Sulphation of lithocholic acid in the colon-carcinoma cell line CaCo-2

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High levels of bile acids in the colon may correlate with an increased risk of colon cancer, but the underlying mechanisms are not known. Proteoglycan structures have been shown to change when human colon cells differentiate *in vitro*. The expression of [^{35}S]sulphated molecules was used as a phenotypic marker to study the effects of bile acids on the human-colon-carcinoma cell line CaCo-2. [^{35}S]sulphated compounds were isolated from the medium of cell fractions of cells metabolically labelled with [^{35}S]sulphate in the absence and presence of cholic acid, deoxycholic acid, chenodeoxycholic acid and lithocholic acid (LA). Labelled molecules were analysed by gel chromatography, HPLC and SDS/PAGE in combination with chemical and enzymic methods. The expression of ^{35}S-labelled proteoglycans was not affected by any of the bile acids tested. However, the level of sulphated metabolites increased 7–18-fold in different experiments during a 22 h labelling period in the presence of an LA concentration of 10 μg/ml (26.6 nmol/ml) compared with controls. Further analyses showed that this was due, at least in part, to the sulphation of LA itself. This sulphation of LA was a rapid process followed by secretion back to the medium. Brefeldin A did not reduce the sulphation of LA, indicating that this conversion takes place in the cytosol, rather than in the Golgi apparatus of the CaCo-2 cells. LA in colon may be sulphated efficiently by the colonocytes to reduce the toxic effects of this particular bile acid. Sulphation may possibly be an important protective mechanism in the colon.

Key words: bile acids, sulpho-lithocholate, intestine, cancer, cytosolic sulphation.

INTRODUCTION

The risk of colorectal cancers may be influenced by a number of factors, including diet, smoking, physical activity, and genetic background. A high fat intake has been shown to increase the risk for colon cancer in epidemiological studies [1]. The intestinal uptake of fat is, among other factors, dependent on bile acids. Primary and secondary bile acids have been suggested to contribute to the development of colon cancer [2–4]. Increased levels of secondary bile acids, in particular, lithocholic acid (LA), in the colon is related to colorectal neoplasia [5]. However, the molecular mechanisms underlying this phenomenon remain poorly understood. Primary bile acids such as cholic acid (CA) and chenodeoxycholic acid (CDA) are conjugated with taurine or glycine and secreted from the liver into the intestine via the bile duct. To some extent they are dehydroxylated into the secondary bile acids deoxycholic acid (DCA) and LA by the intestinal microflora. In colon primary and secondary bile acids are present in either the conjugated or deconjugated forms, and the physiological concentration of bile acids in the gut has been reported to be approximately 4 mM [6], but more than 95% of this is reabsorbed in the ileum. Sulphated conjugates of LA are poorly absorbed from the intestine and pass into the colon. There they are desulphated and deconjugated to some extent. The intralumenal amount of bile acids has been reported to vary from 110 to 690 mg per day [7] and the concentration of LA will depend on the concentration of the primary bile acids precursors CDA and ursodeoxycholic acid [8].

Changes in the expression of sulphated molecules such as proteoglycans (PG), mucins and other glycoconjugates have been demonstrated in transformed-colon-epithelial cells. A typical finding is that sulphation of mucins [9] and PG [10] expressed by transformed-colon cells is decreased compared to their normal counterparts. Furthermore, specific structural changes in heparan sulphate have been demonstrated when human-colon adenoma progress to carcinoma *in vitro* [11]. Changes in the pattern of heparan sulphate expressed by CaCo-2 colon-carcinoma cells affect the ability of heparan sulphate proteoglycans to interact with growth factors [12]. To analyse if primary or secondary bile acids would induce phenotypic changes in CaCo-2 colon-carcinoma cells, the expression of sulphated molecules was studied. For this reason we labelled cells with [^{35}S]sulphate and harvested [^{35}S]molecules released to the medium or remaining in the cell fraction. Sulphation of PG was not affected by any of the bile acids tested. However, one secondary bile acid, LA, induced a large increase in secretion of sulphated molecules from CaCo-2 cells. This increase was primarily due to a rapid sulphation of LA after cellular uptake, followed by secretion back into the medium.

MATERIALS AND METHODS

Chemicals

LA, CDA, CA, DCA, Blue Dextran, 2,4-dinitrophenylalanine and insulin were purchased from Sigma Chemical Co. (St. Louis,

Abbreviations used: BFA, brefeldin A; CA, cholic acid; CDA, chenodeoxycholic acid; DCA, deoxycholic acid; DMEM, Dulbecco's modified Eagle's medium; FCS, foetal-calf serum; LA, lithocholic acid; PG, proteoglycans.

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MO, U.S.A.). Dulbecco's modified Eagle's medium (DMEM), non-essential amino acids, penicillin, streptomycin and foetal-calf serum (FCS) were obtained from Bio Whittaker (Wakersville, MD, U.S.A.). Sulphate-free RPMI-1640 culture medium was purchased from Gibco (Paisley Ltd., Scotland), and trypsin was purchased from Difco Laboratories (Detroit, MI, U.S.A.). All tissue culture dishes and flasks were supplied by Costar (Cambridge, MA, U.S.A.). [^{35}S]sulphate, [24-^{14}C]LA and [^{3}H]leucine were obtained from Amersham (Buckinghamshire, U.K.). Chondroitinase ABC (C-ABC, EC 4.2.2.4) was purchased from Seikagaku Kogyo Co. (Tokyo, Japan). Sephadex G-50 fine, Superose 6 and DEAE-Sephacel were purchased from Amersham Pharmacia (Uppsala, Sweden).

Cell cultures

The CaCo-2 intestinal cell line was obtained from A.T.C.C. (Rockville, MD, U.S.A.). These cells were chosen as a model system for studying effects and metabolism of bile acids in human-intestinal cells. The cell line was maintained in DMEM medium containing 20 % (v/v) FCS, 2 mM L-glutamine, insulin (10 μg/ml), penicillin (50 units/ml), streptomycin (50 μg/ml) and 0.001 % non-essential amino acids. For subculture, the cell medium was removed and the cells were released with 0.25 % trypsin (in PBS containing EDTA at 0.2 g/l). The trypsinization was stopped by adding FCS, and the cells were re-seeded at approx. 40000/cm^2 in new flasks. When plating cells for experiments, approx. 500000 cells were seeded per well (35 mm diameter), and they were grown to 90 % confluence before the experiment was started.

Bile acids

The sodium salts of LA, CDA, CA and DCA were solubilized in DMEM : ethanol (1 : 1, v/v), and during experiments control cells were exposed to the same concentration of ethanol as cells treated with the different bile acids.

Viability of CaCo-2 cells

The viability of CaCo-2 cells exposed to increasing concentrations of LA was examined by measuring the ability of the cells to incorporate [^{3}H]leucine into cell-associated proteins. After confluency, the cells were preincubated for 20 h with medium containing 10 % (v/v) FCS supplemented with LA at concentrations from 0–20 μg/ml. New media also containing 5 μCi/ml [^{3}H]leucine were then added to the cells and the incubation was continued for 2 h. Thereafter, the cells were washed six times with 95 % (v/v) ethanol on ice and solubilized in 0.2 M NaOH. The total protein-associated radioactivity was measured in a Packard Scintillation Counter, and aliquots were taken for protein determination using the bicinchoninic acid (BCA) Protein Assay (Pierce Laboratories Inc., Rockford, IL, USA). The results are presented as d.p.m./mg protein.

Labelling of cells

The medium used during labelling experiments was RPMI-1640 depleted of sulphate but containing 10 % (v/v) FCS, L-glutamine and non-essential amino acids. However, no antibiotics were added to reduce the level of unlabelled sulphate during labelling with [^{35}S]sulphate. Before labelling the cells were washed once with sulphate-depleted RPMI-1640. Thereafter, labelling medium with [^{35}S]sulphate (200 μCi/ml) was added to the cells. Concomitantly, the cells were exposed to LA, CDA, CA or DCA at concentrations from 1–50 μg/ml (2.7–133 nmol/ml). Control cells were given the corresponding concentration of ethanol without bile acids. The cells were labelled in the absence and presence of bile acids for time periods extending from 5 min up to 48 h. At different time points media and cell fractions were harvested. Cells and debris were removed from the respective medium fractions by centrifugation at 167 ***g*** for 5 min. The adherent cells were extracted in 4 M guanidinium chloride with 2 % (w/v) Triton X-100 in 0.05 M sodium acetate buffer pH 6.0. To remove free [^{35}S]sulphate, the cell extracts and medium fractions were subjected to Sephadex G-50 fine gel chromatography. Material eluting in the void volume was collected and used for further analyses.

Sulphation of [^{14}C]LA

[^{14}C]LA (100000 c.p.m./ml; 55 μCi/mmol), obtained from Amersham, was added to CaCo-2 cells in the labelling medium, which also contained unlabelled LA at a concentration of 5 μg/ml (13.3 nmol/ml). At different time points cell and medium fractions were harvested as previously described. An equal volume of isopropanol was added to the cell-free medium fractions to precipitate proteins. The protein pellet was removed after centrifugation at 3023 ***g*** for 15 min, and aliquots of the supernatants were analysed on a Beckman 126 HPLC instrument using an octadecasilane (ODS) column (5 μm particle size, 10 mm × 25 cm) (Beckman Instruments Inc., Fullerton, CA, U.S.A.) and a Beckman 172 radioisotope detector. The elution was performed with 60 % (v/v) isopropanol in 50 mM potassium phosphate buffer, pH 7.0. Flo-Scint II scintillation solution (Packard) was used for counting of the radioactivity.

Sulphation of LA

CaCo-2 cells were incubated with 5 μg/ml LA for 20 h. In the same time period the cells were labelled with [^{35}S]sulphate as described previously. The medium was collected and free [^{35}S]sulphate removed by Sephadex G-50 fine gel chromatography. Thereafter the sample was subjected to HPLC analysis as described above.

Brefeldin A treatment

Brefeldin A (BFA), a fungal macrocyclic lactone, is a potent inhibitor of membrane trafficking and vesicular transport. It induces disassembly of the Golgi complex and retrograde transport of Golgi back to the endoplasmic reticulum, thereby blocking formation of transport vesicles and protein secretion [13]. BFA has also been shown to inhibit sulphation of PG [14]. To examine whether the Golgi complex was involved in the sulphation processes, CaCo-2 cells were exposed to 5 μg/ml BFA (obtained from Epicentre Technologies, Madison, WI, U.S.A.) 30 min before LA (5 μg/ml) and [^{35}S]sulphate (200 μCi/ml) were added to the cells. The cells were then incubated for up to 18 h before the medium was harvested as described above.

PG labelling

For labelling of PG, cells were seeded in 35 mm wells, and washed with sulphate-depleted RPMI 1640 medium after reaching confluence. The cells were labelled with [^{35}S]sulphate (100 μCi/ml) for 20 h in labelling medium after which the medium was collected and centrifuged to remove non-adherent cells. The medium was finally frozen until further analyses. The adherent

cells were extracted, and free [^{35}S]sulphate was removed as described above.

To define the structure of [^{35}S]PG from medium and cell fractions, aliquots were subjected to treatment for 15 h at 37°C with chondroitinase ABC (0.01 unit per incubation) in 0.05 M Tris/HCl, pH 8.0, containing BSA at 0.1 mg/ml and 0.025 M sodium acetate. Untreated and enzyme-treated samples were analysed by Sepharose CL-6B gel chromatography using Blue Dextran and 2,4-dinitrophenylalanine as markers for the void and total volume of the gel, respectively. The column was run in 0.05 M Tris/HCl, pH 8.0, containing 0.15 M NaCl. To determine the amount of heparan sulphate samples were subjected to HNO_2 treatment at pH 1.5 [15] and analysed by Sepharose CL-6B gel chromatography. One ml fractions were collected and analysed for radioactivity by scintillation counting using a Wallac (Wallac, Turku, Finland) scintillation counter.

^{35}S-labelled material was also chromatographed on DEAE-Sephacel ion exchange columns (Amersham Pharmacia). Material was loaded on to the column, and the column was washed with 0.05 M Tris/HCl, pH 8.0, containing 0.15 M NaCl. Material binding to the column was eluted with a gradient extending from 0.15 to 1.5 M NaCl in the same buffer. Fractions of 1 ml were collected and counted for radioactivity in a scintillation counter.

SDS/PAGE and autoradiography

To separate proteoglycans and sulphated proteins ^{35}S-labelled material was subjected to SDS/PAGE using 4–20% (w/v) acrylamide gradient gels from Novex (Encinitas, CA, U.S.A.). After separation, the gels were dried and analysed with a Phosphor Imager (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

Statistics

Results are presented as averages ± S.D. or as averages with a range. The non-parametric Mann–Whitney U-test was used to test for significant differences between groups.

RESULTS

Effect of bile acids on the expression of [^{35}S]sulphate-labelled molecules

Initial experiments were performed to test whether both primary and secondary bile acids would affect the expression of [^{35}S]sulphated molecules in cultured CaCo-2 cells. LA, CA, CDA and DCA were tested in concentrations ranging from 0.1–10 μg/ml (2.7–26.6 nmol/ml). The level of ^{35}S-labelled compounds released into the culture medium was only affected by exposure to an LA concentration of 10 μg/ml. In the present experiment CaCo-2 cells released approx. 7 times more ^{35}S-labelled compounds than untreated control cells, as can be seen in Figure 1, upper panel. In the cell fraction there was a 2-fold increase in the level of recovered ^{35}S-labelled compounds after treatment with LA (10 μg/ml). The other bile acids tested did not promote significant increases in [^{35}S]sulphation, neither in the media nor the cell fractions. The increase observed after LA treatment was found to vary, and in some experiments it was up to 18 times after 22 h treatment with LA. Furthermore, the stimulation of [^{35}S]sulphate incorporation was a rapid process, as can be seen in Table 1. In this particular experiment the increase was 7 times after 30 minutes and increased further up to 4 h of incubation.

The increase observed after LA treatment was pronounced at a concentration of 10 μg/ml but lacking at 1 μg/ml. The effect of

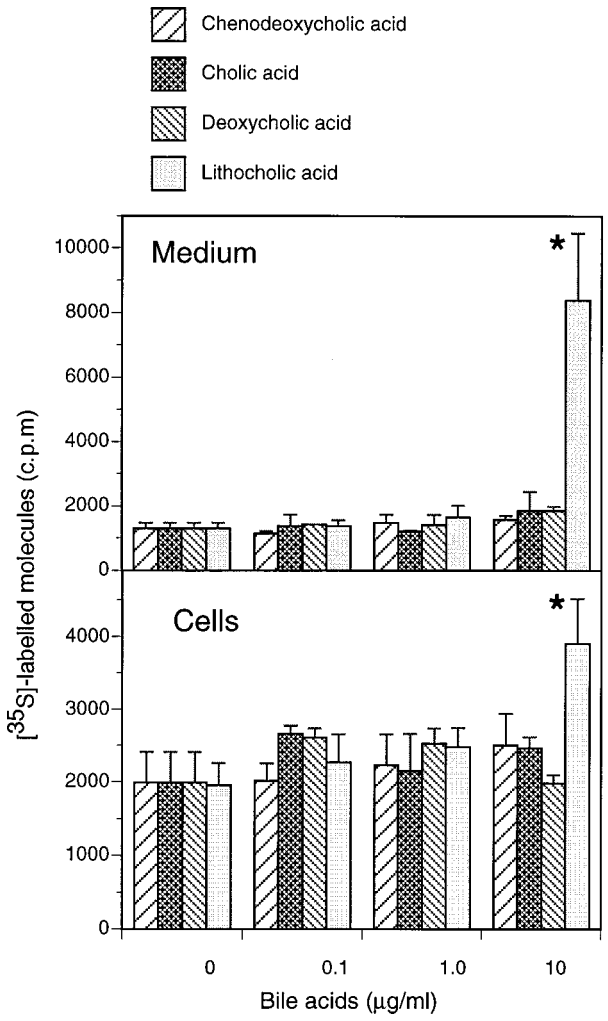


Figure 1 The effect of bile acids on the expression of [^{35}S]sulphated molecules in CaCo-2 cells

CaCo-2 cells were incubated in the absence and presence of different concentrations of CA, LA, DCA and CDA. The cells were labelled for 20 h with [^{35}S]sulphate, the cell and medium fractions harvested and subjected to Sephadex G-50 gel chromatography. The data presented are the means ± S.D. of four parallel cell dishes. *Significant difference between material from control cells and cells treated with LA ($P < 0.05$, Mann–Whitney U-test).

Table 1 Time-dependent expression of [^{35}S]sulphated molecules

Cells were incubated in the absence and presence of LA at 5 μg/ml. After harvesting the cells at different time points the medium was subjected to gel chromatography and aliquots of the void-volume fractions were counted for radioactivity (c.p.m./100 μm). The results presented are means ± S.D. of six separate cell dishes. *Significant difference between material from control cells and cells treated with LA ($P < 0.05$, Mann–Whitney U-test).

Time (h)	Control	LA	LA/Control
0.5	497 ± 24	3737 ± 815*	7.5
4.0	7274 ± 1337	125979 ± 5603*	17.3
22.0	68766 ± 12949	886742 ± 86087*	18.1

LA in concentrations ranging from 1-50 μg/ml was therefore investigated. As can be seen in Figure 2 the level of ^{35}S-labelled molecules recovered from the medium fraction increased in a

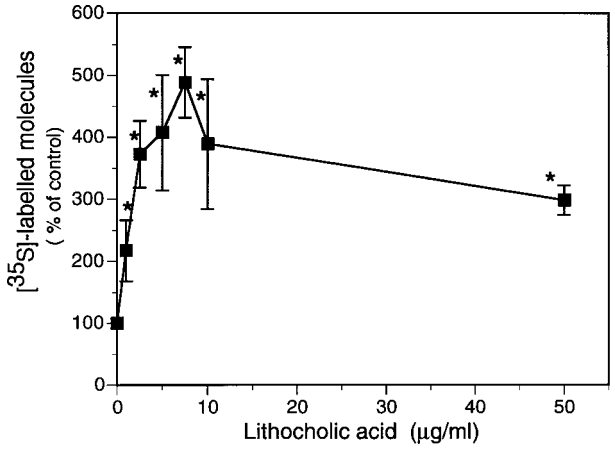


Figure 2 The effect of LA on the expression of [^{35}S]sulphated molecules in CaCo-2 cells

CaCo-2 cells were incubated with different concentrations of LA and labelled with [^{35}S]sulphate for 20 h. The media fractions were harvested, subjected to gel chromatography to remove free [^{35}S]sulphate and analysed for radioactivity. The data presented are the means ± S.D. from six separate cell dishes. *Significant difference between material from control cells and cells treated with LA ($P < 0.05$, Mann–Whitney U-test).

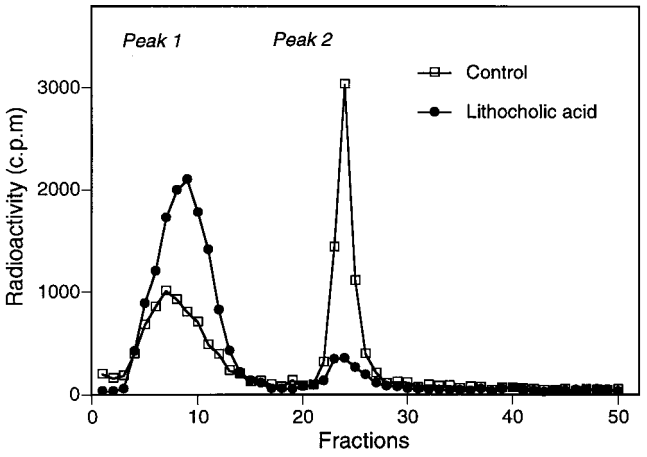


Figure 3 Preparative-ion-exchange chromatography

CaCo-2 cells were labelled with [^{35}S]sulphate in the absence and presence of LA at 10 μg/ml. After 20 h the two medium fractions were harvested, subjected to gel chromatography and then to DEAE-ion-exchange chromatography as described in the Materials and methods section. Aliquots of each fraction were counted for radioactivity, and material corresponding to the indicated peak 1 (fractions 4–14) and peak 2 (fractions 22–26) were pooled, desalted and subjected to further analyses (see Figure 4).

Table 2 Incorporation of [^{3}H]leucine into cell-associated protein

Protein synthesis was measured as described in the Materials and methods section. Data are presented as the average ± S.D. from 6 separate cell cultures.

LA (μg/ml)	Protein synthesis (d.p.m./mg of protein)
0	70939 ± 6113
5	74875 ± 5731
10	72172 ± 5548
20	68113 ± 5407

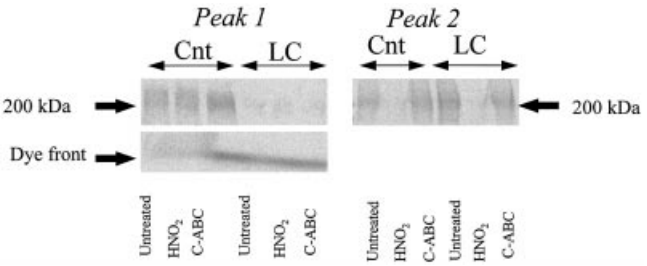


Figure 4 SDS/PAGE of [^{35}S]sulphated material

Material from preparative DEAE-ion-exchange chromatography (see Figure 3) from control cells (Cnt) and LA-treated cells (LC) was subjected to SDS/PAGE under reducing conditions. Material from both peak 1 and 2 was electrophoresed untreated and after HNO_2 or chondroitinase ABC treatment. The migrations of molecular mass markers in kDa are indicated. The upper and lower part of the gel is shown in the figure.

dose-dependent manner up to 7.5 μg/ml. In this particular experiment the level of ^{35}S-labelled compounds was five times higher with 7.5 μg/ml and four times higher with 10 μg/ml after 20 h labelling. No further increase was seen with LA at 50 μg/ml. In subsequent experiments LA concentrations of either 5 or 10 μg/ml were used. By microscopic inspection the viability and morphology of the CaCo-2 cells were not affected by increasing concentrations of LA. To analyse further possible deleterious effects of LA the ability of the cells to synthesize proteins was analysed by measuring the incorporation of [^{3}H]leucine into cell-associated proteins. As can be seen from Table 2 none of the LA concentrations used affected the level of [^{3}H]leucine incorporation in CaCo-2 cells.

Characterization of ^{35}S-labelled molecules

To further characterize the ^{35}S-labelled molecules released following exposure of CaCo-2 cells to LA, medium material was subjected to preparative DEAE ion-exchange chromatography, as shown in Figure 3. Material corresponding to peaks 1 and 2 was pooled and subjected to SDS/PAGE prior to and after chondroitinase ABC or HNO_2 treatment. From Figure 4 it is evident that the peak 1 material in the control consists mostly of a high molecular mass component, which most likely contains sulphated proteins or glycoproteins. These have not been further characterized. These sulphated macromolecules can also be seen in the peak 1 material from cells treated with LA. However, in this fraction the most prominent constituent is a low molecular mass component, migrating below the 14 kDa standard. Both the high molecular mass and low molecular mass components were resistant to treatment with both chondroitinase ABC and HNO_2, clearly showing that the peak 1 material from LA treated cells is not of PG nature.

Material obtained from peak 2 in both controls and cells treated with LA appeared as a broad smear, a feature typical of PG, as is evident from Figure 4. Furthermore, this material was resistant to chondroitinase ABC treatment but almost completely depolymerized after treatment with HNO_2, demonstrating that the material from both sources contains heparan sulphate PG in the peak 2 fraction.

The low molecular mass [^{35}S]sulphated component

To further identify the material eluting in peak 1 from the LA-treated cells, we wanted to investigate whether some of this

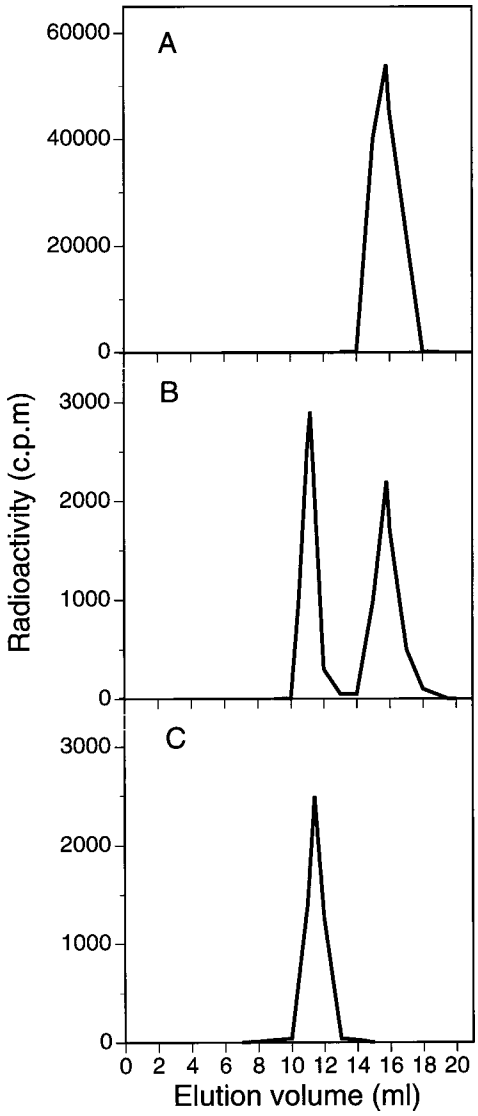


Figure 5 HPLC analysis of LA

[^{14}C]LA was subjected to HPLC analysis after incubation with CaCo-2 cells for 0 h (**A**) and 20 h (**B**). In addition, material from the medium of cells treated with LA at 5 μg/ml and labelled with [^{35}S]sulphate for the same time period was subjected to HPLC analysis (**C**). Fractions were collected and counted for radioactivity. LA is eluted at 15–17 ml and sulphated LA at 10–12 ml.

material could be LA itself. For this purpose CaCo-2 cells were incubated in the presence of [^{14}C]LA and LA at 5 μg/ml (13.3 nmol/ml). After incubation for 0 h and 20 h (without [^{35}S]sulphate labelling) these media were harvested, processed and subjected to HPLC. The ^{14}C radioactivity in the extract from the 0 h sample eluted as one distinct peak after 15–17 ml, the same as the [^{14}C]LA standard (Figure 5A). After 20 h incubation with the cells, approx. 50 % of the ^{14}C radioactivity was incorporated into a more polar product, as shown by the appearance of a peak eluting in 10–12 ml (Figure 5B). After a parallel 20 h incubation with unlabelled LA and [^{35}S]sulphate, an ^{35}S-labelled product was formed that eluted in the same position (10–12 ml) as the polar ^{14}C-labelled product, as can be seen in Figure 5(C). Material eluting in this position was in both cases formed after incubation with CaCo-2 cells. The unlabelled LA could only be detected after incubation with cells also exposed to

Table 3 Time-dependent sulphation of [^{14}C]LA

CaCo-2 cells were incubated with [^{14}C]LA, harvested and processed as described in the Materials and methods section. The samples were then analysed on a HPLC system, and the degree of sulphation calculated on the basis of the size of the two peaks (see Figures 5A and 5B) and related to the amount of material added to the cell cultures. Values are presented as the average from two separate cell dishes and the range is given in parentheses ().

Time (h)	Degree of sulphation (% sulphation after 1 h)
1	0
3	1.8 (1.0–2.5)
6	7.4 (7.3–7.5)
12	14.4 (13.0–15.8)
22	29.1 (28.8–29.3)
48	53.8 (51.2–56.3)

Table 4 The effect of BFA on the expression of [^{35}S]sulphated molecules

CaCo-2 cells were preincubated for 30 min with or without BFA (5μg/ml) and then cultured for a further 20 h with [^{35}S]sulphate in the absence and presence of LA (5μg/ml). The media were harvested and chromatographed on Sephadex G-50 columns. Aliquots of the void-volume fractions were counted for radioactivity. Results presented are the means $\pm$ S.D. from three separate cell cultures. *Significant difference between control cells and BFA-treated cells. #Significant difference between LA-treated cells and cells treated with both LA and BFA. ($P < 0.05$, Mann–Whitney U-test).

	Radioactivity (c.p.m)	
Cell preincubation	LA-untreated	LA-treated
Control	10675 $\pm$ 828	21096 $\pm$ 869
BFA	1828 $\pm$ 488*	11171 $\pm$ 1455#

[^{35}S]sulphate, suggesting that both the unlabelled (Figure 5C) and the ^{14}C-labelled material (Figure 5B) eluting after 10–12 ml are the sulphated LA. Glycine or taurine conjugates of LA are not expected to be formed, and would have been eluted in earlier fractions than sulphated LA (B. F. Kase, unpublished work).

CaCo-2 cells were further incubated with [^{14}C]LA, and medium was harvested at different time points. The medium fractions were processed and analysed by HPLC. The degree of sulphation was calculated on the basis of the amount of material eluting in the peaks corresponding to the elution positions for LA and sulphated LA. From Table 3 it is evident that the sulphation of LA is a rapid and time-dependent process.

During isolation of the [^{35}S]sulphated molecules the sulphated LA was recovered in the void fractions after Sephadex G-50 gel chromatography. This observation suggests that LA is bound to a carrier, possibly albumin [16] or bile acid binding protein (BABP) [17] during secretion, or associates with such components after release from the cells.

Sulphation of LA

Sulphation of PG takes place in the Golgi apparatus. It has been demonstrated that this process can be inhibited in cultured cells when they are incubated with the fungal isoprenoid metabolite BFA [14,18]. To analyse the sulphation of LA further, CaCo-2 cells were treated with BFA during exposure to [^{35}S]sulphate and LA. After treatment with BFA the incorporation of [^{35}S]sulphate was reduced by 83 % in control cells and 47 % in cells treated with LA (Table 4). The material was further analysed by ion-exchange chromatography. The control material (Figure 6, upper

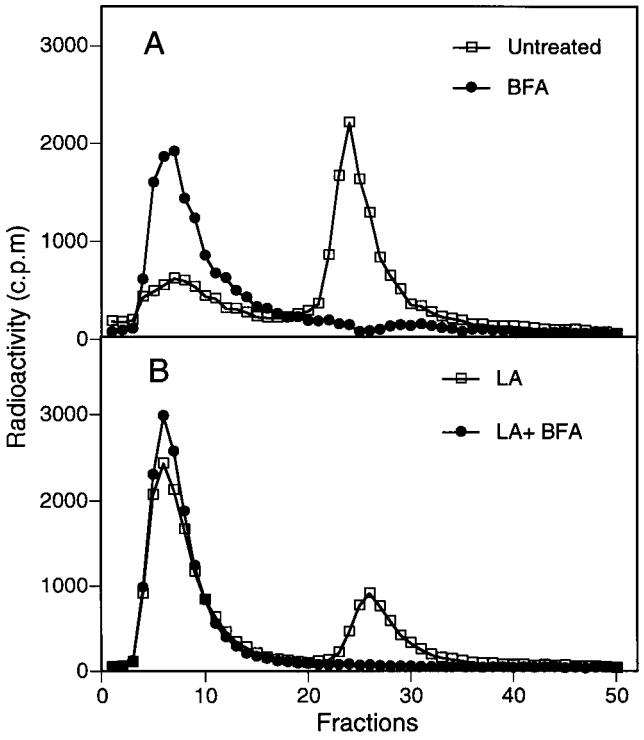
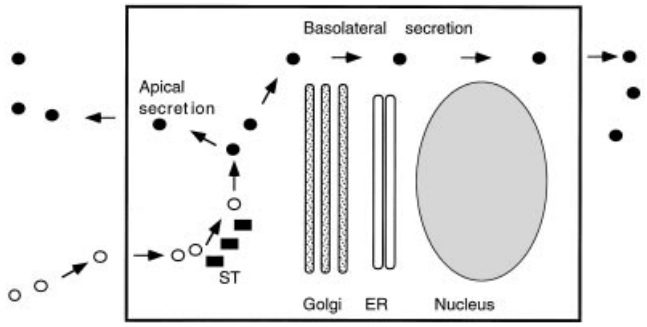


Figure 6 Ion-exchange chromatography

Material from CaCo-2 cells cultured in either the absence or presence of BFA (5 μg/ml) and labelled with [^{35}S]sulphate was subjected to DEAE-ion-exchange chromatography. Cells were cultured either with (**B**) or without (**A**) LA (5 μg/ml). Column running conditions were as described in the Materials and methods section.



Scheme 1 A tentative scheme of LA sulphation in enterocytes

LA (○) can be taken up by enterocytes and sulphated in the cytosol in a BFA-resistant process. Sulphated LA (●) is then secreted from the cells. ST, cytosolic sulphotransferase (■); ER, endoplasmic reticulum.

panel) was affected by BFA treatment such that the sulphation of PG was almost completely inhibited, as shown by the disappearance of the second [^{35}S]PG-containing peak. CaCo-2 cells treated with LA expressed [^{35}S]sulphated material, the major part of which was eluted in the early fractions. However, this fraction was not further reduced after BFA treatment. In contrast, the expression of [^{35}S]PG was almost completely abolished, as shown by the disappearance of the second peak after ion-exchange chromatography. No PG material was found in the early fractions, suggesting that a lower degree of PG sulphation after BFA treatment cannot account for the changes in the elution profiles observed. These results therefore indicate that the sulphation of the non-PG fraction is not affected by BFA treatment.

DISCUSSION

In the present paper we present evidence for sulphation of unconjugated LA in the colon-carcinoma cell line CaCo-2. To the best of our knowledge this is the first report of LA sulphation in human-colon cells. The process is rapid, BFA-resistant and the sulphated LA is released from the cells back into to the culture medium. Of the four bile acids tested, LA was the only one observed to induce a dramatic increase in the incorporation of [^{35}S]sulphate.

Sulphation of bile acids has been addressed in several studies where the main emphasis has been on such processes in the liver [19]. Cytosolic sulphotransferases in the liver are important (e.g. to promote the renal elimination of drugs and xenobiotic compounds). It is, however, interesting to note that human-intestinal tissues contain substantial amounts of the sulphate donor adenosine 3′-phosphate 5′-phosphosulphate (PAPS) [20], suggesting that the intestine has a high capacity for sulphation. The possibility that human-colon-epithelial cells can sulphate LA is important in relation to the effects of bile acids on the processes leading to colon cancer. Of the four bile acids used in the present study, LA has been shown to be the most cytotoxic to colonic cell lines [21]. Furthermore, an increase in the level of secondary bile acids, LA in particular, was found in faeces from patients with colorectal neoplasia when compared with control subjects [22,5]. In the former study a higher proliferative capacity was also demonstrated in biopsies from patients with tumours compared to healthy controls. Higher levels of both primary and secondary bile acids may increase the activity of protein kinase C in human-colonic tissue [23] and transcription factor AP-1 in a colon-carcinoma cell line [24], thereby influencing cellular signalling processes important for the regulation of cell proliferation and differentiation.

It has been shown that the LA/DCA ratio is increased in patients with colon cancer, and this ratio has been proposed to be a valuable diagnostic tool [25]. Dietary-intervention studies have shown that increased calcium intake may increase the level of calcium-bound bile acids in faeces and thereby have protective effects on the development of colon cancer [26,27]. Increased intake of dietary fibre will also increase the efflux of bile acids from the colon and decrease the incidence of colon cancer [28]. Reduced levels of bile acids, or reduced time of exposure to bile acids in the colon may, therefore protect against the development of colon cancer.

The results presented here using the human-colon-carcinoma cell line CaCo-2 suggest that the colon-epithelial cells have an additional mechanism for protection against the potentially deleterious effects of LA. The results outlined show that LA can be sulphated by intestinal cells themselves, to increase water solubility and reduce its hydrophobic and possibly also its cytotoxic properties [29,30].

A tentative scheme of a rapid sulphation of LA in intestinal-epithelial cells is presented in Scheme 1. Sulphation in plasma membrane fractions has not been detected [31], although functions in compartments other than the Golgi apparatus and the cytosol cannot be excluded, as sulphotranferases have been purified from cell culture media [32].

CaCo-2 cells have been cultured on semipermeable filters to induce polarization, and they have also been kept on filters for two weeks to induce differentiation into enterocytes [33]. Neither

undifferentiated nor differentiated CaCo-2 cells displayed polarized secretion of sulphated LA (B. Halvorsen, unpublished observation). There was, furthermore, no difference between differentiated and undifferentiated CaCo-2 cells in the capacity to sulphate LA. This indicates that the ability to sulphate LA could be a property of normal intestinal cells.

Sulphation of bile acids may be an important protective process in epithelial cells in the colon. Furthermore, it may be such that an increased sulphation of bile acids may have secondary effects on the sulphation of macromolecules such as mucins or PG. In the present study the sulphation of LA did not affect the expression level of sulphated PG of the heparan-sulphate type. Bile acids have, however, been shown to induce changes in the expression of mucin in cultured-colon cells [34]. A decreased sulphation of PG or mucins, due to competition with the sulphation of LA for the supply of adenosine 3′-phosphate 5′-sulphatophosphate, may lead to events favouring tumour development. These possibilities are the subject of further investigations.

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