Modulation of lipid synthesis, apolipoprotein biogenesis, and lipoprotein assembly by butyrate

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Marcil, Valérie, Edgard Delvin, Ernest Seidman, Lucie Poitras, Monika Zolowska, Carole Garofalo, and Emile Levy. Modulation of lipid synthesis, apolipoprotein biogenesis, and lipoprotein assembly by butyrate. Am J Physiol Gastrointest Liver Physiol 283: G340–G346, 2002; 10.1152/ajpgi.00440.2001.—Short-chain fatty acids (SCFAs) constitute the predominant luminal anions in the colon (39). They are directly derived from milk, dairy products, and the anaerobic fermentation of complex carbohydrates by endogenous bacterial flora (38, 39). In addition to their vital role in maintaining colonic integrity and metabolism, SCFAs display the ability to inhibit DNA synthesis and cell growth (47), influence cellular DNA through posttranslational modifications, i.e., acetylation, methylation, phosphorylation (10, 20), alter gene expression (7, 8, 35), induce cell differentiation (2), and modify both cell morphology and ultrastructure (5, 16). Although nutritionally nonessential, SCFAs present beneficial effects toward maintaining general health as well as disease prevention/protection (32). Clinically, SCFAs have been studied as possible therapeutic agents in diversion colitis, ulcerative colitis, radiation proctitis, pouchitis, and antibiotic-associated diarrhea (32). It has also been suggested that some of the beneficial effects of high-carbohydrate, high-fiber diets on lipid metabolism and atherosclerosis are mediated by the metabolism of SCFAs in the liver (3, 13, 50, 51). In this conjecture, SCFAs, produced as an energy source by colonic cells, may inhibit hepatic cholesterol synthesis and lower cholesterol concentrations in all lipoprotein fractions (51). These effects on circulating cholesterol may be elicited by bile acid excretion, which is considered to be associated with the hypocholesterolemic action of dietary soluble fiber (33). Surprisingly, to our knowledge, there are no data on the ability of SCFAs to influence the synthesis and secretion of lipoproteins by the intestine, which is considered as one of the major players in lipid homeostasis (4, 9, 19, 24, 48). Only an indirect effect was suggested for butyrate in view of its conversion to ketone bodies and other metabolites in the gut, which are then capable of modifying lipid metabolism (17, 38). The aim of the present study was to determine whether SCFAs play a key role in the absorption of alimentary fat. With the use of a Caco-2 cell line, an established intestinal model (11, 23), we could demonstrate that butyrate modulates the cellular events governing the assembly and delivery of lipoproteins.

Short-chain fatty acids (SCFAs) constitute the predominant luminal anions in the colon (39). They are directly derived from milk, dairy products, and the anaerobic fermentation of complex carbohydrates by endogenous bacterial flora (38, 39). In addition to their vital role in maintaining colonic integrity and metabolism, SCFAs display the ability to inhibit DNA synthesis and cell growth (47), influence cellular DNA through posttranslational modifications, i.e., acetylation, methylation, phosphorylation (10, 20), alter gene expression (7, 8, 35), induce cell differentiation (2), and modify both cell morphology and ultrastructure (5, 16). Although nutritionally nonessential, SCFAs present beneficial effects toward maintaining general health as well as disease prevention/protection (32). Clinically, SCFAs have been studied as possible therapeutic agents in diversion colitis, ulcerative colitis, radiation proctitis, pouchitis, and antibiotic-associated diarrhea (32). It has also been suggested that some of the beneficial effects of high-carbohydrate, high-fiber diets on lipid metabolism and atherosclerosis are mediated by the metabolism of SCFAs in the liver (3, 13, 50, 51). In this conjecture, SCFAs, produced as an energy source by colonic cells, may inhibit hepatic cholesterol synthesis and lower cholesterol concentrations in all lipoprotein fractions (51). These effects on circulating cholesterol may be elicited by bile acid excretion, which is considered to be associated with the hypocholesterolemic action of dietary soluble fiber (33). Surprisingly, to our knowledge, there are no data on the ability of SCFAs to influence the synthesis and secretion of lipoproteins by the intestine, which is considered as one of the major players in lipid homeostasis (4, 9, 19, 24, 48). Only an indirect effect was suggested for butyrate in view of its conversion to ketone bodies and other metabolites in the gut, which are then capable of modifying lipid metabolism (17, 38). The aim of the present study was to determine whether SCFAs play a key role in the absorption of alimentary fat. With the use of a Caco-2 cell line, an established intestinal model (11, 23), we could demonstrate that butyrate modulates the cellular events governing the assembly and delivery of lipoproteins.
**MATERIALS AND METHODS**

**Cell culture.** Caco-2 cells (American Type Culture Collection, Rockville, MD) were grown at 37°C in MEM (GIBCO-BRL, Grand Island, NY) containing 1% penicillin-streptomycin and 1% MEM nonessential amino acids (GIBCO-BRL) supplemented with 10% decomplemented fetal bovine serum (FBS; Flow, McLean, VA). Caco-2 cells (passages 30–40) were maintained with 5% CO₂ in T-75-cm² flasks (Corning, NY). Cultures were split (1:6) when they reached 70–90% confluence using 0.05% trypsin-0.5 mM EDTA (GIBCO-BRL). For individual experiments, cells were plated at a density of 1 × 10⁶ cells/well on 24.5-mm polycarbonate Transwell filter inserts with 0.4-μm pores (Costar, Cambridge, MA) in MEM (as above) supplemented with 5% FBS. The inserts were placed into six-well culture plates, permitting separate access to the upper and lower compartments of the monolayers. Cultures were maintained for 20 days, a period at which we observed that the cells are highly differentiated and suitable for studies on lipid synthesis (30, 31). The medium was refreshed every second day.

**Measurement of lipid synthesis and secretion.** Caco-2 cells lipid synthesis and secretion were assayed as we described previously (31, 43). Briefly, radiolabeled [¹⁴C]oleic acid (sp act 53 mCi/mmol, Amersham, Oakville, Ontario, Canada) was added to unlabeled oleic acid and then solubilized in fatty acid-free BSA (BSA/oleic acid 1:5 mol/mol). The final oleic acid concentration was 0.7 mM (0.45 μCi/well). Cells were first washed with PBS (GIBCO), and the [¹⁴C]oleic acid-containing medium was added to the upper compartment. Butyric acid, at a concentration of 20 mM, was added to the upper chamber in serum-free MEM. At the end of a 24-h incubation period, cells were washed, then scraped with a rubber policeman in a PBS solution containing antiproteases (phenylmethylsulfonyl fluoride, pepstatin, EDTA, aminocaproic acid, chloramphenicol, leupeptin, glutathione, benzamidine, dithiothreitol, sodium azide, and trisylol, all at a final concentration of 1 mM). An aliquot was taken for lipid extraction by standard methods (27) in the presence of unlabeled carrier phospholipids (PL), monoglycerides, diglycerides (DG), triglycerides (TG), free fatty acids, free cholesterol, and cholesterol ester (CE).

The various lipid classes synthesized from [¹⁴C]oleic acid were then separated by thin-layer chromatography (TLC) using the solvent mixture hexane-ether-acetic acid [80:20:3 (vol/vol)] as previously described (27, 29). The area corresponding to each lipid was scratched off of the TLC plates, and the silica powder was placed in a scintillation vial with Ready Safe counting fluid (Beckman, Fullerton, CA). Radioactivity was then measured by scintillation counting (Beckman LS 5000 TD, Ontario, Canada). Cell protein was quantified by the Bradford method, and results were expressed as mg of cell protein per cm² of cell surface area.

**Cholesterol biogenesis.** Cholesterol biogenesis was evaluated employing [¹⁴C]acetate as a precursor (53.9 Ci/mmol) after a 24-h incubation period as described previously (22, 28). Separation of free cholesterol and CE was performed by TLC.

**Lipid carrier.** Blood was drawn 2 h after the oral intake of a fat meal by human volunteers, and postprandial plasma was prepared to serve as a carrier for the lipoproteins synthesized by Caco-2 cells. The TG-enriched plasma was incubated at 56°C for 1 h to inactivate enzymatic activity in the presence of antiproteases.

**Isolation of lipoproteins.** For the determination of secreted lipoproteins, Caco-2 cells were incubated with the lipid substrate as above in the presence or absence of butyric acid. The medium supplemented with antiproteases (as above) was first mixed with a plasma lipid carrier [4:1 (vol/vol)] to efficiently isolate de novo lipoproteins synthesized. The lipoproteins were then isolated by sequential ultracentrifugation using a TL-100 ultracentrifuge (Beckman Instruments, Montreal, Quebec, Canada) as described previously (27, 29). Briefly, chylomicrons were isolated following an ultracentrifugation (20,000 rpm, 20 min). Very low-density lipoproteins (VLDL; 1.006 g/ml) and low-density lipoproteins (LDL; 1.063 g/ml) were separated at 100,000 g for 2.26 h with a tabletop ultracentrifuge 100.4 rotor at 4°C. The high-density lipoprotein (HDL) fraction was obtained by adjusting the LDL infranatant to density at 1.21 g/ml and centrifuging for 5.5 h at 100,000 g. Each lipoprotein fraction was exhaustively dialyzed (24 h) against 0.15 M NaCl and 0.001 M EDTA, pH 7.0 at 4°C.

**De novo apolipoprotein synthesis.** The effect of butyric acid on newly synthesized and secreted apolipoproteins (apo) A-I, A-IV, B-48, B-100, and E was assessed as we previously described (25). To first induce apo synthesis, cells were incubated apically with unlabeled oleic acid bound to albumin in serum-free medium for 24 h before [³⁵S]methionine incubation. The concentration of the unlabeled lipid was equivalent to the labeled substrate described above. During this time, butyric acid was again added to the apical chamber. Thereafter, cells as well as the outer chambers were rinsed twice with PBS. The apical compartment was replaced with 1.5 ml of methionine-free medium containing the unlabeled substrate, [³⁵S]methionine (100 μCi/ml; Amersham Life Sciences, 50 mCi/mmol), and 20mM butyrate. After a 20-h incubation period at 37°C with 5% CO₂, the medium was collected from the basolateral compartment and cells were scraped off of the inserts in cell lysis buffer supplemented with the antiprotease cocktail, as above. Aliquots were precipitated with 20% trichloroacetic acid (TCA). The precipitates were then washed three times with 5% TCA before the radioactivity was determined in a Beckman liquid scintillation spectrometer.

**Immunoprecipitation of apolipoproteins.** Immunoprecipitation was performed in the presence of excess polyclonal antibodies to human apolipoproteins (Boehringer Mannheim, Mannheim, Germany) at 4°C overnight (25, 26). Samples were then washed with Nonidet (0.05%). They were subsequently centrifuged and resuspended in sample buffer (1.2% SDS, 12% glycerol, 60 mM Tris, pH 7.3, 1.2% betamercaptoethanol, and 0.003% bromophenol blue) and analyzed by a linear 4–15% polyacrylamide gradient preceded by a 3% stacking gel, as described previously (25, 26, 28). Radioactive molecular weight standards (Amersham Life Sciences) were run with the same conditions. Gels were sectioned into 2-mm slices and counted after an overnight incubation with 1 ml BSA-450 (Beckman) and 10 ml of liquid scintillation fluid (Ready Organic, Beckman).

**Statistical analysis.** All values were expressed as the means ± SE. Differences among control and sodium butyrate-treated Caco-2 cells were assessed using Student’s t-test with the level of significance set at 5% (P < 0.05).

**RESULTS**

**Caco-2 cell integrity.** The effect of butyrate on monolayer integrity and paracellular permeability was examined. As assessed by lactic dehydrogenase and...
Measurement of lipid esterification with $[^{14}\text{C}]$oleic acid. To elucidate the role of butyrate in lipid esterification and delivery, Caco-2 cells were cultured for 20 h with $[^{14}\text{C}]$oleic acid in the presence of this SCFA. As illustrated in Fig. 1, lipid changes were recorded in cells and media. The addition of butyrate led to increased TG cellular content without marked alterations in the other lipid classes (Fig. 1A). On the other hand, in the presence of butyrate, the amount of total labeled lipids secreted over a period of 20 h was significantly decreased (22%; $P < 0.01$) (Fig. 1B). This decrease was accounted for by a reduction in TGs (27%; $P < 0.003$) and PLs (25%; $P < 0.01$). The composition of all lipid fractions was also studied, which collectively represented 100% (Table 2). The intracellular content of TG tended to increase, whereas a significant decrease characterized the fractions of PLs and DGs following butyrate supplementation. Other differences were noted in the composition of lipid fractions secreted into the media in the presence of butyrate. A greater proportion of free cholesterol and DGs accompanied the percent decline in TGs.

Measurement of de novo cholesterol synthesis with $[^{14}\text{C}]$acetate as a precursor. The ability of Caco-2 cells to synthesize and export cholesterol under the influence of butyrate was tested with the incorporation of $[^{14}\text{C}]$acetate. The supplementation of Caco-2 cells with this SCFA provoked a slight but not significant reduction in the production of cellular free cholesterol (Fig. 2). However, the release of both forms of cholesterol was inhibited by butyrate, and only the delivery of CE was significantly diminished (49%; $P < 0.002$).

Apolipoprotein biogenesis. The subsequent step was to examine the modulation of apolipoprotein production by butyrate. For this purpose, Caco-2 cells were cultured with $[^{35}\text{S}]$methionine for 24 h, and the delivery of newly synthesized apolipoproteins in the medium was analyzed by NaDoSO$_4$ PAGE following immunoprecipitation. On the incubation of Caco-2 cells with butyrate, a decrease in the secretion of apo B and apo A-I was recorded (Fig. 3). Although both types of apo B were affected, only apo B-48 was significantly reduced (28%; $P < 0.02$). Butyrate also diminished apo A-I by $\sim$32% ($P < 0.004$) without altering the output of $[^{35}\text{S}]$methionine-labeled apo A-IV.

Lipoprotein secretion. To determine whether butyrate is able to regulate lipid transport, Caco-2 cells were incubated with $[^{14}\text{C}]$oleic acid for 20 h, media were collected, and lipoprotein fractions were immediately separated by ultracentrifugation. Figure 4 shows that the addition of butyrate to Caco-2 cells resulted in a consistent reduction in chylomicrons (13%; $P < 0.02$) and VLDL (20%; $P < 0.03$). However, only a slight decrease characterized LDL and HDL fractions.

**DISCUSSION**

The intestine is the essential site for the transport of alimentary fat in the form of lipoprotein structure. Huge amounts of cholesterol, PLs, lipid-soluble vitamins, and, particularly, TGs are transferred to peripheral tissues for energy production or for storage on a daily basis. Evidently, modulators of intestinal fat transport could have an impact on circulating lipoprotein concentrations and metabolism. Therefore, extensive work was performed to identify nutritional and hormonal factors involved in the control of the assimilation of digestive products by intestinal absorptive cells and the numerous intricate events guiding the assembly and release of lipoproteins (9, 24, 36). The present investigation documents, for the first time, several aspects of the action of butyrate on intestinal fat transport. Overall, an inhibitory effect of butyrate

Table 1. Effect of butyrate on Caco-2 cell integrity

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Butyrate</th>
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<tbody>
<tr>
<td>Transepithelial resistance, Ω·cm$^2$</td>
<td>1,211 ± 63</td>
<td>1,118 ± 44</td>
</tr>
<tr>
<td>Lactic dehydrogenase, U/l</td>
<td>159 ± 13</td>
<td>163 ± 17</td>
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Values are means $\pm$ SE of 4 wells. Transepithelial resistance and lactic dehydrogenase were determined after a 20-h incubation of Caco-2 cells with or without 20 mM butyrate.
was consistently observed on TG export, apo B-48 secretion, and chylomicron and VLDL output. In our experiments, butyrate was selected among SCFA, because it has been shown to induce multiple biological effects by regulating the expression of various genes (40). It is considered the most powerful modifi
cer and, therefore, represents an attractive tool for biological studies. Moreover, the present experimental conditions include butyrate at a concentration of 20 mM, given that previous reports described a luminal range of 20–30 mM in the intestinal lumen (32). We also chose a 20-h incubation period for Caco-2 cell culture with butyrate, because previous studies documented that this time length was suitable for the study of lipid synthesis and lipoprotein assembly (31, 43, 45). Finally, the determination of lactate dehydrogenase and transepithelial resistance allowed us to conclude that butyrate was not toxic to Caco-2 cells and did not disrupt membrane integrity.

To investigate the modulation of the formation and release of newly synthesized lipids by butyrate, we incubated Caco-2 cells with two different lipid precursors. Our results with [14C]oleate indicate that butyrate was particulary effective in retaining TGs intracellularly and reducing their export into the medium. With [14C]acetate, a more appropriate substrate for cholesterol synthesis, the secretory process of free and esterified cholesterol was affected. Butyrate has been shown in some cells to simultaneously regulate the activity of some membrane-associated enzymes and change membrane PLs (12, 14, 20, 34, 37). It has, therefore, been suggested that this SCFA alters the fluidity of the endoplasmic reticulum, where the synthetic lipid enzymes are located. Alternatively, it could

| Lipid composition of cell homogenates and media |
|------------------|---|---|---|---|
| \( \text{PL, \%} \) | \( \text{FC, \%} \) | \( \text{DG, \%} \) | \( \text{TG, \%} \) | \( \text{CE, \%} \) |
| **Homogenates** | | | | |
| Control | 24.52 ± 1.15 | 0.35 ± 0.03 | 0.35 ± 0.02 | 76.43 ± 2.41 | 0.95 ± 0.04 |
| Butyrate | 18.37 ± 1.04† | 0.29 ± 0.02 | 0.27 ± 0.02† | 79.89 ± 1.04 | 0.94 ± 0.06 |
| **Media** | | | | |
| Control | 11.20 ± 0.66 | 3.13 ± 0.12 | 2.90 ± 0.09 | 75.60 ± 0.70 | 3.20 ± 0.07 |
| Butyrate | 11.00 ± 0.70 | 4.00 ± 0.09† | 3.70 ± 0.19† | 72.70 ± 0.88* | 4.10 ± 0.06† |

Values are means ± SE. Caco-2 cells were incubated with [14C]oleic acid substrate for 20 h. Lipids of cell homogenates and media were then extracted with chloroform-methanol (2:1), separated by thin-layer chromatography, and quantitated as described in MATERIALS AND METHODS. Results represent the percentage of total 14C-lipid distribution for \( n = 5 \). * \( P < 0.003 \); † \( P < 0.01 \). DG, diglycerides; TG, triglycerides; CE, cholesterol ester; FC, free cholesterol.

Fig. 2. Effect of butyrate (hatched bars) on newly synthesized and secreted cholesterol. Differentiated Caco-2 cells were incubated with [14C]oleate in the presence of 20 mM butyrate for 20 h at 37°C. Samples of cell homogenates and basolateral media were analyzed essentially as described in MATERIALS AND METHODS. Results are expressed as dpm/mg cell protein, and values represent means ± SE of 5 experiments. ** \( P < 0.002 \). Filled bars, control; FC, free cholesterol; CE, cholesterol ester.

Fig. 3. Effect of butyrate (hatched bars) on de novo apolipoprotein synthesis. After a 20-h incubation of Caco-2 cells with [35S]methionine in the presence or absence of butyrate, apolipoproteins (B-100, B-48, A-IV, and A-I) in basolateral media were immunoprecipitated and analyzed by SDS-PAGE. Results are expressed as %trichloroacetic acid (TCA)-precipitable/mg cell protein, and values represent means ± SE of 6 experiments. * \( P < 0.02 \); ** \( P < 0.004 \). Filled bars, control.
simply modify gene expression, as documented in the liver by various studies. In fact, butyrate was capable of upregulating PL transfer protein in HepG2 cells at the transcriptional level (15). Similarly, butyrate had an enhancing effect on the CE transfer protein (CETP) owing to the upregulation of CCAAT/enhancer binding protein (C/EBP) expression (44), which activates the CETP gene promoter (1). Moreover, the treatment of HepG2 cells by butyrate resulted in a decline in lecithin-cholesterol acyltransferase (LCAT) activity, which was accompanied with a reduction in LCAT mRNA via a posttranscriptional mechanism (42). More specifically, SCFAs showed considerable effectiveness in decreasing 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase in pig liver slices (6) and rat liver microsomes (21). Consistent with this literature review and the current data, one may suggest that SCFAs influence circulating cholesterol by acting on the two major organs active in cholesterogenesis: the liver and the intestine. One can also reasonably propose that the hypocholesterolemic effects of fibers are, to some extent, due to SCFAs that depress HMG-CoA reductase activity. Nevertheless, additional studies are required to establish the exact status of key enzymes in sterol metabolism, including HMG-CoA reductase and acyl-coenzyme A: cholesterol acyltransferase (ACAT), in the small gut following the administration of butyrate.

In our experiments, the effects of butyrate on lipids, apolipoproteins, and lipoproteins were specific, because butyrate 1) particularly reduced the transfer of TGs (among the various lipid fractions) to the medium; 2) diminished the release of newly synthesized apo B and apo A-I without affecting the delivery of apo A-IV; and 3) decreased chylomicron and VLDL secretion without altering LDL and HDL fractions. With regard to apolipoproteins, one should point out that divergent observations were recorded in HepG2 cells. When Kaptein et al. (18, 19) incubated this hepatoma cell line with butyrate, they noted an accelerated accumulation of apo A-I and apo B-100 in the medium. The discrepancy between their results and ours may be due to numerous factors, including cell origin, butyrate concentrations, and other experimental conditions.

It has previously been shown that TGs protect newly synthesized apo B from intracellular degradation and stimulate the output of TG-rich lipoproteins (46). In our studies, there was a fall in apolipoprotein biogenesis in butyrate-treated Caco-2 cells despite the raised concentrations of intracellular TGs. A possible explanation for this observation is that TGs are located in intracellular pools other than the secretory pathway, which, under the influence of butyrate, do not transfer TGs to the endoplasmic reticulum for lipoprotein assembly. In this respect, triacylglycerol “cycling” is necessary in hepatic (41) and intestinal (50) tissues for...
their recruitment in the endoplasmic reticulum and their association with apo B, allowing the preservation of apo B and the active secretion of apo B-containing lipoproteins. Furthermore, butyrate may affect microsomal TG transfer protein (MTP), an obligatory factor in lipoprotein assembly (48). On the basis of several studies, it has been suggested that MTP most likely assists in the increased translocation of nascent apo B from the membrane to the lumen of the endoplasmic reticulum by adding lipid molecules to apo B. Thus abnormal lipidation of apo B represents a limiting step for synthesis and transport of TG-rich apo B lipoproteins. Additional studies are needed to determine the actual contribution to the reported cholesterol-lowering effects of complex carbohydrates.

In summary, butyrate, a metabolite of the natural fermentation of carbohydrates or a component of milk and dairy products, exhibited a regulatory role in lipid transport by Caco-2 cells. Further work is, however, necessary to confirm the physiological action of butyrate in fat absorption in vivo and to evaluate its effect on other determinants of lipoprotein synthesis and transport by Caco-2 cells. Further work is, however, necessary to confirm the physiological action of butyrate in fat absorption in vivo and to evaluate its actual contribution to the reported cholesterol-lowering effects of complex carbohydrates.

We are grateful to D. St-Cyr for secretarial assistance. This study was supported by grants from the Canadian Institutes of Health Research, Crohn's and Colitis Foundation of Canada, Natural Sciences Eng. Research Council of Canada, and the Dairy Farmers of Canada.

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