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\textsuperscript{-}), 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 (NO\textsubscript{x}) 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 NO\textsubscript{x} generation was determined. Nitric oxide synthase activity was monitored by the conversion of \textsuperscript{3}H-L-arginine to citrulline. Median NO\textsubscript{x} generation by inflamed colon 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 NO\textsubscript{x} generation is significantly decreased by methylprednisolone and ketotifen. The decrease in NO\textsubscript{x} 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 NO\textsubscript{x} 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.

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 vasoconstrictor, 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 such 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 still not 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 selected 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. 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 — NO\textsubscript{x}, 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. N\textsuperscript{\textwedge}-nitro-L-arginine methyl ester, L-arginine, L-citrulline, N\textsuperscript{\textwedge}-nitro-L-arginine, NADPH,
calcium ionophore, dithiothreitol, phenylmethylsulphonyl fluoride, EDTA, EGTA, ketotifen, 5-aminosalicylic acid (5-ASA), acetyl-5-ASA, methylprednisolone, and cyclosporine (Sigma Chemical); 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-1-naphthyl-ethylene diamine hydrochloride (Aldrich Chemical).

Patients
For determination of colonic NO\textsubscript{x} 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.

Colonoscopy biopsy specimens were 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.\textsuperscript{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°C and within 15 minutes after excision were cultured as previously described.\textsuperscript{11} In brief, the tissue was weighed, oriented on metal grids, and organ cultured for 24 hours at 37°C, 95% O\textsubscript{2}, 5%\textsubscript{2} 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\textsuperscript{-}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\textsuperscript{9} 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 volumes of 1% sulphanilamide in 0.5 N HCl. After five minutes 50 µl 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 \textsuperscript{[3H]}-L-arginine to citrulline in the presence of 1 mM N\textsuperscript{-}nitro-L-arginine, according to Bush et al.\textsuperscript{12} Colonoscopic biopsy specimens (10–15 mg) were homogenised for 30 seconds at 4°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 20,000×g for 30 minutes at 4°C and the supernatant was used as the source of NO synthase. 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 CaCl\textsubscript{2}, 0.20–0.40 mg supernatant proteins, other test agents as indicated, and about 200,000 dpm of \textsuperscript{[3H]}-arginine (77 Ci/mmol; Amersham) to a final volume of 100 µl. 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 6×1 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±2%.

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

Results
NO\textsubscript{x} generation by cultured mucosal explants obtained from patients with active ulcerative
Discussion

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

NO has been proposed as a mediator of bactericidal, tumorstatic, and tumorocidal activity of macrophages.\textsuperscript{1,3} These last two properties are ascribed to the simultaneous generation by macrophages of superoxide and NO yielding peroxynitrite, which decomposes to \textit{OH} and NO\textsubscript{2} or other related decomposing products. Peroxynitrite and the free radicals, \textit{OH} and NO\textsubscript{2}−, oxidise sulphydryl groups and react with metal ions.\textsuperscript{4,13} In addition, NO also has immunoregulatory properties, such as inhibition of lymphocyte proliferation.\textsuperscript{14} 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
activation of these inflammatory cells is expressed in various ways, including synthesis and release of certain cytokines, inflammatory mediators such as leukotriene B₄ and platelet activating factor, and the release of reactive oxygen metabolites. Recently, these mediators were also shown to induce the production of NO by phagocytic leucocytes. Moreover, macrophages and inflammatory neutrophils were shown to contain a calcium and calmodulin independent NO synthase that is activated by agents such as lipopolysaccharide and interferon. Endothelial cells, fibroblasts, or mast cells, or all three may also contribute to NO generation in inflamed tissues; however, a large component of their activity is generally regarded as calcium dependent.

In this study colonic NO synthase activity was determined by monitoring the conversion of L-arginine to citrulline in the presence of Nω-nitro-L-arginine, as previously described. In this assay labelled products not reflecting NO synthase activity can pass through the Dowex columns and, therefore, only NO synthase activity that is inhibited by an L-arginine analogue represents real NO synthase activity. Arginine is one of the enzymes that may contribute to conversion of L-arginine to citrulline. In this study valine, which inhibits arginase activity, decreased the conversion of L-arginine to citrulline by 32%, similar to the extent of decrease in the conversion of L-arginine to citrulline induced by Nω-nitro-L-arginine. Nω-nitro-L-arginine does not affect arginase activity, thus suggesting that the difference in the conversion of L-arginine to citrulline, in the absence and presence of the L-arginine analogue, may represent arginase activity. The enzyme activity seen in colonic mucosa of ulcerative colitis and Crohn's colitis patients, therefore, represents a real stimulation of the enzyme activity in inflamed mucosa.

The colon was found to express the constitutive NO synthase, as evidenced in this study by its NO response to changes in calcium flux and by the reduced enzyme activity detected in active ulcerative colitis in the presence of EGTA. Geller et al. recently identified a calcium dependent inducible isoform in human hepatocytes. Thus, these findings of a significant degree of inhibition of NO synthase activity by EGTA suggest that a component of activity may be that of a constitutive isoform, or alternatively, that we are identifying a calcium dependent inducible isoform. The lack of effect of calcium sequestration on colonic NO synthase activity in Crohn's colitis suggests that in these patients most of the enhanced enzyme activity is calcium independent. Calcium did not induce a further increase in NO generation by inflamed mucosa of patients with ulcerative colitis, showing that in these patients calcium independent enzyme activity is overwhelming by comparison with the modest calcium dependent activity, even when the second activity is fully stimulated. Only cloning of NO synthase from isolated colonic epithelial and inflammatory cells will show the exact contribution of each cell type to the herewith shown enhanced NO generation and NO synthase activity in human colonic inflammation.

In patients with active ulcerative colitis and Crohn's disease, mucosal generation of nitrates reflecting NO synthase and NO synthase activity were found to be significantly increased in the colonic mucosa of normal colonic mucosa. Nitrates are end products of the oxidative metabolism of the labile NO in vivo and their quantification is regarded as an indicator of NO generation. Recently, plasma concentration of nitrates was found to be increased three weeks after induction of granulomatous colitis by intramuscular injection of peptidoglycan-polysaccharide into the distal colon of genetically susceptible rats and nitrite concentration was found to be increased in the lavage of trinitrobenzene sulphonic acid induced ileitis in guinea pigs. Furthermore, L-NNAME was found, in this species, to ameliorate trinitrobenzene sulphonic acid induced ileitis, further suggesting that enhanced NO synthase promotes mucosal injury in this model. Organ culture was verified in this study to be a useful method to study colonic generation of inflammatory mediators and cytokines in inflammatory bowel disease, as previously used to show enhanced generation of eicosanoids, platelet activating factor, and interleukin 1. Recently, increased NO synthase activity was reported in the inflamed colonic mucosa of six ulcerative colitis patients, and also in rats, within one week after induction of colitis with trinitrobenzene

**TABLE I**

<table>
<thead>
<tr>
<th>Calcium ionophore</th>
<th>Nω-nitro-L-arginine</th>
<th>NO generation (µM)</th>
<th>Basal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal colonic</td>
<td>197 (3)</td>
<td>26 (11)</td>
<td></td>
</tr>
<tr>
<td>Ulcerative colitis</td>
<td>145 (18)</td>
<td>27 (10)</td>
<td></td>
</tr>
<tr>
<td>Crohn's disease -</td>
<td>390 (129)*</td>
<td>Not tested</td>
<td></td>
</tr>
<tr>
<td>Crohn's disease -</td>
<td>Not tested</td>
<td>57 (40)*</td>
<td></td>
</tr>
</tbody>
</table>

Colonscoposcopic biopsy specimens were obtained from inflamed mucosa of patients with active ulcerative colitis and were cultured for 24 hours in the presence of absence of the various drugs. NO, accumulation in the medium was determined as described in Methods. Basal NO generation in the absence of drugs in 5-ASA, acetyl-5-ASA, ketotifen, cyclosporine, and methylprednisolone was 690 (270), 526 (250), 722 (344), 390 (117), and 953 (304) µmol/L/24 h, respectively, and was regarded as 100%. Results are mean (SEM). Significantly different from basal p < 0.05. (Mann-Whitney test).
the enzyme activity was stimulated to a greater extent in ulcerative colitis. Enhanced colonic NO\textsubscript{X} 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, N\textsuperscript{\textdagger}arginine, decreased NO\textsubscript{X} 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.\textsuperscript{1}

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\textsuperscript{1} and, therefore, may have only decreased the level of NO\textsubscript{X} generation and the extent 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.\textsuperscript{25}

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 NO\textsubscript{X} generation by organ cultured explants of inflamed colonic mucosa, whereas 5-ASA, acetylated 5-ASA, and cyclosporin had no effect. Corticosteroids were shown to inhibit the induction of NO synthase activity\textsuperscript{1} and their effect herefrom with shown on colonic NO\textsubscript{X} generation suggests that, during the 24 hours of culture, the inducible isoform is induced. Moreover, their induced decrease in colonic NO\textsubscript{X} 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, show to prevent mucosal damage along the gastrointestinal tract\textsuperscript{26} on the one hand, and cyclosporin, currently used successfully in the treatment of ulcerative colitis patients\textsuperscript{27} 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 NO\textsuperscript{X} 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.\textsuperscript{28} However, in extreme amounts it may have deleterious effects. The enhanced NO\textsuperscript{X} generation by the inflamed colonic mucosa may amplify the extent of tissue

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**Figure 3: Colonic NO synthase activity.**

N\textsuperscript{\textdagger}arginine (1 mM) inhibitable mucosal NO synthase activity in colonoscopic biopsy specimens obtained from patients with active ulcerative colitis, Crohn's colitis, and normal mucosa, from healthy subjects determined, as described in Methods. The conversion of L-arginine to citrulline in the absence of N\textsuperscript{\textdagger}arginine was 4.8 (0.5, 13.7 (2.0)), and 8.9 (7.4 (1.0) mmol/min in normal, ulcerative, and Crohn's colitis, respectively. Results are mean (SEM).

*Significantly different from normal p<0.05 (Student's t test for unpaired data).

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sulphonic acid.\textsuperscript{24} Significantly higher concentrations of citrulline were also found in rectal biopsy specimens of patients with active ulcerative colitis than in those with disease in remission or in normal controls.\textsuperscript{9}

Colonic mucosal NO synthase activity, however, in four patients with Crohn's colitis was not found to be stimulated.\textsuperscript{8} In this study both NO\textsubscript{X} 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\textsuperscript{8} 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 colitis 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.\textsuperscript{8} 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.\textsuperscript{23} 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 NO\textsubscript{X} generation. Whereas colonic NO\textsubscript{X} generation was higher in Crohn's colitis,
inflammation and injury. Moreover, the enhanced colonic NO3 generation may produce carcinogenic nitrosamines shown to be generated by neutrophils during active intestinal inflammation.9 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.

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4 Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci USA* 1990; 87:1620–5.