Role of an *N*-Ethylmaleimide-Sensitive Factor in the Selective Cellular Uptake of Low-Density Lipoprotein Free Cholesterol†

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ABSTRACT: Low-density lipoprotein (LDL) was the major contributor to an influx of free sterol from plasma which balances high-density lipoprotein (HDL)-mediated efflux from cultured skin fibroblasts. When HDL was absent, the uptake of LDL free cholesterol was associated with an increase in total cell cholesterol, due in part to accumulation of esterified cholesterol. This influx was mediated by a high-capacity, low-affinity pathway whose magnitude was similar in normal and LDL receptor-deficient cells. In the presence of HDL, some of the interiorized labeled LDL free cholesterol became available for HDL-mediated efflux and some was interiorized, as a result of a transport mechanism which was sensitive to *N*-ethylmaleimide (NEM) and nitrate ion but resistant to progesterone, azide, or vanadate. We suggest that normal free cholesterol homeostasis in these cells includes the initial binding of LDL followed by the selective transfer of free cholesterol to a compartment from which it is either returned to the membrane for efflux or internalized for storage or further metabolism within the cell. In the presence of NEM, LDL-derived free cholesterol remained mostly accessible for efflux from the cell surface. This free cholesterol pathway may function physiologically to stabilize plasma membrane cholesterol levels against the effect of varying concentrations of HDL and LDL.

Most of the cholesterol contained in mammalian cells is localized to the plasma membrane. Its concentration there is tightly regulated, and changes in plasma membrane free cholesterol (FC)[1] content are associated with major effects on the rates of transmembrane transport of ions such as Na^+, K^+, and Ca^{2+} (Yeagle et al., 1988; Shouffani & Kanner, 1990) and transmembrane signaling (Whetton et al., 1983). The transport of solutes transferred by other pathways, such as those mediated by plasmalemmal or clathrin-coated vesicles, is also sensitive to plasma membrane FC content (Rothberg et al., 1990; Smart et al., 1994; Xie et al., 1986). Cell FC levels are normally well buffered against changes in external concentrations of lipoprotein FC through a variety of influx and efflux mechanisms (Fielding, 1992). High-affinity low-density lipoprotein (LDL) receptor-mediated endocytosis represents one important mechanism for the regulation of cellular sterol; but in most nonhepatic cells these receptors are markedly down-regulated by even low concentrations of LDL, as is the synthesis of new cholesterol (Goldstein et al., 1985). A selective transfer of esterified cholesterol (EC) from LDL has been demonstrated (Reaven et al., 1986), but this pathway seems to be active mainly in steroidogenic cells. Nevertheless, despite the low rates of cholesterol uptake expected via these pathways, high rates of FC efflux continue to be observed in the presence of plasma lipoproteins (Fielding & Fielding, 1981; Johnson et al., 1991; Kawano et al., 1993). These considerations suggest there is substantial recycling of FC between the cell surface and the extracellular medium.

Several laboratories have observed that the initial efflux of FC from peripheral cells to medium lipoprotein acceptors is primarily to high-density lipoproteins (HDL) (Francone et al., 1990; Huang et al., 1993). The origin of this FC and the mechanism by which it may have originally entered the cell have been little studied, even though this pathway could be a significant contributor to cholesterol homeostasis. Each of the major plasma lipoproteins (HDL, LDL, and very low density lipoprotein, VLDL) is a potential source of FC for influx. Simple diffusion or molecular collision accounts for the transfer of cholesterol between lipoproteins and erythrocytes (Steck et al., 1988; Johnson et al., 1991), but additional mechanisms seem likely to contribute in fibroblasts, vascular smooth muscle cells, and macrophages. In these cells (unlike erythrocytes) efflux is markedly inhibited by protease pretreatment of the cell surface (Kawano et al., 1993).

In this research the mechanism and specificity of FC influx from plasma and plasma lipoproteins into cultured cell monolayers were investigated. The results obtained suggest the existence of a high-capacity, ATPase-dependent pathway by which LDL FC taken up by the cell is first collected to a membrane compartment or domain, from which it either is lost by efflux to HDL or interiorized for further metabolism or storage.

EXPERIMENTAL PROCEDURES

Blood was taken into ice-cooled tubes from normolipemic volunteers who had fasted overnight. Streptokinase (Sigma Chemical Co., St. Louis, MO, final concentration 150 U/mL) was included as anticoagulant (Miida et al., 1990). Plasma was obtained as supernatant following centrifugation (2000*g*,

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[1] Abbreviations: LDL, low-density lipoprotein; HDL, high-density lipoprotein; FC, free cholesterol; EC, esterified cholesterol; NEM, *N*-ethyl maleimide.

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30 min, 0 °C) and used immediately in the studies described below.

Preparation of ^{3}H-FC-Labeled Plasma and Lipoprotein Fractions. To compare the influx of FC from different lipoproteins, native plasma was labeled with 1,2-^{3}H-cholesterol (40−50 Ci/mmol) (New England Nuclear, Boston, MA). A 0.5−1.0 mCi amount of isotope was brought to 10−20 μL in ethanol and injected slowly with stirring into 20−30 mL of plasma at room temperature. The plasma was then incubated at 37 °C for 1−2 h to equilibrate the FC label between lipoprotein fractions. Dithiobis(2-nitrobenzoic acid) (2 mM) was included to inhibit the in vitro esterification of labeled cholesterol (Fielding & Fielding, 1981). FC equilibration was confirmed from the cholesterol specific activity of each lipoprotein class following fractionation as described below. In other experiments labeling of LDL was carried out following fractionation of native unlabeled plasma on columns (2.5 × 10 cm) of heparin−agarose (Pharmacia, Piscataway, NJ) which had been previously equilibrated in phosphate-buffered saline (PBS) (pH 7.4). A 6−10 mL amount of plasma was added at 0−2 °C. The nonabsorbed fraction contained HDL and other plasma proteins (Fielding & Fielding, 1986). VLDL and LDL were eluted together with 3M NaCl, 0.01 M phosphate (pH 7.4). Individual lipoproteins were isolated from these fractions by ultracentrifugal flotation (Havel et al., 1955). Lipoproteins were dialyzed into PBS containing 0.1 mM sodium EDTA (pH 7.4). Immediately before use in individual experiments the medium was brought to 1 mM Ca^{2+}, 1 mM $MgCl_2$. If necessary, individual lipoprotein fractions were reconcentrated to their original plasma volume with ultrafiltration membranes (Macrosep: Filtron, Northborough, MA) at 0 °C. Recovery (>95% in these experiments) was assessed by comparing the FC content of the original plasma with the sum of the free cholesterol concentration of the individual fractions recovered.

More than 98% of label in the nonabsorbed fraction in 0.15 M NaCl was recovered as HDL between density limits 1.063 and 1.21 g/mL. More than 95% of label in the fraction (VLDL + LDL) eluted with 3 M NaCl was recovered as LDL (1.019 < d < 1.063 g/mL). The ratio of protein and FC mass in LDL was 2.9 ± 0.1 (Fielding et al., 1984).

In most experiments, LDL was labeled directly with ^{3}H-FC by exchange from albumin−agarose covalent complex essentially as previously described (Miida et al., 1990). Briefly, recrystallized human serum albumin (Sigma Chemical Co, St. Louis, MO) was covalently linked to CNBr-activated Sepharose 6B (Pharmacia). 1,2-^{3}H-FC in ethanol (0.1−1.0 mCi, 10−20 μL/mL of gel suspension) was added, and the mixture was incubated with gentle stirring for 60 min at 37 °C. The labeled suspension was centrifuged (500g, 1 min), and the gel was equilibrated (60 min, 37 °C) with unlabeled LDL prepared as described above. Finally the gel was removed by centrifugation, leaving labeled LDL in the supernatant. LDL total and FC mass were determined fluorimetrically with cholesterol oxidase (Heider & Boyett, 1978) in the presence or absence of cholesterol esterase. EC mass was obtained by difference. LDL ^{3}H radioactivity was measured by liquid scintillation spectrometry. FC specific activity in these experiments was 3 × 10^4−5 × 10^5 cpm μg^{-1}.

In other experiments LDL was labeled with ^{125}I by the iodine monochloride method (MacFarlane, 1958). More than 98% of label was TCA-precipitated. Lipid-bound label was <5% when LDL was extracted with $CHCl_3$. The specific activity of ^{125}I-labeled LDL in these experiments was (2−3) × 10^5 cpm/μg of protein, equivalent to (6−9) × 10^5 cpm of protein label/μg of LDL FC.

Cell Culture. Normal skin fibroblasts, two lines of LDL receptor-deficient fibroblasts (American Type Culture Collection, ATCC GM 0701 and GM 2000) and a receptor internalization-defective line (GM 2408) were cultured in 10% fetal bovine serum in Dulbecco's modified Eagle's medium (DMEM). For individual experiments, cells were cultured in 3.5 cm plastic dishes until nearly confluent; 24 h before use in individual experiments, dishes were transferred into DMEM containing 7%−80% of human plasma.

The viability of cells in DMEM containing 7%−80% human plasma was compared to cells in PBS or in DMEM−10% fetal bovine serum in terms of the release of label into each medium from cells prelabeled (60 min, 37 °C) with ^{14}C-adenine (New England Nuclear, 1 μCi/mL of medium) (Shirhatti & Krishna, 1985). In each case, ^{14}C label released into PBS or human serum was the same as or lower than that released into the standard growth medium containing 10% fetal calf serum−DMEM (8%−10% over 120 min).

Determination of FC Influx. Cell monolayers were washed with PBS (×4) at 37 °C and then incubated for 5−120 min with 1 mL of ^{3}H-labeled plasma or the same volume of purified labeled lipoprotein in PBS with Ca^{2+} and Mg^{2+} (complete PBS) on an orbital shaker (1 cycle/s). The protein concentration of isolated lipoproteins was usually 0.5−1.0 mg mL^{-1}. The inclusion of irrelevant protein (purified goat IgG, 4 mg mL^{-1}) was without effect on FC flux in these experiments.

Following incubation, each monolayer was washed with human albumin solution (recrystallized, 4 mg mL^{-1}) in complete PBS, then 4× with complete PBS. For lipid analysis or fractionation, the washed monolayers were digested with 1 mL of 0.2 N NaOH (24 h, room temperature) before extraction with equal volumes of chloroform and methanol. Portions of chloroform phase were taken for chemical analysis. To determine cell-associated ^{3}H-FC label only, cell monolayers were dissolved directly in 4 mL of liquid scintillation cocktail (RPI, Mount Prospect, IL). Recovery of label under these conditions was >99%. Influx was linear for at least 10 min under these conditions. In some experiments sodium heparin, chloroquine, isobutyl methylxanthine (IBMX), or forskolin (all from Sigma) was included in the influx medium. In other experiments the cells were preincubated with proteinase K (final concentration 10 μg mL^{-1}) in PBS for 8−10 min prior to measurement of influx.

The FC and EC mass of cell monolayers was determined before and after incubation with lipoprotein as described above. Portions of the chloroform phase were analyzed for total and free cholesterol. Except where indicated each data point represents the mean of triplicate dishes. The coefficient of variation was <5% of the means in these experiments.

Determination of FC Efflux. Fibroblast monolayers were equilibrated with ^{3}H-FC-labeled native plasma or with isolated ^{3}H-labeled LDL, as specified for each experiment. Following incubation, the dishes were washed with albumin-complete PBS and then (4×) with complete PBS. They were then incubated for 3 min with unlabeled plasma or lipoprotein fractions, and the rate of appearance of radioactivity in the

Table 1: Effects of Medium Plasma Concentration on Rates of Cholesterol Influx and Efflux

	7% v/v[a]	50% v/v[a]	80% v/v[a]
cell FC μg dish^{-1}	13.2 ± 0.2	14.8 ± 0.2	15.0 ± 0.2
cell EC μg dish^{-1}	<0.1	1.6 ± 0.6	3.0 ± 0.7
FC influx[b]	27.1 ± 4.6	107.0 ± 19.9	133.0 ± 20.8
FC efflux[b]	25.1 ± 0.5	102.9 ± 12.8	156.6 ± 20.3

[a] Percent native plasma v/v in DMEM. [b] Cholesterol influx and efflux are expressed as ng of FC transferred per minute between the cell monolayer and 1 mL of plasma medium. Cell monolayers in 3.5 cm dishes were preincubated with unlabeled plasma−DMEM (influx) or with ^{3}H-cholesterol-labeled plasma−DMEM (efflux) for 24 h. The unlabeled cells were then incubated with ^{3}H-FC plasma at the same plasma dilution, and the cell-associated label was determined as described under Experimental Procedures. The ^{3}H-cholesterol-labeled cells were incubated at the indicated dilution of unlabeled plasma and efflux determined from medium radioactivity as described under Experimental Procedures. Each value represents the mean ± 1 standard deviation of six determinations.

medium was determined. Samples of medium (100 μL) were immediately chilled in ice water and centrifuged (10 min, 2000g) at 0−2 °C. ^{3}H-cholesterol radioactivity in the supernatant was either assayed directly or fractionated by agarose gel electrophoresis. FC efflux was linear as previously reported (Kawano et al., 1993).

For electrophoresis, 20 μL portions of labeled medium were added to strips of 0.75% w/v agarose in 0.025 M barbital buffer (pH 8.6) and separated as previously described (Fielding et al., 1991). The 2.5 mm gel fractions were then collected, and the radioactivity was measured. The location of major lipoprotein classes was determined from strips run simultaneously with whole native plasma equilibrated (60 min, 37 °C) with ^{3}H-FC.

RESULTS

Contributions of Plasma Lipoproteins to Cellular Cholesterol Influx and Efflux. Rates of FC influx and efflux between medium and fibroblast monolayers preincubated for 24 h with 7%−80% native human plasma in DMEM were determined. Cells equilibrated in 80% compared to 7% v/v plasma contained 35%−40% more total cholesterol but only 10%−15% more FC. In 50% v/v plasma, cholesteryl ester levels were lower than in 80% plasma but FC levels were almost the same. Rates of transfer of FC between the cells and their extracellular medium were determined for each plasma dilution. Influx was measured as the rate of transfer of ^{3}H-FC radioactivity from labeled native plasma to unlabeled cells. Efflux was determined as the rate of transfer of radioactivity from uniformly labeled cells to unlabeled native plasma medium. As shown in Table 1, both influx and efflux increased almost in parallel by 6-fold when medium FC content was increased 11-fold. Maximal rates of influx and efflux reached as much as 1% of cell FC min^{-1}. These data show that there is a rapid bidirectional transfer of FC between the cell monolayers and medium lipoproteins, whose rate is strongly dependent on medium FC concentration.

Cellular FC efflux to plasma media is mainly mediated by HDL (Francone et al., 1990). The pre-β-migrating fraction of small HDL appears to be particularly active in this pathway (Castro & Fielding, 1988; Huang et al., 1993). The contributions of HDL and LDL to FC influx were determined and compared to influx catalyzed by unfractionated native plasma (Table 2). Each lipoprotein was tested at its original plasma concentration. As shown in Table 2, the greatest influx was obtained from LDL (81% of the rate with native plasma). This value exceeded the proportion of total FC in plasma associated with LDL. While HDL contributed on average 35% of plasma FC, the rate of FC influx when only HDL was present represented only about 15% that determined with native plasma. The sum of influx catalyzed by HDL and LDL was similar to that measured with native plasma. These data indicated that most FC entering the cells from media containing native plasma originated from LDL.

Table 2: Contributions of LDL and HDL to Influx from Native Plasma

	native plasma		LDL		HDL	
	FC[a]	influx[b]	FC[a]	influx[b]	FC[a]	influx[b]
expt 1	409	79.0	282	58.9	102	14.6
expt 2	367	63.6	231	55.9	118	15.0
expt 3	432	58.0	284	51.5	149	11.5
means	403 ± 33	69 ± 11	266 ± 30	56 ± 4	123 ± 24	14 ± 2

[a] FC concentrations of plasma and plasma fractions were determined enzymatically and are expressed as μg of FC mL^{-1} of original plasma volume. [b] Rate of ^{3}H-cholesterol influx is expressed as ng min^{-1} following determination of cell-associated ^{3}H-cholesterol radioactivity over 5 min. Influx was linear over this time course.

To determine whether cellular factors contributed to the increased influx of LDL FC with increasing LDL concentration, cells were equilibrated with unlabeled 7% v/v plasma in DMEM and then transferred to unlabeled 80% v/v plasma medium. At zero time and at intervals thereafter up to 24 h in the 80% medium, triplicate dishes of cells were washed, and ^{3}H-FC-labeled LDL (94 μg of FC mL^{-1}) added for 10 min at 37 °C to determine the rate of influx of FC. The initial rate of influx into 7% plasma−DMEM medium was 39.8 ± 7.0 ng min^{-1}. This rate was almost unchanged at the end of 24 h in 80% plasma−DMEM medium (49.4 ± 8.3 ng min^{-1}) (difference not significant). This result indicates that the increase in cholesterol influx as a function of medium plasma FC content shown in Table 1 was solely a function of medium LDL concentration over the 7%−80% plasma range.

The concentration dependence of influx from ^{3}H-FC-labeled LDL is shown in Figure 1. The data illustrate a saturable pathway with a maximum velocity of 80−100 ng min^{-1} (three experiments) and a Km_{app} of 250 ± 20 μg of LDL-FC mL^{-1}, equivalent to 0.8 mg of LDL protein mL^{-1} (Fielding et al., 1984).

Cells were incubated (10 min) with ^{3}H-FC-labeled LDL. These were then transferred for 3 min to unlabeled native plasma. Samples of this plasma were then fractionated by agarose gel electrophoresis (Figure 2). Almost the whole of radioactivity was recovered in those fractions which comigrated with pre-β- and α-HDL, consistent with earlier findings (Fielding et al., 1991; Miida et al., 1990). None was detected comigrating with the LDL or albumin fractions of plasma. These data indicated that transfers of FC occurring at the cell surface were represented for the most part by the uptake of FC from LDL into the cell and by the release of cellular FC to HDL in the medium.

Receptor-Mediated Endocytosis and Free Cholesterol Transfer. The rate of delivery of FC by the endocytosis of

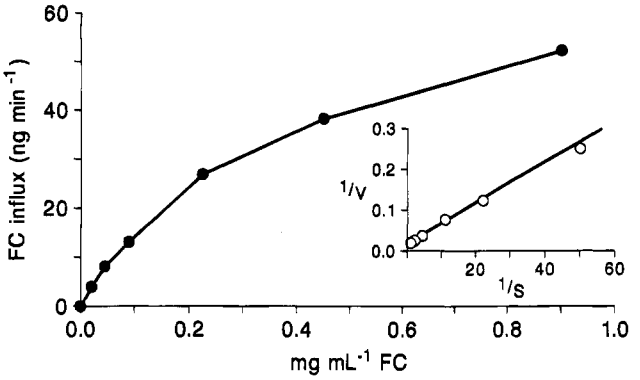

FIGURE 1: Concentration dependence of LDL ^{3}H-FC uptake by fibroblast monolayers. Unlabeled monolayers equilibrated with 7% plasma–DMEM were washed with PBS (4×), incubated (10 min, 37 °C) with LDL at the indicated concentration, and then washed with PBS–albumin (4 mg mL^{-1}) and then with PBS (4×). The washed monolayers were then extracted as described under Experimental Procedures, and the cell-associated label was determined. Each data point represents the mean of triplicate dishes. Insert: double-reciprocal plot of the experimental data.

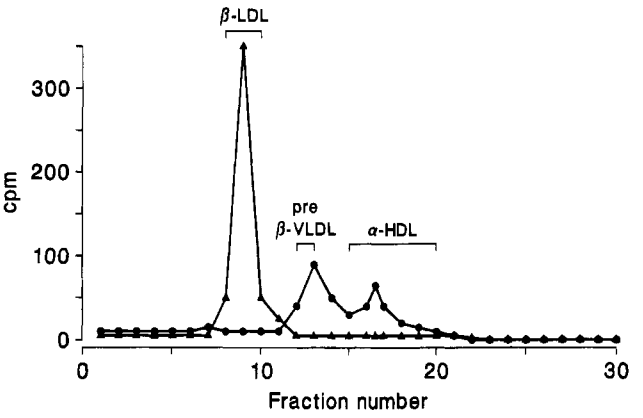

FIGURE 2: Efflux of radioactivity from cells prelabeled with ^{3}H-FC-labeled LDL. Unlabeled cells equilibrated in 7% plasma–DMEM were washed, incubated with labeled LDL (10 min, 37 °C), and then washed with PBS–albumin and PBS as described in the legend to Figure 1. Unlabeled native plasma was then added for 1 min at 37 °C, and then a sample (20 μL) was taken for immediate agarose gel electrophoresis. The 2.5 mm wide gel strips were collected after separation and analyzed for contained radioactivity. Closed circles, label recovered from plasma incubated with the cell monolayer. Closed triangles, original ^{3}H-FC-labeled LDL run in a separate agarose strip. The positions of major lipoprotein species were determined from a sample of whole plasma prelabeled with ^{3}H-FC and electrophoresed simultaneously. FC efflux was 17.8 ng min^{-1} in this experiment. Label recovered in total HDL was >90% of that applied.

intact LDL was determined from the rate of appearance of TCA-soluble ^{125}I-radioactivity from ^{125}I-labeled LDL. The rates of influx of ^{3}H-FC label from LDL to normal fibroblasts and to several lines of LDL receptor-deficient cells were also compared under the same conditions and assayed as described under Experimental Procedures.

The appearance of TCA-soluble label from ^{125}I-LDL was measured over 3 h at 37 °C. Its rate was linear, and its magnitude (1.2 ± 0.3 ng of LDL protein min^{-1}; three experiments) represents an LDL FC transfer to the cells of 0.4 ± 0.1 ng of LDL free cholesterol min^{-1} from the FC/protein mass ratio of LDL determined experimentally. Under the same conditions the rate of selective transfer of ^{3}H-FC label from LDL was 32.4 ± 3.5 ng min^{-1}, about 80-fold greater.

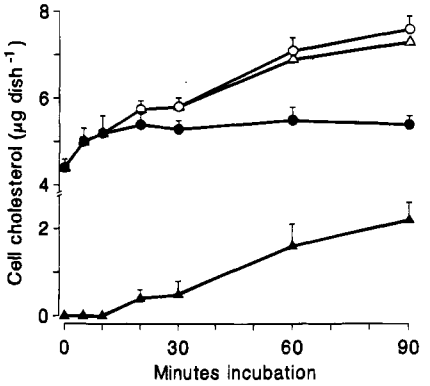

FIGURE 3: Cell cholesterol mass and radioactivity in unlabeled cells incubated with ^{3}H-FC-labeled LDL. Closed circles, cellular FC mass determined enzymatically; open circles, total cholesterol mass; closed triangles, EC mass determined as the difference between total and free values; open triangles, predicted total cholesterol mass based on the sum of the measured initial content of the cells (4.4 ± 0.2 μg dish^{-1}) together with cell-associated ^{3}H-cholesterol from labeled LDL based on a measured specific radioactivity of 5.8 × 10^4 cpm μg^{-1}. Data points are the means of six measurements in each case.

The rate of FC transfer from ^{3}H-FC-labeled LDL to normal, receptor-deficient and internalization-deficient fibroblast monolayers was compared. In a representative experiment at an LDL FC concentration of 46 μg mL^{-1}, the rate of transfer to normal cells was 15 ± 3 ng min^{-1}; while the rate of influx from the same preparation to receptor-deficient cells was 16 ± 3, 15 ± 3 and 19 ± 3 ng min^{-1}, respectively, for GM 2000, GM 0701, and GM 2408 lines.

These data suggest that the influx of LDL FC to cell monolayers maintained in the presence of human plasma was largely independent of the receptor-mediated endocytosis of intact LDL.

Cellular Effects of LDL-Mediated FC Influx. ^{3}H-FC-labeled LDL was incubated with fibroblast monolayers at 37 °C for up to 90 min. At intervals, cells were washed, and assayed for FC and EC mass and radioactivity. Before the addition of LDL, the cells contained no detectable EC (Figure 3). Transfer to LDL solution was associated with an increase in cellular FC mass without the appearance of EC over the first 10 min of incubation. Beyond this point, as cell-associated cholesterol label continued to increase, FC mass remained almost constant while EC mass began to accumulate.

Cell-associated cholesterol radioactivity and the increase in cell total cholesterol mass were compared. As shown in Figure 3, when the specific activity of LDL was used to convert the increase in cell-associated label to mass, calculated values for cellular cholesterol mass were similar to those determined directly, indicating that in the absence of other lipoproteins almost the whole of FC taken up from labeled LDL was retained within the cells. Since much of this cholesterol was esterified, this finding shows that at least some of the FC internalized from ^{3}H-FC-labeled LDL must be accessible to microsomal acyl CoA:cholesterol acyltransferase (ACAT), the only significant source of EC in these cells (Suckling & Stange, 1985). This was confirmed by determining the specific activities of FC and EC in extracts of cells incubated with LDL for 60 and 90 min. These did not differ significantly, confirming that internalized LDL FC was available for esterification and was in equilibrium with

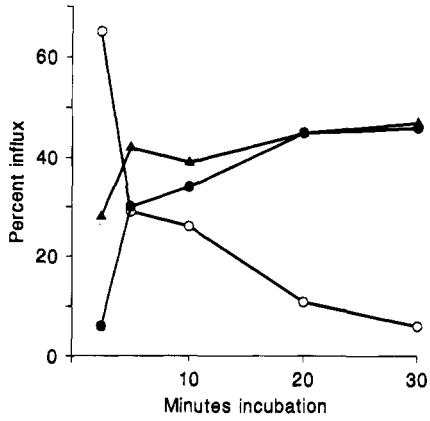

FIGURE 4: Distribution of cell-associated ^{3}H-FC from LDL as a function of time. Label released by unlabeled LDL (open circles); label released by unlabeled HDL but not unlabeled LDL (closed triangles), and label not released with either LDL or HDL (closed circles) expressed as percent of total label released. LDL specific activity was 3.1×10^4 μg mL^{-1}. All fractions are defined in terms of label released by either unlabeled lipoprotein within 5 min of incubation at 37 °C.

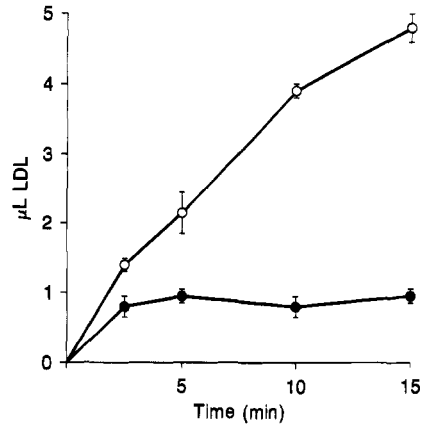

FIGURE 5: Association of LDL ^{125}I-protein and ^{3}H-FC labels with fibroblast monolayers as a function of time. LDL (69 μg of FC mL^{-1}) was labeled with either isotope as described in Experimental Procedures and incubated with unlabeled cell monolayers for the period shown. After washing with PBS−albumin and PBS, cell-associated label was determined following solubilization of the cell monolayers with 0.2 N NaOH. Data are expressed in terms of the volume of LDL solution associated with the cells at each time point to allow direct comparison with the cell association of both labels. Values shown are the means of three different experiments. Open circles, LDL ^{3}H-free cholesterol; closed circles, LDL ^{125}I-protein.

cellular EC. Comparable results were obtained with dishes containing initial FC contents of 8.7 ± 0.2 and 10.0 ± 0.2 μg dish^{-1}.

Kinetics of Cellular FC Influx Mediated by LDL. The mechanism of LDL-mediated influx of labeled FC was studied further by measuring the ability of unlabeled lipoprotein fractions to displace cell-associated label, as a function of the time during which the influx of ^{3}H-FC took place.

Cell monolayers equilibrated with 7% plasma−DMEM were first incubated with ^{3}H-FC labeled LDL as described under Experimental Procedures. At intervals, dishes of cells were washed, then incubated with unlabeled LDL or HDL at their plasma concentrations. A fraction of cell-associated label was rapidly released; maximum recovery in the medium occurred within 10 min at 37 °C. In Figure 4, the proportions of cell label which were LDL-releasable, LDL-resistant but HDL-releasable, and resistant to both LDL and HDL are shown as a function of time.

The major part of cell-associated LDL ^{3}H-FC could initially be dissociated into the medium with cold LDL; but this proportion decreased with time as label accumulated in the cells. The proportion of label resistant to LDL but released by HDL was initially low but reached 40%−60% (three experiments) after 15 min. Label inaccessible to either LDL or HDL over the time course of these experiments was 30%−50% of total label after 15 min of incubation at 37 °C. These data are consistent with a mechanism in which LDL ^{3}H-FC was first bound to the cell surface and then transferred to a compartment from which it was either released by HDL to the medium or transferred into the cell for further metabolism.

Further information on the mechanism of FC influx was obtained by comparing the cell association of LDL labeled in the protein moiety with ^{125}I or in the free cholesterol moiety with ^{3}H-FC. Unlabeled cell monolayers were incubated with the same concentration of either ^{3}H- or ^{125}I-labeled LDL for 2.5−15 min at 37 °C. At each time point the dishes were washed, and bound ^{125}I-protein or ^{3}H-FC radioactivity was determined. As shown in Figure 5, cell-associated ^{125}I label reached a maximum within 2.5 min of incubation that was maintained during a 15 min incubation period. In contrast, ^{3}H-FC label increased nearly linearly over the same period. To allow comparison of protein and free cholesterol labels, the data have been expressed as the uptake of LDL medium volume min^{-1}. In these units the uptake of LDL FC by the cells represented 0.3−0.4 μL of LDL solution min^{-1}, equivalent to 20.7 ng of FC min^{-1}. In four experiments with different LDL preparations the final ratio of ^{3}H/^{125}I label was 5.6 ± 1.5 following 15 min of incubation.

In other experiments, ^{125}I-labeled LDL was incubated (10 min) with unlabeled cell monolayers. These were then washed and transferred to medium containing unlabeled LDL at the same concentration. The whole of bound ^{125}I label was rapidly displaced (Figure 6) with a half-time of 1.5 ± 0.5 min (three experiments).

The data in Figure 5 suggest that LDL first bound to the cell and then transferred part of its FC content to the cell surface before being displaced by new particles. The data shown in Figure 6 indicate that little if any bound LDL was retained at the cell surface.

Regulation of the Uptake and Intracellular Transport of LDL-Derived Free Cholesterol. As shown in Figure 7, the influx of FC from LDL was similarly inhibited by heparin in both normal and LDL receptor-deficient (GM 2000) cells. Approximately 80% inhibition was obtained at 0.1 mg of heparin mL^{-1}. In contrast to LDL receptor-mediated influx (Goldstein & Brown, 1974) or HDL-mediated efflux (Kawano et al., 1993) the uptake of ^{3}H-FC LDL label was only slightly inhibited (7% ± 6%; four experiments) when the cell monolayer was pretreated with proteinase K.

HDL-mediated efflux has been reported dependent upon cellular cAMP levels and activity of signaling intermediates (Oram et al., 1991; Hokland et al., 1993; Voyno-Yasenetskaya et al., 1993). The effect of these agents on the internalization and retention of LDL-derived FC was determined. As shown in Table 3, there was no effect on LDL FC uptake by forskolin or isobutyl methylxanthine under conditions shown

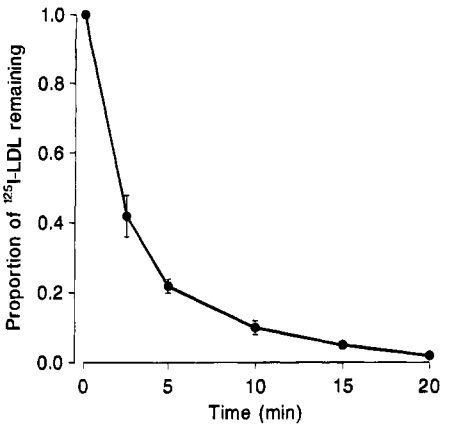

FIGURE 6: Turnover of ^{125}I-labeled LDL at the cell surface after addition of unlabeled LDL. Labeled LDL (70 μg of FC mL^{-1}) was incubated with fibroblast monolayers for 10 min at 37 °C. The cells were then washed and incubated for the time shown with unlabeled LDL at the same concentration. Remaining cell-associated label was determined as a function of time with unlabeled LDL following extraction of the cells with 0.2 N NaOH. Data from three independent experiments is expressed relative to initial cell content of ^{125}I-radioactivity.

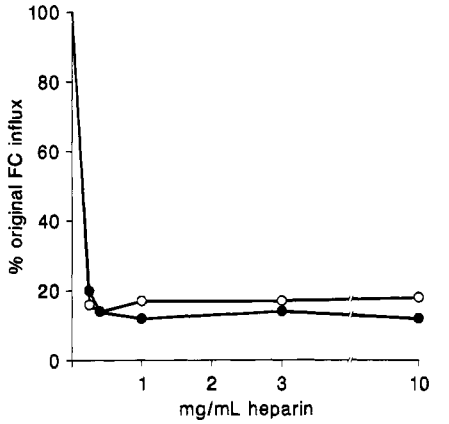

FIGURE 7: Inhibition of LDL ^{3}H-FC influx by sodium heparin. Monolayers of either normal fibroblasts (closed circles) or LDL receptor-deficient cells (GM 2000 line, open circles) were incubated with LDL (150 μg of FC mL^{-1}) for 10 min at 37 °C in the presence of the indicated concentration of heparin. The plates were then washed as described in the legend to Figure 1 and extracted to determine cell-associated radioactivity. Data are expressed as a percent of the label bound in the absence of heparin.

previously to modify HDL-mediated signaling (Oram et al., 1991). Azide and vanadate, effective inhibitors of ATPases catalyzing transmembrane ion transport (Pederson & Carafoli, 1987) were also without effect on LDL FC transfer. There was no effect of progesterone, even at a concentration (30 μM) which would maximally inhibit fibroblast ACAT activity (Goldstein et al., 1978) or cholesterol transport in hepatocytes (Lange, 1994). However *N*-ethylmaleimide (NEM) and KNO_3, inhibitors of the ATPases required for vesicular transport between cell compartments (Pederson & Carafoli, 1987; Tagaya et al., 1993), both strongly (≥70%) inhibited the uptake of LDL FC by these cells.

With cells prelabeled to equilibrium (24 h) with ^{3}H-FC, inhibition by NEM was complete within 30 min of the extracellular addition of inhibitor. Maximal inhibition was obtained at 2−5 mM NEM in intact cells, compared to 1 mM in assays of vesicular transport in vitro (Tagaya et al., 1993).

Table 3: Effects of Metabolic Inhibitors on the Uptake of ^{3}H-Cholesterol from LDL[a]

	concn	FC influx (μg)[b]	%
PBS only	−	1.31 ± 0.05	100.0
sodium azide	1 mM	1.18 ± 0.02	89.5
ammonium vanadate	1 mM	1.36 ± 0.08	103.2
N-ethylmaleimide	2 mM	0.40 ± 0.01	30.0
KNO_3	50 mM	0.28 ± 0.02	21.0
progesterone	30 μM	1.28 ± 0.06	97.3
forskolin	30 μM	1.39 ± 0.01	105.3
IBMX	100 μM	1.46 ± 0.04	110.8
chloroquine	20 μM	1.34 ± 0.04	102.3

[a] Confluent cell monolayers were cultured in unlabeled 7% plasma−DMEM, washed in PBS (4×), pre-equilibrated in PBS (30 min, 37 °C) with the factors shown at the indicated concentration (or with PBS only, in the control dishes), and then incubated (60 min, 37 °C) in ^{3}H-cholesterol-labeled LDL-FC (60.2 μg mL^{-1}). Cells were then washed and extracted as described under Experimental Procedures. Values shown are means ±1 standard deviation for triplicate dishes. [b] Influx is calculated from cell-associated ^{3}H-label (LDL-FC specific activity 2.24 × 10^4 cpm μg^{-1}).

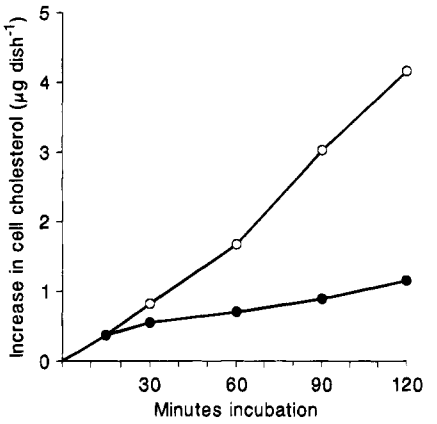

FIGURE 8: Time course of uptake of ^{3}H-FC labeled LDL in the presence or absence of 5 mM NEM. Cells in 7% plasma−DMEM were washed, preincubated (30 min, 37 °C) with PBS or PBS−NEM, and then transferred to ^{3}H-FC LDL solution (91 μg of FC mL^{-1}) for the indicated period. Open circles, without NEM; closed circles, with NEM. Data points represent means of triplicate dishes.

Table 4: Effects of *N*-Ethylmaleimide on Cholesterol Transfer between Cell Monolayers and Plasma Lipoproteins[a]

	control	+NEM
Δ total cholesterol (μg dish^{-1})[b]	4.1 ± 0.3	0.4 ± 0.2
lipoprotein-resistant label[c]	0.57 ± 0.03	0.24 ± 0.03
% efflux to plasma (3 min)[d]	13.3 ± 0.1	32.4 ± 0.2

[a] Values represent data obtained in cells incubated in the presence or absence of 2 mM NEM. [b] Increase in cellular cholesterol mass determined enzymatically over 120 min at 37 °C. Initial cell FC was 5.0 ± 0.2 μg. [c] Proportion of cell cholesterol label retained in the cells following incubation (10 min, 37 °C) with 80% v/v plasma−PBS. [d] Percent loss of label from cells prelabeled (10 min, 37 °C) with ^{3}H-cholesterol-labeled LDL during incubation (3 min, 37 °C) with unlabeled 80% v/v plasma-PBS.

The time course of inhibition of ^{3}H-FC transfer from labeled LDL to unlabeled fibroblast monolayers preincubated (30 min) with NEM is shown in Figure 8. For the first 10−15 min of incubation with LDL, there was no effect of NEM on the cellular uptake of LDL FC. Upon further incubation an almost complete (>80%) inhibition of the uptake of ^{3}H-FC from LDL. This was associated with a parallel inhibition of accumulation of cholesterol mass (Table 4). A comparable time course to that shown in Figure 8 was

obtained for cells preincubated with 50 mM KNO_3 (data not shown).

Further information on the mechanism of NEM-mediated inhibition was obtained by preincubating NEM-blocked or unblocked cells with ^{3}H-FC labeled LDL for 10 min, before the inhibition of ^{3}H-FC transfer was detectable (Figure 8). The initial rate of appearance of cellular ^{3}H-FC in the medium, and the proportion of label resistant to lipoprotein-mediated efflux, were then compared in NEM-blocked and unblocked control cells. As shown in Table 4, NEM mediated an increased rate of efflux and reduced the proportion of cellular label transferred to the lipoprotein-resistant compartment.

DISCUSSION

The experiments in this research describe a high-capacity pathway by which LDL FC was bound and selectively interiorized by cultured skin fibroblasts. It can account for a major part of the influx required to maintain cellular FC in response to cholesterol efflux, mainly to HDL under these conditions (Francone et al., 1990), in cells maintained in the presence of plasma lipoproteins. The rate of FC efflux from the cells, for example in 80% plasma, represents a transfer of about 9.4 μg of FC h^{-1} (mL of medium)$^{-1}$, comparable to the rate of cholesterol esterification by lecithin:cholesterol acyl transferase in this medium (Fielding & Fielding, 1981). However the enzyme transesterifies FC of both cell and plasma lipoprotein origin. Some of the cell-derived FC will be transferred back to LDL (Huang et al., 1993) and become reavailable for selective uptake by the cell. The proportion of FC recycled or esterified in vivo probably depends mainly on the proportions of cellular and lipoprotein FC. LDL is present in all extracellular fluids including lymph where its concentration is about 7% of that in plasma (Hong et al., 1984). As shown in Table 1, selective influx of FC to cells under these conditions will remain significant.

In properties and mechanism this pathway differs substantially from the endocytosis of intact LDL (Goldstein et al., 1985). The rate of selective FC uptake was almost unaccompanied by the uptake of LDL protein label or LDL cholesteryl ester mass. It was similar in normal fibroblasts and several lines of LDL receptor -deficient cells. Compared to receptor-mediated endocytosis LDL-FC influx was mediated by low-affinity, high-capacity pathway whose rate varied with medium LDL levels over the physiological range. Both pathways are inhibited by heparin; this probably reflects in each case formation of soluble charged complexes of LDL and heparin which are less reactive with the cell surface. An earlier study (Slotte et al., 1984) had reported uptake and esterification of LDL FC label by LDL receptor-deficient fibroblasts, probably reflecting the same pathway as described here.

In mammalian cells, the greatest part of FC is located in the plasma membrane (Lange et al., 1989; Schroeder & Nemecz, 1990). Most of this (~85%) was localized to the inner leaflet of the bilayer (Brasaemle et al., 1988; Jefferson et al., 1991; Schroeder et al., 1991), leaving the outer leaflet relatively cholesterol-poor. As a result the initial transfer of FC from LDL to the cell surface may be energetically favorable. When the mass of LDL-FC bound to the cells at equilibrium was 70 ng, 25 ng of FC min^{-1} was transferred to the cell. Some 50% of bound LDL (initial FC content 35 ng) was displaced per minute by cold LDL. As a result up to 70% (25/35) of LDL FC may be transferred to the cell during each binding event. However, only a very small part of medium LDL is cell-associated at any one time. The FC content of LDL particles newly desorbed from the cell's surface may be restored either by transfer from other LDL particles (Lund-Katz et al., 1982; Miida et al., 1990) or from HDL acceptors of cell-derived FC (Huang et al., 1993).

FC transfer did not reflect the simple exchange of cholesterol at the cell surface, for several reasons. There was a marked increase in cellular FC mass in the absence of HDL; indeed LDL-FC was retained in the cells almost quantitatively. Influx was largely LDL-dependent while efflux was HDL-dependent. There was an obvious lag (10 min) between the binding of labeled LDL-FC and its availability for efflux to HDL. The effects of metabolic inhibitors confirmed that the LDL-FC influx pathway described in these studies differed from other mechanisms of FC transport. In human hepatoblastoma (HepG2) cells, progesterone promoted the transfer of FC from the plasma membrane to the interior of the cell (Lange, 1994). In the present research progesterone was without effect on LDL-FC influx, as was chloroquine, an inhibitor of lysosomal transport that blocks endocytotic processing. The transfer of newly-synthesized cholesterol to the plasma membrane of fibroblasts is cAMP-dependent and stimulated by forskolin and IBMX (Hokland et al., 1993); LDL-FC influx was unaffected by these agents. Finally, LDL-FC influx was blocked by inhibitors specific for an ATPase (*N*-ethylmaleimide-sensitive factor, NSF) now broadly implicated in vesicular transport in both yeast and mammalian cells (Pederson & Carafoli, 1987; Sollner et al., 1993; Ferro-Novick & Jahn, 1994). A second potential FC carrier, sterol carrier protein-2 (SCP-2) is also inhibited by NEM although at higher concentrations (10 mM). However, the selective transfer of FC from LDL and its inhibition by NEM and KNO_3 were normal in SCP-2-deficient (Zellweger sydrome) cells (C. Fielding, unpublished experiments), arguing against a role for this factor.

ATPases in the NSF family are characterized by resistance to azide and vanadate and sensitivity to nitrate and NEM. The best-characterized of these enzymes (NEM-sensitive factor, NSF) forms a complex at the inner surface of the plasma membrane with attachment factors and other proteins (Weidman et al., 1989) and plays an essential role in the transfer of solute vesicles between intracellular compartments. ATPases with similar properties have been reported within the plasma membrane. In LDL FC transfer, the effect of NEM and KNO_3 was to prevent the transfer of free cholesterol away from the plasma membrane to the cell interior. As a result, most of the FC transferred to the cells from LDL remained accessible to plasma lipoproteins, and the initial rate of efflux of FC from prelabeled cells to plasma was increased several-fold. We hypothesize that the role of the NEM-sensitive factor in LDL-FC influx is to draw FC into specific plasma membrane microdomains, for which there is considerable independent evidence (Schroeder et al., 1990). From here it can be either interiorized or effluxed to HDL. Further research will be required to localize the site of action of such a factor and to determine if its activity represents NSF or another NEM-sensitive ATPase.

Because of its high capacity and sensitivity to the physiological LDL concentration range the LDL-FC transfer

pathway would be effective as part of a mechanism to stabilize plasma membrane FC as LDL-FC concentration changes in response to nutritional status. Under pathological conditions, including diabetes, renal failure, and some inherited hyperlipidemias, LDL free cholesterol content is unusually high (Fielding, 1984; Bagdade et al., 1990; Dieplinger et al., 1986) while HDL levels are markedly reduced. Changes of this kind in plasma lipoproteins are often associated with an accumulation of both free and esterified cholesterol in the vascular bed. It will be of interest to determine whether LDL FC transfer is also abnormal under these conditions.

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