Coupling of photoactivatable glycolipid probes to apolipoproteins A-I and A-II in human high density lipoproteins 2 and 3

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Abstract High density lipoprotein (HDL) from human serum was subfractionated into HDL_2 and HDL_3 by rate-zonal density gradient ultracentrifugation. The orientation of apoproteins (apo) A-I and A-II in these subfractions was investigated by use of the photosensitive glycolipid probes, 2-(4-azido-2-nitrophenoxy)-palmitoyl[1-^{14}C]glucosamine (compound A) and 12-(4-azido-2-nitrophenoxy)-stearoyl[1-^{14}C]glucosamine (compound B). Both probes were added to the HDL-structures in a ratio of two or three probe molecules per particle and were photoactivated by irradiation at a wavelength above 340 nm. After delipidation the probe-apoprotein adducts were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Both the "shallow" probe (compound A) and the "depth" probe (compound B) were coupled for 10–14% (of the label added) to apoA-I and apoA-II from HDL_3 and for about 6% to apoA-I and apoA-II from HDL_2. By taking into account the relative amounts of apoA-I and apoA-II, it was estimated that the "shallow" probe labeled apoA-I 40% more effectively than apoA-II in both HDL_2 and HDL_3; the "depth" probe labeled apoA-I and apoA-II equally well in both subfractions. The data suggest that towards the surface HDL_2 and HDL_3 contain a relatively larger portion of apoA-I than apoA-II, whilst towards the core both subfractions are occupied by an equal portion of apoA-I and apoA-II. Application of these photolabels has failed to point out differences in the structural organization of HDL_2 and HDL_3. — **Berkhout, T. A., P. H. E. Groot, R. van Belzen, and K. W. A. Wirtz.** Coupling of photoactivatable glycolipid probes to apolipoproteins A-I and A-II in human high density lipoproteins 2 and 3. *J. Lipid Res.* 1985. **26:** 964–969.

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The structure of high density lipoprotein-3 (HDL_3) from human serum has been studied and several models have been suggested (1–6). All these models emphasize the existence of an apolar core covered by a surface film of apoproteins, phospholipids, and unesterified cholesterol. However, a detailed molecular structure of the HDL particles is still lacking. In the present study we have attempted to establish the localization of apoA-I and apoA-II in isolated human HDL_2 and HDL_3 using two photoactivatable glycolipids, 2-(4-azido-2-nitrophenoxy)-palmitoyl[1-^{14}C]glucosamine (compound A), and 12-(4-azido-2-nitrophenoxy)-stearoyl[1-^{14}C]glucosamine (compound B) (**Fig. 1**). These probes insert spontaneously into membranes and will render highly reactive nitrenes upon photoactivation (7, 8). The structural design of compound A enables coupling to lipid and protein near the lipid-water interface, whereas compound B will couple to these segments embedded more deeply in the hydrophobic core of the membrane (9–12). Compound B has already been successfully applied in studies on the penetration of subunits of cholera toxin into membranes (13) and on the topography of membrane proteins of Newcastle-disease virus (14).

Here, we present data to show that both the shallow (compound A) and the depth probe (compound B) couple efficiently but not identically to apoA-I and apoA-II in HDL_2 as well as HDL_3. Possible implications for the localization of apoA-I and apoA-II in these HDL subclasses will be discussed.

MATERIALS AND METHODS

Photolabels

The synthesis of 2-(4-azido-2-nitrophenoxy)-palmitoyl-[1-^{14}C]glucosamine (compound A) and 12-(4-azido-2-nitrophenoxy)-stearoyl[1-^{14}C]glucosamine (compound B) has been described elsewhere (7, 8). The probes (sp act 57 mCi/mol) were stored in ethanol at −20°C in the dark at a concentration of 2 mM.

Isolation and characterization of HDL subclasses

Human serum was obtained from young healthy women and HDL_2 and HDL_3 were isolated by rate-zonal

Abbreviations: HDL, high density lipoproteins; SDS, sodium dodecyl sulfate.

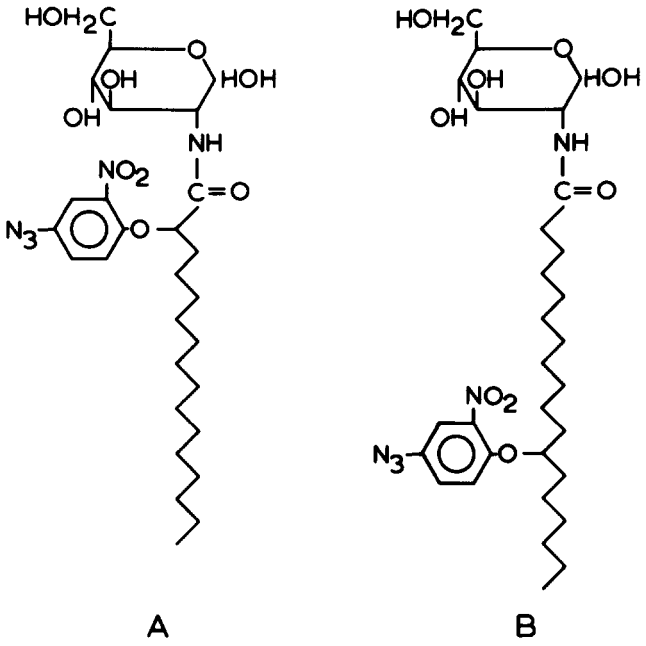

Fig. 1. Chemical structure of 2-(4-azido-2-nitrophenoxy)-palmitoyl-[1-^{14}C]glucosamine (compound A) and 12-(4-azido-2-nitrophenoxy)-steroyl[1-^{14}C]glucosamine (compound B).

density gradient ultracentrifugation as described earlier (15). Lipoprotein fractions were dialyzed against 10 mM Tris-HCl, 1 mM EDTA, and 150 mM NaCl, pH 7.4 (TES buffer). Concentrations of apoA-I and apoA-II in the HDL subfractions were determined by radial immunodiffusion as described elsewhere (15).

Photolabeling procedure

All procedures before irradiation were carried out under yellow-green safety lights. HDL fractions (100 μg of protein) were suspended in 1 ml of TES buffer, saturated with argon. Compounds A or B (2 nmol in 0.001 ml ethanol) were added by injection. After incubation for 10 min at room temperature, the incubation mixtures were photolyzed (10 min) using a Philips HPK 125W lamp. A GWV filter with a cut-off below 340 nm was used. In some experiments, the photoactivation of HDL was performed in the presence of reduced glutathione (10 mM) as a free radical scavenger. The sample was photolyzed as described above.

Delipidation

After photolysis the samples were dialyzed against 20 mM ammonium bicarbonate for 16 hr at 4°C and lyophilized. The fractions were extracted with 2.5 ml ethanol-diethyl ether 3:2 (v/v) for 16 hr at 4°C to remove the bulk of non-coupled probe and lipids (16). After removal of the solvent, this procedure was repeated. The apoprotein samples were dried under a gentle stream of nitrogen. Protein recovery was usually more than 90% (17).

SDS-polyacrylamide gel electrophoresis

The apoprotein fractions were dissolved in 200 μl of sample buffer, containing 2% (w/v) sodium dodecyl sulfate. Aliquots of 40 μl were used for electrophoresis on 12.5% polyacrylamide gels according to Laemlii (18). After staining with Coomassie brilliant blue R-250, the gels were scanned on a Vitatron densitometer and sliced into 5-mm segments. The slices were incubated with 1 ml of Soluene 350 (Packard) for 16 hr at 40°C and the radioactivity was determined by liquid scintillation counting.

Lipid analysis

The ethanol-diethyl ether fractions containing the extracted lipids were combined, dried under vacuo, and the lipid residues were dissolved in 1 ml of chloroform-methanol 95:5 (v/v). Aliquots were taken to determine the radioactivity in the samples. Phospholipids were separated from neutral lipids and non-covalently linked probe by applying the lipids to a silicic acid column as described (8). The phospholipid fraction was analyzed by thin-layer chromatography using the solvent systems chloroform-methanol-ammonia-water 90:54:5.5:5.5 (v/v), and isopropanol-hexane-water 8:6:1.5 (v/v), as described elsewhere (8).

Statistical methods

Data are expressed as mean ± SD. The statistical significance of differences in labeling of apolipoproteins by compounds A and B were evaluated using Student's two-tailed paired *t*-test. Differences between HDL_2 and HDL_3 were evaluated using Student's two-tailed *t*-test.

RESULTS

Isolation of HDL_2 and HDL_3

HDL subclasses were isolated using rate-zonal density ultracentrifugation (15). Serum of five premenopausal women was used as the HDL_2 content is relatively high in these donors. HDL subclass profiles are presented in **Fig. 2**, and pooled as indicated (i.e., HDL_3, fractions 9-20; HDL_2, fractions 24-31). HDL_2 preparations contained approx. 5 times more apoA-I than apoA-II (w/w) and HDL_3 contained approx. 2.5 times more apoA-I than apoA-II. Similar ratios have been reported earlier (15, 19, 20).

Labeling of HDL

Under our conditions of labeling, 2 nmol of compounds A and B was added to 100 μg of HDL protein in 1 ml

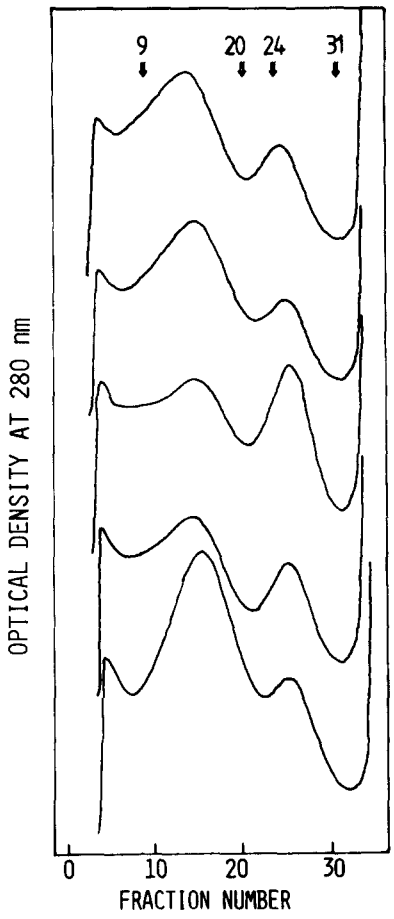

Fig. 2. Separation of human HDL_2 and HDL_3 by rate-zonal density gradient ultracentrifugation. The absorption at 280 nm was continuously monitored. The arrows indicate the boundaries of the pooled HDL_3 (9–20) and HDL_2 (24–31) fractions.

of TES buffer. When we assume that HDL_3 and HDL_2 have molecular weights of 175,000 and 320,000 and protein contents of 55% and 41%, respectively (19, 21), one can estimate that 100 μg of protein is equivalent to 1 nmol of HDL_3 and 0.75 nmol of HDL_2. This indicates that, in case of complete partitioning of the probe, each HDL complex will contain about two or three probe molecules. A complete partitioning of compound B was shown for erythrocytes (8) and vesicles containing M13 coat protein (22). This behavior was confirmed for both compounds A and B using phosphatidylcholine vesicles (T. A. Berkhout, unpublished observation). After irradiation, 50–70% of the probe could be removed by extraction using ethanol–diethyl ether 3:2 (v/v). Fractionation of the lipid extract by column chromatography yielded about 20% of the total radioactivity in the phospholipid fraction. Analysis of this phospholipid fraction by thin-layer chromatography followed by radioautography showed one major spot with an R_f value identical to that of phosphatidylcholine coupled to compound A or compound B (8). From this we conclude that at least 20% of the probe couples to the phospholipids in both HDL_2 and HDL_3.

Labeling of apoA-I and apoA-II

The delipidated apoprotein fractions of HDL were fractionated using SDS-polyacrylamide gel electrophoresis. A densitometer tracing of apoproteins from HDL_3 is shown in **Fig. 3**. In order to obtain information about the amount of probe coupled to apoA-I and apoA-II, the gels were sliced (5 mm) and the radioactivity in the slices was determined. Inspection of the radioactive pattern (compound A) shows that the bulk of the radioactivity coincides with apoA-I and apoA-II (Fig. 3).

In **Table 1** the results of coupling of compounds A and B to the apoproteins of HDL_2 and HDL_3 are summarized. It is evident that both compounds A and B label apoproteins A-I and A-II in HDL_3 more efficiently than in HDL_2. This probably reflects the higher protein-lipid

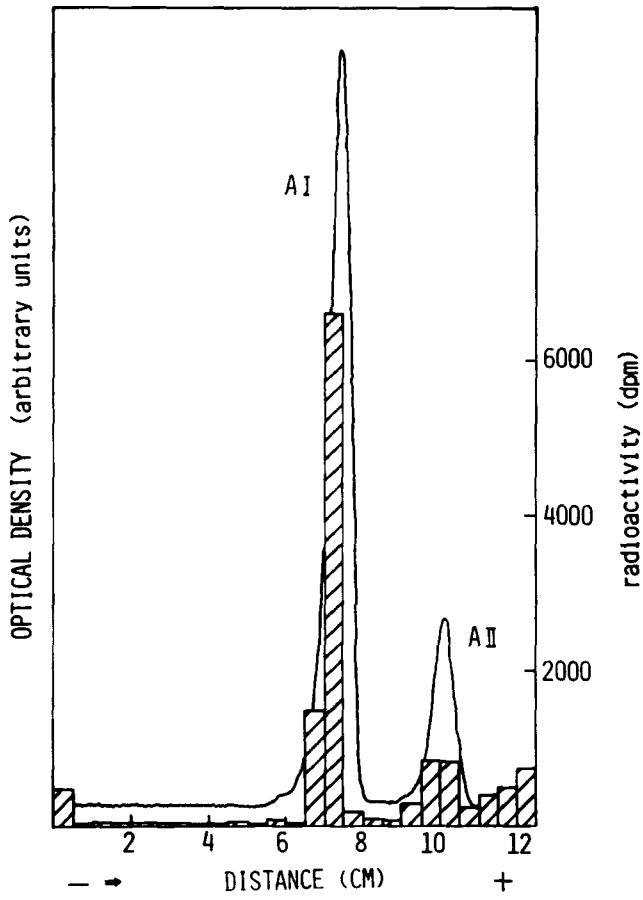

Fig. 3. SDS-polyacrylamide gel electrophoresis of HDL_3 apoproteins labeled with compound A. Two nmol of compound A was added to HDL_3 (100 μg of protein) in 1 ml of TES buffer. After irradiation and delipidation, the labeled apoproteins (20 μg) were separated by a 12.5% SDS-polyacrylamide gel. The Coomassie brilliant blue staining pattern was scanned at 610 nm, after which the gel was sliced and the radioactivity was determined.

TABLE 1. Coupling of probe to HDL subfractions

	Labeling[a]		Protein Ratio[b]	Relative Labeling Ratio[c]	
	ApoA-I	ApoA-II	ApoA-I/ApoA-II	ApoA-I/ApoA-II	
	% of total radioactivity				
Compound A					
HDL_2	5.34 ± 0.50	0.76 ± 0.18	5.6 ± 1.5	1.37 ± 0.32	5
HDL_3	7.37 ± 1.05[d]	1.98 ± 0.37[e]	2.7 ± 0.45	1.41 ± 0.24	5
Compound B					
HDL_2	5.33 ± 1.16	1.03 ± 0.16	5.6 ± 1.5	0.98 ± 0.25[g]	5
HDL_3	9.77 ± 1.61[f]	3.89 ± 0.32[e]	2.7 ± 0.45	0.93 ± 0.09[g]	5

[a] Coupling of compound A and B to apoA-I and apoA-II was determined by measuring the radioactivity associated with protein bands after SDS gel electrophoresis as described in Fig. 3, and expressed as per cent of total radioactivity added.

[b] For each donor the apoA-I and apoA-II protein concentrations (mg/ml) were determined by radial immunodiffusion.

[c] The apoA-I/apoA-II labeling ratio was divided by the apoA-I/apoA-II protein ratio for each donor. The average value of this ratio (± SD) for n donors is presented.

[d] Statistically different from HDL_2, $P < 0.01$, unpaired *t*-test.

[e] Statistically different from HDL_2, $P < 0.001$, unpaired *t*-test.

[f] Statistically different from HDL_2, $P < 0.005$, unpaired *t*-test.

[g] Statistically different from compound A, $P < 0.01$, paired *t*-test.

ratio in HDL_3 as compared to HDL_2. In addition, the labeling of apoA-I is severalfold higher than that of apoA-II for both labels in both HDL subclasses. However, by taking into account the apoA-I and apoA-II protein ratio (w/w), one can calculate the efficiency of labeling corrected for the protein mass, i.e., apoA-I/apoA-II relative labeling ratio (Table 1). This ratio indicates that the "shallow" probe, compound A, couples about 1.4-times better to apoA-I than to apoA-II in both HDL_2 and HDL_3. Coupling of the "depth" probe, compound B, to apoA-I and apoA-II, resulted in an apoA-I/apoA-II relative labeling ratio of 0.98 in HDL_2 and 0.93 in HDL_3. The latter ratio may indicate that in both HDL subclasses compound B encounters an equal mass of apoA-I and apoA-II. The significantly higher ($P < 0.01$) apoA-I/apoA-II relative labeling ratios for the "shallow" probe as compared to the "depth" probe suggest that, relative to apoA-II, more apoA-I is available for labeling near the interface in both HDL subfractions.

In order to obtain information about the specificity of the labeling, glutathione was added as a free radical scavenger during irradiation (23, 24). Under these conditions only the probe in the direct environment of the protein will be able to couple to the protein ("specific labeling"), while labeling by long-living intermediates of the probe or labeling from the aqueous phase ("nonspecific" labeling) will be strongly reduced. In the presence of scavenger, coupling of compound B to apoA-I and apoA-II was about halved in comparison to coupling without scavenger. However, the relative labeling ratio between apoA-I and apoA-II in HDL_2 and HDL_3 did not change.

In other experiments, the incubation and irradiation of the HDL fractions in the presence of probe was performed at 37°C instead of at room temperature. This did not result in a different labeling of apoA-I and apoA-II. Furthermore, in some experiments the concentration of the probes in the HDL subfractions was doubled. No effect on percentages of coupling to apoA-I and apoA-II could be detected, indicating that there is not a special saturable binding site on apoA-I or apoA-II for the probe (24).

DISCUSSION

Glycolipid compounds containing a photoreactive group at distinct positions along the fatty acyl chain can give more direct information on the occurrence of a protein at a certain depth of the membrane structure (7). Recently, compound B was successfully used to study the localization of subunit AI of cholera toxin during the process of entering the membrane (13), and the localization of subunits of the ninth component of the human complement (25). In the present study we have used similar glycolipid probes (see Fig. 1) to investigate the orientation of apoA-I and apoA-II in the HDL subclasses (HDL_2 and HDL_3). Assuming that the apolar groups of the probes are localized at the interface and the fatty acyl chain is directed towards the hydrophobic core of the particle, one can estimate that the nitrenes are generated at a distance of 0–5 Å from the surface of HDL for the "shallow" probe (compound A) as compared to 10–15 Å for the "depth" probe (compound B). From the data of Table 1, we can conclude that, relative to apoA-II, apoA-I is more efficiently labeled by the "shallow" probe than by the "depth" probe in both HDL subclasses. We take this to indicate that towards the surface HDL_2 and HDL_3

contain relatively more apoA-I than apoA-II. By the same token, the mass of apoA-I and apoA-II molecules exposed to the "depth" probe appear equal. On the other hand one has to realize that the relative labeling ratio reflects the availability of reactive groups on the proteins towards the generated nitrenes; this number is not necessarily proportional to the protein mass. However, we may conclude from the primary structure that the content of reactive (polar) amino acid residues is approximately equal in both apoA-I and apoA-II (26). From similar studies using phosphatidylcholine derivatives containing an alkyl-azide moiety at different positions along the acyl chain, Stoffel and Metz (27) concluded that apoA-I penetrated deeper into HDL as compared to apoA-II. This result was also obtained using chemical labels (28). We have no explanation for the discrepancy between their studies and ours, except that Stoffel and co-workers used reconstituted HDL particles using the cholate exchange procedure.

That apoA-I is localized more to the surface than apoA-II agrees with the preferential losses of apoA-I from HDL during sonication (29), in the presence of guanidine-HCl (30), and on heating (31). Furthermore, it has been shown that addition of apoA-II to HDL results in its uptake and an equal loss of apoA-I by HDL (32). On the other hand, addition of apoA-I does not replace apoA-II from HDL (33). Stronger binding of apoA-II to HDL, as compared to apoA-I, could reflect a deeper orientation of apoA-II in the particle.

This study has not revealed evidence for apoA-I and apoA-II having a different orientation in the HDL subclasses, HDL_2 and HDL_3. Differences in substrate specificity between HDL_2 and HDL_3 towards lecithin:cholesterol acyltransferase (34, 35) and hepatic lipase (36, 37) have been reported. The results of the present study suggest that these differences in metabolic properties are probably not explained by differences in orientation of apoA-I or apoA-II in the surface film of HDL_2 and HDL_3. However, possible differences in orientation, not monitored by the probes used in the present study, cannot be excluded.

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