Experimental colitis is ameliorated by inhibition of nitric oxide synthase activity

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Abstract
Enhanced nitric oxide (NO) generation by stimulated NO synthase (NOS) activity may, through its oxidative metabolism contribute to tissue injury in experimental colitis. In this study the possible amelioration of experimental colitis by NG-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NOS activity, was evaluated. Colitis was induced in rats by intracolonic administration of 30 mg trinitrobenzene sulphonic acid (TNB) dissolved in 0.25 ml 50% ethanol or by flushing the colon of capsaicin pretreated rats with 2 ml of 5% acetic acid. In several experiments, L-NAME 0.1 mg/ml was added to the drinking water at the time of colitis induction with TNB or seven days before acetic acid treatment. Rats were killed at various time intervals after induction of colitis. A 10 cm distal colonic segment was isolated, weighed, lesion area measured, and explants organ cultured for 24 hours for determination of NO generation by the Greiss reaction. The rest of the mucosa was scraped for determination of myeloperoxidase and NOS activities and leukotriene generation. In TNB treated rats mean arterial pressure was also determined up to 72 hours after damage induction, with or without cotreatment with nitroprusside. L-NAME significantly decreased the extent of tissue injury in TNB treated rats. Seven days after TNB treatment lesion area was reduced by 55%, colonic weight by 37%, and myeloperoxidase and NOS activity by 59% and 42%, respectively. Acetic acid induced colitis in capsaicin pretreated rats was also significantly decreased by L-NAME. Twenty four hours after acetic acid treatment lesion area was reduced by 61%, colonic weight by 21%, and NOS activity by 39%. Mean (SEM) arterial blood pressure in TNB + L-NAME treated rats was 37.6 (8.1) mm Hg higher than in TNB treated rats, an effect that was only partially abolished by nitroprusside. These results show that inhibition of NO synthesis by an L-arginine analogue significantly ameliorates the extent of tissue injury in two models of experimental colitis, an effect that is not due only to its vasoconstrictor properties. Modulation of NO generation may be a novel therapeutic approach in inflammatory bowel disease.

Keywords: nitric oxide, acetic acid colitis, trinitrobenzene sulphonic acid colitis.

Nitric oxide (NO) has been implicated in diverse biological functions, mediation of macrophage function being one of them. Basal NO synthase (NOS) activity in macrophages is negligible but, once stimulated by bacterial products or cytokines, NO generation is significantly increased. NO generation by activated macrophages has an important role in the defence against infectious agents as well as tumour cells. When actively produced in large amounts, however, NO may induce tissue injury due to its combination with superoxide anion yielding peroxynitrite. We have shown in rats that intracolonic administration of peroxynitrite induces significant colonic injury.

Recently, in patients with inflammatory bowel disease, colonic NO generation and NOS activity were reported to be stimulated. Moreover, stimulated colonic NOS activity was reported in the rat model of trinitrobenzene sulphonic acid (TNB) induced colitis and NO generation was found to be increased in TNB induced ileitis in guinea pigs. It is thus suggested that increased NO generation by stimulated NOS activity may contribute to the pathogenesis of experimental colitis and inflammatory bowel disease. Inhibition of NOS activity may, therefore, ameliorate the extent of tissue inflammation and may be used as a novel therapeutic modality in inflammatory bowel disease patients. Analogue of L-arginine such as NG-nitro-L-arginine methyl ester (L-NAME) and NG-monomethyl-L-arginine (L-NMMA) are potent inhibitors of NOS activity and L-NAME was shown to ameliorate the extent of TNB induced ileitis on guinea pigs. The mechanism, however, whereby L-NAME induces its effect and, especially, its potent vasoconstricting effects in this setting was not established.

The aim of this study was to further evaluate the possible contribution of NO to the pathogenesis of several models of experimental colitis and to assess their possible amelioration through potent inhibition of NOS activity.

Methods

Materials
L-arginine, L-citrulline, N\textsuperscript{\wedge}G-nitro-L-arginine (NNA), L-NAME, L-NMMA, NADPH, calcium ionophore, lipopolysaccharide, dithiothreitol, phenylmethylsulphonyl fluoride, EDTA, and EGTA were purchased from Sigma Chemical, St Louis, MO, USA; Dowex AG50W-X8 (Na form) 100–200 mesh, and TRIS base (electrophoresis grade) were purchased from Bio-Rad Labs, Richmond,
CA, USA; sodium nitrate and sodium nitrite were obtained from Fisher Chemical, Fair Lawn, NJ, USA; opti-fluor was purchased from Packard, the Netherlands; sulphamamide and N-1-naphthylethylene diamine hydrochloride were purchased from Aldrich Chemical, Milwaukee, MI, USA; TNB was purchased from Eastman Kodak, Rochester, NY, USA; rat recombinant interferon γ (IFN γ) was purchased from Gibco BRL, Gaithersburg, MD, USA; nitroprusside was purchased from Hoffman-La Roche, Basle, Switzerland; 3H-citrulline, leukotriene B4 (LTB4), leukotriene C4 (LTC4), radioimmunoassay, were purchased from Amersham, Bucks, UK.

Animals
Male, Sprague-Dawley rats, weighing 200–250 g and fed ad libitum were used in all the studies. All the animal studies described adhere to the standards established by the Guide for The Care and Use of Laboratory Animals.8

TNB induced colitis
Inflammation of the colon was induced under light ether anaesthesia by a single intracolonic administration of 0.25 ml of 50% ethanol containing 30 mg of TNB, as previously described.10 The solution was introduced through a catheter with a 0.3 mm outer diameter placed 7 cm from the anus. Control rats were treated with the vehicle. In several experiments L-NAME (0-1 mg/ml) was added to the drinking water immediately after TNB or vehicle administration. Daily water consumption was monitored per cage with five rats uniformly treated. Drinking behaviour was similar in control and L-NAME treated rats. Estimated daily L-NAME consumption was 30 mg/kg. This oral route of L-NAME administration was previously shown to induce systemic inhibition of NOS activity.11 Rats were killed one, three, and seven days after the induction of colonic injury. The colon was isolated, placed unstretched on a ruler, and a 10 cm segment of distal colon was resected, its lumen rinsed with ice cold saline and weighed. Tissue samples from the most damaged site were obtained for histological assessment and several sections along the 10 cm segment were taken for organ culture. In different rats the mucosa was scraped and samples were processed for determination of lipoxigenase products, myeloperoxidase, and NOS activities.

Determination of mean arterial pressure
In several experiments rats were anaesthetised with ether and a PE-50 cannula was inserted through the femoral artery into the abdominal aorta and exteriorised subcutaneously at the interscapular area. Cannulas were filled with heparinised saline solution (100 µU/ml) and rats were housed individually. Mean arterial pressure was recorded 24 hours after recovery by use of P23 Statham-Gould transducers (Gould, Oxnard, CA, USA) and recorded on Gould physiograph (model 13-34615-52) after 10–20 minutes of equilibration while rats were moving freely in their cages. Mean arterial pressure was measured before, 24, 48, and 72 hours after TNB treatment and during L-NAME administration to the drinking water. In another group of rats after the basal mean arterial pressure measurement, under ether anaesthesia, an osmotic minipump (Alzet, Palo Alto, CA, USA) was implanted intraperitoneally delivering nitroprusside, 0.42 µg/min.

Acetic acid induced colitis
Acetic acid colitis was induced in control rats and also two weeks after completion of capsaicin treatment in which rats were treated subcutaneously with capsaicin in increasing doses (20, 30, and 50 mg/kg) on three consecutive days in a regimen shown to deplete neuropeptides in primary afferent neurons.12 In several experiments L-NAME 0-1 mg/ml was added to the drinking water seven days before, or at the time of colitis induction. Under light ether anaesthesia a midline abdominal incision was made and the junction of the caecum and ascending colon identified. Two ml of 5% acetic acid were injected into the lumen of the colon at its proximal part through a 25 gauge needle, followed by 3 ml of air, which cleared most of the acetic acid from the colon. The midline incision was closed. Twenty four hours later the rats were killed and their colons removed and handled, as with the TNB model.

Determination of mucosal damage
Mucosal damage was measured macroscopically and expressed in mm²/rat. All measurements of damage were performed by two blinded observers using a stereomicroscope. The interobserver variability between the two observers was 7%.

Organ culture
Colonic explants were kept in NaCl 0.15 M at 4°C and within 15 minutes after excision were cultured as previously described.13 In brief, the tissue was weighed, oriented on metal grids, and organ cultured for 24 hours at 37°C, 95% O2, 5% CO2 in AIM-V medium (Gibco) containing penicillin and gentamicin.

Measurement of NO production
NO, quantified by the accumulation of oxides of nitrogen (NOx) in the culture medium, was measured spectrophotometrically using the Greiss reaction,14 with sodium nitrite dissolved in the organ culture medium as a standard. Each determination was controlled to subtract any possible interference of the medium. Briefly, 50 µl of culture supernatants were mixed with equal volume of 1% sulphamamide in 0.5 N HCl. After five minutes, 50 µl of 0-02% N-1-naphthylethylene diamine
Determination of NOS activity

NOS activity was monitored by the conversion of [3H]-L-arginine to citrulline according to Bush et al. Mucosal scrapings (100 mg) were homogenised for 30 seconds at 4°C with a polytron (Kinematica GmbH, Kriens-Luzern, Switzerland) in 0.3 ml of ice cold 50 mM TRIS HCl, pH 7.4 containing 0.1 mM EDTA, 0.1 mM EGTA, 0.5 mM dithiothreitol, 1 mM phenylmethylsulphonyl fluoride. Homogenates were centrifuged at 20,000 g for 60 minutes at 4°C and the supernatant was used as the source of NOS. Enzymatic reactions were conducted at 37°C in 50 mM TRIS HCl, pH 7.4 containing 100 µM L-arginine, 100 µM NADPH, 2 mM CaCl2, 0.2-0.4 mg supernatant proteins, other test agents as indicated, and approximately 200,000 dpm of L-[2,3,4,5-3H]-arginine HCl (77 Ci/mmol; Amersham, UK) to a final volume of 100 µl. Enzymatic reactions were terminated by addition of 2.0 ml of ice cold stop buffer: twenty mM sodium acetate, pH 5.5, 1 mM L-citrulline, 2 mM EDTA, and 0.2 mM EGTA. Citrulline was determined by applying the samples (2.0 ml) prepared as described above, to columns (1 cm diameter) containing 1 ml of Dowex AG50W[X8, Na form, which had been pre-equilibrated with stop buffer. Columns were eluted with 4X1.0 ml of water collected into scintillation vials. Opti-fluor (10 ml) was added to each vial and samples were counted in a Packard Tri-Carb liquid scintillation spectrometer. Citrulline was recovered in the first 4.0 ml of the Dowex column eluate to the extent of 96 (2%). Inter assay variation is 1.0 (1-9)% (mean (SEM); n=55).

Determination of myeloperoxidase activity

Two hundred mg of colonic mucosal scrapings were homogenised three times for 30 seconds at 4°C with a polytron (Kinematica GmbH, Kriens-Luzern, Switzerland) in 1.0 ml of ice cold 0.5% hexadecyltrimethylammonium bromide in 50 mM phosphate buffer, pH 6.0. The polytron probe was rinsed twice with 1.0 ml of the buffer and the washings were added to the homogenate. The homogenate was then sonicated for 10 seconds, freeze thawed three times, and centrifuged for 15 minutes at 40,000 g. An aliquot of the supernatant was taken for determination of the enzyme activity, according to Bradley et al. The correlation coefficient based on 10 standard curves is r=0.98. Inter assay variation is 0 (1-65)% (mean (SEM); n=57).

Determination of lipooxygenase products

One hundred and fifty mg of mucosa were placed in preweighed tubes containing 1.0 ml of phosphate buffer (50 mM, pH 7.4). The mucosa was minced with scissors and centrifuged in an Eppendorf centrifuge for 10 seconds. The pellet was resuspended in 1.0 ml of the above buffer, incubated for one minute in a vortex mixer, indomethacin added, and the tubes centrifuged for 60 seconds. The supernatants were kept at -20°C until radioimmunoassays were performed. The capability of the mucosa to generate LTC4 and LTB4 was expressed as ng/g wet tissue weight.

Measurement of LTB4

LTB4 immunoactivity was determined by a radioimmunoassay kit (Amersham, TRK 940). The assay combines the use of a high specific activity LTB4 tracer, an antiserum specific for LTB4 (cross reactivity 100%), and a leukotriene standard (range 1-6 to 200 pg/tube). The specific binding of tracer is 42-55%, non-specific binding 2-4%. Fifty per cent B/Bo displacement is obtained with 15 pg/tube and 90% B/Bo displacement with 2-2 pg/tube of LTB4. The percentage coefficient of variation (CV) for within assay precision ranges from 8-2 (low) to 8-6 (high). The percentage CV for the precision profile of the assay ranges from 2.3 to 6.35 (n=10). The percentage B/Bo for between assay reproducibility ranges from 92 to 5-9 (standard 1-5-200 pg/tube; n=10).

Measurement of LTC4

LTC4 immunoactivity was determined by radioimmunoassay. The assay combines the use of a high specific activity LTC4 tritiated tracer with a monoclonal antibody specific for LTC4 and LTC4 standard (8-500 pg/tube). The assay uses highly specific LTC4 antiserum (cross reactivity 100%) and has low cross reactivity with leukotriene D4 (LTD4) (<5%). The specific binding of tracer is 40-45% and non-specific binding is 1%-3%. Fifty per cent B/Bo displacement is obtained with 34 pg/tube, and 80% B/Bo displacement with 9-5 pg/tube of LTC4. The percentage CV for within assay precision ranges from 7-68 (low) to 3-94 (high). The percentage CV for the precision profile of the assay ranges from 1-64 to 3.60. The percentage B/Bo for between assay reproducibility ranges from 87 to 5-4 (standard 8-800 pg/tube; n=9).

Morphological studies

Sections of colon were obtained from the same areas of the large intestine during necropsy. They were fixed in phosphate buffered formaldehyde, embedded in paraffin wax, and routine 5 µm sections were prepared. Tissues were routinely stained with haematoxylin and eosin and were evaluated by light microscopy by a pathologist unaware of the experiments being performed. As the histological features of the TNB10 and acetic acid17 induced colitis were previously reported by us, only the histology of the new acetic acid augmented colitis in capsaicin pretreated rats is reported in detail.

Statistical analysis

Data are expressed as mean (SEM). Statistical
TABLE I Effect of L-NAME on colonic lesions and weight in TNB induced colitis

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TNB</th>
<th>TNB+L-NAME</th>
<th>None</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (days)</td>
<td>1 3 7</td>
<td>1 3 7</td>
<td>1 3 7</td>
</tr>
<tr>
<td>Lesions (mm²/rat)</td>
<td>7 14 12</td>
<td>7 14 12</td>
<td>5 9 21</td>
</tr>
<tr>
<td>Median</td>
<td>780 698 710</td>
<td>576 592 98</td>
<td>0</td>
</tr>
<tr>
<td>Range</td>
<td>276–1840 300–950</td>
<td>300–1600 78–1235</td>
<td>0–720 0–966</td>
</tr>
<tr>
<td>Weight (g/10 cm)</td>
<td>8 14 18</td>
<td>5 18 21</td>
<td>8 5 9</td>
</tr>
<tr>
<td>Number</td>
<td>19* 12* 18*</td>
<td>12* 14* 18*</td>
<td>8 18 21</td>
</tr>
<tr>
<td>Mean (SEM)</td>
<td>1.90 (0.5)* 1.28 (0.9)* 1.38 (0.4)*</td>
<td>1.28 (0.5)* 1.28 (0.5)* 1.38 (0.4)*</td>
<td>1.28 (0.5)* 1.28 (0.5)* 1.38 (0.4)*</td>
</tr>
<tr>
<td>Median</td>
<td>1.90 1.28 1.38</td>
<td>1.28 1.28 1.38</td>
<td>1.28 1.28 1.38</td>
</tr>
<tr>
<td>Range</td>
<td>1.24–2.33 1.38–2.84 1.56–3.53</td>
<td>0.93–1.56 0.89–1.82 1.19–2.67</td>
<td>0.48–0.78 0.48–0.78 0.48–0.78</td>
</tr>
</tbody>
</table>

Lesion area and weight of the 10 cm distal colonic segment of rats treated with TNB with or without addition of L-NAME to the drinking water were determined as described in Methods. *Significantly different from TNB (p<0.05).

Results

Effect of L-NAME on TNB induced colitis

Intracolonic administration of TNB/ethanol resulted in extended haemorrhagic and ulcerative damage to the distal colon, as we reported previously.10 By 24 hours the damage was localised with a lesion area of 913 (117) mm² (n=17; mean (SEM)). Three and seven days after damage induction the lesion area was smaller but not significantly different from that seen after 24 hours (Table I). The addition of L-NAME to the drinking water induced a significant decrease in the lesion area, but this was noted only three and seven days after damage induction (Table I). The amelioration of the severity of the inflammatory response in L-NAME treated rats was also reflected by the significant difference in the wet weight of the 10 cm long distal colonic segment in L-NAME and control rats treated with TNB/ethanol (Table I). At all time intervals colonic myeloperoxidase activity in TNB treated rats was several fold higher than its activity in control rats. In L-NAME+TNB treated rats, myeloperoxidase activity three and seven days after TNB treatment was 40% of its activity in rats treated only with TNB (Fig 1). In nitroprusside treated rats mucosal myeloperoxidase activity was 0–5 (0–4) U/g, similar to its activity in control rats. In TNB treated rats, one and three days after its administration, mucosal LTC₄ generation was significantly increased when compared with its generation in control rats. Mucosal LTC₄ generation was significantly increased three and seven days after damage induction. In TNB+L-NAME treated rats mucosal leukotriene generation was lower than in TNB treated rats, but the difference was not statistically significant (Table II).

The accumulation of NO₃ in the medium of cultured colonic segments isolated from control rats increased with time: 14·5 (5·7) (n=6), 39·2 (14·0) (n=4), and 258·0 (27·0) (n=22) µmol/g wet weight after four, eight, and 24 hours, respectively. The small standard errors of colonic NO₃ generation during 24 hour culture of six explants obtained from the same normal rat 196 (25) µmol/g (mean (SEM)) show that explant sampling was reproducible. NO₃ generation by inflamed colonic segments isolated 24 hours after TNB treatment was 11-fold higher than by explants isolated from control rats: 2236 (320) (n=12) and 204 (36) (n=19) µmol/g/24 h, respectively.

One, three, and seven days after TNB treatment colonic NOS activity was 4·5, 22·4, and 18-fold higher, respectively, than in control rats. In TNB+L-NAME treated rats, three and seven days after treatment, NOS activity was significantly lower than in TNB treated rats (Fig 2).

Effect of L-NAME on mean arterial pressure

Figure 3 shows mean arterial pressure measurements in the various treatment groups.

TABLE II Effect of L-NAME on colonic leukotriene generation in TNB induced colitis

<table>
<thead>
<tr>
<th>Treatment</th>
<th>None</th>
<th>TNB</th>
<th>TNB+L-NAME</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (days)</td>
<td>1 3 7</td>
<td>1 3 7</td>
<td>1 3 7</td>
</tr>
<tr>
<td>LTB₄ (ng/g)</td>
<td>5.7 (0.4)  6.6 (1.0)*  3.4 (0.5)*</td>
<td>5.2 (1.7)  3.8 (0.8)  3.1 (0.5)  2.7 (0.3)</td>
<td></td>
</tr>
<tr>
<td>LTC₄ (ng/g)</td>
<td>2.4 (0.1)  3.1 (0.8)  5.1 (0.9)*  4.0 (0.4)*</td>
<td>2.0 (0.4)  4.0 (0.8)  3.7 (0.6)</td>
<td></td>
</tr>
</tbody>
</table>

Mucosal LTB₄ and LTC₄ generation in the 10 cm distal colonic segment of rats treated with TNB with or without addition of L-NAME to the drinking water was determined as described in Methods. Results are mean (SEM). *Significantly different from no treatment (p<0.05).

Figure 1: Effect of L-NAME on colonic myeloperoxidase activity in TNB treated rats. Colonic myeloperoxidase activity was determined one, three, and seven days after colonic TNB administration to control and L-NAME treated rats, as described in Methods. Results are mean (SEM). *Significantly different from control (p<0.05). †Significantly different from TNB treated rats (p<0.05).
Experimental colitis is ameliorated by inhibition of nitric oxide synthase activity

L-NAME induced in TNB treated rats a significant increase in mean arterial pressure of 37.6 (8.1) mm Hg, compared with mean arterial pressure in rats treated only with TNB (p<0.05). Two way analysis of variance indicated differences among mean arterial pressure curves shown in Fig 3, \( F(4;16)=18.8 \), p<0.005. Post hoc analysis showed that the highest curve was in the TNB+L-NAME, specifically greater than that in the TNB+L-NAME+nitroprusside group (p<0.02), showing that nitroprusside attenuated the L-NAME induced hypertension as the TNB and TNB+L-NAME+nitroprusside curves did not differ. The TNB+nitroprusside curve was significantly lower than that in TNB alone (p<0.008), however, pointing to the hypotensive efficacy of nitroprusside. Nitroprusside treatment did not induce any injury but abolished the protective effect of L-NAME on the extent of tissue injury induced by TNB. L-NAME, by itself, did not induce any macroscopic injury (Table III).

**Effect of L-NAME on acetic acid induced colitis**

Twenty four hours after administration of acetic acid the colons were resected and inflamed. The lesion area and the wet weight of the 10 cm distal colonic segment were measured. *Significantly different from TNB+L-NAME (p<0.05). †Significantly different from no treatment (p<0.05). Results are mean (SEM).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number</th>
<th>Lesion area (mm²)</th>
<th>Wet weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>21</td>
<td>0</td>
<td>0.60 (0.02)</td>
</tr>
<tr>
<td>Nitroprusside</td>
<td>3</td>
<td>0</td>
<td>0.80 (0.04)</td>
</tr>
<tr>
<td>L-NAME</td>
<td>9</td>
<td>0</td>
<td>0.70 (0.04)</td>
</tr>
<tr>
<td>TNB</td>
<td>14</td>
<td>702 (53)</td>
<td>2.2 (0.1)*</td>
</tr>
<tr>
<td>TNB+L-NAME</td>
<td>8</td>
<td>384 (102)</td>
<td>1.3 (1.1)</td>
</tr>
<tr>
<td>TNB+L-NAME+nitroprusside</td>
<td>4</td>
<td>807 (32)*</td>
<td>2.0 (0.3)*</td>
</tr>
</tbody>
</table>

Colitis was induced by intracolonic TNB administration. Rats were treated with L-NAME (0.1 mg/ml) added to the drinking water. One of the groups also received intraperitoneal nitroprusside (0.42 µg/ml). Another group was treated only with nitroprusside (0.42 µg/ml). Rats were killed after 72 hours. Lesion area and the wet weight of the 10 cm distal colonic segment were measured. *Significantly different from TNB+L-NAME (p<0.05). †Significantly different from no treatment (p<0.05). Results are mean (SEM).

**Table III**

**Figure 2**: Effect of L-NAME on colonic NOS activity in TNB treated rats. Colonic NOS activity was determined one, three, and seven days after colonic TNB administration, as described in Methods. *Significantly different from control (p<0.05). †Significantly different from TNB (p<0.05).

**Figure 3**: Line graph showing baseline, 24, 48, and 72 hours mean intra-arterial blood pressure in TNB treated rats, TNB+nitroprusside, TNB+L-NAME, TNB+L-NAME+NPS, and NPS alone. Two way ANOVA between the groups shows significance \( F(4;16)=18.8, p<0.004. * TNB+L-NAME \) vs all other groups, p<0.02. †=TNB+L-NAME+NPS v TNB and TNB+nitroprusside, p<0.01. ‡=TNB+nitroprusside v TNB, p<0.01.

In acetic acid treated rats colonic NOx generation by cultured explants and colonic NOS activity were three- and twofold higher, respectively, than in normal colonic mucosa (Fig 5). The augmentation of acetic acid induced colitis in capsaicin pretreated rats is accompanied by further increase in NOx generation and NOS activity. On the other hand, both NOx generation and NOS activity were significantly reduced in L-NAME treated rats when compared with their values in acetic acid+capsaicin treated rats. In control rats, the addition of L-NAME to the drinking water...
induced significant decrease in NOx generation by cultured mucosa, but had no effect on NOS activity.

Colonic NOx generation

In normal rats, colonic NOx generation was inhibited by NNA (Table V). In the presence of calcium ionophore (1 μM), NOx generation during 24 hours of culture was 219 (53)% (n=10) of its basal generation regarded as 100% (p=0.05; t test for paired data), indicating the contribution of the constitutive enzyme to NO generation under basal conditions. The stimulation of NOx generation in normal colonic mucosa by IFN γ indicates expression of the inducible isofrom of NOS (Table V).

NOx generation by inflamed colonic segments was significantly stimulated by lipopolysaccharide and IFN γ and significantly inhibited by NNA. The inhibition of NOx generation induced by NNA was more pronounced in explants isolated from normal rats than that seen in explants isolated from rats with experimental colitis (Table V). Both in normal rats and in rats with experimental colitis, colonic NOS activity was significantly inhibited by NNA and was NADPH dependent (Table VI).

Histological assessment

Sections of the large intestine from rats treated with acetic acid showed multiple, full thickness, mucosal ulcerations with moderate oedema of the submucosa, accompanied by moderate inflammatory cell infiltrate (Fig 6(A)). In rats pretreated with capsaicin before acetic acid treatment, there were widespread ulcerations involving the mucosa and submucosa with extensive haemorrhages, oedema and widespread, acute, inflammatory infiltrate. There was pseudomembrane formation as well as foci of inflammation in the muscularis propria (Fig 6(B)). Cross sections of colonic segments from rats treated with L-NAME before damage induction by capsaicin and acetic acid disclosed multiple small ulcerations confined to the mucosa, some involving the full thickness of the mucosa with mild to moderate oedema accompanied by mild to moderate inflammatory infiltrate (Fig 6(C)).

Discussion

In this study inhibition of NOS activity by L-NAME was found to ameliorate the extent and severity of tissue injury in two models of experimental colitis in which colonic NOx generation and NOS activity were found to be stimulated. These findings support the concentration that NO participates in the pathogenesis of colonic inflammation augmenting and amplifying the extent of tissue injury.

Inflamed colonic mucosa is characterised by the abundance of activated inflammatory cells, such as macrophages and neutrophils. The activation of these inflammatory cells is expressed in a variety of ways, including synthesis and release of certain cytokines, inflammatory mediators, such as leukotrienes and platelet activating factor, and the release of reactive oxygen metabolites. Recently, these mediators were also shown to induce NO generation by phagocytic leucocytes. Moreover, macrophages and inflammatory neutrophils were shown to contain a calcium and calmodulin independent NOS that is activated by agents, such as lipopolysaccharide and IFN γ. NO is produced from L-arginine by the enzyme, NOS. The normal colon expresses the constitutive NOS, as shown in this study, by its response to changes in calcium flux. The high NOx output response of colonic mucosa to cytokines, for example, IFN γ, and to lipopolysaccharide, indicates expression of the inducible isofrom of NOS in the gut. NO has been proposed as a mediator of bacterialidial, tumorous, and tumorcidal activity of macrophages. These last properties are ascribed to the simultaneous generation by macrophages of superoxide and NO, yielding peroxynitrite (ONOO·), which decomposes to OH· and NO2·, or other related decomposing products. OONO· and the free radicals, OH· and NO2·, oxidise sulphydryl groups and react with metal ions, and was recently shown by us to induce severe colonic inflammation. In addition, NO has also immunoregulatory properties, such as inhibition of lymphocyte proliferation.
Figure 5: Effect of L-NAME on colonic NO\textsubscript{X} generation and NOS activity in capsaicin+acetic acid treated rats. Colonic NO\textsubscript{X} generation by cultured explants and colonic NOS activity were determined in rats treated with capsaicin+acetic acid with or without administration of L-NAME, as described in Methods. A=control; B=L-NAME; C=acetic acid; D=capsaicin+acetic acid; E=capsaicin+L-NAME+acetic acid.

\*Significantly different from control (p<0.05). \&Significantly different from acetic acid (p<0.05).

The results obtained in this study show that, in two models of experimental colitis, colonic generation of nitrites reflecting NO generation and colonic NOS activity are significantly increased when compared with the respective NO generation and NOS activity of normal colonic mucosa. Nitrites are end products of the oxidative metabolism of the labile NO in vivo and their quantification is regarded as an indicator of NO generation.\textsuperscript{14} Recently, plasma concentrations of nitrites was found to be increased three weeks after induction of granulomatous colitis by intramural injection of peptidoglycan-polysaccharide into the distal colon of genetically susceptible rats\textsuperscript{24} and the nitrite concentration was found to be increased in the lavage of TNB induced ileitis in guinea pigs.\textsuperscript{25} In the two models of experimental colitis used in this study, the magnitude of stimulation in NOS activity is correlated with the respective increase in NO\textsubscript{X} generation by colonic explants during 24 hours of culture. Moreover, the magnitude of the increase in colonic NO\textsubscript{X} generation and NOS activity were found to correlate with the severity of tissue damage. In TNB induced colitis, both tissue injury and NO\textsubscript{X} generation were higher than in acetic acid induced colitis. Increased NOS activity was previously reported in rats within one week after induction of colitis with TNB\textsuperscript{27} and in inflamed colonic mucosa of inflammatory bowel disease patients.\textsuperscript{6} Significantly higher concentrations of citrulline were found in rectal biopsy specimens of patients with active ulcerative colitis than in those with disease in remission or in normal controls,\textsuperscript{23} further suggesting increased NO synthesis in ulcerative colitis.

Within 24 hours, L-NAME significantly increased mean arterial pressure in TNB treated rats, an effect that lasted throughout the 72 hour study period. This is consistent with other longterm studies in one of which hypertension persisted 13 weeks after the four week L-NAME treatment period had finished.\textsuperscript{26} That acute NO synthesis inhibition causes immediate rise in mean arterial pressure because of widespread vascular constriction is

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
Treatment & Lipopolysaccharide & IPN (\% of basal) & NNA \\
\hline
None & 150 (10)* & 154 (20)* & 20 (9)* \\
Acetic acid & 168 (14)* & 214 (25)* & 50 (9)* \\
TNB & 163 (11)* & 175 (17)* & 77 (1)* \\
\hline
\end{tabular}
\caption{Rat colonic NO\textsubscript{X} generation}
\end{table}

Colonic segments isolated from normal, acetic acid, and TNB treated rats were organ cultured for 24 hours in the presence or absence of lipopolysaccharide (0.5 mg/ml), IPN (10 units/ml) or NNA (1 mM). Basal NO\textsubscript{X} generation was 258 (27), 589 (50), and 1705 (129) \textmu mol/g wet weight in normal untreated, acetic acid, and TNB treated rats, respectively, and was regarded as 100%. Results are mean (SEM) of cultures performed with 8-15 explants obtained from 4-6 rats in each treatment group.

*Significantly different from basal NO\textsubscript{X} generation p<0.05.

Figure 6: Histological sections of the colon isolated from acetic acid treated rats. (A) Treated with 5% acetic acid, showing small, superficial ulcers with a mild inflammatory infiltrate and oedema; (B) after capsaicin denervation and then treatment with 5% acetic acid, showing large, widespread, deep ulcerations with considerable inflammatory cell infiltrate involving all layers of colonic wall; (C) capsaicin denervated and L-NAME treated before 5% acetic acid administration, showing small, superficial ulcerations similar to those seen in (A) - rats treated only with 5% acetic acid.
well described. The activity of the mesenteric blood flow and decreased capillary permeability may have a certain role in the remarkable protective effect of L-NAME in TNB induced colitis. The protective effect provided by L-NAME was accompanied by significant decrease in inflammatory activity and NO generation. The amelioration of experimental colitis by L-NAME supports the contention that enhanced NO generation promotes mucosal injury in the inflammatory models. In a similar way, L-NAME was shown also, to ameliorate TNB induced ileitis in guinea pigs.

In previous studies evaluating the possible modification of gastrointestinal injury by L-NAME, the major haemodynamic changes induced by L-NAME were overlooked. In this study it is clearly shown that reduced mesenteric blood flow and decreased capillary permeability may have a certain role in the remarkable protective effect of L-NAME in TNB induced colitis. The protective effect provided by L-NAME was accompanied by significant decrease in myeloperoxidase activity and in capsaicin augmented acetic acid induced colitis also in LTB\(_4\) and LTC\(_4\) generation. Myeloperoxidase activity is expressed in granulocytes and is a sensitive marker of the severity of colonic inflammation. LTB\(_4\) and LTC\(_4\) are potent proinflammatory mediators and the lack of significant decrease in leukotriene generation in the TNB model despite the effective protection provided by L-NAME, shows that their role is probably secondary to that of NO.

The stimulation of colonic NO generation induced by lipopolysaccharide and IFN\(\gamma\) points to the possible important contribution of macrophages and inflammatory neutrophils to the enhanced NO\(_\gamma\) generation detected in the inflamed colonic segments isolated from acetic acid and TNB treated rats. Endothelial cells, fibroblasts or mast cells, or all three, may also contribute to NO generation. In these cells NO generation is mainly constitutive. Endothelial cells, mast cells, and vascular smooth muscle cells, however, also contain the inducible isoform and, therefore, their contribution to the enhanced NO generation cannot be excluded. Rat colonic NOS activity was found to be NADPH dependent. The L-arginine analogue, NNA, inhibited NOS activity and decreased NO\(_\gamma\) generation measured as nitrite accumulation by organ cultured explants obtained from normal and inflamed rat colonic mucosa (Tables V, VI). The inhibitory effects with this analogue at a concentration of 1 mM may be due to an effect on the inducible, as well as the constitutive isoform, though its activity is more selective against the second.

The protective effect of L-NAME, as shown in this study, is in contrast with findings in the upper gastrointestinal tract where NO donating substances are protective and decrease acute injury. NO is important for organ defense and possesses beneficial antiinflammatory properties. The increased NO generation, as herewith reported, may also represent a protective effect, as it has been shown that lipopolysaccharide induced intestinal damage is enhanced by inhibition of NO formation and decreased by NO donors. In extreme excess, however, it may have deleterious effects. The enhanced NO generation by the inflamed colonic mucosa may amplify the extent of tissue inflammation and injury. Enhanced generation of free NO radical species by stimulated NOS of macrophages, inflammatory neutrophils, and possibly other inflammatory cells may determine the extent and severity of the inflammatory response. Moreover, the enhanced colonic NO generation may produce carcinogenic nitrosamines shown to be generated by neutrophils during active intestinal inflammation. The results obtained in this study encourage the evaluation of the therapeutic effect of modulation of NOS activity as a possible therapeutic modality in patients with inflammatory bowel disease.

The excellent technical help of Mrs J Mekler is gratefully acknowledged.

Experimental colitis is ameliorated by inhibition of nitric oxide synthase activity


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