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Short communication

# Site-specific lesion formation, inflammation and inducible nitric oxide synthase expression by indomethacin in the rat intestine

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**Abstract**

The involvement of nitric oxide (NO) formed by the inducible isoform of NO synthase (iNOS) has been investigated in the development of rat intestinal lesions following indomethacin administration. Over a 72-h period, indomethacin (10 mg $kg^{-1}$, s.c.) provoked a time-dependent increase in expression of iNOS (assessed by the conversion of radiolabelled L-arginine to citrulline) and enhancement of vascular leakage of radiolabelled human serum albumin in the jejunum which commenced 18 h after indomethacin. Similar effects were not observed in the ileum, colon or caecum. In addition, macroscopic lesions were detectable and myeloperoxidase activity (an index of neutrophil recruitment) were increased in the rat jejunum 18–24 h after indomethacin, but remained at basal levels in the ileum and colon. These findings suggest that indomethacin provokes a site-selective expression of iNOS in the rat jejunum which correlates with lesion formation and vascular leakage, whereas both the ileum and colon are spared. 

*Keywords:* Nitric oxide (NO); Inflammation, intestinal; Non-steroid anti-inflammatory drug

## 1. Introduction

The capacity of non-steroid anti-inflammatory drugs to provoke intestinal inflammation and mucosal erosion has been well established (Kent et al., 1969; Fang et al., 1977). The pathology of this intestinal injury in animal models is similar to that found in humans (Bjarnason et al., 1987; Davies et al., 1994). Anti-inflammatory doses of indomethacin in rats are well-known to exert ulcerogenic effects on the rat jejunum (Somogyi et al., 1969) due, in part, to enterohepatic circulation and subsequent co-secretion with bile which elevates the concentrations of the drug at the epithelium of the small intestine (Brodie et al., 1970). The factors involved in the damage by non-steroid anti-inflammatory drugs that have been proposed, include the inhibition of protective prostaglandins (Robert and Asano, 1977; Whittle, 1981), bacteria and bacterial products (Kent et al., 1969; Robert and Asano 1977) or microcirculatory disturbances associated with indomethacin treatment (Miura et al., 1991).

In a number of studies, a correlation has been demonstrated between inducible nitric oxide synthase (iNOS) expression and tissue injury, with inhibition of iNOS decreasing the extent of cell damage (for review, see Whittle, 1997). In a previous study, the expression of iNOS activity following indomethacin administration was demonstrated to be related in a time-dependent manner to the initiation of jejunal microvascular injury, determined by albumin leakage (Whittle et al., 1995). Moreover, the indomethacin-induced vascular leakage was reduced by the administration of NO synthase inhibitors at the time of iNOS expression (Whittle et al., 1995).

In the present study, the site-specific induction of iNOS and macroscopic injury by indomethacin has been investigated in the small and large intestine. As an index of microvascular permeability changes that accompany the intestinal lesions, the leakage of radiolabelled albumin was measured at various times following indomethacin administration, and correlated with the induction of iNOS. Myeloperoxidase (MPO) activity was quantified as a marker of neutrophil recruitment into the intestine.

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## 2. Methods

### 2.1. Macroscopic assessment of non-steroid anti-inflammatory drug-induced jejunal damage

Male Wistar rats (200–250 g) received food and water ad libitum during the course of the experiments. Indomethacin (10 mg $kg^{-1}$), a dose chosen from previous studies (Whittle et al., 1995), dissolved in $NaHCO_3$ (5% w/v) and volume adjusted with saline, was administered subcutaneously.

Photographs of the jejunal mucosa were blindly scored on a scale 1–5 where score 0 = no damage to tissue; 1 = 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 abdominal viscera due to perforating lesions.

### 2.2. NO synthase activity

NO synthase activity was determined as the conversion of L-[$^{14}$C]arginine monohydrochloride to L-[$^{14}$C]citrulline (Boughton-Smith et al., 1993; Tepperman et al., 1993). Tissues were homogenised (15 s) in buffer (250 mg $ml^{-1}$, 4°C, 10 mM HEPES, 32 mM sucrose, 1 mM DTT, 0.1 mM EDTA, 10 μg $ml^{-1}$ soybean trypsin inhibitor, 10 μg $ml^{-1}$ of leupeptin, and 2 μg $ml^{-1}$ of aprotonin, pH 7.4) followed by centrifugation for 20 min at 10,000 × *g* at 4°C. Samples were mixed with Dowex (AG 50W-8; 200–400, 8% cross-linked, $Na^+$ form) 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 reaction buffer comprising (final concentrations) 50 mM $KH_2PO_4$, 1 mM $MgCI_2$, 0.2 mM $CaCI_2$, 50 mM valine, 1 mM DTT, 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. The reaction was arrested by the addition (0.5 ml) of a 1:1 v/v suspension of Dowex:water. After addition of 0.85 ml distilled water and allowing to settle for 30 min, the supernatant was removed for scintillation counting. Protein content was estimated via spectrophotometric assay (Bio-Rad Protein Assay), and iNOS activity was expressed as pmol $min^{-1}$ $mg^{-1}$ protein.

NOS activity was defined as citrulline formation that was abolished by incubation in vitro with $N^G$-monomethyl-L-arginine (L-NMMA, 300 μM). Basal L-NMMA-sensitive activity, that was abolished by EGTA, was taken as calcium-dependent constitutive NO synthase activity, while that not inhibited by EGTA incubation was taken as calcium-independent iNOS activity.

### 2.3. Measurement of MPO activity

MPO activity was measured using a method modified from Bradley et al. (1982). Tissues were weighed and homogenised (30 s) in 0.5% hexadecyltrimethyl-ammonium bromide (HTAB) in 50 mM citrate buffer (pH 4.5). The suspensions (200 mg $ml^{-1}$ w/v) were frozen (−20°C) and thawed (37°C) three times, before centrifugation (10,000 × *g*; 15 min; 4°C). Supernatant (100 μI) was mixed with 2.9 ml of 50 mM citrate buffer containing 0.167 mg $ml^{-1}$ of *O*-dianisidine dihydrochloride and 0.1% hydrogen peroxide. Absorbance was measured immediately (Beckman spec model 25; 460 nm; 1 min). One unit of MPO activity was defined as that degrading one μM of peroxidase per min at 37°C (Bradley et al., 1982).

### 2.4. Albumin leakage

Under transient halothane anaesthesia, [$^{125}$I]labelled human serum albumin (2 μCi $kg^{-1}$) was injected via a tail vein, 2 h before autopsy. The leakage of radiolabelled albumin was subsequently measured in segments of jejunal (3 cm; removed 10–15 cm from the pyloric sphincter), ileal (3 cm terminal region) colonic (3 cm; removed 2–3 cm from the anus) and caecal (3 cm) tissues taken from rats terminally anaesthetised with halothane as described previously (Whittle et al., 1995). Blood was collected from the abdominal aorta into syringes, containing trisodium citrate (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 intestinal tissues was determined in a gamma spectrometer (Nuclear Enterprises NE1600). 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). Intravascular volume was determined in a separate group of rats by the administration of [$^{125}$I]human serum albumin via the tail vein at each time point, 2 min prior to tissue removal.

### 2.5. Materials

[$^{125}$I]labelled human serum albumin and L-[$^{14}$C]arginine monohydrochloride were obtained from Amersham International (UK). All other compounds were from the Sigma (Poole, Dorset, UK)

### 2.6. 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. Intestinal mucosal lesion formation

Indomethacin (10 mg $kg^{-1}$) given as a single subcutaneous dose, produced a progressive inflammation of the proximal jejunum. The appearance of palpable white nodules at 15–18 h was accompanied by small haemorrhagic points along the mesenteric side of the jejunum at 18 h, which developed to mucosal erosion and haemorrhage at 24 h. These mucosal erosions increased in severity, resulting in perforated lesions and extensive intra-intestinal adhesions in 30% of rats by 48 h, and in all rats by 72 h. In contrast, the ileum, colon and caecum showed no detectable changes in macroscopic appearance.

The jejunal tissue damage scores at 6 and 12 h following indomethacin were not significantly different from untreated jejunum ($n = 4$). However, from 18 h onwards, the tissue damage score increased in a time-dependent manner, reaching maximal damage in all rats at 72 h (Fig. 1a).

### 3.2. Effects on intestinal NO synthase activity

No iNOS activity that could be inhibited by L-NMMA but not by EGTA (1 mM), was detected in resting jejunum. Following the administration of indomethacin (10 mg $kg^{-1}$, s.c.), no change in jejunal iNOS activity was seen in samples taken at 3–15 h ($n = 8$) as shown in Fig. 1b. From 18 h onwards, an iNOS activity was detected and continued to increase until the final measurement taken at 72 h ($98 \pm 11$ pmol $min^{-1}$ $mg^{-1}$ tissue; $n = 10$; $P < 0.001$), as shown in Fig. 1b. However, no significant increases in iNOS activity were detected above basal activity after 72 h in either ileum, colon or caecum ($11 \pm 4$, $2 \pm 2$ or $12 \pm 7$ pmol $min^{-1}$ $mg^{-1}$ protein, respectively; $n = 8$).

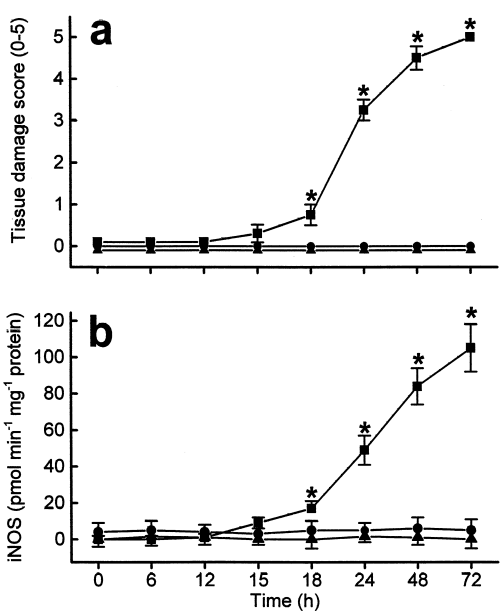


Fig. 1. Time-dependent actions of indomethacin (10 mg $kg^{-1}$, s.c.) on intestinal macroscopic tissue damage (score 0–5; in figure a) and on expression of the inducible nitric oxide synthase (iNOS, expressed as pmol $min^{-1}$ $mg^{-1}$ protein; in figure b) in the jejunum (square), ileum (circle) and colon (triangle) of the rat over 72 h. Data are expressed as mean ± S.E.M., where $n = 4$–8 in each group. Statistical significance is shown as $^{*}P < 0.05$ compared to the untreated control (0 h) group.

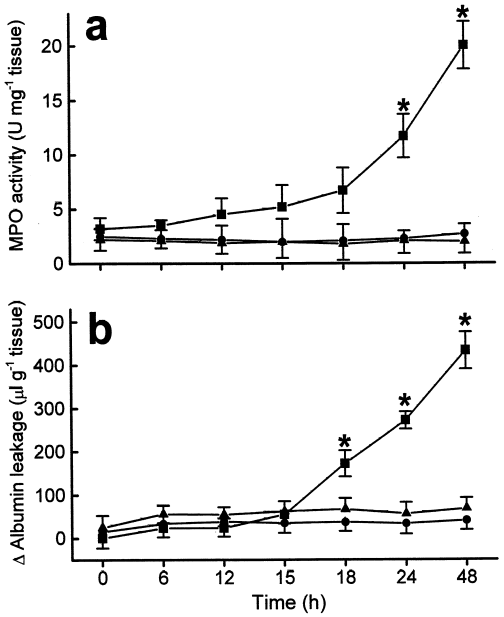


Fig. 2. Time-dependent actions of indomethacin (10 mg $kg^{-1}$, s.c.) on intestinal myeloperoxidase activity (MPO, U $mg^{-1}$ tissue; in figure a) and on vascular leakage of radiolabelled albumin (expressed as Δ albumin leakage, μl $g^{-1}$ tissue; in figure b) in the jejunum (square), ileum (circle) and colon (triangle) of the rat over 48 h. Data are expressed as mean ± S.E.M., where $n = 8$ in each group. Statistical significance is shown as $^{*}P < 0.05$ compared to the untreated control (0 h) group.

### 3.3. Effects on intestinal MPO activity

Basal MPO activity, was detected in rat jejunal homogenates ($3.2 \pm 0.5$ U $mg^{-1}$ tissue; $n = 8$) as demonstrated in Fig. 2a. Following a single dose of indomethacin (10 mg $kg^{-1}$, s.c.), there was a time-dependent increase in MPO activity, reaching significant levels after 24 h, and reaching near-maximal levels after 48 h, which were maintained up to 72 h ($20 \pm 1$ U $mg^{-1}$ tissue; $n = 8$, $P < 0.001$) as shown in Fig. 2a. Basal MPO activity, was also detected in ileal and colonic homogenates ($3 \pm 1$ U $mg^{-1}$ tissue and $3 \pm 1$ U $mg^{-1}$ tissue, respectively). However, throughout the 72-h time-course of jejunal injury after indomethacin administration, there was no significant change in ileal and colonic MPO activity (Fig. 2a).

### 3.4. Effects on intestinal plasma leakage

From 18 h following indomethacin (10 mg $kg^{-1}$, s.c.), there was a significant time-dependent increase in jejunal

plasma extravasation of [$^{125}$I]human serum albumin (Fig. 2b). However, over the 48 h period following indomethacin administration, no significant changes in plasma leakage were observed in the ileum and colon (Fig. 2b), nor in the caecum ($\Delta 3 \pm 24$ μl $g^{-1}$ tissue; $n = 6$).

## 4. Discussion

In the present study, indomethacin induced the time-dependent formation of macroscopically apparent lesions in the upper jejunum of rats, as reported by others (Kent et al., 1969; Somogyi et al., 1969; Thomas et al., 1969). However, while jejunal injury appeared 18 h following a single indomethacin administration, no macroscopic tissue damage was found in either the ileum or colon over the 72 h time-course. The onset and time-dependent increase in macroscopic tissue damage, observed in the jejunum, correlated closely with the onset of the microvascular leakage of radiolabelled albumin, as well as, the expression of iNOS activity. The lack of increased microvascular leakage and expression of an iNOS activity in the rat ileum, colon and caecum following indomethacin, accords with the lack of macroscopic tissue damage in these tissues.

In our present study, a basal MPO activity was found in the jejunum, ileum and colon, which increased in the jejunum 24–72 h following subcutaneous indomethacin administration, but remained unchanged in the ileum and colon over the 72-h period. Yamada et al. (1993), showed increased MPO activity in the distal jejunum and proximal ileum 24 h after subcutaneous indomethacin administration, but also found cases of mucosal permeability with no elevation of MPO activity. While neutrophils are known to participate in non-steroid anti-inflammatory drug-induced gastropathy (Wallace et al., 1991), it is uncertain whether they participate in the small intestinal injury induced by non-steroid anti-inflammatory drugs (Miura et al., 1991; Nygard et al., 1995). Thus, depletion of circulating neutrophils to less than 7% by use of an antisera significantly attenuated indomethacin-induced increases in MPO activity. However, it did not affect intestinal mucosal injury, suggesting that neutrophils do not mediate the acute mucosal injury of non-steroid anti-inflammatory drugs, their recruitment being a consequence of the lesion formation (Yamada et al., 1993). Antibiotics have been found to prevent the development of this enteropathy (Yamada et al., 1993; Whittle et al., 1995), which suggests the possible stimulus of neutrophil infiltration may be gut bacterial flora or their toxins.

The expression of iNOS in the model of non-steroid anti-inflammatory drug-induced enteropathy was observed in the jejunum, but not in the ileum, colon or caecum of the rat. This jejunal expression of iNOS activity became significant at 18 h after indomethacin administration, increasing in a time-dependent manner over the 72-h period. The timing of expression of this iNOS activity correlates with the onset of macroscopic jejunal damage and microvascular leakage.

The results of the present study suggest that indomethacin provokes lesion formation in the rat jejunum while not affecting lower intestinal tissues such as the ileum, colon or caecum. The susceptibility of the jejunum to indomethacin may reflect the enterohepatic circulation which the majority of non-steroid anti-inflammatory drugs undergo (Brodie et al., 1970; Yesiar et al., 1970). Following indomethacin administration, the upper intestinal lumen concentrations of indomethacin and its metabolites have been shown to be extremely high in comparison with the caecum, colon and even plasma levels in rats (Nygard et al., 1995). These jejunal concentrations of indomethacin may inhibit cyclo-oxygenase with possible impairment of the mucosal barrier function (Whittle, 1981). The loss of the epithelial barrier will increase intestinal permeability, allowing tissues to be exposed to the gut luminal contents including antigens and microorganisms (Kent et al., 1969; Satoh et al., 1983; Bjarnason et al., 1991; Zuccato et al., 1992; Whittle et al., 1995). Luminal bacteria, known to be involved in indomethacin-induced enteropathy (Kent et al., 1969; Robert and Asano, 1977; Whittle et al., 1995), may provoke the local release of mediators, such as cytokines, and may also prime or activate inflammatory cells, epithelial cells and vascular cells (Whittle, 1997). Moreover, these bacteria can locally produce endotoxin lipopolysaccharide, which is a potent stimulus for iNOS induction in intestinal tissue (Boughton-Smith et al., 1993). This time-dependent onset and site-selectivity of tissue injury correlates closely with iNOS expression and the increase in microvascular leakage, which is subsequently followed by the increase in MPO activity indicating the infiltration of neutrophils as a response to infection. Such site-selectivity gives further support to the intimate involvement of iNOS expression to the processes underlying enteropathy provoked by non-steroid anti-inflammatory drugs.

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