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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

20 μL of butylated hydrotoluene (0.66 g/100 mL in ethanol) added to 20–50 μL of sample, heated to 90 °C for 1 h, cooled in cold water for 10 min then 300 μL of butanol added, mixed and spun at 7500 rpm (10 min) on a microfuge. The upper butanol phase (with extracted product) was then removed and placed in 96 well-plates together with tetramethoxypropane used as a standard and absorbance measured at 532 nm (BMG Labtech Polarstar Omega).

Fatty acid concentrations were quantified using gas chromatography (Varian CP 8400 GC) as previously performed [12] using tricosanoic acid (23:0) as an internal standard to quantify fatty acids. All data (oxygen consumption, fatty acid samples, lipid hydroperoxide and malondialdehyde content) were all derived from oxygen consumption profiles and samples taken from the same incubation in the order; fatty acid, lipid hydroperoxide and malondialdehyde where oxygen consumption was determined from oxygen consumption traces at the time of fatty acid sampling. Time required for total sampling was ~10 s (note: since the fatty acid sample was the first sample taken and oxygen consumption determined at that instant of sampling there was no offset in these measurements).

3. Results

The maximal rates of peroxidation of five different phosphatiylcholine molecular species as either free in solution or formed into liposomes are shown in Fig. 1. All phosphatidylcholine molecules contained palmitic acid (16:0) at the sn-1 position but differed in the second fatty acid present at the sn-2 position being either; stearic (18:0), oleic (18:1), linoleic (18:2), arachidonic (20:4) or docosahexaenoic acid (22:6). Peroxidation rates are expressed in terms of the number of molecules of oxygen consumed per phospholipid molecule per minute.

As expected, phospholipids with saturated (18:0) and monounsaturated (18:1) fatty acids did not peroxidize during the incubation period examined, whereas those with polyunsaturated fatty acids peroxidized readily. The most obvious difference in rate of peroxidation was that between phospholipids free in solution versus those in liposomes for the three PUFA containing phospholipids. The rate of peroxidation of these phospholipids in liposomes was approximately half that found for the same phospholipids when peroxidized as phospholipids free in solution (Fig. 1). A second major difference was that between the average peroxidation rates of linoleic acid (18:2), with its single bisallyic group, versus arachidonic (20:4) and docosahexaenoic (22:6) acids, with their multiple bisallylic groups.

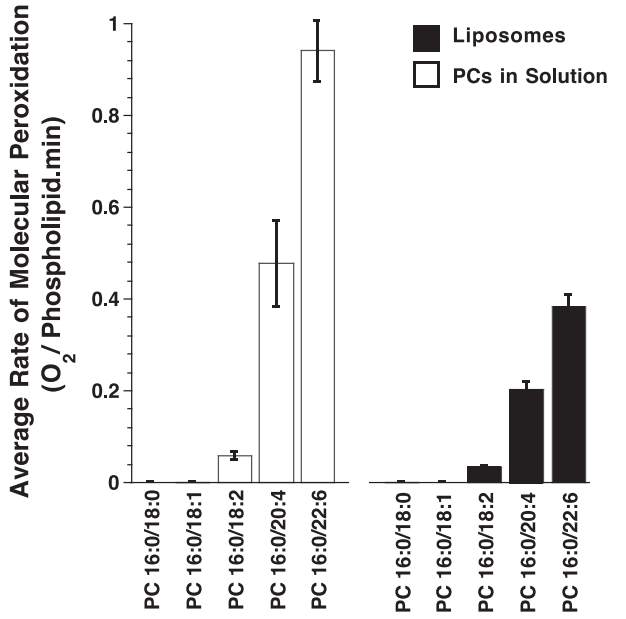

Fig. 1. Maximal rate of peroxidation of five phosphatidylcholine molecules differing in one fatty acid (at the sn-2 position). Peroxidation was performed either as phospholipids free in solution (□) or formed into liposomes (■). Peroxidation rate is expressed as the number of oxygen molecules consumed per phospholipid molecule per minute. $N = 4$–11 for PCs in solution and $N = 6$ for liposomes; values are means ± SEM.

The two highly polyunsaturated fatty acids i.e. arachidonic (20:4) and docosahexaenoic (22:6) acid peroxidized at rates relative to one another in proportion to ~0.5. This value is close to their relative ratio of bisallylic groups (at 3 and 5 respectively giving a proportional rate of 0.6). In contrast, the phospholipid containing linoleic acid i.e. PC 16:0/18:2, with its single bisallylic group, peroxidized at a rate per bisallylic group that was approximately 33% slower as PCs in solution and 44% slower when in liposomes compared to the two other PUFA containing phospholipids (i.e. PC 16:0/20:4 and PC 16:0/22:6). Table 1 shows the average rate of peroxidation per bisallylic bond for phospholipids containing a polyunsaturated fatty acid. The results show both a 1.8-2.5 fold higher rate of peroxidation per bisallylic bond for PCs in solution versus those in liposomes as well as a faster rate (2.0–3.3 times) of peroxidation per bisallylic bond of PCs 16:0/20:4 and 16:0/22:6 compared to PC 16:0/18:2, either in solution or as liposomes.

Fig. 2 shows molecular peroxidation expressed as the amount of oxygen consumed per phospholipid, or as shown in Fig. 3, the amount of secondary product formed per phospholipid molecule peroxidized (i.e. determined by fatty acid disappearance at the sn-2 position) using subsamples from the same phospholipid incubations used to measure rates of peroxidation shown in Fig. 1. Sampling involved a 'snapshot' approach, where oxygen consumed, fatty acid disappearance and the appearance of peroxidation products (lipid hydroperoxide and malondialdehyde) were measured at the same time during maximal rates of peroxidation (determined from each oxygen consumption profile) from the same set of incubations. Fig. 2 shows that phospholipids containing a saturated or a monounsaturated fatty acid did not consume oxygen over the period of incubation (in these cases 20 min) at 38 °C. Phosphatidylcholine molecules in solution with a PUFA consumed 1.3, 2.3 and 3.7 molecules of oxygen for each 18:2, 20:4 and 22:6 fatty acid present on the phospholipid molecule respectively.

The same experiment conducted for the same PCs in liposomes resulted in the number of molecules of oxygen consumed per PUFA molecule for 18:2, 20:4 and 22:6 being 1.7, 3.2 and 4.4 respectively. These values are comparable to the theoretical values of weak bisallylic C – H bonds per fatty acid of 1 for 18:2, 3 for 20:4 and 5 for 22:6 with slightly higher values for 18:2 and slightly lower values for 22:6. The interesting point about these measurements is that they were taken during the reactive cycle (without depleting substrate) rather than at termination of the peroxidation process. Therefore within the incubation, abundant oxygen and ample intact PUFA was available for further peroxidation. Table 2 shows the average percentage of oxygen and PUFA available in the incubations at the time of sampling (i.e. during propagation). For example, Table 2 shows that at the time of sampling (in this case 2 min) 78.6% of PC 16:0/22:6 in liposomes was intact (as determined from GC profiles of 22:6) whereas 21.4% of the phospholipid had consumed 4.4 O_2 molecules per 22:6 or phospholipid molecule (as determined by the disappearance of 22:6 and amount of oxygen consumed at that point in time).

Fig. 3 shows molecular peroxidation in terms of the number of molecules of lipid hydroperoxide and malondialdehyde produced per

Table 1
Average rates of peroxidation per bisallylic group for each phospholipid with polyunsaturated fatty acid incubated as PCs in solution or as liposomes.

Phospholipids	PCs in solution	Liposome
PC 16:0/18:2	0.058 ± 0.008	0.032 ± 0.005
PC 16:0/20:4	0.159 ± 0.031	0.067 ± 0.006
PC 16:0/22:6	0.189 ± 0.014	0.077 ± 0.005

Values are in units of O_2 molecules consumed per bisallylic group per phospholipid molecule per minute, $n = 4$–11.

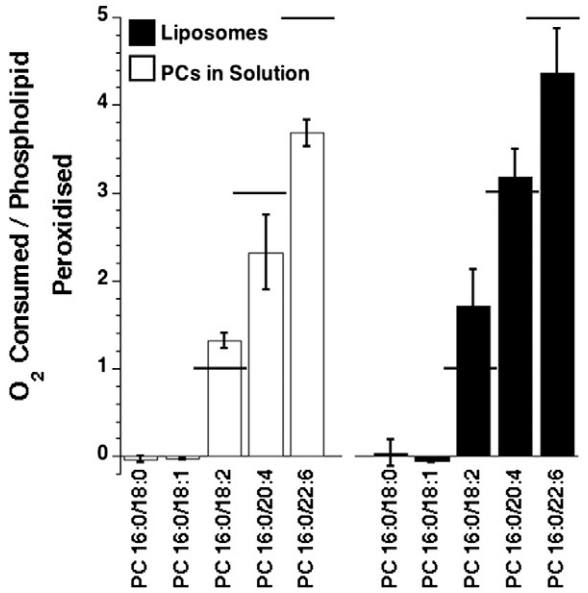

Fig. 2. Molecular peroxidation of five phosphatidylcholine molecules differing in one fatty acid (at the sn-2 position). Peroxidization was performed either as phospholipids free in solution (■) or formed into liposomes (□). Peroxidation is expressed as molecules of oxygen consumed per phospholipid. A theoretical peroxidative value for each phospholipid is shown as a straight bar for each phospholipid. $N = 4$–11 for PCs in solution and $N = 6$ for liposomes; values are means ± SEM.

phosphatidylcholine molecule peroxidized with 18:2, 20:4 and 22:6. Generally, the various phospholipids showed levels of product formation in proportion to their number of bisallylic groups. As previously found [13,14], 18:2 in PC 16:0/18:2 showed that it did not readily produce malondialdehyde. For both PCs in solution and as liposomes the percent conversion of peroxidized PCs (based on O_2 molecules consumed) into lipid hydroperoxides was 32.7 ± 4.5% and for malondialdehyde 13.7 ± 1.2% (Note: excludes results for 18:2 for MDA).

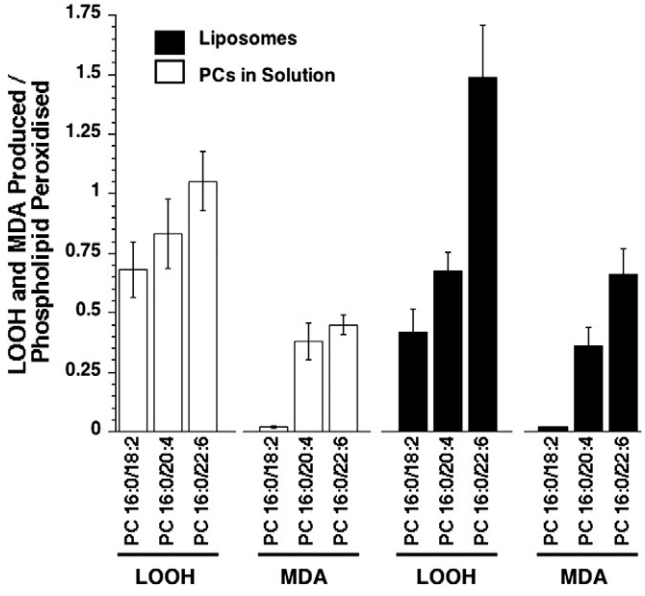

Fig. 3. Molecular peroxidation of three peroxidizable phosphatidylcholine molecules (PC 16:0/18:2, PC 16:0/20:4, PC 16:0/22:6). Each phosphatidylcholine molecule differed by one fatty acid and was peroxidized either as molecules free in solution (■) or formed into liposomes (□). Peroxidation is expressed as the number of molecules of lipid hydroperoxide (LOOH) and malondialdehyde (MDA) produced per phosphatidylcholine molecule (or per peroxidizable fatty acid) peroxidized. $N = 4$–11 for PCs in solution and $N = 6$ for liposomes; values are means ± SEM.

Table 2
Percentage of oxygen and phosphatidylcholine present in the oxygen electrode chamber at the time of sampling to determine molecular peroxidation for PCs in solution and liposomes ($n = 4$–11).

Phospholipid	% oxygen remaining		% phospholipid remaining	
	PCs in solution	*Liposome*	*PCs in solution*	*Liposome*
PC 16:0/18:0	99.9 ± 0.4	100.5 ± 0.5	108.4 ± 5.2	100.5 ± 1.6
PC 16:0/18:1	100.3 ± 0.4	99.7 ± 0.3	103.5 ± 5.8	107.2 ± 2.2
PC 16:0/18:2	73.3 ± 3.5	80.2 ± 5.0	80.8 ± 2.9	88.0 ± 2.7
PC 16:0/20:4	41.9 ± 4.9	51.9 ± 3.3	59.2 ± 8.6	73.7 ± 2.1
PC 16:0/22:6	25.7 ± 4.0	47.8 ± 4.4	49.2 ± 5.7	78.6 ± 2.8

4. Discussion

Membrane peroxidation is commonly viewed as a cascade where lipid peroxidation radiates out from a point of initiation moving from PUFA to PUFA on neighboring phospholipid molecules. This view is perpetuated by schemes and figures that show the propagating step of the peroxidative cascade involving a second fatty acid donating a hydrogen to the peroxyl radical of the original peroxidized species. This should be true for 18:2, as this fatty acid possesses only one bisallylic group. However, the primary finding of the present study is that highly polyunsaturated fatty acids such as 20:4 and 22:6, which possess 3 and 5 bisallylic groups respectively, propagate peroxidation via an intramolecular cascade. Intramolecular, versus intermolecular, peroxidation is indicated by the number of oxygen molecules consumed by each phospholipid molecule being close to the theoretical maximum (as shown in Fig. 2) based on the number of bisallylic groups for each phospholipid (i.e. 1 for 18:2, 3 for 20:4 and 5 for 22:6). Whereas at the same time, in the same incubations other PUFA were intact with abundant oxygen still available indicating that these reactions were not substrate limited in the form of bisallylic hydrogen and oxygen (see Table 2). In essence, peroxidation works up and down the carbon chain of highly polyunsaturated fatty acids, using up all weakly bound hydrogen on the bisallylic groups before jumping to PUFA to neighboring phospholipids.

Differences in the number of oxygen molecules consumed by each peroxidized PUFA compared to their theoretical value (at time of sampling) are likely to indicate molecules caught in partial peroxidation (overall average deficit for arachidonic and docosahexaenoic acid being 16%). PCs in liposomes were closer to their theoretical values and this may reflect environmental differences such as rate constants for hydrogen atom transfer and oxygen solubilities in the different environments [3]. There was also a consistent over-consumption of oxygen for PC 16:0/18:2 (both in solution and in liposomes) compared to its theoretical value. This could partially be explained by the production of dihydroxyperoxides that have been measured for 18:2 in non-enzymatically derived peroxidation [15] that involves the consumption of 2 oxygen molecules per linoleic molecule.

Intramolecular peroxidation could explain the reduced rate of peroxidation (i.e. oxygen consumption rate) per bisalyllic group shown by PC 16:0/18:2 (33% in solution and 44% in liposomes) compared to the highly polyunsaturated phospholipids (see Table 1). Since each PC 16:0/18:2 molecule has only one reactive bisallylic group, in order to peroxidize it needs to be either initiated by ferrous iron or come in contact with a lipid radical from the surrounding medium. Also in the case of dihydroperoxides the addition of the second oxygen molecule will be slower because it is on an allylic carbon and any β-fragmentation of the peroxyl radical [1] would also slow the reaction, and therefore the oxygen consumption rate. On the other hand, each PC 16:0/20:4 and PC 16:0/22:6 molecule can peroxidize 3 and 5 bisallylic carbons respectively without any intermolecular interactions. In essence, docosahexaenoic and arachidonic acid show rates of peroxidation that are in relative proportion to their bisallylic load. PC 16:0/18:2 also produces more lipid hydroperoxide per bisallylic group compared to both PC 16:0/20:4 and 16:0/22:6 (Fig. 3) and this has also been previously reported by Visioli et al. [16]. This is likely to reflect the

higher oxygen consumption per bisallylic carbon exhibited by 18:2 and the fact that lipid hydroperoxides produced from this fatty acid do not go on to form malondialdehyde, as do those formed from 20:4 and 22:6.

Differences in the peroxidation between PCs free in solution versus those in liposomes (Fig. 1) have previously been noted and speculated to be due to slower diffusion rates within the membrane (compare values in Tables 1 and 2 in [3]) or the 'floating of peroxyl radicals' to the surface prevents further propagation from occurring. However, recent modeling by Garec et al. [4] has suggested the absence of a 'floating' effect of peroxyl radicals in membrane phospholipids. Intramolecular propagation of PUFA peroxidation does not require the "floating peroxyl radical" hypothesis to explain the slower rates of peroxidation of PCs in liposomes compare to PCs free in solution. Alternatively, we would suggest that the two-fold difference in rates is more likely due to the fact that only half of the phospholipids are readily available for peroxidation in liposomes. The bilayer structure of liposomes could protect phospholipids on the inner leaflet from coming in contact with ferrous iron and lipid radicals.

Since peroxidation rate is commonly determined by the concentration of the incubation, in the case of liposomes the phospholipids on the internal leaflet may not have been exposed to ferrous iron, potentially halving the available PUFA and halving the rate. This is supported by ~3 fold increase in peroxidation rates when the same incubations of liposomes containing 18:2, 20:4 and 22:6 (with no effect on phospholipids with 18:0 and 18:1) were exposed to 0.05% Triton X-100® as a permalizing agaent. We attribute the increase in peroxidation to the access of ferrous iron to phospholipids previously unavailable on the inner leaflet. The heightened rate of peroxidation (3 versus 2) may be due to the fact that Triton X-100 is known to contain up to 0.22% of hydroperoxide equivalence that would also accelerate peroxidation rate. The potential lack of peroxidation of PUFA on phospholipids in the inner leaflet of liposomes indicates that peroxidation events in the outer leaflet do not readily cross to the inner leaflet. As shown by Garrec et al., [4], although peroxyl radicals may stay deeper in the membrane than previously thought, hydroperoxides are likely to be found at the interfacial outer surface of the membrane protecting the inner sanctum of the cell membrane from hydrogen atom transfer.

Of the four major phospholipids naturally found in vertebrate membranes phosphatidylcholine and sphingomyelin are found primarily in the outer (extracellular facing) leaflet and phosphatidylethanolamine and phosphatidylserine in the inner (cytoplasmic facing) leaflet [17,18]. Phosphatidylethanolamine and phosphatidylserine are the two major phospholipid classes that possess a large proportion of the highly polyunsaturated fatty acids [7]. This differential partitioning, combined with the inability of peroxidation to jump the leaflet divide in biological membranes, would act to protect highly polyunsaturated phospholipids from external initiated peroxidation and provide the cell with some control over peroxidation of phospholipids on the inner leaflet. This could partly explain how natural membranes protect themselves from exposures to initiators of peroxidation such as transition metals that are major players in numerous oxidative based diseases [2,19].

5. Conclusions

The results of this study indicate that phospholipids with polyunsaturated fatty acids with more than two double bonds tend to propagate peroxidation via intramolecular prior to intermolecular reactions. This conclusion is supported by experiments using a 'snap-shot' sampling method showing phospholipids with PUFA undergoing propagation possessed full complements of oxygen bound to each fatty acid molecule, whereas other phospholipids with PUFA in the same incubation were intact. These experiments indicate that even after heavy peroxidation of the external leaflet of liposomes phospholipids on the inner leaflet are likely unaffected. This provides new insight into how peroxidation events occurring in the outer leaflet of membranes may be prevented from spilling over to initiate peroxidation of the more susceptible highly polyunsaturated phospholipids present on the inner leaflet. Intramolecular peroxidation is likely to produce highly localized sites of peroxidation product in membranes that provides a new view of the propagation of peroxidation in cell membranes.

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