

LDL and cAMP cooperate to regulate the functional expression of the LRP in rat ovarian granulosa cells

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Abstract Rat ovarian granulosa rely heavily on lipoprotein-derived cholesterol for steroidogenesis, which is principally supplied by the LDL receptor- and scavenger receptor class B type I (SR-BI)-mediated pathways. In this study, we characterized the hormonal and cholesterol regulation of another member of the LDL receptor superfamily, low density lipoprotein receptor-related protein (LRP), and its role in granulosa cell steroidogenesis. Coincubation of cultured granulosa cells with LDL and *N*⁶,*O*²-dibutyryl adenosine 3',5'-cyclic monophosphate (Bt₂cAMP) greatly increased the mRNA/protein levels of LRP. Bt₂cAMP and Bt₂cAMP plus human (h)LDL also enhanced SR-BI mRNA levels. However, there was no change in the expression of receptor-associated protein, a chaperone for LRP, or another lipoprotein receptor, LRP8/apoER2, in response to Bt₂cAMP plus hLDL, whereas the mRNA expression of LDL receptor was reduced significantly. The induced LRP was fully functional, mediating increased uptake of its ligand, α_2 -macroglobulin. The level of binding of another LRP ligand, chylomicron remnants, did not increase, although the extent of remnant degradation that could be attributed to the LRP doubled in cells with increased levels of LRP. The addition of lipoprotein-type LRP ligands such as chylomicron remnants and VLDL to the incubation medium significantly increased the progesterone production under both basal and stimulated conditions. In summary, our studies demonstrate a role for LRP in lipoprotein-supported ovarian granulosa cell steroidogenesis.—Azhar, S., S. Medicherla, W.J. Shen, Y. Fujioka, L. G. Fong, E. Reaven, and A. D. Cooper. **LDL and cAMP cooperate to regulate the functional expression of the LRP in rat ovarian granulosa cells.** *J. Lipid Res.* 2006. 47: 2538–2550.

Supplementary key words low density lipoprotein • adenosine 3',5'-cyclic monophosphate • low density lipoprotein receptor-related protein

Steroid-producing tissues have special requirements for cholesterol, which is used as a precursor for tissue-specific steroid hormone biosynthesis. Because of this, all steroidogenic tissues have evolved multiple cholesterol delivery

pathways and an efficient intracellular cholesterol transport system to ensure a constant supply and adequate availability of cholesterol. There are four potential sources that could provide cholesterol needed for steroidogenesis: *a*) de novo biosynthesis; *b*) stored cholesteryl esters (CEs) in the form of lipid droplets; *c*) exogenous lipoprotein-supplied cholesterol; and *d*) plasma membrane-derived cholesterol (1, 2). In rodents, blood-borne cholesterol-rich HDLs appear to be the principal suppliers of cholesterol to ovarian and adrenal tissues and cells (1, 2). A nonendocytic process known as the scavenger receptor class B type I (SR-BI)/"selective" CE uptake pathway appears to be the principal uptake mechanism for the bulk delivery of cholesterol (1–4). However, under in vitro cell culture conditions, the classical endocytic [LDL or Apolipoprotein B/apolipoprotein E (B/E)] receptor pathway also becomes unmasked in steroidogenic cells, and in addition to HDL-CE taken in by selective uptake, exogenously provided human or rat LDL can be internalized by the traditional B/E pathway (5, 6). This study was initiated to explore the role of still another member of the LDL receptor superfamily, the low density lipoprotein receptor-related protein (LRP) (7–9), in the lipoprotein-derived cholesterol uptake process.

LRP (independently described as the α_2 -macroglobulin receptor) is a high-molecular-mass (600 kDa) receptor protein for a broad range of biologically diverse soluble ligands (~30) that undergoes rapid and constitutive endocytosis in clathrin-coated pits, delivering most ligands to lysosomes for degradation (7–9). These include activated

Abbreviations: apoE, apolipoprotein E; Bt₂cAMP, *N*⁶,*O*²-dibutyryl adenosine 3',5'-cyclic monophosphate; CE, cholesteryl ester; DRB, 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole; GST, glutathione S-transferase; LRP, low density lipoprotein receptor-related protein; RAP, receptor-associated protein; RPA, RNase protection assay; SR-BI, scavenger receptor class B type I.

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Manuscript received 9 June 2005 and in revised form 2 August 2006 and in re-revised form 18 August 2006.

Published, JLR Papers in Press, August 23, 2006.
DOI 10.1194/jlr.M600349-JLR200

coagulant factors, proteases of the fibrinolytic pathway, natural anticoagulants, and protease inhibitors (e.g., α_2 -macroglobulin and α_1 -antitrypsin) as well as proteins with important functions in lipoprotein metabolism, such as lipoprotein lipase, hepatic lipase, lipoprotein [a], and apolipoprotein E (apoE)-rich lipoproteins (7–9). In addition to soluble ligands, LRP mediates the endocytosis of other plasma membrane proteins, including urokinase-type plasminogen activator, urokinase plasminogen activator receptor, tissue factor, and β -amyloid precursor protein (8, 9). Furthermore, LRP mediates signal transduction by interacting with cytosolic adaptor and scaffold proteins that are involved in the regulation of mitogen-activated protein kinase activity, cytoskeletal reorganization, and cell adhesion. These proteins include Shc, FE65, PSD-95, and c-Jun N-terminal kinase-interacting proteins (8, 9). A 39 kDa protein termed the receptor-associated protein (RAP) is a chaperone for the LRP and acts as a potent inhibitor of all known ligand interactions with the LRP (10). LRP is widely distributed and has been found in every tissue in which it has been sought (11–13).

Considerable evidence now indicates that the expression of LRP and to a large extent its inhibitor RAP is subject to regulation in response to hormones, growth factors, cytokines, and various pathophysiological conditions (7, 14–25). However, little is known about LRP regulation at the molecular level, and the factors that might mediate the action of LRP have not been characterized. The ability of the LRP to mediate lipoprotein binding and uptake, and the manner in which this occurs, are somewhat confusing. Binding of apoE-rich lipoprotein to the LRP has been demonstrated by ligand blotting (26, 27). In cell culture study conditions, apoE must be added to apoE-rich lipoproteins to demonstrate a metabolic effect resulting from the LRP-mediated uptake of lipoproteins (27). On the other hand, antibody neutralization of LRP function in mutant human fibroblasts that lack the LDL receptor results in a failure to remove apoE-rich lipoproteins (28). These observations have led to the suggestion that the lipoproteins bind to another site, such as heparan sulfate proteoglycans/hepatic lipase (29–31), before being “handed off” to the LRP for internalization. To further assess the role of the LRP pathway in the delivery of lipoprotein cholesterol for steroidogenesis, we took advantage of a well-studied hormone-primed rat ovarian granulosa cell model (32). This cell model has very limited lipoprotein-CE uptake by either the selective or endocytic (LDL receptor) pathway before stimulation with trophic hormones or cAMP analogs (5, 6, 33, 34). However, when cultured with trophic hormones or the second messenger analog N^6, O^2' -dibutyryl adenosine 3',5'-cyclic monophosphate (Bt_2cAMP), the cells become luteinized, express high levels of both LDL receptor (5, 6) and SR-BI (35) proteins, and in the presence of lipoproteins take up massive amounts of CEs via the selective pathway. The cells respond with a 1,000- to 2,000-fold increase in secretion of progestins compared with cells grown under basal conditions (32).

In this study, we examined the hormonal and cholesterol regulation of LRP and its role in rat ovarian granulosa cell steroidogenesis. The results show that granulosa

cells have a well-developed functional LRP system. Surprisingly, coincubation of lipoproteins, notably human (h)LDL with Bt_2cAMP , resulted in the upregulation of granulosa cell LRP function through its increased expression of mRNA and protein levels. Incubation of granulosa cells with certain triglyceride-rich, cholesterol-carrying lipoproteins enhanced their steroidogenic potential in an LRP-dependent manner, suggesting that LRP under certain physiological conditions could potentially provide cholesterol needed for steroid hormone biosynthesis. Given that the combined presence of LDL and Bt_2cAMP upregulates LRP in granulosa cells, we also exploited this system to further evaluate the role of LRP in the uptake and metabolism of one of its lipoprotein ligands, the chylomicron remnant.

EXPERIMENTAL PROCEDURES

Materials

$Na^{125}I$ (carrier-free; 643.8 GBq/mg, 17.4 Ci/ μ g) was purchased from NEN Life Science Products (Boston, MA). Bt_2cAMP , human α_2 -macroglobulin, transferrin, estradiol, insulin, fibronectin, actinomycin D, 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB), and methylamine were supplied by Sigma Chemical Co. (St. Louis, MO). Human RAP, expressed in bacteria as a fusion protein with glutathione S-transferase (GST), was extracted and purified as described previously (36). The anti-rat LDL receptor antibodies have been described and characterized, and their monospecificity and lack of cross-reactivity with LRP and VLDL receptors have been documented (37). Rabbit anti-LRP (also known as the α_2 -macroglobulin receptor) and RAP antibodies (13) were kindly provided by Dr. D. K. Strickland (American Red Cross, Rockville, MD). SR-BI blocking antibody was purchased from Novus Biologicals (Littleton, CO). Drs. Guojun Bu and Alan Schwartz (Washington University School of Medicine, St. Louis, MO) generously supplied the partial cDNA clones for rat LRP and RAP (38). The cDNA probes for LDL (B/E) receptor, rat HMG-CoA reductase, and 18S rRNA were obtained as described previously (5, 6). All other reagents used were of analytical grade.

Isolation and culture of rat ovarian granulosa cells

Immature female Sprague-Dawley rats (21–23 days old; Harlan Sprague-Dawley, Indianapolis, IN) were injected subcutaneously with 17 β -estradiol (1 mg) daily for 5 days (32). The animals were euthanized 24 h after their last injection (i.e., on day 6), and granulosa cells were isolated from ovaries and cultured as described previously (32). In brief, cells were cultured in 35 mm plastic dishes coated with human plasma fibronectin (2 μ g/cm²). Cultures were initiated by adding $\sim 1-2 \times 10^5$ cells in 100 ml of culture medium to 1.4 ml of basal culture medium [F12 supplemented with 15 mM HEPES, BSA (1 mg/ml), insulin (2 μ g/ml), transferrin (5 μ g/ml), hydrocortisone (100 ng/ml), human fibronectin (2 μ g/cm²), streptomycin (100 μ g/ml), and penicillin G (100 U/ml)]. After 24 h of culture, the dishes were washed extensively to remove dead and unattached cells. At this stage, the cell viability as measured by 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide assay (39) averaged >97%. Some cultures were subsequently maintained at 37°C for up to 72 h in a basal medium. Other cultures at 72 h were presensitized with or without Bt_2cAMP (2.5 mM) for 24 h followed by incubation with or without Bt_2cAMP and/or lipoproteins (100 μ g/ml hLDL or 500 μ g/ml hHDL₃) for an additional 24 h. Subsequently, cells were used for various measurements as described below.

Lipoprotein-supported progesterin secretion by cultured granulosa cells

After changing basal culture medium and rinsing the dishes thoroughly, the 72 h cultured granulosa cells were presensitized without or with hLDL (100 $\mu\text{g}/\text{ml}$) or hHDL₃ (500 $\mu\text{g}/\text{ml}$) with or without Bt₂cAMP (2.5 mM) for 24 h and then incubated for an additional 24 h in basal culture medium containing chylomicron remnants (80 $\mu\text{g}/\text{ml}$), VLDL (80 $\mu\text{g}/\text{ml}$), hLDL (100 $\mu\text{g}/\text{ml}$), or hHDL₃ (500 $\mu\text{g}/\text{ml}$) with or without Bt₂cAMP (2.5 mM). At the end of the incubation, samples of the incubation medium were frozen and stored until assayed for progesterins. Progesterone and its metabolite 20 α -hydroxyprogesterone were quantified by radioimmunoassay using specific antiserum as described previously (32). The results are expressed as nanograms of progesterone, 20 α -dihydroxyprogesterone, and the sum of progesterone and 20 α -dihydroxyprogesterone formed per microgram of cell DNA. Results presented are means \pm SEM of duplicate determinations from three different dishes.

Preparation of lipoproteins

ApoE-free hHDL₃ and rat VLDL were isolated as described previously (32, 40). Chylomicrons and chylomicron remnants were prepared by a previously standardized method (41, 42). Chylomicron remnants were iodinated using iodine monochloride (41). All chylomicron remnants were screened for biological activity according to the procedure described by Choi and Cooper (41). In addition, the degree of lipid iodination was monitored on batches as described previously and fell within the range reported previously (41). Selected batches were analyzed by PAGE in a system containing 0.5% SDS with or without autoradiography, and apoprotein content and iodination patterns were similar to previously reported values (41, 42).

Preparation of active α_2 -macroglobulin

α_2 -Macroglobulin was activated by methylamine or by trypsin according to the procedure described by Imber and Pizzo (43). The products obtained by the two methods behaved identically. The α_2 -macroglobulin preparations were iodinated using the IODO-GEN (Pierce Chemical Co.) method as described previously (41).

Uptake and degradation of chylomicron remnants and α_2 -macroglobulin by cultured granulosa cells

Granulosa cells cultured for 72 h were treated with or without Bt₂cAMP and or lipoproteins as described above. Subsequently, dishes were washed with the medium to remove nonadherent cells and then incubated in a binding medium (0.5% BSA and 10 mM HEPES, pH 7.4) containing ¹²⁵I-labeled α_2 -macroglobulin (1 $\mu\text{g}/\text{ml}$) or chylomicron remnants (2 $\mu\text{g}/\text{ml}$) in the presence or absence of unlabeled α_2 -macroglobulin, GST-RAP, or the anti-LDL receptor antibody at 37°C for 4 h. Measuring the amount of trichloroacetic acid and silver nitrate-soluble radioactivity in the incubation medium assessed the extent of protein degradation. The small amount of degradation products generated in the absence of cells was also measured and subtracted from the corresponding samples incubated with the cells. The amount of α_2 -macroglobulin and remnants associated with cells was determined by dissolving the cells with 0.1 N NaOH after washing three times with PBS.

Western blot (immunoblot) analysis of LRP and RAP expression

The expression of LRP and RAP was monitored by immunoblotting of cellular lysates (41). Granulosa cells were cultured in

basal culture medium for 72 h and then treated with or without Bt₂cAMP (2.5 mM) for 24 h. Subsequently, cells were treated for an additional 24 h with or without the medium containing lipoprotein (100 $\mu\text{g}/\text{ml}$ hLDL or 500 $\mu\text{g}/\text{ml}$ hHDL₃) with or without Bt₂cAMP 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 15 mM 2-mercaptoethanol), and placed in a 40°C water bath, and the lysate was sonicated briefly to disrupt chromatin (DNA).

The lysate samples containing equal amounts of protein (40–60 mg of protein) were mixed with 2 \times sample buffer (41), boiled for 90 s, and immediately loaded onto a gel. Samples and molecular weight markers were separated by SDS-PAGE (4% running gel for LRP or 7% running gel for RAP) as described previously (41). 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, membranes were incubated at room temperature for 2 h with anti-LRP or anti-RAP in a 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 in a 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 isolation

Total RNA was isolated from granulosa cells using TRIzol[®] (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. The purity and concentration of RNA samples were determined by following absorbance (A_{260}/A_{280}) ratios. The integrity of the purified total RNA samples to be used in the RNase protection assay (RPA) and real-time PCR assay was confirmed by 1.2% formaldehyde-agarose gel electrophoresis.

mRNA quantitation by RPA

LRP, RAP, HMG-CoA reductase, and LDL (B/E) receptor were determined using a sensitive RPA as described previously (5, 6). The antisense [³²P]complementary ribonucleic acid probes were synthesized using [α -³²P]CTP, restriction endonuclease-linearized plasmid (*Eco*RI for LRP and RAP, *Xho*I for LDL receptor, *Hind*III for HMG-CoA reductase, and *Bam*HI for 18S rRNA), and the appropriate T3 or T7 polymerase according to the method described in Stratagene's in vitro transcription kit. Because of their high lability, the riboprobes were always freshly prepared before hybridization. Aliquots of total granulosa cell RNA (10 μg) or control tRNA (10 μg) were hybridized with $\sim 1 \times 10^5$ cpm of specific probe for 18 h at 42°C. The unprotected probe was digested with RNase A (40 $\mu\text{g}/\text{ml}$) and RNase T₁ (2 $\mu\text{g}/\text{ml}$) for 1 h at 30°C, followed by the addition of proteinase K (50 μg) and SDS for 15 min at 37°C (5, 6). After phenol-chloroform extraction and ethanol precipitation, the protected RNA-RNA hybrids were resolved on 6% acrylamide-urea denaturing gels. After electrophoresis, gels were exposed to Kodak XAR-5 film at -70°C with intensifying screens. For strong signals, gels were usually exposed for 6–12 h; for weaker signals, they were usually exposed for up to 48 h.

For quantification, the films were analyzed by densitometry. The data are expressed as a ratio of the intensity of the LRP, RAP, LDL receptor, or HMG-CoA reductase signal to that of 18S rRNA to correct for differences in RNA loading. In these studies, the steady-state levels of 18S rRNA remained constant in response to Bt₂cAMP and/or lipoprotein treatment. More widely used internal controls, such as GAPDH and β -actin, were not used in

these studies because the steady-state levels of these mRNAs are known to be influenced by hormones and growth factors (5, 6).

Measurement of mRNA levels by TaqMan quantitative real-time PCR

The rat-specific primer sets used to detect the mRNA expression of LRP-8, LRP-1, SR-BI, LDL receptor, and 28S rRNA were developed using Primer Express software (Applied Biosystems) according to the recommended guidelines based on sequences accessed through GenBank. **Table 1** shows the primer sequences for each quantitative real-time PCR assay used. cDNAs were synthesized from 1 µg of total RNA using random hexamer primers and Superscript II (Invitrogen Life Technologies) as described previously (44). Amplification of cDNAs was performed with an ABI Prism 7900 system according to the manufacturer's instructions. Each sample consisted of 1 µl of cDNA, 4 mM MgCl₂, 200 mM deoxynucleoside triphosphate, 500 nM of each sense and antisense primer, 2 µl of 10× PCR buffer, TaqMan polymerase, and SYBR® Green in a final volume of 20 µl. As an internal quantitative control for gene expression, 28S rRNA gene expression was also determined. The LRP-8, LRP-1, SR-BI, LDL receptor, and 28S rRNA gene expression of all cDNA samples was determined by fluorescence from SYBR Green. The final data were normalized to 28S rRNA, and the ratios of LRP-8, LRP-1, SR-BI, or LDL receptor to 28S rRNA represented the normalized relative levels of LRP-8, LRP-1, SR-BI, and LDL receptor expression (44). Each sample was measured in triplicate plus a control without reverse transcriptase.

Miscellaneous procedures

The DNA content of the cells was quantified fluorometrically (45). The procedure of Markwell et al. (46) was used to quantify the protein content of lipoproteins. Protein levels in cellular lysates were determined as described by Peterson (47). Cholesterol content of the lipoproteins was determined colorimetrically according to the procedure of Tercyak (48).

Statistical analysis

Statistical analysis was performed by an unpaired *t*-test using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, CA). Differences between groups were considered significant at *P* < 0.05. Values are expressed as means ± SEM.

TABLE 1. Oligonucleotide sequences of primers used for real-time reverse transcription-PCR

Gene	GenBank™ Accession Number	Orientation	Sequence (5'–3')
Rat LRP-1	BC088327	Forward	CGAGCTCTGTGACCAGTG
		Reverse	AGAGCACACAATGCCTTCAC
Rat LRP-8	XM_342877	Forward	AGCACTATGGGAATGAAGGC
		Reverse	GATGATCCCAATGACAGCAG
Rat SR-BI	BC076504	Forward	CCTTCAATGACAACGACACC
		Reverse	CAGGACCAAGATGTTAGGCA
Rat LDL receptor	NM_175762	Forward	CTCTGTTCCGAGAGAAAGGG
28S rRNA		Reverse	TCTTGATCTTGGCAGGTGTC
		Forward	GAATCCGCTAAGGAGTGTGTAAACA
		Reverse	CTCCAGCGCCATCCATTT

LRP, low density lipoprotein receptor-related protein; SR-BI, scavenger receptor class B type I.

RESULTS

Effect of treatment of ovarian granulosa cells with Bt₂cAMP alone or in combination with hLDL on lipoprotein receptors involved in supplying cholesterol to cells

Ovarian granulosa cells, like other steroid-producing cells, are capable of obtaining cholesterol for steroidogenesis from multiple sources, including de novo synthesis, stored lipid droplets, and cholesterol-rich plasma lipoproteins. When challenged to produce more sterol hormone in response to trophic hormone (Follicle-stimulating hormone) or its second messenger (cAMP), the expression levels of SR-BI (35) (which mediates selective cholesterol uptake) (1–4), the LDL receptor (which can increase cholesterol uptake from apoB- and apoE-containing lipoproteins), and HMG-CoA reductase (which determines the rate of de novo cholesterol synthesis) (49, 50) are increased (5, 6). Another member of the LDL receptor superfamily, LRP (7–9), and LRP8/apoER2 (51) also could contribute to the removal of apoE-rich lipoproteins. To learn whether these pathways are coordinately regulated, granulosa cells from hormone-primed immature rats were isolated and cultured in a basal medium followed by culture for 24 h in medium containing Bt₂cAMP and appropriate concentrations of human LDL. The rationale was that the LDL would supply adequate cholesterol to down-regulate those genes that had been stimulated by cholesterol deprivation through the sterol-regulatory element binding protein-mediated pathway (50) but might not affect genes that were not responsive to sterol per se. The cells were harvested, and the levels of mRNA for the LDL receptor, HMG-CoA reductase, RAP, and LRP were determined using ³²P-labeled riboprobes. The abundance of specific mRNA species was determined relative to 28S or 18S rRNA.

As expected, the addition of LDL to the incubation medium reduced the levels of LDL receptor mRNA relative to cells treated with Bt₂cAMP alone (**Fig. 1C**). Likewise, the level of mRNA encoding the rate-limiting enzyme of cholesterol biosynthesis, HMG-CoA reductase, was reduced (**Fig. 1D**), consistent with the fact that the rate of transcription of both of these genes is determined largely by the amount of sterol-regulatory element binding protein reaching the nucleus, which may in turn be controlled by the sterol content of the endoplasmic reticulum (50). In contrast, the levels of mRNA for the LRP were increased >3-fold by the combined treatment (**Fig. 1A**). The level of mRNA encoding the RAP, a chaperone for the LRP, was not changed (**Fig. 1B**). Neither Bt₂cAMP nor hLDL alone induced a change in the level of LRP; only when both were in the medium together was this change induced (**Table 2**). Although these data are consistent with most other instances in which the expression of LRP was not regulated in concert with that of the LDL receptor, this is one of the few instances in which the level of LRP expression was increased so substantially.

To determine the broader effect of hLDL and Bt₂cAMP, mRNA levels of two additional genes involved in lipopro-

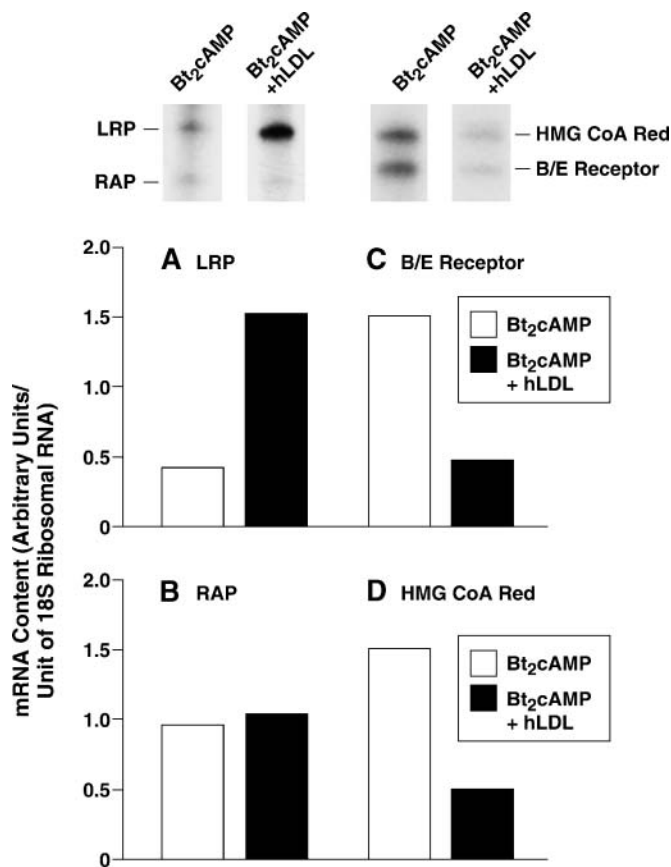


Fig. 1. Effects of *N*⁶,*O*^{2'}-dibutyryl adenosine 3',5'-cyclic monophosphate (Bt₂cAMP) or Bt₂cAMP plus hLDL on low density lipoprotein receptor-related protein (LRP) (A), receptor-associated protein (RAP) (B), LDL (B/E) receptor (C), and HMG-CoA reductase (D) mRNA levels in granulosa cells. Granulosa cells were pretreated with Bt₂cAMP (2.5 mM) for 24 h. Cells were then incubated with Bt₂cAMP (2.5 mM) or with Bt₂cAMP plus hLDL (100 μg/ml) for an additional 24 h. Subsequently, cells were harvested, total RNA was extracted from each sample, and RNase protection assays (RPAs) were performed with 10 μg of total RNA per assay using [α -³²P]CTP-labeled LRP, RAP, LDL (B/E) receptor, and HMG-CoA reductase riboprobes. Details are described in Experimental Procedures. The extent of variation among the data points was between 8.1% and 12.2%.

tein-cholesterol uptake (LRP8/apoER2) were measured by TaqMan quantitative real-time PCR using granulosa cells after 24 h of treatment with vehicle alone (basal), hLDL (100 μg/ml), or hHDL₃ (500 μg/ml) with or without Bt₂cAMP (2.5 mM). As reported previously (35), SR-BI mRNA in the granulosa cells was increased significantly, whereas LRP8 mRNA expression was unaffected. The simultaneous presence of LDL and Bt₂cAMP further increased SR-BI mRNA levels, but mRNA levels of LRP8/apoER2 remained unchanged (Fig. 2). Addition of hLDL or hHDL₃ alone had no detectable effect on either the SR-BI or LRP8/apoER2 mRNA level. In contrast, SR-BI mRNA expression was decreased significantly by hHDL₃ and Bt₂cAMP compared with Bt₂cAMP alone. Overall, these results provide evidence that the combined presence of hLDL and Bt₂cAMP specifically stimulates LRP mRNA

TABLE 2. Quantification of LRP and RAP mRNA levels in cultured granulosa cells pretreated with or without hLDL, Bt₂cAMP, or Bt₂cAMP plus hLDL

Additions	LRP	RAP
	<i>arbitrary units/U 18S rRNA</i>	
None	0.92 ± 0.21	1.13 ± 0.17
hLDL (100 μg/ml)	0.96 ± 0.09	1.33 ± 0.41
Bt ₂ cAMP (2.5 mM)	1.12 ± 0.26	0.81 ± 0.15
hLDL + Bt ₂ cAMP	3.18 ± 0.49 ^a	0.92 ± 0.11

Bt₂cAMP, *N*⁶,*O*^{2'}-dibutyryl adenosine 3',5'-cyclic monophosphate; hLDL, human LDL; RAP, receptor-associated protein. Results are means ± SEM of three separate experiments. Cultured granulosa cells were pretreated with or without the indicated concentrations of hLDL and/or Bt₂cAMP for 24 h. Total cellular RNA preparations were used for the quantification of LRP or RAP mRNA levels using a sensitive RNase protection assay (RPA).

^a*P* < 0.005.

levels, whereas SR-BI expression appears to be primarily stimulated by Bt₂cAMP alone.

Effect of HDL and Bt₂cAMP on LRP mRNA levels

To determine whether the effect of combined hLDL and cAMP was specific to cholesterol delivered through the LDL receptor pathway, the effect of apoE-free hHDL₃ alone and with Bt₂cAMP on LRP mRNA levels was assessed. ApoE-free hHDL₃ delivers cholesterol to cells by a process that does not involve endocytosis of the intact particle and is now referred to as the SR-BI-mediated selective uptake pathway (1–4). Although hHDL₃ was less effective compared with LDL, there was still a substantial increase in LRP mRNA levels when both hHDL₃ and Bt₂cAMP were present in the incubation medium (Table 3), whereas there was no effect with either agent alone. This suggests that it is the combination of increased intracellular cholesterol and stimulation by the second messenger cAMP that results in the increase of the LRP mRNA level.

Time course of the increase in LRP mRNA in response to incubation with both LDL and Bt₂cAMP and dependence on transcription

When cells were incubated with LDL and Bt₂cAMP, the increase in LRP mRNA was apparent within 8 h and persisted for 48 h, with a peak stimulation of >5-fold at 24 h (Fig. 3). As in previous experiments, there was no change in the levels of RAP mRNA. To learn whether the effect on LRP was at the level of mRNA stability, cells were incubated with Bt₂cAMP in the presence or absence of LDL plus the transcription inhibitor actinomycin D or DRB (52, 53). The rate of decay of LRP mRNA levels was the same with either inhibitor in the presence or absence of hLDL (half-life ~ 10.1 h; Fig. 4A, B), suggesting that the induction of the LRP mRNA was attributable to increased transcription and not to decreased rates of mRNA degradation.

Effect of Bt₂cAMP and lipoproteins on the levels of LRP and RAP in the membranes of ovarian granulosa cells

In subsequent experiments, granulosa cells were incubated in basal medium or medium containing Bt₂cAMP alone, hHDL₃ alone, hLDL alone, or Bt₂cAMP plus either

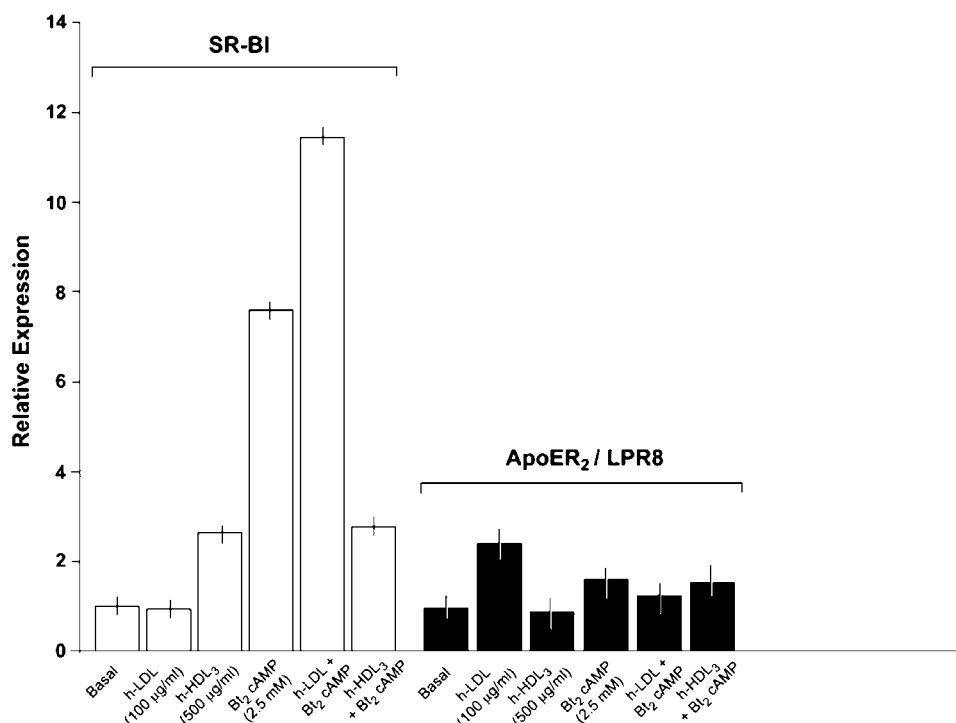


Fig. 2. Scavenger receptor class B type I (SR-BI) and LRP8/apoER2 expression in granulosa cells. SR-BI (left) and LRP8/apoER2 (right) expression was measured by TaqMan quantitative real-time PCR analysis after treatment of cells for 24 h with vehicle (basal), hLDL (100 µg/ml), or hHDL₃ (500 µg/ml) with or without Bt₂cAMP (2.5 mM). Data are presented as means ± SEM from three independent experiments.

hHDL₃ or hLDL. After 24 h, the cells were harvested and lysates were prepared from the cells. Identical amounts of protein were applied to lanes of SDS-polyacrylamide gels, and after electrophoresis, the proteins were transferred to a nylon membrane that was incubated with rabbit anti-rat LRP antibody or rabbit anti-rat RAP antibody. After reaction with goat anti-rabbit IgG coupled to horseradish peroxidase and substrate, chemiluminescence was detected for various times, and the films were subjected to densitometric scanning. None of the lipoprotein additions alone increased the level of LRP relative to that found in

cells grown in a control medium (**Table 4**). However, in good agreement with the effect on LRP mRNA levels, HDL in the presence of Bt₂cAMP doubled and hLDL in the presence of Bt₂cAMP increased by >6-fold the total amount of LRP expressed (**Fig. 5**, **Table 4**). None of the lipoprotein additions in the incubation medium affected the level of RAP (**Fig. 5**, **Table 4**).

Cell association and degradation of α₂-macroglobulin by ovarian granulosa cells expressing either high or low levels of LRP

To establish that the expressed LRP was functional, the association and degradation of ¹²⁵I-α₂-macroglobulin by granulosa cells cultured with Bt₂cAMP alone or with Bt₂cAMP plus hLDL were studied. In the absence of LDL (the condition in which LRP expression was at its minimum), the cell association (**Fig. 6A**) and degradation (**Fig. 6B**) of ¹²⁵I-α₂-macroglobulin were barely detectable. However, after addition of both Bt₂cAMP and LDL (the condition in which LRP was increased), there was a dramatic increase in both the cell association (**Fig. 6A**) and degradation (**Fig. 6B**) of α₂-macroglobulin. These data demonstrate that the expressed LRP is functional when its levels are induced.

Cell association and degradation of chylomicron remnants by ovarian granulosa cells expressing either low or high levels of LRP

Although a role for the LRP in the removal of chylomicron remnants by the liver has now been clearly estab-

TABLE 3. Quantification of LRP mRNA levels in cultured granulosa cells pretreated with or without hLDL, hHDL₃, Bt₂cAMP, Bt₂cAMP plus hHDL₃, or Bt₂cAMP plus hLDL

Additions	LRP mRNA Content <i>arbitrary units/U 18S rRNA</i>
None	0.81 ± 0.13
hHDL ₃ (500 µg/ml)	0.87 ± 0.16
hLDL (100 µg/ml)	0.80 ± 0.09
Bt ₂ cAMP (2.5 mM)	0.89 ± 0.18
hHDL ₃ + Bt ₂ cAMP	1.96 ± 0.42 ^a
hLDL + Bt ₂ cAMP	3.90 ± 0.48 ^b

Results are means ± SEM of three separate experiments. Cultured granulosa cells were pretreated with or without Bt₂cAMP for 24 h. Subsequently, cells were incubated with the indicated concentrations of hHDL₃ or hLDL with or without Bt₂cAMP for an additional 24 h. Total cellular RNA preparations were used for the quantification of LRP mRNA and 18S rRNA levels using a sensitive RPA.

^a *P* < 0.04 versus Bt₂cAMP alone.

^b *P* < 0.002 versus Bt₂cAMP alone.

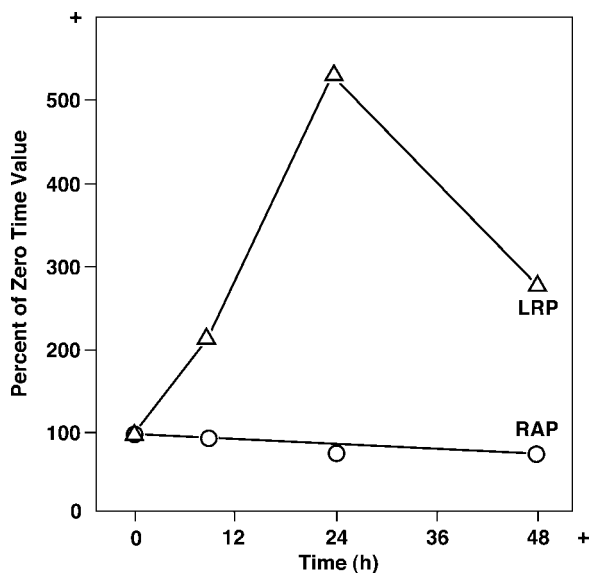


Fig. 3. Time course of LRP and RAP expression in granulosa cells stimulated with Bt_2cAMP plus hLDL. Granulosa cells were treated with Bt_2cAMP (2.5 mM) for 24 h. After treatment, dishes were washed and further incubated with Bt_2cAMP (2.5 mM) with or without hLDL (100 $\mu g/ml$) for the times indicated. Total RNA was extracted, and LRP and RAP mRNA levels were quantified by RPA as described in Experimental Procedures. The data are expressed as percentages of the respective control (Bt_2cAMP -treated alone) values measured at each time point and are means \pm SEM of three independent components. The extent of variation among the data points was between 7.5% and 13.0%.

lished (54), the precise mechanism by which this occurs has not been elucidated. The importance of ancillary molecules such as additional apoE (27, 28), lipoprotein lipase, hepatic lipase, and proteoglycans has been postulated (29–31, 55). Indeed, it has been difficult to demonstrate the removal of plasma lipoproteins in isolated or cultured cells by LRP in the absence of additions to the medium and even with the addition of apoE (56). Our own unpublished observations suggest that the amount of uptake and degradation of remnants attributable to the LRP is modest compared with that mediated by the LDL receptor (J. Chen and A. D. Cooper, unpublished data). Thus, the ability to vary the functional level of LRP on the surface of granulosa cells afforded an opportunity to evaluate this issue further. In the presence of Bt_2cAMP alone, there was significant specific cell association of ^{125}I -chylomicron remnants with the ovarian granulosa cells (Fig. 7). When a monospecific anti-LDL receptor antibody was added to the incubation mixture, the cell association was reduced by $\sim 25\%$ (Fig. 7). RAP at a concentration that inhibits binding to all members of the LDL receptor family also inhibited cell association by 25% (Fig. 7), suggesting that the LDL receptor was the primary molecule in this family responsible for the observed cell association. Consistent with this, addition of both anti-LDL receptor antibody and RAP together did not result in a greater degree of inhibition of cell association of the lipoprotein (Fig. 7). These results suggest that with cAMP alone, the majority of the cell association between chylomicron remnants and

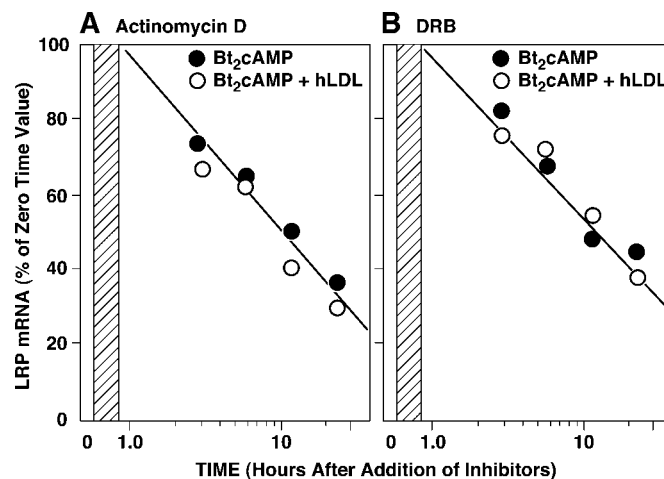


Fig. 4. Effects of Bt_2cAMP with or without hLDL on mRNA stability. Bt_2cAMP -presensitized cells were exposed to Bt_2cAMP (2.5 mM; closed circles) or Bt_2cAMP plus hLDL (100 $\mu g/ml$; open circles) for 24 h before the addition of actinomycin D (10 $\mu g/ml$; A) or 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB) (100 $\mu g/ml$; B) to inhibit gene transcription. The 24 h time point represents the time of maximal increase in LRP transcript levels in granulosa cells, as shown in Fig. 3. Total RNA was isolated from the cells at the indicated times after the addition of transcription inhibitors and processed for LRP mRNA determination by RPA. The data shown, expressed as percentages of the initial LRP content before inhibitor addition, are from a representative experiment, and the mean mRNA half-lives determined from several such experiments are given in the text. The extent of variation among the data points was between 9.1% and 13.7%.

this cell type is independent of members of the LDL receptor family and any cell association attributable to the LDL receptor.

When granulosa cells were cultured in the presence of both Bt_2cAMP and hLDL, the cell-associated ^{125}I -chylomicron remnant decreased slightly (Fig. 7). In this case, the presence of the LDL receptor antibody did not reduce the amount of cell-associated ^{125}I -chylomicron remnants in these cells (Fig. 7). Also, the addition of RAP to the bind-

TABLE 4. Expression of LRP and RAP in response to Bt_2cAMP and/or lipoproteins in cultured rat granulosa cells

Treatment	Arbitrary Units per 50 μg of Protein	Percentage
LRP		
Basal	22.6 \pm 2.9	100
Bt_2cAMP (2.5 mM)	23.3 \pm 3.6	102
hHDL ₃ (500 $\mu g/ml$)	22.5 \pm 100	100
hLDL (100 $\mu g/ml$)	21.9 \pm 2.2	97
Bt_2cAMP + hHDL ₃	48.8 \pm 6.3 ^a	216
Bt_2cAMP + hLDL	136.6 \pm 21.2 ^b	609
RAP		
Basal	46.8 \pm 5.9	100
Bt_2cAMP (2.5 mM)	45.9 \pm 6.3	98
hHDL ₃ (500 $\mu g/ml$)	46.1 \pm 6.6	99
hLDL (100 $\mu g/ml$)	47.3 \pm 4.2	101
Bt_2cAMP + hHDL ₃	47.4 \pm 5.1	101
Bt_2cAMP + hLDL	48.3 \pm 7.1	103

Results are means \pm SEM of three separate experiments.

^a $P < 0.01$.

^b $P < 0.005$.

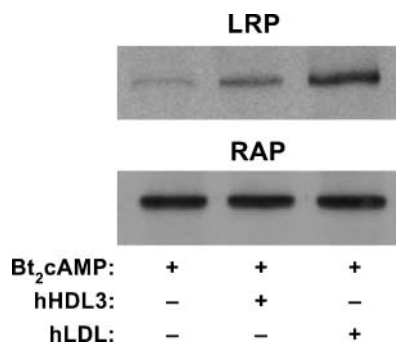


Fig. 5. Immunoblot analysis of LRP and RAP. Crude membranes were prepared from Bt₂cAMP-sensitized granulosa cells and further incubated with Bt₂cAMP (2.5 mM), Bt₂cAMP plus hHDL₃ (500 μg/ml), or Bt₂cAMP plus hLDL (100 μg/ml) as indicated. Samples of the cell lysate (40–50 μg) were applied to a 4% (LRP) or 7% (RAP) polyacrylamide gel containing 0.1% sodium dodecyl sulfate under reducing conditions. Proteins were transferred from the gel to Immobilon membranes, and the blots were incubated first with rabbit anti-LRP or anti-RAP IgG and then developed with a chemiluminescent detection system (ECL; Amersham Corp.). The blot shown is from a representative experiment, and the mean LRP and RAP levels were determined from several such experiments and are given in Table 4.

ing medium of cells that had been cultured under these conditions did not result in any decrease in cell-associated radioactivity, nor did the addition of both RAP and the anti-LDL receptor antibody (Fig. 7). Thus, even when LRP

expression was stimulated, there appeared to be little direct binding to the LRP.

A different pattern was seen when the degradation of the ¹²⁵I-chylomicron remnants was determined. In ovarian granulosa cells that had been cultured in the presence of Bt₂cAMP alone, ~38% of the degradation was inhibited by the use of the anti-LDL receptor antibody and 85% was inhibited by RAP either alone or in combination with the antibody (Fig. 8). This suggests that members of the LDL receptor family mediated most of the specific degradation of chylomicron remnants and that approximately half of this was attributable to the LDL receptor itself. When cells were grown in the presence of both Bt₂cAMP and hLDL, degradation was similar to that of cells grown in the presence of Bt₂cAMP alone (Fig. 8). Thus, the LDL receptor had been replaced by another member that could mediate internalization. Degradation by cells treated with the combination of Bt₂cAMP plus LDL was inhibited almost completely by a high concentration of RAP alone or RAP with the combined anti-LDL receptor antibody (Fig. 8). However, the addition of anti-LDL receptor antibody alone caused significantly less inhibition of chylomicron remnant degradation (~15%; Fig. 8). This is consistent with the reduced level of LDL receptor seen in cells cultured under these conditions and with the replacement of the LDL receptor by another member, presumably LRP, under these conditions. In absolute terms, the amount of degradation attributable to the LRP was almost doubled

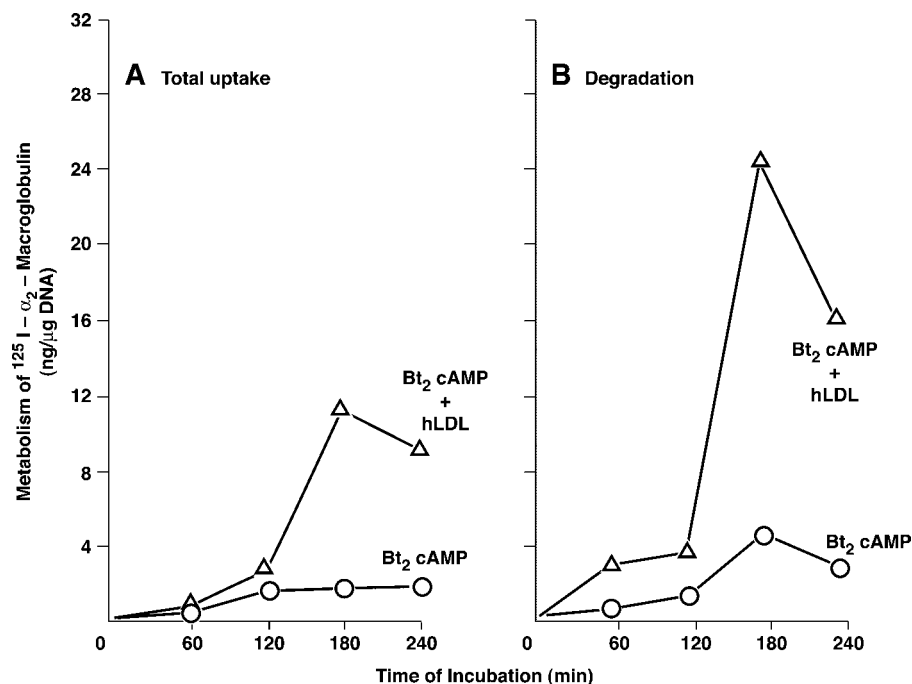


Fig. 6. Stimulation of ¹²⁵I-α₂-macroglobulin uptake and degradation in granulosa cells by Bt₂cAMP plus hLDL. Bt₂cAMP-sensitized granulosa cells were treated with Bt₂cAMP (2.5 mM) plus hLDL (100 μg/ml; open triangles) or maintained in medium containing Bt₂cAMP (2.5 mM; open circles) for 24 h as described in Experimental Procedures. They were then incubated with ¹²⁵I-α₂-macroglobulin (1 μg/ml) for 60–240 min at 37°C. A: After washing as described, the amount of ¹²⁵I-α₂-macroglobulin associated with the cells (total uptake) was determined by dissolving the cells with 0.1 N NaOH. B: Degradation was measured as the radioactivity in the incubation medium that was soluble in 15% trichloroacetic acid. Results are of duplicate determinations. The extent of variation among the data points was between 7.8% and 14.0%.

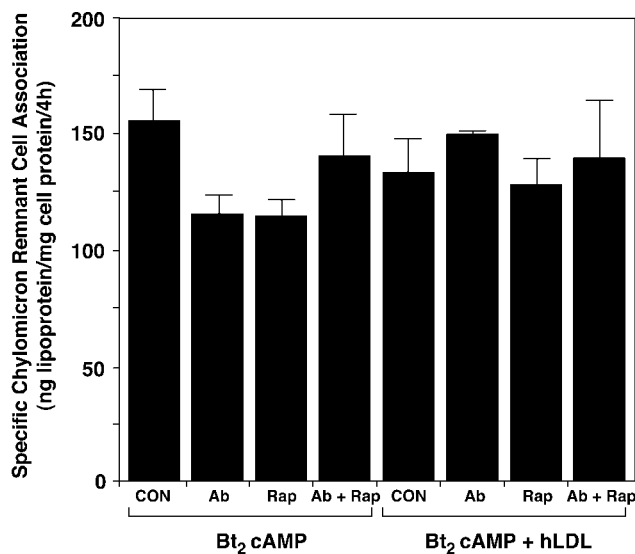


Fig. 7. Binding of ¹²⁵I-chylomicron remnants to granulosa cells. Bt₂cAMP-sensitized granulosa cells were treated with Bt₂cAMP alone or Bt₂cAMP plus hLDL for 24 h as described for Fig. 5. Subsequently, cells were incubated for 4 h at 37°C with ¹²⁵I-chylomicron remnants (10 μg/ml) with or without anti-LDL (B/E) receptor (IgG, 100 μg/ml), *rec*RAP (25 μg/ml), or anti-LDL (B/E) receptor plus *rec*RAP. After washing, the amount of cell-associated radioactivity was determined as described in Experimental Procedures. Nonspecific binding was determined using a 20-fold excess of unlabeled ligand. Results are means ± SEM of triplicate determinations. Ab, antibody; CON, control.

(12 vs. 19 ng lipoprotein/mg cell protein/24 h). This difference between antibody- and RAP-inhibited binding in Bt₂cAMP- compared with Bt₂cAMP plus LDL-treated cells was significant ($P < 0.01$).

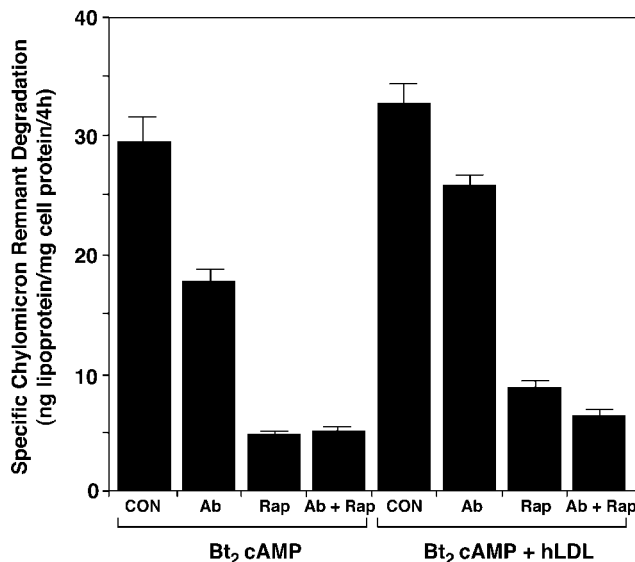


Fig. 8. Degradation of ¹²⁵I-chylomicron remnants by granulosa cells. Experimental conditions were as described for Fig. 6. Degradation was measured as the radioactivity in the incubation medium that was soluble in 15% trichloroacetic acid and silver nitrate. Results are means ± SEM of triplicate determinations. Ab, antibody; CON, control.

Chylomicron remnant- and VLDL-supported progestin production

We also directly measured chylomicron remnant- and VLDL-supported progestin secretion in the presence and absence of Bt₂cAMP (the second messenger of the trophic hormone FSH) to further demonstrate a role of LRP in cholesterol delivery and the regulation of granulosa cell steroidogenesis. As a positive control, we also used hHDL₃ (the most potent lipoprotein ligand for SR-BI-mediated selective cholesterol delivery and progestin production). Granulosa cells were pretreated with Bt₂cAMP and hLDL for 24 h, and the medium was changed and then incubated with chylomicron remnants, VLDL, or hHDL₃ with or without Bt₂cAMP for an additional 24 h. Subsequently, the incubation medium were analyzed for progestin production by specific radioimmunoassays. As shown previously (32), the granulosa cells secrete two types of steroids (progesterone and 20α-dihydroprogesterone) in response to Bt₂cAMP, and the response is amplified in the presence of lipoproteins (Tables 5, 6). Although all three lipoproteins evoked a strong response, that achieved with hHDL₃ was ~2-fold greater compared with that achieved with chylomicron remnants or VLDL at the indicated concentrations (Tables 5, 6). Given that the expression of LRP and to some extent SR-BI is upregulated by Bt₂cAMP and hLDL, we next determined the contribution of LRP in lipoprotein-supported progestin production using SR-BI blocking antibodies. Consistent with a previous study (57), inclusion of SR-BI blocking antibody blocked hHDL₃-supported progestin production by ~60–70% both under basal conditions and in response to Bt₂cAMP stimulation (Table 6). In contrast, coincubation of cells with the SR-BI antibody showed no significant effect on VLDL-supported steroid production, further suggesting that LRP and not SR-BI facilitates VLDL-cholesterol delivery in granulosa cells.

DISCUSSION

These studies continue a line of research directed at elucidating the pathways by which steroid hormone-producing tissues obtain cholesterol, the required precursor for steroid hormone synthesis. Because of the importance of ensuring a constant supply of various steroid hormones under widely varying conditions, it is not surprising that several complementary systems have evolved to carry out this function (1, 2). Although mobilization of stored CEs is probably the most rapid way to support the “acute” stimulation of steroidogenesis (58), the amount of cholesterol precursor supplied by this route is necessarily limited. De novo synthesis under certain conditions may be able to produce the significant amounts to partially meet the demand for steroid production (1, 2, 58), but it does so at a considerable energy cost. Accordingly, the use of lipoprotein-derived cholesterol appears to be quantitatively the most important source to support steroidogenesis (1–4). In rodents, the principal source of this cholesterol is a high density lipoprotein, and it is now well established that this involves the functional participation of the HDL receptor SR-BI,

TABLE 5. Chylomicron remnant-, hHDL₃-, and Bt₂cAMP-stimulated progesterone production by cultured granulosa cells pretreated with Bt₂cAMP plus hLDL

Addition	Progesterone (a)	20 α -Dihydroxyprogesterone (b)	Total Progesterone (a + b)
Basal	0.26 \pm 0.03	0.56 \pm 0.08	0.82 \pm 0.06
Chylomicron remnants (80 μ g/ml)	0.49 \pm 0.04	1.92 \pm 0.34	2.41 \pm 0.41
hHDL ₃ (500 μ g/ml)	1.39 \pm 0.28	5.41 \pm 0.811	6.80 \pm 1.02
Bt ₂ cAMP (2.5 mM)	30.9 \pm 5.4	56.2 \pm 5.1	96.0 \pm 5.9
Chylomicron remnants + Bt ₂ cAMP	308 \pm 52	331 \pm 65	639 \pm 94
hHDL ₃ + Bt ₂ cAMP	962 \pm 52	909 \pm 117	1871 \pm 67

Results are means \pm SEM of three separate experiments. Values are expressed as ng/ μ g DNA/24 h.

which facilitates the selective uptake of CEs from HDL without internalization of the entire particle (1–4). It is also known that endocytosis of lipoproteins can mediate cholesterol delivery into steroidogenic cells (1, 2, 58).

This study was directed at exploring the role of another member of the LDL receptor family, the LRP. In this process, we used primary rat granulosa cells cultured in a serum-free medium as a classical model of lipoprotein-supported steroidogenesis. The surprising finding of this study was that simultaneous addition of both LDL and Bt₂cAMP caused a several-fold increase in the level of LRP mRNA and protein expression. Consistent with this, a change in cholesterol influx into the ovary alone did not affect the level of LRP mRNA or protein. Similarly, treatment of the cells with a potent hormone second messenger analog, Bt₂cAMP, failed to alter the LRP level. This was accompanied by a decrease in the level of LDL receptors, an increase in the uptake of α_2 -macroglobulin, a ligand specific for the LRP, and an increase in the amount of chylomicron remnants degraded by a RAP-sensitive, LDL receptor-independent mechanism, presumably the LRP; LRP can then mediate the endocytosis of apoE-rich particles and support steroidogenesis. We also explored the expression of two other lipoprotein receptors, LRP8/apoER2 and SR-BI. Although the expression of LRP8/apoER2 has been reported in cattle ovarian follicles (59, 60) and confirmed here, we failed to find any alteration

in its expression in response to cotreatment of granulosa cells with Bt₂cAMP and hLDL. On the contrary, we did observe a significant increase in the functional expression of SR-BI. However, this upregulation of SR-BI stimulation appears to be different from that of LRP in two major ways. First, SR-BI expression was induced more robustly by Bt₂cAMP alone, and incubation with LDL resulted in only a modest increase in SR-BI expression (Fig. 2). Second, the use of a blocking SR-BI antibody has clearly indicated that SR-BI does not mediate the delivery of cholesterol from the LRP-specific lipoprotein ligands. These observations thus indicate that SR-BI and LRP are not functionally linked and suggest that the LRP pathway independently facilitates the delivery of lipoprotein-derived cholesterol in the granulosa cell model system.

Relatively little is known about the molecular mechanism involved in the regulation of the LRP. This receptor is expressed early in embryonic development and is required for fetal maturation (7, 11–13, 61). It has been found to be present in every tissue in which it has been sought (7, 11–13, 61). A number of studies now indicate that certain hormones, growth factors, and cytokines as well as altered physiological conditions can modulate LRP expression (7, 14–25). However, to date, instances of its regulation by lipids have not been reported, and in this study, cholesterol loading alone did not affect the level of its expression. Similarly, increasing the requirement of the ovarian cells for cholesterol did not affect its level of expression. These results are consistent with previous studies showing that transcription of LRP, unlike the LDL receptor and other members of this receptor family, was not downregulated by sterols (62). Although another study identified a sequence corresponding to a sterol response element in the 5'-untranslated region of the LRP transcript, it appears to be functionally inactive, given that LRP does not show any sensitivity toward sterols (63). Gauthier et al. (22) also reported no change in LRP mRNA levels when the human liposarcoma cell line SW872 was cultured in a standard fetal calf serum-containing medium versus cells that were cultured in a medium supplemented with lipoprotein-deficient fetal calf serum (22). Together, these findings suggest that the gene encoding LRP is not sensitive to sterol regulation.

Instances of hormone responsiveness have been reported (17, 18), suggesting that the gene may contain hormone response elements (22). How the loss of LDL receptors works in concert with the hormonal signal triggered by

TABLE 6. Effect of anti-SR-BI on hHDL₃- and rat VLDL-supported progesterone production by cultured rat granulosa cells pretreated with Bt₂cAMP plus hLDL

Additions	Progestins
None (basal)	0.50 \pm 0.12
Rat VLDL (80 μ g/ml)	2.47 \pm 0.47
Rat VLDL (80 μ g/ml) + anti-SR-BI (50 μ g IgG/ml)	2.18 \pm 0.35
hHDL ₃ (500 μ g/ml)	4.56 \pm 0.54
hHDL ₃ (500 μ g/ml) + anti-SR-BI (50 μ g IgG/ml)	1.90 \pm 0.49 ^a
Bt ₂ cAMP (2.5 mM)	48.7 \pm 11.89
Bt ₂ cAMP (2.5 mM) + rat VLDL (80 μ g/ml)	549.3 \pm 75.6
Bt ₂ cAMP (2.5 mM) + rat VLDL (80 μ g/ml) + anti-SR-BI (50 μ g IgG/ml)	328.4 \pm 81.0 ^b
Bt ₂ cAMP (2.5 mM) + hHDL ₃ (500 μ g/ml)	1,205 \pm 106
Bt ₂ cAMP (2.5 mM) + hHDL ₃ (500 μ g/ml) + anti-SR-BI (50 μ g IgG/ml)	520.8 \pm 91.9 ^c

Results are means \pm SEM of three separate experiments. Progestins are the sum of progesterone plus 20 α -dihydroxyprogesterone.

^a $P = 0.0211$.

^b $P = \text{NS}$.

^c $P = 0.0205$.

Bt₂cAMP, resulting in the stimulation of its transcription, will require further study. The current results, however, do suggest that there may be a mechanism that can detect a state in which the sterol hormone requirement is increased and the ability to remove lipoproteins by endocytosis is impaired. This state results in the stimulation of the gene, allowing the LRP to increase its functional ability to deliver lipoprotein-derived cholesterol to cells. In addition, increased expression of LRP may also independently facilitate the functioning of the selective CE uptake pathway. For example, we recently showed that LDL- (but not hHDL₃)-derived CE uptake by apoE-expressing mouse Y1-BS1 adrenocortical cells is dependent on apoE and can be inhibited by RAP and α_2 -macroglobulin, raising the possibility that LRP contributes to selective LDL-CE uptake by tethering the lipoprotein particles to the cell surface (64). Likewise, a role for LRP has also been suggested in SW872 liposarcoma cells and primary adipocytes (65). Finally, in macrophages, LRP may mediate the selective transfer of CE from the donor LDL particles to the cell surface-associated plasma membrane pool of CEs (66, 67).

Given that the combination of Bt₂cAMP and hLDL specifically upregulates LRP, we exploited this condition to further delineate the role of LRP in the binding and clearance of chylomicron remnants. Evidence for a physiological role for this receptor in the removal of the highly apoE-enriched chylomicron remnants has come from knockout experiments reported by Herz and colleagues (54). Despite this, the amount of lipoprotein that can be clearly attributed to uptake by the LRP in most tissue culture experiments has been difficult to document. By allowing examination of LRP functions at two levels of expression, our experiments provide some insight into the reason for this. The amount mediated by the LRP was determined as the difference between the cell association of lipoproteins or degradation inhibited by the anti-LDL receptor antibody and by RAP, which inhibits both the LDL receptor and the LRP function at the concentration used. In the basal state, little if any lipoprotein cell association can be attributed to the LRP. It was somewhat surprising that this association was not increased when the expression level of LRP doubled. The newly synthesized LRP was functional, because the cell association of α_2 -macroglobulin was increased to a degree commensurate with the increase in expression. This suggests that relatively little if any binding of chylomicron remnants to the cell surface is mediated by the LRP.

In contrast, even in the basal state, ~50% of remnant degradation could be attributed to LRP. The simplest explanation is that although the LRP is present at a sufficient level to bind and internalize chylomicron remnants in their native form, they are poor ligands for this receptor and better ligands for other receptors, some of which do not lead to internalization. At some point, perhaps after modification, the lipoprotein is handed off to the LRP, where it is rapidly internalized and degraded (67). The observation that degradation is only doubled when the LRP level increases by 6-fold suggests that either the rate of modification of the particle is the rate-determining step or

that the affinity is so low that the process does not occur in the linear range of the kinetic curve for this ligand. There was no detectable secretion of apoE, lipoprotein lipase, or hepatic lipase by these cells (our unpublished observations). Thus, if there is any modification of the remnants after binding, it must be attributable to other molecules. A similar postulate has been put forward to explain remnant uptake by the LRP in the liver and of β -migrating VLDL by vascular smooth muscle cells.

In summary, we report here a unique type of upregulation of LRP in response to increased cellular sterol levels and hormonal stimulation. We also show that enhanced expression of LRP has the potential to provide cholesterol for steroidogenesis and that it may serve as a backup system to ensure the delivery of cholesterol to steroidogenic tissues by the endocytic pathway, as it appears to do in the liver. These studies also suggest that LRP, however, does not serve as a primary binding site but rather serves to internalize particles bound to the cell surface by other means. The results also demonstrate that there is a complex mechanism of regulation of the LRP that warrants further study. ■

This work was supported by National Institutes of Health Grants HL-33881 and DK-56339 and funds from the Office of Research and Development, Medical Research Service, Department of Veterans Affairs.

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