Cholesterol uptake by the 'selective' pathway of ovarian granulosa cells: early intracellular events

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Abstract Although the 'selective' pathway is a major cholesteryl ester (CE) uptake pathway used by steroidogenic cells, essentially nothing is known about the itinerary of the CE once it is extracted from lipoproteins at the cell surface. In the current report we have begun to trace 'selective' pathway internalized-CE using both native and reconstituted human (h) high density lipoproteins ($hHDL_3$) with variously labeled and tagged CEs to provide information from either a biochemical or morphological perspective. It appears that the amount of $hHDL_3$-CE that is internalized and processed through the 'selective' pathway is directly related to the amount of cholesterol used for steroidogenesis at any given time point. There is a time-related correlation between the level of the Bt_2cAMP-stimulated cell steroidogenic response, the level of conversion of freshly obtained $hHDL_3$-CE into progestins, increases in $hHDL_3$-derived CE internalization, $hHDL_3$-CE hydrolysis, re-esterification and/or storage. None of this processing takes place in non-stimulated (non-Bt_2cAMP treated) cells which do not secrete progestins despite the availability of $hHDL_3$ as a cholesterol source. The data suggest that the 'selective' pathway has a special role in steroidogenic cells—one of providing sufficient cholesterol to fuel the required production of steroid hormones.—**Reaven, E., L. Tsai, and S. Azhar.** Cholesterol uptake by the 'selective' pathway of ovarian granulosa cells: early intracellular events. *J. Lipid Res.* 1995. **36:** 1602–1617.

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Mammalian cells have a number of options when it comes to the use of cholesterol. All cells need cholesterol for membrane biosynthesis but some cells must process additional cholesterol for the purpose of synthesizing new products in which cholesterol is used as a precursor molecule (e.g., steroid hormones). To accomplish these needs, cells have developed a number of cholesterol processing systems which rely to different extents on endogenous synthesis of cholesterol or the uptake of exogenous cholesterol or cholesteryl esters (1–11); the newly provided cholesterol can be stored by the cell (1, 4, 7, 12, 13), utilized in cell products (1, 4, 12–15), or returned to the cell surface (7, 12, 16). The cholesterol processing pathways can vary with cell type and function, but also with the animal species and/or characteristics of that species' circulating lipoproteins (1–5, 17–29). These variations in cells, plus complicated intracellular physiological dynamics between cholesterol and cholesteryl esters, as well as difficulties in conducting morphological studies due to problems with lipid extraction, have negatively impacted on cell research having to do with intracellular lipid traffic. As a result, information on how cells process their cholesterol for special purposes is often sketchy (12).

In the current study we have begun to address basic questions relating to one particular cholesterol pathway—that involving the 'selective' uptake and intracellular utilization of cholesteryl esters from circulating lipoproteins. The 'selective' pathway (2, 3, 5, 26–38) is a relatively simple pathway to examine because it primarily involves cholesteryl esters (CE), and this form of cholesterol (unlike free cholesterol) can be specifically labeled in natural lipoproteins or reconstituted particles and traced intracellularly. We use the term 'selective' uptake of cholesterol when surface-associated cholesterol-rich lipoproteins (HDL or LDL, regardless of apolipoprotein composition) release CE directly into cells without the lipoprotein particle itself entering the cells (2, 3, 5, 30).

Rat granulosa cells provide a useful cell model for this project as the bulk of the internalized CE in these cells

Abbreviations: CE, cholesteryl ester; HDL, high density lipoprotein; LDL, low density lipoprotein; $hHDL_3$, human HDL_3; BSA, bovine serum albumin; COE, cholesteryl oleoyl ether; DLT, dilactitol tyramine; *rec*, reconstituted.

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is directed toward synthesizing steroids and not in recycling cholesterol to the cell surface. The cells are extraordinarily responsive to exogenous lipoproteins. When cultured in the presence of both lipoproteins and hormones (or stimulatory agents such as Bt_2cAMP), they produce from 1000–2000 times the progestins made by cells grown under basal conditions (32, 35, 39). Although freshly isolated granulosa cells primarily use the 'selective' pathway for CE uptake (32, 35), after several days of cell culture, or Bt_2cAMP stimulation, the 'endocytic' (B/E receptor) pathway becomes increasingly expressed (35), and lipoprotein-derived cholesterol can be obtained by either the 'selective' or 'endocytic' pathway depending on which lipoproteins are available to the cell (32, 35).

Although the 'selective' pathway is a major CE uptake pathway for steroidogenic cells (3, 26, 28, 32, 39–41), essentially nothing is known about the itinerary of the CE once the cells internalize it, and, it is not known whether the CE itinerary varies under different metabolic conditions. In the current report, we have begun to trace CE through the 'selective' pathway of granulosa cells by asking the following questions. Is newly internalized CE utilized directly for steroidogenesis by these cells? Is the newly internalized CE stored in the cell? If stored, is it stored in fat droplets? If stored in fat droplets, is it directly stored, or hydrolyzed and re-esterified prior to storage? Do these events differ in basal versus stimulated cells, and in what way do the events change depending on the use of apoE-free human HDL_3 [internalized exclusively by the selective pathway (32, 35)], or human LDL [internalized by both the 'selective' and 'endocytic' pathways (32, 35)].

To address these questions, we have used both natural and reconstituted lipoproteins (with variously labeled and tagged CEs) to provide information from either a biochemical or morphological perspective.

EXPERIMENTAL PROCEDURES

Materials

[1α,2α(N)-^{3}H]cholesteryl oleolyl ether (1.85 TBq/mmol; 50 Ci/mmol) was purchased from Amersham Corporation, Arlington Heights, IL. [9,10-^{3}H(N)]oleic acid (2.22 TBq/mmol; 60 Ci/mmol) and cholesteryl [1-^{14}C]oleate, (1.85 GBq/mmol/ 50 mCi/mmol) were obtained from American Radiolabeled Chemicals, St. Louis, MO. [1,2,6,7(N)-^{3}H cholesteryl]cholesteryl oleate (2.9 TBq/mmol; 78 Ci/mmol), [1,2-^{3}H(N)]progesterone (2.1 TBq/mmol; 57 Ci/mmol), [1,2-(N)^{3}H]20α-hydroxypregn-4-ene-3-one (1.9 TBq/mmol, 51.2 Ci/mmol) and Na ^{125}I (carrier-free, 643.8 GBq/mg; 17.4 Ci/mg) were supplied by DuPont Co., NEN Research Products, Boston, MA. The following chemicals were the products of Sigma Chemical Co., St. Louis, MO: N^6, 2′-O-dibutyryl adenosine 3′:5′-cyclic monophosphate (Bt_2cAMP), cholesteryl oleate, triolein, progesterone, 20α-hydroxyprogesterone, and 17β estradiol. Human plasma transferrin, human plasma fibronectin, and insulin were obtained from Collaborative Research, Bedford, MA. Cholesteryl 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoate (cholesteryl BODIPY® FL C12) and Nile Red were purchased from Molecular Probes, Inc., Eugene, OR. All other reagents used were of analytical grade.

Isolation and culture of granulosa cells

Immature female Sprague-Dawley rats (21–23 days old, Bantin and Kingman, Fremont, CA) were injected subcutaneously with 17β-estradiol (1 mg) daily for 5 days. The animals were killed 24 h after their last 17β-estradiol injection (i.e., on day 6) and granulosa cells were isolated from ovaries and cultivated as previously described (39). Unless otherwise noted, the cells were maintained in basal culture medium (DME:F12 supplemented with bovine serum albumin (1 mg/ml), insulin (2 μg/ml), transferrin (5 μg/ml), hydrocortisone (100 ng/ml), and human fibronectin (2 μg/ml) for 72 h. Subsequently, cells were treated with or without Bt_2cAMP (2.5 mM) for an additional 24 h before the addition of other agents.

Lipoprotein preparation

Human (h) low density lipoprotein (hLDL) and apoE-free high density lipoprotein ($hHDL_3$) were isolated as previously described (32, 42). For uptake and internalization studies, lipoproteins were conjugated with residualizing labels, i.e., ^{125}I-labeled dilactitol tyramine (DLT) and [^{3}H]cholesteryl oleolyl ether (COE) (28). Colloidal gold hLDL (Au-hLDL) and gold $hHDL_3$ (Au-$hHDL_3$) were prepared as described earlier (35). Sucrose gradient purified Au-lipoprotein preparations were resuspended in PBS containing defatted bovine serum albumin (1%) and dialyzed overnight against PBS.

Reconstituted (*rec*) cholesteryl BODIPY® HDL particles were prepared by slightly modified procedures described earlier (37, 43–45). In brief, 2.3 μmol egg phosphatidylcholine, 0.6 μmol sphingomyelin, 2.24 μmol cholesteryl BODIPY® FL C12, 0.6 μmol unesterified cholesterol, and 0.34 μmol triolein were dissolved in chloroform, dried under N_2 as a thin film, and lyophilized. The lipid mixture was resuspended in 5 ml of Tris-NaCl-EDTA buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.25 mM EDTA), vortexed, and then sonicated at 52°C using a Fisher Sonic Dismembrator model 300 (Fisher Scientific, Pittsburgh, PA) equipped

with a microtip at a power setting of 40 watts. After sonication for 40 min, the temperature was lowered to 42°C and 5 mg of human HDL_3 apolipoproteins in 1 ml of 2.5 M urea in Tris-NaCl-EDTA buffer was added dropwise over 5 min: sonication continued for an additional 10 min. The sonicated sample was centrifuged for 20 min at 4,000 *g* in cold to sediment tungsten particles. The reconstituted cholesteryl BODIPY®-hHDL particles were isolated by sequential ultracentrifugation between the densities of 1.065 and 1.21 g/ml and dialyzed against PBS-EDTA. Immediately prior to use, the preparation was dialyzed against DME:F12 basal medium. A cholesteryl oleate-labeled hHDL was prepared essentially as described above except 2.24 μmol cholesteryl oleate (500 μCi) replaced the cholesteryl BODIPY® FL C12.

Measurement of steroidogenesis

Secretion of steroids. To assay steroidogenesis, cultured granulosa cells were pre-treated with or without Bt_2cAMP (2.5 mM) for 24 h. Subsequently, triplicate culture dishes were supplemented with ± Bt_2 cAMP (2.5 mM) ± $hHDL_3$ (500 μg protein/ml) or hLDL (100 μg protein/ml) and incubated at 37°C for 5, 9, and 24 h; samples of incubation medium were frozen and stored until assayed for progestins. Progesterone and its metabolite (20α-hydroxyprogesterone) were quantified by RIA using specific antiserum as described previously (3, 32). Results are expressed as ng progestins (the sum of progesterone and 20α-hydroxyprogesterone) produced/μg DNA and represent the mean ± SE of duplicate determinations of three different dishes.

Incorporation of newly derived $hHDL_3$-cholesteryl ester (CE) into progestins. Incorporation of $hHDL_3$-derived [^{3}H]cholesteryl oleate into progestins (progesterone + 20α-hydroxyprogesterone) was measured as described previously (32). Briefly, cultured granulosa cells (pretreated with or without Bt_2cAMP) were incubated in 2 ml basal medium containing 100 μg protein/ml reconstituted [^{3}H]cholesteryl oleate $hHDL_3$ ± Bt_2cAMP (2.5 mM). After incubation at 37°C for 5, 9, and 24 h, samples of incubation medium were removed and saved. The cells were washed extensively with culture medium containing 0.5% bovine serum albumin to remove any absorbed extracellular radioactivity. Suitable aliquots of incubation medium and cells were extracted and quantified for total progestins (i.e., [^{3}H]progesterone plus [^{3}H]20α-hydroxyprogesterone) as described earlier (32). The results are expressed as DPM [^{3}H]cholesterol incorporated into progestins/μg DNA. In each case greater than 99% of the progestins produced were secreted into the incubation medium.

Cholesteryl ester (CE) internalization by granulosa cells

In these experiments medium from 24 h ± Bt_2cAMP-treated cells was replaced with fresh medium containing ± Bt_2cAMP ± $hHDL_3$ or ± hLDL equipped with radiolabeled, non-releasable apolipoprotein and cholesteryl ester tags that would accumulate within the cells even when degraded (28, 32). Incubations were carried out with ^{125}I-labeled dilactitol tyramine-[^{3}H]cholesteryl oleolyl ether ([^{125}I]DLT-[^{3}H]COE) hLDL (100 μg/ml) or hHDL (500 μg/ml) ± Bt_2cAMP (2.5 mM) for 5, 9, and 24 h at 37°C. At the end of incubation, the accumulated cells were washed four times with phosphate-buffered saline–0.1% bovine serum albumin, one time with phosphate-buffered saline, and subsequently solubilized in 2 ml of 0.1 M NaOH. One-ml aliquots were precipitated with an equal volume of 20% trichloroacetic acid to determine insoluble and soluble radioactivity or extracted with organic solvents to determine [^{3}H]radioactivity (28, 32).

Under these conditions, trichloroacetic acid-insoluble ^{125}I radioactivity is assumed to represent ^{125}I-labeled protein remaining bound to the cell surface as part of intact lipoproteins (28, 32); trichloroacetic acid-soluble ^{125}I radioactivity is taken to be internalized, degraded, and accumulated residualizing protein ^{125}I label. As the ^{125}I and ^{3}H labels are on the same lipoprotein particles, it follows that the relative amounts of surface-bound ^{125}I and ^{3}H radioactivity must be equal. Thus, the amount of cholesteryl ester internalized can be computed as the difference between total cholesteryl ester uptake and trichloroacetic acid-insoluble (i.e., surface bound) ^{125}I radioactivity. The results are expressed as pmol ^{125}I- or ^{3}H-labeled protein internalized/μg DNA. To determine the net mass of cholesteryl ester internalized, ^{3}H-labeled protein values are divided by the protein to cholesterol ratio of each lipoprotein (e.g., for $hHDL_3$ and hLDL the respective protein/cholesterol ratios are 2.59 and 0.47).

Intracellular cholesteryl ester (CE) hydrolysis and re-esterification

The in situ esterification of newly released (hydrolyzed) lipoprotein-cholesterol was assayed by following the formation of cholesteryl [^{3}H]oleate (46). Granulosa cells were cultured in standard basal culture medium for 72 h, then treated with or without Bt_2cAMP (2.5 mM) for 24 h. On the day of experiment, ± $hHDL_3$ (500 μg/ml) or ± hLDL (100 μg/ml) and ± Bt_2cAMP (2.5 mM) were added to triplicate dishes, and incubated for 5, 9, and 24 h in 24 ml of basal medium. Each dish also received 60 μl aliquot of [9,10-^{3}H(N)]sodium oleate complexed with fatty acid-free bovine serum albumin (BSA) to given a final concentration of 100 μM oleate/20 μM BSA (45). At the end of incubation, each dish was washed three

times with 2 ml of wash buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, and 2 mg/ml BSA), followed by one wash with wash buffer without BSA. Lipids were extracted from granulosa cell cultures by the in situ procedure described by Radin (47). Two ml of hexane–isopropanol 3:2 was added to each dish and incubated for 30 min at room temperature. The organic solvent was removed and replaced with 1 ml of fresh solvent for a second 10-min extraction. The combined extracts were evaporated under N_2 and dissolved in 100 µl dichloromethane–methanol 2:1 containing internal standards, [^{3}H]cholesteryl oleate (50 µg; 25,000 dpm), unlabeled triolein (50 µg), unlabeled oleic acid (50 µg), and unlabeled cholesterol (50 µg), and 50-µl aliquots were spotted on a Whatman LK 6D Silica Gel 60A thin-layer chromatography plate. The plates were developed in heptane–ethyl ether–acetic acid 90:30:1 (v/v/v) and CE and TG spots were identified with iodine vapor, scraped from the plates, and counted in 10 ml of scintillation fluid (#4a20, Research Products International, Mt. Prospect, IL) using a Beckman LS 6000 IC Scintillation counter. The remaining protein precipitate in the dishes was dissolved in 0.2 N NaOH for 1 h and aliquots were then used for protein determination.

Intracellular triglyceride formation

Experimental conditions were similar to those used for CE formation (described above), except endpoint measurements of triglyceride were carried out.

Ability of cellular cholesteryl esterases to hydrolyze cholesteryl-BODIPY® FL C12

The hydrolysis of cholesteryl-BODIPY® and natural substrate (cholesteryl oleate) was measured at neutral (cytosolic) and acidic (lysosomal) pH using homogenates from adrenal and granulosa cells as an enzyme source.

Preparation of adrenal and granulosa cell homogenates. Adrenal and granulosa cell homogenates were prepared as described earlier by this laboratory (48, 49). In brief, freshly excised adrenals were chilled to 4°C in homogenizing medium (20 mM Tris-HCl, pH 7.4, 0.25 M sucrose, 1 mM EDTA) and cleaned of fatty tissue. The adrenals, weighing about 100 mg, were homogenized in 2 ml homogenization medium at 4°C, centrifuged at 600 *g* for 10 min, and the resulting pellets were resuspended in 1 ml of homogenization buffer and centrifuged as above. The pooled supernate was used as a source of esterases. Similarly, granulosa cells (1×10^7 cells) were homogenized in 1 ml of homogenization buffer, briefly sonicated and centrifuged at 600 *g* for 10 min. The resultant supernatant was used as an enzyme source.

Neutral cholesteryl esterase. Enzyme activity was measured by a modification of previously described procedures (48–50). The incubation medium in a final volume of 0.5 ml contained 100 mM potassium phosphate (pH 7.5), 1 mM EDTA, 2.5 mM 2-mercaptoethanol, 60 µM cholesteryl [1-^{14}C]oleate or 60 µM cholesteryl BODIPY® FL C12 and suitable aliquots of cell extracts (10–100 µg protein). The assay was initiated by the addition of respective cholesteryl ester substrate in 5 µl of acetone. The reaction was stopped and the unhydrolyzed substrate was removed by addition of 8.15 ml of methanol–chloroform–heptane 1.41:1.25:1.00 (v/v/v) followed by addition of 2.65 ml of 50 mM potassium carbonate, 50 mM potassium borate buffer, pH 10 (51). The mixture was vortexed for 5 min, shaken for 30 min, and then centrifuged for 10 min at 1500 *g* to clear two phases. The amount of liberated [1-^{14}C]oleate in the upper aqueous phase was determined by the addition of 1.5 ml aliquot to 15 ml of Safety Solve (Research Products International, Mt. Prospect, IL) and measurement of radioactivity. The amount of released BODIPY® fatty acid in the aqueous phase was quantified spectrofluorometrically at 503 nm excitation and 512 nm emission.

Acid (lysosomal) cholesteryl esterase assay. Enzyme activity associated with lysosomes was measured by a modification of the procedure of Haley et al. (52). The reaction mixture in a final volume of 0.5 ml contained 50 mM sodium acetate (pH 3.9), 2 mM sodium taurocholate, 0.005% digitonin, 60 µM cholesteryl [1-^{14}C]oleate or 60 µM cholesteryl BODIPY® FL C12 and suitable aliquots of cell extracts (10–100 µg protein). The assay was initiated by the addition of substrate in 5 µl acetone and was allowed to proceed for 30 min at 37°C before termination with 8.15 ml of methanol–chloroform–heptane 1.41:1.25:1.00 (v/v/v), followed by the addition of 2.65 ml of 50 mM potassium carbonate, 50 mM potassium borate buffer, pH 10.0 (51). The released [1-^{14}C]oleate or BODIPY® FL C12 was quantified using liquid scintillation counting and spectrofluorometric techniques, respectively, as described above.

Miscellaneous biochemical techniques

The DNA content of the cells was quantified fluorometrically (35). The procedure of Markwell et al. (53) was used to quantify protein content of cellular homogenates and lipoproteins. Cholesterol and cholesteryl ester determinations were carried out as described by Tercyak (54). The delipidated preparation of $hHDL_3$ apolipoproteins was obtained using the ethanol–diethyl ether extraction as described previously (37).

Morphological techniques

Electron microscopy. Granulosa cells were processed for electron microscopy by standard techniques used in this

laboratory (3, 31, 42); in brief, incubated cells were fixed for 10 min with 2% glutaraldehyde, scraped from dishes, pelleted for 30 sec at 10,000 *g*, fixed again in glutaraldehyde followed by osmium, uranyl acetate, dehydration in alcohols, and embedment in plastic. For standard microscopy, thin sections were stained with uranyl acetate and lead and viewed with a JEOL (JEM100CX-II) electron microscope.

For negative staining of gold-labeled lipoproteins, preparations of lipoproteins were dialyzed overnight with ammonium acetate/ammonium carbonate buffer (pH 7.4) then brought to a concentration of 100–200 μg protein/ml with dialysis buffer. One drop of the lipoprotein preparation was placed on the reverse side of a formvar/carbon-coated grid for ~30 sec, wicked off, and stained with filtered 1% sodium phosphotungstate (pH 7.0) for ~30 sec, air dried, and immediately viewed in the electron microscope.

For quantifying gold particle uptake by granulosa cells, two separate preparations of cells incubated with gold-labeled $hHDL_3$ and hLDL (10 μg/ml) for 5 h were processed, sectioned, and stained as for standard electron microscopy. Ten micrographs were taken at random from each preparation at 4,800 × (each micrograph contained portions of ~ 4–5 cells), prints were enlarged to a final magnification of 24,000 ×, and all gold particles observed in each print were counted with the aid of a magnification eyepiece (7 × Mag). Subsequently, the total cell area comprising each micrograph was measured using an image analysis system (Bioquant II, R + M Biometrics, Nashville, TN), and the average number of cell-internalized gold particles/100 μm^2 cell area was assessed.

Fluorescence microscopy. To assess the uptake and direct accumulation of HDL-provided CE, granulosa cells were grown on fibronectin-coated UV-treated glass coverslips (0.3×10^6 cells/coverslip) for 72 h, then treated with or without cAMP for 24 h prior to incubation with *rec*hHDL for varying time periods. After incubation, each coverslip containing unfixed cells was washed 3–4 × with PBS, mounted on a slide containing a 1.5-cm hanging drop filled with PBS, and 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 (Dept. of Molecular and Cellular Physiology, Stanford University) and involved the use of a custom-built, mirror-scanning, single-beam laser confocal microscope designed by Stephen Smith (Stanford University). The equipment uses low light (< 100 μwatt beam power) and is attached via a Nikon inverted microscope to laser-scanned Nomarski DIC. The samples were excited with blue light (488 nm) and observations were made at an emission wavelength of 510–550 nm. The fluorescent signal was filtered by a dichroic mirror (510). A Nikon 60 × (NA 1.4) planapo objective was used. The fluorescent and Nomarski images were stored in a computer and subsequently merged using Adobe Photoshop; the red color was assigned arbitrarily.

For Nile Red fluorescence (55), cells on coverslips [incubated as above, but with native $hHDL_3$ (500 μg/ml)] were washed 3–4 × with PBS, fixed for 5 min with 1.5% glutaraldehyde, washed 3 ×, then incubated with Nile Red (prepared as a stock solution of 10 μg/ml in acetone and used at 1:100 dilution) in PBS for 5 min (protected from light), washed briefly in PBS, and mounted upside-down on a drop of distilled water. The cells were examined and processed with the equipment described above (see Fig. 6, yellow color assigned arbitrarily), or with a Leitz Orthoplan photomicroscope equipped for epi-illumination. With the latter, Nile Red fluorescence was viewed as yellow/orange using a 488 nm band pass exciter filter, a 510 nm center wavelength chromatic beam splitter, and a 528 nm long pass barrier filter. As the stained cells faded rapidly with the high pressure mercury light source, photography using a 40 × planneofluor objective lens was carried out quickly (1–2 sec exposures) using Kodak TMAX (ASA 400) film, push-processed to 1600.

In order to assess lipid droplet accumulation under different conditions, granulosa cells on coverslips treated with or without cAMP and incubated for 24 h without lipoproteins or with native HDL_3 or hLDL were stained with Nile Red as described, and different fields of cells were selected randomly and rapidly photographed. Photographic prints (8 × 10 in) were prepared and all lipid droplets were counted in all of the photographed cells (~400) with the aid of a 7 × magnification eye piece.

RESULTS

When granulosa cells are maintained in culture for 72 h, then treated with cAMP for 24 h with or without lipoproteins, they undergo dramatic shape changes associated with activated cells. In the basal (unstimulated) state, the cells are exceedingly flat and stretched out on the culture dish. After cAMP, the perinuclear region of the cells swells in height as the cells retract much of their peripheral cytoplasm. The newly swollen central portion of stimulated cells includes the Golgi Complex and all of the cell's mitochondria, lysosomes, and fat droplets. The remaining peripheral cytoplasm is stretched so thinly that it becomes almost invisible at the light microscope level, though with Nomarski imaging and/or

electron microscopy it is clear that most cells continue to have peripheral contact with each other.

Steroidogenic response to lipoproteins

In the absence of stimulation and without lipoproteins, granulosa cells make no progestins. When the unstimulated cells are provided with either $hHDL_3$ or hLDL, progestin production is still very low (**Fig. 1A**), but on an expanded scale (insert to Fig. 1A) one sees that hLDL induces the formation of ~3 times more hormone than does $hHDL_3$.

Figure 1B shows that cAMP-treated granulosa cells respond slowly to the availability of lipoproteins, showing only small increases in progestin production for the first 9 h, but large production rates thereafter; by 24 h, cells incubated with $hHDL_3$ (or hLDL) produce 10–20 times the amount of hormone made by cells not given lipoproteins.

That the newly internalized CE from the supplied $hHDL_3$ is actually used for steroid production is seen in **Fig. 2.** When $hHDL_3$ are reconstituted with [^{3}H]cholesteryl oleate and used in incubations with cAMP-stimulated granulosa cells, the progestins formed contain the [^{3}H]CE-derived radiolabel and show the same rate of production as observed in Fig. 1B.

Internalization of lipoprotein-supplied CE

Figure 3 shows the total mass of CE internalized by unstimulated and cAMP-stimulated granulosa cells, while **Table 1** indicates the proportion of CE mass taken up by either the 'selective' or 'endocytic' pathway during incubation experiments in which the granulosa cells were provided with $hHDL_3$, or hLDL double-labeled in the apoprotein or CE positions with the non-hydrolyzable tags, ^{125}I-labeled DLT or [^{3}H]COE. The mass of CE available to the cells in the supplied $hHDL_3$ (500 μg/ml) or hLDL (100 μg/ml) was calculated to be identical. The results are expressed as ng cholesteryl ester internalized by cells/μg DNA.

Without cAMP (Fig. 3A), granulosa cells take up a small mass of $hHDL_3$-CE, but substantial amounts of hLDL-CE during a 5-, 9-, or 24-h incubation interval. During this period, virtually all the $hHDL_3$-CE enters by way of the 'selective' pathway, while hLDL-CE enters using both pathways (Table 1).

Whereas the total mass of CE entering cAMP-stimulated granulosa cells (Fig. 3B) is increased with both ligands, the relative increase in $hHDL_3$-supplied CE is dramatic. By the 9- and 24-h time intervals, total $hHDL_3$-delivered CE in cAMP treated cells is 9- to 16-fold

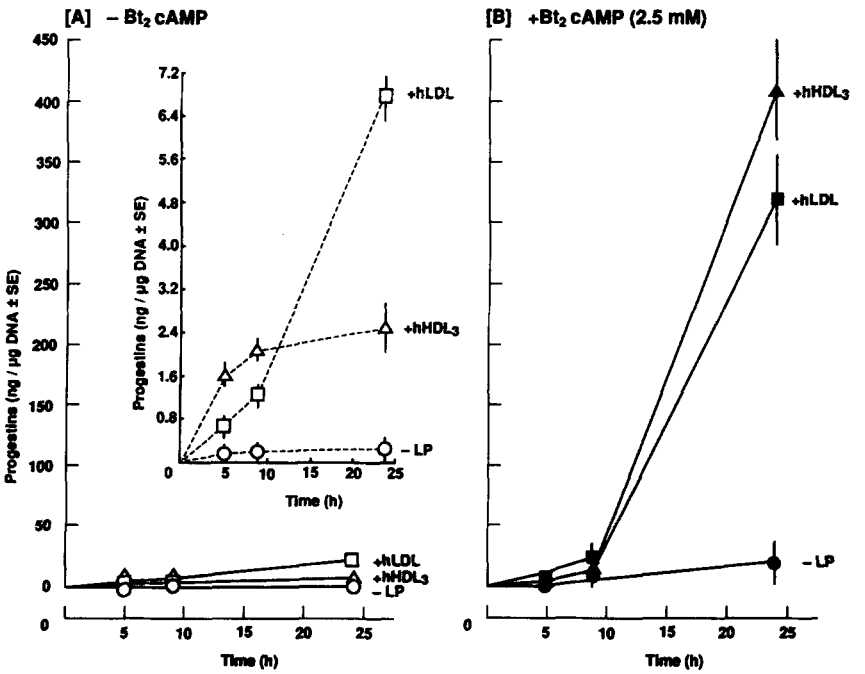

Fig. 1. Time course of Bt_2cAMP and lipoprotein-augmented progestin accumulation. Granulosa cells were cultured in basal medium for 72 h and then sensitized with (B) or without (A) Bt_2cAMP (2.5 mM) for an additional 24 h. Subsequently, cells were cultured without or with $hHDL_3$ (500 μg protein/ml) or hLDL (100 μg protein/ml) ± Bt_2cAMP (2.5 mM) for the duration indicated (5–24 h). Collected media were assayed for their progestin content (progesterone + 20α-hydroxyprogesterone) by RIA. The results represent the mean ± SE of three separate experiments. Inset: expanded scale plot of basal progestin production in response to $hHDL_3$ or hHDL.

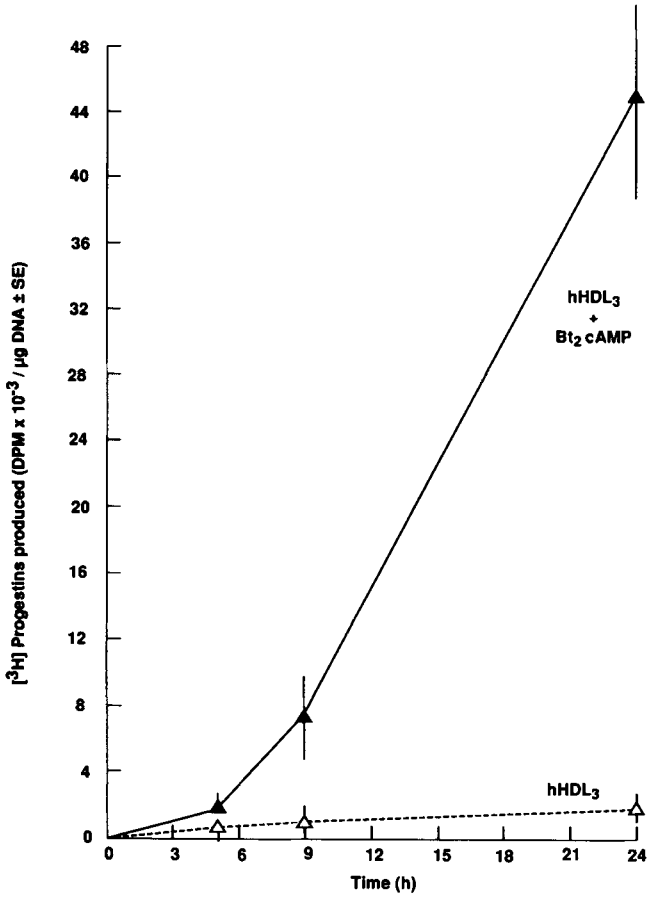

Fig. 2. Conversion of hHDL$_3$-derived [^{3}H]cholesteryl oleate to [^{3}H]progestins. Granulosa cells were cultured and sensitized with or without Bt$_2$cAMP as described under Fig. 1. The cells were then incubated with [^{3}H]cholesteryl oleate-hHDL$_3$ (50 µg protein/ml) + Bt$_2$cAMP (2.5 mM) for an additional 24 h. Each sample (medium + cells) was processed for determination of [^{3}H]cholesteryl oleate incorporation into progestins. Values are the mean ± SE of quadruplicate cultures from a typical experiment. Total progestin production included the sum of [^{3}H]progesterone + [^{3}H]20α-hydroxyprogesterone.

increased over that seen in unstimulated cells, while total hLDL-CE internalized by cAMP stimulated cells is 2-fold that of nonstimulated cells. As seen previously, essentially all (~98%) of the hHDL$_3$-CE enters the cAMP-stimulated cells via the 'selective' pathway (Table 1). Although the impact of the 'selective' pathway in hLDL-CE uptake is less one-sided, it is of interest that with increased incubation times, relatively larger proportions of the internalized hLDL-CE are internalized via this mechanism. These results may be obtained by down-regulation of the LDL-receptor (1, 5).

Internalization of gold-labeled apoproteins

As described in Table 1, 5-h experiments using hHDL$_3$ particles with nonreleaseable radiolabeled tags on apoproteins and CE showed < 1% the uptake of CE via the 'endocytic' pathway seen with hLDL. Because uptake by the endocytic pathway is defined as uptake of the intact particle, and thus, internalization of CE equals internalization of apolipoprotein, we attempted to confirm this observation using a direct method of visualizing lipoprotein-apolipoprotein uptake. cAMP-stimulated granulosa cells were incubated for 5 h with apolipoprotein-complexed gold hHDL$_3$ or hLDL (**Figs. 4A and 4B**), and the internalized gold was quantified in electron micrographs such as Figs. 4C and 4D. Cells incubated with gold-hLDL for 5 h internalized ~7-fold the gold internalized by cells incubated with gold-hHDL$_3$ (mean ± SE = 1,410 ± 120 hLDL-gold particles vs. 209 ± 50 hHDL$_3$-gold particles/100 µm^2 cell area). It is of interest that all of the gold observed in the hHDL$_3$-incubated cells was aggregated into tight clumps in lysosomal-like vacuoles

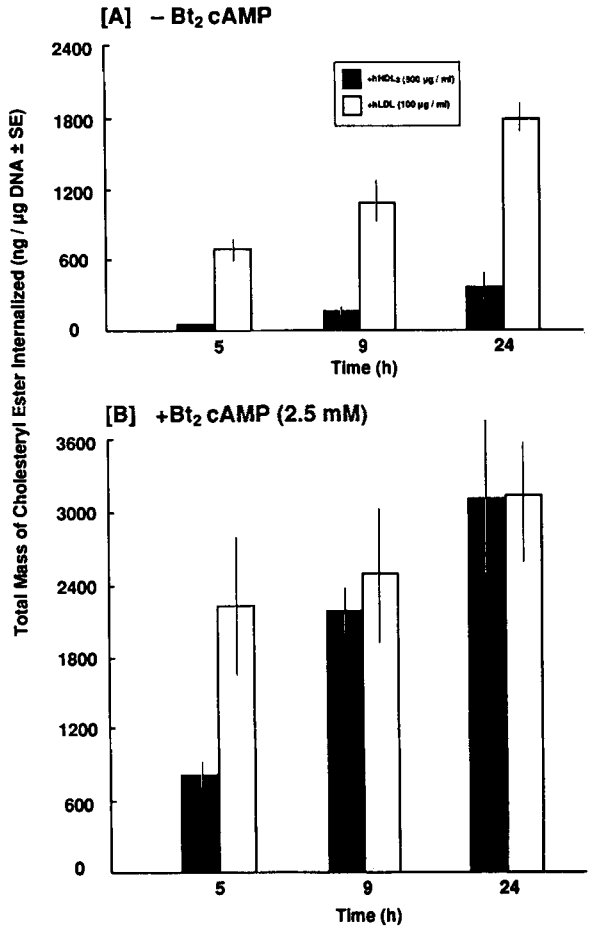

Fig. 3. Effect of Bt$_2$cAMP on the internalization of hHDL$_3$ and hLDL$_3$-derived cholesteryl esters by granulosa cells. The incubation conditions were same as described under Fig. 1 except hHDL$_3$ and hLDL were replaced with [^{125}I]DLT-[^{3}H]COE-hHDL$_3$ (500 µg/ml) and [^{125}I]DLT-[^{3}H]COE-hLDL (100 µg/ml). Total mass of cholesteryl ester internalized was calculated as described previously (28). Values represent mean ± SE of triplicate determinations.

TABLE 1. Effect of incubation time and Bt_2 cAMP on uptake (internalization) of lipoprotein-derived cholesteryl esters by cultured granulosa cells

	Incubation Time	Lipoprotein	Mass of Cholesteryl Ester Internalized	
			Via the Endocytic Pathway	Via the Selective Pathway
	h			
$-Bt_2$cAMP	5	$hHDL_3$	6 ± 1	57 ± 11
		hLDL	477 ± 40	213 ± 85
	9	$hHDL_3$	13 ± 2	144 ± 16
		hLDL	817 ± 143	268 ± 251
	24	$hHDL_3$	17 ± 7	379 ± 145
		hLDL	1370 ± 119	404 ± 106
$+Bt_2$cAMP (2.5 mM)	5	$hHDL_3$	10 ± 1	804 ± 140
		hLDL	1596 ± 366	621 ± 240
	9	$hHDL_3$	54 ± 8	2105 ± 167
		hLDL	1593 ± 281	885 ± 438
	24	$hHDL_3$	72 ± 25	3449 ± 428
		hLDL	2143 ± 383	1011 ± 502

Results are expressed as ng cholesteryl ester internalized/μg DNA ± SE (n = 3). Incubation conditions were the same as described under Fig. 3; in each case, the amount of cholesteryl ester internalized was computed using the formula outlined in Experimental Procedures.

(Figs. 4C and 4E). In contrast, much of the gold observed inside hLDL-incubated granulosa cells was found in endosomal-like compartments (Figs. 4D and 4F) and appeared to be evenly dispersed and associated with an electron dense substance.

Cell storage of internalized lipoprotein-derived cholesteryl esters

Cholesteryl oleate incorporation. To determine whether CE derived from the different lipoproteins is similarly hydrolyzed and stored in granulosa cells, the cells were co-incubated with lipoproteins and [^{3}H]oleate complexed with BSA. If the internalized lipoprotein-CE is, in fact, hydrolyzed, the released free cholesterol can be used for steroidogenesis, or can be re-esterified with the exogenously supplied [^{3}H]oleate acid and identified as cholesteryl [^{3}H]oleate.

Figure 5A shows that without cAMP treatment, granulosa cells do not hydrolyze, re-esterify, and store $hHDL_3$-CE though substantial amounts of LDL-derived CE are processed in this manner.

However, with cAMP and after incubation with either $hHDL_3$ or hLDL, large amounts of cholesteryl esters are hydrolyzed and reesterified (Fig. 5B). Thus, the 'selective' pathway-internalized CE from $hHDL_3$ (see Table 1) is hydrolyzed to free cholesterol and used for steroidogenesis (see Fig. 2), as is LDL-CE, but a substantial amount of the free cholesterol formed from both ligands is also re-esterified and stored within the cell as CE. Of interest is the finding that at the early time point of 5 h, relatively less $hHDL_3$ than hLDL-CE is re-esterified. Whether this is the result of less $hHDL_3$-CE internalization at this time point, slower turnover of the internalized $hHDL_3$-CE, or relatively increased utilization of the internalized CE in progestin production is not clear.

Storage of fluorescent lipids. In an attempt to visualize the processing and storage of newly internalized lipoprotein-CE in granulosa cells, we relied on two separate fluorescent techniques. The most specific approach involved the use of *rec*-HDL made with apolipoproteins, triglyceride, phospholipids, cholesterol, and fluorescent cholesteryl BODIPY® FL C12 as CE. These reconstituted fluorescent particles have both advantages and disadvantages for CE tracing experiments. On the one hand, use of CE-BODIPY®-labeled particles permits direct and specific visualization of lipoprotein-derived CE within the cells. As seen in **Table 2,** the CE-BODIPY®-labeled particle does not act as a substrate for neutral (cytosolic) cholesteryl esterase activity, and, as a result, BODIPY®-labeled sites found within the cells after incubation of granulosa cells with the reconstituted particles likely represent sites of specific, non-hydrolyzed, non-lysosomal, newly accumulated lipoprotein-derived CE. Indeed, with low-light computerized fluorescent images of CE-BODIPY®-incubated cells (represented in **Fig. 6, panel A**), one sees an accumulation of discreet fluorescent spots that correspond in size, location, and number with lipid droplets found on Nomarski images of the same cells (Fig. 6, panel B). Images such as these suggest that some of the internalized CE derived from HDL particles is not immediately hydrolyzed and/or used for steroidogenesis, but can be directly stored as CE in lipid droplets within cAMP-stimulated granulosa cells. For our purposes a clear advantage of the CE-BODIPY®-labeled particle is that it specifically identifies the early

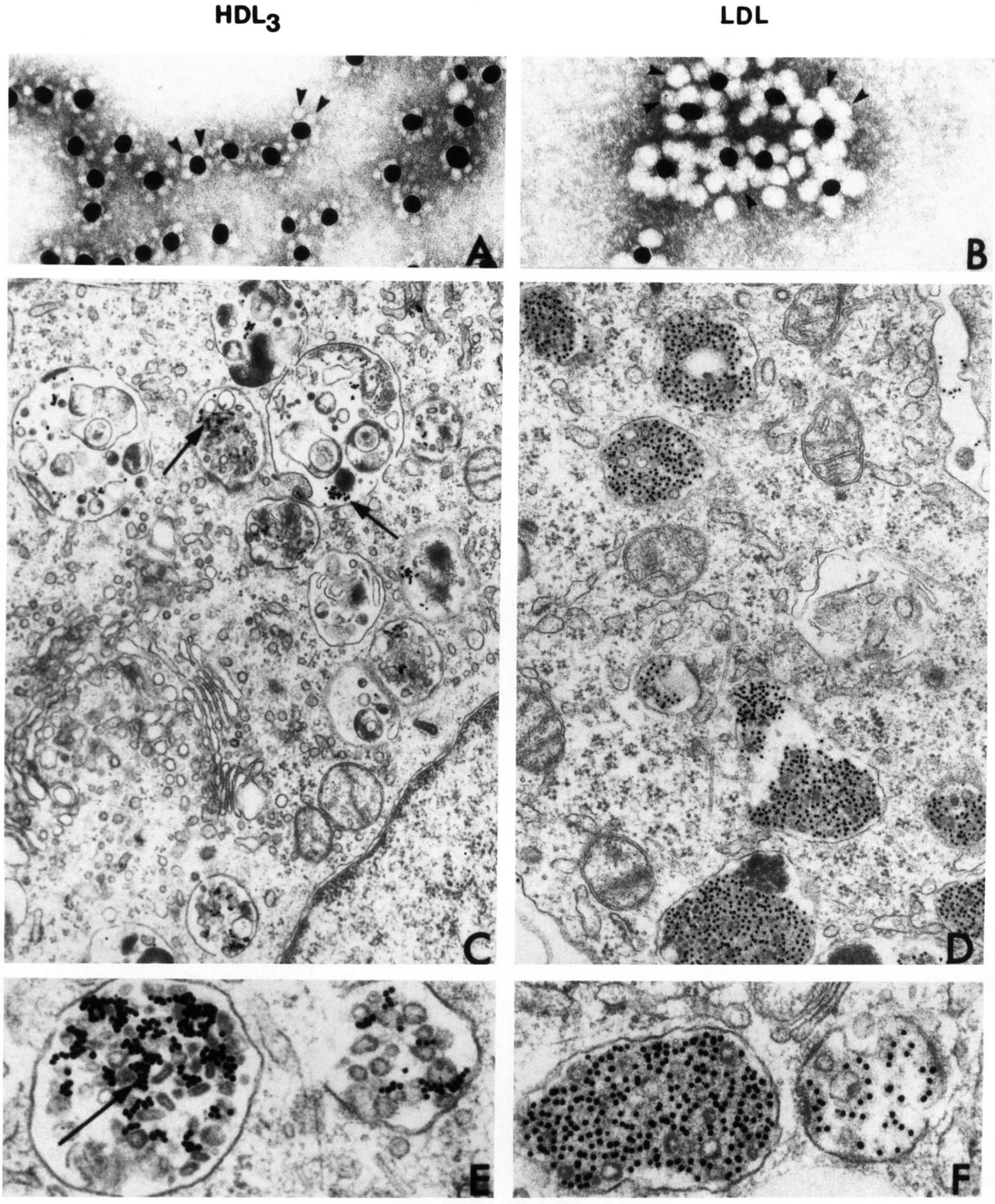

Fig. 4. Uptake of hHDL$_3$- and hLDL-gold complexes as a marker for lipoprotein-apolipoproteins in Bt$_2$cAMP-treated granulosa cells. Figures 4A and 4B illustrate the high quality of the lipoprotein-complexed gold preparations. Figures 4C and 4D illustrate that far less gold is internalized by hHDL$_3$-treated cells (than hLDL-treated cells) during the 5-h incubation period (see text for quantitation data). In hHDL$_3$-treated cells most of the gold is found clumped in multivesicular or lysosomal-like bodies (see 4E); in hLDL-treated cells, much of the gold is located in endosomal-like vacuoles where the gold is evenly dispersed and associated with an amorphous electron dense substance (4F).

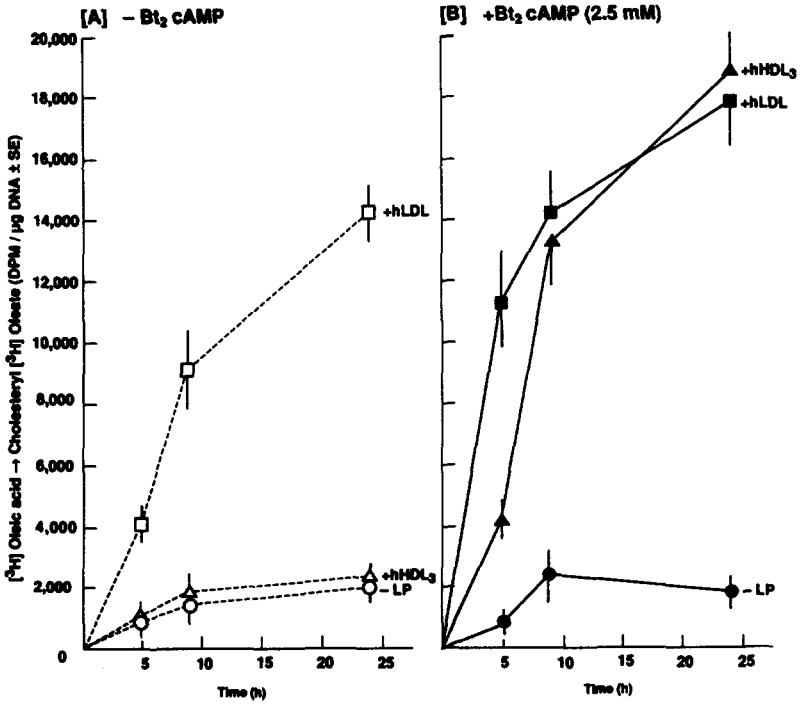

Fig. 5. Stimulation of [^{3}H]oleate incorporation into cholesteryl [^{3}H]oleate by hHDL$_3$ and hLDL in granulosa cells pretreated with or without Bt$_2$cAMP. Granulosa cells cultured in basal medium were pretreated with or without Bt$_2$cAMP (2.5 mM) for 24 h as described in Fig. 1, after which hHDL$_3$ (500 μg protein/ml) or hLDL (100 μg protein/ml) + [^{3}H]oleate-BSA was added for 24 h at 37°C. The cells were assayed for [^{3}H]oleate incorporation into cholesteryl esters by TLC. Each point represents the mean ± SE of triplicate dishes. This figure indicates that granulosa cells must be pre-stimulated with cAMP before hHDL$_3$-derived cholesterol is capable of being reesterified with newly provided (radioactive) cholesteryl oleate.

itinerary of CE that enters cells via the 'selective' pathway. CE-BODIPY® is not a substrate for neutral (non-lysosomal) cholesterol esterases (Table 2), so BODIPY®-labeled CE internalized by the 'selective' pathway would not be hydrolyzed. On the other hand, CE-BODIPY® internalized via the endocytic pathway would be efficiently hydrolyzed by acidic cholesteryl esterase present in lysosomes (see Table 2), and any fluorescent (trace) fatty acid resulting from such hydrolysis would be lost to follow-up due to dilution with endogenous fatty acids.

The primary disadvantage of the CE-BODIPY® technology is that the fluorescent label is self-quenching in lipid depots (e.g., the reconstituted particles express only 1/10th the fluorescence seen after particle extraction), and its rapid quenching in cells requires the availability of low-light computerized imaging. As a result, the CE-BODIPY®-labeled cells cannot be viewed with standard fluorescent microscopic technology, results with changing conditions are difficult to quantify, and fluorescence may be underestimated depending on experimental conditions. It turns out, however, that granulosa cells incubated with standard lipoproteins and subsequently stained with the fluorescent dye, Nile Red, show images remarkably similar to those seen with *rec* HDL specifically labeled for CE. In a timed incubation experiment (using the same computerized double-imaging used for the CE-BODIPY® experiments), we could see that cAMP-treated cells incubated without lipoproteins (Fig. 6C) show only occasional droplets of lipid with Nile Red (shown as yellow). Cells incubated for 5 h with hHDL$_3$ (Fig. 6D) showed little additional accumulation of lipid. However, by 9 h (Fig. 6E), lipid accumulation was substantial, and by 24 h of continuous supply of hHDL$_3$, the cells were filled to capacity with lipid (Fig. 6F).

As the Nile Red methodology permitted the use of standard fluorescent microscopy, it was possible to subject the cells growing on coverslips to various incubation conditions, and afterwards fix the cells, stain with Nile Red, photograph and quantitate the lipid droplets under appropriately random conditions. **Figure 7** (panels A–F) shows representative cells pre-incubated without cAMP (panels A, C, E) or with cAMP (panels B, D, F)

TABLE 2. Measurement of neutral and acidic cholesteryl esterase activity using cholesteryl [1-^{14}C]oleate or cholesteryl ester BODIPY® as substrate

	Cholesteryl [1-^{14}C]oleate	Cholesteryl Ester BODIPY®[b]
Neutral cholesteryl esterase[c]		
Adrenal extract	459	no activity
Granulosa cell extract	109	no activity
Acidic cholesteryl esterase[c]		
Adrenal extract	125	867
Granulosa cell extract	38	345

[a]Values given as pmol · min^{-1} · mg protein ± SE.
[b]Values given as arbitrary units × 10^{-2} · min^{-1} · mg protein^{-1} ± SE.
[c]Note, cholesteryl esterase maximally active at neutral pH is predominantly cytosolic in nature; cholesteryl esterase active at acid pH is exclusively lysosomal (13). Assays were carried out as described under Experimental Procedures using 60 μM cholesteryl [1-^{14}C]oleate or 60 μM cholesteryl ester BODIPY® at both pH 4 and pH 7.5.

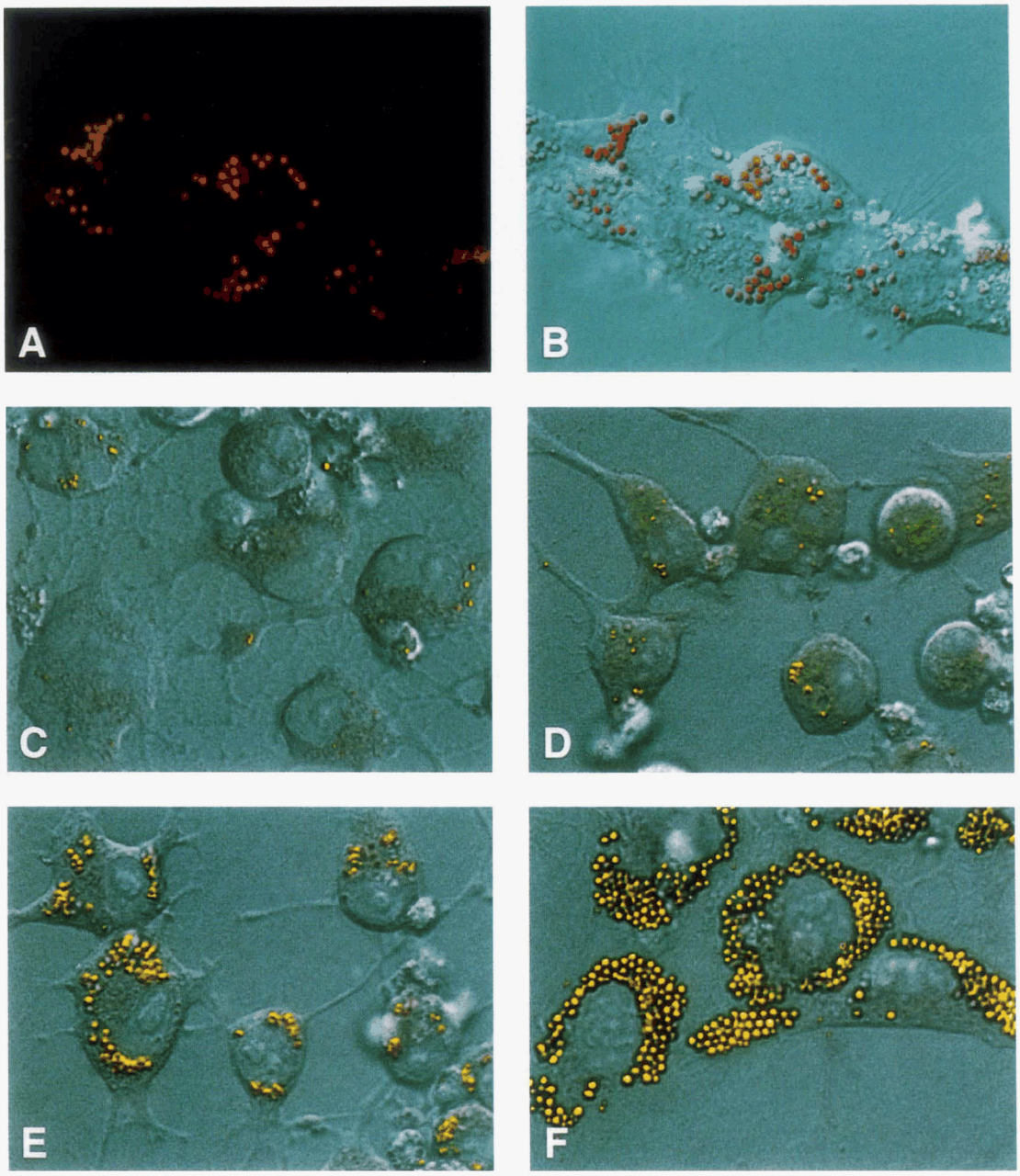

Fig. 6. Demonstration of granulosa cell fluorescence after cell incubation with CE-BODIPY®-labeled HDL (6A, 6B), or native $hHDL_3$ + subsequent staining by Nile Red dye (6C–F). Figures 6A and B show cells pretreated with Bt_2cAMP then incubated for 9 h with *rec* HDL labeled with fluorescent CE-BODIPY®. Figure 6A shows the fluorescent image identifying the internalization and storage of non-hydrolyzed HDL-CE: Fig. 6B shows a composite of this image and a Nomarski DIC image of the same cells. Figures 6C–F show Nile Red staining of cAMP-treated cells incubated without lipoproteins (6C), or with native $hHDL_3$ for 5 h (6D), 9 h (6E), or 24 h (6F). The cells show that lipid droplets accumulate intracellularly after 5 h of incubation with $hHDL_3$, and that these droplets reach an exceptionally large number by 24 h.

then cultured without lipoproteins (panels A, B) or with $hHDL_3$ (panels C, D) or hLDL (panels E, F) for 24 h. **Table 3** provides quantitative data on the lipid stored in such cells treated either with or without cAMP and lipoproteins. The results show that granulosa cells store lipid differently depending on hormonal stimulation and CE source. For example, under basal conditions, without cAMP and without lipoproteins, the average granulosa cell stores very little lipid as judged by the few fluorescent droplets seen per cell. When such unstimulated cells are provided with $hHDL_3$, no additional storage of lipid takes place. On the other hand, when

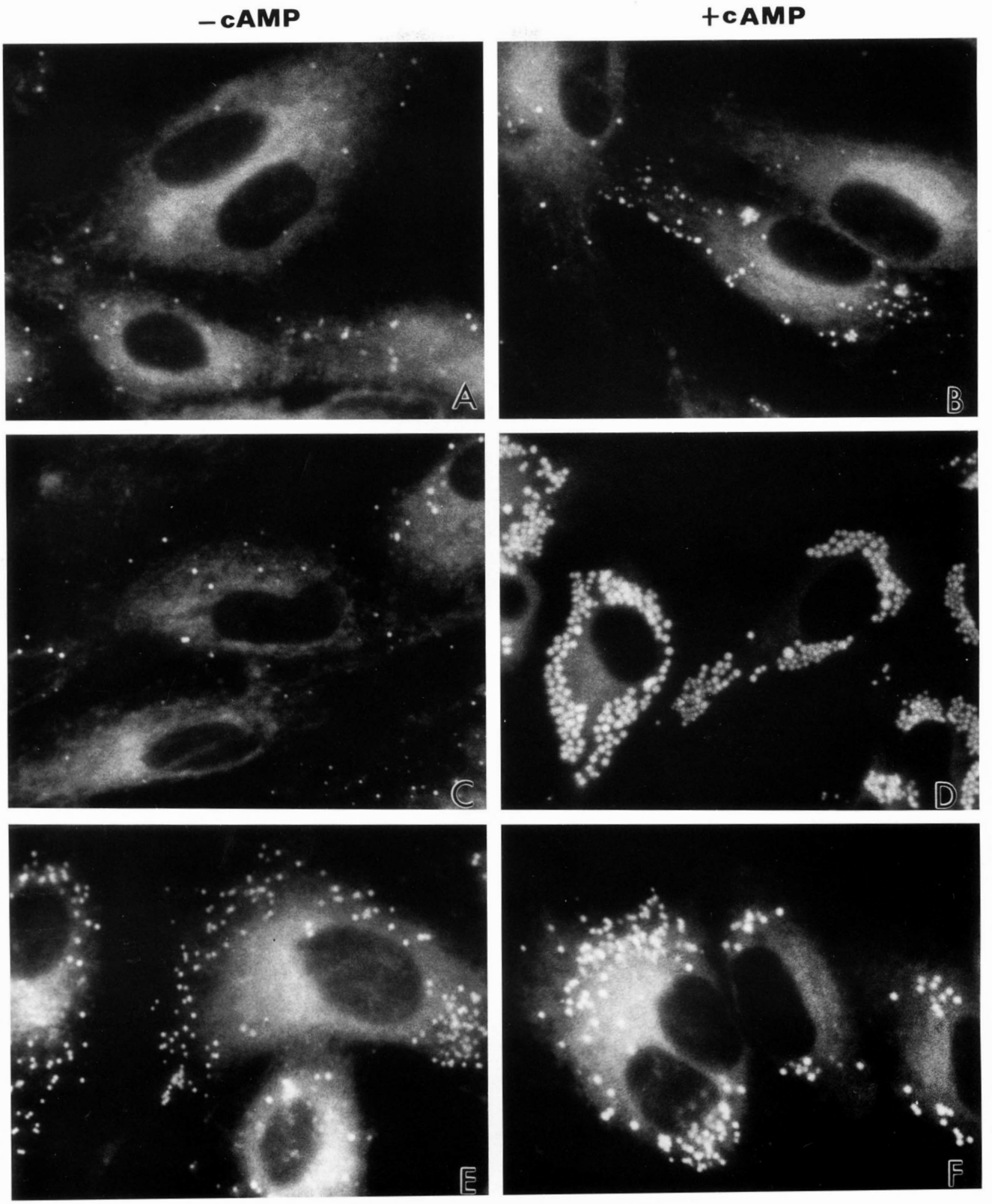

Fig. 7. Storage of intracellular lipid in granulosa cells treated with or without Bt_2cAMP and incubated without lipoproteins (7 A, B), or with native $hHDL_3$ (7 C, D), or hLDL (7 E,F) for 24 h; the cells were subsequently fixed with glutaraldehyde and stained for Nile Red fluorescence. Without cAMP, only cells treated with LDL accumulated lipid droplets (7E). With cAMP but no lipoproteins, the cells accumulate a small additional amount of lipid (7B). With cAMP and either $hHDL_3$ or LDL, the cells accumulate large amounts of lipid; the lipid accumulation with $hHDL_3$ (7D) approximately doubles that seen with hLDL (7F).

TABLE 3. Accumulation of Nile Red lipid droplets by granulosa cells after incubation ± Bt_2cAMP and ± lipoprotein

	-Bt_2cAMP	+Bt_2cAMP
No lipoproteins	9.4 ± 1.5	21.6 ± 4.4
+$hHDL_3$ (500 μg/ml)	7.3 ± 2.0	82.0 ± 3.5[a]
+hLDL (100 μg/ml)	24.0 ± 2.0	41.1 ± 3.9

Granulosa cells were cultured with or without Bt_2cAMP (see text) and then given lipoprotein for an additional 24 h. Values represent number (mean ± SE) of lipid droplets per cell. Approximately 10,000 droplets were counted in 380 cells photographed at random.

[a]In general, the lipid droplets observed in these cells were larger than those seen under other experimental conditions.

these cells are provided with hLDL, increased storage occurs and the cells show more than a doubling of their basal number of lipid droplets.

After stimulation with cAMP, cells without lipoproteins accumulate double the basal cell number of lipid droplets. That value is doubled again when the cells are provided with LDL, but the number is quadrupled when the cells are given $hHDL_3$.

Triglyceride formation. Given the large accumulation of lipid droplets in the stimulated granulosa cells with $hHDL_3$, it was of interest to determine to what extent triglyceride formation could account for the lipid. **Figure 8** shows that triglyceride formation is, in fact, increased after cAMP treatment, but surprisingly, the increase in triglyceride occurs even when lipoproteins are not provided. As cAMP-treated cells treated with lipoproteins accumulate far more Nile Red fluorescence than cells without lipoproteins (see Fig. 7 and Table 3), the fluorescent droplets observed cannot be due to triglyceride formation alone.

DISCUSSION

The results from this study provide new information about the 'selective' cholesterol pathway. We now know that CE that enters cells through this pathway can be directly used for steroid hormone production. Because of the large endogenous cholesterol pool within these cells, it is difficult to quantitatively trace what percentage of the entering CE is directly used for steroidogenesis and what percentage is stored, but it is clear from tracing the conversion of radiolabeled CE into progestins, that there is increasing conversion of CE into hormone with time, and that the rate of conversion of the labeled precursor precisely follows the rate of total hormone production in the same cells. We also now know from morphological observations with CE-BODIPY® that newly internalized HDL-fluorescent-labeled CE can be directly stored (without prior hydrolysis) within the cell, and, for the most part, the storage sites are lipid droplets. Biochemical (cholesteryl oleate formation) data shows that some of the CE entering cells can also be hydrolyzed, and that the free cholesterol released can

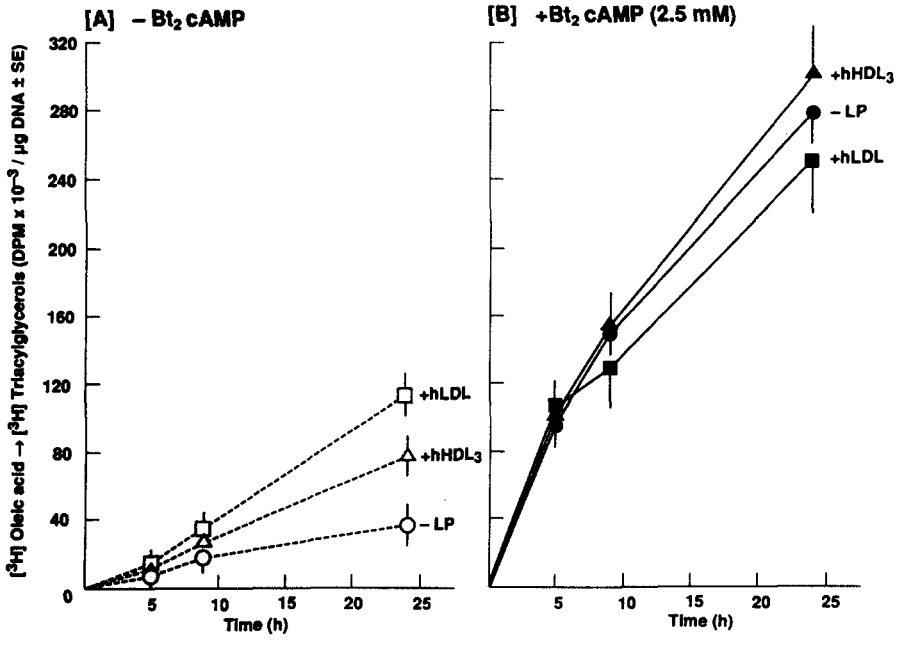

Fig. 8. [³H]oleate incorporation into triglycerides in granulosa cells incubated without or with Bt_2cAMP. Incubation conditions were the same as described under Fig. 1 except TG spots on the TLC plates were counted for ³H radioactivity. This figure illustrates that with cAMP, triglyceride formation is greatly increased but the provision of lipoproteins has no special effect.

be re-esterified and stored in the cells as fat droplets. Although cAMP, by itself, increases triglyceride storage in the granulosa cells, this storage is not increased in the presence of $hHDL_3$ (or hLDL), and as a result, we believe a substantial proportion of the large pool of lipid that accumulates intracellularly after increased internalization of $hHDL_3$-CE is due to the CE itself.

As predicted from previous reports from this laboratory (32, 35, 39), cultured cAMP-stimulated rat granulosa cells provided with lipoproteins as a source of cholesterol, respond by producing large amounts of hormone. They acquire this cholesterol directly as cholesteryl ester by the use of the 'selective' pathway when $hHDL_3$ is the available lipoprotein, or they acquire the cholesterol by internalizing intact lipoproteins through the 'endocytic' pathway (as well as CE through the 'selective' pathway) when hLDL is the available lipoprotein. What is new from the current data is that the amount of $hHDL_3$-CE internalized by granulosa cells is clearly correlated with the amount of cholesterol being used for hormone-regulated steroidogenesis at any given time point, and in a number of respects this relationship between cAMP regulation, CE internalization, and utilization for steroid hormone production is not as obvious when hLDL is the ligand.

Evidence for this thinking about $hHDL_3$ comes from the close time-related correlation between the level of the cAMP-stimulated granulosa cell steroidogenic response, the level of conversion of freshly obtained (labeled) $hHDL_3$-CE into progestins, and the increase in $hHDL_3$-CE internalization by granulosa cells; these events are not impressive at the 5-h time point, but each increases dramatically at subsequent time points. In addition, we see that CE hydrolysis and re-esterification ([^{3}H]cholesteryl oleate data) and CE storage as lipid droplets (accumulation of fluorescent $hHDL_3$-derived lipids) start slowly but increase exponentially with time as more $hHDL_3$-CE is internalized by the cells. Finally, and perhaps most significantly, we see that none of the above events occur in nonstimulated (non-cAMP-treated) granulosa cells that do not secrete progestins despite the availability of $hHDL_3$ as a cholesterol source; i.e., these unstimulated granulosa cells do not convert labeled $hHDL_3$-CE into progestins, they do not internalize CE, they do not hydrolyze and re-esterify $hHDL_3$-CE, and they do not accumulate $hHDL_3$-CE or lipids within the cells with time.

In contrast, identical preparations of granulosa cells appear to handle hLDL-CE quite differently. Although the availability of hLDL in cAMP-treated cells induces a steroidogenic response that follows the same time course as that seen with $hHDL_3$, hLDL-CE internalization does not follow the steroidogenic response (hLDL uptake starts high and does not change during the 24-h study period), hLDL hydrolysis and re-esterification (cholesteryl oleate data) starts relatively high, increases somewhat with time, but saturates quickly, as does the storage of fluorescent lipids. Perhaps most important is the observation that in non-cAMP-stimulated cells, despite the fact that only minimal amounts of progestins are produced (~1% that produced with cAMP), all measurements having to do with hLDL-CE uptake and utilization nevertheless operate at relatively high levels. For example, hLDL-CE internalization is significant at all time points (and at 9 and 24 h represents between one-half and two-thirds that observed after cAMP stimulation), lipid accumulation in cells is substantial, and cholesteryl oleate production approximates that seen after cAMP treatment.

Overall, these data suggest that hLDL-CE, when available to granulosa cells, is constantly being processed at some significant level, and when the cells are programmed by cAMP to produce steroid hormones, the level of hLDL-CE processing is modestly increased to accommodate the need for increased progestin production (32, 35, 39, 56). In this framework, hLDL-CE may be seen as a constitutive substance needed to carry out essential housekeeping chores in granulosa cells such as membrane replenishment, etc., but a potential source of additional cholesterol when the need arises. In contrast, $hHDL_3$-CE appears to fulfill a narrower function. Our evidence suggests that $hHDL_3$-CE is essentially not utilized by granulosa cells unless the cells have been stimulated to secrete steroid hormones. Under these conditions (where cells are stimulated for maximal hormone production), $hHDL_3$-CE utilization is both efficient and dramatic.

Finally, one can speculate as to what extent the specific use of either the 'endocytic' or 'selective' pathway for CE internalization is related to ligand characteristics or to some other variable. Clearly, under all the conditions observed in this study, the use of $hHDL_3$-CE seems to be correlated with the programmed need for steroidogenesis, and it is certain that virtually all the $hHDL_3$-CE used for this purpose enters the cells through the 'selective' pathway. With hLDL, the situation is somewhat different. hLDL-CE clearly uses both cholesterol pathways for CE delivery. Although in every case, more hLDL-CE enters by way of the 'endocytic' rather than the 'selective' pathway, it is of interest that the major impact of cAMP stimulation itself is not on increasing the use of the endocytic pathway but of increasing that proportion of hLDL-CE that enters the granulosa cells through the 'selective' pathway.

Given these various observations, we have begun to view the 'selective' pathway as having a specialized function in steroidogenic cells—one of providing sufficient cholesterol to fuel the required production of steroid

hormones; in this sense, the name 'selective' implies a unique pathway reserved for the bulk transport of cholesterol. This pathway functions in granulosa cells by permitting the cell to directly extract lipoprotein-derived CE at the cell surface and by funneling and/or storing that CE for steroidogenesis.

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