Solubilization of the 17α-ethinyl estradiol-stimulated low density lipoprotein receptor of male rat liver

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Abstract Pharmacological doses of 17α-ethinyl estradiol induce a low density lipoprotein (LDL) receptor in the liver of male rats. Our aim was to solubilize this receptor. Isolated liver membranes (8,000–100,000 *g* fraction) from male rats treated with 17α-ethinyl estradiol and from control rats were solubilized in 1% (w/v) Triton X-100. Using Amberlite XAD-2, more than 90% of the detergent was then removed. Liposomes were prepared by precipitating the solubilized proteins with acetone in the presence of phosphatidylcholine. The receptor activity of these liposomes was assayed using human ^{125}I-labeled LDL. Filtration was used to separate bound from free ^{125}I-labeled LDL. The assay was optimized; 0.25 mM $CaCl_2$, 25 mM NaCl, pH 8.0, were chosen as the standard conditions. Binding of ^{125}I-labeled LDL was dependent on Ca^{2+}. Liposomes containing solubilized membrane proteins from treated rats displayed Ca^{2+}-dependent binding which was 11 times higher than for control rats. The specific binding of ^{125}I-labeled LDL was saturable with a K_d = 18 μg/ml. ^{125}I-Labeled LDL was displaced by unlabeled lipoproteins containing apolipoproteins B and E and by dimyristoylphosphatidylcholine liposomes containing purified apolipoprotein E, but not by HDL_3. The binding was abolished by pronase and was inhibited by suramin. Ligand blotting with ^{125}I-labeled LDL revealed one band of protein with an apparent molecular weight of 133,000 daltons. These properties are characteristic of the low density lipoprotein receptor. — **Roach, P. D., and S-P. Noël.** Solubilization of the 17α-ethinyl estradiol-stimulated low density lipoprotein receptor of male rat liver. *J. Lipid Res.* 1985. **26:** 713–720.

Supplementary key words Triton X-100 • Amberlite XAD-2 • liposomes • soluble receptor assay • lipoproteins • apolipoproteins • pronase • suramin • ligand blotting

The liver plays a consequential role in cholesterol metabolism. It is only via this organ that cholesterol is effectively removed from the circulation and eliminated from the body. Hepatic receptors that mediate specific uptake of cholesterol-containing lipoproteins are, therefore, of prime importance.

The rat liver, which is particularly effective in removing chylomicron remnants (1) and very low density lipoprotein (VLDL) remnants (2) from the circulation, expresses a variety of lipoprotein receptors. A receptor that is specific for apolipoprotein E mediates the uptake of chylomicron remnants (3) and possibly VLDL remnants (4). An apolipoprotein A-1 or high density lipoprotein (HDL) receptor has been characterized in isolated plasma membranes (5) but the liver's contribution to HDL degradation is low (6). A low density lipoprotein (LDL) receptor has also been studied extensively (7–9) in male rats treated with 17α-ethinyl estradiol (17α-EE). This hormone-inducible receptor is very similar to the LDL receptor of human fibroblasts (10). It may contribute to the removal of LDL and remnant lipoproteins from the circulation but the uptake of LDL by the normal rat liver is very slow (11).

These rat hepatic receptors have been characterized mostly using membrane preparations (3–5, 8, 9). In this report, we describe the use of Triton X-100 to solubilize liver membranes from rats treated with 17α-EE and the subsequent use of Amberlite XAD-2 to remove the detergent. Using a filtration assay and ligand blotting, we show that we have solubilized the hormone-induced LDL receptor. The binding of human ^{125}I-labeled LDL was dependent on calcium and was sensitive to pronase and suramin. ^{125}I-labeled LDL was effectively displaced by unlabeled lipoproteins containing apolipoprotein B and/or E, but not by HDL_3. Blotting with ^{125}I-labeled LDL revealed a single band that corresponded to a protein with a molecular weight of 133,000. These are all characteristics of the LDL receptor (8–10, 12, 13).

METHODS

Materials

Male Sprague-Dawley rats weighing 200–500 g were purchased from Charles River Canada Inc. (St. Constant, P.Q.). 17α-EE, Amberlite XAD-2, phenylmethylsulfonyl

Abbreviations: VLDL, very low density lipoproteins; IDL, intermediate density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; 17α-EE, 17α-ethinyl estradiol; DMPC, dimyristoylphosphatidylcholine; EDTA, ethylenediamine tetraacetic acid (disodium salt); SDS, sodium dodecyl sulfate.

fluoride, egg yolk phosphatidyl choline type V-E, dimyristoylphosphatidylcholine (DMPC), and bovine serum albumin (fraction V) were obtained from Sigma Chemical Co. (St. Louis, MO). Suramin was purchased from Mobay Chemical Corp. (New York, NY). Pronase from *Streptomyces griseus* was from Calbiochem-Behring Corp. (La Jolla, CA). Nuflow cellulose acetate membrane filters (N25/45) were purchased from Oxoid Canada Inc. (Ottawa, Ont.). We obtained sodium [^{125}I]iodide from Amersham Canada Ltd. (Oakville, Ont.) and Schleicher and Schuell BA83 nitrocellulose membrane filters (0.2 μm) from Mandel Scientific Co. (Rockwood, Ont.). Cronex Lightning Plus intensifying screens (Dupont) were from Compagnie Générale de Radiologie (Ville St-Laurent, P.Q.). Human blood was obtained from the Canadian Red Cross (Montréal, P.Q.).

Preparation of rat liver membranes

17α-EE was dissolved in 1 ml of ethanol and propyleneglycol was then added to make a 1 mg/ml solution. Daily subcutaneous injections, 5 mg per kg of body weight, were administered for 4 or 6 consecutive days. Control rats received subcutaneous injections of the appropriate volume of propyleneglycol.

Liver membranes were prepared as described by Kovanen, Brown, and Goldstein (8). Fasted rats were anesthetized using diethyl ether and their livers were quickly removed and washed in ice-cold 0.154 M NaCl. Each liver was homogenized with two 10-sec pulses of a Polytron homogenizer (Brinkman Instruments, Canada Ltd, Rexdale, Ont.) at a setting of 10. The homogenization buffer contained 10 mM Tris-HCl (pH 7.5), 0.154 M NaCl, 1 mM $CaCl_2$, and 1 mM phenylmethylsulfonyl fluoride. Ten ml of the buffer was used per gram of tissue. All manipulations in these and subsequent procedures were done at 4°C unless otherwise stated. Homogenates were pooled and centrifuged at 500 *g* for 5 min. The supernatant was centrifuged at 8,000 *g* for 15 min. This supernatant was then centrifuged at 100,000 *g* for 1 hr. The precipitate was resuspended in the same buffer (6 ml per tube), passed ten times through a 21-gauge needle, and recentrifuged at 100,000 *g* for 1 hr. The pellet was quickly aliquoted, frozen in liquid nitrogen, and stored at −70°C.

Solubilization of liver membranes

Liver membranes were solubilized as described by Schneider et al. (12). Liver membranes (120–160 mg) were thawed and resuspended in 10 ml of 250 mM Tris-maleate buffer, pH 6, containing 2 mM $CaCl_2$ and 1 mM phenylmethylsulfonyl fluoride. The suspension was ultrasonicated for two 20-sec pulses with a microprobe (Heat Systems Ultrasonic Inc., Plainview, NY) at position 6. An equal volume of a 2% (w/v) Triton X-100 solution containing 2 mM $CaCl_2$ was then added and the suspension was agitated for 30 min on a rotating wheel. The undissolved material was removed by ultracentrifugation at 100,000 *g* for 1 hr.

Removal of Triton X-100

Amberlite XAD-2 (14) was washed and added to the solubilized membrane solution (500 mg/ml) and the suspension was mixed for 1 hr on a rotating wheel. When the wheel was stopped the Amberlite XAD-2 quickly sedimented. The supernatant was removed and centrifuged at 10,000 *g* for 10 min to pellet insoluble material and remaining Amberlite XAD-2. The clear supernatant was stored at 4°C and used within 2 weeks.

Lipoproteins

Human VLDL (density <1.006 g/ml), intermediate density lipoproteins (IDL, density 1.006–1.019 g/ml), LDL (density 1.025–1.05 g/ml), HDL_2 (density 1.063–1.125 g/ml), and HDL_3 (density 1.125–1.21 g/ml) were prepared by sequential ultracentrifugation (15). All lipoproteins were washed by ultracentrifugation at the appropriate density and dialyzed against 0.154 M NaCl, pH 7.5, containing 0.01% (w/v) disodium ethylenediaminetetraacetate (EDTA). The d > 1.21 g/ml fraction was similarly dialyzed.

ApoE was purified using a modification of the selective extraction procedures described by Holmquist and Carlson (16). Human VLDL was washed twice (100,000 *g* for 1 hr) to remove chylomicron contamination and twice (100,000 *g* for 18 hr) to remove denser lipoproteins and albumin. To a solution of VLDL (1.0 to 1.5 mg/ml) in 0.154 M NaCl containing 0.01% (w/v) EDTA, an equal volume of isopropanol (100%) was added while agitating vigorously on a Vortex mixer. After standing 5 min at room temperature, the solution was centrifuged at 10,000 *g* for 10 min. The supernatant was recovered and filtered through Nuflow cellulose acetate membrane filters (0.45 μm). The isopropanol was then evaporated under a jet of nitrogen. Acetone, 1.2 times the volume of the remaining solution, was added while agitating vigorously on a Vortex mixer. After standing 5 min at room temperature, the solution was centrifuged at 20,000 *g* for 20 min. The supernatant was removed and the precipitate was resuspended in 0.154 M NaCl containing 0.01% (w/v) EDTA. The acetone extraction was then repeated. The precipitate was then delipidated with ethanol–ether 3:1 as described by Scanu and Edelstein (17) and stored at −20°C. ApoE thus prepared was very pure. Traces of albumin and C apolipoproteins were the only contaminants, as seen by electrophoresis on polyacrylamide gels containing SDS and β-mercaptoethanol. The purified apoE was dissolved in 10 mM Tris-HCl buffer, pH 7.6, containing 0.154 M NaCl, 1 mM EDTA, and 5 M urea.

The solution was centrifuged at 10,000 *g* for 10 min to remove undissolved material and then dialyzed against the same buffer containing no urea. ApoE-DMPC liposomes were prepared as described by Roth et al. (18).

LDL was iodinated by the iodine monochloride method of McFarlane (19) as modified for lipoproteins by Langer, Strober, and Levy (20). Na ^{125}I (2.5 mCi), ICl (160 nmol), 1 M glycine–NaOH (0.5 ml), pH 10, LDL (4 mg) were added sequentially while agitating on a Vortex mixer; $Na_2S_2O_5$ (80 mmol) and KI (2 mg) were then added after a 30-sec wait, to give a final volume of approximately 1 ml. The iodinated LDL was then passed through a 1.6 × 50 cm column of Sephadex G-25 (Pharmacia Canada Ltd. Dorval, P.Q.) with 20 mM phosphate, pH 7.5, containing 0.154 M NaCl and 1 mM EDTA as the eluting buffer. The ^{125}I-labeled LDL protein concentration was from 1.0 to 1.7 mg/ml; the specific radioactivity ranged from 160 to 390 cpm/ng protein; the free ^{125}I accounted for 0.5 to 0.7% of the total radioactivity; the incorporation into the lipid moiety was 1.6 to 4.3%; and the iodine to protein ratio (per 100,000 daltons) was 0.7 to 1.3. Ninety-seven percent of the radioactivity was in apolipoprotein B, 0.8% in albumin, 0.35% in apoE, and 1.7% in apoC, as revealed by electrophoresis on SDS-polyacrylamide gels.

Receptor binding assay

The receptor binding assay was as described by Schneider, Goldstein, and Brown (21). Egg yolk phosphatidylcholine was placed in a round-bottom flask and the chloroform–methanol was evaporated under a jet of nitrogen. The phospholipid was then redissolved in diethyl ether (anhydrous) and spread on the walls of the flask while the ether was evaporated under a jet of nitrogen. Tris-maleate buffer (50 mM, pH 6) containing 2 mM $CaCl_2$, 0.5 ml per mg of phosphatidylcholine, was then added and the flask was shaken by hand for 5 min at room temperature. This suspension was stored at 4°C and used within 1 month.

To incorporate the solubilized liver membrane proteins into the phosphatidylcholine liposomes the following solution was prepared in 50 mM Tris-maleate buffer, pH 6: 0.4 mg/ml phosphatidylcholine, 1.0 mg/ml solubilized protein, 0.4 M NaCl, and 2 mM $CaCl_2$. For each ml of this mixture, 0.6 ml of cold acetone (−20°C) was quickly added while agitating vigorously on a Vortex mixer. After 2 min on ice, the solution was centrifuged at 20,000 *g* for 20 min. The pellet was resuspended in 20 mM Tris-HCl, pH 8.0, containing 50 mM NaCl and 1 mM $CaCl_2$ (1 μl of buffer per 10 μg of added membrane protein) and the suspension was passed 50 times through a 30-gauge needle and used immediately in the binding assay.

The standard binding assay contained 55–66 μg of solubilized membrane protein (incorporated into phosphatidylcholine liposomes), 60 mM Tris-HCl (pH 8.0), 25 mM NaCl, 0.25 mM $CaCl_2$, 20 mg/ml bovine serum albumin, and desired concentrations of ^{125}I-labeled LDL, LDL, other lipoproteins, and other substances, all in a final volume of 100 μl. After an incubation on ice for 1 hr, 80 μl of the binding assay solution was filtered on cellulose acetate membrane filters (0.45 μm) using a multi-well filtration apparatus (Bio-Rad Lab. Canada Ltd., Mississauga, Ont.). The filters were incubated in 20 mM Tris-HCl, pH 8.0, containing 25 mM NaCl, 0.25 mM $CaCl_2$, and 1 mg/ml bovine serum albumin for at least 30 min at room temperature and washed once by filtration with the same buffer (4°C) prior to filtration of the assay mixture. After the assay mixture was filtered, the filters were washed three times with 3 ml of the same buffer and counted on a gamma counter (LKB, Sweden). Binding was expressed as μg of ^{125}I-labeled LDL protein bound per mg of solubilized membrane protein in the assay mixture.

Ligand blotting

Electrophoresis of solubilized liver membrane proteins was done on 4–18% polyacrylamide gradient slab gels containing 0.1% (w/v) SDS (22). The samples were not subjected to sulfhydryl reducing agents and were not heated (13). Protein (125 μg) was added to each well and electrophoresis was done at 15 mA/gel for 1 hr followed by 30 mA/gel for 4 hr. Gels were calibrated with the Bio-Rad high molecular weight standards: myosin, 200,000; β-galactosidase, 116,000; phosphorylase b, 94,000; bovine serum albumin, 68,000; and ovalbumin, 43,000.

Proteins were transferred onto nitrocellulose membrane filter paper according to Burnette (23) using a Bio-Rad Transblot apparatus, under the following conditions: 170 mA, 60 V for 17 hr with water-cooling. Some nitrocellulose papers, including those with the molecular weight standards, were stained in 0.2% Coomassie Blue R-250 in 40% methanol and 10% acetic acid for 5 min and destained with 90% methanol and 2% acetic acid for 5 min. The other nitrocellulose papers were incubated in 10 ml of 60 mM Tris-HCl, pH 8.0, containing 25 mM NaCl, 0.25 mM $CaCl_2$, and 50 mg/ml bovine serum albumin for 30 min in a shaking water bath set at 37°C. The buffer was replaced with 10 ml of fresh buffer containing 20 μg/ml of ^{125}I-labeled LDL and the nitrocellulose papers were agitated on a rotating wheel for 1 hr at room temperature in the presence and absence of a 25-fold excess of unlabeled LDL. The papers were then washed once quickly with the same buffer containing no lipoproteins, followed by two 20-min washes, and finally a quick wash. For radioautography, the nitrocellulose papers were dried and exposed to Fuji RX film for 8 hr at −70°C using Cronex Lightning Plus enhancing screens (24).

Other assays

Protein was assayed by the method of Lowry et al. (25) in the presence of SDS for samples containing Triton X-100 (26) or lipids (27). Cholesterol was measured using an enzymatic method described previously (28); phospholipid phosphorus was determined by the method of Fiske and Subbarow (29) after perchloric acid digestion. Triton X-100 was assayed using the method of Garewale (30).

RESULTS

Treatment of the male rats with 17α-EE caused a profound hypocholesterolemia. Serum cholesterol levels decreased from 54 ± 6.1 mg/dl (mean ± SEM) before treatment to 5.1 ± 1.6 mg/dl after treatment, a more than 90% reduction. Control rats that received only propyleneglycol did not exhibit this precipitous fall in cholesterol levels. They had cholesterol concentrations of 59 ± 4.5 mg/dl before treatment and 59 ± 3.6 mg/dl after treatment.

Solubilization of liver membranes

The subcellular fractions obtained from the livers of 17α-EE-treated rats were assayed for ^{125}I-labeled LDL binding as described in Methods, except that the airfuge was used to separate bound ^{125}I-labeled LDL from free as described by Kovanen et al. (8). The pellet thus obtained was then washed once with fetal calf serum using the airfuge. The recovery of binding activity among unwashed subcellular fractions was as follows: 63% in the 500 *g* fraction, 11% in the 8,000 *g* fraction, and 22% in the 100,000 *g* fraction, giving an overall recovery of 96% of the activity found in the unfiltered homogenate. The 100,000 *g* fraction had the highest specific binding activity and represented a twofold purification over the homogenate. This membrane fraction was used for solubilization experiments.

One percent (w/v) Triton X-100 solubilized 65 ± 3.3% of the membrane proteins from control rats and 67 ± 3.0% from 17α-EE-treated rats. The subsequent removal of the Triton X-100 with Amberlite XAD-2 was very efficient. As shown in **Fig. 1,** more than 90% of the detergent was removed after 30 min. Longer incubations were only marginally better, but we routinely incubated for 1 hr to remove as much Triton X-100 as possible. With most of the detergent bound to the Amberlite XAD-2, some of the membrane material became insoluble and caused substantial turbidity. A 10-min centrifugation at 10,000 *g* was sufficient to remove this material. The overall recovery of membrane protein after this centrifugation averaged 45 ± 1.4% for control rats and 43 ± 1.4% for treated rats. Of the solubilized proteins subjected to acetone precipitation in the presence of phosphatidylcholine, 58 ± 2.0% (control rats) and 57 ± 1.0% (treated rats) were recovered in the pellet. After filtration, 80.3 ± 0.9% of the liposomal protein remained associated with the filter, as determined by measuring protein eluted when albumin was omitted or the radioactivity associated with the filter when ^{125}I-labeled solubilized proteins were incorporated into liposomes.

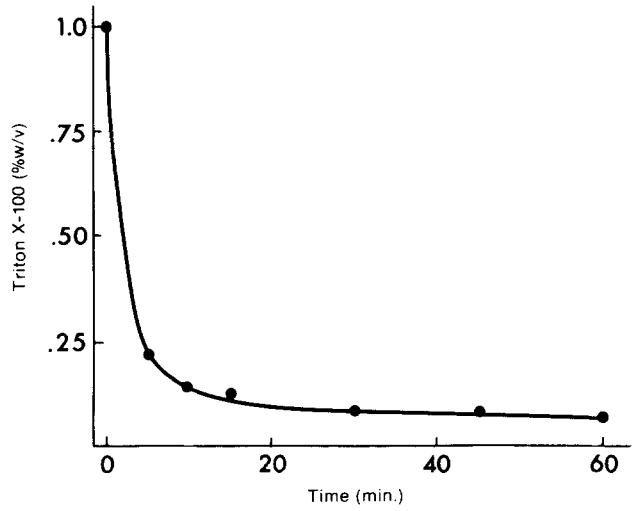

Fig. 1. Removal of Triton X-100 with Amberlite XAD-2. Rat liver membranes solubilized in 1% (w/v) Triton X-100 were incubated at 4°C with Amberlite XAD-2. Aliquots were taken at indicated times and the concentration of Triton X-100 was determined. Values are means of duplicate determinations.

The binding activity in the solubilized proteins (treated rats), recovered in liposomes after precipitation with acetone, represented 65.8 ± 2.9% of the activity originally found in the membranes. The membranes were assayed using the airfuge while the liposomes were assayed by filtration. A 2.7-fold purification was obtained by solubilization which resulted in a 5.3-fold purification over the homogenate.

Receptor binding assay

In 60 mM Tris-HCl buffer, specific binding of human ^{125}I-labeled LDL (binding displaced by a 25-fold excess of unlabeled LDL) to liposomes prepared with solubilized proteins from treated rats, was maximal between pH 7.25 and 8.5 and the optimum concentrations of NaCl were from 0 to 40 mM (data not shown). We chose pH 8.0 and 25 mM as standard assay conditions. Under these conditions, 0.25 mM EDTA was enough to reduce specific binding by 90% (**Fig. 2A**). Furthermore, 1.25 mM $CaCl_2$ sufficed to recover maximum binding in the presence of 1 mM EDTA (Fig. 2B). $CaCl_2$ (0.25 mM) was therefore included in the standard receptor assay.

$CaCl_2$ (0.25 mM) was also optimal for the specific binding of ^{125}I-labeled LDL to liposomes prepared with solubilized proteins from control rats (data not shown). However, in contrast to the specific binding to liposomes containing liver membrane proteins from treated rats which was almost totally dependent on Ca^{2+}, the specific

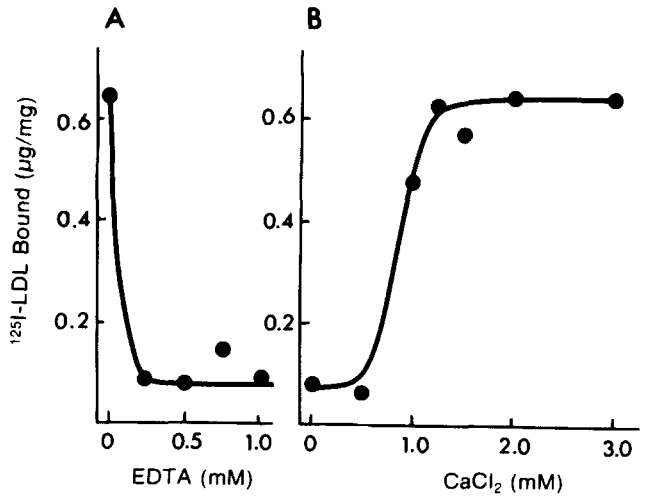

Fig. 2. Effect of EDTA (A) and $CaCl_2$ (B) on the specific binding of ^{125}I-labeled LDL to liposomes containing soluble liver membrane proteins from rats treated with 17α-EE. Assay tubes contained 56 μg of solubilized membrane protein and 75 μg/ml of ^{125}I-labeled LDL (249 cpm/ng). In (A) the $CaCl_2$ concentration was 0.1 mM and in (B) the EDTA concentration was 1 mM. Specific binding was binding obtained in the absence of unlabeled LDL minus binding obtained in the presence of a 25-fold excess of unlabeled LDL. Values are means of duplicate determinations.

binding to liposomes containing solubilized proteins from control rats was only inhibited by 50% in the presence of up to 10 mM EDTA. Ca^{2+}-dependent binding averaged 0.537 ± 15.4 μg/mg of protein from treated rats and 0.049 ± 11.0 μg/mg of protein from control rats. There was therefore an 11-fold increase in Ca^{2+}-dependent binding of human ^{125}I-labeled LDL binding due to treatment with 17α-EE.

In a control experiment, liposomes containing only phosphatidylcholine were prepared by precipitating the phospholipid with cold acetone in the absence of protein. Binding of ^{125}I-labeled LDL to these liposomes could not be detected.

Characterization of the binding induced by 17α-EE

The specific binding of human ^{125}I-labeled LDL to liposomes containing solubilized proteins from treated rats exhibited saturation kinetics (**Fig. 3**). Scatchard analysis (31) of these data gave a K_d of 18 μg/ml and a B_{max} of 0.97 μg/mg. Virtually identical results were obtained when 10 mM EDTA was used to determine nonspecific binding instead of unlabeled LDL at a 25-fold excess (data not shown).

Table 1 shows that different unlabeled lipoprotein fractions had different effects on the binding of ^{125}I-labeled LDL. VLDL at a 10-fold excess and IDL at a 5-fold excess were the most effective at competing for ^{125}I-labeled LDL binding sites. LDL at a 10-fold excess also competed effectively. In contrast, HDL_2 was not very effective and HDL_3 inhibited ^{125}I-labeled LDL binding by only 14%. The d > 1.21 g/ml fraction had no effect. In **Fig. 4**, the effects of increasing concentrations of unlabeled LDL, HDL_3, and apoE-DMPC liposomes are compared. The apoE-DMPC liposomes were the most effective; at a protein concentration equivalent to that of the ^{125}I-labeled LDL, the binding of the iodinated lipoproteins was almost totally inhibited. For unlabeled LDL, a 15- to 40-fold excess was needed to achieve a comparable displacement of ^{125}I-labeled LDL. HDL_3, even at a 40-fold excess in protein concentration, inhibited ^{125}I-labeled LDL binding by only 25%.

The binding sites for ^{125}I-labeled LDL were sensitive to pronase. When liposomes containing solubilized proteins from rats treated with 17α-EE were incubated for 15 min at 37°C in the presence of pronase (25 μg/ml), the specific binding of ^{125}I-labeled LDL was totally abolished (data not shown). The same incubation in the absence of pronase caused less than a 10% loss in binding activity. The ^{125}I-labeled LDL binding was also sensitive to suramin. **Fig. 5** shows that the polysulfated polycyclic hydrocarbon inhibited the binding in a concentration-dependent manner. Suramin (2 mM) decreased the binding almost to the level observed when a 25-fold excess of unlabeled LDL was included in the assay mixture.

After electrophoresis on SDS-polyacrylamide gels in the absence of reducing agents, the solubilized proteins from rats treated with 17α-EE were transferred electrophoretically onto nitrocellulose paper. The transfer was efficient as seen by staining of nitrocellulose strips with

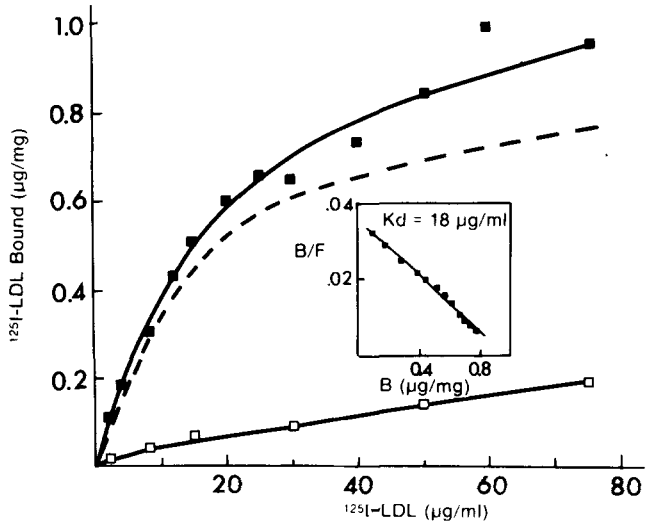

Fig. 3. Saturation curve for the binding of ^{125}I-labeled LDL to liposomes containing soluble liver membrane proteins from rats treated with 17α-EE. Assay tubes contained 66 μg of solubilized membrane protein and the indicated concentrations of ^{125}I-labeled LDL (388 cpm/ng). Specific binding (---) was the binding obtained in the absence of unlabeled LDL (■) minus the binding obtained in the presence of 1.7 mg/ml unlabeled LDL (□). Values are means of duplicate determinations. The insert shows the Scatchard plot of the specific binding data.

TABLE 1. The effect of unlabeled lipoproteins on the binding of ^{125}I-labeled LDL to liposomes containing solubilized liver membrane proteins from rats treated with 17α-EE

Unlabeled Lipoprotein	Fold Excess (Protein)	^{125}I-labeled LDL Bound[a]	
		μg/mg	*% control*
		676	100
VLDL	10	88	13
IDL	5	143	21
LDL	10	196	29
HDL_2	10	366	54
HDL_3	10	581	86
Density > 1.21	10	674	100

[a]The concentration of ^{125}I-labeled LDL (194 cpm/ng) was 55 μg/ml.

Coomassie Blue and comparison with gels stained before or after the transfer (data not shown). After blotting with ^{125}I-labeled LDL, only one band was visualized by radioautography (**Fig. 6A**). To determine the molecular weight of the ^{125}I-labeled LDL binding protein, its relative mobility as visualized by radioautography was compared to the relative mobilities of molecular weight protein standards visualized by Coomassie Blue staining following their transfer onto nitrocellulose paper. Its apparent molecular weight was thus found to be 133,000 ± 2,700 (n = 6). When blotting with ^{125}I-labeled LDL was done in the presence of a 25-fold excess of unlabeled LDL, the intensity of the radioactivity band was dramatically reduced (Fig. 6B).

DISCUSSION

Triton X-100 has been used extensively for solubilizing membrane proteins. Notably, Schneider et al. (12) used the nonionic detergent to solubilize the LDL receptor from bovine adrenal cortex. In the presence of 1% (w/v) Triton X-100 however, the receptor cannot be assayed reliably because a very high nonspecific binding of ^{125}I-labeled LDL is obtained. To overcome this problem, we used Amberlite XAD-2 (14). It proved to be very effective: most of the Triton X-100 was removed (Fig. 1) and we were able to reliably assay for specific binding of ^{125}I-labeled LDL.

Treatment of the male rats with 17α-EE had the expected effects (8, 32). The hormone caused a profound hypocholesterolemia and, more importantly, a substantial increase in the binding of ^{125}I-labeled LDL. The Ca^{2+}-dependent binding was 11 times higher for treated rats than for controls. The yields in protein after solubilization with Triton X-100, after incubation with Amberlite XAD-2, and after precipitation with acetone were very similar for treated versus control rats and therefore did not correlate with the difference observed in the Ca^{2+}-dependent binding.

In addition to the Ca^{2+}-dependency and the stimulation by 17α-EE, the binding of ^{125}I-labeled LDL to liposomes containing solubilized liver membrane proteins from treated rats, exhibited other properties that were similar to those of the LDL receptor studied by Windler et al. (9) using isolated rat liver membranes. The binding was saturable with a K_d of 18 μg/ml (Fig. 3), which is comparable to the K_d of 23 μg/ml reported for the binding of human ^{125}I-labeled LDL to the isolated membranes (9). It is also very similar to the K_d of 17 μg/ml found for the binding of human ^{125}I-labeled LDL to the purified LDL receptor from bovine adrenal cortex (12). The specificity was also as expected: the binding was specific for apolipoproteins B and E. Apolipoprotein B was recognized because human ^{125}I-labeled LDL, which contained almost exclusively B, was bound with high affinity. The binding was not due to the iodination because unlabeled LDL displaced ^{125}I-labeled LDL (Fig. 4). ApoE was also recognized, seemingly with greater affinity than B. Unlabeled liposomes prepared with purified apoE and DMPC were more effective than unlabeled LDL at competing with ^{125}I-labeled LDL for binding sites (Fig. 4). Unlabeled VLDL and IDL, which contain both apolipoproteins B and E, were also more effective (Table 1). A

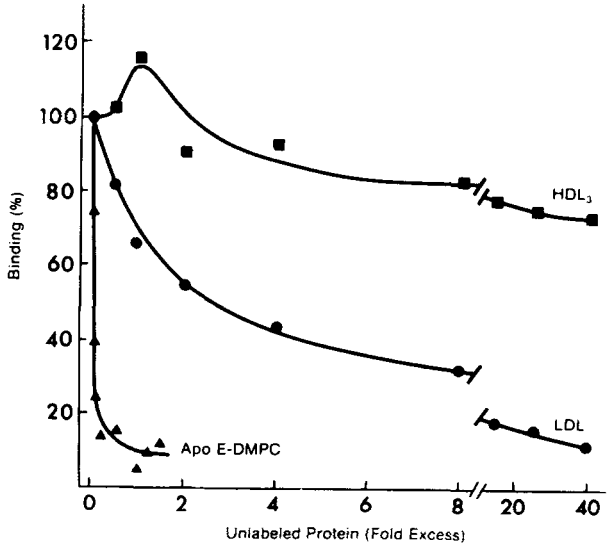

Fig. 4. Comparison of the ability of unlabeled apoE-DMPC liposomes (▲), LDL (●), and HDL_3 (■) to compete with ^{125}I-labeled LDL for binding sites on liposomes containing soluble liver membrane proteins from rats treated with 17α-EE. For the data with LDL and HDL_3, assay tubes contained 66 μg of solubilized membrane protein, 50 μg/ml ^{125}I-labeled LDL (388 cpm/ng), and the indicated fold excess of unlabeled LDL and unlabeled HDL_3. The 100% binding value was the binding of ^{125}I-labeled LDL obtained in the absence of the unlabeled lipoproteins (0.87 μg/mg). For the data with apoE-DMPC liposomes, assay tubes contained 112 μg of solubilized membrane protein, 3 μg/ml ^{125}I-labeled LDL (167 cpm/ng) and the indicated fold excess of unlabeled apoE-DMPC liposomes. The 100% binding value was the binding of ^{125}I-labeled LDL obtained in the absence of unlabeled apoE-DMPC liposomes (0.40 μg/mg). Values are means of duplicate determinations.

higher affinity for human apoE- than for human apoB-containing lipoproteins is not unusual. It is also a characteristic of the LDL receptor of human fibroblasts (33). Furthermore, the fibroblast receptor and the LDL receptors from rat liver (9), dog liver (34), and bovine adrenal gland (12) also have higher affinities for canine apoE- than for human apoB-containing lipoproteins. Our results obtained with HDL_2 and HDL_3 in competition experiments are also consistent with the apoB and apoE specificity. Unlabeled HDL_3, which does not contain any apoE or apoB, was not an effective competitor (Fig. 4). Unlabeled HDL_2, which usually contains small amounts of apoE and may also contain some apoB, was intermediate between unlabeled LDL and HDL_3 in competition effectiveness (Table 1).

Like the binding of human ^{125}I-labeled LDL to isolated membranes from rats treated with 17α-EE (8), the binding to liposomes containing solubilized liver membrane proteins from these rats was very sensitive to pronase, which is also a characteristic of LDL receptors from other sources (10, 12, 34). The ^{125}I-labeled LDL binding was also sensitive to suramin (Fig. 5). This characteristic of the LDL receptor was reported by Schneider et al. (12) for the bovine adrenal gland receptor. Using suramin, they were able to preferentially elute the LDL receptor from an LDL-Sepharose column, thus purifying the receptor to homogeneity. Our results suggest that suramin could be similarly used to purify the LDL receptor from the liver of rats treated with 17α-EE. Recently, suramin was also shown to inhibit the binding of ^{125}I-labeled LDL and ^{125}I-labeled chylomicron remnants to rat hepatoma cells (35).

Finally, we have shown by blotting with ^{125}I-labeled LDL, that the lipoprotein binds to a protein with an apparent molecular weight of 133,000 (Fig. 6). This is very close to the molecular weight of 130,000 found for the

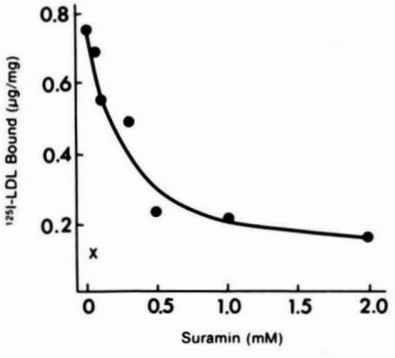

Fig. 5. The effect of suramin on the binding of ^{125}I-labeled LDL to liposomes containing soluble liver membrane proteins from rats treated with 17α-EE. Assay tubes contained 57 μg of solubilized membrane protein and 100 μg/ml of ^{125}I-labeled LDL (156 cpm/ng). Tubes contained the indicated concentrations of suramin (●) or a 25-fold excess of unlabeled LDL (x). Values are means of duplicate determinations.

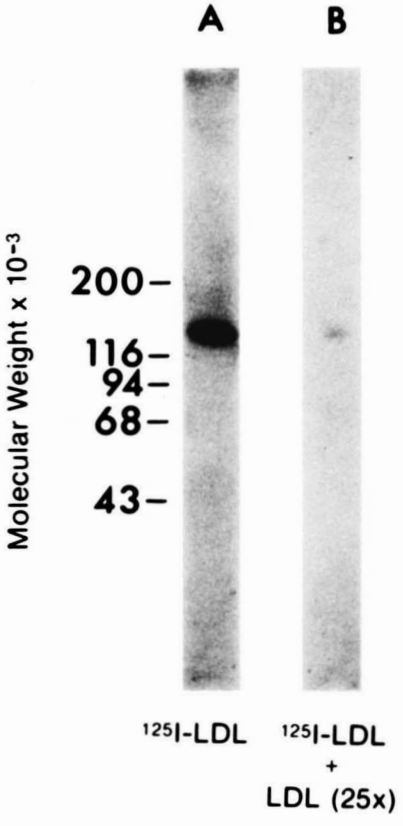

Fig. 6. Ligand blotting of soluble liver membrane proteins from rats treated with 17α-EE. Soluble proteins were subjected to electrophoresis in 4–18% polyacrylamide slab gels containing 0.1% (w/v) SDS. Samples were not exposed to reducing agents and were not heated. Proteins were transferred electrophoretically to nitrocellulose paper and incubated with 20 μg/ml ^{125}I-labeled LDL in the absence (A) and in the presence of a 25-fold excess of unlabeled LDL (B). Nitrocellulose papers were processed by radioautography.

LDL receptor from bovine and rabbit adrenal cortex and human fibroblasts when β-mercaptoethanol was omitted (13). In the presence of the reducing agent, the bovine adrenal cortex receptor has a molecular weight of 164,000 but cannot be visualized by ligand blotting.

In conclusion, we feel we have solubilized the 17α-EE-stimulated LDL receptor of male rat liver which has previously been studied by others using isolated liver membranes (8, 9). The stimulation by 17α-EE, the Ca^{2+}-dependency, the K_d values of 18 μg/ml, the specificity for apoB and apoE, and the pronase sensitivity of the ^{125}I-labeled LDL binding support this conclusion. Moreover, its sensitivity to suramin and its molecular weight of 133,000 are further indications that this LDL receptor is very similar to LDL receptors from other sources (12, 13). ■

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