Vol. 145, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 29, 1987 Pages 350-356

INHIBITION OF CELLULAR CHOLESTEROL ESTERIFICATION CAN DECREASE LOW DENSITY LIPOPROTEIN RECEPTOR NUMBER IN HUMAN FIBROBLASTS

Bruce Middleton

Dept. of Biochemistry, University of Nottingham Medical School, Queen's Medical Centre, Nottingham NG7 2UH, U.K.

Received January 20, 1987

In fibroblasts deprived of exogenous cholesterol to induce low density lipoprotein receptors there is a continuing flux of cholesterol esterification. The structurally unrelated inhibitors of acyl-CoA: cholesterol acyl-transferase, progesterone, trimethylcyclohexanyl mandelate and 3-[decyldimethylsilyl]-N-[2-(4-methylphenyl)-1-phenylethyl] propanamide, (58035), could all inhibit this basal rate of esterification within 1h of addition. Exposure of cholesterol-deprived fibroblasts for 17h to progesterone or trimethylcyclohexanyl mandelate caused decreased specific binding and metabolism of low density lipoprotein. The effect was not a direct inhibition of lipoprotein binding; it was time dependent and followed from the reversible inhibition of cholesterol esterification by these two compounds. The irreversible inhibition of esterification by 58035 left the receptor number unaffected. The results indicate that down regulation of low density lipoprotein receptors is initiated by accumulation of cholesterol in a specific intracellular pool. Inhibition of cholesterol esterification by progesterone and trimethylcyclohexanyl mandelate causes accumulation of cholesterol in this pool but 58035 does not.

Human skin fibroblasts from normal individuals show exquisitely fine control of their cholesterol metabolism. Goldstein and Brown [1] have shown how low density lipoprotein(LDL)-derived cholesterol can regulate LDL receptor number, stimulate cholesterol esterification and inhibit de novo cholesterol synthesis. Agents which could increase endogenous free cholesterol should similarly repress LDL binding and cholesterol synthesis. Such agents could include inhibitors of acyl-CoA: cholesterol acyl-transferase (ACAT) provided that cellular cholesterol esterification occurred at a significant rate in

Abbreviations: LDL, low density lipoprotein; ACAT, acyl-CoA: cholesterol acyl-transferase; 58035, 3-[decyldimethylsilyl]-N-[2-(4-methylphenyl)-1-phenylethyl] propanamide; TMCM, trimethylcyclohexanyl mandelate; LPDS, lipoprotein-deficient serum; FBS, foetal bovine serum; IC_{50}, concentration of inhibitor giving 50% inhibition; HDL, high density lipoprotein.

0006-291X/87 $1.50

relation to processes generating intracellular free cholesterol. Recently [2] it has been reported that ACAT inhibition in J774 macrophages caused LDL receptor down-regulation but that the effect was specific to macrophages and not found in human fibroblasts. The present study shows that only some inhibitors of cellular cholesterol esterification can cause down-regulation of LDL receptors in human skin fibroblasts.

MATERIALS AND METHODS

Trimethylcyclohexanyl mandelate (Cyclospasmol(c)) was a gift from Gist-Brocades n.v., The Netherlands. Progesterone was obtained from Sigma (U.K.) Ltd. 3-[Decyldimethylsilyl]-N-[2-(4-methylphenyl)-1-phenylethyl] propanamide (58035) was a gift from Sandoz Inc. U.S.A. Growth media and foetal bovine serum (FBS) were from Imperial Ltd. U.K. [9,10-^{3}H]-Oleic acid, [^{14}C]-cholesterol and [^{3}H]-H_2O were from Amersham International Plc. U.K. [^{14}C]-Cholesteryl oleate was synthesised as described in [3]. Lipoprotein-deficient serum (LPDS) and LDL were isolated from serum of normal individuals. [^{125}I]-LDL was prepared as described in [4] and was kindly given by Dr A.M.Salter of this department. Skin fibroblasts were from individuals with normal cholesterol metabolism and were grown in Eagle's MEM (alpha modification) supplemented with penicillin (100 units per ml), streptomycin (100µg per ml), glutamine (2mM) and FBS (10%,v/v) in an atmosphere of CO_2 and air (10:90,v/v). Lipoprotein-deficient(LPD) medium contained human LPDS (5mg per ml) in place of FBS. LDL receptors were induced in near confluent monolayers by culture for 2x24h in 2 lots of LPD-medium. Specific binding of LDL to high affinity receptors was then determined at 4° and 37° and metabolism of LDL was measured at 37°; all as described in [4]. Esterification of cellular cholesterol was measured after 48h in LPD-medium (low flux) or 17h after the addition of LDL (100µg per ml) to cells precultured for 31h in LPD-medium (high flux). Monolayers were then incubated with 100µM [9,10-^{3}H]-oleate bound to defatted bovine serum albumin (0.6 mg per ml) for 1h at 37°. The incubation was terminated, cholesteryl esters extracted, purified and counted for radioactivity exactly as described in [4].All inhibitors were added to growth media in ethanol solution to give final ethanol concentrations of 0.5%(v/v). Monolayers were freed of inhibitor-containing medium by washing with growth medium for 15 min at 37° before adding the appropriate assay medium. Use of [^{3}H]-H_2O showed that this procedure removed more than 99% of cell associated counts. Protein was measured by the Folin reagent [5].

RESULTS

This study used three ACAT inhibitors of differing structure: a steroid, progesterone [6]; an ester, trimethylcyclohexanyl mandelate (TMCM) [7] ; an N-acylamide, 3-[decyldimethylsilyl]-N-[2-(4-methylphenyl)-1-phenylethyl] propanamide (58035) [8]. These compounds exert their effects on esterification of cholesterol in intact cells with different potencies: progesterone, IC_{50} 10µM [6]; TMCM, IC_{50} 15µM [B.Middleton, unpublished data]; 58035, IC_{50} 0.1µM [8]. These results were all obtained at high rates of cholesterol

Table 1. Inhibition of cholesterol esterification at low and high flux rates and its reversal in human fibroblasts

Inhibitor	Cholesterol esterification rate (pmol/h/mg protein)		
	Low flux	High flux	High flux (inhibitor removed)
Control	93 ± 6	3961 ± 204	4240 ± 398
58035 (2μM)	35 ± 16**	235 ± 65**	415 ± 33**
Progesterone (100μM)	46 ± 5**	349 ± 28**	4512 ± 466
TMCM (100μM)	50 ± 13**	396 ± 66**	4150 ± 265

**P<0.01 with respect to control

Rates were determined in cells cultured 48h in LPD-medium alone (low flux rates), or after addition of LDL (high flux rates). ACAT inhibitors or ethanol were added 1h before assay and were present during the assay except where reversibility was tested. Results are means of 3 experiments ± SD.

esterification. The first object of this investigation was to seek evidence that the basal rate of cholesterol esterification observed in fibroblasts deprived of exogenous cholesterol was due to ACAT activity and thus inhibitable by these compounds. Table 1 shows that they caused significant inhibition within 1h of addition to cells previously exposed to lipoprotein-deficient medium for 48h. Inhibition ranged from 46% to 62% in cholesterol-deprived cells. This inhibition increased to >90% when esterification rates were raised 43-fold by inclusion of LDL in the medium (Table 1). All compounds were specific in their inhibition of cholesterol ester formation: no effect was observed on phospholipid or protein synthesis under the conditions of Table 1 over periods of 24h. The inhibition exerted by TMCM and progesterone was rapidly and completely reversed by washing the monolayers (Table 1) while inhibition by 58035 remained unaffected. This difference in reversibility indicates that TMCM and progesterone differ from 58035 in their mechanism of inhibition of ACAT.

To determine the effect of ACAT inhibition on expression of LDL receptors, cells were exposed to lipoprotein-deficient medium for at least 31h in absence of the inhibitors to allow full expression of LDL receptors. Inhibitors were then added to the medium 17h or 2h before subsequent measurement of LDL binding. Table 2 shows that exposure of up-regulated cells to TMCM or

Table 2. The effect of ACAT inhibitors on expression of LDL receptors in human fibroblasts

Inhibitors	LDL binding (ng LDL/mg protein) after culture with inhibitors for varying times		
	2h	17h	17h (inhibitor removed)
Control	123 ± 18	107 ± 20	122 ± 4
58035 (2μM)	127 ± 10	104 ± 8	nd
(100μM)	nd	129 ± 22	nd
Progesterone (100μM)	113 ± 6	40 ± 6**	43 ± 8**
TMCM (100μM)	124 ± 9	40 ± 9**	53 ± 8**

**P<0.01 with respect to control; nd = not determined.

LDL receptors were induced by 48h culture in LPD-medium prior to measurement of [^{125}I]-LDL binding at 4°. Inhibitors or ethanol were added to culture medium 17h or 2h before measurement of LDL binding and, unless indicated, were present during the assay. Results are means of 3 experiments ± SD.

progesterone for 17h resulted in the loss of 50-60% of LDL binding. This was not due to a direct effect of the compounds on LDL binding because a) no effect was seen when the compounds were added 2h before the [^{125}I]-LDL and b) inhibition persisted when the drugs were washed out after the 17h exposure but before the addition of [^{125}I]-LDL (Table 2). By contrast, the ACAT inhibitor 58035 did not inhibit LDL binding even at high concentrations (Table 2). The reduction of LDL binding at 4° by TMCM and progesterone was mirrored in their inhibition of binding and subsequent metabolism of [^{125}I]-LDL at 37° (Table 3): compound 58035 was again without effect.

Table 3. The effect of inhibitors of cholesterol esterification on metabolism of LDL by human fibroblasts

Inhibitor	Binding and metabolism of LDL at 37°		
	Binding (ng LDL/mg)	Internalization (ng LDL/mg)	Degradation (ng LDL/h/mg)
Control	210 ± 20	1156 ± 21	845 ± 115
58035 (2μM)	256 ± 95	1332 ± 188	928 ± 99
Progesterone (100μM)	86 ± 16**	561 ± 20**	307 ± 11**
TMCM (100μM)	115 ± 20**	576 ± 102**	621 ± 15*

**P<0.01, *P<0.05 with respect to control

Cells were exposed to inhibitors or ethanol for only the last 17h of culture with LPD-medium (see Table 2). Inhibitors were removed before measuring metabolism and binding of [^{125}I]-LDL (10μg/ml) at 37°. Results are means of 3 experiments ± SD.

DISCUSSION

Reversible inhibition of cholesterol esterification by the ACAT inhibitors progesterone and TMCM caused down regulation of LDL binding and decreased LDL metabolism in human fibroblasts. This effect explains the observation [9] that progesterone abolished the LDL-mediated suppression of HMG-CoA reductase activity in human cells. Lack of effect of 58035 on LDL expression confirms the report of Tabas et al. [2] and indicates that this irreversible inhibitor of ACAT acts at a different site to progesterone and TMCM. The LDL receptor has a $t_{1/2}$ of 12-20h [10] and so the time course of inhibition of LDL binding by progesterone or TMCM is compatible with an inhibition of LDL receptor synthesis. By blocking the esterification of cellular cholesterol, these compounds (Table 1) should increase the concentration of free cholesterol causing repression of the synthesis of the LDL receptor. These effects are predicted by the work of Goldstein and Brown [1] subject to the proviso that cholesterol esterification continues in fibroblasts even in the absence of an influx of LDL. The existence of such basal esterification rates has been reported in normal fibroblasts deprived of lipoprotein and in cells lacking functional LDL receptors [11]. ACAT has also been shown to be active in extracts of such fibroblasts [12]. The data of Table 1 shows that ACAT activity is responsible for at least 50% of the basal rate of cholesterol esterification observed in cholesterol-deprived fibroblasts.

Unlike progesterone and TMCM, 58035 failed to affect LDL receptor numbers in spite of its very potent inhibition of ACAT (Table 1). This apparent discrepancy suggests that there may be two separate pools of free cholesterol in microsomal membranes (as in Fig. 1), with only one of these, pool A, exerting a regulatory effect on the number of LDL receptors. Thus progesterone and TMCM could increase the amount of cholesterol in this regulatory pool by inhibiting the transfer of cholesterol from pool A to the ACAT substrate pool (B), whereas 58035 could interact only with the ACAT active centre. Aulinskas and Oram [13] have reported that ACAT inhibition by 58035 increases the efflux

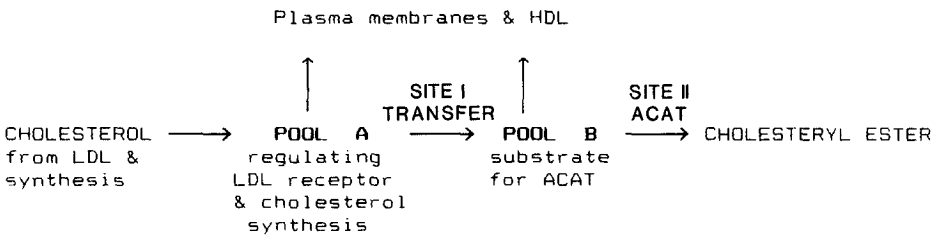

Figure 1. Proposed microsomal pools of free cholesterol and sites for inhibition of cholesterol esterification.
It is suggested that 58035 acts at site II while progesterone and TMCM act at site I. All compounds inhibit cholesterol esterification in vivo and microsomal ACAT activity in vitro. LDL receptor down-regulation in fibroblasts results from expansion of cholesterol pool A after blockage of esterification by progesterone and TMCM.

of cholesterol from fibroblasts via the HDL receptor, so that the excess cholesterol in pool B may be preferentially directed to efflux rather than back into pool A. It is significant that two pools of free cholesterol have been identified in liver microsomal fractions [14] and that progesterone has been shown to inhibit ACAT by action distant from its active centre [15].

As predicted by Fig.1, inhibition of esterification by TMCM also results in decreased supply of [^{3}H]-cholesterol from reconstituted [^{3}H]-cholesteryl linoleate-LDL [7], and in decreased synthesis of cholesterol in liver [16] and fibroblasts [7]. Progesterone has no effect on cholesterol synthesis because it also inhibits oxysterol formation via cytochrome P-450 [17] thereby preventing the accumulated free cholesterol repressing HMG-CoA reductase. Since both progesterone and TMCM cause identical repression of LDL binding, cytochrome P-450-dependent modification of cholesterol [18] may not be required for regulation of LDL receptors.

In conclusion, inhibition of cellular cholesterol esterification in fibroblasts has been shown to cause decreased expression of LDL receptors in an analogous manner to the effect of added LDL or exogenous cholesterol. It is proposed that ACAT inhibitors progesterone and TMCM cause an accumulation of cholesterol in a distinct pool (A in Fig. 1) which regulates cholesterol influx in fibroblasts whereas excess cholesterol resulting from inhibition by

58035 accumulates in a pool which does not control LDL receptor expression in human skin fibroblasts.

REFERENCES

1. Goldstein,J.L. and Brown,M.S. (1977) Annu. Rev. Biochem. 46, 897-930.
2. Tabas,I., Wieland,D.A. and Tall,A.R. (1986) J. Biol. Chem. 261, 3147-3155.
3. Goodman,DeW.S. (1969) Methods Enzymol. 15, 522-535.
4. Goldstein,J.L., Basu,S.K. and Brown,M.S. (1983) Methods Enzymol. 98, 241-260.
5. Lowry,O.H., Rosebrough,N.J., Farr,A.L. and Randall,R.J. (1951) J. Biol. Chem. 193, 265-275.
6. Goldstein,J.L., Faust,J.R., Dygos,J.H., Chorvat,R.J. and Brown,M.S. (1978) Proc. Natl. Acad. Sci. USA 75, 1877-1881.
7. White,D.A., Bell,G.D. and Middleton,B. (1986) in Pharmacological Control of Hyperlipidaemia (Fears,R., Levy,R.I., Shepherd,J. and Miller,N.E. eds.) J.R.Prous, Barcelona, Spain, pp. 389-399.
8. Ross,A.C., Go,K.J., Heider,J.G. and Rothblat,G.H. (1984) J. Biol. Chem. 259, 815-819.
9. Gal,D., MacDonald,P.C. and Simpson,E.R. (1981) J. Clin. Endocrinol. Metab. 53, 29-33.
10. Brown,M.S. and Goldstein,J.L. (1975) Cell 6, 307-316.
11. Goldstein,J.L., Dana,S.E. and Brown,M.S. (1974) Proc. Natl. Acad. Sci. USA 71, 4288-4292.
12. Gavigan,S.J.P. and Knight,B.L. (1983) Biochem. J. 216, 93-100.
13. Aulinskas,T.H. and Oram,J.F. (1984) Arteriosclerosis 4, 535a.
14. Synouri-Vrettakou,S. and Mitropoulos,K.A. (1983) Eur. J. Biochem. 133, 299-307.
15. Synouri-Vrettakou,S. and Mitropoulos,K.A. (1983) Biochem. J. 215, 191-199.
16. Middleton,B., Middleton,A., Miciak,A., White,D.A. and Bell,G.D. (1983) Biochem. Pharmacol. 32, 649-651.
17. Sexton,R., Gupta,A., Shertzer,H. and Rudney,H. (1986) J. Lipid Res. 27, 567.
18. Gupta,A., Sexton,R.C. and Rudney,H. (1986) J. Biol. Chem. 261, 8348-8356.