methods

Lipid analysis of human HDL and LDL by MALDI-TOF mass spectrometry and ^{31}P-NMR

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Abstract The analysis of HDL and LDL is important for the further understanding of atherosclerosis because changes of the protein and lipid moieties occur under pathological conditions. Because destruction of lipids leads to the formation of well-defined products such as lysophospholipids or chlorohydrins, methods that allow their fast and reliable determination would be useful. In this study, matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-TOF MS) was applied for the analysis of the lipid composition of human lipoproteins. These data were compared with high resolution ^{31}P-NMR spectroscopy. Differences between LDL and HDL in sphingomyelin and phosphatidylcholine content could be monitored by NMR and mass spectrometry, and differences with respect to the extraction efficiency were found by MALDI-TOF MS. Additionally, treatment of LDL with hypochlorite and phospholipase A_2 resulted in marked changes (formation of chlorohydrines and lysolipids). Lysophosphatidylcholines were detectable by both methods, whereas MALDI-TOF MS failed to detect chlorohydrines of phospholipids. We conclude that MALDI-TOF MS provides rapidly a reliable lipid profile of lipoproteins. However, a previous lipid separation must be performed to detect lipid oxidation products. NMR can be directly applied, but suffers from lower sensitivity, and provides only limited information on fatty acid composition.—Schiller, J., O. Zschörnig, M. Petković, M. Müller, J. Arnhold, and K. Arnold. **Lipid analysis of human HDL and LDL by MALDI-TOF mass spectrometry and ^{31}P-NMR. *J. Lipid Res.* 2001.** 42: **1501–1508.**

Supplementary key words MALDI-TOF MS • lysolipids • chlorohydrine

High cholesterol levels in blood combined with other factors such as smoking represent the primary risk factors for the development of atherosclerosis. A major aspect is the oxidative modification of LDL that promotes arterial wall alterations. Endothelial cells oxidize LDL, which subsequently accumulate in macrophages (foam cells) in the form of cholesterol and cholesteryl esters (1–3).

LDL can also be oxidized by a number of different agents (3, 4). The modifications induced by copper ions (5) and hypochlorite (6–10) were intensively studied. Because there are two major reaction sites for reactive oxygen species within the lipoprotein (i.e., the protein and lipid moieties), different analytical methods are required for the evaluation of the physiological state of both substance classes. In addition to classical protein analysis techniques such as SDS-PAGE, protein oxidation can conveniently be monitored by changes of UV or fluorescence properties of tryptophane (11) that is highly susceptible to oxidation. Two new approaches for the lipid composition analysis of human blood lipoproteins [matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-TOF MS) and ^{31}P-NMR] are described in this article.

Due to its high sensitivity and its unmatched mass accuracy, mass spectrometry is often used for the investigation of lipids in organic extracts of biological samples. However, due to the low volatility of lipids, the overall fatty acid composition of the sample is mainly determined (12). This is performed subsequently to the hydrolysis of lipids and the derivatization of the released free fatty acids to enhance their volatility. This tedious procedure is required by the limitations of the classical electron impact mass spectrometry (12). Using more sophisticated ionization methods, however, complex lipids can also be analyzed by mass spectrometry. Nowadays, fast atom bombardment (FAB) (13) and electrospray ionization (ESI) mass spectrometry (7, 14) are most successfully applied to this field.

MALDI-TOF MS (15) has also been used for lipid analysis (16–20), although the number of publications in this field is still surprisingly low. MALDI-TOF MS possesses several advantages in comparison with other mass spectrometric methods (12). The preparation of MALDI samples is extremely fast and easy. Any derivatization (e.g., silylation) of samples is not required, and buffer or salt con-

Abbreviations: DHB, 2,5-dihydroxybenzoic acid; ESI, electrospray ionization; FAB, fast atom bombardment; MALDI-TOF MS, matrix-assisted laser desorption and ionization time-of-flight mass spectrometry; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PLA_2, phospholipase A_2; PS, phosphatidylserine; SM, sphingomyelin; TFA, trifluoroacetic acid.

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taminants are, in contrast to ESI, tolerated (12). In comparison with particle beam ionization (FAB), the extent of fragmentation is very low (12). However, the most important advantage of MALDI-TOF MS is that the lipid sample and the matrix compound are readily soluble in organic solvents. Compared with the analysis of proteins and polysaccharides, this provides highly homogeneous cocrystals between the analyte and the matrix (19). Therefore, the reproducibility of MALDI-TOF spectra is excellent. For instance, we have shown in a recent article that diacylglycerols can be accurately analyzed by MALDI-TOF MS. Deviations from "shot to shot" are sufficiently small that even a quantitative analysis could be performed (20).

One further method for phospholipid analysis described in this article is high resolution ^{31}P-NMR. From the technical point of view of the application of NMR, the phospholipid analysis does not confer major problems, but sample preparation is the crucial point. Because phospholipids form bilayers in an aqueous environment and "inverse" micelles in an organic solvent that are both characterized by considerable line broadening, solvent mixtures or detergents have to be used to obtain highly resolved spectra (21–23). It has been found for phospholipid analysis that the use of sodium cholate is superior in comparison with solvent mixtures. By using this detergent method, different lipid classes as well as differences in fatty acid composition can be monitored (22, 23).

The present article deals with the analysis of organic extracts of human LDL and HDL by mass spectrometry and high resolution ^{31}P-NMR spectroscopy. It will be shown that MALDI-TOF MS represents a simple and reliable tool for the estimation of the lipid composition of human lipoproteins and of products generated by phospholipase A_2 (PLA_2) digestion. Unfortunately, the detection of chlorohydrine formation under the influence of HOCl was rather difficult to assess by MALDI-TOF MS. On the other hand, both PLA_2 digestion and chlorohydrin formation caused a characteristic change in ^{31}P-NMR spectra.

MATERIALS AND METHODS

Chemicals

All chemicals for buffer preparation, lipoprotein isolation (NaCl, KH_2PO_4, and Na_2HPO_4), mass spectrometry [2,5-dihydroxybenzoic acid (DHB) and trifluoroacetic acid (TFA)], NMR spectroscopy (sodium cholate, EDTA, and deuterated water with an isotopic purity of 99.6%), as well as all solvents (chloroform, heptane, and methanol) were obtained in highest commercially available purity from Fluka Feinchemikalien GmbH (Neu-Ulm, Germany). Phosphatidylcholine (PC) and sphingomyelin (SM) from egg yolk, as well as cholesterol were also purchased from Fluka. PLA_2 (from pig pancreas) showing an activity of 561 U/mg was obtained as lyophilizate by Fluka. The concentration of a stock solution of sodium hypochlorite (Sigma) was checked spectrophotometrically at pH 11 prior to use ($\varepsilon_{290} = 350\ M^{-1}cm^{-1}$) (24).

Preparation of LDL and HDL

HDL and LDL were isolated from the blood plasma of healthy volunteers by sequential ultracentrifugation according to Havel, Eder, and Brangdon (25) using a Beckman L8-50 ultracentrifuge. The purity of the preparation was checked by Lipidophor gel electrophoresis (Immuno AG, Vienna, Austria). Isolated LDL were dialyzed overnight at 4°C against phosphate buffer (pH 7.4). Subsequently, LDL was concentrated using ultrafiltration equipment (Amicon, Witten, Germany). The concentration of apolipoprotein B-100 was checked turbimetrically using a commercially available test kit (Sigma, Deisenhofen, Germany) according to Winkler et al. (26) and was adjusted to 0.4 or 0.8 mg/ml.

Treatment of lipoproteins with PLA_2 and hypochlorite

Lipoproteins were treated with 0.1 mg/ml PLA_2 and incubated for 2 h at 37°C. Lipids were extracted according to the Bligh and Dyer (27) method [methanol–chloroform–water 1:1:0.9 (v/v/v)] and the water-methanol layer was discarded. MALDI-TOF analysis showed that all lipids were in the chloroform layer under these experimental conditions. Incubations with hypochlorite (10 mM) were carried out in 50 mM phosphate buffer, pH 7.4, for 30 min. Lipids were extracted as described above. In a separate experiment, chloroform-heptane was used instead the chloroform-methanol mixture.

For means of comparison, liposomes from egg yolk PC were also digested by PLA_2 to obtain the corresponding lysophosphatidylcholines (28). Briefly, aliquots of phospholipids dissolved in chloroform were evaporated to dryness in a centrifugal evaporator (Jouan, Germany). Liposomes were prepared by dissolving the resulting lipid film in 50 mM phosphate buffer, pH 7.4, and vortexing vigorously for 30 s. The final lipid concentration was 1 mM in all cases.

MALDI-TOF MS

For all samples, a 0.5 M DHB solution in methanol containing 0.1% TFA (12) was used as matrix. Organic extracts of lipoproteins were directly applied to the sample plate as 1 μl droplets, followed by the addition of 1 μl matrix solution. Subsequently, samples were allowed to crystallize at room temperature under standard conditions. Drying of samples with a moderate warm stream of air improved the homogeneity of crystallization (19).

All MALDI-TOF mass spectra were acquired on a Voyager Biospectrometry DE workstation (PerSeptive Biosystems, Framingham, MA). The system utilizes a pulsed nitrogen laser, emitting at 337 nm. The extraction voltage was 20 kV and the "low mass gate" was turned on to prevent the saturation of the detector by ions resulting from the matrix (12). For each mass spectrum, 128 single laser shots were averaged. The laser strength was kept about 10% above threshold to obtain the best signal-to-noise ratio. To enhance the spectral resolution, all spectra were measured in the reflector mode.

NMR spectroscopy

Organic extracts of lipoproteins were evaporated to dryness in a centrifugal evaporator (Jouan, Germany). The residue was subsequently redissolved in deuterated water containing 200 mM sodium cholate and 5 mM EDTA. After intense vortexing, clear solutions were obtained. This method (21–23) provided much higher resolved NMR spectra than the use of chloroform-methanol as solvents.

^{31}P-NMR spectra were recorded on a Bruker DRX-600 spectrometer operating at 242.94 MHz for ^{31}P. All measurements were performed on 0.6-ml samples in 5-mm NMR tubes using a 5-mm direct broadband probehead at 37°C. Composite pulse decoupling (Waltz-16) was applied to eliminate ^{31}P-^{1}H coupling. Other NMR parameters were as follows: acquisition time: 1 s; data size: 16 K; 60° pulse (5 μs); pulse delay: 2 s. A line broadening of 2 Hz was applied for the processing of the free induction decays of the lipoprotein samples. Chemical shift assignments were externally referenced relative to 85% orthophosphoric acid at 0.00 ppm.

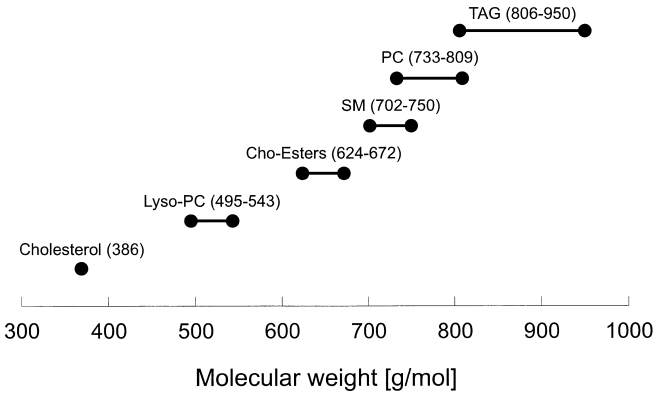

Fig. 1. The molar mass distribution of compounds extracted from lipoproteins with apolar solvents. The ranges of molar masses are caused by a different fatty acid composition (ranging from palmitic acid 16:0, up to arachidonic acid 20:4). Lyso-PC, lysophosphatidylcholine; TAG, triacylglycerides.

RESULTS AND DISCUSSION

Influence of matrix peaks on MALDI-TOF mass spectra

According to the lipid composition of lipoproteins (29), a number of different lipid classes is expected to be detectable in their organic extracts. A rough survey of the molar masses of those lipids is given in **Fig. 1**. All masses were calculated assuming that lipids contain fatty acids ranging between palmitic acid (16:0) and arachidonic acid (20:4). Fig. 1 clearly demonstrates that all considered lipids can be easily distinguished by mass spectrometry. Slight mass overlap exists exclusively between SM and PC, whereas all other lipids show clear mass differences. All of them are easily detectable as positive ions (19).

ESI is mostly regarded as a more powerful ionization method for the analysis of lipids than MALDI. A number of articles on the ESI analysis of lipids appeared (e.g., 7, 8, 14), whereas the applications of MALDI-TOF MS for lipid studies are relatively scarce (16–20, 30). This is caused by the following reasons. First, due to the marked influence of inorganic salts (e.g., NaCl) on the spectra quality, the ESI equipment is usually accompanied by a column to remove these contaminants (12). In this way, the separation of lipids according to their fatty acid composition or their head groups can concomitantly be performed. Second, MALDI-TOF MS uses a relatively small organic molecule as matrix compound. The presence of this matrix is often assumed to complicate the interpretation of mass spectra of lipids because the matrix undergoes photochemical reactions resulting in the formation of additional products.

For our study, we used DHB as matrix, and our very first aim is to show that the matrix peaks do not complicate the interpretation of mass spectra of lipid extracts of lipoproteins because well-defined ions are formed upon laser irradiation. **Figure 2** shows different positive ion laser desorption mass spectra of the applied DHB matrix. Fig. 2A represents DHB dissolved in pure methanol, whereas in Fig. 2B and C, sodium chloride- and potassium chloride-saturated methanol, respectively, was used. The m/z ratios and the corresponding assignments are given in the figure, whereby M denotes the mass of DHB (154 g/mol). In contrast to the spectra of DHB in pure methanol, the presence of higher amounts of ions (especially potassium) obviously leads to an enhancement of the formation of association products of a higher m/z ratio. Fortunately, all these peaks possess only comparably low intensity and, therefore, do not prevent the recording of mass spectra of lipids. This holds true, particularly if the fragmentation pattern of the matrix is known and the low mass gate is turned on. By comparing Figs. 1 and 2, it is evident that only a few matrix peaks overlap with lipid peaks.

Mass spectrometric investigations of lipoprotein extracts

The suitability of MALDI-TOF MS for the analysis of lipid extracts of lipoproteins is demonstrated in **Fig. 3**. The positive ion mode mass spectra of LDL (Fig. 3A) and HDL (Fig. 3B) are compared, and a more detailed peak assignment is given in the insert of Fig. 3. Mainly, two lipid species, SM and PC, are detectable in lipoproteins by MALDI-TOF MS. SM contains mainly palmitic acid (m/z =

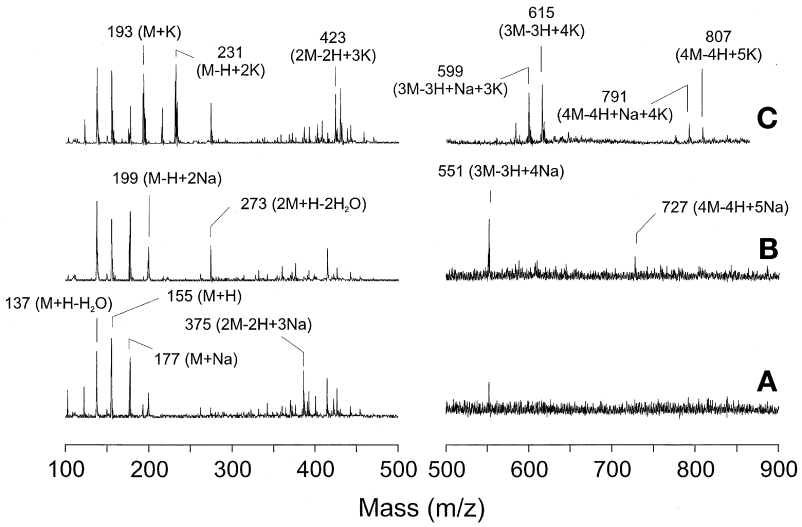

Fig. 2. Positive ion laser desorption mass spectra of the pure DHB matrix, which was used in all experiments as a 0.5 M solution in methanol containing 0.1% TFA. Sample A contained no additional salt, whereas sample B was prepared with sodium chloride-saturated methanol and sample C with potassium chloride-saturated methanol. For better resolution, all spectra were recorded using a reflectron and delayed extraction conditions.

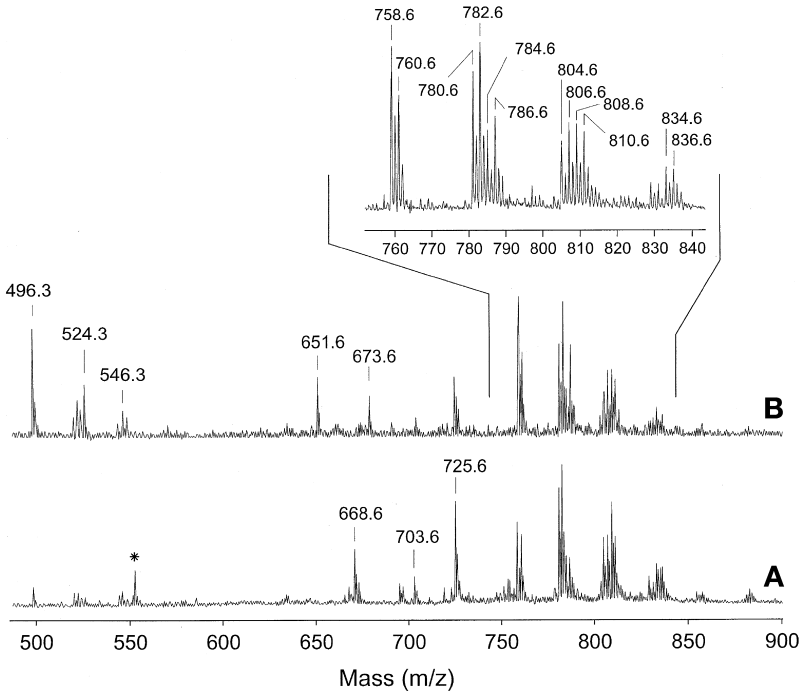

Fig. 3. Typical positive ion MALDI-TOF mass spectra of a chloroform-methanol extract of LDL (A) and HDL (B). The insert emphasizes the most relevant mass range of the HDL sample. The complete assignment of all detected molecular ions is given in Table 1. The peak labeled with an asterisk is caused by the matrix.

703.6 for the protonated and $m/z = 725.6$ for the sodiated form; Fig. 3a), whereas the PC moiety of the lipoproteins exhibits more expressed fatty acid heterogeneity (cf. **Table 1**).

To assign more accurately all peaks to protonated or sodiated molecular ions, exchange experiments (altering the proton/sodium ion ratio) have also been carried out. The enhancement of the NaCl concentration in lipid solutions increases the intensity of sodiated molecular ions and diminishes those of protonated molecular ions (data not shown). This approach is often very helpful for the assignment of ambiguous peaks.

The PC in LDL and HDL are composed mainly of palmitic, stearic, oleic, and linoleic acid residues (cf. Table 1 for peak assignments). The peak at $m/z = 782.6$ corresponds to PC 16:0/20:4. It might, however, also contribute to the sodiated form of PC 16:0/18:1. In contrast, if mainly sodiated molecular ions would be formed, the peak at $m/z = 780.6$ should be more intense than that at 782.6 (758.6 is also more intense than 760.6). Because this is obviously not observed, the peak at 782.6 must be primarily assigned to PC composed of palmitic and arachidonic acid. Some minor peaks may arise from docosahexaenoic acid (22:6). However, the presence of this fatty acid has not yet been reported in lipoproteins (29).

As previously shown (30), the peak at 668.6 contributes to SM after elimination of its choline headgroup ($\Delta = 57$ Da). This seems to be a preferred side reaction of SM, as well as of PC (19). The rather intense peaks at 651.6 and 673.6 in HDL might indicate a fragmentation of the double bonds in PC 16:0/18:2 and in PC 16:0/18:1, with the formation of the corresponding carbonyl compounds. Because such reactions mainly occur at peroxidized lipids (12, 19), one can conclude that oxidatively modified lipids were also present in this HDL preparation.

Cholesterol yields a very intense peak at 369.3 (M+H-H_2O). Spectra were, however, truncated at 485 Da to keep the sensitivity for the detection of other lipids as high as possible. There are also peaks for lysophosphatidylcholine containing one palmitic (496.3) or stearic acid (524.3) residue. Although it is known that the amount of lysophospholipids is very low in lipoproteins, these peaks are quite intense in comparison with PC. However, the applied methodology detects compounds more sensitively the lower their molecular weight is (31, 32); therefore, the lysophosphatidylcholine content is only apparently high.

Despite the fact that the detectability of phospholipids correlates reciprocally with their molecular weights, an at-least-semiquantitative analysis can be performed. However, this requires the a priori knowledge on the peak

TABLE 1. Assignment of molecular ions detectable in lipid extracts of HDL and LDL to different phospholipids

m/z	Assignment
369.3	Cholesterol +H $-H_2O$
651.5	Aldehyde of POPC/LPPC
668.6	SM 16:0 − Cho (+Na)
673.6	Aldehyde of POPC/PLPC (+Na)
703.6	SM 16:0
725.6	SM 16:0 (+Na)
758.6	PC 16:0/18:2
760.6	PC 16:0/18:1
780.6	PC 16:0/18:2 (+Na)
782.6	PC 16:0/20:4 PC 16:0/18:1 (+Na)
786.6	PC 18:0/18:2
804.6	PC 16:0/20:4 (+Na)
806.6	PC 16:0/22:6
808.6	PC 18:0/18:2 (+Na)
810.6	PC 18:0/20:4
834.6	PC 18:0/22:6

Cho, choline, LPPC, linoleoyl-palmitoyl-PC; PLPC, palmitoyl-linoleoyl-PC; POPC, palmitoyl-oleoyl-PC.

identity. If this is known, a master curve can be recorded with isolated lipids. Different peak intensities provide the information required for the correction factor for compensating effects of different molecular weights. Therefore, this drawback of MALDI-TOF methodology does not confer major problems (31). The quantification of individual lipids in lipid mixtures is, however, more difficult (32). The presence of lipids with quaternary ammonia groups (PC and SM) might lead to the suppression of the peaks arising from the further lipids. Although this is not a problem for phosphatidylserine (PS) and phosphatidylinositol (PI) that can also be detected as negative ions, phosphatidylethanolamine (PE) is not detectable at all in the presence of PC and SM (32). Because PC and SM constitute the overwhelming majority of the phospholipids of lipoproteins, limited detectability of further lipid species is not a prevalent problem.

Influence of solvents for the extraction of phospholipids, cholesteryl esters, and triglycerides

It is well known that human lipoproteins contain high amounts of cholesteryl esters and triglycerides (29). However, chloroform-methanol mixtures seem to extract primarily the more polar lipids, whereas less polar lipids are extracted only to a minor extent: the mass spectra of chloroform-methanol extracts of lipoproteins do not show significant amounts of apolar lipids (cf. Fig. 3). Therefore, lipids were additionally extracted from lipoproteins using chloroform-heptane as solvent, and the corresponding mass spectrum is given in **Fig. 4**.

Obviously, some additional lipids are extracted from LDL by the chloroform-heptane solution (Fig. 4) in comparison with the chloroform-methanol extraction procedure (Fig. 3A). Peaks in the high molecular mass range ($m/z > 850$) possess higher intensity and can be attributed to triacylglycerides. It has been recently shown that triacylglycerides do not occur as protonated molecular ions, but exclusively as sodiated molecular ions (31), which minimizes assignment problems. The peaks at 853.8 and 881.8 represent triacylglycerides with two C16 and one C18 chain, whereas triacylglycerides with one C16 and two C18 chains are found at 879.8 and 881.8. Peaks at 671.6 and 695.6 correspond to cholesteryl esters containing one linoleic or arachidonic acid residue, respectively.

In addition to this difference, heptane in the extraction medium causes the change in the relative intensities of the protonated and the sodiated molecular ions, whereby the latter is favored in a less polar solvent. This can be seen from the peaks at 703.6 and 725.6, which represent the proton and the sodium adduct of SM, respectively. Analogous effects are also detectable for the ratio of the corresponding PC peaks at 758.6 (protonated form) and 780.6 (sodiated form).

Detection of modified products of lipoproteins

To demonstrate the capability of MALDI-TOF MS for the analysis of lipoproteins, this method was also applied to artificially modified systems. In **Fig. 5**, the positive ion MALDI-TOF mass spectra of native LDL (Fig. 5A), LDL after digestion with PLA_2 (Fig. 5B), and LDL after treatment with 10 mM sodium hypochlorite (Fig. 5C) are shown. It is obvious that individual spectra differ considerably according to the different effects of the applied reagents: the digestion of the lipoprotein with PLA_2 (Fig. 5B) leads to the formation of lysophosphatidylcholines containing palmitic (protonated form at $m/z = 496.3$ and the sodiated form at $m/z = 518.3$) and stearic acid (protonated form at $m/z = 524.3$ and the sodiated form at $m/z = 546.3$). Peaks of PC are not longer detectable; that is, PC was completely converted into the lyso compounds. The incubation of LDL with PLA_2 leads to the formation of only two products: becausePLA_2 preferentially cleaves fatty acids at position 2 of the glycerol backbone, only lysolipids containing a saturated fatty acid (palmitic or stearic acid) can be detected. The remaining peaks at 703.6 and 725.6 correspond to SM, which is not susceptible to PLA_2 (32).

The incubation of LDL with hypochlorite leads to the formation of chlorohydrins with a 52-Da higher mass than that of the target molecule. The peaks at $m/z = 421.3$ and $m/z = 443.3$ contribute to the chlorohydrine of choles-

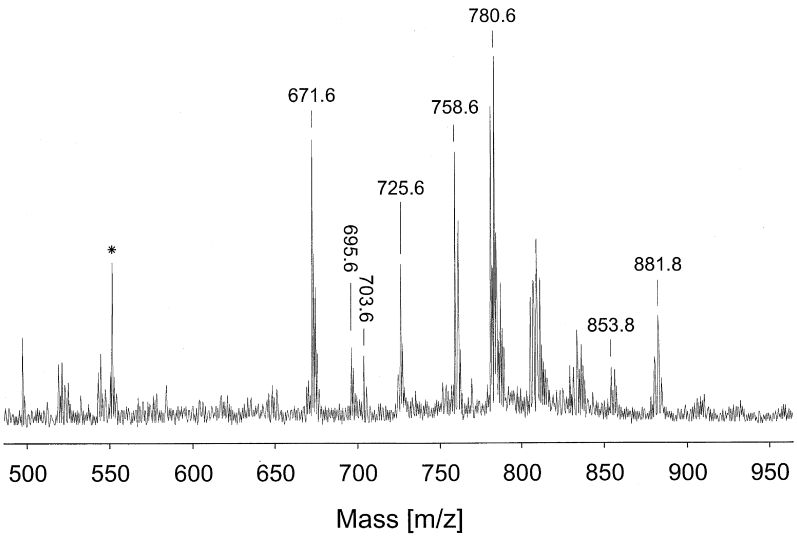

Fig. 4. Positive ion mass spectrum of a chloroform-heptane extract of LDL. The spectrum was recorded with DHB as matrix under delayed extraction conditions. The peak labeled with an asterisk is caused by the matrix.

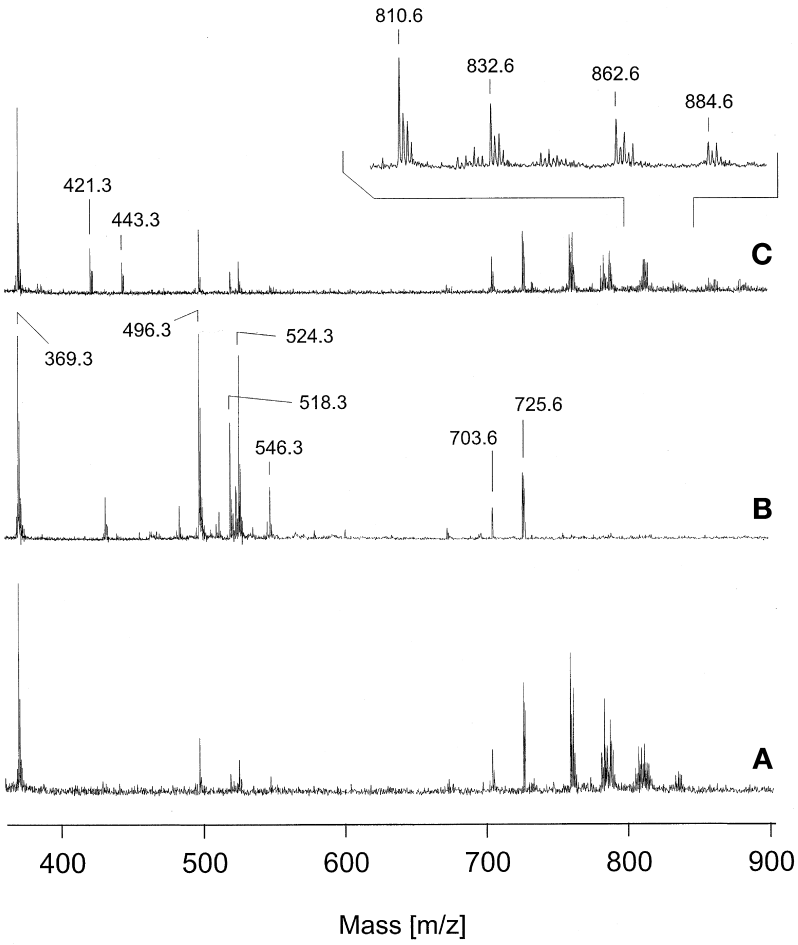

Fig. 5. Positive ion mass spectra of organic extracts of LDL. Spectrum A represents the native control sample. In B, LDL was digested with PLA_2, whereas in C the LDL was incubated with a 10 mM sodium hypochlorite solution. The insert in C shows the spectrum (product region) of a lipid extract of LPPC liposomes treated with sodium hypochlorite.

terol (33), where the first peak represents the protonated form and the second peak, the sodiated form. In contrast, effects of hypochlorite on the PC moiety of LDL are rather weak. Problems are still more pronounced because chlorohydrins may overlap with PC containing higher fatty acid residues. Therefore, only at *m/z* ratios higher than about 850 are some new, but weak, peaks detectable.

The peaks at about 833 are slightly enhanced, but this might be caused by the higher sodium content of the solution subsequent to NaOCl addition. The formation of sodiated instead of protonated products is largely favored in the presence of higher sodium concentrations (as described in the previous section). Astonishingly, the addition of NaOCl also leads to enhanced generations of lysophosphatidylcholines. Because the pH after NaOCl addition was carefully controlled, this cannot be explained by alkaline phospholipid hydrolysis. First results seem to point out that the tendency for the formation of lysophospholipids increases with an increasing content of double bonds within the PC (data not shown). This intriguing reaction is currently under investigation in our laboratory.

On the other hand, the reaction between pure PC 16:0/18:2 and hypochlorite (cf. the insert in Fig. 5C) gives the expected product at 810.6 and 832.6 (protonated and sodiated monochlorohydrine of PC 16:0/18:2), as well as in smaller amounts at 862.6 and 884.6 the corresponding dichlorohydrine. Therefore, the lipids of lipoproteins should also give the same products. However, due to peak overlap and further reaction sites, these products are not so easily detectable by MALDI-TOF MS in such a complex lipid mixture.

^{31}P-NMR spectroscopy of lipoproteins and related products

Although NMR spectroscopy is, by far, less sensitive than mass spectrometry, we recorded also the corresponding NMR spectra of lipid extracts. Unfortunately, proton NMR spectra (even in two dimensions) were not very useful with respect to a complete assignment of all resonances. Because cholesterol, cholesterol esters, and phospholipids are present in high amounts, and due to their high content of magnetically nonequivalent protons (34), the ^{1}H-NMR spectra are dominated by their resonances, and other substances are detectable only to a low extent (35). Thus, proton NMR spectroscopy is not the method of choice for the detection of different lipid classes in organic extracts.

^{31}P-NMR is a very suitable NMR method for the investigation of phospholipid mixtures. In **Fig. 6**, the ^{31}P-NMR spectra of aqueous suspensions of native HDL (Fig. 6A), LDL (Fig. 6B), and LDL after treatment with hypochlorite (Fig. 6C) or PLA_2 (Fig. 6D) are shown. For means of comparison, the spectrum of an equimolar mixture of SM, PE, PS, PI, and PC is also given (Fig. 6E). All spectra were re-

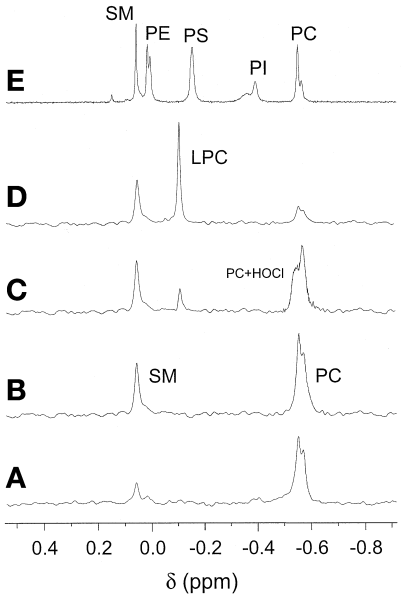

Fig. 6. ^{31}P-NMR spectra of solutions of HDL (A) and LDL (B); C and D represent LDL after incubation with hypochlorite and PLA_2, respectively. For means of comparison, a mixture of different commercially available phospholipids is shown in E. Spectra were recorded in aqueous sodium cholate solutions (200 mM) containing 5 mM EDTA. LPC, lysophosphatidylcholine.

corded in an aqueous solution containing 200 mM sodium cholate and 5 mM EDTA (21–23). This experimental approach provides a very good detectability of different phospholipid classes because the broad lines of lipids in an aqueous environment can be avoided under these conditions (21–23).

In a few cases, we also tried to dissolve lipoproteins directly in the detergent; that is, without a previous isolation of lipids by extraction. However, under these conditions, spectra were less resolved. Therefore, the previous lipid extraction would be regarded as an absolute necessity to obtain best NMR results.

All individual phospholipid classes can be easily differentiated (Fig. 6E), although the phospholipids differ slightly from each other toward their headgroups. One should additionally note that PE and PC do not yield a single resonance, but two different resonances that may be explained by a different content of unsaturated fatty acids. It already has been shown that the presence of unsaturated fatty acids leads to a shift to higher ppm values (23) in comparison with saturated phospholipids.

Both HDL (Fig. 6A) and LDL (Fig. 6B) contain only the phospholipids PC and SM, although the SM moiety is higher in LDL. PC shows a marked heterogeneity in both lipoproteins, as indicated by the splitting of the PC resonance at highest field. SM does not show such heterogeneity, but results in a single resonance. This is in good agreement with the results obtained by MALDI-TOF MS, indicating that mainly palmitic acid is present in SM.

The spectra of LDL treated with sodium hypochlorite (Fig. 6C) or with PLA_2 (Fig.6D) differ considerably from the starting material (Fig. 6B). In both cases, the PC resonance is considerably changed, accompanied by the formation of a new resonance at −0.10 ppm, which was assigned to lysophosphatidylcholine by comparison with the corresponding pure compound (22). The lysophosphatidylcholine resonance (in contrast to PC) does not show any heterogeneity because it contains only completely saturated fatty acid residues. In accordance to MALDI-TOF mass spectra (Fig. 5), no major changes were detectable at the SM residue.

Finally, the PC signal is considerably broadened (Fig. 6C) upon reaction with hypochlorite, which might be caused by the formation of mono- and dichlorohydrins. One further support to this hypothesis comes from the fact that the treatment of pure palmitoyl-linoleoyl-PC with hypochlorite also results in a considerable broadening of the PC resonance (data not shown). This would indicate that such products are actually also formed in lipoproteins and can be easily detected by ^{31}P-NMR.

CONCLUSIONS

It was shown that MALDI-TOF MS and ^{31}P-NMR spectroscopy are powerful methods to characterize the lipid composition in lipoproteins. Although ^{31}P-NMR spectroscopy is able only to differentiate the two major components of lipoproteins (SM and PC), the application of mass spectrometry provides the information on the fatty acid composition of lipoproteins. The higher sensitivity and the fact that even lipids that do not contain phosphate are detectable by mass spectrometry makes MALDI-TOF MS a superior analytical tool compared with NMR spectroscopy. This also holds for the detection of defined products such as lysophospholipids that are formed by the action of PLA_2. As previously shown, quantitative analysis of MALDI-TOF mass spectra can also be accomplished by the application of an internal standard (20) or by the use of the signal-to-noise ratio as a measure of concentration (36).

Mass spectrometry allows the detection of chlorohydrins of cholesterol formed after the treatment of LDL with sodium hypochlorite. On the other hand, this method fails to detect chlorohydrins of PC in LDL samples due to signal overlap. These products broaden the PC resonance in ^{31}P-NMR spectra considerably, whereas the arising lysophospholipids yield sharp resonances.

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