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LC/ESR/MS study of pH-dependent radical generation from 15-LOX catalyzed DPA peroxidation

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Abstract

Docosapentaenoic acid (DPA) is a unique fatty acid that exists in two isomeric forms (n-3 and n-6) which differ in their physiological behaviors. DPA can undergo free-radical mediated peroxidation via lipoxygenase (LOX). 15-LOX, one of the LOX isomers, has received much attention in cancer research due to its very different expression level in normal tissues compared to tumors and some bioactive fatty acid metabolites modulating the tumorigenic pathways in cancer. However, the mechanism linking 15-LOX, DPA-metabolites, and the bioactivities is still unclear, and the free radicals generated in DPA peroxidation have never been characterized. In this study, we have studied radicals formed from both soybean and human cellular (PC3-15LOS cells) 15-LOX-catalyzed peroxidation of DPAs at different pH's using a combination of LC/ESR/MS with the spin trapping technique. We observed a total of three carbon-centered radicals formed in 15-LOX/DPA (n-3) stemming from its 7-, 17- and 20-hydroperoxides, while only one formed from 17-hydroperoxide in DPA (n-6). A change in the reaction pH from 8.5 (15-LOX enzyme optimum) to 7.4 (physiological) and to 6.5 (tumor, acidic) not only decreased the total radical formation but also altered the preferred site of oxygenation. This pH-dependent alteration of radical formation and oxygenation pattern may have significant implications and provide a basis for our ongoing investigations of LOXs as well as fatty acids in cancer biology.

Keywords

ESR spin-trapping; DPA-derived radicals; 15-LOX-catalyzed peroxidation; PC-3-15 LOS cellular peroxidation; LC/ESR; LC/MS; LC/MS²

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INTRODUCTION

Docosapentaenoic acid (DPA), a straight chain 22:5 polyunsaturated fatty acid (PUFA), consists of two isomers found to have particularly interesting bioactivities [1, 2]. All-*cis*-7, 10, 13, 16, 19-docosapentaenoic acid, DPA (n-3), is one of the three major n-3 PUFAs, along with eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3), abundant in fish and marine oils; while all-*cis*-4, 7, 10, 13, 16-docosapentaenoic acid, DPA (n-6) is a major constituent of many algal oils. Dietary supplementation with DPA (n-3) has been reported to be of benefit in the management of a variety of clinical conditions such as coronary artery disease [1, 2], hypertension [3], cancer [4–7], inflammatory states [7], diabetes [8] and renal diseases [9]. Along with other n-3 PUFAs, DPA (n-3) has also been reported to have the ability to reduce the levels of inflammatory eicosanoids, e.g., prostaglandins, thromboxanes, and leukotrienes, that are formed via cyclooxygenase (COX) and lipoxygenase (LOX) pathways [7, 10, 11]. Both DPA (n-6) and algal oils (rich in this DPA isomer) have demonstrated some anti-inflammatory activities [12, 13]. However, the role of DPA (n-6) in cancer has remained unclear. Unlike DPA (n-3), DPA (n-6) has not been observed to exert any protective effect in cell survival and apoptosis [14].

LOX is a lipid peroxidizing enzyme that, like COX, metabolizes dietary and membrane PUFAs through a series of free radical reactions. Among the various LOX isoforms, namely, 5-LOX, 12-LOX, 8-LOX, and 15-LOX, the latter has received much attention in cancer research due to its expression level, which differs significantly in normal tissue compared to colon and prostate tumor tissue, as well as in tumors with different pathological parameters [15–17]. One of the 15-LOX catalyzed linoleic acid metabolites, 13-(S)-HODE, has been reported to act by activation of the peroxisome proliferator receptor activator (PPAR) γ , which plays a key role in cancer cell proliferation, survival and differentiation [18, 19]. Free radicals generated during LOX-mediated peroxidation of DPA have been proposed to play a significant role in its bio-activity [20]. However, the mechanism linking 15-LOX and DPA metabolites to the bioactivity of DPA is still unclear, and free radicals generated in LOX-mediated DPA peroxidation have never been characterized. In order to better understand LOX-DPA biology, we used a combination of LC/ESR and LC/MS coupled with the spin trapping technique to identify free radicals formed from DPA peroxidation catalyzed by soybean 15-LOX and also by human cellular 15-LOX overexpressed in the PC3-15LOS cell line, which is generated from the human prostate cancer cell line PC-3 [18].

Bio-activity of fatty acids can be different if their altered metabolite pattern results from the different oxygenation preference of 15-LOX due to changes in reaction pH. Since tumors generally have a more acidic microenvironment than normal tissues [21], the effect of pH on the alteration of the free radicals and related non-radical metabolites, e.g., hydroxy-DPA (HDPAs), was also tested for the first time using our combined technique of LC/ESR and LC/MS. Soybean 15-LOX metabolized both DPAs optimally at pH 8.5 as observed by the maximum yield of radical adducts and HDPAs. Decreasing the reaction pH shifted the preferred oxygenation site on DPA (n-3), but not DPA (n-6), in both soybean and human cellular (PC3-15LOS) 15-LOX peroxidation. This observation of changes in intensity and shift in site of 15-LOX-catalyzed oxygenation of DPAs at different pH provides a strong basis for our ongoing investigation of the free radical-mediated biological mechanisms by which LOXs metabolize fatty acids in cancer.

MATERIALS AND METHODS

Reagents

Soybean 15-lipoxygenase (15-LOX) and 15-hydroxyeicosatetraenoic acid (HETE) was obtained from Cayman Chemicals (Ann Arbor, MI) and DPAs were purchased from Nu-

Chek-Prep Inc. (Elysian, MN). HPLC grade acetonitrile (ACN) was obtained from Mallinckrodt Baker (Phillipsburg, NJ) and LC/MS grade water was purchased from EMD Chemicals (Gibbstown, NJ). Ethyl alcohol was purchased from Sigma Chemical Co. (St. Louis, MO). Chelex 100 (200–400 mesh sodium form) was bought from Bio-Rad Laboratories (Hercules, CA). High-purity spin trap, α -[4-pyridyl-1-oxide]-N-*tert*-butyl nitron (POBN), was purchased from Alexis Biochemicals (San Diego, CA), and deuterated POBN (d₉-POBN) was obtained from CDN Isotopes (Pointe-Claire, QC, Canada). The human prostate cancer cell line over-expressing the enzyme 15-LOX (PC3-15LOS) was obtained from Mercer University (Savannah, GA). Zeocin was purchased from Invitrogen (Carlsbad, CA). RPMI 1240 medium was purchased from Thermo Fischer Scientific (Logan, Utah), and fetal bovine serum (FBS) and trypsin-EDTA were purchased from GIBCO BRL (Grand Island, NY, USA).

Reaction conditions

The reactions of the 15-LOX catalysis of DPAs were performed in 50 mM (pH 6.5, 7.4, and 8.5) “metal-free” phosphate buffer solutions. Metal ions in the buffer were chelated by treatment with Chelex 100 resin to provide a virtually metal-free buffer solution, which passed an ascorbic acid test [22]. The reaction mixture containing 20 Kunit/mL soybean 15-LOX enzyme, 20 mM POBN, and 1 mM DPA (1% ethyl alcohol, *v/v*, from DPA stock solution) was continuously stirred at 37°C and 600 rpm on a Thermo-Shaker in the absence of light for 30 min. After the incubation, the 15-LOX-catalyzed peroxidation was immediately stopped by mixing with same amount of ACN, centrifuged and condensed for later LC/ESR/MS analysis as described elsewhere [23].

Free radicals from cellular 15-LOX-mediated peroxidation were generated using a prostate cancer cell line (PC3-15LOS) in RPMI 1240 medium supplemented with Zeocin (50 µg/mL) and 10% FBS in an incubator containing a humidified atmosphere of 5% and 20% CO₂, producing microenvironments with pH 7.4 and 6.5, respectively. At 70–80% confluency the cells were trypsinized, harvested and suspended in phosphate buffer saline (PBS) with pH 7.4 and 6.5, respectively, for 10⁷ cells/mL. POBN and DPA (n-3/n-6) at final concentrations of 50 mM and (0.1–1.0) mM, respectively, were then added to the cell suspension to initiate the DPA peroxidation and POBN spin-trapping reactions. After a 30-min incubation, the peroxidation of the cell suspension was stopped with ACN (1:1, *v/v*), and supernatant was collected, centrifuged and condensed for LC/ESR and LC/MS analysis.

Offline ESR measurements

Complete reaction mixtures and relevant controls were directly transferred to the ESR flat cell for offline ESR measurements (e.g. magnetic field scans). ESR spectra were obtained with a Bruker EMX spectrometer equipped with a super high Q cavity operating at 9.78 GHz and room temperature. Other ESR spectrometer settings were: magnetic field center, 3494.4 G; magnetic field scan, 70 G; modulation frequency, 100 kHz; microwave power, 20 mW; modulation amplitude, 1.0 G; receiver gain, 5.0×10^4 ; time constant, 0.655 s; and conversion time, 0.164 s.

Online LC/ESR measurements

The on-line LC/ESR system consisted of an Agilent 1200 series HPLC system and a Bruker EMX ESR system. The outlet of the Agilent UV detector was connected to a highly sensitive Aquax ESR cell with red PEEK HPLC tubing (0.005 i.d.). The POBN radical adducts were monitored *via* UV absorption at 265 nm [23, 24] followed by ESR detection. There was a delay of about 9 s between the UV and the ESR detection in our on-line LC/ESR settings. LC separations were performed on a C18 column (Zorbax Eclipse-XDB, 4.6 × 75 mm, 3.5 µm) and a guard column (Zorbax Eclipse-XDB, 4.6 × 12.5 mm, 5.0 µm).

equilibrated with A (H₂O - 0.1% HOAc). Forty microliters of enzyme-free condensed sample was injected into the HPLC system by autosampler and eluted at a 0.8 mL/min flow rate with a gradient elution: (i) 0 to 40 min: 100% to 30% of solvent A, 0% to 70% of solvent B (ACN-0.1% HOAc); (ii) 40 to 45 min: 30% to 5% of A, 70% to 95% of B; (iii) 45 to 54 min: 5% of A and 95% of B; and (iv) 54 to 60 min: 5% to 100% of A and 95% to 0% of B. On-line ESR monitoring consisted of a time scan with the magnetic field fixed on the maximum of the middle line of the six-line spectrum of the POBN adduct as described elsewhere [23–26]. Other ESR settings were modulation frequency, 100 kHz; modulation amplitude, 3.0 G; microwave power, 20 mW; receiver gain, 4×10^5 ; and time constant, 2.6 s.

Online LC/MS and LC/MS² measurements

The LC/MS system consisted of an Agilent 1200 series HPLC system and an Agilent LC/MSD SL Ion Trap mass system. The outlet of the UV detector in the LC was connected to the MS system with red PEEK HPLC tubing as well. Chromatographic conditions were identical to those used for on-line LC/ESR. However, the LC flow rate (0.8 mL/min) into the MS inlet was adjusted to 40 μ L/min via a splitter. There was a delay of ~35 s between the UV and the MS detection in our on-line LC/MS settings. Electrospray ionization (ESI) in positive ion mode was used for all LC/MS and LC/MS² measurements. Total ion current (TIC) chromatograms in full mass scan mode (m/z 50 to m/z 600) were performed to profile all products formed in the reaction of 15-LOX-catalyzed DPA peroxidation in vitro in the presence of POBN. Other MS settings were capillary voltage, -4500 V; nebulizer press, 20 psi; dry gas flow rate, 8 L/min; dry temperature, 60°C; compound stability, 20%; and number of scans, 50. Extracted ion current (EIC) chromatograms of ions of interest were projected from TIC to acquire MS chromatograms that could match with ESR chromatograms, in which all POBN radical adducts were monitored as structure nonspecific ESR-active peaks. EIC was also performed to determine the number of isomers of given ions. Normally an isolation width of ± 0.5 Da was selected for EICs. The multiple reaction monitoring (MRM) mode of LC/MS² was conducted to confirm structural assignments of POBN adducts. A width of ± 2.0 Da was typically selected to isolate parent ions of interest.

The EIC and LC/MS² analysis of radical products formed from d₉-POBN spin trapping experiments were performed for the peaks of POBN adducts and/or related redox forms whose structures could not be clearly observed by fragmentation pattern of LC/MS² spectra with POBN alone. All reaction conditions of the d₉-POBN spin trap experiments were the same as in the corresponding POBN spin trapping reaction system with POBN replaced by the same amount of d₉-POBN.

LC/MS quantification of radical adducts

Since no radical internal standard can be made due to its highly reactive nature, we used a small and known amount of d₉-POBN (~4.0 and ~1.0 μ g/mL for cell-free and cellular peroxidation, respectively), in our LC/MS quantification, purely as an alternative internal standard as described elsewhere [23–26]. For this purpose, d₉-POBN was added after the analyte (the LOX/DPAs reaction system in the presence of POBN) was mixed with ACN for denaturing and precipitating the LOX enzyme. All redox forms of the POBN radical adduct, including the adduct itself (radical) and the oxidized and reduced adducts, were quantified simultaneously. The different ionization efficiencies of the redox forms of the radical adducts vs. internal standard (d₉-POBN) were normalized using the respective MS response factors as described elsewhere [25, 26]. To comprehensively and accurately quantify the radical formation, concentrations of all redox forms of radical adducts were also corrected for their isotopic contributions [27, 28].

LC/MS quantification of non-radical metabolites

LC/MS quantification of HDPA metabolites formed from soybean 15-LOX-catalyzed DPA peroxidation was also conducted under different reaction pH's of 6.5, 7.4 and 8.5, respectively. LC separations were performed using the same column as that used for radical adducts. Ten microliters of sample were injected into the HPLC by autosampler and eluted at 0.8 mL/min flow rate with a gradient elution: (i) 0 to 5 min: 44% of solvent A (H_2O -0.01% HOAc), 56% of solvent B (ACN-0.01% HOAc); (ii) 6 to 30 min: 44% to 14% of A, 56% to 86% of B; (iii) 31 to 40 min: 14% to 44% of A and 86% to 56% of B. Since no HDPA standard is commercially available, concentrations of HDPA metabolites (e.g. hydroxylation at different sites, determined by diagnostic fragments in MRM with negative mode) were calculated using 15-HETE (1.0 $\mu\text{g/mL}$) as an internal standard in LC/MS. The LC/MS settings were similar to those used for quantification of radical adducts except for a change in nebulizer pressure, 15 psi; dry gas flow rate, 5 L/min and dry temperature, 325°C. The settings of MRM (negative mode) were the same as those used in LC/MS² for POBN adducts, except for the nebulizer pressure, 15 psi; dry gas flow rate, 5 L/min; dry temperature, 325°C; and a width of ± 4.0 Da to isolate parent ions.

RESULTS

When off-line ESR analysis was used to measure free radical adducts in soybean 15-LOX and DPA (n-3) and DPA (n-6) reaction mixtures, we obtained typical six-line ESR signals of POBN adducts with hyperfine coupling constants of $a^{\text{N}} \approx 15.69$ G and $a^{\text{H}} \approx 2.68$ G (Fig 1). However, this off-line ESR measurement can only provide an overall signal intensity of radical adducts, not specific information in terms of types, numbers, and structures, since many of the POBN spin adducts tend to have the same or similar a^{N} and a^{H} hyperfine couplings [23–28].

Identification of radical adducts from soybean 15-LOX-catalyzed DPA (n-3) peroxidation

When we used the combined techniques of on-line LC/ESR and LC/MS to further examine radicals formed from the 15-LOX and DPA (n-3) complete reaction mixture, we observed a total of seven ESR-active peaks corresponding to different POBN radical adducts (include isomers) (Fig 2B). Except for the first ESR-active peak, which was co-eluted with overloaded POBN, the ESR-active peaks (2–7) were all matched with their corresponding UV peaks (Fig 2A). EICs of four protonated molecular ions of m/z 240, m/z 224, m/z 310, and m/z 264 could be projected from the full MS scan (m/z 50 to m/z 600, Fig 2C) of the 15-LOX and DPA (n-3) complete reaction mixture. These EIC peaks matched the profile of ESR-active peaks 2–7, suggesting that there are at least four types of free radicals mainly formed in the 15-LOX-DPA (n-3) system trapped by POBN.

The m/z 240 ion and the corresponding ESR-active peak 2 ($t_{\text{R}} \approx 9.3$ min, Fig 1B) appear to represent a DPA-unrelated radical adduct since 1% ethanol was always used to prepare fatty acid stock solution for our reaction system. Formation of the POBN-trapped hydroxyethyl radical ($^{\bullet}\text{C}_2\text{H}_4\text{OH}$) from oxidation of ethanol corresponded to a protonated molecular ion of m/z 240. The observation of the typical fragmentation pattern of the POBN/ $^{\bullet}\text{C}_2\text{H}_4\text{OH}$ in the LC/MS² spectrum of m/z 240 (Fig 3A) as previously published elsewhere [27, 28] further confirmed its structural assignment.

The ESR-active peak 3 and the corresponding EIC peak ($t_{\text{R}} \approx 12.0$ min, m/z 224, Figs 1B and 1C) represent a POBN adduct of a radical (POBN/ $^{\bullet}\text{C}_2\text{H}_5$) generated from β -scission of the 20-DPA (n-3)-derived alkoxyl radical (Scheme 1A). Structural assignment of this adduct was confirmed from obtaining the typical fragmentation pattern in LC/MS² of m/z 224 (Fig 3B) as published elsewhere [24, 27].

The ESR-active peak 4 and the corresponding EIC peak ($t_R \approx 14.0$ min, m/z 310, Figs 1B and 1C) most likely represented the POBN adduct of $^*C_6H_{11}O_2$ generated from the β -scission of the 7-DPA (n-3)-derived alkoxyl radical as proposed in Scheme 1A. The assignment of its structure was confirmed by the observation of typical fragmentation ions of m/z 310 in LC/MS² (Fig 3C) and of m/z 319 in the LC/MS² from the d_9 -POBN experiment (data not shown).

Instead of observing one isomer each for the m/z 240, m/z 224, and m/z 310 molecules, the ESR-active peaks 5–7 and the corresponding EIC peaks (m/z 264, $t_R \approx 18.2$, 18.5 and 19.3 min, Figs 2B and 2C) represented the three isomers of POBN/ *C_5H_9 . The isomers at $t_R \approx 18.2$, 18.5 of such adduct (asterisked in Figs 2B–2C) could be identified more clearly when the peroxidation and spin trapping experiments were conducted at pH 8.5 (data not shown). This radical appeared to be generated from the β -scission of the 17-DPA (n-3)-derived alkoxyl radical as proposed in Scheme 1A. The typical fragmentation pattern of m/z 264 in LC/MS² (Fig 3D) and the number of its isomers were consistent with previous publications [24, 27]. However, there was not enough evidence from the fragmentation ions in the LC/MS² of peaks 5–7 to distinguish the three isomers.

Since it co-eluted with the large POBN peak (POBN overload, labeled in Fig 2A), the corresponding EIC of ESR-active peak 1 was unable to be projected in Fig 2C. According to previous observations [23–28], the peaks that are co-eluted with POBN most likely represent the POBN adducts of polar fatty acid-derived radicals, e.g. dihydroxyl-type radicals. Thus, the ESR-active peak 1 very likely corresponds to a DPA (n-3) dihydroxyl-type radical (m/z 558) as proposed in Scheme 1A. In fact, in both POBN and d_9 -POBN experiments, trace of m/z 558 and m/z 567 molecules could be observed for the fraction at retention time ~ 7.4 min (data not shown).

Identification of adduct redox forms in soybean 15-LOX-catalyzed DPA (n-3) peroxidation

POBN radical adducts can readily be either reduced or oxidized to their ESR-silent forms, nitron and the hydroxylamine (Scheme 1B). In order to comprehensively quantify the total amount of radical formation, three redox forms of each POBN adduct were measured simultaneously under our reaction conditions. The reduction and oxidation of any given POBN adduct corresponded to a mass change of ($\pm$ Da) of protonated adduct molecules in MS.

A total of two oxidized forms of POBN/ *C_2H_5 (m/z 224) as protonated molecular ions of m/z 223 were profiled at $t_R \approx 5.9$ and 10.8 min, respectively (Fig 4A). The corresponding oxidized forms of d_9 -POBN/ *C_2H_5 as m/z 232 were also detected in the d_9 -POBN spin trapping experiment ($t_R \approx 5.8$ and 10.8 min, Fig 4B). More isomers of the oxidized form were sometimes identified than were present in the original adduct, most likely due to the formation of geometric isomers from the newly formed carbon double bond. The observation of typical fragmentation patterns of m/z 223 and m/z 232 in their LC/MS² analysis (Figs 4B and 4D) from POBN and d_9 -POBN experiments, respectively, further confirmed the structural assignment of the oxidized product of POBN/ *C_2H_5 . However, its reduced form as m/z 225 was under the detection limit.

Both oxidized and reduced forms of POBN/ $^*C_6H_{11}O_2$ (m/z 310) were profiled as m/z 309 ($t_R \approx 13.8$ min (Fig 5A) and m/z 311 ($t_R \approx 9.3$ min, Fig 5C), respectively. The fragmentation patterns in LC/MS² of these ions (Figs 5B and 5D) as well as the corresponding d_9 -POBN experiments (data not shown) supported the structural assignments.

The oxidized and reduced forms of POBN/ *C_5H_9 (m/z 264) were also profiled as m/z 263 and m/z 265 at $t_R \approx 14.3$ and 21.5 min, respectively (Fig 6A and 6C). The structural

assignment was confirmed from their LC/MS² (Fig 6B and 6D) as well as the corresponding EIC and LC/MS² in the d₉-POBN experiment (data not shown).

Identification of radical adducts from soybean 15-LOX-catalyzed DPA (n-6) peroxidation

Using the combined techniques of on-line LC/ESR/MS to examine radicals formed from the 15-LOX and DPA (n-6) complete reaction mixture, we observed only three ESR-active peaks (Fig 7B). Again, due to co-elution with overloaded POBN, the first ESR peak most likely represents a POBN adduct of a dihydroxyl-type radical of DPA (n-6) (m/z 558, Scheme 1C). Just as in the DPA (n-3) system, the ESR-active peak 2 corresponded to the POBN-hydroxyethyl radical (POBN/C₂H₄OH, m/z 240, t_R ≈ 9.3 min). The ESR-active peak 3 appeared to correspond to the POBN adduct of a pentyl radical (POBN/C₅H₁₁, m/z 266, t_R ≈ 21.2 min Fig 7A) stemming from 17-DPA (n-6)-OOH as proposed in Scheme 1C. The observation of typical fragmentation patterns of its LC/MS² and the relevant redox products as published elsewhere [28, 29] confirmed the structure assignments. The oxidized and reduced forms of POBN/C₅H₁₁ were also profiled as LC/MS peaks at t_R ≈ 19.0 min (m/z 265) and 14.6 and 15.1 min (two isomers of m/z 267), respectively (Fig 7C).

pH effect on radical and related HDPA formation in soybean 15-LOX-catalyzed DPA peroxidation

In order to determine pH effect on the activity and oxygenation specificity of soybean 15-LOX-catalyzed DPA (n-3/n-6) peroxidation, quantification of all redox forms of each POBN adduct was performed at reaction pH 6.5, 7.4, and 8.5, respectively. Soybean 15-LOX appears to have its optimum activity at pH 8.5 for DPA (n-3) peroxidation (Figs 8A–8C), where the 17-C and 7-C oxygenation (Figs 8B–8C) corresponded to the two major radical pathways. All redox forms of POBN/C₅H₉ (m/z 263, 264 and 265, stemming from 17-C) and POBN/C₆H₁₁O₂ (m/z 309, 310 and 311, stemming from 7-C) were quantified as ~71.35 μg/mL and ~63.59 μg/mL, respectively, much greater than the product stemming from 20-C (~30.0 μg/mL). However, when the reaction pH dropped, the 17-C oxygenation product became the least abundant, while the 7-C oxygenation remained as the major radical pathway with production even greater than the two others combined (e.g. 17- and 20-C oxygenation). Unlike DPA (n-3), no alteration in the oxygenation site was observed with a change in pH in 15-LOX-catalyzed DPA (n-6) peroxidation. As the reaction pH decreased, the production of pentyl radical (m/z 266), the only β-scission radical stemming from 17-DPA (n-6)-OOH, dropped significantly (Fig 8D).

Considering the different trapping rate constants of free radicals and different decay rates of the radical adducts, the values reported for POBN adducts in our reaction system may not represent the actual amount of radicals formed. Therefore, LC/MS quantification of related non-radical metabolites, e.g., 7-HDPA, 17-HDPA and 20-HDPA, was also performed to further confirm the oxygenation preference of 15-LOX-catalyzed DPA peroxidation at pH 6.5, 7.4, and 8.5. Different sites of hydroxylation between 7-, 17- and 20-HDPA were recognized by observing the diagnostic fragments of m/z 143 in 7-HDPA (n-3), m/z 247 in 17-HDPA (n-3), m/z 287 in 20-HDPA (n-3), and m/z 245 in 17-HDPA (n-6) (Table 1) [12]. Consistent with the radical adduct profile, 7- and 17-HDPA (n-3) were found to be the two major metabolites formed during peroxidation of DPA (n-3), while 17-HDPA (n-6) was the only metabolite formed during peroxidation of DPA (n-6). In addition, the ratios between different HDPAs were very similar to those of corresponding free radicals; e.g., the ratio of 17- vs. 7-HDPA (n-3) ~1.17 (Table 1) was comparable to the value of ~1.13 observed for corresponding radical adducts (Fig 8) in soybean 15-LOX-catalyzed peroxidation of DPA (n-3) at the enzyme optimal pH 8.5.

Identification of DPA-derived radicals from the PC3-15LOS cell line

The genetically modified human prostate cancer cell line PC3-15LOS (from PC-3 cells overexpressing 15-LOX) [18] has enabled the study of bioactive metabolites generated from human cellular 15-LOX-catalyzed peroxidation of various PUFAs. In order to test whether the cellular 15-LOX mediates DPA (n-3/n-6) peroxidation as the proposed mechanisms in Schemes 1A and 1C, we again used our combined techniques of LC/ESR/MS and spin trapping with POBN in the PC3-15LOS cellular peroxidation. Almost all radical metabolites observed in soybean 15-LOX-DPAs reactions, including $\text{POBN}^{\bullet}\text{C}_2\text{H}_5$ (m/z 224), $\text{POBN}^{\bullet}\text{C}_5\text{H}_9$ (m/z 264), and $\text{POBN}^{\bullet}\text{C}_6\text{H}_{11}\text{O}_2$ (m/z 310), all formed from peroxidation of DPA (n-3), and $\text{POBN}^{\bullet}\text{C}_5\text{H}_{11}$ (m/z 266), formed from DPA (n-6), were detected in our cellular 15-LOX-catalyzed reactions (Fig 9). Thus, the mechanism of cellular 15-LOX/DPAs peroxidation is confirmed as proposed in Scheme 1A and 1C. In addition, free radical quantification was also conducted at both physiological and tumor acidic pH values for PC3-15LOS cellular peroxidation (Fig 10). Just as in soybean 15-LOX, the yield of total radical adducts from DPAs decreased as pH dropped, the pathway of radical formation stemming from 7-DPA (n-3)-OOH was dominant at both physiological (7.4) and acidic (6.5) pH, and only one type of radical stemming from 17-DPA (n-6)-OOH was observed.

DISCUSSION

DPA is a unique PUFA that exists in both n-3 and n-6 isomeric forms that differ only in the position of two double bonds in the acyl chain. However, their physiological behavior can differ profoundly [30]. Free radicals generated during 15-LOX-mediated peroxidation of DPA may play a significant role in its bioactivity [20]. In this study, a total of three types of DPA (n-3)-derived radicals were formed and trapped by POBN and characterized by LC/ESR and LC/MS, including radicals formed from β -scission of DPA (n-3)-derived alkoxyl radicals stemming from 7-, 17- and 20-hydroperoxides. However, only one type of DPA (n-6)-derived radical stemming from 17-hydroperoxide was observed in 15-LOX-mediated DPA (n-6) peroxidation. Two POBN radical adducts, $\text{POBN}^{\bullet}\text{C}_5\text{H}_9$ as m/z 264 and $\text{POBN}^{\bullet}\text{C}_6\text{H}_{11}\text{O}_2$ as m/z 310, stemming from 17- and 7-DPA (n-3)-OOH, respectively, were observed as major radical products at the enzyme's optimal pH, 8.5 (Fig 8). However, the radical stemming from 17-C became much less abundant at pH 7.4 and 6.5. Although quantification based on ESR spin trapping may suffer from the limitation of variable trapping and decay rates of free radicals and radical adducts, respectively, it allowed a reliable radical adduct profile in this study (30 min reaction time) to be measured. This quantification is a reasonable reflection of the preference of 15-LOX-catalyzed DPA oxygenation, since the related HDPAs were also determined (Table 1) to have comparable ratios. Our results indicate that at pH 8.5 the preferred site of oxygenation particularly favors the generation of both 7- and 17-carboncentered radicals and non-radical metabolites, while at $\text{pH} \leq 7.4$, the generation of free radicals from 7- DPA-OOH becomes the only favored pathway. Thus, oxygenation specificity of soybean 15-LOX displays its pH dependence mainly on 17-C. The pH dependent changes in product specificity of murine 8-LOX and human 15-LOX-2 reactions of arachidonic acid have been reported and explained by a pH dependent substrate orientation [31]. Our result is consistent with this explanation: e.g., at acidic pH, the hydrophilic carboxylic group assumed to be protonated can penetrate the substrate binding pocket in an inverse head to tail orientation and allow favorable oxygen insertion at C-7; however, as the pH increases, the substrate is likely to become more deprotonated and thus aligned for oxygen insertion at C-17.

A stable human prostate cancer cell line that overexpresses 15-LOX-1 (PC3-15LOS) was generated by transfection with 15-LO-1-sense (15-LOS) and possessed 10-fold the 15-LO-1 activity of parental PC3 cells [18]. Considering that most cellular bioactivity studies of 15-LOX and fatty acid metabolites have been conducted at physiological pH (e.g., in normal

culture medium) where metabolite profiles from oxygenation sites and enzyme activity may not be the same in tumor microenvironments (more acidic), there is a need to study pH dependent fatty acid metabolite profiles from LOX peroxidation and the associated bioactivities.

In PC3-15LOS cells, which we used for LOX-catalyzed studies for the first time, we found that the total radical formation decreases as the pH drops, and just as in soybean 15-LOX, oxygenation on 7-C DPA (n-3) was observed to be the major radical pathway compared to oxygenation on other carbons at both pH 7.4 (~physiological pH) and pH 6.5 (~acidic pH, tumors in general) in DPA (n-3) cellular peroxidation. The pH-dependent decrease in radical formation in our experiments has been attributed to the decrease in activity of 15-LOX, particularly at acidic pH, since all reactions were performed in phosphate buffer or PBS under aerobic conditions.

The yield of radical adducts formed during cellular peroxidation of DPA (n-6) was found to be much lower than all three types of radical products from DPA (n-3), e.g. 7-, 17-, and 20-C, at both pH 7.4 and 6.5. This result suggests that the poorer metabolism of DPA (n-6) during cellular peroxidation may be associated with its reported relatively low bioactivity in cancer. The relationship of the reaction pH and the rate as well as the site of oxygenation of DPAs by 15-LOX may be an important research direction to be further studied for defining the association of bioactivities from fatty acids and their metabolites, including free radicals.

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LIST OF ABBREVIATIONS

ACN	acetonitrile
DPA	docosapentaenoic acid
HDPA	hydroxy-docosapentaenoic acid
EIC	extracted ion current
EPA	eicosapentaenoic acid
ESR	electron spin resonance
HPLC (LC)	high performance liquid chromatography
LOX	lipoxygenase
MRM	multiple reaction monitoring
MS	mass spectrometry
POBN	α -[4-pyridyl-1-oxide]-N- <i>tert</i> -butyl nitron
PUFAs	polyunsaturated fatty acids
TIC	total ion current
t_R	retention time

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Fig 1.

Off-line ESR spectra of the complete reaction systems of soybean 15-LOX and DPA (n-3/n-6) peroxidation and the relevant control experiments. (**A-B**) ESR spectra of the complete reaction system (in 50 mM phosphate buffer, pH 7.4) with reaction conditions: 20 K Unit/mL 15-LOX, 1.0 mM DPAs (n-3 and n-6, respectively), and 20 mM POBN, at 37°C for 30 min. ESR field scans were performed and the hyperfine couplings of the spectra were $a^N \approx 15.69$ G and $a^H \approx 2.68$ G; (**C-E**) ESR spectra of control experiments performed in the absence of DPA, 15-LOX and POBN, respectively.

A. LC/UV of peroxidation of DPA (n-3) and soybean 15-LOX

B. LC/ESR of peroxidation of DPA (n-3) and soybean 15-LOX

C. LC/MS (EIC) of m/z 240, m/z 224, m/z 310 & m/z 264

Fig 2.

On-line LC/ESR and LC/MS spectra of the reaction mixture of soybean 15-LOX and DPA (n-3). Reaction conditions (in phosphate buffer, pH 7.4): 20 K Unit/mL 15-LOX, 1.0 mM DPA, 20 mM POBN at 37°C for 30 min. (A) UV chromatogram at 265 nm with a C₁₈ column (ZORBAX Eclipse-XDB, 3.0 × 75 mm, 3.5 μm) equilibrated with solvent A (H₂O-0.1% HOAc); (B) ESR chromatogram was obtained in an ESR spectrometer equipped with an Aquax ESR cell. A time scan was performed with the magnetic field fixed on the maximum of the middle line of the offline spectra. A total of seven ESR active peaks are labeled with the corresponding retention times. (C) Extracted ion current (EIC) chromatogram of m/z 240, m/z 224, m/z 310, and m/z 264 projected from the total ion current (TIC) chromatogram (m/z 150–600). TIC was obtained with chromatographic

conditions identical to those in on-line ESR. Note that the peaks labeled with „*’ were the two minor isomers of m/z 264, which could be measured at much higher intensity at reaction pH 8.5 (data not shown).

Fig 3. LC/MS² spectra of ESR-active peaks. (A) LC/MS² of POBN/[•]C₂H₄OH (m/z 240) ion for ESR-active peak 2; (B) LC/MS² of POBN/[•]C₂H₅ (m/z 224) for ESR-active peak 3; (C) LC/MS² of POBN/[•]C₅H₉O₂ (m/z 310) for ESR-active peak 4; and (D) LC/MS² of POBN/[•]C₅H₉ (m/z 264) for ESR-active peak 5–7 in Fig 1B. Note that the fragmentation patterns and „a‘, ‘b‘, ‘c‘, and ‘d‘ fragment ions (Scheme 1B) formed from MS ionization of all POBN adducts identified in this study were consistent with the LC/MS² of their POBN adducts published elsewhere [28, 29].

Fig 4.

EIC and LC/MS² of oxidized form of POBN/[•]C₂H₅ (m/z 224). (A–B) EIC and LC/MS² of m/z 223 ion as oxidized form of POBN/[•]C₂H₅; and (C–D) EIC and LC/MS² of m/z 232 ion from the d₉-POBN system. Note that „x’ represents radical-unrelated peak(s) since such peak(s) are unable to be detected in both d₀- and d₉-POBN spin trapping experiments, and the typical fragmentation pattern of the spin adduct could not be observed either.

Fig 5.

EIC and LC/MS² of oxidized and reduced forms of POBN/[•]C₅H₉O₂ (m/z 310). (A–B) EIC and LC/MS² of m/z 309 ion as oxidized form of POBN/[•]C₅H₉O₂; and (C–D) EIC and LC/MS² of m/z 311 ion as reduced form of POBN/[•]C₅H₉O₂. Note that „*’ represents a portion of ESR-active form(s) being reduced/oxidized during MS ionization, and that „x’ represents radical-unrelated peak(s) since such peak(s) are unable to be detected in both d₀- and d₉-POBN spin trapping experiments, and the typical fragmentation pattern of spin adduct could not be observed either.

A. EIC of m/z 263 **B. LC/MS² of m/z 263** **C. EIC of m/z 265** **D. LC/MS² of m/z 265** **Fig 6.**

EIC and LC/MS² of oxidized and reduced forms of POBN/[•]C₅H₉ (m/z 264). (**A–B**) EIC and LC/MS² of m/z 263 ion as oxidized form of POBN/[•]C₅H₉; and (**C–D**) EIC and LC/MS² of m/z 265 ion as reduced form of POBN/[•]C₅H₉. Note that „*’ represents a portion of the ESR-active form(s) being reduced/oxidized during MS ionization and that „x’ represents radical-unrelated peak(s) since such peak(s) are unable to be detected in both d⁰- and d₉-POBN spin trapping experiments, and the typical fragmentation pattern of spin adduct could not be observed either.

A. LC/UV of peroxidation of DPA (n-6) and soybean 15-LOX

B. LC/ESR of peroxidation of DPA (n-6) and soybean 15-LOX

C. LC/MS (EIC) of m/z 266, m/z 265 & m/z 267

Fig 7.

On-line LC/ESR and LC/MS spectra of the reaction mixture of soybean 15-LOX and DPA (n-6). Reaction conditions (in phosphate buffer, pH 7.4): 20 KUnit/mL 15-LOX, 1.0 mM DPA, 20 mM POBN at 37°C for 30 min. (A) UV chromatogram at 265 nm with a C₁₈ column (ZORBAX Eclipse-XDB, 3.0 × 75 mm, 3.5 μm) equilibrated with solvent A (H₂O-0.1% HOAc); (B) ESR chromatogram was obtained in an ESR spectrometer equipped with an Aquax ESR cell. A total of three ESR active peaks are labeled with the corresponding retention times. (C) EIC chromatograms of radical adducts m/z 266, m/z 265 (oxidized form), and m/z 267 (reduced form) were projected from the full scan (TIC, m/z 150–600). Other conditions of LC gradient elution, complete on-line MS and on-line ESR settings were as described in Experimental Methods.

Fig 8.

LC/MS quantification of radical adducts in their all redox forms formed from soybean 15-LOX-catalyzed DPA (n-3/n-6) peroxidation at different reaction pH. **(A)** Quantities of POBN/ C_2H_5 (m/z 224) and its oxidized form (m/z 223) stemming from 20-DPA (n-3)-OOH; **(B)** Quantities of POBN/ $\text{C}_5\text{H}_9\text{O}_2$ (m/z 310) and its oxidized (m/z 309) and reduced forms (m/z 311) stemming from 7-DPA (n-3)-OOH; **(C)** Quantities of POBN/ C_5H_9 (m/z 264) and its oxidized (m/z 263) and reduced forms (m/z 265) stemming from 17-DPA (n-3)-OOH; **(D)** Quantities of POBN/ C_5H_{11} (m/z 266) and its oxidized (m/z 265) and reduced forms (m/z 267) stemming from 17-DPA (n-6)-OOH. Relative quantities are expressed as a ratio of LC/MS POBN peak area vs. that of d_9 -POBN (4.0 $\mu\text{g/mL}$) normalized with the respective ionization efficiencies and isotopic contributions as described in Experimental Methods. Quantanalysis version 1.8 for Agilent 6300 Series Ion trap LC/MS was used to process the integration and calculation of peaks. Data are expressed as means $\pm$ SD from $n \geq 3$. Quantities of oxidized form ($\square$), radical form ($\blacksquare$) and reduced form ($\boxtimes$) at pH 6.5, 7.4 and 8.5 respectively.

A. EIC m/z 224 from PC3-15LOS mediated DPA (n-3) peroxidation **B. EIC m/z 310 from PC3-15LOS mediated DPA (n-3) peroxidation** **C. EIC m/z 264 from PC3-15LOS mediated DPA (n-3) peroxidation** **D. EIC m/z 266 from PC3-15LOS mediated DPA (n-6) peroxidation** **Fig 9.**

EICs of m/z 224, m/z 310, m/z 264 and m/z 266 as their corresponding radical adducts in cellular peroxidation using a human prostate cancer cell line (PC3-15LOS). At about 70–80% confluency the cells were trypsinized, harvested and suspended in phosphate buffer saline ~ pH 7.4 and 6.5 with 10^7 cells/mL each. POBN and DPA (n-3/n-6) at a final concentration of 50 mM and 1.0 mM, respectively (lower concentrations, e.g. 100 and 500 μ M of DPA, were also tested, but even lower EIC intensities were obtained and data is not shown), were then added to the cell suspension to initiate the DPA peroxidation and POBN spin-trapping reactions. After a 30-min incubation, the peroxidation of the cell suspension was stopped using ACN (1:1, v/v), and the supernatant was collected, centrifuged and condensed for LC/MS analysis. (A) EIC of POBN/ C_2H_5 (m/z 224), t_R 12.0 min; (B) EIC of

POBN/C₅H₉O₂ (m/z 310), t_R 14.0 min; (C) EIC of POBN/[•]C₅H₉ (m/z 264), t_R 19.4 min; and (D) EIC of POBN/[•]C₅H₁₁ (m/z 266), t_R 21.1 min. Note that „×’ represents radical-unrelated peak(s) since such peak(s) are unable to be detected in both d₀- and d₉-POBN spin trapping experiments.

Fig 10.

LC/MS quantification of radical adducts formed from cellular PC3-15LOS-catalyzed peroxidation of DPAs at different cellular pH. Relative quantities expressed as a ratio of LC/MS POBN peak area to that of d_0 -POBN (1.0 $\mu\text{g/mL}$), normalized with the respective ionization efficiencies and isotopic contributions as published elsewhere [28, 29]. Quantities of radicals generated from DPA (n-3) stemmed from 7-, 17- and 20-hydroperoxides: redox forms of $\text{POBN}^{\bullet}\text{C}_5\text{H}_9\text{O}_2$ (m/z 309, m/z 310, m/z 311), redox forms of $\text{POBN}^{\bullet}\text{C}_5\text{H}_9$ (m/z 263, m/z 264, and m/z 265), and redox forms of $\text{POBN}^{\bullet}\text{C}_2\text{H}_5$ (m/z 223 and m/z 224); while that from DPA (n-6) stemmed only from 17-hydroperoxide as redox forms of $\text{POBN}^{\bullet}\text{C}_5\text{H}_{11}$ (m/z 265, m/z 266, and m/z 267) at pH 6.5 ($\square$) and pH 7.4 ($\blacksquare$), respectively. Quantanalysis version 1.8 for Agilent 6300 Series Ion trap LC/MS was used to process the integration and calculation of peaks. Data are expressed as means $\pm$ SD from $n \geq 3$.

Scheme 1A

Scheme 1B

Scheme 1C

Scheme 1.

Mechanisms of formation of POBN radical adducts of 15-LOX-catalyzed DPA peroxidation and redox reaction of POBN radical adduct. (A) Proposed mechanism of formation of POBN adducts of DPA (n-3)-derived carbon-centered radicals in 15-LOX-catalyzed peroxidation; (B) Redox reaction of POBN radical adduct and the fragmentation patterns and „a‘, ‘b‘, ‘c‘, and ‘d‘ ions of POBN adduct and its redox forms; and (C) Proposed mechanism of formation of POBN adducts of DPA (n-6)-derived carbon-centered radicals in 15-LOX-catalyzed peroxidation. Note that the proposed mechanisms are based on the free radicals and non-radical metabolites (e.g. HDPAs) observed in our experiments. However,

additional pathways may also exist and generate some minor free radicals that are under the detection limit.

LC/MS quantification of HDPA metabolites formed from soybean 15-LOX-catalyzed DPA peroxidation at different reaction pH. Note that recognition of different HDPAs (7-, 17- and 20-HDPA) was made by observing diagnostic fragments in LC/MS (MRM with negative mode). HPLC and MS settings were described in Methods.

Table 1

HDPA (m/z 345)	t _R (min)	Abundance* (µg/mL)			Diagnostic fragments (LC/MS ²)
		pH 8.5	pH 7.4	pH 6.5	
7-HDPA (n-3)	20.1	25.37 ± 2.11	7.85 ± 1.23	4.93 ± 0.97
17-HDPA (n-3)	14.5	29.82 ± 2.34	2.73 ± 0.56	1.04 ± 0.03
20-HDPA (n-3)	18.9	11.59 ± 1.11	2.80 ± 0.38	1.16 ± 0.01
17-HDPA (n-6)	16.8	19.80 ± 2.31	3.30 ± 0.56	1.34 ± 0.026

* relative quantities were calculated using 15-HETE as an internal standard (1.0 µg/mL, t_R ≈ 12.4 min). Quantanalysis version 1.8 for Agilent 6300 Series Ion trap LC/MS was used to process the integration and calculation of peaks. Data are expressed as means ± SD from n ≥ 3.