Regulation of Cholesterol Responsive Genes in Ovary Cells: Impact of Cholesterol Delivery Systems†

Satyanarayana Medicherla,‡,§ Salman Azhar,‡,§ Allen Cooper,§,|| and Eve Reaven*,§

Geriatric Research, Education and Clinical Center, VA Palo Alto Health Care System, and Department of Medicine, Stanford University School of Medicine, Palo Alto, California 94304, and Research Institute, Palo Alto Medical Foundation, Palo Alto, California 94301

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ABSTRACT: The "selective" cholesterol uptake pathway represents a bulk pathway by which many steroidogenic cells internalize lipoprotein-delivered cholesteryl esters. In the current report, we question whether cholesteryl esters entering cells via this pathway are capable of governing standard cholesterol end product feedback repression mechanisms. Cultured rat ovary granulosa cells which utilize both the "selective" and "endocytic" pathways to internalize lipoprotein-derived cholesteryl esters were used as a model system. ApoE-free $hHDL_3$ was used to deliver cholesteryl esters to the cells exclusively by the selective pathway; hLDL was used as a control ligand which when internalized by the endocytic pathway releases cholesteryl esters which subsequently regulate the expression of the B/E (LDL)-receptor, HMG CoA reductase, and acyl-CoA:cholesterol acyltransferase (ACAT). Whereas trophic hormone (Bt_2cAMP) stimulation by itself increased the activity, mRNA, and protein levels of both B/E-receptor and HMG CoA reductase, pretreatment with either lipoprotein (adjusted for equal cholesterol ester content) down-regulated this expression. Linked with these lipoprotein-related changes was an increase in activity (though not gene expression) of ACAT. The level of change in mRNA levels, protein content, and activity for the examined regulatory proteins was essentially equivalent whether the lipoprotein provided to the cells was hLDL or hHDL3. Thus, similar signals appear to have been received by the cells despite differences in the uptake and processing of the ligand-derived cholesteryl esters, and these signals resulted in identical homeostatic responses by the cells.

Cultured rat ovary granulosa cells can internalize lipoprotein-derived cholesteryl esters (CEs) through the classical B/E-receptor pathway or through an alternative "selective" pathway in which CE is extracted from the lipoprotein particle at the cell surface (Azhar et al., 1990; Reaven et al., 1995). In hormone-stimulated granulosa cells, use of a specific cholesterol pathway is dependent on which lipoproteins are provided to the cells; recent studies have shown that apoE-free human high-density particles ($hHDL_3$) are exclusively processed by the selective pathway (Azhar et al., 1990; Reaven et al., 1995), most human low-density lipoprotein particles (hLDL) are processed by the endocytic B/E-receptor pathway (Reaven et al., 1994) [though they may also be processed by the selective pathway (Reaven et al., 1995)], and rat HDL (rHDL) are processed by both pathways (Leitersdorf et al., 1984; Pittman et al., 1987a,b; Azhar et al., 1990; Richard & Pittman, 1993). It is of interest that the two cholesterol uptake pathways are equally efficient in CE delivery, and in a given 24 h experimental period, $hHDL_3$ and hLDL (with equal amounts of CE to contribute) will deliver an identical mass of CE to granulosa cells (Azhar et al., 1990; Reaven et al., 1994,1995).

Even though the selective cholesterol pathway is a major pathway in the rat, very little is known about it. Even the cellular fate of the newly internalized CE is just now becoming clear. From recent work with rat granulosa cells, we have learned that essentially no CE uptake takes place if the cells have not initially been stimulated with Bt_2cAMP or trophic hormone (Reaven et al., 1995). It appears that HDL_3−cholesteryl esters entering by way of the selective pathway are directly used for steroid hormone production, but if the entering $hHDL_3$−CE exceeds that needed for hormone production (Azhar et al., 1990; Reaven et al., 1994, 1995), any excess CE may be hydrolyzed, re-esterified, and stored or directly stored (without prior hydrolysis) within the cell in lipid droplets (Reaven et al., 1995). The amount of $hHDL_3$−CE which the cells are capable of storing is impressive, being >10 times that stored by nonstimulated cells in a 24 h experimental period (Reaven et al., 1995). Indeed, when visualized with the lipid stain Nile Red or following a 24 h incubation period with reconstituted $hHDL_3$ labeled with a nonhydrolyzable fluorescent CE probe, one finds the granulosa cells virtually bursting with newly stored lipid droplets (Reaven et al., 1995).

While it is understood that this abundant selective pathway-internalized CE is used for steroidogenesis, it is not yet clear whether the cholesterol from this source is capable of regulating cholesterol-sensitive processes within steroidogenic cells (Goldstein & Brown, 1990; Javitt, 1995; Towle, 1995). Does the CE which enters by the selective pathway govern cholesterol end product feedback repression mechanisms, as does LDL−CE which enters cells by the endocytic pathway?

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* Address requests for reprints to Eve Reaven, Ph.D., VA Palo Alto Health Care System (GRECC/182-B), 3801 Miranda Avenue, Palo Alto, CA 94304. Telephone: (415) 858-3933. Fax: (415) 855-9437.

‡ VA Palo Alto Health Care System.

§ Department of Medicine, Stanford University School of Medicine.

|| Palo Alto Medical Foundation.

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It has been well-documented that mammalian cells maintain their intracellular sterol balance, in part by regulating the transcription of apoB/apoE (LDL)-receptor (B/E-receptor) which supplies cholesterol from an external source via the endocytic pathway and in part by enzymes such as HMG CoA reductase and HMG CoA synthase which determine the rate of *de novo* cholesterol synthesis (Goldstein & Brown, 1990; Javitt, 1995; Towle, 1995). The promotor regions of these genes contain sterol regulatory elements (SREs). Two transcription factors, termed sterol regulatory elements binding protein-1 (SREBP-1) and SREBP-2, bind to the SREs and modulate the expression of these genes (Stark et al., 1992; Yokoyama et al., 1993; Hua et al., 1993; Sato et al., 1994; Wang et al., 1994; Sanchez et al., 1995; Towle, 1995; Javitt, 1995). Intracellularly, these SREBPs exist as large precursor proteins attached to membranes of the endoplasmic reticulum and nuclear envelope (Sato et al., 1994; Wang et al., 1995). When cellular sterol levels are low, these SREBPs are cleaved by proteolysis and transported to the nucleus where they bind to SREs in the promotor region of cholesterol-sensitive genes, increasing their transcription (Towle, 1995; Javitt, 1995). Conversely, when sufficient cholesterol is present, the metabolites of cholesterol, particularly hydroxycholesterols, block this proteolytic processing of SREBPs and decrease the availability of "active" SREBPs. These, in turn, cause the down-regulation of cholesterol-sensitive genes (Towle, 1995; Javitt, 1995).

Although the precise mechanism by which the HDL−CE is internalized and transported through cells is still largely unknown, it is unlikely that it is transported by coated vesicles, endosomes, or other membrane-bound organelles which interact with lysosomes (Brown & Goldstein, 1986; Schneider, 1989; Johnson et al., 1991). As such, the influx of HDL−CE vs LDL−CE may provide quite different signals to cholesterol-sensitive genes and may activate different proteins.

In the current study, we have chosen to examine this issue in the responsive rat granulosa cells described above. The objective was to assess changes in cholesterol homeostasis following pretreatment of rat granulosa cells with Bt_2cAMP and/or apoE-free $hHDL_3$, which exclusively utilizes the selective pathway (Azhar et al., 1990; Reaven et al., 1995). Three cholesterol-sensitive cell markers [apoB/apoE (LDL)-receptor protein (B/E-receptor), 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG CoA reductase), and acyl-CoA:cholesterol acyltransferase (ACAT)] were evaluated for changes in activity, mRNA levels, and protein mass. Human LDL was used as a control in the sense that treatment with this lipoprotein (and the resulting uptake of cholesterol) is known to down-regulate the B/E-receptor and HMG CoA reductase genes while up-regulating the ACAT post-translationally (cholesterol esterification and storage) in a variety of cell types (Brown & Goldstein, 1986; Schneider, 1989; Johnson et al., 1991; Suckling & Stange, 1985; Goldstein & Brown, 1990; Chang et al., 1993; Pape et al., 1995; Rea et al., 1995). It was of interest to determine if HDL−CE entering cells by the selective pathway similarly affected cellular mechanisms to control cholesterol uptake, synthesis, and usage.

MATERIALS AND METHODS

Materials. The following radiochemicals were supplied by DuPont Company (NEN Research Products, Boston, MA): $Na^{125}I$ (carrier-free) (specific activity of 644 GBq/mg; 17.4 Ci/mg), [α-^{32}P]dCTP (specific activity of 111 TBq/mmol; 3000 Ci/mmol), [α-^{32}P]CTP (specific activity of 29.6 TBq/mmol; 800 Ci/mmol), DL-$_3$[*glutaryl*-3-^{14}C]-3-hydroxy-3-methylglutarylcoenzyme A (specific activity of 2.2 GBq/mmol; 59.9 mCi/mmol), and mevalonolactone (*RS*)-[5-^{3}H(N)]oleic acid (specific activity of 2.22 TBq/mmol; 60 Ci/mmol). All other reagents used were obtained from the sources described in previous publications (Reaven et al., 1994). For measurement of HMG CoA reductase, an 362 bp rat HMG CoA reductase cDNA was isolated by screening a rat liver, λ Zap II library (Stratagene) with a radiolabeled cDNA fragment (0.4 kb) derived from hamster cDNA (Chin et al., 1984). The nucleotide sequence of the rat HMG CoA reductase showed 84% homology with the sequence reported for hamster cDNA (Chin et al., 1984). Other cDNA probes were generously provided by the following individuals: The rabbit ACAT cDNA clone (pRQV-ACAT/Rb_{14b}) was obtained from Dr. M. E. Pape (Parke-Davis Pharmaceutical Research Division, Ann Arbor, MI) (Pape et al., 1995), a partial coding and the 3′ untranslated region fragment of the rat LDL receptor cDNA (Lee et al., 1989) from Dr. M. Komaromy (Palo Alto Medical Foundation, Palo Alto, CA), and a partial coding and spacer DNA fragment (5.9 kb) of rat 18S ribosomal RNA (Chikaraishi et al., 1983) from Dr. D. M. Chikaraishi (Tufts University School of Medicine, Boston, MA).

Granulosa Cell Preparation and Culture. Rat ovarian granulosa cells were isolated and cultured in DME:F12 medium containing fibronectin, iron-saturated transferrin, insulin, and hydrocortisone (basal medium) as described previously (Azhar et al., 1988). Some cultures were maintained at 37 °C for up to 96 h in basal medium. Other cultures at 72 h were presensitized with Bt_2cAMP (2.5 mM) for 24 h. Subsequently, these cells were treated for an additional 5−24 h with or without medium containing hLDL (100 μg of protein/mL) or $hHDL_3$ (500 μg of protein/mL) with or without Bt_2cAMP (hormonal) stimulation. hLDL and apoE-free $hHDL_3$ were isolated as described earlier (Azhar et al., 1989, 1990; Reaven et al., 1990; Shi et al., 1992).

LDL (B/E)-Receptor Binding Assay. The binding of ^{125}I-labeled hLDL to granulosa cell membranes was carried out by a slight modification of the method of Basu et al. (1978) as previously described (Reaven et al., 1994). The results are expressed as nanograms of [^{125}I]hLDL bound per milligram of protein.

Determination of HMG CoA Reductase Activity. The enzyme activity was measured according to the method of Goldstein et al. (1983) except that 1.25% Brij 96 detergent was used instead of 0.25% Kyro EOB as previously described (Gal et al., 1982). Separation of the product, [^{14}C]-mevalonate, from the substrate was accomplished with an ion exchange resin according to the method of Beg and Stonik (1982). All assays were performed in duplicate, and enzyme activity was linear with respect to protein concentration and incubation time. HMG CoA reductase activity is expressed as picomoles of [^{14}C]mevalonate formed per minute per milligram of cellular protein.

Measurement of Acyl-CoA:Cholesterol Acyltransferase (ACAT) Activity. The incorporation of [^{3}H]oleate into cholesteryl [^{3}H]oleate in granulosa cells was measured as described previously (Reaven et al., 1995) by incubating cells

with a complex of [^{3}H]oleate and albumin for 5−24 h. The cholesteryl [^{3}H]oleate content was determined after thin layer chromatographic isolation (Reaven et al., 1995).

Uptake of Gold-Labeled LDL. Internalization of gold-labeled LDL (apoB protein) was examined morphologically (Reaven et al., 1984, 1995). Granulosa cells were incubated with Au-LDL for 5 h at 37 °C, washed, fixed, and processed for electron microscopy (Reaven et al., 1984, 1995). Quantitation of internalized gold was carried out as described previously from this laboratory (Reaven et al., 1984, 1995).

Western Blotting of LDL (B/E)-Receptor Protein. Granulosa cell membrane preparations were resolved on 7.5% SDS−PAGE (Laemmli, 1970) using 35 μg of protein from each sample. Transfer of resolved proteins to nitrocellulose membranes was performed as described before (Burnette, 1981; Azhar et al., 1994). The blots were blocked and then incubated with appropriately diluted B/E-receptor antiserum and detected using enzyme-conjugated horse anti-rabbit IgG $F(ab)_2$, followed by chemiluminescent detection using the ECL system according to the manufacturer's instructions (Amersham Corp). Note, Western blots examining HMG CoA reductase or ACAT mass were not possible since appropriate antibodies for these proteins were not available to us.

RNA Preparation. RNA was extracted from granulosa cells by the method of Chomczynski and Sacchi (1987).

Construction of Plasmids Expressing Fragments of LDL (B/E)-Receptor, HMG CoA Reductase, and 18S rRNA cDNAs. For LDL receptor, a 1.2 kb PCR-generated rat liver LDL receptor cDNA fragment was subcloned into the *Eco*RI site of pBluescript KSII$^+$ (pBS KSII$^+$) (Stratagene, La Jolla, CA). The insert sequence runs from base 1878 through the 3′-untranslated region of the rat LDL receptor cDNA sequence (Lee et al., 1989). Similarly, a 362 bp rat HMG CoA reductase cDNA was isolated by screening a rat liver λ Zap II library with a radiolabeled cDNA fragment (0.4 kb) derived from hamster cDNA (Chin et al., 1984). This fragment was subcloned into the *Eco*RI site of pBS KSII$^+$. Finally, a 274 bp *Dra*II-*Tha*I (position 134−408) fragment of 18S ribosomal RNA cDNA (Chikaraishi et al., 1983) was subcloned into the *Dra*II-*Eco*RV sites of pBS KSII$^+$. The plasmids were linearized with appropriate restriction endonucleases (*Xho*I for LDL receptor, *Hin*dIII for HMG CoA reductase, and *Bam*HI for 18S ribosomal RNA), extracted twice with phenol and twice with chloroform, precipitated in ethanol, and redissolved in DEPC-treated water to a concentration of 100−250 μg/mL.

Preparation of Riboprobes. The antisense [^{32}P]cRNA probes were synthesized using [^{32}P]rCTP and the appropriate T3 or T7 RNA polymerase following the method supplied in the Strategene's *in vitro* transcription kit. Because of their high lability, the riboprobes were always freshly prepared prior to hybridization.

Messenger RNA Quantitation by RNase Protection Assay. The concentrations of LDL (B/E)-receptor and HMG CoA reductase mRNAs were determined using a "sensitive" "RNase protection assay" as described previously (Reaven et al., 1994; Shilo et al., 1995; Azhar et al., 1995). Aliquots of granulosa cell total RNA (5−20 μg) or control tRNA (5−20 μg) were dried under vacuum and redissolved in 30 μL of hybridization buffer containing 100 000 cpm of each probe [i.e., the radiolabeled LDL receptor plus HMG CoA reductase riboprobes or the 18S ribosomal (r) RNA riboprobe that was used as an internal standard for quantification]. The mixture was incubated for 5 min at 85 °C to denature RNA and was then rapidly transferred to hybridization temperature of 42 °C for incubation overnight (about 18 h). To digest unprotected probe, 350 μL of ribonuclease digestion buffer (10 mM Tris-HCl, 5 mM EDTA, and 0.3 M NaCl) containing 40 μg/mL ribonuclease A and 2 μg/mL ribonuclease T1 was added to the hybridization reaction mixture and the mixture incubated for 1 h at 30 °C. The RNase digestion reaction was terminated by addition of proteinase K (50 μg) and SDS (2 mg) and incubation for 15 min at 37 °C. After phenol−chloroform extraction, the protected RNA−RNA hybrids were ethanol-precipitated using yeast tRNA as a carrier. The pellet was dissolved in 15 μL of loading buffer [80% formamide, 1 mM EDTA (pH 8), 0.1% bromophenol blue, and 0.1% xylene cyanol] and heated for 5 min at 85 °C. The protected fragments were separated 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 and for weaker signals for up to 48 h.

For quantification, the films were anlayzed by densitometry. The data are expressed as fraction of LDL (B/E)-receptor or HMG CoA reductase signal to that of 18S rRNA in order to correct for differences in loading the small amounts of total RNA. In our studies, the steady-state levels of 18S rRNA remained constant in response to various hormonal manipulations. Also, this is in keeping with recent reports recommending the use of rRNA gene expression as a preferred RNA-loading (internal) control for Northern blot analysis in which total RNA is used (Barbu & Dauty, 1989; Perrella et al., 1994; Yang & Tashjian, 1993; DeLeeuw et al., 1989; Bhatia et al., 1994). More widely used internal controls such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin were not employed in the current studies, since the steady-state levels of these mRNAs are known to be greatly influenced by hormones and growth factors (Alexander et al., 1988; Wadsworth et al., 1990; Bray et al., 1991; Sabath et al., 1990; Skinner et al., 1985; Amsterdam & Rotmensch, 1987; Amsterdam et al., 1989; Reaven et al., 1994).

Northern Blot Analysis. ACAT mRNA levels were quantified by Northern blot analysis using standard conditions for gel electrophoresis and blot hybridization (Sambrook et al., 1989). The ACAT probe was generated by digesting the rabbit ACAT cDNA plasmid (Pape et al., 1995) with *Hin*dIII and *Not*I restriction endonucleases and isolating the 919 bp band from low-melting point agarose. The cDNA probe was labeled with [α-^{32}P]dCTP using the random-priming technique (Feinberg & Vogelstein, 1983). A 24-mer oligonucleotide (5′-ACGGTATCTGATCGTCTTC-GAACC-3′) complementary to 18S ribosomal RNA was used to quantitate the amount of 18S RNA (Yoshizumi et al., 1992) present on the blots, and all results were normalized to sample ribosomal RNA levels.

RESULTS

Effect of Bt_2cAMP and Lipoproteins on the Activity of Cholesterol-Sensitive Proteins

(1) B/E-Receptor Activity. *(a) LDL Binding Studies*. Figure 1 describes the binding of exogenously provided

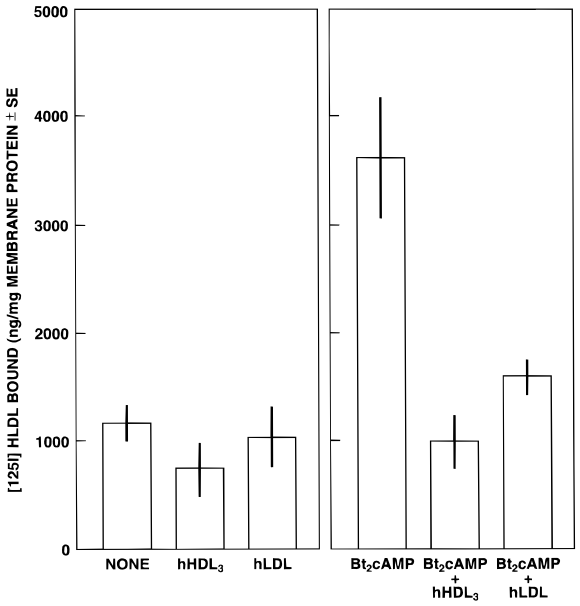


FIGURE 1: High-affinity binding of [^{125}I]hLDL to membranes isolated from granulosa cells pretreated with or without Bt_2cAMP with or without $hHDL_3$ or hLDL. Granulosa cells were presensitized for 24 h without (left panel) or with hormone (right panel) and subsequently incubated with or without lipoproteins for an additional 24 h. At the end of the treatment period, total granulosa cell membranes (100000*g*) were prepared. Each binding reaction mixture contained 30 μg of membrane protein and 33 μg/mL [^{125}I]hLDL (140 cpm/ng) in the absence or presence of 1 mg/mL unlabeled LDL. After incubation at 4 °C for 2 h, the amount of [^{125}I]LDL bound to the membranes was determined. The binding data shown represent values for the high-affinity binding calculated by subtracting the nanograms of [^{125}I]LDL bound in the presence of the excess unlabeled LDL from the nanograms of [^{125}I]LDL bound in the absence of unlabeled LDL. Each value represents the mean ± SE of duplicate assays of three separate experiments.

Table 1: Gold−hLDL Particles Internalized during 5 h of Incubation with Granulosa Cells Pretreated with or without Bt_2cAMP with or without Lipoproteins for 24 h

	number of gold particles per 100 μm^2 of cell cytoplasm	
	(mean ± SE)	%
Bt_2cAMP (2.5 mM)	1040 ± 200	100
Bt_2cAMP + $hHDL_3$ (500 μg of protein/mL)	266 ± 53	25
Bt_2cAMP + hLDL (100 μg of protein/mL)	142 ± 32	14

[^{125}I]LDL to granulosa cells following 24 h of treatment with Bt_2cAMP, $hHDL_3$ or hLDL, or a combination of hormone and lipoproteins. As seen, no changes occur with lipoproteins alone but Bt_2cAMP by itself shows a 3−4-fold increase in binding. A combination of Bt_2cAMP + either hHDL3 or hLDL results in a substantial decrease in hormone-stimulated LDL binding.

(b) LDL-Gold Uptake Studies. The level of function of the B/E-receptor was also assessed by the ability of granulosa cells pretreated with Bt_2cAMP or Bt_2cAMP + lipoproteins for 24 h to internalize gold-labeled hLDL supplied for 5 h. Table 1 indicates that pretreatment with hHDL3 reduces gold−LDL uptake by ~75% while pretreatment with hLDL reduces gold−LDL uptake by ~86%; i.e., pretreatment with either ligand down-regulates B/E-receptor function.

(2) HMG CoA Reductase Activity. In a protocol similar to that used for the B/E-receptor, granulosa cells were pretreated with hormone and/or lipoproteins, and HMG CoA reductase activity was measured. Figure 2 shows that Bt_2cAMP alone substantially increases the activity of this enzyme as compared to basal levels and that lipoproteins alone do not alter the basal levels, but that a combination of Bt_2cAMP + either lipoprotein significantly reduces the activity of this enzyme.

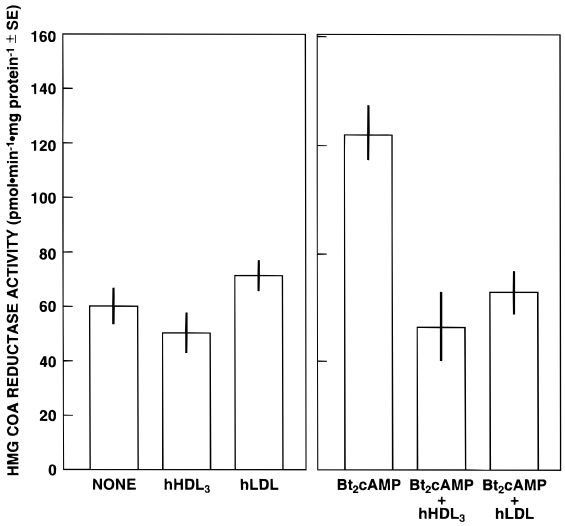


FIGURE 2: Effects of Bt_2cAMP, $hHDL_3$, and hLDL on HMG CoA reductase activity in cultured granulosa cells. Granulosa cells were pretreated with or without Bt_2cAMP (2.5 mM) for 24 h. After this period, $hHDL_3$ (500 μg of protein/mL) or hLDL (100 μg of protein/mL) was added (time zero). Cultures were harvested at 24 h in triplicate and processed for determination of HMG CoA reductase activity as described in Materials and Methods.

Table 2: Stimulation of [^{3}H]Oleate Incorporation into Cholesteryl [^{3}H]Oleate by $hHDL_3$ and hLDL in Granulosa Cells Pretreated with or without Bt_2cAMP[a]

	[^{3}H]oleic acid → cholesteryl [^{3}H]oleate (dpm/μg of DNA ± SE)	
	9 h	24 h
basal	1430 ± 283	2065 ± 337
$hHDL_3$	1849 ± 415	2335 ± 296
hLDL	7136 ± 1320	12232 ± 277
Bt_2cAMP	1796 ± 316	2459 ± 282
Bt_2cAMP + $hHDL_3$	14731 ± 1992	17637 ± 3877
Bt_2cAMP + hLDL	14160 ± 2187	16762 ± 2762

[a] Granulosa cells cultured in basal medium were pretreated with or without Bt_2cAMP (2.5 mM) for 24 h, after which $hHDL_3$ (500 μg of protein/mL) or hLDL (100 μg of protein/mL) + [^{3}H]oleate−BSA was added for 9 or 24 h at 37 °C. The cells were assayed for [^{3}H]oleate incorporation into cholesteryl esters by TLC. The data are mean ± SE of triplicate dishes.

(3) ACAT Activity. ACAT activity was assayed by following the formation of cholesteryl [^{3}H]oleate in granulosa cells treated for 24 h with or without Bt_2cAMP with or without lipoproteins and given tritiated sodium oleate complexed to BSA for either 9 or 24 h. Table 2 indicates that without Bt_2cAMP $hHDL_3$ does not induce the formation of cholesteryl oleate over basal levels, though hLDL is quite effective in this regard, showing a 5−6-fold increase. With Bt_2cAMP stimulation, both ligands induce a 10-fold response. Thus, provision of stimulated cells with either $hHDL_3$ or hLDL induces a large increase in ACAT activity.

Effect of Bt_2cAMP and Lipoproteins on mRNA Levels of Cholesterol Sensitive-Genes

The observed changes in activity of the cholesterol-sensitive proteins in granulosa cells following incubation with

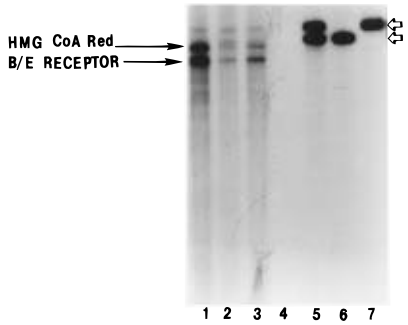


FIGURE 3: RNase protection assay to determine the levels of mRNA for both HMG CoA reductase and B/E-receptor in samples of total cellular RNA from cultured granulosa cells. Cellular RNA was isolated according to the procedure of Chomozynski and Sacchi (1987). Equal amounts of total RNA (10 μg) were hybridized with freshly synthesized anti-B/E (LDL)-receptor + HMG CoA reductase or 18S rRNA-[^{32}P]cRNA transcripts, and RNA−RNA hybrids were digested with RNase A and T1 (Reaven et al., 1994). The protected fragments were resolved by electrophoresis, followed by autoradiography to visualize bands: lanes 1−3; two protected fragments from cell samples in the expected size of HMG CoA reductase and B/E-receptor mRNA; lane 4, tRNA control; lane 5, intact cRNA probes of B/E-receptor + HMG CoA reductase together; lane 6, intact cRNA probe of B/E-receptor alone; and lane 7, intact cRNA probe of HMG CoA reductase alone.

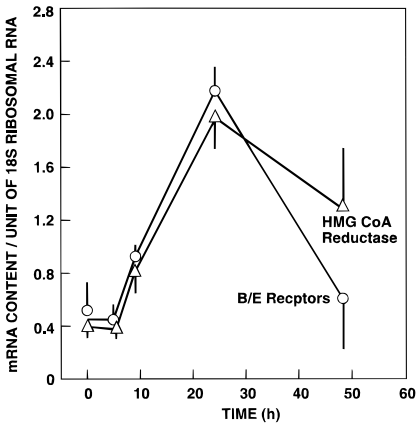


FIGURE 4: Time dependent changes in steady-state levels of B/E-receptor and HMG CoA reductase mRNAs in Bt_2cAMP-treated granulosa cells. Granulosa cells were treated with Bt_2cAMP (2.5 mM) for 0, 6, 9, 24, or 48 h. RNA was isolated from cells, and the levels of B/E-receptor and HMG CoA reductase mRNA were measured by RNase protection assay and normalized to 18S rRNA. Values are the mean of three separate experiments.

either $hHDL_3$ or LDL suggested a change in mRNA levels of the proteins. RNAse protection assay techniques were used to assess B/E-receptor and HMG CoA reductase expression, but in the case of ACAT genes, Northern blotting methodology was used since homologous rat cDNA has not been cloned.

Figure 3 illustrates simultaneous measurement of HMG CoA reductase and B/E-receptor mRNA by use of an RNase protection assay carried out on a single RNA sample. Lanes 1−3 show two protected fragments from the experimental sample in the expected size of HMG CoA reductase and B/E-receptor mRNAs. In lane 4, control tRNA shows no detectable band; lanes 5−7 show the intact probes together or separately.

A 48 h time course of Bt_2cAMP-induced changes in the mRNA of HMG CoA reductase and B/E-receptor was carried out (Figure 4): radioautogram data were corrected for 18S ribosomal RNA values, which remained unchanged during

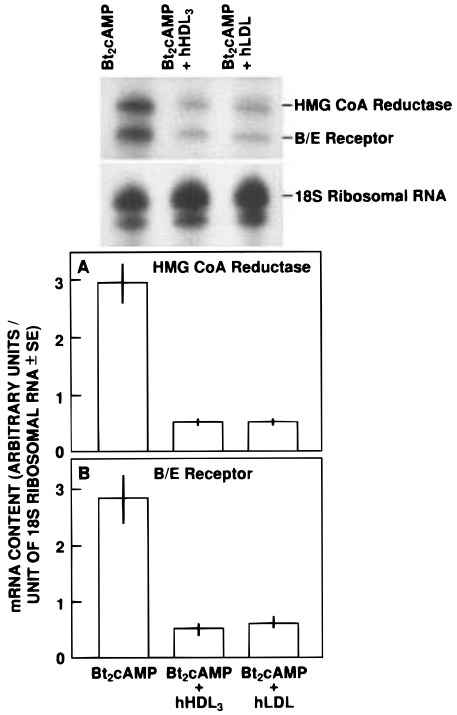


FIGURE 5: Changes in steady-state levels of B/E-receptor and HMG CoA reductase mRNAs in Bt_2cAMP (2.5 mM)-, Bt_2cAMP + $hHDL_3$ (500 μg/mL)-, or Bt_2cAMP + hLDL (100 μg/mL)-treated granulosa cells. Granulosa cells were pretreated with Bt_2cAMP with or without $hHDL_3$ or hLDL as described in Figures 1 and 2. Total cellular RNA was extracted for RNase protection assay. The histograms represent densitometric scanning of the B/E-receptor and HMG CoA reductase bands normalized to 18S rRNA. The top panel shows the respective autoradiograms with the B/E-receptor, HMG CoA reductase, and 18S ribosomal bands labeled.

the 48 h experimental period. Bt_2cAMP induces a dramatic increase in mRNA levels of both proteins, reaching a maximum at 24 h. During the second 24 h segment of Bt_2-cAMP treatment, the message for both proteins falls, that of B/E-receptor protein decreasing more rapidly than that for HMG CoA reductase.

In Figure 5, data were presented for both the B/E-receptor and HMG CoA reductase mRNA in maximally (24 h) stimulated granulosa cells provided with either $hHDL_3$ **or** hLDL. The radioautograms from the RNase protection assay seen in the uppermost panel represent pretreatment with Bt_2-cAMP alone (lane 1), Bt_2cAMP + $hHDL_3$ (lane 2), and Bt_2-cAMP + hLDL (lane 3). Below this is the 18S rRNA depicted for each of the incubations; below this are bar graphs of the mRNA B/E-receptor and HMG CoA reductase data corrected for 18S rRNA.

In order to determine whether the observed down-regulation of B/E-receptor and HMG CoA reductase genes occurs only at points of maximal uptake of CE (i.e., 24 h), a time course was carried out in which mRNA levels of both proteins were measured in samples in which lipoproteins were added to hormone-treated cells for 5, 9, 24, and 48 h. Figure 6 shows the normalized data and illustrates the fact that (1) Bt_2cAMP treatment results in a linear increase in mRNA levels for both proteins, reaching a maximum at 24 h, and (2) the addition of either $hHDL_3$ or hLDL (for any of the indicated time intervals) down-regulates the hormone-induced response.

Table 3 shows the concentration dependent effect of lipoproteins in down-regulating both cholesterol-sensitive

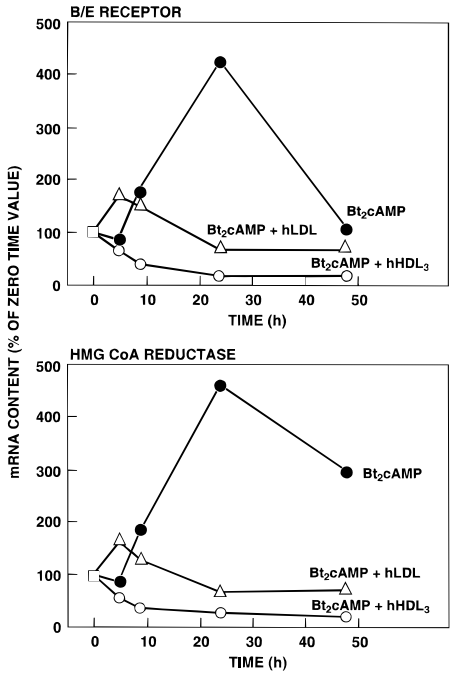


FIGURE 6: Time dependent down-regulation of steady-state levels of HMG CoA reductase and B/E-receptor mRNAs in granulosa cells by $hHDL_3$ and hLDL. Granulosa cells were treated with or without Bt_2cAMP (2.5 mM) with or without $hHDL_3$ (500 μg/mL) or hLDL (100 μg/mL) for 0, 6, 9, 24, or 48 h. RNA was isolated from cells, and the levels of HMG CoA reductase and B/E-receptor were measured by RNase protection assay and normalized to 18S rRNA as described above. The values shown are the average of two separate experiments.

Table 3: Down-Regulation of B/E-Receptor and HMG CoA Reductase mRNAs by Lipoproteins: Effect of Different Concentrations of $hHDL_3$ and hLDL

addition	HMG CoA reductase mRNA[a] (%)	B/E-receptor mRNA[a] (%)
none	1.55 (100)	1.51 (100)
$hHDL_3$ (25 μg/mL)	1.25 (80)	1.24 (82)
$hHDL_3$ (100 μg/mL)	0.98 (63)	1.00 (67)
$hHDL_3$ (500 μg/mL)	0.66 (43)	0.68 (44)
hLDL (10 μg/mL)	1.19 (77)	1.19 (84)
hLDL (25 μg/mL)	1.03 (66)	0.96 (64)
hLDL (100 μg/mL)	0.62 (40)	0.68 (45)

[a] Arbitrary units per unit of 18S rRNA.

Table 4: Down-Regulation of B/E-Receptor and HMG CoA Reductase mRNAs by Hydroxycholesterol

addition	HMG CoA reductase mRNA[a] **(%)**	B/E-receptor mRNA[a] (%)
none	1.55 (100)	1.51 (100)
25-hydroxycholesterol (20 μg/mL)	0.67 (43)	0.68 (45)

[a] Arbitrary units per unit of 18S rRNA.

genes in cells incubated for 24 h. A clear-cut concentration gradient effect is seen with both ligands for both genes.

Table 4 indicates that a freely diffusible form of cholesterol (e.g., 25-hydroxycholesterol) is similarly effective in down-regulating these cholesterol-sensitive genes.

The data of Figure 7 represent changes in ACAT mRNA from experimental protocols identical to that of the proteins in Figure 5, except that measurement of ACAT mRNA was carried out by Northern blotting. Again the uppermost panel (A) represents the uncorrected radioautograms of the mRNA for this enzyme following treatment with Bt_2cAMP (lane 1),

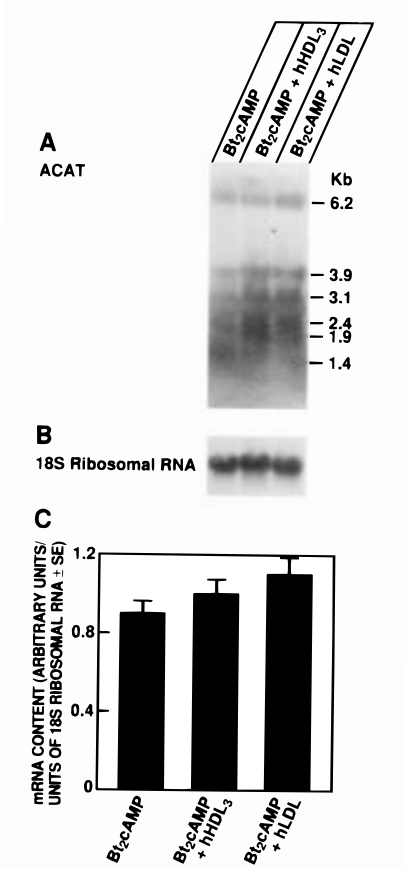


FIGURE 7: Northern blot analysis of steady-state levels of ACAT mRNA. Granulosa cells were incubated with or without Bt_2cAMP (2.5 mM) with or without $hHDL_3$ (500 μg/mL) or hLDL (100 μg/mL) as described earlier. Total cellular RNA was harvested and used for Northern blot analysis. ACAT mRNA species on the membrane were detected with a radiolabeled RNA probe containing sequences from the entire 919 bp rabbit ACAT cDNA Rb_{14b} (Pape et al., 1995). The top panel shows autoradiograms with variably sized species of ACAT mRNA (A) or 18S ribosomal mRNA (B). The histogram (C) represents a sum of densitometric scannings of the ACAT mRNA normalized to the amount of 18S ribosomal RNA per sample.

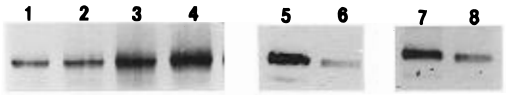
Bt_2cAMP + $hHDL_3$ (lane 2), and Bt_2cAMP + hLDL (lane 3). These radioautograms and the unchanging content of rRNA permit graphing of mRNA for ACAT, showing that pretreatment of hormone-stimulated granulosa cells with either $hHDL_3$ or hLDL has only a minor, nonstatistically significant, impact on the ACAT genes (B).

Effect of Bt_2cAMP and Lipoproteins on B/E-Receptor Protein Mass

Figure 8 depicts the results of Western blotting of the B/E-receptor protein performed on samples from hormone-stimulated granulosa cells pretreated with Bt_2cAMP alone or with $hHDL_3$ or hLDL. The immunoblots shown represent the protein mass observed after 24 h of treatment as indicated by the treatment boxes. Scanning of the blots (Table 5) indicates that Bt_2cAMP alone increases the protein mass of B/E-receptor protein 3-fold and that preincubation of stimulated cells with either $hHDL_3$ or hLDL results in 70% less B/E-receptor protein than seen with hormone treatment alone.

DISCUSSION

In previous reports, the selective cholesterol uptake pathway has been described as a "bulk" cholesterol pathway



None	+	+	-	-		-	-		-	-
Bt_2cAMP	-	-	+	+		+	+		+	+
$hHDL_3$	-	-	-	-		-	+		-	-
hLDL	-	-	-	-		-	-		-	+

FIGURE 8: Immunoblot analysis of the mass of the B/E-receptor protein (~130 kDa). Crude membranes were prepared from granulosa cells pre-treated with Bt_2cAMP (2.5 mM), Bt_2cAMP + $hHDL_3$ (500 μg of protein/mL), or Bt_2cAMP + hLDL (100 μg of protein/mL) as indicated in the treatment box. Samples of the detergent-solubilized membrane (35 μg) were applied to 6% polyacrylamide gel containing 0.1% sodium dodecyl sulfate (SDS) under nonreducing conditions. Proteins were transferred from the gel to immobilon membranes, and the blots were incubated first with rabbit anti-rat B/E-receptor (IgG) and then developed with a chemiluminescent detection system (ECL, Amersham Corp.). Columns 1−4, 5 and 6, and 7 and 8 represent immunoblots from three separate experiments.

Table 5: Effects of Bt_2cAMP, hLDL, and $hHDL_3$ on B/E-Receptor Protein Content

	B/E-receptor protein (arbitrary units per milligram of protein ± SE) (%)
experiment 1	
basal	24.6 ± 3.3 (100)
Bt_2cAMP (2.5 mM)	70.3 ± 11.5 (286)
experiment 2	
Bt_2cAMP (2.5 mM)	70.7 ± 8.9 (100)
Bt_2cAMP + $hHDL_3$ (500 μg/mL)	23.4 ± 1.9 (33)
Bt_2cAMP + hLDL (100 μg/mL)	17.3 ± 2.7 (25)

[a] Results are mean ± SE of three separate experiments.

for steroidogenic cells which have unusually high requirements for cholesterol as a precursor for steroid hormone production. Indeed, the selective pathway is essentially nonexistent in cells which have not been stimulated to produce steroids (Reaven et al., 1995), but responds dramatically to trophic hormone or second messenger signals by internalizing large quantities of lipoprotein-derived cholesteryl esters and filling the cells with lipid droplets (Reaven et al., 1995). It was of interest to determine to what extent CE entering cells by this pathway activates standard cholesterol homeostatic mechanisms.

We found that two powerful weapons cells have to regulate cholesterol content (the B/E-receptor which mediates uptake of lipoprotein−cholesterol and the cholesterol-synthesizing enzyme, HMG CoA reductase, which controls the *de novo* synthesis of cholesterol) were activated when the cells were stimulated with Bt2cAMP alone, showing substantial increases in activity, mRNA, and (in the case of the B/E-receptor) protein mass. However, when the cells were also provided with lipoproteins, these cholesterol-sensitive genes were down-regulated. This was the case with hLDL which was used here as a control ligand since LDL is well-known in other cell systems (Brown & Goldstein, 1986; Schneider, 1989; Johnson et al., 1991; Goldstein & Brown, 1977, 1990; Brown et al., 1979; Mahley & Innerarity, 1983; Ponec et al., 1985; St. Clair et al., 1986; Yoshimura et al., 1987) to bind to the cell surface B/E-receptor, enter cells primarily through the endocytic pathway, and deliver its cholesterol via lysosomes for further processing, while the released cholesterol in turn regulates the activity and genetic expression of cholesterol-associated proteins. Linked with these LDL-related changes was an increase in activity (though not gene expression) of ACAT, which is known from previous studies to increase cholesterol storage via nontranscriptional/nontranslational mechanisms (Goldstein & Brown, 1977, 1990; Doolittle & Chang, 1982; Drevon et al., 1980; Rea et al., 1995; Pape et al., 1995).

Of importance was the fact that this down-regulation of B/E-receptor and HMG CoA reductase proteins and the increase in activity of ACAT occurred also when the lipoprotein used to pretreat granulosa cells was $hHDL_3$, a ligand from which cholesteryl esters are extracted at the cell surface and directly enter cells in a manner quite separate from that described for LDL (Azhar et al., 1990; Reaven et al., 1994, 1995). The level of change in messenger RNA, protein content, and activity for the examined regulatory proteins was essentially equivalent whether the lipoprotein provided to the cells was hLDL or $hHDL_3$. Thus, similar signals appear to have been received by the cells despite differences in the uptake and processing of the ligand-derived cholesteryl esters, and these signals resulted in identical homeostatic responses by the cells.

Although the identity of the intracellular mediator regulating cholesterol biosynthesis in cells is not yet certain, there is evidence in the literature to suggest that the cell concentration of free (unesterified) cholesterol or that of oxygenated cholesterol derivatives such as hydroxycholesterols (Goldstein & Brown, 1977, 1990; Kandutsch & Chen, 1974; Chang et al., 1981; Rudney & Sexton, 1986; Gupta et al., 1986; Metherall et al., 1989; Axelson & Larsson, 1995) may play this role. Were this the case with granulosa cells, it could explain how $hHDL_3$ and hLDL (as well as 25-hydroxycholesterol) led to identical cholesterol regulatory responses in the current study. That is, when $hHDL_3$ or hLDL−lipoproteins (with equal cholesteryl ester content) are incubated with granulosa cells, essentially identical amounts of intracellular free cholesterol become available [as measured by the amount of cholesterol which can be re-esterified with radiolabeled oleic acid (Reaven et al., 1995)]. Together, these events suggest that it is the level of the intracellular cholesterol itself, and not the method of delivery or processing of this cholesterol, which activates or represses the genes regulating cellular cholesterol.

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