Faecal free fatty acids in tropical sprue and their possible role in the production of diarrhoea by inhibition of ATPases

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SUMMARY Faecal excretion of fatty acids is increased in patients with tropical sprue because of unabsorbed dietary fatty acids. The excretion of fatty acids correlates well with faecal wet weight. *In vitro* unsaturated fatty acids inhibited Na K-ATPase and Mg-ATPase isolated from basolateral membranes of enterocytes and colonocytes. These findings are a possible explanation for the observed abnormalities in water and electrolyte absorption by the colon in patients with tropical sprue and steatorrhoea.

Colonic water and electrolyte absorption is defective in patients with tropical sprue.¹ Faecal free fatty acids could play a role in the pathogenesis of this functional defect of the colon in patients with tropical sprue and steatorrhoea. The precise role of fatty acids in the genesis of diarrhoea associated with steatorrhoea is not clear² but it has been suggested that hydroxy fatty acids could produce a secretory diarrhoea.³⁻⁵ This paper reports the results of the analysis of faecal fatty acids in a group of patients with tropical sprue and appropriate controls. To investigate the possible role of fatty acids in producing colonic water malabsorption, their effect on Mg⁺⁺ and Na⁺K⁺ adenosine triphosphatase (Mg-ATPase and Na K-ATPase) isolated from the basolateral membranes of enterocytes and colonocytes were investigated *in vitro*.

Methods

PATIENTS

CLINICAL STUDY

Eighteen patients with tropical sprue (all with steatorrhoea and defective xylose absorption) admitted consecutively to a metabolic ward from rural North Arcot District, Tamil Nadu, and 12 age, sex, matched controls without malabsorption were studied. Intestinal function was tested as previously described.⁶ The patients and controls were on the

ward diet containing 50 g fat per day and the analysis was done after at least one week on this diet. The dietary fat was mainly groundnut oil and analysis of a typical 24 hour diet showed that palmitic (16:0), oleic (18:1), and linoleic (18:2) acids were the major fatty acids (Fig. 1).

Faeces were passed into polyethylene containers and kept immediately by the patients in a freezer (-20°C). The samples obtained during 72 hours were mixed and homogenised while still cold.

Fig. 1 Comparison of dietary fatty acids with faecal fatty acids in control subjects and patients with tropical sprue. C10 to C18 fatty acids only were taken for calculating 100% distribution.

Aliquots of homogenate were quantitatively analysed for total fatty acids⁷ and for total free fatty acids.⁸ Aliquots of a toluene extract were dried and free fatty acids methylated and extracted as described by Morrison and Smith.⁹ Hydroxy fatty acids were separated as described,¹⁰ except that the liquid phase, SE-30, was used at 3% on GAS-CHROM Q80-100 (Applied Science Labs, Pa, USA) using a Pye 104 gas chromatograph. For other fatty acids, the method of Matthys, Christophe, and Verdonk¹¹ was followed under isothermal conditions. The percentage distribution of each fatty acid was determined by the peak height and retention time method.¹² Fatty acids were identified by the use of pure standards obtained from Sigma Chemical Co., USA.

IN VITRO STUDIES

White rats weighing 150–200 g were killed by a blow on the head, the intestines removed and washed with ice cold saline and mucosa scraped from small intestine and colon separately. All procedures were carried out at 4°C unless otherwise specified.

Small intestinal enterocyte basolateral membrane was prepared essentially as described by Quigley and Gotterer.¹³ Briefly, a 5% homogenate in 5 mM EDTA, pH 7.4, prepared in a Sorvall omnimixer at a speed of 140 for 25 seconds, was filtered through nylon mesh and centrifuged at 700 g for 10 minutes. The supernatant was further centrifuged at 10 000 g for 10 minutes and the mitochondrial pellet obtained was processed after ageing and separation on a sucrose density gradient. Final preparations of basolateral membrane was suspended in 10 mM Tris, 2.5 mM EDTA, 0.25 mM sucrose, pH 7.2, with the protein concentration adjusted to 1.5 to 2.5 mg/ml, and stored at –20°C till further use. Na K-ATPase was enriched 20–30 fold over original homogenate in this preparation.

Experiments showed that without minor modification, the recovery of Na K-ATPase from the colonic mucosa after the Quigley and Gotterer¹³ procedure was low. The initial homogenising buffer, therefore, was modified as follows: 0.25M sucrose, 30 mM L-histidine, 2.0 mM EDTA, pH of 7.0 adjusted with Tris. For homogenisation the Sorvall

omnimixer was set at a speed of 180 and the homogenisation done for 60 seconds. Subsequent steps for purification were identical with the small intestinal scrapings. Na K-ATPase was enriched 10–12 fold by this method in the basolateral membrane preparation from colonocytes. Proteins were determined by the method of Lowry *et al.*¹⁴

ATPase was assayed as described by Quigley and Gotterer¹³ with slight modifications. The assay medium consisted of 60 mM Tris imidazole buffer, pH 7.0, 6 mM ATP (di-sodium salt grade II, Sigma, pH adjusted to 7.0 with Tris), 10 mM MgCl₂, 120 mM NaCl, and 20 mM KCl. Basolateral membrane (50 µg protein) was added to give the final volume of 1.5 ml. The reaction was started either by the addition of the enzyme or by the addition of ATP and magnesium chloride together. Incubation was at 37°C for 20 minutes in a shaking water bath and the reaction was stopped by the addition of 0.5 ml of 46% TCA. Inorganic phosphate released was measured by the method of Taussky and Shorr.¹⁵

ATPase activity was measured under three conditions: in the presence of Mg²⁺, Na⁺, and K⁺ (total ATPase), in the presence of Mg²⁺ and Na⁺ (Mg-ATPase), and in the presence of Mg²⁺, Na⁺, K⁺, and 1 mM ouabain (ouabain insensitive ATPase). The Na K-ATPase was calculated by subtracting the appropriate values from the total ATPase activity. Fatty acid inhibition studies were carried out by adding fatty acids as solutions of 100 times the final concentration in 90% ethanol.¹⁶ In all such assays, controls contained the same level of ethanol without fatty acids.

Results

FAECAL FATTY ACIDS IN TROPICAL SPRUE

The mean total fatty acids in the faeces of controls was 3.5 g/24 h (SEM 0.09) and 92% of these were free fatty acids (3.2 g). In contrast, in patients with tropical sprue the total fatty acids were increased (11.1 g/24 h, SEM 0.3) and free fatty acids while only 77.5% of total were more (8.6 g/24 h) than in controls. Palmitic (16:0), stearic (18:0), and oleic (18:1) acids were the predominant faecal fatty acids in both groups (Table 1). Unsaturated fatty acids

Table 1 Amounts of individual stool free fatty acids in control and tropical sprue patients

	n		12:0	14:0	16:0	18:0	18:0*	20:0	22:0	18:1	18:2	20:4
Controls	12	g/24 h	0.19	0.23	0.84	0.82	0.09	0.05	0.14	0.53	0.14	0.12
		SEM	(0.02)	(0.01)	(0.03)	(0.03)	(0.01)	(0.003)	(0.008)	(0.02)	(0.01)	(0.01)
Tropical sprue	18	g/24 h	0.28	0.43	2.65	2.62	0.13	0.23	0.31	1.37	0.32	0.15
		SEM	(0.01)	(0.01)	(0.08)	(0.10)	(0.01)	(0.007)	(0.01)	(0.06)	(0.01)	(0.01)

* Hydroxystearic acid.

amounted to 25% and 21% of the total fatty acids in the controls and patients with tropical sprue respectively. Arachidonic acid (20:4), which is virtually absent from the south Indian diet, was present in similar amounts in both groups and may represent the loss of endogenous fat. The major dietary fatty acids are in the C10–C18 series. The similarity of the fatty acids profile in diet and faeces suggests that in controls and in patients with tropical sprue most of the faecal fat is of dietary, rather than endogenous, origin (Fig. 1).

Hydroxystearic acid was present in measurable amounts in eight of 12 controls and in seven of 18 patients with tropical sprue. The largest amount of hydroxystearic acid was 1.0 g/24 h in a tropical sprue patient with a 24 hour stool weight of 869 g. Hydroxystearic acid excretion varied from 0.02–1.0 g/24 h and stool weight from 151–869 g/24 h in the 15 subjects in whom hydroxystearic acid could be detected. Stool weight for the 18 patients with tropical sprue ranged from 130–1244 g/24 h. There was no significant correlation between stool weight and excretion of hydroxy fatty acids in the 30 subjects included in the study. The three day mean stool wet weight showed a highly significant correlation with total stool fat ($r=0.73$) and with unsaturated fatty acid excretion ($r=0.74$) (Fig. 2). A significant correlation between total stool fat and unsaturated fatty acids was also observed ($r=0.85$).

EFFECT OF FATTY ACID ON ATPASES *in vitro*

Inhibition of basolateral membrane Na K-ATPase by unsaturated fatty acids (0.05 mM) ranged from 50.5% (oleic) to 69.5% (arachidonic) for the small intestinal and 43.9% (oleic) to 75.2% (linoleic) for

Fig. 2 Correlation of stool wet weight and faecal excretion of unsaturated free fatty acids in control subjects and patients with tropical sprue.

the large intestinal preparations. Saturated fatty acids, hydroxy fatty acids, and methylester of oleic acid produced considerably lower inhibition. The inhibition by various fatty acids of Mg-ATPase was similar to that for Na K-ATPase except that the percentage inhibition was in general less (Table 2).

The K_m for ATP for the colonocyte Na K-ATPase was higher than that of the enterocyte enzyme. The colonic Na K-ATPase K_m was altered in the presence of 0.16 molar ethanol, but no such alteration occurred for the enterocyte enzyme. Similarly, the K_i for oleic acid was higher for the colonocyte enzyme. The pH optimum and optimum concentration of Na^+ and K^+ were identical for the two enzymes (Table 3).

Na K-ATPase from the colon or the small

Table 2 Percentage inhibition of rat small intestinal and colonic ATPases by different fatty acids at 0.05 mM concentration

Fatty acids	Chain length	Percentage inhibition			
		Na K-ATPase		Mg-ATPase	
		Small intestine	Colon	Small intestine	Colon
Octanoic	8:0	5.5±1.2	0.0	9.5±2.8	0.0
Lauric	12:0	12.2±1.4	0.0	10.6±2.2	0.0
Myristic	14:0	32.2±0.6	21.4±1.0	23.1±1.6	5.0±0.1
Palmitic	16:0	21.2±1.0	3.0±1.0	0.0	11.2±0.4
Stearic	18:0	11.8±0.5	6.1±0.3	0.0	14.8±0.5
Arachidic	20:0	9.6±1.0	32.2±0.7	3.9±0.5	26.7±0.9
Oleic	18:1	50.5±1.0	43.9±0.4	33.5±1.5	40.3±0.6
Methyl oleate	18:1	17.2±1.0	*	11.7±0.6	*
Linoleic	18:2	68.2±1.0	75.2±0.3	51.6±0.8	50.9±0.3
Linolenic	18:3	56.7±1.1	58.3±0.4	41.8±1.2	41.9±0.0
Arachidonic	20:4	69.5±0.9	68.5±0.7	69.7±1.8	53.3±0.2
Ricinoleic	18:1-OH	33.5±0.9	37.3±1.0	13.3±1.8	10.3±0.4
12-Hydroxystearic	18:0-OH	18.8±0.6	10.0±0.7	28.5±2.4	12.5±0.3

* Not tested. The results are expressed as mean and SEM for three determinations with duplicates.

Table 3 Comparison of properties of enterocyte and colonocyte Na K-ATPase

	Na K-ATPase	
	Small intestine	Colon
K _m for ATP	9×10^{-4} M	16×10^{-4} M
K _m for ATP in presence of 0.16 M ethanol	9×10^{-4} M	54×10^{-4} M
K _i for oleic acid in presence of 0.16 M ethanol	4×10^{-5} M	11×10^{-5} M
Optimum pH	7.0	7.0
Optimum Na ⁺	120 mM	120 mM
Optimum K ⁺	20 mM	20 mM

intestine was inhibited to a greater extent by oleic acid at different levels than the Mg-ATPase from the same source (Fig. 3). The inhibition of Na K-ATPase by oleic acid was noncompetitive in type (Fig. 4), whereas the inhibition of Mg-ATPase was uncompetitive (Fig. 5).

Discussion

The percentage distribution of the major fatty acids in faeces in control subjects and in patients with

tropical sprue was similar to the composition of dietary fatty acids (Fig. 1), indicating that non-absorption of dietary fat is the major factor contributing to faecal fat in both groups. Only a small proportion of the faecal fat in subjects without steatorrhoea in southern India is of endogenous origin, in contrast with reports from temperate regions.¹⁷ This difference may be related to the high prevalence of tropical enteropathy with decreased absorptive capacity of the small intestine for fat, xylose, and other nutrients.¹⁸ The presence of similar amounts in faeces of controls and patients of arachidonic acid, an uncommon dietary fat in southern India, is probably a reflection of the small endogenous loss.

In tropical sprue, as in postileal resection,¹⁹ hydroxy fatty acids do not appear to play a

Fig. 3 Concentration dependent inhibition of rat enterocyte and colonocyte ATPases by oleic acid. Percentage activity was determined from control incubations without oleic acid. Values are expressed as the average of three different experiments with duplicates. ○—○ enterocyte Na K-ATPase, ●—● enterocyte Mg-ATPase, ▲—▲ colonocyte Na K-ATPase, △—△ colonocyte Mg-ATPase.

Fig. 4 Noncompetitive inhibition of enterocyte Na K-ATPase by oleic acid, △—△ control, ○—○ in the presence of 0.05 mM oleic acid. Ratio of 2:1 for Mg²⁺ to ATP was maintained in assay medium.

Fig. 5 Uncompetitive inhibition of colonocyte Mg-ATPase by oleic acid. △—△ control, ○—○ in presence of 0.06 mM oleic acid.

significant role in the pathogenesis of diarrhoea. The failure to detect hydroxystearic acid in half of the subjects is unlikely to be because of technical problems as the gas chromatographic method should detect hydroxy fatty acids at 1% of the total fatty acids, a level lower than in earlier reports.⁵ Although the two factors believed necessary for production of faecal hydroxystearate, sufficient amounts of free oleic acid and bacteria capable of hydrating the oleic acid to hydroxystearate²⁰ were present, the absence of hydroxystearate in the faeces of several subjects suggests that other factors may be necessary for its production. These may be related to the nature of the dietary fat⁵ or the induction of the specific bacterial hydroxylases.

Faecal wet weight correlated well with total faecal fat and saturated and unsaturated fatty acids in the stool but not with hydroxy fatty acids. Intestinal perfusion studies showed that ricinoleic acid and oleic acid inhibit the absorption of water and solutes in the human intestine.²¹⁻²⁴ Hydroxy fatty acids cause secretory diarrhoea by stimulation of adenylate cyclase²⁵ while oleic acid does not stimulate this enzyme while producing watery diarrhoea.^{25, 26} It has been suggested that the effect of fatty acids may be related to decreased activity of the sodium pump.²⁷ Na K-ATPase is considered the biological counterpart of the sodium pump, maintaining the sodium gradient and electrical potential difference across cell membranes, although the exact role of this enzyme in sodium and water absorption is not yet fully understood.^{28, 29} Significant reduction of water and electrolyte absorption was found when loops of small intestines were perfused *in vivo* with solutions containing ouabain, a potent inhibitor of Na K-ATPase.³⁰ In children with active postenteritis syndrome, coeliac disease, and acute viral enteritis, a reduction in jejunal mucosal Na K-ATPase activity has been reported.³¹⁻³³ The effect of oleic acid in perfusion studies was less than that of ricinoleic acid and recovered rapidly suggesting a reversible inhibition of an enzyme system.²³

Fatty acids are known to inhibit a variety of enzymes.^{16, 34-36} The results reported here extends the range of enzymes inhibited by fatty acids to include Na K-ATPase of the basolateral membranes of enterocytes and colonocytes. Unsaturated fatty acids were more inhibitory compared with saturated or hydroxy fatty acids (Table 2). Blocking the free carboxyl group in oleic acid by methylation reduced, but did not totally remove, its inhibitory effect. Mg-ATPase of the basolateral membranes was also inhibited although to a lesser degree. This confirms earlier observations from this laboratory of fatty acid inhibition of enterocyte brush border Mg-

ATPase (unpublished observation), an enzyme thought to have a role in the maintenance of the mucosal acid microclimate.³⁷ The mechanism of Na K-ATPase inhibition by fatty acids was non-competitive and is possibly brought about by changes in membrane characteristics.

Although fatty acids are known to reduce water and electrolyte absorption in the human jejunum,²¹⁻²³ it is possible that in tropical sprue jejunal contents are not in contact with the enterocyte for a sufficient period of time to produce an effect. Small intestinal *in vivo* perfusion studies in patients with tropical sprue in southern India have not shown water malabsorption sufficient to explain the severity of the diarrhoea³⁸ and out of keeping with the enterocyte damage, characteristic of the disease.³⁹ Colonic contents are, however, in intimate contact with the colonic epithelium for longer periods of time and their level is further increased by absorption of water, the normal physiological role of the colon. In patients with tropical sprue, a defect in colonic sodium and water absorption¹ is accompanied by increased amounts of faecal unsaturated fatty acids, especially oleic acid. The results of these *in vitro* studies, correlation between faecal wet weight and faecal fatty acids, and the ability of unsaturated fatty acids to inhibit the colonocyte basolateral membrane ATPases, suggest one possible explanation for the pathogenesis of the colonic functional abnormality in tropical sprue. The extrapolation of *in vitro* results to the clinical situation, however, has to be made with caution especially since the abnormality of water and electrolyte absorption was shown in a situation where fatty acids were not present in the perfusion fluid.

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