Suppression of Pro-Inflammatory Cytokine Release by Selective Inhibition of Inducible Nitric Oxide Synthase in Mucosal Explants from Patients with Ulcerative Colitis

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Background: In ulcerative colitis (UC), inflammatory damage is associated with increased production of pro-inflammatory cytokines and nitric oxide through the inducible nitric oxide synthase (iNOS) pathway. In an animal model of acute experimental colitis we have previously shown amelioration of inflammation with the highly selective iNOS inhibitor 1400 W. The aim of the present study was to investigate the effects of selective iNOS inhibition on the production of pro-inflammatory cytokines by the colon mucosa in UC.

Methods: Inflamed and uninflamed mucosa from patients with severe UC were incubated with a highly selective iNOS inhibitor N-[3-(aminomethyl)benzyl]acetamidine (1400 W), with a relatively selective cNOS inhibitor N(G)-nitro-L-arginine-methyl-ester (L-NAME), or with an NO-donor, S-nitroso-acetylpenicillamine (SNAP). Cytokine concentrations in the incubation medium were quantitated with ELISA.

Results: Compared to uninflamed mucosa there was an increase in iNOS protein and nitrotyrosine levels in inflamed mucosal samples. Immunolocalization of iNOS and nitrotyrosine showed their expression in inflammatory cells in the lamina propria. Expression of iNOS was also found in the epithelial brush border. Selective inhibition of iNOS suppressed the release of tumour necrosis factor alpha (TNF-α) by 66% and interleukin-6 (IL-6, by 27%). The NO-donor, SNAP, augmented the secretion of TNF-α, IL-6 and IL-1-β by 62%, 52% and 175%, respectively and decreased the release of IL-1 receptor antagonist (IL-1Ra, by 34%) by the inflamed mucosa. Moreover, in uninflamed samples, 1400 W suppressed the production of TNF-α (by 69%) and incubation with SNAP decreased IL-6 concentrations by 48%. The cNOS over iNOS selective inhibitor l-NAME had no significant effects on the accumulation of cytokines. Conclusion: Selective inhibition of iNOS suppresses mucosal TNF-α and IL-6 release in active UC, whereas NO seems to exacerbate the inflammatory response. These results suggest that selective iNOS inhibition may have therapeutic promise in the treatment of UC.

Key words: Anti-inflammatory drugs; inducible nitric oxide synthase; nitric oxide; NOS inhibitors; pro-inflammatory cytokines; ulcerative colitis

The colon mucosa in ulcerative colitis (UC) produces increased amounts of pro-inflammatory cytokines such as interleukin-1 beta (IL-1β), tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) (1–3). The main cellular sources of these cytokines are activated macrophages (4–6). The increased production of these pro-inflammatory cytokines correlates with disease activity in inflammatory bowel diseases (1, 7) and their synthesis is implicated in the pathogenesis of the disease (6). In addition, inflamed tissue produces cytokine antagonists to endogenously control their actions and to limit inflammation (8). One of these cytokine antagonists is the IL-1 receptor antagonist (IL-1Ra), which binds to the IL-1 receptor and thereby suppresses IL-1 signalling (9).

Active inflammation in UC is also associated with increased expression of the inducible, calcium-independent nitric oxide (NO) synthase (iNOS, NOS-2) (10). iNOS is capable of producing high amounts of NO (11) and is...
regarded as the principal source of NO in gut inflammation (12, 13). The expression of iNOS is induced by certain pro-inflammatory stimuli such as IL-1β, TNF-α and bacterial products (11, 12). Increased iNOS activity and NO production contribute to inflammation and oxidative stress through the reaction of NO with superoxide leading to the formation of a highly cytotoxic oxidant, the peroxynitrite anion (14, 15). Small amounts of NO are produced for physiological purposes by the constitutively expressed, calcium-dependent, enzymes: endothelial (eNOS, NOS-3) and neuronal NOS (nNOS, NOS-1) (11). NO contributes to several central functions in immune regulation (16), and an NO-releasing aminosalicylate-derivative has been shown to exert beneficial effects on colon inflammation in experimental models (17, 18).

Recently, we showed that the inflammatory process associated with induction of iNOS in an acute model of rat colitis (19) was suppressed by a highly selective inhibitor of iNOS, 1400 W (20, 21). Our finding agrees with other reports on the advantageous effects of NOS inhibitors in the treatment of experimentally induced colitis (22–27). In the present study we provide further insight into these mechanisms and investigate the effects of a selective iNOS inhibitor 1400 W on the release of pro-inflammatory cytokines from colon mucosa of patients with severe UC.

Materials and Methods

Patients

The patient characteristics are given in Table I. All patients were on oral steroids and 5-aminosalicylic acid (5-ASA). They underwent elective proctocolectomy because of severe UC, the patients’ mean blood leucocyte count at the time of operation was 10.2 × 10^9 l⁻¹. Inflammation, the patients’ mean blood leucocyte count at adverse drug effects. As an indicator of the ongoing disease, failure of conventional treatment, or because of

They underwent elective proctocolectomy because of severe UC, diagnosed with pancolitis; the other patients had diagnosed inflammation of descending colon only. At time of collecting the mucosal samples, the descending colon was most inflamed by eye in all cases, whereas macroscopically uninflamed mucosa was present in the ascending and transverse colon.

Incubation of colon mucosal samples

Immediately after removal, the colon was longitudinally opened and 4 cm² samples of mucosa were cut from inflamed and uninflamed regions. The samples were placed in separate containers containing 500 mL of ice-cold phosphate buffered saline (PBS) for transport. Thereafter (approximately after 15 min) the tissues were washed in fresh ice-cold PBS. Mucosal samples were cut with a curette (4 mL in diameter). Each sample was placed in 800 μL PBS in a cell culture plate. After 30 min pre-incubation, the PBS was replaced with 400 μL of culture medium (RPMI 1640 containing antibiotics: penicillin 100 U mL⁻¹, streptomycin 0.1 mg mL⁻¹, and amphotericin B 250 ng mL⁻¹) supplemented with the test compounds. Both uninflamed and inflamed mucosal samples were divided into four groups, one group with vehicle (culture medium only), one with l-NAME (1 mM), one with 1400 W (1 mM), and one group with SNAP (500 μM). The concentrations were chosen for sufficient mucosal tissue sample (50–60 mg) penetration by the drugs from the relatively small volume (400 μL) of incubation medium. The culture plates were kept in a humidified atmosphere consisting of 95% air and 5% CO₂ at 37°C for 5 h. After incubation, the medium and mucosal samples were collected and immediately frozen in liquid nitrogen and stored at −70°C until analysed.

Enzyme-linked immunosorbent assay (ELISA)

Cytokines IL-1β, IL-6 and TNF-α were analysed by ELISA using reagents supplied by Central Laboratory of The Netherlands Red Cross (CLB), The Netherlands (FeliPair reagent sets). IL-1Ra was analysed by ELISA using reagents from R&D Systems, USA (Quantikine®).

Immunohistochemistry

Mucosal samples were embedded in Tissue Tek O. C. T. Compound (Sakura Finetek Inc., Zoeterwoude, The Netherlands). Cryostat sections were air dried and fixed in ice-cold methanol. Endogenous peroxidase activity was quenched with subsequent incubation in PBS containing 0.3% hydrogen peroxide.

Table I. Patient characteristics

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<th>#</th>
<th>Male (M)/Female (F)</th>
<th>Age</th>
<th>Years from diagnosis</th>
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<th>5-ASA* mg/day</th>
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*5-ASA = 5-aminosalicylic acid.
peroxide. The sections were then washed three times with ice-cold PBS, incubated in normal blocking serum, washed and incubated overnight with the primary antibody. Thereafter the sections were processed according to the avidin-biotin-peroxidase procedure (Vectastain ABC Elite-kit, Vector Laboratories, Burlingame, Calif., USA). Monoclonal anti-iNOS at a dilution of 1:2000 (Transduction Laboratories, Franklin Lakes, N.J., USA) and polyclonal anti-nitrotyrosine at a dilution of 1:500 (Upstate Biotechnology, Lake Placid, NY, USA) were used as primary antibodies. Respective non-immune serum was used instead of primary or secondary antibody as negative controls. Rabbit/mouse IgG of the same sub-type as the primary antibody but with an irrelevant specificity was used as an additional negative control. The sections were counterstained with haematoxylin.

Western blot
Western blots for iNOS and nitrotyrosine were carried out using previously described protocols (21). The nitrotyrosine antibody was from Upstate Biotechnology, the monoclonal eNOS and nNOS antibodies were from Transduction Laboratories, and the monoclonal iNOS antibody was from Santa Cruz Biotechnology (Santa Cruz, Calif., USA). Densitometric analysis was carried out using specific computer programs GeneSnap Version 2.60.0.14 and GeneTools Version 2.10.03 (Synoptics, Cambridge, UK).

Materials
L-NAME and SNAP were purchased from Sigma Chemical Co. (St. Louis, Mo., USA). 1400 W was kindly provided by Dr. Richard G. Knowles, Glaxo SmithKline (Stevenage, UK). RPMI-1640 and the antibiotics were purchased from Gibco BRL (NY, USA). Culture plates were from Nunc (Copenhagen, Denmark) and curettes from Stiefel Laboratories Ltd. (Sligo, Ireland).

Results
The expression of iNOS was about 10-fold higher in the inflamed samples of colon mucosa as compared to macroscopically healthy mucosa (Fig. 1). In contrast, no differences in the expressions of the constitutive NOS isoforms, eNOS and nNOS, were found between the inflamed and the quiescent mucosa (Figs. 2 and 3). Nitrotyrosine was measured as a marker of peroxynitrite production. Immunoblotting of nitrotyrosine expression in the inflamed mucosa revealed scantily, but non-significantly elevated levels of nitrotyrosine in inflamed mucosa as compared to the uninflamed samples (data not shown). Localization of iNOS and nitrotyrosine by immunohistochemistry showed their predominant expression in infiltrated inflammatory cells. iNOS was also localized to the apical epithelial brush border in some areas (Fig. 4).

All mucosal samples produced detectable amounts of IL-6, TNF-α and IL-1β into the culture medium. Incubation of the inflamed mucosa with the highly selective iNOS inhibitor 1400 W decreased the accumulation of IL-6 by 27% and TNF-α by 66% (Fig. 5), but had no effect on IL-1β or IL-1Ra secretion as compared to untreated inflamed control samples.

**Fig. 1.** (a) Expression of inducible nitric oxide synthase (iNOS) and (b) densitometry of iNOS expression in macroscopically inflamed and uninflamed, quiescent mucosal samples from the seven patients with ulcerative colitis. In (b), data are expressed as mean ± s.d. (SE), **P < 0.01 as compared to uninflamed mucosa.

**Fig. 2.** (a) Expression of endothelial nitric oxide synthase (eNOS) and (b) densitometry of eNOS expression in macroscopically inflamed and uninflamed, quiescent mucosal samples from the seven patients with ulcerative colitis. In (b), data are expressed as mean ± s.d. (SE).
The relatively cNOS over iNOS selective inhibitor L-NAME had no significant effects on cytokine accumulation. In contrast, treatment with NO-donor SNAP increased the production of TNF-α (by 62%), IL-6 (by 52%) and IL-1β (by 175%). SNAP also inhibited the production of IL-1Ra by 34% (Figs. 5 and 6) suggesting pro-inflammatory action of NO-supplementation in inflamed colitic mucosa.

Incubation of macroscopically uninflamed, quiescent mucosal samples with the selective iNOS inhibitor, 1400 W, decreased TNF-α concentrations by 69% (Fig. 7), but had no effect on IL-6 (Fig. 7) or IL-1β (data not shown) concentrations. Interestingly, treatment of uninflamed mucosa with the NO-donor SNAP decreased IL-6 accumulation by 48%, but had no effect on TNF-α (Fig. 7) or IL-1β (data not shown). Treatment with L-NAME showed no significant effect on cytokine production by the uninflamed mucosa.

Fig. 3. (a) Expression of neuronal nitric oxide synthase (nNOS) and (b) densitometry of nNOS expression in macroscopically inflamed and uninflamed, quiescent mucosal samples from the seven patients with ulcerative colitis. In (b), data are expressed as mean ± s.e. (SE).

Fig. 4. Immunohistochemical localization of inducible nitric oxide synthase (iNOS) and nitrotyrosine in inflamed colon mucosa. (a) iNOS positive inflammatory cells in lamina propria, (b) iNOS positive staining in apical epithelial cell brush border, (c) nitrotyrosine positive staining in cells of lamina propria, and (d) negative control section incubated with non-immune serum instead of primary antibody.

Fig. 5. The effects of L-NAME, 1400 W and SNAP on concentrations of (a) tumour necrosis factor-alpha (TNF-α), and (b) interleukin-6 (IL-6) in incubation medium of inflamed colon mucosa from the seven patients with UC. Data are expressed as mean ± s.e. (SE), *P < 0.05, ***P < 0.001 as compared to untreated control.
Discussion

Recently, we reported a beneficial effect of selective iNOS inhibition in a rat model of acute colitis (21). In the present study we investigated the effect of the highly selective iNOS inhibitor, 1400 W, on the production of pro-inflammatory cytokines TNF-α (a) and IL-1β (b) in mucosal samples from patients with UC. Data are expressed as mean ± s.e.m (SE), *P < 0.05, **P < 0.01 as compared to untreated control.

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Immunoblotting for iNOS, and marginally also for nitrotyrosine, showed their increase in the inflamed mucosa as compared with samples from uninfamed areas. The expression of iNOS and presence of nitrotyrosine were found predominantly in inflammatory cells of the lamina propria. iNOS was also found in the brush border of the epithelial cells. This pattern of iNOS and nitrotyrosine expression is supported by previous findings in inflammatory bowel disease (28, 29). Tyrosine nitration has been widely used as a marker of peroxynitrite formation. Despite the fact that peroxynitrite-independent mechanisms of tyrosine nitration exist (30), the formation of nitrotyrosine reflects the overall amount of local oxidative and nitrosative stress, and the production of different nitrating species (31). It is feasible to assume that an increased iNOS expression in the inflamed mucosa, as found in the present study, is capable of high-output generation of NO, which rapidly reacts with the superoxide anion produced by inflammatory cells leading to the formation of peroxynitrite and nitrotyrosine. In inflammatory conditions, iNOS may also produce superoxide and thus contribute directly to oxidative stress (32).

Incubation of inflamed mucosa with 1400 W reduced the release of IL-6 and TNF-α, suggesting that in the colitic mucosa of UC, iNOS activity stimulates and drives pro-inflammatory cytokine release. SNAP, on the other hand, increased the secretion of IL-1β, IL-6, TNF-α, whereas it decreased IL-1Ra release by the inflamed mucosa. These findings are in concert with the previously reported positive feedback mechanism of nitric oxide on cytokine release (33) and on TNF-α production in activated human neutrophils and mononuclear cells (34–36). In tissue devoid of macroscopic inflammation, treatment with the NO-donor SNAP significantly inhibited the accumulation of IL-6 showing the opposing anti-inflammatory potential of NO. Thus, the detrimental effects of NO can be enhanced in active disease, while in inactive disease NO may have anti-inflammatory activity.

Inflamed tissues produce IL-1Ra, a cytokine antagonist, which acts as an endogenous suppressor of the pro-inflammatory responses of IL-1 (37). Imbalanced production of IL-1 and IL-1Ra in UC aggravates the inflammatory reaction (38). In the inflamed colon mucosa, as seen in the present study, exogenous NO increased the production of IL-1β and decreased IL-1Ra. In light of these results, NO may exacerbate active mucosal inflammation by disrupting the IL1/IL1Ra balance even further.

The mechanisms through which the high-output NO generation by iNOS regulates cytokine release are not clear. The mitogen activated protein kinase (MAPK) cascade can be activated by peroxynitrite (39) and NO (40, 41) resulting in enhanced activity of transcription factors such as NF-κB and activating protein-1 (AP-1) (42, 43). The activation of NF-κB is associated with increased production of IL-6 and TNF-α (44–46), and the AP-1 family of transcriptional activators functions in cooperation with NF-κB contributing to for example increased transcription of TNF-α (42). It was
recently reported that NO may augment cytokine-induced AP-1 activation also through cyclic-GMP-dependent protein kinase (47), suggesting that NO may have both direct and indirect actions on activation of pro-inflammatory cytokine genes (48). Also a more direct action of NO and oxidative stress has been reported on TNF-α. They have been shown to activate the TNF-α-converting enzyme, thus increasing TNF-α release (49, 50). These data are well in concert with the present results, since selective inhibition of iNOS, leading to decreased production of NO and peroxynitrite, inhibited TNF-α release from both inflamed and uninflamed samples.

We have previously shown that selective inhibition of iNOS with 1400 W decreases macroscopic mucosal lesions, inflammatory edema, and granulocyte infiltration in experimentally induced acute colitis in the rat (21). In that study, the NOS-inhibitor L-NAME, the selectivity of which favors cNOS over iNOS, produced no significant anti-inflammatory effects. The results of the present study further suggest that anti-inflammatory benefits are provided through selective inhibition of iNOS rather than inhibition of the constitutive NOS isoforms in colon inflammation. However, the detrimental role of iNOS in gut inflammation has been disputed (51, 52). Data from experimental models suggest that induction of iNOS may also contribute to resolution of inflammation (52), and that a basal, physiological induction of iNOS in the gut is required for prevention of bacterial overgrowth (53). Even though iNOS seems to have protective effects in gut mucosa (54), the pathological actions, e.g. oxidative and nitrosative stress related NO-metabolism and persistent T-cell activation in chronic inflammatory disease may overcome or prevent these beneficial actions.

In conclusion, our results support the hypothesis that selective inhibition of iNOS down-regulates mucosal inflammation by suppressing TNF-α and IL-6 production in UC-mucosa. These results warrant further investigation of NO-related therapies, and suggest that selective inhibitors of iNOS have therapeutic potential in the treatment of UC.

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