Intestinal function in cholestasis and essential fatty acid deficiency
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CHAPTER 5

Essential fatty acid deficiency in mice impairs lactose digestion

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Submitted
ABSTRACT
Background: Essential fatty acid (EFA) deficiency in mice induces fat malabsorption. We previously reported indications that the underlying mechanism is located at the level of the intestinal mucosa.

Aim: To characterize the effects of EFA deficiency on small intestinal morphology and function.

Methods: Mice were fed an EFA-deficient or control diet for 8 weeks. A 72 h fat balance, the EFA status, and small intestinal histology were determined. Carbohydrate absorptive and digestive capacities were assessed by stable isotope methodology after administration of U-13C-glucose and 1,13C-lactose. Concentrations of the EFA linoleic acid (LA), and the enzyme activity and mRNA expression of lactase, were measured in small intestinal mucosa.

Results: Mice fed the EFA-deficient diet were markedly EFA-deficient with a profound fat malabsorption. EFA deficiency did not affect the histology or proliferative capacity of the small intestine. Blood 13C6-glucose appearance and disappearance were similar in both groups, indicating unaffected monosaccharide absorption. In contrast, blood appearance of 13C-glucose, originating from 13C-lactose, was delayed in EFA-deficient mice. EFA deficiency profoundly reduced lactase activity (-58%, p<0.01) and mRNA expression (-55%, p<0.01) in mid small intestine. Both lactase activity and its mRNA expression strongly correlated with mucosal LA concentrations (r=0.89 and 0.79, resp., p<0.01).

Conclusions: EFA deficiency in mice inhibits the capacity to digest lactose, but does not affect small intestinal histology. These data underscore the observation that EFA deficiency functionally impairs the small intestine, possibly mediated by low LA levels in the enterocytes.
INTRODUCTION

Essential fatty acid (EFA) deficiency can occur in cholestatic liver diseases as a consequence of fat malabsorption. However, EFA deficiency itself also induces fat malabsorption. The underlying mechanism of EFA-deficient fat malabsorption remains unclear. Absorption of fat involves lipolysis, solubilization and intestinal translocation from the lumen into the mucosa, chylomicron assembly and transport into the lymph. Previous studies in EFA-deficient mice have indicated that impaired lipolysis or bile formation do not cause the fat malabsorption in EFA deficiency. Recently we reported data to suggest that EFA deficiency in mice affects fat absorption at the level of the small intestinal mucosa. However, it has not been proven that EFA deficiency impairs the mucosal phase of fat absorption.

In contrast to fat absorption, the absorption of di- and monosaccharide carbohydrates exclusively depends on mucosal function. The monosaccharide glucose is actively transported across the brush border membrane in the small intestine by the brush-border transporter SGLT1 (Sodium-dependent glucose transporter). The disaccharide lactose is first hydrolyzed by the mucosal membrane anchored lactase-phlorizin hydrolase (lactase, LPH) into glucose and galactose, prior to their active transport across the brush border by SGLT1. Besides being an important enzyme in lactose hydrolysis, lactase is a marker of enterocyte differentiation. Throughout development total intestinal lactase activity remains similar to that found in newborns. This characteristic makes lactase a good marker for functional assessment of the small intestine in adult animal.

Essential fatty acids (EFAs) are structural components of membrane phospholipids. Enterocyte membrane phospholipids are particularly rich in linoleic acid (LA, C18:2n-6) which is necessary for modulations of a wide variety of biological functions and for physiochemical adaptations of the membrane lipid matrix to alterations in membrane fluidity. The lipid matrix influences the conformation and function of proteins embedded in the inner and/or outer leaflet of the membrane. Recently, an additional role of EFAs in alterations of bilayer elastic properties and lipid composition in lipid rafts have been reported. Through activation of peroxisome proliferator-activated receptors (PPARs), EFAs can regulate transcriptional activity of several genes, including of those involved in fatty acid transport and metabolism.

In the present study we characterized the effects of EFA deficiency on small intestinal morphology and function in mice. Korotkova et al. have shown that EFA deficiency affects the fatty acid composition in the phospholipids of the rat small intestinal mucosa by decreasing the jejunal concentrations of linoleic acid. However, no studies have been performed on the effect of EFA deficiency on the small intestinal function. We assessed the absorption of glucose, a major source of metabolic energy for mammalian cells, and lactose, as appropriate functional markers of the small intestine, in a previously developed and characterized murine model of EFA deficiency. We applied stable isotope methodology, since this approach allows extension to similar studies in patients with EFA deficiency, cholestasis, or other forms of malabsorption. U-13C-labeled glucose and 1-13C-labeled lactose were administered to EFA-deficient and control mice. Blood appearance of labels derived from administered glucose (13C6-glucose) and lactose (13C-glucose) into the blood glucose fraction was subsequently quantified. We also determined the activity and expression of lactase, as well as the concentration of LA, in the mucosa along the proximal-to-distal axis of the small intestine.
Our data show that EFA deficiency is associated with impaired lactose digestion in mice. This functional observation corresponds with lower lactase mRNA expression and enzyme activity in the mid small intestine of EFA-deficient mice, accompanied by low LA concentrations.

MATERIALS AND METHODS

Mice and housing
Wild type mice with a free virus breed (FVB) background were obtained from Harlan (Horst, The Netherlands). Male mice (25-35 g) were housed in a light-controlled (lights on 6 AM-6 PM) and temperature-controlled facility and were allowed tap water and chow (AB diets, Woerden, Netherlands) ad libitum. The experimental protocol was approved by the Ethics Committee for Animal Experiments, Faculty of Medical Sciences, University of Groningen, the Netherlands.

Materials
U-\(^{13}\)C-glucose and \(^{1-13}\)C-lactose were obtained from Isotec Inc. (Miamisburg, Ohio, USA) with isotopic enrichments of 99%. Unlabeled lactose was obtained from Fluka (Buchs, Switzerland).

Experimental diets
Similar to previous studies, we used high-fat EFA-deficient and EFA-sufficient (control) diets (16 wt% and 34 energy% fat), in order to mimic more closely the human diet composition\(^4\). The diets were custom synthesized by Arie Bloks BV (Woerden, the Netherlands, diet codes EFA deficient #4141.08 and EFA-sufficient #4141.07). The EFA-deficient diet contained 64 mol% palmitic acid (C16:0), 18 mol% stearic acid (C18:0), 13 mol% oleic acid (C18:1n-9) and 5 mol% linoleic acid (C18:2n-6). The isocaloric EFA-sufficient diet contained 36 mol% C16:0, 5 mol% C18:0, 31 mol% C18:1n-9 and 29 mol% C18:2n-6. Fatty acid contents of the diets were analyzed by extracting, hydrolyzing and methylating total dietary fatty acids as described by Muskiet et al. and subsequent separation and quantification of fatty acid methyl esters was performed by gas chromatography as described previously\(^4,24\).

Experimental procedures
Induction of EFA deficiency. Mice were fed standard laboratory chow containing 6 weight% fat from weaning, and were switched to EFA-deficient or control diet at eight weeks of age. At the end of eight weeks-period on EFA-deficient or control diet, fat absorption was assessed by measuring food intake and collecting feces for 72 h. Following this eight weeks-period the mice underwent a glucose/lactose absorption test with U-\(^{13}\)C-glucose and \(^{1-13}\)C-lactose (details see below)\(^24,25\). After the test the mice were anesthetized and sacrificed by obtaining a large blood sample through cardiac puncture for determination of erythrocyte EFA-status by the triene/tetraene (C20:3n-9/C20:4n-6) ratio\(^4\). The small intestine was excised, flushed with ice-cold PBS and divided into a proximal, mid and distal segment of similar size. Smaller parts from the middle of each small intestinal segment were harvested for histology and gene expression. The remaining part of the small intestine was opened lengthwise and the mucosa was removed by scrubbing the luminal surface with a glass
coverslip. Mucosa was homogenized in buffer (see below for details) and used for the determination of enzyme activity, proteins and LA concentrations in mucosal phospholipids. **Glucose/lactose absorption.** Glucose absorption and lactose digestion were determined by a combined U-\(^{13}\)C-glucose/1-\(^{13}\)C-lactose absorption test. After an overnight fast, mice received 0.5 mg U-\(^{13}\)C-glucose, 5 mg 1-\(^{13}\)C-lactose and 5 mg naturally enriched lactose in 300 \(\mu\)L PBS via gastric gavage. Before and at time points 7.5, 15, 30, 45, 60, 90, 120 and 180 min. after administration, blood samples were obtained by blood spot technique from the tail for determination of blood concentrations of (total) glucose, \(^{13}\)C-glucose (glucose originating from U-\(^{13}\)C-glucose) and \(^{13}\)C-glucose (originating from 1-\(^{13}\)C-lactose) \(^{25}\). For reasons of clarity, we will address “blood” \(^{13}\)C-glucose and \(^{13}\)C-glucose as “plasma” in the Results and Discussion sections.

**Analytical methods**

- **Lipid absorption, triene/tetraene ratio, blood glucose and serum insulin concentrations.** Lipid absorption and erythrocyte triene/tetraene ratio were determined as described previously \(^4,27\). Blood glucose levels were measured with a Lifescan EuroFlash glucose meter (Lifescan Benelux, Beerse, Belgium). Insulin was measured in a solid phase two-site enzyme immunoassay in which two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule (Ultrasensitive Mouse Insulin kit; Merodia, Uppsala, Sweden).

- **Histology and villus length along the small intestinal axis.** Morphology of proximal, mid and distal small intestine was assessed by hematoxylin and eosin staining of formalin-fixed material. Proliferating cells were detected by staining of nuclear Ki-67 antigen. Morphometrical analysis of small intestinal samples was performed as described by evaluation of 4, 6 and 7 villi per proximal, mid and distal intestinal segment, respectively, of 4 to 6 animals per group. The digitized images were evaluated at 10 x magnification using the calibrated image analysis system (Leica Quantimet 570 C; Leica Qwin Pro V 2.8). The epithelial surface lining was demarcated and measured as a parametrical length, whereby 1 pixel was equal to 0.544 \(\mu\)m.

- **Glucose/lactose absorption.** The analysis of \(^{13}\)C-glucose and \(^{13}\)C-glucose concentrations from blood spots was performed according to Van Dijk *et al.* by gas chromatography-mass spectrometry (SSQ700, ThermoFischer B.V., Breda, The Netherlands) \(^{25}\).

- **Disaccharidase activity assay in mucosal homogenates.** A portion of small intestinal mucosa (from the proximal, mid and distal part) was homogenized with PBS buffer containing protease inhibitors (Roche, Indianapolis, USA) in order to make 4% homogenates for use in enzyme activity assay. Enzyme activity level of lactase was measured in freshly scraped intestinal mucosa as described previously by Dahlqvist \(^{28}\). Activity levels were normalized to protein levels, measured by the BCA method as described by the manufacturer (Pierce, Rockford, IL).

- **Measurement of mRNA expression by real-time PCR (Taqman).** mRNA expression of the differentiation marker and lactose digesting enzyme (lactase) was measured in proximal, mid and distal small intestine by real-time PCR as described previously \(^{29}\). In addition, mRNA expression levels of intestine-specific transcription factors (Cdx-2, Gata-4 and Hnf-1\(\alpha\)) were measured by real-time PCR in the mid part of the small intestine. PCR results were normalized to \(\beta\)-actin mRNA levels. The sequences of the primers and probes are listed in Table 1.
LA determination in phospholipids of intestinal mucosa. Thirty mg of intestinal mucosa was homogenized in 200 µl of 0.9% NaCl and lipids were extracted according to Bligh and Dyer after the addition of the fatty acid internal standard (C17:0) and anti-oxidant (BHT). Lipid extracts were fractionated into phospholipids and other lipids using TLC (20 x 20 cm, Silica gel 60 F254; Merck) with hexane/diethyl ether/acetic acid (80:20:1, v:v:v) as running solvent. Phospholipid spots were scraped and phospholipids were extracted by methanol/chloroform. Phospholipid LA ratio was determined according to Muskiet et al. as described previously.

Statistical analysis
Values represent means ± SD for the indicated number of mice per group. Using SPSS version 12.0.2 statistical software (Chicago, IL, USA), we calculated significance of differences with the Mann-Whitney U-test. P-values below 0.05 were considered statistically significant.

RESULTS
Body weight and food ingestion were assessed every two weeks and there were no significant differences in basal or final body weight, nor in food intake (data not shown) between EFA-deficient and control mice.

Pronounced EFA deficiency of EFA-deficient mice
After eight weeks of treatment, in mice fed the EFA-deficient diet, the triene/tetraene ratio in red blood cell membranes was strongly increased compared with the control group (0.23 ± 0.06 vs. 0.01 ± 0.00; respectively, p<0.01). Conform previous observations, the mice fed the EFA-deficient diet also showed other characteristics of EFA deficiency including increased bile flow (+78%, p<0.05), biliary secretion rates of bile salts (+212%, p<0.01) and phospholipids (+82%, p<0.05), and higher levels of triene/tetraene ratio in plasma (0.55 ± 0.20 vs. 0.01 ± 0.00, p<0.01), compared with control mice (data not shown). Fat balance during 72 hours revealed a decreased total fat absorption in EFA-deficient compared to control mice (81% vs. 99%, resp., p<0.01; fig 1). The absorption of saturated fatty acids, palmitic (C16:0) and stearic (C18:0) acids, was affected to a greater extent than that of the unsaturated fatty acids oleic (C18:1n-9) and linoleic acid (C18:2n-6). Together, these observations indicated that the mice fed the EFA-deficient diet had profound EFA deficiency after 8 weeks on the experimental diet.
EFA deficiency in mice not associated with alterations in intestinal morphology

Hematoxylin/eosin (data not shown) and Ki67 staining of the three segments of the small intestine revealed no clear differences in morphology or proliferative capacity between EFA-deficient and control mice (fig 2). The villus lengths were similar in EFA-deficient and control mice, as determined by morphometrical measurements in the three segments of the small intestine (fig 2).

EFA deficiency is associated with delayed glucose clearance

Basal blood glucose concentrations were similar in EFA-deficient and control mice. After intragastric administration of the glucose/lactose bolus, glucose concentrations rapidly increased in control mice, with a maximum concentration at 30 min. after administration (fig 3). In EFA-deficient mice, the increase in blood glucose levels was similar to that in control mice up to 30 min., but then continued to increase, reaching a maximum concentration at 60 min. The glucose concentrations between 60 and 180 min. were slightly, but significantly higher in EFA-deficient mice compared to controls (+10-15%, p<0.05). Accordingly, the area under the curve was higher for the EFA-deficient mice compared with controls (+15%, p<0.01).
p<0.05, data not shown). Based on the apparently delayed glucose clearance, we determined insulin concentrations at the end of the experiment (at ~180 min.). In EFA-deficient mice insulin concentrations were significantly higher than in control mice (0.55 µg/ml vs. 0.35 µg/ml, respectively, p<0.01).

Similar glucose absorption but delayed lactose digestion in EFA-deficient mice

To assess the competence of monosaccharide absorption in EFA deficiency, we determined blood appearance of $^{13}$C$_6$-glucose (fig 3). After the administration of the bolus, blood $^{13}$C$_6$-glucose concentration rapidly increased with a maximum at 45 min. for both groups. After 45 min., $^{13}$C$_6$-glucose rapidly disappeared until 120 min., after which the rate of disappearance decreased in both EFA-deficient and control mice. Thus, the $^{13}$C$_6$-glucose appearance and disappearance was similar in EFA-deficient and control mice, supporting unaffected monosaccharide absorption in the former.

In order to measure the competence of disaccharide digestion and absorption, we determined blood appearance of $^{13}$C-glucose, originating from the administered $^1$-$^{13}$C-lactose (fig 3). $^{13}$C-glucose reached a maximum concentration in control mice at 45 min. after bolus administration. The $^{13}$C-glucose disappeared from the blood within the next 2 hours, with the slowest disappearance during the last hour after the bolus administration. Blood appearance of $^{13}$C-glucose in EFA-deficient mice, however, increased to a slower extent and reached its maximal concentration at approximately 60 min. after the bolus administration. Thus, the $^{13}$C-lactose uptake was delayed in EFA-deficient compared to control mice.
Decreased mRNA expression and lactase activity in mid small intestine of EFA-deficient mice

Lactase is a critical disaccharidase during early postnatal life and a sensitive intestinal marker for functional changes occurring in the small intestine of the adult animal. Its activity relatively decreases during weaning to low adult levels, thus the total lactase activity remains the same during the adulthood\(^3\). Measurement of the enzyme activity of lactase along the proximal-to-distal axis of the small intestine revealed a lower activity in the mucosa of the mid part of the small intestine of EFA-deficient compared to control mice (fig 4). The decreased lactase activity corresponded with lower mRNA levels of lactase, as shown by quantitative PCR (fig 4). We determined if reduced lactase mRNA expression levels were regulated at the transcriptional level. However, the mRNA expression of transcription factors involved in regulation of the lactase mRNA expression, namely Cdx-2, Gata-4 and Hnf-1α (fig 4), was not different between EFA-deficient and control animals.

Decreased mRNA levels in the mid intestine indicate that the intestinal impairment can not exclusively be the result of alterations in membrane composition and fluidity.

EFAs are involved in regulation of membrane fluidity and alterations in membrane lipid matrix. Therefore, it has been proposed that EFAs indirectly influence normal conformation and functioning of the proteins embedded in the inner and/or outer leaflet of the membrane\(^14\). For this reason we tested if lactase activity in the mid segment of the small intestine correlated with LA levels. Phospholipid LA concentration was determined the mucosa of the three segments of the small intestine (fig 5). LA concentrations were highest in the mid part of the small intestine in control mice. Interestingly, LA concentration was significantly lower in the mid part of the small intestinal mucosa of EFA-deficient compared to control mice (26 mol% vs. 16 mol%, respectively, p<0.01). LA concentrations in proximal and distal part were similar in both groups. In the mid small intestine LA concentrations positively correlated with lactase activity (r=0.88, p<0.001) and mRNA expression of lactase (r=0.79, p<0.01) (fig 5). Decreased mRNA levels in the mid intestine indicate that the intestinal impairment can not exclusively be the result of alterations in membrane composition and fluidity.
DISCUSSION

Our previous studies suggested that EFA deficiency in mice affects fat absorption at the level of the small intestinal mucosa. We now explored the effects of EFA deficiency in mice on mucosal histology and on a physiological function of the small intestine, carbohydrate digestion and absorption. Our data demonstrate that EFA deficiency is not only associated with fat malabsorption, but also with impaired lactose digestion in the murine model of EFA deficiency. The impaired lactose digestion coincided with a ~50% reduced lactase activity and mRNA expression in mid small intestine of EFA-deficient mice. Intestinal lactase activity and mRNA expression strongly correlated with mucosal linoleic acid concentrations, which were depressed in EFA deficiency, particularly in mid-intestine.

As expected from previous studies, our murine model of EFA deficiency was clearly deficient, as indicated by elevated triene/tetraene ratios in erythrocytes and plasma, fat malabsorption, and other biochemical signs of EFA deficiency, like increased bile flow and biliary output. EFA deficiency in mice did not affect morphology or proliferative capacity of the small intestine. As far as we know, our study is the first to describe the effects of EFA deficiency on the intestinal morphology in mice. Christon et al. have shown that low dietary linoleic acid levels were associated with alterations in villi and crypt sizes in rats. We did not observe differences in villus length between EFA-deficient and control mice using morphometrical evaluation of the villus length in the proximal-to-distal axis of the small intestine. These results indicate that EFA deficiency associated malabsorption of fats and disaccharides is not associated with morphological alterations in small intestine of mice.

To assess small intestinal function in EFA-deficient mice, we studied carbohydrate absorption, using stable isotope methodology. The advantage of stable isotope methodology is that it can easily be extrapolated to patient studies. EFA-deficient mice
had higher total blood glucose levels from 60 min. after the administration of the glucose/lactose bolus. High total glucose levels in blood could theoretically be explained by lower blood glucose clearance (slower postprandial uptake of glucose by the peripheral tissues), rather than by disturbed intestinal absorption. This hypothesis is supported by higher insulin concentrations at the end of the experiment in EFA-deficient compared with control mice. This observation is in accordance with previous studies suggesting a relationship between EFA deficiency and insulin resistance. However, we cannot exclude that the increased content of saturated fats in the EFA deficient diet contributes to this phenomenon, independently from EFA deficiency.

Measurement of the absorption of $^{13}$C$_6$-glucose, originating from the administered $^{13}$C-glucose, revealed similar appearance and disappearance of the labeled glucose in both groups. This observation indicates that EFA deficiency does not affect the absorption of the monosaccharide glucose in mice. The blood appearance of $^{13}$C-glucose originating from lactose, however, was significantly delayed in EFA-deficient mice. The discrepancy in the effect of EFA deficiency on glucose and lactose absorption could be explained by the diverse intestinal fates of these carbohydrates. Unlike glucose, which is directly transported by the glucose transporters across the brush border membrane of the enterocyte, lactose first needs to be hydrolyzed by the enzyme lactase. In order to investigate whether our functional results corresponded with altered lactase activity or expression, we measured these parameters in EFA-deficient and control mice. Lactase is the critical enzyme for hydrolysis of lactose and a good marker of functional changes in the small intestine. The delayed lactose digestion corresponded with an approximate 50% reduction in both lactase activity and mRNA expression compared to control mice. The mRNA levels of relevant transcription factors for lactase mRNA expression were unaffected in EFA-deficient mice, suggesting that the expression of lactase is regulated in a post-transcriptional manner during EFA deficiency.

Under physiological conditions phospholipids of the small intestinal mucosa contain considerable amounts of LA (C₁₈:2n-6) and of its long-chain polyunsaturated fatty acid metabolite arachidonic acid (AA, C₂₀:₄n-6). During EFA deficiency the levels of this major dietary EFA are decreased in intestinal mucosa. We observed LA deficiency in mucosal phospholipids, particularly the mid part of the small intestinal mucosa, which strongly correlated with reduced lactase activity and mRNA expression. We speculate that the decreased levels of LA in this intestinal segment correspond to the predominant location of nutrient absorption. It is tempting to speculate that low levels of LA in phospholipids of cellular membranes lead to structural and physiological changes in the lipid membrane, eventually causing functional changes in membrane anchoring lactase enzyme. Since not only lactase activity but also its mRNA expression was decreased in EFA deficiency, it is likely that altered membrane fluidity is not the single factor involved.

Our present results indicate that EFA deficiency has functional consequences for small intestinal function in mice, and it provides indirect support for the hypothesis that reduced mucosal function is involved in fat malabsorption in EFA deficiency. EFA deficiency in (pediatric) cholestatic patients seems primarily caused by fat malabsorption due to bile deficiency. Recently, we reported that cholestasis per se does not affect carbohydrate digestion or absorption, using a rat model of short-term cholestasis. Our present study indicates, however, that EFA deficiency aggravates the malabsorption of fat, and decreases the small intestinal capacity to digest carbohydrates. Decreased levels of LA in the mid part of the small intestine seem to play a pathophysiological role in the diminished mucosal
function in EFA deficiency. Our findings imply that the nutrition of cholestatic patients encountering EFA deficiency should accommodate the decreased capacity to absorb fat, the EFA deficiency (possibly by using LA-rich phospholipids) 40, and the reduced capacity to digest disaccharides.

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