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The effect of (−)-hydroxycitrate on the activity of the low-density-lipoprotein receptor and 3-hydroxy-3-methylglutaryl-CoA reductase levels in the human hepatoma cell line Hep G2

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(−)-Hydroxycitrate, a potent inhibitor of ATP citrate-lyase, was tested in Hep G2 cells for effects on cholesterol homoeostasis. After 2.5 h and 18 h incubations with (−)-hydroxycitrate at concentrations of 0.5 mM or higher, incorporation of [1,5-^{14}C]citrate into fatty acids and cholesterol was strongly inhibited. This most likely reflects an effective inhibition of ATP citrate-lyase. Cholesterol biosynthesis was decreased to 27% of the control value as measured by incorporations from ^3H_2O, indicating a decreased flux of carbon units through the cholesterol-synthetic pathway. After 18 h preincubation with 2 mM-(−)-hydroxycitrate, the cellular low-density-lipoprotein (LDL) receptor activity was increased by 50%, as determined by the receptor-mediated association and degradation. Measurements of receptor-mediated binding versus LDL concentration suggests that this increase was due to an increase in the numbers of LDL receptors. Simultaneously, enzyme levels of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase as determined by activity measurements increased 30-fold. Our results suggest that the increases in HMG-CoA reductase and the LDL receptor are initiated by the decreased flux of carbon units in the cholesterol-synthetic pathway, owing to inhibition of ATP citrate-lyase. A similar induction of HMG-CoA reductase and LDL receptor was also found after preincubations of cells with 0.3 μM-mevinolin, suggesting that the underlying mechanism for this induction is identical for both drugs.

INTRODUCTION

(−)-Hydroxycitrate is a potent inhibitor of ATP citrate-lyase (EC 4.1.3.8, Watson *et al.*, 1969; Cheema-Dhadli *et al.*, 1973; Sullivan *et al.*, 1972, 1977*b*). It has been demonstrated that under certain conditions (−)-hydroxycitrate suppresses biosynthesis of both fatty acids and cholesterol in rat hepatocytes (Sullivan *et al.*, 1977*a*; Mathias *et al.*, 1981; Pullinger & Gibbons, 1983) and in rat liver (Lowenstein, 1971; Sullivan *et al.*, 1972; Triscari & Sullivan, 1977; Lowenstein & Brunengraber, 1981). The underlying mechanism of this suppression is likely to be a decrease in the supply of acetyl-CoA units, which feed into both synthetic pathways.

Inhibition of endogenous cholesterol synthesis by 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase inhibitors such as compactin (Endo *et al.*, 1976) results in an increase of the activity of the low-density-lipoprotein (LDL) receptor (Brown *et al.*, 1978). It is now well accepted that the diminished level of LDL cholesterol *in vivo* after treatment with HMG-CoA reductase inhibitors is at least partially related to an increase in hepatic LDL receptor levels (Kovanen *et al.*, 1981). At present, it is unknown if LDL receptor activity will increase similarly when ATP citrate-lyase inhibitors such as (−)-hydroxycitrate are used to decrease endogenous cholesterol synthesis. If this were the case, ATP citrate-lyase inhibitors may have hypocholesterolaemic properties. As a first step, we studied the effect of (−)-hydroxycitrate on the LDL receptor *in vitro*. For these studies we used Hep G2 cells, since they share many of the properties of human hepatocytes in terms of their cholesterol homoeostasis (Havekes *et al.*, 1983; Wang *et al.*, 1988).

Both the LDL receptor and HMG-CoA reductase are, at least partially, under the same metabolic control through feedback repression of the genes encoding these proteins via the cholesterol regulatory pool (Sudhof *et al.*, 1987; Osborne *et al.*, 1988; Dawson *et al.*, 1989). Therefore we studied the effect of inhibition of ATP citrate-lyase on activity levels of HMG-CoA reductase. It was demonstrated by Pullinger & Gibbons (1983) that, in rat hepatocytes incubated with (−)-hydroxycitrate, HMG-CoA reductase enzyme levels were increased. This increase is likely to result not only from a decrease in the cholesterol regulatory pool but also from both a decreased formation of non-sterol and sterol metabolic intermediates in the cholesterol-synthetic pathway (Havel *et al.*, 1979; Rudney & Sexton, 1986; Panini *et al.*, 1986; Kempen *et al.*, 1987; Boogaard *et al.*, 1987).

Here, we present evidence that (−)-hydroxycitrate inhibits cholesterol synthesis in Hep G2 cells and show that this effects an increase of HMG-CoA reductase enzyme levels, as determined by activity measurements, and an up-regulation of the LDL receptor activity.

MATERIALS AND METHODS

Materials

Brij 96, mevalonolactone, dithiothreitol, HMG-CoA, human serum albumin (HSA), ATP, mevinolin, glucose-6-phosphate dehydrogenase (type VII), D-glucose 6-phosphate and NADP$^+$ were obtained from Sigma (St. Louis, MO, U.S.A.). Na^{125}I (0.5 GBq/μg), 3-hydroxy-3-methyl[3-^{14}C]glutaryl-CoA ([^{14}C]HMG-CoA; 2 GBq/mmol), ^3H_2O, [9, 10 (n)-^{3}H]oleic acid (74 GBq/mmol), [7 (n)-^{3}H]cholesterol (185 GBq/mmol), [1,5-^{14}C]citric acid (4 GBq/mmol) and *RS*-[2-^{3}H]mevalonolactone (18.5 GBq/mmol) were purchased from Amersham International

Abbreviations used: LDL, low-density lipoprotein; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; HSA, human serum albumin; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline.

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(Amersham, Bucks., U.K.). Bond-Elut 3 cc silica and aminopropyl columns were from Analytichem International (Harbor City, CA, U.S.A.). Polyacrylamide (PAA 2-16) gradient gels and the ATP assay kit based on bioluminescence were purchased from Pharmacia (Uppsala, Sweden). The culture medium consisted of Dulbecco's modified Eagle's medium (DMEM) including 20 mM-Hepes, bicarbonate (10 mM) and 10% (v/v) fetal-calf serum (Flow Laboratories, Irvine, Scotland, U.K.). Phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS) was from Gibco, Paisley, Scotland, U.K.). Cell-culture dishes were from Costar (Cambridge, MA, U.S.A.). (−)-Hydroxycitrate was prepared from *Garcinia cambogia* fruit (Lewis & Neelakantan, 1965) by Dr. A. Gribble of the Medicinal Chemistry Department of Smith Kline and French Research Ltd., Welwyn, Herts., U.K.

Preparation of LDL

Human serum was prepared from freshly collected blood obtained from healthy donors. LDL was isolated by density-gradient centrifugation by the method of Redgrave *et al.* (1975), followed by tube slicing. A narrow-density fraction was used (density 1.03–1.05 g/ml). LDL was routinely checked for apolipoprotein E content by SDS/polyacrylamide-gel electrophoresis on a 2–16% gradient gel (Havekes *et al.*, 1986). LDL (2 mg of protein/ml) was iodinated to a specific radioactivity of 50–200 d.p.m./ng as described by Havekes *et al.* (1986).

Incubation conditions of the Hep G2 cells

Hep G2 cells were cultured in 24-well cell culture plates in DMEM culture medium containing 10% fetal-calf serum. For experiments, the fetal-calf serum was replaced by 1% fatty-acid-free human serum albumin (DMEM/HSA; Havekes *et al.*, 1986). A solution of mevinolin (0.2 mM) was prepared by heating the lactone form in 0.4 M-NaOH at 60 °C for 2 h. The solution was neutralized with HCl. (−)-Hydroxycitrate was prepared as a free acid solution (200 mM) by hydrolysing the lactone in 3.3 equiv. of NaOH for 30 min at 90 °C, followed by neutralization to pH 7.0. For experiments, the compounds were added to culture medium supplemented with 1% HSA (incubation medium) and sterilized by filtration.

[1,5-^{14}C]Citrate experiments

The cells were allowed to re-equilibrate in the incubation medium for 1 h. Various doses of (−)-hydroxycitrate or vehicle (media) were then added to the cell-culture media and the cells were incubated for a further 2.5 to 18 h. The incorporation of acetyl-CoA units, from citrate, into cholesterol and fatty acids was determined by the addition of [1,5-^{14}C]citrate (100 μCi/μmol; final concn. 45 μM) to the cultures during the final 90 min of the incubation. After this, the cells were washed with 500 μl of PBS and removed from the multi-well plates with 1.8 ml of 1 M-NaOH. [^{3}H]Cholesterol was added as a recovery marker for cholesterol and the cells were saponified at 95 °C for 1 h. The samples were acidified by the addition of 200 μl of concentrated HCl and extracted into 3 × 5 ml of light petroleum (b.p. 40–60 °C). These extracts were evaporated to dryness under N_2, redissolved in 3 ml of hexane, and the cholesterol and fatty acid fractions were separated and isolated by a method similar to that of Kaluzny *et al.* (1985). Aminopropyl columns, used in this method, were primed with 1 ml of hexane. The total lipid extract was then pipetted on the column and the column was washed with 5 ml of hexane, followed by 6 ml of hexane/diethyl ether/dichloromethane (89:1:10, by vol.). Next, cholesterol and fatty acids were eluted with 7 ml of hexane/diethyl ether (1:1, v/v), and 10 ml of 2% acetic acid in diethyl ether, respectively. To each fraction 10 ml of Instagel scintillation fluid was added, and the ^{14}C and ^{3}H contents were determined by scintillation spectrometry. The ^{14}C label incorporated into cholesterol was corrected for recovery by using the [^{3}H]cholesterol internal marker. Incorporation data for the fatty acid fraction were used without correction for recovery during the separation procedure.

Incorporation of ^3H_2O

Hep G2 cells were grown on 2 cm^2 wells to 90% confluency in the DMEM culture medium, and on the day of the experiment this medium was replaced by the DMEM/HSA medium. After 1 h this medium was supplemented with ^3H_2O to a sp. radioactivity of 73.1 μCi/mmol in the absence or presence of 2 mM-(−)-hydroxycitrate, and the cells were incubated for a further 18 h. The incubation was terminated and cells were extracted by a method similar to that of Gibbons *et al.* (1983). Briefly, cells were washed in PBS and then removed from the plate in 6.5 ml of 1.15 M-NaOH and saponified at 95 °C for 1 h. The non-saponifiable sterol fraction was extracted with 3 × 8 ml of light petroleum (b.p. 60–80 °C). The aqueous phase was acidified with 1.25 ml of conc. H_2SO_4 and the fatty acids were extracted as above. The combined organic phases containing either sterols or fatty acids were washed three times with water of pH 12 or pH 2 respectively. Both the sterol and the fatty acid extracts were evaporated to dryness under N_2 and dried overnight under vacuum with P_2O_5. The sterol fractions were redissolved in chloroform and applied to silica-gel t.l.c. plates. The plates were developed in chloroform containing 1% ethanol, and the band corresponding to the main peak (cholesterol) was scraped off and counted for radioactivity. The fatty acid fractions were redissolved in light petroleum (b.p. 60–80 °C) and the radioactivity was measured. Recoveries of cholesterol and fatty acids were determined in parallel experiments in which known amounts of [^{3}H]cholesterol and [^{3}H]oleate were added to non-treated cells before the saponification step. The recoveries of cholesterol and fatty acids were 81 ± 1% and 59 ± 8% respectively (mean ± S.D., $n = 3$). The rate of cholesterol and fatty acid synthesis was calculated by assuming that 23 g-atoms of ^{3}H are incorporated/mol of cholesterol (Dietschy & Spady, 1984) and 14 g-atoms of ^{3}H/mol of fatty acids (Jungas, 1968).

Measurement of binding, association and degradation of ^{125}I-LDL

Hep G2 cells were preincubated for 18 h with different concentrations of (−)-hydroxycitrate or mevinolin as described above. The medium was discarded and the cells were washed with DMEM/HSA. The LDL-receptor-mediated association (degradation) was defined as the total association (degradation) of ^{125}I-LDL to the cells minus the association (degradation) of ^{125}I-LDL in the presence of an excess of unlabelled LDL, measured as described by Havekes *et al.* (1986). Briefly, the experiment was started by adding 0.3 ml of incubation medium, containing 10 μg of ^{125}I-LDL/ml in the absence or presence of 300 μg of unlabelled LDL/ml. After 3 h of incubation at 37 °C, each cell dish was cooled on ice, the medium was removed and trichloroacetic acid-soluble radioactivity ([^{125}I]iodotyrosine) was measured as described by Goldstein *et al.* (1983). After seven consecutive washes with PBS, the cell-associated radioactivity and the cell protein content (Lowry *et al.*, 1951) were determined. Receptor-mediated binding of different concentrations of ^{125}I-LDL was measured at 4 °C during 2.5 h incubations in the absence or presence of a 20-fold excess of unlabelled LDL. Cells were further treated as described above.

HMG-CoA reductase assay

Hep G2 cells were preincubated for 18 h with (−)-hydroxycitrate as above. Cells were then washed (2 × 10 min)

with ice-cold PBS and exposed to 100 μl/well of ice-cold assay buffer containing 0.1 M-sucrose, 40 mM-KH_2PO_4, 30 mM-EDTA, 100 mM-KF, 50 mM-KCl and 5 mM-dithiothreitol at pH 7.4 (Smith *et al.*, 1986; Balasubramaniam *et al.*, 1976), in which 0.25% (v/v) of Brij 96 was dissolved (Goldstein *et al.*, 1983). The cell suspensions were kept on ice for 20 min, after which the cell extracts were transferred to a micro-centrifuge tube. The wells were washed with 100 μl of assay buffer and combined with the extracts. Samples were centrifuged for 1 min in a Microfuge (12000 *g*). The supernatant was used for protein determination (Bradford, 1976) and for measuring HMG-CoA reductase 'expressed' activity (Brown *et al.*, 1978). A sample of this extract (40 μl) was supplemented with 50 μl of assay buffer and 50 μl of the NADPH-generating system in assay buffer. This final assay mixture contained 20 mM-glucose 6-phosphate, 2.5 mM-$NADP^+$ and 3 units of glucose-6-phosphate dehydrogenase (EC 1.1.1.49)/ml. The assay samples were preincubated for 10 min at 37 °C. The assay was initiated by adding 10 μl of [^{14}C]HMG-CoA (15 d.p.m./pmol, final concn. 100 μM) and terminated after 30 min by addition of 10 μl of 6 M-HCl. Then 10 μl of [^{3}H]mevalonolactone (250 d.p.m./nmol) was added to a final concentration of 6.7 mM as a recovery marker. Samples without NADPH-generating cocktail and samples without cell extracts were used as controls. The samples were incubated for another 30 min in order to ensure complete formation of mevalonolactone. Denatured protein was removed by centrifugation at 10000 *g* for 2 min. The supernatant (100 μl) was applied to a silica column (Bond-Elut) that was primed with 1 ml of the eluting solvent mixture toluene/acetone (3:1, v/v). After the aqueous sample was applied to the column, by using the plunger of a syringe, the column was eluted with 3 ml of the solvent. The eluate was discarded and a further 4 ml of the toluene/acetone mixture was applied to the column to elute the mevalonolactone. This fraction was collected in a scintillation vial for measuring ^{3}H and ^{14}C radioactivity. Recoveries of [^{3}H]mevalonate marker from the columns ranged between 75% and 95%, whereas ^{14}C control blank values were usually 200 d.p.m. or less.

ATP measurements

Hep G2 cells were preincubated for 18 h in the presence of (−)-hydroxycitrate, after which the cells were exposed to 0.5% trichloroacetic acid for 20 min. The extracts were measured for ATP content by a bioluminescence method as described by the manufacturer of the assay kit (see under 'Materials').

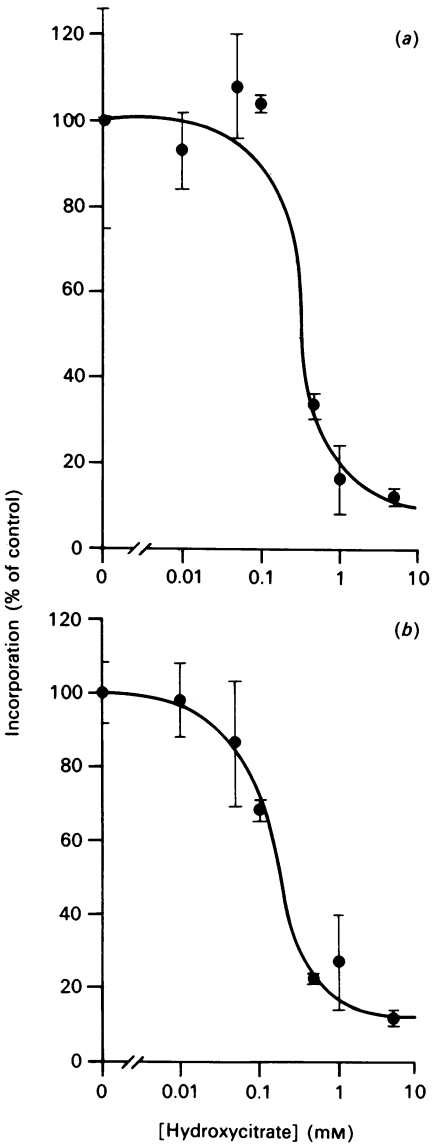

Fig. 1. Effect of increasing concentrations of (−)-hydroxycitrate on the incorporation of [1,5-^{14}C]citrate into fatty acids (*a*) and cholesterol (*b*) in Hep G2 cells

Cells were preincubated with (−)-hydroxycitrate for 2.5 h and treated as described in the Materials and methods section. Symbols represent means ± S.D. of triplicate observations. In the control dishes a total of 1734 ± 448 d.p.m. was incorporated into cholesterol and 8091 ± 718 d.p.m. into the fatty acid fraction.

RESULTS

Effect of (−)-hydroxycitrate on lipogenesis and cholesterogenesis

The effect of (−)-hydroxycitrate on lipogenesis and cholesterogenesis in Hep G2 cells was studied in three different experiments. First, the immediate effect of (−)-hydroxycitrate addition on the flux of label from [1,5-^{14}C]citrate into lipids was investigated. Fig. 1 illustrates that incorporation of ^{14}C label into both fatty acids and cholesterol was strongly inhibited after a 2.5 h preincubation with (−)-hydroxycitrate, with IC_{50} (concn. giving 50% inhibition) between 0.1 and 0.5 mM, consistent with a block at the level of ATP citrate-lyase.

Sometimes blockage of a pathway can be overcome by metabolic compensatory mechanisms. However, in a second experiment we found that the decrease in lipogenesis and cholesterogenesis was also maintained after a 18 h period of preincubation with 0.5 mM-(−)-hydroxycitrate (results not shown). Incubation with (−)-hydroxycitrate may change the specific radioactivity of the citrate and acetyl-CoA precursors in the cell, and consequently incorporations of ^{14}C label from [1,5-^{14}C]citrate into lipids may not reflect the real flux through the pathway. Furthermore, the inhibitor may compete with the uptake of citrate in the cell. Therefore in a third experiment ^3H_2O was applied as the radiolabelled precursor. Incorporation of label into cholesterol and fatty acids was measured during 18 h with or without (−)-hydroxycitrate (1 mM). Incorporation of ^3H_2O into cholesterol was significantly decreased by 73% (Table 1), whereas the incorporation of label into fatty acids was decreased non-significantly. Clearly, cholesterol synthesis is consistently decreased in the presence of (−)-hydroxycitrate in all three experiments.

Table 1. Effect of (−)-hydroxycitrate on the formation of cholesterol and fatty acids in Hep G2 cells

Hep G2 cells were incubated for 18 h with ^3H_2O in the absence or presence of 1 mM-(−)-hydroxycitrate. The ^3H label in 3β-hydroxy sterols and fatty acids of these cells was then determined, and the quantity of cholesterol and fatty acid per well synthesized after 18 h was calculated (see the Materials and methods section). Results are expressed as means ± S.D. with the numbers of observations in parentheses. Value marked * is significantly different from control at $P < 0.001$, by Student's two-tailed t test.

[(−)-Hydroxycitrate]	Cholesterol (nmol/18 h)	Fatty acids (nmol/18 h)
0	12.85 ± 0.43 (4)	68.1 ± 17.3 (4)
1 mM	3.5 ± 0.5 (3)*	44.8 ± 16.4 (3)

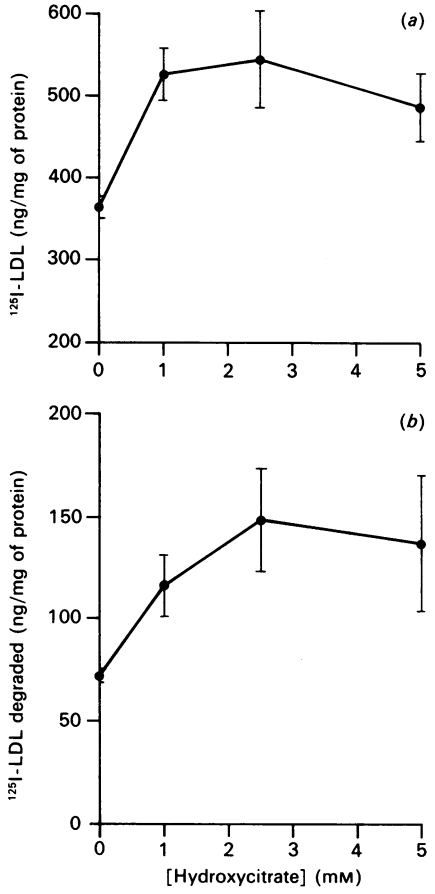

Fig. 2. Dose–response curve showing the concentration of (−)-hydroxycitrate versus the LDL-receptor-mediated association (*a*) and degradation (*b*) of human ^{125}I-LDL in Hep G2 cells

Cells were preincubated for 18 h with (−)-hydroxycitrate and incubated for a further 3 h in the presence of ^{125}I-LDL (10 μg/ml). The radioactivity associated with the cells (*a*) and the non-trichloroacetic acid-precipitable fraction in the medium (*b*) were determined as in the Materials and methods section. This Figure represents a typical example out of a range of six independent experiments. Results are means ± S.D. ($n = 3$).

Effect on LDL receptor activity

After we had established that (−)-hydroxycitrate decreases cholesterol synthesis, we were interested in the effect of an 18 h incubation with this compound on the activity of the LDL receptor (Fig. 2). In cells treated with 1–3 mM-(−)-hydroxycitrate

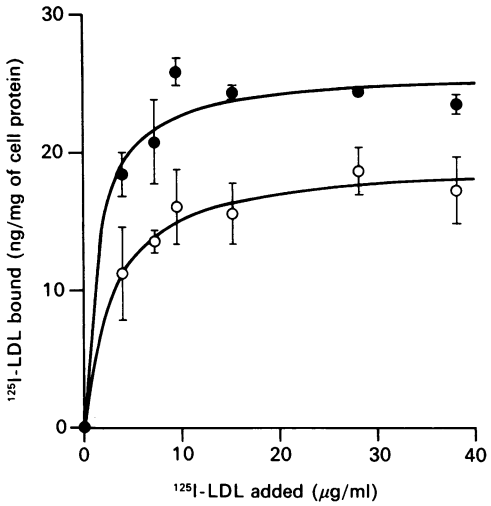

Fig. 3. Effect of 2 mM-(−)-hydroxycitrate on the LDL-receptor-mediated binding

Cells were preincubated with (−)-hydroxycitrate for 16 h and further incubated with different concentrations of human ^{125}I-LDL for 2.5 h at 4 °C. Receptor-mediated binding was measured as described in the Materials and methods section. Results are means ± S.D. for three observations: ○, control; ●, treated.

the receptor-mediated association was increased by 50% over the control values. A very similar result was obtained when the receptor-mediated degradation of the ^{125}I-LDL was measured (Fig. 2*b*), which was about twice the activity in control cells. This indicates that (−)-hydroxycitrate does not impair the degradation pathway of internalized ^{125}I-LDL. The increases in both the LDL-receptor-mediated association and degradation were significant over six independent studies [$P < 0.001$ and $P < 0.05$ at 1 mM-(−)-hydroxycitrate respectively]. Receptor-mediated association increased from 273 ± 77 to 401 ± 86 ng of ^{125}I-LDL/mg of cell protein, whereas receptor-mediated degradation increased from 73 ± 32 to 106 ± 55 ng of ^{125}I-LDL/mg of cell protein. At higher doses of (−)-hydroxycitrate (10 mM), the induction of the LDL-receptor-mediated association and degradation becomes less pronounced, whereas ATP levels in these cells were decreased to 75% of the control values, indicating a loss of viability of the cells under these incubation conditions. ATP levels were found to be unchanged for the concentrations of (−)-hydroxycitrate used in Fig. 2. To compare the effect of inhibition at the level of ATP citrate-lyase with inhibition of HMG-CoA reductase, we treated Hep G2 cells with mevinolin (0.3 μM). This resulted in maximal increases of 50% and 48% in receptor-mediated association and degradation respectively (results not shown), which was lower than the increase of 150% obtained with compactin (Cohen *et al.*, 1984).

Fig. 3 shows the effect of preincubation with 2 mM-(−)-hydroxycitrate on the receptor-mediated binding of LDL to Hep G2 cells. Scatchard (1949) analysis of these data showed that incubation of the Hep G2 cells with (−)-hydroxycitrate resulted in an increase in the maximum specific binding, from 19.6 to 29.3 ng LDL/mg of cell protein, whereas the affinity of the receptor for LDL remained unaffected.

HMG-CoA reductase activity in Hep G2 cells

LDL receptor and HMG-CoA reductase activities may be co-ordinately regulated. We therefore studied the effect of increasing concentrations of (−)-hydroxycitrate on the HMG-CoA reductase levels in the cell. Data are shown in Fig. 4. Between

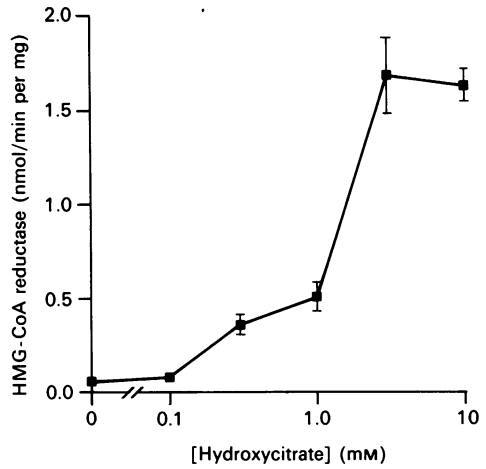

Fig. 4. Effect of increasing concentrations of (−)-hydroxycitrate on HMG-CoA reductase levels in Hep G2 cells

Cells were preincubated for 18 h with the drug, lysed in Brij 96 in the presence of 50 mM-fluoride, and the HMG-CoA reductase activity was measured (see the Materials and methods section). Results are means ± S.D. ($n = 3$).

0.1 mM- and 0.5 mM-(−)-hydroxycitrate the amount of HMG-CoA reductase activity starts to increase, until the maximum activity is reached at a concentration of 3 mM. The amount of HMG-CoA reductase is increased about 30-fold, e.g. from 57 to 1680 pmol/min per mg of protein. This result indicates that a decrease in cholesterol synthesis in these cells results in a compensatory up-regulation not only of the LDL receptor activity but also of the level of HMG-CoA reductase. In this particular situation the HMG-CoA reductase activity is a measure of the number of potentially active HMG-CoA reductase molecules and not the actual turnover of substrate in the cell. Dephosphorylation, and thereby activation of existing enzyme molecules, is unlikely to play a significant role in this up-regulation process, as the 'expressed' HMG-CoA reductase activity in control Hep G2 cells (measured in the presence of fluoride) is nearly 80% of the 'total' HMG-CoA reductase activity (measured in the absence of fluoride). In parallel experiments, inhibition of cholesterol biosynthesis at the level of HMG-CoA reductase by mevinolin also resulted in an increase of HMG-CoA reductase levels of about 30-fold.

DISCUSSION

The aim of the present study was to investigate the effects of inhibition of ATP citrate-lyase on the lipid and cholesterol homoeostasis, by using the human hepatoma cell line Hep G2. Interest in this area is aroused by the possibility of ATP citrate-lyase being a target for treatment of hypolipidaemia. The most significant finding in the present work is that overnight exposure of Hep G2 cells to the ATP citrate-lyase inhibitor (−)-hydroxycitrate results in an up-regulation of LDL receptor activity and LDL-receptor-dependent LDL catabolism.

Cholesterogenesis and lipogenesis in mammals derive their precursor, acetyl-CoA, from the cytosolic acetyl-CoA pool (Kornacker & Lowenstein, 1965). ATP citrate-lyase is thought to be the major enzyme in supplying this pool with substrate from citrate cleavage. However, under certain conditions such as fasting, hepatic cytosolic acetyl-CoA may also be derived from cytosolic activation of acetoacetate, thereby by-passing the ATP citrate-lyase (Buckley & Williamson, 1975; Mathias *et al.*, 1981; Bergstrom *et al.*, 1984; Gibbons *et al.*, 1986), and in liver in fasting this activity has been reported to be high enough to maintain the cholesterogenesis (Lowenstein & Brunengraber, 1981). In the present study we have found that (−)-hydroxycitrate, a potent inhibitor of the ATP citrate-lyase, inhibits the incorporation of [1,5-^{14}C]citrate into fatty acids and cholesterol to near completion in a parallel dose-dependent way. Contrary to this, measurements of absolute rates by using ^3H_2O revealed that cholesterogenesis was not completely inhibited, and also that cholesterogenesis was far more affected than lipogenesis (73% versus 34% inhibition). Sources of cytosolic acetyl-CoA not derived from ATP citrate-lyase (see above) or an overestimation of the extent of inhibition of ATP citrate-lyase, based on the [1,5-^{14}C]citrate experiments, may underly these findings. The less efficient inhibition of fatty acid synthesis than of cholesterol synthesis in the ^3H_2O experiments may be explained by allosteric stimulation of acetyl-CoA carboxylase activity by (−)-hydroxycitrate, as shown by Sugden *et al.* (1984) and Triscari & Sullivan (1977).

There is a large discrepancy between the K_i of (−)-hydroxycitrate for the purified ATP citrate-lyase (52 nM; Sullivan *et al.*, 1977*b*) and the IC_{50} for [1,5-^{14}C]citrate incorporation into lipids that we found in Hep G2 cells (0.2–0.5 mM). This suggests that intracellular concentrations of (−)-hydroxycitrate are low, owing to a limited penetration into the cell as reported by Sullivan *et al.* (1977*a*).

It has been demonstrated by several laboratories that the receptor-mediated uptake of LDL and HMG-CoA reductase enzyme levels increase after preincubation with the HMG-CoA reductase inhibitor compactin (Brown *et al.*, 1978; Leichtner *et al.*, 1984; Cohen *et al.*, 1984). Our studies in Hep G2 cells with mevinolin confirm these results. The dramatic increase in HMG-CoA reductase levels by overnight incubation of the cells with (−)-hydroxycitrate further substantiates the concept that inhibition of cholesterol synthesis by acetyl-CoA availability results in similar responses to those found with the HMG-CoA reductase inhibitors. However, in contrast with mevinolin, (−)-hydroxycitrate showed no acute effect on the activity of HMG-CoA reductase when added to the HMG-CoA reductase assay mixture, excluding the possibility that the compound is itself a HMG-CoA reductase inhibitor. An increase in HMG-CoA reductase activity after hydroxycitrate (and compactin) exposure has been reported by Pullinger & Gibbons (1983) in primary cultures of rat hepatocytes. The much lower stimulation of only 2–3-fold may be due to the length of the incubation periods (4 h) in their experiments.

An increase in LDL receptor activity would be, if found in human liver, a favourable response resulting in a hypocholesterolaemic effect in the blood. However, at present it is not known if other pathways that would generate acetyl-CoA units in the cytosol of human liver are active as well.

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