Butyrate stimulates the secretion of apolipoprotein (apo) A-I and apo B100 by the human hepatoma cell line Hep G2

Induction of apo A-I mRNA with no change of apo B100 mRNA

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Addition of sodium butyrate to the culture medium of the human hepatoma cell line Hep G2 resulted in a time- and dose-dependent increase in the secretion of apolipoprotein A-I (apo A-I) and apolipoprotein B100 (apo B100). After a 24 h preincubation period, a 2.4- and 2.2-fold increase in the secretion of apo A-I and apo B100 respectively was obtained during the next 24 h in the presence of 2 mM-sodium butyrate. Secretion of albumin, fibrinogen or [³⁵S]methionine-labelled newly synthesized proteins was unaffected or only marginally affected, indicating that the effect of butyrate on apo A-I and apo B100 is not part of a general effect on protein synthesis and secretion. In structure-function studies, butyrate was found to be the most potent inducer among various straight-chain carboxylic acids. Hydroxylated, aminated and otherwise modified butyrate derivatives were inactive. The enhanced accumulation of apo A-I and apo B100 in the culture medium could not be explained by changes in the uptake and degradation of the synthesized apolipoproteins or by alterations in the secretion of possible intracellular pools. In addition, [³⁵S]methionine incorporation studies indicated that synthesis and/or secretion of newly synthesized apo A-I and apo B100 is enhanced in the presence of butyrate. The apo A-I mRNA level was increased 2.3-fold upon treatment with 2 mM-butyrate for 48 h, suggesting regulation at (post-)transcriptional level. In contrast, no change in the level of apo B100 mRNA in butyrate-treated cells was observed, indicating regulation at translational or co- or post-translational level. We propose that the effect of butyrate on the secretion of apo A-I and apo B100 by Hep G2 results from two different regulatory mechanisms.

INTRODUCTION

Plasma levels of low-density lipoprotein (LDL) cholesterol and high-density lipoprotein (HDL) cholesterol are, respectively, positively and negatively related to the development of atherosclerosis [1–3]. The proteins which are part of these lipoproteins, i.e. the apolipoproteins, are also used in risk assessment for cardiovascular disease. The plasma levels of apolipoprotein B100 (apo B100) and apolipoprotein A-I (apo A-I) have been reported to be even more discriminatory in determining the risk of developing coronary heart disease than the cholesterol concentration in the lipoprotein from which they are a constituent, i.e. LDL and HDL [4–6].

A vast number of reports describe regulation of the synthesis and secretion of apo A-I and apo B100, as a way of altering the amounts and composition of lipoproteins in the blood. Since the liver is the central organ in the metabolism of these two apolipoproteins, many of the studies were done with perfused livers and primary cultures of hepatocytes from animal origin, owing to the limited availability of human liver. More recently, the human hepatoma cell line Hep G2 has been used as a model to study the apolipoprotein and lipoprotein synthesis and catabolism in human hepatocytes. This cell line has been shown to synthesize a number of apolipoproteins, including apo A-I and apo B100 [7]. Apo A-I is secreted as a HDL-like particle, but is also found in a lipid-poor or lipid-free form, whereas apo B100 is secreted as a constituent of a LDL-like particle enriched in triacylglycerol [8]. In this respect, secretion of apo B100 differs from the situation *in vivo*, possibly as a result of the decreased amount of smooth endoplasmic reticulum in Hep G2 cells [8], which may lead to a defective lipoprotein assembly and secretion in these cells [9]. Furthermore, the availability of fatty acids may

be of importance [10]. Nevertheless, the Hep G2 cell line was found to be a suitable model for investigating the regulation of the synthesis and/or secretion of apo A-I and apo B100 in response to lipoproteins [11,12], fatty acids [9,10,13–15], insulin [9,13,14] and steroid hormones [16].

In many of these reports, supply of lipids and changes in the assembly of lipoproteins are thought to be responsible for the alterations in the synthesis and secretion of apo A-I and apo B100. In a search for compounds which may affect the synthesis and/or secretion of these two apolipoproteins by another regulatory mechanism, we investigated the effect of butyrate. Butyrate is known to induce specific alterations in different cell types, including hepatoma-cell lines and hepatocytes (for a review see Kruh [17]). One of the most evident changes brought about is the hyperacetylation of histones [18], owing to inhibition of the enzyme histone deacetylase [19]. This process has been shown to affect gene expression [20–23].

In this paper we describe the effects of butyrate on apo A-I and apo B100 secretion by Hep G2 cells. The secretion of these proteins into the medium was found to be enhanced more than 2-fold upon incubation with butyrate. This increase was accompanied by a comparable stimulation of the apo A-I mRNA, without a change in the apo B100 mRNA level.

MATERIALS AND METHODS

Materials

[α -³²P]dCTP (3000 Ci/mmol), [³⁵S]methionine (> 1000 Ci/mmol) and Na¹²⁵I (100 mCi/ml) were obtained from Amersham International (Amersham, Bucks., U.K.). Sodium butyrate and butyrate analogues (as listed in Table 1 in the Results section) were purchased from Sigma Chemical Co. (St. Louis, MO,

Abbreviations used: LDL, low-density lipoprotein; HDL, high-density lipoprotein; apo, apolipoprotein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DMEM, Dulbecco's modified Eagle's medium; FBS, foetal-bovine serum.

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U.S.A.), except for sodium acetate which was purchased from Merck (Darmstadt, Germany). Dulbecco's modified Eagle's medium (DMEM) was purchased from Flow Laboratories (Irvine, Scotland, U.K.), and foetal-bovine serum (FBS), penicillin and streptomycin were from Boehringer Mannheim (Mannheim, Germany).

Cell culture

The established Hep G2 cell line, derived from a human liver tumour, was obtained from Dr. B. B. Knowles (Wistar Institute of Anatomy and Biology, Philadelphia, PA, U.S.A.). The cells were cultured as described [24] at 37 °C, under air/CO₂ (19:1), in 25 cm² flasks containing 0.1–0.2 ml of culture medium/cm². Standard medium used was DMEM supplemented with 20 mM-Hepes, 10 mM-NaHCO₃, 10% (v/v) FBS (heat-inactivated for 30 min at 56 °C), 2 mM-glutamine, penicillin (100 units/ml) and streptomycin (100 µg/ml). The medium was renewed twice a week. At 4–5 days before the start of the experiment, the cells were treated with trypsin and transferred to six-well-cluster plates (10 cm²/well; Costar, Cambridge, MA, U.S.A.) with a split ratio of 1:6, and were grown to confluency before starting the experiments, in order to study the effect of butyrate on protein synthesis, other than due to arrest of cell growth.

Except where otherwise described, the effect of sodium butyrate and related analogues was studied over a 24 h period, after a 24 h preincubation with the same compound at the same concentration. At the end of the incubation period, medium was collected and centrifuged for 1–2 min in an Eppendorf centrifuge (type 5414) to remove detached cells and debris. The supernatant was frozen immediately in liquid nitrogen and stored at –20 °C, until measurement of apo A-I and apo B100 concentrations. Cells were washed three times with cold phosphate-buffered saline (sodium/potassium phosphate buffer; 11 mM, pH 7.5) containing 150 mM-NaCl and were harvested by scraping in water. The cellular protein was determined as described by Lowry *et al.* [25].

E.l.i.s.a. of apo A-I

Specific IgGs against human apo A-I were isolated from goat antiserum (raised in our Institute) by affinity chromatography using purified apo A-I cross-linked to Sepharose 4B (Pharmacia), as described by Bury & Rosseneu [26]. Part of the isolated anti-(apo A-I) IgG was conjugated to horseradish peroxidase (EC 1.11.1.7) by the SPDP [*N*-succinimidyl 3-(2-pyridyldithio)propionate] method, essentially according to the Pharmacia procedure. Immunoblotting of human serum and FBS after SDS/PAGE showed that anti-(apo A-I) IgG was monospecific for human apo A-I.

The isolated anti-(apo A-I) and the peroxidase-anti-(apo A-I) conjugate were used in a 'sandwich' e.l.i.s.a., in accordance with the procedure described by Bury & Rosseneu [26], with a few modifications. In short, polystyrene micro-titre plates ('Nunc Immuno' plates, 'high-binding' quality; Intermed, Roskilde, Denmark) were coated with anti-(apo A-I) IgG (10 µg/ml in phosphate-buffered saline) containing NaN₃ (1 g/l) (120 µl/well). Blocking of the residual binding sites was done with 0.1% (w/v) casein in phosphate-buffered saline (150 µl/well). Samples for apo A-I determination were diluted in the same buffer as used for blocking (100 µl of diluted sample added per well). Peroxidase-anti-(apo A-I) conjugate was diluted in blocking buffer containing 0.05% (w/v) Tween 20 (100 µl of diluted conjugate added per well). The final IgG concentration of the peroxidase-anti-(apo A-I) conjugate used in the assay was 25 ng/ml. The peroxidase-labelled conjugate was detected by using 3,3',5,5'-tetramethylbenzidine and H₂O₂ as the substrate mixture [27].

The working range of the e.l.i.s.a. is 20–150 ng/ml and as little as 10 ng of apo A-I/ml can be detected. The standard curves for purified apo A-I, apo A-I in human serum, and apo A-I in the medium of Hep G2 cell cultures were parallel. Apo A-I concentrations in sera measured by the e.l.i.s.a. were identical with values found with radial immunodiffusion [28].

E.l.i.s.a. of apo B100

Specific IgGs against human apo B100 were isolated from rabbit antiserum (raised in our Institute) by affinity chromatography, exactly as described above for the purification of anti-(apo A-I) IgG, by using purified apo E-free LDL cross-linked to Sepharose 4B (Pharmacia). LDL was purified from human serum by density-gradient ultracentrifugation as described by Redgrave *et al.* [29]. The LDL was aspirated from the gradient between densities of 1.030 and 1.050 g/ml, adjusted with KBr to a density of 1.21 g/ml, layered under a KBr solution in water, of the same density, and ultracentrifuged to purify further and to concentrate LDL. SDS/PAGE of the LDL showed that apo B100 was the only protein present in the isolated LDL, as determined by silver staining as described by Paleologue *et al.* [30]. SDS/PAGE was performed under reducing conditions by the method of Laemmli [31], with resolving gels containing a gradient of 4–20% (w/v) acrylamide and stacking gels of 3.5% (w/v) acrylamide. Immunoblotting of human serum and FBS, after SDS/PAGE, showed that the isolated antibodies were monospecific for human apo B.

Part of the isolated anti-(apo B) IgG was conjugated to horseradish peroxidase and used together with anti-(apo B) in a 'sandwich' e.l.i.s.a. The e.l.i.s.a. procedure was similar to that for apo A-I, except that the final IgG concentration in the peroxidase-anti-(apo B) conjugate was 50 ng/ml. The working range of the assay was 100–1000 ng of apo B/ml, and as little as 50 ng/ml could be detected. The standard curves for LDL (isolated from human serum by density-gradient ultracentrifugation), apo B in human serum, and apo B in medium of Hep G2 cells were parallel. Apo B concentrations measured in sera by the e.l.i.s.a. showed a linear correlation with values found with radial immunodiffusion [32].

Association and degradation of apo B100 (LDL) and apo A-I (HDL)

Association and degradation of HDL and LDL by Hep G2 cells were measured as described by Dashti *et al.* [33] and Goldstein *et al.* [34] respectively, over a 24 h period. The concentration of ¹²⁵I-labelled apo B100 (in LDL) and apo A-I (in HDL) added to the medium were 2.0 and 2.5 µg/mg of cell protein respectively, as measured by the e.l.i.s.a. procedure. These concentrations are comparable with the amounts of apo A-I and apo B100 secreted by Hep G2 cells during a 24 h incubation in control medium.

Measurement of the secretion of albumin and fibrinogen

Accumulation of albumin in the medium of Hep G2 cell cultures was measured by rocket immunoelectrophoresis by the method described by Laurell [35] by using rabbit anti-(human albumin) antiserum (Dakopatts, Glostrup, Denmark).

Accumulation of fibrinogen in the medium was measured by an e.l.i.s.a. procedure [36]. Degradation of fibrinogen was prevented by adding trasylol and heparin to the medium, both at a concentration of 10 units/ml. This addition did not affect basal or butyrate-stimulated secretion of apo A-I and apo B100 in the culture medium.

Protein synthesis

Overall secretion of newly synthesized proteins was measured essentially as described by Kooistra *et al.* [37]. The metabolically radiolabelled proteins secreted in the medium were analysed by SDS/PAGE under reducing conditions as described above.

Protein molecular-mass standards (Bio-Rad, Richmond, CA, U.S.A.) were used for calibration of the gel. For autoradiography the gel was treated with an autoradiography enhancer (Enhance; NEN DuPont, Boston, MA, U.S.A.) in accordance with the manufacturers' instructions, dried and placed on X-ray film (Kodak X-Omat AR films; Eastman-Kodak Co., Rochester, NY, U.S.A.), and stored at -80°C for the appropriate time.

RNA hybridization

Total RNA was isolated from Hep G2 cells by the method of Chomczynski & Sacchi [38]. After washing the RNA pellets with 70% (v/v) ethanol, RNA samples were dissolved in water. The RNA concentration in each sample was determined spectrophotometrically, assuming that one A_{260} unit is 40 μg of RNA/ml.

Equal amounts of total RNA from different incubations were fractionated by electrophoresis in a 0.8% (w/v) agarose gel containing 1 M-formaldehyde, and transferred to Hybond N (Amersham) in accordance with the manufacturer's instructions. RNA blots were hybridized with different probes at 65°C in a sodium phosphate buffer (0.5 M, pH 7.5), containing 7% (w/v) SDS and 1 mM-EDTA. DNA fragments used as probes were isolated from low-melting-point agarose [39]. One blot was hybridized with 25 ng of probe, labelled by the random-primer method (Multi-prime, Amersham) to approx. 2×10^8 – 10^9 c.p.m./ μg of DNA.

After hybridization, the blots were washed twice with $2 \times \text{SSC}/0.1\%$ SDS (30 min at 65°C) ($1 \times \text{SSC} = 0.15$ M-NaCl/0.015 M-sodium citrate, pH 7.0), twice with $1 \times \text{SSC}/0.1\%$ SDS (30 min at 65°C), and twice with $0.1 \times \text{SSC}/0.1\%$ SDS (30 min at 65°C), successively. The blots were exposed to Hyperfilm MP (Amersham), together with an intensifying screen (Eastman-Kodak Co.), for 15–75 h at -80°C . For quantification of the relative amounts of mRNA, the autoradiographs were scanned with a Shimadzu CS 910 chromatograph scanner,

and areas under the peaks were integrated and plotted with the aid of a data processor (Shimadzu Corp., Kyoto, Japan). The mRNA levels were quantified by using three different amounts of total RNA loaded on the gel, giving a linear relation between the specific mRNA signal and the amount of RNA applied. For apo A-I and albumin mRNA, linearity was present with 1.5, 3 and 6 μg of total RNA. For apo B100 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA there was linearity with 3, 6 and 12 μg of total RNA.

The following DNA fragments were used as probes in the hybridization experiments: a 2.2 kb *Pst*I fragment of the human apo A-I genomic DNA (kindly provided by Dr. S. E. Humphries [40]), a 2.7 kb *Hind*III fragment located at the 3' end of human apo B100 cDNA (kindly provided by Dr. J. Scott [41]), a 740 bp *Hind*III/*Pst*I fragment located at the 3' end of human serum albumin cDNA, and a 1.2 kb *Pst*I fragment of rat GAPDH cDNA (kindly provided by Dr. R. Offringa [42]). The human serum albumin clone was obtained from Dr. H. Pannekoek, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands.

Statistical analysis

Statistical significance of differences was calculated by Student's *t* test for paired data with the level of significance selected as $P < 0.05$. Values are expressed as means $\pm$ S.D.

RESULTS

Time course and dose-dependency of the effect of butyrate on the accumulation of apo A-I and apo B100 in the cellular medium of HepG2 cell cultures

In Fig. 1 a typical experiment is shown of the time course of the butyrate effect on apo A-I and apo B100 secretion by Hep G2 cells. With control medium, apo A-I and apo B100 increased linearly with time over a 48 h period. With 2 mM-sodium butyrate present in the medium, an accelerated accumulation of apo A-I and apo B100 was observed after a lag time of 16–24 h. In two additional time-course experiments an identical lag time was seen for both apo A-I and apo B100 (results not shown). Therefore,

Fig. 1. Time course of the effect of butyrate on apo A-I (a) and apo B100 (b) accumulation in the medium of Hep G2 cells

Hep G2 cells were cultured for 48 h on standard medium without (○) and with (●) sodium butyrate (2 mM). At various times, samples of medium were taken and analysed for apo A-I and apo B100. Apo A-I and apo B100 concentrations in the culture medium were measured as described in the Materials and methods section. Data are from one representative of three independent experiments. The values are means $\pm$ range of triplicate assays of two separate wells.

Fig. 2. Effect of butyrate on the secretion of apo A-I and apo B100 in the medium of Hep G2 cell cultures

Hep G2 cells were incubated for 24 h with various concentrations of sodium butyrate, after a 24 h preincubation with the same concentration of sodium butyrate. Apo A-I and apo B100 concentrations in the culture medium were measured as described in the Materials and methods section. Results were normalized for cell protein in culture dishes and are expressed as a percentage of control, and are means $\pm$ s.d. for three to five independent experiments. Apo A-I ($\bullet$) and apo B100 ($\circ$) production by Hep G2 in control medium was 2.9 ± 1.2 and 1.9 ± 0.3 $\mu\text{g}/24$ h per mg of cell protein respectively (means $\pm$ s.d. for five experiments): * indicates a significant difference ($P < 0.05$) between control and treated cells.

in subsequent experiments the effect of butyrate on Hep G2 cell cultures was studied over a 24 h period, after a 24 h preincubation period in the presence of the same butyrate concentration.

As shown in Fig. 2, addition of increasing amounts of butyrate resulted in an increase in the secretion of apo A-I and apo B100 in the medium of Hep G2 cell cultures. The effects on apo A-I and apo B100 were comparable in magnitude for the various butyrate concentrations. A significant increase was only found with concentrations of 1 mM-sodium butyrate or higher (paired t test, $P < 0.05$). Judging from the changes in cell morphology and loss of cells from the culture dishes, butyrate concentrations ≥ 3 mM were cytotoxic to Hep G2 cell cultures. Therefore, further experiments were performed at a concentration of 2 mM-butyrate. At this concentration no adverse effect was observed on the cell morphology, on the cell protein present in the culture dishes at the end of the incubation, or on the release of the cytoplasmic enzyme lactate dehydrogenase.

Butyrate stimulates the secretion of newly synthesized apo A-I and apo B100

Since Hep G2 cells are capable of binding and internalizing LDL [43] and HDL [33], inhibition of the uptake and degradation might have caused the observed changes in the accumulation of apo B100 and apo A-I in the culture medium. The results listed in Table 1 for the accumulation and for the binding and degradation of apo B100 and apo A-I show that uptake and degradation is far smaller than the accumulation in the medium of both apolipoproteins over a 24 h period. Addition of butyrate (2 mM) to the medium resulted in decreased binding and degradation of apo A-I and apo B100, but this does not explain the increases found in the accumulation.

In addition, changes in the secretion of possible intracellular storage pools of apo A-I and apo B100 can also not explain the

Table 1. Effect of butyrate on the secretion and on the association and degradation of apo B100 (LDL) and apo A-I (HDL) by Hep G2 cells

The secretion, binding and degradation of apo A-I and apo B100 were measured over a 24 h period in the absence or presence of 2 mM-sodium butyrate after a 24 h preincubation on the same medium. In binding and degradation experiments 2.0 and 2.5 μg of apo B100 (in LDL) or apo A-I (in HDL)/mg of cell protein, respectively, were added to the medium of Hep G2 cells. * Means $\pm$ s.d. for five experiments; † means $\pm$ s.d. for one experiment performed in quadruplicate.

	DMEM/FBS	DMEM/FBS + 2 mM- butyrate
Apo B100 secretion* (μg of apo B100/24 h per mg of cell protein)	1.9 ± 0.3	4.2 ± 0.7
^{125}I -apo B100 (LDL) cell-associated† (μg of apo B100/ mg of cell protein)	0.17 ± 0.01	0.10 ± 0.01
^{125}I -apo B100 (LDL) degradation† (μg of apo B100/24 h per mg of cell protein)	0.24 ± 0.01	0.16 ± 0.01
Apo A-I secretion* (μg of apo A-I/24 h per mg of cell protein)	2.9 ± 1.2	7.0 ± 1.3
^{125}I -apo A-I (HDL) cell-associated† (μg of apo A-I/ mg of cell protein)	0.019 ± 0.001	0.016 ± 0.001
^{125}I -apo A-I (HDL) degradation† (μg of apo A-I/24 h per mg of cell protein)	0.007 ± 0.003	0.004 ± 0.001

Table 2. Effect of butyrate on the secretion by Hep G2 cells of albumin, fibrinogen and [^{35}S]methionine-labelled proteins

Hep G2 cells were incubated for 24 h in the absence or presence of 2 mM-sodium butyrate after a 24 h preincubation in the same medium. Results are means $\pm$ range of two independent experiments, performed in duplicate.

	DMEM/FBS	DMEM/FBS + 2 mM-butyrate
Albumin secretion ($\mu\text{g}/24$ h per mg of cell protein)	16.9 ± 1.3	13.2 ± 0.2
Fibrinogen secretion ($\mu\text{g}/24$ h per mg of cell protein)	0.64 ± 0.03	0.77 ± 0.09
[^{35}S]Methionine incorporation in secreted proteins (% of incubation in standard medium)	100 %	92 ± 7 %

differences in accumulation caused by butyrate. After a 24 h incubation of Hep G2 cells in standard medium, the apo A-I and apo B100 concentrations were measured in the culture medium and in the cells. The cells were solubilized in phosphate-buffered saline containing 0.5 % Triton X-100. Apo A-I and apo B100 were measured by the c.l.i.s.a. procedure (addition of Triton X-100 had no effect on the c.l.i.s.a. results). For apo A-I and apo B100 we found that 95 % and 85–90 %, respectively, of the total amount of the apolipoproteins was present in the medium. These experiments indicate that the effect of butyrate on the accumulation of apo A-I and apo B100 is caused by increases in the secretion of newly synthesized apolipoproteins.

Fig. 3. SDS/PAGE and autoradiography of metabolically radiolabelled proteins in the medium of Hep G2 cell cultures

Hep G2 cells were incubated for 24 h in standard medium with or without 2 mM-sodium butyrate, containing 50 μ Ci of [35 S]methionine/ml, after a 24 h preincubation in the same medium without labelled methionine. Molecular-mass standards (kDa) are indicated on the right of the autoradiograph. At the left side of the autoradiograph the positions of apo A-I and apo B100 and of other proteins with altered intensity after butyrate treatment are indicated. + or - indicates incubation with or without butyrate respectively.

Specificity of the stimulation of apo A-I and apo B100 secretion by butyrate

To see whether the effect of butyrate on the secretion of apo A-I and apo B100 is part of a general effect of butyrate on Hep G2 cell cultures, secretion of albumin and fibrinogen in the culture medium was measured. The results are shown in Table 2. Only minor differences were found for the secretion of albumin and fibrinogen upon incubation of cells in standard medium with 2 mM-butyrate.

In addition to the effect of butyrate on the albumin and fibrinogen secretion, the total protein secretion by Hep G2 cell cultures was studied by using [35 S]methionine incorporation in newly synthesized and secreted proteins. Little effect was observed on the secretion of total newly synthesized proteins (see Table 2). To explore the possibility that butyrate may alter the pattern of the proteins secreted into the culture medium, the medium of the experiment described above was separated by SDS/PAGE and autoradiographed (Fig. 3). Hep G2 cells incubated with butyrate secreted a similar pattern of proteins in the culture medium as in control cells, except for a few proteins (indicated by the arrows in Fig. 3) which showed an altered intensity. After specific immunoprecipitation, the proteins with molecular masses of approx. 500 and 28.5 kDa were identified as apo B100 and apo A-I respectively.

Structural specificity studies

Table 3 shows the effects of various butyrate analogues, all tested at a concentration of 2 mM, on the secretion of apo A-I

Table 3. Structural specificity of butyrate

Hep G2 cells were incubated with different butyrate analogues (2 mM) for 24 h, after a 24 h preincubation with the same compound at the same concentration. The concentrations of apo A-I and apo B100 in the medium were measured as described in the Materials and methods section and normalized for the amount of cell protein. Results are expressed as percentages of control, and are means $\pm$ range for two experiments; for butyrate, valerate and propionate results are means $\pm$ S.D. for five experiments. Compounds indicated by * were dissolved in dimethyl sulphoxide (DMSO) giving a final concentration of DMSO in the medium of 0.1% (v/v), and values with this are expressed as a percentage of those for control medium containing 0.1% DMSO. Apo A-I and apo B100 secretion by Hep G2 cells in control medium was 2.9 ± 1.2 and 1.9 ± 0.3 μ g/24 h per mg of cell protein respectively (means $\pm$ S.D. for five experiments). † indicates a significant difference ($P < 0.05$) between control and treated cells.

Compound added to the medium	Secretion (% of control)	
	apo A-I	apo B100
Acetate	101 $\pm$ 10	102 $\pm$ 13
Propionate	128 $\pm$ 13†	131 $\pm$ 22†
Butyrate	239 $\pm$ 45†	218 $\pm$ 33†
Valerate*	173 $\pm$ 23†	181 $\pm$ 47†
Hexanoate*	129 $\pm$ 26	124 $\pm$ 1
2-Hydroxybutyrate	101 $\pm$ 11	105 $\pm$ 11
3-Hydroxybutyrate	114 $\pm$ 12	106 $\pm$ 14
4-Hydroxybutyrate	120 $\pm$ 18	132 $\pm$ 18
2-Aminobutyrate	103 $\pm$ 10	94 $\pm$ 3
3-Aminobutyrate	98 $\pm$ 8	83 $\pm$ 9
4-Aminobutyrate	107 $\pm$ 1	89 $\pm$ 9
Isobutyrate*	116 $\pm$ 26	125 $\pm$ 14
Crotonate*	106 $\pm$ 15	126 $\pm$ 20
Butanol	128 $\pm$ 29	123 $\pm$ 3
Butyrolactone*	110 $\pm$ 25	108 $\pm$ 6
2-Oxobutyrate	125 $\pm$ 5	128 $\pm$ 36
Succinate	98 $\pm$ 12	115 $\pm$ 15

and apo B100 in the medium of Hep G2 cell cultures. The results for the various butyrate analogues used were comparable for apo A-I and apo B100. Butyrate was the most potent inducer. Other straight-chain unsubstituted carboxylic acids, propionate (C_3) and valerate (C_5), also gave a significantly increased secretion of both apolipoproteins. They were, however, less effective than butyrate. The straight-chain carboxylic acids acetate (C_2) and hexanoate (C_6) were either ineffective or only marginally effective. No effects were found with hydroxylated or aminated butyrate derivatives, isobutyrate (the branched-chain analogue of butyrate), crotonate (which contains a double bond between the C-2 and C-3 as compared with butyrate), butanol, γ -butyrolactone (intramolecular lactone), 2-oxobutyrate and succinate, all differently oxidized C_4 analogues.

Effect of butyrate on apo A-I and apo B100 mRNA levels

To observe whether the accelerated secretion of apo A-I and apo B100 results from changes in mRNA levels, the effect of butyrate on the mRNA level of both apolipoproteins was assessed by Northern-blot hybridization (Fig. 4). Apo A-I mRNA levels increased with time, as compared with the albumin and GAPDH mRNA levels, which were not affected. A significant increase in the apo A-I mRNA level was seen when Hep G2 cells were treated with 2 mM-sodium butyrate for 16 h, and reached maximally 2.3-fold stimulation after 48 h. In contrast with the apo A-I mRNA level, the apo B100 mRNA level was not significantly affected by butyrate during the 48 h incubation period.

Fig. 4. Effect of butyrate on apo A-I and apo B100 mRNA levels in Hep G2 cells

(a) Autoradiograph of a Northern blot hybridized with ^{32}P -labelled probes for apo A-I, apo B100, albumin and GAPDH mRNA in a single-step hybridization, with $3\text{ }\mu\text{g}$ of total RNA applied to the agarose/formaldehyde gel. The hybridized blot was exposed for 16 h, at -80°C , to Hyperfilm, by using an intensifying screen. Total RNA was isolated from Hep G2 cultured for various times (indicated on top of the autoradiograph) in culture medium containing 2 mM-sodium butyrate. (b) Time course of the butyrate effect on apo A-I (●) and apo B100 (○) mRNAs. The intensity of the bands on autoradiographs (as shown, for example, in a) was assessed by densitometric scanning. Albumin was used as an internal standard to correct for differences in the amount of total RNA applied to the gel. The results are expressed as percentages of those in the incubation without butyrate and are means $\pm$ S.D. for three independent experiments, with analysis of three RNA concentrations in each experiment. * indicates a significant difference ($P < 0.05$) between control and treated cells. Similar results were found when corrected for the GAPDH mRNA signal (results not shown).

DISCUSSION

In this paper we show that butyrate increases the secretion of apo A-I and apo B100 by the human hepatoma cell line Hep G2, in a time- and dose-dependent way. This is not due to a decreased uptake and degradation of HDL (apo A-I) and LDL (apo B100) in the presence of butyrate, nor to differences in the secretion from possible intracellular pools of apo A-I and apo B100. It indicates that butyrate stimulates the synthesis and/or the secretion of newly synthesized apolipoproteins. This was confirmed by the [^{35}S]methionine incorporation experiment (Fig. 3).

As illustrated by the secretion of albumin, fibrinogen and [^{35}S]methionine-labelled proteins, the effect of butyrate on apo A-I and apo B100 was not part of a general effect on proteins secreted by Hep G2 cells. In addition, the stimulatory effect was specific for butyrate. Only propionate and valerate, the C_3 and C_5 straight-chain carboxylic acids respectively, induced a significant increase in the secretion of apo A-I and apo B100, although less markedly than butyrate. Similar quantitative differences were found with butyrate and a limited set of other straight-chain carboxylic acids tested on various functions in different cell types [17]. Kooistra *et al.* [37] studied a large set of butyrate analogues for their effect on the tissue plasminogen activator ('t-PA') synthesis by cultured human endothelial cells. The stimulatory effect on this synthesis was in the order butyrate > valerate > propionate. Our results on structure-function relationship are comparable with the reports mentioned above, suggesting that a common mechanism may be responsible for the effects described.

Such a mechanism may be acetylation and deacetylation of histones, which play a role in the regulation of the expression of specific genes by introduction of changes in the chromatin structure. Butyrate treatment of HeLa cells and Friend erythroleukaemia cells [18] resulted in a hyperacetylation of the histones. Similar findings were described for a rat hepatoma cell line

(HTC) by Sealy & Chalkley [19]. These authors showed that this hyperacetylation was due to inhibition of the enzyme histone deacetylase by butyrate. An effect of butyrate on gene expression may also be an explanation for the enhanced secretion of apo A-I by Hep G2 cells.

Another possibility may be interference by butyrate with lipid synthesis and secretion. It has been reported that butyrate stimulates synthesis of fatty acids and triacylglycerols in rat hepatocytes [44,45] and hepatoma cells [46], with only a marginal effect on sterol synthesis [44,45]. Supply of triacylglycerols or fatty acids leading to an increased triacylglycerol synthesis did, however, not affect apo A-I secretion [13,47,48] by hepatocytes or the apo A-I mRNA level [14] in Hep G2 cells. Furthermore, apo A-I secretion was variably affected by cholesterol-rich liposomes and only slightly increased upon incubation with β -VLDL [11], indicating that the supply of cholesterol has no or little effect on apo A-I secretion by Hep G2 cells. This is supported by the absence of an effect of the 3-hydroxy-3-methylglutaryl-CoA reductase inhibitor CS-514 on apo A-I secretion [49] and the increase of only 20% in the apo A-I mRNA level during supplementation of the medium with LDL [12]. In our experiments with butyrate we found a 2.3-fold increase in the apo A-I mRNA level after 48 h, which is comparable with the 2.4-fold increase in the secretion. In addition, the accelerated accumulation of apo A-I, as shown in Fig. 1, is in agreement with the time course of the effect found on the mRNA level (Fig. 4b). These findings, together with the above-mentioned observations, do not favour the hypothesis that butyrate stimulates apo A-I secretion via a possibly altered lipid secretion, and suggest an effect of butyrate on gene expression. However, an increase in the stability of the apo A-I mRNA cannot be excluded.

Although the overall effects of butyrate and butyrate analogues on the secretion of apo A-I and apo B100 were similar, this need

not result from the same regulatory mechanism. In contrast with apo A-I, butyrate did not affect the apo B100 mRNA level. Synthesis of apo B100 and/or secretion of newly synthesized apo B100 was increased in butyrate-treated Hep G2 cells. After separating the culture medium, by density centrifugation, into lipoproteins ($d < 1.20$) and a lipid-poor or lipid-free fraction ($d > 1.20$), we found that cells incubated with or without butyrate secreted apo B100 only as a constituent of lipoproteins. Apo A-I was found both in the lipoprotein-rich and in the lipid-poor or lipid-free fraction (results not shown). The latter finding may be explained by dissociation of apo A-I from the native discoidal lipoprotein particle after secretion, owing to a decreased affinity of apo A-I for this particle in the absence of lecithin:cholesterol acyltransferase activity or as the result of direct secretion of lipid-poor apo A-I in the medium itself [8]. The increase in the secretion of apo A-I upon butyrate treatment was seen in both fractions. The observed distribution of apo B100 over lipoproteins and of apo A-I over the lipoprotein-rich and the lipid-poor fraction, secreted by Hep G2 cells, was in accordance with results of Thrift *et al.* [8]. The increased secretion of apo B100 may be regulated translationally (increased synthesis) or by the co- or post-translational processing of apo B100 or assembly of apo B100 into the lipoprotein. This co- or post-translational regulation has been described as important in determining the amount of apo B100 secreted by Hep G2 and hepatocytes [50–53]. Lipid supply, i.e. fatty acid, triacylglycerols and cholesterol, was found to be of particular importance in this process by increasing apo B100 secretion [9,15,54]. Although we did not measure lipid synthesis, the finding that butyrate stimulates fatty acid and triacylglycerol synthesis in rat hepatocytes and hepatoma cells may provide a plausible explanation for the enhanced secretion of apo B100. However, as we show in the structure-specificity studies, propionate also increased the apo B100 secretion significantly, and acetate had no effect. Nishina *et al.* [44] and Wright *et al.* [55] reported that propionate inhibited fatty acid synthesis and had no or an inhibitory effect on sterol synthesis, whereas acetate stimulated fatty acid synthesis and inhibited sterol synthesis [44,56]. These conflicting data suggest that alterations in lipid synthesis need not be the only explanation for the increased apo B100 secretion.

Butyrate is present in human nutrition, e.g. dairy products, but it is unlikely that it is a suitable agent to affect apo A-I and apo B100 secretion by hepatocytes *in vivo*. Plasma concentrations of butyrate remain low in general, because it is metabolized rapidly. However, the experiments with butyrate provide interesting data which may lead to a better understanding of the mechanism which controls the synthesis and secretion of apo A-I and apo B100 by hepatocytes.

We thank Dr. Louis M. Havekes for the antisera against human apo A-I and apo B100, Dr. Willem Nieuwenhuizen for his helpful support in the measurement of fibrinogen, and Mrs. C. Horsting-Been and Miss M. Horsting for typing the manuscript. This work was made possible by financial support from the Netherlands Heart Foundation (Grant 87.035).

REFERENCES

- Lipid Research Clinics Programme: The Lipid Research Clinics Coronary Primary Prevention Trial Results (1984) *J. Am. Med. Assoc.* **251**, 351–364
- Miller, G. J. & Miller, N. E. (1975) *Lancet* **i**, 16–19
- Gordon, T., Castelli, W. P., Hjortland, M. C., Kannel, W. B. & Dawber, T. R. (1977) *Am. J. Med.* **62**, 707–714
- Glueck, C. J., Gartside, P., Fallat, R. W., Sielsk, J. & Steiner, P. M. (1976) *J. Lab. Clin. Med.* **88**, 941–957
- De Backer, G., Rosseneu, M. & Deslypere, J. P. (1982) *Atherosclerosis* **42**, 197–203
- Brunzell, J. D., Sniderman, A. D., Albers, J. J. & Kwiterovich, P. O., Jr. (1984) *Arteriosclerosis* **4**, 79–83
- Zannis, V. I., Breslow, J. L., SanGiacomo, T. R., Aden, D. P. & Knowles, B. B. (1981) *Biochemistry* **20**, 7089–7096
- Thrift, R. N., Forte, T. M., Cahoon, B. E. & Shore, V. G. (1986) *J. Lipid Res.* **27**, 236–250
- Pullinger, C. R., North, J. D., Teng, B.-B., Rifichi, V. A., De Brito, A. E. R. & Scott, J. (1989) *J. Lipid Res.* **30**, 1065–1077
- Ellsworth, J. L., Erickson, S. K. & Cooper, A. D. (1986) *J. Lipid Res.* **27**, 858–874
- Craig, W. Y., Nutik, R. & Cooper, A. D. (1988) *J. Biol. Chem.* **263**, 13880–13890
- Monge, J. C., Hoeg, J. M., Law, S. W. & Brewer, H. B., Jr. (1989) *FEBS Lett.* **243**, 213–217
- Dashti, N. & Wolfbauer, G. (1987) *J. Lipid Res.* **28**, 423–436
- Dashti, N., Williams, D. L. & Alaupovic, P. (1989) *J. Lipid Res.* **29**, 1365–1373
- Moberly, J. B., Cole, T. G., Alpers, D. H. & Schonfeld, G. (1990) *Biochim. Biophys. Acta* **1042**, 70–80
- Tam, S.-P., Archer, T. K. & Deeley, R. G. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 3111–3115
- Kruh, J. (1982) *Mol. Cell. Biol.* **2**, 65–82
- Riggs, M. G., Whittaker, R. G., Neumann, J. R. & Ingram, V. M. (1977) *Nature (London)* **268**, 462–464
- Sealy, L. & Chalkley, R. (1978) *Cell* **14**, 115–121
- Partington, G. A., Yarwood, N. J. & Rutherford, T. R. (1984) *EMBO J.* **3**, 2787–2792
- Philippe, J., Drucker, D. J., Chick, W. L. & Habener, J. L. (1987) *Mol. Cell. Biol.* **7**, 560–563
- Rius, C., Cabañas, C. & Aller, P. (1990) *Exp. Cell Res.* **188**, 129–134
- Tichonicky, L., Kruh, J. & Defer, N. (1990) *Biol. Cell* **69**, 65–67
- Havekes, L. M., De Wit, E. C. M. & Princen, H. M. G. (1987) *Biochem. J.* **247**, 739–746
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Bury, J. & Rosseneu, M. (1985) *Clin. Chem.* **31**, 247–251
- Bos, E. S., Van der Doelen, A. A., Van Rooij, N. & Schuur, A. H. W. M. (1981) *J. Immunoassay* **2**, 187–204
- Albers, J. J., Wahl, P. W., Cabana, V. G., Hazzard, W. R. & Hoover, J. J. (1976) *Metab. Clin. Exp.* **25**, 633–644
- Redgrave, T. G., Roberts, D. C. K. & West, C. E. (1975) *Anal. Biochem.* **65**, 42–49
- Paleologue, A., Reboud, J. P. & Reboud, A. M. (1988) *Anal. Biochem.* **169**, 234–238
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Havekes, L., Hemmink, J. & De Wit, E. (1981) *Clin. Chem.* **27**, 1829–1833
- Dashti, N., Wolfbauer, G. & Alaupovic, P. (1985) *Biochim. Biophys. Acta* **833**, 100–110
- Goldstein, J. L., Basu, S. K., Brunschede, G. J. & Brown, M. S. (1976) *Cell* **7**, 85–95
- Laurell, C. B. (1966) *Anal. Biochem.* **15**, 57–65
- Hoegge-de Nobel, E., Voskuilen, M., Briët, E., Brommer, E. J. P. & Nieuwenhuizen, W. (1988) *Thromb. Haemostasis* **60**, 415–418
- Kooistra, T., Van Den Berg, J., Töns, A., Platenburg, G., Rijken, D. C. & Van Den Berg, E. (1987) *Biochem. J.* **247**, 605–612
- Chomczynski, P. & Sacchi, N. (1987) *Anal. Biochem.* **162**, 156–159
- Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor
- Kessling, A. M., Horsthemke, B. & Humphries, S. E. (1985) *Clin. Genetics* **28**, 296–306
- Knott, T. J., Rall, S. C., Jr., Innerarity, T. L., Jacobson, S. F., Urdea, M. S., Levy-Wilson, B., Powell, L. M., Pease, R. J., Eddy, R., Nakai, H., Byers, M., Priestly, L. M., Robertson, E., Rall, L. B., Betsholtz, C., Shows, T. B., Mahley, R. W. & Scott, J. (1985) *Science* **230**, 37–43
- Fort, P., Marty, L., Piechaczyk, M., El Sabrouy, S., Dani, C., Jeanteur, P. & Blanchard, J. M. (1985) *Nucleic Acids Res.* **13**, 1431–1432
- Havekes, L., Van Hinsbergh, V. W. M., Kempen, H. J. & Emeis, J. J. (1983) *Biochem. J.* **214**, 951–958

44. Nishina, P. & Freedland, R. A. (1990) *J. Nutr.* **120**, 668–673
45. Carmona, A. & Freedland, R. A. (1989) *J. Nutr.* **119**, 1304–1310
46. Wright, P. S., Dudley, D. T. & Chalkley, R. (1986) *Arch. Biochem. Biophys.* **248**, 243–252
47. Patsch, W., Tamai, T. & Schonfeld, G. (1983) *J. Clin. Invest.* **72**, 371–378
48. Davis, R. A. & Boogaerts, J. R. (1982) *J. Biol. Chem.* **257**, 10908–10913
49. Sato, R., Imanaka, T. & Takano, T. (1990) *Biochim. Biophys. Acta* **1042**, 36–41
50. Davis, R. A., Thrift, R. N., Wu, C. C. & Howell, K. E. (1990) *J. Biol. Chem.* **265**, 10005–10011
51. Borén, J., Wettsten, M., Sjöberg, A., Thorlin, T., Bondjers, G., Wiklund, O. & Olofsson, S.-O. (1990) *J. Biol. Chem.* **265**, 10556–10564
52. Chuck, S. L., Yao, Z., Blackhart, B. D., McCarthy, B. J. & Lingappa, V. R. (1990) *Nature (London)* **346**, 382–385
53. Gibbons, G. F. (1990) *Biochem. J.* **268**, 1–13
54. Fuki, I. V., Preobrazhensky, S. N., Misharin, A. Y., Bushmakina, N. G., Menschikov, G. B., Repin, V. S. & Karpov, R. S. (1989) *Biochim. Biophys. Acta* **1001**, 235–238
55. Wright, R. S., Anderson, J. W. & Bridges, S. R. (1990) *Proc. Soc. Exp. Biol. Med.* **195**, 26–29
56. Beynen, A. C., Buechler, K. F., Van der Molen, A. J. & Geelen, M. J. H. (1982) *Int. J. Biochem.* **14**, 165–169

Received 19 December 1990/17 April 1991; accepted 2 May 1991