Increased activity and expression of iNOS in human duodenal enterocytes from patients with celiac disease

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Received 24 July 2001; accepted in final form 20 February 2002

Murray, Iain A., Ian Daniels, Kathryn Coupland, Julie A. Smith, and Richard G. Long. Increased activity and expression of iNOS in human duodenal enterocytes from patients with celiac disease. Am J Physiol Gastrointest Liver Physiol 283: G319–G326, 2002. First published February 27, 2002; 10.1152/ajpgi.00324.2001.—The activity of nitric oxide synthase (NOS) was assayed in enterocytes isolated from human duodenal biopsies to determine its role in celiac disease. Patients were categorized into groups with irritable bowel syndrome, iron-deficiency anemia, B12/folate deficiency, and treated and untreated celiac disease. Enterocytes isolated from all groups showed 1400W-inhibitable Ca2+-inhibitable NOS activity with a pH level and temperature optimum of 9.4 and 37°C, respectively. Western blotting showed that enterocytes expressed the inducible NOS protein and proteins with nitrated tyrosine residues, the latter being indicative of nitric oxide-driven peroxynitrite and/or free-radical damage. Endothelial NOS was seen only in the lamina propria. Patients with celiac disease had higher NOS activity than other patient groups. Treatment of the condition led to a fall in activity. Enzyme-linked immunosorbent assay demonstrated cGMP production by the enterocyte fraction, but cGMP levels did not correlate with NOS activity. These results suggest that inducible NOS is constitutively expressed in human duodenal enterocytes, is increased in patients with untreated celiac disease, and is partially corrected when such patients are treated. We found no evidence to support a role for nitric oxide in the formation of cGMP within the small intestine. Furthermore, we were unable to demonstrate a role for peroxynitrite/free radical damage in the pathophysiology of celiac disease.

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NITRIC OXIDE (NO) synthase (NOS) catalyzes the production of NO and l-citrulline from l-arginine. Three separate isoforms of the enzyme have thus far been described. Neuronal (nNOS; NOS I) and endothelial NOS (eNOS; NOS III) are calcium dependent, produce small (nanomolar) levels of NO, and are constitutively expressed, primarily in neuronal and endothelial cells, respectively. The third isoform, inducible NOS (iNOS; NOS II), is calcium independent and, when transcription is induced, will produce large (micromolar) quantities of NO for sustained periods of time. The enzyme is classically activated (induced) by cytokine and/or endotoxin stimulation of inflammatory cells such as macrophages. It has become increasingly apparent that NO has a dichotomous role in nature, exerting both beneficial and detrimental effects within cells. However, the classic viewpoint that low levels of NO formed via the constitutive enzymes (eNOS and nNOS) are essential for normal physiological function and are therefore good, whereas prolonged excessive generation by iNOS is detrimental (23, 25), has recently been challenged (15). A number of studies now suggest that increased activity in constitutive NOS (cNOS) could, in some circumstances, be responsible for pathological tissue changes, whereas, in contrast, NO released by iNOS may play a protective role (18). Studies with genetically modified knockout mice with experimentally induced colitis show that animals deficient in eNOS and nNOS develop gastric dilation and hypertension and lack vasodilatory responses to injury, whereas iNOS-deficient animals are more susceptible to inflammatory damage but are more resistant to septic shock (19).

NO generated by NOS will stimulate soluble guanylyl cyclase (sGC) to produce cGMP, resulting in the initiation of a variety of signal cascades such as the gating of ion channels, modulation of cAMP, and regulation of Ca2+-homeostasis by activation of G protein kinase (17). However, in some cases, NO will also react with free radicals such as superoxide, which are cogenerated during its formation (4). The peroxynitrite formed as a consequence of these reactions is a highly reactive, short-lived species [half-life (T1/2) = 1.9 s at pH 7.4] that will oxidize a variety of molecules such as sulfhydryls, thiols, and ascorbate and trigger cytotoxic processes such as DNA damage and lipid peroxidation (5). Of particular relevance is the ability of peroxynitrite to attack the tyrosine residues of proteins. This reaction results in the formation of ortho-nitrotyrosine residues that are considered to be good indicators of peroxynitrite-driven cellular damage (20).

To date, the majority of studies on NO in the human gut has concentrated on NOS activity in the colon of patients with inflammatory bowel diseases such as...
ulcerative colitis and Crohn’s disease. Most studies suggest that NOS activity is elevated in these conditions when the diseases are “active” and are reduced when quiescent (22, 29). The role of NOS in diseases of the small bowel, for example celiac disease, is less well defined. With this in mind, we have assayed NOS activity in biopsies isolated from the small intestine of patients suffering various disease conditions including celiac disease. Furthermore, we have attempted to identify the isoform of the enzyme activated and have examined the fate of the NO formed.

MATERIALS AND METHODS

Patients. One hundred seventy patients with unexplained diarrhea and iron, B12, or folate deficiency underwent endoscopic biopsy from the second part of the duodenum for histological assessment. Patient demographic details are outlined in Table 1. Additional biopsies were taken for this study. Patients with giardiasis or gastroduodenal ulceration were excluded. All patients categorized as having irritable bowel syndrome (IBS) had normal duodenal histology and were considered similar to normal controls. The Nottingham City Hospital Ethics Committee approved the study. All patients gave full written informed consent for the additional biopsies, and no significant complications occurred.

Cell isolation. Enterocytes were isolated using a modification of a method previously described (30). Biopsies were collected and washed twice in prewarmed sterile citrate buffer (in mM: 1.5 KCl, 96 NaCl, 27 Na citrate, 8 KH2PO4, pH 7.4) before being transferred to a calcium-chelating buffer (in mM: 1.5 EDTA, 0.5 1,4-dithiothreitol, 10 NaH2PO4, 154 NaCl) and incubated at 37°C for 30 min. Cells released from the biopsies remained in suspension and were carefully removed from the remaining lamina propria before being washed twice in cold endotoxin-free sterile PBS (in mM: 10 NaH2PO4, 2.7 KCl, and 140 NaCl, pH 7.4). Cells were further washed in homogenization buffer (25 mM Tris, pH 7.4, 1 mM EDTA, and 1 mM EGTA) before being suspended at 1 × 10⁶ per milliliter in the same buffer. In some cases, cell homogenates were prepared by mechanically disrupting the cells using an Ultra-Turrax (Ika-Werke, Germany; 1,000 rpm for 10 s). Protein concentrations of whole cell suspensions and homogenates were determined using a modified Lowry technique (16, 26).

Histology of the cell population. Cytospin preparations were made from 1 × 10⁵ cells and stained with peroxidase-labeled monoclonal antibodies to the cytokeratin marker MNF116, CD45 (leucocyte common antigen), CD34, and actin (Dako, Ely, Cambridge, UK). Background staining was with hematoxylin.

NOS assay. NOS activity was assayed by measuring the conversion of [3H]L-arginine to [3H]L-citrulline (7). Briefly, 25 μl of whole cell suspension or homogenate were added to 110 μl of reaction mixture (25 mM Tris, pH 7.4, 200,000 dpm [3H]arginine, 12 mM L-arginine, 3 mM tetrahydrobiopterin (BH4), 1 mM flavin adenine dinucleotide, 1 mM flavin mononucleotide, 100 nM calmodulin, 1 mM NADPH, and 545 mM CaCl2 (equivalent to 75 μM free Ca²⁺)) and incubated for 60 min at 37°C. The reaction was terminated by the addition of 2 ml of ice-cold stop buffer (50 mM HEPES, pH 5.5, 5 mM EDTA, and 1 mM L-citrulline). In experiments involving whole cells, the cells were lysed by freeze thawing. Samples were loaded onto a 2-mL cation-exchange column (Dowex AG50W-X8, Na form; Bio-Rad, Hemel Hempstead, Herts, UK) preequilibrated with stop buffer, and the eluant was collected. Columns were washed twice with a further 2 ml of stop buffer, and the eluants were combined and mixed with 16 ml of scintillation cocktail (Ultima Gold, Packard Canberra, Pangbourne, Berks, UK) before being assayed for radioactivity (Minaxi Tri-Carb 4000 Series; United Technologies, Packard Canberra, Pangbourne, Berks, UK). Results were corrected for the radioactivity not retained by the columns by performing parallel experiments without tissue. All experiments were performed in duplicate.

In experiments in which inhibitors were used, isolated cells were washed twice in PBS before a single wash in L-arginine-depleted reaction mixture. Cells were suspended in arginine-depleted reaction mixture containing 1–1,000 μM of inhibitor and incubated at 37°C for 15 min before the addition of 100 μl of reaction mixture containing 200,000 dpm [3H]L-arginine and 12 mM L-arginine. The reaction was continued for a further 60 min as previously described.

Western blot analysis of NOS isoforms and nitrotyrosine. Washed whole enterocytes and lamina propria were suspended in lysis buffer (25 mM Tris, pH 7.2, 1 mM Na orthovanadate, 0.1% (vol/vol) Triton X-100 and Complete protease inhibitor cocktail (Boehringer-Mannheim, Mannheim, Germany) and disrupted by controlled sonication on ice (4 × 4-s bursts; power setting 2) using a cell disrupter (Rapidis 50, Ultrasonics, London, UK). Cell homogenates were assayed for protein (16, 26) before an equal volume of 4× Laemmli buffer was added. The samples were heated at 95°C for 5 min, and 50 μg of total cellular protein were loaded to a 7.5% (wt/vol) SDS polyacrylamide gel. Separated proteins were transferred to nitrocellulose membranes (BioTrace NT, Gelman Sciences, Northampton, UK), blocked for 4 h in 5% (wt/vol) skimmed milk in PBS + 0.1% (vol/vol) Tween 20, and

| Table 1. Patient demographics showing the split between biopsies used in the form of homogenates and those used as whole cells |
|----------------|---------------------|----------------|
| Disease        | Homogenates         | Whole Cells     |
|                | n       | Ratio | Age Range | n       | Ratio | Age Range |
| IBS            | 26      | 12:14 | 21–64      | 39 ± 22 |
| IDA            | 17      | 3:14  | 33–86      | 71 ± 30 |
| B12/folate DA  | 3       | 0:3   | 28–84      | 54 ± 28 |
| Celiac         | 2       | 1:1   | 66–72      | 69 ± 3 |
| Celiac (GFD)   | 7       | 2:5   | 37–70      | 48 ± 13 |

Patients were categorized into those diagnosed as having irritable bowel syndrome (IBS), iron deficiency anemia (IDA), B12/folate-deficient anemia (B12/folate DA), newly diagnosed celiac disease, and celiac disease patients who had been maintained on a gluten-free diet (GFD). The n value indicates the number of patients in each group, ratio indicates the split between male and female, age ranges are in years, and parentheses are median ± interquartile range.
were incubated with 440/H9262 reprobed with primary and secondary antibodies as required.

RESULTS

Assay of cGMP One hundred microliters of cell suspension were incubated with 440 μl of reaction mixture [25 mM Tris, pH 7.4, 12 mM L-arginine, 3 mM BH4, 1 mM flavin adenine dinucleotide, 1 mM flavin mononucleotide, 100 nM calmodulin, 1 mM NADPH, 545 mM CaCl2 (equivalent to 75 μM free Ca2+) and 2 mM isobutylmethyl xanthine] at 37°C for 60 min. The reaction was stopped by snap freezing in liquid nitrogen. Before assay of cGMP, samples were lyophilized and resuspended in 1.0 ml of assay buffer [0.05 M Na acetate, 0.02% (wt/vol) bovine serum albumin, and 0.5% (wt/vol) preservative]. cGMP production was determined using a commercially available enzyme immunoassay (Amersham International, Buckinghamshire, UK).

Statistics. Results are expressed as means ± SE. Statistical comparisons used a one-way ANOVA or, where appropriate, Student’s paired t-test. Correlation coefficients were calculated according to Pearson’s product moment correlation. A P value of ≤0.05 was considered significant.

RESULTS

Patient demographics. The patient demographics shown in Table 1 demonstrate that the patients included in this study were from a wide age range, with female predominance in all disease groups. No age- or sex-related differences were observed between any of the parameters measured (data not shown).

Histology. The calcium chelation method of cell isolation employed in this study yielded a near pure population of human enterocytes. A percentage of the cells (92.5 ± 2.2%) were identified as epithelial cells, staining positive for the cytokeritin marker MNF116 (n = 20). Another percentage of the cells (2.6 ± 0.8%) were leukocytes, staining positive for the common leukocyte antigen CD45 (n = 20), whereas no cells stained positive for CD34.

NO activity. All patient groups demonstrated linear citrulline production with respect to time (up to 120 min, r = 0.811, P < 0.0005 for homogenate, r = 0.988, P < 0.0005 for whole cells) and protein concentration (between 20 and 1, 120 μg/ml, r = 0.882, P < 0.0005 for homogenate, r = 0.9932, P < 0.0005 for whole cell Pearson’s correlation coefficient).

All of the enterocyte preparations studied displayed NO activity. A summary of the results for those patients presenting with diarrhea (diagnosed as IBS), iron-deficiency anemia (IDA), B12/folate deficiency, and treated and untreated celiac disease is shown in Fig. 1. Assays were performed on both whole cell enterocyte preparations and on enterocyte homogenates. Whole enterocyte preparations have the advantage that they represent a very “physiological” model, whereas homogenates allow experimental manipulation of the system without impediment of molecules, such as inhibitors, by the cell membrane. In all cases, we observed no significant difference between NO activity assayed in whole cell preparations compared with homogenates. This observation remained true for all subsequent experiments. There was also no significant difference in NO activity among the IBS, IDA, and B12/folate deficiency anemia disease groups (6.22 ± 0.59, n = 72; 10.17 ± 1.93, n = 60; 10.05 ± 1.91, n = 10 nmol citrulline·g total protein−1·min−1, respectively, for combined homogenate plus whole cell preparations). Patients who presented with newly diagnosed celiac disease had highly elevated NO activity with respect to other patient groups (94.1 ± 13.6, n = 11, P < 0.005 compared with all 3 groups, ANOVA). NO activity in patients who were already maintained on a gluten-free diet (GFD) was significantly lower (18.96 ± 5.84, n = 17) than newly diagnosed celiac patients (P < 0.005, ANOVA) but remained significantly higher than the other patient groups (P < 0.05, ANOVA).

Activity in all nonceliac patient groups remained similar even when calcium was excluded from the system. Figure 2 is a representative experiment showing the effect of calcium on NO activity in six patients presenting with IBS, in which values of 7.8 ± 1.2 vs. 8.0 ± 1.3 nmol citrulline·g protein−1·min−1 with and without 75 μM calcium, respectively (P = 0.9, Student’s t-test), were recorded. Increasing exogenous free calcium from 0 to 750 μM did not significantly alter activity [7.9 ± 0.9 vs. 8.10 ± 1.8 nmol citrulline·g protein−1·min−1 (P = 0.7, Student’s t-test)]. NO activity in patients presenting with celiac disease showed similar Ca2+ independence.
In all patients, NOS activity was temperature dependent, with optimal activity observed at 37°C. At 19°C, activity was reduced to 82.6 ± 18.6% of that seen at 37°C (n = 4). Further reduction of the incubation temperature to 2°C significantly dropped NOS activity to 2.3 ± 2.3% of that observed at 37°C (P < 0.005; Student’s t-test).

NOS activity in all patients was pH dependent. Figure 3 is representative of the results obtained from studying 11 patients presenting with IBS. Activity (expressed as a percentage of that seen at pH 7.4) was inhibited at acidic pH (by 46.2 ± 11.4% at pH 6.4, n = 11) and stimulated at alkaline pH (by 59.4 ± 32.4% at pH 9.4, n = 11).

When 1–100 mM of the NOS inhibitor Nω-nitro-L-arginine methyl ester (L-NAME) was added to the reaction mixture, citrulline production in both homogenates and whole cell preparations of all patient groups was inhibited in a dose-dependent manner. Figure 4 shows representative results obtained from studying the effect of L-NAME on six patients presenting with IBS. In our system, the compound displayed an IC50 of ~6.96 mM. Inhibition of citrulline production was also observed with the broad-range NOS inhibitor Nω-nitro-L-arginine (L-NNA; IC50 = 10 μM) and the selective iNOS inhibitor 1400W (IC50 = 5 μM).

NOS activity was highly dependent on the cofactor NADPH. Omitting this from the reaction mixture significantly reduced NOS activity by 59.9 ± 8.2% (n = 7, P < 0.0005, Student’s t-test). The individual removal of...
FAD, FMN, BH₄, or calmodulin had no significant effect on NOS activity (Fig. 5).

Assay for cGMP. cGMP was assayed in enterocytes isolated from 16 patients presenting with IBS and 10 patients with IDA. In the presence of 2 mM IBMX, the cells produced 11.60 ± 1.70 nmol cGMP·g total protein⁻¹·h⁻¹ (mean ± SE). When NOS activity was assayed in these same samples, a mean activity of 6.56 ± 0.68 nmol citrulline·g total protein⁻¹·min⁻¹ was recorded. We were, however, unable to demonstrate a statistically significant correlation between cGMP generation and NOS activity (r = 0.22, Pearson correlation coefficient; data not shown).

Western blot analysis of NOS isoforms and peroxynitrite damage. Enterocyte preparations from all patients were negative for eNOS. Conversely, the lamina propria remaining after enterocyte removal consistently showed a 140-kDa band corresponding to eNOS (Fig. 6A). Laser densitometry of the eNOS band in lamina propria indicated that the intensity remained constant irrespective of disease condition (Table 2). When blots were stripped and reprobed with a monoclonal antibody to the 130-kDa iNOS protein, the enterocyte preparations consistently stained positive (Fig. 6B). In some instances (8 samples from a total of 30), weak positive staining was also observed in the lamina propria. This staining probably represents the incomplete removal of the enterocyte population from the lamina propria. Densitometry analysis of band intensities (Table 2) clearly showed that enterocytes isolated from patients presenting with newly diagnosed celiac disease expressed more iNOS protein than other patient groups. Furthermore, the amount of protein expressed was reduced in patients who had been maintained on a GFD (Table 2). Neither enterocyte preparations nor lamina propria stained for the nNOS isoform.

Table 2. Densitometry results of scanning Western blots for the lamina propria-localized 140-kDa eNOS protein and the enterocyte-localized 130-kDa iNOS protein

<table>
<thead>
<tr>
<th></th>
<th>IBS</th>
<th>IDA</th>
<th>B₁₂/folate DA</th>
<th>CD</th>
<th>CD (GFD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>eNOS</td>
<td>108.6 ± 8.6</td>
<td>99.9 ± 0.5</td>
<td>107.1 ± 15.0</td>
<td>102.1 ± 5.1</td>
<td>94.6 ± 2.2</td>
</tr>
<tr>
<td>iNOS</td>
<td>67.8 ± 13.2</td>
<td>52.2 ± 24.0</td>
<td>59.7 ± 22.3</td>
<td>163.6 ± 12.0</td>
<td>123.2 ± 6.3</td>
</tr>
</tbody>
</table>

Results are expressed in arbitrary units ± SE with respect to the expression of a control protein (actin). Patients were categorized into those having IBS (n = 14), IDA (n = 6), B₁₂/folate DA (n = 4), newly diagnosed celiac disease (CD, n = 4), and celiac disease patients who had been maintained on a gluten free diet (CD GFD, n = 2). eNOS, endothelial NOS; iNOS, inducible NOS.
All enterocyte preparations showed positive staining for nitrotyrosine (Fig. 7A). Staining was particularly marked between 40 and 70 kDa in all samples tested. Patients newly diagnosed with celiac disease (lane 1) did not appear to express more nitrated proteins than enterocytes isolated from other patient groups such as IDA (lanes 2–4) and IBS (lanes 5–7). Enterocyte nitrotyrosine staining was completely blocked by the addition of 10 mM nitrotyrosine to the primary antibody mix. When lamina propria samples were probed with the polyclonal anti-nitrotyrosine antibody, all samples were negative (Fig. 7B).

**DISCUSSION**

In this study, we demonstrated that in a nearly pure population of enterocytes isolated from human duodenal biopsies, NOS activity is expressed. More than 90% of cells isolated by the calcium chelation method employed were identified as epithelial cells, with preparations routinely containing <3% leukocytes. We have previously demonstrated that a highly purified population of enterocytes was produced using the same isolation technique and a different cytokeratin marker (30). It, therefore, seems most likely that the NOS activity measured in these preparations is derived from enterocytes with minimal or no contribution from contaminating leukocytes.

Characterization of NOS activity in enterocytes showed it to be independent of Ca2+, have a pH optimum of 9.4, a temperature optimum of 37°C, and to require exogenous NADPH. Activity was inhibited by L-NMMA and L-NNA, two broad-spectrum, widely used NOS inhibitors (1, 6). In this study, we report an IC50 for L-NNA that is nearly 700-fold less than that of L-NMMA (10 μM vs. 6.9 mM, respectively). The reason for this difference in sensitivity toward L-NMMA and L-NMMA lies with the fact that the potency of L-NMMA is greatly increased by the action of cellular esterases that hydrolyze the compound to the more active L-NMMA (12). At concentrations of 1 mM, L-NMMA will inhibit both iNOS and cNOS. There is a moderate increase in selectivity toward cNOS at lower concentrations of the compound (24); however, it is generally accepted that L-NMMA shows no useful NOS isofrom specificity (1).

NOS activity was also inhibited by 1400W, a slow, tight-binding selective inhibitor of iNOS (11). 1400W is more than 5,000- and 200-fold more selective for iNOS relative to cNOS and nNOS, respectively. We estimate the IC50 of this compound for human duodenal enterocytes to be 5 μM, which is in good agreement with the IC50 reported to induce contraction in endothelium-removed, LPS-treated rat aortic rings (IC50 0.8 ± 0.3 μM) (11). These data, together with the Ca2+-independent nature of enterocyte NOS activity, strongly suggest that it is a result of iNOS activation.

Western blotting confirmed that the NOS activity seen in duodenal enterocytes was accompanied by increased expression of the iNOS protein. Monoclonal antibodies detected a clear 130-kDa band in all enterocyte preparations that comigrated with an iNOS-positive control (IFN-γ-stimulated mouse macrophage cell lysate). Previous workers have reported constitutive expression of iNOS mRNA and protein in the ileum (but not jejunum or colon) of mice (13, 21). More recent studies also suggest constitutive expression of this enzyme in the normal human colon (28). It is accepted that iNOS protein expression correlates well with inflammation; however, conditions such as IBS, IDA, and B12/folate deficiency are not classified as inflammatory conditions. The fact that the epithelial cells of the gut are constantly exposed to foreign antigens may explain why these histologically normal biopsies express iNOS protein and would indicate that the protein has a role in normal intestinal homeostatic function.

iNOS is recognized as an enzyme that is activated in response to stimuli such as cytokines and endotoxin (25), the protein generally requires 6–8 h for full activation (27). The possibility should therefore be considered that iNOS was induced in samples during their removal and/or enterocyte stripping. Biopsies were prepared under sterile conditions using endotoxin free reagents and were also processed within 2 h of removal to minimize this possibility. When the lamina propria was examined after enterocyte removal, ~70% (22 of 30) of the samples tested showed no iNOS staining. The remaining eight samples showed only weak iNOS staining. Enterocyte or leukocyte contamination may explain the presence of low levels of iNOS in these samples.

In this study, for ethical reasons, we used biopsies removed from patients with IBS as a “disease” control group. In animal models, NO has been shown produce both contraction and dilation in the small intestine (2, 14). As IBS is associated with disordered gut motility, there is a possibility that enterocyte iNOS expression seen in these patients was secondary to their disorder. This would, however, seem unlikely for two reasons. First, this study involved examination of surface epithelial cells rather than the underlying muscle cells. Second, patients with both iron and B12/folate deficiency anemia (who showed no indications of disorders) also express iNOS.

Of the other two isoforms of NOS, only the lamina propria of samples stained positive for the 140-kDa eNOS protein, whereas neither enterocytes nor lamina propria stained for the 155-kDa nNOS protein. These data suggest that NOS isoforms have a clearly defined tissue distribution within the gut mucosa.

One of the most important findings of this study is the increase in NOS activity seen in enterocytes isolated from patients with celiac disease compared with other disease groups. This increased activity of NOS was accompanied by increased expression of the iNOS protein (as shown by Western blotting and densitometry). When patients were maintained on a GFD, iNOS activity and protein expression were reduced compared with untreated patients but still remained higher than in other disease groups. The increase in iNOS activity and protein expression in enterocytes isolated from celiac patients was specific for the inducible isoform and specific to the enterocyte population. eNOS expres-
sion in the lamina propria was unaffected by the presence of celiac disease. The patients studied in this group were taking a GFD for periods ranging from 6 mo to several years. Whether or not long-term adherence to a GFD would result in a return to “normal” iNOS activity is a matter for further study.

Other groups have previously reported on the generation of NO and the distribution of different NOS isoforms in celiac patients using other methods. Beckett et al. (3) have demonstrated that cultured celiac biopsies generate more nitrite than control biopsies; furthermore, this nitrite production can be increased by coculture with a peptic/tryptic digest of gluten and reduced by incubation with the NOS inhibitor l-NMMA. The data presented in this study support that of Steege et al. (31), whose immunohistochemical study demonstrated that duodenal biopsies removed from children suffering with celiac disease show colocalization of iNOS and nitrotyrosine within the enterocyte population. Our data also suggests that nitrotyrosine staining is localized with iNOS within the enterocyte population. If this is so, the source of superoxide required for peroxynitrite generation is questionable. Because the superoxide anion has such a short $T_{1/2}$ (<1 s), it is doubtful that it is generated by infiltrating immune cells within the lamina propria because it would be destroyed before it was able to reach the site of NO generation (i.e., the enterocytes). Deitch et al. (9) have presented evidence to suggest that intestinal epithelial cells may be capable of superoxide production. This offers an attractive explanation for the localized iNOS/nitrotyrosine staining reported by us and others (31). In this study, we report nitrotyrosine staining in enterocytes isolated from all patient groups, but we were unable to demonstrate a positive relationship between nitrotyrosine levels and NOS activity. This observation questions either the contribution of peroxynitrite to tissue damage or the relationship between peroxynitrite and nitrotyrosine. The fact that recent evidence suggests that ortho-nitrotyrosine may be formed from a number of sources that do not necessarily involve peroxynitrite, e.g., the reaction between nitrite and hypochlorous acid (10, 32), may go in some way to explain our findings.

Physiologically, perhaps the most relevant action of NO is the activation of sGC by nitrosation of its heme moiety (17). The subsequent increase in cGMP alters the activity of three main target proteins: cGMP-regulated ion channels, cGMP-regulated phosphodiesterases, and cGMP-dependent protein kinases. In turn, a number of signaling cascades is operated that remain essential for correct cellular function (17). Of particular relevance to this study is the fact that elevated cGMP levels can lead to hypersecretion and diarrhea; both of these symptoms are closely associated with celiac disease (8). In this study, however, we were unable to demonstrate a positive correlation between NOS activity and cGMP generation in isolated duodenal enterocytes. Our in vitro system consisted predominantly of enterocytes with some goblet cells. Our observations may be explained by the fact that the guanylyl cyclase in enterocytes is mainly particulate, a form not activated by NO. The relatively low level of cGMP that we were able to detect in our enterocyte preparations may result from the activity of goblet cells that contain soluble guanylyl cyclase, a form activated by NO. We did not, however, routinely estimate the amount of goblet cells in our enterocyte preparations; therefore, a direct comparison between goblet cell number and cGMP was not performed.

In summary, we have shown that histologically normal human duodenal enterocytes express NOS activity. Activity is increased in patients with untreated celiac disease and is partially corrected when patients are maintained on a GFD. Increased and decreased NOS activity is paralleled by increased and decreased expression of the iNOS protein. We were unable to demonstrate that NOS activity was associated with cGMP generation in all of the patient groups that we studied. We did find evidence to suggest nitrated proteins within the enterocyte population but again failed to find evidence that this was correlated to NOS activity.

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