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European Journal of Pharmacology 461 (2003) 63–71
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Role of bacteria and inducible nitric oxide synthase activity in the systemic inflammatory microvascular response provoked by indomethacin in the rat

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Received 23 October 2002; received in revised form 23 December 2002; accepted 24 December 2002

Abstract

The role of bacteria and nitric oxide (NO), formed by the inducible isoform of NO synthase (iNOS), in a widespread systemic inflammatory microvascular response that follows indomethacin administration, has been investigated in the rat. Subcutaneous administration of indomethacin (10 mg kg^{-1}) daily for 2 days produced an increase in microvascular leakage of radiolabelled albumin accompanied by expression of iNOS activity in the lung, liver, spleen and kidney, as well as in the jejunum, caecum, colon and ileum. Pretreatment with dexamethasone (1 mg kg^{-1} day^{-1}, s.c.) reduced indomethacin-provoked microvascular leakage and the expression of iNOS activity in all the tissues studied. The widespread microvascular leakage and iNOS activity was also inhibited by pretreatment with ampicillin (200 mg kg^{-1} day^{-1}, p.o.), metronidazole (200 mg kg^{-1} day^{-1}, p.o.) or by polymyxin B (15 mg kg^{-1} day^{-1}, s.c.). Administration of the highly selective iNOS inhibitor GW 273629 (3-{[2-(ethanimidoylamino)ethyl]sulphonyl}-L-alanine; five doses of 5 mg kg^{-1}, s.c. over 48 h) substantially inhibited the microvascular leakage in the affected organs. Such findings suggest the involvement of indigenous gut bacteria, lipopolysaccharide and iNOS expression following indomethacin-induced enteropathy in this widespread systemic inflammatory microvascular response.

Keywords: Indomethacin; Microvascular albumin leakage; Gram-negative bacteria; Multiple organ failure; Inflammatory response syndrome, systemic; GW 273629; Nitric oxide (NO) synthase; Nitric oxide (NO) synthase-II; Enteropathy; Injury, intestinal

1. Introduction

Multiple organ dysfunction syndrome or multiple organ failure defines a condition in which individual organs or organ systems fail to perform their life-sustaining functions (Baue, 1975; Goris et al., 1985; Deitch, 1992). In multiple organ failure, the failing organs are not necessarily those with the primary disease or infection or those directly injured by the inciting pathogen, while a time-lag separates the initial injury and the onset of failure in the organs (Polk and Shields, 1977; Deitch, 1992).

The term gut-origin sepsis has arisen from clinical and experimental studies that have designated the gut as a reservoir for bacterial involvement in systemic infections (Rush et al., 1988; Deitch, 1990; Mainous et al., 1991; Pape et al., 1994). Bacterial translocation and the subsequent development of an uncontrolled infection-driven systemic inflammatory response syndrome has been implicated in the pathogenesis of sepsis, multiple organ failure and adult respiratory distress syndrome (Rush et al., 1988; Deitch, 1990; Sheng et al., 1991; Qureshi et al., 2001).

In a model of enteropathy involving gut bacteria, a single dose of indomethacin has been shown to induce a localised inflammation in the small intestine over 72 h with recovery within 1 week (Robert and Asano, 1977; Whittle, 1981; Yamada et al., 1993; Nygard et al., 1994; Reuter et al., 1997). Mechanistic studies indicated that these effects of indomethacin on the jejunum involved the localised expression of the inducible nitric oxide synthase isoform, iNOS (NOS-II), through actions of lipopolysaccharide derived from the luminal bacteria (Whittle et al., 1995; Konaka et al., 1999; Chen et al., 1999). It is also known that severe intestinal injury can result in substantial bacterial translocation from the gut lumen into the mucosa and portal blood, hence providing access to other organs (Mainous et

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doi:10.1016/S0014-2999(02)02959-X

al., 1991). Indeed, when administered over 2 days, indomethacin can produce extensive inflammation in rat small intestine, accompanied by bacterial translocation, with bacteria being found in the mesenteric lymph nodes, liver and spleen (Yamada et al., 1993). However, the consequences of such actions in producing a systemic microvascular inflammatory response have not been reported.

The present study thus investigates the effects of two sequential daily doses of indomethacin, which produce extensive jejunal enteropathy, to assess the repercussion in other regions of the gut and in other organs including the lung, heart, kidney and spleen, in terms of iNOS induction and microvascular injury. Such actions could provide the basis for a possible model of multiple organ failure or systemic inflammatory response syndrome. To evaluate the role of bacteria, the effects of the antimicrobials, ampicillin and metronidazole, on microvascular leakage of radiolabelled albumin and the induction of iNOS have been determined. The effect of polymyxin B, which binds and inactivates lipopolysaccharide, has also been determined. To assess the role of iNOS in the microvascular injury, the actions of a potent, highly selective iNOS inhibitor, GW 273629 (3-{[2-(ethanimidoylamino)ethyl]sulphonyl}-L-alanine; Knowles et al., 1999; Young et al., 2000) was evaluated.

2. Materials and methods

Male Wistar rats (200–250 g) received food and water ad libitum during the course of these experiments. From previous studies, the dose of indomethacin (10 mg kg^{-1}, s.c.) was selected to produce macroscopic jejunal injury. Indomethacin (Sigma-Aldrich Chemicals, Poole, UK) was first dissolved in sodium bicarbonate (5% w/v), before volume adjustment with saline, then administered subcutaneously (0.5 ml).

2.1. Albumin leakage

Under transient halothane anaesthesia, [^{125}I] human serum albumin (2 μCi kg^{-1}, s.c.; Amersham, UK) was injected via a tail vein, 2 h before autopsy. The leakage of [^{125}I] human serum albumin was subsequently determined in segments of jejunal tissues (3 cm; removed 10–15 cm from the pyloric sphincter) as well as tissue from the ileum, caecum, colon, lung, heart liver, spleen and kidney, taken from rats terminally anaesthetised with halothane. Blood was collected from the abdominal aorta into syringes, containing trisodium citrate (Sigma-Aldrich Chemicals; final concentration 0.318%) and centrifuged (10,000 × *g*, 10 min, 4 °C). The [^{125}I] human serum albumin content of the plasma (100 μl) and segments of the tissue were determined in a gamma spectrometer (Nuclear Enterprises NE1600) and the albumin content in the tissues was calculated. Values from control tissue were subtracted from the values of treated tissue and the data were expressed as plasma leakage, Δ plasma μl g^{-1} tissue, corrected for intravascular volume as previously described (Boughton-Smith et al., 1993). In a separate group of rats, intravascular volume was assessed in terms of the [^{125}I] human serum albumin in tissues after a 2-min circulation. Throughout these studies, intravascular volumes in the indomethacin-treated rats did not differ from saline control rats, the values being: jejunum 31 ± 2, ileum 31 ± 2, colon 36 ± 3, lung 368 ± 36, heart 250 ± 31, kidney 103 ± 10, liver 97 ± 10 μl g^{-1} tissue (n = 6).

2.2. Nitric oxide synthase activity

Nitric oxide synthase activity was determined as the conversion of L-[^{14}C] arginine monohydrochloride (Amersham) to L-[^{14}C] citrulline (Knowles et al., 1990). 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 NaOH) 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 NaOH four times and then washing with distilled 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 NADPH, 3 μM FAD, 3 μM FMN, 3 μM tetrahydrobiopterin and 0.17 μM of [^{14}C] L-arginine (110,000 dpm 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 for 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 nitric oxide synthase activity as pmol min^{-1} mg $protein^{-1}$.

Nitric oxide synthase activity was defined as citrulline formation that was abolished by incubation in vitro with N^G-monomethyl-L-arginine (300 μM) and was further characterised by the effects of incubation in vitro with EGTA (1 mM). Thus, basal N^G-monomethyl-L-arginine-sensitive activity, which was abolished by EGTA, was taken as calcium-dependent constitutive nitric oxide synthase, while

that not inhibited by EGTA incubation was taken as calcium-independent iNOS activity. All reagents were obtained from Sigma-Aldrich Chemicals.

2.3. Histology

Rats were terminally anaesthetised with pentobarbitone and 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 hot plate. 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. All reagents were obtained from Sigma-Aldrich Chemicals.

2.4. Effects of dexamethasone

Dexamethasone (Perbio Science, Cheshire, UK; 1 mg kg^{-1}, s.c.) was administered concurrently with each indomethacin dose. The dose of dexamethasone was derived from previous studies (Boughton-Smith et al., 1993). Tissues were taken for evaluation of iNOS and vascular leakage, 48 h after the initial dose of indomethacin.

2.5. Effects of antimicrobials

The antibacterial agents, ampicillin or metronidazole (Sigma-Aldrich Chemicals; both at 200 mg kg^{-1} day^{-1},

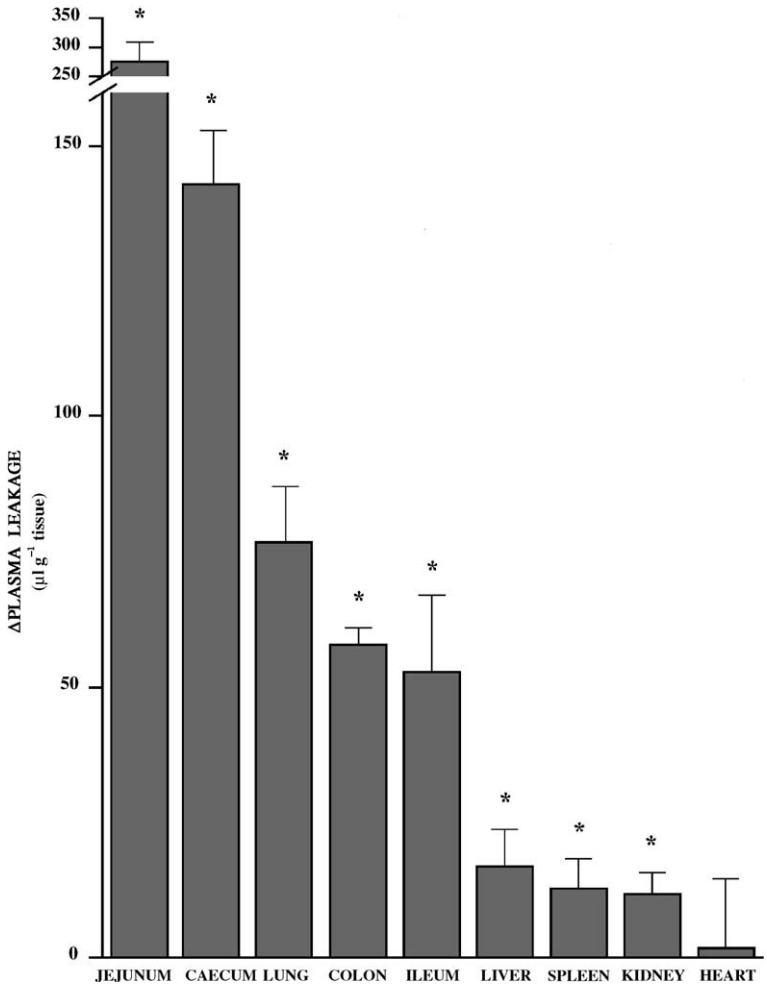

Fig. 1. Microvascular leakage of radiolabelled albumin (expressed as Δ plasma leakage, μl g^{-1} tissue) in rat organs following two daily doses of indomethacin (10 mg kg^{-1}, s.c.). Data determined 48 h after the first dose of indomethacin are expressed as mean ± S.E.M., where $n=6$ for all groups, and statistical significance is shown as *($P<0.05$) in comparison with organ-respective basal leakage values.

p.o., 1 ml), were administered as a suspension via a rubber gastric tube, 24 h before the first dose of indomethacin and again immediately before the second dose. The dose of ampicillin was derived from previous studies (Evans and Whittle, 2001). Tissues were taken for evaluation of iNOS and vascular leakage, 48 h after the initial dose of indomethacin.

2.6. Effects of polymyxin B

Polymyxin B nonapeptide (Sigma-Aldrich Chemicals; 15 mg kg^{-1}) was administered subcutaneously, concurrently with each dose of indomethacin. The dose of polymyxin B was derived from previous studies (Evans and Whittle, 2001). Tissues were taken for evaluation, 48 h after the initial dose of indomethacin.

2.7. Effects of GW 273629

The relatively short acting but highly selective iNOS inhibitor GW 273629 (3-{[2-(ethanimidoylamino)ethyl]sulphonyl}-L-alanine Knowles et al., 1999; Young et al., 2000) was administered (5 mg kg^{-1}, s.c.) 15 h after the administration of indomethacin (10 mg kg^{-1}, s.c.) and every 8 h thereafter (total of five doses in 48 h). Tissues were taken for evaluation, 48 h after the initial dose of indomethacin.

2.8. Macroscopic assessment of jejunal damage

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.

2.9. Statistical analysis

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

3. Results

3.1. Effects of indomethacin on the jejunum

Two doses of indomethacin (10 mg kg^{-1}) were administered subcutaneously to male Wistar rats (200–250 g) 24 h apart. This regimen produced sedated behavioural change and piloerection after 48 h. Following laparotomy at 48 h after the initial dose of indomethacin, jejunal lesions (tissue damage score, 4.7 ± 0.2) were found to extend toward the distal jejunum and severe adhesions were found around both the small and large intestines.

Sections of jejunum taken from rats 48 h after the initial dose of indomethacin were mounted and Gram-stained. Mucosal integrity was severely disrupted and high-power magnification of the jejunal mucosa revealed high numbers of adhering and invading bacteria, the Gram-negative bacteria (which stained red) being the predominant species (data not shown).

3.2. Widespread changes in microvascular leakage

Plasma leakage in rats that had received two doses of indomethacin, determined 48 h after the initial dose of indomethacin, was significantly increased in the jejunum, caecum, lung, colon, ileum, liver, spleen and kidney, but not in the heart (Fig. 1).

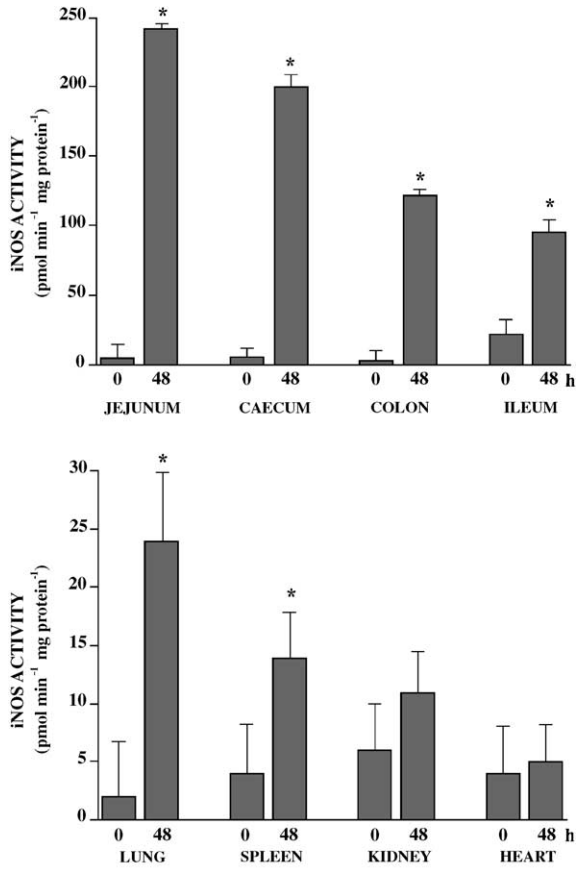

Fig. 2. The iNOS activity (pmol min^{-1} mg^{-1} protein) in rat organs following two daily doses of indomethacin (10 mg kg^{-1}, s.c.). Data determined 48 h after the first dose of indomethacin are expressed as mean ± S.E.M., where $n = 6$ for all groups, and statistical significance is shown as *($P < 0.05$) in comparison with iNOS levels at 0 h.

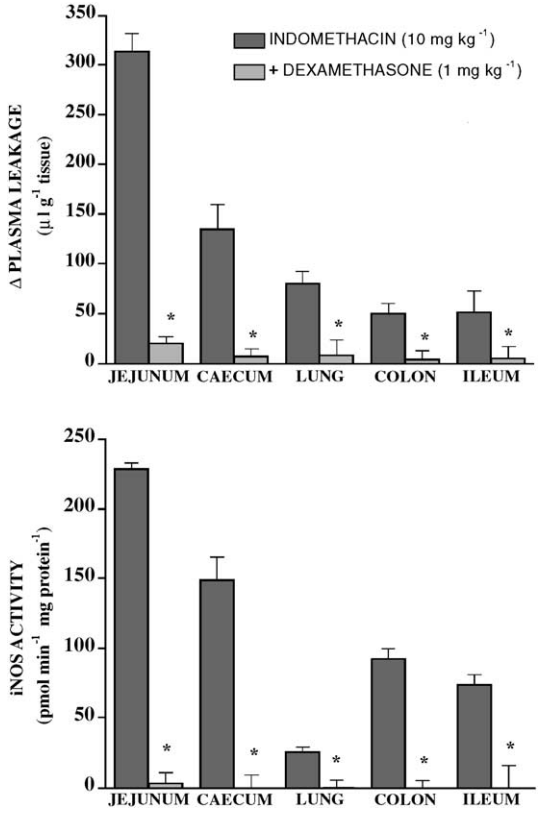

Fig. 3. Inhibition by dexamethasone (1 mg kg^{-1}, s.c. concurrently with indomethacin, and daily thereafter) of the induction of microvascular leakage (expressed as Δ plasma leakage, µl g^{-1} tissue) and iNOS activity (pmol min^{-1} mg^{-1} protein) in rat organs following two daily doses of indomethacin (10 mg kg^{-1}, s.c.). Data determined 48 h after the first dose of indomethacin are expressed as mean ± S.E.M., where $n=6$ for all groups, and statistical significance is shown as *($P<0.05$) in comparison to groups receiving indomethacin alone.

3.3. Widespread induction of iNOS activity

In rats that received the two doses of indomethacin, significant iNOS activity was detected in the jejunum, caecum, lung, colon, ileum and spleen, 48 h after the initial indomethacin administration (Fig. 2). As with microvascular leakage, no significant iNOS activity could be detected in the heart following indomethacin treatment and this tissue was not studied further. In control untreated or saline-treated rats, no significant levels of iNOS activity were detected in these tissues ($n=8$). Measurement of nitric oxide synthase activity in liver homogenates was highly variable, possibly due to the high levels of arginase enzymes (Schimke, 1967) not being fully inhibited by the presence of L-valine and hence this tissue was not used in further studies. In addition, because of the relatively low and variable levels of iNOS, as well as plasma leakage detected in the spleen and kidney following indomethacin treatment, these tissues were not routinely studied further.

3.4. Effects of dexamethasone pretreatment

Dexamethasone, when administered concurrently with each indomethacin dose, decreased the jejunal macroscopic injury determined 48 h after the initial dose of indomethacin (tissue damage score reduced from 4.7 ± 0.2 to 1 ± 0.3; $n=8$; $P<0.001$). Rats administered dexamethasone (1 mg kg^{-1}, s.c., 0.2 ml) alone showed no changes in the jejunal macroscopic appearance after 48 h ($n=8$).

Dexamethasone administered concurrently with each indomethacin dose, significantly inhibited the increases in plasma leakage in the jejunum, caecum, lung, colon and ileum (by 93 ± 7%, 94 ± 6%, 89 ± 10%, 91 ± 9% and 90 ± 10%, respectively; $n=6$; $P<0.05$), measured 48 h after the initial indomethacin administration (Fig. 3).

This dose regimen of dexamethasone also significantly reduced iNOS activity in the jejunum, caecum, lung, colon and ileum, measured 48 h after the initial indomethacin administration (by 98 ± 5%, 99 ± 4%, 97 ± 13%, 99 ± 5%, 100 ± 17% and 91 ± 8%, respectively; $n=6$; $P<0.05$) as shown in Fig. 3.

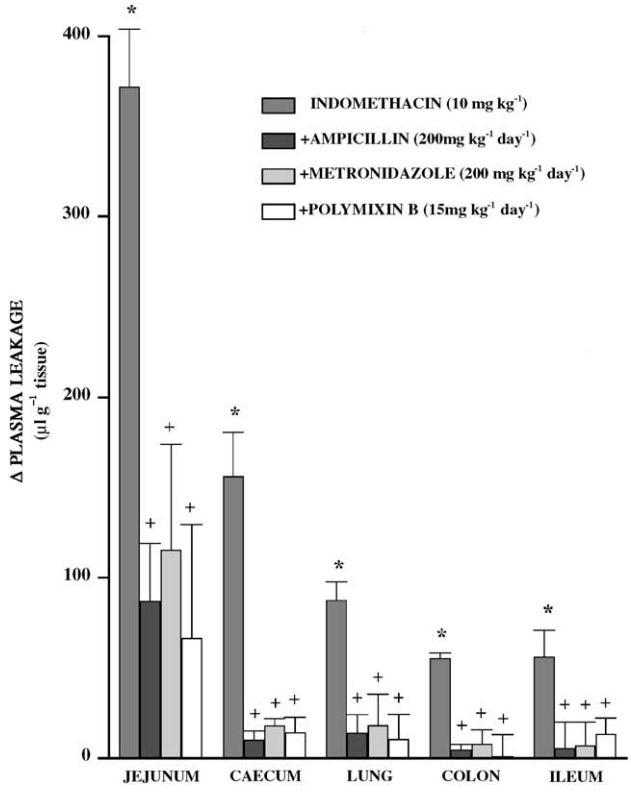

Fig. 4. Effect of antibacterial agents ampicillin (200 mg kg^{-1}, p.o.), metronidazole (200 mg kg^{-1}, p.o.), and polymixin B nonapeptide (15 mg kg^{-1}, s.c.) on microvascular leakage (expressed as Δ plasma leakage, µl g^{-1} tissue) after two daily doses of indomethacin (10 mg kg^{-1}, s.c.). Data determined 48 h after the first dose of indomethacin are expressed as mean ± S.E.M., where $n=8$, and statistical significance is shown as $^{+}$($P<0.05$) in comparison to groups receiving indomethacin alone.

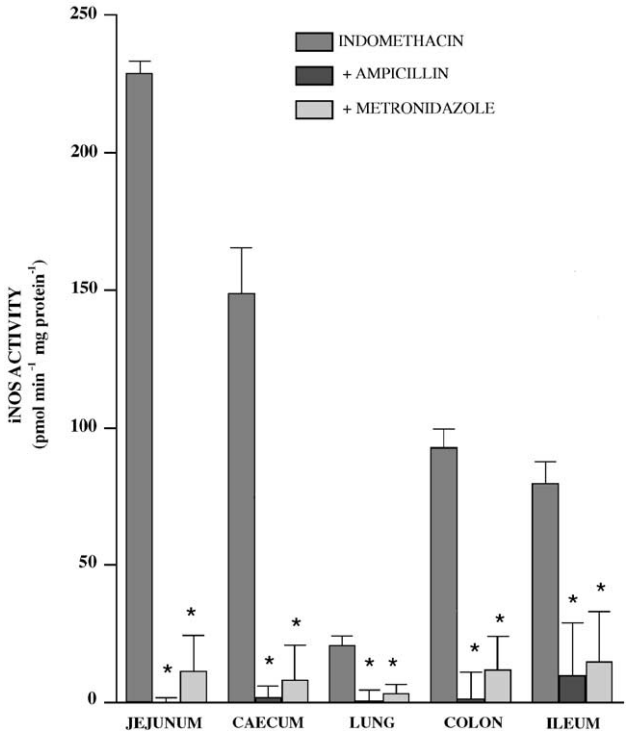

Fig. 5. Effect of antibacterial agents ampicillin (200 mg kg^{-1}, p.o.) or metronidazole (200 mg kg^{-1}, p.o.), on iNOS activity (pmol min^{-1} mg^{-1} protein) induced after two daily doses of indomethacin (10 mg kg^{-1}, s.c.). Data determined 48 h after the first dose of indomethacin are expressed as mean ± S.E.M., where $n=8$, and statistical significance is shown as *($P<0.05$) in comparison to groups receiving indomethacin alone.

3.5. Effects of antibacterial pretreatment

Pretreatment with either ampicillin (200 mg kg^{-1}, p.o.) or metronidazole (200 mg kg^{-1}, p.o.) alone caused no significant changes in either jejunal damage score, plasma extravasation or the expression of iNOS activity ($n=8$; data not shown). However, the jejunal mucosal damage induced by indomethacin after 48 h was significantly reduced by ampicillin or metronidazole pretreatment (tissue damage score, 0.3 ± 0.2 and 0.8 ± 0.2, respectively; $n=8$, $P<0.001$).

Pretreatment with these doses of ampicillin or metronidazole significantly inhibited the plasma extravasation in the jejunum (77 ± 9% and 67 ± 8% inhibition, respectively; $n=8$; $P<0.001$), caecum (93 ± 3% and 88 ± 3% inhibition, respectively; $P<0.001$), lung (84 ± 11% and 79 ± 19% inhibition, respectively; $P<0.01$), colon (92 ± 5% and 86 ± 14% inhibition, respectively; $P<0.001$) and ileum (90 ± 25% and 88 ± 21% inhibition, respectively; $P<0.05$) 48 h after the initial dose of indomethacin (Fig. 4).

Pretreatment with either ampicillin or metronidazole also significantly reduced the iNOS expression in the jejunum (100 ± 2% and 95 ± 6% inhibition, respectively; $n=8$; $P<0.001$), caecum (99 ± 3% and 94 ± 9% inhibition, respectively; $P<0.001$), lung (96 ± 17% and 84 ± 16% inhibition, respectively; $P<0.01$), colon (98 ± 10% and 87 ± 13% inhibition, respectively; $P<0.001$) and ileum (87 ± 25% and 80 ± 24% inhibition, respectively; $P<0.05$) 48 h following the initial dose of indomethacin (Fig. 5).

3.6. Effects of polymyxin B

Polymyxin B nonapeptide (15 mg kg^{-1}) alone, administered subcutaneously, caused no significant changes in jejunal tissue damage score nor plasma extravasation ($n=8$). Subcutaneous administration of polymyxin B nonapeptide, concurrently with indomethacin, prevented the jejunal injury (tissue damage score 0.5 ± 0.2; $n=8$; $P<0.001$) and significantly reduced the plasma extravasation in the jejunum, caecum, lung, colon and ileum (by 82 ± 16%, 91 ± 5%, 88 ± 15%, 98 ± 20%, 76 ± 15% inhibition, respectively; $n=8$; $P<0.05$) seen 48 h after the initial indomethacin administration (Fig. 4).

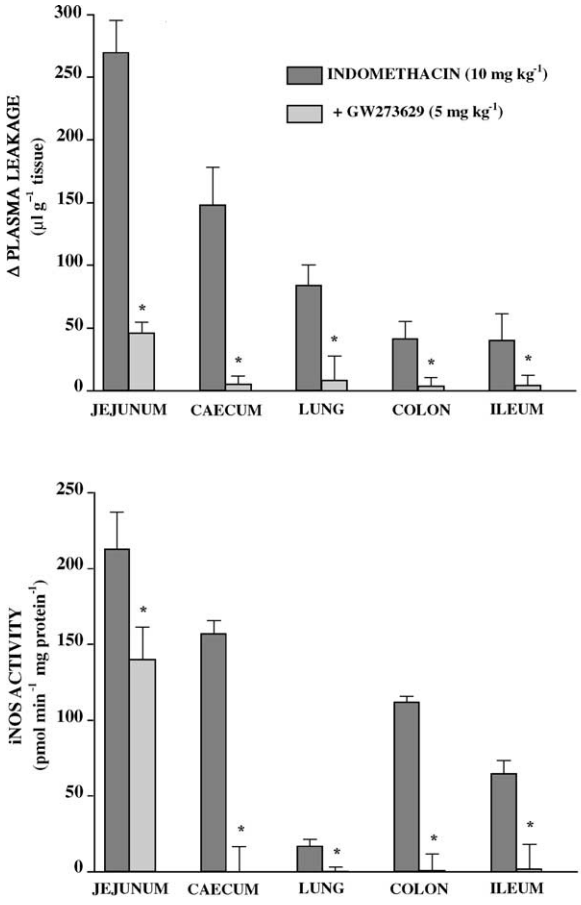

Fig. 6. Effect of GW 273629 (5 mg kg^{-1}, s.c.) on microvascular leakage (expressed as Δ plasma leakage, μl g^{-1} tissue) and iNOS activity (pmol min^{-1} mg^{-1} protein) induced in rat organs following two daily doses of indomethacin (10 mg kg^{-1}, s.c.). Data determined 48 h after the first dose of indomethacin are expressed as mean ± S.E.M., where $n=8$, and statistical significance is shown as *($P<0.05$) in comparison to groups receiving indomethacin alone.

3.7. Effects of GW 273629

Administration of GW 273629 (5 mg kg^{-1}, s.c.) alone, at 15 h following the start of the study and every 8 h thereafter, caused no detectable intestinal pathology or plasma leakage ($n=4$). However, GW 273629 significantly inhibited the macroscopic jejunal damage seen 48 h after the initial dose of indomethacin (tissue damage score, 1.2 ± 0.3; $n=6$; $P<0.001$). Likewise, GW 273629 significantly reduced the plasma leakage in the jejunum, caecum, lung, colon and ileum ($83 \pm 3\%$, $97 \pm 4\%$, $89 \pm 22\%$, $92 \pm 15\%$ and $89 \pm 16\%$ inhibition, respectively; $n=8$; $P<0.05$) measured 48 h after the initial dose of indomethacin (Fig. 6).

This dose regime of GW 273629 also reduced the iNOS activity in the jejunum, caecum, colon, lung, ileum ($34 \pm 9\%$, $100 \pm 10\%$, $99 \pm 9\%$, $94 \pm 12\%$ and $97 \pm 24\%$ inhibition, respectively; $n=8$; $P<0.05$), induced 48 h after the initial indomethacin administration (Fig. 6).

4. Discussion

In this study, subcutaneous daily administration of indomethacin for 2 days provoked microvascular leakage of albumin in the lung, liver, spleen and kidney, as well as in the jejunum, caecum, colon and ileum. This slowly developing widespread microvascular inflammation outside of the gut thus contrasts with the localised tissue and microvascular leakage of albumin seen only in the jejunum and not in other gut regions, following a single dose of indomethacin (Evans et al., 2000). Such events were associated with the widespread expression of iNOS in all of these organs both within and outside the gut. Previous studies have demonstrated only the localised expression of iNOS protein and enzyme activity in the jejunum following indomethacin administration (Whittle et al., 1995; Konaka et al., 1999; Chen et al., 1999).

In this present study, pretreatment with the antibacterial agents, metronidazole or ampicillin, significantly inhibited microvascular injury and the expression of iNOS activity in the organs studied, indicating the involvement of bacteria in these events. In an earlier study, metronidazole normalised the small-intestinal bacteria count and decreased the bacterial translocation following indomethacin administration in rats (Yamada et al., 1993). Polymyxin B, which binds lipopolysaccharide and can also reduce bacterial translocation (Yao et al., 1995), also reduced the widespread microvascular leakage in the current work, supporting a role for lipopolysaccharide in this injurious process. Previous studies have shown that polymyxin B reduced both iNOS activity and plasma leakage in the jejunum following a single dose of indomethacin, while none of these agents themselves directly inhibited the iNOS enzyme in vitro (Evans and Whittle, 2001).

Dexamethasone pretreatment prevents the expression of iNOS activity and the development of intestinal microvascular injury induced by lipopolysaccharide challenge in the rat (Boughton-Smith et al., 1993). In the present study, dexamethasone administration significantly reduced the widespread microvascular leakage and the expression of iNOS seen after the second dose of indomethacin. The inhibition of microvascular injury in the organs remote from the jejunum is likely to reflect the direct action of dexamethasone on the expression of iNOS in those organs. However, the inhibition of iNOS expression, microvascular injury and macroscopic damage in the jejunum by dexamethasone, which would reduce the loss of mucosal integrity, could also impede the access of bacteria or bacterial products involved in iNOS induction, into the systemic circulation. Indeed, recent studies have shown that iNOS knockout mice exhibit a resistance to development of gut bacterial translocation, microvascular alterations and the subsequent systemic inflammatory response induced by zymosan (Cuzzocrea et al., 2001), by reperfusion injury (Suzuki et al., 2000a) or by lipopolysaccharide challenge (Mishima et al., 1997, 1998; Suzuki et al., 2000b). In addition, removal of NO by use of a scavenger reduced the bacterial translocation and intestinal injury in rats induced by endotoxin challenge (Dickinson et al., 1999).

In the present study, GW 273629 significantly inhibited the microvascular injury in the involved tissues, suggesting a role of iNOS in the process of tissue injury provoked by indomethacin. Previous work has characterised that GW 273629 is a highly selective inhibitor of iNOS both in vitro and in vivo (Knowles et al., 1999) with a selectivity ratio of over 125 between human iNOS and eNOS (Young et al., 2000), while it has been shown to inhibit jejunal iNOS in vitro and reduce jejunal plasma leakage in vivo provoked by single dose of indomethacin (Evans and Whittle, 2001). It is not known why the degree of iNOS inhibition ex vivo in the jejunum was less than in the other tissues studied in the present work, but this may reflect the higher iNOS activity, a greater degree of washout of this reversible inhibitor in this preparation in vitro or perhaps some differences in tissue access or specificity of the agent in vivo. The ability of GW 273629 to prevent the systemic inflammatory response could suggest the usefulness of such iNOS inhibitors in preventing the development of these gut-origin responses.

There appeared to be an association between the extent of the expression of iNOS and the degree of microvascular leakage within the gut, with the rank order of tissues for both parameters being the jejunum, caecum, colon and ileum. In the spleen and kidney, lower levels of both iNOS expression and of microvascular leakage were observed. The lung, by contrast, exhibited substantial microvascular leakage but only moderate iNOS expression. Although the microvascular leakage in all organs was reduced by GW 273629, it is likely that the combination of superoxide and NO to form the cytotoxic peroxynitrite radical may be responsible for the tissue injury (Beckman et al., 1990), a superoxide dismutase preparation having been shown to

abolish indomethacin-induced enteropathy (Evans and Whittle, 2001). Thus, the correlation between iNOS expression and the extent of microvascular injury between organs could be expected to vary depending on the prevailing superoxide levels, as could the degree of inhibition of iNOS needed to reduce such injury. This difference could also reflect the levels of enzymatic and non-enzymatic antioxidants, which vary considerably between different tissues (Grisham et al., 1990), although the involvement of other local injurious factors independent of iNOS expression in this process cannot as yet be entirely excluded.

The referred induction of iNOS in organs remote from the jejunal septic focus also has implications for interpreting previous findings. Low doses of indomethacin over a 7-week period resulted in inflammation of the rat caecum (Nygard et al., 1994), which may reflect the subsequent induction of iNOS, following the initial jejunal injury, while the high caecal bacterial content may perpetuate the inflammation. It is also feasible that such referred induction of iNOS in the colon may possibly contribute to the potential for chronic NSAID administration to activate quiescent colitis (Kaufmann and Taubin, 1987).

The loss of intestinal barrier function precedes the development of multiple organ failure in patients (Nieuwenhuijzen et al., 1996), while urinary nitrate levels have been considered as a marker of bacterial translocation in the gut (Oudenhoven et al., 1994). Thus, multiple organ failure may be mediated by widespread iNOS expression following bacterial translocation across an impaired mucosal barrier. The circulatory failure and liver dysfunction in a rat endotoxin model of multiple organ dysfunction has been shown to be attenuated by the iNOS inhibitors, aminoethyl-isothiourea or 1-amino-2-hydroxy-guanine (Thiemermann et al., 1995; Ruetten et al., 1996). The microvascular injury and gut barrier dysfunction and subsequent systemic inflammatory response induced by lipopolysaccharide challenge can also be prevented by the iNOS inhibitors, *S*-methylisothiourea and aminoguanidine (Unno et al., 1997; Kavulku et al., 1998), as well as by the potent and highly selective iNOS inhibitor, 1400W (Garvey et al., 1997; Matejovic et al., 2001). The current study has not addressed the pathological processes that promote the initial mucosal barrier dysfunction allowing subsequent bacterial translocation in the gut, but these are likely to involve the early inhibition of cyclooxygenase isoforms.

The present study has thus shown that administration of indomethacin to rats for only 2 days is sufficient to initiate a widespread systemic inflammatory microvascular response of gut origin. The provocation of microvascular leakage as a consequence of the expression of iNOS in organs remote from the initial site of injury in the jejunum is likely to be dependent on bacteria, bacterial endotoxins or lipopolysaccharide entering the systemic circulation from the impaired gut mucosa. Because a single dose of indomethacin gives rise to only local response in the jejunum, it is concluded that this widespread effect following multiple doses of indomethacin reflects the more extensive injury in the gut barrier, promoting bacterial translocation. The response may also involve the production and release into the circulation of pro-inflammatory and stimulatory mediators such as cytokines by the rat-inflamed jejunum (Bertrand et al., 1998), a species in which iNOS is readily expressed. Further studies will establish if this model of systemic inflammatory response syndrome involving indigenous bacterial products will be useful in the evaluation of novel pharmacological interventions.

Acknowledgements

This work was supported in part by a grant from the William Harvey Research Foundation, London.

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