Enhanced colonic nitric oxide generation and nitric oxide synthase activity in ulcerative colitis and Crohn’s disease

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Abstract
Recent studies have suggested that nitric oxide (NO), the product of nitric oxide synthase in inflammatory cells, may play a part in tissue injury and inflammation through its oxidative metabolism. In this study the colonic generation of oxides of nitrogen (NOₓ) and nitric oxide synthase activity was determined in ulcerative colitis and Crohn’s disease. Colonic biopsy specimens were obtained from inflammatory bowel disease patients and from normal controls. Mucosal explants were cultured in vitro for 24 hours and NOₓ generation was determined. Nitric oxide synthase activity was monitored by the conversion of [3H]-L-arginine to citrulline. Median NOₓ generation by inflamed colonic mucosa of patients with active ulcerative colitis and Crohn’s colitis was 4-2- and 8-1-fold respectively higher than that by normal human colonic mucosa. In ulcerative colitis and Crohn’s colitis nitric oxide synthase activity was 10-0- and 3-8-fold respectively higher than in normal subjects. Colonic NOₓ generation is significantly decreased by methylprednisolone and ketotifen. The decrease in NOₓ generation by cultured colonic mucosa induced by methylprednisolone suggests that NO synthase activity is induced during the culture and the steroid effect may contribute to its therapeutic effect. Enhanced colonic NOₓ generation by stimulated nitric oxide synthase activity in ulcerative colitis and Crohn’s disease may contribute to tissue injury.

Keywords: ulcerative colitis, Crohn’s disease, nitric oxide.

Nitrergic nitric oxide (NO) has been implicated in a variety of diverse cellular functions and biological responses. In addition to its classic functions as a neurotransmitter and vasodilator, NO has an important role in inflammatory processes, being a mediator of macrophage function. Under basal conditions, NO synthase activity in macrophages is negligible but stimulation with lipopolysaccharide or cytokines produces effective increase in NO generation. NO is synthesised from L-arginine by the enzyme NO synthase, which is present in several molecular isoforms. The two important subtypes encompass a constitutive form (calcium dependent) and an inducible form (calcium independent). NO secreted by activated macrophages is an important cytotoxic molecule in the defence against various infectious agents as well as tumour cells. High NO concentrations, however, may be toxic and may damage healthy tissue. Tissue injury may result from a combination of NO with superoxide anion, which are both actively produced in inflammatory settings, yielding the highly cytotoxic species, peroxynitrite. Peroxynitrite induces tissue injury through mechanisms entailing direct lipid peroxidation and sulphhydril oxidation. We have recently established a new model of colonic inflammation induced by intracolonic administration of peroxynitrite. NO is a highly unstable molecule and its direct determination is difficult; however, quantification of its metabolic end products, nitrates, or nitrates, or both and determination of NO synthase activity can be used to assess NO generation.

The aetiology of inflammatory bowel disease is not still known. Various mediators have been shown as possible participants in the pathogenesis of the inflammatory response. Both Crohn’s disease and ulcerative colitis are characterised by an abundance of activated macrophages and granulocytes in the inflamed tissue. Generation and secretion of high NO concentrations by these cells may lead to perpetuation of local tissue damage, irrespective of the trigger of the inflammatory response. Recently, NO synthase activity in colonic mucosa resected from six patients with active ulcerative colitis was reported to be eightfold higher than its activity in normal colonic mucosa, whereas the enzyme activity in colonic mucosa of four Crohn’s colitis patients was found to be unstimulated. Increase in rectal biopsy tissue concentrations of citrulline, a coproduct of NO synthase activity, in ulcerative colitis is also compatible with increased NO generation in this disease. In view of the limited available data and the unexplained reported discrepancy in NO synthase activity in ulcerative colitis and Crohn’s disease, in this study colonic generation of oxides of nitrogen—NOₓ, NO synthase activity and its drug modulation were determined in a large group of inflammatory bowel disease patients and normal controls.

Methods

Chemicals

The following chemicals were obtained: Nω-nitro-L-arginine methyl ester, L-arginine, L-citrulline, Nω-nitro-L-arginine, NADPH,
calcium ionophore, dithiothreitol, phenylmethylsulphonyl fluoride, EDTA, EGTA, ketotifen, 5-aminosalicylic acid (5-ASA), acetyl-5-ASA, methylprednisolone, and cyclosporine (Sigma Chemicals); Dowex AG50W-X8 (Na form) 100–200 mesh, and TRIS base (electrophoresis grade) (Bio-Rad, Richmond, CA, USA); sodium nitrite and sodium nitrate (Fisher Chemical); aquasol-2 (DuPont Co/NEN Research); sulphanilamide, and N-1naphthyl-ethylene diamine hydrochloride (Aldrich Chemical).

Patients
For determination of colonic NO_{\text{a}} generation colonoscopic biopsy specimens were obtained from inflamed and uninfamed sites of inflammatory bowel disease patients and from normal controls. Biopsy specimens were obtained from 14 patients with active Crohn’s colitis, M:F 10:4, mean age 39 years; and from 29 patients with active ulcerative colitis, M:F 15:14, mean age 29 years. The disease was diagnosed colonoscopically and confirmed histologically. All but two patients with active disease were treated at the time of tissue sampling with sulphasalazine 2-0–3-0 g/day or with one of the 5-ASA drugs 1-5–2-0 g/day. Ten of the patients with active ulcerative colitis and four of those with active Crohn’s disease were treated with oral corticosteroids. Only one patient with active ulcerative colitis was treated with an immunosuppressive drug. The control group consisted of 28 subjects, M:F 14:14, mean age 42 years, undergoing colonoscopy for various reasons and in whom no abnormality was found in the colon.

Colonic NO synthase activity was determined in colonoscopic biopsy specimens obtained from 13 patients with active ulcerative colitis, M:F 8:5, mean age 33 years, all treated with 5-ASA; and from seven patients with active Crohn’s colitis, M:F 4:3, mean age 37 years, all treated with corticosteroids. NO synthase activity was also determined in colonoscopic biopsy specimens obtained from 20 normal subjects, M:F 9:11, mean age 46 years, undergoing colonoscopy for various reasons and in whom no abnormality was found. In each subject one specimen, fixed in phosphate buffered formaldehyde and stained with haematoxylin and eosin, was assessed for the degree of inflammation on a 0–3 scale, as previously described.\(^\text{10}\) The degree of inflammation was similar in ulcerative colitis (six, severe; four, moderate; three, mild) and Crohn’s colitis (four, severe; three, moderate; one, mild). All subjects gave informed consent and the study was approved by the institutional human studies committee.

Organ culture
Colonic explants were kept in NaCl 0-15 M at 4\(^{\circ}\)C and within 15 minutes after excision were cultured as previously described.\(^\text{11}\) In brief, the tissue was weighed, orientated on metal grids, and organ cultured for 24 hours at 37\(^{\circ}\)C, 95% O\(_2\), 5%\(_{\text{v}}\) in AIM-V medium (Gibco) containing penicillin and gentamycin. In several experiments explants were also organ cultured in the presence of calcium ionophore or N\(^{\text{O}}\)-nitro-L-arginine, 5-ASA, acetyl-5-ASA, ketotifen, methylprednisolone or cyclosporine.

Measurement of NO production
NO, quantified by the accumulation of nitrite in the culture medium, was measured spectrophotometrically using the Greiss reaction\(^\text{6}\) 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 \(\mu\)l of culture supernatants were mixed with equal volumes of 1% sulphanilamide in 0.5 N HCl. After five minutes 50 \(\mu\)l of 0.1% N-1-naphthylethylene-diamine-dihydrochloride was added and 10 minutes later absorbance was measured at 570 nm.

Determination of NO synthase activity
NO synthase activity was monitored by the conversion of [\(^{\text{14}}\)H]-L-arginine to citrulline in the presence of 1 mM N\(^{\text{O}}\)-nitro-L-arginine, according to Bush \textit{et al.}\(^\text{12}\) Colonoscopic biopsy specimens (10–15 mg) were homogenised for 30 seconds at 4\(^{\circ}\)C with a polytron (Kinematica, 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 20000\(\times\)g for 30 minutes at 4\(^{\circ}\)C and the supernatant was used as the source of NO synthase. Enzymatic reactions were conducted at 37\(^{\circ}\)C in 50 mM TRIS HCl, pH 7-4, containing 100 \(\mu\)M L-arginine, 100 \(\mu\)M NADPH, 2 mM CaCl\(_2\), 0-20–0-40 mg supernatant proteins, other test agents as indicated, and about 200 000 dpm of L-[2,3,4,5-\(^{\text{3}}\)H]-arginine HCl (77 Ci/mmol, Amersham) to a final volume of 100 \(\mu\)l. Enzymatic reactions were terminated by addition of 2 ml of ice cold ‘stop buffer’ – 20 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 ml) prepared as described to columns (1 cm diameter) containing 1 ml of Dowex AG50W-X8, Na form, that had been pre-equilibrated with ‘stop buffer’. Columns were eluted with 6\(\times\)1 ml of water collected into scintillation vials. Aquasol-2 (10 ml) was added to each vial and samples were counted in a Beckman LS 3801 liquid scintillation spectrometer. Citrulline was recovered in the first 3 ml of the Dowex column eluate to the extent of 96\(\pm\)2%.

Statistical analysis
Data are expressed as mean (SEM). Statistical analysis for significant differences was performed according to the Student’s \(t\) test for paired data and the non-parametric Mann-Whitney \(U\) test.

Results
NO\(_{\text{a}}\) generation by cultured mucosal explants obtained from patients with active ulcerative...
colitis and Crohn's colitis was 4·2- and 8·1-fold higher than by mucosal explants obtained from normal subjects (Fig 1). Median NOx and range of generation in normal subjects were 133 (0·787) μmol/g/24 h, in ulcerative colitis patients 565 (28·5848) μmol/g/24 h, and in Crohn's colitis patients 1183 (174–12430) μmol/g/24 h. In seven ulcerative colitis patients NOx generation by explants obtained from healthy, uninfamed mucosa was significantly lower (p<0·05) than by inflamed mucosa obtained from the same subjects (Fig 2). The L-arginine analogue, Nω-nitro-L-arginine, significantly decreased NOx generation both by normal mucosa and inflamed colonic mucosa of ulcerative colitis and Crohn's disease patients. Calcium ionophore, on the other hand, induced significant increase in NOx generation by uninfamed colonic mucosa of ulcerative colitis patients and by uninfamed colonic mucosa of healthy subjects. The magnitude of stimulation of NOx generation by calcium ionophore was more pronounced in uninfamed mucosa of ulcerative colitis patients than in controls (Table I), though the difference was not statistically significant. The addition to the culture medium of methylprednisolone and ketotifen significantly decreased colonic NOx generation. 5-ASA, acetyl-5-ASA, and cyclosporin had no significant effect on colonic NOx generation (Table II).

Colonic NO synthase activity was almost not detected in normal colonic mucosa. Colonic NO synthase activity in patients with active ulcerative colitis and Crohn's colitis was 10·00 (0·23) and 3·80 (0·56) nmol/g/min, respectively (Fig 3). Colonic NO synthase activity in uninfamed mucosa obtained from three patients with Crohn's colitis was 1·7 (0·1) nmol/g/min. In the absence of NADPH (100 μM) colonic synthase activity in patients with active ulcerative or Crohn's colitis was 23·6 (1·6)% (n=4) and 40·0 (10·0)% (n=4), respectively, of the basal activity. In inflamed mucosa of patients with active ulcerative colitis, sequestration of calcium with EGTA (1 mM) and EDTA (1 mM) resulted in a significant decrease of 65 (13)% (n=5) of the basal colonic NO synthase activity. Sequestration of calcium had no effect on the enzyme activity in Crohn's colitis, which was 95·2 (13·0)% (n=6) of the basal activity. The addition of valine (60 mM) to the assay mixture induced 32 (4)% (n=3) decrease in the conversion of L-arginine to citrulline by inflamed colonic mucosa.

**Discussion**

In this study normal human colonic mucosa was shown to express both calcium dependent and independent subtypes of NO synthase activity, the second isofrom being responsible for most of NOx generation under basal conditions. In active inflammatory bowel disease colonic NOx generation is enhanced and may contribute to tissue injury.

NO has been proposed as a mediator of bactericidal, tumorstatic, and tumorocidal activity of macrophages. These last two properties are ascribed to the simultaneous generation by macrophages of superoxide and NO yielding peroxynitrite, which decomposes to OH· and NO2; or other related decomposing products. Peroxynitrite and the free radicals, OH· and NO2; oxidise sulphhydril groups and react with metal ions. In addition, NO also has immunoregulatory properties, such as inhibition of lymphocyte proliferation. NO may, therefore, participate in the pathogenesis of inflammatory bowel disease, amplifying and augmenting the extent of tissue injury and damage, irrespective of the unknown aetiology of inflammatory bowel disease.

Inflamed colonic mucosa is characterised by the abundance of activated inflammatory cells such as macrophages and neutrophils. The
Colonoascopic biopsy specimens were obtained from nine normal subjects, from the inflamed mucosa of five Crohn’s colitis patients, and from the inflamed and uninfamed mucosa of 14 and four ulcerative colitis patients, respectively. Explants were organ cultured for 24 hours and NO accumulation in the medium was determined in the presence or absence of calcium ionophore (1 μM) or Nω-nitro-arginine (1 mM), as described in Methods. Basal NO generation for normal colon, inflamed and uninfamed ulcerative colitis and Crohn’s colitis was 169 (34), 799 (166), 281 (96), and 3027 (142) μmol/g, respectively, and was regarded as 100%. Basal NO generation by normal colon did not differ significantly from basal NO generation by uninfamed mucosa of ulcerative colitis patients. *Significantly different from basal (100%) p<0.05.

| Colonoscopic biopsy specimens obtained from inflamed mucosa of patients with active ulcerative colitis and were organ cultured for 24 hours in the presence or absence of the various drugs. NO accumulation in the medium was determined as described in Methods. Basal NO generation in the absence of drugs for 5-ASA, acetazolamide, ketotifen, cyclosporine, Nω-nitro-L-arginine, and Nω-nitro-L-arginine methyl ester was 690 (270), 526 (250), 722 (344), 390 (117), and 953 (204) μmol/g, respectively, and was regarded as 100%. Results are mean (SEM). *Significantly different from basal p<0.05 (Mann-Whitney test).

<table>
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<tr>
<th>Drug</th>
<th>No mg/ml</th>
<th>NO generation (% of basal)</th>
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<tbody>
<tr>
<td>None</td>
<td></td>
<td>100</td>
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<tr>
<td>5-ASA</td>
<td>3 0-1</td>
<td>141 (22)</td>
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<tr>
<td>Acetazolamide</td>
<td>4 0-1</td>
<td>108 (70)</td>
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<tr>
<td>Ketotifen</td>
<td>4 0-1</td>
<td>58 (7)*</td>
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<tr>
<td>Cyclosporine</td>
<td>5 0-1</td>
<td>127 (25)</td>
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<tr>
<td>Methylprednisolone</td>
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<td>69 (15)*</td>
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<tr>
<td>Methylprednisolone methyl ester</td>
<td>5 0-1</td>
<td>49 (13)*</td>
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Colonic mucosal NO synthase activity, however, in four patients with Crohn’s colitis was not found to be stimulated. In this study both NOX generation and mucosal NO synthase activity in colonic mucosa of a large group of patients with active Crohn’s disease were found to be significantly increased. There is no important difference in the nature and type of inflammatory cells present in the inflamed mucosa of ulcerative colitis and Crohn’s colitis patients. The reported low NO synthase activity in four patients with Crohn’s colitis is, therefore, probably due to the small number of subjects and to the great variability in the severity of mucosal inflammation in surgically resected segments of Crohn’s disease patients. The low rate of NO synthase activity in colonoscopic biopsy specimens obtained from uninfamed sites of patients with active Crohn’s disease further supports out finding of enhanced enzyme activity in inflamed sites.

In this study mucosal NO synthase activity in ulcerative colitis patients was twofold higher than in patients with active Crohn’s colitis despite a similar degree of histologically assessed inflammation. A similar difference was previously reported in tissue obtained from surgical resections in five and four ulcerative colitis and Crohn’s colitis patients, respectively. The difference in the enzyme activity between ulcerative and Crohn’s colitis patients may point to an important difference in the pathogenesis of these diseases as previously reported with respect to interleukin 1. The possible principal difference in the pathogenesis of Crohn’s colitis and ulcerative colitis is also reflected in this study by the opposite magnitude of stimulation in NO synthase activity and NOX generation. Whereas colonic NOX generation was higher in Crohn’s colitis, the enzyme activity was stimulated to a greater extent in ulcerative colitis. Enhanced colonic NOX generation during organ culture represents ex vivo induction, which is apparently different in the two diseases.

Colon NO synthase activity was found to be NADPH dependent. The L-arginine analogue, Nω-nitro-L-arginine, decreased NOX generation measured as nitrite accumulation by organ cultured explants obtained from normal and inflamed colonic mucosa (Table I). The inhibitory effects with this analogue at a concentration of 1 mM may be caused by an effect on the inducible, as well as the constitutive isoform, though its activity is more selective against the second.

Several of the patients with active inflammatory bowel disease from whom biopsy specimens were obtained were treated with corticosteroids at the time of tissue sampling. Glucocorticosteroids inhibit the induction of NO synthase and, therefore, may have only decreased the level of NOX generation and the decreased of NO synthase activity as determined in this study. 5-ASA, with which several of the inflammatory bowel disease patients were treated at the time of tissue sampling, was also shown to inhibit the activity of NO synthase.

Modulation of enhanced NO generation in the inflamed mucosa may be a potential approach to decrease the possible contribution of NO to the amplification of tissue injury. The effect of several drugs on colonic mucosal NO generation was, therefore, evaluated. Methylprednisolone and ketotifen were found to significantly decrease colonic NOX generation by organ cultured explants of inflamed colonic mucosa, whereas 5-ASA, acetyl-5-ASA, and ciclosporin had no effect. Corticosteroids were shown to inhibit the induction of NO synthase activity and their effect herewith shown on colonic NOX generation suggests that, during the 24 hours of culture, the inducible isofrom is induced. Moreover, their induced decrease in colonic NOX generation may be an additional mechanism to explain their therapeutic effects in inflammatory bowel disease. The opposite effects of methylprednisolone and ketotifen, a mast cell stabiliser, shown to prevent mucosal damage along the gastrointestinal tract on the one hand, and ciclosporin, currently used successfully in the treatment of ulcerative colitis patients and 5-ASA, on the other hand, show that drug induced modulation of mucosal NO generation is not the major mechanism to explain the beneficial effects of these drugs in experimental and clinical gastrointestinal inflammation.

NO is important for organ defence. It possesses bactericidal and cytostatic properties. The increased NOX generation, as here- with 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. However, in extreme amounts it may have deleterious effects. The enhanced NOX generation by the inflamed colonic mucosa may amplify the extent of tissue
inflammation and injury. Moreover, the enhanced colonic NO\textsubscript{3} generation may produce carcinogenic nitrosamines shown to be generated by neutrophils during active intestinal inflammation.\textsuperscript{22} Formation of nitrosamines may contribute to the increased risk of malignancy in chronic inflammatory bowel disease patients. The results obtained in this study encourage further evaluation of the therapeutic effect of modulation of NO synthase activity as a possible therapeutic modality in patients with inflammatory bowel disease.

This work was supported by a grant from the National Institutes of Health (5P30 DK 43351).

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*Gut* 1995 36: 718-723
doi: 10.1136/gut.36.5.718

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