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Unexpected products of the hypochlorous acid-induced oxidation of oleic acid: A study using high performance thin-layer chromatography–electrospray ionization mass spectrometry

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ABSTRACT

Reactive oxygen species (ROS) play important physiological roles and are of particular relevance in the pathogenesis of inflammatory diseases. At inflammatory conditions, the enzyme myeloperoxidase generates hypochlorous acid (HOCl) which adds to the double bonds of fatty acyl residues of (phospho)lipids under the formation of chlorohydrins. This may lead to the development of many inflammatory diseases, such as atherosclerosis or arthritis, if the ROS generation exceeds a certain extent. Using oleic acid as the simplest unsaturated fatty acid which contains just a single double bond, as a model system, we investigated all products – including the chlorohydrin – after its reaction with HOCl by a combination of thin-layer chromatography and electrospray ionization mass spectrometry. Unlike the general acceptance, the reaction of oleic acid and HOCl leads not exclusively to the formation of chlorohydrin (isomers) but is much more complex: there are also considerable amounts of dimeric and (to a minor extent) trimeric products which can be assigned to isomeric ethers and esters. The obtained products after oleic acid chlorination were also compared with the reaction products of 1-palmitoyl-2-oleoyl-*sn*-phosphatidylcholine (POPC) and HOCl. The reasons why different products are obtained will be discussed and the involvement of the carboxylic acid emphasized.

1. Introduction

Lipids are important constituents of the human body, especially in cell membranes, to guarantee both the correct functionality and the environmental separation of the cells [1]. Additionally, lipids are involved in energy storage and signal transduction [2]. The activities of many enzymes, which are often membrane proteins, depend on the regulation of the lipid composition. Only slight modifications of these lipid compositions can be associated with important changes of the enzyme activity [3]. Nonetheless, lipid modifications by oxidation are essential steps in human physiology [4]. On the one hand, the addition of oxygen to the double bonds of lipids generates messenger molecules such as leukotrienes or thromboxanes and on the other hand oxygen is converted by the organism into more reactive oxygen species (ROS) which are important (in addition to certain enzymes) for the immune defense of the organism in order to kill invading microorganisms [5]. Therefore, ROS represent a Janus face because they may also trigger the development of many inflammatory diseases, such as atherosclerosis or arthritis, if their generation exceeds a certain extent [6]. Inflammation is always correlated with the occurrence of macrophages and neutrophilic granulocytes. Both cells possess the enzyme myeloperoxidase (MPO), which constitutes about 5% of the total protein content of granulocytes and converts the cellular waste product hydrogen peroxide (in the presence of chloride ions) into hypochlorous acid (HOCl) [7]:

$$H_2O_2 + Cl^- \rightarrow HOCl + HO^-$$

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Due to their strongly oxidizing and chlorinating abilities, HOCl and its salt (NaOCl) are important constituents to kill bacteria and pathogens [8]. HOCl is a very reactive compound in mammalian physiologic processes, but it discriminates between different functional groups [9]: whereas thiol and thioether groups of amino acids are the most rapidly affected groups, amino groups and, finally, the double bonds of lipids are less reactive. Despite the relatively poor reactivity of the double bonds in (phospho) lipids, their high concentration in biological samples renders them important targets for HOCl [10].

While the reaction between HOCl and phospholipids with free amino groups (such as phosphatidylethanolamines) is much more complex due to the limited stability of the initially generated chloramines [11], the reaction products of lipids with inert headgroups (such as phosphatidylcholines) is presumably rather simple. The latter case leads, if the reaction is carried out at conditions of moderate salt concentrations, to the addition of HOCl to the double bond under formation of a chlorohydrin (CH) as dominating product. This has been shown in many previous investigations [12] by a variety of different analytical methods such as liquid chromatography coupled to mass spectrometry (LC–MS) and nuclear magnetic resonance (NMR) [13,14]. It is also well known that the presence of CHs destabilizes cellular membranes [15] and CHs are additionally more sensitive to hydrolysis than the native phospholipids. Especially higher unsaturated phospholipid CHs (such as arachidonic or docosahexaenoic acid) [16] result in the formation of the corresponding lysophospholipids (LPL) which represent detergents and might be therefore one important reason for the decreased membrane stability.

Compared to lipids, the HOCl-induced oxidation of free fatty acids (FFA) have been investigated in much lesser detail [17]. Although the *in vivo* concentration of FFA is normally rather small in comparison to lipids, in some organs (particularly the liver) FFA are highly abundant and are able to activate pro-inflammatory signaling pathways [18]. Since FFA are present in relatively small amounts in the whole human body due to the continuous turnover of lipids, their detection is still challenging and requires often time consuming derivatization steps prior to long-lasting chromatographic separation methods [19].

We have studied the product diversity of the reaction between oleic acid (OA) and HOCl by different chromatographic and mass spectrometric methods without any derivatization. It will be shown that there are much more products than the CHs and it will be emphasized that there are also dimers and trimers which were not described and discussed yet. A mechanism to explain the generation of these surprising products, ethers and esters, will be suggested and potential physiological consequences discussed.

2. Materials and methods

2.1. Chemicals

Oleic acid, methyl oleate, decanoic acid, nonanal, aqueous sodium hypochlorite (NaOCl) solution, sodium hydroxide (NaOH), butylated hydroxytoluene (BHT), 2,5-dihydroxybenzoic acid (DHB), 2,4-dinitrophenylhydrazine (DNPH), chloroform, methanol, diethylether, hexane, acetic acid, triethylamine and the dye primuline were purchased from Sigma Aldrich (Taufkirchen, Germany) in the highest available purity. 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was obtained from AVANTI Polar Lipids (Alabaster, AL, USA) and used without further purification. 2-Propanol, ethanol, acetonitrile and phosphomolybdic acid were from Merck Millipore (Darmstadt, Germany) and sodium dihydrogen phosphate monohydrate ($NaH_2PO_4{\cdot}H_2O$) was purchased from Roth (Karlsruhe, Germany).

2.2. Incubation of phospholipids or oleic acid with HOCl

An aliquot of POPC or free OA (both dissolved in chloroform) was evaporated to dryness. Vesicles were prepared according to [12,20] by suspending the dried lipid film in phosphate buffer (50 mM $NaH_2PO_4{\cdot}H_2O$, pH = 6.5). A stock solution of NaOCl was kept in the dark at 4 °C. Its concentration was determined at pH 12 using $\varepsilon_{290} = 350\ M^{-1}\ cm^{-1}$ [21] and diluted with phosphate buffer immediately prior to use.

POPC or OA were incubated separately with varying amounts of NaOCl for 360 min at 37 °C and thoroughly shaken on a mixing block thermostate (Biostep, Jahnsdorf, Germany). The incubation was stopped by adding chloroform/methanol (1/1, v/v, +0.05% BHT) in the same volume as the aqueous phase to extract the lipids according to Bligh and Dyer [22]. The organic (lower) layer was isolated by a Hamilton syringe. One part of the organic layer was immediately analyzed by electrospray ionization or matrix-assisted laser/desorption ionization mass spectrometry, whereas the remaining part was subjected to thin-layer chromatography separation and (in the majority of cases) MS analysis. Selected experiments were also performed in an identical way as described above with either methyl oleate or oleic acid in the presence of decanoic acid (DA) to monitor the impact of the carboxyl group.

2.3. High performance thin-layer chromatography (HPTLC)

The products of interest were separated either by normal phase (HPTLC silica gel 60 F254 MS-grade glass plates, 20 × 10 cm, Merck Millipore, Darmstadt, Germany) or reversed phase (HPTLC silica gel 60 RP18 F254S MS-grade glass plates, 20 × 10 cm, Merck Millipore, Darmstadt, Germany) HPTLC. Using a Linomat 5 (CAMAG, Switzerland) combined with a standard Hamilton syringe, 5 μl of each standard solution (c = 1 mg/ml) and sample (containing about 1 mg/ml of the analyte) was applied to the HPTLC plate with 0.5 cm space between the spots and a distance of 1.5 cm from the bottom edge of the plate. The plates were developed in commercially available vertical TLC chambers (CAMAG, Switzerland) with chamber saturation using either hexane/diethylether/acetic acid (30/70/1, v/v/v) according to [17] or (10/90/1, v/v/v) [23] as acidic mobile phases or chloroform/ethanol/water/triethylamine (30/35/7/35, v/v/v/v) as alkaline solvent mixture [24] (normal phase) and methanol/acetonitrile (1/2, v/v) [25] at reversed phase conditions. The HPTLC run was performed at room temperature (22 ± 2 °C), 50 ± 5% relative humidity and a run time of about 45 min and 15 min for normal and reversed phase chromatography, respectively. The total length of the run was about 6 cm. Subsequent to development, TLC-plates were dried in a stream of air and the analytes (after normal phase TLC) were visualized by spraying with a solution of primuline (Direct Yellow 59) according to [26]. Upon irradiation by UV light (366 nm), analytes become detectable as violet spots which were scanned by a videodensitometric device (Biostep GmbH, Jahnsdorf, Germany). Reversed phase TLC-plates were stained with phosphomolybdic acid according to [27] and heated up to 80 °C (TLC Plate Heater III, CAMAG, Switzerland) to visualize the analytes as blue spots.

2.4. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)

The samples of interest were mixed either with an equal volume of DHB-solution (0.5 M in methanol) or DNPH [28] and spotted onto the MALDI steel target. All spectra were acquired on an Autoflex I MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) working in positive or negative ion mode to detect the chlorohydrin of POPC or OA, respectively. A pulsed 50 Hz nitrogen laser, emitting at 337 nm, and an extraction voltage of 20 kV were