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Intestinal function in cholestasis and essential fatty acid deficiency

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Publisher's PDF, also known as Version of record

Publication date:

2007

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Citation for published version (APA):

Los, E. L. (2007). *Intestinal function in cholestasis and essential fatty acid deficiency*. s.n.

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CHAPTER 4

Cholestatic conditions and intestinal cell proliferation and differentiation

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ABSTRACT

Cholestasis in children impairs their nutritional status and growth. Recently we demonstrated in a rat model of cholestasis that increased plasma bile salt concentrations did not affect intestinal carbohydrate digestion or absorption. We addressed to what extent cholestatic conditions affect proliferation or differentiation of enterocytes *in vitro*, and what underlies their apparent resistance to bile salts. We studied proliferation and differentiation of an intestinal cell line, Caco-2, exposed to bile salts in cholestatic concentrations. Exposure of proliferating or short-term differentiated cells to cholestatic conditions did not affect DNA content, cell number or sucrase activity. In contrast, exposure of long-term differentiated cells to cholestatic conditions reduced sucrase activity, coinciding with increased expression of bile salt transporter ASBT. Unconjugated CDCA reduced sucrase activity earlier in the differentiative phase. Present data suggest that enterocytes are protected from bile salt-induced effects through absence of bile salt transporter ASBT. These data were confirmed *in vivo*.

INTRODUCTION

Cholestatic liver disease negatively affects nutritional status and growth in children. For cholestatic children requiring liver transplantation, a poor nutritional status imposes an increased risk of morbidity and mortality. Malnutrition and growth retardation in cholestatic disorders are usually ascribed to insufficient fat solubilization and absorption due to reduced presence of bile components in the intestinal lumen¹⁻³.

Cholestasis is also characterized by elevated bile salt plasma concentrations, which theoretically could affect intestinal function. Lower concentrations of bile salts have been shown to induce proliferation, differentiation or apoptosis in intestinal epithelial cell lines, depending on hydrophobicity and cell line⁴⁻⁶. In contrast to *in vitro* data, we recently demonstrated that carbohydrate digestion and absorption was maintained in a rat model of cholestasis, but we could only speculate about the mechanism underlying the functional protection against cholestatic conditions⁷.

To address this issue, we exposed the human colon carcinoma cell line Caco-2 to cholestatic concentrations of conjugated bile salts and assessed bile salt transport parameters, proliferation, differentiation and apoptosis. Caco-2 cells develop small intestinal features upon reaching confluency⁸. We separately studied proliferating Caco-2 cells, which can be regarded as a model for proliferating crypt cells, and differentiated Caco-2 cells, which can be regarded as a model for differentiated villous cells of the small intestine. Results were compared to those of unconjugated chenodeoxycholic acid (CDCA) since it can enter the cells independent of transporters as opposed to conjugated bile salts.

Finally, we studied the interaction between nutrient absorption and bile salt absorption *in vivo* by looking at the correlation between expression of proteins involved in carbohydrate digestion and absorption in the ileal part of the small intestine and plasma bile salt concentrations in cholestatic, bile-deficient and control rats.

Present data indicate that exposure of proliferating and short-term differentiated cells to conjugated bile salts in cholestatic concentrations did not affect Caco-2 cell proliferation or differentiation. Exposure of long-term differentiated cells to cholestatic conditions reduced differentiation, coinciding with increased expression of bile salt transporter ASBT.

MATERIALS AND METHODS

Materials

The bile salts glycocholic acid (GCA) and CDCA, (sodium salts), were from Calbiochem (San Diego, CA, USA). Taurocholic acid (TCA), glycochenodeoxycholic acid (GCDCA) and taurochenodeoxycholic acid (TCDCA), (sodium salts), were from Sigma (St. Louis, MI, USA). All bile salts were dissolved in demineralized H₂O in 50 mM stock solutions and mixed with the medium before addition to the cells.

Cell culture

The human colon carcinoma cell line Caco-2 was obtained from the American Type Tissue Culture Collection (Manassas, VA, USA) and maintained in DMEM (Gibco BRL, USA) supplemented with 10% (vol/vol) FBS, 100 IU/ml penicillin, 100 µg/ml streptomycin, 1% non-essential amino acids and 0.25% human transferrin in a humidified atmosphere of 95% air–5% CO₂ at 37°C. For the experiments, cells were used between passage 15 and 30.

Experimental design

Caco-2 cells were seeded at 5×10^4 cells per well in 6-well plates (Corning Costar, Cambridge, MA, USA). Caco-2 cells were exposed to 0, 150, 300 and 450 $\mu\text{mol/L}$ of a bile salt mixture containing GCA, TCA, GCDCA and TCDCA in a 2:2:1:1 molar ratio or to 250 $\mu\text{mol/L}$ CDCA. The effects of bile salts on proliferating and differentiated Caco-2 cells were assessed by exposing the cells to bile salts 24h after seeding and after confluency was reached (~8 days), respectively. Proliferating cells were harvested at day 0, 2, 4, 6 and 14 (P0, P2, P4, P6 and P14) and differentiated cells were harvested at day 0, 7, 14 and 21 (D0, D7, D14 and D21). The effect of exposure to CDCA was assessed at P4, P14, D7, D14 and D21. Caco-2 cells in Transwell plates were harvested at day 7, 14 and 21 (D7, D14 and D21). Medium with or without bile salts was changed every 2-3 days.

Analyses

Cell numbers were counted manually with the Burker Cell System. DNA was quantified with a DNA quantification kit (Sigma, St. Louis, MI, USA) according to the manufacturer's instructions. Caspase-3 activity was measured with the CaspACE™ Assay System (Promega, Madison, USA) according to the manufacturer's instructions. mRNA expression levels were measured by real-time PCR, as described previously⁹. PCR results were normalized to GAPDH mRNA levels. The sequences of the primers and probes are listed in Table 1. Enzyme activity levels of sucrase were measured as described by Dahlqvist¹⁰. Activity levels were normalized to protein levels, measured by the BCA method as described by the manufacturer (Pierce, Rockford, IL, USA). Lactate dehydrogenase (LDH) in the medium was quantified as described by Smit *et al.*¹¹.

Table 1. Primer and probe sequences

Gene	GenBank	Forward Primer	Reversed Primer	TaqMan® probe
GAPDH	NM_002046	GGT GGT CTC CTC TGA CTT CAA CA	GTG GTC GTT GAG GGC AAT G	ACA CCC ACT CCT CCA CCT TTG ACG C
FXR	AF384555 ($\alpha 1$) NM_005123 ($\alpha 2$)	TTC TGA AAA TTT ATT TGG TGT TTT AAC AGA	TGG AAT AAT AGG ATG ACG AGG AAA TC	CAT TGC TGT ATT GCG AGT ATG GTT CCA CTT CC
ASBT	NM_000452	CAC GCA GCT ATG TTC CAC CAT	GAG CCG AAG GTG AAT ACG A	CAG CTC TCC TTC ACT CCT GAG GAG CTC A
IBABP	NM_001445	AAG GCC CGC AAC TTC AAG AT	GGA GTA GTG CTG GGA CCA AGT G	CCA TCC TGC TGC ACC TCC GTG A
GPBAR1	NM_001077191.1	CGT CTA CTT GGC TCC CAA CTT C	GGC CTC AGG ACT GCC ATG TA	CTC TCC CTG CTT GCC AAC CTC TTG C
SI	NM_001041	TGT GGA TAA ATG AGT CAG ATG GAA G	GCC CAC CAA TCA ATG CAG TT	ACT GTT AAT CCT GGC CAT ACG TCT CCA ATA A
CDX-2	NM_001265	CTC GGC AGC CAA GTG AAA AC	TGG TGA TGT AGC GAC TGT AGT GAA	CCT TCT CCA GCT CCA GCC GCT G
GATA-4	NM_002052.2	GGA GAT GCG TCC CAT CAA GA	CTG ACT GAG AAC GTC TGG GAC A	CAT CTC ACT ACG GGC ACA GCA GCT CC

Gene expression and plasma bile salt concentrations in cholestatic, bile-deficient and control rats

mRNA levels of SI, Sgl-1 and Glut-2 and plasma bile salt concentrations in cholestatic, control and bile-deficient rats were obtained and statistically tested as described previously⁷.

Statistical analysis

Values represent means \pm SEM of 3-6 experiments. Using SPSS version 12.0.2 statistical software (Chicago, IL, USA), we calculated significance of differences with the Mann-

Whitney *U*-test. Statistical information is provided regarding differentiative (D0) vs. proliferative cells (P0), alterations during the differentiative phase (D0 vs. D21) and control cells vs. conjugated bile salts in cholestatic concentrations (450 $\mu\text{mol/L}$) or CDCA (250 $\mu\text{mol/L}$). $P < 0.05$ was considered significant.

RESULTS

Cholestatic conditions increase IBABP expression in proliferating and differentiated cells.

First, we assessed key parameters regarding to bile salt transport and signaling; bile salt-activated farnesoid X receptor (FXR), apical sodium-dependent bile salt transporter (ASBT), ileal bile acid-binding protein (IBABP) and G protein-coupled membrane bile acid receptor (GPBAR).

In Figure 1 is shown that the expression of *FXR* was increased (~5-fold; $P < 0.05$) in differentiated cells (D0) compared to proliferating cells (P0). *FXR* expression was further increased during the differentiative phase (D0 vs. D21) of Caco-2 cells (~11-fold; $P < 0.01$). Similarly, *ASBT* expression was increased in differentiative cells (~3-fold; $P < 0.05$) and during the differentiative phase (~7-fold; $P < 0.01$). In contrast, *IBABP* expression and *GPBAR1* expression (data not shown) were not induced during the differentiative phase, but were increased in differentiative cells compared to proliferative cells (~4-fold; $P < 0.01$ and ~1.5-fold; $P < 0.05$, respectively; Fig 1).

Exposure to conjugated bile salts in cholestatic concentrations significantly increased *IBABP* expression at day 6 (~2-fold; $P < 0.01$) and 14 (~20-fold; $P < 0.05$) in proliferating cells and at day 7-21 (~20-35 fold; $P < 0.01$) in differentiated cells, while exposure to conjugated bile salts in cholestatic concentrations did not affect *FXR*, *ASBT* or *GPBAR1* expression.

In contrast to conjugated bile salts in cholestatic concentrations, CDCA significantly reduced *ASBT* expression in differentiated cells (by ~40% at day 14 and by ~75% at day 21; both $P < 0.05$, Fig 1). *IBABP* expression was strongly induced upon exposure to CDCA in differentiated cells (Fig 1).

Conjugated bile salts in concentrations of 150 and 300 $\mu\text{mol/L}$ generated similar results as 450 $\mu\text{mol/L}$. For sake of clarity, we only present results of cells exposed to 450 $\mu\text{mol/L}$ conjugated bile salts or 250 $\mu\text{mol/L}$ CDCA compared to control cells.

Cholestatic conditions do not affect DNA content or cell number in proliferating or differentiated cells.

DNA content and cell number were quantified to assess cell proliferation under cholestatic conditions. DNA content in differentiative cells was ~9-fold ($P < 0.01$) higher compared to proliferative cells, independent of the presence or absence of conjugated bile salts. During the differentiative phase, however, DNA content remained similar. CDCA decreased DNA content in proliferating cells, but not in differentiated cells (Fig 2).

Cell number showed a similar pattern during differentiation. Cholestatic bile salt concentrations did not affect cell number at any time point, but CDCA reduced cell number in proliferating cells (~99%, $P < 0.01$, data not shown).

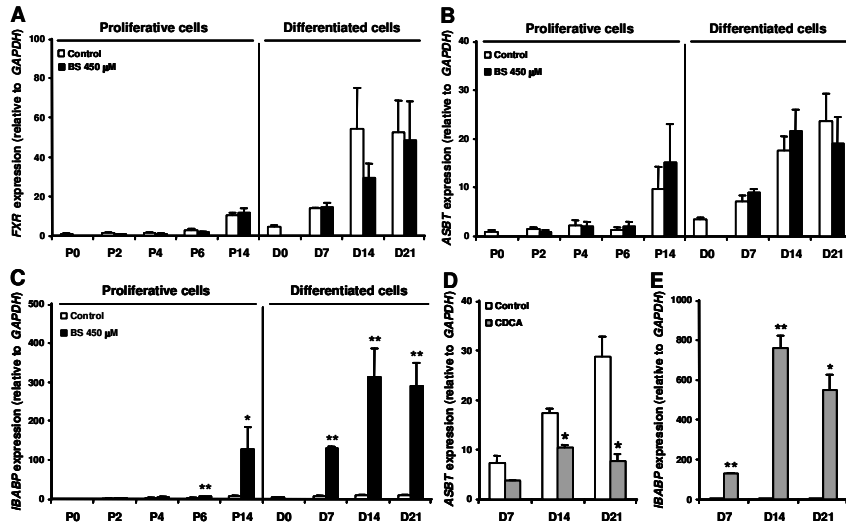


Figure 1. Bile salt transporter/receptor gene expression in Caco-2 cells exposed to conjugated bile salts in cholestatic concentrations (450 μmol/L) or CDCA (250 μmol/L) compared to control cells. (A) FXR, (B) *ASBT*, (C) *IBABP* expression, normalized to *GAPDH*, in cells exposed to conjugated bile salts in cholestatic concentrations compared to control cells. (D) *ASBT*, (E) *IBABP* expression, normalized to *GAPDH*, in cells exposed to CDCA compared to control cells. P0, P2, P4, P6 and P14 represent proliferating cells at day 0, 2, 4, 6 and 14 after exposure to bile salts and D0, D7, D14 and D21 represent differentiated cells at day 0, 7, 14 and 21 after exposure to bile salts. **P*<0.05 and ***P*<0.01.

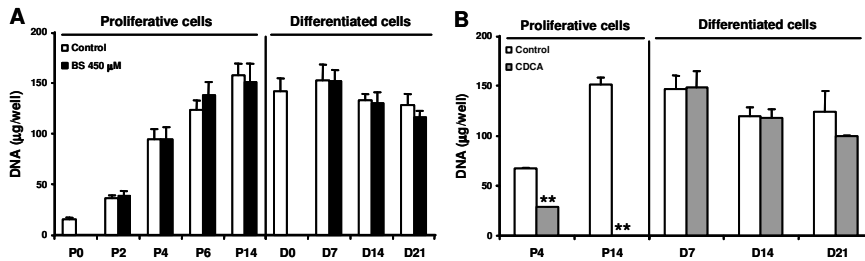


Figure 2. DNA content of Caco-2 cells exposed to conjugated bile salts in cholestatic concentrations (450 μmol/L) or CDCA (250 μmol/L) compared to control cells. (A) DNA content of cells exposed to conjugated bile salts in cholestatic concentrations compared to control cells. (B) DNA content of cells exposed to CDCA compared to control cells. P0, P2, P4, P6 and P14 represent proliferating cells at day 0, 2, 4, 6 and 14 after exposure to bile salts and D0, D7, D14 and D21 represent differentiated cells at day 0, 7, 14 and 21 after exposure to bile salts. ***P*<0.01.

Cholestatic conditions do not affect caspase-3 activity in proliferating or differentiated cells.

Caspase-3 activity was quantified to assess the occurrence of apoptosis. Caspase-3 activity was very low and similar in control cells and in cells exposed to conjugated bile salts in cholestatic concentrations. Unconjugated CDCA increased caspase-3 activity by ~50% in proliferating cells (*P*<0.05, data not shown).

LDH leakage in the medium was quantified to assess potential bile salt-induced cytotoxicity. Neither in proliferating nor differentiated cells, LDH leakage was induced by exposure to conjugated bile salts in cholestatic concentrations or unconjugated CDCA (data not shown).

Cholestatic conditions reduce sucrase enzyme activity in long-term differentiated cells.

Sucrase enzyme activity and sucrase-isomaltase (SI) mRNA levels were quantified as markers for Caco-2 cell differentiation. Sucrase enzyme activity was markedly induced (~27-fold; $P<0.05$) in differentiative cells compared to proliferative cells and further during the differentiative phase (~16-fold; $P<0.01$) as also described by Van Beers *et al.*¹². Exposure to conjugated bile salts in cholestatic concentrations did not affect sucrase enzyme activity in proliferating cells or in differentiated cells up to day 7. Conjugated bile salts significantly reduced sucrase enzyme activity in differentiated cells after day 14 ~20-25%; $P<0.05$; Fig 3). Unconjugated CDCA abolished sucrase enzyme activity in proliferating cells at day 14, and in differentiated cells from day 14 on (by ~25-45%; $P<0.01$; Fig 3). The expression pattern of SI mRNA during differentiation was comparable to that of sucrase enzyme activity. *SI* expression was strongly increased in differentiative cells compared to proliferative cells (~81-fold; $P<0.01$) and further increased during the differentiative phase (~5-fold; $P<0.01$). Conjugated bile salts in cholestatic concentrations significantly reduced *SI* expression in proliferating cells at day 2 (by ~65%; $P<0.05$). In differentiative cells, *SI* expression was also reduced upon exposure to conjugated bile salts, but this did not reach statistical significance (Fig 3).

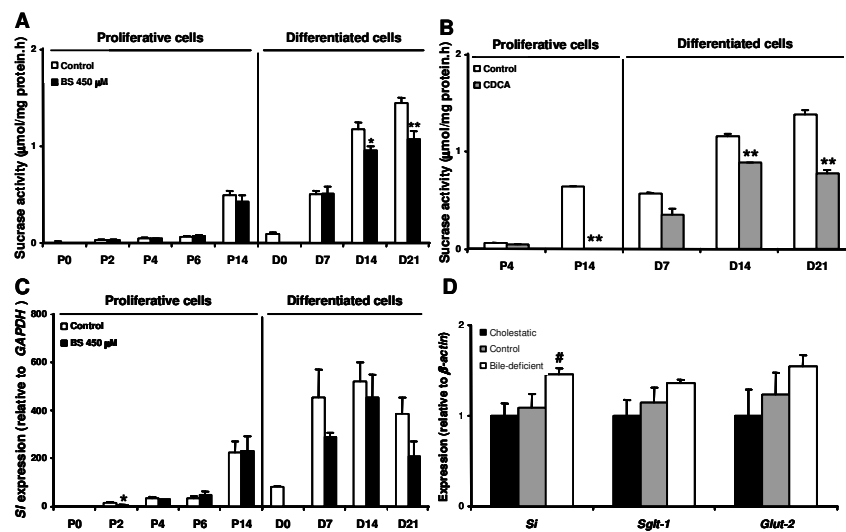


Figure 3. Sucrase enzyme activity and sucrase-isomaltase (SI) expression in Caco-2 cells exposed to conjugated bile salts in cholestatic concentrations (450 μmol/L) or CDCA (250 μmol/L) compared to control cells. (A) Sucrase enzyme activity in cells exposed to conjugated bile salts in cholestatic concentrations compared to control cells. (B) Sucrase enzyme activity in cells exposed to CDCA compared to control cells. (C) *SI* expression, normalized to GAPDH, in cells exposed to conjugated bile salts in cholestatic concentrations compared to control cells. P0, P2, P4, P6 and P14 represent proliferating cells at day 0, 2, 4, 6 and 14 after exposure to bile salts and D0, D7, D14 and D21 represent differentiated cells at day 0, 7, 14 and 21 after exposure to bile salts. * $P<0.05$ and ** $P<0.01$. (D) Ileal expression of *SI*, *Sglt-1* and *Glut-2* in cholestatic (black bars), control (grey bars) and bile-deficient (white bars) rats, normalized to β-actin. Values of cholestatic rats are set to 1. Plasma bile salt concentrations cover a range from 187 ± 20 μmol/L in cholestatic rats, $10\pm 4^{**}$ μmol/L in control rats to $1.0\pm 0.5^{***\#}$ μmol/L in bile-deficient rats of which ** $P<0.01$ vs. cholestatic group and # $P<0.01$ vs. control group. # $P=0.064$ (Kruskal-Wallis H) and $P=0.032$ (Mann-Whitney U) vs. control group.

Plasma bile salt concentrations correlate inversely with ileal SI, Sglt-1 and Glut-2 expression in rats.

As stated before, we previously demonstrated that intestinal carbohydrate absorption was maintained in cholestatic rats⁷. Figure 3D shows mRNA levels of SI, and intestinal glucose transporters Sglt-1 (apical) and Glut-2 (basolateral) in the ileal segment of the small intestine of cholestatic, control and bile-deficient rats. The average plasma bile salt concentrations in cholestatic, control and bile-deficient rats were 187 ± 20 , 10 ± 4 ($P < 0.01$ vs. cholestatic rats) and 1.0 ± 0.5 ($P < 0.01$ vs. cholestatic and control rats) $\mu\text{mol/L}$, respectively. Though not significantly different among the groups, a clear pattern can be observed. *SI*, *Sglt-1* and *Glut-2* expression showed a trend towards being higher in bile-deficient rats compared to control and cholestatic rats. *SI* expression was increased by $\sim 45\%$ in bile-deficient rats compared to cholestatic rats (Kruskal-Wallis H: $P = 0.064$ and Mann-Whitney U: $P = 0.028$; Fig 3).

Cholestatic conditions do not affect CDX-2 or GATA-expression in proliferating and long-term differentiated cells.

CDX-2 and GATA-4 are intestine-specific transcription factors known to cooperatively regulate sucrase-isomaltase gene transcription¹³. Expression of *CDX-2* and *GATA-4* was, respectively, ~ 3 -fold ($P < 0.05$) and ~ 5 -fold ($P < 0.01$) higher in differentiative cells compared to proliferative cells. During the differentiative phase, CDX-2 and GATA-4 expression showed similar patterns, specifically slight inductions of expression at day 7 and 14, returning to base level at day 21 (Fig 4).

CDX-2 expression was not changed upon exposure to conjugated bile salts. Conjugated bile salts in cholestatic concentrations did not affect *GATA-4* expression in proliferating or differentiated cells, except for day 7 in differentiated cells ($\sim 35\%$; $P < 0.05$, Fig 4).

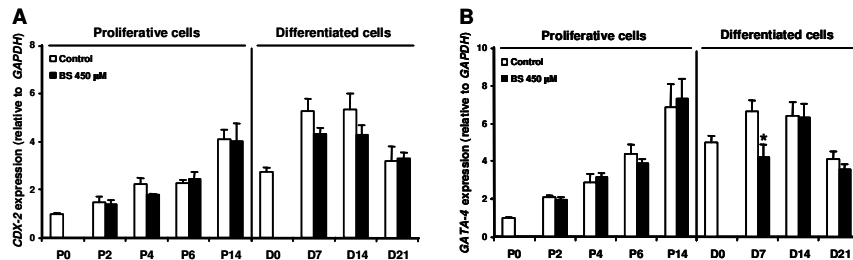


Figure 4. Gene expression of intestine-specific transcription factors in Caco-2 cells exposed to conjugated bile salts in cholestatic concentrations (450 $\mu\text{mol/L}$) compared to control cells. (A) *CDX-2*, (B) *GATA-4* expression, both normalized to *GAPDH*, in cells exposed to conjugated bile salts in cholestatic concentrations compared to control cells. P0, P2, P4, P6 and P14 represent proliferating cells at day 0, 2, 4, 6 and 14 after exposure to bile salts and D0, D7, D14 and D21 represent differentiated cells at day 0, 7, 14 and 21 after exposure to bile salts. * $P < 0.05$.

DISCUSSION

We recently demonstrated that carbohydrate digestion and absorption is maintained in a rat model of cholestasis⁷. In this study we set out to find an explanation for enterocyte protection against cholestatic conditions. We found that exposure of proliferating and short-term differentiated Caco-2 cells to conjugated bile salts in cholestatic concentrations did not affect proliferation or differentiation, whereas exposure of long-term differentiated cells to cholestatic conditions reduced differentiation, coinciding with increased expression of bile salt transporters.

The lifespan of an enterocyte between crypt cells entering the villous-crypt junction, migrating upwards reaching the villous tip and eventually shedding off, ranges from approximately 2 to 3 days in mice and rats to 6 days in humans^{14,15}. Based on this physiological knowledge, one would consider short-term exposure as physiologically most relevant. We realized, however, that a cell model may not mimic physiological conditions in this respect. We first determined relevant features of Caco-2 cells at different developmental stages, including long-term differentiated cells, to find the condition most relevant to study our research question.

Our results clearly showed a discrepancy with respect to proliferating–short-term differentiated cells and long-term differentiated cells, i.e. exposure to cholestatic conditions in long-term differentiated cells reduced differentiation in contrast to proliferating and short-term differentiated cells. This discrepancy might be due to the higher quantity of the bile salt transporters in long-term differentiated cells compared to proliferating and short-term differentiated cells. Corresponding with this, our data indicate that ASBT mRNA levels are strongly increased in long-term differentiated cells compared with proliferative and short-term differentiative cells, coinciding with increased FXR mRNA levels as previously described by De Gottardi *et al.*¹⁶. Though bile salts seem to be able to enter cells with relatively low ASBT expression, indicated by reduced *IBABP* expression upon exposure to conjugated bile salts in proliferating and short-term differentiated cells, the quantity is most likely to low to exert effects on sucrase activity. Unconjugated CDCA can cross the cellular membrane independent of active transporters. The observation that exposure to CDCA already reduces sucrase activity earlier in the differentiative phase is compatible with the theory stated above.

Bile salts, however, do not need to enter the cells to activate cellular signaling. Marayuma *et al.* identified a cell surface G protein-coupled receptor, GPBAR1, responsive to low concentrations of extracellular bile salts¹⁷. Human GPBAR1 mRNA has been shown to be expressed in the small intestine and activation by bile salts leads to the activation of cellular signaling routes¹⁸. The elevated plasma bile salt level during cholestasis could theoretically activate signaling routes via GPBAR1. Since GPBAR1 was present in proliferating and differentiated Caco-2 cells, cholestatic bile salt concentrations might be able to influence enterocyte proliferation or differentiation via GPBAR1. The observation that reduced activity and mRNA levels of sucrase did not coincide with changes in GPBAR1 expression, however, does not support this theory.

Effects of bile salts on human intestinal cell proliferation range from inhibition to induction, usually related to the hydrophobicity of the bile salt. Unconjugated CDCA induces profound apoptosis in human intestinal cells, while similar concentrations of conjugated bile salts induce proliferation⁵. Effects of bile salts on intestinal cell differentiation have not been studied extensively. Ursodeoxycholic acid (UDCA) has been found to induce differentiation in a HCT116 cell line, derived from human colon carcinoma cells⁴. Interestingly, in culture,

hepatocytes seem to be much more sensitive to bile salt exposure than enterocytes. Glycochenodeoxycholic acid already induces apoptosis at 50 $\mu\text{mol/L}$ in hepatocytes, while similar concentrations do not affect enterocytes *in vitro*⁶. We cannot appoint a cause for the differences in behavior between hepatocytes and enterocytes exposed to bile salts, but we can speculate about the underlying basis. First, hepatocytes possibly possess more bile salt uptake transporters than enterocytes, with possible higher affinity. Second, cell signaling pathways might be differentially regulated.

Nutrient absorption is most efficient in the jejunal part of the small intestine, while active bile salt absorption is restricted to the terminal ileum¹⁹. This observation would suggest that enterocytes involved in nutrient absorption are protected from bile salts. The observation that reduced sucrase enzyme activity in long-term differentiated cells exposed to conjugated bile salts in cholestatic concentrations coincided with high expression of the bile salt transporter ASBT, supports this theory. Thus, bile salts could potentially be harmful for enterocyte function when nutrient absorption and bile salt absorption would have been localized in the same intestinal segment. The significance of different intestinal segments involved in nutrients absorption and bile salt reabsorption has been well defined by Bosse *et al*²⁰. They identified Gata-4 as an essential mediator of the maintenance of jejunal-ileal identities in mice. Synthesis of a transcriptionally inactive Gata-4 mutant in the mouse jejunum resulted in an attenuation of expression of genes involved in nutrient absorption and an induction of genes involved in bile salt absorption²⁰. Moreover, results from our *in vivo* study showed reduced sucrase activity in rats with higher plasma bile salt concentrations (bile-deficient > control > cholestatic rats) in the ileal part of the small intestine, while this pattern was not observed in the duodenal and jejunal part of the intestine⁷. The effect could possibly be more pronounced in the terminal ileum of rats exposed to even higher plasma bile salt concentrations, as seen in human cholestasis.

Since Caco-2 cells are derived from colon carcinoma cells it is impossible to ascribe features gained upon differentiation to any part of the small intestine. However, since differentiated Caco-2 cells express proteins involved in nutrient absorption as well as proteins involved in bile salt absorption, it is the ideal model to study interactions between these processes.

In conclusion, our data indicate that intestinal epithelial cells involved in nutrient absorption are resistant to exposure to conjugated bile salts in cholestatic concentration and composition. The maintenance of intestinal cell proliferation and differentiation under cholestatic conditions suggests that the absorptive function of the small intestine is preserved, which is most likely related to the fact that nutrient absorption and bile salt absorption are localized in different parts of the intestine.

ACKNOWLEDGEMENTS

This study is supported by the Dutch Digestive Disease Foundation (MLDS). A.B. is supported by an unrestricted research grant from the Nutricia Research Foundation. E.H.H.M.R. is supported by a fellowship of the Royal Netherlands Academy of Arts and Sciences (KNAW). The authors would like to thank Martijn Hoeke, Hilda van der Molen and Fjodor van der Sluijs for excellent technical assistance.

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