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An antioxidant-like action for non-peroxidisable phospholipids using ferrous iron as a peroxidation initiator

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ABSTRACT

The degradation of phospholipids containing polyunsaturated fatty acids, termed peroxidation, poses a constant challenge to membranes lipid composition and function. Phospholipids with saturated (e.g. PC 16:0/16:0) and monounsaturated fatty acids (e.g. PC 16:0/18:1) are some of the most common phospholipids found in membranes and are generally not peroxidisable. The present experiments show that these non-peroxidisable phospholipids, when present in liposomes with peroxidisable phospholipids (i.e. those containing polyunsaturated fatty acids) such as PC 16:0/18:2 and Soy PC, produce an inhibitory effect on rates of peroxidation induced by ferrous-iron. This inhibitory effect acts to extend the duration of the lag phase by several-fold. If present in natural systems, this action could enhance the capacity of conventional antioxidant mechanisms in membranes. The results of this preliminary work suggest that non-peroxidisable phospholipids may exert an antioxidant-like action in membranes.

1. Introduction

The deterioration of lipids in oxygen, commonly referred to as peroxidation, is a process that all organisms must accommodate in order to survive. Peroxidation is a constitutive process in living systems and is not only part of healthy function but also pathology, ageing and death [1,2]. Although the chemistry of phospholipid peroxidation is complex [3–5], peroxidation in membranes can be mainly attributed to polyunsaturated fatty acids (PUFAs) present in phospholipids. PUFAs are particularly prone to peroxidation due to the presence of bis-allylic methylene groups. These methylene groups are those that reside between consecutive double bonds (i.e. –CH═CH–**CH_2**–CH═CH–) and therefore are only present in PUFA. The bis-allylic position makes the hydrogen atoms attached to the carbon of these methylene groups more readily abstracted by free radicals. Monounsaturated fatty acids (MUFA) that possess only allylic methylene groups (methylene groups on either side of the single double bond; i.e. CH–**CH_2**–CH═CH–), have hydrogen that bind far more strongly to their carbon on the fatty acid chain. Subsequently, MUFA are far less prone to peroxidation than PUFA. Saturated fatty acids, with no double bonds, have strong carbon–hydrogen bond energies throughout the molecule making these fatty acids the most resistant to peroxidation [6]. As a result of these differences in the bond energies of hydrogen to the carbons of fatty acids, those membrane phospholipids that contain SFA and/or MUFA are resistant to peroxidation whereas those that contain PUFA are highly prone to peroxidation. In this study, phospholipids that contain SFA and/or MUFA are referred to as non-peroxidisable phospholipids (non-PPLs) whereas those that contain PUFA are referred to as peroxidisable phospholipids (PPLs).

Peroxidation is a free radical reaction that when measured in vitro can progress through three well-recognised phases; i) lag, with a slow rate of reaction (R_{Lag}) and product formation, ii) propagation, with a fast rate of reaction (R_{Max}) and product formation and, iii) termination (Fig. 1) [7]. From a biological perspective, the lag phase is important as it offers the opportunity to prevent peroxidation entering into the more damaging propagation phase. It is within the lag phase that antioxidants and enzymes can stop the peroxidative process, with the duration of the lag phase commonly considered a measure of the antioxidant status of a membrane [8].

Irrespective of the challenge PUFAs present to living organisms in terms of peroxidation, PPLs are prevalent in membranes. In mammals, for example, membrane phospholipids typically possess between 30 and 60% of their total fatty acids as PUFAs [9,10] making PPLs a major portion of membrane phospholipids. The current research examines if non-PPLs (those that contain SFA and MUFA) can protect PPLs (those that contain PUFA) from peroxidising. This work examines the separate peroxidation of two PPLs (a natural phospholipid, soy phosphatidylcholine, and a synthetic phospholipid phosphatidylcholine, PC 16:0/18:2) in the presence of one of two different non-PPLs (PC 16:0/16:0 and PC 16:0/18:1) to determine if any protection occurs. Peroxidation was

Abbreviations: PPLs, peroxidisable phospholipids; non-PPLs, non-peroxidisable phospholipids; PC, phosphatidylcholine; 16:0, palmitic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, linolenic acid; BHT, butylated hydroxytoluene; T_{Lag}, duration of the lag phase; R_{Lag}, rate of peroxidation during the lag phase; R_{max}, maximal rate of peroxidation during the propagation phase; A_{Lag}, total amount of oxygen consumed during the lag phase

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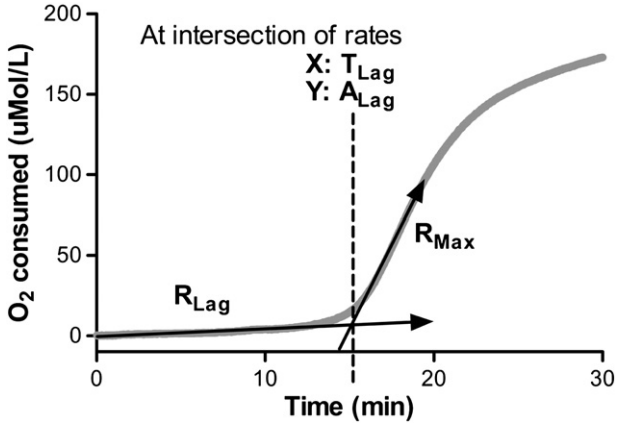

Fig. 1. A sample profile of an in vitro peroxidation reaction of 1.0 mM Soy PC and 1.5 mM PC 16:0/18:1 measured using oxygen consumption. The profile shows a lag phase transitioning into propagation phase with movement towards termination at the end of the profile. The profile indicates the characteristic parameters measured in the present study that were: the duration of the lag phase (T_{Lag}), the rate of peroxidation during the lag phase (R_{Lag}), the maximal rate of peroxidation during the propagation phase (R_{max}) and the total amount of oxygen consumed during the lag phase (A_{Lag}).

induced by low levels of ferrous iron (rather than the more common high concentrations of azo-initiator such as AAPH) in order to produce a more natural peroxidative stimulus. Although the mechanism by which iron initiates lipid peroxidation is still under debate [11] poorly-ligated iron is a problem common to a number of pathologies [12]. The results of this study suggest that the presence of non-PPLs attenuates the rates of peroxidation resulting in an extension of the duration of the lag phase through an antioxidant-like action.

2. Methods

2.1. Materials

Phosphatidylcholines, PC 16:0/16:0 (1,2-dipalmitoyl-sn-glycero-3-phosphocholine), PC 16:0/18:1 (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine), PC 16:0/18:2 (1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphocholine) and Soy PC (L-α-phosphatidylcholine, acyl composition 14.9% 16:0, 3.7% 18:0, 11.4% 18:1, 63% 18:2, 5.7% 18:3 and 1.2% unknown), without added butylated hydroxytoluene (BHT) as antioxidant, were purchased from Avanti Polar Lipids (Alabaster, USA). Ammonium ferrous sulphate ($(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$), sodium sulphite and butylated hydroxytoluene (BHT) were purchased from Sigma-Aldrich (St Louis, USA). Methanol (HPLC grade) and sulphuric acid (98%) were purchased from Crown Scientific (Rowville, Australia). All chemicals used were of analytical grade.

2.2. Liposome preparation

Stock solutions of phospholipids (125 mM) of both peroxidisable (PC 16:0/18:2 and Soy PC) and non-peroxidisable (PC 16:0/16:0 or PC 16:0/18:1) phosphatidylcholines were made-up in methanol and stored at −20 °C under nitrogen. Phospholipids from each stock solution were combined to produce the required phosphatidylcholine mixtures. Each phospholipid mixture was dried down under a stream of nitrogen for a minimum of 90 min (or until completely dry) at 42 °C, made-up to 10 mM using deionised water (42 °C, pH 3), stirred continuously for 45 min (42 °C) then passed nine times across a 0.1 μm pored polycarbonate membrane (Avanti Polar Lipids) using a Mini-Extruder (Avanti Polar Lipids) at (42 °C). Following extrusion, samples were diluted to 2.5 mM. Samples of liposomes were taken from each preparation, before and after peroxidation, in order to measure liposome size (with BHT added to prevent further peroxidation). In addition, lipid hydroperoxide (LOOH) levels of each liposomal preparation was measured (as previously described; [13]) prior to initiation of peroxidation and only those preparations with no measureable levels of LOOH were used.

2.3. Measurement of lipid peroxidation and liposome size

Peroxidation was measured using oxygen consumption as a measure of peroxyl formation. Oxygen consumption was measured using a Clarke type microelectrode system (Strathkelvin Instruments) with up to six electrodes used simultaneously. Oxygen consumption measurements were made over 2 periods at 37 °C using a RC-650 six-electrode respirometer. Oxygen consumption data was acquired each second from each microelectrode using a Six-Channel Oxygen Meter (Strathkelvin Instruments). Oxygen electrodes were prepared fresh daily using high sensitivity, fast response membranes (YSI Life Sciences, Morningside, Australia). Care was taken to remove any trace of lipid hydroperoxides adhering to the respiration wells between experiments. Prior to any measurement microelectrodes were allowed to stabilize and thermally equilibrate (to 37 °C) for a minimum period of 30 min. Incubations were stirred continuously during experimentation. Microelectrode calibration was performed as per manufacturers instruction using air saturated, deionised water with sodium sulphite used to determine oxygen range. At 37 °C the oxygen concentration used was 6.73 mg or 210.3 μmol of oxygen/L of water at prevailing atmospheric pressure.

Peroxidation was initiated using 10 μM ferrous iron derived from ammonium ferrous sulphate (pH 3–4). Liposomes were made up to a final concentration of 2.5 mM, with PPLs present at between 2.5 and 0.5 mM and non-PPLs added from 0.5 to 2.0 mM (20–80%). Plots shown in Fig. 2 decrease in PPL and increase in non-PPL from left to right. The inset in each figure shows the same experiment in liposomes composed of the same amount of PPL only (2.5–0.5 mM). Except for T_{Lag}, all measurements are normalised for PPL concentration. Measurement made during peroxidation included: rate of peroxidation during the lag phase (R_{Lag}), maximum rate of peroxidation during the propagation phase (R_{Max}), duration of the lag phase (T_{Lag}) and total amount of oxygen consumed during the lag phase (A_{Lag}), as shown in Fig. 1. Rates of peroxidation (during lag and propagation phases) were determined using segmental linear regression analysis (GraphPad Prism 5.04) which divided the data into R_{Lag} and R_{Max} segments for each replicate using an iterative process to determine the best fit for each segment. The duration of the lag period (T_{Lag}) was taken as the period between the point of ferrous iron addition to the point of intersection between the derived R_{Lag} and R_{Max} segments [7].

Liposome size was determined using a Malvern Zetasizer (Malvern, UK) at a refractive index of 1.46 at 37 °C. Measurements were taken before and after each experiment with liposomes found to vary in size between 140 and 155 nm. No significant change in liposome size was detected as a result of time of incubation (120 min), phospholipid composition or peroxidation (results not shown). All results were analysed using 2-way ANOVAS with Bonferroni post-hoc tests using GraphPad Prism 5.04.

3. Results

In liposomes undergoing peroxidation, the duration of the lag phase (T_{Lag}) of peroxidisable phospholipids (PPLs) was increased when non-peroxidisable phospholipids (non-PPLs) were present. Specifically, the duration of the lag phase (T_{Lag}) of liposomes composed of PPLs, Soy PC and PC 16:0/18:2 was extended by the presence of non-PPLs, PC 16:0/16:0 or PC 16:0/18:1. This is shown in Fig. 2A for Soy PC and in Fig. 2B for PC 16:0/18:2 (all liposome preparations with non-PPLs were at a final concentration of 2.5 mM). The insets in each figure show T_{Lag} in the absence of non-PPLs at the same concentration of PPL as in the main graphs (2.5 mM down to 0.5 mM). In the absence of non-PPLs both Soy PC and PC 16:0/18:2 (2.5–0.5 mM) show no change in the duration of their lag phases (Fig. 2A and B insets). The presence of either

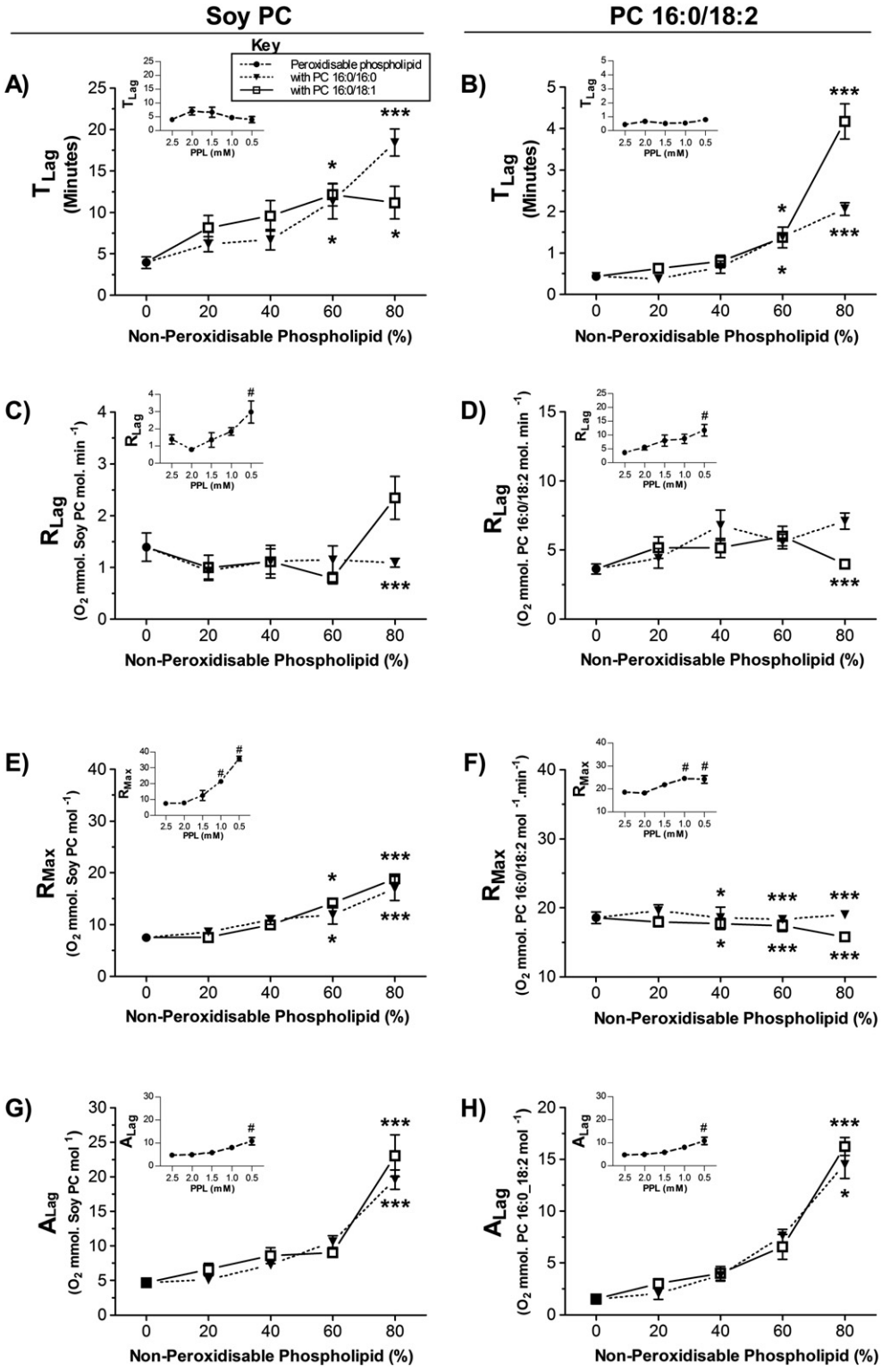

Fig. 2. The peroxidation of the peroxidisable phospholipids (PPLs) Soy PC and PC 16:0/18:2 in the presence of the non-peroxidisable phospholipids (non-PPLs) PC 16:0/16:0 (▼) and PC 16:0/18:1 (□). Parameters measured for Soy PC and PC 16:0/18:2 respectively are, T_{Lag} shown in A) and B), R_{Lag} shown in C) and D), R_{max} shown in E) and F), and A_{Lag} shown in G) and H). Parameters measured for PPLs at concentrations between 2.5 and 0.5 mM, with non-PPLs added to make all final concentrations of phospholipid 2.5 mM (i.e. at between 0.5 and 2.0 mM to make non-PPL at 20–80% of total phospholipid content). With the exception of T_{Lag} all parameters measured are normalised for differences in the concentration of PPL. The inset in each figure shows the response of each PPL (●) for each parameter in the absence of any non-PPL across the same concentration range (2.5–0.5 mM). Significant differences are those comparing PPLs in the presence and absence of non-PPLs where * $p < 0.05$ and *** $p < 0.001$. In the insets, # indicates significant differences at $p < 0.05$ (or less) if any values are statistically different from the original PPL concentration of 2.5 mM.

non-PPL with Soy PC or PC 16:0/18:2 increased the duration of the lag phase, becoming significant when present at 60% and 80% of liposomal phospholipid (Fig. 2A and B). In the presence of PC 16:0/16:0, the lag phase of Soy PC increased by up to 4.7 times (Fig. 2A) and that of PC 16:0/18:2 by up to 2.6 times (Fig. 2B). The presence of PC 16:0/18:1 increased the duration of the lag phase of Soy PC by up to 3.1 fold (Fig. 2A)

and that of PC 16:0/18:2 by up to 5.3 fold (Fig. 2B). One major difference between the two PPLs was the duration of T_{Lag} in the absence of non-PPLs which was approximately 9 fold longer for Soy PC compared to PC 16:0/18:2 (3.9–7.0 min for Soy PC compared to 0.43–0.79 min for PC 16:0/18:2).

The ability of non-PPLs to increase the duration of the lag phase of PPLs appears to be due to their capacity to reduce rates of peroxidation (note: with the exception of T_{Lag} all other parameters were normalised to account for the differences in PPL concentration). This is shown in the rates of peroxidation during the lag (R_{Lag}; Fig. 2C and D) and propagation (R_{Max}; Fig. 2E and F) phases of Soy PC and PC 16:0/18:2 respectively. During the lag phase, R_{Lag} of Soy PC, in the absence of non-PPLs, increased by 2.1 fold (from 1.4 to 3.0 mmol of O_2 per mole of Soy PC per minute, between 2.5 to 0.5 mM; Fig. 2C inset) and that of PC 16:0/18:2 increased by 3.2 fold (from 3.6 to 11.7 mmol of O_2 per mole of PC 16:0/18:2 per minute, Fig. 2D inset). Similarly, in the absence of non-PPLs, the R_{Max} of Soy PC increased 2.1 fold (Fig. 2E inset) and that of PC 16:0/18:2 by 1.3 fold (Fig. 2F inset). The ability of non-PPLs to inhibit the R_{Lag} was supported by measurements of the total amount of oxygen consumed during the lag phase (A_{Lag}) by Soy PC and synthetic PC 16:0/18:2. The presence of non-PPLs increased the total amount of oxygen consumed during the lag phase of both Soy PC (Fig. 2G) and PC 16:0/18:2 (Fig. 2H), being statistically significant when present at 80% of liposomal phospholipid. This inhibition is likely due to the inability of each PPL to reach a critical level of peroxidation product (presumably peroxyl radicals) needed to transition into the propagation phase.

The increases in rates of peroxidation of PPLs can be explained by changes in the Fe^{2+}:PPL ratio as the concentration of each PPL decreased. This occurs because 10 μM ferrous iron does not produce a maximal peroxidation response (a maximal peroxidation response would be non-physiological and overwhelm any physiological antioxidant defence; see Discussion) and as the concentration of each PPL decreased (in the absence of non-PPLs) the relative strength of the iron stimulus increased (from 1:250 to 1:50 Fe^{2+}:PPL). To test this idea, the R_{Max} of Soy PC was measured at both variable (1:250 to 1:50) and constant (1:250) Fe^{2+}:PPL ratios. The result shows (Fig. 3) that increases in the R_{Max} were removed once the Fe^{2+}:PPL ratio was held constant.

4. Discussion

Antioxidant molecules and enzymes are considered key defences in controlling the radical generating reactions of peroxidation. These defence mechanisms act to lengthen the lag phase of peroxidation giving more time to bring the reaction under control and prevent its transition into the more damaging propagation phase [8]. The duration of the lag phase is commonly considered to reflect the antioxidant status of membranes [8]. Many factors influence peroxidation including both physical and chemical aspects of the lipid and their packing within membranes, with polyunsaturated fatty acid (PUFA) content particularly important [5]. Our work suggests that non-peroxidisable phospholipids (non-PPLs) that do not contain PUFA can produce an antioxidant-like action that protects peroxidisable phospholipids (PPLs) that do contain PUFA, therefore preventing the propagation of peroxidation in membranes. This action appears to be accomplished by decreasing the overall rate of peroxidation during the lag phase and impeding the buildup of peroxidation product necessary to enter into propagation phase. As a result, the presence of non-PPLs extends the duration of the lag phase which is likely to be the most important antioxidant-like action produced by these phospholipids in membranes. Non-PPLs commonly account for about half of the phospholipid molecules found in membranes [10] and it is at this level that the significant antioxidant-like activity of non-PPLs extends the lag phase by 2.5–5.3 fold.

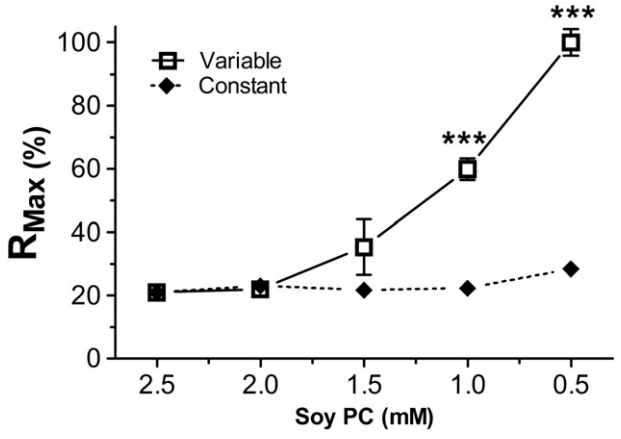

Fig. 3. The relative maximal rates of peroxidation (R_{Max}) of Soy PC at either a constant (1:250; ♦) or variable (1:250 to 1:50; □) Fe than PC 16:0/18:2:Soy PC ratio. Statistical differences show comparisons between measurements at the same Soy PC concentration but different Fe^{2+}:Soy PC ratios, *** indicates $p < 0.001$.

The antioxidant-like action of non-PPLs was found to have a greater influence on the extracted Soy PC than the synthetic PC 16:0/18:2 even though these two phospholipids have a similar acyl composition. It is known that natural Soy lecithin (i.e. PC) has a high level of antioxidants [14] and therefore the extended lag phase of Soy PC versus the synthetic PC 16:0/18:2 is likely due to the presence of antioxidants co-extracted with Soy PC. If this is the case, the greater influence of non-PPLs on Soy PC than PC 16:0/18:2 suggests that there is a synergistic action of the antioxidants in Soy PC with the antioxidant-like action of the non-PPLs. If this is occurring, the antioxidant-like action of non-PPLs could reduce the levels of antioxidant needed in membranes to control peroxidation reactions by providing more time for antioxidants to be recycled and enzymes to work.

Indications of some antioxidant-like actions for non-peroxidisable phospholipids have been previously reported by Lee et al. [15] who examined peroxidation in liposomes made-up of phosphatidylcholines with a constant proportion of 18:2 (37.5%) and varying amounts of 18:1 and 16:0. This study found that "when 16:0 was replaced by 18:1 there was a marked increase in the lag time". The study of Lee et al. did not vary the concentration of non-PPLs and emphasised the role of oleic acid. In the present study the concentration of non-PPLs varied and PC 16:0/16:0 and 16:0/18:1 were both found to be capable of extending the lag phase of Soy PC and PC 16:0/18:2. An antioxidant-like action for PC 16:0/16:0 has also been shown in the study of Soto-Arriza et al. [16] examining the peroxidation of liposomes made-up of egg PC and varying amounts of PC 16:0/16:0 (0–60 mol%). This study found that PC 16:0/16:0 inhibited peroxidation "beyond that expected from the unsaturated lipid dilution". The antioxidant action was interpreted as being due to a rigidification of the bilayer and 'caging' of initiating radicals by steric hindrance. It is likely that the presence of non-PPLs like PC 16:0/16:0 and PC 16:0/18:1 affect how PPLs are packed within bilayers, which in turn is likely to influence the likelihood of these PPLs to undergo peroxidation [5]. The peroxidative cascade requires lipid radicals to come in contact with new lipid in the plane of the monolayer in order to continue peroxidation and this requires diffusion as peroxidation appears not to cross monolayer within the bilayer [17]. The presence of non-PPL may 'cage' lipid radicals within the monolayer and thus slow rates of peroxidation. The ability of different non-PPL to perform this task is likely to depend upon their individual structure and acyl composition. This mechanism appears not to be a simple linear dilution effect as shown for PC 16:0/18:2 where a 20% increase in PC 16:0/18:1 (60–80%) increased lag duration 3-fold.

One reason the antioxidant-like action of non-PPL has only been recognised in a small number of studies [15,16] is that most peroxidation studies use high levels of initiator (e.g. AAPH, Cu^{2+}, Fe^{2+}, or radiation) to maximize the peroxidation response. The levels of ferrous iron needed to produce a maximal peroxidation response (100–1000 μM) would have overwhelmed any protective effect of the non-PPL. In the

present study, the use of a submaximal ferrous iron concentration (10 μM) added extra complexity due to variable Fe^{2+}:PPL ratios, but did allow non-PPL to exhibit their antioxidant capacity. From a biological standpoint, a 10 μM ferrous iron peroxidation stimulus is likely to be extreme, and therefore the strength of the antioxidant-like action of non-PPL in the membrane of normal working cells is likely to be biological relevant.

5. Conclusion

Phospholipids with saturated and monounsaturated fatty acids are some of the most common forms of phospholipid found in membranes. These phospholipids are highly resistant to peroxidation and provide the fundamental structure of bilayers. This study shows that non-peroxidisable phospholipids may play an important role in peroxidation by extending the lag phase as measured using ferrous iron initiated peroxidation. This action is achieved through decreasing the rate of peroxidation that acts to extend the lag phase presenting a novel antioxidant-like action for non-peroxidisable phospholipids in membranes.

Acknowledgements and declaration of interest

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