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The effect of non-receptor-mediated uptake of cholesterol on intracellular cholesterol metabolism in human skin fibroblasts

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Unilamellar lipid vesicles of various cholesterol:phosphatidylcholine molar ratios were used to alter, via passive exchange at the plasma membrane, the cellular free cholesterol content of cultured human skin fibroblasts which had been preincubated in lipoprotein-deficient serum. The effects of these net surface transfers of cholesterol on cellular cholesterol biosynthesis, cholesterol esterification and low density lipoprotein (LDL) binding were determined and were compared with the effects of cholesterol delivered to the cell interior via the receptor-mediated endocytosis of LDL. Both LDL and cholesterol-rich lipid vesicles increased cell cholesterol within 6 h. Cells exposed to LDL also showed, within 6 h, decreased cholesterol synthesis, decreased LDL binding and increased cholesterol esterification. Cells incubated with the cholesterol-rich vesicles showed similar changes but these were delayed and did not occur until 24 h. Fibroblasts incubated with cholesterol-free phosphatidylcholine vesicles had decreased cell cholesterol, increased cholesterol synthesis, increased LDL binding, and decreased esterification, but only after 24 h of incubation. These results suggest that passive net transfers of cholesterol occurring at the cell surface can with time modulate intracellular cholesterol metabolism. These findings are consistent with the idea that the movement of cholesterol from the cell surface to the cell interior is a limited and relatively slow process.

INTRODUCTION

The factors which determine the movement of cholesterol between cells and serum are complex. LDL can efficiently deliver free cholesterol and cholesteryl ester to cultured cells such as fibroblasts via an energy-dependent, receptor-mediated endocytic pathway (Brown & Goldstein, 1979). The internalization and lysosomal degradation of LDL delivers free cholesterol to the cell and has several important metabolic consequences: (1) a suppression of cholesterol synthesis *de novo*, achieved through a reduction in the activity of the microsomal enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase, (2) a stimulation of cholesterol esterification, achieved through an increase in the activity of the microsomal enzyme acyl-CoA:cholesterol acyl-transferase, and (3) a decrease in the synthesis and thus the number of LDL receptors.

Free cholesterol can also move between a variety of lipid membranes via a passive, surface transfer process that does not require metabolic energy. Cholesterol exchange has been shown to occur between lipid vesicles and human skin fibroblasts (Poznansky & Czekanski, 1982) or vesicles and rat arterial smooth muscle cells (Slotte & Lundberg, 1983*a*). It has also been demonstrated that free cholesterol in the outer shell of LDL will exchange with the cellular cholesterol of fibroblasts (Shireman & Remsen, 1982; Goldstein *et al.*, 1979) and adipocytes (Angel *et al.*, 1981).

Net movements of cholesterol between model lipid and various biological membranes can also occur via passive exchange. For relatively simple systems, such as lipid vesicles or erythrocytes, the driving force for these net transfers appears to be related to the difference in the cholesterol:phospholipid (C/P) molar ratios of the donor and acceptor membranes (Lange & D'Alessandro, 1977). For more physiological systems, involving metabolically active cells cultured in serum-containing medium, the situation is complicated by the LDL receptor pathway and by potential sterol fluxes occurring via exchange to or from serum components. The contribution of these kinds of non-receptor-mediated surface transfer movements of cholesterol to the overall sterol flux between serum lipoproteins and cells remains unclear, but various studies have suggested that LDL cholesterol may move to cells independently of the lipoprotein particle both *in vitro* (Shireman & Remsen, 1982) and *in vivo* (Portman *et al.*, 1980). Also it has been proposed that high density lipoprotein may be involved in the removal of cholesterol from peripheral tissues (Glomset, 1968), possibly via some sort of surface transfer process. This hypothesis has been intensively investigated (for a review see Tall & Small, 1980).

In the present study we have investigated the role of non-receptor-mediated movements of cholesterol in the regulation of cholesterol homeostasis in cultured human skin fibroblasts. In order to avoid the complicating additional fluxes of cholesterol occurring via the LDL receptor-mediated uptake mechanism we have carried out these experiments in the absence of serum. We have used cholesterol/phospholipid vesicles of various compositions to alter the cellular cholesterol content, and then have examined the effects on cellular cholesterol synthesis, cholesterol esterification, and LDL binding.

MATERIALS AND METHODS

Human skin fibroblasts

Normal human skin fibroblasts of the Detroit 551 line (American Type Culture Collection, Rockville, MD, U.S.A.) were cultured in monolayer in Eagle's minimum

Abbreviations used: LDL, low density lipoprotein; C/P, cholesterol:phospholipid molar ratio; LPDS, lipoprotein-deficient serum.

essential medium (MEM) supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), 24 mM-$NaHCO_3$ and 10% (v/v) fetal bovine serum. The cells were plated at a density of 5×10^5 cells/25 cm^2 flask on day 0, and fresh medium was added on day 3. On day 6 the growth medium was replaced with medium which contained 10% (v/v) human LPDS and the confluent cell cultures were used 24 h later on day 7.

Lipoproteins

Human LDL (density 1.019–1.063 g/ml) and human LPDS (density > 1.215 g/ml) were prepared by differential density ultracentrifugation of human plasma obtained from healthy volunteers (Hatch & Lees, 1968). The isolated fractions were dialyzed against 0.15 M-NaCl/0.3 mM-EDTA (pH 7.4) and sterilized by filtration prior to use. Protein concentrations were determined by the method of Lowry *et al.* (1951). ^{125}I-LDL was prepared by the iodine monochloride method of Macfarlane as modified by Bilheimer *et al.* (1972). $Na^{125}I$ (carrier free) was obtained from Edmonton Radiopharmaceutical Center (Edmonton, Alberta, Canada).

Lipid vesicles

To prepare unilamellar vesicles, various amounts of cholesterol were added to 10 mg of egg phosphatidylcholine (Sigma) to produce vesicles with C/P ratios of 0, 0.6, and 2. The lipids were co-lyophilized from benzene, dispersed in 10 ml of Eagle's minimum essential medium and sonicated at 4 °C under N_2 with a Branson W185 probe sonicator as described previously (Poznansky & Czekanski, 1982). For some experiments, tracer amounts of [^{14}C]cholesteryl oleate (New England Nuclear) were added to the lipids prior to lyophilization.

Cholesterol and phosphate determinations

Cell lipids were extracted according to the procedure of Bligh & Dyer (1959), and lipid phosphorus was determined as described by Chalvardjian & Rudnicki (1970). Free cholesterol was quantified on a Hewlett Packard 5730A gas chromatograph with 3% OV-17 columns operated isothermally at 250 °C and with 5α-cholestane as an internal standard.

Incubation of cells with lipid vesicles

On day 7 following a 24 h incubation in minimum essential medium containing 10% LPDS, cell monolayers were washed with Dulbecco's phosphate buffered saline and the medium was replaced with 2 ml of minimum essential medium which contained the lipid vesicles. The final concentration of vesicle phospholipid was 0.5 mg/ml, which was a 10–15-fold excess over cell phospholipid. The cells were incubated in the presence of the vesicles for 6 or 24 h at 37 °C before determinations of LDL binding, cholesterol synthesis and cholesterol esterification were made.

Acetate incorporation into cholesterol

After the experimental incubation of 6 or 24 h, 10 μCi of [^{14}C]acetate (New England Nuclear) was added to each flask and the incubation was continued at 37 °C for 2 h. The cells were washed with phosphate-buffered saline and were harvested from the flasks with 0.25% trypsin. The lipids were extracted and the radiolabelled cholesterol was isolated by t.l.c. on Sil G plastic backed plates (Brinkmann Canada, Rexdale, Ontario, Canada) which were developed in hexane/diethyl ether/acetic acid (85:20:2, by vol.). The incorporation of labelled acetate into cholesterol was determined by liquid-scintillation counting of the cholesterol spot and was corrected for total cell protein.

Oleate incorporation into cholesteryl esters

Cell monolayers were incubated with 10 μCi of [^{3}H]oleate for 4 h at 37 °C. The cells were harvested as above, their lipids were extracted and were chromatographed on Sil G plates. The incorporation of labelled oleate into cholesteryl esters was determined by liquid-scintillation counting of the cholesteryl ester spot.

Binding of LDL

The binding of ^{125}I-LDL was determined as described by Brown & Goldstein (1975). Following the experimental incubations, the medium was removed and the monolayers were washed with cold Dulbecco's phosphate-buffered saline. Then 2 ml of cold Eagle's minimum essential medium containing 10% LPDS and 5 μg of ^{125}I-LDL/ml were added to each flask and the flasks were incubated with rotary shaking at 4 °C for 2 h. The medium was then removed and the monolayers were washed extensively as described. After addition of 1 ml of 0.1 M-NaOH to the cells, samples were removed for determination of cell protein and of ^{125}I-LDL radioactivity (in a Beckman Gamma 4000 counter). All ^{125}I-LDL binding data were corrected for non-specific binding which was determined from the binding assay done in the presence of 250 μg of unlabelled LDL/ml.

Statistical analysis of the data

The data were analysed by a one-way analysis of variance and statistical significance was determined by the Duncan–Bonnor test (Duncan, 1955). Statistical significance was accepted if the P value was < 0.05.

RESULTS

Fibroblast monolayers were incubated for 24 h in Eagle's minimum essential medium containing 10% LPDS. The cells were then exposed for 6 or 24 h to medium containing LDL, or lipid vesicles with C/P ratios of 0, 0.6 or 2. Cell viability was assessed by exclusion of 0.1% nigrosin and remained > 95% for all incubations up to 24 h. Cell protein averaged 250 μg/flask and this did not vary significantly with different incubation conditions. To ensure that cholesterol movements from the vesicles into the cells occurred via exchange at the plasma membrane rather than via fluid phase pinocytosis or vesicle–cell fusion, we carried out control incubations with vesicles containing tracer amounts of [^{14}C]cholesteryl oleate, a non-exchangeable marker. Even with prolonged incubations of up to 24 h, less than 0.1% of this vesicle marker was associated with the cells.

Table 1 illustrates the effects of the various incubation media on the cholesterol content of the fibroblasts. The results are similar whether expressed as cholesterol/mg of protein or as a C/P ratio. The stock cells cultured in 10% fetal bovine serum had an average of 31.7 μg of cholesterol/mg of cell protein, corresponding to a C/P of 0.421. The 24 h preincubation of the cells in minimal essential medium containing 10% LPDS significantly reduced these values to 26.3 μg of cholesterol/mg of protein with C/P = 0.316. Adding LDL (80 μg/ml) back

Table 1. Cholesterol content of fibroblasts following incubation with media of various compositions

Fibroblasts were grown in 10% fetal calf serum as described in the Materials and methods section and then preincubated for 24 h in medium containing 10% LPDS. The medium was then removed and replaced with medium containing either 80 μg of LDL/ml or 0.5 mg of lipid vesicles/ml composed of egg phosphatidylcholine or cholesterol and egg phosphatidylcholine with a C/P of 0.6 or 2. The cells were incubated at 37 °C for 6 or 24 h before determinations were made. Values are means ± S.E.M. with the number of experiments indicated in parentheses: *significantly different from control (LPDS), $P < 0.01$; †significantly different from control (LPDS), $P < 0.05$.

Addition to the medium		Cholesterol content (μg/mg of protein)	C/P
Fetal calf serum		31.7 ± 0.9* (7)	0.421 ± 0.028* (7)
LPDS		26.3 ± 0.4 (10)	0.316 ± 0.006 (10)
LDL	6 h	37.8 ± 2.1* (4)	0.414 ± 0.015* (5)
	24 h	30.1 ± 0.4† (4)	0.318 ± 0.007 (5)
Egg phosphatidylcholine vesicles	6 h	27.1 ± 1.2 (4)	0.314 ± 0.006 (4)
	24 h	21.3 ± 1.3* (4)	0.220 ± 0.019* (4)
C/P = 0.6 vesicles	6 h	28.3 (2)	0.326 ± 0.052 (4)
	24 h	28.0 ± 0.4 (4)	0.316 ± 0.013 (5)
C/P = 2 vesicles	6 h	33.0 (2)	0.364 ± 0.016 (3)
	24 h	31.7 ± 1.3* (4)	0.367 ± 0.012 (5)

to the cells resulted in a rapid increase in cell cholesterol after 6 h which returned the cells to a similar cholesterol content as when grown in whole serum. Incubation of cells with egg phosphatidylcholine vesicles had no significant effect on fibroblast cholesterol content after 6 h but after 24 h the cell cholesterol was significantly reduced. Lipid vesicles composed of cholesterol and egg phosphatidylcholine at C/P = 0.6 ratio had no significant effect on fibroblast cholesterol content, even after a 24 h incubation. However incubations of cells with cholesterol-rich (C/P = 2) vesicles rapidly increased the cell cholesterol to about 32 μg/mg of protein, a value similar to that for fibroblasts grown with whole serum or incubated in a medium containing LDL.

Fig. 1 demonstrates the effect of the incubation with various media on the cholesterol synthesis activity of the fibroblasts. The results are expressed as a percentage of the activity of the control cells incubated in LPDS. When LDL was added back to the cells, cholesterol synthesis declined rapidly to 15% of the control value after 6 h. This rapid down-regulation of cholesterol synthesis by LDL was no doubt due to the receptor-dependent pathway described by Brown *et al.* (1974). When egg phosphatidylcholine vesicles were incubated with the cells, cholesterol synthesis increased slightly after 6 h and then increased to almost twice the control value after 24 h. Under the conditions of this incubation, we have shown a decrease in the fibroblast cholesterol content (Table 1) which we believe occurred as a result of a net efflux of cholesterol from the cell plasma membrane to the egg phosphatidylcholine vesicles. We interpret the stimulation of endogenous cholesterol synthesis to be a consequence of this efflux and the resulting decrease in cell cholesterol content. Incubation of the fibroblasts with vesicles containing cholesterol at C/P = 0.6 ratio resulted in a small but significant decrease in cholesterol synthesis after 24 h to about 65% of control values. In the case of the cholesterol-rich (C/P = 2) vesicles the synthesis activity was significantly reduced to less than 10% of the control after 24 h. Under these experimental conditions we expect that cholesterol is delivered to the fibroblasts via exchange from the vesicle to the plasma membrane. This resulted in the increase in cell cholesterol shown in Table 1, and it appears that these cells respond by down-regulating their endogenous cholesterol production. Using a similar protocol, Slotte & Lundberg (1983*b*) have also demonstrated decreased cholesterol synthesis in rat aortic smooth muscle cells. The fact that the C/P = 0.6 vesicles showed some limited effect on cholesterol synthesis probably indicates that these vesicles may also have some ability to deliver cholesterol to the cells.

Fig. 2 demonstrates the effects of the incubation of cells with either LDL or vesicles on the cholesterol esterification activity of the fibroblasts. When LDL was added to the cells, esterification activity (measured as the incorporation of [^{14}C]oleate into cholesteryl ester) was increased 3-fold after 6 h but had returned after 24 h to values not significantly different from control. This has been previously demonstrated for the receptor-mediated LDL pathway (Goldstein *et al.*, 1974). After 6 h, none of the different vesicles showed any significant effect on the fibroblast esterification activity. But after 24 h of incubation, the various vesicles had exerted significant effects which appear to be directly related to the cholesterol content of the vesicles. The egg phosphatidylcholine vesicles decreased esterification activity, the C/P = 0.6 vesicles had no effect, and the cholesterol-rich C/P = 2 vesicles markedly stimulated the esterification activity by 2.5-fold. These data suggest that cholesterol delivered in net amounts to the plasma membrane can within 24 h stimulate the cholesterol esterification machinery of the cell.

Fig. 3 shows the effects of the incubation with different media on the binding of ^{125}I-LDL to the fibroblasts. The average LDL binding (following the standard 24 h incubation of cells in LPDS) was 118 ng/mg of cell protein, in agreement with literature values (Brown & Goldstein, 1975). When LDL was added back to cells, we

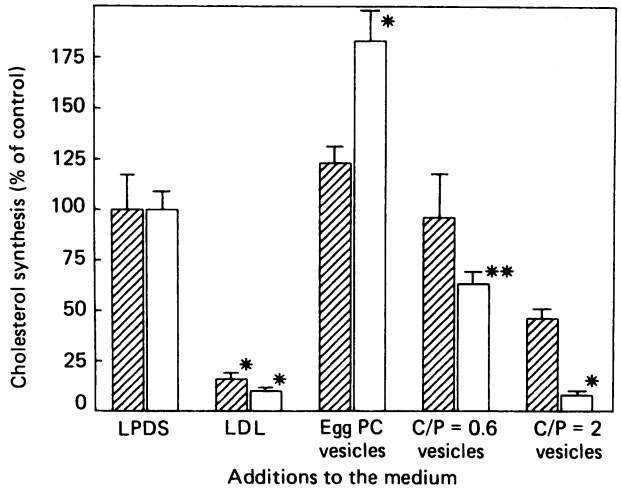

Fig. 1. Effects of various incubation media on cholesterol synthesis

The fibroblasts were cultured as described in the Materials and methods section and then preincubated for 24 h in minimum essential medium containing 10% LPDS. The medium was then removed and replaced with minimum essential medium containing either 80 μg of LDL/ml (LDL), 0.5 mg of egg phosphatidylcholine vesicles/ml (egg PC vesicles), 0.5 mg of cholesterol/egg phosphatidylcholine vesicles/ml with C/P = 0.6 (C/P = 0.6 vesicles); or 0.5 mg of cholesterol/egg phosphatidylcholine vesicles/ml with C/P = 2 (C/P = 2 vesicles). The cells were incubated for either 6 h (hatched bars) or 24 h (open bars) at 37 °C before cholesterol synthesis activity was determined. Results are expressed as a percentage of control (LPDS) synthesis, ±S.E.M. Results are from four to six experiments. Significant difference from control: $*P < 0.01$, $**P < 0.05$.

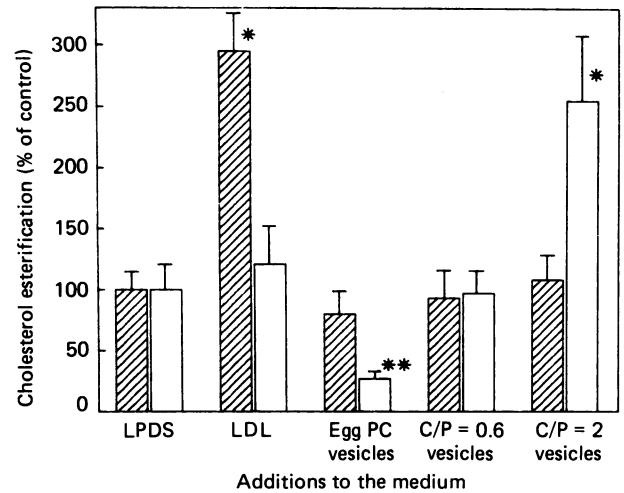

Fig. 2. Effects of various incubation media on cholesterol esterification

The experimental conditions were as described in Fig. 1. Following the incubations for 6 h (hatched bars) or 24 h (open bars), cholesterol esterification activity was determined. Results are expressed as a percentage of control (LPDS) incorporation of [^{3}H]oleate into cholesteryl esters, ±S.E.M. Results are from six to eight experiments, except for LDL at 6 h (three experiments) and 24 h (two experiments). Significant difference from control: $*P < 0.01$, $**P < 0.05$.

observed typical down-regulation of the LDL receptors toward basal levels by 24 h. Incubation of cells for 6 h with any of the various lipid vesicles had no significant effect on the binding of LDL. However, after 24 h of incubation we observed a significant decrease in ^{125}I-LDL binding in cells exposed to cholesterol-rich vesicles to levels that were similar to those obtained for cells that were incubated with LDL. Cells exposed to C/P = 0.6 vesicles showed a small, but not significant, decrease in LDL binding after 24 h. Fibroblasts incubated with egg phosphatidylcholine vesicles showed small, but not statistically significant, increases in LDL binding.

DISCUSSION

Each of the three key activities which are involved in the regulation of cellular cholesterol levels (cholesterol synthesis activity, cholesterol esterification activity, and LDL receptor number) responds to cholesterol delivered to the cell via the LDL pathway (Brown & Goldstein, 1975; Brown *et al.*, 1974; Goldstein *et al.*, 1974). Our results indicate that these activities also respond to cholesterol that enters the cellular pool via passive exchange at the plasma membrane. Incubation of fibroblasts with either LDL or cholesterol-rich vesicles resulted in similar changes in cell cholesterol content, cholesterol synthesis and esterification activities, and LDL receptor number. However, although both LDL and the vesicles increased cell cholesterol after 6 h there were differences in the time course of other effects. LDL exerted significant effects on synthesis, esterification, and binding within 6 h. The effects of the C/P = 2 vesicles were delayed compared with those of LDL, with clear changes in the various activities not occurring until 24 h.

The mechanism by which cholesterol exchanged into the cell at the plasma membrane is able to participate in the regulation of intracellular cholesterol metabolism is unknown. Estimates of the half-time for the translocation of newly synthesized cholesterol from the endoplasmic reticulum to the plasma membrane range from 10 min to about 1 h (De Grella & Simoni, 1983; Mills *et al.*, 1984; Lange & Matthies, 1984). In contrast, however, the movement of cholesterol from the plasma membrane to the cell interior seems to be a much more slower or limited process. Cholesterol introduced into the plasma membrane of human fibroblasts via exchange does not readily equilibrate with endogenously synthesized cholesterol (Poznansky & Czekanski, 1982), nor does it become rapidly available for microsomal esterification (Poznansky & Czekanski, 1982; Shireman & Remsen, 1982). In rat smooth muscle cells there is a similar situation; only 2–3% of cholesterol introduced via exchange from lipid vesicles is esterified within 24 h (Slotte & Lundberg, 1983*a*). Kaplan *et al.* (1984) have also reported that newly synthesized cholesterol, once transported to the plasma membrane, remains there and does not return to the cell interior.

Since it can be estimated that cultured cells such as fibroblasts internalize the equivalent of their surface area approximately every 1 h (Steinman *et al.*, 1983), it is unclear how this separation of plasma membrane and intracellular cholesterol pools is maintained. Bretscher

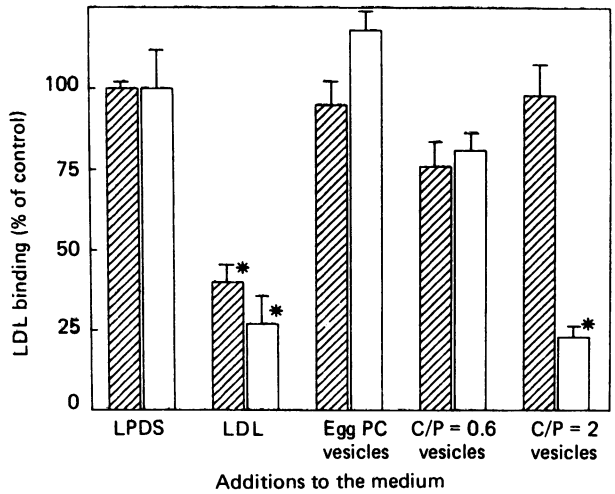

Fig. 3. Effects of various incubation media on LDL binding

Experimental incubation conditions are described in Fig. 1. Following the incubations at 37 °C for 6 h (hatched bars) or 24 h (open bars), the fibroblast monolayers were washed and incubated with ^{125}I-LDL at 4 °C for 2 h. Specific binding was determined as described in the Materials and methods section. Results are expressed as a percentage of control (LPDS) specific LDL binding, ±S.E.M. Results are from four to six experiments, except for egg phosphatidylcholine vesicles at 6 h (three experiments) and for LDL at 24 h (nine experiments). Significant difference from control: $*P < 0.01$.

(1976) has proposed that coated pits, which are the sites of internalization of cell plasma membrane, act as 'molecular filters' which may exclude various membrane components, including cholesterol. Another possibility is that plasma membrane cholesterol may be internalized into the cell but then at some point become segregated from the endocytic pathway and recycled to the cell surface, as has been demonstrated for several cell-surface receptors, including the LDL receptor (reviewed by Brown *et al.*, 1983). Our results are consistent with either of these hypotheses; it may well be that cholesterol delivered directly to the cell interior via the endocytosis and degradation of LDL may gain access to any intracellular regulatory pool(s) more rapidly than cholesterol exchanged into the cell at the plasma membrane. This would explain why cholesterol delivered to the cell by exchange from vesicles requires a longer period of time than does LDL cholesterol to effect changes in intracellular cholesterol metabolism. Under these conditions it may be that the movement of cholesterol from the plasma membrane to the cell interior is rate-limiting.

Conversely, the results from experiments involving cellular cholesterol efflux to egg phosphatidylcholine vesicles imply that, in this case, the efflux from the plasma membrane is the rate-limiting step, since the changes in both cell cholesterol content and intracellular metabolism occur together after 24 h of incubation. The fact that the cholesterol efflux to egg phosphatidylcholine vesicles appears to be a slower process than the influx from the cholesterol-rich (C/P = 2) vesicles (24 h versus 6 h) is not unexpected, considering that the cells were preincubated in LPDS which had already reduced the cell cholesterol content.

In summary, we have demonstrated that cholesterol exchange processes occurring at the plasma membrane of human skin fibroblasts will modulate at least three intracellularly regulated activities; cholesterol synthesis, cholesterol esterification, and LDL receptor number. These effects occur in the absence of any serum proteins. Our results are consistent with the idea that cholesterol transport from the cell surface to intracellular sites is a much slower and limited process than sterol movement in the opposite direction.

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