Studies of gut mucosal protein synthesis in a non-steroidal anti-inflammatory drug (NSAID) model of inflammatory bowel disease

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Abstract

The extent to which defects in protein synthesis occurred in an experimental indomethacin induced rat model of non-steroidal enteropathy has been examined. Male rats (nine) were fed indomethacin (8 mg/kg/day) for three days mixed with a powdered form of chow. The control group of rats (nine) were fed the same diet for three days without indomethacin. After the feeding period, both groups were fed a normal solid diet for four days. At the end of this period, the fractional rates of intestinal protein synthesis was determined by the 'flooding dose' technique. The mucosal protein, RNA and DNA contents in the proximal ileum of animals with enteropathy were not significantly different from controls ($p > 0.05$). Experimental enteropathy induced selective increases in the fractional rates of protein synthesis (26% increase, $p < 0.03$) and RNA activities (23% increase, $p < 0.04$). There were no significant changes in any of these variables in the duodenum ($p > 0.05$ in all instances). These changes may partly reflect the activity of those processes responsible for the pathogenic changes in NSAID enteropathy.

Small bowel mucosal protein synthesis is of importance in inflammatory bowel disease for a variety of reasons. First, the pathogenesis of stricturing and, in NSAID enteropathy, sub-mucosal diaphragms may well be the result of a differential imbalance in synthetic rates between gut structural proteins, collagen, and other stromal proteins, as well as mucosal soluble proteins. Second, the relationship between nutrition, body mass indices and small bowel mucosal absorptive function is important in inflammatory bowel disease. For example, many patients with inflammatory bowel disease are nutritionally compromised with enhanced nitrogen excretion and muscle wasting. The relationship between changes in mucosal proteins in the gastrointestinal tract and nutrient absorption in inflammatory bowel disease is, however, poorly understood. Third, recent work in cutaneous thermal injury in animals has shown increases in small gut paracellular permeability¹ and a reduction in small intestinal mucosal weight and nutrient absorption early in the postburn period.² Furthermore, in this model, there were decreases in protein, RNA and DNA synthetic rates with the greatest effect in the jejunum.³ Potentially, factors initiating the acute inflammatory reaction may directly inhibit nucleic acid and protein synthesis and

lead to alterations in nutrient absorption and intestinal barrier function in experimental indomethacin induced enteropathy.

For all these reasons a detailed characterisation of small intestine protein turnover was carried out in indomethacin induced enteropathy. An established and well documented technique using a 'flooding dose' of phenylalanine was used to measure protein synthesis.⁴ This method overcomes problems of precursor specific radioactivity and other errors arising from the breakdown of labelled protein that are encountered when tracer amounts of amino acids are used.⁵ This method has been used with effect in the study of small bowel protein synthesis in starvation, various surgical stress situations, such as, partial hepatectomy^{6,7} and metabolic toxicity studies – for example, ethanol.⁸

Methods

ANIMALS AND CHEMICALS

L-[4-³H]phenylalanine was obtained from Amersham International (Amersham, UK), and the other reagents were obtained from The Sigma Chemical Company Ltd (Poole, Dorset, UK). Male rats, Sprague Dawley strain, were obtained from an inhouse breeding colony at weaning, housed in a temperature controlled environment on a 12 hour light/dark cycle commencing at 0700 h, and fed a commercial diet (Labsure, Manea, Cambridgeshire, UK) *ad libitum* until 140–160 g body weight was achieved. The rats were divided into two groups of equal mean body weight.

EXPERIMENTAL PROTOCOL

The experimental group of rats were fed indomethacin (8 mg/kg/day) for three days mixed in a powdered form with the laboratory chow. The control group of rats (nine) were fed the same diet for three days without indomethacin – that is, standard rat chow alone *ad libitum*. After the feeding period, both groups were fed normal solid diet for four days. As previously described, at the end of this period, the development of enteropathy is demonstrable by *in vivo* ⁵¹Cr EDTA test administration by gavage.⁹ Experimental animals all have >5% ingested dose of ⁵¹Cr excreted in their urine, while controls have <5% excreted.⁹

Rats were injected with L-[4-³H]phenylalanine; 150 mmol/l; 150 µmol/100 g body weight through a lateral tail vein.⁴ At two and 10 minutes after injection of isotope, rats were decapitated (three and six, for rats killed at

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two and 10 minutes, respectively) and blood collected for subsequent extraction of plasma. Briefly, mucosa was dissected out from the duodenum and proximal ileum/terminal jejunum and placed into ice cold plastic tubes, weighed, and the mucosa frozen on solid CO₂ and stored at -70°C.

After an initial precipitation with 0.2 mol/l HClO₄ (to obtain S_i, the specific activity of free phenylalanine after neutralisation), pellets were washed twice in 12–14 ml 0.2 mol/l HClO₄. Protein pellets were then digested in 10 ml 0.3 mol/l NaOH, and a sample was removed for protein and DNA determination.^{10 11} After reprecipitation of the protein with 2 mol/l HClO₄, tubes were spun and the supernatants used to measure RNA in the supernatant.¹² Protein pellets were washed repeatedly (that is, eight times, 12–14 ml each) in 0.2 mol/l HClO₄. After heating the protein pellets in 3 ml 6 mol/l hydrochloric acid (36 hours, 105°C), the hydrolysates were dried *in vacuo* over solid NaOH and P₂O₅. The residue was suspended in citrate buffer (1.5 mol/l; pH 6.3) for determination of S_B, the specific radioactivity of phenylalanine in tissue protein. The specific radioactivity of free phenylalanine in plasma, S_p, was determined in the supernatant after precipitation of the proteins with 0.2 mol/l perchloric acid and subsequent neutralisation with saturated tripotassium citrate.⁴ Phenylalanine specific radioactivities in tissue, plasma and protein were converted to 2-phenylethylamine, and assayed by fluorimetry and radioactivity by scintillation spectrophotometry.⁴

Fractional rates of protein synthesis (defined as the percentage of tissue protein renewed each day by synthesis – that is, k_s, %/day) were calculated from the formula:

$$k_s = \frac{S_B \times 100}{\bar{S}_i \times t} \text{ (%/day)}$$

where ‘ $\bar{S}_i$ ’ is the mean specific radioactivity of the free phenylalanine in acid supernatants of tissue homogenates during synthesis measurement (calculated from S_i at two and 10 minutes

after injection of isotope), ‘S_B’ is the specific radioactivity of phenylalanine in the tissue protein at the end of the labelling period, and ‘t’ is the labelling time in days.

The above calculation assumes that proteins are labelled from free phenylalanine in the intracellular compartment. As there is no direct experimental evidence to support this, the k_s was also calculated assuming that the free label in extracellular pools was incorporated into protein, ie by substituting $\bar{S}_i$ with $\bar{S}_p$ in the above formula, where $\bar{S}_p$ was the mean plasma specific radioactivity as determined from S_p values in rats killed at two and 10 minutes.⁴

Rates of protein synthesis were also calculated in terms of RNA – that is, the RNA activity (k_{RNA}, g protein/day/g RNA), and were obtained by dividing k_s by the RNA/protein ratios.^{6 7}

STATISTICAL ANALYSIS

All data are shown as mean (SD) of six observations in each group. Differences between control and treated data were assessed by Student’s unpaired *t* tests (two tailed) with significance at the level of p≤0.05.

Results

PROXIMAL ILEAL MUCOSA (TABLE I)

The data are shown for both control and enteropathic animals seven days after the start of the experiment – that is, four days after cessation of indomethacin treatment. The rat weights, tissue weights, total mucosal proteins, and nucleic acid contents – that is, total amounts per 10 cm, as well as protein and nucleic acid concentrations (amounts per unit wet weights) did not differ significantly between the groups. The mean $\bar{S}_i$ was reduced by 10% in enteropathic rats but this change was not significant (p=0.204). In contrast, the specific radioactivities of phenylalanine in proximal ileal proteins were increased (by approximately 10%) as was k_s calculated from $\bar{S}_i$ (by 26%). Translational efficiencies (amount of synthesis per unit RNA, k_{RNA}) were also increased in enteropathic rats. Similar qualitative results were obtained when k_s and k_{RNA} were calculated from $\bar{S}_p$ (Table I).

The synthetic rates were corrected for the slight gradient of S_i and S_p between animals killed at two and 10 minutes. This acts as a methodological ‘fail-safe’ mechanism to correct for the decline in S_i during the labelling period.^{4 5}

DUODENAL MUCOSA (TABLE II)

Again, the tissue composition and protein synthetic rates are shown for both control and enteropathic animals four days after cessation of indomethacin treatment. There were no changes in protein, RNA or DNA composition. No significant differences were seen in any of the protein synthesis measurements between the two groups of animals (Table II).

TABLE I Protein, RNA and DNA composition and rates of protein synthesis in the proximal ileal mucosa of control and enteropathic animals

	Control	Enteropathic	% Change	p value
<i>Weights and tissue composition</i>				
Rat weight (g)	234 (10)	237 (12)	+1	0.648
Tissue weight/10 cm (mg)	331 (69)	328 (62)	-1	0.935
Total protein (mg)	35.9 (8.4)	34.5 (6.7)	-4	0.752
Protein concentration (mg/g)	108 (4)	105 (4)	-3	0.244
Total RNA (mg)	2.29 (0.48)	2.24 (0.31)	-2	0.835
RNA concentration (mg/g)	6.92 (0.22)	6.89 (0.54)	0	0.902
Total DNA (mg)	2.00 (0.20)	2.11 (0.41)	+6	0.568
DNA concentration (mg/g)	6.23 (1.34)	6.59 (1.61)	+6	0.683
<i>Protein synthetic data</i>				
Intracellular specific activity ($\bar{S}_i$, dpm/nmol)	162 (12)	146 (26)	-10	0.204
Plasma specific activity ($\bar{S}_p$, dpm/nmol)	166 (4)	164 (11)	-1	0.838
Terminal bound specific radioactivity (S _B , dpm/nmol)	1.48 (0.10)	1.64 (0.12)	+10	0.042
k _s from $\bar{S}_i$ (%/day)	113 (14)	143 (26)	+26	0.032
k _s from $\bar{S}_p$ (%/day)	110 (7)	125 (16)	+13	0.064
k _{RNA} from $\bar{S}_i$ (g protein/day/g RNA)	17.6 (2.4)	21.7 (3.5)	+23	0.041
k _{RNA} from $\bar{S}_p$ (g protein/day/g RNA)	17.1 (0.8)	19.0 (1.6)	+11	0.029

All data are mean (SD), six observations in each group. Differences between means were assessed by Student’s *t* test for unpaired samples. $\bar{S}_i$ =specific radioactivity of free phenylalanine in acid supernatants of tissue homogenates. $\bar{S}_p$ =specific radioactivity of free phenylalanine in plasma. S_B=specific radioactivity of protein bound phenylalanine. k_s=fractional rates of protein synthesis. k_{RNA}=RNA activity.

Discussion

The results indicate that experimental indo-

TABLE II Protein, DNA and RNA composition and rates of protein synthesis in the duodenal mucosa of control and enteropathic animals

	Control	Enteropathic	% Change	p value
<i>Weights and tissue composition</i>				
Tissue weight/10 cm (mg)	414 (55)	410 (66)	-1	0.648
Total protein (mg)	45.8 (6.1)	45.2 (7.9)	-1	0.886
Protein concentration (mg/g)	111 (6)	110 (11)	0	0.985
Total RNA (mg)	2.90 (0.42)	2.94 (0.48)	+1	0.881
RNA concentration (mg/g)	7.00 (0.28)	7.20 (0.77)	+3	0.563
Total DNA (mg)	2.00 (0.20)	2.11 (0.41)	+6	0.568
DNA concentration (mg/g)	4.85 (0.36)	5.15 (0.57)	+6	0.291
<i>Protein synthetic data</i>				
Intracellular specific activity ($\bar{S}_i$, dpm/nmol)	164 (5.0)	162 (11)	-2	0.694
Terminal bound specific activity ($\bar{S}_b$, dpm/nmol)	1.61 (0.15)	1.60 (0.06)	0	0.947
k_s from $\bar{S}_i$ (%/day)	120 (12)	124 (8)	+3	0.512
k_s from $\bar{S}_p$ (%/day)	119 (12)	122 (8)	+2	0.621
k_{RNA} from $\bar{S}_i$ (g protein/day/g RNA)	19.0 (2.4)	19.0 (1.2)	0	1.00
k_{RNA} from $\bar{S}_p$ (g protein/day/g RNA)	18.9 (2.4)	18.7 (1.2)	-1	0.859

All data are mean (SD), six per group. Experimental details are contained in the methods section and the legend to Table I. Differences between means were assessed by Student's *t* test for unpaired samples. For abbreviations see Table I.

methacin-induced enteropathy produces selective changes in gut mucosal protein synthesis. Rates of protein synthesis in the proximal ileum mucosa were markedly increased. This was primarily a consequence of raised translational efficiencies, as ascertained by the increase in k_{RNA} . As there was no discernible increase in mucosal protein composition, we can conclude that protein degradation was also increased. It could be argued that the increased rates of protein synthesis and degradation were a direct consequence of a raised rate of cell turnover. Indomethacin treatment has, however, previously been shown to decrease cell turnover in the jejunal mucosa.¹³

The failure of our experimental model to induce changes in the duodenum implies that the enteropathy is a specific pathological state affecting selective regions of the small intestine. These findings are not directly the result of the pharmacological action of indomethacin as these changes would appear to be progressive and occurred several days after cessation of indomethacin administration.

The observed changes in protein turnover in ileal muscularis propria could reflect the activity of those processes (as yet not characterised) responsible for the submucosal fibrosis observed in chronic NSAID enteropathy.¹⁴ It is important to emphasise, however, that the increase in overall mean protein turnover does not unequivocally imply that the experimental treatment increased the quantity of any individual or groups of mucosal protein. Furthermore, it is possible that the development of strictures (in both *de novo* and relapsing disease) may be the combined consequence of changes in gut mucosal protein synthesis (and/or degradation) together with luminal bile acid and bacterial damage.

Similar studies on small intestinal protein and nucleic acid synthesis have been carried out in a rat model of thermal injury where reduced rates of DNA, RNA and protein synthesis were shown.³ Their conclusion was that factors initiating the acute inflammatory reaction may directly inhibit nucleic acid and protein synthesis and lead to alterations in nutrient absorption and intestinal barrier function. The findings described in the present paper are analogous. Caution is required, however, in interpreting

some of the data of Carter *et al.*³ In their studies they did not determine the specific radioactivity of the free labelled amino acid during synthesis measurement.³ The interpretational errors arising from such techniques have been clearly described by the classical studies of Waterlow and colleagues (reviewed in⁵).

The mechanisms of the changes in protein synthesis in both indomethacin induced enteropathy (Table I) and thermal injury are complex; some of the changes in the latter may be prevented by the trophic hormone bombesin.¹⁵ There is some evidence that the changes seen with thermal injury are produced by intestinal ischaemia, as a result of injury-induced acute splanchnic vasoconstriction,¹⁶ although this is refuted by Jones *et al.*¹⁷

There is evidence that DNA synthesis may be at least partially dependent on protein synthesis in the small intestine, as shown by the use of cycloheximide *in vivo*. Verbin *et al.*¹⁸ have shown that drastic reductions in protein synthesis (such as an 80% reduction) eliminates DNA synthesis in the intestine. It is possible that protein synthesis is diminished by this degree in some selective regions of the intestinal mucosa, and that this directly inhibits DNA synthesis locally.

Our results indicate that protein degradation is increased in enteropathic ileal mucosa. Pertinent in this regard are studies on formyl-methionyl-leucyl-phenylalanine (FMLP)-induced ileitis in Sprague Dawley rats which suggest that neutrophils may cause mucosal damage by non-oxidative mechanisms involving enhanced proteolytic activity.^{19,20} Von Ritter *et al.*²¹ have shown in the FMLP ileitis model that the FMLP-induced increase in ⁵¹Cr EDTA permeability is attenuated both by pre-treatment with soya bean trypsin inhibitor and post treatment with the specific elastase inhibitor Eglin C. Other evidence for altered proteolysis in the pathogenesis of the mucosal lesions observed in inflammatory bowel disease is controversial – for example, normal or even decreased tissue mucosal levels of proteolytic enzymes.^{22,23}

It is important to reemphasise the validity of our protein synthesis measurements for two reasons. First, the studies of Preedy and Peters²⁴ recently showed that there was a lack of equilibration between the intracellular ($\bar{S}_i$) and extracellular ($\bar{S}_p$) pools of free labelled amino acids in rats chronically fed liquid diets containing ethanol or isocaloric glucose.²⁴ In the present study there was an excellent equilibration between the extracellular and intracellular pools. In control and enteropathic rats the mean $\bar{S}_i/\bar{S}_p$ ratio in the proximal ileum was 0.98 and 0.89, respectively. In the duodenum the corresponding $\bar{S}_i/\bar{S}_p$ values were 0.99 and 0.99 respectively. These values compare with a corresponding mean $\bar{S}_i/\bar{S}_p$ ratio of between 0.34 and 0.36 in the study of Preedy and Peters.²⁴ Second, some studies have injected the isotope intraperitoneally and used free and protein bound phenylalanine specific radioactivities at 15 minutes to calculate rates of protein synthesis in various tissues (see Jepson *et al.*)²⁵ based on the assumptions that the rise of $\bar{S}_i$ to plateau is rapid and unaffected by the experimental treatment being investigated.²⁵ We have recently shown, however, that the rise of

intestinal S_i to plateau was significantly retarded by chronic ethanol toxicity.²⁶ In the current study, we injected the isotope intravenously and killed rats at two and 10 minutes after the injection to accurately define the time course changes of S_i and S_p . Therefore, we feel our present data to be reliable and accurate. Although k_s in the proximal ileum was altered by approximately a quarter, and p ranged from 0.03 to 0.04, we consider this significant, and these observations may have profound physiological implications.

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