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Docosahexaenoic and arachidonic acid peroxidation: It's a within molecule cascade

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ABSTRACT

Peroxidation is a well-known natural phenomenon associated with both health and disease. We compared the peroxidation kinetics of phosphatidylcholine (PC) molecules with different fatty acid compositions (i.e. 18:0, 18:1n-9, 18:2n-6, 20:4n-6 and 22:6n-3 at the sn-2 and 16:0 at sn-1 position) either as molecules free in solution or formed into liposomes. Fatty acid levels, oxygen consumption plus lipid hydroperoxide and malondialdehyde production were measured from the same incubations, at the same time during maximal elicitable peroxidation. PCs with highly peroxidizable fatty acids (i.e. 20:4n-6 and 22:6n-3) in the same incubation were found to be either fully peroxidized or intact. Rates of peroxidation of PCs with multiple bisallylic groups (i.e. 20:4n-6 and 22:6n-3) peroxidized at 2-3 times the rate per bisallylic bond than the same phospholipid with 18:2n-6. The results suggest that propagation of peroxidation (H-atom transfer) is firstly an intramolecular process that is several-fold faster than intermolecular peroxidation. PCs in solution peroxidized twice as fast as those in liposomes suggesting that only half of the phospholipids in liposomes were available to peroxidize i.e. the outer leaflet. Experiments on liposomes suggest that even after heavy peroxidation of the outer leaflet the inner leaflet is unaffected, indicating how cells may protect themselves from external peroxidation and maintain control over internal peroxidation. Intramolecular peroxidation may produce highly concentrated, localized sites of peroxidation product that together with internal control of peroxidation of the inner leaflet of membranes provide new insights into how cells control peroxidation at the membrane level.

1. Introduction

Membranes are the quintessential structure of life. They separate the living from non-living and extracellular environments plus define the internal working precincts of the cell. The fundamental biomolecules of membranes are phospholipids that naturally form bilayer structures. Yet many phospholipids have a problem, they 'go off' in the presence of oxygen producing both toxic and damaging products. This rancidification is known as peroxidation by biologists and autoxidation by chemists [1] and is primarily due to polyunsaturated fatty acids (PUFA). PUFA possess methylene groups that reside between two double bonds known as bisallylic groups (– CH═CH – **CH**$_2$ – CH═CH –) that have hydrogen with weakened bond energies due to the attraction of their electrons to the double bonds on either side [2]. Since monounsaturated fatty acids possess allylic rather than bisallylic methylene groups, their carbon–hydrogen bond energies are stronger and less prone to cleavage whereas the methylene groups of saturated fats, with a total lack of double bonds have even stronger carbon–hydrogen bond energies and are highly resistant to peroxidation [2].

Peroxidation of PUFA normally begins with the removal of a hydrogen atom from a bisallylic carbon by a free radical. This sets off a sequence of reactions that normally leads to the binding of oxygen and the formation of a lipid peroxyl radical (LOO •; including cyclized peroxyl radicals on PUFA with three or more double bonds [1]). The formation of this second radical propagates the reaction as the peroxyl radical seeks to abstract a second hydrogen atom [3] with lipid hydroperoxides and aldehydes (e.g. malondialdehyde) formed as downstream products.

General schemes of the lipid peroxidation mechanism propose that this second hydrogen atom is most likely to come from a second bisallylic group on a neighboring PUFA [4]. Yet, in the case of fatty acids containing more than one bisallylic group (i.e. 20:4n-6 and 22:6n-3 versus 18:2n-6) this hydrogen atom can potentially come from the same original molecule. Peroxyl radical cyclization has been shown to occur in fatty acids and esters with three or more double bonds. Here the "carbon framework offers intermediate peroxyl radicals

Abbreviations: LOOH, lipid hydroperoxide; MDA, malondialdehyde; BHT, butylated hydroxytoluene

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an appropriate double bond target for serial intramolecular addition" of O_2 through cyclization [1].

Previous measurements have shown that the rates of peroxidation of PUFA occur in proportion to the total number of bisallylic groups available and this has provides a relative scale of peroxidation known as the peroxidation index. The peroxidation index ranges from 1 to 5 for polyunsaturated fatty acids with 2 to 6 double bonds respectively [5]. In mammalian membranes the two most prevalent PUFAs found in mammalian membranes are arachidonic (20:4n-6) and docosahexaenoic acid (22:6 n-3) [6,7]. These PUFA are also the most biologically active and prone to peroxidation with 4 and 6 double bonds respectively. Yet in studies and descriptions of peroxidation kinetics, linoleic acid (18:2n-6) has been the preferred fatty acid examined based on its simpler structure [8–10].

The present study examines the relative peroxidizabilities of different fatty acids, when attached to the most prevalent phospholipid i.e. phosphatidylcholine found in mammalian membranes [7]. Phosphatidylcholine molecules were examined either as molecules free in solution or formed into liposomes (artificial lipid bilayer microspheres). Peroxidation was measured during maximal elicitable rates of peroxidation during propagation phase by taking a 'snapshot' of this process via samples taken during this most active phase. Continuous oxygen consumption profiles (associated with peroxyl formation) were used to visualize the peroxidation process during the propagation phase with samples taken for fatty acid analysis, as well as for measurement of secondary product formation i.e. lipid hydroperoxide (LOOH) and malondialdehyde (MDA). One of the major finding of this study was that peroxidation of PUFA with more than one bisallylic group is primarily an intramolecular process rather than an intermolecular process. The implications of intramolecular peroxidation of these highly polyunsaturated fats in membranes are that they are likely to produce localized high concentrations of peroxidation product that provides a new mode of action for these biologically active fats.

2. Materials and methods

2.1. Materials

Synthetic phospholipids (1-palmitoyl-2-stearoyl-*sn*-glycero-3-phosphocholine; PC 16:0/18:0, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; PC 16:0/18:1, 1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphatidylcholine; PC 16:0/18:2, 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine; PC 16:0/20:4 and 1-palmitoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine; PC 16:0/22:6) all without added antioxidant in the form of butylated hydroxytoluene (BHT), together with the natural phosphatidylcholine extract Soy PC were purchased from Avanti Polar Lipids Alabama USA. Xylenol orange was purchased from Sigma-Aldrich (USA). All chemicals used were of analytical grade.

2.2. Preparation of phospholipids and liposomes

Prior to their use phospholipids were made into stock solutions (at either 25 or 12.5 mM) and used immediately. Stock solutions where made up in methanol and kept under nitrogen at −80 °C. Phospholipids were measured either as phospholipids in solution (90% water, and 10% methanol) or as liposomes. Phospholipids in solution showed no discernable population of particles when examined using a Zetasiser®. For phospholipids in solution and as liposomes, final working concentrations ranged from 1 mM to 62.5uM for PC 16:0/18:0 to PC 16:0/22:6. This concentration range was to allow for measurement of maximum rates of peroxidation (as determined by rates of oxygen consumption) as phospholipids became increasing polyunsaturated. Liposomes were made from the same stock solutions as used for phospholipids in solution. Liposomes were prepared by drying down each phospholipid for 45 min under a stream of nitrogen, rehydrating using warm nitrogen saturated distilled water (5–10 mL at 45 °C) followed by vigorous vortexing under nitrogen (25 Hz) for 30 min. Solutions were then sonicated on ice (Misonix, NY USA) using a fine tipped probe for 6 × 10 s dispensing a total energy level of 420 J with 30 s rest periods between sonication bouts. Liposomes were used immediately following their preparation.

2.3. Measurement of lipid peroxidation

Oxygen consumption was measured at 38 °C using a Qubit® oxygen electrode system connected to a LabPro® Vernier data logger (Version 3). Continuous oxygen consumption measurements provided the ability to follow the kinetic activity of phospholipids undergoing peroxidation. These were used to ensure that all samples (fatty acids, LOOH and MDA) were taken during maximal rates of peroxidation during the propagation phase of peroxidation for each phospholipid. Peroxidation was initiated using ammonium ferrous sulfate (at pH ~4) to produce ferrous iron at a final incubation concentration of 50uM (preliminary experiments using Soy PC were conducted to determine the ferrous concentration required to elicit maximal peroxidation within the concentration range used for each phospholipid). Fresh ammonium ferrous sulfate was prepared every few hours to ensure that iron remained in the ferrous state at pH 4 and avoid any yellowing of the solution indicating ferric conversion. For all incubations, samples were taken at the start of the incubation immediately prior to iron addition and during maximum rates of peroxidation for each phospholipid (as determined using oxygen consumption profiles). All samples were immediately mixed with BHT 0.01% (*w*/*w*) to prevent further peroxidation. The ability of BHT to stop peroxidation was tested by adding BHT (at 0.01% *w*/*w*) directly to the oxygen consuming incubations containing highly peroxidizable phospholipids (e.g. PC 16:0/22:6) during maximal peroxidation. The effect of BHT addition (at 0.01% *w*/*w*) was immediate causing the instant stoppage of oxygen consumption, indicating the effectiveness of BHT to stop propagation of peroxidation.

In order to ensure maximization of rates of peroxidation (i.e. during propagation) the phospholipids were tested for their ability to peroxidize from least to the most peroxidizable at reducing concentrations. Once a reduction in concentration caused no further increase in the peroxidation rate for that phospholipid it was subsequently tested at that concentration. This resulted in phospholipid concentrations being continuously reduced down from 1000 μM to 62.5 μM to ensure that maximal rates of peroxidation were achieved. Each phospholipid incubation was sampled before and part-way through maximal peroxidation (i.e. during propagation). The final incubation conditions for each PC in solution/liposomes being measured at concentration (in μM) and at time of sampling during maximal rate of peroxidation (in minutes) are: for PC 16:0/18:0 (500 μM) at 20 min/20 min, PC 16:0/18:1 (500 μM) at 20/20 min, PC 16:0/18:2 (500/250 μM) at 5 min/10 min and for both PC 16:0/20:4 (250/125 μM) at 2-4 min/5 min and PC 16:0/22:6 (125/62.5 μM) at 2 min/2 min. A correction factor for the adherence of phospholipids (in solution and as liposomes) to the walls of the oxygen chamber was made using the difference in concentration of the non-peroxidizable fatty acid i.e. 16:0 present in all phospholipids measured at time zero versus that used during sampling of maximal peroxidation. Average correction factors were determined as 12% for PCs in solution and in liposomes. All data was taken from the same set of incubations for all experiments.

Lipid hydroperoxide levels were measured using the xylenol orange based FOX2 assay (as previously described, [11]) with the final assay medium consisting of 0.25 mM $(NH_4)_2Fe(SO_4)_26H_2O$, 0.25 mM H_2SO_4, 100 μM xylenol orange and 1 mM of butylated hydroxytoluene (as antioxidant) in methanol with tert-butyl hydroperoxide used as standard (linear range between 0 and 20 nmol; $r^2 = 0.999$). Absorbance was measured at 560 nm using a Biochrom Libra 512 spectrophotometer. Malondialdehyde levels were measured using the TBARS method with 250 μL of thiobarbituric acid (0.4 g/100 mL in 10% acetic acid) and