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Investigations of the lysophospholipid composition of human neutrophils under different stimulation conditions by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

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Matrix-assisted laser desorption / ionization time-of-flight mass spectrometry (MALDI-TOF MS) is usually used for the analyses of proteins, carbohydrates and oligonucleotides. In spite of the number of advantages that MALDI-TOF MS exhibits for lipid analysis, this method has not often been applied in this field. In this paper we have extended our previous studies on the suitability of MALDI-TOF MS for the investigation of changes in the content of lipid-derived second messengers in organic extracts of human neutrophils. Qualitative differences in the lysophospholipid composition in organic extracts of the human neutrophils under different stimulation conditions could be easily observed by MALDI-TOF MS. Although there are still some methodological problems to be solved before this method can be routinely applied for the quantification of different lipid classes in complex biological mixtures (such as organic extracts of human neutrophils) it is shown here that MALDI-TOF MS possesses the capability to be used as a simple screening method for the investigation of the content of lipid-derived second messengers and of signalling pathways in cells.

Keywords: lysophospholipids, MALDI-TOF MS, neutrophils, phospholipase, phospholipids, signal transduction.

INTRODUCTION[*]

Human polymorphonuclear leukocytes (shorter: "neutrophils"or PMNs) are immune cells involved in the non-specific immune response. Neutrophils are highly spe-

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* *Abbreviations:* AACOCF3: arachidonoyltrifluoromethyl ketone; CL: chemiluminescence; DAG: diacylglycerol; fMLP: *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; LPA: lysophosphatidic acid; LPC: lysophosphatidylcholine; LPE: lysophosphatidylethanolamine; LPL: lysophospholipid; LY294002: 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one; MALDI-TOF MS: matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; NADPH: nicotinamide adenine dinucleotide phosphate, reduced form; PA: phosphatidic acid; PC: phosphatidylcholine; PI3-kinase: phosphoinositide 3-kinase; PI: phosphatidylinositol; PLA_2: phospholipase A_2; PL: phospholipid; PMN: polymorphonuclear leukocytes; PE: phosphatidylethanolamine; PPI: (poly-)phospho-inositide; PS: phosphatidylserine; ROS: reactive oxygen species; SM: sphingomyeline; TAG: triacylglycerol.

cialized cells for the defence of the host, particularly against bacterial and fungal infections. They possess a highly complex enzyme, NADPH oxidase, whose activation is the starting point for the production of a variety of reactive oxygen species (ROS). Upon stimulation at the site of infection, neutrophils produce ROS – especially the superoxide anion radical, hydrogen peroxide, hypochlorous acid and hydroxyl radicals and they also release a number of enzymes that are all involved in the defence against the invading microorganisms.[1,2] On the other hand, the ROS and enzymes released from stimulated PMNs can contribute to tissue injury in pathologies such as atherosclerosis[3] or rheumatoid arthritis.[4–6]

Signalling pathways leading to the activation of the NADPH oxidase are complex and involve the simultaneous activation of various phospholipases that generate lipid second messengers.[7] Lipid second messengers are further involved in the activation and regulation of other proteins, such as protein kinase C or the recently discovered phosphatidic acid- activated protein kinase (PAPK).[8–11]

Investigations of the enzyme activity and the production of lipid-derived second messengers as a part of the intracellular communication system can help dissecting signalling pathways involved in a particular cell function. The analysis of lipid-derived second messengers usually requires either selective radioactive labelling, derivatisation of lipids or previous separation by HPLC or TLC.[12–14] Methods that would allow lipid analysis in a single step are rather scarce. After the introduction of the so-called "soft ionization" techniques, mass spectrometry has gained huge interest for the investigation of signalling events, since derivatisation of lipids is no longer required.[15]

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is an established tool for the analysis of proteins, carbohydrates and nucleic acids.[16–19] However, MALDI-TOF MS also possesses a number of advantages for the analysis of lipids, but this method has only recently been used in lipid and phospholipid research.

Recently, it was shown that all classes of lipids can be easily and accurately analysed by MALDI-TOF MS, and that 2,5-dihydroxybenzoic acid (2,5-DHB) is the matrix of choice.[20–22] An important advantage of MALDI-TOF MS and the application of 2,5-DHB as the matrix for lipid analysis is that both the analyte and the matrix are readily soluble in organic solvents. This results in extremely homogeneous analyte/matrix co-crystals that provide highly reproducible spectra with an excellent signal-to-noise ratio.[20] Lipid spectra can be simply interpreted, since each molecule yields mainly singly-charged ions, and, therefore, MALDI-spectra are by far less crowded by peaks than the corresponding ESI spectra.[20] Very recently it was shown that multiply-charged ions are simultaneously generated upon the process of desorption and ionization.[23] However, these multiply-charged ions more easily undergo neutralization than singly-charged ones and, therefore, they are usually not detectable in the spectra. Another very important advantage of MALDI-TOF MS for lipid analysis is that it is possible to obtain information on the lipid class and on the fatty acid composition of a given lipid in a single measurement. Finally, by the introduction of a suitable internal standard, even the quantification of lipids is possible by MALDI-TOF MS.[22]

In our previous reports, the spectra of individual lipids and phospholipids (PLs) were analysed,[20,22,24] and it could be shown that there are some limitations for their de-

tection.[25] Especially, it was shown that the presence of phosphatidylcholine (PC) can prevent the detection of other lipid classes, that the detectability correlates reciprocally with the molecular weight of the lipid and that the number of charges on a given analyte influences its detectability. Nevertheless, lipid mixtures of biological origin were shown to be easily screened for their lipid composition by MALDI-TOF MS.[26,27] After having found the most suitable parameters for the quantification of various lysophospholipid (LPL) classes,[28] the aim of this study was to check whether MALDI-TOF MS can be used for the investigation of changes in lipid composition of human PMNs under different stimulation conditions. It will be shown that, on the negative side, there are some limitations for such an application of MALDI-TOF MS but that the method is, on the positive side, sufficiently sensitive for the detection of changes in the LPL composition of PMNs upon stimulation. These changes will be additionally explained by the inhibition of different signalling pathways by selective enzyme inhibition.

EXPERIMENTAL

Materials

The lipids, namely 1-stearoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine, PC (18:0, 18:2), and 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphate, LPA (16:0), were purchased from Avanti Polar Lipids Inc. (Alabama, USA). Both were obtained as 10 mg/ml chloroform solutions. DAG (16:0, 16:0) (1,2-dipalmitoyl-glycerine) was obtained as a powder from Fluka (Seelze, Germany) and was dissolved in chloroform (10 mg/ml).

Chemicals for the neutrophil purification, *i.e.,* Hank's balanced salt solution (HBSS), Ficoll-Hypaque, dextran, heparin, and the stimulators, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) and phorbol myristoyl acetate (PMA) were purchased from Sigma (Deisenhofen, Germany). Luminol (5-amino-2,3-dihydrophthalazine-1,4-dione) was a product from Boehringer-Mannheim (Germany). DAG kinase II inhibitor (R59949), wortmannin and 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002), inhibitors of phosphoinositide 3-kinase, as well as arachidonoyltrifuoromethyl ketone (AACOCF3), an inhibitor of cytosolic phospholipase A_2, were purchased from Calbiochem-Novabiochem (Bad Soden, Germany). The site of action of these inhibitors will also be discussed later in the text.

All solvents (chloroform and methanol), 2,5-dihydroxybenzoic acid (DHB), dimethyl sulfoxide (DMSO) and trifluoroacetic acid (TFA) were purchased from Fluka (Seelze, Germany) in the highest commercially available purity and were used without any further purification.

Cell preparation

Polymorphonuclear leukocytes (PMNs) were isolated from heparinised (10 U/ml) blood of healthy volunteers as described elsewhere.[29] Briefly, after dextran-enhanced sedimentation of erythrocytes, the supernatant was applied to Ficoll-density gradient centrifugation. The remaining erythrocytes in the pellet were lysed three times for 1 min with distilled water. The resulting cell suspension was centrifuged and the PMNs contained in the pellet were resuspended in HBSS and stored on ice until use. The PMNs were used within 2 h after purification.

Chemiluminescence measurements

All chemiluminescence (CL) measurements were performed on a microplate luminometer MicroLumat LB 96 P (EG & G Berthold, Wildbad, Germany). PMNs were preincubated with luminol (5×10^{-5} M) and with varying concentrations of different inhibitors (ranging from 1×10^{-9} M to 1×10^{-5} M). The inhibitors were stored as 10^{-2} M solutions in DMSO (–20 ºC), and were diluted with HBSS prior to the addition. As control, cells were preincubated with DMSO. After preincubation, the corre-

sponding stimulator of the respiratory burst, *i.e.,* fMLP (10^{-6} M, final concentration) was injected into the cell suspension and the CL response was monitored over 25 min. CL measurements were carried out with all cell preparations used for lipid extraction and MALDI-TOF mass spectrometry.

Extraction of lipids from human neutrophils

PMNs were preincubated at 37 ºC for 10 min and afterwards stimulated with fMLP (10^{-6} M, final concentration). In the case of the control sample, the same volume of HBSS was added. Separately, PMNs were preincubated with various inhibitors at a concentration of 10^{-6} M (wortmannin) or 10^{-5} M (R59949, LY294002 and AACOCF3) at 37 ºC for 10 min and afterwards stimulated with fMLP. The reaction was terminated after 10 min by the addition of a chloroform / methanol mixture, and the organic extract was recovered according to the Bligh and Dyer method.[30] These experiments were repeated with ten different cell preparations, and only those changes detected in at least 6 preparations were considered.

Matrix and sample preparation for MALDI-TOF mass spectrometry

For MALDI-TOF MS, a 0.5 M 2,5-dihydroxybenzoic acid (DHB) solution in methanol containing 0.1 % v/v trifluoroacetic acid (TFA) was used. TFA was used because small amounts of TFA improve the signal-to-noise ratio of the lipid spectra.[20] No degradation of PLs was observed in the presence of TFA at the concentration used in our experiments.

Chloroform was removed from the organic extracts of PMNs prepared as described above or from the lipids diluted to a 1 mg/ml concentration by vacuum centrifugation (Vacuum Centrifugal Evaporator, Jouan, Germany). Subsequently, 20 µl of 0.5 M DHB solution in methanol was added and the sample was vigorously mixed. All samples were applied as 1.8 µl droplets onto the sample plate and were rapidly dried under a moderate warm stream of air.

Mass spectrometry

All MALDI-TOF mass spectra were acquired on a Voyager Biospectrometry Workstation (PerSeptive Biosystems, Framingham, MA, USA). The system utilizes a pulsed nitrogel laser, emitting at 337 nm. The pressure in the ion chamber was maintained between 1×10^{-7} and 4×10^{-7} Torr. A two stage acceleration device allows the application of delayed extraction conditions (DE-mode), improving both the mass resolution and the mass accuracy.[31] The formed ions were accelerated by a 20 kV accelerating voltage within the ion source. In order to enhance the spectral resolution, the device was used in the reflector mode, so that the total field-free time-of-flight distance was 2 m. An internal calibration was performed by setting the peak of the protonated DHB-matrix to its appropriate value (155.034 Da). For all samples, 128 single shots from the laser were averaged for each mass spectrum and each sample was analysed in duplicate. The laser power was maintained about 10 % over the threshold in order to obtain the best signal-to-noise ratio. All lipid spectra (besides those for calibration) were acquired using the "low-mass gate" at 400 Da to prevent detector saturation by ions arising from matrix peaks.[24]

RESULTS AND DISCUSSION

MALDI-TOF mass spectrometry of individual lipids

Among a number of different matrices available for MALDI-TOF MS, 2,5-DHB is recommended for PL analysis due to its rather low yield of fragmentation products.[20,21] However, even this matrix gives a number of peaks that correspond to various oligomerization products in the positive, as well as in the negative ion mode.[24] Although less expressed than with other matrices, the mass region below $m/z = 400$ in the positive ion mode is crowded with matrix peaks and, therefore, 2,5-DHB is not useful for the analyses of such low molecular weight compounds. In order to prevent detector

saturation with matrix peaks, all measurements were carried out with a "low mass gate" at *m*/*z* = 400. This means that peaks arising from compounds with a molecular weight lower than 400 Da are suppressed. However, there are still some peaks arising from the matrix that are found in the mass region of interest, but their presence does not markedly complicate the detection of the analyte peaks in the positive ion mode. Moreover, matrix peaks can be completely suppressed when the lipid concentration is sufficiently high. On the other hand, the matrix gives a higher number of peaks in the negative ion mode,[24] which most probably contributes to the somewhat lower sensitivity of MALDI-TOF MS for the detection of PLs as negative ions.

Since MALDI-TOF MS was only recently applied for the analysis of lipids and PLs, our first aim was to demonstrate its capability for lipid analyses and to demonstrate the characteristic spectral pattern of individual lipid and PL species. For this purpose, selected commercially available lipids and PL were used.

Figure 1 represents the positive ion MALDI-TOF mass spectra of PC (18:0, 18:2) (a), DAG (16:0, 16:0) (b), and LPA (16:0) recorded in the positive and in the negative ion mode (c and d, respectively). All these lipids are easily detectable as positive ions by MALDI-TOF MS. LPA is also easily detectable as a negative ion due to its acidic properties.

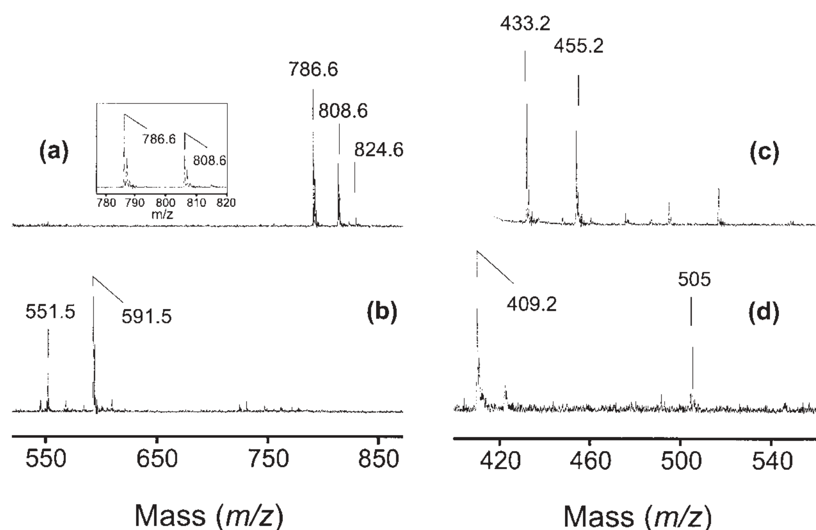

Fig. 1. The positive ion MALDI-TOF mass spectra of (a) PC (18:0, 18:2), (b) DAG (16:0, 16:0) and (c) LPA (16:0). In (d) the negative ion mass spectrum of LPA (16:0) is presented. The inset represents an expanded region (*m*/*z* ≈ 780–820) of the PC spectrum to show that the peaks are isotopically-resolved. The peaks are labelled according to their *m*/*z* ratios. All the spectra are recorded with a reflectron under delayed extraction conditions and with a *m*/*z* = 400 "low mass gate" to prevent saturation of the detector by ions arising from the matrix.

Each individual peak in the spectra is actually a group of isotopically-resolved peaks due to the presence of different isotopes (*cf.* inset in Fig. 1a). The most intense peak in each group corresponds to the exclusive presence of the most abundant iso-

topes, *i.e.*, ^1H, ^{12}C, ^{14}N, ^{16}O, ^{31}P, whereas all other peaks are due to the occurrence of higher isotopes which are much less abundant. The positive ion MALDI-TOF mass spectrum of PC (18:0, 18:2) consists of three peaks: The peak at m/z = 786.6 arises from the proton adduct of PC ($[M+H]^+$), the peak at m/z = 808.6 corresponds to the sodium adduct ($[M+Na]^+$), and the smallest peak at m/z = 824.6 is due to the potassium adduct ($[M+K]^+$). It can be easily proven that the intensity ratio of the different adducts reflects mainly the composition and the abundance of inorganic ions already present in the solvents and in the matrix. The ion content can be easily changed by the addition of salts.[24] Since PC is a neutral phospholipid it requires only one positive ion to become detectable in the positive ion mode. Compensation could be achieved by H^+ (favoured under our experimental conditions due to the addition of TFA to the matrix solution), Na^+ or K^+. In some cases, for the analysis of PL mixtures, the addition of an excess of inorganic salts, for instance CsCl, can help to confirm the identity of a peak.[32] This especially holds when there are doubts if one peak represents a proton or a sodium adduct of lipids with different fatty acid compositions (*e.g.,* PC (16:0, 18:1) and PC (16:0, 20:4)).

Degradation products of PC can also be observed, but usually only to a very low extent, even if highly unsaturated PCs are investigated. The most characteristic fragmentation product of PC is generated upon the loss of the choline head group, as well as upon dimerization of PC.[20] It has additionally been demonstrated, that certain PLs are more susceptible towards degradation than others.[21,24] However, the extent of fragmentation reactions can be easily controlled by carefully adjusting the laser intensity.

The second species, whose spectrum is given in Fig. 1b, is DAG (16:0, 16:0). The positive ion MALDI-TOF mass spectrum consists of one peak at m/z = 591.5 which corresponds to the sodium adduct of DAG. As previously shown, DAGs and TAGs are exclusively cationized by sodium or, if the potassium concentration is high, by K^+ ions,[22,33] whereas they never occur as negative ions. The peak at m/z = 551.5 corresponds to a fragmentation product of DAG generated upon loss of NaOH which occurs after cationisation with Na^+.[22] A rather small, but still detectable, peak at m/z = 551.1 corresponds to an oligomerization product of the matrix.

The third analysed species is LPA (16:0) – an acidic PL – which can be easily detected as either a positive or as a negative ion. The corresponding positive and negative ion MALDI-TOF mass spectra are given in Fig. 1c and 1d, respectively. LPA bears two negative charges and, therefore, requires three positive ions to be detectable in the positive ion mode (two for charge compensation and one for cationisation). The following peaks of LPA (16:0) were detected in the positive ion mode: the peaks at m/z = 433.2 and at m/z = 455.2 which correspond to the ions generated upon addition of two protons and one sodium ($[M+2H+Na]^+$) and two sodium ions and one proton ($[M+H+2Na]^+$), respectively. The three-proton adduct, expected at m/z = 411.2, is suppressed by a strong matrix peak at $m/z \approx$ 413. Finally, the adduct containing three sodium ions (at m/z = 477.2) was detectable only at increased Na^+ concentrations (data not shown). Besides these discussed peaks of LPA, there were some unidentified peaks at higher m/z ratios, which most probably arise from impurities in the sample (these peaks are not labelled in the spectrum).

The negative ion MALDI-TOF mass spectrum of LPA is given in Fig. 1d, and it is – despite the poor signal-to-noise ratio – easier to interpret than the positive ion mode

spectrum (1c); LPA requires only one additional cation to be detectable as a negative ion. Upon the addition of one H^+, LPA (16:0) gives one peak at *m*/*z* = 409.2. Although expected, the sodium adduct was not detected under the employed experimental conditions. One additional peak arising from the matrix is, however, detectable at *m*/*z* = 505 and the matrix generally yields much more intense peaks in the negative ion mode.[24] Therefore, a higher noise level is observed in the negative ion mode (1d) than in the positive ion mode spectra (1c).

The above described species were chosen as representatives for the lipid and PL classes present in PMNs. Other PL classes found in PMNs behave in accordance to their nature: they are neutral as SM and PE, or acidic as PS, PI and PPIs. The spectra of other PL classes have been discussed in more details elsewhere.[20,24,25]

Mass spectrometry of organic extracts of human neutrophils

It has been demonstrated that the quality of MALDI-TOF mass spectra depends on the lipid concentration, as well as on the lipid composition of the analyte.[24,26,27] Our prime aim was to investigate the suitability of MALDI-TOF MS for the determination of changes in the content of lipid-derived second messengers under different stimulation conditions of the PMNs. An overview of the lipid composition of PMNs is given in a report by Marinetti and Cattieu.[12] The organic extract of PMNs contains high amounts of cholesterol (up to 40 % of the total lipids). Neutral PLs, the main constituents of biological membranes, PC and PE, are equally abundant, whereas SM and especially PS are less abundant. According to those data, DAGs, LPLs and PAs are only very minor lipids. Some of them, such as PAs and DAGs are important second messenger molecules and their concentration should increase only after the PMNs are stimulated.[8,9]

Besides the lipid class, the fatty acid composition of the lipids and PLs plays an essential role for the detectability of a given species, since it determines the molecular weight of the molecule. This is of importance, since it is well known for the MALDI-TOF method that the sensitivity for the detection of a given metabolite decreases at higher molecular weights.[33,34]

According to the data of Marinetti and Cattieu,[12] PMNs contain high amounts (about one third) of saturated fatty acids, such as palmitic (16:0) and stearic (18:0) acids. Besides them, the monounsaturated fatty acid, oleic acid (18:1), is also highly abundant, whereas higher unsaturated fatty acids contribute to the PMN lipids to a significant but lower extent. Finally, 18:0-dimethylacetal, (18:0) DMA, makes up about 5 % of the fatty acid composition of human PMNs, although its sources are not well clarified.[12]

According to these data, it can be calculated that the main cellular lipids will be located in the mass region between *m*/*z* ≈ 700 and *m*/*z* ≈ 850. Although the most abundant of all lipids in the organic extract of PMNs is cholesterol with a molecular weight of 386.3 Da, which yields a peak at *m*/*z* = 369 after cationization by one proton and the subsequent elimination of H_2O,[27] this compound is not detectable due to the low mass gate at *m*/*z* = 400.

The positive ion MALDI-TOF mass spectra of organic extracts of unstimulated (a) and the fMLP-stimulated (b) PMNs are shown in Fig. 2. In the mass region between *m*/*z* ≈

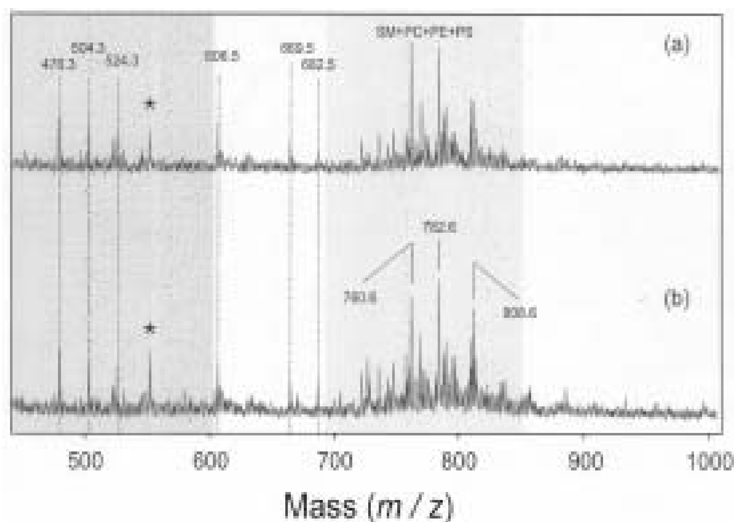

Fig. 2. Positive ion MALDI-TOF mass spectra of organic extracts of unstimulated (a) and fMLP-stimulated (b) PMNs. The PMNs were purified from the blood of healthy volunteers, and organic extracts were prepared as described in the experimental section. Both the spectra were recorded with 2,5-DHB as the matrix which provides a characteristic peak at *m*/*z* = 551.1 (labeled with an asterisk). Two mass regions are emphasized by grey bars: The lower mass range corresponds to the region where the LPLs are found, and the higher mass range according to the molecular weights of the most abundant cellular lipids. Selected peaks are labelled according to their *m*/*z* ratio and their identity is given in the text. Abbreviations: SM: sphingomyeline; PC: phosphatidylcholine; PS: phosphatidylserine; PE: phosphatidylethanolamine.

440 and *m*/*z* ≈ 600 (this region is emphasized by a grey bar) mainly LPLs are detectable. Of particular interest is LPC (18:0) which yields two peaks: one at *m*/*z* = 524.3 and one at *m*/*z* = 546.3, which correspond to the proton and the sodium adduct, respectively. These peaks are discussed in more details elsewhere.[35] LPE (16:0) also gives a peak in that mass region (the corresponding sodium adduct is detected at *m*/*z* = 476.3, and represents the most intense peak of all LPE adducts).[28] One additional LPE peak, but with a stearoyl fatty acid residue, is detectable at *m*/*z* = 504.3. A certain amount of LPLs is always present in the organic extract of human PMNs,[12] but the peak intensity of those species does not properly reflect their concentration in comparison to other cellular lipids, due to the higher sensitivity of MALDI-TOF MS for the detection of low molecular weight compounds. Besides these defined LPLs, there are also some peaks from degradation products, arising especially from PS or PE, which are more susceptible to fragmentation upon laser irradiation than other PLs, as was demonstrated in a previous report,[24] as well as the peak arising from the matrix (at *m*/*z* = 551.1, labelled with an asterisk in both spectra).

The second region emphasized by a grey bar is the region where the most abundant cellular lipids – SM, PC, PE, and PS – are found (the region between *m*/*z* ≈ 700 and *m*/*z* ≈ 850, Fig. 2b). This region is overcrowded with peaks and it is rather difficult to analyse it comprehensively and unequivocally. However, the most expressed peaks have already been assigned, although this assignment is not clear in some cases.[20] For

instance, the peak at m/z = 760.6 might arise from the proton adduct of PC (16:0, 18:2) but also from the sodium adduct of PE (16:1, 20:4). The peak at m/z = 782.6 arises from the sodium adduct of PC (16:0, 18:1). Since PC (16:0, 20:4) might, however, also contribute to that peak, the addition of CsCl would be useful, since no peak overlap may occur between different Cs adducts and, hence, an unambiguous assignment of the fatty acid composition would result.[32]

Of course, a different combination of fatty acids might also exist, for instance, the peak at m/z = 782.6 might also correspond to PC (16:1, 18:0) and in such cases, MALDI-TOF MS can provide only a coarse overview on the fatty acid composition and the most probable combination of fatty acid residues must be estimated. Finally, the peak at m/z = 808.6 arises from the sodium adduct of PC (18:0, 18:2) which confirms the assignment given above. Additional problems for the analysis of that region arise from the marked isotopic distribution of the individual PLs and the resulting peak overlap. Finally, different PLs exhibit a different response to MALDI. Therefore, a PA that gives a weak response in the positive ion mode can be completely suppressed by peaks arising from PC that gives the strongest response of all PLs. As a consequence of these difficulties, a detailed assignment of the peaks in that mass region was not attempted.

The region between the above described mass regions, approximately between $m/z \approx 600$ and $m/z \approx 700$ is the region where DAGs – if generated – would be found. Although there are some peaks in that region, they occur at m/z ratios that differ to those expected for DAGs. Three peaks were detected: the peak at m/z = 606.5 most probably arises from a fragmentation product of PS. The identity of this peak was not determined, but it was observed in the positive ion MALDI-TOF mass spectra only of the PS-mixture.[24] Two other peaks at m/z = 669.5 and at m/z = 682.5 correspond to the impurities arising from the pipette tips.[22]

Due to their rather low concentrations and their properties, PIs and especially PPIs were not detected at all in the organic extract of PMNs.[25] These lipids also possess higher molecular masses than the above described PLs. All these facts together lead to their very low detectability as positive ions.[25]

Investigations of lysophospholipids in the organic extracts of human neutrophils under different stimulation conditions

It is clear from the above described results that MALDI-TOF MS exhibits a great potency for application in the analyses of changes in the lipid composition of PMNs upon stimulation. However, on the other hand, even upon careful analyses of the spectra presented in Fig. 2, no significant changes could be observed with respect to the lipid composition between the organic extract of the fMLP-stimulated (2b) and the unstimulated PMNs (2a). It is highly probable that the lipid-derived second messengers are present at low concentrations below the detection limit of MALDI-TOF MS. Another possibility is that they are quickly metabolized after their generation. Therefore, in the next step we wanted to check if the inhibition of selected enzymes, which are known to be activated after stimulation of PMNs, would lead to an accumulation of certain lipid-derived messenger molecules leading to their improved detectability by MALDI-TOF MS.

A rough overview of literature data[7–11] representing a part of the signalling pathways resulting in NADPH oxidase activation is shown in Fig. 3. After binding of fMLP to its re-

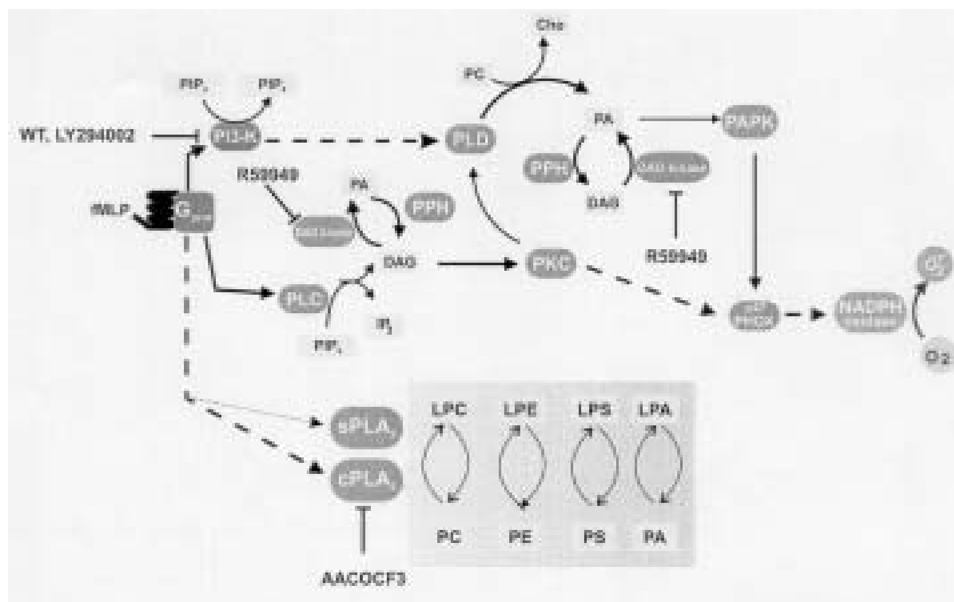

Fig. 3. Overview of the signalling pathways triggered by the chemotactic tripeptide, fMLP, leading to the activation of NADPH oxidase. The solid lines indicate direct interactions between enzymes or lipid second messengers, whereas dashed or dotted lines refer to multi-step activation processes. Sites of the action of the inhibitors used in this study are indicated: Wortmannin (WT) and LY294002 inhibit PI3-kinase; AACOCF3 phospholipase A_2, and R59949 inhibits DAG kinase II. For a detailed description see the text. Abbreviations: AACOCF3: arachidonoyltrifluoromethyl ketone; Cho: choline; $cPLA_2$: cytosolic phospholipase A_2; DAG: diacylglycerol; fMLP: *N*-formyl-L- methionyl-L-leucyl-L-phenylalanine; $G_{prot.}$: heterotrimeric G-protein; IP_3: inositol-tris-phosphate; LPA: lysophosphatidic acid; LPC: lysophosphatidylcholine; LPE: lysophosphatidylethanolamine; LPS: lysophosphatidylserine; PA: phosphatidic acid; PAPK: phosphatidic acid-activated protein kinase; PI3-kinase: phosphatidylinositol 3-kinase; PIP_2: phosphatidylinositol-bis-phosphate; PIP_3: phosphatidylinositol-tris-phosphate; PKC: protein kinase C; PLC: phospholipase C; PLD: phospholipase D; PC: phosphatidylcholine; PPH: phosphatidate phosphohydrolase; $sPLA_2$: secretory phospholipase A_2; WT: wortmannin.

ceptor and the subsequent activation of the heterotrimeric G protein ($G_{prot.}$), at least three different phospholipases are activated: phospholipase C (PLC), phospholipase A_2 (PLA_2) and phosphoinositide 3-kinase (PI3-kinase). PLC produces two further lipid second messengers, DAG and inositol tris-phosphate (IP_3) from membranous phosphatidylinositol-bis-phosphate (PIP_2), which directly interact with protein kinase C and with the IP_3 receptor, respectively. PI3-kinase, an enzyme that possesses dual lipid and protein kinase activity, produces phosphatidylinositol-tris-phosphate (PIP_3) from PIP_2, and the increase in the PIP_3 concentration is connected with the activation of phospholipase D (PLD). PLD digests PC and produces PA which can be converted by phosphatidate phosphohydrolase (PPH) into DAG. DAG can, on the other hand, also be converted into PA by the action of DAG kinase. All PLs, *i.e.,* those that are structural membrane elements as well as those that are exclusively generated after cell stimulation, are themselves substrates for the several types of PLA_2 present in PMNs, producing free fatty acids and the corresponding LPLs.

Although oversimplified, since an unknown number of regulatory mechanisms and cross-connections are involved in this complex signalling cascade, this scheme should give the reader at least a rough idea why the employed enzyme inhibitors were used in this study (also indicated in Fig. 3).

PMNs were preincubated with R59949, a potent inhibitor of DAG-kinase,[36] AACOCF3, an inhibitor of the cytosolic phospholipase A_2,[37] wortmannin, a covalent inhibitor of PI3-kinase[38] and LY294002, a non-covalent inhibitor of the latter enzyme.[39] After preincubation with the corresponding inhibitor, the PMNs were stimulated with fMLP. The lipids were extracted as described above and the positive ion mass spectra recorded. No changes in the lipid composition in the mass region between $m/z \approx 600$ and $m/z \approx 800$ were observed (data not shown). This result was not so unexpected, since it has been shown previously that, due to the simultaneous presence of high amounts of neutral PLs which are very easily detectable by MALDI-TOF MS and especially due to a number of overlapping peaks in that mass region, the detection of small amounts of, for example, PA would be very difficult. The corresponding peaks are most probably suppressed, as it has been already shown for PL mixtures in the presence of high PC concentrations.[24]

On the other hand, some qualitative changes in the mass region where LPLs are usually found were observed. The positive ion mass spectra of the LPL region of the lipid extract of PMNs preincubated with various enzyme inhibitors, and stimulated with fMLP are presented in Fig. 4: in (a) the PMNs were preincubated with R59949, whereas in (b) and (c) the organic extracts of PMNs were treated with wortmannin and LY294002, respectively. The PMNs whose spectra are presented in (d) were preincubated with AACOCF3, and in (e) a combination of AACOCF3 and LY294002 was applied. The action and the efficiency of all inhibitors used in this study were tested by luminol-amplified CL,[36] and all of them were effective towards the modulation of the ROS production subsequent to the fMLP-stimulation. Briefly, R59949 and AACOCF3 enhanced the first and the second phase of the CL response of PMNs, respectively, whereas wortmannin and LY294002 showed a strong concentration-dependent inhibition of both CL phases (data not shown). This strongly indicates the involvement of these enzymes in pathways leading finally to the activation of the neutrophil NADPH oxidase.

The spectra presented in Fig. 4 are representative data of ten independent measurements and indicate those peaks that were detected in the organic extract of at least 6 cell preparations. The restriction became necessary since PMNs vary from donor to donor, whereas the reproducibility of MALDI-TOF mass spectra of lipids and phospholipids is excellent as was demonstrated in recent publications.[20–22,24,25] Even the signal-to-noise ratio varied by not more than 10 % (at the same sample concentration) allowing also a quantification of the LPLs.[28] Therefore, it can be stated that differences in the LPL composition of human neutrophils indeed arise from the different lipids present in their organic extract. Since the PMNs were most probably prestimulated (primed) to a different extent before purification, this is also accompanied by a different lipid composition. This was also confirmed by a different CL intensity upon fMLP-stimulation between the individual cell preparations (unpublished observation).

The identities of the peaks detected in the spectra presented in Fig. 4 are given in Table I. In the spectrum of the organic extract of PMNs inhibited by R59949, the inhibitor itself yields an intense peak at $m/z = 490.9$ which most probably prevented the detec-

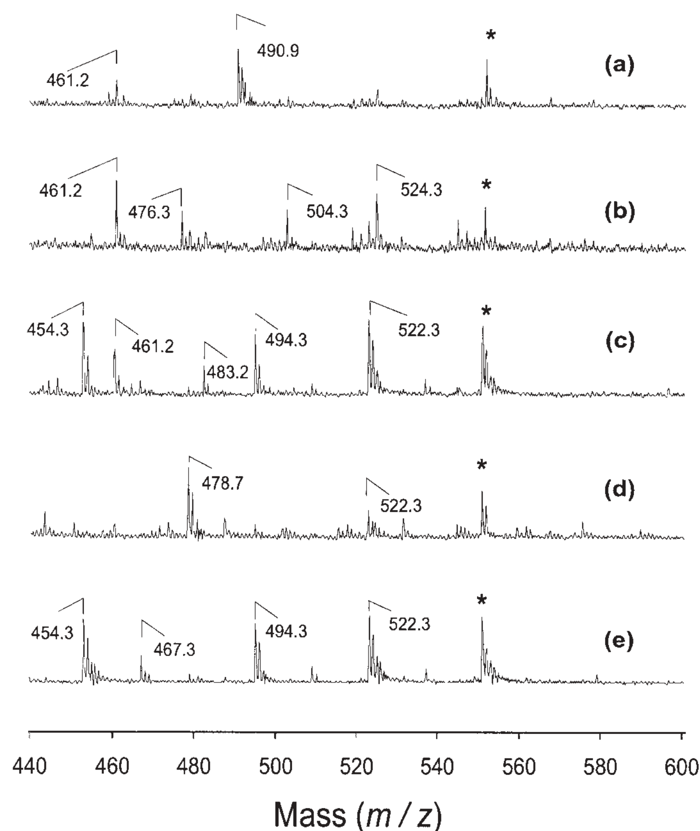

Fig. 4. Expanded region of the positive ion MALDI-TOF mass spectra of organic extracts of PMNs treated with inhibitors of the selected signalling pathways: (a) R59949, (b) wortmannin, (c) LY294002, (d) AACOCF3, (e) LY294002 and AACOCF3. All the PMN preparations were preincubated for 10 min at 37 ºC with the indicated inhibitors, and were subsequently stimulated with fMLP for 10 min prior to lipid extraction.

tion of peaks in its close vicinity (4a). The peak at $m/z = 461.2$ was detected in the same spectrum as well as in the spectra of the PMNs treated with wortmannin and LY294002 (4b and 4c, respectively). This peak corresponds to the two proton and one sodium adduct of LPA (18:0). A two sodium and one proton adduct of the same LPA was detected only in the spectrum of the PMNs treated with LY294002 (at $m/z = 483.2$, 4c). Since the intensity of the individual peaks depends strongly on the lipid as well as the ion concentration,[24] it is possible that this peak is suppressed in the spectra presented in Fig. 4a and 4b. Besides these peaks, in the spectra of the PMNs preincubated with wortmannin (4b), there is also a peak at $m/z = 476.3$, corresponding to the sodium adduct of LPE (16:0) as well as the sodium adduct of LPE (18:0) at $m/z = 504.3$. The presence of these two peaks probably reflects the high sodium content of the sample. One peak at $m/z = 524.3$ corresponding to the proton adduct of LPC (18:0) is also present in the spectrum. In contrast, this peak was missing in the spectra presented in Figs. 4c, 4d, and 4e, or was only detectable as a very small peak. An intense peak arising from

the proton adduct of LPC (18:1) at m/z = 522.3 was detected in all spectra besides the spectrum shown in Fig. 4a. The presence of this peak might indicate that the inhibition of the function of PMNs with LY294002 and AACOCF3, as well as with a combination of both these inhibitors, followed by fMLP stimulation, led to a marked modulation of the PLA_2 activity. PMNs possess at least three types of this enzyme (two of them are indicated in Fig. 3, namely the secretory and the cytosolic PLA_2),[40] and, therefore, it is possible that their activity is controlled by different regulatory mechanisms. These enzymes might also possess different preferences for fatty acid residues they cleave. This could be additionally confirmed, since besides LPC (18:1) also a peak corresponding to LPC (16:1) at m/z = 494.3 is detectable in the spectra of the PMNs treated with LY294002 (4c and 4e). We are currently conducting a study in order to characterize in more detail the enzyme(s) with PLA_2 activity in human PMNs.

TABLE I. Assignment of the peaks detected in the positive ion mass spectra of the organic extracts of PMNs stimulated with fMLP arranged in order of the m/z ratio. The table encompasses all the peaks detected in Fig. 4

Peak position (m/z)	Peak identity
454.3	$[LPE (16:0) + H]^+$
461.2	$[LPA(18:0) + Na + 2H]^+$
467.2	Not defined
476.3	$[LPE(16:0) + Na]^+$
478.7	Possibly degradation product of DAG (18:0, 20:4)
483.2	$[LPA(18:0) + 2Na + H]^+$
490.9	DAG kinase inhibitor $[R59949 + H]^+$
494.3	$[LPC(16:1) + H]^+$
504.3	$[LPE(18:0) + Na]^+$
522.3	$[LPC(18:1) + H]^+$
524.3	$[LPC(18:0) + H]^+$
551.1	Matrix

In the spectrum of the PMNs preincubated with LY294002, there are also peaks arising from LPE (16:0), and from LPA (*cf.* Table I). LPE (16:0) and LPE (18:0) are also detectable in the spectrum presented in Fig. 4e (PMNs inhibited with AACOCF3 and LY294002), whereas these peaks are missing in the spectrum of the PMNs treated with AACOCF3 (4d). Besides the above mentioned peaks, there are also some peaks the identity of which need to be finally confirmed (*cf.* Table I).

The differences in the spectra obtained after inhibition of the PI3-kinase by wortmannin and LY294002 can be explained by different inhibition mechanisms.[38,39] Since this enzyme possesses a rather complex dual kinase activity (lipid and serine protein kinase),[41] it is possible that the corresponding inhibitors also act in different ways.

Unfortunately, to the best of our knowledge, similar investigations by other methods, with which our data could be compared, have not been performed. Also, the signalling pathways of the PMNs are not yet completely clear which prevents more exten-

sive interpretation of our results on the changes of the LPL composition. It can only be speculated that, besides the known mechanisms of regulation of PLA_2 activity in human PMNs, PI3-kinase is most probably also involved in this process.

Summarizing, in this paper it has been demonstrated that MALDI-TOF MS possesses a great potency towards lipid analysis, as well as for the screening of the lipid and phospholipid composition of crude biological mixtures. The method has a number of advantages in comparison to other well-established methods in lipid research, and it might become a powerful tool for the investigation of lipid-derived messenger molecules in PMNs. However, there are still some methodological problems to overcome, especially those caused by the different sensitivity of the method towards various lipid classes. At this moment, it can be stated with certainty that at least the monitoring of qualitative changes of the lysophospholipid composition of human PMNs under different stimulation conditions can be easily accomplished by MALDI-TOF MS.

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И З В О Д

ИСПИТИВАЊЕ САСТАВА ЛИЗОФОСФОЛИПИДА У ХУМАНИМ НЕУТРОФИЛИМА ПОД РАЗЛИЧИТИМ УСЛОВИМА СТИМУЛАЦИЈЕ MALDI-TOF МАСЕНОМ СПЕКТРОМЕТРИЈОМ

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MALDI-TOF (matrix-assisted laser-desorption / ionization time-of-flight) масена спектрометрија је до сада била углавном коришћена за анализу протеина, угљених хидрата и олигонуклеотида. Иако поседује низ предности за анализу липида, MALDI-TOF масена спектрометрија се ретко примењује у овој области. У овом смо раду наставили истраживања могућности да се MALDI-TOF масена спектрометрија користи за одређивање промена у саставу липида интрацелуларних "гласника" у хуманим неутрофилима. У органском екстракту хуманих неутрофила су MALDI-TOF масеном спектрометријом детектоване квалитативне промене у зависности од услова стимулације. Пре него што се овај метод почне рутински примењивати за одређивање концентрације индивидуалних липида у смесама биолошког порекла (као што је органски екстракт хуманих неутрофила) неопходно је решити још неке методолошке проблеме. Ми смо показали да је MALDI-TOF масена спектрометрија погодан и једноставан метод за одређивање састава смесе разних липида и у вези тога за студије сигналних молекула у хуманим ћелијама.

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