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

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Butyrate, an end product of dietary fermentation, may influence lipid metabolism in the gut by an as yet undefined mechanism. To determine whether butyrate modulates lipid synthesis and apolipoprotein biogenesis in the human intestinal cell line Caco-2, we studied the effect of butyrate (0.5 mM) on lipid metabolism over a 30-day period. We also investigated the effect of butyrate on the regulation of gene expression involved in lipid metabolism. Surprisingly, butyrate significantly decreased (P < 0.01) the synthesis of [14C]oleic acid by Caco-2 cells, suggesting a potential regulatory action of butyrate on intestinal lipid metabolism. However, butyrate did not alter the synthesis of [35S]methionine-labeled apolipoprotein B-48 and A-I, two major cholesterol carriers. A [14C]oleic acid pulse in permeabilized Caco-2 cells revealed that butyrate inhibited butyryl-CoA:acyl carrier protein O-acyltransferase, the enzyme responsible for the synthesis of cholesteryl ester. These results indicate that butyrate may affect the absorption of dietary lipid and contribute to the hypocholesterolemic action of dietary fiber. In addition, butyrate may have a potential effect on enteral lipid absorption. Further studies are needed to elucidate the underlying mechanisms.
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).

Measurement of lipid synthesis and secretion. Caco-2 cell 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 unlabelled 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 trasyol, all at a final concentration of 1 mM). An aliquot was taken for lipid extraction by standard methods (27) in the presence of unlabelled 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/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 dpm per milligram of cell protein. Lipids secreted in the basolateral compartment were analyzed and quantified, as above, after centrifugation (2,000 rpm, 30 min, 4°C) to remove cell debris.

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 was harvested, and the plasma was exhaustively dialyzed at 4°C against 150 mM NaCl and 0.001 M EDTA, pH 7.0

RESULTS

Caco-2 cell integrity. The effect of butyrate on monolayer integrity and paracellular permeability was examined. As assessed by lactate dehydrogenase and...
Measurement of lipid esterification with [14C]oleic acid. To elucidate the role of butyrate in lipid esterification and delivery, Caco-2 cells were cultured for 20 h with [14C]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.

Apolipoprotein biogenesis. The subsequent step was to examine the modulation of apolipoprotein production by butyrate. For this purpose, Caco-2 cells were cultured with \([35S]\)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 \([35S]\)methionine-labeled apo A-IV.

Lipoprotein secretion. To determine whether butyrate is able to regulate lipid transport, Caco-2 cells were incubated with [14C]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>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Butyrate</th>
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</thead>
<tbody>
<tr>
<td>Transepithelial resistance, (\Omega)-cm(^2)</td>
<td>1,211 \pm 63</td>
<td>1,118 \pm 44</td>
</tr>
<tr>
<td>Lactic dehydrogenase, U/l</td>
<td>159 \pm 13</td>
<td>163 \pm 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.

Fig. 1. Effect of butyrate (hatched bars) on lipid esterification. Differentiated Caco-2 cells were incubated with [14C]oleic acid in the presence of 20 mM butyrate for 20 h at 37°C. Lipids from cell homogenates (A) and basolateral media (B) were extracted with chloroform-methanol (2:1), separated by TLC, and quantitated, as described in MATERIALS AND METHODS. Results are expressed as dpm/mg cell protein, and values represent means \pm SE of 5 experiments. \(* P < 0.03; ** P < 0.02; *** P < 0.01; **** P < 0.003\). Filled bars, control.
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 modifier 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 particularly 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

### Table 2. Lipid composition of cell homogenates and media

<table>
<thead>
<tr>
<th></th>
<th>PL, %</th>
<th>FC, %</th>
<th>DG, %</th>
<th>TG, %</th>
<th>CE, %</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Homogenates</strong></td>
<td></td>
<td></td>
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<tr>
<td>Control</td>
<td>24.52 ± 1.15</td>
<td>0.35 ± 0.03</td>
<td>0.35 ± 0.02</td>
<td>76.43 ± 2.41</td>
<td>0.95 ± 0.04</td>
</tr>
<tr>
<td>Butyrate</td>
<td>18.37 ± 1.04†</td>
<td>0.29 ± 0.02</td>
<td>0.27 ± 0.02†</td>
<td>79.89 ± 1.04</td>
<td>0.94 ± 0.06</td>
</tr>
<tr>
<td><strong>Media</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Control</td>
<td>11.20 ± 0.66</td>
<td>3.13 ± 0.12</td>
<td>2.90 ± 0.09</td>
<td>75.60 ± 0.70</td>
<td>3.20 ± 0.07</td>
</tr>
<tr>
<td>Butyrate</td>
<td>11.00 ± 0.70</td>
<td>4.00 ± 0.09†</td>
<td>3.70 ± 0.19†</td>
<td>72.70 ± 0.88*</td>
<td>4.10 ± 0.06†</td>
</tr>
</tbody>
</table>

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.
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 in the physiological action of butyrate, since it may impair the production of lipoproteins. Furthermore, butyrate may affect microvillar brush border 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 (Fig. 5).

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 actual contribution to the reported cholesterol-lowering effects of complex carbohydrates.

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