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Increased Activity and Expression of Inducible Nitric Oxide Synthase in Human Duodenal Enterocytes from Patients with Celiac Disease

by

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Short title: Nitric oxide synthase in human duodenal enterocytes

Abbreviations:

sGC- soluble guanylate cyclase; cGMP – cyclic guanosine 3’, 5’ monophosphate; NO – nitric oxide; NOS – nitric oxide synthase.

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Abstract:

Background & Aims: The activity of nitric oxide synthase was assayed in enterocytes isolated from human duodenal biopsies to determine its role in celiac disease. Patients were categorized into those with irritable bowel syndrome, iron deficiency anemia, B_{12}/folate deficiency and treated and untreated celiac disease.

Methods & Results: Enterocytes isolated from all groups showed 1400W-inhibitable, Ca^{2+}-independent nitric oxide synthase activity with a pH and temperature optimum of 9.4 and 37°C respectively. Western blotting showed that enterocytes expressed the inducible nitric oxide synthase protein and proteins with nitrated tyrosine residues, the latter being indicative of nitric oxide-driven peroxynitrite and/or free radical damage. Endothelial nitric oxide synthase was seen only in the lamina propria. Patients with celiac disease had higher nitric oxide synthase activity than other patient groups. Treatment of the condition led to a fall in activity. Enzyme-linked immunosorbent assay demonstrated cyclic guanosine monophosphate (cGMP) production by the enterocyte fraction but cGMP levels did not correlate with NOS activity.

Conclusion: These results suggest that inducible nitric oxide synthase 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.

Key words: small bowel, malabsorption, nitric oxide, peroxynitrite, cyclic GMP

Introduction

Nitric oxide synthase (NOS) catalyzes the production of nitric oxide (NO) and L-citrulline from L-arginine. Three separate isoforms of the enzyme have so far been described. nNOS (NOS I) and 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, 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 classical viewpoint that low levels of NO formed via the constitutive enzymes (e and nNOS) are essential for normal physiological function and are therefore good, whilst prolonged excessive generation by iNOS is detrimental [1,2] has recently been challenged [3]. A number of studies now suggest that increased activity in cNOS could, in some circumstances, be responsible for pathological tissue changes, whilst in contrast NO released by iNOS may play a protective role[4]. Studies with genetically modified knock out mice with experimentally-induced colitis show that animals deficient in e and n NOS develop gastric dilation, hypertension and lack vasodilatory responses to injury whereas iNOS deficient animals are more susceptible to inflammatory damage but are more resistant to septic shock[5].

NO generated by NOS will stimulate soluble guanylyl cyclase (sGC) to produce cyclic guanosine monophosphate (cGMP) resulting in the initiation of a variety of signal cascades such as the gating of ion channels, modulation of cAMP and regulation of Ca^{2+} homeostasis by activation of G protein kinase[6]. However, in some cases NO will also react with free radicals such as superoxide that are co-generated during its formation[7]. The peroxynitrite formed as a consequence of these reactions is a highly reactive, short-lived species ($T_{1/2}$=1.9

secs at pH 7.4) that will oxidise a variety of molecules such as sulfhydryls, thiols and ascorbate and trigger cytotoxic processes such as DNA damage and lipid peroxidation[8]. 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[9].

To date the majority of studies on NO in the human gut have concentrated upon NOS activity in the colon of patients with inflammatory bowel disease 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[10,11]. 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 & Methods

Patients: 170 patients with unexplained diarrhea, iron, B_{12} 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 gastro-duodenal 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 we have previously described[9]. Biopsies were collected and washed twice in pre-warmed sterile citrate buffer (1.5mM KCl, 96mM NaCl, 27mM Na citrate, 8mM KH_2PO_4, 5.6mM Na_2HPO_4, pH7.4) before being transferred to a calcium-chelating buffer (1.5mM EDTA, 0.5mM DTT, 10mM NaH_2PO_4, 154mM NaCl) and incubated at 37°C for 30 mins. 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 phosphate buffered saline (10mM NaH_2PO_4, 2.7mM KCl, 140mM NaCl, pH 7.4 - PBS). Cells were further washed in homogenization buffer (25mM Tris, pH 7.4, 1mM EDTA, 1mM EGTA) before being counted and suspended at $1x10^6$ ml^{-1} in the same buffer. In some cases cell homogenates were prepared by mechanically disrupting the cells using an Ultra-Turrax (Ika-Werke, Germany - 1000 rpm for 10 secs). Protein concentrations of whole cell suspensions and homogenates were determined using a modified Lowry technique[13,14].

Histology of the cell population: Cytospin preparations were made from $1x10^5$ cells and stained with peroxidase-labelled monoclonal antibodies to the cytokeratin marker MNF116,

CD45 (leucocyte common antigen), CD34 and actin (Dako Ltd., Ely, Cambs. UK). Background staining was with hematoxylin.

Nitric Oxide Synthase Assay: NOS activity was assayed by measuring the conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline[15]. Briefly 25µL of whole cell suspension or homogenate was added to 110µL of reaction mixture (25mM Tris, pH 7.4, 200,000 dpm [^{3}H]-arginine, 12mM L-arginine, 3mM tetrahydrobiopterin (BH_4), 1mM flavin adenine dinucleotide , 1mM flavin mononucleotide, 100nM calmodulin, 1mM NADPH and 545mM $CaCl_2$ (equivalent to 75µM free Ca^{2+})) and incubated for 60 mins at 37°C. The reaction was terminated by the addition of 2mls of ice cold stop buffer (50mM HEPES, pH 5.5, 5mM EDTA, 1mM L-citrulline). In experiments involving whole cells, the cells were lysed by freeze thawing. Samples were loaded onto a 2ml cation exchange column (Dowex AG50W-X8, Na form, Bio-Rad, Hemel Hempstead, Herts, UK.) pre-equilibrated with stop buffer and the eluant collected. Columns were washed twice with a further 2mls of stop buffer and the eluants combined and mixed with 16ml of scintillation cocktail (Ultima Gold, Packard Canberra, Pangbourne, Berks, UK.) before being assayed for radioactivity (Minaxi Tri-Carb 4000 Series, United Technologies, Packard Canberra). 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 where 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 1-1000µM of inhibitor and incubated at 37°C for 15 mins before the addition of 100µL of reaction mixture containing 200,000 dpm [^{3}H]-L-arginine and 12mM L-arginine. The reaction was continued for a further 60 mins as previously described.

Western Blot Analysis of NOS isotypes and Nitrotyrosine: Washed whole enterocytes and lamina propria were suspended in lysis buffer (25mM Tris, pH 7.2, 1mM Na orthovanidate, 0.1%[vol/vol] triton X-100 and COMPLETE^{TM} protease inhibitor cocktail (Boehringer Meinheim GmbH, Meinheim, Germany)) and disrupted by controlled sonication on ice (4 x 4 second bursts: power setting 2) using a cell disrupter (Rapidis 50, Ultrasonics, London, England, UK). Cell homogenates were assayed for protein[13,14] before an equal volume of x4 Laemmli buffer was added. The samples were heated at 95°C for 5 mins and 50μg of total cellular protein was loaded to a 7.5%[wt/vol] SDS polyacrylamide gel. Separated proteins were transferred to nitrocellulose membranes (BioTrace NT, Gelman Sciences, Northampton, England, UK), blocked for 4 hour in 5%[wt/vol] skimmed milk in PBS plus 0.1%vol/vol Tween-20, and probed with the desired antibodies. Monoclonal antibodies to eNOS, iNOS and nNOS (Transduction Laboratories, Lexington, KY, USA) were used at 1:1000, polyclonal anti-nitrotyrosine (Upstate Biotechnology, Lake Placid, NY, USA) was used at 2μgml^{-1}, monoclonal anti-actin (Santa Cruz Biotechnology, Santa Cruz, California, USA) was used at a concentration of 1:500. Secondary horse radish peroxidase-linked antibodies were used at 1:5000 (Transduction Laboratories, Lexington, KY, USA). Bound antibodies were detected by enhanced chemiluminescence (Supersignal, Pierce, Rockford, IL, USA). Once visualized blots were stripped of the primary:secondary antibody complex by exposure to 62.5mM tris-HCl (pH 6.7) containing 100mM mercaptoethanol and 2%[wt/vol] SDS for 20 mins at 50°C. Stripped blots were re-probed with primary and secondary antibodies as required.

Assay of cGMP: 100μL of cell suspension was incubated with 440μL of reaction mixture (25mM Tris, pH 7.4, 12mM L-arginine, 3mM tetrahydrobiopterin (BH_4), 1mM flavin adenine dinucleotide , 1mM flavin mononucleotide, 100nM calmodulin, 1mM NADPH, 545mM $CaCl_2$ (equivalent to 75μM free Ca^{2+}) and 2mM isobutylmethyl xanthine) at 37°C for 60

mins. The reaction was stopped by snap freezing in liquid nitrogen. Before assay of cGMP samples were lyophilised and resuspended in 1.0ml of assay buffer (0.05M 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 mean±SEM. Statistical comparisons used a one-way analysis of variance (ANOVA) or where appropriate Student's paired t-test. Correlation coefficients were calculated according to Pearson's product moment correlation. A value of $p \leq 0.05$ 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. 92.5±2.2% of cells were identified as epithelial cells, staining positive for the cytokeritin marker MNF116 (n=20). 2.6±0.8% of the cells were leukocytes, staining positive for the common leukocyte antigen CD45 (n=20) whilst no cells stained positive for CD34.

Nitric Oxide Activity: All patient groups demonstrated linear **c**itrulline production with respect to time (up to 120 mins, r=0.811,p<0.0005 for homogenate, r=0.988,p<0.0005 for whole cells) and with respect to protein concentration (between 20-1120μgml^{-1}, 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 NOS activity. A summary of the results for those patients presenting with diarrhoea (diagnosed as irritable bowel syndrome - IBS), iron deficiency (IDA), B_{12}/folate deficiency, treated and untreated celiac disease are shown in figure 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, whilst homogenates allow experimental manipulation of the system without impedance of molecules, such as inhibitors, by the cell membrane. In all cases we observed no significant difference between NOS activity assayed in whole cell preparations compared with homogenates - this observation remained true for all subsequent experiments. There was also no significant difference in NOS activity between the IBS, IDA and B_{12}/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^{-1} total protein min^{-1} respectively for combined homogenate plus whole cell preparations). Patients who presented with newly diagnosed celiac disease had highly elevated NOS activity with respect to other patient groups (94.1±13.6, n=11, $p<0.005$ when compared to all three groups, ANOVA). NOS activity in patients who were already maintained on a gluten free diet 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 non-celiac patient groups remained similar even when calcium was excluded from the system. Figure 2 is a representative experiment showing the effect of calcium upon NOS activity in 6 patients presenting with IBS in which values of 7.8±1.2 v 8.0±1.3 nmole citrulline g^{-1}protein min^{-1} with and without 75µM calcium respectively, p=0.9, Student t-test) were recorded. Increasing exogenous free calcium from 0 to 750µM did not significantly alter activity (7.9±0.9 v 8.10±1.8 nmole citrulline g^{-1}protein min^{-1}, p=0.7, Student t test). NOS activity in patients presenting with celiac disease showed similar Ca^{2+} 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 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 % 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 1-100mM of the NOS inhibitor N^{ω}-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 6 patients presenting with IBS. In our system the compound displayed an IC_{50} (concentration producing an inhibition of 50% of that seen in the absence of inhibitor) of approximately 6.96mM. Inhibition of citrulline production was also observed with the broad-range NOS inhibitor L-NNA (N^{ω}-nitro L-arginine) (IC_{50}=10µM) and the selective iNOS inhibitor 1400W (IC_{50}=5µM).

NOS activity was highly dependent upon the co-factor NADPH. Omitting this from the reaction mixture significantly reduced NOS activity by 59.9±8.2% (n=7, $p<0.0005$, Student t test). The individual removal of FAD, FMN, BH_4 or calmodulin had no significant effect upon NOS activity (figure 5).

Assay for Cyclic GMP: Cyclic GMP was assayed in enterocytes isolated from 16 patients presenting with IBS and 10 patients with IDA. In the presence of 2mM IBMX the cells produced mean±SEM of 11.60±1.70 nmol cGMP g^{-1} total protein $hour^{-1}$. When NOS activity was assayed in these same samples a mean activity of 6.56±0.68 nmol citrulline g^{-1}total protein 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 140kDa band corresponding to eNOS (figure 6.A.). 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 130kDa iNOS protein the enterocyte preparations consistently stained positive (figure 6.B.). 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 gluten free diet (table 2). Neither enterocyte preparations nor lamina propria stained for the nNOS isoform.

All enterocyte preparations showed positive staining for nitrotyrosine (figure 7.A.). Staining was particularly marked between 40 and 70kDa 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 iron deficiency anaemia (lanes 2-4) and IBS (lanes 5-7). Enterocyte nitrotyrosine staining was completely blocked by the addition of 10mM nitrotyrosine to the primary antibody mix. When lamina propria samples were probed with the polyclonal anti-nitrotyrosine antibody all samples were negative (figure 7.B.).

Discussion.

In this study we demonstrate that in a near 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 less than 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[12]. 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 Ca^{2+}, have a pH optimum of 9.4, a temperature optimum of 37°C and to require exogenous NADPH. Activity was inhibited by L-NAME and L-NNA two broad-spectrum, widely used NOS inhibitors[16,17]. In this study we report an IC_{50} for L-NNA that is nearly 700 fold less than that of L-NAME (10μM versus 6.9mM respectively). The reason for this difference in sensitivity towards L-NNA and L-NAME lies with the fact that the potency of L-NAME is greatly increased by the action of cellular esterases that hydrolyse the compound to the more active L-NNA[18]. At concentrations of 1mM L-NNA will inhibit both iNOS and cNOS. There is a moderate increase in selectivity towards cNOS at lower concentrations of the compound[19], however it is generally accepted that L-NNA shows no useful NOS isoform specificity[17]. NOS activity was also inhibited by 1400W, a slow, tight binding selective inhibitor of iNOS[20]. 1400W is more than 5000 and 200-fold more selective for iNOS relative to eNOS and nNOS respectively. We estimate the IC_{50} of this compound for human duodenal enterocytes to be 5μM which is in good agreement with the IC_{50} reported to induce contraction in endothelium removed, LPS-treated rat aortic rings (IC_{50} 0.8±0.3μM)[20]. This

data, together with the Ca^{2+}-independent nature of enterocyte NOS activity strongly suggests 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 130kDa band in all enterocyte preparations that co-migrated with an iNOS positive control (IFNγ-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[21,22]. More recent studies also suggest constitutive expression of this enzyme in the normal human colon[23]. It is accepted that iNOS protein expression correlates well with inflammation, however conditions such as IBS, IDA and B_{12}/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 recognised as an enzyme that is activated in response to stimuli such as cytokines and endotoxin[1], the protein generally requires 6-8 hours for full activation[24]. 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 two hours of removal in order to minimise this possibility. When the lamina propria was examined after enterocyte removal, approximately 70% (22 out of 30) of the samples tested showed no iNOS staining. The remaining 8 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[25,26]. 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. Firstly, this study involved examination of surface epithelial cells rather than the underlying muscle cells and secondly, patients with both iron and B_{12}/folate deficiency anaemia (who showed no indications of disordered gut motility) also express iNOS.

Of the other two isoforms of NOS only the lamina propria of samples stained positive for the 140kDa eNOS protein while neither enterocytes nor lamina propria stained for the 155kDa 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 to 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 gluten free diet (GFD) iNOS activity and protein expression was reduced compared to 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 expression in the lamina propria was unaffected by the presence of celiac disease. The patients studied in this group were taking a gluten free diet for periods ranging from 6 months to several years. Whether or not long-term adherence to a gluten free diet would result in a return to “normal” iNOS activity is a matter for further study.

Other groups have previously reported upon the generation of NO and the distribution of different NOS isoforms in celiac patients using other methods. Beckett *et al*[27] have demonstrated that cultured celiac biopsies generate more nitrite than control biopsies,

furthermore this nitrite production can be increased by co-culture with a peptic/tryptic digest of gluten and reduced by incubation with the NOS inhibitor L-NMMA. The data presented in this study supports that of Forget *et al* whose immunohistochemical study[28] demonstrated that duodenal biopsies removed from children suffering with celiac disease show co-localization 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. Since the superoxide anion has such a short half life ($T_{1/2}$=<1 sec) 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 (ie. the enterocytes). Deitch *et al*[29] have presented evidence to suggest that intestinal epithelial cells may be capable of superoxide production. This offers an attractive explanation for the localised iNOS/nitrotyrosine staining reported by us and others[28]. 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 eg. the reaction between nitrite and hypochlorous acid[30,31] 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[6]. 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 signalling cascades are operated that remain essential for correct cellular function[6]. Of particular relevance to this study is the fact that elivated cGMP levels can lead to hypersecretion and diarrhoea - both of these symptoms

are closely associated with celiac disease[32]. 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 GC 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 GC, a form activated by NO. We did not however routinely estimate the amount of goblet cells in our enterocyte preparations so 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 gluten free diet. 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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Figure Legends

Figure 1: NOS activity in tissue biopsies removed from the small intestine of patients with irritable bowel syndrome (IBS), iron deficiency anemia (IDA), B_{12}/folate deficient anemia (B_{12}Folate DA), newly diagnosed celiac disease (untreated) and celiac patients that had been maintained on a gluten free diet (GFD). NOS activity was assayed in both whole enterocytes (hatched bars) and in enterocyte homogenates (solid bars) by the conversion of [^{3}H]-arginine to [^{3}H]-citrulline. The results, expressed as nmol of citrulline produced g^{-1} total protein min^{-1}, are presented as means±SEM, the number of patients in each sample group (n) is given above each bar.

Figure 2: The effect of free Ca^{2+} on NOS activity in enterocyte homogenates isolated from patients presenting with irritable bowel syndrome (n=6). The results, expressed as the % of citrulline generated at 75µM free Ca^{2+} are presented as means±SEM. Similar results were obtained using both whole enterocytes and/or enterocyte homogenates isolated from other patient groups.

Figure 3: The effect of pH upon NOS activity in enterocyte homogenates isolated from patients presenting with irritable bowel syndrome (n=11). The results, expressed as the % of citrulline generated at pH 7.4 are presented as means±SEM. Similar results were obtained using both whole enterocytes and/or enterocyte homogenates isolated from other patient groups.

Figure 4: The effect of the NOS inhibitor L-NAME on NOS activity in enterocyte homogenates isolated from patients presenting with irritable bowel syndrome (n=6). The results, expressed as the % of citrulline generated in the absence of L-NAME are presented as

means±SEM. Similar results were obtained using both whole enterocytes and/or enterocyte homogenates isolated from other patient groups.

Figure 5: The effect of omitting NADPH, flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), tetrahydrobiopterin (BH_4) and calmodulin upon NOS activity in enterocyte homogenates isolated from patients presenting with irritable bowel syndrome (n=7). The results, expressed as the % of citrulline generated with all co-factors present (column 1) are presented as means±SEM. An asterix indicates a statistically significantly different value from control ($p<0.05$). Similar results were obtained using both whole enterocytes and/or enterocyte homogenates isolated from other patient groups.

Figure 6: Western blot analysis of NOS isoforms in enterocytes and lamina propria isolated from 4 patients. Enterocytes (lanes 1, 3, 5 and 7) and lamina propria (lanes 2, 4, 6 and 8) were prepared as described in Materials & Methods. Proteins were separated on a 7.5% polyacrylamide gel and transferred to nitrocellulose before being probed with monoclonal antibodies to eNOS (figure 6.A.) and iNOS (figure 6.B.). The blots demonstrate the results of 4 representative patients (from a total of 30). Patients b and c were diagnosed as having celiac disease, whilst patients a and d were diagnosed as having IBS.

Figure 7: Western blot analysis of nitrated protein tyrosine residues in enterocytes (figure 7.A.) and lamina propria (figure 7.B.). Samples were prepared as described in Materials & Methods. Blots were probed with a polyclonal antibody to nitrotyrosine residues. The blots demonstrate the results of 7 representative patients (from a total of 30). Patient 1 was diagnosed as having celiac disease, patients 2 and 3 iron deficiency anaemia and patients 4, 5, 6 and 7 IBS.

Table 1: Patient demographics showing the split between biopsies used in the form of homogenates and those used as whole cells.

	Homogenates			Whole Cells		
Disease	**n**	**Ratio (m:f)**	**Age(years) range (median ± IQR)**	**n**	**Ratio (m:f)**	**Age(years) range (median ± IQR)**
IBS	26	12:14	21-64 (39 ± 22)	46	19:27	22-77 (43 ± 27)
IDA	17	3:14	33-86 (71 ± 30)	43	17:26	21-78 (59 ± 23)
B_{12}/folate DA	3	0:3	28-84 (54 ± 28)	7	0:7	28-77 (48 ± 17)
Celiac	2	1:1	66-72 (69 ± 3)	9	3:6	30-55 (47 ± 11)
Celiac (GFD)	7	2:5	37-70 (48 ± 13)	10	2:8	27-72 (50 ± 22)

Patients were categorized into those diagnosed as having irritable bowel syndrome (IBS), iron deficiency anaemia (IDA), B_{12}/folate deficient anaemia (B_{12}/folate DA), newly diagnosed celiac disease and celiac disease patients who had been maintained upon a gluten free diet (GFD). The n value indicates the number of patients in each group, ratio (m:f) indicates the split between male and female, IQR indicates the inter-quartile range.

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

	IBS	**IDA**	**B_{12}/folate DA**	**CD**	**CD (GFD)**
eNOS	108.6±8.6	99.9±0.5	107.1±15.0	102.1±5.1	94.6±2.2
iNOS	67.8±13.2	52.2±24.0	59.7±22.3	163.6±12.0	123.2±6.3

Results are expressed in arbitrary units±SEM with respect to the expression of a control protein (actin). Patients were categorized into those having irritable bowel syndrome (IBS) (n= 14), iron deficiency anaemia (IDA) (n= 6), B_{12}/folate deficient anaemia (B_{12}/folate DA) (n= 4), newly diagnosed celiac disease (CD, n=4) and celiac disease patients who had been maintained upon a gluten free diet (CD GFD, n=2).

Figure 1:

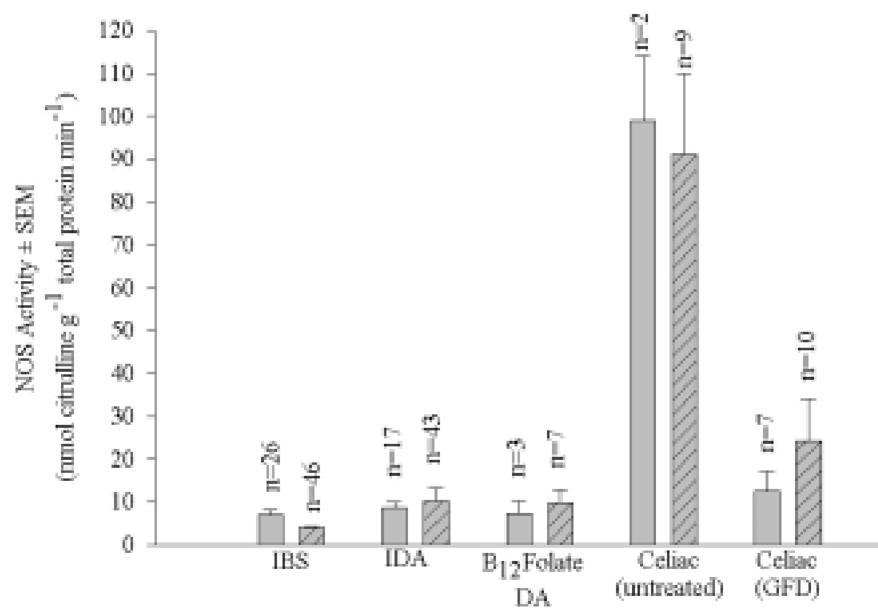

Figure 2:

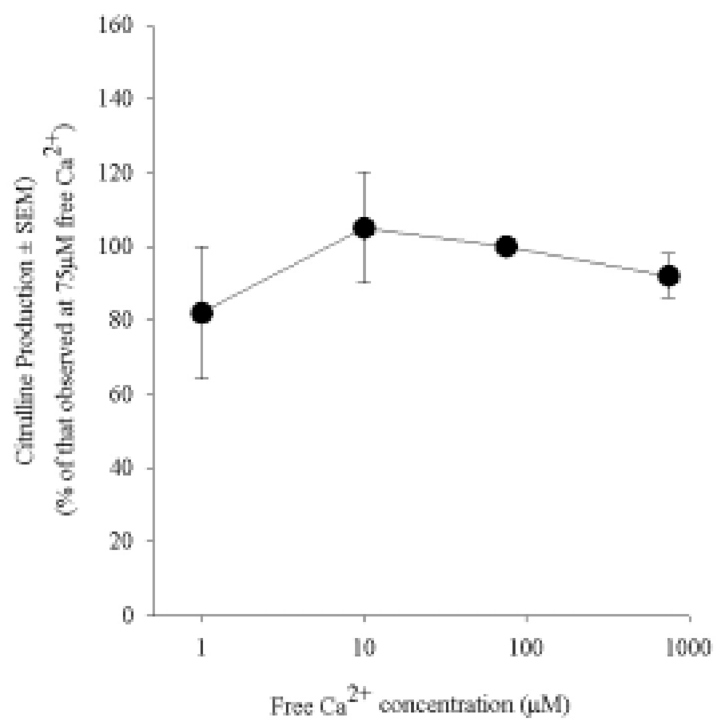

Figure 3:

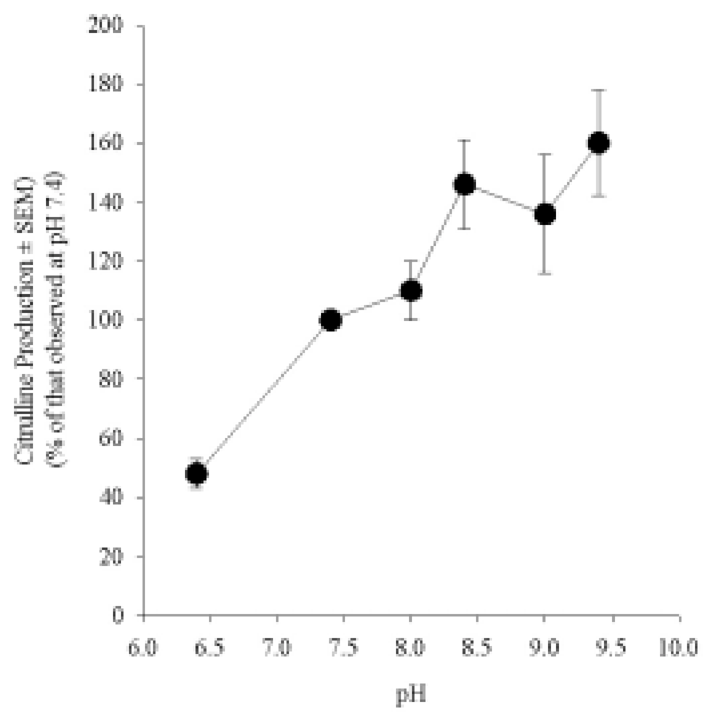

Figure 4:

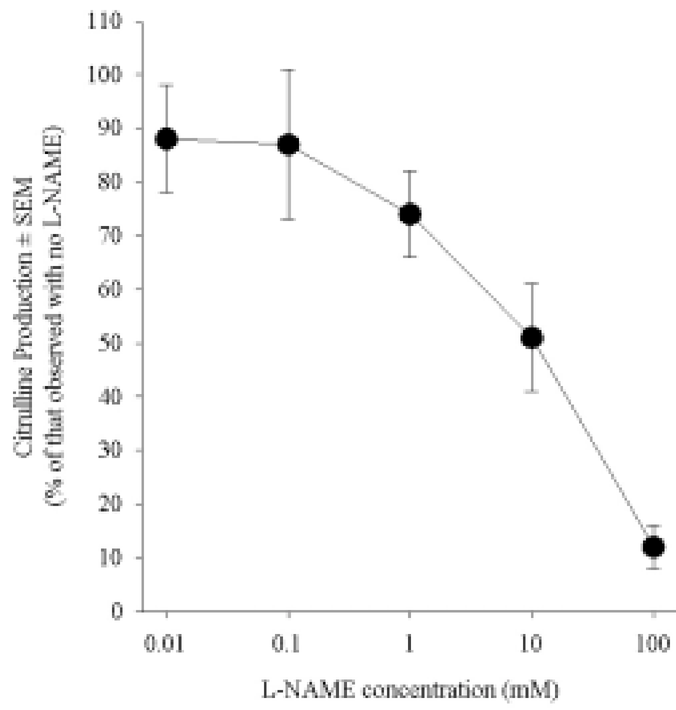

Figure 5:

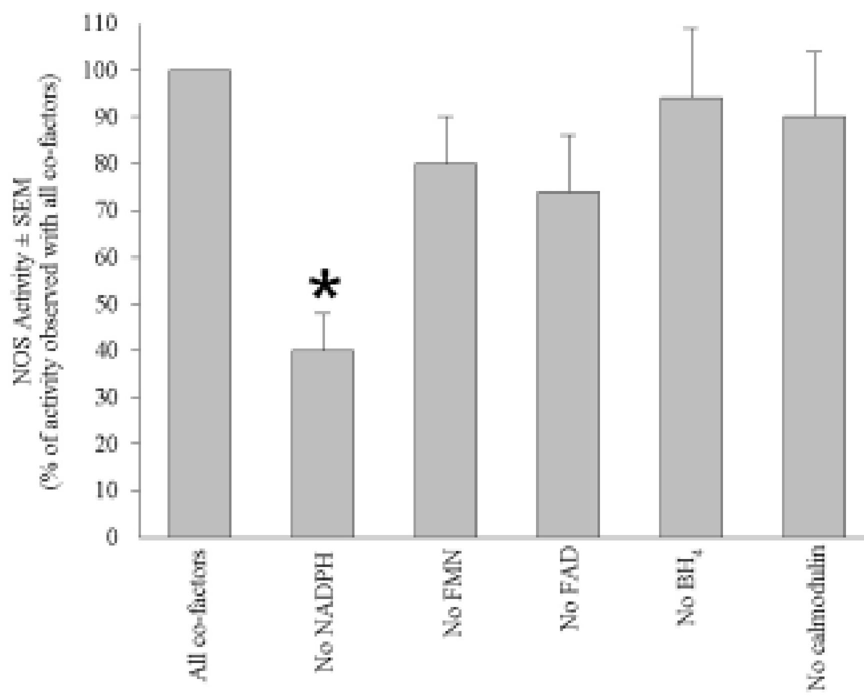

Figure 6:A and B:

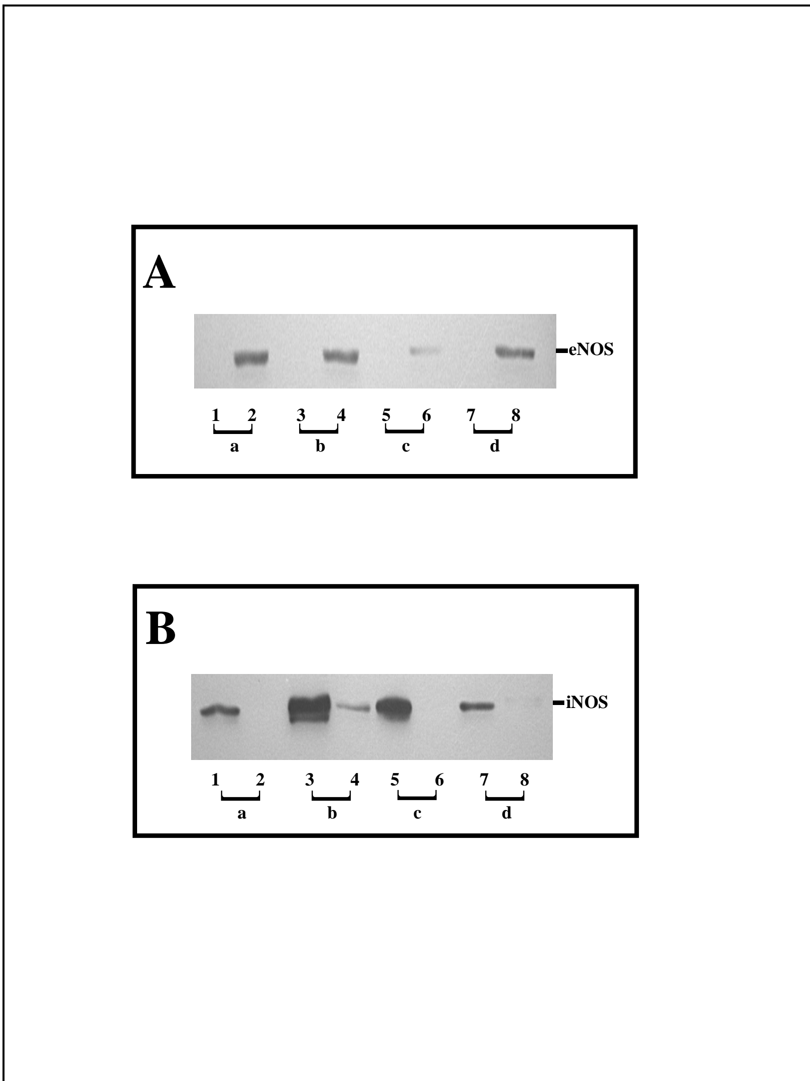

Figure 7: A and B: