Intracellular Events in the “Selective” Transport of Lipoprotein-derived Cholesteryl Esters*

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The current study utilizes human, apoE-free high density lipoprotein reconstituted with a highly specific fluorescent-cholesteryl ester probe to define the initial steps and regulatory sites associated with the “selective” uptake and intracellular itinerary of lipoprotein-derived cholesteryl esters. Bt2cAMP-stimulated ovarian granulosa cells were used as the experimental model, and both morphological and biochemical fluorescence data were obtained. The data show that cholesteryl ester provided through the selective pathway is a process which begins with a temperature-independent transfer of cholesteryl ester to the cell’s plasma membrane. Thereafter transfer of the lipid proceeds rapidly and accumulates prominently in a perinuclear region (presumed to be the Golgi/membrane sorting compartment) and in lipid storage droplets of the cells. The data suggest that lipid transfer proteins (or other small soluble proteins) are not required for the intracellular transfer of the cholesteryl esters; nor is an intact Golgi complex or an intact cell cytoskeleton (although the transfer is less efficient in the presence of certain microtubule-disrupting agents). The intracellular transfer of the cholesteryl esters is also somewhat dependent on an energy source in that a glucose-deficient culture medium or a combination of metabolic inhibitors reduces the efficiency of the transfer. A protein-mediated event may be required for cholesteryl ester internalization from the plasma membrane, in that N-ethylmaleimide dramatically blocks the internalization phase of the selective uptake process. Taken together these data suggest that the selective pathway is a factor-dependent, energy-requiring cholesteryl ester transport system, in which lipoprotein-donated cholesteryl esters probably flow through vesicles or intracellular membrane sheets and their connections, rather than through the cell cytosol.

In the intact rat ovary and adrenal, blood-borne cholesteryl ester-rich high density lipoproteins (HDL)1 supply the major share of the cholesterol used for progesterone and corticosterone production (1–4). The circulating lipoproteins are trapped in an intricate microvillar channel compartment present on the surface of the steroidogenic cells (2, 4, 5–9), and with time, cholesteryl esters (CEs) are extracted from the lipoproteins and interiorized by the cells, leaving behind the remaining lipoprotein components (2, 4, 5–8). This nonendocytic delivery of cholesteryl esters to cells has been termed the “selective” cholesteryl ester pathway (10–14) and is described as an efficient, high capacity, regulatable cholesterol delivery system (2, 4–6, 9–18). Although the pathway is especially prominent in steroidogenic cells which require large amounts of exogenous cholesterol as a fuel for steroidogenesis, this pathway appears also to operate in liver of several mammalian species and in a variety of cultured cells including fibroblasts, hepatocytes, steroidogenic cells, and various tumor cell lines from rodents, rabbits, cattle, and humans (for review, see Refs. 14–18).

The selective pathway differs from the classical B/E (LDL) receptor pathway (19, 20) for cholesterol uptake in several important respects. Most prominent is the fact that, while the cells are internalizing lipoprotein-derived lipids by the selective process, the intact lipoprotein itself is not internalized (2, 4–8, 12, 14, 21, 22). Perhaps because proteins (apo proteins) are not being interiorized, the selective pathway uses an extralysosomal route, e.g. selective pathway-interiorized CEs are directly stored in lipid droplets without prior hydrolysis by acid hydrolases found in lysosomes (14, 23–25). It is also of interest that the selective pathway shows no specificity for apoproteins, and LDL and amino acid-modified lipoproteins (as well as HDL) can utilize the pathway (2, 4–6, 10–14, 21, 22, 26). In the rat, where the predominant circulating lipoprotein is HDL, the pathway recognizes rat HDL containing apoproteins A1 + E and rat LDL containing apoB + apoE, as well as apoE-free human hHDL3 (containing only apoA1) and human LDL (containing only apoB) (2, 4–6, 10–14, 21, 22). Some primary cell cultures are able to utilize the selective pathway for internalizing CEs, even when the microvillar channel system is not present (e.g. ovarian granulosa cells) (14, 21) and even when the receptor-mediated LDL pathway is also available to the cells for cholesterol uptake (14, 21). Recent work from this laboratory on steroidogenic cells suggests that the selective pathway is a unique pathway for the bulk transport of cholesterol during steroidogenesis (14). Unlike the LDL pathway, which is constantly processing lipoprotein-derived CEs in these cells at some significant level, selective pathway function is minimally detectable in unstimulated cells; once stimulated, however, granulosa cell utilization of lipoprotein CEs by the selective pathway is both efficient and dramatic (14).

1 The abbreviations used are: HDL, high density lipoprotein; LDL, low density lipoprotein; HDL, human HDL3; CE, cholesteryl ester; V-type ATPases, vacuole-type proton-translocating ATPases; P-type ATPases, ATPases which form a covalent phosphorylated (hence the symbol “P”) intermediate as part of their reaction cycle; F-type ATPases, mitochondrial proton ATPases of the F,F1 variety which participate predominantly in ATP synthesis; BFA, brefeldin A; rec, a modified Krebs Ringer’s medium; NSF, NEM-sensitive factor; BODIPY FL C12, 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoate; SL-O, steryllysine 0.
Currently, little is known about how the lipoprotein-derived CE is transported through the plasma membrane of target cells, and virtually nothing is understood about how the internalized CE makes its way to its intracellular sites of metabolism, hydrolysis, or storage. In contrast to recognized ground rules governing intracellular protein traffic (27–30), lipid transport in cells may vary depending on characteristics of the molecule being transported: these characteristics relate to lipid charge, solubility in lipid or aqueous media, and to fatty acid chain length, composition, and saturation (31–39). In general, lipid molecules are thought to move between intracellular membranes either by vesicle budding and fusion, by lateral diffusion between organelles through membrane connections, or by protein-mediated transport through the cytosol (31–39). To date, there is no evidence to support any of these possibilities with regard to CE movement in biological systems.

Also, studies of lipid traffic have not lent themselves to the myriad technical advances which have been successfully used in defining intracellular protein traffic in cells. For certain lipids (e.g. unesterified cholesterol), spontaneous exchange or protein-mediated lipid transfer between intracellular membranes poses problems in homogenization and fractionation techniques causing uncertainty as to whether isolated membrane fractions accurately reflect the composition of their in situ membrane counterparts (40–42). These same worries accompany morphological studies of lipids and are exaggerated by the fact that lipids are soluble in many of the agents used in standard electron microscopy. With cryomicroscopy, a lack of specific antibodies and probes for most lipids has limited the ability to distinguish lipid type.

Fortunately, a new and specific fluorescent cholesteryl ester tag is making it possible to circumvent some of the above problems. Recently, we reported that we have been able to visualize the direct uptake and storage of lipoprotein-provided CEs in rat ovarian granulosa cells (14). This was possible through the incubation of the cells with reconstituted (rec) human HDL prepared with a fluorescent CE (BODIPY® FL C12) marker. The fluorescent BODIPY labeled used in the recHDL of these studies is found on the fatty acid component of the CE (14). The molecule is not hydrolizable with neutral cholesteryl esterases of the cell (14), and as such, the probe specifically identifies selective pathway-internalized CE (i.e. any small amount of HDL-CE brought in via endocytic pathways is routed through lysosomal compartments where the acidic pH actively hydrolyzes the probe) (14). The BODIPY label rapidly bleaches, but with the use of computerized, low light, confocal microscopy, it is easily captured (14). Early experiments with the specific HDL-CE-BODIPY probe demonstrated that hormone-stimulated granulosa cells can internalize a massive amount of HDL-derived CE through the selective pathway (14). The underlying message of this early study is that newly internalized HDL fluorescent-labeled CE can be directly stored (without prior hydrolysis) within the cell, and, that for the most part, the storage sites are lipid droplets. When native HDL were used in similar experiments (and the lipid accumulation was identified with the nonspecific lipid fluorescent dye, nile red), identical images were obtained. These control experiments provided evidence that the BODIPY tag on the HDL-cholesteryl ester does not modify the way in which the cells process the cholesteryl ester itself.

In the current study, we have begun to take advantage of this useful probe to define sequential steps and certain regulatory phenomena associated with the intracellular flux of CEs through cells. Ovarian granulosa cells have been utilized throughout as a responsive and uncomplicated cell model, i.e. the cells need to take in large amounts of cholesterol to be used for progesterone production, and, as such, the cholesterol flux is unidirectional, i.e. the lipoprotein-derived CE is interiorized by the cells and either stored or utilized directly in hormone production. In general, internalized cholesterol in these cells is not effluxed back to the cell surface, as is the case with unesterified cholesterol in many cell types (14).

The questions addressed in this study resulted from an early time course carried out with the CE-BODIPY probe from which it became clear that unhydrolyzed CEs from reconstituted lipoproteins entered the granulosa cells and were distributed throughout the cell very rapidly (<5 min), and within 15 min a perinuclear compartment was clearly labeled and lipid droplets were beginning to accumulate. Subsequent studies were carried out to address the issue of the importance of the Golgi compartment and the cytoskeleton to the selective CE uptake process, to question the need for cytosolic factors, and to begin to understand the role of a variety of other regulatory factors.

**EXPERIMENTAL PROCEDURES**

**Materials**

Brefeldin A was obtained from Epicentre Technologies (Madison, WI). Nocodazole was purchased from Aldrich. Okadaic acid and wortmannin were the products of L. C. Services Corp. (Woburn, MA). L-trunculin B and bafilomycin A were supplied by Calbiochem-Novabiochem Corp. (La Jolla, CA). Streptolysin O was purchased from Murex Diagnostics, Inc. (Narcox, GA). The following chemicals were obtained previously (14, 21), and sensitized with Bt2cAMP (2.5 mM) for 24 h. dexamethasone (100 ng/ml), and human fibronectin (2 

**Methods**

Isolation and Culture of Granulosa Cells—Immature female Sprague-Dawley rats (21–23 days old, Harlan Sprague-Dawley, Indianapolis, IN) were injected subcutaneously with 17β-estradiol (1 mg) daily for 5 days (21). The animals were killed 24 h after their last injection, and granulosa cells were isolated from ovaries, cultured for 72 h in Dulbecco’s modified Eagle’s medium (supplemented with bovine serum albumin (1 ng/ml), insulin (2 μg/ml), transferrin (5 μg/ml), hydrocortisone (100 ng/ml), and human fibronectin (2 μg/ml) as described previously (14, 21)), and sensitized with Bt2cAMP (2.5 mM) for 24 h.

Lipoprotein Preparation—ApoE-free high density lipoproteins (hHDL) were isolated as described previously (4, 9, 14, 21). These human-derived lipoproteins were used exclusively in this report because they are not recognized by the classical receptor-mediated LDL pathway. CE uptake and internalization studies were done as described previously (4, 14, 21, 22).

The BODIPY CE molecule as supplied by Molecular Probes Inc. is shown in Fig. 1. recHDL particles with the CE-BODIPY probe are prepared as outlined in Fig. 2 (14). The BODIPY particle is actively hydrolyzed by acid cholesteryl esterases (lysosomal), but is not a substrate for cholesteryl esterase at neutral pH (14). As a result, any visible fluorescence is considered to be intact (nonhydrolyzed) CE internalized by cells through a nonendocytic, nonlysosomal (i.e. selective) route.

Confocal Microscopic Visualization of CE-BODIPY Fluorescence—To illustrate the localization of CE-BODIPY fluorescence in rat granulosa cells, and the results were published in Proc. Natl. Acad. Sci. U.S.A. 91: 12,898 (1994). The BODIPY CE molecule as supplied by Molecular Probes Inc. is shown in Fig. 1 (14).

**Fig. 1. BODIPY-cholesteryl ester used in reconstituted HDL.** The molecule is not hydrolyzed by neutral cholesteryl esterases of the cell, but is a substrate for acidic cholesteryl esterases.

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2 E. Reaven, L. Tsai, and S. Azhar, unpublished results.
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assess the uptake and accumulation of HDL-provided CE, granulosa cells were grown on fibronectin-coated sterile glass coverslips for 72 h, treated with Bt2cAMP for 24 h prior to incubation with reHDL (50 μg/ml) for varying time periods. After incubation, each coverslip containing unfixed cells was washed three or four times with PBS, fixed with 1.5% glutaraldehyde for 5 min, washed again in PBS three or four times, and subsequently mounted on a slide containing a 1.5-cm hanging drop filled with PBS. The slide was immediately positioned in an upside-down position on an inverted microscope stage; the coverslip adhered tightly to the slide without mounting media. Imaging was performed at the Cell Science Imaging Facility (Department of Molecular and Cellular Physiology, Stanford University) and involved the use of a custom-built, mirror-scanning, single beam laser confocal microscope designed by Dr. Stephen Smith (Stanford University). The equipment uses low light (~100 microwatt beam power). The samples were excited with blue light (488 nm) and observations were made at an emission wavelength excited with blue light (488 nm) and observations were made at an emission wavelength of 512 nm. The results are expressed as arbitrary units/mg of protein/ml for 10 min at 37 °C at 37 °C. The cells were then washed, permeabilized for 10 min at 37 °C in buffer containing 0.2 unit/ml SL-O, and fixed. Preparation of Rat Luteal Cytosol—Rat luteal cytosol was prepared from pregnant mare serum gonadotropin-human chorionic gonadotropin-primed rat ovaries (2, 4, 5) using a modification of the procedure of Miller and Moore (45). In brief, day 7 luteinized ovaries were homogenized in buffer (25 mM Heps-KOH, pH 7.2, 0.1 mM potassium-glutamate, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 1 μM pepstatin, 0.5 mM 1,10-phenanthroline, 2 μg/ml soybean trypsin inhibitor, and 0.5 mM benzamidine) in a Dounce homogenizer. The homogenate was centrifuged at low speed (10,000 g) for 15 min, and the resulting supernatant was centrifuged at 100,000 × g for 90 min at 4 °C, desalted by chromatography on Bio-Gel P6 column (preequilibrated with the transport buffer), and the protein effluent was centrifuged at 100,000 × g before aliquoting and storing in liquid nitrogen. The average concentration of cytosol (protein) was ~12 mg/ml.

ATP Depletion—To study the involvement of cellular energy in the uptake and intracellular transport of cholesteryl BODIPY-CE transport, cultured granulosa cells were depleted of their intracellular ATP stores (46). Bt2cAMP primed cultured granulosa cells dishes were incubated in a glucose-free modified Krebs Ringer’s (MRK) medium (46) (115 mM NaCl, 25 mM NaHCO3, 5 mM K2HPO4, 2 mM MgSO4, 1 mM EGTA, 5 mM free Mg2+). After 30 min preincubation in the presence of metabolic inhibitors, reHDL (50 μg/ml) was added, and incubations were continued for an additional 60 or 180 min. At the end of incubation, the dishes were washed extensively, extracted with hexane-isopropanol alcohol (3:2, v/v) as described previously (14). The organic extracts were quantified by fluorometry using a Perkin-Elmer 650-40 fluorescence spectrophotometer at an excitation wavelength of 503 nm and emission wavelength of 512 nm. The results are expressed as arbitrary units/μg of DNA. When necessary, cell preparations were extracted and assayed for the ATP and ADP levels using a ultrasensitive bioanalyzer assay. In some studies, the modified Krebs Ringer’s modified buffer was replaced with the regular culture medium. Also, some incubations were performed in the presence of iodoacetate and 1,10-phenanthroline or dithiothreitol (100 μM each).

Electron Microscope Techniques—Granulosa cells were processed for electron microscopy by standard techniques used in this laboratory (2, 4, 9, 21). In brief, incubated cells were fixed for 10 min with 2% glutaraldehyde, dehydrated, and embedded in plastic. Quantification of cell GdG2i area and of microtubule content was carried out as described previously (22).

RESULTS

Although the selective pathway utilizes both HDL and LDL, only reconstituted (rec) apoE-free HDL3 were prepared with the CE-BODIPY agent and utilized in the present study. The choice of HDL was made to simplify experiments by reducing the possibility of lipoprotein-derived CE uptake via receptor-mediated LDL (19, 20) or LDL receptor-related protein (47, 48) pathways.

Uptake and Intracellular Transport of Cholesteryl Esters to Lipid Droplets—The specificity and rapid uptake of CE-BODIPY HDL by granulosa cells are shown in Fig. 3. No fluorescence is observed if stimulated (Bt2cAMP-treated) granulosa cells are incubated with a free fatty acid (lauric acid) BODIPY-BSA complex instead of CE-BODIPY HDL (Fig. 3A), or with native (non-BODIPY-incorporated) HDL (Fig. 3B). The
Fig. 3. Uptake and intracellular transport of recHDL-BODIPY-CE to lipid droplets. A, free cholesterol control. Granulosa cells were incubated for 1 h with free fatty acid (lauric acid) BODIPY-BSA complex to show that unesterified cholesterol present in cells will not be esterified with released BODIPY fatty acids to become visible by fluorescent techniques. B, hHDL$_3$, control. Granulosa cells were incubated with native (nonfluorescent) hHDL$_3$ for 1 h. No cell fluorescence is visible. C, +recBODIPY-CE HDL (5 min incubation). All granulosa cells in the preparation showed a very low level (greenish/yellow) fluorescence throughout the cytoplasm, with occasional cells, as shown in this figure, beginning to accumulate lipid droplets. D, +recBODIPY-CE HDL (15-min incubation). Granulosa cells in this preparation showed prominently fluorescent (yellow) Golgi regions (see asterisk), and developing lipid droplets (yellow or red fluorescence). Favorable slices through cells showed plasma membrane staining (yellow). Background cytoplasmic staining (greenish/yellow) was more intense than that seen at 5 min. E, +recBODIPY-CE HDL (1-h incubation). The distribution of fluorescence in 1-h incubated cells was identical to that seen at 15 min, but the intensity of the fluorescence in each compartment was increased. Thus, Golgi areas (asterisks) were more intensely yellow, and more lipid droplets and more fluorescent (red) lipid droplets were observed. F, +recBODIPY-CE HDL (3-h incubation). Lipid droplets are more numerous and highly fluorescent at this time point, but Golgi areas (asterisks) and background cytoplasm of cells are similar to that seen at 1 h.

BODIPY-lauric acid control provides evidence that any small amount of BODIPY-fatty acid which may have resulted from hydrolysis of CEs would not be esterified with free cholesterol and visualized by our fluorescent technique. Additional evidence that this is the case comes from the fact that cells continued to show uptake and storage of the BODIPY-CE despite the use of an acyl-CoA:cholesterol O-acyltransferase inhibitor (Sandoz compound 58-035; 50 μg/ml, data not shown) (15).

Granulosa cells incubated with the CE-BODIPY HDL molecule for very short periods (5 min) show a faint (greenish/yellow) fluorescence throughout the cytoplasm (Fig. 3C). Occasionally one sees cells showing some medium (yellow) fluorescence in the form of droplets. Identification of these structures as lipid droplets is based on combined fluorescent/Nomarski images described in a previous report (14.) Accumulation of fluorescence in cell plasma membranes is not prominent. By 15 min of incubation with recHDL, all cells in the preparation are well labeled (Fig. 3D). Many show prominently stained areas adjacent to the nucleus (see asterisks), as well as yellow and red (highly fluorescent) lipid droplets. An occasional cell plasma membrane is seen. By 1 h (Fig. 3E) and 3 h (Fig. 3F) of incubation with the recHDL, lipid droplets in the cells are increasingly labeled and more highly fluorescent (red) lipid droplets are seen throughout. In the 1- and 3-h incubated cells, the fluorescent perinuclear regions continue to be prominent, and background color is high, the latter suggesting increased membrane and/or cytosolic staining.

On the basis of their cellular location and their identical location in granulosa cell preparations provided in vivo with BODIPY-ceramide for 15 min (49) or stained with an antibody to Golgi membrane coat protein, β-COP (22), the fluorescent perinuclear regions were assumed to belong to the Golgi compartment. For convenience this region of the cells will be referred to as the Golgi complex.

Effect of Various Golgi and Cytoskeletal Inhibitors on the Uptake and Distribution of BODIPY-CE from recHDL—In order to determine to what extent an intact Golgi compartment is required for continued selective pathway uptake, intracellular transport, and storage of HDL-BODIPY-donated CEs in granulosa cells, cell preparations were pretreated with several agents known to disrupt Golgi structure and function (50–55). These included brefeldin A (BFA), which directly affects elements of the Golgi complex (50–55), as well as nocodazole and colchicine, which interfere with microtubule assembly (52, 53), but also disrupt Golgi membranes, and okadaic acid, which is a potent inhibitor of serine type protein phosphatases 1 and 2A (54), but also disrupts Golgi membranes (22, 55).

In addition, cytochalasin D (56, 57) and latrunculin B (58) were used to interfere with actin-type filaments, and acrylamide (59, 60) was used to interfere with intermediate filament action. Wortmannin (61) was used to inhibit phosphatidylinositol 3-kinase (62), but wortmannin has also been shown to interact with tubulin in cells (63). Cells treated with the various inhibitors were examined by electron microscopy to determine whether the inhibitors had,
in fact, morphologically altered the cytoskeleton and/or the Golgi compartment. In general, the inhibitors functioned as previously reported, i.e., treatment with BFA, nocodazole, colchicine, and okadaic acid resulted in 70–80% smaller Golgi compartments measured as Golgi area/cytoplasmic area. In addition, nocodazole and colchicine reduced the number of cytoplasmic microtubule segments by >80%; latrunculin B and cytochalasin D disrupted the microfilament pattern, and acrylamide altered the intermediate filament pattern normally found in granulosa cells.

With each cytoskeletal inhibitor, total cellular BODIPY fluorescence (measured fluorometrically in organic extracts of cells) is described in Table I. Under the conditions used, only nocodazole and colchicine were associated with a significant drop in total BODIPY fluorescence in granulosa cells. Cell uptake with the other inhibitors was comparable to that seen in controls of the same cell preparation.

With the use of confocal microscopy, only nocodazole- and colchicine-treated cells appeared to show a reproducible loss in the amount of stored BODIPY-CE following incubation with CE BODIPY HDL. However, disruption of Golgi membranes by other inhibitors, or rearrangement of microfilaments or intermediate filaments with the other inhibitors (which often produced dramatic shape changes in cells) had no noticeable effect on either the uptake or the intracellular distribution of the BODIPY-CE marker. Curiously, treatment with okadaic acid (which disrupted some microtubules as well as disrupting Golgi membranes) in granulosa cells, had little adverse effect on the internalization or storage of reCHDL-derived cholesteryl ester. Fig. 4 provides confocal images illustrating the effect of nocodazole (Fig. 4B) and BFA (Fig. 4C) as compared to control cells (Fig. 4A) incubated for 1 h with reCHDL. Both the nocodazole and BFA-treated cells showed ample uptake and lipid droplet storage of BODIPY-cholesteryl esters, although the overall amount of storage in noaddazole-treated cells appeared to be less. Golgi areas in the nocodazole-treated cells were no longer prominently fluorescent; Golgi areas in the BFA cells were much reduced in size. Of special interest is the fact that pretreatment with glutaraldehyde (Fig. 4D), used here as a means of nonspecifically denaturing and cross-linking membrane proteins, was found to completely inhibit the plasma membrane uptake and internalization of the BODIPY-CE marker.

Effect of Cold Temperature Incubation on the Uptake and Distribution of BODIPY-CE from reCHDL—In an attempt to alter membrane fluidity (and/or decrease cellular energy utilization), some granulosa cell preparations (Fig. 5) were incubated with reCHDL at 4°C for one hour, then warmed to 37°C and examined at 1 min, 15 min, and 1 h. At 4°C, BODIPY fluorescence is found only in the granulosa cell plasma membrane (Fig. 5A); i.e., no BODIPY-CE is internalized by the cells. However, once the cells are warmed, BODIPY-CE is rapidly internalized by the cells (Fig. 5, B–D), and its intracellular distribution is identical to that seen in control cells (see Fig. 3).

**Table I**

Effect of various cytoskeletal inhibitors on BODIPY cholesteryl ester delivery to cultured granulosa cells

Granulosa (1 × 10⁶) cells were cultured in a serum-free Dulbecco's modified Eagle's (DME):F12 medium for 72 h. Cells then received DME:F12 medium containing Bt,cAMP (2.5 mM) to sensitize the cells. Subsequently, cells were pretreated with indicated concentrations of various inhibitors for appropriate times (as noted below) prior to the addition of reCHDL (50 μg/ml) for 60 min. BODIPY-CE was extracted and analyzed as described under “Experimental Procedures.” Values shown represent the mean for duplicate samples in one experiment which was repeated on two further occasions with virtually the same results.

| Inhibitorsa | BODIPY-CE internalized (U × 10⁻³)b | % |
|-------------|-----------------------------------|---|
| None (control) | 11.76 | 100 |
| BFA (35 μM) | 10.36 | 88 |
| Colchicine (10 μM) | 7.76 | 66 |
| Nocodazole (30 μM) | 7.12 | 61 |
| Okadaic acid (100 nM) | 10.37 | 88 |
| Latrunculin B (0.35 μg/ml) | 11.44 | 97 |
| Cytochalasin D (20 μM) | 13.20 | 112 |
| Acrylamide (5 mM) | 11.96 | 102 |
| Wortmannin (100 nM) | 12.20 | 104 |

a Pretreatment time = BFA, 10 min; nocodazole, colchicine, 90 min; okadaic acid, 180 min; latrunculin B and cytochalasin D, 60 min; wortmannin, 90 min; and acrylamide, 360 min.

b U = arbitrary units.

**Fig. 4. Effect of cytoskeletal inhibitors and glutaraldehyde on BODIPY-CE uptake.** Granulosa cells were pretreated with glutaraldehyde or inhibitors for specified times, then incubated with reCHDL for 1 h. A, control cells; B, nocodazole (30 μM, 90 min); C, BFA (35 μM, 10 min); D, prefixation with 1.5% glutaraldehyde for 5 min. Cells treated with nocodazole or BFA showed very little fluorescence in the Golgi region. Nocodazole-treated cells showed a diminished number of fluorescent lipid storage droplets. Glutaraldehyde-treated cells did not take up BODIPY-CEs.
Effect of Cytosolic Factors on the Uptake and Distribution of BODIPY-CE from reCHDL—To rid cells of small sterol-type transfer factors (33) and other cytosolic peptide/protein factors ≤150 kDa (28–30), some granulosa cells were permeabilized with streptolysin O (43–45). Experiments indicated that >85% of the cells were permeabilized as determined by staining of nuclei with the membrane-impermeable dye, propidium iodide (45). Functionally, this resulted in loss of >80% of lactic dehydrogenase stores in cells.

Fig. 6 shows propidium iodide stained intact (−SL-O) and semi-intact (−SL-O) granulosa cells at low magnification (A and B), or high magnification (C and D), or the same cells visualized by phase microscopy (E and F). Fig. 6, G and H, shows that both preparations of cells actively take up, internalize, and transport the BODIPY-CE to lipid droplets. Under the conditions of these experiments, no differences in quantity or distribution of the fluorescent probe can be detected between intact and semi-intact cells. The addition of ATP (in the presence of a ATP-regenerating system), and/or the addition of cytosol (prepared from luteinized ovaries) prior to treatment with the reCHDL did not alter the uptake of the tracer.

The Role of ATPases on the Uptake and Distribution of BODIPY-CE from reCHDL—Granulosa cells were preincubated with various V-type ATPase inhibitors, NEM, bafilomycin, KNO₃ (64–67), and general P-type ATPase inhibitors (e.g., vanadate, molybdate) (64, 65) prior to incubation with reCHDL. They were examined for total fluorescence biochemically and/or by confocal microscopy. Table II shows the effect of treatment with the various agents on total BODIPY-CE delivery. Of the inhibitors used, only NEM showed a reduction in BODIPY-CE uptake. However, when viewed by confocal microscopy (Fig. 7) it becomes clear that NEM has an additional effect. With this agent, BODIPY-CE can enter the plasma membrane of cells to some extent, but subsequent internalization steps are totally blocked (Fig. 7B). Provision of a sulfhydryl agent (e.g. dithiothreitol), prevented the NEM effect, and such cells transported the BODIPY-CE normally. In contrast, cells preincubated with P-type ATPase inhibitors, vanadate (Fig. 7C) or molybdate, showed no changes in BODIPY-CE content and distribution.

The Effect of Energy Inhibitors on the Uptake and Distribution of BODIPY-CE from reCHDL—In order to assess the importance of cellular energy to the selective pathway, cells were preincubated with MKR medium and various inhibitors of glycolysis (2-deoxyglucose and iodoacetate) and/or oxidative phosphorylation (carbonyl cyanide m-chlorophenylhydrazone, azide). Table III indicates that all of these agents have some effect on total cell BODIPY fluorescence, with iodoacetate and a combination of azide + 2-deoxyglucose showing major changes. The data of Table IV which measure CE uptake using double-radiolabeled particles (i.e. 125I-labeled dilactitol tyramine-3H-labeled cholesteryl oleoyl ether-hHDL), agree with these data showing a 50% decrease in CE uptake with iodoacetate or azide + 2-deoxyglucose. Confocal images (Fig. 8) show some diminished uptake of the BODIPY-CE in cells incubated in a glucose-free MKR medium (compare Fig. 8, A and B) and an additional loss of fluorescence in similarly maintained cells pretreated with the metabolic inhibitors, azide + 2-deoxyglucose before incubation with reCHDL (Fig. 8C). Under these conditions, cellular ATP levels were reduced 60–80%. It is of interest that Golgi regions are generally not visible in azide + 2-deoxyglucose-treated cells, but otherwise the distribution pattern of fluorescence in these energy-deprived cells looks much like that of control cells.

DISCUSSION

In the current study, a fluorescent BODIPY-CE (extracted from HDL at the granulosa cell surface) was used as a marker for the selective uptake and intracellular processing of lipoprotein-derived cholesteryl esters. The distribution of this marker in cells was recorded by fluorescent microscopy under varying conditions, and quantitative information on the total cellular uptake of the marker under similar conditions was assessed fluorometrically in cell extracts. The combined use of these techniques overcame inherent problems in both methods, i.e. difficulties in acquiring accurate quantitative information microscopically because of photobleaching of the samples and quenching of the BODIPY label in lipid-rich environments and difficulties in obtaining site-specific information about the BODIPY-CE label when using the biochemical approach.

Data from this study have provided the following insights into how lipoprotein-derived CEs are selectively internalized and transported intracellularly in stimulated rat ovarian granulosa cells. (a) The uptake of HDL-derived CE through the selective uptake pathway is a process which begins with the transfer of CE to the plasma membrane; this transfer proceeds at 4 °C, but is less efficient than at higher temperatures. (b) Cell internalization of the plasma membrane-transferred lipid does not occur at 4 °C, but proceeds rapidly at permissive temperatures and CEs accumulate prominently in a Golgi (perinuclear) compartment of cells and in lipid storage droplets. (c) Studies on permeabilized cells suggest that lipid transfer proteins (or other small soluble proteins) are probably not required for the intracellular transport of the CEs (although a requirement for large (preassembled?) and/or membrane-associated protein complexes was not ruled out). Also, (d) inhibitor studies with various agents suggest that an intact Golgi compartment is not an absolute requirement for CE transport, nor (e) an intact cytoskeleton, although the use of nocodazole and colchicine appear to diminish the efficiency of CE intracellular trafficking. Our data suggest that the intracellular portion of the selective pathway is to some extent dependent on an energy source in that an energy-deficient culture medium, or a combination of metabolic inhibitors reduces the efficiency of the intracellular transfer of CEs. That these conditions (or the loss of ATP in permeabilized cells) do not absolutely block the pathway suggests that the intracellular movement of CEs and accumulation into lipid droplets may require a low level of
energy, perhaps that which is always available from mitochondrial sources. Our data suggest also (g) that some control for the internalization and intracellular flow of cholesteryl esters (which may involve a protein with sulfhydryl groups or the action of an NEM-sensitive factor (NSF) protein) resides at the plasma membrane level, i.e. inhibition with NEM permits some transfer of lipoprotein cholesteryl esters into the plasma membrane but completely blocks the further processing of this cholesteryl ester by the cell.

Taken together, these data suggest that the selective pathway is a factor-dependent, energy-requiring CE transport system, in which the lipoprotein-donated CEs probably flow through vesicles or intracellular membrane sheets and their connections, rather than through the cytosol. The fact that

### Table II

| Inhibitor               | BODIPY-CE internalized (U < 10^{-5}) | % |
|-------------------------|--------------------------------------|---|
| None (control)          | 11.76                                | 100|
| Bafilomycin A (250 nM)  | 12.44                                | 106|
| NEM (1 mM)              | 5.84                                 | 50 |
| KN04 (50 mM)            | 10.88                                | 93 |
| Vanadate (50 μM)        | 11.40                                | 97 |
| Molybdate (250 μM)      | 12.20                                | 104|

*U = arbitrary units.*
membranes of the cell interior, uptake of the BODIPY-CE has different requirements than do ess. It is of interest that the granulosa cell plasma membrane some level of order is required for different stages of the proc-
onto membranes and flows in an unrestricted way, but that
CE whatsoever, suggests that the lipid is not simply adsorbed
proteins prevents any cellular uptake of the BODIPY-labeled
glutaraldehyde cross-linking and/or denaturation of membrane
proteins prevents any cellular uptake of the BODIPY-labeled CE whatsoever, suggests that the lipid is not simply adsorbed
into isolated fractions of rat ovary plasma membrane continues to take place in the cold, albeit at a reduced level (68). Whether
difference in membrane permeability to the BODIPY-CE is due to differences in the composition or physical properties of
plasma membranes versus endomembranes (such as differ-
ences in the content of free (unesterified) cholesterol which
could affect the fluidity of the membranes at low tempera-
tures), or is due to low temperature interference with some essential lipid translocase (69) or NSF (with a role in translo-
cating lipids from one membrane compartment to another) (31), is not yet clear.
In considering the possibility that the internalized BODIPY-
CEs flow either through membrane vesicles or through sheets of endomembranes of granulosa cells, one has to assume that the lipids can both enter and exit membranes and flow through them laterally. We know that lipids flow readily in the mid-
plane of membrane bilayers where lipid hydrocarbon chains have considerable motional freedom (31, 34, 35, 39), but the ease of entering and exciting membranes depends to a large extent on the specific properties of the lipid being transported. Theoretically, the neutral CEs would have greater ease in
flowing through membranes than would highly charged lip-
ids. But, in addition, CEs can exist in a liquid crystalline state (as evidenced by the fact that they form liquid crystals in the interior of serum lipoproteins or in atherosclerotic plaques), and depending on conditions (temperature, fatty acid chain length, saturation, and composition), they can undergo phase transitions which could facilitate their diffusion into mem-
branes (31, 70–72). However, in our laboratory experience with various CE-rich particles exhibiting variations in fatty acid

![A](image1.png) ![B](image2.png) ![C](image3.png)

**Fig. 7.** Effect of NEM and vanadate on uptake of HDL-derived BODIPY-CEs. Control cells were treated with reHDL for 1 h (A), preincubated (B) with NEM (1 μM, 20 min), or preincubated (C) with vanadate (50 μM, 20 min) prior to incubation with reHDL for 1 h. Whereas NEM-inhibited cells showed no interiorization of BODIPY-

**TABLE III**

Effect of metabolic inhibitors on BODIPY-CE delivery to cultured granulosa cells
Granulosa cells were sensitized with Bt2cAMP (2.5 mM) for 24 h prior to incubation in Dulbecco's modified Eagle's (DME):F12 medium or MKR medium for 3 h. Subsequently, cells were pretreated with various metabolic inhibitors for 30 min prior to the addition of reHDL (50 μg/ml) for 1 h. Other details were same as described under Table I and “Experimental Procedures.”

| Inhibitor | BODIPY-CE internalized (μg CE/μg DNA)* |
|-----------|----------------------------------------|
| DME:F12 medium (control) | 11.76 (100) |
| + Iodoacetate (0.2 mM) | 7.20 (61) |
| MKR medium | 8.30 (71) |
| + 2-Deoxyglucose (50 mM) | 8.00 (68) |
| + Azide (3 mM) | 7.56 (64) |
| + 2-Deoxyglucose + azide | 6.61 (56) |
| + m-Cl-CCP (20 μM) | 7.32 (62) |
| + Iodoacetate (0.2 mM) | 4.84 (41) |

* U = arbitrary units.

**TABLE IV**

Effect of metabolic inhibitors on uptake (internalization) of HDL-derived cholesteryl esters
Results are expressed as nanograms of CE internalized/μg of DNA; changes as percent of control are shown in parentheses. Incubation conditions were the same as described under Table III except glucose free Dulbecco's modified Eagle's (DME):F12 culture medium contained 0 μg H-cholesterol ester-dilactitol tyramine-2H-labeled cholesteryl oleoyl ether-hHDL 3 ± indicated concentrations of various inhibitors. All incubations were performed for 5 h at 37°C. Other details were the same as described under “Experimental Procedures.” The protein to cholest-

| Inhibitor | Mass of cholesteryl esters internalized (nanograms CE/μg DNA) |
|-----------|---------------------------------------------------------------|
| None (control) | 28 (100) |
| Carbonyl cyanide m-chlorophenylhydrazine | 22 (80) |
| Azide (3 mM) | 21 (76) |
| 2-Deoxyglucose (50 mM) | 22 (80) |
| Iodoacetate (0.2 mM) | 13 (48) |
| Azide + 2-deoxyglucose | 14 (49) |

Inhibitor Mass of cholesteryl esters in-
membrane domains (e.g. sphingomyelin-rich areas) are themselves capable of gel to liquid crystal phase transitions which could affect the rate of lipid diffusion through the bilayers (31–39). Such considerations may explain how certain intracellular membrane compartments in the granulosa cells of the current study (e.g. Golgi regions) acquire particularly prominent BODIPY-CE fluorescence.

The pattern of BODIPY-CE flow through the granulosa cells is of interest. The BODIPY-CE uptake by granulosa cells is extremely rapid, and once inside, entire cells appear to be uniformly labeled within minutes. Perinuclear (Golgi) regions are clearly labeled by 15 min, but lipid droplets appear to be forming almost simultaneously with the Golgi labeling. Several questions come to mind. Are there two intracellular pathways for the moving CE? One, in which CE goes directly from plasma membrane to endoplasmic reticulum sites from which lipid storage droplets emerge, and another, in which CE flows through the Golgi to become incorporated into Golgi-associated membranes? Or is there simply a lipid mass transported spontaneously through all cell membranes, a flow which highlights the Golgi region with a fluorescent probe because there is a large concentration of membranes in that region, or because Golgi membrane composition provides the organelle with a unique affinity for the fluorescent probe? The fact that agents like BFA, nocodazole, colchicine, and okadaic acid disrupt the Golgi compartment (and reduce Golgi region fluorescence in these cells), but do not have a major effect on the ability of the cells to store CEs, seems to suggest that the storage part of the pathway does not require an intact Golgi compartment. The fact that in normal cells, lipid droplet formation and Golgi compartment labeling occurs simultaneously, rather than sequentially, fits with this thinking, as does the fact that Golgi regions remain uniformly labeled after pulse labeling with HDL BODIPY-CE, and Golgi fluorescence is not diminished as lipid droplet storage increases with time.

Despite the use of a large number of inhibitory agents, only one, NEM, results in a block of the internalization phase of the selective uptake of BODIPY-CEs; i.e. in granulosa cells treated with 1 mM NEM and incubated with HDL BODIPY-CE, the resulting fluorescence is limited to granulosa cell plasma membranes. It may be that NEM interferes with a required translocation of plasma membrane-absorbed BODIPY-CEs across the bilayer to the cytoplasmic leaflet, or NEM may interfere with the exit of BODIPY-CE from the inner leaflet of the bilayer to the cell interior. In either scenario a protein-mediated event would seem to be a requirement for CE internalization. Membrane translocases in other systems have been found to be ATP-dependent (73) and NEM-sensitive, and V-type ATPases (e.g. NSF proteins) have been found both within the plasma membrane and at the inner surface of the plasma membrane where they are presumably involved in vesicular traffic between the plasma membrane and endomembrane compartments of cells (74–76). However, V-type ATPases and NSF proteins are characteristically resistant to azides and vanadate (64, 65, 76, 78), but sensitive to agents such as NEM, baflomycin, and KNO3 (64–67, 77, 78). In this study, only NEM is effective in blocking the movement of plasma membrane CE, and it appears unlikely that a V-ATPase is involved. However, the fact that co-incubation of cells with dithiothreitol + NEM prevents the NEM effect, and that diminished BODIPY-CE uptake occurs also with the use of iodoacetate (Table IV), suggests that sulfhydryl groups are important to this step and that a sulfhydryl-requiring protein may be required for CE internalization and intracellular transport.

To our knowledge, the current study represents the first time that CEs have been traced as a pure lipid class (apart from

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**Fig. 8. Effect of metabolic inhibitors on uptake of HDL-derived BODIPY-CEs.** Control cells (A) were grown in standard medium and incubated with reHDL for 1 h. The cells of panel B were grown in a glucose-free medium, and cells of panel C were grown in the glucose-deficient medium, then incubated with azide (3 mM, 30 min) and 2-deoxyglucose (50 mM, 30 min) prior to incubation with reHDL for 1 h. In general, cells grown with the energy-deficient medium incorporated less BODIPY-CE than did control cells, but the distribution of the internalized CE was similar to that seen in controls. Cells incubated with the metabolic inhibitors showed even less CE uptake than with the deficient medium, i.e. fewer lipid droplets were seen, but, in addition, Golgi regions were not fluorescent, and general cytoplasmic staining was diminished in the cells.
association with proteins) in intact cells. As such, it is of interest to compare the BODIPY-CE transport observed in granulosa cells with the intracellular transport for another BODIPY-labeled lipid, glucosylceramide as reported by Martin and Pagano (79), and the vesicle-related efflux trafficking of endogenously synthesized free (unesterified) cholesterol as reported by Urbani and Simoni (80). In many respects, the scenarios are similar, i.e. lipid transport in all the systems appears to be vesicle or membrane mediated. Transport is rapid, not generally affected by agents disrupting Golgi or cytoskeletal networks, or by ATP depletion. Free cholesterol movement appears sensitive to temperature, and like the internalization of vesicle or membrane mediated. Transport is rapid, not gener-

similiar, by Urbani and Simoni (80). In many respects, the scenarios are

genously synthesized free (unesterified) cholesterol as reported

Pagano (79), and the vesicle-related efflux trafficking of endo-

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