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Short communication

A simple method to generate oxidized phosphatidylcholines in amounts close to one milligram

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ABSTRACT

Oxidized (phospho)lipids are of paramount interest from different reasons: beside their significant *in vivo* relevance, these products are often needed in the laboratory to study the response of selected cells to oxidized lipids. Unfortunately, the commercial availability of oxidized lipids is limited and scientists interested in studying the physiological impact of oxidized lipids are normally forced to prepare the required compounds by themselves.

We will show here that chain-shortened products of oxidized phosphatidylcholines (PCs) such as aldehydes and carboxylic acids can be easily (and in nearly quantitative yields) generated by the Fenton reaction ($H_2O_2 + Fe^{2+}$) or the $KMnO_4$-induced oxidation of the PC. Using the Fenton reaction and physiological saline, chlorinated oxidation products such as chlorohydrins are also readily available. Additionally, it will be shown that preparative thin-layer chromatography (TLC) is a convenient but simple method to isolate the individual oxidation products in reasonable yields and high purities: all relevant products could be successfully identified by matrix-assisted laser desorption and ionization (MALDI) mass spectrometry and the amounts of the oxidized products determined by a simple colorimetric assay.

1. Introduction

Phospholipids (PLs) are (beside cholesterol and selected membrane proteins) important constituents of all cellular membranes. Because of their amphiphilic properties PLs enable the formation of stable bilayers that protect the interior of the cells but enable also selective transport processes. Additionally, selected PLs such as phosphatidic acids or poly-phosphoinositides are molecules with important regulatory functions serving as lipid second messengers (Martelli et al., 2004).

The oxidation of PLs by reactive oxygen species (ROS) is a very important reaction and many oxidized PLs are of considerable biological significance (Fuchs et al., 2011). This particularly applies at inflammatory conditions that are accompanied by increased ROS generation by immune cells such as neutrophilic granulocytes. Lipid oxidation is involved in the pathogenesis of many diseases such as asthma (Olopade et al., 1997), rheumatoid arthritis (Schiller et al., 2003; Fuchs et al., 2005), atherosclerosis (Victor et al., 2009) and Parkinson's disease (Mattson, 2009). Finally, selected oxidized PLs (for instance, oxidatively-modified phosphatidylcholines and lyso-phosphatidylcholines) are known to possess biological activities similar to platelet-activating factors (PAF) (Marathe et al., 2000) and are likely to destabilize the structures of biological membranes.

Phosphatidylcholines (PCs) are the most abundant PLs in the human body as well as in virtually all mammalian cells (Henry et al., 1998). This – in combination with their commercial availability – renders PCs interesting compounds to study their oxidation products. The related oxidation reactions are not only of interest regarding mechanistic considerations (e.g., to investigate the stabilities of the oxidation products) but as well to study the response of selected cells towards selected oxidation products. Unfortunately, oxidized (phospho)lipids are scarcely commercially available and, thus, scientists are often forced to synthesize the desired oxidation products by themselves.

The oxidation of unsaturated PCs such as PLPC (1-palmitoyl-2-linoleoyl-*sn*-phosphatidylcholine) leads to per-oxidized compounds (endo- and hydroperoxides) as primary products which decay subsequently under generation of aldehydes as well as carboxylic acids as secondary products, i.e., a scission of the double bonds occurs (Niki, 2009; Fuchs et al., 2011). Although oxidation reactions are commonly used to determine the positions of double

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bonds within an unsaturated fatty acyl chain, many details of lipid oxidation processes remain to be elucidated (Fuchs et al., 2011).

The present work has two different aims: on the one hand, different oxidizing agents (atmospheric oxygen, $KMnO_4$ and the Fenton reagent) are compared (a) regarding their ability to induce lipid oxidation and (b) the generated product patterns. On the other hand, preparative thin-layer chromatography (TLC) will be used to isolate the most abundant lipid oxidation products: although the separation quality achievable by TLC is rather poor in comparison to HPLC (high performance liquid chromatography), the required TLC equipment is inexpensive and simple and the method can be, thus, easily established in virtually all laboratories. PLPC will be exclusively used here because it represents in these author's opinion an excellent compromise: due to its two double bonds (within the linoleoyl residue) it is more readily oxidized in comparison to POPC (1-palmitoyl-2-oleoyl-*sn*-phosphatidylcholine) that contains only a single double bond. On the other hand, the variety of products is much smaller in comparison to higher unsaturated PLs (Reis et al., 2004).

The characterization of the generated oxidation products will be performed by matrix-assisted laser desorption and ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS) (Fuchs et al., 2010), which is a simple, but powerful method to characterize lipids and their oxidation products. Finally, the yields of the different oxidation products will be determined by using a simple colorimetric assay.

2. Materials and methods

2.1. Chemicals

All chemicals and all solvents (methanol, ethanol, chloroform, isopropanol, acetonitrile, water, triethylamine and glacial acetic acid) were obtained in the highest commercially available purity from Sigma–Aldrich (Taufkirchen, Germany) and used as supplied.

PLPC was purchased from AVANTI Polar Lipids (Alabaster, AL, USA) as 10 mg/ml chloroform solution and used as supplied. Chemicals used for lipid oxidation were purchased either from Fluka ($KMnO_4$ and H_2O_2) or from Sigma–Aldrich ($FeCl_2$).

The dye primuline (for monitoring the presence of lipids on the TLC plate) was obtained from Sigma–Aldrich (Taufkirchen, Germany) and used as previously described (Fuchs et al., 2008; White et al., 1998). Phospholipase A_2 (PLA_2) from hog pancreas was also obtained from Sigma–Aldrich.

2.2. Oxidation of PLPC at room temperature (296 K)

An aliquot of about 66 nmol PLPC (50 μg, dissolved in 10 μl $CHCl_3$) was evaporated to dryness and the resulting lipid film subsequently exposed to atmospheric oxygen in a small glass vessel (the area of the lipid film exposed to oxygen was estimated to be about 19.6 mm^2). The oxidation was performed for "0" (control), 2 and 7 days at 296 K. The incubation was stopped by the addition of the MALDI matrix and the resulting solution immediately analyzed by MALDI–TOF MS (vide infra).

2.3. Oxidation of PLPC by $KMnO_4$

An aliquot of 33 nmol PLPC (25 μg, dissolved in 10 μl $CHCl_3$) was evaporated to dryness. Oxidation was induced by the addition of 100 μl of a 1.58 mol/l $KMnO_4$ (250 mg/ml dissolved in H_2O) solution and incubated for 10 min at 37 °C. The oxidation was stopped by the addition of organic solvents (chloroform/methanol (1:1 (v/v); 200 μl)) to extract the apolar lipids from the aqueous phase, whereby the $KMnO_4$ remains nearly quantitatively in the aqueous phase (the organic phase was nearly colorless, while the aqueous phase was violet).

2.4. Oxidation of phospholipids by the Fenton reagent (H_2O_2 and $FeCl_2$)

An aliquot of 33 nmol PLPC (25 μg dissolved in 10 μl $CHCl_3$) was evaporated to dryness. The resulting lipid film was incubated with a fixed amount of 50 μl of a 500 mM aqueous solution of H_2O_2 (spectrophotometrically determined ($\varepsilon_{230} = 74\ M^{-1}\ cm^{-1}$)) (Beers and Sizer, 1952) and 50 μl of a 50 mM aqueous $FeCl_2$ solution. These conditions resulted in our hands in a maximum yield of oxidation products. Incubations were performed for 1 h, 2 h or over night (about 16 h). The incubation was stopped by adding chloroform/methanol (1:1 (v/v)) to extract the lipids (Bligh and Dyer, 1959) and to reduce the amounts of inorganic salts.

2.5. Large scale oxidation of PLPC with H_2O_2 and $FeCl_2$

An aliquot of 1.32 μmol PLPC (1 mg dissolved in 100 μl $CHCl_3$) was evaporated to dryness. The resulting lipid film was incubated with a fixed amount of 50 μl H_2O_2 (500 mM dissolved in H_2O) and 50 μl $FeCl_2$ (50 mM dissolved in H_2O). Incubations were performed over night (about 12 h). The incubation was stopped by adding chloroform/methanol (1:1 (v/v)) to extract the lipids (Bligh and Dyer, 1959). Six different reaction batches were combined and used for preparative TLC. The total amount of lipid was, thus, 6 mg corresponding to about 7.9 μmol.

2.6. Lipid extraction

Lipid extraction was performed in all cases according to Bligh and Dyer (1959). After addition of the organic solvent mixtures (aqueous phase/chloroform/methanol = 1:1:1 (v/v/v)) the sample was vigorously vortexed and the mixture centrifuged at 1000 × *g* for 5 min (296 K) to expedite the separation of the organic and the aqueous phase. The lower (chloroform) phase was carefully isolated by using a Hamilton syringe whereas the upper phase (aqueous methanol) was discarded. Samples were directly used for subsequent MALDI–TOF MS characterization or TLC separation.

2.7. Thin-layer chromatography (TLC)

Oxidized lipid extracts were applied onto HPTLC silica gel 60 plates (10 × 10 cm in size with aluminum backs (Merck, Darmstadt, Germany)), using a Linomat 5 device (CAMAG; Berlin, Germany), and developed in a vertical TLC chamber with $CHCl_3$, methanol, water (60:30:5 (v/v/v)) as the mobile phase (Kupke and Zeugner, 1978). Lipids were visualized by spraying the plate with primuline (Direct Yellow 59), which is known to bind non-covalently to the apolar fatty acyl residues of PLs without affecting the molecular weights (White et al., 1998). Upon illumination with UV light (366 nm), individual lipid classes are detectable as colored spots. These spots were assessed using a digital image system in combination with the program Argus X1 (BioStep, Jahnsdorf, Germany).

In one selected case, two-dimensional TLC (using commercially available diol-modified (Merck, Darmstadt, Germany) HPTLC silica plates) was also applied in an attempt to improve the separation quality. Chloroform, methanol, water, ethanol, triethylamine (13/4/2/7/7, v/v/v/v/v) was used in the first dimension and (after careful drying of the TLC plate) chloroform, methanol, acetic acid, water (45/20/6/1, v/v/v/v) in the second dimension.

2.8. Phospholipase A_2 digestion

Selected PL oxidation mixtures were digested by the enzyme PLA_2 to obtain the corresponding lysolipids and to confirm peak