Tetranitromethane modification of human high density lipoprotein (HDL_3): inactivation of high density lipoprotein binding is not related to cross-linking of phospholipids to apoproteins

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Abstract Treatment of human high density lipoprotein (HDL) with tetranitromethane (TNM) inhibits its binding to HDL-specific binding sites of cells and isolated membranes. The mechanism of this inhibition, however, is not known; during treatment of HDL with TNM, in addition to the expected nitration of tyrosine residues, cross-linking of lipids to apoproteins and of apoproteins to one another occurs. In order to determine whether the cross-linking of lipids to apoproteins occurs through the carbon–carbon double bonds in the acyl chains, and to determine whether the cross-linking of phospholipids to apoproteins is a possible mechanism of inhibition of binding, we have prepared a reconstituted HDL_3 in which the native phospholipids were replaced with dimyristoyl phosphatidylcholine (DMPC). As a control, a reconstituted HDL_3 (C-r-HDL_3) was also prepared using the total apoproteins and the total lipid constituents of native HDL_3. The reconstituted DMPC-containing HDL_3 (DMPC-r-HDL_3) was similar to native HDL_3 and to C-r-HDL_3 in its agarose gel electrophoretic mobility, in its chemical composition, and in its binding to rat liver plasma membranes. When treated with TNM, DMPC-r-HDL_3, like the native HDL_3 and C-r-HDL_3, lost its ability to bind to the HDL binding sites of rat liver plasma membranes, as determined by competitive binding assays with ^{125}I-labeled human HDL_3 as the tracer. Nitrated DMPC-r-HDL_3 contained only traces of phospholipids covalently linked to apoproteins, whereas 21–26% of the total phospholipids were cross-linked to apoproteins of nitrated C-r-HDL_3 and nitrated native HDL_3. It is concluded from these results that, during treatment of human HDL_3 with TNM, cross-linking of lipids to apoproteins occurs through double bonds in the acyl chains, and that the inhibition of HDL binding by treatment of HDL with TNM is not due to cross-linking of phospholipids to apoproteins.—**Chacko, G. K., S. Lund-Katz, W. J. Johnson, and J. B. Karlin.** Tetranitromethane modification of human high density lipoprotein (HDL_3): inactivation of high density lipoprotein binding is not related to cross-linking of phospholipids to apoproteins. *J. Lipid Res.* 1987. **28:** 332–337.

Supplementary key words DMPC • plasma membranes

High-affinity saturable binding sites for apoprotein E-free high density lipoproteins (HDL) have been detected in a variety of cells and membranes (1, 2). They have been implicated in several physiological processes, especially in the flux of unesterified cholesterol from cells (3, 4) and in the preferential transfer of cholesteryl esters into rat liver, adrenal, and ovarian cells (5–7). Recent studies have shown that treatment of human HDL_3 with tetranitromethane (TNM) inhibits its binding to HDL binding sites of cells and isolated membranes (4, 8–10). The mechanism of this inhibition, however, is not known. During treatment of HDL_3 with TNM, in addition to the expected nitration of tyrosine residues of apoproteins, cross-linking of lipids to apoproteins and of apoproteins to one another occurs (10). It seemed likely that the cross-linking of lipids to apoproteins was mediated by the unsaturated carbon–carbon bonds in the fatty acyl chains. Therefore, the objectives of the present studies were: *1)* to test the hypothesis that the cross-linking of lipids to apoproteins occurs through the unsaturated centers of the acyl chains; and *2)* to determine the possible role of cross-linking of phospholipids to apoproteins in the inhibition of the binding of HDL. We prepared a reconstituted human HDL_3 in which the native phospholipids were replaced with dimyristoyl phosphatidylcholine (DMPC). Nitration of the reconstituted DMPC-containing HDL_3 (DMPC-r-HDL_3)

Abbreviations: HDL, high density lipoprotein; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; DMPC, dimyristoyl phosphatidylcholine; TNM, tetranitromethane; C-r-HDL_3, control reconstituted HDL_3; DMPC-r-HDL_3, DMPC reconstituted HDL_3; N-HDL_3, native HDL_3.

with TNM did not result in the cross-linking of phospholipids to apoproteins. The nitrated DMPC-r-HDL_3, like the nitrated native HDL_3, did not bind to the HDL binding sites of rat liver plasma membranes.

Materials

Human HDL_3 ($1.125 < d < 1.21$ g/ml) was isolated by differential ultracentrifugation (11), from fresh human plasma that had been treated with 5 mM N-ethylmaleimide to inhibit lecithin:cholesterol acyl transferase (12). It was further processed by heparin-Sepharose affinity chromatography (13) to remove any traces of apoprotein E, if present. Apoprotein A-I constituted about 70% of the human HDL_3 apoproteins as determined by SDS-polyacrylamide gel electrophoresis (PAGE) followed by Coomassie blue staining and densitometric scanning (14); the other apoprotein components were apoprotein A-II and the C apoproteins. HDL_3 was labeled with ^{125}I using the iodine monochloride procedure (15). The specific activities ranged from 70 to 100 cpm/ng protein. No more than 2% of the label was associated with lipids. Dimyristoyl phosphatidylcholine, A grade, was obtained from Calbiochem (LaJolla, CA). No impurities were detected when it was analyzed by thin-layer chromatography. Tetranitromethane was purchased from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals were of reagent grade.

Methods

Isolation of membrane fractions. Fischer 344 strain male rats weighing 100–150 g were used. Liver plasma membranes were isolated according to the procedure of Ray (16), as described previously (17). The membranes were suspended in 10 mM Tris-HCl buffer, pH 7.4 (containing 0.15 M NaCl and 0.5 mM $CaCl_2$) at a protein concentration of 5 mg/ml and used for the binding studies.

Binding of ^{125}I-labeled human HDL_3 to isolated membranes. The binding of ^{125}I-labeled human HDL_3 to isolated membranes was determined according to the procedure described previously (18). Briefly, aliquots of rat liver plasma membranes (200 μg of protein) were incubated with ^{125}I-labeled human HDL_3 at room temperature for 1 hr in a total volume of 0.2 ml, containing 10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 0.5 mM $CaCl_2$, and 1% BSA (incubation medium). After incubation, 0.175-ml aliquots of the incubation mixture were centrifuged in a Beckman 42.2 Ti rotor at 30,000 rpm for 15 min to recover the membranes. The membrane pellets were washed once by adding 0.175 ml of the incubation medium from the top of the tubes through the sides, followed by centrifugation and aspiration of the supernatant. The tubes containing the membranes were assayed for radioactivity in a Beckman Model 300 gamma scintillation spectrometer with a ^{125}I counting efficiency of 66%. The effect of nitration of HDL_3 on the binding of HDL_3 to the membranes was investigated by studying its ability to compete for the binding of ^{125}I-labeled HDL_3 to the membranes.

Preparation of reconstituted lipoproteins. The total apoproteins from HDL_3 were prepared by delipidation with ethanol–ethyl ether 3:2 (v/v) at 0°C (19). The lipid extract was evaporated to dryness under vacuum and the lipid components were quantitated. For the preparation of DMPC-r-HDL_3, an aliquot of the lipid extract was fractionated into nonpolar and polar fractions by silicic acid column chromatography; chloroform was used to elute the nonpolar fraction and methanol the polar fraction. The lipid components in the fractions were quantitated. An amount of DMPC equivalent to the weight of phospholipid in the polar fraction was added to the nonpolar fraction and this mixture was used to prepare the DMPC-substituted-reconstituted HDL_3, according to the procedure of Hirz and Scanu (20). The ratio of lipid to apoprotein was 1:1. The reconstituted HDL_3 was isolated by ultracentrifugation in the density range of 1.125–1.21 g/ml. Similarly, the total lipid extract from HDL_3, at a ratio of lipid–apoprotein of 1:1, was used to make the control-r-HDL_3. The yield of the DMPC and control reconstituted HDL_3 was about 50%, based on apoprotein recovery.

Preparation of nitrated lipoproteins. Lipoproteins were nitrated with TNM, as previously described (10). The lipoprotein (10 mg of protein/ml) was incubated with a 10-fold molar excess of TNM in 0.05 M Tris-HCl buffer, pH 8.0, containing 0.1 M NaCl for 1 hr at 22°C. The sample was then chromatographed on a Bio-Gel P-6 DG desalting column. The nitrated HDL_3, eluting as the first yellow component from the column, was recovered in nearly 100% yield.

Chemical and physical analyses. Protein was determined by the method of Lowry et al. (21); protein in nitrated HDL_3 was determined according to the biuret procedure (22). BSA was used as the standard. Polyacrylamide gel electrophoresis of apolipoproteins was performed on 10% gels containing 0.1% SDS (14).

Total lipids were extracted from lipoprotein preparations using the procedure of Bligh and Dyer (23). Lipid phosphorus was measured according to the procedure of Rouser, Siakotos, and Fleischer (24). For the determination of phosphorus linked to apoproteins, the lipids were extracted from the lipoprotein samples by the methanol–chloroform–ethyl ether extraction procedure of Lux, John, and Brewer (25) and the apoprotein residues were analyzed for phosphorus by the procedure of Rouser et al. (24). Cholesterol was determined by gas–liquid chromatography using coprostanol as an internal standard. Unesterified and total cholesterol represented cholesterol determined, respectively, before and after saponification (26). Esterified cholesterol was obtained by the difference. For the determination of esterified cholesterol covalently linked to apoproteins, the lipoprotein samples were first delipidated, ac-

cording to the procedure of Lux et al. (25), the apoprotein residues were saponified (26), and the extracts were analyzed for cholesterol by gas–liquid chromatography.

Agarose gel electrophoresis of lipoprotein samples was carried out in barbital buffer, pH 8.6, in the apparatus and according to the direction of Bio-Rad Laboratories (Richmond, CA). After electrophoresis, the lipoproteins were visualized by staining with Fat Red B. For determination of the amino acid composition, the control and the respective nitrated lipoproteins were hydrolyzed in 6 N HCl under vacuum for 24 hr at 110°C and applied on the Beckman 6300 amino acid analyzer.

RESULTS

Preparation and characterization of reconstituted lipoproteins

Three preparations of HDL_3 were used in the present studies: *1)* native HDL_3 (N-HDL_3), as isolated from fresh human plasma; *2)* control-r-HDL_3 (C-r-HDL_3), reconstituted from the total apoproteins and total lipid constituents of native HDL_3; and *3)* DMPC-r-HDL_3, reconstituted from the total apoproteins and total lipids from which the native phospholipids were replaced by an equal amount of DMPC. The same pooled batch of HDL_3 was used for the preparation of all the lipoproteins.

The chemical composition of the three HDL_3 preparations was similar (**Table 1**). The apoprotein patterns of the reconstituted HDL_3 samples, as seen on SDS-PAGE, were similar to that of the native HDL_3. All three preparations had similar electrophoretic mobility on agarose gel electrophoresis (**Fig. 1**).

Preparation and characterization of the nitrated lipoproteins

The conditions used for the nitration of lipoproteins with TNM were the same as used previously (10), namely 10 molar excess of reagent, pH 8.0, and an incubation time of 1 hr at room temperature. These conditions of nitration were shown to completely inhibit the binding of HDL_3 to isolated rat liver plasma membranes (10). As is shown in Fig. 1, nitration of the native and reconstituted HDL_3 samples resulted in a product that moved as a single band, ahead of the unnitrated sample on agarose gel electrophoresis. On SDS-PAGE, a marked reduction in apoproteins was seen for the nitrated samples. A series of Coomassie blue-staining bands appeared in the high molecular weight region of the gel, indicating covalent cross-linking of apoproteins to high molecular weight polymers. These results were similar to those published previously for the native HDL_3 (10).

It was previously predicted (10) that cross-linking of phospholipids to apoproteins during TNM treatment of native HDL_3 occurred through unsaturated centers of fatty acyl chains. Analysis of the apoprotein residues from the nitrated HDL_3s for the presence of phosphorus confirmed this prediction. In **Table 2** is shown the lipid phosphorus bound to apoprotein residues in the unnitrated and nitrated samples. Whereas the apoprotein residues of nitrated native and nitrated control-r-HDL_3 contained about 21–26% of phospholipid phosphorus, only traces of phospholipid phosphorus were found in the case of nitrated DMPC-r-HDL_3.

Effect of nitration of lipoproteins on the binding to rat liver plasma membranes

Competitive binding assays were used to compare the binding characteristics of the unnitrated and nitrated HDL_3 samples to the HDL binding sites of rat liver plasma membranes. Membranes (200 μg of protein) were incubated with a constant, 10 μg/ml of ^{125}I-labeled HDL_3, and various amounts of either unlabeled unnitrated or nitrated HDL_3 (**Fig. 2**). The unnitrated C-r-HDL_3 and DMPC-r-HDL_3 competed effectively, and in a manner similar to that of N-HDL_3, for the binding sites, with an apparent K_d of about 10 μg/ml. This value agrees well with previously published results (18). In contrast, all of the nitrated samples, including nitrated DMPC-r-HDL_3 in which the phospholipids were not linked to apoproteins, failed to compete for the binding of the ^{125}I-labeled HDL_3. Thus the inability of nitrated HDL_3 to compete for the binding is probably related to factor(s) other than the cross-linking of phospholipids to apoproteins.

TABLE 1. Chemical composition of native and reconstituted lipoproteins[a]

	Protein	Phospholipid	Unesterified Cholesterol	Cholesteryl Ester	Triglycerides
			% by weight		
Native HDL_3	57.1 ± 0.1	20.6 ± 1.1	1.8 ± 0.1	17.6 ± 1.2	2.8 ± 0.8
Control-r-HDL_3	55.4 ± 0.5	24.7 ± 1.4	2.1 ± 0.1	15.4 ± 0.1	2.4 ± 0.9
DMPC-r-HDL_3	50.5 ± 2.3	24.9 ± 1.0	1.3 ± 0.02	19.3 ± 0.3	4.0 ± 0.1

[a]Values represent mean ± SD of three determinations.

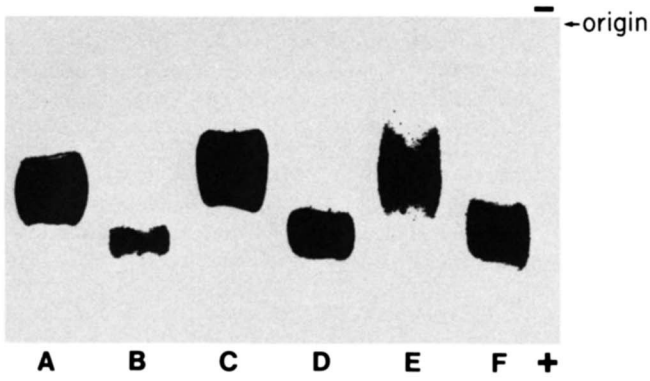

Fig. 1. Agarose gel electrophoresis of unnitrated and nitrated lipoproteins. A, native HDL_3; B, nitrated native-HDL_3; C, control-r-HDL_3; D, nitrated control-r-HDL_3; E, DMPC-r-HDL_3; F, nitrated DMPC-r-HDL_3.

DISCUSSION

In contrast to the LDL receptor, very little is known about the nature of or the component in HDL that is involved in the binding to the putative HDL receptor (or binding sites). Results from a number of studies on the characterization of the binding sites, such as enzymatic modification of binding sites (27–30), effect of cation (31, 32) as well as the effect of chemical modification of HDL (18, 33, 34) on its binding, can be interpreted to indicate that the binding sites are non-protein in nature. On the other hand, isolation of a HDL-specific receptor protein from rat adrenal cortex has recently been reported (35). In addition, cycloheximide inhibition of the cholesterol-induced up-regulation of HDL binding sites in human fibroblasts suggests that the HDL binding sites constitute a receptor protein (3). Regarding the ligand involved in the binding, there is some evidence to suggest that the HDL binding sites recognize the apoprotein A-I, the predominant apoprotein in HDL (32, 17). However, recent data suggest that other apoproteins, including apoprotein A-II and the C apoproteins, may also be recognized (18, 28, 36). Furthermore, there are reports that show that the binding of HDL to human endothelial cells, smooth muscle cells, and fibroblasts is not mediated by a specific ligand or receptor, but rather involves interaction between the lipoprotein and the membrane lipids (37).

TABLE 2. Covalent cross-linking of phospholipids to apoproteins in nitrated lipoproteins[a]

Lipoprotein	Non-Extractable Phosphorus[b] (% of total HDL phosphorus)
Native-HDL_3	2.1 ± 0.1
Nitrated native-HDL_3	25.5 ± 2.5
Control-r-HDL_3	1.5 ± 0.4
Nitrated control-r-HDL_3	21.0 ± 2.9
DMPC-r-HDL_3	0.5 ± 0.5
Nitrated DMPC-r-HDL_3	0.4 ± 0.1

[a]Samples were delipidated by the methanol–chloroform–ethyl ether extraction procedure of Lux et al. (25). The apoprotein residues were analyzed for phosphorus (24).

[b]Values represent mean ± SD of three determinations.

It has recently been shown that treatment of HDL with TNM inhibits the binding of HDL to the HDL binding sites of isolated membranes (8–10) and cultured cells (4, 8, 9). The expected reation is the nitration of tyrosine residues of apoproteins. However, two major side reactions also occur during the treatment of HDL with TNM, i.e., the covalent cross-linking of apoprotein to one another and of lipids to apoproteins. Therefore, possible mechanisms by which the TNM treatment inhibits the binding of HDL are: *1)* nitration of tyrosine residues; *2)* cross-linking of apoproteins to one another; or *3)* cross-linking of lipids, especially the surface phospholipids to apoproteins. A major objective of the present study was to test the last possibility, namely the cross-linking of phospholipids to apoproteins as a mechanism of inhibition of HDL binding. It was hypothesized that cross-linking of phospholipids to apoproteins occurred via unsaturated centers of the phospholipid acyl chains. Thus, it was expected that a reconstituted HDL_3, in which the native phospholipids were replaced with DMPC, would result in tyrosine modification and apoprotein-to-apoprotein cross-linking, but not apoprotein-to-phospholipid cross-linking, during nitration.

In chemical composition and in electrophoretic mobility on agarose gel (Table 1 and Fig. 1), the DMPC-r-HDL_3 resembles the N-HDL_3 and the C-r-HDL_3. In addition, all three HDL_3 samples have similar apoprotein patterns on SDS-PAGE and have similar ability to compete for the binding of ^{125}I-labeled HDL_3 to the binding sites of rat liver plasma membranes. When treated with TNM, DMPC-r-HDL_3, like N-HDL_3 and C-r-HDL_3, lost its ability to compete for the binding sites of rat liver membranes with ^{125}I-labeled HDL_3 (Fig. 2), even though no cross-linking of phospholipid to apoprotein occurred during the reaction. In the nitrated N-HDL_3 and nitrated C-r-HDL_3, about 21–26% of the total phospholipids were cross-linked to apoproteins (Table 2), similar to our previous results (10). Except for the absence of cross-linking of phospholipids to apoproteins, the nitrated DMPC-r-HDL_3 was similar to the other two nitrated lipoproteins. Thus, tyrosine residues in all the nitrated lipoproteins were nitrated, as evidenced by their increase in cathodic electrophoretic mobility on agarose gel and by a reduction of more than 95% of the tyrosine residues upon amino acid analyses (data not shown). Cross-linking of apoproteins to one another and cross-linking of 10–15% of the cholesteryl esters to apoproteins (data not shown) were found in all three samples.

In summary, it is concluded that the cross-linking of lipids to apoproteins that occurs during the TNM treatment of HDL is through the double bonds of acyl chains, and that the inhibition of binding of HDL is not due to cross-linking of phospholipids to apoproteins. The inhibi-

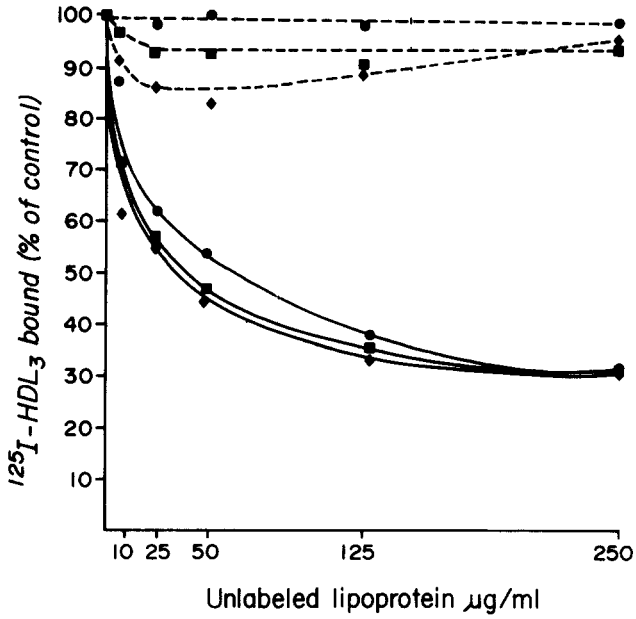

Fig. 2. Effect of unnitrated and nitrated lipoproteins on the binding of ^{125}I-labeled human HDL$_3$ to isolated rat liver plasma membranes. Each incubation mixture contained membranes (200 μg of protein), 10 μg of ^{125}I-labeled HDL$_3$ protein/ml in 0.2 ml of incubation medium (10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 0.5 mM $CaCl_2$, and 1% BSA) and indicated concentrations of various lipoproteins: native-HDL$_3$, ■—■; nitrated native-HDL$_3$, ■--■; control-r-HDL$_3$, ●—●; nitrated control-r-HDL$_3$, ●--●; DMPC-r-HDL$_3$, ◆—◆; nitrated DMPC-r-HDL$_3$, ◆--◆. After incubation at 22°C for 1 hr, the amount of ^{125}I-labeled HDL$_3$ bound to the membranes was determined as described in Materials and Methods. Data in this figure are representative of three separate experiments.

tion is more likely due to nitration of tyrosine residues or due to cross-linking of apoproteins to one another. It is also possible that the cross-linking of non-phospholipid lipids, such as cholesteryl esters, to apoproteins is responsible for the loss of binding activity. These possibilities are now under investigation.

The authors wish to thank Ms. Jane Salkoff for her excellent technical assistance and Drs. R. Angeletti and J. F. Strauss III, University of Pennsylvania, for the amino acid analyses. A research resources grant from NIH, RR01412, provided the microsequencing and amino acid analyzer equipment. This research was supported by AHA, PA Affiliate, NIH HL 22633, and the W. W. Smith Charitable Trust.

Manuscript received 25 August 1986 and in revised form 4 November 1986.

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