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Characterization of oxidation products from 1-palmitoyl-2-linoleoyl-*sn*-glycerophosphatidylcholine in aqueous solutions and their reactions with cysteine, histidine and lysine residues

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ABSTRACT

This report focuses on studies of lipid peroxidation products reactivity towards the side chains of cysteine, histidine, and lysine residues in structurally unordered peptides. Thus we have analyzed linoleic acid peroxidation products (LaPP) obtained by incubating 1-palmitoyl-2-linoleoyl-*sn*-glycerophosphatidylcholine (PLPC) overnight with or without H_2O_2 in the presence or absence of CuCl. In total, 55 different LaPP were identified with 26 containing reactive carbonyl groups. The strongest oxidation conditions (H_2O_2 and Cu(I), i.e. a Fenton-like reagent) yielded 51 LaPP, whereas air oxidation produced only 12 LaPP. Independent of the oxidation conditions, around half of all LaPP were short-chain (oxidative cleavage) and the others long-chain (oxygen addition) PLPC oxidation products. The stronger oxidation conditions increased the number of LaPP, but also oxidized the added peptide Ac-PAAPAAPAPAEXTPV-OH (X = Cys, His or Lys) very quickly, especially under Fenton conditions. Thus, PLPC was oxidized by milder conditions (air or Cu(I)), incubated with the peptide and the peptide modifications were then analyzed by nano-RPC-ESI-Orbitrap-MS. Ten LaPP-derived peptide modifications were identified at lysine, whereas nine products were identified for cysteine and only three for histidine. Three high molecular weight LaPP still esterified to the GPC backbone were detected on Lys-containing peptide. Furthermore, three LaPP-derived mass shifts were obtained at cysteine, which have not previously been reported.

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1. Introduction

Oxidative stress is characterized by an imbalance between reactive oxygen species (ROS) produced in a cell and cellular antioxidants, which eliminate these reactive species and thereby prevent further damage to the cells or the surrounding tissue, such as structural and functional alterations caused by oxidation of biomolecules. High levels of ROS and oxidative stress in general are recognized as pathogenic mechanisms of numerous (particularly inflammatory) diseases and physiological disorders, such as asthma, atherosclerosis, Alzheimer's disease, Parkinson's disease and cellular aging (Wood et al., 2003; Barnham et al., 2004; Singh and Jialal, 2006; Berlett and Stadtman, 1997). Many intra- and extracellular proteins have been shown to be oxidized, which can occur at any of the 20 proteinogenic amino acids, although a few side chains are more susceptible to oxidation, with the oxidation tendency decreasing in the following order: methionine, cysteine, tryptophan, proline, histidine, lysine and threonine residues (Stadtman, 1993). In particular, however, lipids with polyunsaturated fatty acyl residues (PUFA) are also easily oxidized. Lipid peroxidation can be mediated by enzymes (e.g. lipoxygenase) or non-enzymatically by ROS (e.g. hypochlorite, hydroxyl radicals, or superoxide anion radicals and peroxides) and transition metal ions (Parthasarathy et al., 1990; Yoshida and Kisugi, 2010; Niki, 2009). The reactivity of lipids is attributed to the C—H-bond of the

Abbreviations: CID, collision induced dissociation; DNPH, 2,4-dinitrophenylhydrazine; EDTA, ethylenediaminetetraacetic acid; FDP, 3-formyl-3, 4-dehydropiperidino)lysine; GPC, glycerophosphatidylcholine; LA, linoleic acid; LPP, lipid peroxidation products; LaPP, linoleic acid peroxidation products; PC, phosphatidylcholine; PLPC, 1-palmitoyl-2-linoleoyl-sn-glycerophosphatidylcholine; ROS, reactive oxygen species; RPC, reversed phase chromatography; RT, room temperature; UPLC, ultra performance liquid chromatography.

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methylene group between two adjacent double bonds, e.g. position 11 in linoleic acid with its two double bonds at positions 9 and 12 in cis-configuration (*cis*, *cis*-9,12-octadecadienoic acid). Radicals can abstract this hydrogen atom, thereby inducing a bond rearrangement followed by addition of molecular oxygen yielding hydroperoxides. Lipid hydroperoxides can be further oxidized by ROS resulting in C—C-bond cleavages yielding, for example, alkenals, hydroxyl/oxo-alkenals, epoxy-alkenals, and γ-ketoaldehydes (Spiteller, 1998). The oxidized compounds, especially unsaturated carbonyl compounds, are referred to as lipid peroxidation products (LPP). Carbonylated LPP represent very strong electrophils and can readily react with nucleophilic groups in proteins, e.g. the side chains of lysine, cysteine and histidine, to form predominantly Schiff bases or Michael adducts (Fenaille et al., 2003). These irreversible modifications, and the high stability of the products, make lipid peroxidation-derived carbonyl compounds even more harmful than primary ROS.

Several studies on LPP and other reactive carbonyl compounds have investigated the reactivity of different LPP in vitro to reveal their reactions with nucleophilic protein side chains in model peptides or proteins (Shibata et al., 2011; Doorn and Petersen, 2003; LoPachin et al., 2009; Isom et al., 2004; Doorn and Petersen, 2002). These studies relied mostly on α,β-unsaturated aldehydes, such as 4-hydroxy-2-nonenal (HNE) and 4-oxo-2-nonenal (ONE), which can covalently modify Lys-, His-, Cys- or Arg-residues forming, for example, Schiff bases, Michael adducts, ketoamides, pyrrolinones and pyrrole cross-links. Several research groups also studied in situ oxidation of PUFA and PL. There is, however, a significant lack of data regarding LPP-derived modifications still containing the PL esters (Reis et al., 2006). This is also true for a recent report (Silva et al., 2011) that did not identify any LPP-derived products still containing a PC-ester when incubating angiotensin II with LPP preformed by oxidation of 1-palmitoyl-2-arachidonoyl-*sn*-glycerophosphatidylcholine (PAPC) under Fenton conditions. One explanation for this lack of complex, high-mass LPP-derived products might be the harsh oxidation conditions (Fenton reagent) and long incubation times (72 h) compared to the in vivo conditions expected in living organisms. Such mild or early LPP products should be formed in vivo at significant quantities and thus modifying proteins with severe structural and functional consequences, as reported for many LPP-derived modifications (Isom et al., 2004; Stewart et al., 2009). Especially zwitterionic PL molecules can significantly alter the protein structure via the reactive carbonyl assembly, as shown for example for nitrated PUFA, which can change the subcellular localization of proteins (Batthyany et al., 2006). Recently Mahalka et al. (2011) revealed the role of oxidized 1-palmitoyl-2-(9-oxononanoyl)-*sn*-glycerophosphatidylcholine in gelsolin-driven amyloidosis. Thus several hypotheses link these LPP-derived modifications to the progression of different diseases or claim that they even cause such diseases during aging. This shows also that a deeper understanding of the underlying processes demands the simultaneous analysis of the LPP formed in situ, the LPP-derived protein modifications, and possible oxidative modifications of the peptides, which was accomplished only in very few studies (Reis et al., 2006).

Here we studied the reactivity of LPP produced by oxidation of 1-palmitoyl-2-linoleoyl-*sn*-glycerophosphatidylcholine (PLPC) or free linoleic acid (LA) towards the side chains of peptide containing Cys, His or Lys at a certain position. Starting from air oxidation, the oxidation conditions were successively increased by adding hydrogen peroxide and then additionally Cu(I)-ions, or a combination of both (Fenton-like reagent). Based on the formed linoleic acid peroxidation products (LaPP), mild (air oxidation) and intermediate oxidation conditions (Cu(I)-ions) were then selected to incubate the LaPP mixtures individually with each of the three peptides. Thus, we could identify 16 low and three high molecular weight LaPP-modified peptides.

2. Experimental

2.1. Reagents

Copper(I)chloride, EDTA, hydrogen peroxide, and 2,4-dinitrophenylhydrazine (DNPH) were purchased from Fluka Chemie GmbH (Buchs, CH). Chloroform was from Merck KGaA (Darmstadt, Germany). Linoleic acid and formic acid were supplied by Sigma–Aldrich GmbH (Taufkirchen, Germany). Acetonitrile (ULC/MS grade) was obtained from Biosolve BV (Valkenswaard, Netherlands) and 1-palmitoyl-2-linoleyl-*sn*-phosphatidylcholine (PLPC) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). The concentration of the H_2O_2 stock solution was checked spectrophotometrically in water ($\varepsilon_{230} = 74\,M^{-1}\,cm^{-1}$) (Beers and Sizer, 1952).

2.2. Peptide synthesis

Peptides Ac-PAAPAAPAPAE**K**TPV-OH, Ac-PAAPAAPAPAE**C**TPV-OH, and Ac-PAAPAAPAPAE**H**TPV-OH were synthesized on a multiple peptide synthesizer (SYRO 2000, MultiSynTech GmbH, Witten, Germany) using 9-fluorenylmethoxycarbonyl/*tert*-butyl (Fmoc/^{t}Bu) chemistry and in situ activation with di-isopropyl carbodiimide (DIC) in the presence of 1-hydroxy-benzotriazole (HOBt) (Todorovski et al., 2011; Bollineni et al., 2011a,b).

2.3. Oxidation of LA and PLPC and co-incubation with model peptides (Reis et al., 2004a)

LA or PLPC (0.5 mmol/L in water) were oxidized with air, H_2O_2 (50 mmol/L in water), CuCl (0.8 mmol/L in water) or a combination of H_2O_2 and CuCl (50 mmol/L and 0.8 mmol/L in water, respectively; further referred to as Fenton reagent) at 37 °C on an orbital shaker (750 rpm) overnight. Samples were stored at −20 °C or the model peptides were added directly (12 μmol/L final concentration). The mixture was incubated on an orbital shaker (30 min, 37 °C, 750 rpm) and the reaction then stopped by addition of EDTA (5 mmol/L final concentration). All experiments were performed in triplicates.

2.4. Derivatization with DNPH (Yuan et al., 2007)

The oxidized PLPC samples were mixed with 2,4-dinitrophenylhydrazine (DNPH) solution (10 mmol/L in a mixture of acetonitrile, water and formic acid (10:9:1 by vol.)) at an equal volume and incubated on an orbital shaker (2 h, 37 °C, 550 rpm). The lipids were extracted according to the Bligh and Dyer protocol (Bligh and Dyer, 1959). Briefly, the derivatized sample (100 μL) was mixed with equal volumes of methanol (100 μL) and chloroform (100 μL), vortexed (10 s), and centrifuged (10 min, room temperature (RT), 500 × *g*). The organic phase was dried under vacuum and dissolved in a mixture of chloroform and methanol (1:2 by vol.) containing ammonium formate (5 mmol/L).

2.5. ESI-MS/MS analysis of oxidized PLPC

Samples (~10 pmol) were dissolved in ammonium formate buffer (pH 6; 5 mmol/L) and analyzed by ESI-MS/MS (static mode) on a LTQ-Orbitrap XL (Thermo Fisher Scientific GmbH, Bremen, Germany) equipped with a nanoESI source operated in positive ion mode using the following setup: capillary temperature 200 °C, capillary voltage 36 V, tube lens voltage 150 V, spray voltage 0.5–1.0 kV, and FT-MS scan mode (*m*/*z* 150–2000) with a resolution of 60,000.

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