Effect of corticosteroids on nitric oxide production in inflammatory bowel disease: are leukocytes the site of action?

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NITRIC OXIDE (NO) is synthesized by a family of enzymes referred to as the NO synthases (NOS) from the amino acid l-arginine. Three isoforms of the enzyme have so far been identified, two constitutive forms involved in homeostasis [endothelial (eNOS) and neuronal NOS (nNOS)] and one inducible form [inducible NOS (iNOS)]. iNOS expression is highly regulated by cytokines and is predominantly expressed in areas of inflammation, where it is responsible for the production of large quantities of NO (58).

NO is produced at many different sites in the gastrointestinal tract and has been associated with both physiological and pathological events depending on the quantity and timing of NO production (13). Studies in both animal models and humans indicate that NO is involved in gastrointestinal inflammation and has an important role in the pathogenesis of inflammatory bowel diseases (IBD) (12, 36, 45). We have shown that the main cellular source of NO during intestinal inflammation is the colonic epithelial cell (30) probably via the induction of the inducible isoform of NOS by proinflammatory cytokines (29). iNOS protein and nitrotyrosine, a marker of cellular protein nitrosylation by peroxynitrate, have both been immunohistochemically localized exclusively in the colonic epithelial cells in patients with active IBD. The use of immunostaining, iNOS has been localized to the epithelial cells in both active ulcerative colitis (UC) and Crohn’s disease (CD) as well as in infectious colitis and diverticulitis, indicating that iNOS expression is a feature of intestinal inflammation rather than specifically IBD (30, 56).

The role of NO production in intestinal inflammation remains controversial (18). It seems that excess production of NO after induction of iNOS from microbial products and/or cytokines is harmful for the colonic mucosa (38). In contrast, constitutive NO production is considered to be essential in gut homeostasis and to have a protective role during intestinal inflammation (32, 33). Although the steroids prednisolone and budesonide are widely and successfully used in the treatment of colitis, the exact way of action and the cell type on which they are primarily acting are unknown. There is evidence to suggest that corticosteroids might regulate human iNOS expression and subsequent NO production. In patients with asthma, exhaled NO levels are reduced by corticosteroids treatment (26), and dexamethasone inhibited nitrite production in cells from human joint (17) and lung epithelial cells (51). However, the exact mechanism of steroid action on NO production has not been fully elucidated.

In this study, we examined the effect of prednisolone and budesonide on NO production and iNOS expression in colonic epithelial cells and colonic specimens from patients with active, newly diagnosed UC. In addition, we studied the possible role of peripheral blood mononuclear monocytes on the regulation of NO production in an in vitro model of intestinal inflammation.

MATERIALS AND METHODS

Materials. Recombinant human IL-1α and TNF-α were gifts from Glaxo (Greenford, Middlesex, UK) and Bayer (Slough, Berks, UK), respectively. Recombinant human IFN-γ was purchased from Roche Diagnostics (Lewes, UK), and LPS was from Sigma (Poole, UK). Cell culture reagents and FBS were from GIBCO-BRL (Paisley, UK).

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Table 1. The oligonucleotide sequence and product size for each gene specific primer pair used

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>Annealing Temperature</th>
<th>Product Size</th>
</tr>
</thead>
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<tr>
<td>iNOS</td>
<td>sense: ACGAGGCGCTTTAAGCTGC</td>
<td>60.5°C</td>
<td>232 bp</td>
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<tr>
<td></td>
<td>antisense: TTCTCCAGGGGACACAG</td>
<td>60.9°C</td>
<td></td>
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<tr>
<td>β-act</td>
<td>sense: GGTGGGTTTAAAGTGCCAG</td>
<td>62.9°C</td>
<td>161 bp</td>
</tr>
<tr>
<td></td>
<td>antisense: ACTGGAGCGTTGAAGTGCAG</td>
<td>63.6°C</td>
<td></td>
</tr>
</tbody>
</table>

iNOS, inducible nitric oxide synthase.

Oligonucleotide primers were synthesized by PE Applied Biosystems (Warrington, UK). Sodium nitrite and 2,3-diaminonaphthalene were purchased from Sigma and Lancaster Synthesis (Newgate, UK), respectively. Standard reagents were purchased from Sigma.

Cell cultures. HT-29 cell line was purchased from European Collection of Animal Cell Cultures. This cell line is a well-characterized epithelial cell line derived from a primary colon tumor, which has characteristics of normal intestinal epithelium such as epithelial polarity, presence of the actin-binding protein villin, and the occurrence of an enteroctytic differentiation (8). These cells have been chosen because we have previously shown their ability to produce NO and to upregulate this production following proinflammatory cytokine stimulation via the expression of iNOS similarly to the normal colonic intestinal epithelium (28). Cell cultures were maintained in McCoy’s 5A medium supplemented by 10% FBS, penicillin-streptomycin (10 U/ml and 10 mg/ml), and fungizone (0.5 μg/ml) and incubated at 37°C in an atmosphere of 5% CO2 until confluent. Confluent cell cultures were treated with the appropriate concentrations of stimuli, as we have previously described (29).

Colonic biopsies. Multiple colonic biopsies were taken from the sigmoid colon of patients who underwent colonoscopy at the Royal United Hospital (Bath, UK). We used colonic samples from patients with newly diagnosed UC before any treatment and normal controls who underwent colonoscopy for other reasons, e.g., abdominal pain where the examination was found to be normal. Consent was obtained from patients in all cases, and the local research ethics committee granted approval for these studies.

Biopsy cultures. Biopsies were cultured as previously described (31). Briefly, colonic biopsies taken at diagnostic colonoscopy were immediately placed in transport medium of Hanks’ balanced salt solution (pH 7.4) supplemented with antibiotics. Colonic biopsies were incubated at 37°C in an atmosphere of 5% CO2 in the presence of appropriate stimuli up to 30 h. Supernatants were then collected for nitrite measurement, and colonic specimens were used for RNA extraction and/or estimation of total protein per each well. Colonic biopsy specimens, after treatment, were used for estimation of total protein per well, using the Bio-Rad protein microassay (6), and individual nitrite content of each well was expressed as picomoles per 100 micrograms of total protein.

Coculture of mixed mononuclear cells and HT-29 cells. Mixed mononuclear cells (MMC) were isolated from peripheral blood from healthy human volunteers, using a standard protocol based on a method originally described by Boyum (5). Briefly, heparinized samples were diluted 1:1 with RPMI 1640, layered onto the appropriate amount of Lymphoprep (Nycomed, Oslo, Norway), and centrifuged to give a layer of mixed mononuclear cells. Cells were isolated, washed in RPMI 1640, and resuspended in McCoy’s 5A medium. Then, 0.5 × 10^6/ml of healthy MMCs were cocultured with growth-arrested confluent monolayers of HT-29 cells.

Fluorometric assay for the measurement of nitrite. NO production by human colonic biopsies and cell cultures was determined by measuring in culture supernatants the stable-end product nitrite (163.66 ± 9.38 nmol/10^6 cells). Treatment with 1 μM of either prednisolone or budesonide had no effect on basal nitrite production by HT-29 cells. Stimulation with cytokimix: IL-1α (10 ng/ml), TNF-α (100 ng/ml), and IFN-γ (300 U/ml) added in combination produced a highly significant increase in nitrite production of 990.66 ± 48.60 nmol/10^6 cells (P < 0.001, n = 3). Pretreatment with either prednisolone or budesonide, added a Photon Technology International dual-wavelength spectrophotometer, as we have previously described (29). The sensitivity of the assay was 10 nM.

RT-PCR. RT-PCR was performed on total RNA extracted from colonic biopsies as previously described (24). Samples were homogenized into RNAzol within 1 h, and total RNA was extracted. RTPoly (A+) RNA was purified using a Quick Prep micro purification kit (Pharmacia) as described by the manufacturer. Purified mRNA was reverse transcribed with Superscript II (GIBCO-BRL) at 42°C for 60 min. cDNA was PCR amplified using High Fidelity Expand polymerase (Roche, Lewish, Sussex, UK). The oligonucleotide sequence, annealing temperature, and product size for each gene specific primer pair used are shown in Table 1. Coamplification of β-actin and iNOS was done on the same samples. To control for genomic contamination, an identical parallel PCR reaction (RT negative) was performed for each sample containing starting material, which had not been reverse transcribed. PCR products were resolved by electrophoresis on 2% agarose gels and visualized by ethidium bromide staining. The ratio of PCR product, as measured by densitometry between iNOS and β-actin at a constant volume of RT product, was used for cross-sample comparison.

Statistical analysis. Data were analyzed by one-way ANOVA. In the case of a significant difference in variances between groups, further analysis was performed using the independent-samples Student’s t-test or the paired-samples Student’s t-test when appropriate. Data are expressed as means ± SE of n number of experiments. Significance was established at P < 0.05.

RESULTS

Effect of corticosteroids on nitrite production by HT-29 cells. Growth-arrested monolayers of HT-29 cells when stimulated with vehicle for 48 h produced a basal level of nitrite (163.66 ± 9.38 nmol/10^6 cells). Treatment with 1 μM of either prednisolone or budesonide had no effect on basal nitrite production by HT-29 cells. Stimulation with cytokimix: IL-1α (10 ng/ml), TNF-α (100 ng/ml), and IFN-γ (300 U/ml) added in combination produced a highly significant increase in nitrite production of 990.66 ± 48.60 nmol/10^6 cells (P < 0.001, n = 3). Pretreatment with either prednisolone or budesonide, added...
in concentrations up to 1 μM, had no significant effect on cytokine-induced nitrite production (Fig. 1).

Effect of corticosteroids on nitrite production in inflamed colonic biopsies. Biopsies from inflamed areas of large bowel from colitic patients were cultured up to 30 h. The basal nitrite production in biopsies was 1046.77 ± 170.71 pmol/100 μg protein. Incubation for 30 h of paired biopsies with 30 nM of prednisolone or 30 nM of budesonide significantly reduced the nitrite production to 480.77 ± 87.81 (P < 0.01, n = 9) and 424.11 ± 95.72 pmol/100 μg protein, respectively (P < 0.01, n = 9). There was no significant difference between the two steroids at the dose studied (Fig. 2). Similar paired biopsies from inflamed areas of the colon in patients with colitis were incubated with either vehicle or 30 nM prednisolone or budesonide for 30 h. At the end of this period, the mRNA from the biopsies was extracted and underwent multiplex RT-PCR for -actin and iNOS. iNOS mRNA expression was present in untreated biopsies (basal), whereas we observed a decreased level of iNOS mRNA expression in biopsies treated for 30 h with either 30 nM of prednisolone (54.4 ± 3.1%, n = 9, P < 0.05) or budesonide (59.2 ± 3.42%, n = 9, P < 0.05) compared with the basal expression (Fig. 3A). Representative blots from a patient are shown (Fig. 3B).

Effect of corticosteroids on nitrite production by stimulated colonic biopsies. Colonic biopsies from histologically normal areas of the large bowel in patients (n = 12) with UC were cultured for 30 h. At 30 h, basal nitrite production in noninfamed biopsies from patients without colitis was 335 ± 70.52 pmol/100 μg protein. After stimulation with cytokin (3C), this basal production significantly rose to 1732.66 ± 431.33 pmol/100 μg protein (P < 0.01, n = 12). Pretreatment of paired biopsies with prednisolone or budesonide at 30 nM for 2 h before stimulation with 3C significantly decreased the nitrite production to 730.33 ± 193.53 pmol/100 μg protein (P < 0.05, n = 12) and to 706.16 ± 215.54 pmol/100 μg protein (P < 0.05, n = 12), respectively (Fig. 4). There was no significant difference between the two steroids at the dose studied. Similarly, colonic biopsies from normal controls (n = 6) were cultured for 30 h, and basal nitrite production was 347 ± 98.66 pmol/100 μg protein. After stimulation with the three cytokines (3C) IL-1α (10 ng/ml), TNF-α (100 ng/ml), and IFN-γ (300 U/ml), added in combination, this basal production sign-

![Fig. 2. Nitrite production by cultured colonic biopsies taken from inflamed areas from patients (n = 9) with colitis. Basal is the amount of nitrite produced by untreated biopsies in 30 h. Paired biopsies were also incubated with either prednisolone or budesonide at a concentration of 30 nM. Each bar is the mean ± SE of 9 experiments.](image)

![Fig. 3. Inducible nitric oxide synthase (iNOS) mRNA expression in biopsies from inflamed areas of large bowel following incubation for 30 h with either vehicle (basal) or 30 nM of prednisolone or 30 nM of budesonide. A: mean densitometry readings for PCR product of iNOS measured as %maximum iNOS mRNA expression in untreated colitic mucosa (basal). Each bar is the mean ± SE of 9 different patients. B: representative blot from a patient showing iNOS and -actin mRNA expression in inflamed colonic mucosa cultured in the presence of vehicle or 30 nM of either prednisolone or budesonide. The gel shows fluorescence of ethidium bromide-stained PCR products resolved by electrophoresis. Bands were detected under ultraviolet light and compared with the 100-kb pair ladder in the first lane.](image)

![Fig. 4. Nitrite production by cultures of colonic biopsies taken from histologically normal areas in patients (n = 12) with ulcerative colitis. Basal is the amount of nitrite produced after 30 h incubation without any treatment. Pretreatment of paired biopsies with prednisolone or budesonide at 30 nM for 2 h before stimulation with 3C significantly decreased the nitrite production to 354.25 ± 117 (P < 0.01, n = 6) and 367 ± 58.7 pmol/100 μg protein (P < 0.01, n = 6), respec-

* p < 0.05 vs basal
** p < 0.05 vs 3C stimulated tissue
* p < 0.01 vs basal

![Diagram showing nitrite production by cultured colonic biopsies.](image)
and IFN-γ-stimulated MMCs (conditioned media) was removed after 6 h and transferred to HT-29 confluent cultures. After a 48-h incubation period, the media were removed and analyzed for nitrite. Conditioned media caused a significant increase in nitrite production by HT-29 cells up to 1,592 ± 96.82 nmol/10^6 cells (P < 0.01, n = 6; Fig. 6).

**Effect of steroids on nitrite production by coculture of HT-29 cells and MMCs.** To examine the effect of steroids on nitrite production by a coculture of HT-29 cells and MMCs, the cocultures were pretreated for 2 h with different concentrations of either prednisolone or budesonide (3–30 nM) and then stimulated with LPS and IFN-γ for 48 h. Both corticosteroids were found to inhibit nitrite production in this model in a concentration-dependent manner. After treatment with 30 nM of prednisolone or budesonide, nitrite production was decreased to 630 ± 64.51 (P < 0.01, n = 6) and 549 ± 14.15 nmol/10^6 cells (P < 0.01, n = 6), respectively (Fig. 7). To examine the level of the inhibitory effect of corticosteroids in this model, we pretreated HT-29 cells with either prednisolone or budesonide (30 nM) for 2 h before stimulation with conditioned media from MMCs stimulated with a combination of LPS and IFN-γ. Pretreatment with prednisolone or budesonide (30 nM) had no effect on the conditioned media-induced nitrite production by the HT-29 cells (Fig. 8A). Interestingly, stimulation of HT-29 with conditioned media from MMCs pretreated with either prednisolone or budesonide (30 nM) for 2 h before stimulation with LPS/IFN-γ resulted in a significantly lower nitrite production (630 ± 28.47 and 728 ± 63.84 nmol/10^6 cells, respectively) compared with the nitrite production induced by conditioned media from LPS/IFN-γ-stimulated MMCs (1,592 ± 96.82 nmol/10^6 cells, P < 0.01, n = 6; Fig. 8B). This indicates that the effect of corticosteroids on nitrite production by the HT-29/MMCs cocultures was mediated via the inhibition of production of unidentified soluble mediator(s) originating from the MMCs.

![Nitrite production by coculture of HT-29 cells and MMCs](image)

**Fig. 5.** Nitrite production by cultures of colonic biopsies taken from normal controls (n = 6). Basal is the amount of nitrite produced after 30 h incubation without any treatment. Paired biopsies were also incubated with 3C. Paired biopsies incubated with 3C were also pretreated with either prednisolone or budesonide at a concentration of 30 nM for 2 h. Each bar is the mean ± SE of 6 different experiments.

![Nitrite production by HT-29 cell](image)

**Fig. 6.** Nitrite production by HT-29 cell, cultured at 37°C for 48 h, after stimulation with a combination of LPS (5 μg/ml) and IFN-γ (300 U/ml) in coculture with mixed mononuclear cells (MMCs) from normal volunteers (n = 6) or stimulation with conditioned media from LPS (5 μg/ml)- and IFN-γ (300 U/ml)-stimulated MMCs (n = 6). Each bar is the mean ± SE of 6 different experiments.

![Nitrite production by coculture of HT-29 cells and MMCs](image)

**Fig. 7.** Nitrite production by a coculture of HT-29 cells and MMCs from normal volunteers (n = 6). The cocultures were pretreated with prednisolone or budesonide for 2 h before stimulation with LPS (5 μg/ml) and IFN-γ (300 U/ml). Supernatant was removed at 48 h and analyzed for nitrite concentration. Basal is the amount of nitrite produced by cultures of HT-29 cells in the absence of any stimuli. Each bar is the mean ± SE of 6 different experiments. Significance from the cocultures not pretreated with steroids: *P < 0.05, **P < 0.01, ***P < 0.001.
First, because HT-29 cells are corticosteroid insensitive. Previous studies (9, 25) have shown prednisolone and budesonide to regulate cell cycle progression and to induce apoptosis in HT-29 cells. Second, NO production by colonic epithelial cells in colitis is unaffected by steroids, or the HT-29 cells are different from their the normal counterparts with regard to the effect of steroids. Finally, there is another cell type that is sensitive to steroids, which acted directly on the stimulation of colonic epithelial cells by the proinflammatory cytokines.

Previous studies in human immune-mediated diseases such as asthma, multiple sclerosis, cardiac and liver allograft rejection, and arthritis have shown an inhibitory effect of corticosteroids on NO production and iNOS expression (1, 17, 26, 52, 54, 57, 59), suggesting a significant effect of steroids in the regulation of NO production. Other studies have shown that corticosteroids inhibit iNOS expression in vascular endothelial cells (49) and NO production in organ culture explants from UC patients (48) and reduce circulating levels of NO metabolites (50). However, other investigators (16, 27) have failed to observe any differences in iNOS expression, NO production (4, 35), and peroxynitrite formation (27) in patients with UC following treatment with corticosteroids. A possible explanation is that corticosteroids may affect NO generation if given at the very early stage of inflammation, whereas they lose their effect once the inflammation is already set up (16, 20). This suggestion could partly explain why high levels of iNOS expression were unaffected by corticosteroid treatment, in steroid-resistant IBD patients (34). However, the influence of corticosteroids on iNOS activity and consequent NO generation in the settings of UC is still ambiguous.

In the present study, cultured biopsies from patients with active newly diagnosed UC produced a significantly increased amount of nitrite compared with tissue from normal controls. Interestingly, treatment with both prednisolone and budesonide significantly reduced this production. RT-PCR demonstrated increased expression of iNOS mRNA in tissue from colitic patients, which was downregulated following incubation with either prednisolone or budesonide. Effective steroid concentrations were in the range of the plasma levels typically achieved during treatment with steroids for active UC (3, 21, 37).

To further examine whether the cytokine stimulation used in the HT-29 cell model was creating conditions where steroids were unable to affect iNOS expression and NO production by colonic epithelial cells, we cultured colonic biopsies from histologically normal mucosa. This model showed that normal colonic tissue, when exposed to IL-1, TNF-α, and IFN-γ, produced large quantities of NO, and this production was significantly suppressed when pretreated with both steroids. We have similarly shown an inhibitory effect of the anti-inflammatory cytokine IL-13 on colonic NO production in the same experimental settings (31). These results suggest that steroids may regulate NO production in the colonic mucosa and that prednisolone and budesonide might exert their anti-inflammatory effects in part by reducing colonic NO production. As we mentioned above, the influence of standard therapeutic agents for IBD on NOS activity and consequent NO generation is still controversial (19). A possible explanation for these controversial results might be that steroids perhaps influence NO production only if given at the early stage of the inflammatory process, whereas their effect is diminished once the inflammation is established or refractory to clinical treat-
ment (16, 19, 34). One of the interesting differences in our study compared with other studies is that all of our patients were newly diagnosed colitic patients who had not received any treatment before the experiments.

With the data presented above, it is apparent that the HT-29 cells behave differently from both inflamed and stimulated biopsies. However, in inflamed tissue, the epithelial cells are not in isolation and are likely to be influenced by cytokines and other substances produced by gut immune and inflammatory cells in the inflammatory reaction (44, 47). Intraepithelial lymphocytes (IEL) are the most likely cells to be involved (2, 40). Although peripheral mixed mononuclear cells are likely to be different in some respects from IELs, they are the easiest population of similar cells to obtain in large enough numbers to perform multiple coculture experiments. Therefore, we decided to see whether a coculture of HT-29 and mixed mononuclear cells could more easily reproduce the results from the biopsies.

In the present study, we found no increase in NO production when HT-29 and MMCs were cultured together in the absence of any stimulation or separately in the presence of LPS and IFN-γ. However, when HT-29 and MMCs were cultured in the presence of LPS and IFN-γ, they do produce large quantities of NO. Pretreating the coculture with low doses of prednisolone or budesonide easily inhibited this production. Interestingly, HT-29 cells and MMCs did not need to be in contact for nitrite production, because media from IFN-γ and LPS-stimulated MMCs induced a significantly increased nitrite production by HT-29. This would suggest that NO production by the epithelial cells is dependent on the presence of soluble mediators by other cellular sources. We also report that steroid treatment did not affect the conditioned media-induced NO production by HT-29 cells, whereas conditioned media from steroid-treated MMCs induced a dramatically lower NO production by the HT-29 cells. This strongly suggests that steroids might regulate colonic NO by affecting cells other that the colonic epithelial cells with mononuclear monocytes being a plausible candidate.

Mononuclear monocytes have been found to produce an array of soluble inflammatory mediators after stimulation with microbial products and proinflammatory cytokines such as LPS and IFN-γ (15, 22, 23, 41). Corticosteroids have been found to modulate this production (14). Thus, in our model, MMCs may be the target of both inflammatory stimuli and steroids resulting in the production of soluble mediators that regulate NO production and iNOS expression in colonic epithelial cells. Previous studies have shown that intestinal epithelial cells can become antigen-presenting cells (11, 55) and express molecules that activate a subpopulation of CD8+ regulatory lymphocytes whose function is to suppress immune responses (7) and that corticosteroids inhibit cytokine-induced major histocompatibility complex class II expression on the intestinal epithelial cells (53). This, in theory, could suggest that the MMCs are interacting directly with the HT-29 cells once they, in turn, have become antigen-presenting cells and produce cytokines, whereas corticosteroids could inhibit this cell interplay. This would appear unlikely in our study, because the time period in which the two cell populations were cultured together was very short. Furthermore, the effect of MMCs and steroids on NO production by the HT-29 was reproduced using conditioned media from the MMC cultures, which clearly shows that the two populations of cells do not actually need to be in physical contact.

These data suggest that corticosteroids downregulate colonic iNOS expression and NO production during UC. This effect is not directly exerted on colonic epithelial cells, which is the major site of NO production, but rather indirectly by affecting soluble mediator production by other cell units of the colonic mucosa, possibly of the mononuclear cells system. This highlights the importance of cell-to-cell interplay during immune-mediated inflammation and adds new information to the possible mechanisms by which steroids exert their anti-inflammatory actions on IBDs.

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GRANTS

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REFERENCES


