Inhibition of Lipoprotein Binding to Cell Surface Receptors of Fibroblasts following Selective Modification of Arginyl Residues in Arginine-rich and B Apoproteins*

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Treatment of human low density lipoproteins (LDL) with 0.1 mM 1,2-cyclohexanedione in borate buffer selectively modified half of the arginyl residues of the apolipoproteins and almost totally abolished the binding of the LDL to the high affinity cell surface receptors of human fibroblasts. Except for the modification of the arginyl residues, there were no apparent alterations in the other amino acid residues, lipid composition, size and morphologic appearance, or apoprotein pattern. Removal of cyclohexanedione by incubation of the modified LDL with 0.5% hydroxylamine for 7 h restored more than 80% of the original activity as determined by competitive binding, internalization, and degradation studies with 125I-LDL. Likewise, selective modification with cyclohexanedione of the arginyl residues of certain canine lipoproteins (LDL, HDL, and HDL) prevented their interaction with the cell surface receptors. In particular, the binding activity of the cholesterol-induced HDL, which contain the arginine-rich apoprotein as the only detectable protein was abolished by cyclohexanedione. The only detectable alteration in the canine lipoproteins was a modification of the arginyl residues. Several conclusions are suggested by these studies: (a) the recognition site on the lipoprotein which determines specificity for binding to the cell surface receptors resides with the apoprotein; (b) both the B and arginine-rich apoproteins can react with the receptor; (c) a structural sequence or similarly charged (sterespecific) region may be common to both apoproteins; and (d) a limited number of arginyl residues are functionally significant in or near the recognition site on the lipoproteins.

The specificity for binding certain classes of plasma lipoproteins to the high affinity cell surface receptors on fibroblasts appears to be determined by the apoprotein content of the lipoproteins. Previously, it has been shown that lipoproteins containing apolipoprotein B, as LDL (1), or the arginine-rich apoprotein, as HDL (2, 3), were bound to the same cell surface receptor on human fibroblasts. Typical HDL, which lacked the B and arginine-rich apoprotein, were not bound. Additional evidence that the arginine-rich apoprotein was involved in the interaction of lipoproteins with the receptor came from studies of the differential precipitation of subpopulations of canine and swine HDL, following heparin/manganes treatment (4). The fractions of HDL, which were most readily precipitable with heparin were the most effective in competing with LDL for binding, internalization, and degradation by human fibroblasts. By comparison, the subpopulation of HDL which was not precipitated with heparin did not displace LDL from the cell surface receptors. Characterization of the highly reactive and precipitable HDL, revealed that the arginine-rich apoprotein was a major apoprotein constituent, whereas the nonprecipitable, nonreactive fraction lacked detectable arginine-rich apoprotein. Since heparin has been shown to displace LDL from fibroblasts by binding to the lipoproteins (5), presumably at sites of positive charge (6), it was proposed that specific regions of positive charge on LDL and HDL may be important for binding to cell surface receptors. It is possible that these regions of similar positive charge are associated with a primary structural sequence common to both the B and arginine-rich apoproteins (4).

One of the components of a lipoprotein which is positively charged at physiologic pH is the amino acid arginine. Because arginine is a prominent constituent of the arginine-rich apoprotein and present in the B apoprotein, studies were undertaken to modify selectively the arginyl residue of the various apolipoproteins which have been shown to bind to the cell surface receptor. A recently described reagent which reacts specifically with arginine or the arginyl residues of proteins under very mild conditions is 1,2-cyclohexanedione. It has been demonstrated that cyclohexanedione reacts only with the guanido groups of arginine and not with other amino acid residues (7-9). The product is stable but cyclohexanedione

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The abbreviations used are: LDL, low density lipoproteins; HDL, high density lipoproteins; DME media, Dulbecco's modified Eagle's media; SDS, sodium dodecyl sulfate; TLC, thin layer chromatography.

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may be released quantitatively by incubation of the modified protein with hydroxylamine. Thus, it has been possible to block selectively the activity of certain enzymes, such as pancreatic ribonuclease A, which have arginine in or near the active site of the molecule and then to regenerate most of the enzymatic activity following the removal of the 1,2-cyclohexanedione derivative.

In the present studies, cyclohexanedione was used to modify the arginyl residues of the apolipoproteins of human LDL and certain canine lipoproteins, and the effects of this modification upon competitive binding, internalization, and degradation of the lipoproteins by human fibroblasts were assessed. The canine lipoproteins LDL, HDL$_r$, and HDL$_s$ were studied because all three have been shown to be bound to the high affinity cell surface receptors of fibroblasts despite differences in apoprotein constituents (4). LDL and HDL$_s$, (an $\alpha_2$-migrating lipoprotein) obtained from control dogs have similar chemical compositions but differ in that the HDL$_s$ lack detectable $B$ apoprotein and contain both the A-1 and arginine-rich apoproteins (10). When dogs were fed a semisynthetic diet containing coconut oil and cholesterol, an $\alpha_2$-migrating lipoprotein similar to the HDL$_s$, (referred to as HDL$_s$ in the cholesterol-fed dog) became a major carrier of the plasma cholesterol. The canine HDL$_s$ contained the arginine-rich apoprotein as the major, sometimes exclusive, apoprotein (3, 11). The effect of arginine modification by cyclohexanedione on the binding of human LDL and canine LDL, HDL$_r$, and HDL$_s$, to cell surface receptors in human fibroblasts will be presented.

**EXPERIMENTAL PROCEDURES**

**Materials**—Dulbecco's modified Eagle's media (Catalogue No. H-21), fetal calf serum, trypsin/EDTA solution, Dulbecco's phosphate-buffered saline (Catalogue No. K-13), potassium penicillin G, and streptomycin sulfate were purchased from Gibco (Grand Island, N.Y.). Sodium [3H]iodide (carrier-free) in NaOH was obtained from Amersham/Biocode (Arlington Heights, Ill.) and cyclo[14C]hexanone (18.7 mCi/mmol) was obtained from New England Nuclear (Boston, Mass.). Analytical reagent grade 1,2-cyclohexanedione (Aldrich), hydroxyamine hydrochloride, mannitol, and sodium borate (Fisher) were used. Chloroform, methanol, and acetone used for lipid analyses were glass-distilled (Burdick and Jackson).

**Plasma Lipoprotein Isolation—Human LDL.** The LDL and HDL$_s$ were prepared by ultracentrifugation in a 60 Ti rotor at 59,000 rpm for 48 h. The second time with chloroform:methanol:H$_2$O (1:2:0.8). Equal volumes of water and chloroform were then added to the combined extracts and the upper aqueous phase was removed. Folch solvent upper phase was used to wash the side walls of the tube and the interface was discarded. The second time with chloroform:methanol:H$_2$O (1:2:0.8). Equal volumes of water and chloroform were then added to the combined extracts and the upper aqueous phase was removed. Folch solvent upper phase was used to wash the side walls of the tube and the interface was discarded.

**Regeneration of Lipoproteins by Removal of Cyclohexanedione**

An aliquot of the cyclohexanedione-modified lipoprotein was mixed with an equal volume of 1 M hydroxylamine and 0.3 M mannitol at pH 7. The solution was incubated at 38°C for 7 h and then dialyzed for 12 to 24 h against 0.15 M NaCl and 0.01% EDTA, pH 7, at 4°C. The procedure was based on previously described methods (7, 9).

**Amino Acid Analysis—** Solutions of lipoproteins for amino acid analysis were lyophilized and lipid was extracted with chloroform:methanol:ether (2:1:1). Hydrolysis of samples of 0.5 mg was performed in 1 ml of 6 N HCl in the presence of 20 $\mu$mol of mercuric chloride at 110°C for 24 h. Lyophilized 100 mmol of nitrogen were used. To establish the stability of the cyclohexanedione derivative of arginine, N7,N1(1,2-dihydroxy)cyclohex-1,2-ylenearginine hydrochloride was prepared (7) and subjected to hydrolysis conditions. In the presence of mercaptoacetic acid, the cyclohexanedione derivative of arginine was converted to a neutral product without regeneration of arginine as described by Patthy and Smith (7). Amino acids were quantitated on an automated analyzer equipped with sample applicator. The data were arbitrarily normalized assuming 250 amino acid residues/mole of LDL protein and 290 amino acid residues/mole of HDL protein.

**Cyclo[14C]hexanone Synthesis—** 1,2-Cyclo[14C]hexanone was prepared from cyclo[1]hexanone by a modification of the procedure of Hach et al. (21). Cyclo[14C]hexanone (5.3 mg, 54 $\mu$mol) was oxidized with an equimolar quantity of selenium acid. Following the oxidation, unlabeled 1,2-cyclohexanedione (1.6 g, 14.3 mmol) was added to the reaction mixture and the labeled product was purified by vacuum distillation. The product was stored under nitrogen at –40°C until use.

**Lipid Analyses—** Lipids were extracted as described by Kates (22). Chloroform:methanol (1:2) was added to the lipoproteins in solution. The solvent was removed and the residue extracted with a second time with chloroform:methanol:H$_2$O (1:2:0.8). Equal volumes of water and chloroform were then added to the combined extracts and the upper aqueous phase was removed. Folch solvent upper phase was used to wash the side walls of the tube and the interface (23). In the experiments using the cyclo[14C]hexanone-labeled...
lipoproteins, it was possible to account for greater than 90% of the original activity in both the lipid and non-lipid fractions obtained by the procedure described above.

Phospholipids were separated by one- or two-dimensional thin layer chromatography. One-dimensional TLC was performed on silica Gel H containing 1 mm Na₂CO₃ and development with chloroform:methanol:acetic acid:H₂O (25:15:4:2) (24). Two-dimensional TLC was performed on Silica Gel H containing 7.5% magnesium acetate (25). Plates were activated for 1 h at 120°. Development in the first dimension was with chloroform:methanol:ammonia (65:25:5) and in the second dimension with chloroform:acetone:methanol:acetic acid:H₂O (5:2:1:0.5). Visualization of the chromatograms for qualitative evaluation and for phospholipid phosphorus analysis was by charring at 175° after spraying with 0.6% potassium dichromate vapors prior to scraping for ³¹C quantitation. Phospholipid composition was determined by phosphorus analysis as described by Bartlett (26).

**RESULTS**

The specificity and reversibility of the reaction of 1,2-cyclohexanedione with the guanido group of the arginyl residues of several proteins have been reported (7-9). The reaction occurs most effectively in a borate buffer at pH 8 to 9 at 25-40°. Removal of cyclohexanedione and quantitative regeneration of the arginyl residues is accomplished by incubation at 37° in hydroxylamine buffer for 7 to 16 h (7-9). Using the conditions described by Dietl and Tschesche (9), we found that the treatment of human LDL with 0.1 M cyclohexanedione for 2 h at 35° markedly inhibited the ability of this lipoprotein to compete with ¹²⁵I-LDL for binding, internalization, and degradation by human fibroblasts (Fig. 1, top curve). Binding activity was restored to the modified LDL by removal of the cyclohexanedione (Fig. 1). The untreated LDL (bottom curve) gave a typical competitive displacement curve for binding, internalization, and degradation by fibroblasts at 37°. Likewise, when the study was performed at 4°, the modified LDL were incapable of displacing the ¹²⁵I-LDL from the high affinity receptor sites, indicating a direct effect of the cyclohexanedione specifically on the binding process (Fig. 2).

To determine the most effective and yet mildest conditions for the reaction with the lipoproteins, different concentrations of cyclohexanedione and different times for the reaction of cyclohexanedione with human ¹²⁵I-LDL were compared with respect to the propensity of the modified ¹²⁵I-LDL to be bound, internalized, and degraded by human fibroblasts. The time required for optimal reaction of cyclohexanedione with human LDL was examined by incubating ¹²⁵I-LDL with 0.05 M cyclohexanedione at 35° for variable times from 0 min to 2 h. There was a decrease in the ability of the cyclohexanedione-treated ¹²⁵I-LDL to be bound, internalized, and degraded by the fibroblasts with increasing time of incubation up to 2 h (Fig. 3). In subsequent experiments, incubation with cyclohexanedione was performed for 2 h at 35°.

To determine the optimal concentration of cyclohexanedione...
in the reaction mixture, human \( {^{125}}\text{I-LDL} \) were incubated for 2 h at 35°C with varying concentrations of cyclohexanedione from 0.05 to 0.1 M. The binding and degradation of cyclohexanedione-treated \( {^{125}}\text{I-LDL} \) were decreased with increasing concentrations of cyclohexanedione (Fig. 4). At a concentration of 0.1 M cyclohexanedione, only 1% of the modified \( {^{125}}\text{I-LDL} \) was bound and internalized by the fibroblasts as compared to the untreated \( {^{125}}\text{I-LDL} \) (see inset, Fig. 4). Since 0.05 and 0.075 M cyclohexanedione were less effective, subsequent experiments were performed with 0.1 M cyclohexanedione.

To determine the optimal time for removal of cyclohexanedione from the arginyl residues of LDL, the modified \( {^{125}}\text{I-LDL} \) were incubated with hydroxylamine for 2 to 8 h (Fig. 5). Seventy per cent of the binding capacity could be restored by a 7- to 8-h incubation. In other experiments to be shown later, it was possible to regenerate more than 80% of the original reactivity. Incubation for 16 h did not restore any additional activity to the LDL. Therefore, an incubation time of 7 to 16 h was used to release the cyclohexanedione from the lipoproteins in subsequent experiments.

Reaction of human LDL with cyclohexanedione and the subsequent release of the cyclohexanedione under the conditions described above did not alter the physical or chemical properties of the lipoproteins except for their electrophoretic mobility. On paper electrophoresis (Fig. 6), the cyclohexanedione-treated LDL were relatively more negative and migrated further toward the anode than the untreated or regenerated LDL. Both the untreated and regenerated LDL had typical \( \beta \) mobility. By negative-staining electron microscopy, the untreated LDL, cyclohexanedione-treated LDL, and regenerated LDL (cyclohexanedione treatment followed by cyclohexanedione removal) were of the same size and had the same morphologic appearance (Fig. 7). The chemical composition (Table I) and apoprotein pattern on SDS-polyacrylamide gel electrophoresis were likewise identical for the untreated, treated, and regenerated LDL. These results in combination with the competitive binding and degradation studies shown in Fig. 1 indicated that the reaction was mild, that it did not alter the physical or chemical properties, and that it was almost completely reversible.

To determine the extent of the modification of the apoproteins by the cyclohexanedione treatment, the amino acid compositions of the lipoprotein proteins were compared before and after reaction with cyclohexanedione and after the release of cyclohexanedione with hydroxylamine treatment. Previously, it was reported by Putthy and Smith (7) that the product of the reaction of 1,2-cyclohexanedione with arginine was stable under acid hydrolysis conditions in the presence of mercaptoacetic acid. We have confirmed these results. Since under acidic conditions cyclohexanedione-modified arginine did not undergo conversion to the unmodified amino acid, it was possible to determine by subtractive amino acid analysis the number of arginine residues which reacted with cyclohexanedione. Amino acid analyses of the LDL revealed that the only amino acid modified was arginine (Table II). Untreated LDL contained 9 residues of arginine per mol, assuming 250 amino acid residues per mol, as compared to 5 residues following the reaction with cyclohexanedione (i.e. 4 of 9

![Fig. 4. Binding, internalization, and degradation of human \( {^{125}}\text{I-LDL} \) after reaction with various concentrations of cyclohexanedione. Human \( {^{125}}\text{I-LDL} \) protein (240 \( \mu \)g) was incubated for 2 h at 35°C with the indicated concentrations of 1,2-cyclohexanedione in 0.166 M borate buffer (pH 8.1). The \( {^{125}}\text{I-LDL} \) was incubated with the cells at a lipoprotein protein concentration of 5 \( \mu \)g/ml with a specific activity of 340 cpm/\( \mu \)g. In the inset, the 100% bound represents the untreated (native) \( {^{125}}\text{I-LDL} \) bound and internalized (total bound) minus the nonspecific total binding (19.9 \( \mu \)g/mg) which was arrived at by incubating the native \( {^{125}}\text{I-LDL} \) with 300 \( \mu \)g/ml of unlabeled LDL.](http://www.jbc.org/)

![Fig. 5. Recovery of the ability of the cyclohexanedione-modified \( {^{125}}\text{I-LDL} \) (\( \bullet \)) to be bound and degraded by fibroblasts after removal of cyclohexanedione by incubation with hydroxylamine for the indicated times. Human \( {^{125}}\text{I-LDL} \) (535 \( \mu \)g) was treated with 0.1 M 1,2-cyclohexanedione in 0.166 M borate buffer (pH 8.1) for 2 h at 35°C. After 2 h, half of the modified \( {^{125}}\text{I-LDL} \) was added to an equal volume of 1 M hydroxylamine, 0.3 M mannitol (pH 7.0), and aliquots were removed at the indicated times. The regenerated \( {^{125}}\text{I-LDL} \) was added at a protein concentration of 10 \( \mu \)g/ml with a specific activity of 279 cpm/\( \mu \)g. The value for binding and degradation of the untreated \( {^{125}}\text{I-LDL} \) (\( \circ \)) at a concentration of 10 \( \mu \)g/ml is indicated. Other conditions were as described in Fig. 1.](http://www.jbc.org/)

![Fig. 6. Paper electrophoretograms of untreated (native) human LDL (top pattern), LDL treated with cyclohexanedione (LDL + 1,2-CHD), and LDL from which the cyclohexanedione was removed (LDL - 1,2-CHD). Conditions for the modification and regeneration were as described in Fig. 1.](http://www.jbc.org/)
residues were modified. After the release of cyclohexanedione with hydroxylamine, 8 residues of arginine were detected by amino acid analysis (Table II). An aliquot of the same untreated, treated, and regenerated LDL subjected to amino acid analyses was utilized in the competitive binding and degradation studies. The results were essentially identical with those depicted in Fig. 1 and revealed that the modification of 4 arginine residues virtually abolished the capability of the LDL to compete for binding and degradation. Eighty per cent of the original activity was recovered after the removal of the cyclohexanedione by hydroxylamine incubation.

There was a direct correlation between the number of arginyl residues modified and the ability of the LDL to competitively inhibit the binding, internalization, and degradation of $^{125}$I-LDL. When 0.05 M cyclohexanedione was allowed to react with LDL for 15, 30, 60, and 120 min, the number of modified arginyl residues increased with time (Table III). Competitive binding and degradation studies revealed a progressive decrease in the capability of the modified LDL to compete with the $^{125}$I-LDL for both binding and degradation. As shown in Fig. 8, modification of 2 arginine residues resulted in a 50% decrease in the capacity of the modified LDL to inhibit degradation of the $^{125}$I-LDL as compared to the untreated LDL at a concentration of 20 $\mu$g/ml of LDL protein. The modification of approximately half of the arginyl residues (4 out of 9 residues) resulted in the abolishment of greater than 85% of the activity of the LDL (Fig. 8).

**TABLE I**

| Lipid and protein composition determined on duplicate samples of untreated (native) LDL, LDL modified by incubation with cyclohexanedione (CHD-treated), and LDL from which the cyclohexanedione was removed (regenerated). Conditions for the modification and regeneration were as described in Fig. 1. |

| Lipid and protein composition | Untreated | CHD-treated | Regenerated |
|-----------------------------|-----------|-------------|-------------|
| Triglyceride                | 7.2       | 8.4         | 8.5         |
| Total cholesterol           | 41.9      | 40.7        | 40.4        |
| Phospholipid                | 26.6      | 25.9        | 25.0        |
| Protein                     | 25.2      | 25.0        | 26.1        |

**TABLE II**

| Amino acid composition of human LDL |
|------------------------------------|
| Amino Acids | Untreated | CHD-treated | Regenerated |
| Aspartic acid | 27.1 (27)* | 27.4 (27) | 27.2 (27) |
| Threonine | 14.0 (14) | 14.9 (15) | 13.4 (13) |
| Serine | 20.9 (21) | 20.5 (21) | 20.5 (21) |
| Glutamic acid | 30.8 (31) | 31.4 (31) | 31.0 (31) |
| Proline | 10.1 (10) | 10.6 (11) | 10.5 (11) |
| Glycine | 12.1 (12) | 12.3 (12) | 11.9 (12) |
| Alanine | 15.3 (15) | 15.9 (16) | 15.6 (16) |
| Valine | 13.6 (14) | 14.0 (14) | 14.0 (14) |
| Methionine | 3.9 (4) | 3.7 (4) | 3.8 (4) |
| Isoleucine | 14.4 (14) | 14.5 (15) | 14.5 (15) |
| Leucine | 30.6 (31) | 30.6 (31) | 30.4 (30) |
| Tyrosine | 8.4 (8) | 8.4 (8) | 8.3 (8) |
| Phenylalanine | 12.4 (12) | 12.4 (12) | 12.3 (12) |
| Lysine | 21.2 (21) | 20.0 (20) | 20.8 (21) |
| Histidine | 6.3 (6) | 6.2 (6) | 6.1 (6) |
| Arginine | 8.6 (9) | 5.2 (5) | 8.1 (8) |

* Amino acid residues calculated per mol assuming 250 residues per mol. Value in parentheses is rounded off to nearest whole number. Conditions for the modification and regeneration were as described in Fig. 1.
Although the chemical reactivity of 1,2-cyclohexanedione would appear to preclude a reaction with the lipids, it was important to exclude this possibility, particularly a reaction with the phospholipids which might alter the surface properties of the lipoproteins. The phospholipid classes from the untreated, treated, and regenerated LDL were separated by thin layer chromatography, visualized by charring, and quantitated by phosphorus determinations. Since a minor modification of phospholipids changes their relative mobility on thin layer chromatography, the reaction of cyclohexanedione with phospholipid might be expected to alter the chromatographic pattern and/or distort the quantitation. A comparison of the one- and two-dimensional chromatograms of the phospholipids of untreated and regenerated LDL with the pattern obtained for the cyclohexanedione-treated LDL indicated that they were essentially identical, as was the quantitation (Table IV).

To quantitate the amount of cyclohexanedione which occurred in the lipid extract, cyclo[14C]hexanedione was allowed to react with human LDL and then the lipids were extracted with chloroform:methanol. Of the total 14C activity associated with the modified LDL, only 1.6 and 1.8% appeared in the lipid extract in two separate experiments. After chloroform:methanol extractions of the free, untreated cyclo[14C]hexanedione reagent, approximately 4% of the total activity appeared in the organic phase. Therefore, a portion of the 1.6 or 1.8% detected in the lipid extract of the LDL could have been free cyclohexanedione. Two-dimensional chromatographic separation of the lipid extract which contained the 14C activity from the LDL revealed that the activity moved with the solvent front in the second dimension. The unreacted, free cyclo[14C]hexanedione also migrated with the solvent front in an identical position. No activity was detected in any of the phospholipids.

Since specific canine lipoproteins (LDL, HDL1, and HDL2) have been shown to interact with the same cell surface receptor on human fibroblasts as human LDL (4), it was of interest to determine whether cyclohexanedione treatment of these lipoproteins prevented their binding and degradation, thus suggesting a common property through which these reactive lipoproteins interact with the receptor. The ability of untreated canine LDL to compete with canine 125I-LDL for binding and degradation by human fibroblasts was compared to that of cyclohexanedione-modified LDL. The modification eliminated the ability of HDL1 and HDL2 (primarily apoprotein B) and canine HDL3, and HDL4 (primarily arginine-rich and A-I apoproteins), modification of the HDL1, and HDL2 by cyclohexanedione also prevented the interaction of these lipoproteins with the receptors. The ability of the control HDL and the cholesterol-induced HDL to compete with 125I-LDL for binding and degradation was compared with those of the cyclohexanedione-modified HDL, and HDL4. The modification eliminated the ability of HDL, and
HDL$_1$ to displace the $^{125}$I-LDL from the receptors as shown by the binding, internalization, and degradation assays (Figs. 10 and 11). As was observed with human and canine LDL, the activities of the HDL$_1$ and HDL$_2$ were restored by the release of cyclohexanedione from these lipoproteins (Figs. 10 and 11).

The HDL from a coconut oil-cholesterol-fed dog used in the above experiment contained the arginine-rich apoprotein as the only detectable apoprotein. The occurrence of such a lipoprotein had been previously reported to occur in dogs on this diet (3). Amino acid analyses of the total HDL$_1$ protein confirmed that the arginine-rich apoprotein was the major apoprotein constituent, since the arginine content was determined to be approximately 10 mol% and the complete analysis was essentially identical with that of the isolated and purified arginine-rich apoprotein previously described (3). When the calculations were based on 290 amino acid residues per mol of protein (assuming a molecular weight of ~35,000), the analyses showed that the untreated HDL$_1$ contained 27 residues of arginine per mol of protein. The HDL$_1$, treated with 0.1 M cyclohexanedione for 120 min had 10 arginine residues (i.e. 17 residues modified). Following the regeneration of the HDL$_1$ with the removal of the cyclohexanedione, 25 arginine residues were determined by amino acid analysis. The other amino acid residues were unchanged by the treatment. Comparison of the untreated and regenerated HDL$_1$ revealed no alteration in the chemical composition, morphologic appearance by negative-staining electron microscopy, or apoprotein pattern by SDS-polyacrylamide gel electrophoresis.

**DISCUSSION**

The observations that certain plasma lipoproteins bind to specific cell surface receptors on human fibroblasts and that this initiates a series of events which regulates cellular cholesterol metabolism advanced the understanding of lipoprotein metabolism (for a review see Ref. 27). The initial observation that LDL, but not HDL, were bound to high affinity receptor sites suggested a possible specificity of the receptors for the B apoprotein (1). Our studies with lipoproteins from cholesterol-fed dogs and swine indicate that not only the B apoprotein but also the arginine-rich apoprotein might be involved in the interaction of lipoproteins with fibroblasts and arterial smooth muscle cells. The cholesterol-induced HDL$_1$ of the dog, which contain only the arginine-rich apoprotein, or the HDL$_2$ of the swine, which contain the arginine-rich and A-I apoproteins (and lack apoprotein B), are as active as LDL in the suppression of 3-hydroxy-3-methylglutaryl CoA reductase activity in smooth muscle cells and fibroblasts (2, 28), in the accumulation of cellular cholesterol (3) and in binding to the same cell surface receptors (2, 4).

Recent studies of subfractions of canine and swine lipoproteins separated on the basis of differential heparin/manganese precipitation further indicate that the B and arginine-rich apoproteins are involved in receptor specificity and suggest that there is a structural sequence or similarly charged region common to both apolipoproteins (4). Fractions of HDL$_1$ which are readily precipitated by heparin are the most effective in competing with LDL for binding, internalization, and degradation and are the fractions which contain the most arginine-rich apoprotein. The nonprecipitable HDL$_1$ do not bind to the receptors and contain little or no detectable arginine-rich...
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that the surface charge resides on the apolipoproteins and that both the B and arginine-rich apoproteins are capable of reacting with the receptor. It also appears that a similar positively charged region of the B and arginine-rich apoprotein on the lipoprotein surface is involved and that the arginyl residues are a functionally significant part of the recognition site. However, the precise mechanism by which the cyclohexanedione modification of the B and arginine-rich apoproteins prevents the binding of the lipoproteins awaits more detailed information on the nature of lipoprotein-cell receptor interactions and more detailed characterization of the recognition site.

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REFERENCES

1. Brown, M. S., and Goldstein, J. L. (1974) Proc. Natl. Acad. Sci. U. S. A. 71, 788-792
2. Bersot, T. P., Mahley, R. W., Brown, M. S., and Goldstein, J. L. (1976) J. Biol. Chem. 251, 2395-2398
3. Mahley, R. W., Innerarity, T. L., Weisgraber, K. H., and Fry, D. L. (1977) Am. J. Pathol. 87, 205-226
4. Mahley, R. W., and Innerarity, T. L. (1977) J. Biol. Chem. 252, 3980-3986
5. Goldstein, J. L., Basu, S. K., Brunachedy, G. Y., and Brown, M. S. (1976) Cell 7, 85-95
6. Iverius, P. H. (1972) J. Biol. Chem. 247, 2607-2613
7. Patthy, L., and Smith, E. L. (1975) J. Biol. Chem. 250, 557-564
8. Patthy, L., and Smith, E. L. (1976) J. Biol. Chem. 251, 555-569
9. Dietl, T., and Tachesche, H. (1976) Hoppe-Seyler's Z. Physiol. Chem. 357, 657-665
10. Mahley, R. W., and Weisgraber, K. H. (1974) Circ. Res. 35, 713-721
11. Mahley, R. W., Weisgraber, K. H., and Innerarity, T. (1975) Circ. Res. 35, 722-733
12. Mahley, R. W., Weisgraber, K. H., and Innerarity, T. (1974) Biochemistry 13, 1964-1968
13. Mahley, R. W., Weisgraber, K. H., Innerarity, T., Brewer, H. J. R., and Assmann, G. (1975) Biochemistry 14, 2817-2823
14. Abell, L. L., Levy, R. B., Brodie, B. B., and Kendall, F. E. (1952) J. Biol. Chem. 195, 357-366
15. Sperry, W. M., and Webb, M. (1960) J. Biol. Chem. 197, 97-106
16. Fletcher, M. J. (1968) Clin. Chim. Acta 22, 393-397
17. Zilversmit, D. B., and Davis, A. K. (1950) J. Lab. Clin. Med. 35, 155-160
18. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275
19. Bilheimer, D. W., Eisenberg, S., and Levy, R. I. (1972) Biochim. Biophys. Acta 260, 212-221
20. Goldstein, J. L., and Brown, M. S. (1974) J. Biol. Chem. 249, 5153-5162
21. Hach, C. C., Banks, C. V., and Eiseh, H. (1963) in Organic Synthesis (Rabjohn, N., ed) Vol. 4, pp. 229-232, John Wiley and Sons, Inc., New York
22. Katoe, M. (1972) Techniques of Lipidology, Isolation, Analysis, and Identification of Lipids, pp. 351-352, American Elsevier Publishing Co., New York
23. Foleh, J., Leeu, M., and Sloane Stanley, G. H. (1967) J. Biol. Chem. 229, 497-508
24. Spitski, V. P., Peterson, R. F., and Barclay, M. (1964) Biochem. J. 90, 374-377
25. Nelson, G. J. (1975) in Analysis of Lipids and Lipoproteins,
| No. | Reference |
|-----|-----------|
| 26. | Bartlett, G. R. (1959) *J. Biol. Chem.* 234, 466-468 |
| 27. | Brown, M. S., and Goldstein, J. L. (1976) *Science* 191, 150-154 |
| 28. | Assmann, G., Brown, B. G., and Mahley, R. W. (1975) *Biochemistry* 14, 3996-4000 |
| 29. | Nishida, T., and Cogan, U. (1970) *J. Biol. Chem.* 245, 4689-4697 |
| 30. | Day, C. E., and Levy, R. S. (1975) *Artery* 1, 150-164 |
| 31. | Riordan, J. F., McElvany, K. D., and Borders, C. L., Jr. (1977) *Science* 195, 884-886 |
Inhibition of lipoprotein binding to cell surface receptors of fibroblasts following selective modification of arginyl residues in arginine-rich and B apoproteins.

R W Mahley, T L Innerarity, R E Pitas, K H Weisgraber, J H Brown and E Gross

J. Biol. Chem. 1977, 252:7279-7287.