Plasma Membrane Caveolae Mediate the Efflux of Cellular Free Cholesterol†

Phoebe E. Fielding*,‡ and Christopher J. Fielding§

Cardiovascular Research Institute and Departments of Medicine and Physiology, University of California, San Francisco, California 94143

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ABSTRACT: Caveolae are clathrin-free cell-surface organelles implicated in transmembrane transport. A fibroblast caveolar membrane fraction was isolated by sucrose density gradient ultracentrifugation and its identity confirmed by protein markers (caveolin, annexin II). When ^{3}H-labeled free cholesterol was selectively transferred to the cells from labeled low density lipoprotein to increase cell free cholesterol ~15%, there was a 6-fold increase in label in the caveolar fraction above baseline levels. Subsequent incubation of these cells with unlabeled native plasma or plasma high density lipoprotein selectively unloaded caveolar free cholesterol into the medium. Okadaic acid, which decreased caveolar activity as measured by cholera toxin binding and uptake, decreased cholesterol efflux in parallel. Cholesterol newly synthesized from [^{3}H]mevalonate was also preferentially incorporated into the caveolar fraction and selectively released by plasma into the medium. Together these data indicate that caveolae represent a major site of efflux of both newly synthesized and low density lipoprotein-derived free cholesterol in these cells.

Caveolae and clathrin-coated pits represent alternative cell-surface organelles specialized for binding and internalizing extracellular solutes (Hansen et al., 1991). Caveolae are clathrin-free invaginations (40−80 nM diameter) of many cell surfaces. The caveolar membrane fraction is enriched in GPI-anchored proteins, src-family tyrosine kinases, and Ca^{2+}-ATPase (Rothberg et al., 1990; Stefanova et al., 1991; Brown & Rose, 1992; Dupree et al., 1993). Annexins, GTPases implicated in vesicle-mediated exocytosis (Creutz, 1992), are also located in the caveolar fraction (Fiedler et al., 1995). Highly purified caveolae contain proteins involved in both the budding and fusion of cytoplasmic vesicles (Schnitzer et al., 1995).

The caveolar membrane fraction purified from cell homogenates is rich in sphingolipids and free cholesterol (FC)[1] (Hagmann & Fishman, 1982; Smart et al., 1994). In contrast clathrin-coated vesicles contain little FC and are rich in lecithin (Pearse, 1976; Montesano et al., 1979). Although representing only a small fraction of total membrane surface, the caveolae contain much of the FC in the outer leaflet of the plasma membrane bilayer (Smart et al., 1994), and FC plays a key role in maintaining the structural and functional integrity of caveolae at the plasma membrane surface (Chang et al., 1992). A membrane-associated protein, caveolin, is associated with the cytoplasmic side of many caveolae. The uncoating of caveolin from caveolae as these vesicles are released into the cytoplasm may be required for their further processing (Scherer et al., 1994) although the functional role of caveolin in the regulation of caveolar activity remains controversial (Kandror et al., 1995; Gorodinsky & Harris, 1995).

We recently described a novel pathway by which low density lipoprotein (LDL) FC was selectively transferred to the cell surface and then interiorized via an *N*-ethyl maleimide (NEM) sensitive pathway (Fielding & Fielding, 1995b). Because of the high capacity of this pathway and the proposed regulatory role of FC in caveolar function (Rothberg et al., 1990; Chang et al., 1992; Schnitzer et al., 1994), we investigated whether caveolae played a role either in the initial binding of LDL or in the subsequent efflux of LDL-derived FC to HDL.

EXPERIMENTAL PROCEDURES

Normal skin fibroblast monolayers were cultured to near confluence in 10% fetal calf serum−DMEM in 3.5 or 10 cm plastic dishes and then switched to DMEM containing 7% human plasma 24 h before each experiment. The plasma was obtained from the blood of fasting normal donors which had been anticoagulated with streptokinase (final concentration 150 units mL^{-1}) (Fielding et al., 1991).

In some experiments cholesterol synthesis was measured with [^{3}H]mevalonolactone (33 Ci mmol^{-1}; NEN/Dupont, Boston, MA). Dishes of cells were transferred into phosphate-buffered saline (PBS) containing 4 mg mL^{-1} of recrystallized human serum albumin (Sigma, St Louis, MO), 0.2 mM mevalonolactone (Sigma), and 5 μCi mL^{-1} of [5-^{3}H]-mevalonolactone. After 3 h at 37 °C, the labeled medium was removed, and the cells were used in individual experiments described below. In other experiments the cells were uniformly labeled with [1,2-^{3}H]cholesterol (40−50 Ci/mmol)(NEN/Dupont) in 7% human plasma−DME medium (24−48 h, 37 °C)(Fielding et al., 1991). Final [^{3}H]-cholesterol specific activity was 4−8 × 10^3 dpm μg^{-1} FC. Alternatively, LDL was obtained from fasting human plasma following heparin-agarose column chromatography (Fielding & Fielding, 1986). LDL-FC was selectively labeled with [^{3}H]cholesterol via albumin−agarose covalent complex (Miida et al., 1990) and diluted to its original plasma volume with PBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (complete PBS) immediately before use in individual experi-

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‡ Department of Medicine.

§ Department of Physiology.

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

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ments. LDL FC specific activity in these experiments was $2-5 \times 10^4$ cpm μg^{-1}. FC mass was measured enzymatically (Heider & Boyett, 1978) and FC label by liquid scintillation spectrometry. [^{3}H]FC-labeled LDL was incubated with unlabeled cell monolayers for 0.05−1 h at 37 °C. High density lipoprotein (HDL) was quantitatively recovered in the nonadsorbed fraction of heparin-agarose chromatography (Fielding & Fielding, 1986).

Caveolae were separated from Triton-extracted fibroblast monolayers using a published technique (Brown & Rose, 1992; Sargiacomo et al., 1993; Smart et al., 1994). Briefly, the cells were collected into 25 mM methanesulfonic acid and 0.15 M NaCl, pH 6.5, containing phenylmethanesulfonyl fluoride, soybean trypsin inhibitor, and benzamidine to inhibit proteolysis. The cells were centrifuged, and the pellet was resuspended at 4 °C in the same buffer containing 1% (v/v) Triton X-100. After 20 min on ice the cells were homogenized, and an equal volume of 85% (w/v) sucrose was added. The sample (2.0 mL) was layered under a 7 mL 10−30% sucrose gradient in the same buffer without Triton. Centrifugation was carried out in a SW-41 rotor (Beckman Instruments, Palo Alto, CA) for 5 h at 4 °C and 35 000 rpm. Fractions (0.65 mL) collected from these gradients were analyzed for [^{3}H]FC radioactivity and FC mass as described above. The proportion of label in the caveolar peak was determined by triangulation. The location of caveolae in the gradient was confirmed with antibodies against caveolin and annexin II (Transduction Laboratories, Lexington, KY). Portions of each fraction were analyzed on 12% SDS−polyacrylamide gels. The gels were electrotransferred to nitrocellulose (0.2 μm pore size, S & S, Keene, NH) and immunoblotted for the proteins indicated using conditions specified by the antibody supplier. The blots were developed with IgG-horseradish peroxidase using SuperSignal CL-HRP substrate (Pierce, Rockford, IL), according to the manufacturer's instructions. FC specific radioactivity was calculated as the ratio between FC label and FC mass in each fraction.

The caveolar fraction of cholesterol was also measured in terms of its susceptibility to cholesterol oxidase in unfixed cells. Cell monolayers prelabeled by incubation with [^{3}H]-FC LDL as described above were chased with unlabeled LDL to displace surface-bound label (Fielding & Fielding, 1995b) and then incubated with cholesterol oxidase (Boehringer-Mannheim, Indianapolis, IN) (1 unit mL^{-1} in PBS, 60 min, 37 °C) to convert caveolar [^{3}H]FC to labeled cholest-4-en-3-one (Smart et al., 1994). After being washed with PBS, the cells were dissolved in 0.2 N NaOH. Total lipid was extracted with methanol and chloroform. Portions of chloroform phase, together with authentic lipid standards (FC, cholest-4-en-3-one, Sigma), were taken for thin layer chromatography on silica gel layers (Whatman PE Sil G) developed in petroleum ether/diethyl ether/acetic acid 80/20/1 (v/v). ^{3}H label in each fraction was determined by liquid scintillation spectrometry.

Cholera toxin (CT) is internalized via clathrin-free vesicles following its interaction with caveolar gangliosides (Montesano et al., 1982). *Vibrio cholerae* toxin (Inaba 569B strain, CalBiochem, La Jolla, CA) was labeled with ^{125}I (Markwell, 1982) and its rate of internalization determined from PBS-albumin medium (pH 7.4). Okadaic acid reduces the number of cell-surface caveolae, an effect reversed by staurosporine (Parton et al., 1994). The effect of okadaic acid and staurosporine (both from CalBiochem) was determined under the same conditions both on the rate of efflux of [^{3}H]FC to plasma and on the rate of internalization of ^{125}I-labeled CT.

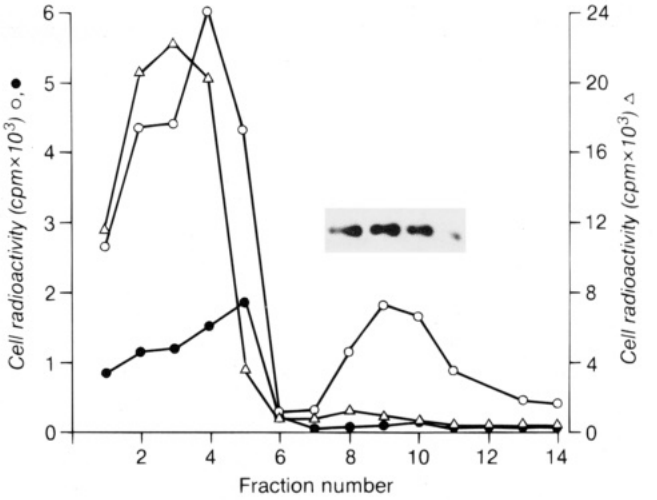

FIGURE 1: Distribution of [^{3}H]FC between caveolae and bulk membrane fractions in cells either preequilibrated (24 h) with [^{3}H]-FC as described under Experimental Procedures or incubated with [^{3}H]FC LDL (60 min, 37 °C) in the presence or absence of 5 mM NEM. The cells from two 10 cm dishes for each gradient were washed, then chased with unlabeled LDL (15 min, 37 °C), and finally homogenized at 4 °C in methanesulfonic acid buffer containing 1% (v/v) Triton X-100. Sucrose density gradient centrifugation was carried out as described under Experimental Procedures. Open triangles, equilibrium-labeled cells; open circles, cells labeled by selective FC transfer from LDL; closed circles, cells labeled by selective transfer in the presence of 5 mM NEM. Values given are cpm per 0.1 mL of fraction. In the equilibrium-labeled cells FC specific activity was 4.2×10^3 cpm μg^{-1}; in the cells labeled with [^{3}H]FC LDL (LDL-FC 90 μg mL^{-1}) FC specific activity was 2.0×10^4 cpm μg^{-1}. Insert: caveolin as determined with anti-caveolin polyclonal antibody. There was no detectable signal in any other gradient fraction. In some gradients low levels of [^{3}H]FC label were recovered at the top of the gradient (fractions 13 and 14) where caveolin was not detectable.

RESULTS

Effects of Cholesterol Transfer from LDL on Caveolar Cholesterol. As shown in Figure 1 and consistent with previous reports (Brown & Rose, 1992; Sargiacomo et al., 1993; Smart et al., 1994), a Triton-insoluble caveolar membrane fraction was isolated on sucrose gradients and identified by its content of cholesterol and the localization to this fraction of protein markers (caveolin, annexin II). The rest of the cellular FC was recovered in a more dense membrane fraction. This fraction showed no reactivity with antibodies to caveolin or annexin II. The caveolar fraction contained 3.0 ± 0.4% of gradient FC mass and a similar proportion (3.4 ± 0.6%) of total label in extracts of cells prelabeled to equilibrium with [^{3}H]FC (Figure 1). When monolayers labeled to equilibrium with [^{3}H]FC were incubated with cholesterol oxidase to selectively modify caveolar FC, 3.5 ± 0.7% of label was recovered in the cholest-4-en-3-one fraction.

When the cells were preincubated with [^{3}H]FC-labeled LDL (60 min, 37 °C) in the absence of HDL, FC mass increased by 15 ± 3%. After removal of surface-associated [^{3}H]LDL with cold LDL (15 min, 37 °C), the distribution of label between caveolar and bulk membrane fractions was determined both by sucrose-density gradient analysis and in

Table 1: Effects of Plasma Factors on Caveolar FC Content and FC Oxidation[a]

	% caveolar FC	% FC oxidized
control[b]	3.4 ± 0.6	3.6 ± 0.4
LDL FC-labeled[c]	21.6 ● 3.0	22.7 ± 2.2
+ plasma[d] (3 min, 37 °C)	10.0 ± 2.0	12.0 ± 1.3
+ HDL[d] (3 min, 37 °C)	13.0 ± 3.2	15.0 ± 2.8

[a] The proportion of [^{3}H]cholesterol in the caveolar fraction was determined following density gradient centrifugation, as described under Experimental Procedures. The proportion of FC reactive with cholesterol oxidase was determined from the recovery of labeled FC and cholest-4-en-3-one after cholesterol oxidase treatment for 60 min at 37 °C. [b] Uniformly labeled cells after 24 h at 37 °C with [^{3}H]cholesterol labeled human plasma- DMEM. [c] Following incubation (60 min, 37 °C) with [^{3}H]FC LDL. [d] Following incubation (3 min, 37 °C) with 80% (v/v) native plasma or the HDL fraction of plasma at the same dilution. Values shown are means ± one SD for three independent experiments.

terms of reactivity with cholesterol oxidase. There was an approximately 6-fold increase in the proportion both of [^{3}H]-FC associated with the caveolar fraction (to 21.6 ± 3.0%) and a parallel increase in the proportion of [^{3}H]cholest-4-en-3-one measured by thin-layer chromatography following incubation with cholesterol oxidase (to 22.7 ± 2.2%) (Table 1). The proportion of FC mass in the caveolar fraction was increased to 11.0 ± 2.0% of total FC. These data show that a disproportionate increase in caveolar FC mass followed the selective transfer of LDL-FC to the cells under these conditions, together with an exchange of FC label between the caveolar and bulk membrane fractions.

Unloading of Caveolar FC by Plasma and Plasma Lipoproteins. When cells preincubated with [^{3}H]FC-labeled LDL were subsequently incubated with native plasma, there was a rapid and specific unloading of the caveolar FC fraction into the medium. A reduction of about 50% in FC label in the caveolar fraction and a similar decrease in oxidizable FC label were observed. Caveolar FC reached original levels within 15−25 min of incubation with plasma (Figure 2). The HDL-containing fraction of plasma was also effective in the unloading of caveolar cholesterol, while neither the LDL fraction at its plasma concentration or albumin (5 mg mL^{-1}) had any effect. Caveolar FC was 28.1 ± 0.4% and 27.6 ± 2.3% of total label after 5 min incubation with LDL or albumin, respectively, compared to an initial value of 29.4 ± 2.8% (difference not significant).

Effects of Metabolic Inhibitors on FC Influx and Efflux. *N*-Ethyl maleimide (NEM) did not inhibit the initial transfer of LDL-derived FC to the plasma membrane but blocked its further processing either for internalization or for specific efflux to HDL (Fielding & Fielding, 1995b). As shown in Figure 1, NEM (5 mM) almost completely (>90%) blocked the accumulation of [^{3}H]FC label in the caveolar fraction isolated by sucrose density gradient ultracentrifugation, when fibroblasts were incubated with labeled LDL. There was also a much lower total uptake of label by the cells. These findings are consistent with the role proposed for NEM as inhibitor of a vesicular transport pathway carrying LDL-FC into the cells.

The activity and number of caveolae at the cell surface is reduced by the presence of okadaic acid, an inhibitor of protein phosphatases 1 and 2A. This effect is blocked by staurosporine, a potent protein kinase inhibitor (Parton et al., 1994). As shown in Table 2, comparable changes were

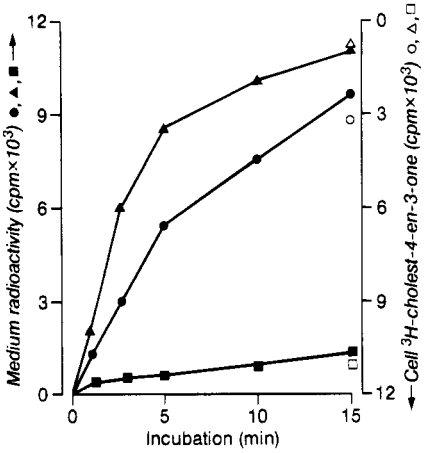

FIGURE 2: Rate of unloading of FC label from [^{3}H]FC-LDL loaded fibroblasts. Cells preincubated as described in the legend to Figure 1 were incubated with plasma (80% v/v) or with HDL or LDL fractions purified from plasma by heparin-agarose affinity chromatography and brought to the same dilution. Unloading was at 37 °C in each case. (Left axis) Medium FC radioactivity: closed triangles, native plasma; closed circles, HDL fraction of plasma; closed squares, LDL fraction of plasma. (Right axis) Label recovered in the cholest-4-en-3-one fraction of cellular total lipids after cholesterol oxidase treatment (60 min, 37 °C) at zero time or after 15 min of incubation. Open triangle, cells incubated with plasma; open circle, cells incubated with the HDL fraction of plasma; open square, cells incubated with the LDL fraction of plasma. The similarity of medium and cell radioactivities after 15 min confirms that the caveolar fraction was the major or only source of label released into the medium under these conditions.

Table 2: Effects of Okadaic Acid and Staurosporine on [^{125}I]CT influx and [^{3}H]FC Efflux[a]

inhibitors	[^{125}I]CT uptake (ng min^{-1})	[^{3}H]FC efflux (ng min^{-1})
none	24.3 ± 0.7	51.5 ± 3.0
OKA (1 μM)	14.4 ± 0.5	30.8 ± 3.5
OKA (1 μM) + STA (2 μM)	26.0 ± 1.7	51.9 ± 3.5

[a] Confluent fibroblast monolayers were washed with PBS and then incubated in PBS−human serum albumin (4 mg mL^{-1}, pH 7.4) for 60 min at 37 °C in the presence or absence of okadaic acid (OKA) or okadaic acid + staurosporine (STA) at the indicated concentration. Cells for [^{3}H]FC efflux had been prelabeled by equilibration (24 h, 37 °C) with [^{3}H]FC as described under Experimental Procedures. FC efflux was determined over 3 min following the replacement of albumin−PBS medium with unlabeled native plasma. To determine uptake of ^{125}I-labeled CT, the labeled toxin (final concentration 0.5 μg mL^{-1}) was added in PBS−albumin medium for 30 min at 37 °C, before washing with only PBS−albumin as described under Experimental Procedures. In the presence of a 100-fold excess of unlabeled CT (50 μg mL^{-1}), uptake of CT label was <5% of its original value, indicating the virtual absence of low affinity CT binding to these cells.

observed when [^{3}H]FC efflux into unlabeled native plasma and the cellular uptake of ^{125}I-CT were measured in the presence and absence of okadaic acid and staurosporine. [^{3}H]-FC efflux was reduced 50 ± 6% by okadaic acid (1 μM) (three experiments) and fully protected by staurosporine (2.5 μM). Staurosporine alone was without effect. Similar changes were observed in the uptake of CT under these conditions (Table 2).

Efflux of Newly Synthesized FC. Cells were preincubated with [^{3}H]mevalonolactone to label newly synthesized cholesterol. The distribution of [^{3}H]FC label was then determined following sucrose density gradient ultracentrifugation, as described above for LDL-derived FC. As shown in Figure 3, the caveolar fraction was labeled to a significantly greater

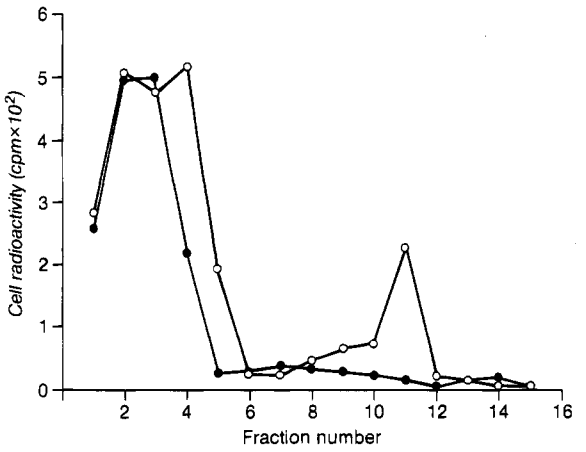

FIGURE 3: Distribution of newly synthesized cholesterol from [^{3}H]-mevalonolactone between infranatant membrane and caveolar fractions. Fibroblast monolayers were prelabeled for 3 h at 37 °C as described under Experimental Procedures. Following labeling, cells were either directly homogenized, or homogenized after preincubation with plasma (5 min, 37 °C). Sucrose density gradients were prepared and centrifuged as described under Experimental Procedures. Open circles, labeled cells processed directly. Closed circles, labeled cells processed following incubation with native plasma.

extent than in the bulk membrane fraction. When mevalonolactone-labeled cells were incubated (5−30 min) with unlabeled native plasma, the caveolar fraction was selectively released into the medium, and there was little or no loss of label from the stripped membrane fraction. These data show that newly synthesized FC, like FC internalized by selective transfer from LDL, was mainly or exclusively exteriorized to plasma via the caveolae of the plasma membrane.

DISCUSSION

Caveolae have recently been identified as integral components of a major independent pathway available in most cells for the concentration and specific transfer of solutes across the plasma membrane (Anderson et al., 1992). While the main focus of research has been on the role of caveolae in endocytosis, a potential additional role for these organelles in exocytosis is suggested by the localization there of annexins, Ca^{2+}-dependent phospholipid-binding proteins increasingly recognized for their role in membrane fusion during exocytosis (Creutz, 1992; Gruenberg & Emans, 1993).

The present results identify the caveolae as a major intermediate for the efflux of FC from cultured fibroblasts to native plasma and in particular to HDL. This conclusion is based on three types of evidence. First, labeled FC originating either by selective transfer from LDL or from new synthesis was overrepresented in the caveolar fraction of cell homogenates fractionated by sucrose density gradient, and the caveolar fraction of [^{3}H]FC label was selectively unloaded into plasma. Second, caveolar FC was specifically modified in unfixed cells by cholesterol oxidase (Smart et al., 1994). FC accessible to cholesterol oxidase was increased during [^{3}H]FC loading with LDL and preferentially decreased in the presence of plasma or HDL, in parallel to the changes found by sucrose gradient fractionation. Third, okadaic acid, an inhibitor of protein phosphatases, reduces caveolar-mediated internalization (Parton et al., 1994). In the present studies okadaic acid proportionately reduced the uptake of CT, a marker for cell surface caveolae (Montesano et al., 1982), and the efflux of [^{3}H]FC. Together, these results strongly suggest a major role for the caveolae in the efflux of FC. The unusually high concentration of FC in the caveolae (Simionescu et al., 1983; Smart et al., 1994) and copurification of components of the vesicular exocytosis pathway (Schnitzer et al., 1995) are consistent with this concept. However, the present findings appear to be the first directly implicating the caveolae in exocytosis.

FC efflux from the plasma membrane involves both a rapid, protease-dependent selective efflux of FC to pre-β-migrating, apo A-I-only HDL, and a slower, protease-independent nonspecific (probably diffusional) component to lipoproteins generally (Fielding & Fielding, 1995a). Apo A-I-specific FC efflux has been reported in adipocytes, fibroblasts, macrophages, and vascular smooth muscle cells (Barbaras et al., 1987; Castro & Fielding, 1988; Hara & Yokoyama, 1991; Ohta et al., 1992; Li et al., 1993) all of which contain relatively high levels of caveolae (Chang et al., 1994; Scherer et al., 1994; Schnitzer et al., 1995). The present research suggests a direct link between the activity of caveolae and the rate of FC efflux in normal fibroblasts. Further research will be required to establish the relationship in other cell lines, but the level and activity of cell surface caveolae may be an important factor in determining the FC content of cells.

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