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Essential fatty acid deficiency and the small intestine

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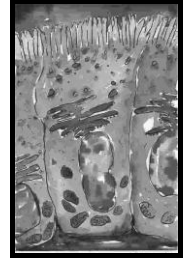
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CHAPTER 5



FUNCTIONAL CHARACTERIZATION OF AN *IN VITRO* MODEL OF ESSENTIAL FATTY ACID DEFICIENCY IN INTESTINAL EPITHELIAL CELLS

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ABSTRACT

In vivo studies in mice have indicated that essential fatty acid (EFA) deficiency negatively affects small intestinal function. It has remained difficult, however, to unravel the molecular mechanism(s), during EFA deficiency, which affect(s) small intestinal epithelium *in vivo*. The aim of the present study was to develop and characterize an *in vitro* model for EFA deficiency in small intestinal cells, which could allow unraveling the (intra)cellular molecular mechanism(s) with respect to the effect on small intestinal function.

Intestinal epithelial cells (Caco-2) were cultured in medium containing normal or delipidated fetal calf serum (FCS) for at least 1 week post-confluence. We characterized fatty acid profiles, morphology and mRNA expression of relevant small intestinal markers lactase and sucrase isomaltase in EFA-deficient and control Caco-2 cells. Cellular permeability was assessed by transepithelial electrical resistance (TER) and by determination of the mRNA expression of relevant tight junction components and localization of ZO-1. To study the reversibility and specificity of the effects of EFA deficiency, we cultured the EFA-deficient cells in medium supplemented with linoleic acid (LA).

The culturing of Caco-2 cells with delipidated FCS decreased the concentration of LA by 81% ($p < 0.01$) and increased the triene:tetraene ratio (+187.5%; $p < 0.01$), compared with control cells and represents EFA deficiency in these cells. The morphology of the cells was not severely affected, although mRNA expression of relevant differentiation markers of the brush border membrane of the small intestine was severely reduced in EFA-deficient Caco-2 cells. The cellular permeability was significantly increased as assessed by TER. However, the mRNA expression of tight junction components and localization of ZO-1 were not affected in EFA-deficient Caco-2 cells. Although the LA supplementation to EFA-deficient Caco-2 cells was incorporated into cellular phospholipids to a similar extent as in the control cells, it did not restore reduced mRNA expression of small intestinal differentiation markers or the increased permeability.

Caco-2 cells exposed to delipidated FCS rapidly demonstrate EFA deficiency with characteristics mimicking EFA deficiency in the small intestine *in vivo*. EFA deficiency severely reduced expression of relevant brush border markers of the small intestine, and impaired cellular permeability. Interestingly, these effects were not immediately reversible by LA supplementation to EFA-deficient Caco-2 cells.

INTRODUCTION

Essential fatty acid (EFA) deficiency is a frequent complication of diseases in which fat malabsorption occurs, mainly during cholestatic liver diseases in pediatric patients with limited fat storage. Interestingly, EFA deficiency by itself can also induce fat malabsorption and impair small intestinal function, probably by intracellular mechanism(s), in rats and mice.^{1,2,3} Studies on the intracellular consequences of EFA deficiency in small intestinal enterocytes have been scarce.¹ Spalinger et al. demonstrated in EFA-deficient Caco-2 cells *in vitro* that supplementation with structured triglycerides could increase cellular concentrations of linoleic acid (LA, C18:2 ω -6) and its metabolite arachidonic acid (AA, C20:4 ω -6), resulting in the correction of the biochemical marker of EFA deficiency (triene:tetraene ratio).¹ However, the functional consequences of EFA deficiency or those of structural triglyceride supplementation to EFA-deficient cells were not explored in this *in vitro* model. EFA deficiency in mice and rats has been associated with reduced fat and disaccharide absorption,^{2,3} reduced expression and enzyme activity of the small intestinal differentiation marker (lactase) and impaired negative feedback regulation of bile salt synthesis.⁴ To understand the mechanism(s) by which EFA deficiency influences small intestinal function, we reasoned that further characterization of an *in vitro* model is essential. Therefore, in present study we further characterized an *in vitro* model of EFA deficiency in Caco-2 cells, adapted from the protocol of Spalinger et al.¹ We validated our model by analyzing the fatty acid profiles and triene:tetraene ratio in EFA-deficient Caco-2 cells. Furthermore, we compared relevant functional parameters in the *in vitro* model with our previous *in vivo* findings in EFA-deficient mice. It has been debated whether EFA deficiency affects small intestinal permeability.^{5,6} Therefore, in our functional characterization, we included the analysis of the permeability of small intestinal cells. Cellular permeability was assessed by determination of the transepithelial electrical resistance (TER) and by analysis of tight junction components. Our previous studies in EFA-deficient mice pointed out a strong correlation between LA concentrations in mucosal phospholipids and lactase mRNA expression and enzyme activity. To analyze the robustness and specificity of the EFA deficiency in our *in vitro* model, we re-supplied LA to EFA-deficient cells. We determined whether LA supplementation leads to uptake and incorporation of LA into phospholipids, and whether it can reverse the effects of EFA-deficient phenotype.

We clearly show that EFA-deficient Caco-2 cells are a useful model to study in more detail the effects of EFA deficiency. Our *in vitro* data indicate that EFA deficiency negatively affects the cellular permeability and the presence of typical differentiation markers of small intestinal cells, and that these effects are not rapidly reversible by LA supplementation.

MATERIAL AND METHODS

Delipidation of FCS

FCS was delipidated by means of di-iso-propylether and butanol extraction, according to the protocol of Cham and Knowles.⁷ This protocol is known to eliminate over 90% of all fatty acids in a solution. Subsequent to delipidation, both delipidated and control FCS

were dialyzed for 72 hours in 0.9% NaCl at 4°C by means of the Spectra/Por3 molecular porous membrane tubing (MW 3500; Spectrumbioscience, Rancho Dominguez, CA, USA).

Coomassie blue staining

Protein concentration was determined according to the manufacturer's protocol (BCA kit; Pierce Biotechnology Inc., Rockford, Ill, USA). 10 µg of protein from the FCS was analyzed on gel by means of the standard protocol for Coomassie Blue staining to determine the amount and possible selectivity of protein loss by the delipidation protocol.⁸

Cell culture

Human Caco-2, an immortalized line of heterogeneous human epithelial colorectal adenocarcinoma, cells from the American Type Tissue Culture Collection (Manassas, VA) were cultured in Dulbecco's modified Eagle's medium containing 10%, penicillin (100 units/mL)/streptomycin (100 µg/mL), 1% non-essential amino acids and 0.25% human transferrin in an atmosphere of 5% CO₂-95% air at 37 °C. Cells were subcultured at 90% confluence (approximately every 5 days) by trypsin. For the experiments, cells between passages 20 and 40 were used. Caco-2 cell line was used in the experiments because this is the only immortalized cell line which differentiates *in vitro* into small-intestinal enterocyte-like cells, expressing the hydrolases lactase and sucrase-isomaltase.^{9,10} These characteristics make Caco-2 cells a valid model to study the function of the small intestinal enterocytes.

Induction of EFA deficiency in Caco-2 cells

Cells were made EFA-deficient according to the adapted protocol of Spalinger et al.¹ Shortly, medium was replaced by DMEM supplemented with dialyzed FCS (control cells) or with delipidated FCS (EFA-deficient cells) 1 day after seeding. Seven days after reaching complete confluence, the cells were harvested for several analytic procedures described below (*Morphology and Immunofluorescent stainings, Thin layer chromatography-TLC, Fatty acid methylation and Gas chromatography, RNA isolation and Quantitative PCR*). By this time, cells were cultured in EFA-deficient medium for ten days.

LA supplementation to EFA-deficient Caco-2 cells

In the first experiment, at day 7 after plating control and EFA-deficient cells were supplemented with 50 µM LA and cells were harvested at day 0, 2 and 6 after supplementation for linoleic acid analysis in total cell lysates or thin layer chromatography (TLC) was performed for fatty acid analysis in different lipid classes (phospholipids and triglycerides; see below section *Thin layer chromatography* for details).

In the second experiment, at day 7 after plating EFA-deficient Caco-2 cells were randomly separated in two groups. One group continued in culture with EFA-deficient medium, while the other group received DMEM supplemented with 50 µM LA (Sigma Chemical Co., St. Louis, MO, USA). Cells were harvested at day zero and every two days afterwards for 6 days. Subsequently, harvested cells were used for RNA isolation.

In a separate, similar experiment, control and EFA-deficient cells were separated and half of control cells was treated with 50 μM LA and half of the EFA-deficient cells was treated with 50 μM LA. The other half of the cells received regular (control) or EFA-deficient medium. Cells were harvested at day 0, 4 and 6 after LA supplementation. After lipid extraction, TLC was performed for fatty acid analysis in different lipid classes (phospholipids and triglycerides).

Transelectrical epithelial resistance (TER)

For TER measurements, cells were cultured in sterile polystyrene transwell plates (Costar, Corning, NY, USA), for a period of 2 weeks. TER was measured every second day after by means of the EVOM meter (World Precision Instruments, Sarasota, FL, USA). For the experiments with LA supplementation, TER was measured for a period of one week. Control cells (cultured in DMEM with normal FCS) were set at 100%.

Analytical methods

Fatty acid methylation and Gas chromatography

Cells were washed twice with PBS and harvested for fatty acid extraction, hydrolysis and methylation according to the protocol of Muskiet et al.¹¹ Subsequent separation and quantification of fatty acid methyl esters was performed by gas chromatography. Heptadecaenoic acid (C17:0) was added to all samples as an internal standard before extraction and methylation procedures, and butylated hydroxytoluene was added as an antioxidant.

Thin layer chromatography (TLC)

Total triglycerides and phospholipids were separated in harvested cells, after lipid extraction,¹² by means of TLC, as described previously.¹³ Subsequent to TLC, lipid fractions were scraped and fatty acid extraction, hydrolysis and methylation was performed as described above, followed by fatty acid analysis by gas chromatography.¹³ For the separation of different phospholipid classes, chloroform/methanol/acetic acid/water was used as the running solvent. For the separation of different lipid classes (triglycerides and phospholipids) hexane/diethyl ether/acetic acid was used as the running solvent.

Morphology and Immunofluorescent stainings

Morphology of Caco-2 cells was assessed by hematoxylin and eosin staining of formalin-fixed cells grown in 6-well cell culture plates.

Immunocytochemical staining for tight junction component ZO-1 was performed in Caco-2 cells. Rabbit anti-ZO-1 antibody was from Zymed Laboratories Inc. (South San Francisco, CA, USA). Cells were seeded and grown on cover slips in DMEM with control or delipidated (EFA-deficient) serum. At the end of the experiment, Caco-2 cell monolayers were washed in PBS and fixed in acetone at -20°C for 15 min. Cell monolayers were washed with PBS afterwards on room temperature for 15 min. on a shaker. Subsequently, the cells were incubated with primary antibodies for 2h at room temperature (1:100 dilution in 1% BSA/PBS) followed by three washes with PBS. The incubation with the secondary antibody (FITC-conjugated goat anti-rabbit, 1:400 dilution

in 1% BSA/PBS) was performed at room temperature for 30 min. Cells were mounted on a slide and the fluorescence was examined using a fluorescent microscope (Zeiss Hal100; Carl Zeiss BV, Sliedrecht, the Netherlands) Images were stacked using the software, Zeiss Axio Vision (Release 4.6.3; Carl Zeiss BV, Sliedrecht, the Netherlands).

RNA isolation and Quantitative PCR

At the end of the experiment, cells were washed in PBS and RNA isolation was performed using TRIzol reagent (Invitrogen, Breda, the Netherlands), followed by quantitative PCR analysis of mRNA expression (TaqMan) as previously described.¹⁴ PCR results were normalized to the mRNA expression of the housekeeping gene *GAPDH*. Primer and probe sequences for the Q-PCR analysis have been published (www.LabPediatricsRug.nl: *Realtime PCR Primers & Probes Database*).

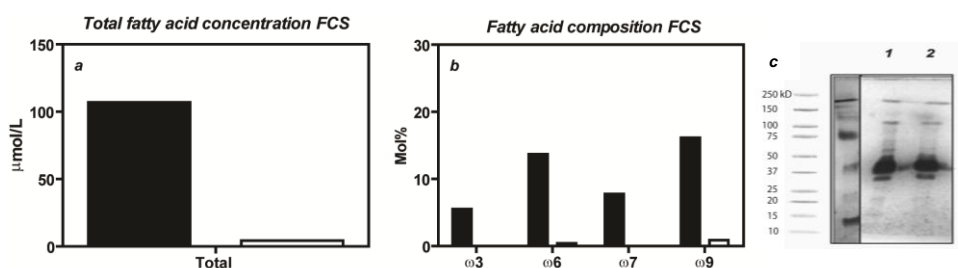


Figure 1 (a) Total fatty acid concentrations and (b) molar concentrations of fatty acid families in control (black bars) and delipidated FCS (white bars). (c) Coomassie blue staining of the protein content in control (lane 1) and delipidated FCS (lane 2).

Statistical analysis

Data were statistically analyzed by Student's two-tailed *t*-test. For all experiments, *p*-values below 0.05 were considered statistically significant.

RESULTS

Delipidation

Cells were cultured in medium containing delipidated FCS or control FCS. Fatty acids were removed almost completely by the delipidation of FCS (Figure 1a). There was no major difference in classes of fatty acids removed; both essential (ω -3 and ω -6) and non-essential (ω -7 and ω -9) fatty acids were virtually absent in delipidated FCS (Figure 1b). Protein content was not significantly altered in concentration or composition by the delipidation, as indicated by the Coomassie blue staining, indicating specificity of the procedure for removal of fatty acids, rather than for proteins (Figure 1c).

Fatty acid profiles

One week after the full confluence, cultured cells were harvested for fatty acid analysis by means of gas chromatography. In EFA-deficient cells the biochemical marker for EFA deficiency, triene:tetraene ratio, was significantly higher compared with control cells (Figure 2a). Total fatty acid concentration was lower in EFA-deficient cells, but the

difference did not reach the significant difference (Figure 2b). While the molar concentrations of monounsaturated fatty acids (MUFA) tended to be higher in EFA-deficient cells, the molar concentrations of polyunsaturated fatty acids (PUFA) tended to be lower in EFA-deficient cells (Figure 2c). However, both differences did not reach the statistical significance. The molar concentrations of saturated fatty acids (SAFA) were similar in both EFA-deficient and control cells (Figure 2c).

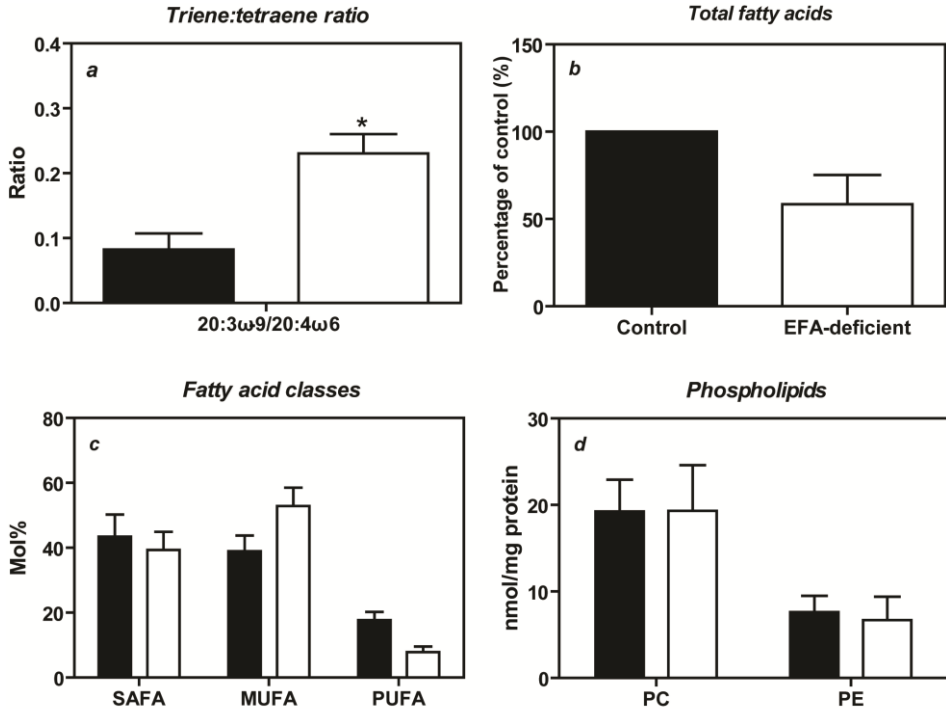


Figure 2 (a) Triene:tetraene ratio, (b) total fatty acid concentrations, (c) different fatty acid classes and (d) phosphatidylcholine/phosphatidylethanolamine concentrations in control (black bars) and EFA-deficient (white bars) Caco-2 cells. Cells were cultured in control or EFA-deficient medium for one week after complete confluence. Data represent means \pm SEM of four independent experiments and * $p < 0.05$ is the significant difference between the EFA-deficient and control cells.

Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are major phospholipids in mammalian membranes. Li et al. recently proposed the PC/PE ratio as a marker for cell membrane integrity in hepatic tissue.¹⁵ We evaluated whether EFA deficiency in Caco-2 cells leads to reduced phospholipid concentrations and alterations in the PC/PE ratio. There is no significant difference in PC or PE concentrations (or the ratio of these two, data not shown) between EFA-deficient and control cells (Figure 2d).

Morphology

Cell morphology was assessed every two days of culture to monitor the growth and confluence of the cells. There was no significant difference observed in the morphology of the cells at day 0 and day 4 post-confluence between EFA-deficient and control cells.

EFA-deficient cell at day 7 post confluence seemed to have several large structures, possibly gaps or vacuoles (indicated by the white arrows in Figure 3).

Permeability

Transcellular permeability of the cells was assessed by the TER measurements every two days after complete confluence, for two weeks. EFA-deficient cells followed the same increasing trend of TER during the first three days of culture (Figure 4a). At day 3, EFA-deficient cells had even higher TER values than control cells. However, from day 3 TER values decreased drastically in EFA-deficient cells down to 50% of control values at day 5-6 (Figure 4a), down to basal levels measured at day 1 (~100 Ω , equal to an empty transwell). These data indicated increased transcellular permeability in EFA-deficient Caco-2 cells compared with control cells. The values of control cells were stable from day 14 and further post-confluence (~300 Ω ; data not shown).

In addition to the transcellular route, transport of molecules from the apical to the basolateral compartment can occur by means of paracellular transport. Paracellular permeability across the epithelia is regulated by the tight junctions.¹⁶ We analyzed the mRNA expression three relevant tight junction components, ZO-1, occludin and claudin 1. In addition, localization of ZO-1 was assessed by immunofluorescent staining. Quantitative PCR analysis revealed similar mRNA expression of all three tight junction markers (Figure 4b), indicating that EFA deficiency does not affect transcriptional regulation of these tight junction components *in vitro*. Immunofluorescent staining was performed to study the protein expression and localization of ZO-1 (Figure 4c). As expected, in both control and EFA-deficient Caco-2 cells ZO-1 was expressed at the intracellular junctions of the monolayers.

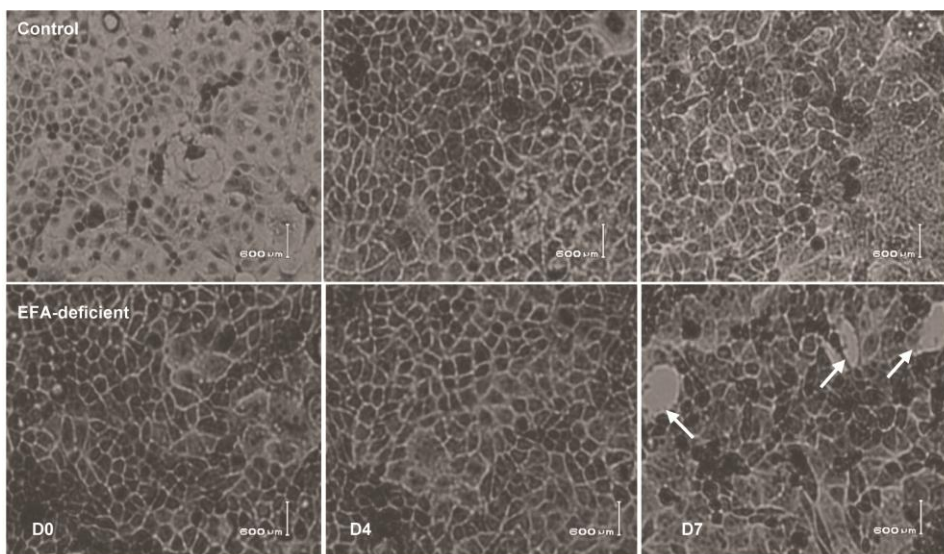


Figure 3 Representative picture of the hematoxylin/eosin staining of EFA-deficient (lower panel) and control (upper panel) Caco-2 cells at day 0, 4 and 7 after complete confluence. The staining was performed twice in two independent experiments.

In EFA-deficient Caco-2 cells ZO-1 the expression is not only restricted to the intracellular junctions, but seemed to be present in the intracellular compartment as well (Figure 4c).

Small intestinal markers/differentiation

In order to determine if EFA deficiency affects small intestinal differentiation *in vitro*, we measured mRNA expression of relevant enterocyte markers lactase and sucrase isomaltase. Upon confluence, Caco-2 cells normally differentiate from the colonic towards the small intestinal phenotype. This differentiation process is accompanied by an increasing mRNA expression of lactase and sucrase isomaltase. Despite the relatively normal morphology, EFA-deficient Caco-2 cells showed almost absent expression of lactase and sucrase isomaltase (Figure 5a), while the expression in control cells was comparable to previous studies in post-confluent Caco-2 cells.¹⁰ Lactase and sucrase isomaltase are expressed at the apical cell compartment, at the brush border membrane. The mRNA expression of another intestinal marker villin, which is not exclusively located at the brush border membrane, but also intracellularly, was not affected by EFA deficiency (Figure 5a). Fatty acids are known as the endogenous ligands for peroxisome proliferator-activated receptors (PPARs). We determined whether EFA-deficient Caco-2 cells cultured in medium with delipidated serum expressed lower

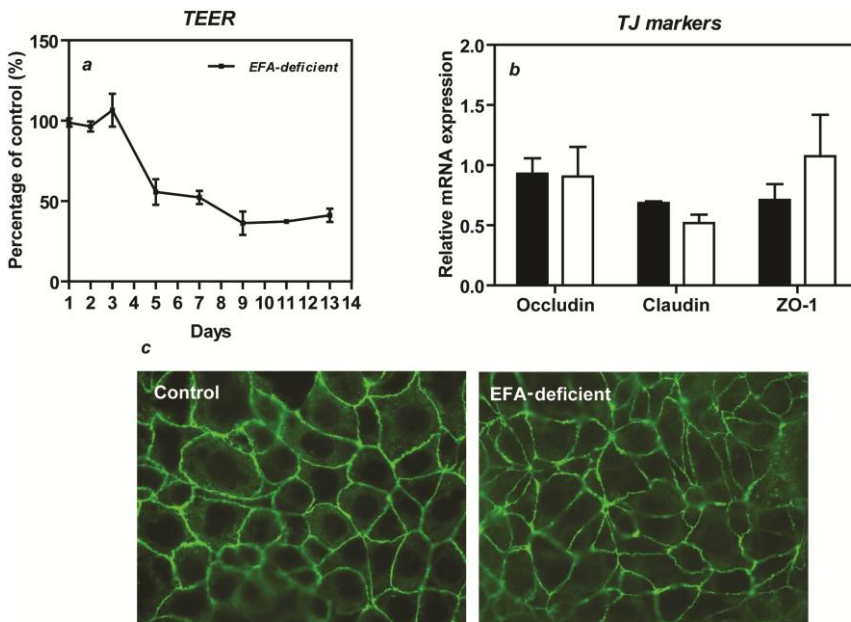


Figure 4 (a) Transepithelial electrical resistance (TER) in EFA-deficient Caco-2 cells as a percentage of control cells over a period of 2 weeks of culture (control cells reached the maximum TER values of 350 Ohm, while the EFA-deficient Caco-2 cells had maximum TER values of 250 Ohm). (b) mRNA expression of tight junction components occludin, ZO-1 and claudin in EFA-deficient (white bars) and control cells (black bars). (c) Fluorescence staining for ZO-1 in control (left panel) and EFA-deficient (right panel) cells. Data represent means \pm SEM and $*p < 0.05$ is the significant difference between the EFA-deficient and control cells. The experiments were performed at least in duplicate.

mRNA expression of PPARs. EFA-deficient Caco-2 cells showed reduced mRNA expression of PPAR α (Figure 5b) compared with control Caco-2 cells, while the expression of PPAR δ and PPAR γ was similar. Function of PPARs requires heterodimerization with the retinoid X receptor (RXR). There was no difference in RXR expression between EFA-deficient and control Caco-2 cells (Figure 5b).

Effects LA supplementation to EFA-deficient Caco-2 cells

Fatty acid profiles in our *in vitro* and *in vivo* studies revealed that EFA deficiency in the small intestinal epithelium mostly affected the concentrations of the LA (specifically in the phospholipids). Therefore, we aimed to study the specificity and reversibility of the effects of EFA deficiency. For this reason we cultured the EFA-deficient cells in medium supplemented with LA.

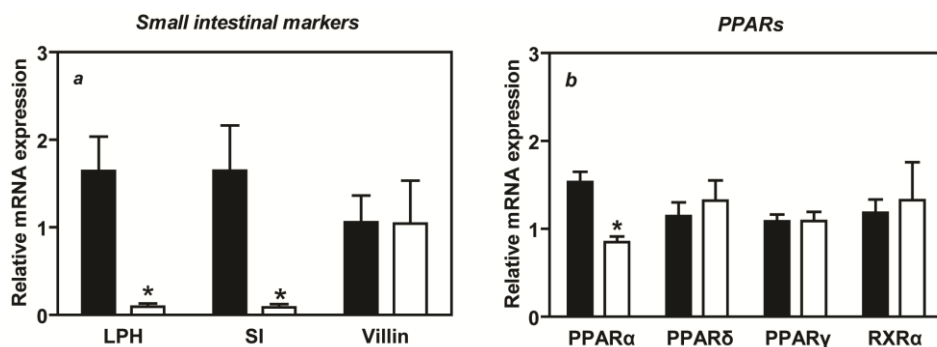


Figure 5 (a) mRNA expression of relevant small intestinal enterocyte markers lactase, sucrose isomaltase and villin was measured in EFA-deficient (white bars) and control (black bars). **(b)** mRNA expression of three types of PPARs (α , δ and γ) and of RXR α in EFA-deficient (white bars) and control (black bars). Data represent means \pm SEM of four independent experiments and * $p < 0.05$ is the significant difference between the EFA-deficient and control cells.

Supplementation with LA resulted in increasing concentrations of LA in total cell lysates of both control and EFA-deficient Caco-2 cells (Figure 6a). This would be beneficial if LA was incorporated into the phospholipids, which are normally retained within the enterocytes and mobilized towards the membrane. Triglycerides, on the other hand, are not maintained within the enterocytes, but are mainly secreted at the basolateral membrane after synthesis. Therefore, we determined the LA concentrations in both phospholipid and triglyceride fractions of control and EFA-deficient cells. LA concentrations in phospholipids were significantly lower in EFA-deficient compared with control Caco-2 cells at day 0 (before the start of the LA supplementation; Figure 6b), supporting our previous *in vivo* findings in jejunum of EFA-deficient mice.² LA concentrations in total cell lysates and in triglycerides fractions were similar between the EFA-deficient and control mice at all time points (Figure 6a, 6c). After the LA supplementation, LA was taken up by both control and EFA-deficient cells to a similar extent in both total cell lysates as in phospholipids (Figure 6a, 6b). There was no increase in LA concentration in time after the LA supplementation in EFA-deficient or the control cells, indicating that LA was not taken up by the triglycerides (Figure 6c).

To functionally study if LA supplementation reverses the effects of EFA deficiency, we analyzed the mRNA expression of lactase, sucrase isomaltase and PPAR α . In addition, we determined the effects of LA supplementation on TER in EFA-deficient Caco-2 cells. Preliminary data demonstrate that LA supplementation did not increase the mRNA expression of lactase, sucrase isomaltase or PPAR α in EFA-deficient Caco-2 cells (Figure 7a, 7b, 7c). TER values were significantly higher in EFA-deficient Caco-2 cells supplemented with LA on day 2 and 4 during the LA supplementation (Figure 7d). However, after 4 days there TER values decrease to a similar extent in LA treated and untreated EFA-deficient cells and EFA-deficient cells supplemented with LA, towards the baseline values (equal to empty wells; negative controls) (Figure 7d).

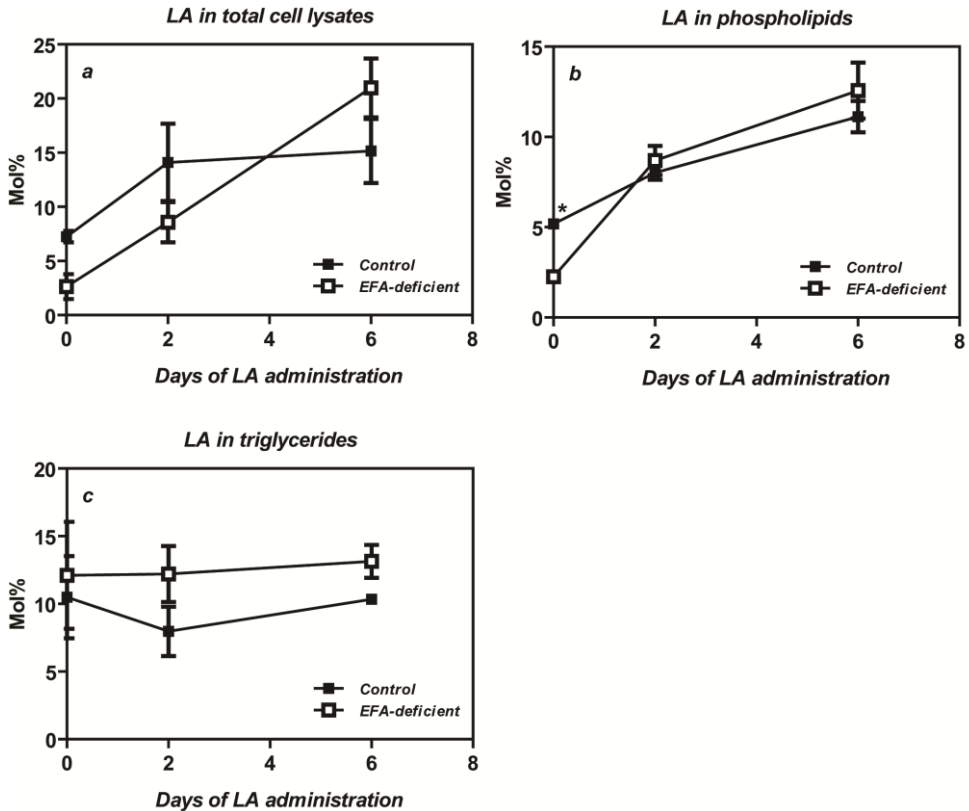


Figure 6 Molar concentrations of LA in (a) total cell lysates, (b) phospholipids and (c) triglycerides in EFA-deficient cells (white squares) and control cells (black squares) treated with 50 μ M LA. Data represent means \pm SEM of two independent experiments and * $p < 0.05$ is the significant difference between the EFA-deficient and control cells.

DISCUSSION

We aimed to develop an *in vitro* model of EFA deficiency, which would be helpful to identify the (intra)cellular (molecular) mechanism(s) underlying the negative effects of

EFA deficiency on the small intestinal function level.^{2,17} Our data show that Caco-2 cells cultured in medium with delipidated FCS rapidly develop biochemical EFA deficiency and have a phenotype that resembles several aspects of EFA deficiency in the small intestine in mice *in vivo*. Caco-2 cells cultured in delipidated medium show an elevated triene:tetraene ratio in their fatty acid profile, a biochemical marker of EFA deficiency. The difference in triene:tetraene ratio between the control and EFA-deficient Caco-2 cells after 10 days of culture were not as large as observed previously in the *in vitro* model of Spalinger et al. and in EFA deficient mice.^{1,17}

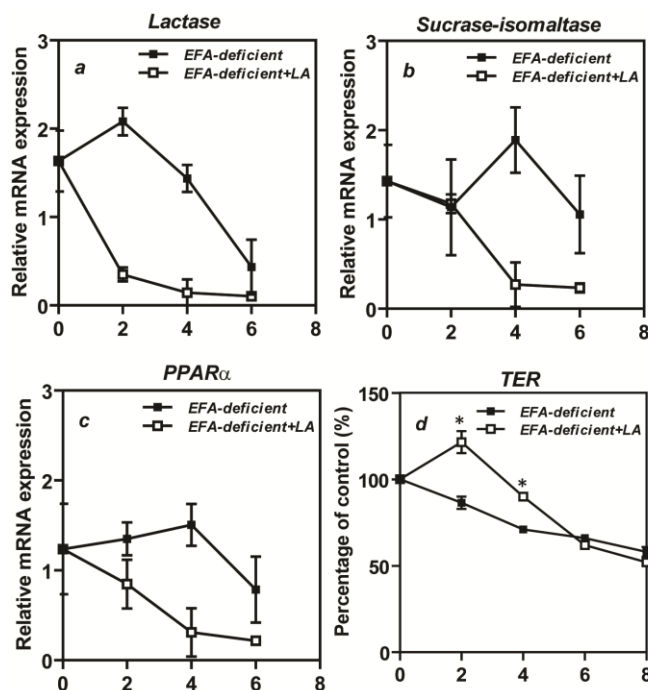


Figure 7 Preliminary data on mRNA expression of (a) lactase, (b) sucrase isomaltase and (c) PPAR α in untreated EFA-deficient cells (black squares) and EFA-deficient cells treated with 50 μ M LA (white squares) harvested every two days. Data represent means \pm SD of three wells per condition. (d) TER was measured every two days in untreated EFA-deficient Caco-2 cells (black squares) and EFA-deficient Caco-2 cells treated with 50 μ M LA (white squares) as a percentage of control (untreated) cells over a period of 8 days in culture. Data represent means \pm SEM of two independent experiments and * p <0.05 is the significant difference between the EFA-deficient and control cells.

Explanation for the observed difference between our *in vitro* and *in vivo* models could be related to differences in timeframe of the exposure to the EFA-deficient condition. Mice received an EFA-deficient diet for 8 weeks, whereas Caco-2 cells were exposed to EFA-deficient medium for approximately ten days. As expected, EFA-deficient Caco-2 cell had decreased molar concentrations of essential fatty acids in their fatty acid profiles. Another difference between our *in vivo* and *in vitro* model is that Caco-2 cells did not receive almost any of the fatty acids, while the mice received reduced concentrations of dietary fatty acids. Thus, there is a possibility that, at least, a part of the phenotype we

observed in Caco-2 cells might be due to a complete, rather than reduced, fatty acid deficiency. However, similar to small intestinal enterocytes, Caco-2 cells are capable of *de novo* synthesis of non-essential fatty acids. Therefore, it is not likely that the phenotype observed in Caco-2 cells is mainly due to the total fatty acid deficiency. This is supported by the observation that several phenotypic features of the *in vivo* model of EFA deficiency are present in our *in vitro* model in Caco-2 cells.

In agreement with studies in rats and mice, Caco-2 deficient cells show significantly reduced concentrations of LA in the phospholipids.^{2,18} Furthermore, the mRNA expression of lactase was reduced in EFA-deficient Caco-2 cells. We previously showed in EFA-deficient mice that lactase mRNA and enzyme activity were reduced by more than 50%, associated with impaired lactose digestion.² Lactase is an apical disaccharide and a relevant marker for the differentiation of Caco-2 cells differentiating towards the small intestinal phenotype.¹⁰ The mRNA expression of sucrase isomaltase, another small intestinal differentiation marker, was also significantly reduced in EFA-deficient Caco-2 cells. Similar to lactase, sucrase isomaltase is localized in the brush border membrane of the enterocytes.¹⁹ Interestingly, the expression of villin, which is an intracellular enterocyte marker, was not affected by EFA deficiency in Caco-2 cells. Combined, these data suggest an impaired differentiation of EFA-deficient cells at the level of transcription of brush border membrane anchored proteins. Possibly, the (ultra)structure of the brush border membrane is impaired by the EFA deficiency in the small intestinal enterocytes. This idea is supported by previous studies in EFA-deficient piglets and rats which show several EFA deficiency associated alterations in the intestinal brush border membrane.^{20,21} Whether EFA deficiency in mice and Caco-2 cells leads to (ultra)structural changes in the brush border membrane, remains to be elucidated. Electron microscopy analysis seems warranted to determine whether EFA deficiency affects the brush border membrane in Caco-2 cells and *in vivo* in EFA-deficient mice. However, possible negative effects on the brush border membrane by EFA deficiency cannot explain the decreased mRNA levels of the brush border membrane enzymes. Possibly, EFA deficiency affects the mRNA synthesis and transport of the brush border membrane enzymes. The mechanism underlying the specific effects of EFA deficiency on the expression of lactase and sucrase isomaltase is presently unclear. In a transcriptome analysis, no differences were detected in the biological processes involved in RNA synthesis and transport in the microarray analysis of EFA-deficient Caco-2 cells (unpublished data).

Previous studies revealed possible role of EFA deficiency on the barrier function in different tissues among different species.^{5,20,22,23} Our data demonstrate that EFA-deficient Caco-2 cells are more permeable, as demonstrated by increased TER. The increased permeability in the EFA-deficient Caco-2 cells was accompanied by unaffected (mRNA) expression of the tight junction components. Localization of the ZO-1 tight junction component, furthermore, was not significantly different from control Caco-2 cells. Since tight junction components are mainly involved in the paracellular permeability, we suggest that EFA deficiency mainly affects the transcellular permeability, while the paracellular permeability is preserved during EFA deficiency. Additional measurements of cascade blue dextran, used as a marker for paracellular permeability, uptake in Caco-2 cells, will further help elucidate whether paracellular permeability is affected by the

EFA deficiency.^{24,25} *In vivo* studies (e.g. lactulose/mannitol permeability test) in models for EFA deficiency might further help elucidate *in vivo* whether small intestinal permeability is increased in EFA-deficient mice. Furthermore, Proksch et al. suggested that LA may play a direct role in the epidermal permeability barrier and that impaired epidermal barrier function might be reversed by local LA administration.⁵ Several attempts have been made in order to reverse the effects of EFA deficiency, mainly in pediatric cystic fibrosis, including LA supplementation in form of corn oil, safflower oil and LA-monoacylglycerides.^{26,27,28} Although the plasma concentrations of LA were normalized, the effects of LA supplementation on restoring the phenotype of EFA deficiency remained poor. To address to what extent the observed phenotype of EFA deficiency (decreased mRNA expression of the brush border enzymes, increased permeability) depends on LA concentration, short term LA re-supplementation experiment was performed. Our results indicate that the EFA-deficient Caco-2 cells take up the administered LA, based on our measurements of LA concentrations in both total cell lysates, as well as in phospholipids fractions. Interestingly, however, the re-supplementation of LA did not restore relevant parameters of the EFA-deficient phenotype, such as the decreased lactase expression. However, these data are preliminary since the experiment was performed only once. Therefore, further confirmation by is required in order to demonstrate if LA supplementation has any positive effects on mRNA expression of lactase, sucrase isomaltase and PPAR α . LA supplementation had a very short, acute effect as demonstrated by the increasing TER in EFA-deficient cells during the first 4 days of supplementation. After this time point, the TER values decreased and were similar to untreated EFA-deficient Caco-2 cells. These data suggest that different tissues are more or less susceptible to the LA supplementation, since Proksch et al. demonstrated positive effects of local LA administration in the epidermis.⁵ Further studies with longer exposure to, and higher concentrations of LA are necessary.

In the fatty acid analysis in EFA-deficient Caco-2 cells, we did not measure any relevant difference in alpha-linolenic acid (ALA) concentrations between EFA-deficient and control Caco-2 cells (data not shown). However, it would be relevant to determine whether additional supplementation with an ω -3 fatty acid would help to reduce the EFA-deficient phenotype in EFA-deficient Caco-2 cells.

Our previous studies in EFA-deficient mice revealed impaired fatty acid absorption and lactose digestion.^{2,17} Furthermore, we have showed that EFA deficiency leads to impaired bile salt metabolism in mice and EFA-deficient Caco-2 cells.⁴ Future studies with stable isotope labeled nutrients in transwell system with EFA-deficient Caco-2 cells will reveal whether nutrient absorption is impaired in this *in vitro* model.

Overall, we have further characterized an *in vitro* model of EFA deficient small intestinal cells. In several aspects the phenotype corresponds with *in vivo* EFA deficiency of the small intestinal epithelium in mice. We expect that this model will allow performing more detailed studies on the underlying mechanism(s) of the EFA-deficient phenotype in the small intestinal enterocyte. Understanding the mechanism(s) by which EFA deficiency affects the small intestine will hopefully contribute to develop more rational therapies to improve the nutritional status of patients with EFA deficiency.

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