Action of low-density lipoprotein and compactin, a competitive inhibitor of 3-hydroxy-3-methylglutaryl-CoA reductase, on the synthesis of dolichol-linked oligosaccharides and low-density-lipoprotein receptor in human skin fibroblasts

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To explore whether there is an inter-relationship between the rate of low-density (LD) lipoprotein binding to its receptor and the formation of dolichol-linked oligosaccharides, experiments were performed with human fibroblasts where the synthesis of lipoprotein receptor and dolichyl saccharides was under control of LD lipoprotein and compactin. Pretreatment of the cells with nonlabelled LD lipoprotein resulted in a suppression of both the binding of ^{125}I -labelled LD lipoprotein to the receptor and the synthesis of dolichyl saccharides from ^{14}C acetate and ^3H mannose, but not from ^3H mevalonolactone. Compactin, in contrast, inhibited only the formation of dolichol-linked oligosaccharides. Mevalonolactone ($1\text{ }\mu\text{M}$) abolished the inhibitory effect of LD lipoprotein on dolichyl saccharide formation, but was not able to restore the receptor-binding capacity, thus suggesting that the synthesis of lipoprotein receptor is not coupled to the formation of dolichyl saccharides.

Cultured human skin fibroblasts have been shown to possess receptor sites on their surface that specifically bind LD lipoprotein (Goldstein & Brown, 1974). The fine chemical structure of these receptors is not known as yet but has been proposed to be a glycoprotein (Basu *et al.*, 1978; Chatterjee *et al.*, 1978; DiFerrante *et al.*, 1978). Tunicamycin, which is known to interfere specifically with the synthesis of *N*-glycosidically linked oligosaccharides (Tkacz & Lampen, 1975; Struck & Lennarz, 1977) was found to strongly reduce the receptor-mediated binding and uptake of LD lipoprotein in human skin fibroblasts (Chatterjee *et al.*, 1979; Filipovic & von Figura, 1980). This leads to the conclusion that the expression of LD lipoprotein receptor on the cell surface depends on the synthesis of glycoproteins.

The synthesis of LD lipoprotein receptor in cultured cells is under feed-back control of LD lipoprotein in the surrounding medium (Brown & Goldstein, 1975). LD lipoprotein also regulates HMG-CoA reductase (Brown *et al.*, 1978). This microsomal enzyme catalyses the formation of

mevalonate, which is the common metabolic precursor not only of cholesterol, but also of non-sterol polyprenoid compounds such as ubiquinone-10 and dolichols (Mills & Adamany, 1978; Faust *et al.*, 1979; Nambudiri *et al.*, 1980). The latter have been shown to be involved in the synthesis of glycoproteins by transferring oligosaccharides to the polypeptide chain (Struck & Lennarz, 1977). Because of the function of dolichol in the formation of cellular glycoproteins, a study was undertaken to explore whether there is a possible connection between the formation of dolichol-linked oligosaccharides and the binding capacity of LD lipoprotein receptor. To establish a metabolic state where only the formation of mevalonate is suppressed we used compactin, a potent competitive inhibitor of HMG-CoA reductase. This inhibitor (also called ML-236B) is a product of several fungal strains, including *Penicillium brevicompactum* (Brown *et al.*, 1976) and *Penicillium citrinum* (Endo *et al.*, 1976). The structure of compactin resembles the structure of the lactone form of mevalonic acid, the product of the HMG-CoA reductase reaction. At low concentrations compactin blocks cholesterol synthesis in cultured cells, but does not suppress the activity of the LD-lipoprotein receptor (Brown *et al.*,

Abbreviations used: LD lipoprotein, low-density lipoprotein; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA.

1978). Results obtained indicate that under the experimental conditions used the expression of LD lipoprotein receptor on the cell surface is not coupled to the synthesis of dolichol-linked oligosaccharides.

Experimental

Materials

[1-¹⁴C]Acetic acid, sodium salt (sp. radioactivity 54 mCi/mmol), [2-³H]mannose (sp. radioactivity 4.1 Ci/mol), DL-[2-³H]mevalonic acid lactone (sp. radioactivity 745 mCi/mmol) and ¹²⁵I (carrier free) were obtained from The Radiochemical Centre. Compactin was the gift of Dr R. B. Fears of Beecham Pharmaceuticals.

Human LD lipoprotein (density 1.02–1.055 g/ml) was isolated and labelled with ¹²⁵I as described previously (Filipovic *et al.*, 1979). Lipoprotein-deficient serum was obtained by centrifugation of human serum at density of 1.22 g/ml.

Human skin fibroblasts were cultured from skin explant of a healthy donor. Cells were grown in plastic dishes in Eagles' minimal essential medium supplemented with 10% (v/v) foetal calf serum (LS Labor Service, München, Germany) as described by Cantz *et al.* (1972).

Methods

All experiments were performed with cells grown to confluency in 25 cm² plastic dishes. On days 5–8 the growth medium was replaced by 3 ml of fresh medium containing 10% (v/v) lipoprotein-deficient human serum, 25 µg of LD lipoprotein/ml or 2 µg of compactin/ml or both. After 24 h the medium was replaced by 3 ml of fresh medium containing 5 µg of ¹²⁵I-labelled LD lipoprotein/ml, 20 µCi of [1-¹⁴C]-acetate/ml, 50 µCi of [2-³H]mannose/ml or 50 µCi of [2-³H]mevalonolactone/ml and the incubation was continued for further 6 h. Then cells were washed eight times with ice-cold buffered saline containing 0.2% bovine albumin. Binding plus uptake of LD lipoprotein were determined according to the method described by Goldstein & Brown (1974). High-affinity binding was determined by incubating the cells with labelled LD lipoprotein either in the absence or presence of 350 µg of non-labelled LD lipoprotein/ml and by subtracting the radioactivity bound and incorporated in the presence of non-labelled LD lipoprotein from that obtained in the absence of non-labelled lipoprotein. For determination of ¹⁴C and ³H incorporation into dolichyl saccharide, glycopeptide and cholesterol, cells were scraped off with a rubber 'policeman' and collected by centrifugation at 800 g in a laboratory centrifuge. Radioactive lipids were extracted from the cell pellet according to the procedure described by Schmitt & Elbein (1979). Dolichol-linked oligosaccharides were then chromatographed on a

column (18 cm × 1.9 cm) of DEAE-cellulose equilibrated with chloroform/methanol/water (1:1:0.3 by vol.). Trace amounts of neutral lipids and cholesterol were eluted with 75 ml of chloroform/methanol/water (1:1:0.3 by vol.) and dolichyl saccharide with 75 ml of 100 mM-ammonium acetate in chloroform/methanol/water (1:1:0.3 by vol.). The delipidated cellular protein was digested with Pronase (Calbiochem) and glycopeptides were separated on Bio-Gel P-6 as described by Mills & Adamany (1978). Cholesterol was purified by t.l.c. on silica gel G plates in the solvent system hexane/diethyl ether/acetic acid (60:40:1 by vol.). Lipid fractions were visualized by iodine vapour and the cholesterol fraction was scraped off for counting. Quench corrections were performed by co-chromatography of small amounts of [³H]- or [¹⁴C]cholesterol. Protein was determined according to the method described by Kaltwasser *et al.* (1965).

Results and discussion

In order to ascertain whether the inhibitory effect of LD lipoprotein on its own receptor could be a result of an insufficient synthesis of dolichol-linked saccharides and hence of glycoproteins, we established a protocol in which human skin fibroblasts were preincubated with LD lipoprotein or compactin so as to block the endogenous synthesis of mevalonate.

The data listed in Table 1 shows that a 24 h preincubation of the cells in the presence of 25 µg of LD lipoprotein/ml resulted in a decrease of the binding and internalization of ¹²⁵I-labelled LD lipoprotein by about 60%, whereas the incorporation of [¹⁴C]acetate and [³H]mannose into dolichyl saccharide and glycopeptide was diminished only by 25–30%. Cholesterol synthesis from [¹⁴C]-acetate was suppressed to 10% of the control values. Compactin (2 µg/ml) was found to be a stronger inhibitor of radioactivity incorporation into dolichyl saccharides and cholesterol than was LD lipoprotein, but compactin enhanced the binding and uptake of ¹²⁵I-labelled LD lipoprotein by the cells. However, this effect was almost totally abolished when the cells were preincubated with compactin along with LD lipoprotein. This indicates that the observed stimulation of the lipoprotein binding to the cells can not be attributed to compactin itself but to the abolition of the feed-back inhibition of receptor by cholesterol. The fact that the receptor activity is enhanced even though compactin suppresses the synthesis of dolichyl saccharides and glycoproteins is remarkable. A possible explanation for this phenomenon is that the newly synthesized receptor is only partly glycosylated and still able to fulfil its functional role. In a recent communication (Filipovic & von Figura, 1980) we showed that treatment of the cells with

Table 1. *Effect of LD lipoprotein and compactin on the binding and internalization of 125 I-labelled LD lipoprotein and on the synthesis of cholesterol, dolichol-linked oligosaccharides and glycopeptide in human skin fibroblasts*

On day 6 the growth medium was replaced by fresh medium containing 10% (v/v) lipoprotein-deficient human serum and the indicated concentrations of non-labelled LD lipoprotein or compactin. After 24 h the medium was replaced by fresh medium containing 5 μ g of 125 I-labelled LD lipoprotein, 20 μ Ci of [14 C]acetate, 50 μ Ci of [3 H]mevalonolactone or 50 μ Ci of [3 H]mannose/ml and the incubation was prolonged for additional 6 h. The binding and internalization of LD lipoprotein and the incorporation of 14 C and 3 H radioactivity into cholesterol, dolichyl saccharides and glycopeptide were determined as described in the Methods section. Data are mean values ($\pm$ S.D.) for four experiments.

Addition (μ g/ml)	Binding and uptake of 125 I-labelled LD lipoprotein (ng of LD lipoprotein/6 h per mg of cell protein)	$10^{-3} \times$ Amount of radioactivity incorporated (c.p.m./6 h per mg of cell protein) from						
		[1- 14 C]Acetate		[3 H]Mevalonolactone		[3 H]Mannose		
		Dolichyl-saccharide	Cholesterol	Dolichyl-saccharide	Cholesterol	Dolichyl-saccharide	Glycopeptide	
Com- LDL	0	580 $\pm$ 46	2.53 $\pm$ 0.36	417.65 $\pm$ 51	0.61 $\pm$ 0.07	114.78 $\pm$ 10	19.24 $\pm$ 1.8	392.16 $\pm$ 43
0	25	203 $\pm$ 19	1.86 $\pm$ 0.23	38.17 $\pm$ 4	1.66 $\pm$ 0.18	50.32 $\pm$ 6	14.35 $\pm$ 1.2	289.53 $\pm$ 31
0	0	754 $\pm$ 83	1.18 $\pm$ 0.16	29.53 $\pm$ 4	0.97 $\pm$ 0.09	98.16 $\pm$ 11	9.56 $\pm$ 0.8	271.86 $\pm$ 27
25	2	249 $\pm$ 27	1.42 $\pm$ 0.16	16.48 $\pm$ 2	1.84 $\pm$ 0.25	37.43 $\pm$ 4	8.41 $\pm$ 1.1	225.14 $\pm$ 25

Table 2. *Binding and internalization of 125 I-labelled LD lipoprotein and the incorporation of [3 H]mannose into dolichol-linked oligosaccharides and glycopeptide in human skin fibroblasts as influenced by non-labelled LD lipoprotein and exogenous mevalonolactone*

Incubation conditions are the same as in Table 1. Data are mean values ($\pm$ S.D.) for three experiments performed in duplicate. Numbers in parentheses represent the percentage of activity of control cells.

Addition		Binding and uptake of 125 I-labelled LD lipoprotein (ng of LD lipoprotein/6 h per mg of cell protein)	$10^{-3} \times$ [3 H]Mannose incorporation (c.p.m./6 h per mg of cell protein) into	
LDL (μ g/ml)	Mevalonolactone (μ M)		Dolichyl saccharide	Glycopeptide
0	0	601 $\pm$ 54 (100)	18.79 $\pm$ 2.1 (100)	386.85 $\pm$ 45 (100)
25	0	218 $\pm$ 26 (36)	13.52 $\pm$ 1.6 (72)	271.43 $\pm$ 31 (70)
25	0.5	204 $\pm$ 17 (34)	16.22 $\pm$ 1.7 (86)	317.77 $\pm$ 38 (82)
25	1.0	183 $\pm$ 16 (30)	18.11 $\pm$ 1.9 (96)	394.56 $\pm$ 41 (102)

tunicamycin resulted in an inhibition of [3 H]-mannose incorporation into glycoproteins by 90%, whereas the binding capacity of LD-lipoprotein receptor was diminished only by 50% as compared with control cells.

To trace the metabolic fate of mevalonate we used small amounts of [3 H]mevalonolactone. When the experiments were performed in the absence of LD lipoprotein and compactin the bulk of the lipid-associated radioactivity was found in cholesterol. In the cells pretreated with lipoprotein or compactin the total radioactivity incorporation into lipids was diminished and the relative proportion of the radioactivity in cholesterol and dolichyl saccharides was altered. Compactin, and especially LD lipoprotein, stimulated [3 H]mevalonolactone incorporation preferentially into dolichol instead of incorporation into cholesterol as occurs under normal conditions. The reciprocal changes in dolichol and cholesterol synthesis from mevalonolactone caused by LD lipoprotein suggest that the

point of regulation of cholesterol synthesis is located above farnesyl pyrophosphate, which is the last common intermediate in cholesterol and dolichol synthesis. Faust *et al.* (1979) reported that LD lipoprotein added to human skin fibroblasts suppressed squalene synthetase by about 90%. Inhibition of this enzyme establishes a metabolic state where the intracellular concentration of farnesyl pyrophosphate is enhanced, thus favouring the synthesis of nonsterol polyprenoids such as co-enzyme Q and dolichols. This might be the reason for the relatively low inhibitory effect of LD lipoprotein on dolichol synthesis from labelled acetate observed in our experiments. Considering the fact that under these conditions the formation of mevalonate is limited, one can assume that the rate-limiting enzyme in the dolichol pathway is saturated by a relatively low level of farnesyl pyrophosphate. This assumption might be supported by the studies of Gold & Olson (1966) on incorporation of [3 H]mevalonate into cholesterol

and coenzyme Q, showing an apparent K_m for cholesterol and coenzyme Q of 1 mM and 0.2 mM, respectively.

To explore whether a restored synthesis of dolichyl saccharides and glycopeptide would result in a normalization of lipoprotein binding and internalization we incubated cells in the presence of LD lipoprotein along with exogenous mevalonolactone. As shown in Table 2 1 μ M-mevalonolactone was able to restore the synthesis of dolichyl saccharides and glycopeptide to the normal levels, but intensified the suppression of LD lipoprotein receptor. This finding indicates that the suppression of LD lipoprotein receptor by LD lipoprotein cannot be due to the concomitant slight inhibition of the synthesis of dolichol-linked oligosaccharides and hence of glycoproteins.

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