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Inhibition of receptor-mediated clearance of lysine and arginine-modified lipoproteins from the plasma of rats and monkeys

(lipoprotein catabolism/protein modification/cell receptors/protein turnover)

ROBERT W. MAHLEY*, KARL H. WEISGRABER*, GEORGE W. MELCHIOR†, THOMAS L. INNERARITY*, AND KATHLEEN S. HOLCOMBE†

†Laboratory of Experimental Atherosclerosis, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20205; and *Gladstone Foundation Laboratories for Cardiovascular Disease, University of California, San Francisco, California 94110

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ABSTRACT **Reductive methylation of at least 30% of the lysine residues or 1,2-cyclohexanedione modification of 45% of the arginine residues prevented low density lipoproteins (LDL) from binding to cell surface receptors of fibroblasts *in vitro*, without significantly altering other physical or chemical properties of the LDL. When rat or human LDL with more than 30% of the lysine residues methylated were injected intravenously into rats, the clearance of these lipoproteins from the plasma was slowed considerably. The half-life of the reductively methylated LDL was approximately twice that obtained for control (unmodified) LDL, and the value for the fractional catabolic rate was approximately half that of the control. Furthermore, when human LDL modified by reductive methylation were injected into rhesus monkeys, the rate of clearance was similarly retarded, and the value for the fractional catabolic rate was reduced by approximately 50% as compared with the value for control LDL. A dual isotope labeling technique (^{125}I and ^{131}I) was used to compare the disappearance of the control and modified LDL in the same animal. It was demonstrated that not only modification of lysine residues but also modification of the arginine residues with 1,2-cyclohexanedione retarded the plasma clearance of the rat LDL. However, the cyclohexanedione modification was spontaneously reversible at 37°C, whereas reductive methylation of the lysine residues was stable. It is concluded that the selective chemical modification of lysine or arginine residues of LDL interferes with the normal uptake of these lipoproteins *in vivo* as well as by fibroblasts *in vitro*. These data provide an estimation of the level of receptor-mediated clearance of LDL from the plasma, a value that may be as high as 50% in rats and monkeys.**

The protein moieties of certain plasma lipoproteins have been shown to be involved in the control of various aspects of lipoprotein metabolism. It has been reported that the recognition site on lipoproteins responsible for their binding to the cell surface receptors of cultured fibroblasts resides with specific apoproteins (1–4). The B apoprotein of low-density lipoproteins (LDL) and the E apoprotein of certain high-density lipoproteins (HDL_1, HDL_c) react with the same receptors on the cell surface (1, 3). The modification of a limited number of arginine and lysine residues has been shown to prevent these lipoproteins from reacting with the apo-B,E receptor sites (5, 6).

Arginine residues were selectively modified with 1,2-cyclohexanedione by a mild procedure that did not otherwise significantly alter the physical or chemical properties of the LDL (5). The lysine residues were modified by acetoacetylation and reductive methylation (6). Both procedures were shown to be selective and mild. An important difference between the two procedures used for lysine modification was that acetoacetylation neutralized the positive charge on the ϵ-amino group, whereas reductive methylation did not alter the charge.

Because modification of the arginine or lysine residues abolished the ability of LDL to react with the cell surface receptors, it was postulated that if these modified lipoproteins were injected intravenously, they would be removed slowly from the plasma. This would demonstrate that the modification had interfered with receptor-mediated uptake *in vivo*. On the contrary, however, after injection into dogs or rats, acetoacetylated LDL were rapidly cleared from the plasma (7, 8). It has been shown that acetoacetylation triggers the rapid removal of LDL by Kupffer cells in the liver (7), and that these modified lipoproteins are also avidly taken up and degraded by macrophages in culture (8). Similar results with macrophages have been reported for acetylated LDL (9). It appears that the alteration in charge resulting from acetoacetylation may be a stimulus for the rapid clearance. Moreover, as will be shown in this paper, reductive methylation, which does not alter the charge on the ϵ-amino group of lysine, does not activate the Kupffer cell removal system. Thus it is possible to obtain data that will estimate the peripheral cell component of LDL clearance from the plasma by comparing the difference in clearance of control and reductively methylated LDL.

MATERIALS AND METHODS

Isolation and Iodination of Lipoproteins. Human LDL (ρ = 1.02–1.05 g/ml) were obtained from the plasma of a fasted male subject by sequential ultracentrifugation at 59,000 rpm in a 60 Ti rotor (Beckman). The LDL were obtained after 18 hr of centrifugation and washed at ρ = 1.050 g/ml with an additional centrifugation. Rat LDL were isolated by ultracentrifugation (ρ = 1.006–1.063 g/ml) of plasma from fasted rats and purified by Geon-Pevikon block electrophoresis as described (10). Human and rat LDL were iodinated by the iodine monochloride method (11). Lipid labeling of the iodinated LDL accounted for less than 2% of the total activity. Greater than 90% of the radioactivity in the dose and in the plasma at all time intervals was trichloroacetic acid precipitable. Sodium [^{131}I]- and [^{125}I]iodide (carrier free) were purchased from Amersham. The specific activities of the ^{125}I-LDL and ^{131}I-LDL ranged from 30 to 225 and 120 to 300 cpm/ng of protein, respectively. The purity of the LDL preparations was monitored by paper electrophoresis, sodium dodecyl sulfate/polyacrylamide gel electrophoresis, and negative staining electron microscopy, as described (12, 13). Dual isotope counting was accomplished with a Beckman model 8000 counter.

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Abbreviations: LDL, low-density lipoproteins; HDL_1 and HDL_c, high-density plasma lipoproteins in dogs, rats, and swine that are distinguished from typical HDL by the presence of the E apoprotein and ability to react with the fibroblast receptors; FCR, fractional catabolic rate; $t_{1/2}$, half-life of the lipoprotein in the plasma compartment.

Reductive Methylation of LDL. Reductive methylation of radiolabeled LDL (protein at ≈ 2 mg/ml) in 0.15 M NaCl/0.01% EDTA, pH 7.0, was performed at 0°C by the addition of 1 mg of sodium borohydride followed by six additions over 30 min of 1 μl of 37% (wt/vol) aqueous formaldehyde. Additions of formaldehyde were made at zero time, 6, 12, 18, 24, and 30 min. The sequence was repeated for more extensive modification. The methodology has been described in detail (6). The level of modification (% of the total lysine residues) was determined by the trinitrobenzenesulfonic acid colorimetric assay. Previously, it was shown that the colorimetric assay gave results that were in good agreement with amino acid analysis (6).

Cyclohexanedione Modification of LDL. The effective conditions for this modification procedure have been described fully (5). Lipoprotein protein (2–5 mg) in 1 ml of 0.15 M NaCl/0.01% EDTA was mixed with 2 ml of 0.15 M 1,2-cyclohexanedione in 0.2 M sodium borate buffer (pH 8.1) and incubated at 35°C for 2 hr. The sample was then dialyzed for 16 hr against 0.15 M NaCl/0.01% EDTA at 4°C. This procedure, under the conditions described, consistently resulted in the modification of 50% of the arginine residues of LDL (5).

Assays for Binding, Internalization, and Degradation. The binding and degradation assays were performed with human fibroblasts by the methods of Goldstein and Brown (14) with minor modifications as described (3, 15).

***In Vivo* Studies.** Male Sprague-Dawley rats (200–250 g) were lightly anesthetized with ether and injected via an exposed saphenous vein with the ^{131}I- and ^{125}I-labeled lipoproteins at a dose of 40–100 μg of protein. Each rat used in the study was weighed just prior to injection, and the plasma volume of each rat was estimated as 4% of the body weight. At specified time intervals after lipoprotein injection, the rats were exsanguinated through the abdominal aorta.

Rhesus monkeys (5 to 10 kg) of both sexes were immobilized with ketamine hydrochloride (Vetalor, Parke Davis) and injected intravenously with 500 μg of human LDL protein (control ^{131}I-LDL and modified ^{125}I-LDL). Blood samples were obtained through the saphenous vein from awake animals in restraining chairs. The animals were accustomed to the chairs. Each animal was weighed, and a plasma volume of 4% of the body weight was used for the calculations. The value was determined experimentally in three monkeys by the Evans blue dye method (16). All data were plotted as a percentage of the total injected dose that remained in the plasma at each time interval. The curves were analyzed by the standard curve-peeling technique, and the fractional catabolic rate was calculated by using the slopes and intercepts of the two exponentials as described by Matthews (17). The values for the half-life $t_{1/2}$ are reported for the second exponential.

Reversibility of the Modification of Lysine and Arginine Residues. Seventy-nine percent of the lysine residues were reductively methylated with [^{14}C]formaldehyde. [^{14}C]Cyclohexanedione was used to label the arginine residues; approximately 60% of the arginine residues were modified (LDL incubated with cyclohexanedione for 2.5 hr). Both preparations were incapable of displacing ^{125}I-LDL from the cell receptors of fibroblasts in binding assays conducted at 4°C. In one study the modified LDL were incubated with whole human serum or 0.1 M phosphate buffer, pH 7.4, for 24 hr at 37°C. After the incubation, the modified LDL were dialyzed against 0.15 M NaCl/0.01% EDTA for 24 hr at 4°C to remove the labeled reactants that were liberated by the incubation. Reversibility was determined by liquid scintillation counting of the activity associated with the modified LDL prior to the serum or buffer incubation. Values are reported as a percentage of the original activity that was present after the final dialysis.

In an additional study, the reductively methylated and cyclohexanedione-modified human LDL were incubated at 37°C for various time intervals with human $\rho > 1.21$ g/ml ultracentrifugal fraction (lipoprotein-deficient serum). At the specified times, aliquots were taken and their abilities to compete with control ^{125}I-LDL were compared in the competitive binding assay conducted with human fibroblasts at 4°C as described (3).

RESULTS

Previously, we observed that reductive methylation of 30% or more of the lysine residues of LDL totally prevented the binding of these lipoproteins to the high-affinity cell surface receptors of fibroblasts in culture (5). We now report that reductive methylation of the lysine residues of rat or human LDL resulted in a marked retardation in the clearance of these lipoproteins from the plasma of rats after intravenous injection. As shown in Fig. 1 for a representative experiment using rat lipoproteins, the control (unmodified) ^{131}I-LDL were cleared from the plasma more rapidly than were the reductively methylated ^{125}I-LDL. At each time interval, the activities (^{125}I and ^{131}I) in the plasma were obtained in three individual rats by dual isotope counting. The $t_{1/2}$ of the second exponential and the fractional catabolic rate (FCR) for the control and modified LDL are compiled in Table 1.

Rat LDL, equivalent to human LDL, were isolated from the

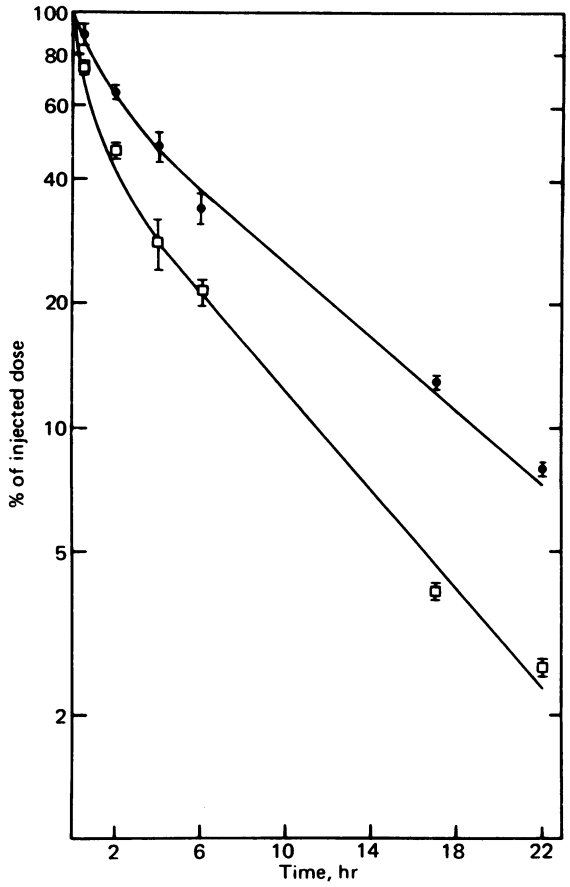

FIG. 1. Percent of the total injected dose of control rat ^{131}I-LDL (□) and reductively methylated rat ^{125}I-LDL (●) that remained in the plasma as a function of time after intravenous injection into rats. The mean ± SD (bar) represents values obtained in three rats by dual isotope counting at each time point. Each rat received 20 μg of control and methylated LDL protein. The reductively methylated LDL had 95% of the lysine residues modified.

Table 1. Simultaneous injection of control ^{131}I-LDL and methylated ^{125}I-LDL into rats

LDL	Rat LDL injected $t_{1/2}$, hr	Rat LDL injected FCR	Human LDL injected $t_{1/2}$, hr	Human LDL injected FCR
Control	4.7*	0.256*	7.3	0.113
Methylated	7.0	0.133	10.0	0.069

* In an additional study, a different preparation of control rat ^{125}I-LDL had a $t_{1/2}$ of 5.0 hr and an FCR of 0.230. These results confirm the values in the table.

ρ = 1.006–1.063 g/ml ultracentrifugal fraction and purified by preparative Geon-Pevikon block electrophoresis. Previously, we reported that this ultracentrifugal fraction was composed of two distinctly different lipoproteins, which could occur in approximately equal concentrations. These were the apo-B-containing LDL and the apo-E-containing lipoprotein referred to as HDL_1 (10, 18). The purified rat LDL used in the above studies were enriched in apo-B (approximately 80–85% of the protein as estimated from stained sodium dodecyl sulfate/polyacrylamide gels), but also invariably contained some low molecular weight (apo-C) apoproteins. To avoid the complication of the presence of C apoproteins, we investigated the possibility of using human LDL (greater than 95% apo-B). We previously used rat and human LDL in rats with qualitatively similar results (7). As described above with rat LDL, modification of 30% or more of the lysine residues of human LDL resulted in a retardation of the clearance of the LDL from the plasma of rats (Fig. 2). Although the absolute values for the $t_{1/2}$ of rat and human LDL were different, the FCRs were similarly decreased by 40 to 50% after modification of the lysine residues by reductive methylation (Table 1).

In six separate experiments with human LDL injected into rats, we observed a similar retardation in clearance of LDL in every case in which more than 30% of the lysine residues were reductively methylated. As shown in Fig. 3, the rates of plasma clearance of LDL with 35, 62, and 91% of their lysine residues modified were essentially identical. All preparations of the modified LDL used in this study were incapable of interacting with the cell surface receptors of fibroblasts in culture.

To establish that decreased clearance of LDL after reductive methylation was not peculiar to metabolism in the rat, we injected rhesus monkeys with control and modified human LDL. As shown in Fig. 4, modification of the lysine residues retarded the clearance of intravenously injected LDL from the plasma

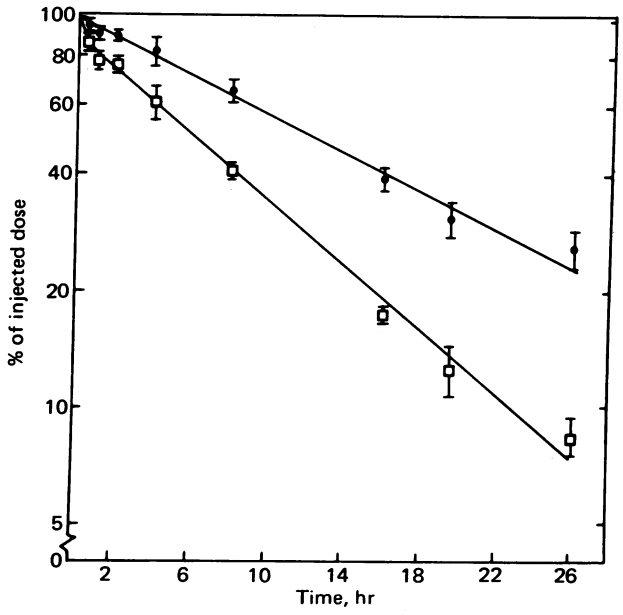

FIG. 2. Percent of the total injected dose of normal human ^{131}I-LDL (□) and reductively methylated human ^{125}I-LDL (●) that remained in the plasma as a function of time after intravenous injection into rats. Three rats were used at each point, and each rat received 50 μg of each lipoprotein. The methylated LDL had 90% of the lysine residues modified. Similar results were obtained in a separate study in which the labeled lipoproteins were reversed (normal human ^{125}I-LDL and methylated ^{131}I-LDL).

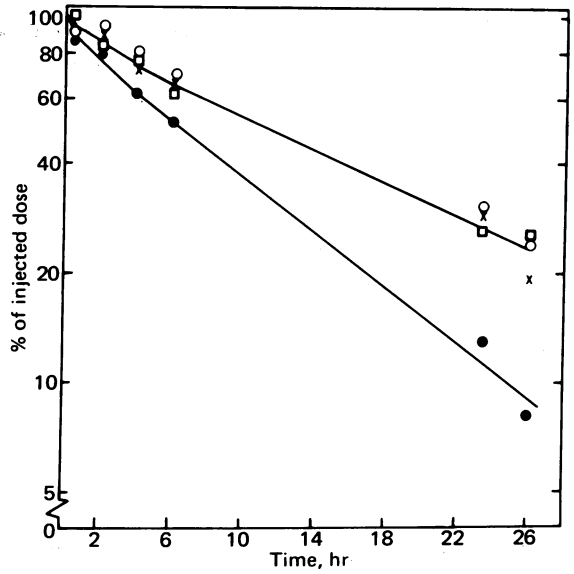

FIG. 3. Percent of the total injected dose of control human ^{125}I-LDL (●) and methylated human ^{125}I-LDL [35% (X), 62% (○), and 91% (□) of the lysine residues modified] that remained in the plasma of the rats. Each point represents the value obtained in a single rat (100 μg of lipoprotein protein injected per rat).

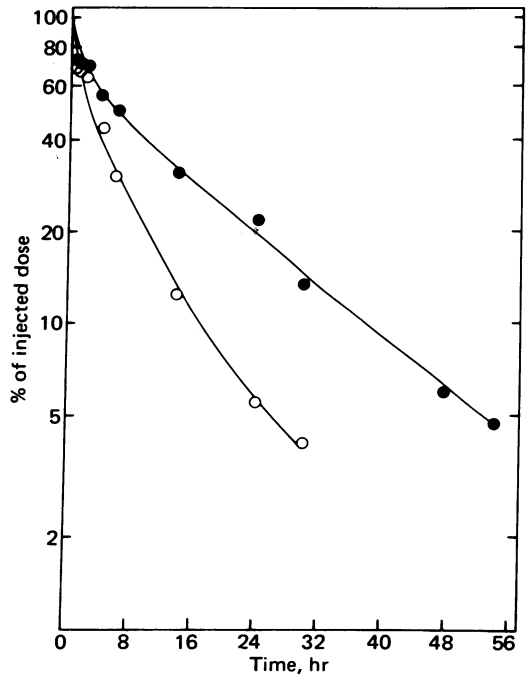

FIG. 4. Percent of the total injected dose of control human ^{131}I-LDL (○) and methylated human ^{125}I-LDL (●) that remained in the plasma of a rhesus monkey after 500 μg of each lipoprotein had been simultaneously injected. The methylated LDL had 90% of the total lysine residues modified. A 5-kg normal rhesus female was the recipient.

Table 2. Simultaneous injection of control human ^{131}I-LDL and methylated human ^{125}I-LDL into rhesus monkeys

		Control LDL		Methylated LDL		
Exp.	Sex	$t_{1/2}$, hr	FCR	$t_{1/2}$, hr	FCR	Lysines modified, %
I*	♂	9.2	0.086	21.3	0.041	77
II*	♂	6.5	0.123	13.5	0.068	85
III*	♀	6.8	0.135	13.3	0.056	90
Mean		7.5	0.115	16.0	0.055	
±SD		±1.5	±0.026	±4.6	±0.014	

* Three different monkeys and three different preparations of LDL were used.

of a monkey ($t_{1/2}$ of second exponential: control, 6.8 hr; modified, 13.3 hr). This observation was confirmed in additional studies and the results are compiled in Table 2. The modified LDL had a longer $t_{1/2}$ (approximately twice that of control LDL; calculated from the second exponential) and a smaller FCR (≈50% less).

Because we had previously demonstrated that binding of LDL to the cell surface receptors of fibroblasts could also be prevented by modification of arginine residues (5), studies were conducted *in vivo* with control LDL and LDL modified by treatment with 1,2 -cyclohexanedione. As shown in Fig. 5, modification of approximately 50% of the arginine residues of rat LDL caused a retardation in their clearance from the plasma of rats. However, it has been reported (19) that the formation of the cyclohexanedione derivative of arginine is spontaneously reversible at 37°C.

To determine the stability of both the arginine modification with cyclohexanedione and the lysine modification with reductive methylation, [^{14}C]cyclohexanedione and [^{14}C]methyl derivatives of LDL were prepared, and the reversals with time were determined in serum or phosphate buffer. Methylation of the lysine residues was found to be a stable modification. After a 24-hr incubation at 37°C with whole serum, >95% of the label was still associated with the LDL. However, only 67% of the label was associated with the LDL after [^{14}C]cyclohexanedione-modified LDL were incubated in serum for 24 hr at 37°C. Furthermore, the modification was 53% reversible when [^{14}C]cyclohexanedione-modified LDL were incubated in phosphate for 24 hr at 37°C. This spontaneous reversibility of the cyclohexanedione modification of the LDL arginine residues was found to be progressive with increasing incubation time in $\rho > 1.21$ g/ml lipoprotein-deficient sera at 37°C. The regeneration of the arginine residues was determined by measuring the abilities of the various lipoprotein preparations incubated for different times to compete with control ^{125}I-LDL for receptor binding activity in fibroblasts. As shown in Fig. 6, cyclohexanedione-modified LDL that were not incubated at 37°C prior to the experiment were incapable of displacing the ^{125}I-LDL from the receptors. However, there was a progressive restoration of binding activity with time from 5 to 48 hr of incubation. By contrast, incubation of reductively methylated LDL for 48 hr in the $\rho > 1.21$ g/ml lipoprotein-deficient sera at 37°C did not restore binding activity to these lipoproteins (Fig. 6).

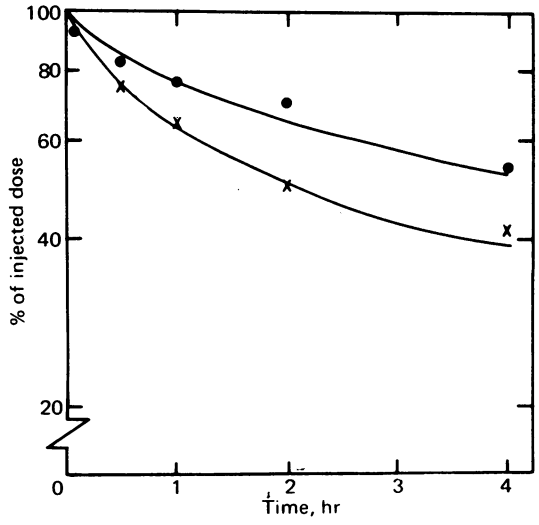

FIG. 5. Percent of the total injected dose of control rat ^{125}I-LDL (X) and cyclohexanedione-modified rat ^{125}I-LDL (●) that remained in the plasma as a function of time. Each point represents the value obtained from an individual rat (100 μg of lipoprotein protein injected). The extent of the level of modification of the arginine residues by cyclohexanedione is estimated at 50%, on the basis of previous experience (5). The modified LDL lacked the ability to bind to the cell receptors of cultured fibroblasts.

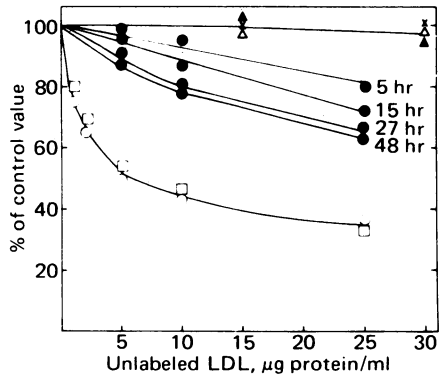

FIG. 6. Competitive displacement of human ^{125}I-LDL from the cell surface receptors of human fibroblasts by various control and modified lipoproteins. □, Control LDL; ○, control LDL incubated for 48 hr in $\rho > 1.21$ g/ml lipoprotein-deficient sera at 37°C; △, methylated LDL kept at 4°C for 48 hr; ▲, methylated LDL incubated in $\rho > 1.21$ g/ml sera at 37°C for 48 hr; X, cyclohexanedione-modified LDL kept at 4°C for 48 hr; and ●, cyclohexanedione-modified LDL incubated in $\rho > 1.21$ g/ml sera at 37°C for 5, 15, 27, or 48 hr as indicated. The binding study was performed at 4°C for 2 hr (2 μg of ^{125}I-LDL protein, 123 cpm/ng of protein). Percent of control value determined by binding in the absence of added lipoprotein (maximal binding 40 ng of ^{125}I-LDL per dish).

DISCUSSION

The observation that the modification of a limited number of lysine or arginine residues of the B apoprotein of LDL prevented their binding to the cell surface receptors of fibroblasts *in vitro* (5, 6) led to the postulate that the plasma clearance of these chemically modified lipoproteins would be retarded. If LDL are at least partially metabolized via a receptor-mediated uptake process by peripheral cells, such as fibroblasts, *in vivo*, as is strongly supported by the studies of Goldstein, Brown, and coworkers (20, 21), the rate of disappearance of the modified LDL, as compared with control LDL, would serve as an approximation of the magnitude of this process. However, interpretation of these data should be made with some reservation. The value obtained for the peripheral cell component of LDL plasma clearance could represent an overestimate, because the liver might also remove LDL by an uptake process that could also be blocked by the chemical modification of the lipoproteins. Nevertheless, studies with chemically modified lipoproteins are useful to probe the mechanisms and cell types responsible for lipoprotein catabolism.

When 30% or more of the lysine residues of LDL were modified by reductive methylation, the plasma clearance was markedly reduced, as determined by following the plasma activity of control and modified LDL. This was observed with both rat and human LDL injected intravenously into rats and with human LDL injected into rhesus monkeys. The values for the FCR were reduced by 40–50% in the rat and by 50% in the monkey by comparison with the values obtained for control LDL. The retardation in clearance of the lysine-modified LDL is compatible with the interpretation that methylation prevents LDL from interacting with receptor-mediated uptake processes in the whole animal. On the basis of the turnover data for LDL obtained by Simons *et al.* (22) and Bilheimer *et al.* (23), it has been speculated that two-thirds of the LDL degraded daily in man are degraded by the receptor-mediated pathway (21). Data from the present study for rats and monkeys would indicate that approximately half of the plasma clearance of LDL is mediated by the receptor process.

It was also demonstrated that modification of the arginine residues of the B apoprotein of LDL with cyclohexanedione retarded plasma clearance of these lipoproteins. However, the cyclohexanedione modification of the arginine residues was not stable at 37°C and was slowly reversed over a period of a few hours. The spontaneous reversibility has been reported previously (19). Modification of the lysine residues by reductive methylation was stable at 37°C.

In conclusion, it has been shown that selective chemical modification of specific amino acid residues of LDL resulted in an altered metabolism *in vivo*. The changes suggest a role *in vivo* for cell surface receptors previously described for cells grown in culture.

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