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IDENTIFICATION OF GONADOTROPIN INDUCIBLE, HIGH DENSITY LIPOPROTEIN RECEPTORS IN THE SOLUBILIZED MEMBRANES FROM RAT OVARY

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Specific receptors for high density lipoproteins (HDL_3) were solubilized from membranes of rat corpus luteum using different detergents. Among the detergents tested, octyl-β-D-glucoside (40 mM) was most effective with respect to recovery of binding activity. The receptor activity released into 105,000 x g supernatant, can be assayed directly or with the precipitate obtained after dilution of the soluble supernatant. The ^{125}I-HDL_3 binding activity in the precipitated extract was linear with time, proportional to the amount of protein in the incubation mixture and saturable with increasing concentrations of ^{125}I-HDL_3. The solubilized receptor has an equilibrium dissociation constant (Kd) of 21.2 μg/ml and the binding activity was insensitive to Ca^{+2}, EDTA and NaCl. These properties are similar to the membrane associated receptor. Administration of gonadotropin induced the HDL_3 receptor in the solubilized membranes, suggesting that this receptor represents the physiologic receptor in the ovary.

High affinity binding sites for high density lipoprotein (HDL_3) have been characterized in adrenal, testis and luteal cells from rat (1-7). Recently we have demonstrated the specific binding of HDL_3 and apolipoproteins A I and A II complexed with DMPC vesicles to HDL_3 receptors in rat luteal cell membranes and stimulation of this binding by *in vivo* treatment of rats with gonadotropin (8, 9). Although low density lipoproteins (LDL) receptors have been well characterized in several systems, similar information about HDL_3 receptors is not available. The luteal cell is an ideal model to study the HDL_3 receptor for several reasons. First, its existence has been well characterized. Second, after binding, HDL_3 derived cholesterol has been shown to be utilized for progesterone production and finally HDL_3 receptors are found to be induced by gonadotropin injection. In this communication, we show evidence that solubilized membrane fractions from highly luteinized rat ovaries contain a protein fraction which recognizes HDL_3 devoid of E apolipoprotein and retain all the binding properties that are observed with native membranes.

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MATERIALS AND METHODS

Human HDL_3 (d 1.125 - 1.21 g/ml) was isolated from normal human blood by ultracentrifugation after adjusting the density with KBr as described by Havel et al. (10) and further purified as described earlier (8). Iodination of HDL_3 was performed as described previously (8, 11, 12).

Twenty-one day old Sprague-Dawley female rats made pseudopregnant by treatment with 50 IU PMSG followed 56 h later by 25 IU of hCG was the model system employed. Following hCG injection, highly luteinized ovaries were collected on day 5. In some experiments where induction of receptors was tested, rats received 25 IU of hCG 12 h before sacrifice. Membranes were prepared from the isolated ovaries following homogenization at 4°C in a buffer containing 25 mM Tris-HCl, 0.25 M sucrose, 1 mM $CaCl_2$, pH 7.4, by the procedure described by Gospodarowicz (13). The relative purification of membrane preparations was ascertained by assaying 5'-nucleotidase activities (14). Membranes were than resuspended at a protein concentration of 5.0 - 7.5 mg/ml in a buffer containing 125 mM Tris-HCl, 0.25 M sucrose, 1 mM $CaCl_2$, and 0.15 M KCl, adjusted to pH 7.4 (solubilization buffer) and varying concentrations of detergents (octyl-β-D-glucoside, CHAPS [3-[(3-cholamido propyl)dimelthyl-ammonio]-1-propane sulfonate] or sodium cholate). The mixture was agitated by vortexing for 1 min followed by incubation for 30 to 45 min on ice with occassional stirring and then centrifuged at 105,000 x g for 60 min to separate the insoluble fraction (pellet) from the solubilized membrane fraction (supernatant).

Precepitation by dilution : The concentration of octyl-β-D-glucoside in the solubilized supernatant was reduced to 4 mM by the addition of a buffer containing 50 mM Tris-HCl, 1 mM $CaCl_2$, pH 7.4 and the precipitate was collected by centrifugation at 105,000 x g for 1 h. The precipitate was resuspended in 25 mM Tris-HCl, 0.25 M sucrose, 1 mM $caCl_2$, pH 7.4 and used for measurement of HDL_3 receptor activity.

Binding assays : All binding assays were performed in glass tubes (12 x 75 mm) which had been precoated overnight with 10% BSA. The incubation mixture contained 300,000 cpm ^{125}I-HDL_3 (specific activity 350-500 cpm/ng protein), solubilized membranes (60 to 100 μg protein) in a final volume of 0.3 ml adjusted with buffer A (25 mM Tris-Hcl, 1 mM $CaCl_2$ and 0.1% BSA, pH-7.4). All incubations were performed at 37°C for 90 min. Nonspecific binding was determined by including 600 μg of unlabeled HDL_3 in the incubation mixture. After incubation, free [^{125}I]HDL_3 was separated from the bound ligand by filtration through 0.45 μ milipore filters followed by washing with buffer A according to the procedure described by Schneider et al (15). The filter papers were assayed for ^{125}I radioactivity by counting in an automatic gamma counter. The specific binding represents the difference between total and nonspecific binding. Protein was measured according to Lowry et. al (16).

RESULTS

Attempts were made to solubilize HDL_3 receptors from plasma membranes of rat luteal cells using various detergents. The ideal detergent saught to solubilize the receptors was one possessing the following properties: high critical micelle concentration, low micelle molecular weight and non-interference in the assay of receptor activity. Of all the detergents tested, octyl-β-D-glucoside, CHAPS and sodium cholate yielded solubilization with appreciable recovery of binding activity. As shown in Fig. 1, CHAPS and sodium cholate were used at concentrations ranging from 0.75 to 1.5% W/V and octyl-β-D-glucoside concentration varied from 30 to 60 mM. Of the three detergents tested, octyl-β-D-glucoside yielded optimal results at 40 mM concentration with 88% recovery of the HDL_3 binding activity in the soluble

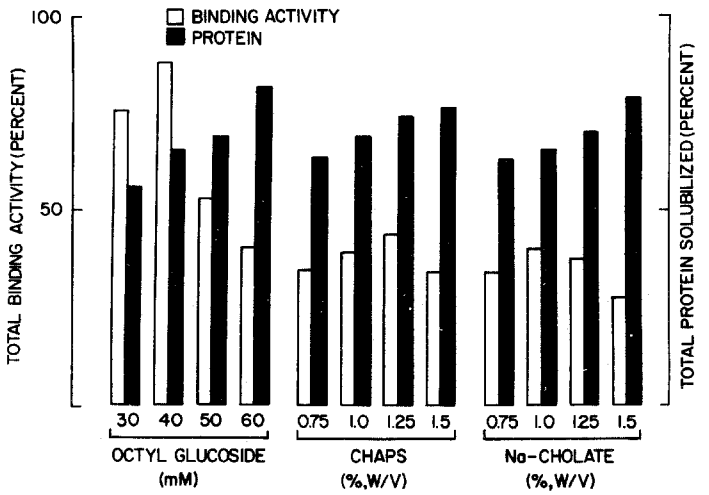

Fig. 1. Comparison of the solubilization of HDL_3 receptor activity in the presence of increasing concentrations of octyl-β-D-glucoside, CHAPS and Sodium cholate. The total binding activity in the membranes refers to 100% which is equivilent to 2.8 ug ^{125}I-HDL_3 bound per 5 mg protein at 37°C for 90 min after subtraction of non-specific binding. In all cases HDL_3 binding activities in aliquots of the detergent soluble fractions obtained after centrifugation at 105,000 x g were compared.

supernatant and 1.5 fold increase in specific activity. At 1.25% (W/V) concentration of CHAPS and 1% (W/V) sodium cholate, the yield of the soluble receptor activity was 43%. Thus 40 mM octyl-β-D-glucoside was selected as the detergent of choice. In the binding assay, the presence of octyl-β-D-glucoside did not influence the extent of nonspecific binding. The solubilized HDL_3 receptor activity was not displaced by LDL or VLDL, but completely inhibited by unlabeled HDL_3 (data not shown). The effect of octyl-β-D-glucoside concentration on the solubilization of HDL_3 receptor activity at two different membrane concentrations is shown in Fig. 2A. The binding activity increased with increase in detergent concentration, with maximum activity at 40 mM. At higher concentration, octyl-β-D-glucoside produced inhibitory effect. The binding data expressed as a function of detergent to protein ratio is shown in Fig. 2B. It can be seen that the detergent to protein ratio was slightly higher at protein concentration of 5 mg/ml than that observed with 7.5 mg/ml membrane protein.

The yield of protein and the binding activity after solubilization with 40 mM octyl-β-D-glucoside is shown in Table 1. The yield of soluble receptor was 84% with 1.5 fold increase in specific activity when the binding activity was tested directly using the solubilized supernatant. When the HDL_3 receptor activity from the

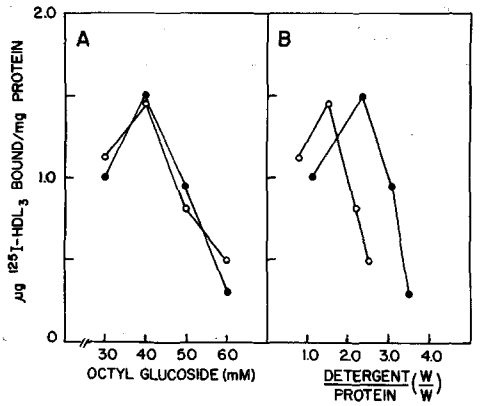

Fig. 2. Solubilization of ^{125}I-HDL$_3$ binding activity from rat ovarian membranes by octyl-β-D-glucoside at two different initial protein concentrations as (A) a function of detergent concentration and (B) a function of the ratio of detergent to protein. o---o, 7.5 mg/ml and ●---●, 5.0 mg/ml.

supernatant fraction was precipitated after dilution with buffer to decrease the detergent concentration below its critical miceller concentration (25 mM for octyl-β-D-glucoside), the yield of the total binding activity remained essentially unchanged, but the specific activity increased 4 fold. The precipitate obtained when the solubilized membrane extract was diluted and centrifuged at 105,000 x g is referred to as the precipitated extract.

Table 1. ^{125}I-HDL$_3$ binding to plasma membranes, solubilized supernatants and its precipitated extract after dilution

Treatment	Total Protein (mg)	Protein Yield (%)	Specific Binding[a]	Total Binding[b] activity (μg ^{125}I-HDL$_3$ bound)	Yield Of binding activity (%)
Plasma Membranes	7.5	100	0.5 ± 0.04	3.75 ± 0.3	100
Solubilized Supernatant	4.5	60	0.70 ± 0.06	3.15 ± 0.2	84.0
Precipitated Extract	1.37	18	2.20 ± 0.12	3.014 ± 0.2	80.3

One gram of rat corpora lutea was the starting material. Preparation of plasma membranes, solubilization procedure and precipitation of the solubilized receptor, and binding assay are described under "Materials and Methods". Each value represents mean ± standard deviation calculated from three experiments.

[a]Specific binding represents μg ^{125}I-HDL$_3$ bound per mg protein at 37°C for 90 min after subtracting the non-specific binding.

[b]Total binding activity was calculated by multiplying specific binding activity with total protein content.

Table 2. Effect of EDTA $CaCl_2$ and NaCl, on ^{125}I-HDL_3 binding activity in precipitated extract from solubilized membranes

Experiment	^{125}I-HDL_3 Specific binding[a]
Control	0.92 ± 0.03
EDTA 2 mM	0.90 ± 0.02
EDTA 4 mM	0.85 ± 0.06
Control	0.82 ± 0.05
$CaCl_2$ 2 mM	0.85 ± 0.07
$CaCl_2$ 4 mM	0.79 ± 0.08
Control	0.75 ± 0.06
NaCl 0.5 mM	0.85 ± 0.05
NaCl 1.0 mM	0.77 ± 0.06

To determine the effect of Ca^{2+} membranes were isolated in the presence of 1 mM EDTA. When the effect of EDTA was tested on the binding activity, the membranes were isolated in presence of 1 mM $CaCl_2$. The binding activity was determined as described in the text.

[a]Specific binding refers to μg ^{125}I-HDL_3 bound per mg protein at 37°C for 90 min after subtracting the non-specific binding. Each value represents mean ± standard deviation calculated from three experiments.

Since the HDL_3 receptor has been well characterized in the membrane fraction with respect to protease sensitivity, divalent cation requirement and apparent Kd (8), attempts were made to determine whether the same properties were exhibited by the solubilized receptors when assayed in the precipitated extract. As expected, the solubilized HDL_3 receptor activity was insensitive to EDTA, Ca^{+2} and NaCl (Table 2). When the receptor activity was exposed to pronase and trypsin up to 10 min, no inhibition of ^{125}I-HDL_3 binding was observed. Exposure for longer periods of time (30 min), however, produced slight inhibition (Table 3). The protease sensitivity seen with the soluble membrane fractions may be due to the loss of lipids from the membranes (and the resultant loss of membrane integrity) thereby exposing the receptor protein to proteotytic digestion. The receptor activity was linear with time and proportional to the amount of protein in the binding assay (Fig. 3A and B).

The binding of ^{125}I-HDL_3 to the precipitated extract or native membranes exhibited comparable equilibrium dissociation constants when analyzed by the method

Table 3. Effect of Pronase and Trypsin on ^{125}I-HDL_3 binding activity in precipitated extract prepared from solubilized membranes

Experiment[a]	^{125}I-HDL_3 specific binding[b]	
	10 min	30 min
Control	0.69 ± 0.04	0.72 ± 0.02
Pronase 20 μg/ml	0.75 ± 0.03	0.60 ± 0.04
Pronase 40 μg/ml	0.72 ± 0.06	0.55 ± 0.06
Trypsin 50 μg/ml	0.72 ± 0.04	0.62 ± 0.03
Trypsin 100 μg/ml	0.63 ± 0.03	0.49 ± 0.03

[a]Aliquots of precipitated extract (0.5 to 0.75 mg protein) were preincubated with indicated concentration of pronase and trypsin at 37°C for 10 and 30 min. The reaction was stopped by the addition of 1 volume of 20% BSA and aliquots (100 ul) were incubated with ^{125}I-HDL_3 (1.3 μg/ml; 350 cpm/ng protein) in binding assay solution (0.3 ml) for 90 min at 37°C in absence (total) and presence of unlabeled HDL_3 (1.0 mg/ml; non-specific binding). Each value represents mean ± standard deviation calculated from three experiments.

[b]Specific binding represents μg ^{125}I-HDL_3 bound per mg protein at 37°C for 90 min after subtracting the non-specific binding.

of Scatchard (17) (21.2 μg/ml for precipitated extract and 17.3 μg/ml for native membranes, Fig. 4), whereas the specific binding increased after solubulization (3.6 μg bound/mg protein for native membranes and 12.0 μg/mg protein for precipitated extract). Our previous studies (8) have shown that hCG treatment induces the

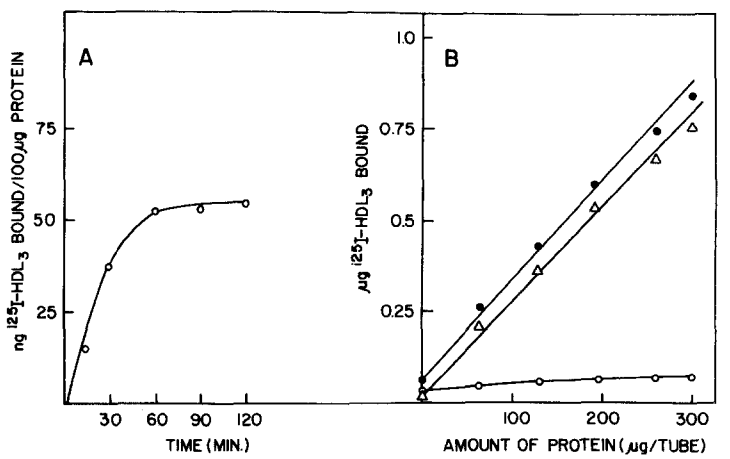

Fig. 3. The binding of ^{125}I-HDL_3 to the precipitated extract derived from solubilized supernatant as a function of (A) time and (B) amount of added protein. The amount of ^{125}I-HDL_3 used in the time course experiment (A) was 1.3 μg/ml, while the ligand concentration in panel B was 7.5 μg/ml. ● -total, △ -specifc and ○ -non-specific binding.

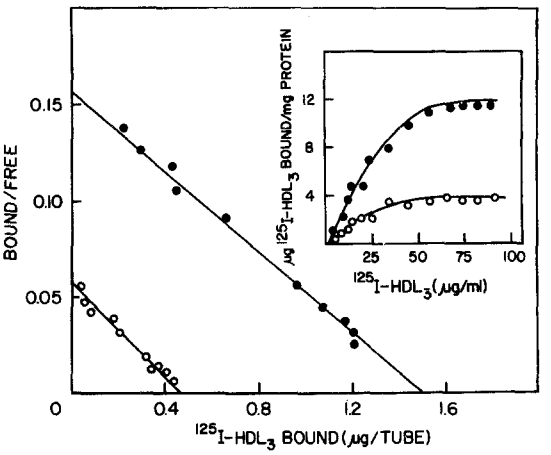

Fig. 4. Analysis of ^{125}I-HDL$_3$ specific binding to plasma membranes and precipitated extract of the solubilized membranes. Aliquots (125 ug) of both plasma membranes and precipitated extract were incubated with varying concentrations of ^{125}I-HDL$_3$ (from 2 to 90 μg ^{125}I-HDL$_3$ protein/ml, 10 cpm/ng of HDL$_3$ protein) in a total volume of 0.45 ml of binding assay buffer at 37°C for 90 min (total binding). For estimation of nonspecific binding, unlabeled HDL$_3$ was added at a final concentration of 6 mg/ml. The insets show the binding data as a function of ^{125}I-HDL$_3$ concentration. ○-plasma membranes, ● -precipitated extract.

receptors for HDL$_3$ in the rat corpus luteum. We therefore examined whether the inducibility of receptors by treatment with hCG can be demonstrated in the solubilized membrane preparation also. The amount of ^{125}I-HDL$_3$ bound per mg of

Table 4. ^{125}I-HDL$_3$ binding activities in plasma membrane and its solubilized preparation from both hCG and saline treated rat ovary

Experiment	Total Protein (mg)	^{125}I-HDL$_3$ Specific binding[a]	Total Binding Activity[b] (μg ^{125}I-HDL$_3$ bound)
Plasma membranes:			
hCG	7.5	0.8 ± 0.02	6.0 ± 0.15
Saline	7.5	0.4 ± 0.012	3.4 ± 0.89
Solubilized Supernatant:			
hCG	4.7	1.15 ± 0.07	5.4 ± 0.33
Saline	4.4	0.7 ± 0.04	2.9 ± 0.18

Membranes (7.5 mg protein) from both hCG (25 IU) and saline treated rat ovaries were solubilized using 40 mM octyl-β-D-glucoside in a total volume of 1 ml.

[a]Specific binding of ^{125}I-HDL$_3$ determined as described under "Materials and Methods" and represents μg ^{125}I-HDL$_3$ bound per mg protein at 37°C for 90 min after subtracting non-specific binding. Each value represents mean ± standard deviation calculated from three experiments.

[b]Total binding activity was calculated by multiplying specific binding activity with total protein content.

membrane protein was about 2 fold higher in intact membranes prepared from the hCG treated rat ovary than those membranes prepared from saline treated rat ovary. The same relative difference was observed when the membranes from the two treatment groups were solubilized and assayed for ^{125}I-HDL$_3$ receptor activity (Table 4).

DISCUSSION

The present communication describes the successful solubilization of ^{125}I-HDL$_3$ receptor from luteinized rat ovary. In the present experiments the properties of ^{125}I-HDL$_3$ binding activity studied in the solubilized preparation reflected the behaviour of native HDL$_3$ receptor of intact membranes (8) such as the *in vitro* requirments for the binding activity and the inducibility by hormone. For these reasons, we believe that the ^{125}I-HDL$_3$ binding activity that is solubilized with octyl-β-D-glucoside actually represents the physiologic HDL$_3$ receptor. Rat luteal cells are a particularly useful model to study HDL$_3$ receptors since the cholesterol derived from HDL$_3$ has been shown to be used for progesterone production and this process is hormonally regulated (6, 8).

Solubilization of HDL$_3$ receptor is the first step towards purification of the receptor activity and this would further delineate the role of HDL$_3$ receptor in cholesterol transport in the ovary. This situation is different from non-endocrine tissue such as endothelial cells (18) where the existence of specific HDL$_3$ receptors has been questioned. However, it has been shown that in the endocrine gland HDL$_3$-associated cholesterol transport mediated by HDL$_3$ receptors is critical for hormone regulated steroid synthesis (2,4,6,7). Further purification of the receptors will shed light on the elucidation of HDL pathway which is presently little understood.

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