Biochimica et Biophysica Acta 1848 (2015) 1303–1307

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

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

Colin H. Cortie *, Paul L. Else

School of Medicine (IHMRI), University of Wollongong, 2522, Australia

ARTICLE INFO

Article history:
Received 11 December 2014
Received in revised form 11 February 2015
Accepted 4 March 2015
Available online 11 March 2015

Keywords:
Fatty acids
Peroxidation
Membrane
Liposomes
Ferrous iron

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

* Corresponding author at: School of Medicine, University of Wollongong, Wollongong, NSW 2522, Australia. Tel.: +61 2 42213496; fax: +61 2 42215945.
E-mail address: chc159@uowmail.edu.au (C.H. Cortie).

http://dx.doi.org/10.1016/j.bbamem.2015.03.002
0005-2736/

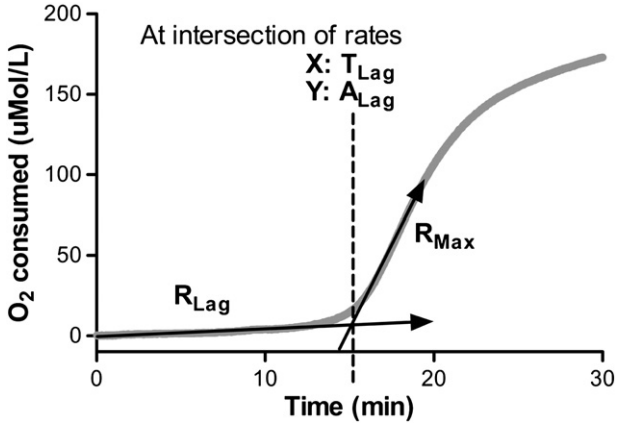

Fig. 1. A sample profile of an in vitro peroxidation reaction of 1.0 mM Soy PC and 1.5 mM PC 16:0/18:1 measured using oxygen consumption. The profile shows a lag phase transitioning into propagation phase with movement towards termination at the end of the profile. The profile indicates the characteristic 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