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Evidence for Distinct Behaviour of Phosphatidylcholine and Sphingomyelin at the Low Density Lipoprotein Surface

Helena C. Murphy,* Mika Ala-Korpela,†‡ John J. White,* Alia Raoof,* Jimmy D. Bell,† Maria L. Barnard,† Shamus P. Burns,* and Richard A. Iles*,[1]

**Medical Unit (Cellular and Molecular Mechanisms Research Group), St. Bartholomew's and The Royal London School of Medicine and Dentistry, London E1 1BB, United Kingdom; †The Robert Steiner NMR Unit, Hammersmith Hospital, London W12 OHS, United Kingdom; and ‡A. I. Virtanen Institute for Molecular Sciences, University of Kuopio, 70211 Kuopio, Finland*

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This study demonstrates that the use of high field ^{1}H NMR spectroscopy permits individual detection of phosphatidylcholine and sphingomyelin molecules at the surface of native low density lipoprotein (LDL) particles. Distinct behaviour was observed for the choline head group $-N(CH_3)_3$ resonances of these different phospholipids revealing preferential immobilisation for phosphatidylcholine. This suggests the existence of reversible and irreversible phosphatidylcholine-apolipoprotein B interactions and is consistent with microdomain formation at the surface monolayer of LDL. The novel resonance assignment and results show that ^{1}H NMR can provide efficient and practical means for future studies on the structure and dynamics at the LDL surface.

Lipoprotein particles are the transport vehicles of water insoluble lipids in the circulation. The low density lipoprotein (LDL) fraction is a key element in cholesterol metabolism and its high plasma concentration is an independent risk factor for coronary heart disease (1). Although the overall metabolism and composition of LDL is fairly well known, the structural and dynamic aspects of the particles remain partly unclear (2,3).

The LDL particles have a micellar structure with a hydrophobic core of nonpolar triglyceride (TG) and cholesteryl ester (CE) molecules surrounded by a monolayer of phospholipids (PL), predominantly phosphatidylcholine (PC) and sphingomyelin (SM). A large apolipoprotein B-100 (apoB) molecule, which is thought to have a pentapartite structure, covers approximately 30 per cent of the particle surface monolayer volume (3,4). Unesterified cholesterol (CHO) molecules are found in both the core and surface regions of the particles and small amounts of TG and CE also penetrate into the surface monolayer (3,5-9). Despite the in-depth knowledge of LDL composition, the lipid-lipid and lipid-protein interactions and the organisation of the molecules at the LDL surface are not well understood. This is partly due to methodological difficulties in studying these complex molecular assemblies with high molecular specificity but without inducing structural perturbations into the system. In general, the NMR approach is favourable because the probes are part of the natural molecular structure of the lipoprotein lipids and thus the data can be obtained from native particles.

We present a novel finding that high resolution ^{1}H NMR spectroscopy permits individual detection and quantification of PC and SM components at the surface monolayer of native LDL particles. Based on this methodology we introduce and discuss findings that show distinct behaviour for PC and SM and suggest the existence of microdomains at the LDL surface.

MATERIALS AND METHODS

LDL and Phospholipid SUVs

Isolation of native LDL. Venous blood was collected from nine, 12 h fasted male volunteers aged from 30 to 56 years. The plasma total cholesterol, HDL-cholesterol, and triglyceride concentrations were measured for each of the volunteers and LDL-cholesterol levels were calculated using the Friedewald approximation (10). All volunteers were normolipidaemic (WHO). The blood used for the LDL isolation procedure was immediately placed in tubes containing 1 mg/ml EDTA and 0.1 mg/ml sodium azide and the plasma was separated by centrifuging at 900 *g* for 20 min at 4 °C. LDL was isolated by equilibrium density ultracentrifugation. The plasma density of the plasma was adjusted to 1.3 g/ml by the addition of sodium bromide andbromide, underlaid in centrifuge

[1] To whom correspondence should be addressed. Fax: + 44-171-3777 636. E-mail: R.A.Iles@mds.qmw.ac.uk.

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tubes containing 0.9 % saline solution. The tubes were, and then centrifuged at 40,000 rpm for 3 h in a Sorvall ultracentrifuge using a fixed angle T-865 Sorvall rotor. The upper portion of the centrifuge tube containing the VLDL fraction was discarded and t (Du Pont, Sorvall, UK). The 'orange' band corresponding to the crude LDL fraction at a density of 1.025 - 1.055 g/ml was removed and placed in a second ultracentrifuge tube containing 5 ml of 18 % NaBr solution and topped up with 8 % NaBr solution and recentrifuged for 17 h at 40,000 rpm. All solutions used during the isolation procedure contained 37.2 mg/l Na_2EDTA and 0.1 g/l NaN_3. All reagents used were Analar grade (BDH, UK) unless otherwise stated. The washed LDL at the top of the tube was removed by aspiration and dialysed using Centricon-100 diafiltration tubes (Amicon, UK), against deuterated phosphate buffered saline (99 % D_2O) containing 10 mM phosphate, 0.137 M NaCl, 2.7 mM KCl and 10 μM Na_2EDTA, pD 7.0. All deuterated solvents were purchased from Aldrich Chemical Co, Dorset, UK. The concentration of the dialysed LDL was determined using the Peterson's modification of the Lowry procedure (11) (Sigma Diagnostics, Sigma, Poole, UK) and adjusted to 1.67 mg/ml protein. The LDL fraction was stored at 4 °C and the NMR measurements were performed within one week. TSP was added as an internal chemical shift standard (final TSP concentration 2 mM). The final LDL concentration in the NMR samples was 1.5 mg/ml protein.

Treatment of LDL with trypsin. Human LDL was prepared from several volunteers as described above. The pooled LDL sample was dialysed further using Centricon-100 tubes against 150 mM NaCl and 10 mM $NaHCO_3$ (99 % D_2O). The LDL protein concentration was measured as above and adjusted to 5.0 mg/ml protein. Three different LDL preparations were used: control LDL (no trypsin added), LDL + 0.08 mg trypsin in 5 μl of 0.01 M HCl added to 0.6 ml of LDL (as used by Yeagle *et al.* (12)) and LDL + 1.52 mg trypsin in 95 μl of 0.01 M HCl added to 0.6 ml of LDL. All preparations were incubated at 25 °C for 2.25 h in a water bath, after which soybean trypsin inhibitor was added to the trypsin modified preparations (0.28 and 5.32 mg, respectively). The samples were stored at 4 °C and the ^{1}H NMR measurements performed the following day.

Phospholipid SUV preparation. Egg yolk phosphatidylcholine and bovine brain sphingomyelin were purchased from Sigma UK. The small unilamellar vesicle (SUV) samples were prepared as described by Barenholtz *et al.* (13). Ten milligrams of phospholipids were dissolved in 2 ml of $CHCl_3/CH_3OH$ (v/v); SUVs with PC/SM-ratios of 0.89, 2.33 and 9 were assembled. The lipids were dried down under nitrogen and placed in a dessicator for 3-4 h to obtain a dry film of lipid. This lipid film was resuspended in 10 ml of oxygen purged KCl solution (50 mM) and the resulting lipid suspension then sonicated using a Lucas Dawe Soniprobe for approximately 1.5 h under nitrogen maintaining the solution temperature at 5 °C for the SUVs composed of PC only and at 40 °C for SUVs containing SM (i.e. above the of T_c of SM). The SUVs were then centrifuged in a Sorvall Ultracentrifuge at 40 000 rpm at 15 °C for 45 min. The top two thirds of the SUVs from the centrifuge tube were harvested and then dialysed using Centricon-100 diafiltration tubes (Amicon, UK), against 50 mM KCl solution (99 % D_2O). The concentration of the lipids present in the sample were estimated to be approximately 6 mg/ml total lipid. The samples were kept at 4 °C until subsequent ^{1}H NMR measurements which were performed within two days of sample preparation. TSP was added as an internal chemical shift standard.

Lipid extraction. Organic extracts of the LDL and the SUV samples were prepared according to the modified Folch procedure (14). The organic layer of the extracted lipids was dried down under N_2 gas and resuspended in 2:1 v/v $CDCl_3/CD_3OD$, with approximately 2 mM TMS as the chemical shift reference.

NMR Spectroscopy

Experimental. ^{1}H NMR spectroscopy of the native and trypsin treated LDL were carried out at 600 MHz and the SUV samples at 400 MHz using Bruker AMX spectrometers. All NMR measurements were carried out at 310 K using 5 mm (o.d.) tubes and data were acquired using a pulse width of 45 degrees, 128 scans, 16 k data points, and a pulse repetition time of 5 s. In the case of the organic extract samples of LDL the ^{1}H NMR measurements were done using either a 400 or 600 MHz Bruker AMX spectrometer. The data were acquired at 298 K using a pulse width of 45 degrees, 128 scans, 16 k data points, and a pulse repetition time of 5 s. The measured FIDs were Fourier transformed without apodisation to the frequency domain spectra.

Data analysis. Lineshape fitting analysis was applied to resolve the areas of the phospholipid $-N(CH_3)_3$ resonances in the ^{1}H NMR spectra. Program FITPLA^C using Lorentzian lineshapes was used (15,16).

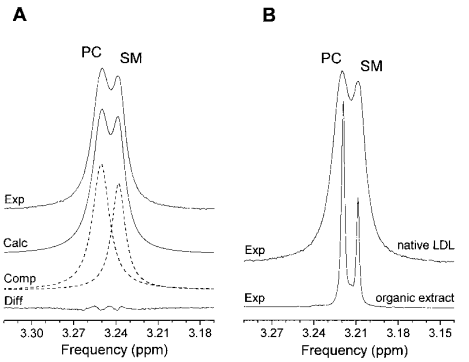

FIG. 1. (A) An expansion of the phospholipid region from a ^{1}H NMR spectrum of native LDL (volunteer B; Table 1) and the result of lineshape fitting analysis that was applied to fully resolve the $-N(CH_3)_3$ choline head group resonances of phosphatidylcholine (PC) and sphingomyelin (SM). Exp refers to the experimental spectrum, Calc to the calculated (two Lorentzian components plus a linear baseline), Comp to the individual Lorentzians, and Diff to the difference spectrum (Exp-Calc). (B) The phospholipid spectral region of native LDL is drawn again here together with the same region in the ^{1}H NMR spectrum of the organic lipid extract of the same LDL sample. Note that the chemical shift of the native LDL spectrum is adjusted in order to illustrate the match with the two resonances observed in the extract spectrum.

RESULTS

PC and SM Resonance Assignment

Native LDL. Fig. 1A shows an expansion of the phospholipid $-N(CH_3)_3$ choline resonance region (3.17 - 3.32 ppm) from a typical ^{1}H NMR spectrum of native LDL together with the separation of the peak into two individual resonances at 3.24 and 3.25 ppm by the aid of lineshape fitting analysis. Similar resonances can be observed in the organic extract spectrum (Fig. 1B). In order to assign these resonances, a standard solution of PC (70%) and SM (30%) in an organic solvent (2:1 $CDCl_3/CD_3OD$) was raun at 600 MHz. The PL head group resonances were almost separate at 3.21 and 3.22 ppm and had an expected ratio of 2.3 to 1, the high frequency resonance being the largest and thus corresponding to the PC (data not shown). The chemical shifts of the PL head group resonances in the organic extract samples of LDL were also found to be identical to those in the standard PC-SM solution (Fig.

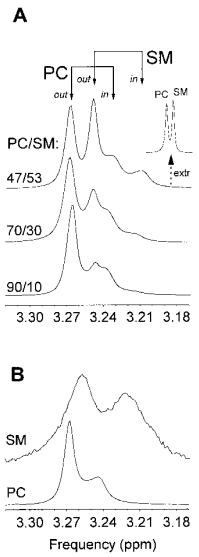

FIG. 2. (A) An expansion of the phospholipid region from the ^{1}H NMR spectra of small unilamellar vesicles (SUV) prepared with PC and SM in the ratios of 0.89, 2.3, and 9 (47/53, 70/30, and 90/10, respectively). PC denotes phosphatidylcholine and SM denotes sphingomyelin; *out* refers to the outer and *in* to the inner monolayer of the SUVs. In the top inset the phospholipid region from the ^{1}H NMR spectrum of the organic extracts from the SUVs with the PC/SM-ratio of 0.89 is illustrated (extr). Note the clear change in the PC/SM resonance ratios according to the SUV composition. (B) An expansion of the phospholipid region from the ^{1}H NMR spectra of pure SM and PC SUVs. Note the more restricted mobility of the choline head groups in the pure SM SUVs and the slight variation in the chemical shifts in the different SUVs (17).

1B). Thus, the resonances at 3.24 and 3.25 ppm in the ^{1}H NMR spectra of native LDL originate from the $-N(CH_3)_3$ choline head groups of SM and PC, respectively.

Independent confirmation of the resonance assignment was obtained from SUVs prepared from either PC or SM and from SUVs with PC and SM in the ratios of 0.89, 2.3 and 9. The choline region of the ^{1}H NMR spectra for these SUVs is illustrated in Fig. 2. For the SUVs composed of only PC or SM, the phospholipid region is made up of two separate resonances (Fig. 2B). This is expected since it is well established that there is a clear chemical shift difference for the $-N(CH_3)_3$ resonance of PL molecules in the inner and outer monolayer of the SUVs (17). It is seen from Fig. 2A that in the spectra from SUVs comprised of both PC and SM, there are four resonances instead of two as is the case for the native LDL particles. It is evident that the ratio of the PC and SM resonances changes according to the composition of the SUVs.

NMR Visibility of PC and SM Resonances

The area of the phospholipid peaks were fully resolved using Lorentzian lineshape fitting analysis. Table 1 shows the PC/SM-ratio for each of the 9 volunteers obtained from the spectra of the native LDL and from the spectra of the organic extracts of the same LDLs. The mean PC/SM-ratio resulting from the organic extracts of LDLs was 2.39 ± 0.14 (ranging from 1.97 to 3.26), which is similar to the literature values calculated from various sources and ranging from 2.2 to 2.8 (18). Thus, the PC/SM-ratio of 1.73 ± 0.13 obtained for the native LDL samples provides evidence for internal mobility restrictions rendering a pool of PL molecules NMR invisible in the native LDL particles.

Additional information was obtained from the ^{1}H NMR measurements of trypsin treated LDL samples as illustrated in Fig. 3. It is evident, that the trypsin treatment causes a clear increase in the PC $-N(CH_3)_3$ resonance while there is only a minor or no effect on the corresponding SM resonance. In comparison with native LDL, the increasing level of trypsin modification led to a significant change in the PC/SM-ratio: 1.67 for the pooled control LDL sample and 2.18 and 2.61 for the samples that were treated with 0.08 and 1.52 mg of trypsin, respectively. The PC/SM-ratio of 2.61 obtained after extensive trypsin treatment was consistent with the corresponding ratios in the organic extract spectra (Table 1) giving independent support for the distinct interactions of PC and SM in the native LDL particles. Since trypsin treatment results in extensive degradation of apoB (19), *i.e.* destroys the integrity of the local structural domains of apoB, these experiments suggested that there are preferential interactions between apoB and PC molecules in native LDL.

DISCUSSION

In this study we have shown that high resolution ^{1}H NMR spectroscopy can be used to examine the phospho-

TABLE 1

The PC/SM-Ratios and the Percentage Change in Phosphatidylcholine (PC) Relative to Sphingomyelin (SM) for Nine Normolipidaemic Volunteers Obtained from the ^{1}H NMR Spectroscopy Analysis of the Native LDL and Organic Extracts of the Same Samples

	PC/SM-ratio		
Volunteer	Native LDL	Organic extracts	% change in PC relative to SM
A	1.34	2.11	+36.49
B	1.79	2.13	+15.96
C	1.63	2.53	+35.57
D	1.25	2.26	+44.69
E	1.48	1.97	+24.87
F	2.52	3.26	+22.70
G	1.68	1.97	+14.72
H	2.01	2.72	+26.10
I	1.87	2.53	+26.09
Mean	1.73	2.39	+27.47
SEM	0.13	0.14	3.27

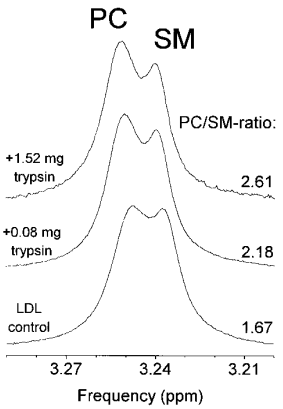

FIG. 3. An expansion of the phospholipid region from the ^{1}H NMR spectra of native and trypsin treated LDL. Note that the trypsin treatment, leading to the loss of the structural integrity of apolipoprotein B, causes a clear increase in the phosphatidylcholine (PC) $-N(CH_3)_3$ resonance, while there is only a minor or no effect on the corresponding sphingomyelin (SM) resonance.

lipid pools at the surface of native LDL particles. In particular, it allows for the separation of the information from PC and SM choline head groups making it possible to probe their individual molecular environments. To the best of our knowledge, this is the first time that such detailed studies of the LDL surface phospholipid interactions have been reported via ^{1}H NMR. As ^{1}H is experimentally much more favourable than the ^{31}P nucleus (20) this new approach made it practical to study the surface structure of native LDL.

Both the trypsin treated LDL and the comparison between the PC/SM-ratios for the native LDL and their extracted lipids showed distinct behaviour for PC and SM at the surface of the LDL particles. Firstly, the evidence from the trypsin treatment of LDL showed that disruption of the apoB integrity resulted in an increase in the PC resonance while the SM resonance was not significantly affected (Fig. 3). This indicates preferential binding of PC and apoB and suggests much weaker interactions between SM and apoB. However, our data cannot completely rule out the possibility that a small portion of SM molecules may be bound to apoB. If we assume the total SM pool as being NMR visible in the native LDL particles, a value of 19 % is obtained for the total PL pool that is NMR invisible, resulting from the immobilisation of 27 % of the PC molecules (Table 1). Independent support for our results was obtained from a recent fluorescence study that provided evidence for preferential interactions between apoB (and apolipoprotein (a)) and the PCs and suggested that the SMs reside in a bulk lipid phase (21).

Studies of the LDL surface structure using ^{2}H, ^{13}C and ^{31}P NMR spectroscopy have been reported previously (6,7,12,22). Only the ^{13}C NMR results by Lund-Katz and Phillips (6) can be directly compared with our ^{1}H NMR data presented here since in both studies the observable atomic probe is in the $-N(CH_3)_3$ group of the PL phosphocholine head group. The observed loss of about 20 % in the intensity of the $-N(CH_3)_3$ ^{13}C resonance from the PL pool is in good accordance with our value of 19 % for the invisibility of the ^{1}H resonance from the same molecular group. The ^{31}P NMR results by Yeagle *et al.* (12), probing the $-PO_4-$ group of the phosphocholine head group, have also shown that approximately 20 % of the phospholipids at the LDL surface are immobilised by the interactions with apoB. However, PC and SM were observed to behave in a similar manner and the authors concluded that both of these PL molecules bind strongly to the apoB. As our trypsin treatment of the LDL sample (+0.08 mg trypsin resulting in a PC/SM-ratio of 2.18; Fig. 3) was carried out as in ref. (12) this discrepancy may suggest different interactions between the apoB and the $-N(CH_3)_3$ and the $-PO_4-$ regions of the phosphocholine head groups. An additional complication for the comparison with our data (or the ^{13}C data by Lund-Katz and Phillips (6)) and the studies by Yeagle *et al.* (12) is that they carried out the measurements at 23 °C. The ^{2}H NMR studies of Chana *et al.* (7) and their conclusions suggesting two distinct PL domains with different order parameters (S_{CD}) at the LDL (and VLDL) surface cannot be directly compared with our ^{1}H NMR based findings. This is because the ^{2}H probes are placed at specific methylene groups of the fatty acid chains, thus leading to S_{CD} values that describe the molecular interactions of these deuterated $-CH_2-$ groups at the hydrocarbon region of the surface. As also noted by Chana *et al.* (7), the immobilisation of PL fatty acid chains by protein interactions is not expected (23).

Our data has indicated distinct behaviour for PC and SM head groups at the surface of LDL, consistent with the general picture about the structure of lipoprotein surfaces (3-9,12,18,20-22,24) and is in agreement with recent findings and hypotheses on the possible importance of microdomain formation on membrane functions (25,26). Recent theoretical work has given evidence for a pentapartite NH_3-α_1-β_1-α_2-β_2-α_3-COOH structure for apoB at the LDL surface (4). This model suggests irreversible lipid association properties for the β_1- and β_2-clusters, a conclusion which is in fact supported by IR spectroscopic findings from protease treated LDL particles: the apoB peptides that remain associated with the particle after proteolysis are rich in β-sheet structure and at least part of these β-sheets are in contact with the LDL PLs (27). Based on a fluorescence study of LDL and reconstituted LDL, Kroon (8) has also derived a model for apoB-PL interactions that involves the phospholipid head groups.

The behaviour of PC and SM is thus consistent with an LDL surface structure in which approximately 27 % of the PC molecules are tightly associated with the β-sheet clusters of apoB: this binding involves the im-

mobilisation of the $-N(CH_3)_3$ groups of the PC phosphocholines. On the other hand, it is known that CHO molecules have a preferential interaction with SMs that is likely to lead to the formation of CHO-SM rich microdomains at the LDL surface (28,29). In addition to the preferential interactions and microdomain formation via irreversible PC binding to the β-sheet structures of apoB and energetically advantageous hydrophobic associations between CHO and SM, the structural constraints and composition of the LDL particles are also likely to favour reversible molecular association between the α-helical domains of apoB and PLs as well as between PL molecules themselves.

There is accumulating evidence that microdomain formation could have important implications on membrane functions (25,26). At lipoprotein surfaces the specific lipid-lipid and lipid-protein interactions may directly influence lipoprotein metabolism. The regions dominant in PC rather than the CHO enriched regions are likely to be the natural domains for the penetration of LDL core lipids into the particle surface to allow for efficient lipolysis and lipid transfer reactions. There is also evidence that SM may regulate the uptake and intracellular processing of LDL (30,31). Since our results show some clear inter-individual variation in the apoB-PC interactions (within the normolipidaemic volunteers; Table 1) it is inviting to suggest that compositional heterogeneity, through changes in the relative content and characteristics of the surface microdomains, may directly impact on LDL metabolism *in vivo*.

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