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Preferential binding of [^{3}H]cholesteryl linoleyl ether-HDL$_3$ by bovine adrenal membranes

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Membranes isolated from bovine adrenal cortex, incubated with human high-density lipoproteins (HDL$_3$), labeled with ^{125}I and [^{3}H]cholesteryl linoleyl ether, showed preferential binding of [^{3}H]cholesteryl linoleyl ether. The preferential binding was Ca^{2+} independent, temperature sensitive and was slightly increased after phospholipase C or pronase treatment. Reduction of membrane phosphatidylcholine by phospholipase A$_2$ resulted in a marked increase in the binding of the entire HDL$_3$ particle and a relative decrease in preferential binding of [^{3}H]cholesteryl linoleyl ether. These findings suggest that the presence of intact phospholipid in the membrane plays an important role in the magnitude of the preferential binding.

High-density lipoproteins (HDL) are considered to play an important role in reverse cholesterol transport [1], i.e., the transport of cholesterol from peripheral tissues to the liver. In recent years it has been shown that interaction between HDL and the liver results in a preferential uptake of the cholesteryl ester moiety which exceeds that of the protein by a factor of 2 to 4 [2–4]. Preferential uptake of HDL cholesteryl ester as determined by its non-hydrolyzable analog cholesteryl linoleyl ether is even more prominent in rat and bovine adrenal cells studied in culture [4,5]. Since the preferential uptake of cholesteryl ester does not require metabolic energy [6], it seemed of interest to study this process in membranes isolated from adrenal glands.

In this communication, we show that bovine adrenal membranes exposed to HDL bind cholesteryl linoleyl ether in excess of HDL protein (= preferential binding of ^{3}H-cholesteryl linoleyl ether) and that treatment of the membranes with phospholipase A$_2$ results in a relative reduction of the preferential binding while increasing markedly the binding of the entire HDL particle.

Bovine adrenals were obtained in a local slaughterhouse within 1 h of slaughter, chilled to 4°C and dissected. Homogenization was carried out in 5 volumes of buffer (150 mM $NaCl_2$, 1 mM $CaCl_2$ and 10 mM Tris (pH 7.5)) using a Polytron homogenizer (Brinkman Instruments). The homogenate was subjected to sequential centrifugation at $500 \times g$ for 5 min, $8000 \times g$ for 15 min and $100\,000 \times g$ for 60 min, followed by resuspension and resedimentation [7]. The pellets were kept at −190°C till use. In some instances, partial delipidation of lyophilized membranes was carried out with 20 volumes of heptane, twice, for 30 min at 4°C. Human HDL$_3$ was isolated from plasma at $d = 1.12$–1.21 g/ml by ultracentrifugation for 48 h at $100\,000 \times g$, and washed by reflotation at $d = 1.21$ g/ml. Labeling of HDL with ^{125}I or with

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[^{3}H]cholesteryl linoleyl ether was carried out as described before [5]; the ^{125}I-HDL was passed through the entire procedure of HDL labeling with [^{3}H]cholesteryl linoleyl ether. [^{3}H]Cholesteryl linoleyl ether was synthesized according to Halperin and Gatt [8].

On the day of the experiment, the membrane pellet was resuspended and incubated with either ^{125}I- or ^{3}H-labeled HDL in 50 mM Tris, 1 mM $CaCl_2$, 100 mM NaCl and fatty acid poor albumin (20 mg/ml), final volume 300 μl. Pretreatment with enzymes was carried out as follows: about 4 mg membrane protein were incubated at 37°C with 100 μg phospholipase A_2 or with 0.1 unit phospholipase C (3.4 units/mg) for 20 min or with 30 μg pronase for 10 min in 250–350 μl Tris buffer (pH 7.5) 1 mM $CaCl_2$ and 50 mM NaCl. After pretreatment, the membranes were chilled to −4°C, resuspended in 10 ml of Tris buffer and the membranes reisolated by centrifugation for 30 min at 100000 × g. The surface of the pellet was washed, the membranes were resuspended, the amount of protein was determined and the membranes were used for the binding of HDL. Incubations were carried out at 37°C for 45 min with agitation. At the end of incubation, a 25 μl aliquot was removed for the determination of total radioactivity. The 250 μl of the membrane suspension were layered over 1.8 ml whole rat serum or 4% bovine serum albumin and centrifuged in 40.3 rotor at 100000 × g at 4°C for 30 min using appropriate adaptors; both procedures gave essentially similar results. All supernatants were collected and the pellet was rewashed twice by recentrifugation with 10% serum or 0.4% albumin. Protein was determined according to Lowry et al. [9], lipid phosphorus according to Bartlett [10], and cholesterol by a cholesterol oxidase-cholesterol esterase procedure [11]. All radioactive materials were from Amersham Int., U.K. Albumin, fatty acid poor, and snake venom were from Sigma, U.S.A. Pronase, grade B and phospholipase C (*Clostridium perfringens*) were from Calbiochem, U.S.A.

Membranes prepared from bovine adrenal glands were incubated with HDL_3 labeled with [^{3}H]cholesteryl linoleyl ether or with ^{125}I for 45 min at 37°C as in preliminary experiments no measurable degradation of ^{125}I-HDL protein occurred during 1 h of incubation. The binding of the labeled HDL to the membranes varied with different membrane preparations but in all more ^{3}H than ^{125}I label was bound and the ratio of the % of each tracer bound to the membranes (^{3}H/^{125}I ratio) ranged between 2 to 8 (Table I). Since a change in the cholesterol content of the membrane could have contributed to the variation in HDL binding, some membranes were lyophilized and partially delipidated with heptane. This procedure resulted in a 40–60% reduction in membrane cholesterol content, but this treatment did not affect preferential binding of [^{3}H]cholesteryl linoleyl ether derived from HDL by the membrane (Table II). Addition of a 50-fold excess of unlabeled HDL during incubation reduced the binding by 89.3 ± 1.3% for the ^{3}H and 81.4 ± 2.7% for ^{125}I.

The preferential binding of [^{3}H]cholesteryl linoleyl ether derived from HDL did occur also in the absence of Ca^{2+} in the medium and in the presence of EDTA (Table III). Higher concentrations (10 mM) of Ca^{2+} increased the binding of the whole particle, but did not affect the preferential binding of the HDL-cholesteryl ester moiety. When the incubation of HDL with lyophilized and partially delipidated membranes was carried out at 22°C or 4°C, the binding of both ^{125}I-HDL and [^{3}H]cholesteryl linoleyl ether-HDL (HDL labeled with [^{3}H]cholesteryl linoleyl ether) was reduced markedly (Table IV). Similar results were

TABLE I

BINDING OF HUMAN HDL_3 TO BOVINE ADRENAL MEMBRANES

Adrenal membranes 500–700 μg protein were incubated with labeled HDL_3 50 μg protein for 45 min at 37°C. Thereafter, the membranes were reisolated as described in Methods and radioactivity was determined directly. Values are means ± S.E. of quadruplicates. CLE, cholesteryl linoleyl ether.

Membrane batch	% HDL bound/100 μg membrane protein		^{3}H/^{125}I
	[^{3}H]CLE-HDL	[^{125}I]HDL	
A	2.09 ± 0.3	0.49 ± 0.09	4.3
B	4.60 ± 0.8	0.58 ± 0.16	7.9
C	0.58 ± 0.07	0.28 ± 0.02	2.1

TABLE II

COMPARISON OF FRESH, LYOPHILIZED AND PARTIALLY DELIPIDATED ADRENAL MEMBRANES ON HDL_3 BINDING

Adrenal membranes 500–700 μg protein were used either fresh after lyophilization alone or lyophilization followed by partial delipidation. The cholesterol content after delipidation was reduced by half. Values are means ± S.E. The numbers in parentheses represent the number of determinations.

Membrane	% HDL bound/100 μg membrane protein		$^3H/^{125}I$
	[^{3}H]CLE-HDL	^{125}I-HDL	
Fresh (4)	1.46 ± 0.09	0.29 ± 0.03	5.0
Lyophilized (25)	1.43 ± 0.07	0.20 ± 0.03	7.2
Partially delipidated (16)	1.86 ± 0.19	0.31 ± 0.05	6.0

obtained also using fresh adrenal membranes.

In the next experiments, the membranes were pretreated with pronase, phospholipase A_2 and phospholipase C, and incubated with the labeled HDL, keeping the amount of membrane protein equal to that of the control membrane. In pronase-treated membranes (Table V), there was a slight increase in ^{125}I-HDL binding, while the binding of [^{3}H]cholesteryl linoleyl ether-HDL increased by 66–116%. Preincubation of the membranes for 20 min with 0.1 u/ml of phospholipase C caused a loss of about 50% of membrane lipid phosphorus and resulted in either a 30% increase or even a slight decrease in the binding of ^{125}I-HDL. On the other hand, there was a 70–150% increase in the binding of [^{3}H]cholesteryl linoleyl ether-HDL (Table V).

TABLE III

EFFECT OF Ca^{2+} AND EDTA ON BINDING OF HDL_3 BY ADRENAL MEMBRANES

Lyophilized adrenal membranes, 700 μg protein were incubated with HDL_3 in the absence or presence of $CaCl_2$. Values are means ± S.E. of quadruplicates.

Conditions	HDL bound/100 μg membrane protein		$^3H/^{125}I$
	[^{3}H]CLE-HDL	^{125}I-HDL	
EDTA, 2 mM	1.02 ± 0.16	0.14 ± 0.01	7.4
$CaCl_2$, 1 mM	1.33 ± 0.14	0.15 ± 0.02	8.8
$CaCl_2$, 10 mM	1.45 ± 0.14	0.24 ± 0.03	6.0

TABLE IV

EFFECT OF TEMPERATURE ON BINDING OF HDL_3 BY ADRENAL MEMBRANES

Lyophilized and partially delipidated membranes, 600 μg protein were incubated with labeled HDL_3 at the indicated temperature. Values are means of duplicate incubations which agreed within 10%.

Temperature (°C)	HDL bound/100 μg membrane protein		$^3H/^{125}I$
	[^{3}H]CLE-HDL	^{125}I-HDL	
37	2.35	0.33	7.1
22	1.30	0.23	5.6
4	0.69	0.13	5.3

Pretreatment of the membranes for 20 min with phospholipase A_2 caused hydrolysis of about 50% of the phosphatidylcholine to lysophosphatidylcholine and resulted in a pronounced increase in the binding of the whole HDL particle as evidenced by the binding of ^{125}I-labelled protein (Table V). In addition, there was also an increase in [^{3}H]cholesteryl linoleyl ether binding in excess of the protein binding. The increment, presented in Table V, shows to which extent the enzyme treatment of the membranes affected either the binding of the whole HDL particle or the preferential binding of [^{3}H]cholesteryl linoleyl ether. Thus, when the $^3H/^{125}I$ ratio of control and enzyme treated membranes as well as the $^3H/^{125}I$ ratio of the increment were compared (Table V), it could be seen that in pronase and phospholipase C treated membranes, the ratio increased while with phospholipase A_2 it decreased. With the first two enzymes the $^3H/^{125}I$ ratio of the increment was higher than that of the untreated membranes indicating only a slight effect on the binding of the entire particle and some increase in preferential binding of [^{3}H]cholesteryl linoleyl ether. On the other hand, the $^3H/^{125}I$ ratio of the increment after phospholipase A_2 treatment was lower than in the untreated membranes, which permitted to conclude that the treatment caused an increase in the binding of the entire particle and therefore the preferential binding of [^{3}H]cholesteryl linoleyl

TABLE V

EFFECT OF ENZYME TREATMENT ON BINDING OF HDL_3 BY ADRENAL MEMBRANES

Adrenal membranes were pretreated with pronase (30 μg) for 10 min, with phospholipase C (0.1 u) for 20 min and phospholipase A_2 (100 μg) for 20 min. Values are means of duplicate incubations which agreed within 10%. Increment = enzyme treated membrane − control value.

Batch of membrane	Expt.	% of added label/100 μg membrane protein								
		[^{3}H]CLE-HDL			[^{125}I]HDL			$^3H/^{125}I$		
		Control	Treated	Increment	Control	Treated	Increment	Control	Treated	Increment
Pronase										
A	I	1.55	3.35	1.80	0.33	0.46	0.13	4.7	7.2	13.8
	II	1.15	1.92	0.77	0.15	0.19	0.04	7.7	10.0	19.3
Phospholipase C										
D	I	5.63	7.04	1.41	0.88	0.74	−0.14	6.4	9.5	–
C	II	0.62	1.54	0.92	0.31	0.40	0.09	2.0	3.9	10.3
	III	0.69	1.17	0.48	0.33	0.43	0.10	2.1	2.7	4.8
Phospholipase A_2										
A	I	2.60	6.75	4.15	0.65	3.70	3.05	4.0	1.8	1.4
B	II	3.25	5.45	2.20	0.30	1.33	1.03	10.9	4.1	2.1
C	III	0.62	2.10	1.48	0.31	1.40	1.09	2.0	1.5	1.3

ether became relatively lower.

Membranes isolated from the adrenal gland cortex of various species were shown to bind plasma lipoproteins such as LDL and HDL [7,12–14]. The binding of LDL was shown to proceed through a specific receptor, which had been later isolated and characterized [15]. The binding of HDL to membranes isolated from the adrenal [12–14], liver [16], kidney [17], testis [14], and ovary [18] was shown to differ from that of the binding of LDL in respect to the requirement of divalent cations and sensitivity to proteolytic enzymes. Another major difference in the interaction between LDL and HDL with various cultured cells is that while the uptake of LDL represents the uptake of the entire lipoprotein particle [19,20], the uptake of HDL in the above mentioned organs is accompanied by a preferential uptake of cholesteryl ester [2–5]. The mechanism of the preferential uptake of cholesteryl ester from HDL had not been elucidated so far, but it was shown not to require metabolic energy [6]. In the present study, incubation of the membranes with labeled HDL_3 resulted in the recovery of more [^{3}H]cholesteryl linoleyl ether than ^{125}I-labeled protein, the latter representing binding of the entire lipoprotein particle. In analogy to the findings with membranes derived from human fetal adrenal [12], bovine [13], or rat adrenal [14], liver [16] or kidney [17], the binding of HDL_3 protein did not require Ca^{2+} and could be demonstrated also in the presence of EDTA. The chelating agent or high Ca^{2+} concentrations had no effect on the preferential binding of [^{3}H]cholesteryl linoleyl ether. The presently studied binding of HDL_3 had a distinct temperature sensitivity, as was shown also for membranes isolated from luteal cells [18] or hepatocytes [16]. As the ratio of $^3H/^{125}I$ in the membranes incubated with labeled HDL at 37 °C was higher than in membranes incubated at 4 °C or 22 °C, it seems tempting to speculate that the greater fluidity of membrane phospholipids at 37 °C might contribute towards the preferential binding of cholesteryl ester.

In another system in which preferential transfer of cholesteryl ester from chylomicrons to cells was shown to be mediated by lipoprotein lipase, the binding of the lipase to the cell surface was of paramount importance for this transfer [21]. Since the uptake of [^{3}H]cholesteryl linoleyl ether-HDL by adrenal cells could be reduced by delipidated HDL or by AI or C apolipoproteins [5], it seems that the binding of the HDL particle, which is required for the transfer of cholesteryl ester, is

mediated by certain apoproteins. Similar conclusions with respect to the ligand involved in HDL binding have been reached also in studies with membranes isolated from rat kidney [17], liver and testis [16]. All studies involved with the characterization of the HDL 'receptor', have shown insensitivity of the binding of HDL to proteolytic treatment of the membrane. Thus, binding of HDL to membranes derived from human adrenal [12], testis [14] or liver [16], was shown to be pronase resistant. Closer examination of results obtained with testicular membranes suggested even a slight increase in binding with 12 μg/ml of pronase or 2.5 mg/ml of trypsin [14]. Similar results were seen when ovarian membranes had been exposed to 10 μg/ml of pronase [18]. Presently, we have also encountered a slight increase in binding of ^{125}I-HDL in pronase treated membranes, which was accompanied by a relatively greater increase in the binding of [^{3}H]cholesteryl linoleyl ether-HDL resulting in a rise of $^{3}H/^{125}I$ ratio with enzyme treatment (Table V). A similar trend can be seen in phospholipase C-treated membranes, in which some increase in binding of [^{3}H]cholesteryl linoleyl ether-HDL was observed. The importance of membrane phospholipid in the preferential binding of [^{3}H]cholesteryl linoleyl ether is indicated by the data obtained with phospholipase A_2. This treatment resulted in a very marked increase in the binding of the ^{125}I-labeled protein, representing binding of the whole particle. This was accompanied by a relative decrease in the preferential binding of [^{3}H]cholesteryl linoleyl ether and a fall in the $^{3}H/^{125}I$ ratio in the treated membranes, as well as in the increment. These findings could indicate that indeed the binding site for the ^{125}I-labeled protein of HDL is embedded in the membrane phospholipid. This has been proposed in the study on liver membranes [16] in which the binding of HDL became pronase sensitive when the membrane had been pretreated by phospholipase A_2. Thus, it appears that while the presence of membrane phospholipid is important for the preferential transfer of cholesteryl ester from HDL, a loss of some phospholipid polar head groups resulting in the presence of diacylglycerol could even facilitate this process to some extent.

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