Simultaneous induction of an HDL receptor protein (SR-BI) and the selective uptake of HDL-cholesteryl esters in a physiologically relevant steroidogenic cell model

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Abstract **This study addresses the question of whether the level of expression of SR-BI (an HDL receptor) is linked to the expression of selective lipoprotein-cholesteryl ester delivery in a steroidogenic cell model. Rat ovarian granulosa cells are physiologically normal cells which show no selective uptake of HDL-cholesteryl esters and no progestin production until luteinized by trophic hormones or adenylate cyclase stimulators, after which expression of the selective cholesterol pathway and production of steroid hormone is dramatically up-regulated. The current study demonstrates that at every cell stage studied, the protein content and level of expression of SR-BI mRNA are linked to changes that occur in HDL-cholesteryl ester uptake; i.e., SR-BI is not present in basal (non-luteinized) cells, develops slowly (from 6–9 h) after hormone treatment, increases robustly from 9–48 h after stimulation, and remains high after incubation with HDL. In contrast, another structural protein, caveolin, did not follow this pattern; caveolin expression showed an inverse relationship to selective cholesteryl ester uptake, and was most prominent in basal cells and least prominent in luteinized, HDL-incubated cells. Morphologically, SR-BI appears to be associated with cell surface sites showing high levels of cholesteryl ester uptake (after luteinization and/or incubation with HDL labeled with fluorescent cholesteryl esters), and at the electron microscope level, SR-BI is most clearly associated with microvillar regions on the cell surface which also bind HDL-labeled with colloidal gold. Thus, induction of the SR-BI receptor system and induction of the HDL-selective cholesterol uptake pathway in rat granulosa cells appear to be linked morphologically, biochemically, and functionally.—**Azhar, S., A. Nomoto, S. Leers-Sucheta, and E. Reaven. **Simultaneous induction of an HDL receptor protein (SR-BI) and the selective uptake of HDL-cholesteryl esters in a physiologically relevant steroidogenic cell model. *J. Lipid Res.* 1998.** 39: **1616–1628.**

Supplementary key words HDL receptor • scavenger receptor, SR-BI • caveolin • selective cholesterol uptake • rat ovary steroidogenic cells • microvillar channels

The 'selective' pathway for cellular uptake of lipoprotein-derived cholesteryl esters (CEs) represents a high capacity, hormone-inducible cholesterol delivery system for cells that require exogeneous cholesterol for product synthesis (1–17). In the selective uptake process, lipoproteins [e.g., high density (HDL) or low density (LDL) lipoproteins] that bind to the surface of cells are not directly interiorized by the cells [as they are in the classical endocytic B/E (LDL) receptor pathway (18)], but the lipoproteins selectively release their CE content to the target cells; i.e., CEs enter the target cells unaccompanied by the ligand apoproteins (3, 7, 9, 12, 15, 19–22). Recent findings have strongly suggested that the scavenger protein, SR-BI [a class B, type I murine scavenger receptor also described as the HDL receptor (23)] mediates such 'selective' cholesterol uptake events.

SR-BI and the selective pathway have many features in common that make their association likely. First, the selective uptake of cholesterol and the presence of the SR-BI receptor are most dramatically expressed in identical tissues, adrenal, ovary and, to a lesser extent, liver (24–26). In addition, the SR-BI receptor and the selective pathway do not show specificity for apolipoproteins; thus, not only HDL, but low density lipoproteins (LDL) (5, 8, 9, 12, 27–29), amino acid-modified LDL (30), and intermediate density lipoproteins [IDL, (31)] both bind SR-BI and contribute CEs via the selective pathway (32–34). But, perhaps, the most compelling association between the two processes has to do with their similar response to cholesterol loading. The recognized function of the selective pathway is to efficiently supply cells with cholesteryl esters for use in hormone or product synthesis (for review, see 15, 22). Likewise for SR-BI, Acton et al. (23) initially showed that SR-BI-transfected CHO cells bind HDL with high affinity and take up both radiolabeled and fluorescent lipid markers (though the identity of the lipids is uncertain). The idea

Abbreviations: HDL, high density lipoprotein(s); LDL, low density lipoprotein(s); LCAT, lecithin:cholesterol acyltransferase; apoA-I, apolipoprotein A-I; SR-BI, scavenger receptor class B type I; ACAT, acyl-CoA:cholesterol acyltransferase; CE(s), cholesteryl ester(s).

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that SR-BI expression in cells is related to lipid uptake was reinforced by subsequent observations showing that SR-BI is specifically associated with tissues and/or cells that are programmed to utilize large quantities of cholesterol (23–26), that hormone treatment or other factors which increase the demand for this cholesterol will likely increase the expression of the SR-BI protein (23–26), and that in mice, deletion of the gene encoding SR-BI results in higher circulating levels of lipoprotein-cholesterol and substantially lower amounts of stored tissue cholesterol (35).

Despite these correlations, there remain various unresolved issues concerning the association between SR-BI and the selective pathway. For example, the studies described above use storehouses of cholesterol as an endpoint, but cholesterol plasma/tissue levels per se may not be good indicators of steroid response or for cholesterol transport into cells. For example, mice lacking certain cholesterol-related genes (A-I, ACAT, LCAT) all show dramatically reduced adrenal cholesterol storage levels, but nevertheless show normal adrenal steroid responses (26, 36–38), and in the case of A-I and LCAT knockout mice, have been shown to have increased adrenal SR-BI protein and mRNA expression (37, 38). Indeed, it is not clear whether selective cholesterol uptake is increased or decreased in these models. Also, studies to date in which lipid uptake or selective CE uptake was actually measured, have all been carried out in tumor cell lines or genetically engineered cells. Although such models are useful in many ways, one must consider the possibility that the normal physiological relationship between SR-BI and the selective pathway may have been lost or altered.

Finally, there are undoubtedly other players in the relationship between SR-BI and the selective pathway which have not yet been seriously explored. Among these are cell structures that may link the HDL receptor to the selective pathway in steroidogenic cells. These include microvilli and microvillar channels which increase the extracellular lipoprotein/membrane association process and appear to facilitate the CE uptake process in steroidogenic cells (39–42). The relationship of the microvillar compartment to the localization of SR-BI in cells has not previously been described. Also, as SR-BI is itself fatty acylated with high affinity for the protein caveolin (43) and as caveolin has been related to cholesterol transport processes and appears to co-exist with SR-BI in surface associated structures (caveolae) of several cell lines (43), its distribution and expression in linking SR-BI with the selective uptake of CEs is of interest.

In the current study, we deal with several of the unresolved issues in the HDL receptor (SR-BI)-selective CE uptake story. Using a phyiologically normal rat granulosa cell model, we are able to compare the expression of SR-BI with actual measurements of the selective uptake of CEs, under changing conditions of hormonal activation and cholesterol provision. We subsequently examined the relationship of caveolin to this SR-BI/selective pathway association, and, in addition, determined whether the distribution of SR-BI on the surface of these reactive cells is related to the microvillar compartment.

Rat granulosa cells provide an ideal cell model for the current study. Initially, immature animals are pretreated with estradiol to stimulate ovarian follicle growth (no gonadotropic effect is induced) and granulosa cells are released into medium after follicle rupture without the use of chemical agents (12). Such basal cells do not internalize lipoprotein CEs via the selective pathway and do not synthesize progestins (12, 15). However, when cultured with trophic hormones (or second messenger, Bt_2-cAMP) the cells become luteinized (12, 15, 22), and in the presence of lipoproteins they take in massive amounts of CEs via the selective pathway, and respond by producing from 1000–2000 times the progestins made by cells grown under basal conditions (12, 15). In using the granulosa cell model, we can address the question of whether the content, level of expression and localization of SR-BI receptor protein, or caveolin, are tightly linked to the expression of the selective cholesterol pathway (as determined by the uptake of both radioactive and fluorescent lipoprotein-CE), and, we are able to do so under changing hormonal and environmental conditions in a physiologically relevant cell model.

MATERIALS AND METHODS

Materials

[1α, 2α (N)-^{3}H]cholesteryl oleolyl ether (1.78 TBq/mmol; 48.0 Ci/mmol) and ECL Western Blotting reagents were purchased from Amersham Corporation (Arlington Heights, IL). [1, 2-^{3}H(N)]progesterone (1.9 TBq/mmol; 50.0 Ci/mmol), 20α-[1, 2-^{3}H(N)]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 NEN Life Science Products (Boston, MA). 8 Br-cAMP, Bt_2-cAMP, cholchicine, cholesteryl oleate, egg phosphatidyl choline cholesterol, progesterone, and 20α-hydroxyprogesterone, were obtained from Sigma Chemical Co.(St. Louis, MO). Cholesteryl BODIPY FL C_{12} (BODIPY-CE) was purchased from Molecular Probes, Inc. (Eugene, OR). Polyclonal anti-caveolin was purchased from Transduction Laboratories (Lexington, KY). All other reagents used were of analytical grade.

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 (12, 15). The animals were killed 24 h after their last injection (i.e., on day 6) and granulosa cells were isolated from ovaries and cultured as previously described (12). The cells were maintained at 37°C for up to 72 h in basal 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/cm^2), then incubated with or without Bt_2-cAMP (2.5 mm) or Bt_2-cAMP + HDL (500 μg/ml) for an additional 24 h.

Lipoprotein preparation. ApoE-free high density lipoproteins ($hHDL_3$) were isolated as described previously (15, 22). These human-derived lipoproteins were used exclusively because they are not recognized by the LDL receptor-mediated pathway. For uptake and internalization studies, $hHDL_3$ preparations were conjugated with residualizing labels, i.e., ^{125}I-labeled dilactitol tyramine (DLT) and [^{3}H]cholesteryl oleolyl ether [COE, (9)].

Reconstituted (*rec*) cholesteryl BODIPY HDL particles were prepared as described earlier (15, 22).

Secretion of steroids. To assay steroidogenesis, cultured granulosa cells were pretreated with or without Bt_2-cAMP (2.5 mm) for 24 h. Subsequently, triplicate culture dishes were supplemented ± Bt_2-cAMP (2.5 mm) and ± $hHDL_3$ (500 μg/ml), and incubated at 37°C for an additional 24 h; samples of incubation medium were frozen and stored until assayed for progestins. Progesterone and its metabolite (20α-hydroxyprogesterone) were quantified by radioimmunoassay using specific antiserum as described previously (12). 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.

Uptake and internalization of lipoprotein-derived cholesteryl esters. For these experiments, medium from 24 h ± Bt_2-cAMP-treated cells was replaced with fresh medium ± Bt_2-cAMP, ± $hHDL_3$ and radiolabeled, non-releaseable apolipoprotein and cholesteryl ester tags that would accumulate within the cells even when degraded (9, 15). Incubations were carried out with ^{125}I-labeled DLT-[^{3}H]COE-$hHDL_3$ (100 μg protein/ml) ± Bt_2-cAMP (2.5 mm) for 24 h at 37°C. At the end of incubation, the accumulated cells were washed and then solubilized in 2 ml of 0.1 N NaOH. One-ml aliquots were precipitated with an equal volume of 20% trichloroacetic acid to determine insoluble and soluble ^{125}I radioactivity or extracted with organic solvents to determine ^{3}H radioactivity (9). The amount of CE internalized was computed as the difference between total CE uptake and trichloroacetic acid-insoluble (i.e., surface bound) ^{125}I radioactivity. The net mass of CE internalized was determined (12, 15) by dividing the ^{3}H-labeled protein values by the protein:cholesterol ratio of $hHDL_3$ (i.e., 2.73).

SR-BI antibody. A polyclonal antibody raised against a peptide to the carboxy terminus of mouse SR-BI [amino acids 489–509; AYSESLMSPAAKGTVLEQEAKL (23)] was prepared in rabbits using standard procedures. This antibody is fully functional in immunoblotting and immunohistochemical assays.

Western blot analysis of SR-BI and caveolin protein expression. The expression of SR-BI and caveolin protein was monitored by immunoblotting of cellular lysates. Granulosa cells were cultured in standard basal culture medium for 72 h, then treated with or without Bt_2-cAMP (2.5 mm) for 24 h, unless otherwise stated. In some cases, follicle-stimulating hormone (FSH), cholera toxin, pertussis toxin, forskolin, 8 Br-cAMP, 8 CPT-cAMP, or 1-methyl-3-isobutyl xanthine was used in place of Bt_2-cAMP. Other Bt_2-cAMP presensitized cells were treated for an additional 24 h with or without medium containing $hHDL_3$ (500 μg protein/ml) with or without Bt_2-cAMP (hormonal) stimulation. Treated cells were washed twice in ice-cold PBS, lysed directly with 0.2 ml of lysis buffer (125 mm Tris-HCl, pH 6.8, 2% SDS, 5% glycerol, and 1% mercaptoethanol), placed at 40°C for 15 min, and the lysate was sonicated briefly to disrupt chromatin (DNA).

The lysates containing equal amount of protein (35–60 μg protein) were mixed with 2× Lammeli buffer, boiled for 90 sec, and immediately loaded onto a gel. Samples and molecular weight markers were separated by SDS/polyacrylamide gel electrophoresis (10% running and 4% stacking gels) as described previously (17, 44). The proteins were electrophoretically transferred onto a nylon membrane and membranes were blocked in PBS–0.02% Tween 20 containing 5% powdered milk and 5% fetal bovine serum at room temperature while shaking. Subsequently, blots were incubated at room temperature for 2 h with anti-SR-BI (1:1000) or anti-caveolin (1:2000) in blocking solution. The blots were washed once for 15 min and twice for 5 min with PBS–0.02% Tween 20 and then incubated with goat anti-rabbit IgG coupled to horseradish peroxidase (1:20,000) in blocking solution. The blots were washed as described above, radiographic chemiluminescence was detected at various times (3–10 min), and appropriate films were subjected to densitometric scanning.

RNA preparation and reverse transcription-polymerase chain reaction (RT-PCR) of SR-BI mRNA. Total RNA was isolated from 60-mm plates using the method of Chomczynski and Sacchi (45). Total RNA from control or cAMP-treated cells was reverse transcribed using oligo $dT_{12–18}$ primer and Superscript II reverse transcriptase (Life Technologies, Inc.). Complementary DNA samples were subjected to 30 cycles of PCR in the presence of trace amounts of [α-^{32}P]dCTP (46) using mouse (m) SRBI primers (23) designed to contain a *Sal I* restriction endonuclease sites at the 5′ end or primers against rat ribosomal protein L19 (46). The mouse SR-BI primers are complementary to rat SR-BI sequence (47) except a single base 'G' (shown in bold, upper primer) is changed to 'T'.

mSR-BI	(upper primer)	5′-gtcgac CAC GCG GAC ATG G**G**C GTC AGC-3′
	(lower primer)	5′-gtcgac GTC TGA CCA AGC TAT CAG GTT-3′
Rat L19	(upper primer)	5′-CTG AAG GTC AAA GGG AAT GTG-3′
	(lower primer)	5′-GGA CAG AGT CTT GAT GAT CTC-3′

Reaction products were ethanol precipitated overnight, resuspended in TE buffer (10 mm Tris-HCl and 1 mm EDTA, pH 8.0), and subjected to polyacrylamide gel electrophoreses on a 5% native gel. Bands were detected by autoradiography using Kodak Omat film.

Fluorescence microscopy of HDL-derived BODIPY CE. For fluorescence microscopy of CEs, granulosa cells were grown on 25 mm-diameter glass coverslips coated with fibronectin and treated with or without Bt_2-cAMP and HDL_3 as described above. After washing, cells were incubated with *rec* HDL-BODIPY-CE (50 μg/ml) at 37°C for 1–3 h and processed for confocal fluorescence microscopy (15, 22).

Immunofluorescence microscopy and immunohistochemistry. For immunofluorescent confocal microscopy and immunohistochemistry of SR-BI and caveolin, granulosa cells were grown on fibronectin coated glass coverslips under basal culture conditions. After the appropriate treatment, cells were fixed for 10 min in 4% paraformaldehyde in PBS, washed with several changes in PBS, treated with ethanolamine, permeabilized with 0.2% Triton-X100, placed in 5% normal goat serum and 5% nonfat dry milk in PBS, for 1 h at 37°C, and incubated with anti-SR-BI (1:1000–1500) or anti-caveolin (1:400) at 4°C overnight. Subsequently, the cells were washed free of antibody and incubated at room temperature in a buffer containing biotinylated goat anti-rabbit IgG (1h), followed by FITC–avidin for 1 h. After washing, coverslips were mounted on slides using Fluoromount G (Fisher Biotech; Pittsburgh, PA) and viewed by confocal fluorescent microscopy (Cell Science Imaging Facility, Stanford University, Stanford).

To detect SR-BI immunoreactivity by immunohistochemical techniques, antibody-treated cells were incubated (30 min) with goat anti-rabbit IgG conjugated to biotin, followed by incubation for 1 h with alkaline phosphatase (AP)-conjugated avidin (Vector Laboratories, Burlingame, CA). AP activity was detected using an alkaline phosphatase substrate kit (Vector Laboratories, Burlingame, CA) as described by the manufacturer. Coverslips were mounted on glass slides and photographed with a Leitz Orthoplan Microscope.

Immunoelectron microscopy. Granulosa cells grown on 60 mm petri dishes were treated with or without Bt_2cAMP for 24 h, then with 30–35 nm colloidal gold-labeled HDL for 5 h (15). After labeling with HDL, the cells were briefly fixed (4% paraformaldehyde + 0.5% glutaraldehyde in PBS), scraped from the dishes,

pelleted, then fixed overnight, processed and embedded in LR Gold resin (Ted Pella, Redding, CA) as described by Berryman and Rodewald (48). Ultrathin sections were blocked with 5% goat serum (1 h, 22°C) and immunostained with rabbit pre-immune serum or polyclonal anti-SR-BI (1:100) overnight at 4°C, followed by BBI International goat anti-rabbit IgG-10nm gold (1:50, 1 h; Ted Pella, Redding, CA).

Miscellaneous techniques. The DNA content of the cells was quantified fluorometrically (49). The procedure of Markwell et al. (50) was used to quantify protein content of $hHDL_3$ and reconstituted HDL preparations. Protein in the cellular lysates and membrane fractions was determined by a modification of the procedure of Lowry et al. (51) as described by Peterson (52). Cholesterol content of the $hHDL_3$ and reconstituted HDL was determined colorimetrically according to the procedure of Tercyak (53).

RESULTS

Bt_2-cAMP induction of selective CE uptake

Non-luteinized (basal) rat granulosa cells, even when grown in the presence of $hHDL_3$, produce essentially no progestins (**Fig. 1A**) and take up no lipoprotein CEs via the selective pathway [as measured by double-radiolabeled ligands (Fig. 1B) or by the uptake of fluorescent (BODIPY)-CEs (**Fig. 2A**)].

When such granulosa cells are treated with Bt_2-cAMP for 24 h, they become luteinized. During this process, granulosa cells typically become more rounded in shape although cell to cell communication is retained through long cytoplasmic extensions that reveal a number of surface microvilli and microvillar channels (39, 40). When provided with Bt_2-cAMP and $hHDL_3$, such luteinized granulosa cells produce 1000–2000 times the progestins produced by basal cells (Fig. 1A) and take in massive amounts of HDL-provided CE via the selective pathway (Figs 1B and 2B). Previous studies from this laboratory using luteinized granulosa cells (15) have shown conclusively that radiolabeled HDL-CE (internalized via the selective pathway) can be directly utilized in steroid hormone production.

As such, rat granulosa cells represent a unique cell sys-

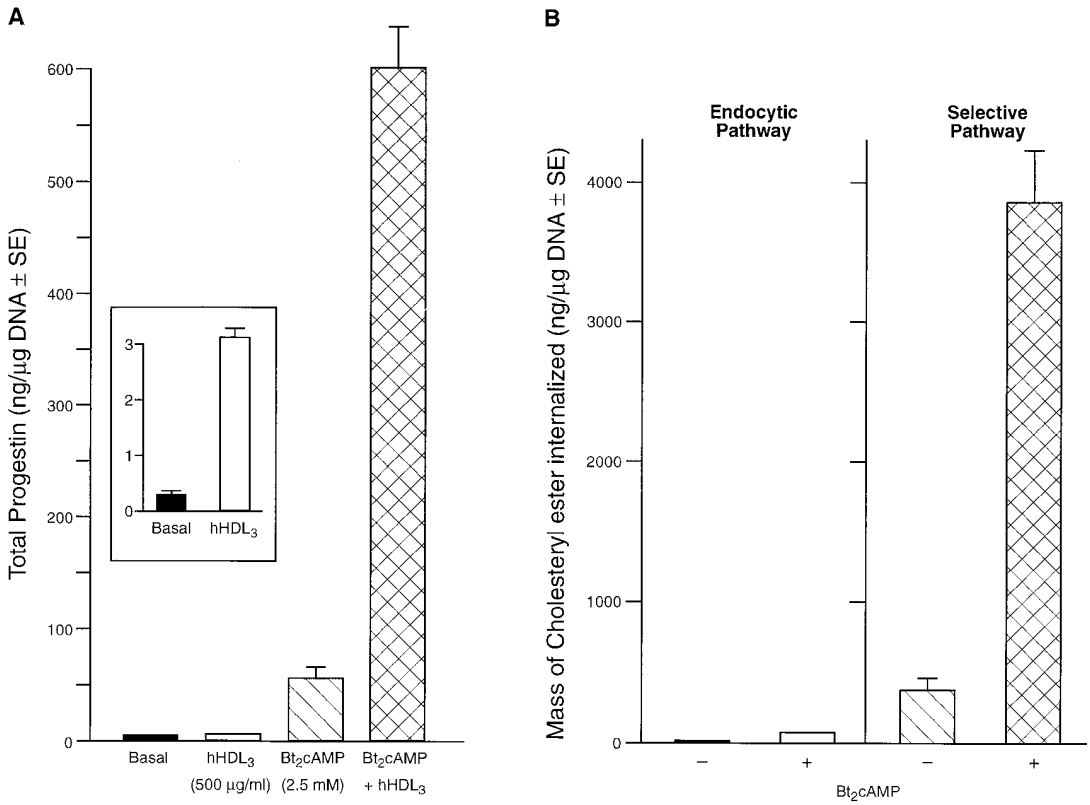


Fig. 1. (A) Bt_2-cAMP and lipoprotein stimulated progestin production in granulosa cells. Granulosa cells were cultured in basal medium for 72 h and then sensitized with or without Bt_2-cAMP (2.5 mm) for an additional 24 h. Subsequently, cells were cultured ± $hHDL_3$ (500 μg protein/ml), ± Bt_2-cAMP (2.5 mm) for 24 h. Collected media were assayed for 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 $hHDL_3$ (B) Effect of Bt_2-cAMP on the internalization of $hHDL_3$-derived cholesteryl esters by granulosa cells. The incubation conditions were the same as described under Fig. 1A except $hHDL_3$ was replaced with ^{125}I-labeled DLT-[^{3}H]COE-$hHDL_3$ (100 μg/ml). At the end of the incubation period, the samples were processed for the determination of ^{125}I and ^{3}H radioactivity. Endocytic uptake is calculated from TCA-soluble ^{125}I label only. Initially, a double radiolabeled residualizing lipoprotein is used with cells: i.e., ^{125}I-labeled DLT [^{3}H]-COE-hHDL/LDL. Subsequently, homogenates are counted for total ^{125}I and ^{3}H activity (after extraction with organic solvents), and TCA-soluble ^{125}I activity. The difference between total and TCA-soluble activity is taken as the surface associated ^{125}I radioactivity. As both ^{125}I and ^{3}H are on the same particle, the surface bound ^{125}I is also equal to the surface bound ^{3}H. Thus, total ^{3}H minus surface bound ^{3}H equals the total amount of ^{3}H internalized. Finally, to calculate mass of CE internalized, these values are divided by protein:cholesterol ratios of each lipoprotein. Values represent mean ± SE of triplicate determinations.

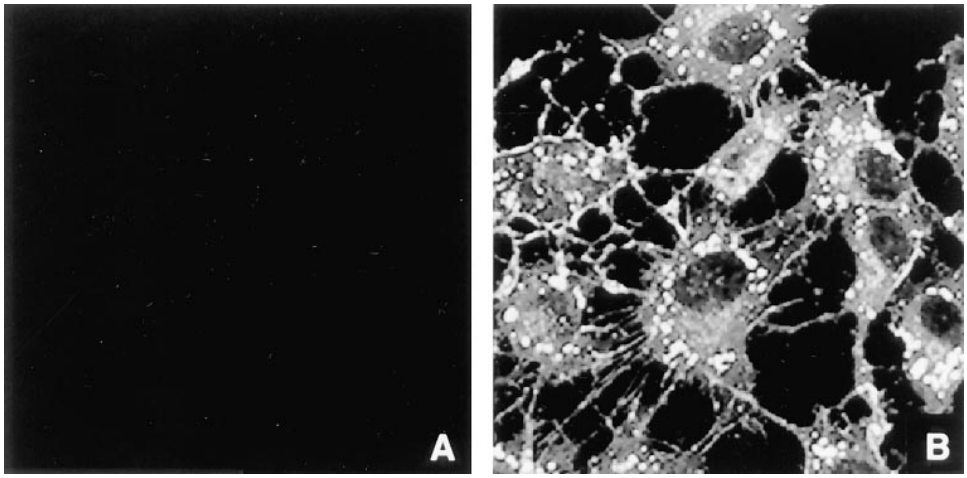


Fig. 2. Effect of luteinization on granulosa cell uptake of HDL-BODIPY CE. Non-luteinized granulosa cells do not take up fluorescent-CE even after 3 h of incubation with HDL-BODIPY CE **(**2A**)**. However, after luteinization (Bt_2-cAMP for 24 h) and incubation with HDL-BODIPY CE for 3 h, granulosa cells show extensive uptake of the fluorescent CE on the surface of cells, in cell extensions, and intracellularly in Golgi regions and especially in lipid droplets (2B).

tem: i.e., a hormone-regulatable system in which selective lipoprotein-CE uptake is virtually nonexistent in the basal state, but highly expressed and highly functional under luteinizing conditions.

Bt_2-cAMP induction of SR-BI expression

The dramatic differences observed in selective cholesterol uptake by basal and luteinized rat granulosa cells led to the question of whether the expression of SR-BI protein would follow the same pattern.

SR-BI Protein and mRNA. Western blot analyses identifying SR-BI protein content in the two groups of cells indicated that basal cells show no trace of the protein (even in overloaded gels), whereas gels of homogenate from 24 h cAMP-treated luteinized cells showed great quantities of protein (**Fig. 3A**). Likewise, RT-PCR analyses indicate that mRNA for SR-BI is barely detectable in basal granulosa cells, but is present in large amounts in 24 h cAMP-treated (luteinized) cells (Fig. 3B). Expression of constitutively active ribosomal protein (L19) was similar in basal and cAMP-treated cells.

SR-BI immunohistochemistry. An immunohistochemical examination of basal and luteinized granulosa cells stained for SR-BI indicates that granulosa cells do not express SR-BI in the basal state (**Fig. 4A**) but luteinized granulosa cells are highly reactive (Fig. 4B). In the cAMP-treated cell preparations, cells appear to show some variation in the overall level of intensity of stain obtained (perhaps relating to the level of luteinization of different cells), but also in the level of expression of SR-BI at different focus levels within individual cells; in general, the focal plane corresponding to cell plasma membranes adhering to the glass coverslip showed the most intense staining.

Development of SR-BI with time and concentration of Bt_2-cAMP treatment. Western blots were used to describe the cAMP-induced development of SR-BI in homogenates from granulosa cells over a 72 h period (**Fig. 5A**), and with a range of Bt_2-cAMP doses over a 24-h interval (Fig. 5B). Figure 5A indicates that SR-BI can be seen initially 6–9 h after the onset of Bt_2-cAMP treatment; beyond this point, SR-BI content increases dramatically with time and does

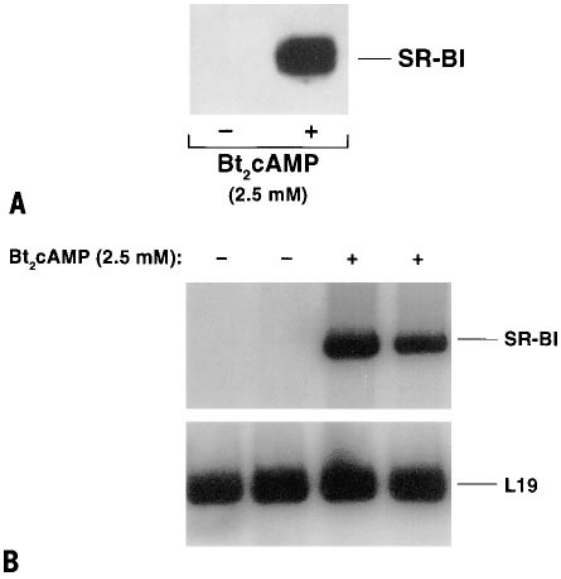


Fig. 3. Inducible expression of SR-BI protein and mRNA in granulosa cells with Bt_2-cAMP. (A) Western blot analysis of cell lysates; 72 h cultured granulosa cells were treated ± Bt_2-cAMP (2.5 mm) for 24 h. Total cell lysates were prepared as described under Materials and Methods and 50 μg of the lysate from each group was subjected to Western blot analysis using rabbit anti-SR-BI peptide antibody. The SR-BI protein was visualized by chemiluminescence. (B) RT-PCR analysis of SR-BI mRNA. Total RNA was isolated from control and Bt_2-cAMP-treated cells; 10 μg of RNA from each group was subjected to RT-PCR using primers specific for rat SR-BI and constitutively expressed rat ribosomal protein L-19 and in the presence of trace amount of [α-^{32}P] dCTP. The reaction products were separated by polyacrylamide gel electrophoresis followed by radioautography.

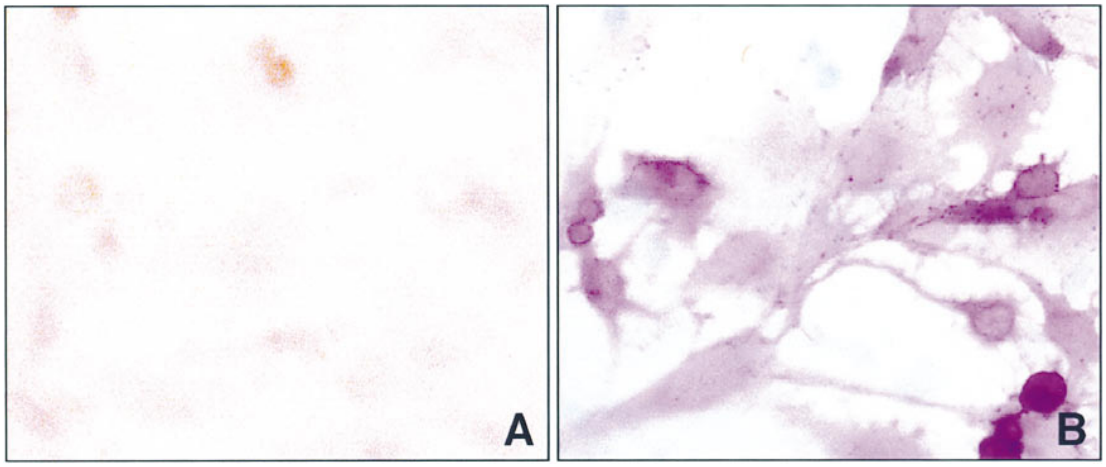


Fig. 4. Immunohistochemical staining of SR-BI protein in granulosa cells. Granulosa cells were cultured for 72 h on fibronectin-coated glass coverslips in basal medium, and then treated ± Bt_2-cAMP (2.5 mm). After 24 h, the cells were fixed and stained with anti-SR-BI antibody followed by biotin-conjugated anti-rabbit IgG, alkaline phosphatase-avidin plus substrate as described in Materials and Methods. (A) Basal cells. (B) Bt_2-cAMP-treated cells. Representative fields are shown.

not appear to saturate over a 72 h experimental period. Immunofluorescent staining of granulosa cell preparations treated with cAMP provided precisely the same information; i.e. immunofluorescence with antibody to SR-BI is faint after 6 h of Bt_2-cAMP treatment but continued to increase with time over a 48-h period (data not shown). The effective dose of Bt_2-cAMP is found to be between 1–2 mm (Fig. 5B).

Effect of Bt_2-cAMP analogs on SR-BI expression. Our data indicate that not only Bt_2-cAMP, but a variety of agents that increase cellular levels of cAMP also increase the expression of SR-BI protein in basal granulosa cells: thus, cAMP analogs such as Bt_2-cAMP, 8CPT-cAMP, 8Br-cAMP (12, 54, 55), cyclic nucleotide phosphodiesterase inhibitor [1-methyl-3-isobutylxanthine (56, 57)], trophic hormone [oFSH (12, 54, 55)], G-protein ADP-ribosylating toxins [cholera toxin and pertussis toxin (55, 58)], and a direct activator of adenylate cyclase [forskolin (59)] all stimulate granulosa cell luteinization in a 24-h period and, at the same time, increase SR-BI content (Fig. 5C).

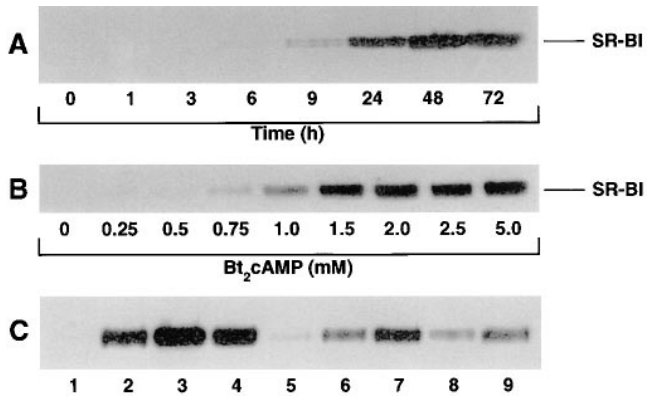


Fig. 5. Induction of SR-BI protein expression by Bt_2-cAMP, other cAMP analogs, gonadotropins, and specific stimulators of cAMP. (A) Basal granulosa cells (72 h) were exposed to 2.5 mm Bt_2-cAMP for 1, 3, 6, 9, 24, 48, or 72 h (B) Alternatively, basal cells were exposed for 24 h to 0.25, 0.50, 0.75, 1.0, 1.5, 2.0, 2.5, or 5.0 mm concentrations of Bt_2-cAMP. (C) In some cases, basal granulosa cells were treated for 24 h with no addition (basal) or Bt_2-cAMP (2.5 mm), 8CPT-cAMP (2.5 mm), 8Br-cAMP (2.5 mm), 1-methyl-3-isobutylxanthine (0.5 mm), o-FSH (250 ng/ml), cholera toxin (1 μg/ml), pertussis toxin (100 ng/ml), or forskolin (0.1 mm) [Note, lane 1 = basal, 2 = Bt_2cAMP, 3 = 8CPT-cAMP; 4 = 8Br-cAMP; 5 = 1-methyl-3-isobutylxanthine; 6 = o-FSH; 7 = cholera toxin; 8 = pertussis toxin; 9 = forskolin]. In each case, total cellular lysate was prepared and subjected to Western blot analysis performed using an anti-SR-BI polyclonal peptide antiserum as described under Materials and Methods.

Relationship between selective uptake of cholesteryl esters and SR-BI expression

To assess a functional link between the expression of SR-BI in cells and the actual uptake of lipoprotein CEs through the selective pathway, preparations of granulosa cells were examined as (A) basal (non-cAMP-treated) cells, as (B) luteinized (+cAMP), or as (C) HDL-treated (+cAMP + $hHDL_3$) cells. The extent of selective CE uptake in A–C was determined by accumulation of non-hydrolyzable HDL-donated BODIPY-CE under the various cell conditions. The extent to which SR-BI expression had been altered in the same cell preparations was monitored separately by immunofluorescent staining and by analysis of Western blotting.

The confocal images of **Fig. 6** were taken through the nuclear regions of cells from the different preparations and indicate various levels of CE uptake by low, medium, and high levels of accumulated BODIPY-CE (viewed respectively as dark gray, light gray, or white in the greyscale images displayed here). Thus, basal granulosa cells are seen not to take up HDL-BODIPY CE (Fig. 6A), but luteinized granulosa cells internalize and store substantial amounts of the trace BODIPY-CE during a 1-h incubation period (Fig. 6B). Luteinized cells, even when pre-loaded with native HDL for a 24-h period, are able to internalize and store vast amounts of the BODIPY-CE trace during the 1-h experiment (Fig. 6C). In the above images, BODIPY-CE

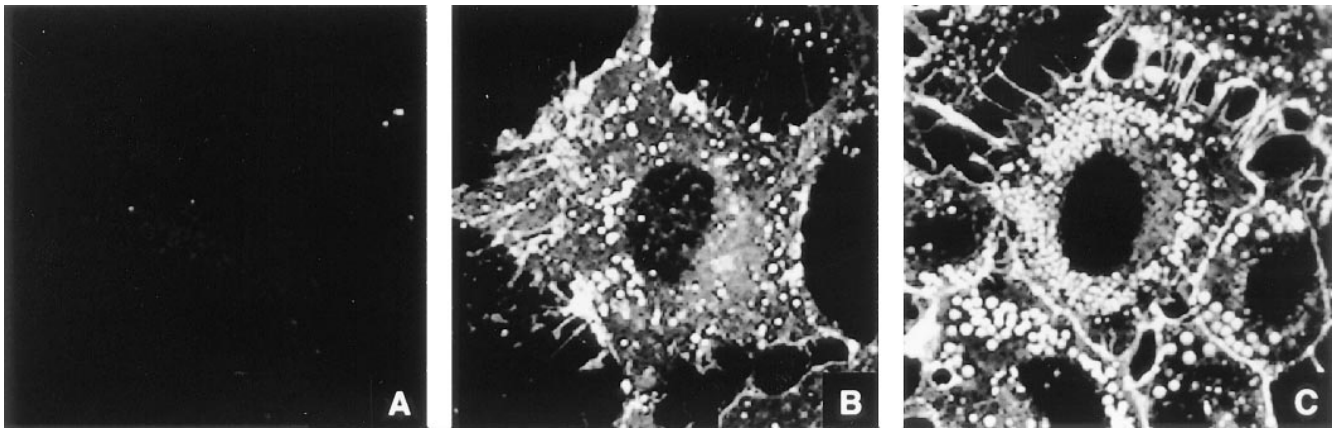
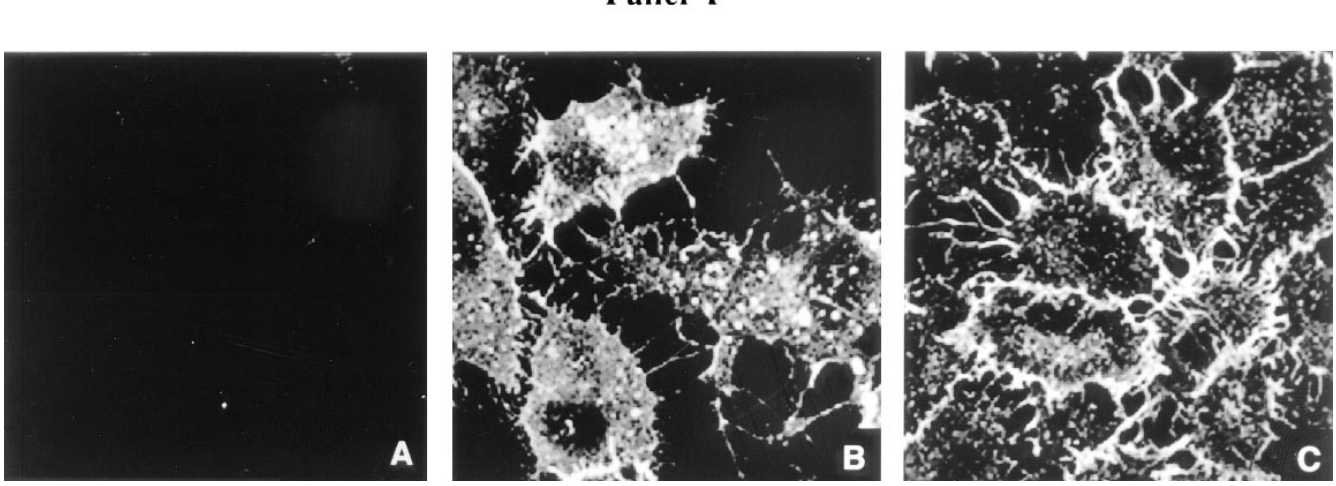


Fig. 6. Effects of luteinization and HDL (cholesterol-loading) on granulosa cell uptake of HDL-BODIPY CE. Granulosa cells were cultured for 72 h on fibronectin coated coverslips and incubated ± Bt_2-cAMP (2.5 mm) for 24 h. Next, cells were treated ± Bt_2-cAMP (2.5 mm), ± HDL_3 (500 μg/ml) for an additional 24 h and exposed to *rec* HDL-bodipy-CE (50 μg/ml) for 1–3 h at 37°C. The coverslips were subsequently processed for confocal microscopy as described under Materials and Methods. (Panel A) basal cells; (Panel B), Bt_2-cAMP-treated cells; (Panel C), Bt_2-cAMP + $hHDL_3$-treated cells. Levels of fluorescence are indicated in grayscale; dark gray, light gray, and white represent low level, medium level, and high level of fluorescence, respectively.

is generally associated with plasma membranes, lipid droplets, and membranes of the Golgi compartment (22).

The confocal images of **Fig. 7** (Panel I) show SR-BI staining in the same three preparations of granulosa cells seen in Fig. 6. As with the selective uptake of CE, SR-BI staining is not seen in basal (non-luteinized) cells (Fig. 7A), but is highly expressed in luteinized cells and in cells preloaded with native HDL (Fig. 7B, 7C). In the HDL-preloaded cells (Fig. 7C), SR-BI appears particularly associated with cell surface membranes.

Western blots quantifying SR-BI protein in the same cell preparations (Fig. 7, Panel II), indicate that basal granulosa cells have no SR-BI protein, that luteinized granulosa cells contain large quantities of the protein, and that in cells preloaded with HDL, high SR-BI levels are maintained.



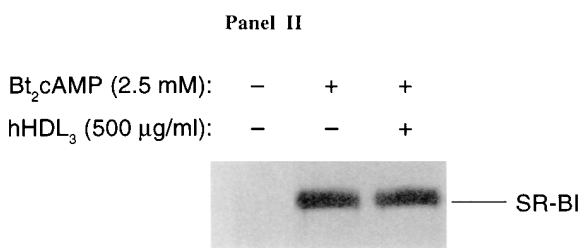


Fig. 7. Immunofluorescence and Western blot analysis of SR-BI expression in luteinized and HDL (cholesterol-loaded) granulosa cells. Immunofluorescent staining of SR-BI (panel I-A, B, C); granulosa cells were left untreated (A) or treated with Bt_2-cAMP (2.5 mm, (B), or Bt_2-cAMP (2.5 mm) plus $hHDL_3$ (500 μg protein/ml, (C) as described under Fig. 6. Cells were fixed, processed, and probed with polyclonal antibodies to SR-BI and visualized with biotin-conjugated goat anti-rabbit IgG and FITC-avidin as described under Materials and Methods. In Western blots (Panel II), cells were treated as above and analyzed for immunoreactivity using polyclonal SR-BI antibody.

Surface localization of BODIPY-CE and SR-BI

Fluorescent confocal microscopy. Optical slices taken directly through the surface (plasma membrane) plane of luteinized granulosa cells exposed to HDL-BODIPY-CE or granulosa cells immunostained for SR-BI, show remarkably similar fluorescent patterns. **Figure 8A** represents such a surface slice through a cell which has selectively taken up BODIPY-CE and Fig. 8C represents a surface slice through an SR-BI-stained cell When viewed in the indexed color mode (low, medium, and high levels of fluorescence showing as green, yellow, or red fluorescence, respectively), these surface slices of BODIPY-CE and SR-BI-stained cells appear quite similar; i.e., en face views of the cell surfaces reveal irregular patches of highly fluorescent material surrounded by areas with little or no fluorescence. In the center cuts through the same cells (Fig. 8B, 8D), this similarity between the cells is lost, and storage lipid droplets stand out in the BODIPY-CE-stained cells (compare with Fig. 2), and diffuse intracellular cytoplasmic staining is prominent in SR-BI stained cells.

Combined SR-BI-immunogold and HDL–colloidal gold electron microscopy. To identify surface areas of luteinized granulosa cells that bind intact HDL and, at the same time, express sites for the SR-BI receptor, luteinized granulosa cells

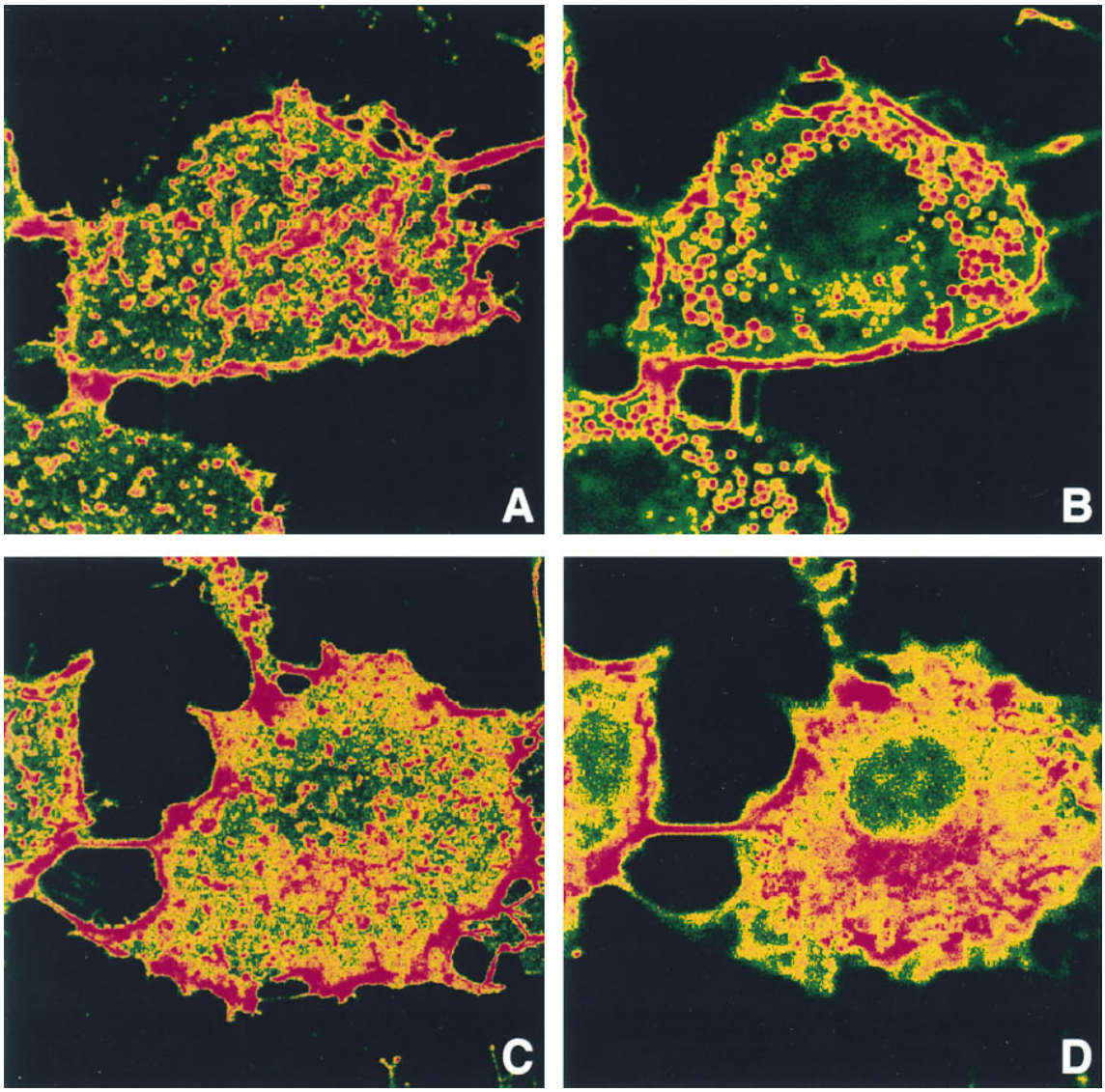


Fig. 8. Variations in the cell surface and intracellular distribution of BODIPY-CE and SR-BI in luteinized granulosa cells. These images were obtained from cell preparations in which cells were treated with Bt_2-cAMP + HDL and stained for BODIPY-CE (8A, 8B) or SR-BI (8C, 8D) as described in Figs. 6 and 7. In each case, optical cuts were taken through the plane of the cells' plasma membrane adhering to the coverslip (10A, 10C) or through a central, nucleated area (8B, 8D), of the stained cells. The indexed color describes low, medium, and high levels of fluorescence as green, yellow, and red, respectively. The plasma membrane sections (8A, 8C) show remarkably similar distributions of BODIPY-CE and SR-BI, the highest level of fluoresence (red color) in both preparations seen at the cell borders, cell extentions, and spiral-shaped folds or crevices in en face views of the plasma membrane. In central cuts (8B, 8D), the distribution of CE and SR-BI is quite different, the former being located primarily in lipid droplets and the latter in a diffuse cytoplasmic pattern especially prominent in perinuclear (Golgi?) regions of the cells.

were pre-incubated with 30–35 nm colloidal gold-labeled HDL particles, and subsequently processed for immunocytochemical staining of SR-BI and 10 nm gold second antibody labeling techniques (**Fig. 9**). Gold-labeled HDL bound tightly, and with great abundance, to microvilli and microvillar channels present on the cell surface. Ultrathin sections pre-labeled with HDL–gold were subsequently immuno-labeled with preimmune (PI) serum or with antisera to SR-BI (SR-BI). PI-stained sections showed essentially no immunogold labeling (small gold particles), despite ample labeling of microvilli with HDL–gold (large gold particles). In contrast, SR-BI-immunostained sections showed a mixture of large and small gold particles in microvillar regions of the cell surface (arrows). Relatively fewer (30–40%) gold particles/cell area were found in the interior of the cells (arrows) where SR-BI is believed to be synthesized and processed in preparation for transport to the cell surface. Non-microvillar surface areas of the cells showed essentially no large HDL–gold particles, and scattered labeling with small gold particles depicting SR-BI distribution.

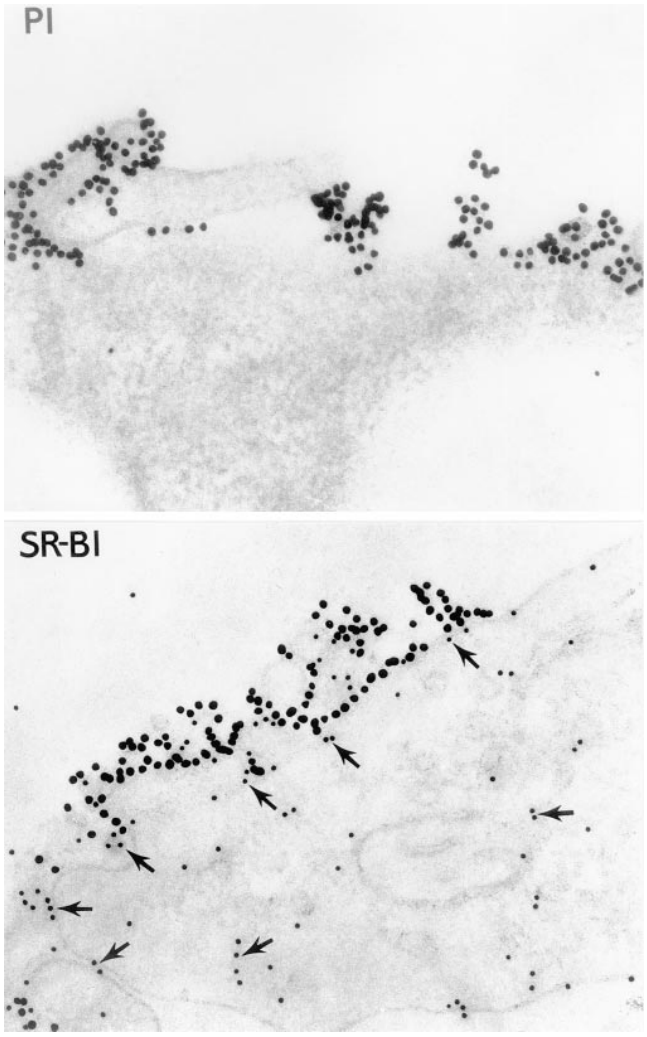


Fig. 9. Electron microscopic localization of SR-BI on cell surface of luteinized granulosa cells pre-incubated with HDL–gold particles. Luteinized granulosa cells were incubated with HDL–colloidal gold (30 nm) in vivo to target HDL binding sites, and subsequently fixed and processed for immunostaining with preimmune serum (PI) or SR-BI antiserum (SR-BI) and labeled with 10 nm gold IgG. Cells labeled with preimmune serum showed only the large HDL–colloidal gold bound at the surface; cells labeled with SR-BI antisera showed both large and small (SR-BI) gold particles (see arrows) associated with microvilli and microvillar channels of the cell surface.

Caveolin expression in granulosa cells

Given the reported relationship between SR-BI and caveolin in other cell systems (41), it was of interest to assess caveolin expression in the current study, in which SR-BI (and the uptake of HDL-CE) are up-regulated by Bt_2cAMP.

The same granulosa cell preparations used above to identify selective CE uptake and SR-BI expression were used to assay caveolin expression; these preparations included basal (non-luteinized) cells, luteinized cells, and luteinized cells preloaded with native HDL. Western blots were analyzed to determine the caveolin protein content in the three groups of cells (**Fig. 10**, panel I). The Western blots show that the total protein level of caveolin is highly expressed in basal granulosa cells; however, the caveolin content of the cells appears to decrease as the cells luteinize, and is found to decrease further when the luteinized cells are pretreated with high levels of HDL. A similar result is obtained with confocal fluorescent microscopy. Non-luteinized granulosa cells show a high level of expression of caveolin staining (Fig. 10, Panel II-A); luteinized cells show less stain (Fig. 10, Panel II-B); and the majority of the staining is lost in the HDL-treated luteinized cells (Fig. 10, Panel II-C). Note, punctate structures labeled for caveolin are found more or less evenly distributed throughout the cells as viewed in optical slices taken through the flat bottom (A), central (B) and top (C) planes of basal (non luteinized) cells pictured in Fig. 10, Panel III.

DISCUSSION

This report confirms earlier work showing that the selective cholesterol pathway does not exist in basal, non-luteinized rat granulosa cells, but can be induced by hormone (Bt_2cAMP or analogs) treatment, and in 24 h develops into a highly expressed CE-uptake system in which large amounts of CE are internalized to provide fuel for progestin production and secretion. As such, granulosa cells provide a physiologically relevant model system in which to study the relationship between selective cholesterol uptake and an HDL receptor under varied metabolic situations.

The study shows that the development of the selective pathway (and its functional corollary represented by the uptake of HDL-CE) is tightly linked to the development of the scavenger receptor, SR-BI, which is believed to represent an HDL receptor. We have found that no SR-BI protein is found in non-luteinized cells, that SR-BI protein and message is highly expressed after B_2cAMP treatment, that the timing of SR-BI development and selective pathway development is identical (12, 15), and that various surface-stimulating agents (affecting cAMP production)

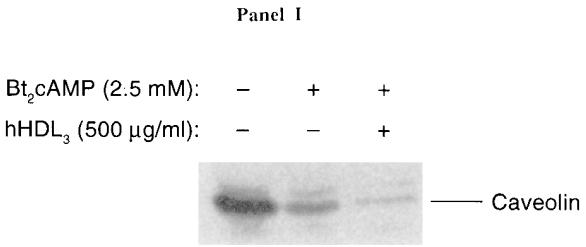
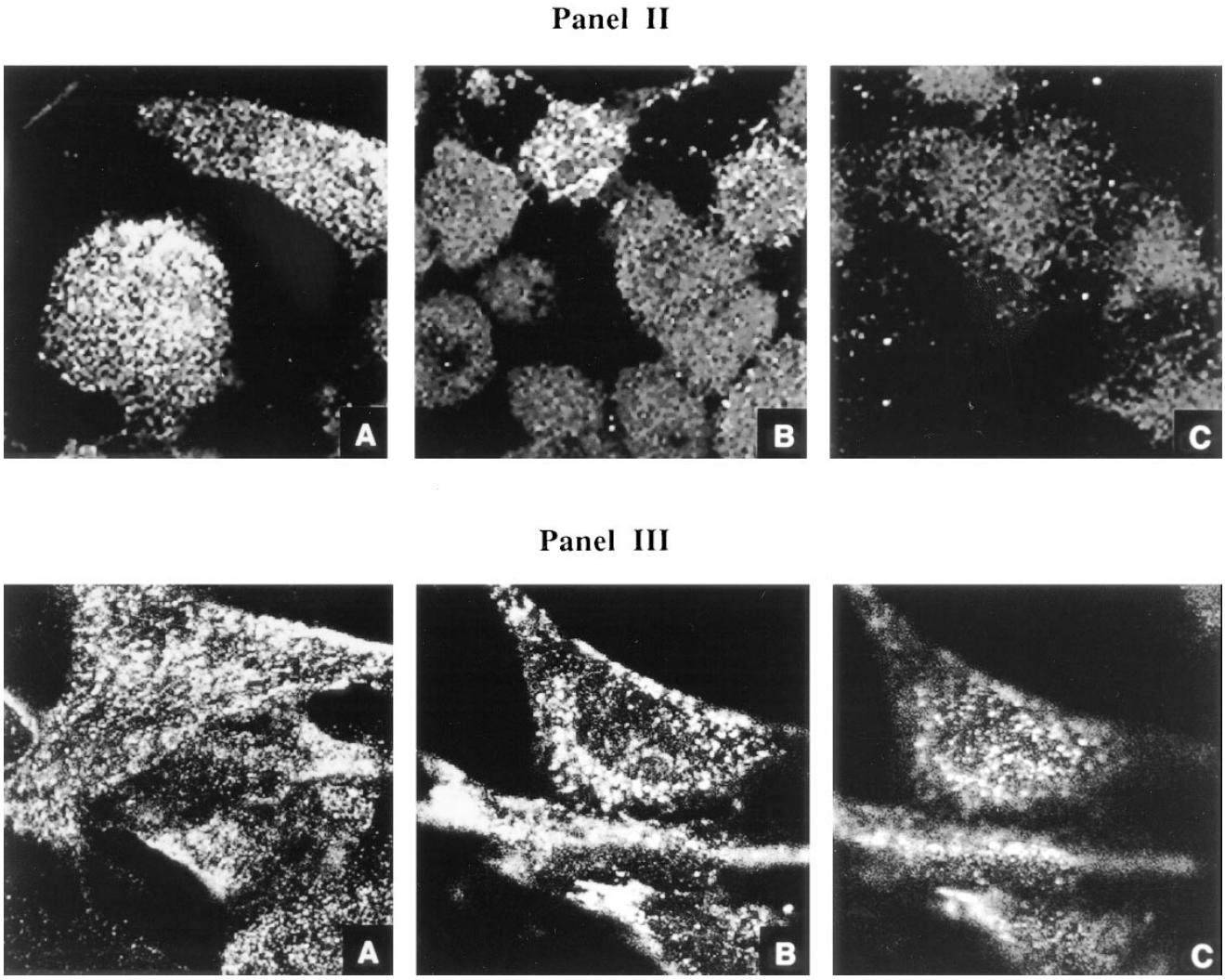


Fig. 10. Immunofluorescence and Western blot analysis of caveolin expression in luteinized and HDL (cholesterol loaded) granulosa cells. (Panel I) Western blot analysis of caveolin protein under the same conditions described under Figs. 8 and 9: caveolin showed highest expression in basal cells and progressively less expression after hormone or hormone + HDL treatment. (Panel II) Similarly prepared cells were probed with polyclonal antibodies to caveolin and visualized with biotin-conjugated goat anti-rabbit IgG and FITC-avidin as described under Materials and Methods. Again, the highest expression was seen in basal cells (II-A) and lowest expression in cells after treatment with hormone + HDL (II-C). In panel III, confocal optical slices were taken of a group of caveolin-stained basal cells through cell surface membranes adhering to the coverslip (III-A), through a central nuclear region (III-B), and through the rounded top surfaces of the same cells (III-C).



act similarly on the development of SR-BI and on the development of the selective CE pathway.

Perhaps most importantly, expression of the selective CE pathway and the expression of the SR-BI protein in the luteinized granulosa cells are shown to be physiologically linked through their sustained up-regulation after HDL loading. In this situation, luteinized granulosa cells were preloaded with native HDL for 24 h, after which there was a dramatic increase in the internalization and storage of HDL-provided CE and a maintenance of high SR-BI levels. This is a novel finding. In most cell systems, lipoprotein and/or other means of cholesterol loading results in a down-regulation of the classic LDL receptor and other cholesterol-sensitive genes (17); in the steroidogenic cell model used here, HDL pre-loading results in continued increase in HDL-CE uptake, and the CE increase is associated with continued high level of expression of the HDL-receptor protein itself.

A further link between the selective cholesterol pathway and the existence of SR-BI in granulosa cells may be seen in the similar localization of newly incorporated CE and SR-BI protein. HDL-newly donated fluorescent (BODIPY)-CE is reported here to be prominent not only in the interior of cells (as lipid storage droplets), but also on the surface of luteinized granulosa cells (and on cell extensions) in what appear to be irregular patches of fluorescence. The surface localization of SR-BI appears to be identical to that described for BODIPY-CE. Intracellularly, however, the distribution of SR-BI and BODIPY-CE is different. One may speculate that much of the intracellularly localized SR-BI is protein in the process of being synthesized, processed, and exported to the cell surface (where it is most highly expressed), whereas intracellular BODIPY-CE is lipid going in the other direction in the cell, that is, lipid which we catch in the process of being interiorized and stored in lipid droplets (where it is most highly expressed).

Ultrastructural studies were helpful in co-localizing cell surface sites of high SR-BI expression to sites of HDL binding. In these experiments, luteinized granulosa cells were incubated with colloidal gold–HDL in an effort to mark sites where HDL particles bind. For the most part, the HDL particles labeled surface microvilli and crowded into narrow invaginations of the cultured cell surface, many of which resembled microvillar channels found in intact ovary and adrenal tissues (3, 5, 39, 40). Such microvillar regions occupy ~20% of the cell surface of rat granulosa cells and probably represent the irregular SR-BI and BODIPY-CE fluorescent patches noted with confocal microscopy. Immunostaining of SR-BI in thin sections taken from the colloidal gold–HDL-incubated blocks indicated that much of the SR-BI was localized in the same sites where the HDL was bound; i.e., microvilli and microvillar channel areas. Based on these various morphological experiments, it seems likely that SR-BI protein, HDL particles, and HDL-derived fluorescent CE colocalize to the same plasma membrane sites on stimulated granulosa cells and, as such, it is reasonable to suspect that SR-BI and HDL interact functionally in directing cholesterol uptake through the selective pathway. Obviously, further studies are necessary to determine the nature of this interaction and to identify the involvement of other potential players in the scenario, but the current data utilizing a hormone-regulateable cell system provides strong evidence for the idea that SR-BI is tightly linked to the selective CE uptake process.

Finally, there is the issue of how caveolin is related to these processes. First, the substantial amount of caveolin present in non-luteinized, basal granulosa cells is surprising given the relatively few caveoli identified in these cells; in this regard it may be of importance that the localization of caveolin is not restricted to the cell surface in these cells, but appears instead to be uniformly distributed throughout the cell. More to the point, however, is the fact that metabolically, caveolin expression turns out to be negatively associated with either the development of the selective CE pathway or the development of the SR-BI protein in the granulosa cells: i.e., caveolin expression is high in basal, non-luteinized cells, and is lower in cAMP-stimulated (luteinized) cells, and still lower in HDL-primed cells. Indeed, it appears that caveolin content is highest when the cell is functioning to keep cholesterol out of the cell, rather than assisting in cholesterol internalization. In this regard, our findings with caveolin may reflect a situation more in keeping with one reported recently in fibroblasts in which caveolin was found associated with selective cholesterol efflux, not influx (60, 61, 62). Or, perhaps the association between SR-BI and caveolin is not similar in all cell models, but varies in accordance with genetic makeup and physiological need. In any event, the significance of caveolin to SR-BI function and to the selective uptake of cholesterol is probably more complex than originally stated (43), and needs to be re-examined under a variety of experimental conditions.

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