Induction of high-density-lipoprotein receptors in rat corpus luteum by human choriogonadotropin

Evidence of protein synthesis *de novo*

Dipak K. GHOSH and K. M. Jairam MENON

Endocrine Laboratory, Departments of Obstetrics and Gynecology and Biological Chemistry, University of Michigan Medical School, Ann Arbor, MI 48109, U.S.A.

The present studies investigated the specific binding of ^{125}I -labelled high-density lipoprotein (^{125}I -HDL) to plasma membranes, Golgi, rough endoplasmic reticulum and mitochondria/lysosomes, prepared from ovaries of rats injected with human choriogonadotropin (hCG) or 0.9% NaCl. Treatment *in vivo* with hCG resulted in 2–3-fold induction of ^{125}I -HDL binding activity in all the subcellular organelles. The specific binding of HDL to various subcellular organelles was dependent on the amount of protein, lipoprotein concentration and incubation time. Equilibrium-binding studies revealed comparable K_d values (13–22 μg of HDL protein/ml) for HDL binding in all the subcellular organelles tested. Treatment with cycloheximide (2.0 mg/kg body wt.) before hCG administration abolished the induction of HDL receptors, suggesting the involvement of a protein-synthesis-dependent process in receptor induction. Analysis of equilibrium dissociation constants (K_d) for ^{125}I -HDL binding in membranes from hCG-, cycloheximide- and saline-treated animals suggests that the increase in binding was due to an increase in the number of binding sites rather than a change in the affinity. Additionally, pretreatment with tunicamycin, an inhibitor of *N*-linked glycosylation, had no effect on hCG-mediated receptor induction, suggesting that glycosylation of the receptor may not be necessary for the interaction of HDL with its receptors.

INTRODUCTION

Steroidogenic cells require cholesterol for plasma-membrane synthesis and as a precursor for steroid-hormone production. Luteal cells are capable of synthesizing cholesterol *de novo* from acetate (Savard *et al.*, 1965), or they may utilize blood-borne cholesterol by a specific receptor-mediated process, primarily by a mechanism described by Goldstein & Brown (1977). The latter process involves the binding of lipoproteins to specific receptors on the cell surface, followed by internalization and processing of the lipoproteins by lysosomes, resulting in the release of free cholesterol. It has been shown that the lipoprotein-derived cholesterol is the preferred substrate for steroid production in the rat ovary (Anderson & Dietschy, 1978). The existence of specific high-affinity binding sites for HDL (high-density-lipoprotein fraction 3 devoid of E apolipoprotein, HDL₃) has been reported in the rat luteal cell membranes (Schuler *et al.*, 1981; Rajendran *et al.*, 1983; Hwang & Menon, 1983). Furthermore, treatment *in vivo* of superovulated rats with gonadotropin (hCG) has been shown to increase the specific binding of both HDL and apolipoproteins AI and AII complexed with dimyristoyl phosphatidylcholine vesicles to HDL receptor in luteal cell membranes (Hwang & Menon, 1983, 1985). More recently the solubilization of a gonadotropin-inducible HDL receptor from rat ovarian membranes has also been reported (Ghosh & Menon, 1986). Although LDL receptors have been well characterized in several systems, information about HDL receptors is still scanty. Unlike LDL, which primarily donates cholesterol to

cells, HDL is probably involved in reverse cholesterol transport from some tissues (Glomset, 1968) and delivery of steroid to other steroidogenic tissues (Anderson & Dietschy, 1978; Chen *et al.*, 1980; Gwynne & Hess, 1980). Thus the processing of HDL is more complex and may involve different mechanisms for different cells.

The present study was undertaken to determine a possible mechanism by which hCG increases the HDL receptors in the luteal cell membranes. In this regard, the possibility of a redistribution of receptors among the various intracellular organelles and the involvement of protein synthesis *de novo* were considered. The present paper describes the distribution of HDL receptors in various subcellular organelles prepared from rat ovaries, induction of these receptor activities by gonadotropin and the effect of inhibitors of both protein synthesis and *N*-linked glycosylation on the induction process. The results indicate that the induction of HDL receptors by gonadotropic hormones involves synthesis of proteins *de novo*.

MATERIALS AND METHODS

Materials

Na^{125}I (carrier-free aqueous solution, pH 8–10), [^3H]glucosamine and [^3H]leucine were purchased from New England Nuclear, Boston, MA, U.S.A. QAE (quaternary aminoethyl)-Sephadex A-25 was obtained from Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A. Pregnant-mare serum gonadotropin, human chorio-

gonadotropin (hCG), cycloheximide, tunicamycin, thiamin pyrophosphate (co-carboxylase), Tris, cytochrome *c* (type VI, from horse heart), NADPH (type IX) and glucose 6-phosphate (monosodium salt) were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Bovine serum albumin (fraction V) was from Reheis Chemical Co., Phoenix, AZ, U.S.A., and phenol reagent from Fisher Scientific Co., Detroit, MI, U.S.A. All other chemicals used were of analytical-reagent grade and obtained from local sources.

Female Sprague-Dawley rats (21 days old) were purchased from Harlan/Sprague-Dawley, Madison, WI, U.S.A. Human serum, collected from healthy donors, was obtained from the blood bank, University of Michigan, Ann Arbor, MI, U.S.A.

Preparation of HDL fraction

Human HDL (HDL₃ fraction, *d* 1.125–1.21) was isolated from normal human blood by ultracentrifugation after adjusting the density with KBr as described by Havel *et al.* (1955). For purification of HDL, the lipoprotein was washed by underlayering HDL below an equal volume of low-density solution (*d* = 1.060). After centrifugation for 24 h at 45000 rev./min in a Beckman ultracentrifuge (model L5-65B) fitted with a Ti 60 rotor, the isolated HDL was subjected to a second wash by layering it on top of high-density solution (*d* = 1.26), followed by centrifugation at 45000 rev./min for 24 h. During centrifugation, the temperature of the chamber was maintained at 4 °C.

Iodination of lipoproteins

HDL was labelled with ¹²⁵I by the ICl procedure of McFarlane (1958), and purified by ion-exchange chromatography on a QAE-Sephadex A-25 column as previously described (Hwang & Menon, 1983; Shepherd *et al.*, 1976). The specific radioactivity of ¹²⁵I-HDL averaged 400–500 c.p.m./ng of protein. More than 95% of the radioactivity in ¹²⁵I-HDL preparations was precipitable by 10% trichloroacetic acid, and less than 3% was extractable with chloroform/methanol (2:1, v/v). On electrophoresis in 0.1% SDS/12%-polyacrylamide gels in the presence of β-mercaptoethanol (Laemmli, 1970) followed by autoradiography (Hofmann *et al.*, 1981), only ¹²⁵I-apolipoprotein AI (*M_r* = 28 300) (Baker *et al.*, 1974) and ¹²⁵I-apolipoprotein AII (*M_r* = 8700 monomer) (Lux *et al.*, 1972a,b) were detectable in ¹²⁵I-HDL preparations.

Animals and treatments

Female rats (21 days old) were made pseudopregnant by injection with 50 i.u. of pregnant-mare serum gonadotropin, followed 56 h later by 25 i.u. of hCG (Parlow, 1958). On day 5 after hCG administration, the animals received a second injection of 25 i.u. of hCG to induce lipoprotein receptors, as we have reported previously (Hwang & Menon, 1983). Control animals were injected with saline (0.9% NaCl) subcutaneously. Rats were killed 12 h after the second injection, and the ovaries were removed. In the experiments in which cycloheximide (2 mg/kg body wt.) or tunicamycin (2–10 mg/kg body wt.) was administered *in vivo*, the animals were injected with 0.1 ml of 0.15 M-NaCl containing the inhibitors 1 h before hCG administration.

Preparation of subcellular organelles

Highly luteinized ovaries from both hCG- and saline-treated rats were homogenized in 8 vol. of 10 mM-Tris/HCl (pH 7.3)/0.25 M-sucrose/1 mM-CaCl₂ in a glass homogenizer fitted with a Teflon pestle. All subsequent procedures were carried out at 4 °C. A portion of the homogenate was kept, and the remainder was filtered through two layers of cheesecloth. The homogenates were then subjected to differential and discontinuous sucrose-gradient centrifugation for the preparation of different subcellular organelles. The plasma membranes and lysosomes/mitochondria were prepared as described by Gospadarowicz (1973), and Golgi and rough endoplasmic reticulum were prepared essentially as described by Ehrenreich *et al.* (1973). After isolation and washing, all subcellular fractions were resuspended in the homogenizing buffer, and stored in 1 ml batches at –20 °C until use. Protein was measured as described by Lowry *et al.* (1951), with bovine serum albumin as the standard. The yield of medium and light Golgi (interface between homogenizing buffer and 20% sucrose or between 20 and 30% sucrose, respectively) isolated separately was not sufficient for characterization and binding studies. Therefore, they were pooled and mixed with heavy Golgi (interface between 30 and 40% sucrose) and termed the 'G' fraction. The two plasma-membrane fractions, PL₁ and PL₂ (interface between 30 and 36% sucrose for PL₁ and 36% and 40% sucrose for PL₂), were pooled, since these two fractions showed identical lipoprotein-binding characteristics, and designated as 'PM' fractions. The lysosomes were not separated from mitochondria in the present studies. Thus this fraction was termed the 'L + M' fraction. Great care was taken during the collection of fractions from different density interfaces to avoid cross-contamination.

Assay of marker enzymes

The marker enzymes were assayed in different subcellular fractions as follows: cytochrome *c* oxidase (Cooperstein & Lazarow, 1951), 5'-nucleotidase (Emmelot & Bos, 1966), thiamin pyrophosphatase (Morre, 1971), NADPH-cytochrome *c* reductase (Williams & Kamin, 1962). Marker enzymes were assayed under optimal conditions and the specific activities were calculated from linear rate data. The P_i released by 5'-nucleotidase and thiamin pyrophosphatase was measured as described by Fiske & Subbarow (1925) and Martin & Doty (1956) respectively.

Binding assays

Suitable samples of the subcellular fractions (50–400 μg of protein) were incubated in disposable glass tubes (12 mm × 75 mm) which had been precoated overnight with 10% (w/v) bovine serum albumin, in a total volume of 0.25 ml of buffer A [25 mM-Tris/HCl (pH 7.4)/1 mM-CaCl₂/0.1% albumin] containing 1.1–1.3 μg of ¹²⁵I-HDL protein/ml (400–500 c.p.m./ng of protein) to determine total binding. Non-specific binding was determined by including 600 μg of unlabelled HDL in the incubation mixture. The incubations were performed at 37 °C for 90 min. After incubation, free ¹²⁵I-HDL was separated from the bound fraction by centrifugation at 3000 *g* for 20 min at 4 °C in an IEC (DPR-6000) centrifuge fitted with a 949 rotor, as described previously (Hwang & Menon, 1983). The

Table 1. Marker-enzyme distribution in different subcellular fractions prepared from hCG-treated rat ovaries

Each value represents means $\pm$ S.D. for four determinations: N.D., not determined.

Fraction	Enzyme			
	5'-Nucleotidase*	NADPH-cytochrome <i>c</i> reductase†	Thiamin pyrophosphatase*	Cytochrome oxidase‡
Homogenate	8.5 $\pm$ 0.75	17.5 $\pm$ 1.25	5.5 $\pm$ 0.75	87.5 $\pm$ 6.25
Plasma membranes	165.0 $\pm$ 8.0	10.1 $\pm$ 0.45	N.D.	45.3 $\pm$ 4.2
Rough endoplasmic reticulum	8.0 $\pm$ 6.2	160.25 $\pm$ 18.09	5.2 $\pm$ 0.34	10.16 $\pm$ 0.87
Lysosomes/mitochondria	6.0 $\pm$ 0.48	25.5 $\pm$ 2.05	N.D.	414.09 $\pm$ 30.2
Golgi	22.1 $\pm$ 2.7	15.7 $\pm$ 2.18	69.5 $\pm$ 4.5	42.2 $\pm$ 3.2

* Activity expressed as nmol of P_i released/min per mg of protein.

† Activity expressed as nmol of cytochrome *c* reduced/min per mg of protein.

‡ Activity expressed as nmol of reduced cytochrome *c* oxidized/min per mg of protein.

separation of the bound from the free ligand for the rough endoplasmic reticulum and Golgi fractions was accomplished by filtration through 0.45 μ m-pore-size Millipore filters, followed by washing with buffer A, in accordance with the procedure described by Schneider *et al.* (1979). The background binding of labelled lipoproteins to filters or tubes averaged 0.2–0.3% of the total radioactivity applied in the absence of added proteins. This represents 2.0–3.0% of that bound to the membrane fraction. After removal of the supernatants, the pellets and/or filter papers were counted for radioactivity in an automatic γ -radiation counter (Tracor Analytic). The specific binding represents the difference between total and non-specific binding. The marker-enzyme assays and binding experiments were done simultaneously in the fractions and were completed within 1 week after preparation. Each experimental value represents the mean $\pm$ S.D. of four independent observations.

Measurement of protein synthesis and glycosylation *in vitro*

Ovaries isolated from both inhibitor- and saline-injected rats were minced and placed in 3 ml of Earle's balanced salt solution containing 0.1% albumin and 3 μ Ci of [³H]leucine (sp. radioactivity 120 μ Ci/mmol) for protein-synthesis measurement or 35 μ Ci of [³H]glucosamine (sp. radioactivity 39.6 μ Ci/mmol) for glycosylation reaction and incubated at 37 °C in the presence of O₂/CO₂ (19:1) for 2 h. They were then homogenized and precipitated with trichloroacetic acid (final concn. 5%, w/v). The precipitates were washed four times with 5% trichloroacetic acid. Finally the precipitates were dissolved in 0.2 ml of 0.1 M-NaOH and incubated for 1 h at 37 °C. After incubation, the samples were cooled, neutralized with 0.1 M-HCl, and proteins were precipitated by addition of trichloroacetic acid (5%). The samples were then filtered through GF/C glass-fibre filters. The filters were washed twice with 10 ml of 5% trichloroacetic acid and once with 10 ml of diethyl ether/ethanol (1:1, v/v). The radioactivity associated with the dried filters was counted after the addition of 10 ml of Biofluor scintillation fluid (New England Nuclear). The *N*-glycosylation reaction, sensitive to tunicamycin, was measured after removal of *O*-linked oligosaccharides by alkali hydrolysis (Rosen *et al.*, 1979).

RESULTS

Distribution of marker enzymes in different subcellular fractions

Table 1 shows that 5'-nucleotidase activity, a marker for plasma membranes, was greatly enriched in plasma-membrane fractions, and was detectable to varying extents in other fractions. NADPH-cytochrome *c* reductase activity, a marker for rough endoplasmic reticulum, was enriched in this fraction. Cytochrome *c* oxidase, a marker for mitochondria, was enriched in the lysosome/mitochondrial fraction, but detectable only in low concentrations in other fractions. Similarly thiamin pyrophosphatase activity, a marker for Golgi (G fraction), was the highest in this fraction, but was either undetectable or present in low activity in other fractions. These data revealed that the subcellular fractions obtained were comparatively pure, with little or no cross-contamination.

Characterization of HDL receptors in different subcellular organelles

Fig. 1 shows that the specific binding of ¹²⁵I-HDL to different organelles was dependent on the amount of protein used. The specific binding of ¹²⁵I-HDL was linear up to 500 μ g of protein per assay tube. The time course of HDL binding to various organelles revealed that saturation was reached by 90 min of incubation (Fig. 2).

The specific binding of ¹²⁵I-HDL to all the subcellular organelles, prepared from both hCG- and saline-treated rat ovaries, was dependent on the concentration of ¹²⁵I-HDL in the incubation media and was found to be saturable (Figs. 3a, 3b and 3c). The binding data obtained for different subcellular fractions were analysed by the procedure of Scatchard (1949). The specific binding of ¹²⁵I-HDL to all the subcellular organelles indicates the presence of a single class of binding sites (Fig. 3). Both the binding affinity (*K_d*) and the number of binding sites (*N_B*) were comparable in all organelles.

As shown in Table 2, the binding of HDL to all subcellular fractions tested was insensitive to EDTA and Ca²⁺ ions. The effect of ionic strength on the binding of ¹²⁵I-HDL to various organelles is shown in Table 2. NaCl (1 M) did not influence ¹²⁵I-HDL binding. There were marked differences in the sensitivity to Pronase treatment of HDL binding to various subcellular organelles (Table

Fig. 1. Specific binding of ^{125}I -HDL to various subcellular organelles as a function of added protein

^{125}I -HDL ($7.5 \mu\text{g}$ of ^{125}I -HDL protein/ml, 40 c.p.m./ng of HDL protein) was incubated with various concentrations of different subcellular preparations (Δ , Golgi; $\circ$, rough endoplasmic reticulum; $\bullet$, lysosomes/mitochondria) at 37°C for 90 min in a total volume of 0.3 ml of binding-assay buffer either alone (total binding) or together with 3 mg of unlabelled HDL/ml (non-specific binding). The specific binding was calculated by subtracting the non-specific binding from the total binding.

Fig. 2. ^{125}I -HDL specific binding to different subcellular organelles as a function of time

^{125}I -HDL ($1.1 \mu\text{g}$ of ^{125}I -HDL protein/ml, 400 c.p.m./ng of HDL protein) was incubated with $200 \mu\text{g}$ of protein of different subcellular fractions (Δ , Golgi; $\circ$, rough endoplasmic reticulum; $\bullet$, lysosomes/mitochondria) in 0.3 ml of binding assay buffer at 37°C in the absence (total binding) or presence (non-specific binding) of unlabelled HDL at a final concentration of 1 mg/ml for the indicated times. The specific binding was calculated by subtracting the non-specific binding from total binding.

2). The HDL binding to rough endoplasmic reticulum, lysosomes/mitochondria and Golgi fractions was inhibited by Pronase (40–55%), in contrast with plasma membranes, where the HDL binding was insensitive to Pronase treatment. Thus the binding characteristics of HDL to different subcellular organelles were similar to those observed with plasma membranes (Hwang & Menon, 1983), except for the sensitivity to Pronase treatment.

Effect of hCG treatment on specific binding of ^{125}I -HDL to various subcellular organelles

After characterization of HDL receptors associated with different subcellular organelles, attempts were made to determine whether induction of HDL receptors by hCG injection involves redistribution of receptors in such a way that the increase in plasma-membrane receptors is paralleled by a decrease in the intracellular receptors. To examine this possibility, the HDL receptors were assayed in different cellular organelles isolated from both saline- and hCG-treated rats. The results of the experiment, shown in Fig. 3, indicate that hCG administration increased HDL binding in all subcellular fractions. When the binding data (Fig. 3) were analysed by the method of Scatchard, it was apparent that the increase in binding seen after hCG injection was due to an increase in binding capacity (N_B) rather than affinity (K_d). The pattern of increase in ^{125}I -HDL binding activity in all fractions suggests that the hCG-induced increase in HDL binding is not due to redistribution of the intracellular receptors. If hCG injection causes redistribution of receptors, then there would be a diminution of binding activity in intracellular organelles and a concomitant increase in plasma-membrane binding. The results clearly indicate that hCG treatment results in an increase in total binding activity, and this increase was distributed throughout all the organelles. This observation suggests that hCG causes an increase in total binding, probably involving new receptor synthesis or activation of receptors from previously unavailable forms.

Effects of the inhibition of protein synthesis and of the glycosylation reaction on lipoprotein-receptor induction

Since the experiments described in the previous section suggested that hCG stimulated the synthesis of new receptors, further studies were carried out to examine this possibility, by using cycloheximide, an inhibitor of protein synthesis. The initial dose of cycloheximide chosen for this experiment was 2 mg/kg body wt. At this dose, the incorporation of $[^3\text{H}]$ leucine into trichloroacetic acid-precipitable protein was inhibited by 60–70% at 120 min when measured in the isolated ovaries. The time course of ^{125}I -HDL binding in cycloheximide-pretreated and hCG-injected rat ovarian membranes was then compared with that of animals treated with hCG alone. As shown in Fig. 4, pretreatment with cycloheximide abolished the hCG-induced increase in HDL receptors. The inhibitor had no effect on HDL receptors in saline-treated controls. Membranes isolated from animals treated with cycloheximide before hCG exhibited HDL binding comparable with that seen in the saline-injected group. As expected, hCG treatment produced a time-dependent increase in ^{125}I -HDL binding. At 10 h after hCG injection, when the induction of lipoprotein receptors reached a maximum, pretreatment with cycloheximide abolished the hCG response. This experiment suggests that a protein-synthesis-dependent process is involved in the up-regulation of lipoprotein receptors after hCG treatment. Analysis of ^{125}I -HDL binding by the method of Scatchard in cycloheximide-, hCG- and saline-treated rat ovarian plasma membranes revealed that the K_d values for the three groups remained identical (Fig. 5). However, cycloheximide treatment before hCG injection decreased the number of binding sites as compared with the hCG-treated control. Whereas K_d for

Fig. 3. Effect of hCG injection of ^{125}I -HDL binding to Golgi (a), rough endoplasmic reticulum (b), lysosomes/mitochondria (c) and plasma membranes (d), depicted as Scatchard plots

Rats were killed 12 h after subcutaneous injection with saline (control, ●) or 25 i.u. of hCG (○), and different organelles were prepared from the isolated ovaries as described in the text. Samples (100 μg of protein) of each subcellular organelle from both control and hCG-treated groups were incubated with various concentrations of ^{125}I -HDL (2–80 μg/ml, 40 c.p.m./ng of HDL protein) in a total volume of 0.45 ml of binding-assay solution at 37 °C for 90 min (total binding). For estimation of non-specific binding, unlabelled HDL was added at a concentration of 6 mg/ml. The specific binding was calculated by subtracting non-specific binding from the total binding. The insets show the binding as a function of ^{125}I -HDL concentrations. The equilibrium dissociation constants (K_d) for HDL binding to lysosomes/mitochondria, Golgi, rough endoplasmic reticulum and plasma-membrane fractions are 13.2, 18.5, 21.9 and 17.3 μg of HDL protein/ml respectively for the hCG-treated group. The corresponding values for saline-treated groups are 11.6, 18, 17.8 and 18.6 μg of HDL protein/ml. The binding capacities (N_B) for the above four fractions are 7.7, 11.4, 12.1 and 7.0 μg of HDL protein/mg of protein respectively for the hCG-treated group, and 2.45, 4.7, 5.5 and 3.4 μg of HDL protein/mg of protein for the saline-treated groups.

^{125}I -HDL binding remained the same for all three groups (17.4, 19.2 and 18.6 μg/ml for hCG, saline and cycloheximide plus hCG respectively), the binding capacity for HDL was decreased after cycloheximide pretreatment, suggesting that the decrease in HDL binding is not due to a change in the binding affinity, but rather to a decrease in the number of receptors.

Since membrane receptors are generally thought to be glycoprotein in nature, the effect of pretreatment with tunicamycin, an inhibitor of the *N*-linked glycosylation reaction, on this induction process was also investigated.

Treatment with tunicamycin *in vivo* at doses of 2, 4, 6 and 10 mg/kg body wt. did not abolish HDL-receptor induction when the binding was measured in isolated membranes (Table 3). When the glycosylation reaction was measured by the incorporation *in vitro* of [^3H]glucosamine into proteins, animals treated with tunicamycin (10 mg/kg body wt.) showed 56–68% and 43–50% inhibition at 4 h and 12 h respectively (Table 4). In addition, no change in glycosylation activity was observed after hCG treatment when compared with the saline-treated control ovary (Table 4).

Table 2. Effect of EDTA, CaCl₂, NaCl and Pronase treatment on ¹²⁵I-HDL specific binding to various subcellular fractions

Samples (100–150 µg of protein) of organelles were incubated for 2 h at 37 °C with ¹²⁵I-HDL (3.0 µg/ml; 350 c.p.m./ng of protein) in the absence and the presence of the indicated concentration of ions and EDTA in 0.3 ml of assay buffer without CaCl₂. For experiments with Ca²⁺ or EDTA, the subcellular organelles were prepared from hCG-treated rat ovary in the presence of 1 mM-EDTA or 1 mM-Ca²⁺ respectively. The effect of Pronase was tested by preincubating different subcellular fractions with indicated concentrations of Pronase at 37 °C for 10 min, followed by stopping the reaction by the addition of 1 vol. of 20% bovine serum albumin. A sample (100 µl) was used for ¹²⁵I-HDL binding assay. For estimation of non-specific binding, unlabelled HDL was added at a final concentration of 1.0 mg/ml. Specific binding was expressed as µg of ¹²⁵I-HDL bound/mg of protein of the subcellular fractions. Values are means ± S.D., *n* = 4; N.D., not determined.

Addition	Fraction . . .	¹²⁵ I-HDL bound (µg/mg of protein)			
		Plasma membranes	Lysosomes/mitochondria	Golgi	Rough endoplasmic reticulum
EDTA	None	0.50 ± 0.04	0.60 ± 0.014	0.75 ± 0.03	0.60 ± 0.07
	2 mM	0.51 ± 0.02	0.62 ± 0.018	0.72 ± 0.04	0.66 ± 0.05
	4 mM	0.47 ± 0.046	0.55 ± 0.018	0.68 ± 0.03	0.57 ± 0.08
CaCl ₂	None	0.55 ± 0.07	0.72 ± 0.02	0.70 ± 0.03	0.55 ± 0.045
	4 mM	0.57 ± 0.05	0.69 ± 0.005	0.73 ± 0.05	0.58 ± 0.06
NaCl	None	0.52 ± 0.06	0.78 ± 0.04	0.67 ± 0.04	0.50 ± 0.02
	1 M	0.50 ± 0.04	0.72 ± 0.07	0.72 ± 0.07	0.52 ± 0.04
Pronase	None	0.43 ± 0.017	0.61 ± 0.06	0.54 ± 0.07	0.47 ± 0.07
	20 µg/ml	N.D.	0.40 ± 0.006	0.35 ± 0.008	0.26 ± 0.013
	40 µg/ml	0.39 ± 0.02	0.29 ± 0.004	0.33 ± 0.009	0.24 ± 0.013

Fig. 4. Time course of the effect of cycloheximide treatment on hCG-induced increase in HDL receptors in rat ovarian plasma membranes

Cycloheximide was injected at a dose of 2 mg/kg body wt. 1 h before hCG injection. Preparation of plasma membranes from the isolated ovaries of rats injected with cycloheximide plus hCG (□), hCG (▨) and saline (▤), and measurement of the ¹²⁵I-HDL specific binding, were as described in the Materials and methods section.

Fig. 5. Scatchard-plot analysis of ^{125}I -HDL specific binding to plasma membranes prepared from ovaries of rats injected with cycloheximide plus hCG (Δ), hCG ($\circ$) and saline ($\bullet$)

Samples (150 μg) of membrane protein were incubated with various concentrations of ^{125}I -HDL (2–80 μg of ^{125}I -HDL protein/ml; 40 c.p.m./ng of HDL protein) in a total volume of 0.45 ml of binding-assay buffer at 37 °C for 90 min (total binding). For estimation of non-specific binding, unlabelled HDL was added at a final concentration of 6 mg/ml. The inset shows the binding data as a function of ^{125}I -HDL concentration.

DISCUSSION

Our previous studies have shown that injection of pseudopregnant rats with hCG results in a dose- and time-dependent increase in HDL receptors in luteal cell membranes (Hwang & Menon, 1983). This increased receptor activity is necessary to meet the increased demand for cholesterol needed in steroidogenesis. The present study was undertaken to determine the mechanism by which hCG increased HDL receptors in rat ovaries. Several possibilities were considered which may explain this increase. The first possibility is the redistribution of the intracellular receptor pool by translocation of receptors from the intracellular pool to plasma membranes. However, the results showed that the binding of HDL to subcellular organelles was higher in hCG-treated rat ovary than in saline-treated ovary (Fig. 3), and there was no clear alteration in the pattern of distribution of binding activities after hormone

Table 3. Effect of tunicamycin on HDL binding to rat ovarian membranes

Preparation of plasma membranes and the ^{125}I -HDL binding assays were performed as described in the Materials and methods section. Each value represents mean $\pm$ S.D., $n = 4$. Tunicamycin was injected at a dose of 6* or 10†mg/kg body wt. 1 h before hCG injection.

Treatment	^{125}I -HDL bound (ng/mg of membrane protein)
Saline	37.3 $\pm$ 3.0
hCG	80.5 $\pm$ 8.0
Tunicamycin* + hCG	78.5 $\pm$ 8.2
Tunicamycin† + hCG	81.5 $\pm$ 9.5

Table 4. Effect of tunicamycin treatment on incorporation of [^3H]glucosamine into the ovarian proteins *in vitro*

Tunicamycin was injected at a dose of 10 mg/kg body wt.

Treatment	Duration of treatment	^3H Glucosamine incorporated (pmol/mg of ovarian protein)	
		Expt. 1	Expt. 2
Saline	10 h	0.140 $\pm$ 0.012	0.169 $\pm$ 0.019
hCG	10 h	0.142 $\pm$ 0.017	0.175 $\pm$ 0.015
Tunicamycin	10 h	0.090 $\pm$ 0.006	0.085 $\pm$ 0.007
Tunicamycin	4 h	0.067 $\pm$ 0.005	0.057 $\pm$ 0.007

treatment. This ruled out the possibility of redistribution of the intracellular receptor pool as being responsible for hCG-induced enhancement of lipoprotein receptors.

Another finding was that, in addition to plasma membranes, a significant amount of HDL binding was observed in other subcellular organelles. The data presented on the differences and similarities in properties between HDL-binding sites in plasma membrane and in intracellular organelles shows that specific binding sites are present in these organelles. The organelles were free of contamination from plasma membranes, as evidenced by the relative absence of the plasma-membrane marker enzyme, 5'-nucleotidase. Although the presence of HDL-binding sites in plasma membranes is consistent with its function, the significance of its presence in different intracellular organelles is not at present understood. One may visualize the presence of HDL-binding sites in rough endoplasmic reticulum, Golgi and lysosome/mitochondrial fraction as their biosynthetic, maturational and degradative itinerary.

Since the binding of labelled HDL in various subcellular organelles is an intrinsic activity that is increased after hCG injection, the question of whether this increased binding activity is due to increased protein synthesis was addressed. Cycloheximide, a protein-synthesis inhibitor, when injected before hCG administration, prevented the induction of HDL receptors caused by hCG (Fig. 4). This clearly indicates that the ovarian response to hCG by increasing lipoprotein receptors is dependent on the synthesis of new protein. Cycloheximide treatment, however, did not inhibit the binding of hCG to plasma-membrane receptors. Thus the abolition of the hCG effect on lipoprotein receptors is not a result of the inhibitor acting to prevent hCG from exerting its effect on the ovary. The present results thus clearly demonstrate the requirement for protein synthesis. The Scatchard analysis of the HDL binding before and after cycloheximide treatment revealed that the decreased binding observed after the treatment was due to a change in the receptor number rather than in binding affinity (Fig. 5).

Since the lipoprotein receptors have been shown to be glycoprotein in nature, the effect of pretreatment with tunicamycin, an inhibitor of *N*-linked glycosylation, on the hCG-stimulated HDL-receptor induction was also tested. Pretreatment with tunicamycin before hCG injection did not prevent the hCG-induced up-regulation of lipoprotein receptors (Table 3). Glycosylation activity, when measured in both hCG- and saline-treated rat ovary, remained unchanged (Table 4). However, the functional significance of glycosylation of lipoprotein receptors is not known. The present results of lipoprotein-binding studies performed after tunicamycin treatment are different from that reported with fibroblast cultures, where treatment with tunicamycin has been shown to result in a diminution of available LDL receptors (Chatterjee *et al.*, 1981). A major difference in experimental methodologies should be noted, however. In our studies tunicamycin was injected into the animals, followed by binding studies using isolated membranes, whereas studies with the fibroblasts involved treatments *in vitro* (Chatterjee *et al.*, 1981). Although receptor function in some systems seems to be impaired in the absence of *N*-linked oligosaccharide, there have been instances in which *N*-linked glycosylation is shown to be not essential for receptor binding (Ronnet & Lane, 1981; Soderquist & Carpenter, 1984; Gilman *et al.*, 1984; Breitfeld *et al.*, 1984).

The molecular mechanism by which hCG induces lipoprotein receptors is not understood. From the results of the cycloheximide experiment, it appears that protein synthesis *de novo* is required for this induction process. This receptor induction cannot be attributed to exposure of cryptic receptors or to simple recycling of receptors, since these processes are thought to be independent of protein synthesis (Brown *et al.*, 1983). Receptor induction also does not appear to be due to increased receptor turnover. If this were true, then inhibition of protein synthesis may result in an accumulation of cell-surface receptors, as has been shown for insulin receptors (Kadle *et al.*, 1983), rather than an inhibition, as observed in the present work. Studies *in vitro* with cultured human granulosa cells (Golos & Strauss, 1985) have shown increased incorporation of [³⁵S]methionine into mature LDL receptor when the cells were treated with hCG and/or 8-bromo-cyclic AMP. These results are

consistent with the present studies *in vivo*. In conclusion, it appears that synthesis *de novo* of HDL receptors, but not recycling or redistribution, is responsible for the increase in lipoprotein binding observed after hCG treatment.

This work was supported by National Institute of Health Grant HD-06656. We are also grateful to Dr. Jaulang Hwang for discussions and to Ms. Melissa Hyma for secretarial assistance.

REFERENCES

- Anderson, J. & Dietschy, J. (1978) *J. Biol. Chem.* **253**, 9024–9032
- Baker, H. N., Delahunty, T., Gotto, A. M. & Jackson, R. L. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 3631–3634
- Breitfeld, P. P., Rup, O. & Schwartz, A. L. (1984) *J. Biol. Chem.* **259**, 10414–10421
- Brown, M. S., Anderson, R. G. H. & Goldstein, J. L. (1983) *Cell* **32**, 663–667
- Chatterjee, S., Selkeski, C. G. & Kwitorovich, P. O. (1981) *Eur. J. Biochem.* **120**, 435–441
- Chen, Y. I., Kraemer, F. B. & Raeven, G. M. (1980) *J. Biol. Chem.* **255**, 9162–9167
- Cooperstein, S. J. & Lazarow, A. (1951) *J. Biol. Chem.* **189**, 665–670
- Ehrenreich, J. H., Bergeron, J. J. M., Siekevitz, P. & Palade, G. E. (1973) *J. Cell Biol.* **59**, 45–62
- Emmelot, P. & Bos, C. T. (1966) *Biochim. Biophys. Acta* **120**, 369–382
- Fiske, G. H. & Subbarow, Y. (1925) *J. Biol. Chem.* **66**, 375–400
- Ghosh, D. K. & Menon, K. M. J. (1986) *Biochem. Biophys. Res. Commun.* **134**, 1006–1014
- Gilman, P. B., Keane, P. & Martinez, J. (1984) *J. Biol. Chem.* **259**, 3248–3253
- Glomset, J. A. (1968) *J. Lipid Res.* **9**, 155–167
- Goldstein, J. L. & Brown, M. (1977) *Annu. Rev. Biochem.* **46**, 897–930
- Golos, T. G. & Strauss, J. F. (1985) *J. Biol. Chem.* **260**, 14399–14402
- Gospadarowicz, D. (1973) *J. Biol. Chem.* **248**, 5050–5056
- Gwynne, J. T. & Hess, B. (1980) *J. Biol. Chem.* **255**, 10875–10883
- Havel, R. J., Eder, H. A. & Bragdon, J. G. (1955) *J. Clin. Invest.* **34**, 1345–1353
- Hofmann, D., Ji, T. H., Miller, B. & Steiner, D. F. (1981) *J. Supramol. Struct. Cell. Biochem.* **15**, 1–13
- Hwang, J. & Menon, K. M. J. (1983) *J. Biol. Chem.* **258**, 8020–8027
- Hwang, J. & Menon, K. M. J. (1985) *J. Biol. Chem.* **260**, 5660–5668
- Kadle, R., Kolter, V. G., Raizda, M. K. & Fellows, R. E. (1983) *J. Biol. Chem.* **258**, 13116–13119
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Lux, S. E., John, K. M. & Brewer, H. B. (1972a) *J. Biol. Chem.* **247**, 7510–7518
- Lux, S. E., John, K. M., Roman, R. & Brewer, H. B. (1972b) *J. Biol. Chem.* **247**, 7519–7527
- Martin, J. B. & Doty, D. M. (1949) *Anal. Chem.* **71**, 965–967
- McFarlane, A. S. (1958) *Nature (London)* **182**, 53
- Morre, D. J. (1971) *Methods Enzymol.* **22**, 130–148
- Parlow, A. F. (1958) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **17**, 402
- Rajendran, K. G., Hwang, J. & Menon, K. M. J. (1983) *Endocrinology (Baltimore)* **112**, 1746–1753
- Ronnet, G. V. & Lane, M. D. (1981) *J. Biol. Chem.* **256**, 4704–4707

- Rosen, C. M., Chin, C. H., Fung, C. & Rubin, C. S. (1979) *J. Cell. Physiol.* **99**, 37–42
- Savard, K., Marsch, J. & Rice, B. (1965) *Recent. Prog. Horm. Res.* **21**, 285–365
- Scatchard, G. (1949) *Ann. N.Y. Acad. Sci.* **51**, 660–672
- Schneider, W. J., Basu, S. K., Mcphaul, M. J., Goldstein, J. L. & Brown, M. S. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 5577–5581
- Schuler, L. A., Langenberg, K. K., Gwynne, J. T. & Strauss, J. F. (1981) *Biochim. Biophys. Acta* **664**, 583–601
- Shepherd, J., Bedford, D. K. & Morgan, H. G. (1976) *Clin. Chim. Acta* **66**, 97–109
- Soderquist, A. M. & Carpenter, G. (1984) *J. Biol. Chem.* **259**, 12586–12594
- Williams, C. H. & Kamin, H. (1962) *J. Biol. Chem.* **237**, 587–597

Received 29 September 1986/29 January 1987; accepted 23 February 1987