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^{1}H NMR at 800 MHz facilitates detailed phospholipid follow-up during atherogenic modifications in low density lipoproteins

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Abstract

The structure of low density lipoprotein (LDL) particles and, particularly, the enzymatic and oxidative modifications of their surface is crucial in the initiation of atherosclerosis. Due to the structural complexity of LDL, there is a lack of suitable methods for dynamic follow-up studies of the molecular mechanisms in native and modified particles in physiological conditions. Here, we report that phosphatidylcholine (PC), lysophosphatidylcholine (lyso-PC), and sphingomyelin (SM) can all be identified and quantified in LDL particles by ^{1}H NMR spectroscopy at 800 MHz. The signal assignment for the lyso-PC is novel and we illustrate the applicability of the methodology in the case of lipid peroxidation that is generally considered as one of the key proatherogenic modifications of LDL. It was found, somewhat surprisingly, that the LDL-associated phospholipase A_2 is activated in the very beginning of the formation of PC-hydroperoxides. The (patho)physiological rationale of the resulting lyso-PC generation is also briefly discussed.

Keywords: LDL; Lipoproteins; NMR spectroscopy; Phosphatidylcholine; Lysophosphatidylcholine; Sphingomyelin; Phospholipase A_2; Lipoprotein-associated phospholipase A_2; Lipid peroxidation; Atherosclerosis; Lipoprotein accumulation; Lipoprotein modifications

Cholesterol in atherosclerotic lesions is derived from lipoproteins that have entered the arterial intima. Most of the cholesterol in the bloodstream is carried by low density lipoprotein (LDL) particles and thus most of the cholesterol in the arterial wall is thought to be derived from LDL [1]. Initiation of atherosclerosis is characterized by the appearance of extracellular lipid droplets in the proteoglycan-rich subendothelial layer. Experimental models have shown that similar droplets can be formed directly from LDL *in vivo* [2]. Moreover, both chemical analyses and measurements of the size of the extracellular lipid droplets in human arterial lesions suggest that the majority of the droplets originate from LDL particles. Since native LDL particles do not fuse into such lipid droplets, they must undergo modification in the intima. Indeed, the lipid droplets isolated from the arterial intima have features suggesting that they are derived from plasma LDL through extensive enzymatic modifications [2–4].

Therefore, understanding of the molecular mechanisms that initiate the atherosclerotic lipid accumulation calls for dynamic physicochemical studies of LDL particles [5]. These kinds of studies, however, are limited by two fundamental aspects.

First, the native LDL particles, of average diameter of 22 nm, are complex assemblies of approximately 3000 lipid molecules encircled by an apolipoprotein B-100 (apoB-100) molecule of over 4000 amino acids [1,6]. The particle core consists mainly of hydrophobic lipids such as triglycerides

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and cholesterol esters as well as some unesterified cholesterol (UC). The surface monolayer comprises a single copy of apoB-100 together with phospholipid and UC molecules. The phospholipid components are phosphatidylcholine (PC), sphingomyelin (SM), and lysophosphatidylcholine (lyso-PC). The nanoenvironments and the interplay between apoB-100 and the surface PC, lyso-PC, SM, and UC are known to play key roles in the structural integrity and interactions of the particles [1,5,6].

Second, there is a lack of specific techniques that would facilitate a non-destructive follow-up of dynamic molecular processes in a native LDL sample under an enzymatic attack in a water-based, physiological environment. NMR spectroscopy has been applied to study both native lipoprotein structures and also some characteristics of their atherosclerotic modifications, such as fusion [2,5,7,8]. While ^{13}C NMR leads to more detailed molecular data than ^{1}H NMR, for the follow-up studies of enzymatically modified LDL, ^{1}H NMR appears the only feasible choice [5,7,8].

Previously, we have followed LDL particle interactions and the accompanied molecular changes during various potentially atherogenic modifications at 500 and 600 MHz ^{1}H NMR spectroscopy [1,2,5,8]. We have also indicated that the use of ^{1}H NMR enables distinction between PC and SM at the LDL particles [9]; a finding that allowed a detailed characterization of LDL surface nanoenvironments [10]. Our present studies on the dynamic effects of lipolytic enzymes, such as phospholipase A_2 (PLA_2), on LDL particle structure and interactions led us to apply ^{1}H NMR spectroscopy at 800 MHz. Surprisingly, and in contrast to data measured at 500 and 600 MHz, the PLA_2-induced generation of lyso-PC in LDL is directly distinguishable from the spectra. This appears the first time that lyso-PC is assigned and quantified in the ^{1}H NMR spectra of lipoprotein particles.

Materials and methods

Isolation of low density lipoprotein particles. Human LDL ($d = 1.019$–1.050 g/ml) was isolated from plasma of healthy volunteers by sequential ultracentrifugation in the presence of 3 mM EDTA [11,12]. Briefly, solid KBr was added to plasma to adjust its density to 1.019 g. Very low and intermediate density lipoproteins were removed, and the density of the bottom fractions was adjusted to 1.050 g/ml with solid KBr. After ultracentrifugation for 72 h at 35,000 rpm, LDL was recovered from the top of the centrifuge tubes, recentrifuged ($d = 1.060$ g/mL) for 24 h at 35,000 rpm, and dialyzed extensively against LDL buffer (1 mM EDTA and 150 mM sodium chloride in water, pH 7.4, adjusted with sodium hydroxide). The amounts of LDL are expressed in terms of their protein concentrations, which were determined by the method of Lowry et al. with bovine serum albumin as a standard [13].

NMR spectroscopy. The ^{1}H NMR spectra were acquired at 37 °C on a Varian Unity INOVA 800 MHz spectrometer equipped with either a conventional (PLA_2 data) or cryogenically cooled (oxidation data) $^{1}H/^{15}N/^{13}C$ triple-resonance 5 mm probehead at the NMR Laboratory, Institute of Biotechnology, University of Helsinki (Helsinki, Finland). A reference tube (outer diameter 1.7 mm, supported by a Teflon adapter) containing sodium 3-trimethylsilyl[2,2,3,3-D_4] propionate (TSP) (40 mM) and $MnSO_4$ (0.6 mM) in 99.8% D_2O was placed concentrically into an NMR tube to be used as an external chemical shift and concentration reference.

Lipolysis of LDL with phospholipase A_2. PLA_2 (from bee venom) was from Sigma–Aldrich (Chemie GmbH, Steinheim, Germany). The NMR measurements were carried out in NMR-buffer containing 137 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM Na_2HPO_4/NaH_2PO_4, pD 7.4 in D_2O. Prior to the experiment, LDL was gel-filtered through PD-10 column equilibrated and eluted with the NMR-buffer. The sample contained 0.7 mg/ml of LDL and 20 ng/ml of PLA_2. The lipolysis of the LDL was followed continuously for 11 h by measuring a standard proton spectrum (number of transients was 96, acquisition time 5 s, relaxation delay 1 s, and flip angle 90°) at every 20 min. The experiment was repeated twice with two different LDL preparations in similar conditions with very similar outcome.

In a parallel incubation, hydrolysis of LDL was also followed by determining the amounts of free fatty acids (FFAs) and the phospholipid composition of aliquots taken at various time points. The amounts of FFAs were determined with the NEFA-C-kit (Waco Chemicals Inc., Dalton, GA). The lipid compositions of the lipid extracts of the LDL samples were analyzed by high-performance thin-layer chromatography (HP-TLC) using chloroform/methanol/concentrated acetic acid/H_2O (50:30:8:3.5, vol/vol/vol/vol) [14]. Individual lipid classes were visualized by dipping the TLC-plate into $CuSO_4$ (3%)/H_2PO_4 (8%) and then heating the plate for 10–20 min at 150 °C. The bands were scanned with an automatic plate scanner (CAMAG TLC Scanner No.3). One set of results is shown in Supplementary Fig. S1.

Oxidation of LDL. The NMR measurements were carried out both in D_2O- and H_2O-environments. The deuterium-based NMR-buffer contained 137 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM Na_2HPO_4/NaH_2PO_4, pD 7.4 in D_2O. To remove EDTA and to change H_2O to D_2O, LDL was gel-filtered prior to the experiments through PD-10 column equilibrated and eluted with the NMR-buffer. The sample contained 1.0 mg/ml of LDL and oxidation was carried out with 30 μM $CuSO_4$ in the sample tube. Oxidation process was monitored for 17 h by measuring a standard proton spectrum (number of transients was 96, acquisition time 5 s, relaxation delay 1 s, and flip angle 90°) at every 10 min.

The other NMR measurements were carried out in standard phosphate-buffered saline (PBS). EDTA was removed from LDL in a PD-10 desalting column equilibrated with PBS. The experiments were done with two LDL samples from two apparently healthy volunteers. The samples contained 1.0 mg/ml of LDL and oxidation were carried out with 50 μM $CuSO_4$. Oxidation process was followed continuously for 7 h by measuring a standard proton spectrum without water suppression (number of transients was 128, acquisition time 5 s, relaxation delay 1 s, and flip angle 90°) at every 15 min.

After the NMR measurements, the degree of oxidation was determined by measuring the amounts of thiobarbituric acid-reactive substances (TBARS) in the samples using malondialdehyde (MDA) as a standard [15]. The amounts of TBARS in native LDL were <3 nmol MDA/mg LDL. During the NMR measurements, the TBARS in the samples increased and after the incubations were 31–49 nmol MDA/mg LDL.

Processing and analysis of the data. All the data processing was done with the PERCH NMR software (PERCH Solutions Ltd., Kuopio, Finland) [16–18]. Prior to Fourier transformation, the measured FIDs with 80,000 data points were zero filled and multiplied by an exponential window function with a line-broadening of 0.5 Hz. All the spectra were scaled according to the area of the corresponding TSP reference signal. Signal areas were determined using lineshape fitting analysis [16–18]. A model lineshape-based approach was adopted in the case of the peroxidation experiments to improve the systematic accuracy of the molecular trends. This kind of analysis is often referred to "use of biochemical prior knowledge" and its use is recommended to decrease the mathematical uncertainties with overlapping resonances [17,18]. It can be applied in situations where the molecular components within the spectral region of interest are known. This applies now for the choline $-N(CH_3)_3$ resonance at around 3.2 ppm since it consists only of three single components, namely phosphatidylcholine, lysophosphatidylcholine, and sphingomyelin. This

information was used as prior knowledge by analyzing the last spectrum of the enzymatic modification series of LDL first (since it contains the maximum information on the weakest lyso-PC component) and then making a relative fixing for the linewidths and chemical shifts of all these phospholipid resonances to be applied in the final analyses of the entire spectral data set. However, some softness was accepted in the relative fixings of the half-linewidths and chemical shifts to allow adjustment for potential (and unknown) biophysical differences between the various stages of oxidized LDL particles. It is notable that we studied only the early stages of LDL peroxidation and thus all the stages studied are likely to be biophysically rather close to each other. The data analysis without the use of biochemical prior knowledge produced essentially the same molecular trends but with more scatter and increased SDs. In the experiments where the PLA_2-enzyme was added to the LDL samples (in order to verify the resonance assignment for the lyso-PC) the spectral changes were much more pronounced than for the peroxidation-initiated activation of LDL-associated PLA_2 and thus no prior knowledge was used in the spectral analyses of those data sets.

Results and discussion

The function of PLA_2 is to catalyze the hydrolysis of the *sn*-2 bond in phospholipids, i.e., to generate a free fatty acid and a lyso-PC [1,2]. The results of the lineshape fitting analyses in Fig. 1 for one set of spectra from a single sample of LDL incubated with PLA_2 in the magnet fully reproduce the expected molecular behavior: the lyso-PCs formed due to the action of the enzyme originate entirely from the hydrolyzed PCs. This unequivocally confirms the assignment of the new signal at around 3.198 ppm for lyso-PC. Similar results were obtained in two other ^{1}H NMR data sets and the behavior was also confirmed by SM, PC, and lyso-PC measurements with TLC (Supplementary Fig. S1).

As the first test for the applicability of the ^{1}H NMR methodology at 800 MHz we studied oxidation of LDL. Oxidized LDL (oxLDL) is thought to play an important role in the initiation and progression of atherosclerosis [19]. During LDL oxidation both the lipid molecules and apoB-100 undergo a variety of chemical changes and modifications due to radical-mediated reactions. These changes include the peroxidation of polyunsaturated fatty acids at the *sn*-2 position of surface PC into lipid hydroperoxides, which are further cleaved to aldehydes as well as to lyso-PC [20].

The lyso-PC in oxLDL is generated by the activity of lipoprotein-associated PLA_2 (Lp-PLA_2) which is an enzyme that is produced by inflammatory cells and co-travels mainly

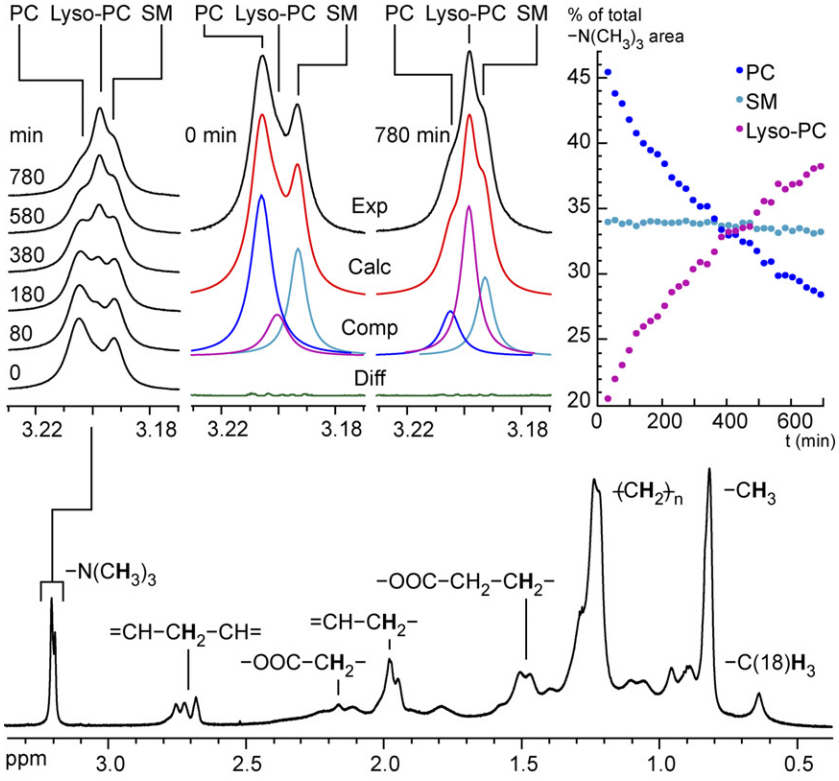

Fig. 1. A characteristic aliphatic region of a ^{1}H NMR spectrum of LDL at 800 MHz is shown at the bottom together with the assignments of the main lipid resonances [8]. The inset on the top left corner illustrates the phospholipid $-N(CH_3)_3$ resonances at around 3.2 ppm at different time points in a single sample in which LDL is continuously modified by PLA_2 in the magnet. The quantification of these phospholipid resonances at the beginning and at the end of the experiment via lineshape fitting analysis is illustrated in the middle. The signal for PC is shown in blue, for lyso-PC in fuchsia, and for SM in cyan. The difference between the experimental (Exp) and calculated (Calc) spectrum is shown in green at the bottom of the insets (Diff). The results from the lineshape fitting analyses for the spectra during the PLA_2 lipolysis of LDL are given at the top right corner. The decrease of the PC resonance is clearly accompanied with an increase in the lyso-PC resonance while the SM resonance remains virtually unchanged. PC, phosphatidylcholine; lyso-PC, lysophosphatidylcholine; SM, sphingomyelin; $-C(18)H_3$, cholesterol backbone resonance. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

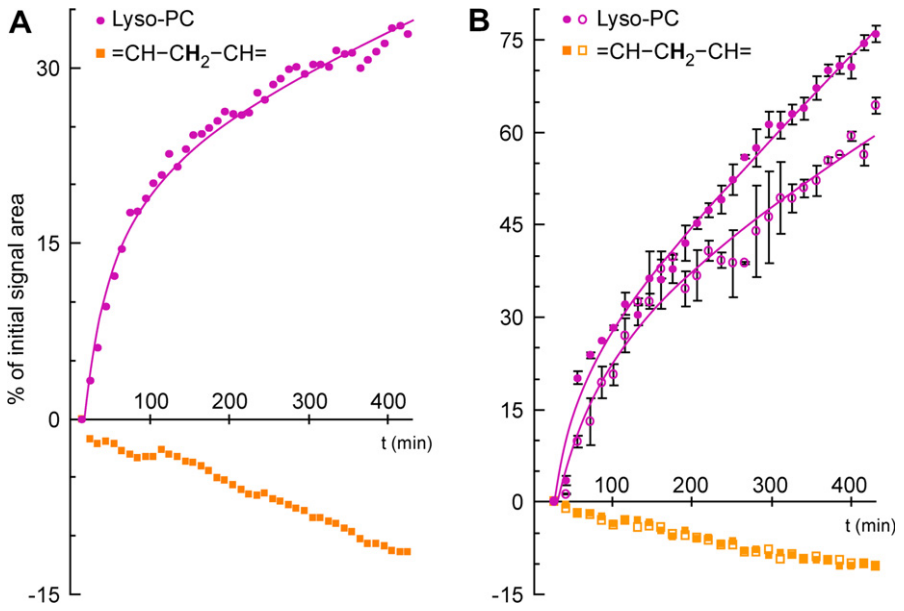

Fig. 2. The behavior of the area of lyso-PC and bisallylic resonances in the ^{1}H NMR spectra at 800 MHz during the oxidation of (A) an LDL sample in D_2O NMR-buffer, and of (B) two different LDL samples (open and closed bullets) in H_2O-buffer. The signal areas are from lineshape fitting analyses similar to those illustrated in Fig. 1. The instantaneous generation of lyso-PC is evident in both experiments even though the bisallylic protons indicate only minor oxidation of LDL lipids. The values in (B) for both individuals are the mean values of two replicates ±SD. Error bars for the bisallylic resonance areas are not shown for clarity since their size is approximately the size of the symbols. The fits to a modified enzyme kinetics equation $y = cx/(d + x) + bx + a$; propose that the hydrolysis reaction approaches zeroth order kinetics after a fast initiation.

with circulating LDL [21]. The products of the action of Lp-PLA$_2$ can affect various atherosclerotic processes. In fact, lyso-PC upregulates the expression of monocyte-chemo-attractant protein-1 and adhesion molecules on endothelial cells and vascular smooth muscle cells, thereby enhancing the recruitment of inflammatory cells to the intima [21]. This suggests a pro-inflammatory and pro-atherogenic role for Lp-PLA$_2$.

The oxidation of LDL first affects the phospholipids at the particle surface, then spreading to apoB-100 and the lipid molecules in the core of the particles [22]. However, it is unclear at what stage of LDL peroxidation the Lp-PLA$_2$ is activated. Previously, this question has been problematic to address since traditional methods, such as TLC, lack the sufficient accuracy to detect such small changes, but also because the commonly used oxidation marker, thiobarbituric acid-reactive substances (TBARS), does not relate to the phospholipid changes in LDL [23].

Fig. 2 shows that activation of Lp-PLA$_2$ is instantaneous for the LDL peroxidation. The behavior of the $=CH-CH_2-CH=$ resonance gives a good general measure for the peroxidation state of the particles [24]. Thus, only minor oxidation of the surface PCs seems sufficient to activate the Lp-PLA$_2$ leading to the concomitant formation of lyso-PC. The activation behavior of Lp-PLA$_2$ in all LDL preparations was very similar.

The observed immediate activation of Lp-PLA$_2$ due to the peroxidation of surface PCs in LDL can be seen to support the current view of Lp-PLA$_2$ as a pro-atherogenic component [25]. It seems that even minor oxidation of LDL lipids *in vivo*, occurring as a host response to infection and inflammation [26], will be sufficient to induce production of additional inflammatory mediators, such as lyso-PC. Alternatively, the instantaneous activation of Lp-PLA$_2$ due to lipid peroxidation may also be seen as an anti-atherogenic response since lyso-PCs also act as inhibitors of macrophage-mediated oxidation of LDL and thereby attenuate foam cell formation and atherosclerotic lesion growth [27].

To conclude, this communication presents a novel signal assignment for lyso-PC in LDL and a ^{1}H NMR methodology at 800 MHz to facilitate dynamic information on molecular mechanisms in LDL particles under atherogenic disturbances.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.06.058.

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