Vitamin A Deficiency Changes Jejunal Mucosal Fatty Acid Profile in Rats

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Summary Changes in intestinal epithelial integrity have been postulated to explain the association between vitamin A status and diarrhoeal disease in children living in areas where vitamin A deficiency is a major public health problem. Fatty acids are an integral component of normal enterocyte structure, and changes to their profile in the intestinal mucosa may impact on the efficacy of the mucosal barrier. The aim of this study therefore was to determine the effect of vitamin A deficiency on intestinal mucosal fatty acid profile. Weanling male specific pathogen free rats were maintained on a vitamin A deficient diet for either 40–42 (subclinical deficiency) or 60–63 days (clinical deficiency), and compared with rats pair fed or allowed free access to the same diet, but supplemented with vitamin A (vitamin A sufficient). Fatty acid profile of jejunal mucosal homogenates was determined using gas chromatography. While subclinically vitamin A deficient rats had only minor differences in fatty acid profile as compared with pair fed and vitamin A sufficient rats, there were marked differences in a number of fatty acids in the clinically vitamin A deficient rats. Clinically vitamin A deficient rats had more arachidonic acid (C20:4n-6) than pair fed rats, and less linoleic acid (C18:2n-6) than both pair fed and vitamin A sufficient rats. These findings suggest that vitamin A deficiency modifies intestinal mucosal fatty acid composition such that there is an increase in pro-inflammatory eicosanoid precursors. This may contribute towards our understanding of a biological mechanism of the epidemiological association between vitamin A status and diarrhoeal disease.

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Improving the vitamin A status of children living in areas where vitamin A deficiency is a major public health problem, has been found to reduce both their overall mortality rate, and that specific to diarrhoeal disease [1–3]. In order to determine a physiological explanation for these findings, we have previously compared the effect of vitamin A deficiency on rat jejunal structure and function with pair-fed controls, and found that subclinical deficiency does not affect gross morphology, disaccharidase activities or glucose transport, whereas clinically deficient rats had mild villous atrophy and reduced disaccharidase activities (although this did not result in diarrhoea) [4]. However, in the presence of a noxious agent such as methotrexate [5] or rotavirus [6], vitamin A deficient rodents develop a more severe intestinal injury than controls treated with these agents. We have postulated that these findings may be due to impaired intestinal mucosal barrier function caused by vitamin A deficiency [5].

Indeed, altered intestinal epithelial integrity has been proposed as a possible mechanism for the epidemiological findings of an association between vitamin A status and diarrhoeal disease mortality in young children [1]. Epithelial integrity is an important factor in the maintenance of the intestinal mucosal barrier, which prevents the movement of pathogens, endotoxins, and antigens from the lumen into the systemic circulation and distant organs [7, 8].

Enterocyte cell structure (and function) is a component of intestinal epithelial integrity [8], and is influenced by membrane lipid composition [9, 10]. The physicochemical and transport properties of both the brush border and basolateral membranes can vary with fatty acyl chain composition, cholesterol and phospholipid head group content [9, 11]. In turn, these components, and hence structural and metabolic activities of enterocytes, can be influenced by fasting and dietary manipulation [9, 10, 12–15]. While there has been interest in the possible interaction of vitamin A status and membranes for many years [16–19], and young rats given large doses of vitamin A have been found to have greater total intestinal lipid content and monoglycerides than controls [20, 21], we are not aware of any studies conducted to investigate intestinal epithelial lipid composition in vitamin A deficiency.

The aims of these experiments were therefore to investigate the effect of both subclinical and clinical vitamin A deficiency on jejunal fatty acid composition.

MATERIALS AND METHODS

Animal models. An established rat model of subclinical and clinical vitamin A deficiency was used [4]. Specific pathogen free (SPF) weanling male Wistar rats (obtained direct or bred from the Animal Resources Centre, Perth, Australia), aged 3 weeks, were separated into three groups. Animals designated VA– were fed a pelleted vitamin A deficient diet (Catalog no: 960220, ICN Biomedicals, Sydney). Because consumption of this diet results in reduced food intake [4], the rats in the second group (PF)

received the same diet, but were pair fed to those in the VA– group and supplemented with 150 mg vitamin A (Rovimix A 500W, Roche Australia, Sydney) added to their drinking water (volume adjusted to thirst) every second day. To control for any effect of the vitamin A deficient diet, a third group (VA+) received the vitamin A deficient diet in unrestricted quantities, with their drinking water similarly supplemented with vitamin A. Rats were maintained on diets for either 40–42 days (subclinical deficiency) or 60–63 days (clinical deficiency) [4].

Rats were individually housed in wire bottom cages in a single room in a conventional animal house. The temperature was maintained at 20–22°C, with a light-dark cycle of 12 h. All animals had free access to water. Animals were observed for clinical evidence of vitamin A deficiency and the presence of diarrhoea. Rats were weighed on the day of study. All procedures were approved by the Animal Care and Ethics Committee, The University of Newcastle.

Mucosal fatty acid analysis. Fatty acid analyses were conducted on mucosal samples from a subset of a series of rats previously reported in studies of vitamin A deficiency and intestinal function and methotrexate enteropathy in rats [4, 5]. On the day of study, animals were anaesthetized with intraperitoneal thiopentone (0.16 g/kg) and blood collected by intracardiac puncture. Jejunum from the ligament of Trietz to mid jejunum was removed and flushed with cold phosphate-buffered saline. Mucosa was scraped off the distal half of this segment using two glass slides, homogenized in 2.5 mM EDTA (1:10, weight:volume), snap frozen with liquid nitrogen and stored at –80°C until analyzed.

For fatty acid determination, mucosal homogenate was thawed, and fatty acids were directly methylated *in situ* with acetyl chloride in toluene : methanol (4:1, v/v), using a modification of the method of Lepage and Roy [22]. Fatty acid methyl esters were extracted and injected into a gas chromatograph (5890A, Hewlett Packard) equipped with a 30 m fused carbon-silica capillary column (internal diameter: 0.25 mm) coated with cyanopropylphenyl (DB-225, 25 mm thickness, 25% w/w coating; J & W Scientific, CA), and connected to an integrator (3392A, Hewlett Packard). The initial temperature of 170°C was maintained for 2 min, then increased to 210°C at a rate of 5°C/min, then to 212°C at a rate of 0.2°C/min, and finally to 220°C at 10°C/min. Fatty acid methyl ester peaks were identified by injecting standard mixtures of fatty acid methyl esters (Nu Chek Prep Inc., Elysian, MN), and estimated using nonedecanoic acid (C19:0, Nu Chek Prep Inc.) as an internal standard. Contents of fatty acids are expressed as %w/w. The Unsaturation Index (U.I.) was calculated as the sum of the number of double bonds in a given fatty acid multiplied by the amount (%w/w) of that fatty acid present [12].

Retinol analyses. Serum retinol was measured by reversed-phase HPLC with UV detection as described previously [4].

Statistical analyses. Values are depicted as mean $\pm$ SEM. For each of the subclinical and clinical studies, all groups were compared using one way analysis of variance or Kruskal-Wallis tests (for non parametric data). Where differences were detected, group means were compared using a Tukey-Kramer multiple comparison test or for non-parametric data, medians were compared using a Mann-Whitney test [23]. Differences between the subclinical and clinical studies were examined using two way analysis of

variance, then either a Student *t*-test or Mann-Whitney test if differences were detected.

RESULTS

The clinical features of the groups of rats of which the animals in this study are a subset, have previously been described [4, 5]. Rats consuming the vitamin A deficient diet for 60–63 days without vitamin A supplementation appeared dishevelled and emaciated. No rats from any other diet group showed similar signs of clinical vitamin A deficiency [4]. There was no significant difference in body weight between the subclinically VA– rats (278 ± 17.7 g), PF (299.2 ± 21.4 g) and VA+ (310 ± 21.0 g) controls. Clinically VA– rats weighed (225.7 ± 16.5 g) significantly less than both PF (259.3 ± 10.6 g, $p < 0.05$) and VA+ (313.9 ± 11.0 g, $p < 0.001$) controls.

Serum retinol

Serum retinol was negligible in subclinically VA– rats (0.08 ± 0.03 $\mu\text{mol/liter}$), and was significantly less than PF (2.19 ± 0.13 $\mu\text{mol/liter}$) and VA+ (2.10 ± 0.10 $\mu\text{mol/liter}$) rats ($p < 0.05$). Clinically VA– rats had no detectable serum retinol, significantly less than both PF (1.95 ± 0.10 $\mu\text{mol/liter}$) and VA+ (1.77 ± 0.24 $\mu\text{mol/liter}$) rats ($p = 0.02$). There were no significant differences in serum retinol concentrations between the sub-clinical and clinical studies.

Table 1. Effects of subclinical vitamin A deficiency on fatty acid profile of jejunal mucosa.

Fatty acid %w/w	Vitamin A deficient (VA–)	Pair fed (PF)	Vitamin A sufficient (VA+)
	<i>n</i> =9	<i>n</i> =9	<i>n</i> =10
C14:0	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1
C15:0	0.6 ± 0.1^a	0.4 ± 0.1^b	0.5 ± 0.0
C16:0	23.5 ± 0.6^a	21.5 ± 0.5^b	21.3 ± 0.3^b
C16:1n-7	2.2 ± 0.5	2.1 ± 0.4	2.2 ± 0.3
C17:0	0.8 ± 0.1	0.7 ± 0.1	0.7 ± 0.1
C18:0	13.5 ± 1.2	13.9 ± 1.3	14.9 ± 0.9
C18:1n-9	13.7 ± 1.4	14.5 ± 1.6	13.6 ± 1.2
C18:1n-7	2.5 ± 0.2	2.4 ± 0.1	2.6 ± 0.1
C18:2n-6	23.6 ± 0.8	26.4 ± 2.0	25.4 ± 1.0
C20:0	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0
C20:1n-9	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
C20:2n-6	0.3 ± 0.0	0.4 ± 0.0	0.4 ± 0.0
C20:3n-6	0.6 ± 0.1	0.8 ± 0.1	0.8 ± 0.1
C20:4n-6	14.4 ± 1.2	13.0 ± 2.1	13.6 ± 1.4
C22:0	0.3 ± 0.1	0.4 ± 0.1	0.5 ± 0.1
C22:4n-6	0.9 ± 0.1	1.1 ± 0.1	1.1 ± 0.1
C22:6n-3	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.0
C24:0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0
Unsaturation index	132.6 ± 3.1	133.3 ± 4.5	133.7 ± 3.1

Values are mean $\pm$ SEM. Values in a column with a different superscript letter are significantly different ($p < 0.05$).

Jejunal mucosal fatty acid analysis

In the subclinical study, no major differences were detected in jejunal mucosal fatty acid profile (Table 1). However VA– mucosal homogenate did have significantly higher percentages of C15:0 and C16:0 fatty acids than PF ($p<0.05$, Table 1), and significantly higher percentages of C16:0 fatty acids than VA+ ($p<0.05$, Table 1). There were no differences in U.I. between groups (Table 1).

In clinical vitamin A deficiency, major differences were detected between groups in fatty acid profile: VA– mucosal homogenate had significantly less C18:2n-6 than PF and VA+ ($p<0.02$, Table 2) and significantly more C20:4n-6 than PF ($p<0.02$, Table 2). VA– mucosal homogenate also had significantly more C15:0, C16:0, C18:1n-7, C20:0, C20:1n-9, C22:4n-6, C22:6n-3 and C24:0 than PF ($p<0.02$, Table 2). Mucosal homogenate from VA– group rats also had significantly more C14:0, C16:0, C20:0, C20:1n-9, C22:0, C22:4n-6, C22:6n-3 and C24:0; and significantly less C18:2n-6 and C20:2n-6 than VA+ ($p<0.02$, Table 2). PF animals had significantly less C18:1n-7 than VA+ ($p<0.02$, Table 2). There were no differences in U.I. between groups (Table 2).

DISCUSSION

Clinical vitamin A deficiency caused major alterations in jejunal mucosal fatty acid profile. Specifically, there was more arachidonic acid (C20:4n-6) and less linoleic acid

Table 2. Effects of clinical vitamin A deficiency on fatty acid profile of jejunal mucosa.

Fatty acid %w/w	Vitamin A deficient (VA–)	Pair fed (PF)	Vitamin A sufficient (VA+)
	n=8	n=8	n=8
C14:0	0.9±0.1 ^a	0.7±0.1 ^{ab}	0.7±0.0 ^b
C15:0	0.7±0.1 ^a	0.4±0.0 ^b	0.6±0.1 ^{ab}
C16:0	23.6±0.6 ^a	21.3±0.6 ^b	19.8±0.4 ^b
C16:1n-7	2.2±0.2	1.5±0.2	1.7±0.2
C17:0	0.8±0.1	0.6±0.1	0.8±0.1
C18:0	13.4±0.7	10.4±1.7	13.4±0.4
C18:1n-9	13.8±1.3	14.3±0.8	12.8±0.6
C18:1n-7	2.8±0.1 ^a	2.1±0.2 ^b	2.6±0.1 ^a
C18:2n-6	18.7±0.9 ^a	33.8±1.9 ^b	29.2±0.9 ^b
C20:0	0.6±0.0 ^a	0.5±0.0 ^b	0.5±0.0 ^b
C20:1n-9	0.3±0.0 ^a	0.2±0.0 ^b	0.2±0.0 ^b
C20:2n-6	0.4±0.0 ^a	0.4±0.0 ^a	0.5±0.0 ^b
C20:3n-6	0.7±0.1	0.6±0.0	0.6±0.0
C20:4n-6	17.2±1.6 ^a	10.8±0.9 ^b	14.0±0.6 ^{ab}
C22:0	0.4±0.0 ^a	0.4±0.0 ^a	0.3±0.0 ^b
C22:4n-6	1.7±0.1 ^a	1.0±0.1 ^b	1.2±0.1 ^b
C22:6n-3	0.5±0.1 ^a	0.2±0.0 ^b	0.3±0.0 ^b
C24:0	0.5±0.0 ^a	0.4±0.0 ^b	0.3±0.0 ^b
Unsaturation index	139.1±4.5	137.4±2.0	141.8±0.9

Values are mean±SEM. Values in a column with a different superscript letter are significantly different ($p<0.02$).

(C18:2n-6) than in pair fed controls. In contrast, a tendency of more arachidonic acid and less linoleic acid was detected in subclinical vitamin A deficient rats.

Interestingly, we can also hypothesize that there may be a functional link between our findings of altered intestinal mucosal fatty acid profile and disaccharidase activities in the clinically deficient rats. Changes in fatty acid composition of microsomal membrane phospholipids may influence alterations to membrane cholesterol and phospholipid biosynthesis, which are important membrane (and hence enterocyte) structural and functional determinants [11]. Although we have previously postulated that changes observed in disaccharidase activities in clinically vitamin A deficient rats may be attributed to vitamin A's role in gene expression [4], changes in membrane-order parameters have also been noted to alter membrane-bound hydrolytic enzyme activity [11], and therefore should be considered as a possible mechanism for our earlier findings.

Although clinically vitamin A deficient rats were more malnourished than subclinically deficient rats [4], it is unlikely that our findings at each stage of deficiency are due to any excess effect of malnutrition, as we have controlled for the effect of reduced intake due to consumption of a vitamin A deficient diet [4] by pair-feeding one group of controls. Although no studies have examined the effect of protein energy malnutrition on intestinal fatty acid profile, malnourished rabbits have an altered intestinal microvillous membrane lipid composition [13], and short-term fasting in rats has been found to modify the fatty acid profile of intestinal microsomes [12]. Furthermore, by feeding the same diet to all groups of rats, we have also controlled for the effects of any other nutrients which may influence intestinal mucosal fatty acid composition [9, 10, 12, 14, 15].

The differences in fatty acid profile in clinically vitamin A deficient rats may have a major impact on the nature of eicosanoids synthesized in the intestinal mucosa. Clinically vitamin A deficient rats have significantly more arachidonic acid (C20:4n-6) than PF rats. Arachidonic acid is a precursor of eicosanoids such as prostaglandins (eg. PGE₂), thromboxane, and leukotrienes; all implicated in the pathogenesis of intestinal inflammation [24]. Although the few studies addressing the addition of retinoids to mesenchymal or endothelial cell cultures have found either no difference [25], or a decrease [26] in PGE₂ production, no studies have investigated the effect of vitamin A deficiency on eicosanoid production.

Clinically vitamin A deficient rats also have less linoleic acid (C18:2n-6) than both pair fed and vitamin A sufficient rats. Linoleic acid is converted to linolenic acid by Δ^6 desaturase, and the same enzyme is also the rate limiting step in arachidonic acid synthesis from linoleic acid [9]. Thus it is likely that intestinal Δ^6 desaturase is upregulated during vitamin A deficiency. Δ^6 Desaturase has been found in intestinal epithelia [12], and its activity in rat small intestinal mucosal microsomes has been shown to be altered by both short term fasting and dietary fat manipulation [12]. Vitamin A deficient rats have been found to have increased liver microsomal Δ^9 , but not Δ^6 , desaturase activity [27], and reduced arachidonic, but increased palmitic acid content as compared with controls [28]. However, Δ^6 desaturase activity appears to be regulated differently in the liver than in the intestine [11], and additionally, Alam and Alam [28] did not use pair fed controls in their studies.

Others [19, 29] have also reported changes in liver microsomal fatty acid profile in vitamin A deficient rats, although the changes differed between studies. Furthermore, both of these studies also examined microsomal membrane fluidity, and found that it was decreased in vitamin A deficiency. Grolier *et al.* [19] postulate that this may be due to observed changes in phospholipid to cholesterol ratio and phospholipid content. Given that high doses of vitamin A have been found to alter total intestinal lipid content [20, 21], vitamin A deficiency may also cause changes in intestinal epithelial membrane fluidity, potentially resulting in changes in the membrane's barrier function.

A tendency to increase production of pro-inflammatory eicosanoids, predisposing the mucosa to inflammatory injury, may explain our previous findings and those of others that vitamin A deficiency exacerbates methotrexate enteropathy [5] and rotavirus enteritis [6]. Moreover, in children whose intestines are regularly insulted with pathogens and other antigens, an overproduction of pro-inflammatory eicosanoids causing increased susceptibility to intestinal mucosal damage, is a possible biological explanation for the epidemiological association of vitamin A deficiency and diarrhoeal disease.

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