Measurement of receptor-independent lipoprotein catabolism using 1,2 cyclohexanedione-modified low density lipoprotein

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Abstract The utility of 1,2 cyclohexanedione-modified low density lipoprotein (CHD-LDL) as a marker for the measurement of receptor independent LDL catabolism has been assessed by examining its metabolic properties in cultured human fibroblasts and in rabbits. Cell culture studies showed that the inhibition of high affinity membrane receptor binding produced by the modification could be partially reversed by prolonged incubation of the CHD-LDL at 37°C. Pre-exposure of the complex to alkaline pH (pH 10.5) prevented this and yielded a product that was apparently stable. Despite its regained ability to bind to the fibroblast receptor, ^{125}I-labeled CHD-LDL incubated at 37°C for 24 hr either in vivo or in vitro was removed from rabbit plasma in the same manner as freshly prepared ^{131}I-labeled CHD-LDL and as ^{131}I-labeled CHD-LDL that had been treated at pH 10.5. However, its plasma clearance was significantly faster than that of reductively methylated LDL. We believe that this may result from differential catabolism of these modified lipoproteins rather than from susceptibility of the CHD-LDL to receptor-directed catabolism.—**Slater, H. R., C. J. Packard, and J. Shepherd.** Measurement of receptor-independent lipoprotein catabolism using 1,2 cyclohexanedione-modified low density lipoprotein. *J. Lipid Res.* 1982. **23:** 92–96.

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Human low density lipoproteins (LDL) bind specifically to a high affinity cell membrane receptor, initiating a sequence of events which culminates in their catabolism (1). The binding process seems to be dependent on the presence of a number of functionally significant arginyl or lysyl residues on the lipoprotein surface. When these are subjected to a specific chemical modification with 1,2 cyclohexanedione (CHD) or formaldehyde–sodium borohydride (MET), respectively, receptor-lipoprotein interactions are abolished (2, 3) and the plasma clearance of the modified products is delayed (4, 5).

In an earlier publication (5) we quantified the extent of the catabolic delay induced by CHD treatment of human LDL in order to assess the importance of the receptor pathway in vivo. This stratagem presupposes first that the modified lipoprotein is not metabolized by the receptor pathway and second that its clearance by other routes is unaffected by modification. In this paper we examine the extent to which CHD-LDL meets these criteria.

METHODS

Isolation and chemical modification of LDL

LDL was prepared from fasting normal human plasma by a zonal centrifugation technique described previously (6). The pooled LDL peak fractions were dialyzed against 0.15 M NaCl/0.01% disodium EDTA (pH 7.0), concentrated, and labeled with ^{125}I or ^{131}I (7). The arginyl residues on the labeled lipoprotein were modified by treatment with 1,2 cyclohexanedione (Fluorochem, Glossop, U.K.), at pH 8.2, resulting in greater than 55% conversion (as measured by amino acid analysis) to a CHD-arginine complex (2). Unreacted cyclohexanedione was removed by gel filtration through a column of G-10 Sephadex using 0.15 M NaCl/0.01% Na_2EDTA, pH 7.0, for elution. The labeled, modified preparation was stored at 4°C and used within 2 hr. In some studies intramolecular rearrangement of the CHD-arginine complex was achieved by incubating the modified lipoprotein under sterile conditions in 0.1 M phosphate buffer, pH 10.5 for 2 hr at 37°C. The product of this rearrangement (CHD-LDL (pH 10.5)) is apparently stable and does not undergo spontaneous reversal (8).

Reductive methylation of LDL was performed as de-

Abbreviations: LDL, low density lipoprotein; CHD-LDL, 1,2 cyclohexanedione-modified low density lipoprotein; MET LDL, low density lipoprotein which has been reductively methylated by treatment with formaldehyde–sodium borohydride.

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scribed by Weisgraber, Innerarity, and Mahley (3) and resulted in modification of more than 75% of the free amino groups on the lipoprotein as detected by titration with trinitrobenzene sulfonic acid (3).

Cell culture studies

Interaction of the modified LDL with the high affinity membrane receptors on cultured normal human fibroblasts was examined in cells obtained from Flow Laboratories, Irvine, U.K. using standard methods (9). The cells were maintained in a humidified incubator (5% CO_2) at 37°C in Basal Eagle's medium (Flow Laboratories) supplemented with fetal calf serum (10%), glutamine (2 mM), non-essential amino acids (1%), $NaHCO_3$ (1.68 g/l), penicillin (100 u/ml), and streptomycin (100 μg/ml). Confluent monolayers were dissociated with 0.5% trypsin/0.02% Na_2EDTA and transferred to flat based culture tubes for use in experiments. Non-confluent cultures grown 48–72 hr in medium containing 10% lipoprotein-deficient serum were used in all experiments.

Binding studies were performed at 4°C as described by Goldstein et al. (9).

Animal studies

Four-month-old male New Zealand White rabbits were maintained on commercial chow (S.G.1, Oxoid Ltd., Basingstoke, Hampshire, U.K.) and were given 0.1 g/liter KI in their drinking water for 2 days before and throughout each turnover study.

Approximately 10 μCi (200 μg protein) of radioiodinated and chemically modified LDL was sterilized by membrane filtration (0.22 μ filters, Millipore Corp., Bedford, MA) and injected into a marginal ear vein. Blood samples were then collected from the opposite ear on frequent occasions over the next 48 hr. Plasma decay curves were constructed and the fractional catabolic rates of the labeled tracers were calculated by the procedure of Matthews (10).

In some studies, biological screening of the labeled lipoprotein was achieved by injecting a large dose of the material (200 μCi approximately) into rabbits. After 24–48 hr the animals were exsanguinated and the remaining labeled LDL in their plasma was reisolated by rate zonal ultracentrifugation and used in metabolic studies as described above.

RESULTS

Competitive binding studies using cultured human fibroblasts have shown that the interaction of LDL with high affinity cell membrane receptors depends on a number of arginyl and lysyl residues on the particle. Chemical modification of these residues inhibits the interaction (**Table 1**) but, whereas reductive methylation of the lysyl residues is apparently irreversible (4), prolonged incubation of the CHD-modified LDL at 37°C regenerates some binding ability in vitro. This regeneration is accelerated (2) in the presence of strong nucleophiles (e.g., hydroxylamine) which almost completely restore receptor-lipoprotein interaction (Table 1).

Incubation of 1,2 CHD-modified arginine at pH 10.5 results in rearrangement of the complex (8). When CHD-LDL, prepared at pH 8.2 as described previously, was treated in this way, a product was formed which was apparently stable in that it failed to interact with the receptor, even after incubation for 24 hr at 37°C (Table 1). This procedure neither altered the number of arginyl residues modified nor blocked any lysyl residues on the particle as determined by amino acid analysis and trinitrobenzene sulfonic acid titration (3).

In parallel with the cell culture studies, we examined the metabolism of the modified LDL in rabbits. Five separate experiments were performed (**Table 2**). In the first, ^{125}I-labeled CHD-LDL was screened for 24 hr in a rabbit, reisolated by zonal centrifugation, and its

TABLE 1. Binding of chemically modified LDL to fibroblast receptors

Addition to Culture	Binding, n = 4	*t* Test versus Control
100 μg/ml	*ng LDL/mg cell protein*	
None	637 ± 20	
Native LDL	10 ± 5	$P < 0.001$
4°C CHD-LDL	613 ± 34	NS
37°C CHD-LDL	541 ± 46	$P < 0.05$
$HONH_2$-treated CHD-LDL	135 ± 25	$P < 0.01$
MET-LDL	630 ± 45	NS
4°C CHD-LDL, pH 10.5	604 ± 48	NS
37°C CHD-LDL, pH 10.5	595 ± 48	NS

Ability of various modified LDL to compete with human ^{125}I-labeled-LDL for binding to normal human fibroblasts. Cells of non-confluent cultures were incubated 72 hr in medium containing 10% lipoprotein-deficient serum. ^{125}I-Labeled-LDL (10 μg protein, 100 cpm/ng protein) and unlabeled lipoprotein (100 g/ml) were then added and the cultures were incubated at 4°C. After 2 hr the medium was removed and cell monolayers were washed six times with ice-cold buffer (0.15 M NaCl, 0.01 M phosphate buffer, pH 7.4). Cells were dissolved in 0.1 M NaOH and radioactivity and protein were measured. Cell-free blanks contained only trace amounts of radioactivity and protein. Human LDL was modified with CHD as described in Methods. Such LDL was then either kept for 24 hr at 4°C (4°C CHD-LDL) or incubated under sterile conditions for 24 hr at 37°C (37°C CHD-LDL). Reversal of CHD modification (2) was carried out by incubating CHD-LDL with 1.0 M hydroxylamine in 0.3 M mannitol at pH 7.0 for 16 hr. The lipoprotein preparation ($HONH_2$-treated CHD-LDL) was then dialyzed against 0.15 M NaCl–0.01% Na_2EDTA, pH 7.0. LDL was methylated (MET-LDL) by treatment with $NaBH_4$–HCHO (3). CHD-LDL was incubated at 37°C for 2 hr at pH 10.5; the pH was then reduced to 7.0 by dialysis against 0.1 M phosphate buffer, pH 7.0, and the preparations were incubated for 24 hr at either 4°C (4°C CHD-LDL, pH 10.5) or 37°C (37°C CHD-LDL, pH 10.5).

TABLE 2. Metabolism of chemically modified human LDL in rabbits

Experiment	Tracers	ApoLDL Fractional Catabolic Rate[a]
1	^{125}I-CHD-LDL	0.64 ± 0.03
(n = 3)	^{131}I-CHD-LDL	0.68 ± 0.03
2	^{125}I-CHD-LDL (37°)	0.65 ± 0.08
(n = 3)	^{131}I-CHD-LDL	0.68 ± 0.15
3	^{125}I-CHD-LDL (pH 8.2)	0.66; 0.66
(n = 2)	^{131}I-CHD-LDL (pH 10.5)	0.66; 0.69
4	^{125}I-CHD-LDL	0.80 ± 0.22[b]
(n = 8)	^{131}I-MET-LDL	0.65 ± 0.25
5	^{125}I-CHD-LDL	0.59; 0.42
(n = 2)	^{131}I-MET-LDL	0.45; 0.29

[a] Derived from plasma decay curves by the procedure of Matthews (10).

[b] Paired t test, CHD-LDL vs MET-LDL, $P < 0.01$.

Experiment 1: A 200-μCi aliquot of ^{125}I-labeled CHD-LDL was injected into the marginal ear vein of a rabbit. After 24 hr the animal was exsanguinated by cardiac puncture and LDL was isolated by rate zonal ultracentrifugation. Aliquots (10 μCi) of this material were then mixed with an equal amount of ^{131}I-labeled CHD-LDL, made fresh from the same original lipoprotein preparation (which had been stored concurrently at 4°C) and were injected into the marginal ear veins of three other rabbits. Blood samples were then collected from the opposite ear into 1.0 mg/ml Na_2EDTA at frequent intervals over the next 48 hr. Plasma radioactivities were measured and used to construct clearance curves for each isotope. The fractional clearance rates of the labeled tracers from the plasma were obtained by curve peeling (10).

Experiment 2: ^{125}I-labeled CHD-LDL was incubated at 37°C under sterile conditions for 24 hr in 0.15 M NaCl–0.01% Na_2EDTA, pH 7.0. Approximately 200 μg of protein (10 μCi) of this was then mixed with an equal amount of freshly prepared ^{131}I-labeled CHD-LDL and injected into the marginal ear vein of three rabbits. Plasma decay curves were obtained and used to calculate the fractional clearance rate of each isotope.

Experiment 3: The plasma clearance rate of ^{125}I-labeled CHD-LDL, prepared as described by Mahley et al. (2), was compared in two rabbits with that of ^{131}I-labeled CHD-LDL which had been further treated by incubation at pH 10.5 for 2 hr. Fractional catabolic rates were determined as before.

Experiment 4: Eight rabbits received, in a rapid sequence via an intravenous cannula placed in a marginal ear vein, 0.5 ml of ^{125}I-labeled CHD-LDL (10 μCi in 0.2 mg of protein) and an equivalent amount of ^{131}I-labeled MET-LDL, separated by a 1.0-ml bolus of 0.15 M NaCl. Plasma decay curves and their derived kinetic parameters were obtained.

Experiment 5: Two hundred μCi each of ^{125}I-labeled CHD-LDL and ^{131}I-labeled MET-LDL were screened together in two rabbits by the procedure outlined in Experiment 4. The animals were then exsanguinated and their LDL isolated by rate zonal ultracentrifugation for injection into two other recipients. The metabolic handling of the tracers was measured in these animals.

plasma clearance was subsequently compared to that of freshly prepared ^{131}I-labeled CHD-LDL in three other animals. Identical decay rates were observed for the fresh and screened lipoproteins (**Fig. 1,** Experiment 1 in Table 2). In a second study, ^{125}I-labeled CHD-LDL was incubated in vitro at 37°C for 24 hr prior to comparing its clearance to that of freshly prepared ^{131}I-labeled CHD-LDL. Again, indistinguishable decay curves were

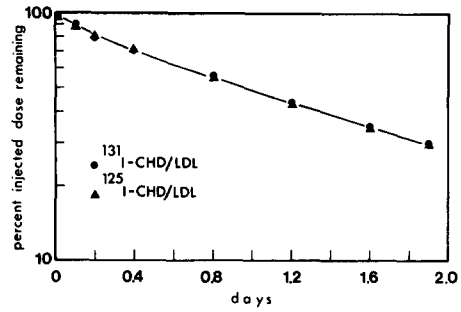

Fig. 1. Effect of pre-screening on the plasma clearance rate of CHD-LDL. Approximately 200 μCi of human ^{125}I-labeled CHD-LDL was screened for 24 hr in a rabbit. The animal was then exsanguinated and LDL (with labeled tracer) was isolated by rate zonal ultracentrifugation. Aliquots (10 μCi) of this material were mixed with equal amounts of ^{131}I-labeled CHD-LDL made fresh from the same original LDL preparation and were injected into three secondary recipient animals. The mean decay curves for both isotopes in this group are presented; ▲, ^{125}I-labeled CHD-LDL; ●, ^{131}I-labeled CHD-LDL.

obtained for both tracers (Experiment 2, Table 2). The third experiment involved comparison of the metabolism of ^{125}I-labeled CHD-LDL (pH 8.2) with that of ^{131}I-labeled CHD-LDL (pH 10.5) which does not interact with the high affinity LDL receptor (Table 1). Both tracers were cleared from the plasma at the same rate (Experiment 3, Table 2).

However, MET-LDL, which is also stable (4), was handled differently by the animals (**Fig. 2,** Experiment 4 in Table 2). Its fractional clearance rate was consistently and significantly slower ($P < 0.01$) than that of CHD-LDL and this difference was unaffected by biological screening (**Fig. 3,** Experiment 5 in Table 2).

DISCUSSION

Chemical modification of LDL designed to block lipoprotein-receptor interaction is a potentially useful tool

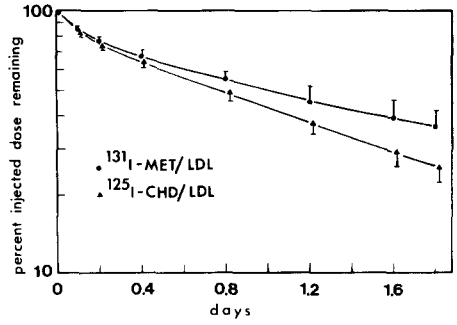

Fig. 2. Comparison of the plasma clearances of human CHD-LDL and MET-LDL in rabbits. Eight rabbits received, via an intravenous cannula, sequential injections of ^{125}I-labeled CHD-LDL and ^{131}I-labeled MET-LDL (10 μCi in 0.2 mg protein) separated by a 1.0-ml bolus of 0.15 M NaCl. Plasma decay curves for each isotope were constructed from blood samples taken over a 48-hr period; ▲, ^{125}I-labeled CHD-LDL; ●, ^{131}I-labeled MET-LDL.

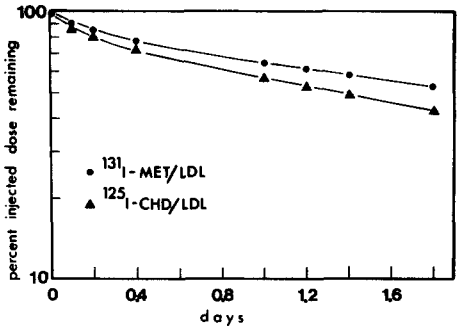

Fig. 3. Plasma clearance rates of CHD-LDL and MET-LDL following 48 hr of biological screening. Two rabbits were injected with 200 μCi each of ^{125}I-labeled CHD-LDL and ^{131}I-labeled MET-LDL as described in the legend for Fig. 2. After 48 hr, the animals were exsanguinated by cardiac puncture and their LDL was isolated separately by rate zonal ultracentrifugation. The plasma clearance rates of 20-μCi aliquots of this screened material were measured in two other recipient animals. The mean decay curve for each isotope is presented; ▲, ^{125}I-labeled CHD-LDL; ●, ^{131}I-labeled MET-LDL.

for quantifying the high affinity receptor pathway in vivo. This approach presupposes that the modified lipoprotein is not degraded via the receptor pathway and at the same time it is treated as native LDL by other catabolic processes. Two LDL modifications, involving reductive methylation of lysyl or 1,2 CHD-treatment of arginyl residues on the particle have been used for this purpose (2, 3, 5). On theoretical grounds, the former appears to be better since it completely blocks receptor recognition and is stable (4). However, when injected into man it is rapidly eliminated from the plasma,[2] suggesting that it is recognizably foreign to the recipient. Cyclohexanedione-treated LDL does not suffer this fate, but is known to be susceptible to spontaneous hydrolysis, at least in vitro. Mahley et al. (4) have shown, and we confirm here, that incubation of CHD-LDL for 24 hr at 37°C partially restores its interaction with the high affinity membrane receptor of cultured fibroblasts. Regeneration however appears to be limited to about 50% (see Fig. 6 in ref. 4), and even prolonged in vitro incubation of the modified lipoprotein fails to revert its electrophoretic mobility to that of native LDL (5).

These in vitro observations, if applicable in vivo would preclude CHD-LDL as a tool for the quantitative measurement of receptor activity and would restrict its use to the provision of qualitative information. However, the data presented here and in previous publications (5, 11) strongly suggest that regeneration of receptor binding observed during in vitro incubation of CHD-LDL is not expressed in vivo as a progressive increase in receptor-mediated catabolism of the modified particle. Although incubation in vitro did partially restore the ability of the particle to bind to the receptor on cultured cell membranes (Table 1), it did not increase its plasma clearance rate over that of freshly prepared CHD-LDL (which showed no binding to the fibroblast receptor) (Table 1). Similarly, biological screening of CHD-LDL did not affect its catabolism (Fig. 1, Experiment 1 in Table 2).

In an alternative approach, we exposed CHD-LDL to alkaline conditions (pH 10.5) known to promote rearrangement of CHD-arginine to a stable derivative (8). The resulting material, even after prolonged incubation at 37°C, did not bind to the fibroblast receptor (Table 1) and was metabolically identical to CHD-LDL prepared in the normal way. Therefore the evident hydrolysis of CHD-LDL in vitro did not influence its in vivo metabolism. One possible explanation for this apparent inconsistency is that the partially regenerated particle, although able to bind to the receptor, fails to be internalized and catabolized via the high affinity pathway.

Cyclohexanedione-modified LDL is cleared consistently faster from the plasma of rabbits than the reductively methylated lipoprotein (Fig. 2). If, in light of our observations above, it is accepted that CHD-LDL is not handled by the receptor pathway in vivo, then some other explanation must be sought for the difference in the metabolic behavior of these two tracers. With this in mind, we have examined the effects of two perturbations of lipoprotein metabolism on the plasma clearance rates of native LDL, CHD-LDL, and MET-LDL. Dietary cholesterol supplementation suppresses receptor-mediated LDL catabolism from the plasma of rabbits and causes native LDL and CHD-LDL to be cleared at virtually the same rate. Nevertheless, even under these circumstances of suppressed receptor activity, the difference between CHD-LDL and MET-LDL clearance is maintained. However, there are conditions under which the difference is minimized. When reticuloendothelial activity is inhibited by ethyl oleate infusion (12), CHD-LDL and MET-LDL are removed from the plasma at similar rates (13), even though this treatment does not consistently affect the function of the high affinity LDL receptor (14). These data imply, first, that CHD-LDL is not handled in vivo by the receptor pathway and, second, that the difference in clearance of CHD-LDL and MET-LDL is not dependent on receptor activity but rather on some other metabolic process, possibly involving the reticuloendothelial system. At present our ignorance of the mechanisms involved in the catabolism of both chemically modified lipoproteins requires that we proceed with caution in the quantitation of receptor-independent catabolism using these probes. When our understanding of their catabolism is clarified, each may yield information on different aspects of the receptor-independent pathway.

[2] Packard, C. J., H. R. Slater, and J. Shepherd. Unpublished observations.

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