma RMCP-II concentrations measured 1 h after administration of LPS (3 mg kg^{-1}, i.p.). Data are presented as mean$\pm$s.e.mean ($n=8$), where $^{**}P<0.01$ for difference from control and $\dagger\dagger P<0.01$ for difference from LPS-treated, by analysis of variance and Newman Keuls test for multiple comparisons.

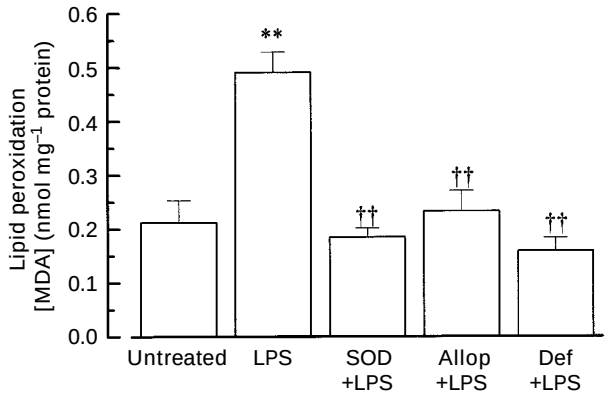


Figure 7 Effect of pretreatment with the antioxidant enzyme superoxide dismutase (SOD; 2000 u kg^{-1}, i.p.; 15 min before the administration of LPS) the xanthine oxidase inhibitor allopurinol (Allop; 100 mg kg^{-1}, i.p.; 1 h before the administration of LPS) and the peroxyl scavenger deferoxamine (Def; 10 mg kg^{-1}, i.p; 15 min before the administration of LPS), on lipid peroxidation measured 4 h after administration of LPS (3 mg kg^{-1}, i.p.) Data are presented as mean$\pm$s.e.mean ($n=8$), where $^{**}P<0.01$ for difference from control and $\dagger\dagger P<0.01$ for difference from LPS-treated, by analysis of variance and Newman Keuls test for multiple comparisons.

observed in biopsy samples from patients with ulcerative colitis (Bhaskar *et al*., 1995), and also in samples of colonic mucosal tissue removed from adult rats with experimental colitis (Loguercio *et al*., 1996). In this study, the onset of elevated iNOS activity and lipid peroxidation were temporally related, supporting data from other studies in adult animals demonstrating that iNOS activity and lipid peroxidation were elevated in the intestine following LPS challenge (Chamulitrat *et al*., 1996).

It is conceivable that NO is not the final mediator of mucosal damage following LPS-challenge since interactions between NO and other cytotoxic reactive oxygen metabolites, such as the superoxide anion, have been observed (Beckman *et al*., 1990). Furthermore, it has been demonstrated that both myeloperoxidase and xanthine oxidase activities are maximal

Table 1 Effect of the selective iNOS inhibitor aminoguanidine (0.05–25 mg kg^{-1}, i.p.) on LPS (3 mg kg^{-1}, i.p.)-induced increase in plasma nitrite concentrations

Treatment	*[Plasma nitrite]* (μmol ml^{-1} plasma)	n
Control	15.0$\pm$1.5	8
Aminoguanidine (25 mg kg^{-1})	16.5$\pm$1.6	8
LPS (3 mg kg^{-1}, i.p.)	79.8$\pm$3.9**	10
LPS + aminoguanidine (0.05 mg kg^{-1}, i.p.)	69.9$\pm$4.9	10
LPS + aminoguanidine (0.5 mg kg^{-1}, i.p.)	54.3$\pm$3.5†	10
LPS + aminoguanidine (5 mg kg^{-1}, i.p.)	24.5$\pm$2.1††	10
LPS + aminoguanidine (25 mg kg^{-1}, i.p.)	19.1$\pm$2.7††	9

Aminoguanidine was administered immediately following LPS and plasma nitrite was determined 4 h later. Data are presented as mean$\pm$s.e.mean, where $^{**}P<0.01$ for difference from control and $\dagger\dagger P<0.01$ and $\dagger P<0.05$ for difference from LPS-treated by analysis of variance and a Newman Keuls test for multiple comparisons.

in the intestine of neonatal rats between 5 and 15 days of age (Musemeche *et al*., 1993). Xanthine oxidase catalyzes the reaction of hypoxanthine (metabolic product of ATP metabolism) and oxygen to form the superoxide anion which sets the stage for further free radical generation and which under conditions of high NO or nitrite/nitrate availability (e.g. following LPS treatment) will include the peroxynitrite anion. Peroxynitrite is a highly reactive oxidizing agent that has been shown to initiate lipid peroxidation and thus produce extreme cellular membrane damage (Beckman *et al*., 1990). Indeed, peroxynitrite has previously been used to produce an animal model of colitis (Rachmilewitz *et al*., 1993). In this study, a number of agents, namely aminoguanidine, SOD, allopurinol and deferoxamine, attenuated the LPS-associated increase in lipid peroxidation, suggesting that a number of reactive oxygen species are important in mediating this response.

Tissue lipid peroxidation and iNOS activity following LPS-challenge have been shown to cause oxidant stress in various tissues including the gastrointestinal tract (Chamulitrat *et al*., 1996), supporting the hypothesis that iNOS activity is associated with lipid peroxidation. Indeed, results from one of our previous studies demonstrated that LPS-mediated initiation of iNOS activity was associated with colonic mucosal damage in neonatal rats and that the damage was associated with tissue lipid peroxidation (Brown & Tepperman, 1997a). Furthermore, the degree of damage was greater in suckling rats when compared to weaned littermates. Since the increase in iNOS activity and lipid peroxidation occur at similar times following administration of LPS, these present findings suggest that these two events are linked.

One potential candidate for the cellular source of iNOS following LPS challenge is the neutrophil. A number of investigators have demonstrated that administration of LPS to adult rats results in polymorphonuclear leukocyte extravasation from the vasculature to the mucosa which involves CD11/CD18 interactions and superoxide production (Harris *et al*., 1994). In the present study, myeloperoxidase activity was assessed as an index of neutrophil infiltration in samples of neonatal rat colon. While the neutrophil content was not determined histologically, measurement of MPO activity has frequently been used as a reliable and valid index of the tissue neutrophil content (Krawisz *et al*., 1984; Wallace, 1987). This study has extended this work since we have demonstrated an

association between the induction of iNOS activity and an increase in myeloperoxidase activity. The increase in MPO activity occurred during a similar time frame to the increase in iNOS activity and lipid peroxidation, with all three parameters being significantly elevated above control levels 2 h after administration of LPS suggesting that these events may be related. Furthermore, the increases in iNOS activity and lipid peroxidation were abolished if the animals were made neutropenic before the administration of LPS. Data from this study further support a role for neutrophils in mediating colonic damage following LPS challenge. A role for NO in this response is also suggested by the observations that iNOS activity and lipid peroxidation could be inhibited by the steroidal anti-inflammatory dexamethasone. Although, due to the myriad of non-specific anti-inflammatory actions of dexamethasone such as inhibition of neutrophil extravasation (McCafferty *et al*., 1995), other mechanisms cannot be excluded. Indeed, dexamethasone inhibited the LPS-associated increase in MPO activity. However, it is likely that NO is at least in part involved in mediating the lipid peroxidation, since administration of the selective iNOS inhibitor aminoguanidine attenuated the LPS-associated increases in iNOS activity and plasma nitrite concentration. A selective inhibition of iNOS activity by aminoguanidine is suggested by the finding that aminoguanidine had no effect upon basal nitrite concentrations. This supports data from other groups who have shown that aminoguanidine, at the dose used in this study, had no effect upon peripheral blood pressure (Ferraz *et al*., 1997). In combination with data from this study, these observations suggest that the dose of aminoguanidine used in this study is selective for inhibiting iNOS with little or no inhibitory effect on the endothelial or neuronal isoforms of NOS.

The increases in lipid peroxidation, iNOS and MPO activity could all be attenuated by administration of the mast cell stabilizer doxantrazole. Mast cell degranulation is often associated with localized inflammatory responses including those affecting the gastrointestinal tract, such as ischaemia-reperfusion type injuries (Kanwar & Kubes, 1994). Results from this study suggest that mucosal mast cells are involved in the mechanism(s) responsible for neutrophil recruitment, increased iNOS activity and lipid peroxidation, since the mast cell stabilizer doxantrazole attenuated all of these events in response to LPS-challenge. The mucosal mast cell is likely to be the type of mast cell that degranulates in response to LPS-challenge, since the selective connective tissue mast cell stabilizer ketotifen had no effect upon plasma RMCP-II levels. Furthermore, this protease is found only in rat mucosal mast cells and release into serum is a specific marker of mucosal mast cell activation (Woodbury & Miller, 1982). The source of RMCP-II could of course be any of the vast number of epithelium within the rat including the gastric and small intestinal epithelium. To determine if colonic mucosal mast cells account for the increase in plasma RMCP-II concentrations following LPS-challenge, we examined alcian blue and safranine O stained sections of whole colon following LPS challenge. Indeed, colons from LPS-challenged animals had a lower number of mucosal mast cells than vehicle-treated animals. Furthermore, the relative reduction in the number of mucosal mast cells was attenuated by doxantrazole but not ketotifin, suggesting that the colonic epithelium is at least in part the source of RMCP-II. These findings support results from studies in adult rats which demonstrated that gastric mucosal mast activation occurred in response to immunological challenge (Andre *et al*., 1978). We have also demonstrated that mucosal mast cell degranulation precedes the increase in tissue neutrophil extravasation, increased iNOS activity and lipid peroxidation following LPS-challenge. This sequence of events is suggested since the plasma concentration of RMCP-II was significantly greater than that which was observed in control animals within 30 min of LPS challenge, whereas the tissue MPO activity was not significantly greater than control until 2 h after LPS administration. In addition, the mechanisms involved in mast cell degranulation were also examined. A stabilizing effect of NO upon mast cells has previously been proposed (Kanwar & Kubes, 1994). However, this is probably mediated by the comparatively small amounts of NO produced by the constitutively expressed isoforms of NOS, and may involve the superoxide scavenging properties of NO (Kubes *et al*., 1993). It has been suggested that superoxide activates mast cells and causes subsequent degranulation (Kanwar & Kubes, 1994). Data from the present study support this hypothesis, since prior administration of the antioxidant enzyme, SOD, and the xanthine oxidase inhibitor, allopurinol, before the LPS-challenge, inhibited the LPS-associated increase in mucosal mast cell degranulation. In combination, these findings lead us to suggest that LPS-challenge causes mast cell degranulation via a mechanism which involves reactive oxygen metabolites. However, the precise mechanism through which this occurs is at present unclear.

In summary, to the best of our knowledge we are the first group to demonstrate a relationship between mast cell degranulation, neutrophil infiltration, iNOS activity and lipid peroxidation following LPS-challenge in neonatal rats. Indeed, we would like to propose a mechanism describing the sequence of events, which ultimately leads to colonic inflammation in neonatal rats. Administration of LPS firstly results in mucosal mast cell degranulation within 30 min of LPS-challenge. Secondly, the degranulation of mucosal mast cells results in a time related increase in the tissue neutrophil content of the colon that leads to an increase in iNOS activity and finally lipid peroxidation. This may have therapeutic implications since the use of mucosal mast cell stabilizers, antioxidant enzymes, xanthine oxidase inhibitors or nitrosyl scavengers may prove to be effective treatments for colitis, particularly in the neonate.

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