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Interactive roles of superoxide and inducible nitric oxide synthase in rat intestinal injury provoked by non-steroidal anti-inflammatory drugs

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Abstract

The role of nitric oxide (NO) formed by inducible NO synthase (iNOS), superoxide and the lipopolysaccharide from luminal bacteria in non-steroidal anti-inflammatory drug-induced intestinal injury was investigated in the rat. Administration (s.c. or p.o.) of indomethacin (10 mg kg^{-1}), flurbiprofen (40 mg kg^{-1}) or diclofenac (40 mg kg^{-1}) increased the vascular leakage of radiolabelled albumin in the jejunum, determined after 24 h, associated with the induction of iNOS, assessed by the conversion of radiolabelled L-arginine. Pre-treatment with ampicillin (200 mg kg^{-1} day^{-1}, p.o.), metronidazole (200 mg kg^{-1} day^{-1}, p.o.), or polymixin B (15 mg kg^{-1} day^{-1}, s.c.), inhibited indomethacin-induced lesion formation, reduced microvascular leakage and prevented the expression of iNOS activity. Administration of the highly selective iNOS inhibitor, GW273629 ((*R*)-2-amino-4,4-dioxo-6(1-iminioethylamino)-4-thiahexanoic acid; 5 mg kg^{-1}, s.c.), 18 h after indomethacin, likewise prevented the intestinal lesions and attenuated the microvascular leakage. Superoxide dismutase conjugated with polyethylene glycol (3000 U kg^{-1}, i.v.), inhibited the indomethacin-induced lesions and microvascular leakage, but not the expression of iNOS activity. These findings suggest that non-steroidal anti-inflammatory drugs compromise mucosal integrity, leading to luminal bacterial translocation. This provokes iNOS induction, leading to microvascular injury involving both NO and superoxide.

Keywords: Nitric oxide (NO); Antibiotic; Bacterial invasion; Inflammation, intestinal; Selective inhibitor; GW273629; Polymixin B; Superoxide

1. Introduction

The ability of non-steroidal anti-inflammatory drugs (NSAIDs) to initiate intestinal inflammation and erosion in animals and humans has been recognised for many years (Whittle, 1981; Robert and Asano, 1977; Kent et al., 1969; Bjarnason et al., 1992; Sigthorsson et al., 1998). While the mechanisms by which NSAIDs can induce gastric damage have been well characterised, (Wallace, 1997; Whittle, 1992, 2000), the mechanisms leading to NSAID-induced intestinal injury are more complex. Pathogenic factors that have been proposed from early studies as initiating events include the inhibition of protective prostaglandins in the intestine (Whittle, 1981; Robert and Asano, 1977), as well as the enterohepatic circulation of NSAIDs (Yesair et al., 1970). The effectiveness of antibiotics in reducing NSAID-induced intestinal lesions and the resistance of germ-free rats to indomethacin-induced intestinal injury suggest that bacteria and bacterial products may also be an inflammatory stimulus in NSAID enteropathy (Robert and Asano, 1977; Kent et al., 1969).

The inducible isoform of nitric oxide (NO) synthase (iNOS) can be detected in many tissues including the intestinal organs following bacterial endotoxin challenge (Whittle, 1997; Salter et al., 1991). Expression of iNOS in intestinal tissue following challenge with lipopolysaccharide has been shown to be accompanied by microvascular leakage (Laszlo and Whittle, 1997; Boughton-Smith et al., 1993) as well as epithelial cell injury (Tepperman et al., 1993; Lamarque et al., 2000). These observations could reflect the sustained or excessive synthesis of NO, and could involve the interaction of NO with the superoxide anion to produce the reactive peroxnitrite moiety or the formation of other radicals (Beckman et al., 1990; Hogg et al., 1992).

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In a previous study, the time-dependent expression of iNOS activity following subcutaneous administration of indomethacin was demonstrated to be accompanied by jejunal microvascular injury (Whittle et al., 1995). Both the iNOS activity and microvascular leakage was reduced by dexamethasone pretreatment, which prevents iNOS expression, and by high doses of the broad-spectrum antibiotic, ampicillin (Whittle et al., 1995). This microvascular injury was also attenuated by an isoform non-selective NO synthase inhibitor, when administered at the time of iNOS expression (Whittle et al., 1995). Other more recent studies have confirmed that subcutaneous indomethacin administration leads to of iNOS expression in the rat small intestine, which is associated with intestinal lesions (Konaka et al., 1999; Chen et al., 1999). In addition, it has been recently demonstrated that the induction of iNOS after single subcutaneous dose of indomethacin is site-specific, occurring in the jejunum, the region that ulceration is primarily noted (Evans et al., 2000).

In the present study, the relationship between NSAID-provoked jejunal lesions, luminal bacteria, superoxide and NO synthesis has been further determined. Thus, the actions of either oral or subcutaneous administration of indomethacin, diclofenac and flurbiprofen on iNOS expression and jejunal injury were investigated. To evaluate the role of luminal bacteria and their products, the effects of the antimicrobials ampicillin, and metronidazole, as well as polymixin B which binds and inactivates endotoxin lipopolysaccharide, on macroscopic injury, microvascular leakage of radiolabelled albumin, and the induction of a iNOS have been determined. The involvement of superoxide in this macroscopic and microvascular injury has been assessed using superoxide dismutase, conjugated with polyethylene glycol for in vivo stability (Lamarque and Whittle, 1995; Lamarque et al., 2000). To assess the role of iNOS in the later phase of the NSAID-induced macroscopic and microvascular injury in the jejunum, the actions of the recently described highly selective iNOS inhibitor GW273629 (Knowles et al., 1999) were evaluated.

2. Materials and methods

L[U-^{14}C]arginine monohydrochloride and ^{125}I-labelled human serum albumin were obtained from Amersham International (UK). The superoxide dismutase-polyethylene glycol conjugate was obtained from Oxis International (New York, USA). Primary anti-mac iNOS monoclonal antibody (#N32020), and horseradish peroxidase-labelled anti-mouse antibody were obtained from Transduction Laboratories (UK). (*R*)-2-Amino-4,4-dioxo-6(1-iminioethylamino)-4-thiahexanoic acid (GW273629) was synthesised by GlaxoSmithKline Research and Development, Stevenage. All other reagents and compounds were from the Sigma (Poole, Dorset, UK).

Male Wistar rats (200–250 g) received food and water ad libitum during the course of these experiments. From preliminary studies, the doses of indomethacin (10 mg kg^{-1}, s.c.), diclofenac (40 mg kg^{-1}, s.c.) and flurbiprofen (40 mg kg^{-1}, s.c.) were chosen to provide similar and reproducible levels of macroscopic jejunal injury after 24 h. These doses were also used to evaluate the actions of these agents after oral administration. NSAIDs were freshly dissolved in sodium bicarbonate solution (5% w/v), before volume adjustment with saline and administered subcutaneously, or orally (0.5 ml).

2.1. Nitric oxide synthase activity

NOS activity was determined as the conversion of L[^{14}C]arginine monohydrochloride to L[^{14}C]citrulline (Knowles et al., 1999). Tissues were homogenised (15 s; ultra-turrax homogeniser; 5 mm blade) in ice-cold buffer (250 mg ml^{-1}, 4 °C), 10 mM HEPES, 32 mM sucrose, 1 mM dithiothreitol, 0.1 mM EGTA, 10 μg ml^{-1} soybean trypsin inhibitor, 10 μg ml^{-1} of leupeptin, and 2 μg ml^{-1} of aprotonin, adjusted to pH 7.4 (using 8 M sodium hydroxide) followed by centrifugation for 20 min at 10,000 × *g* at 4 °C. To remove endogenous arginine, supernatants were separated from tissue pellets and added to a water-free pellet of Dowex resin (AG 50W-8; 200–400, 8% cross-linked, Na^+ form, prepared by washing the H^+ form of the resin with 1 M sodium hydroxide four times and then washing with water until the pH was less than 7). Samples were vortexed (4 s) to ensure mixing with the Dowex resin, followed by centrifugation for a further 10 min at 10,000 × *g* at 4 °C.

Sample supernatant (40 μl) was incubated for 10 min at 37 °C in 110 μl of reaction buffer comprising (final concentrations) 50 mM KH_2PO_4, 1 mM $MgCl_2$, 0.2 mM $CaCl_2$, 50 mM valine, 1 mM dithiothreitol, 15.5 nM L-arginine, 1 mM L-citrulline, 0.3 mM β-nicotinamide adenine dinucleotide phosphate, 3 μM FAD, 3 μM FMN, 3 μM tetrahydrobiopterin, and 0.17 μM of [^{14}C]arginine (110,000 d.p.m. ml^{-1}). The reaction was arrested via the removal of the substrate L-arginine by the addition (0.5 ml) of a 1:1 v/v suspension of Dowex/distilled water. The mixture was dispersed and diluted via the addition of 0.85 ml distilled water (total volume 1.5 ml). After allowing the resin to settle 30 min, the supernatant was removed (0.97 ml) for the estimation of the radiolabelled products by scintillation counting (2 ml Pico-Fluor). Sample protein content was estimated via spectrophotometric assay (Bio-Rad Protein Assay), allowing expression of NOS activity as pmol min^{-1} mg protein^{-1}.

NO synthase activity was defined as citrulline formation that was abolished by incubation in vitro with N^G-monomethy-L-arginine (300 μM) and was further characterised by the effects of incubation in vitro with EGTA (1 mM). Thus basal NO synthase activity, that was abolished by EGTA, was taken as Ca^+-dependent constitutive NOS,

while that not inhibited by EGTA incubation was taken as iNOS activity.

2.2. Western blotting

Tissue samples measured for protein content (Biorad protein assay) were prepared for electrophoresis by 1:2 dilution in Tris–glycine sodium dodecyl sulphate with 5% dithiothreitol, before denaturing at 105 °C for 5 min. Samples were diluted to 50 $\mu g\ ml^{-1}$ protein before being stained. For electrophoretic protein separation, denatured samples were loaded into precast Novex 6% Tris–glycine gels, and run at 100 V constant voltage. Protein was transferred to Hybond-ECL (Amersham) nitro-cellulose membrane in transfer buffer at 30 V constant voltage for 180 min. The membrane was blocked at 4 °C in 5% non-fat dried milk in Tris-buffered saline with 0.1% Tween, 10 h before incubation (1 h) with primary anti-mac iNOS monoclonal antibody (Transduction Laboratories #N32020) in Tris-buffered saline with 0.1% Tween with 5% non-fat dried milk. After rinsing (1×15 and 4×5 min), the nitro-cellulose membrane was incubated (1 h) with horseradish peroxidase-labelled anti-mouse antibody (Transduction Laboratories) in Tris-buffered saline with 0.1% Tween with 5% non-fat dried milk. After a repeat rinsing, the nitro-cellulose paper was incubated with a mixture of luminol and phenol which provided an enhanced chemiluminescence, the latter being detected by a short exposure to blue-light sensitive Kodak autoradiography film.

2.3. Albumin leakage

Under transient halothane anaesthesia, ^{125}I-labelled human serum albumin (2 $\mu Ci\ kg^{-1}$) was injected via a tail vein, 2 h before autopsy. The leakage of radiolabelled albumen was subsequently determined in segments of jejunal tissue (3 cm; removed 10–15 cm from the pyloric sphincter) taken from rats terminally anaesthetised with halothane as described before (Boughton-Smith et al., 1993; Whittle et al., 1995). Blood was collected from the abdominal aorta into syringes, containing trisodium citrate (final concentration, 0.318%) and centrifuged ($10{,}000 \times g$, 10 min, 4 °C). The radiolabelled albumen content of the plasma (100 μl) and segments of the jejunum was determined in a gamma spectrometer (Nuclear Enterprises NE1600) and the albumin content in the tissues was calculated. Values from control jejunal tissue were subtracted from the values of treated tissue and the data were expressed as plasma leakage, Δplasma $\mu l\ g^{-1}$ tissue, corrected for intravascular volume as previously described (Boughton-Smith et al., 1993). Intravascular volume was determined in a separate group of rats by the administration of radiolabelled albumen via the tail vein at each time point, 2 min prior to tissue removal.

2.4. Macroscopic assessment

Photographs of the jejunal mucosa were blindly scored on a scale of 1–5. Scoring values were: 0 = no damage to tissue; 1 = the appearance of palpable white nodules and single haemorrhagic segment extending less than 1 cm; 2 = single zone of mucosal erosion and ulceration extending less than 10 cm with haemorrhage; 3 = single zone of mucosal erosion and ulceration extending more than 10 cm with haemorrhage; 4 = multiple zones of mucosal erosion and ulceration with adhesions and luminal bleeding; 5 = extensive adhesions of bloated abdominal viscera due to perforating lesions, intestinal content black in colour due to blood in lumen.

2.5. Histology and Gram stain

Pentobarbitone-anaesthetised rats were perfused via the abdominal aorta, with heparinised saline (10 $U\ ml^{-1}$) for 5 min, before perfusion fixing with 4% paraformaldehyde for 20 min. Tissues were excised and placed into 4% paraformaldehyde for a further 40 min, before being temporarily stored in phosphate buffered saline. Tissues were embedded in paraffin wax and frozen. Tissue sections were cut while frozen and dried on a hotplate. Paraffin wax was removed using xylene. Gram-positive bacteria were stained intense blue–black by 30 s exposure to Hucker–Conn ammonium oxalate–Crystal violet (20 ml 95% alcohol; 0.8 g ammonium oxalate; distilled water 80 ml). Gram-negative bacteria were stained red by a 20-s exposure to Weigert's iodine (2 g potassium iodide; 1 g iodine crystals; 100 ml distilled water). Sections were decolourised by gentle agitation in acetone for 2–3 s. Sections were counterstained in filtered 1% neutral red (10 $mg\ ml^{-1}$) for 1 min.

2.6. Effects of ampicillin or metronidazole

The antibacterial agents, ampicillin or metronidazole (200 $mg\ kg^{-1}\ day^{-1}$; 1 ml) were administered orally as a suspension via a rubber gastric tube, 24 h prior to study, and again at time of NSAID administration.

2.7. Effects of polymixin B

Polymyxin B nonapeptide (15 $mg\ kg^{-1}$) was administered subcutaneously, concurrently with indomethacin.

2.8. Effects of GW273629

The relatively short-acting but highly selective iNOS inhibitor GW273629 (5 $mg\ kg^{-1}$, s.c.) was administered 18 h after the administration of indomethacin (10 $mg\ kg^{-1}$, s.c.). Plasma leakage was determined 6 h later, i.e. 24 h after indomethacin administration. This dose and timing of administration has been selected from recent

dose–response studies with this agent (Knowles et al., 1999).

2.9. Effects of superoxide dismutase

The superoxide radical scavenger, superoxide dismutase linked to polyethylene glycol for in vivo stability (superoxide dismutase-polyethylene glycol conjugate), was administered intravenously (3000 U kg^{-1}) concurrently with indomethacin. The dose of superoxide dismutase-polyethylene glycol conjugate was derived from previous studies with this compound (Lamarque and Whittle, 1995).

2.10. Statistics

The data are expressed as the mean ± S.E.M. of (n) rats per experimental group. Statistical analysis was performed on raw data using non-parametric Student's t-test for unpaired data where $P < 0.05$ was taken as significant.

3. Results

3.1. Effects of indomethacin on intestinal mucosal lesion formation

Indomethacin (10 mg kg^{-1}, s.c.), produced a time-dependent progression of inflammation of the proximal jejunum, with the appearance of palpable white nodules after 15 h was followed by small haemorrhage points along the mesenteric side of the jejunum at 18 h, which developed to mucosal erosion and haemorrhage by 24 h. Using the criteria for macroscopic assessment of damage, the jejunal tissue damage scores at 6 and 12 h following indomethacin were not significantly different from untreated jejunum ($n = 4$). However from 15 h onwards, the tissue damage score increased in a time-dependent manner, reaching maximal damage in all rats at 72 h (5 ± 0, $n = 6$, $P < 0.001$).

3.2. Histology

Microscopic examination of the jejunum ($n = 8$) confirmed the macroscopic findings, with lesions developing on the mesenteric side of the jejunum at 18 h, with intact but contracted mucosa surrounding the lesion. After 24 h, epithelial cell injury was apparent throughout the jejunal section circumference, and lesion area had increased with severe lesions involving the muscle layer. At the time point of 72 h, over 50% of the mucosa was involved in the lesion, with the development of perforations in the serosa (data not shown).

3.3. Gram stain

Sections of jejunum from control rats and rats 24 h after the administration of indomethacin were mounted and Gram-stained ($n = 8$). Control rats showed no disruption of the mucosa, with dark blue-stained Gram-positive bacteria seen associated luminal contents. High-power magnification of the luminal side of the mucosa of indomethacin-treated rats revealed severe cellular disruption in the jejunum from all eight rats studied. This injured mucosa contained high numbers of translocating bacteria, which had penetrated the mucosa,. These bacteria were stained red, denoting Gram-negative bacteria (data not shown).

3.4. Effects of NSAIDs on intestinal plasma leakage

Jejunal plasma extravasation was increased 24 h following a single subcutaneous administration of either indomethacin (10 mg kg^{-1}), diclofenac (40 mg kg^{-1}) or flurbiprofen (40 mg kg^{-1}) as shown in Fig. 1.

Jejunal plasma extravasation was also increased 24 h after a single oral administration of indomethacin (Fig. 2), and this was comparable to that seen after subcutaneous administration (Fig. 1). Diclofenac (40 mg kg^{-1}) and

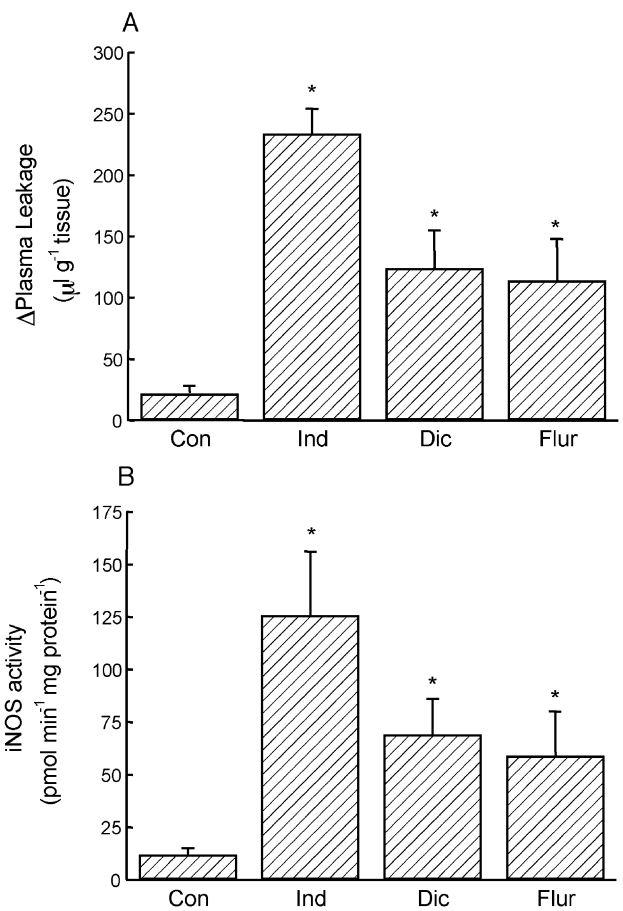

Fig. 1. Jejunal vascular leakage (Δplasma leakage, μl g^{-1} tissue, A) and iNOS activity (pmol min^{-1} mg^{-1} protein, B) induced by subcutaneously administered indomethacin (Ind, 10 mg kg^{-1}), diclofenac (Dic, 40 mg kg^{-1}), and flurbiprofen (Flur, 40 mg kg^{-1}), over 24 h. Data are expressed as mean ± S.E.M., where $n = 8$ for all groups, and statistical significance is shown as * ($P < 0.01$) in comparison to control (untreated) groups.

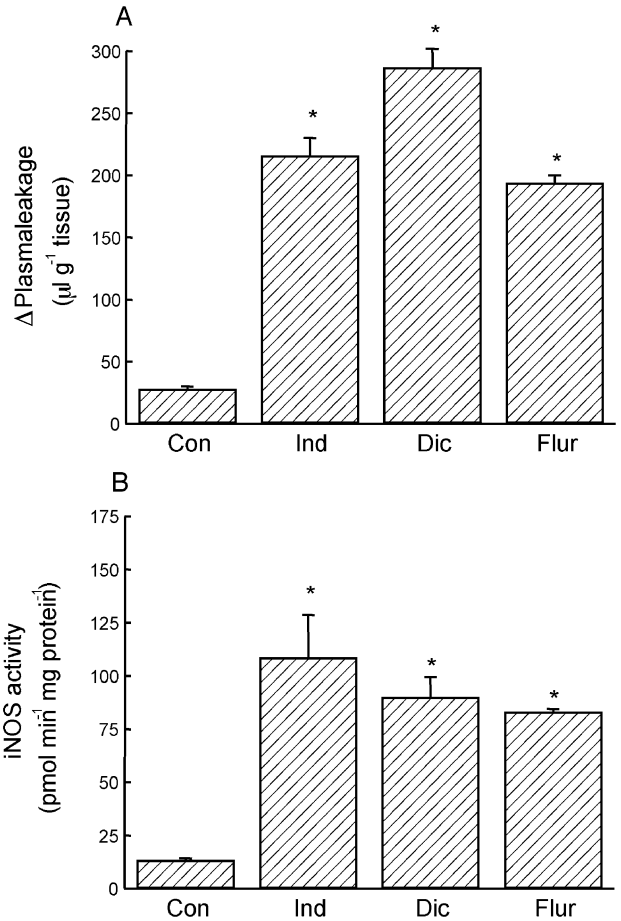

Fig. 2. Jejunal vascular leakage (Δplasma leakage, μl g^{-1} tissue, A) and iNOS activity (pmol min^{-1} mg^{-1} protein, B) induced by orally administered indomethacin (Ind, 10 mg kg^{-1}), diclofenac (Dic, 40 mg kg^{-1}), and flurbiprofen (Flur, 40 mg kg^{-1}), over 24 h. Data are expressed as mean ± S.E.M., where $n = 8$ for all groups, and statistical significance is shown as * ($P < 0.01$) in comparison to control (untreated) groups.

flurbiprofen (40 mg kg^{-1}) when administered orally, provoked jejunal plasma extravasation to a greater extent than when administered subcutaneously ($P < 0.05$) as shown in Figs. 1 and 2.

3.5. Effects of NSAIDs on nitric oxide synthase activity

Subcutaneous administration of indomethacin, diclofenac and flurbiprofen at doses of 10, 40 and 40 mg kg^{-1}, respectively, induced the expression of iNOS, determined after 24 h in the jejunum (Fig. 1).

Oral administration of indomethacin, diclofenac and flurbiprofen at doses of 10, 40 and 40 mg kg^{-1}, respectively, likewise induced the expression of iNOS activity in the jejunum ($P < 0.001$ for each) as shown in Fig. 2.

3.6. Western blot analysis of iNOS expression

Jejunal tissue from control rats and inflamed jejunum from rats administered a single dose of indomethacin (10 mg kg^{-1}, s.c., 24 h), were denatured and electrophoresed, before being probed by an anti-macrophage iNOS antibody. The antibody did not bind to any proteins from the sample prepared from normal jejunum. However, in the inflamed jejunal preparation, the antibody bound to a band at 130 kDa, which corresponds to the iNOS gene product, as shown in Fig. 3.

3.7. Effects of antimicrobials

The role of bacteria in indomethacin-induced inflammation was assessed by pretreating rats with either ampicillin (200 mg kg^{-1}, p.o.) or metronidazole (200 mg kg^{-1}, p.o.), 24 h prior to study, and again at time of NSAID administration. Pretreatment with either ampicillin or metronidazole alone, caused no significant changes in either tissue integrity ($n = 8$), plasma extravasation ($n = 8$), or jejunal iNOS activity ($n = 8$). However, the jejunal mucosal damage induced by indomethacin after 24 h ($P < 0.001$), was diminished by ampicillin or metronidazole pretreatment ($n = 12$, $P < 0.001$) as shown in Fig. 4.

Pretreatment with ampicillin or metronidazole also prevented the induction of plasma extravasation by indomethacin over 24 h (by 88 ± 12% and 80 ± 15% inhibition, respectively; $n = 12$; $P < 0.001$) as shown in Fig. 4. Likewise, pretreatment with either ampicillin or metronidazole prevented the indomethacin-induced expression of iNOS activity over 24 h (98 ± 3% and 98 ± 9% inhibition, respectively; $n = 12$; $P < 0.001$) as shown in Fig. 4.

In order to assess the possibility that any of the antimicobial agents caused their effect via direct inhibition of nitric oxide synthase activity, the activity of each agent on nitric oxide synthase activity in preparations of rat jejunal tissue taken from control and indomethacin-teated rats was assessed in vitro. No significant inhibition of constitutive NOS activity was observed when incubated (1 h) with ampicillin (0.28 ± 7.0% inhibition), metronidazole (2.2 ± 9.0% inhibition), polymyxin B (2.8 ± 6.9% inhibition), or

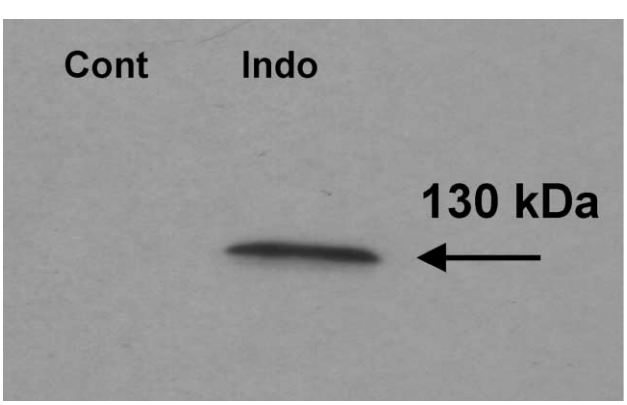

Fig. 3. Detection of iNOS in jejunal mucosa by Western blot analysis using iNOS monoclonal antibody, 24 h following a single administration of indomethacin (10 mg kg^{-1}, s.c.).

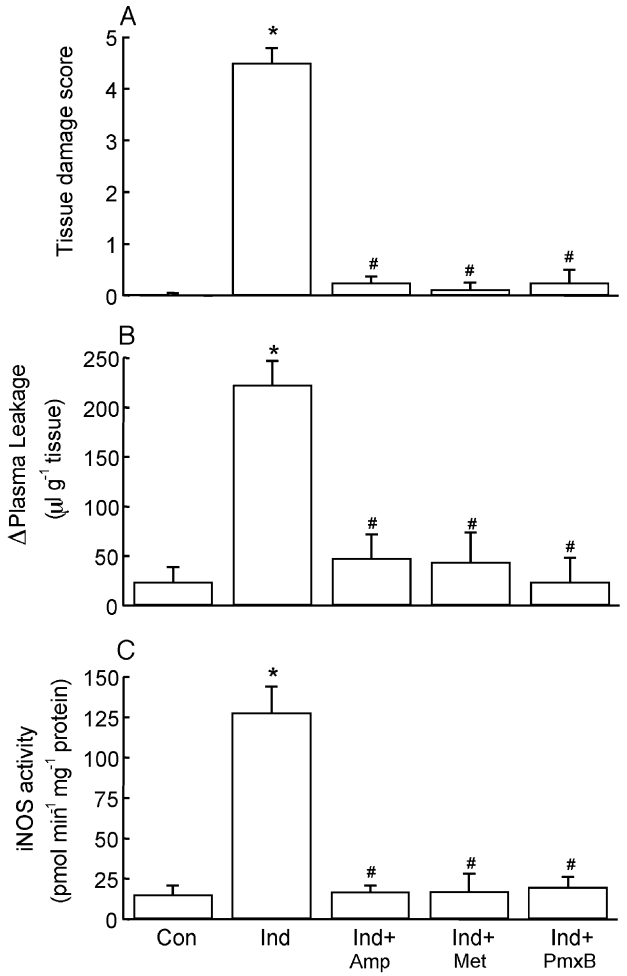

Fig. 4. Inhibition by ampicillin (Amp, 200 mg kg^{-1}, p.o.), metronidazole (Met, 200 mg kg^{-1}, p.o.) or polymixin B nonapeptide (PmxB 15 mg kg^{-1}, s.c.) on tissue damage (A), vascular leakage of radiolabelled albumin (expressed as Δplasma leakage, μl g^{-1} tissue, B), and iNOS activity (as pmol min^{-1} mg^{-1} protein, C) induced by indomethacin (INDO, 10 mg kg^{-1}) over a period of 24 h. Data are expressed as mean ± S.E.M., where $n = 6–12$, and statistical significance is shown as * ($P < 0.001$) in comparison with control values and + ($P < 0.001$) in comparison with INDO treated groups.

indomethacin (2.2 ± 11.4% inhibition), all at 1 mM concentration. Likewise, no significant inhibition of indomethacin-induced jejunal iNOS activity was observed when preparations were incubated with 1 mM concentrations of ampicillin (5.5 ± 5.1% inhibition), metronidazole (7.9 ± 10.5% inhibition), polymyxin B (3.9 ± 9.2% inhibition), or indomethacin (2.3 ± 6.6% inhibition). However, in the same preparation, GW273629 (250 μM) inhibited the iNOS activity by 88 ± 8% ($n = 6$; $P < 0.001$), with an IC_{50} of 71 μM.

3.8. Effect of polymixin B

Polymyxin B (3 mg kg^{-1}) administered subcutaneously ($n = 6$), caused no significant changes in either jejunal integrity, plasma extravasation or the expression of NOS (Fig. 4). Administration of polymyxin B, concurrently with indomethacin, prevented the jejunal injury ($n = 12$, $P < 0.001$), and plasma extravasation ($n = 12$, $P < 0.001$) seen at 24 h (Fig. 4).

Concurrent administration of polymyxin B with indomethacin, also prevented the induction of the expression of iNOS activity after 24 h (97 ± 1% inhibition, $n = 8$, $P < 0.001$) as shown in Fig. 4.

3.9. Effects of selective inos inhibitor GW273629

GW273629 administered subcutaneously at 18 h, abolished the tissue damage seen 24 h after indomethacin challenge ($n = 10$, $P < 0.001$) as shown in Fig. 5. Simi-

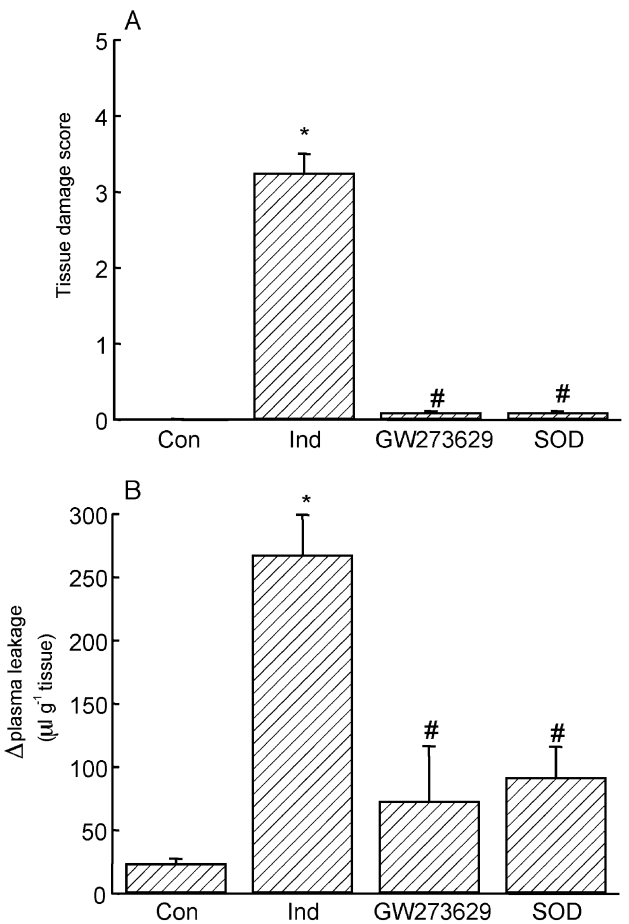

Fig. 5. Inhibition by the iNOS selective inhibitor GW273629 (5 mg kg^{-1}, s.c., 18 h after challenge) or concurrent administration of superoxide dismutase-polyethylene glycol conjugate (SOD, 3000 U kg^{-1}, i.v.) on tissue damage score (A), and the vascular leakage (as Δplasma leakage, μl g^{-1} tissue, B), in the rat jejunum 24 h following a single administration of indomethacin (INDO, 10 mg kg^{-1}, s.c.). Data are shown as the mean ± S.E.M, where $n = 10–12$, and statistical significance is shown as * ($P < 0.05$) in comparison to control values, and # ($P < 0.05$), in comparison to groups treated with indomethacin alone.

larly, subcutaneous administration of GW273629, administered 18 h after indomethacin, significantly decreased the plasma extravasation seen at 24 h (Fig. 5).

3.10. Effects of superoxide dismutase-polyelthylene glycol conjugate

Superoxide dismutase-polyethylene glycol conjugate administered intravenously concurrently with indomethacin, abolished the tissue damage seen 24 h after indomethacin challenge ($n = 6$; $P < 0.001$) as shown in Fig. 5. Likewise, superoxide dismutase-polyethylene glycol conjugate, administered concurrently with indomethacin, dose-dependently inhibited the plasma extravasation induced by indomethacin over 24 h ($76 \pm 10\%$ inhibition, $n = 6$, $P < 0.001$) as shown in Fig. 5. When administered intravenously superoxide dismutase-polyethylene glycol conjugate (3000 U kg^{-1}) itself caused no change in jejunal plasma leakage over 24 h ($n = 6$).

In the rats which received superoxide dismutase-polyethylene glycol conjugate (3000 U kg^{-1}) concurrently with indomethacin, the level of jejunal iNOS activity remained significantly elevated (93 ± 12 pmol min^{-1} mg $protein^{-1}$, $n = 8$, $P < 0.05$) above control values and were not significantly different from those of rats given indomethacin alone ($n = 8$, $P < 0.05$).

4. Discussion

In the present study, single oral or subcutaneous doses of either indomethacin, flurbiprofen, or diclofenac induced iNOS activity in the rat jejunum over the 24 h period. Western blot analysis also demonstrated the expression of iNOS protein in the rat jejunal mucosa 24 h after indomethacin administration. Such findings on intestinal iNOS induction confirm previous studies with subcutaneous administration of indomethacin (Whittle et al., 1995; Konaka et al., 1999) or flurbiprofen (Bertrand et al., 1998).

Indomethacin, diclofenac and flurbiprofen, when administered orally or subcutaneously increased vascular leakage of albumin, an index of microvascular injury, in the rat jejunum determined 24 h after challenge. The time-dependent enhancement of albumin leakage and iNOS expression following indomethacin has previously been shown to occur from 18 h after the administration of indomethacin (Whittle et al., 1995). In the present study, indomethacin exhibited comparable activity in provoking jejunal vascular leakage over a 24-h period when given either orally or subcutaneously. However, the induction of jejunal iNOS and vascular leakage over 24 h by both diclofenac and flurbiprofen was somewhat greater when these agents were administered orally than when given by subcutaneous administration.

Indomethacin, diclofenac and flurbiprofen have all been shown to undergo enterohepatic circulation in the rat (Seitz and Boelsterli, 1998; Yesair et al., 1970; Reuter et al., 1997; Eeckhoudt et al., 1997). The relative extent of this process may account for some of these differences between the oral and parenteral potency of these agents in causing injury. Such enterohepatic recirculation would produce a more prolonged exposure of the jejunal mucosa to high concentrations of the agents and their metabolites, and hence increase the propensity to produce local epithelial injury and barrier dysfunction. Diclofenac given orally has been shown to cause small-intestinal enteropathy in humans (Gut et al., 1996). Flurbiprofen has been demonstrated to increase human small-intestinal epithelial permeability to a labelled marker (Davies et al., 1996). The loss of the epithelial barrier would increase intestinal permeability, allowing underlying tissues to be exposed to the gut-luminal contents including antigens and micro-organisms. In this present study, high power magnification of the jejunum from rats, 24 h after indomethacin administration showed predominantly Gram-negative bacteria adhering and invading to the mucosa. These observations support the suggestion that indomethacin-induced enteropathy involves the overgrowth of Gram-negative bacteria (Kent et al., 1969; Yamada et al., 1993), and that these bacteria, unrestricted by the epithelial barrier, migrate into the mucosal tissue.

In the present study, pretreatement with the broad-spectrum antibiotic ampicillin, or the anti-anaerobic metronidazole, prevented the development of intestinal mucosa injury and microvascular leakage over the 24-h period studied. In an early study where bacterial overgrowth of *Escherichia coli*, *Bacteroides* and *Clostridium* was observed, reduction in lesion severity was achieved using a mixture of neomycin, polymyxin B and bacitracin (Kent et al., 1969). Studies in humans have also demonstrated that metronidazole can reduce the small-intestinal injury from NSAIDs (Bjarnason et al., 1992). All such findings suggested that the intestinal microvascular injury induced by NSAIDs such as indomethacin is not simply a result of direct drug action on the vasculature, but rather, a complex process involving local bacterial infection initiated by the NSAID. In the present study, either ampicillin or metronidazole also prevented the jejunal expression of iNOS. This supports the suggestion that the translocation of luminal bacteria into the small-intestinal mucosa may act as the inflammatory stimulus for the localised expression of iNOS. This also corresponds with findings that show that urinary nitrate, a marker of increased NO production, can also be considered as a marker of intestinal bacterial translocation in rats (Oudenhoven et al., 1994).

Failure of the intestinal barrier, with the subsequent facilitation of intestinal bacterial ingress, has been implicated in necrotising enterocolitis and has been shown to involve the expression of iNOS (Ford et al., 1996). The localised NO synthesis by iNOS under these conditions may also have a beneficial bacteriostatic function and reflect initiation of a host-defence mechanism. The impor-

tance of such a general bacterial defence may explain why iNOS knockout mice have shown susceptibility to infection, in comparison with their normal counterparts (Wei et al., 1995; MacMicking et al., 1995). In contrast however, iNOS gene-deleted mice exhibit an increased resistance to intestinal injury and bacterial translocation following ischemia–reperfusion (Suzuki et al., 2000), suggesting that the products of iNOS expression could be involved in the aggravation of epithelial barrier dysfunction. Indeed, delayed apoptosis occurs after bacterial invasion in human intestinal epithelial cell lines (Kim et al., 1998), while expression of iNOS in rat intestinal epithelial cells is associated with the induction of apoptosis in these cells (Lamarque et al., 2000). However, the initiating process that leads to acute epithelial barrier disruption and bacterial translocation from the gut lumen following administration of NSAIDs is not clear, but appears to involve pro-inflammatory mediators such as platelet-activating factor (Laszlo and Whittle, 1998). The release of such mediators may follow the initial fast-onset inhibition of prostanoid production by cyclo-oxygenase (Whittle, 1981). It is thus likely that inhibition of the cyclo-oxygenase-1 isoform, found extensively in intestinal tissue, is important in this process, but may not be the sole factor (Whittle, 2000). Clinical studies with novel selective inhibitors of the isoform identified predominantly at inflammatory site, cyclo-oxygenase-2, suggest that they have minimal effect on intestinal integrity in humans (Sigthorsson, 2000).

In the present study, polymyxin B given subcutaneously prevented the induction of iNOS activity following indomethacin administration. This agent, as with the antibacterials used in this study, did not directly affect the activity of the iNOS enzyme following in vitro incubation. Such findings not only confirm that the induction of iNOS and the subsequent enteropathy is due to indigenous bacteria from the gut lumen, but also suggests that the lipopolysaccharide fraction of these bacteria is the stimulus which initiates the expression of iNOS. Polymyxin B also prevented the induction of microvascular leakage and the onset of tissue injury in the current investigation. In other studies, polymyxin B has also been found to inhibit the production of tumour necrosis factor-α and interleukin-1β, and the development of inflammation in a haemorrhagic model of gut inflammation, involving bacterial translocation (Yao et al., 1995). It is possible the local release of the lipopolysaccharide from the translocated bacteria in the intestinal mucosa, is also a stimulus for the observed local production of the cytokines, including tumour necrosis factor-α, in the tissue following NSAIDs (Bertrand et al., 1998), these pro-inflammatory mediators being well known to be potent inducers of iNOS expression. Moreover, the agent R 167653 which inhibits cytokine production, has been shown to reduce indomethacin-induced intestinal injury (Konaka et al., 1999).

In the present study, the iNOS selective inhibitor GW273629 (Knowles et al., 1999) prevented the jejunal mucosal erosions and microvascular injury seen following indomethacin treatment. The effect of this highly selective inhibitor confirms that the expression of iNOS, and the subsequent NO synthesis, plays a key role in the tissue erosion and microvascular dysfunction of NSAID enteropathy.

The mechanism by which NO can mediate this microvascular injury and mucosal damage may involve its interaction with superoxide radicals, to generate the more reactive peroxynitrite radical, which is capable of subsequent homolytic cleavage to hydroxyl radicals (Beckman et al., 1990; Hogg et al., 1992). In this present study, pretreatment with the superoxide scavenger, superoxide dismutase, conjugated with polyethylene glycol to reduce clearance from the circulation, significantly inhibited the microvascular injury induced by indomethacin over 24 h. Previous studies with this agent has demonstrated that it can also prevent tissue injury from local infusion of NO donors, as well as the iNOS-associated injury to duodenal epithelial cells following challenge with the lipopolysaccharide from *Helicobacter pylori* (Lamarque and Whittle, 1995; Lamarque et al., 2000). Other studies have also shown that the damage induced by intravenous challenge with *E. coli* lipopolysaccharide in rat small intestinal epithelial cells, can be attenuated by a superoxide dismutase-mimetic or agents that act as peroxynitrite decomposition catalysts (Salvemini et al., 1999). Furthermore, the inhibitor of xanthine oxidase (a potential source of superoxide), allopurinol, which can reduce the cytotoxic actions of local NO on the mucosa (Lamarque and Whittle, 1995), has been demonstrated to reduce the intestinal injury provoked by indomethacin (Konaka et al., 1999). Antioxidants have been shown to affect iNOS expression in vascular smooth muscle cells (Hecker et al., 1996). However, in the present study, the iNOS activity induced by indomethacin was not significantly altered by pretreatment with the superoxide dismutase-polyethylene glycol conjugate. Thus, the present findings suggest that NO alone, produced from iNOS, does not itself provoke the tissue injury in this model in the absence of the superoxide moiety.

The results of the present study thus suggest the adherence and translocation of Gram-negative bacteria at the site of NSAID-induced enteropathy. The actions of the antimicrobial agents ampicillin, metronidazole and polymixin B suggest that bacterial translocation with generation and release of lipoploysaccharide, is the stimulus for the induction of iNOS activity. Use of the selective iNOS inhibitor GW273629 has identified NO synthesised by iNOS to be a key mediator in the microvascular leakage and lesion formation in this model. Selective inhibitors of iNOS may thus have potential therapeutic benefit in the treatment of NSAID-induced enteropathy. The findings with the superoxide dismutase-polyethylene glycol conjugate show the involvement of superoxide, supporting the key interaction in vivo of NO and superoxide in the pathogenesis of NSAID-induced jejunal injury.

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