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Lysophosphatidylethanolamine is – in contrast to – choline – generated under *in vivo* conditions exclusively by phospholipase A_2 but not by hypochlorous acid

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ABSTRACT

Inflammatory liver diseases are associated with oxidative stress mediated particularly by neutrophilic granulocytes. At inflammatory loci, hypochlorous acid (HOCl) is generated by myeloperoxidase. HOCl reacts with a large variety of molecules and induces (among other reactions) the formation of lysophosphatidylcholine (LPC) from polyunsaturated phosphatidylcholines (PC).

As liver tissue contains huge amounts of polyunsaturated PC species enhanced LPC concentrations are detectable under these conditions. However, human liver contains also major amounts of polyunsaturated phosphatidylethanolamine (PE). It is so far widely unknown, if PE oxidation by HOCl leads to the generation of LPE in a similar way as observed in the case of PC. Using MALDI-TOF mass spectrometry (MS) and ^{31}P NMR spectroscopy, LPC generation from unsaturated PC could be verified in the presence of HOCl. In contrast, unsaturated PE led exclusively to chlorohydrins and other oxidation products but not to LPE.

Although these data were obtained with a quite simple model system, it is obvious that LPC is a much more suitable biomarker of oxidative stress than LPE: LPC is more readily generated and also more sensitively detectable by means of mass spectrometry and other spectroscopic methods. Nevertheless, it will also be shown that the nitrile of LPE is also generated. However, this compound is exclusively detectable as negative ion.

1. Introduction

It is commonly accepted that the concentration of lysophospholipids (LPL) – in particular that of lysophosphatidylcholine (LPC) – increases under inflammatory conditions. This has been shown, for instance, for patients suffering from rheumatoid arthritis [1], atherosclerosis [2] and lung infection [3]. The prevalent opinion is that LPLs are generated under *in vivo* conditions by the release and/or activation of the enzyme phospholipase A_2 (PLA_2) that is present, for instance, in neutrophilic granulocytes, important "cellular" mediators of inflammation. However, neutrophils do not only secrete PLA_2 upon activation, but are also capable of generating reactive oxygen species (ROS) [4]. Among other ROS, hypochlorous acid (HOCl) seems primarily responsible for the increased levels of LPC under pathological conditions [5]. HOCl is generated under *in vivo* conditions from H_2O_2 and Cl^- ions under the catalytic influence of the enzyme myeloperoxidase (MPO) [6] that is predominantly found in neutrophils (where it constitutes about 5% of the total protein mass) and in much smaller amounts in macrophages. As the number of neutrophilic granulocytes increases massively under inflammatory conditions, the role of MPO and its products are obvious [7].

The generation of LPC from phosphatidylcholine (PC) under *in vivo* conditions is normally discussed by an increased activity of PLA_2 in the presence of HOCl [8]. However, it could be shown on a model level that LPC is also generated in the absence of PLA_2, i.e. under the influence of HOCl alone: Using PC vesicles with different fatty acyl compositions it could be proven by mass spectrometry that LPC is an abundant reaction product if PC reacts with either HOCl alone or with the products of the complete $MPO/H_2O_2/Cl^-$ system [9]. Although chlorohydrins (addition products of HOCl to the double bonds of the unsaturated fatty acyl residues) are the first detectable products, LPC is also generated in dependence on the number of double bonds in the fatty acyl residues [9,10]. Saturated LPC species are exclusively generated by this reaction indicating that the unsaturated oxidatively modified, but not the saturated fatty acyl residue is fragmented from the lipid backbone: The yield of LPCs increases if the saturation degree of the applied PC decreases. A mechanism to explain this behavior has recently been proposed [10]: Oxygen and chlorine are rather electronegative elements and weaken the ester bond of lipids by the

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withdrawal of electrons (−I effect). Therefore, the acyl residues in the chlorohydrins are more sensitive to hydrolysis than the unmodified acyl residues of the original lipids [10].

Chlorohydrins and LPCs were also obtained in significant yields if e.g. lipoproteins from human blood were incubated with HOCl [11]. However, due to the high protein content of lipoproteins and the considerable reactivity of the thiol and amino groups of proteins with HOCl, a considerable excess of HOCl was necessary to induce alterations of the lipid constituents [12].

Surprisingly, LPL were not obtained if phosphatidylethanolamine (PE) or phosphatidylserine (PS) vesicles were treated with HOCl, even if a significant excess of HOCl over the PL was used [13]. This is a clear indication that the above-mentioned mechanism does not represent the complete truth (LPL generation would be expected independent of the head group of the PL as soon as oxidative modification of the fatty acyl residues occurs) and that further factors (maybe also the structure of the PL head group) determine the LPL yield. Obviously, amino groups possess a much higher reactivity with HOCl than the double bonds of the fatty acyl residues [14]. Unfortunately, the prime reaction products that are generated under these conditions (mono- and di-chloramines) are not completely stable and decompose in a time-dependent manner complicating the evaluation of the products. Additionally, side reactions between the chloramines and the unsaturated fatty acyl residues cannot be completely ruled out.

Finally, the mass spectrometric detection as well as the supramolecular structure of PEs is much more difficult. This might be an additional reason why PEs were so far less intensively investigated than PCs [10].

Besides some other diseases, different chronic liver diseases are also characterized by persistent inflammatory processes and, thus, associated with increased oxidative stress [15]. As human liver tissue contains considerable amounts of polyunsaturated PC and PE species the LPLs derived thereof could represent important biomarkers of oxidative stress and inflammation. Therefore, this paper has two aims: (a) to investigate inflamed liver tissues towards the LPL content and (b) to determine the extent of LPL generation if unsaturated PC and PE species react with HOCl. Due to the complexity of the *in vivo* system, matrix-assisted laser desorption and ionization time of flight (MALDI-TOF) mass spectrometry (MS) [16–18] and ^{31}P NMR spectroscopy [19] were simultaneously applied as they are completely independent methods.

2. Materials and methods

2.1. Chemicals and liver samples

All chemicals for NMR spectroscopy (sodium cholate, EDTA, and deuterated water with an isotopic purity of 99.6%), buffer preparation ($NaH_2PO_4 \times H_2O$, $Na_2HPO_4 \times 2\ H_2O$ and trishydroxymethylaminomethane, TRIS), and matrix preparation (*p*-nitroaniline, PNA, 9-aminoacridine, 9-AA and dihydroxy benzoic acid, DHB) as well as all solvents (chloroform and methanol) were obtained in highest commercially available purity from Fluka Feinchemikalien GmbH (Taufkirchen, Germany). Phospholipase A_2 (PLA_2) (from porcine pancreas) was also purchased from Fluka.

1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (PLPC) and 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanolamine (PLPE) were purchased as 10 mg/ml solutions in $CHCl_3$ from Avanti Polar Lipids (Alabaster, MA, USA) and used without further purification.

Human liver samples from autopsies of normal controls ($n = 4$) and patients with inflammatory liver disease ($n = 3$) were kindly provided by the Leibniz Research Centre for Working Environment and Human Factors, Dortmund, Germany (Prof. J.G. Hengstler) and the Institute of Pathology, University Clinics of Leipzig, Germany (Prof. C. Wittekind), in agreement with national laws and ethical guidelines. Tissue portions were fixed in formaldehyde and embedded in paraffin, and 6 μm thick sections were stained with Hematoxylin and Eosin (HE). The severity of the inflammatory state of the liver sections was estimated by light microscopy.

2.2. Incubation of isolated phospholipids with hypochlorous acid

An aliquot of each PL dissolved in chloroform was evaporated to dryness. Vesicles (1.5 mmol/l phospholipid in total) were prepared by dissolving the phospholipid film in 100 mmol/l phosphate buffer (pH 7.4) and vortexing vigorously for 30 s. In order to mimic physiological conditions and to generate defined vesicles, a 1:1 mixture between PC 16:0/18:2 and PE 16:0/18:2 was additionally reacted with HOCl.

A stock solution of NaOCl was kept in the dark at 4 °C. Its concentration was determined at pH 12 using $\varepsilon_{290} = 350\ M^{-1}cm^{-1}$ for ^{-}OCl [20]. NaOCl was diluted with phosphate buffered saline (100 mmol/l) immediately prior to use. Liposomes were incubated with varying concentrations of sodium hypochlorite for 1 h at pH 7.4 at 37 °C. In order to extract the lipids the aqueous suspension was treated with the double volume of a chloroform/methanol mixture (2:1, v/v) [21]. The organic layer was used for further analysis.

For comparative purposes, PLs were also digested with PLA_2 according to the method essentially described in [22].

2.3. Phospholipid analysis by MALDI-TOF MS

Positive and negative ion MALDI-TOF mass spectra were acquired on a Bruker Daltonics Autoflex workstation (Bruker, Germany). The system utilizes a pulsed nitrogen laser emitting at 337 nm. The extraction voltage was 20 kV, the "low mass gate" was turned on at $m/z = 400$ and 128 single laser shots were averaged for each mass spectrum. In order to enhance the spectral resolution, spectra were recorded in the reflector mode under "delayed extraction" conditions [16,17].

The organic lipid extracts were normally applied onto the MALDI target using DHB as matrix for the positive ion mode and PNA for the negative ion mode [23]. Selected experiments were also performed in the presence of 9-AA that was recently suggested as a matrix with superior properties for the analysis of PLs [24]. All spectra were processed using the software "Flex Analysis" version 2.2 (Bruker Daltonics, Germany).

2.4. Liver extraction and liver phospholipid analysis by ^{31}P NMR spectroscopy

The liver lipid extraction was performed according to Folch [25] by suspending the liver tissue (sliced in approximately $5 \times 5 \times 5$ mm pieces) in 0.9% NaCl solution and the subsequent addition of chloroform/methanol (2:1:1, chloroform:methanol:water, final volume ratio). After separation of the chloroform and the aqueous phases, the chloroform layer was evaporated to dryness in a centrifugal evaporator (Jouan, Germany).

The dried organic residues were dissolved in 50 mmol/l TRIS (pH 7.65) containing 200 mmol/l sodium cholate and 5 mmol/l EDTA [26]. After intense vortexing of the 0.5 ml samples ^{31}P NMR spectra were recorded in 5 mm NMR tubes on a Bruker AVANCE-600 spectrometer operating at 242.88 MHz for ^{31}P. All measurements were performed using a direct ^{31}P/^{1}H NMR probe at 30 °C (303 K) with composite pulse decoupling (Waltz-16) to eliminate ^{31}P-^{1}H coupling. ^{31}P NMR spectra were additionally recorded at 60 °C (333 K). At this temperature the phosphatidylglycerol (PG)

resonance shifts to lower ppm values and the lysophosphatidylethanolamine (LPE) resonance can be clearly resolved [27].

Other NMR parameters were as follows: experiment time: 2–4 h, data size: 8 K, 60° pulse (6.7 μs), pulse delay 2 s, and a line broadening of 1 Hz. All peak assignments were confirmed by comparison with the shift of commercially available reference compounds. Spectra were processed using the software "1D WIN NMR" version 6.2® (Bruker Analytische Messtechnik GmbH, Rheinstetten, Germany).

3. Results

In Fig. 1 representative histological changes of three different liver specimens affected by inflammatory diseases in comparison to healthy human liver (trace a) are depicted. Different disease patterns, namely incipient micronodular cirrhotic disease probably due to chronic alcoholism, minor fatty degeneration and cholestasis in a patient with Lupus erythematodes, and hemorrhagic liver necrosis in a graft failure seven days after liver transplantation were included in this study. Under all three conditions, the inflammation was largely confined to the portal fields and of mixed, predominantly lymphocytic nature. Locally more dense infiltrates were detectable in the first specimen (trace b; patient X), loose but more generalized infiltrates in the third (trace d; patient Z), and very sparse infiltrates with an admixture of eosinophils in the second (trace c; patient Y). Acute inflammatory, purulent or granulomatous infiltrates were not present.

Fig. 2 shows the ^{31}P NMR spectra of the organic extracts of differently inflamed liver biopsies from the different patients recorded at 303 K (left hand side) and 333 K (right hand side). These different temperatures were chosen in order to demonstrate the significant NMR spectroscopic resolution increase under slightly different conditions. Differences in the signal-to-noise ratios of the individual ^{31}P NMR spectra are stemming from the different number of scans averaged for each spectrum. In trace (a) on the left hand side a representative spectrum of the lipid extract of a healthy human liver is shown as reference. This spectrum exhibits six resonances of strongly varying intensities representing phosphatidylcholine (PC; δ = 0.60 and 0.62 ppm), phosphatidylinositol (PI; δ = 0.48 ppm), phosphatidylserine (PS; δ = 0.26 ppm), phosphatidylethanolamine (PE; δ = 0.06 and 0.04 ppm), sphingomyelin (SM; δ = 0.01 ppm) and phosphatidylglycerol (PG; δ = 0.38 ppm) and is in good agreement with the known phospholipid (PL) composition of human liver [28]. Although details of the fatty acyl compositions and linkage types (diacyl, alkyl–acyl and alkenyl–acyl) cannot be completely resolved by ^{31}P NMR, splittings of the PE and PC resonances clearly indicate a rather complex fatty acyl composition [29].

In comparison to the spectrum of the lipid extract of the healthy human liver three lipid extracts of different inflamed human livers (cf. Fig. 1) are also shown in traces (b), (c) and (d) of Fig. 2. It is evident that a new, but small resonance arises at δ = 0.15 ppm corresponding to lysophosphatidylcholine (LPC). The formation of LPC under inflammatory conditions can be explained, for instance, by the hydrolysis of PC under the catalysis of phospholipase A_2 (PLA_2) [30]. In most cases PLA_2 is not specific for PC but is also active toward PE (as well as other PL, but the monitoring of the corresponding LPL species is difficult due to their rather small concentrations). This explains the relative PE decrease in inflammatory livers (traces (b) and (d)). The reason why in trace (c) no decrease of the PE resonance intensity occurs is unfortunately unknown so far. Although there are also effects regarding the PI resonance, changes of this resonance will not be considered because the focus of this study will be on changes of the PE and PC. Unfortunately, the resonance of PG overlaps with the peak of lysophosphatidylethanolamine (LPE) released by PLA_2 activity from PE if the ^{31}P NMR spectra are recorded at 303 K. Therefore, all ^{31}P NMR spectra were recorded a second time at 333 K as well (Fig. 2 right hand side). At 333 K the LPE peak (δ = 0.40 ppm), can be easily differentiated from the PG resonance (δ = 0.35 ppm) (trace (b), (c) and (d) right hand side). From the spectra recorded at 333 K it is evident that the LPE resonance is observed only in the case of the inflamed livers but not the healthy human liver (trace (a) right hand side).

Fig. 3 compares the positive ion MALDI-TOF mass spectra of the organic extracts of the healthy (trace a) and one inflammatory (trace b) human liver tissue (patient Y; cf. Figs. 1 and 2). All *m/z* values are given on the top of the corresponding peaks and all peak assignments are given in Table 1. The positive ion MALDI-TOF mass spectra exhibit primarily signals of SM 16:0, different PCs (thus, particularly PLs with quaternary ammonia groups) and triacylglycerol (TAG) species are detectable. One of the most abundant PC species present in the human healthy liver extract is PC 16:0/18:2 (*m/z* = 758.6) with two double bonds at the *sn*-2 position of the glycerol backbone. Of course, unambiguous assignments of the fatty acyl residues to the *sn*-1 and *sn*-2 positions cannot be

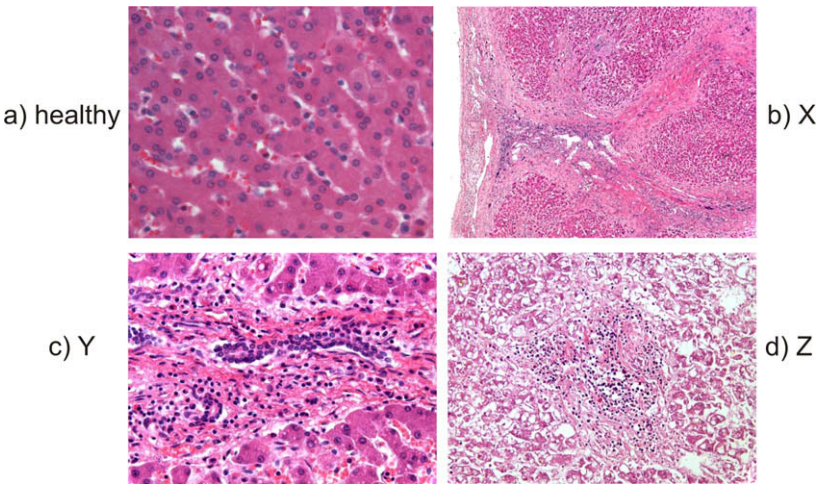

Fig. 1. Light microscopic images of autopsy liver samples, showing mild to moderate chronic inflammatory infiltrates in the portal spaces, in addition to other changes under three different conditions: (a) healthy liver; (b) micronodular cirrhosis due to chronic alcoholism, HE × 50; (c) minor fatty degeneration and cholestasis in Lupus erythematodes, HE × 400; (d) liver necrosis in graft failure seven days after transplantation.

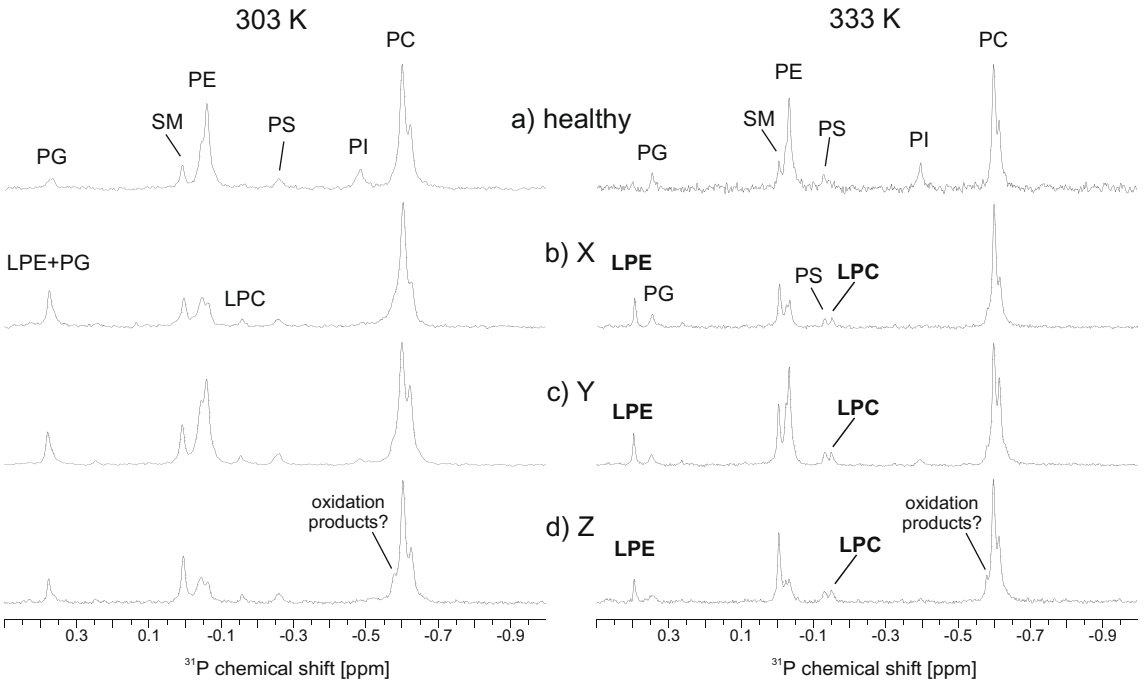

Fig. 2. 242.88 MHz ^{31}P NMR spectra of the organic extracts of human liver representing the phospholipid composition of human healthy liver (a) and three different human inflammatory livers (patients X, Y, Z; traces b–d). Spectra were scaled according to the intensity of the most intense resonance. Spectra at the left were recorded at 303 K and at the right at 333 K in order to clearly differentiate PG and LPE. Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin.

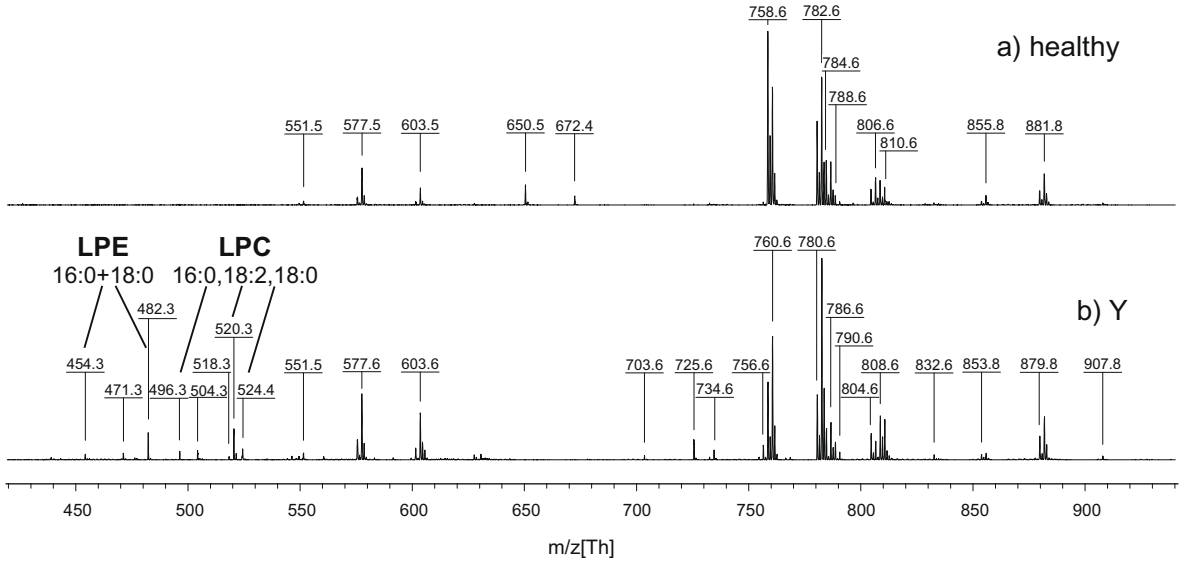

Fig. 3. Positive ion MALDI-TOF mass spectra of the organic extracts of healthy (a) and inflammatory (patient Y; b) human livers, while DHB served as matrix. *m/z* Values are indicated, for *m/z* assignments please see Table 1.

made by recording simple mass spectra, but assignments can be easily confirmed by recording post source decay (PSD) mass spectra [31]. Please also note that the peaks at *m/z* = 551.5, 577.5 and 603.5 are caused by the loss of one fatty acyl residue from the different triacylglycerol as well as PE species although PE as such is not detectable in the positive ion spectra in the presence of PC [17 and references cited therein] – at least if complex mixtures are investigated. Please also note that the signals at *m/z* = 650.5 and 672.4 are caused by oxidation of the oleoyl residue in PC 16:0/18:1 under generation of the aldehyde [32]. In contrast to the healthy, the inflammatory liver extract (trace b) exhibits additional LPL species (LPC and LPE) in the mass region between *m/z* = 450 and 530. Concomitantly the relative contents of the higher unsaturated PCs decrease in the inflammatory liver extracts (cf. PC 16:0/18:1; *m/z* = 760.6 and PC 16:0/18:2; *m/z* = 758.6). It is remarkable that in addition to saturated LPCs there is also a peak corresponding to LPC 18:2 (*m/z* = 520.3 for the H^+ adduct). Normally, PLs contain in *sn*-1 position the saturated and in *sn*-2 position the unsaturated fatty acyl residue [5] and this should lead to the exclusive generation of saturated LPCs under the influence of PLA_2 and ROS. As we do not yet have convincing explanation of LPC 18:2 generation, this compound will not be further discussed.

Table 1
Assignments of the positive ion signals obtained from the MALDI-TOF mass spectra of the organic extracts of human livers.

Mass (*m*/*z*)	Peak assignment
454.3	[LPE 16:0 + H]$^+$
471.3	Not identified
482.3	[LPE 18:0 + H]$^+$
496.3	[LPC 16:0 + H]$^+$
504.3	[LPE 18:0 + Na]$^+$
518.3	[LPC 16:0 + Na]$^+$
520.3	[LPC 18:2 + H]$^+$
524.3	[LPC 18:0 + H]$^+$
551.0	DHB matrix peak
577.5	TAG/PE fragment
603.5	TAG/PE fragment
650.5	Aldehyde from PC 16:0/18:1
672.4	Aldehyde from PC 16:0/18:1
703.6	[SM 16:0 + H]$^+$
725.6	[SM 16:0 + Na]$^+$
734.6	[PC 16:0/16:0 + H]$^+$
756.6	[PC 16:0/16:0 + Na]$^+$
758.6	[PC 16:0/18:2 + H]$^+$
760.6	[PC 16:0/18:1 + H]$^+$
780.6	[PC 16:0/18:2 + Na]$^+$
782.6	[PC 16:0/20:4 + H]$^+$
784.6	[PC 18:0/18:3 + H]$^+$
786.6	[PC 18:0/18:2 + H]$^+$
788.6	[PC 18:0/18:1 + H]$^+$
790.6	[PC 18:0/18:0 + H]$^+$
804.6	[PC 16:0/20:4 + Na]$^+$
806.6	[PC 18:0/18:3 + Na]$^+$
808.6	[PC 18:0/20:5 + H]$^+$
810.6	[PC 18:0/20:4 + H]$^+$
832.6	[PC 18:0/20:4 + Na]$^+$
853.8	[TAG (50:2) + Na]$^+$
855.8	[TAG (50:1) + Na]$^+$
879.7	[TAG (52:3) + Na]$^+$
881.7	[TAG (52:2) + Na]$^+$
907.8	[TAG (54:3) + Na]$^+$

Nevertheless, the positive ion MALDI-TOF mass spectra basically confirm the results obtained by ^{31}P NMR: the LPC and LPE contents increase significantly under inflammatory conditions in the human liver tissue.

Additionally, negative ion MALDI-TOF mass spectra of the organic extracts of healthy and inflammatory human liver tissues were recorded (data not shown). The negative ion MALDI-TOF mass spectra exhibit signals of different diacyl-GPE, alkenyl-linked GPE, PG, PS and PI species confirming the results obtained by ^{31}P NMR. One of the most abundant PE species present in the liver extracts from the healthy control is PE 18:0/18:2 with two double bonds at the *sn*-2 position of the glycerol backbone. Furthermore, the negative ion spectra of the inflammatory liver extracts do not show other LPL peaks beside LPE 16:0 and 18:0 (cf. Fig. 3). Concomitantly the relative content of the stronger unsaturated PEs decreases in the inflammatory liver extracts. However, if the LPLs would be generated exclusively by the action of PLA_2 it must be anticipated that this enzyme is specific towards the PC and PE species because no lysolipids of other PL classes are detectable: Although their contributions are expected to be much lower in comparison to LPE and LPC due to the lower concentration of the original PLs, LPLs such as lysophosphatidylglycerol or serine would be more sensitively detectable as negative ions due to their increased negative charge.

However, there is one additional mechanism by which at least LPC may be generated from PC at inflammatory loci [7]. The hypochlorous acid-mediated oxidation of PC at inflammatory loci and the subsequent release of LPC from unsaturated PC are well-documented [9]. Nevertheless, it is not yet known if other unsaturated PLs behave similarly under inflammatory conditions and generate lysophospholipids (LPL) as well. Therefore, the chemical behavior of PE upon hypochlorous acid (HOCl) treatment was compared with PC in model experiments using isolated PL species. In both cases, the unsaturated PL species with two double bonds at the *sn*-2 position of the glycerol backbone, namely 1-palmitoyl-2-linoleoyl-PL (16:0/18:2) – as one of the main PC and PE species in the human liver – were chosen in order to simplify the complexity of the oxidation products. PE and PC species are exclusively considered in this paper because they are most abundant in the human liver, while all other PL species contribute only to a much smaller extent. After incubation of the individual PL with different HOCl concentrations the reaction products were investigated by MALDI-TOF MS. ^{31}P NMR spectra gave only broadened resonances and were, thus, not really helpful regarding detailed product analysis (data not shown).

Selected examples of positive ion MALDI-TOF mass spectra of the extracts of the reaction mixtures of PLPC and HOCl are shown in Fig. 4. All *m*/*z* values are given on the top of the peaks and were assigned according to Table 2. The spectrum of untreated PLPC is shown in trace (a) as control. PLPC gives two major peaks at *m*/*z* = 758.6 and 780.6 corresponding to the H^+ and Na^+ adduct, respectively. The smaller peaks are most probably caused by impurities but could not be assigned. Both PLPC peaks decrease upon treatment with 2.5 and 5 mmol/l HOCl (trace b and c) and disappeared completely upon treatment with 10 mmol/l HOCl (trace d). In trace (b) the expected oxidation products – epoxides, chlorides [33] and chlorohydrins – appear as new peaks in the higher mass range. Furthermore, in the lower mass region new peaks arise at *m*/*z* = 496.3 and 518.3 corresponding to the H^+ and Na^+ adduct of LPC 16:0. With increasing HOCl concentrations (trace c) the intensities of all peaks of the oxidation products increase and further peaks of oxidation products (particularly dichlorides) arise. After a further increase of the HOCl concentration (trace d) the peaks at *m*/*z* = 844.6 and 866.6 (dichloride/epoxide) and *m*/*z* = 862.6 and 884.6 (bis-chlorohydrin) represent the main oxidation products beside other minor products and the peaks corresponding to the degradation product, LPC 16:0, reach their maxima. All these products are not surprising and in agreement with previous studies. Therefore, the generation of LPC in the diseased liver could be explained – beside PLA_2-induced cleavage of PC – also by HOCl-induced oxidation.

In Fig. 5 the positive ion MALDI-TOF mass spectra of the extracts of the reaction mixtures of PLPE reacted with different amounts of HOCl are shown. In contrast to PC, PE contains a "free" amino group exhibiting high reactivity with HOCl. Thus, additional products in comparison to PC (discussed above) can be expected. All *m*/*z* values are given on the top of the peaks and were assigned according to Table 3. The spectrum of untreated PLPE is shown in trace (a). PLPE gives three peaks at *m*/*z* = 716.5, 738.5 and 760.5 corresponding to the H^+, Na^+ and $H^+ + 2Na^+$ adduct, respectively. Furthermore, the untreated PLPE gives two different fragmentation products at *m*/*z* = 575.5 corresponding to the loss of the head group and *m*/*z* = 601.5 that is likely to represent a product of a rearrangement [31]. The presence of these fragments (in the gas phase) is a clear difference in comparison to PC. The intensities of the PLPE peaks decreased upon treatment with 2.5 mmol/l HOCl (trace b) and disappeared completely upon treatment with 5 and 10 mmol/l HOCl (trace c and d). While in trace (b) no peaks of certain products appear in trace (c) the oxidation products – chlorides, chlorohydrins, dichlorides, epoxides – arise as new peaks in the higher mass range. All oxidation products are also obvious subsequent to fragmentation of the head group (cf. Table 3). After a further increase of the HOCl concentration (trace d) no additional peaks of further oxidation products appear but the peaks at *m*/*z* = 802.5 and 824.5 (dichloride/epoxide), *m*/*z* = 820.5 and 842.5 (bis-chlorohydrin) and their fragmentation products at *m*/*z* = 661.5 and 679.5 (dichloride/epoxide and bis-chlorohydrin) represent

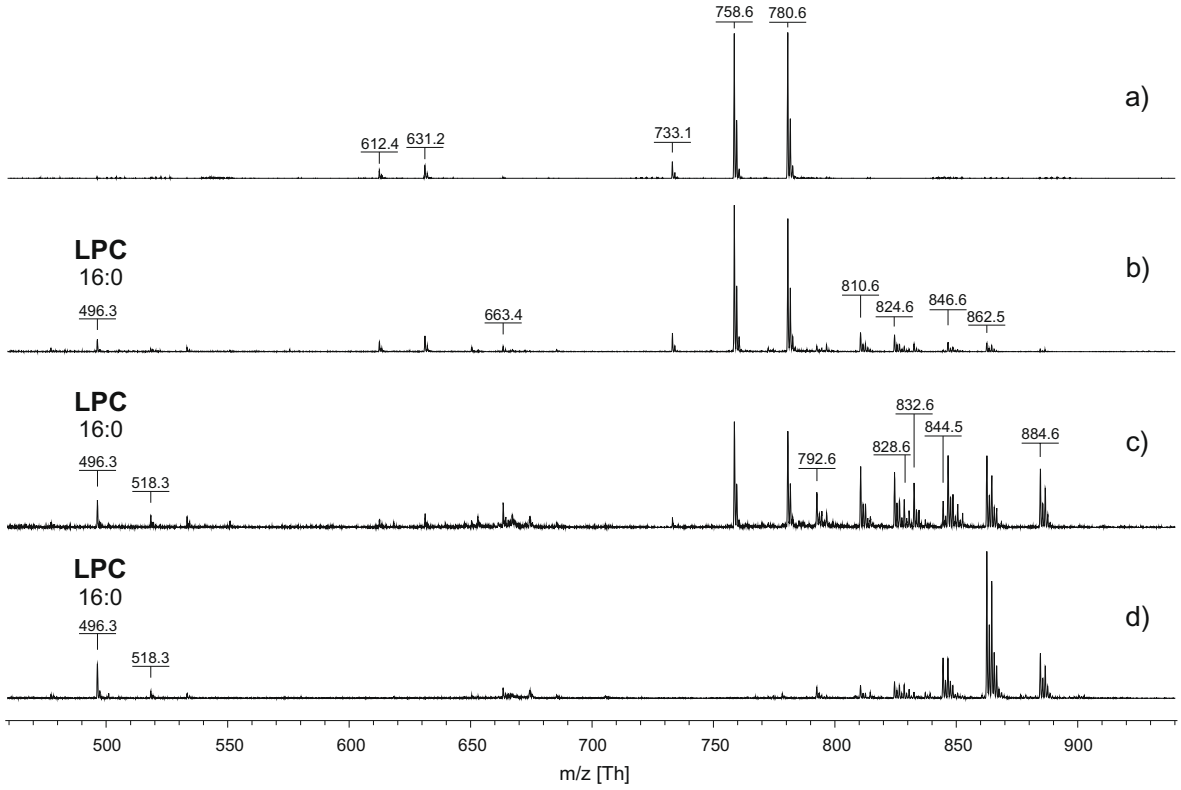

Fig. 4. Positive ion MALDI-TOF mass spectra of the organic extracts of PLPC (1.5 mmol/l each) after incubation with: (a) buffer, (b) 2.5, (c) 5 and (d) 10 mmol/l HOCl for 1 h at 37 °C. *m*/*z* Values are indicated, for *m*/*z* assignments please see Table 2. DHB was used as matrix. For details see text.

Table 2
Assignments of the positive ion signals obtained from the MALDI-TOF mass spectra of the organic extracts of PLPC after incubation with HOCl.

Mass (*m*/*z*)	Peak assignment
496.3	$[LPC16:0 + H]^+$
518.3	$[LPC16:0 + Na]^+$
612.4	Unidentified
631.2	Unidentified
663.4	Impurity from pipette tip
733.1	Unidentified
758.6	$[PC\ 16:0/18:2 + H]^+$
780.6	$[PC\ 16:0/18:2 + Na]^+$
792.6	$[PC\ 16:0/18:2 + chlorine + H]^+$
810.6	$[PC\ 16:0/18:2 + chlorohydrin + H]^+$
824.6	$[PC\ 16:0/18:2 + chlorine + bis\text{-}epoxid + H]^+$
828.6	$[PC\ 16:0/18:2 + dichloride + H]^+$
832.6	$[PC\ 16:0/18:2 + chlorohydrin + Na]^+$
844.6	$[PC\ 16:0/18:2 + dichloride + epoxid + H]^+$
846.6	$[PC\ 16:0/18:2 + chlorine + bis\text{-}epoxid + Na]^+$
862.6	$[PC\ 16:0/18:2 + bis\text{-}chlorohydrin + H]^+$
884.6	$[PC\ 16:0/18:2 + bis\text{-}chlorohydrin + Na]^+$

the main oxidation products beside other minor products. It was, however, not the aim of this work to evaluate all – even minor products – in more detail. Obviously (and in clear contrast to PC) the HOCl oxidation of unsaturated PLPE does not lead to a significant formation of LPE 16:0. LPE 16:0 would be expected at *m*/*z* = 454.3, 476.3 and 498.3 corresponding to the H^+, Na^+ and $H^+ + 2Na^+$ adducts, respectively.

Unfortunately, it is well-known that the tendency of PC and PE to generate defined supramolecular structures is different as the PE forms lamellar phases [34]. This is not a major problem in the case of physiological PL mixtures because there is normally an excess of PC that forms defined vesicles, where the PEs are entrapped, but is a potential problem if pure PE is investigated. Therefore, an additional experiment was performed, where a 1:1 mixture between PLPC and PLPE was used. A relatively high moiety of PE was used because the liver contains high amounts of PEs [28]. This system was also used to illustrate the effects of PLA_2. In Fig. 6 some selected MALDI-TOF mass spectra subsequent to PLA_2 digestion (a) and reaction with HOCl (b–d) are shown and the used matrices as well as the polarities of the measurements are given in the individual traces.

In trace (6a) the effect of PLA_2 on the PC/PE mixture is shown. In addition to the peaks of PE 16:0/18:2 and PC 16:0/18:2 there are intense signals of both expected LPLs. Although the PC and LPC signals are stronger in comparison to the PE and LPE peaks, it is obvious that both species are detectable in the positive ion mass spectra of the mixture. Of course, the ratio between the PLs and the LPLs can be easily altered by the applied PLA_2 activity or the incubation time. The peaks of both LPLs are marked by vertical dotted lines in order to emphasize their presence or absence in the individual traces.

Traces (b) and (c) represent the same sample but were recorded in the presence of different matrix compounds. As a significant excess of HOCl (1:10 in comparison to the PLs) was used, the expected product, i.e. the bis-chlorohydrine of PLPC is easily detectable in the positive ion spectra. This is particularly evident if the spectra are recorded in the presence of 9-AA (6c) because under these conditions primarily the H^+ (*m*/*z* = 862.5) but not the Na^+ adducts are detectable. In the presence of DHB (6b) the same product cationized by H^+ as well as Na^+ (*m*/*z* = 884.5) are detectable. However, in addition to the bis-chlorohydrin of PLPC, the bis-chlorohydrin of PLPE is also detectable. It is not surprising that the PE is exclusively detectable in the positive ion spectrum in the presence of DHB because DHB is a stronger acid in comparison to 9-AA. Nevertheless, in both cases, the expected LPC but not even small amounts of the LPE are detectable (marked by the dotted vertical line) although a considerable excess of HOCl was used.

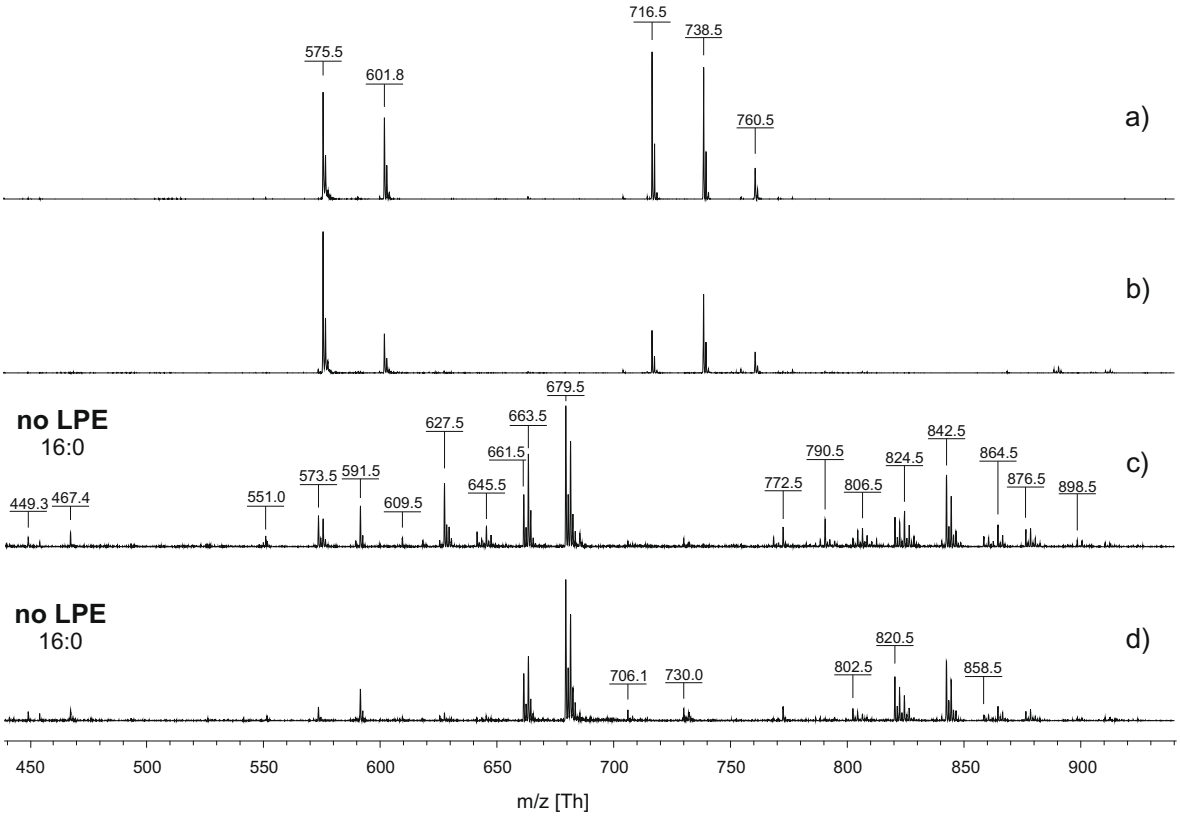

Fig. 5. Positive ion MALDI-TOF mass spectra of the organic extracts of PLPE (1.5 mmol/l each) after incubation with: (a) buffer, (b) 2.5, (c) 5 and (d) 10 mmol/l HOCl for 1 h at 37 °C. *m*/*z* values are indicated, for *m*/*z* assignments please see Table 3. The same conditions as indicated in Figs. 3 and 4 were used.

Table 3
Assignments of the positive ion signals obtained from the MALDI-TOF mass spectra of the organic extracts of PLPE after incubation with HOCl.

Mass (*m*/*z*)	Peak assignment
551.0	DHB matrix peak
575.5	Fragment-head group
591.5	[Fragment from epoxid + H]$^+$
609.5	[Fragment from glycol + H]$^+$
627.5	[Fragment from chlorohydrin + H]$^+$
645.5	[Fragment from dichloride + H]$^+$
661.5	[Fragment from dichloride + epoxid + H]$^+$
663.4	Impurity from pipette tip + H$^+$
679.5	[Fragment from 2 × chlorohydrin + H]$^+$
685.4	Impurity from pipette tip + Na$^+$
716.5	[PE 16:0/18:2 + H]$^+$
738.5	[PE 16:0/18:2 + Na]$^+$
760.5	[PE 16:0/18:2-H$^+$ + 2Na]$^+$
772.5	[PE 16:0/18:2 + chlorine + Na]$^+$
790.5	[PE 16:0/18:2 + chlorohydrin + Na]$^+$
802.5	[PE 16:0/18:2 + dichloride + epoxid + H]$^+$
806.5	[PE 16:0/18:2 + bis-chlorine + Na]$^+$
820.5	[PE 16:0/18:2 + bis-chlorohydrin + H]$^+$
824.5	[PE 16:0/18:2 + dichloride + epoxid + Na]$^+$
842.5	[PE 16:0/18:2 + bis-chlorohydrin + Na]$^+$
858.5	[PE 16:0/18:2 dichloride + chlorine + epoxid + Na]$^+$
864.5	[PE 16:0/18:2 + bis-chlorohydrin − H$^+$ + 2Na]$^+$
876.5	[PE 16:0/18:2 dichloride + epoxid + chlorohydrin + Na]$^+$
898.5	[PE 16:0/18:2 dichloride + epoxid + chlorohydrin − H$^+$ + 2Na]$^+$

9-AA is a highly suitable matrix to record negative ion spectra and the corresponding spectrum is shown in trace (6d). It is obvious that the most intense peak (*m*/*z* = 814.5) does not correspond to the expected negative ion of the bis-chlorohydrin of PLPE (expected at *m*/*z* = 818.5) but differs for 4 amu. Therefore, as already previously shown [13], this peak corresponds to the nitrile of the bis-chlorohydrine of PE 16:0/18:2. It is not astonishing that this compound is particularly well detectable in the negative ion mode spectrum because the nitrile has lost the positively charged quaternary ammonia group but retained the negatively charged phosphate residue. Therefore, the nitrile is not detectable in the positive ion spectra. Accordingly, the nitrile of LPE 16:0 is detectable at *m*/*z* = 448.3.Please also note that the peak marked by "*x*" is stemming from PC that is detectable in the presence of 9-AA as negative ion subsequent to loss of a methyl group. For further information see [35].

Thus, the positive ion MALDI-TOF mass spectra of PC 16:0/18:2 and PE 16:0/18:2 indicate a comparable behavior upon HOCl treatment and the same addition products of HOCl to the two double bonds at the *sn*-2 position of the glycerol backbone are obvious. It does obviously not matter whether both PLs are independently investigated or in a mixture. A pronounced difference is, however, the LPC formation from PC upon HOCl treatment. Due to the presence of the additional amino group that exhibits high reactivity with HOCl, the generation of head group-modified oxidation products of PE is most likely [13,36], but their analysis was not the aim of this study because it is much more difficult and depends considerably on the applied matrix. Nevertheless, the generation of LPE in the positive ion mode can be certainly excluded under the used experimental conditions. The LPE is not detectable as negative ion, too. However, the corresponding nitrile of the LPE is detectable in the negative ion mode (cf. 6d) due to its excessive negative charge.

4. Discussion and conclusions

LPC and LPE formation could be detected by ^{31}P NMR spectroscopy and MALDI-TOF MS under *in vivo* conditions in different human inflammatory liver tissues. Both species may be generated

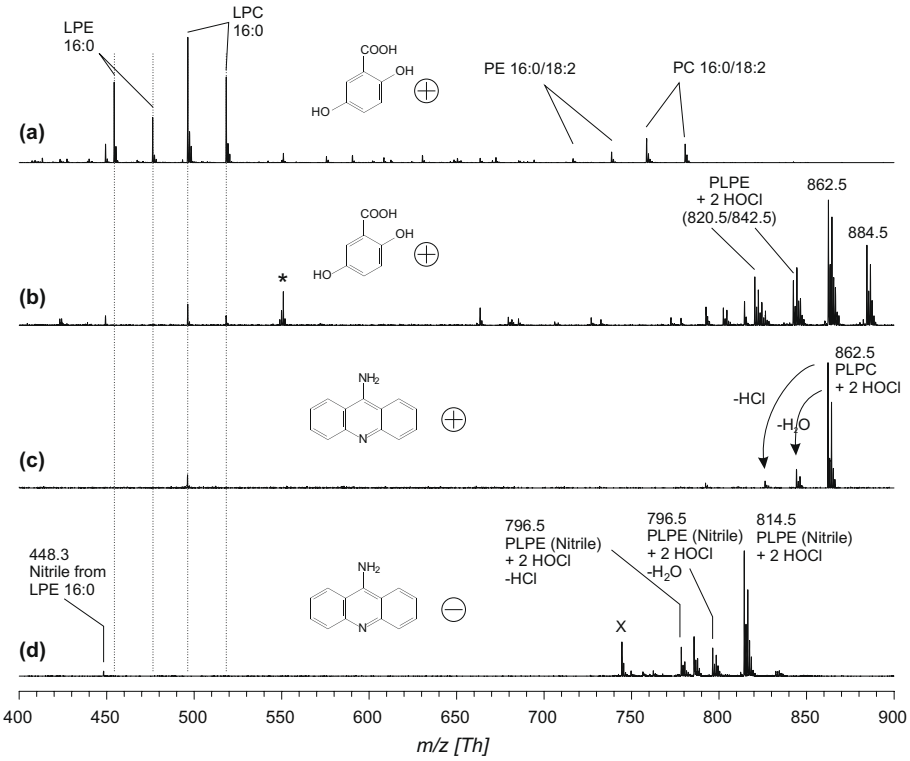

Fig. 6. Positive and negative ion MALDI-TOF mass spectra of the organic extracts of a PLPE/PLPC mixture (1:1) after incubation with PLA_2 (a) and a tenfold excess of HOCl (b-d). The used matrices and the polarities of the individual spectra are given directly in the figure. The assignments of the most abundant products are also given directly in the figure. The vertical dotted lines indicate the H^+ and Na^+ adducts of LPE 16:0 and LPC 16:0. For further details see the text.

by the enzyme PLA_2. It is, however, also known that HOCl is capable to generate LPC from unsaturated PC under inflammatory conditions. The LPC formation from unsaturated PCs has been already well documented [10]. Therefore, MALDI-TOF MS analysis has been used to clarify in more detail the potential LPL generation from PE by HOCl. Both PL classes – PC and PE – have been compared to clarify which PL class leads to LPL generation under the oxidative influence of HOCl. Only the diunsaturated species (linoleoyl residues) – as one of the main PC and PE species in the human liver – have been chosen for all incubations with HOCl in order to reduce the resulting complexity of the products and because monounsaturated species (with e.g. oleoyl residues) do not yield major amounts of LPLs [10]. Relatively large amounts of HOCl have been used to mimic the continuous generation of HOCl by MPO under inflammatory conditions [4].

It could be shown that both PL classes give the expected chlorination/oxidation of the double bonds in the unsaturated fatty acyl residue under chlorohydrin, dichloride and epoxide formation. In contrast, PC (but not PE) is also characterized by subsequent LPL formation upon treatment with HOCl.

Therefore, it is assumed that the PL head group exhibits a major influence on the tendency of the PL to LPL formation upon HOCl oxidation. In this respect it is also a striking result that PE gives intense fragmentation indicating head group losses during positive ion MALDI-TOF MS measurements. Concomitantly, PE does not form LPE in the presence of HOCl. Browning [37–39] found that the PC head group is more flexible than the PE head group and discussed the differences in head group rigidity in terms of the capacity of the various head groups to bind noncovalently to their neighbors. It was assumed that PC cannot form a proton transfer complex ($-PO_2^- \cdots H-{}^+NH_2-$) with neighboring phosphates [40] and can only interact electrostatically that appears to be quite weak and accounts for the more flexible nature of the choline head group [37]. In contrast, the proton transfer complex described for the PE head groups may account for their rigidity [38,39]. Thus, this existence of a network of noncovalent bonds and the consequential rigidity of the head group may account on the one hand for the typical fragmentation behavior of PE and may stabilize the negative inductive effect caused by the chlorohydrin formation in the aliphatic hydrocarbon chain on the other hand.

Summarizing, it is obvious that the LPC formation from unsaturated PC and the accumulation in inflammatory liver tissue can be caused by both, PLA_2-activity and oxidation by hypochlorous acid whereas LPE formation and accumulation results exclusively from PLA_2-activity. It is, thus, concluded that LPC represents a useful biomarker of oxidative stress that can be easily determined by MS – even in complex mixtures and without the need of previous separation. Finally, however, it is also obvious that under conditions of negative ion detection, the nitrile of LPE is detectable. As this compound is not detectable in the positive ion spectra, a quantitative assessment of this compound is rather difficult and it could not be the aim of this study to clarify the contribution of the nitrile with reference to the LPE. Nevertheless, clarifying this problem is a current research topic in our laboratory.

A correlation of liver lipidomics and histological changes has also not been attempted so far. Although we could show exemplarily clear differences between diseased and healthy liver samples, more stringent statements with potential diagnostic relevance must await investigations of larger patient series.

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