Analytical Biochemistry 474 (2015) 50–58

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Identification of oxidized phospholipids in bronchoalveolar lavage exposed to low ozone levels using multivariate analysis

Ann-Charlotte Almstrand [a,b], Dennis Voelker [c], Robert C. Murphy [a,*]

[a] Department of Pharmacology, University of Colorado Denver, Aurora, CO 80045, USA
[b] Department of Public Health and Community Medicine, University of Gothenburg, SE-40530 Gothenburg, Sweden
[c] Department of Medicine, National Jewish Health, Denver, CO 80206, USA

ARTICLE INFO

Article history:
Received 17 October 2014
Received in revised form 23 December 2014
Accepted 24 December 2014
Available online 7 January 2015

Keywords:
Pulmonary surfactant
Oxidized lipids
Mass spectrometry
Lung
Ozone
Principal component analysis

ABSTRACT

Chemical reactions with unsaturated phospholipids in the respiratory tract lining fluid have been identified as one of the first important steps in the mechanisms mediating environmental ozone toxicity. As a consequence of these reactions, complex mixtures of oxidized lipids are generated in the presence of mixtures of non-oxidized naturally occurring phospholipid molecular species, which challenge methods of analysis. Untargeted mass spectrometry and statistical methods were employed to approach these complex spectra. Human bronchoalveolar lavage (BAL) was exposed to low levels of ozone, and samples with and without derivatization of aldehydes were analyzed by liquid chromatography electrospray ionization tandem mass spectrometry. Data processing was carried out using principal component analysis (PCA). Resulting PCA scores plots indicated an ozone dose-dependent increase, with apparent separation between BAL samples exposed to 60 ppb ozone and non-exposed BAL samples as well as a clear separation between ozonized samples before and after derivatization. Corresponding loadings plots revealed that more than 30 phosphatidylcholine (PC) species decreased due to ozonation. A total of 13 PC and 6 phosphatidylglycerol oxidation products were identified, with the majority being structurally characterized as chain-shortened aldehyde products. This method exemplifies an approach for comprehensive detection of low-abundance, yet important, components in complex lipid samples.

Ambient ozone is a common air pollutant produced from photochemical reactions between nitrogen oxides and volatile organic compounds that originate from both anthropogenic and natural sources. The lung toxic properties of ozone were recognized more than a century ago [1], and the detrimental health effects, mainly with regard to pulmonary function, are well described today [2]. Challenges remain to understand the complex biological response and the chemical mechanisms mediating these effects [3]. It is known that the toxicity of ozone in the lungs partly involves reaction with unsaturated fatty acids esterified to glycerophospholipids present in cell membranes of the lung airway cells and the lung surfactant. The lung surfactant is vital for maintaining airway patency but is also involved in the airways' barrier and defense against inhaled toxic components [4,5]. Removal of unsaturated phospholipids by oxidation has been shown to affect the ability of the surfactant to decrease surface tension [6]. In addition, oxidation of these lipids leads to production of potentially harmful oxidative species, lipid–ozone reaction products. The mechanism of formation of these products starts with the chemical reaction of ozone with the fatty acid double bond, leading to the formation of an ozonide. The ozonide decomposes into an aldehyde and an intermediate hydroxyl hydroperoxide product that further decomposes to a second aldehyde and hydrogen peroxide [7,8] (scheme provided as Supplemental Fig. 1 in online supplementary material). In this context, lipid–ozone products such as 1-palmitoyl-2-(9′-oxo-nonanoyl)-*sn*-glycero-3-phosphocholine (PC 16:0_9:0al)[1] and 1-hydroxy-1-hydroperoxynonane have been shown to act as signal transduction molecules inducing both pro-inflammatory cytokine and chemokine production [9,10].

* Corresponding author. Fax: +1 303 724 3357.
E-mail address: robert.murphy@ucdenver.edu (R.C. Murphy).

[1] *Abbreviations used*: PC 16:0_9:0al, 1-palmitoyl-2-(9′-oxo-nonanoyl)-sn-glycero-3-phosphocholine; MS/MS, tandem mass spectrometry; PC, phosphatidylcholine; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PI, phosphatidylinositol; MS, mass spectrometry; PCA, principal component analysis; BAL, bronchoalveolar lavage; LC, liquid chromatography; MOX, methoxime; HPLC, high-performance liquid chromatography; PCVG, principal component variable grouping; CID, collision-induced dissociation; PC 16:0_18:1, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; PC 16:0_16:1, 1-palmitoyl-2-palmitoleoyl-sn-glycero-3-phosphocholine; PC 16:0_18:2, 1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphocholine; PG 18:0_9:0al, stearoyl-(9′-oxo-nonanoyl)-glycerophosphoglycerol.

http://dx.doi.org/10.1016/j.ab.2014.12.018
0003-2697/

Previous studies of ozone and surfactant lipids typically employed rather high concentrations of ozone (200–2000 ppb) with the aim to detect major products [11,12]. The detection of oxidized phospholipids was usually based on predicted structures, often made with the help of previous studies of similar pure compounds [13] or aimed at isolating active compounds of interest and elucidating the structure using tandem mass spectrometry (MS/MS) [9]. In human lung surfactant, phosphatidylcholine (PC) is the dominating lipid class (~80% of the total lipid), with saturated dipalmitoylphosphatidylcholine making up approximately 45% of the PC pool [14]. However, the diversity of surfactant lipids is significant. When different chain lengths and numbers of double bonds are taken into consideration, there are more than 20 documented unsaturated diacyl phospholipids in the lung surfactant counting the most abundant species of PC, phosphatidylglycerol (PG), phosphatidylethanolamine (PE), and phosphatidylinositol (PI) [15]. In addition, plasmanyl and plasmenyl species of PC and PE also exist [16]. Therefore, an approach with the ability to detect small changes after exposure to relevant concentrations of ozone, similar to the average and peak concentrations observed in ambient air today, in such a complex biological sample is attractive.

The use of mass spectrometry (MS) for comprehensive and untargeted lipid profiling in biological samples generates a large amount of data. During recent years, to simplify data processing, multivariate analysis techniques such as principal component analysis (PCA) and partial least squares of latent structures have been used extensively in MS applications with the aim to find quantitative or qualitative differences between groups [17,18].

Here, we present a new approach to identify components for further structural studies using electrospray ionization MS/MS in combination with PCA for global and unbiased data processing. Several oxidized phospholipids in bronchoalveolar lavage (BAL) were identified from the mass spectral data of samples exposed to low concentrations of ozone.

Materials and methods

Materials

1-Palmitoyl(D_{31})-2-hydroxy-*sn*-glycero-3-phosphocholine was obtained from Avanti Polar Lipids (Alabaster, AL, USA). Methoxylamine hydrochloride was obtained from Acros Organics (Geel, Belgium). Solvents (methanol, acetonitrile, and methylene chloride) and ammonium acetate were obtained from Fisher Scientific (Fair Lawn, NJ, USA).

Isolation and determination of total lavage phospholipids

Healthy non-smoking volunteers, between 21 and 65 years of age, were recruited for BAL using a protocol reviewed and approved by the National Jewish Health institutional review board. Lavage was performed with a bronchoscope wedged in a segment of the right middle lobe, and four 60-ml aliquots of sterile saline were instilled and sequentially recovered, with a recovery of approximately 70% of starting volume. The crude lavage fluid was centrifuged at 200*g* for 10 min to remove cells. The resultant supernatant was typically frozen at −20 °C and subsequently processed for analysis of pulmonary surfactant phospholipids. The phospholipids were extracted from thawed lavage supernatants using a Bligh–Dyer procedure [19]. The total phospholipid content of the lipid extract was determined by measuring the inorganic phosphate produced after perchloric acid digestion of the sample [20]. The phospholipid concentration of the recovered lavage was 20 nmol/ml. The BAL was pooled for liquid chromatography (LC)–MS analysis.

Ozonation of BAL

Ozone was generated from ambient air with an ozone calibrator source (model 306, 2B Technologies, Boulder, CO, USA). Exposure of the pooled BAL sample to ozone was accomplished by bubbling the ozone flow, held at a concentration of approximately 60, 150, or 300 ppb, through 1 ml of BAL sample for 60 min. Outgoing ozone concentrations were measured using an ozone monitor (model 202, 2B Technologies) before and after each exposure. To each 1-ml sample of BAL was added 0.14 μg (0.27 nmol) of 1-palmitoyl(D_{31})-2-hydroxy-*sn*-glycero-3-phosphocholine as internal standard before ozone exposure. After exposure, the BAL sample was immediately treated as described in the sample preparation section below. The laboratory air concentration of ozone was 25 to 30 ppb during these experiments.

Sample preparation

Non-ozonized and ozonized BAL samples were either subject to direct lipid extraction or treated with methoxylamine prior to lipid extraction by adding 500 μl of 0.2 M methoxylamine to the BAL sample (samples exposed to 60, 150, or 300 ppb ozone). The samples were incubated in a water bath overnight at 37 °C. During this procedure, the methoxylamine reacts with ketone or aldehyde groups present on the oxidized phospholipid and forms a methoxime (MOX) derivative [21]. Phospholipids in untreated and methoxylamine-derivatized BAL samples were extracted using a modified Bligh–Dyer extraction [19] by adding 1.2 ml of methanol and 1.2 ml of dichloromethane. The sample was mixed thoroughly and centrifuged, after which the dichloromethane phase was transferred to a glass test tube. The extraction was repeated with chloroform. The solution was mixed again and centrifuged. The organic phase with phospholipids was evaporated to dryness under N_2 and resuspended in mobile phase A.

Electrospray ionization MS

Reversed phase LC and MS was performed on an AB Sciex API 3200 triple quadrupole mass spectrometer with an electrospray ionization source (AB Sciex, Concord, Canada). Chromatography was performed on a Shimadzu LC20-AD high-performance liquid chromatography (HPLC) system equipped with a Gemini 5U C18 110A column (150 × 2.00 mm, 5 μm, Phenomenex). For acquisition of full scan data, the gradient mobile phase was composed of A (methanol/acetonitrile/water [60:20:20, v/v/v] with 2 mM ammonium acetate) and B (methanol with 2 mM ammonium acetate). The flow rate was 0.2 ml/min. Initial conditions were 40% A for 1 min, followed by a linear gradient from 40 to 100% B within 50 min, after which 100% B was held for 5 min, followed by reequilibration for 8 min. Each sample was injected in duplicate to improve the statistical analysis.

For untargeted analysis of lipids in BAL samples, mass spectra were acquired in full scan mode. Full scans were carried out in both positive and negative modes where a range of m/z 400 to 1000 was employed. The orifices were set at +58 and −50 V in positive and negative modes, respectively. Data acquisition was carried out by Analyst software version 1.6.1.

Data processing and statistical analysis

Data extraction (peak finding and peak alignment) and PCA were performed on 14 separate LC–MS data sets using MarkerView software version 1.2.1.1 (AB Sciex). The sample sets corresponded to four separate experiments with pooled BAL exposed to 0, 60, 150, and 300 ppb ozone before and after derivatization by methoximation, with each of the eight samples run in duplicate