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Effect of cholesterol in the hydrolysis of oxidized phospholipids in different phosphatidyl choline membrane by PLA_2

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Abstract
In humans oxidative stress leads to the development of certain class of cancer. Upon exposure of cell membranes to oxygen radicals obtained under oxidative stress, phospholipids are the most promissing substrates to oxidation as they owe oxidatively vulnerable polyunsaturated fatty acids predominantly at the *sn-2* position. The oxidized phospholipids are suggested to be repaired by the action of phospholipase A_2. It is because of PLA_2 preferentially hydrolysis oxidized membranes over unoxidized membranes. PLA_2 been hydrolysed PLPC-OOH on incorporation of different mole % of cholesterol into the Soy and egg phosphatidyl choline liposomal membrane. The study emphasizes that amount of cholesterol required for the hydrolysis of PLPC-OOH by PLA_2 will be depending on the type and nature of availability of fatty acid on liposomal membrane.

Keywords: Phospholipase A_2, cholesterol, liposomal membrane

1. Introduction
In humans oxidative stress is thought to be involved in the development of cancer (Halliwell, *et al*., 2007; Brooks M. Hybertson *et al*., 2011) [1, 2], Parkinsons disease (Valko, M., 2007) [3], Alzheimers disease (Pohanka, M., 2013) [4], atherosclerosis, heart failure (Singh, N., 1995) [5], myocardial infraction (Ramond A, *et. al.,* 2011, Dean OM, *et. al.,* 2011) [6, 7]. When cell membranes are exposed to oxygen radicals produced under oxidative stress, phospholipids are the most susceptible substrates to oxidation since they contain oxidatively vulnerable polyunsaturated fatty acids mostly at the *sn-2* position. The oxidized phospholipids are suggested to be repaired by the action of phospholipase A_2 (EC 3.1.1.4, PLA2) (Glende *et al.,* 1986) [8]. PLA_2 preferentially hydrolyzes oxidized membranes over unoxidized membranes (Sevanian *et al.,* 1981; Sevanian *et al.,* 1988; Salgo *et al.,* 1992; Salgo *et al.,* 1993; van Denberg *et al.,* 1993; Rashba-Step *et al.,* 1997) [9-13]. Membrane fluidity is also affected by the presence of cholesterol. The plasma membranes of most cells contain large amounts of cholesterol - up to 50% of the total membrane lipid on a molar basis (Huang *et al.,* 1999: Bach and Wachtel, 2003) [14, 15]. Cholesterol displaces the hydrophilic hydroperoxyl moieties to the surface interface of the liposomal membrane where they are more accessible to PLA_2 hydrolysis (Y. Kambayashi *et. al.,* 1998) [16]. There is a need to understand the amount of cholesterol needed to displace the hydrophilic hydroperoxyl moieties to the surface interface of different liposomal membranes. The present study reports the hydrolysis of 1-palmitoyl-2-linoleoyl phosphatidyl choline (PLPC) hydro peroxide (PLPC – OOH) in cholesterol incorporated Soy-phosphatidyl choline and egg-phosphatidyl choline, liposomal membrane by Crotalus adamanteus venom PLA_2.

2. Materials and Methods
2.1 Determination of Composition of phospholipids by HPLC
The composition of phospholipids was determined by HPLC. The HPLC column was a 4.6mm x 15cm microporasil. The HPLC mobile phase was acetonitrile: methanol: 85% 0-phosphoric acid (780: 10: 0.9) and the flow rate 0.5 ml/min (Hurst and Martin, 1984) [17].

2.2 Purification of soybean lipoxygenase
Soybean LOX was isolated according to the method of Axelrod *et al.,* (1981) [18] with some modifications as described by Sudharshan and Appu Rao, (1997) [19].

2.3 Substrate preparation

2.4 Tween – 20 solubilised substrate

Linoleic acid or phosphatidyl choline with equal amount of Tween – 20 (w/w) were weighed and dissolved in 4.0 ml of oxygen – free water. The resulting suspension was clarified by adding the 0.2 M NaOH. Final volume was made upto 25 ml with 0.2 M borate buffer. This was stored in 1.5 ml capacity eppendorf tubes under nitrogen at 4 ºC until used (Axelord *et al.,* 1981) [18].

2.5 Assay of lipoxygenase

It was assayed by following the appearance of conjugated diene hydroperoxide absorbing at 234 nm using UV 160 Aº spectrophotometer and a molar extinction coefficient of 25,000 M^{-1} cm^{-1}. One unit of enzyme was defined as the formation of 1 µmole of product per min at 25 ºC under assay conditions. The protein concentration was determined using the value $E^{1\%}_{280}$ = 14.0 (Axelrod *et al.,* 1981) [18].

2.6 Preparation of lipid hydroperoxides

PLPC hydroperoxide (PLPC – 00H) was prepared by the aerobic oxidation of PLPC with lipoxygenase as described by Nagata and Yamamoto (1996) 6 µmol of PLPC was dissolved in 20 ml of 0.1 mg sodium borate buffer of p^H 9 containing 3 mM sodium deoxycholate and mixed with 2 mg of soybean lipoxygenase. The solution was stirred for 45 mins at room temperature and purified by HPLC (Superiorex ods column 20 x 250 mm) using 0.02% triethylamine in methanol as mobile phase with a flow rate of 8 ml / min.

2.7 Preparation of unilamellar liposomes

Small unilamellar liposomal suspension was prepared by drying under reduced pressure from a methanolic solution of phosphatidyl choline and butylated hydroxy toluene (BHT) followed by the addition of 50 mM Tris – HCl (pH 7.4) pretreated with chelex 100 shaking vigorously and sonication on an ice bath for 3 min. Final concentration of phosphatidyl choline and butylated hydroxy toluene were 1 mM and 100 µM, respectively. When required 5 mM calcium chloride ($CaCl_2$) was also added.

3. Result

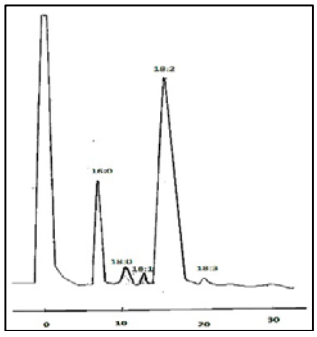
The phosphatidyl choline used in the present study consists of fatty acid as given in table 1. The major fatty acids found in soy phosphatidyl choline were palmitic acid (C 16:0) constituting around 15%, stearic acid (C 18:0) constituting around 8%, oleic acid (C 18:1) constituting around 6%, linoleic acid (C 18:2) constituting around 65% and linolenic acid (C 18:3) constituting around 5%. The major fatty acids found in egg phosphatidyl choline were palmitic acid (C 16:0) constituting around 40%, stearic acid (18:0) constituting around 12%, oleic acid constituting around 45%, linoleic acid constituting around 15% and linolenic acid constituting around 1%. The fatty acid composition of soy and egg phosphatidyl choline was analyzed by gas chromatography after methylation (Fig 1 and Fig 2).

Table 1: Percentage composition of fatty acids in lipids from soy and egg phosphatidyl choline

	16 : 0	18:0	18:1	18:2	18:3
Soy PC	15	8	6	65	5
Egg PC	40	12	45	15	1

3.1 (a) Kinetic studies – in the hydrolysis of oxidized PC in soy and egg PC liposomal membranes by Crotalus adamanteus venom PLA_2

The substrates and products analysis before and after the hydrolysis of soy and egg PC (1 mM) unilamellar liposomal membrane containing PLPC – 00H (18 µM) by Crotalus adamanteus venom PLA_2 (1.0 unit/ml) under aerobic conditions at 37 ºC was carried out. The soy and egg PC liposomes were dispersed in 50 mM Tris HCl containing 5 mM $CaCl_2$ and BHT (100 µM) was added to prevent oxidation during hydrolysis. The decay of Soy-phosphatidyl choline, egg-phosphatidyl choline and PLPC – OOH was measured by normal – phase HPLC analysis using a silica gel column.



Time (mins)

Fig 1: G.C. Profile of Fatty Acid Composition of Soy PC

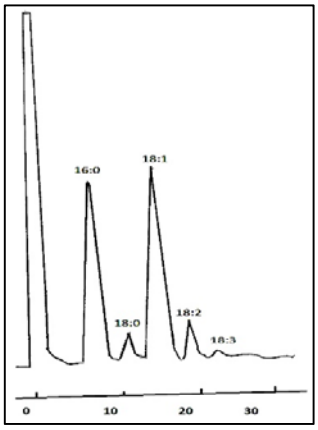


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Fig 2: G.C. Profile of Fatty Acid Composition of Egg PC

Soy-phosphatidyl choline and egg-phosphatidyl choline was quantified by its absorbance at 210 nm and PLPC – OOH was measured at 234 nm. The formation of fatty acids was also measured by UV detection at 210 nm and 18:2 – OOH at 234 nm respectively after separation by reversed – phase chromatography. Fig. 3 and 5 respectively shows the kinetics of the Soy-phosphatidyl choline and egg-phosphatidyl choline liposomes hydrolysis reaction. The quantities of Soy-phosphatidyl choline, egg-phosphatidyl choline and their fatty acids (Fig 3A and 5A) and those of PLPC – OOH and 18:2-OOH (Fig. 3B and 5B) were found to be stable for each sampling. Soy-phosphatidyl choline, egg-phosphatidyl choline and PLPC – OOH were quantitatively hydrolyzed at *sn-2* position to fatty acids and 18:2-OOH, respectively.

3.2 (b) Effect of cholesterol – in the hydrolysis of oxidized PC in soy and egg PC liposomal Membranes by Crotalus adamanteus venom PLA_2

Fig. 4 and 6 respectively shown the effect of cholesterol on the formation of fatty acids and 18:2 OOH during the hydrolysis of Soy-phosphatidyl choline and egg-phosphatidyl choline (1mM) unilamellar liposomal membrane containing PLPC – OOH (57 μM) by Crotalus adamanteus venom PLA_2 (1.0 unit / ml) in 50 mM

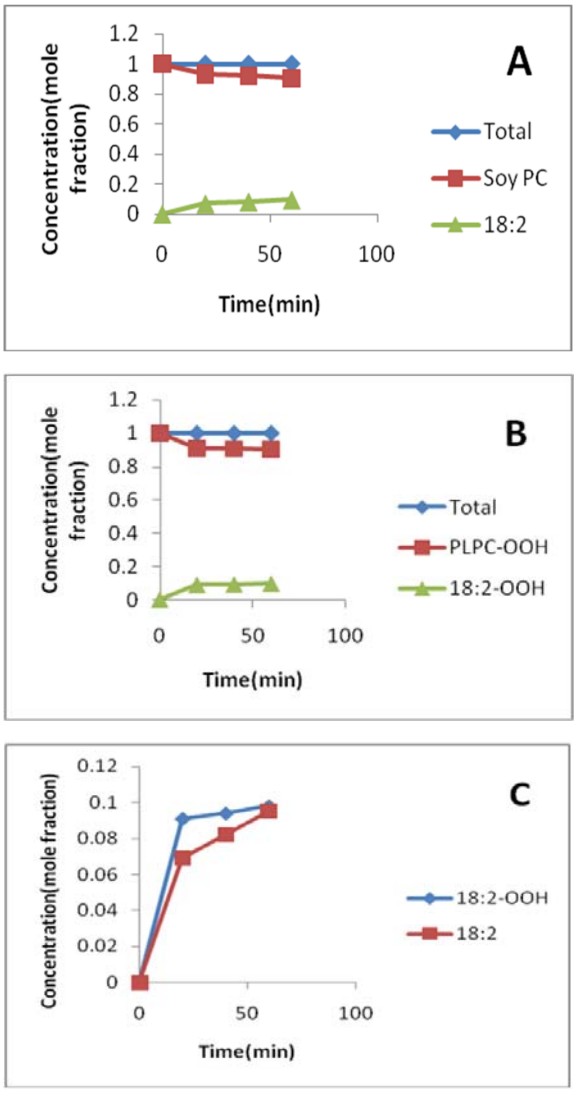

Fig 3: Kinetic studies in the hydrolysis of PLPC-OOH in Soy-PC liposomal membrane by PLA_2

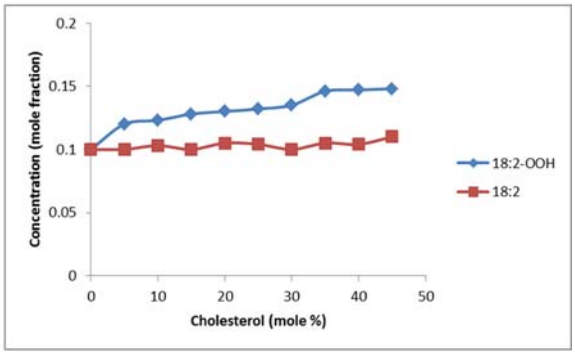

Fig 4: Effect of cholesterol in the hydrolysis of PLPC-OOH in Soy PC liposomal membrane by PLA_2

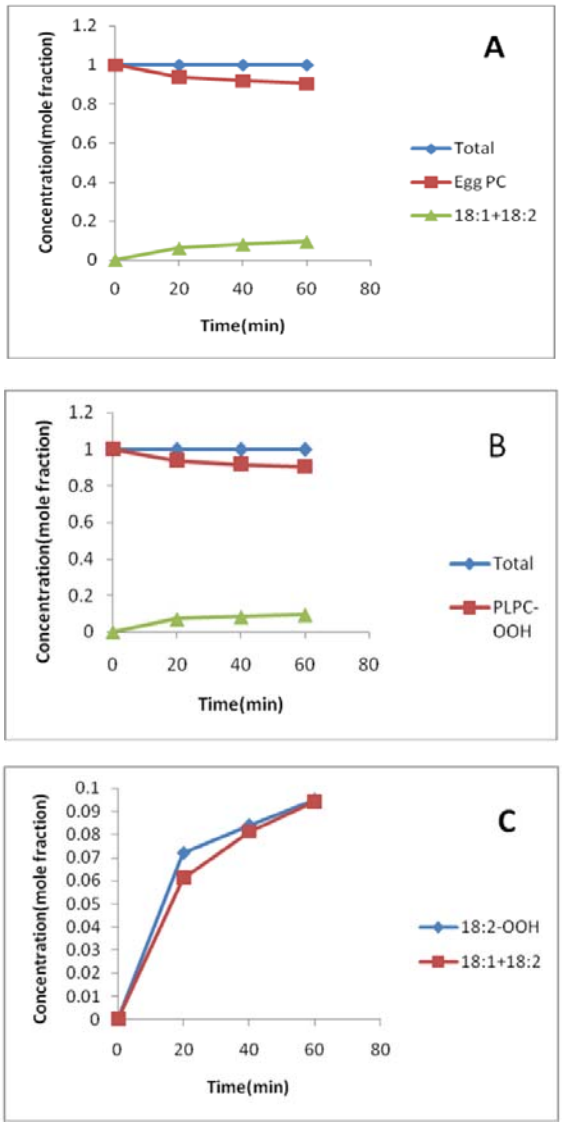

Fig 5: Kinetic studies in the hydrolysis of PLPC-OOH in Egg PC liposomes by PLA_2

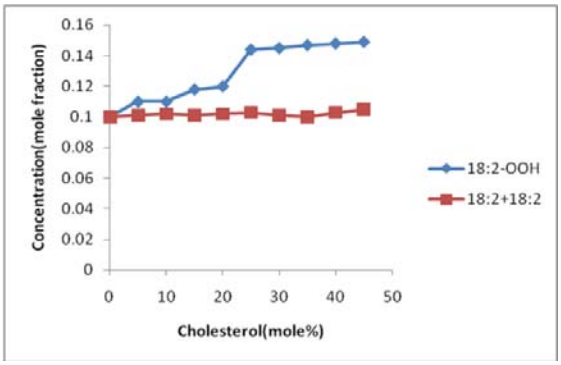

Fig 6: Effect of cholesterol in the hydrolysis of PLPC-OOH in Egg PC liposomal membrane by PLA_2

Tris – HCl (P_H 7.4) with 5 mM $CaCl_2$ for 30 min under aerobic conditions at 37 °C. PLA_2 had a preference to hydrolyze PLPC – OOH over soy PC when more than 35 mole% of cholesterol was incorporated into soy PC liposomal membrane, more than 25 mole % cholesterol is required to hydrolyze PLPC OOH over egg PC in egg PC liposomal membrane.

4. Discussion

The result obtained from the current study indicates the quantities of Soy-phosphatidyl choline and egg-phosphatidyl choline and their fatty acids and those of PLPC– OOH and 18:2 – OOH were stable for each sampling, suggesting that the oxidation of Soy-phosphatidyl choline, egg-phosphatidyl choline and the metal – catalyzed decomposition of hydroperoxides did not occur in the absence of cholesterol. Soy-phosphatidyl choline, egg-phosphatidyl choline and PLPC – OOH were quantitatively hydrolyzed at *sn-2* position to 18:1, 18:2, 18:3 and 18:2-OOH, respectively. Hydrolysis did not proceed in the absence of either PLA_2 or $CaCl_2$. Calcium ion is essential for Crotalus adamanteus venom PLA_2 to bind reactive substrates (Okimasu *et al.,* 1984 and Demmel *et al.,* 1972) [20, 21]. If PLA_2 can preferentially recognize PLPC – OOH over soy and egg PC as a substrate, then the yield of 18:2-OOH (ratio of 18:2 – OOH to initial PLPC – OOH) would be expected to be greater than that of 18:1, 18:2 or 18:3 (ratio of fatty acids to initial soy or egg PC).The yields that PLA_2 did not discriminate between Soy-phosphatidyl choline, egg-phosphatidyl choline as substrate. PLA_2 had a potential to hydrolyze PLPC – OOH over Soy-phosphatidyl choline, egg-phosphatidyl choline when more than 35 mole% of cholesterol incorporated into soy PC liposomal membranes, like wise more than 25 mole% of cholesterol incorporated into egg PC liposomal membrane. This may be due to the interaction of cholesterol depends on the unsaturation and the distribution of the double bonds between the acyl chain and it may also depend on the acyl chain length.

5. Conclusion

The major fatty acids found in soy phosphatidyl choline were palmitic acid (15%), stearic acid (8%), oleic acid (6%), linoleic acid (65%) and linolenic acid (5%). Egg phosphatidyl choline phosphatidyl choline contains palmitic acid (40%), stearic acid (12%), oleic acid (45%), linoleic acid (15%) and linolenic acid (1%). Kinetic studies in the hydrolysis of oxidized phosphatidyl choline in soy and Egg phosphatidyl choline liposomal membranes by Crotalus adamanteus venom PLA_2 were carried out. PLA_2 did not discriminate between soy and egg phosphatidyl choline substrate. PLA_2 had a potential to hydrolyze soy and egg phosphatidyl choline when more than 35 mole % of cholesterol incorporated into soy PC liposomal membrane, like wise more than 25 mole % of cholesterol incorporated into egg PC liposome. Amount of cholesterol required depends on the nature of fatty acids in the membrane.

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