Cholesterol esterification plays a major role in determining low-density-lipoprotein receptor activity in primary monolayer cultures of rat hepatocytes

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1. We have previously shown that the capacity for specific binding of human ¹²⁵I-labelled low-density lipoprotein (LDL) to rat hepatocytes increases with time in culture [Salter, Bugaut, Saxton, Fisher & Brindley (1987) *Biochem. J.* **247**, 79–84]. 2. In the present study we show that this up-regulation is accompanied by a rise in the cholesterol ester content of the cells. 3. Inhibition of cholesterol esterification with the drug 58-035 (Sandoz) significantly decreases the time-dependent 'up-regulation' of LDL receptors. 4. Incubation of hepatocytes with LDL itself has little effect on subsequent LDL binding. However, when cholesterol esterification is inhibited, incubation with LDL decreases binding below that attained with the drug alone. 5. Inhibition of cholesterol synthesis with Lovastatin significantly increases LDL binding and antagonizes the effect of 58-035. 6. We conclude that in hepatocytes the rate of cellular cholesterol esterification can become the major determinant of LDL-receptor activity.

INTRODUCTION

Plasma LDL concentrations vary widely between different species [1]. However, independent of the plasma concentrations, in all species yet studied the liver has been shown to be the major site of catabolism of LDL [2–4]. Thus it is apparent that LDL-receptor expression in the liver cannot simply be governed by prevailing extracellular LDL concentrations.

We have demonstrated the presence of two specific binding sites for human LDL on rat hepatocytes in monolayer culture [5,6]. One of these sites, Site 1, probably represents the 'classical' LDL receptor, which was originally described by Goldstein & Brown on cultured fibroblasts [7]. We have shown that the expression of this receptor is very low when hepatocytes are first isolated, and increases to a maximum after about 48 h in culture [6]. This 'up-regulation' is enhanced by the addition to the culture medium of newborn-calf serum (NBCS) [6], human HDL₃ [6], insulin [8,9] and tri-iodothyronine [9], but is retarded by the synthetic glucocorticoid dexamethasone [8–10]. Unlike many other cell types [11], preincubation of hepatocytes with LDL itself does not result in 'down-regulation' of receptor activity [6,12].

A similar resistance to down-regulation of LDL receptors by preincubation with LDL itself has been found in two diverse transformed cell lines, the human hepatocarcinoma line, HepG2 [13] and, at least in one laboratory, the mouse macrophage line J774 [14]. Responsiveness to down-regulation can be conferred on to both cell types by inhibition of ACAT activity with the Sandoz compound 58-035 [14,15]. In the present study

we have looked at the effects of 58-035 on cholesterol esterification and LDL binding and metabolism in cultured rat hepatocytes. We have found that, compared with HepG2 cells, 12 times the concentrations of 58-035 are required to inhibit ACAT maximally. Unlike HepG2 cells, 58-035 alone significantly decreases LDL binding to the 'classical' LDL receptor. However, like HepG2 and J774 cells, a sensitivity to down-regulation by preincubation with LDL is conferred on rat hepatocytes by inhibition of ACAT. We conclude that cholesterol esterification plays a major role in regulating LDL uptake by the liver.

EXPERIMENTAL

Animals and materials

The sources of rats and most of the materials have been described elsewhere [5,16,17]. [³H]- and [¹⁴C]-oleic acid were obtained from Amersham International, Amersham, Bucks., U.K. Compound 58-035 was a gift from Dr. J. G. Heider, Sandoz Research Institute, Sandoz Inc., East Hanover, NJ, U.S.A.

Preparation and incubation of hepatocytes

Hepatocytes were prepared [15] and attached to collagen-coated tissue-culture plates [18] in a modified [19] Leibowitz L-15 medium containing 10% (v/v) NBCS. After incubation for 1 h, cells were transferred to fresh serum-containing medium for a further 20 h and then to serum-free medium supplemented with 2 g of fatty-acid-poor bovine serum albumin/l. Where indicated, 58-035 and Lovastatin were added to this

Abbreviations used: LDL, low-density lipoprotein; HDL, high-density lipoprotein; ACAT, acyl-CoA:cholesterol acyltransferase; NBCS, newborn-calf serum.

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medium after being dissolved in dimethyl sulphoxide. Equivalent amounts of dimethyl sulphoxide were added to control incubations.

¹²⁵I-LDL binding and degradation assays

The binding of ¹²⁵I-LDL to hepatocytes at 4 °C and 37 °C was performed in serum-free Leibovitz L-15 medium supplemented with 2 g of fatty-acid-poor bovine serum albumin/l as previously described [5,6]. At 4 °C ¹²⁵I-LDL released by dextran sulphate was defined as that bound to Site 1, and that remaining associated with the cells as that bound to Site 2. At 37 °C, ¹²⁵I-LDL released by dextran sulphate was also defined as being bound to Site 1, but that cell-associated was assumed to be a combination of internalized LDL and that bound to Site 2. Non-specific binding was measured in parallel incubations in the presence of a 30-fold excess of unlabelled LDL. Specific binding was calculated by subtracting non-specific binding from total.

The degradation of ¹²⁵I-LDL was determined as previously described [6]. This was performed in the presence of 1 mM-3-iodo-L-tyrosine to inhibit deiodinase activity [6].

Measurement of cellular cholesterol and cholesterol ester

Cellular free and esterified cholesterol were extracted in hexane/propan-2-ol (3:2, v/v) as described by Goldstein *et al.* [20]. An internal standard of [¹⁴C]-cholesterol ester was added to each extract. Extracts from triplicate plates were combined and evaporated to dryness under N₂. A further 1 ml of propan-2-ol was added to each tube; this was evaporated and finally lipids were dissolved in 70 µl of chloroform. A 50 µl portion of this extract was loaded on to silica gel 60 t.l.c. plates, and cholesterol and cholesterol ester were separated with the solvent system light petroleum (b.p. 60–80 °C)/diethyl ether/acetic acid (90:30:1, by vol.). Cholesterol and cholesterol ester bands were cut from the plates and extracted twice with chloroform/methanol (2:1, v/v). Extracts were evaporated to dryness, 100 µl of propan-2-ol was added to each tube and cholesterol content was measured enzymically with a Boehringer cholesterol kit (C-System). Results were corrected for recovery by using the internal standard.

Measurement of cholesterol esterification

Cholesterol esterification was measured essentially as described by Goldstein *et al.* [20] but with [³H]oleate. Preliminary experiments (results not shown) showed that at an oleate concentration of 300 µM esterification rate remained constant for at least 30 min. These conditions were therefore routinely used.

RESULTS

Effect of 58-035 on cholesterol esterification in hepatocytes

Incubation of hepatocytes for 24 h with 60 µg of 58-035/ml resulted in a maximum inhibition of esterification of approx. 90% (Fig. 1) and had no adverse effects on cell viability.

Table 1 shows the effect of preincubation with two different concentrations of LDL, in the presence and absence of 58-035, on cholesterol esterification. In these experiments hepatocytes were incubated for 20 h in medium containing 10% (v/v) NBCS and then for a

Fig. 1. Inhibition of cholesterol esterification in hepatocytes by 58-035

Hepatocytes were incubated for 20 h in medium containing 10% (v/v) NBCS and then for a further 24 h in fresh serum-containing medium supplemented with the indicated amounts of 58-035. The incorporation of [³H]oleate into cholesterol ester was then measured over 30 min at an oleate concentration of 300 µM. Results are means ± range of duplicate determinations and are expressed relative to incorporation in the absence of 58-035, which was 2.7 ± 0.07 nmol of oleate incorporated/h per mg of cell protein.

further 24 h in serum-free medium containing the indicated concentrations of LDL and 58-035. As we have previously found [6], a concentration of 100 µg of LDL protein/ml stimulated esterification, but 58-035 almost completely inhibited esterification even in the presence of LDL.

To investigate whether cells continue to esterify cholesterol in serum-free medium, even in the absence of exogenous fatty acid, hepatocytes were incubated with tracer (0.0185 MBq/plate) [¹⁴C]oleate only for 4 h after a 20 h preincubation in serum-free medium with or without 60 µg of 58-035/ml. Fig. 2 shows the accumulation of

Table 1. Effect of preincubation of hepatocytes with 58-035 with or without LDL on the rate of cholesterol esterification

Hepatocytes were incubated for 20 h in medium containing 10% (v/v) NBCS and then for a further 24 h in serum-free medium with or without 58-035 and LDL as indicated. The incorporation of [³H]oleate into cholesterol ester was measured over 30 min at an oleate concentration of 300 µM. Results are means ± s.d. of triplicate determinations.

Treatment	[³ H]Oleate incorporated (nmol/mg of cell protein)	
	Expt. 1.	Expt. 2
Control	2.85 ± 0.15	1.03 ± 0.06
58-035 (60 µg/ml)	0.25 ± 0.03	0.18 ± 0.02
LDL*	2.93 ± 0.15	1.73 ± 0.06
58-035 + LDL	0.31 ± 0.02	0.20 ± 0.01

* LDL concn: Expt. 1, 20 µg/ml; Expt. 2, 100 µg/ml.

Fig. 2. Incorporation of [¹⁴C]oleate into triacylglycerol and cholesterol ester after preincubation with 58-035

Hepatocytes were incubated for 20 h in medium containing 10% (v/v) NBCS and then for a further 24 h in serum-free medium supplemented with 2 g of fatty-acid-free bovine serum albumin with (▲) or without (○) 60 μg of 58-035/ml. Fresh serum-free medium was then added containing 9.25 kBq of [¹⁴C]oleate/ml. Hepatocytes were incubated for the indicated lengths of time, and incorporation of [¹⁴C]oleate into (a) triacylglycerol and (b) cholesterol ester was determined.

Fig. 3. Effect of time in culture on free and esterified cholesterol concentrations in hepatocytes

Hepatocytes were maintained in culture for the length of time indicated. At 20 h they were changed to serum-free medium with or without 60 μg of 58-035/ml as indicated. At the times stated, intracellular free cholesterol (a) and cholesterol ester (b) were measured as described in the Experimental section. Results are means ± range of duplicate determinations, and were reproduced in a further independent experiment.

[¹⁴C]oleate into both triacylglycerol (a) and cholesterol ester (b). Approximately 10 times more oleate was incorporated into triacylglycerol than into cholesterol ester; incorporation into triacylglycerol was unaffected by preincubation with 58-035. In contrast, 58-035 markedly inhibited the incorporation of oleate into cholesterol ester. This confirms the fact that 58-035 is specifically inhibiting cholesterol esterification through its effects on ACAT.

Fig. 3 shows the free and esterified cholesterol content of hepatocytes at different times in culture. A 2-fold increase in cholesterol ester concentration can be seen between 20 and 44 h of culture. This is not a result of the change from serum-containing to serum-free medium, as similar effects were seen when cells were maintained in 10% NBCS throughout the culture period (results not shown). The inclusion of 60 μg of 58-035/ml in the final 24 h incubation completely inhibited the rise in cholesterol ester concentration. Throughout the culture period little change was seen in free cholesterol concentrations.

Effect of 58-035 on LDL binding

Fig. 4 shows the effect of preincubation of hepatocytes for 24 h with different concentrations of 58-035 on the subsequent specific binding of [¹²⁵I]-LDL to Site 1 and Site 2. Concentrations of 10–100 μg of 58-035/ml inhibited binding to Site 1, i.e. the classical LDL receptor, by approx. 50%. In contrast, a maximum inhibition of only 23% of binding to Site 2 was brought about by 100 μg of 58-035/ml. Table 2 shows the combined results of a number of experiments in which the effects of preincubation with 60 μg of 58-035/ml on LDL binding at both 4 °C and 37 °C were measured. At both temperatures dextran sulphate-releasable binding (Site 1) was significantly decreased after preincubation with the ACAT inhibitor. No significant effect was seen on binding to Site 2 (cell-associated at 4 °C), whereas, presumably as a result of decreased internalization of LDL, cell-associated [¹²⁵I]-LDL measured at 37 °C was significantly decreased. In fact, the inhibition of binding to Site 1 by 58-035 was an inhibition of the increase in binding that occurs during the 24 h incubation in the absence of the drug. In six experiments binding was measured at 4 °C to

Fig. 4. Effect of preincubation with 58-035 on the subsequent specific binding of human ^{125}I -LDL to Site 1 and Site 2 on rat hepatocytes

Hepatocytes were incubated for 20 h in medium containing 10% (v/v) NBCS and then for a further 24 h in serum-free medium containing the indicated amounts of 58-035. Plates were cooled to 4 °C, washed with serum-free medium and incubated with 10 µg of ^{125}I -LDL protein/ml for 4 h at 4 °C. Non-specific binding was measured in parallel incubations in the presence of 300 µg of unlabelled LDL protein/ml, and this was subtracted from total binding to give the specific binding shown. Binding to Site 1 (□) and Site 2 (●) was determined as described in the Experimental section. Results are expressed relative to binding in the absence of 58-035 and represent mean $\pm$ range of duplicate determinations. Similar results were obtained in three further independent experiments.

hepatocytes after 20 h in culture in the presence of 10% (v/v) NBCS, and then again after a further 24 h in serum-free medium with or without 60 µg of 58-035/ml. In the absence of the drug, binding increased from 6.5 ± 0.6 to 17.3 ± 3.2 ng of ^{125}I -LDL protein/mg of cell

protein (mean $\pm$ S.E.M., $P < 0.001$), whereas when 58-035 was present in the second incubation binding was 8.0 ± 1.7 ng/mg, which was not significantly different from that before incubation. Thus it appears that in cells incubated for 24 h in serum-free medium there is a significant increase in the expression of LDL receptors which can be decreased by the inhibition of cholesterol esterification.

Effect of preincubation with 58-035 and LDL on subsequent binding and degradation of LDL

We have previously reported that preincubation of hepatocytes with LDL itself does not significantly affect the subsequent binding of ^{125}I -LDL to Site 1 [5]. The results shown in Table 3 confirm these findings. However, preincubation with both 58-035 and LDL decreases binding to a level below that which could be attributed to an additive effect. A similar trend was seen in cell-associated ^{125}I -LDL. Binding was only measured at 37 °C, as this ensured that all binding sites were available and not blocked by unlabelled LDL, which would presumably be internalized at this temperature. Fig. 5 shows the effects of preincubation with different concentrations of LDL in the presence and absence of 60 µg of 58-035/ml on the subsequent binding of ^{125}I -LDL to Site 1. At each concentration LDL was more potent at down-regulating binding in the presence of the ACAT inhibitor. The effect of preincubation of hepatocytes with 58-035 and LDL on the subsequent degradation of LDL was also measured. Cells were incubated with or without 58-035 (60 µg/ml) and LDL (20 µg/ml) as above. Specific degradation of ^{125}I -LDL to ^{125}I -tyrosine was then measured at a ligand concentration of 10 µg/ml after incubation at 37 °C for 2, 4 and 6 h, and the rate was then calculated. In the absence of any additions this was 15.3 ng of LDL protein degraded/h per mg of cell protein. This was decreased to 5.1 after incubation with 58-035 and LDL. Thus, as expected, the decrease in binding was accompanied by a comparable decrease in degradation.

Table 2. Effects of preincubation of hepatocytes with 58-035 on the subsequent binding of LDL at 4 and 37 °C

Hepatocytes were incubated for 20 h in 10% NBCS, followed by a further 24 h in serum-free medium with or without 60 µg of 58-035/ml. Specific binding of human ^{125}I -LDL was measured at a ligand concentration of 10 µg of LDL protein/ml for 4 h at either 4 or 37 °C as indicated. At 4 °C dextran sulphate-released binding represents Site 1, and cell-associated Site 2. At 37 °C cell-associated LDL represents Site 2 and internalized LDL. Results are means $\pm$ S.E.M. Significantly different from control by paired t test: * $P < 0.025$, ** $P < 0.01$, *** $P < 0.001$.

Temp.	Addition	LDL (ng of protein/mg of cell protein)	
		Dextran sulphate-released	Cell-associated
4 °C ($n = 14$)	None	15.3 ± 1.8	8.8 ± 1.5
	58-035	$7.4 \pm 0.9^{***}$	7.8 ± 1.1
37 °C ($n = 12$)	None	12.2 ± 1.8	39.3 ± 4.4
	58-035	$8.3 \pm 1.5^{**}$	$31.0 \pm 3.8^*$

Table 3. Effect of preincubation of hepatocytes with 58-035 with or without LDL on the subsequent binding of LDL at 37 °C

Hepatocytes were incubated as indicated in Table 2. 58-035 (60 µg/ml), LDL (20 µg/ml) or both were included in the second incubation as indicated. Binding was measured at 37 °C as described in Table 2. Results are means $\pm$ S.E.M. ($n = 10$). * Significantly different from control ($P < 0.05$); ** significantly different from control, ($P < 0.005$), 58-035 alone ($P < 0.02$) and LDL alone ($P < 0.001$); *** significantly different from control ($P < 0.005$) and LDL alone ($P < 0.005$).

Additions	LDL (ng of protein/mg of cell protein)	
	Dextran sulphate-released	Cell-associated
None	12.7 ± 2.3	37.2 ± 5.1
58-035	$9.0 \pm 1.7^*$	31.1 ± 4.5
LDL	11.1 ± 1.5	$34.8 \pm 4.8^*$
LDL + 58-035	$5.2 \pm 0.8^{**}$	$24.4 \pm 2.6^{***}$

Fig. 5. Effect of preincubation with 58-035 and LDL on the subsequent specific binding of ^{125}I -LDL to Site 1 on rat hepatocytes

Rat hepatocytes were maintained for 20 h in the presence of 10% NBCS. They were then incubated for a further 24 h in serum-free medium supplemented with the indicated amount of LDL with (●) or without (○) 60 μg of 58-035/ml. Specific binding to Site 1 was measured at 37 °C as described in Fig. 4. Results are means of duplicate determinations and are expressed relative to binding after incubation in the absence of LDL, which was 20.6 ± 1.3 and 12.5 ± 0.4 ng of ^{125}I -LDL protein bound/mg of cell protein in the absence and presence of 58-035 respectively.

Effect of Lovastatin on LDL binding

Lovastatin is a specific inhibitor of 3-hydroxy-3-methylglutaryl-CoA reductase, the rate-limiting enzyme in cholesterol synthesis. In rat hepatocytes we have shown that 10 μM -Lovastatin inhibits the incorporation

Fig. 6. Effect of preincubation with Lovastatin and 58-035 on the subsequent binding of human ^{125}I -LDL to Site 1 on rat hepatocytes

Rat hepatocytes were incubated for 20 h in the presence of 10% (v/v) NBCS. Specific ^{125}I -LDL binding to Site 1 was measured at 4 °C in some plates (as in Fig. 4), and others were changed to serum-free medium containing 58-035 (60 μg /ml), Lovastatin (10 μM) or both, as indicated. After 24 h, specific ^{125}I -LDL binding to Site 1 was determined. Results are means $\pm$ S.E.M. of five independent experiments, and are expressed relative to binding measured after 20 h in culture. * Significantly different from control ($P < 0.025$); ** significantly different from Lovastatin alone ($P < 0.005$).

of [^{14}C]acetate into cholesterol by over 99% (A. M. Salter, N. Ekins & B. Middleton, unpublished work). This concentration of Lovastatin also caused a 47% inhibition of the incorporation of [^{14}C]oleate into cholesterol ester (results not shown). Fig. 6 shows the effect of 24 h incubation with Lovastatin alone or in combination with 58-035 on the dextran sulphate-releasable binding of ^{125}I -LDL at 4 °C. Binding was measured both before and after the incubation, and results are expressed as a percentage change from that before incubation. As described above, incubation in serum-free medium results in a substantial 'up-regulation' of binding which was inhibited by 58-035. In contrast, Lovastatin significantly enhanced the up-regulation. This effect was reversed when 58-035 was also included in the incubation.

DISCUSSION

The results presented in this paper show that the time-related increase in LDL-binding capacity of rat hepatocytes in culture is accompanied by an accumulation of cholesterol ester within the cell. Inhibition of ACAT with 58-035 not only inhibits the accumulation of cholesterol ester but also decreases the rise in LDL binding. It seems likely that this increase in esterification is not matched by an increase in cholesterol synthesis. This would result in a fall in the intracellular free cholesterol concentration in the pool which regulates receptor activity and thus lead to an increased receptor expression. Although this is not reflected in any significant change in cellular free cholesterol concentrations, this may simply be because the vast bulk of cholesterol resides in the plasma membrane and may not be readily available to contribute to the regulatory pool. In addition, it appears that inhibition of cholesterol synthesis may further deplete the regulatory free cholesterol pool, as indicated by decreased esterification of cellular cholesterol. We find this results in further up-regulation of LDL receptors.

As in the transformed cell lines HepG2 [15] and J774 [14], 58-035 induces a LDL-mediated down-regulation of LDL-receptor activity in cultured rat hepatocytes. These cells do, however, behave differently from HepG2 cells in a number of respects. First, the rate of cholesterol esterification, under basal conditions, appears to be approximately an order of magnitude higher than that reported for HepG2 cells [15]. Furthermore, ACAT activity measured with [^3H]oleoyl-CoA in hepatocyte homogenates was 0.23 ± 0.08 nmol of oleate incorporated into cholesterol ester/min per mg of homogenate protein (mean $\pm$ S.D. for four different preparations) (M. Al-Seeni & B. Middleton, unpublished work), some 13-fold higher than that reported for HepG2 cells [21]. Secondly, 12 times as much 58-035 was required to give maximum inhibition of esterification as compared with HepG2 cells [15]. Although this may simply reflect the much higher ACAT activity found in these cells, it may also reflect differences in the rate at which the drug is transported into the cells and/or on to the endoplasmic reticulum. Other investigators [22] have reported a similar insensitivity of rat hepatocytes to inhibition of cholesterol esterification by 58-035. Despite these differences it appears that the lack of response of LDL receptors to preincubation with LDL and the appearance of such sensitivity on the inhibition of ACAT is not just a

characteristic of certain transformed cell lines, but may be constitutive to hepatocytes.

Thus it appears that LDL-receptor activity increases in rat hepatocytes in response to a high cholesterol esterification rate which is responsible for the rapid removal of both endogenously and exogenously derived cholesterol from the intracellular free cholesterol pool which regulates receptor activity. Results in skin fibroblasts, where there is no possibility of further metabolism of cholesterol and where ACAT activity is approximately one-twentieth of that in hepatocytes, support this hypothesis. Thus the direct inhibition of fibroblast ACAT by 58-035 has no effect on LDL-receptor activity [23], whereas fibroblast LDL receptors are very sensitive to down-regulation by even low concentrations (5 µg/ml) of LDL [24]. In common with HepG2 cells [15] and a specific strain of J774 macrophages [14], this appears to be the explanation for their relative resistance to down-regulation of receptors in response to extracellular concentrations of LDL. Should this occur *in vivo*, then it may explain why hepatic LDL receptors remain active [2-4] and hepatic cholesterol synthesis remains high [25] even in the presence of relatively high circulating plasma LDL concentrations.

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