Regulation by vitamin E of phosphatidylcholine metabolism in rat heart

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INTRODUCTION
The principal catabolic pathway for phosphatidylcholine, the major phospholipid in mammalian tissues [1], is through the hydrolytic action of phospholipase A₁ or A₂, which releases an acyl group and results in the formation of lysophosphatidylcholine [2]. Lysophosphatidylcholine can be further decylated by lysophospholipase or, alternatively, it can be reacylated to the parent phospholipid through the reacylation reaction. The decylation–reacylation process is not only an important mechanism for the remodelling of the acyl groups in phospholipids [3], but also serves as the principal pathway by which lysophospholipids are removed from the cell. Since all lysophospholipids are cytolytic at high concentrations, the maintenance of normal cellular function necessitates a rigid control of its transient presence [4]. This is achieved by the combined action of phospholipase A₁, lysophospholipase and acyl-CoA acyltransferase.

A significant increase in lysophosphatidylcholine was observed in the liver of vitamin E-deficient rats [5]. Such an increase was attributed to an increase in mitochondrial phospholipid metabolism [5]. More recently, we demonstrated a direct inhibitory effect of vitamin E on platelet phospholipase A₂ [6]. These studies clearly show that vitamin E may act as a modulator of phospholipid metabolism in mammalian tissues. Although the effect of vitamin E on cardiac phospholipid metabolism had not been studied, electrocardiac abnormalities and cardiac necrosis as a direct result of vitamin E deficiency in the rabbit heart have been well documented [7]. Abnormalities in membrane conductivity and subsequent necrosis appeared to originate from the membrane defects that might result from altered phospholipid metabolism. In the present study we provide evidence that cardiac phospholipase A activity and, consequently, the level of lysophosphatidylcholine in the heart, can be regulated by dietary vitamin E levels.

MATERIALS AND METHODS
Materials
1-[1-¹⁴C]Palmitoyllysophosphatidylcholine (58.5 mCi/mm), 1-stearyloyl-2-[¹⁴C]arachidonylglycerophosphocholine (60.1 mCi/mm), and aqueous counting scintillant were obtained from Amersham International. [Me-³H]Choline chloride was obtained from New England Nuclear Corp. Phosphatidylcholine (from pig liver), lysophosphatidylcholine (from pig liver) and other lipid standards were purchased from Serdary Research Laboratory (London, Ont., Canada). CM-cellulose (CM-11) was the product of Whatman. All other chemicals and t.l.c. plates (Redi-Plate silica-gel G) were obtained from the Fisher Scientific Co. The synthetic vitamin E (α-DL-tocopherol) and natural vitamin E (α-D-tocopherol acetate) were purchased from Sigma Chemical Co.

Animals and diet
Weaning male Sprague–Dawley rats were fed a purified diet consisting of (% by wt.): vitamin-free casein (20), DL-methionine (0.3), cornstarch (10.2), dextrose (50), Alphacel fibre (5), salt mix 4164 (3.5), AIN (American Institute of Nutrition) vitamin mix without tocopherol (1.0) and stripped corn oil (10.0) as described previously [8]. A batch of 20 rats was separated into three groups and fed on this diet containing (a) no added vitamin E, (b) 50 p.p.m. of vitamin E or (c) 5000 p.p.m. of vitamin E as α-D-tocopherol acetate. After 4 months of feeding, vitamin E deficiency or sufficiency was verified by plasma tocopherol values and pyruvate kinase activity [8,9]. The animal was put under light diethyl ether

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anaesthesia and a sample of blood was obtained by aortic puncture [6]. The heart was rapidly removed and stored at −70 °C until used.

Preparation of subcellular fractions from rat heart

Each rat heart was cut into small pieces and homogenized in 10 ml of 20 mM-Tris/HCl buffer, pH 7.5, containing 0.25 M-sucrose and 1 mM-EDTA at 4 °C. The subcellular fractions were prepared by differential centrifugation and the cross-contaminations between fractions were determined as described previously [10].

Preparation of labelled phosphatidylcholine

Labelled phosphatidyl[Me-3H]choline was prepared by perfusion of [Me-3H]choline into the isolated rat heart as described previously [11]. Before perfusion, the lipids were extracted from the heart and the labelled phosphatidylcholine was separated by t.l.c. The specific radioactivity of phosphatidyl[Me-3H]choline obtained was 8000–10000 d.p.m./nmol.

Phospholipase A assay

Phospholipase A activity was assayed with phosphatidyl[Me-3H]choline as substrate [11]. The reaction mixture (0.5 ml) contained 20 mM-Tris/HCl, pH 8.5, 5 mM-CaCl₂ and 200–500 nmol of labelled phosphatidylcholine (3000–4000 d.p.m./nmol) dispersed in water by sonication. Owing to the presence of lyso phospholipase activity in some subcellular fractions, 200 nmol of unlabelled lysophosphatidylcholine was also included in the reaction mixture. As demonstrated previously, the presence of lysophosphatidylcholine effectively inhibited the further hydrolysis of lysophosphatidyl[Me-3H]choline formed, but did not significantly inhibit the hydrolysis of phosphatidylcholine [11]. The assay was initiated by the addition of enzyme (0.3–0.5 mg of subcellular fractions) to the mixture containing the buffer and substrate, and the reaction mixture was incubated at 37 °C for 30 min. The reaction was terminated by the addition of 1.5 ml of chloroform/methanol (2:1, v/v). Water (0.25 ml) was added to cause phase separation. The lysophosphatidylcholine in the lower phase was isolated by t.l.c. with a chloroform/methanol/water/20% acetic acid (70:30:4:2, by vol.) solvent system. When the enzyme activity was assayed in the presence of vitamin E, the vitamin was first dissolved in 6% (v/v) dimethyl sulphoxide [6] and 0.1 ml (containing the appropriate amount of vitamin E) was added to the assay mixture and preincubated for 10 min at 37 °C before the addition of the labelled substrate. The reaction was then initiated by the addition of the labelled substrate. Enzyme activity in the presence of 0.1 ml of 6% dimethyl sulphoxide was used as control.

Determination of phospholipase A₁ and phospholipase A₂ activities

To assay for phospholipase A₁ and phospholipase A₂ activities, 1-stearoyl-2-[14C]arachidonoylglycerophosphocholine was used as substrate [11]. The conditions of the assay were identical with those described in the preceding sub-section. After the reaction, radioactivities in the fatty acid and lysophosphatidylcholine fractions were determined. Phospholipase A₁ activity was calculated from the amount of radioactivity in the lysophosphatidylcholine fraction, whereas phospholipase A₂ activity was estimated in the same manner from the fatty acid fraction.

Lyso phospholipase assay

Lyso phospholipase activities in microsomal and cytosol fractions of rat hearts were assayed with 1-[1-14C]-palmitoyl-sn-glycero-3-phosphocholine [12]. The reaction mixture (0.5 ml) contained 20 mM-Tris/HCl (pH 7.0), 0.2 mM labelled lyso phosphatidylcholine (1200 d.p.m./nmol) and 0.3–0.5 mg of enzyme protein. The reaction was initiated by the addition of microsomal or cytosol preparation. After incubation at 37 °C for 30 min, the reaction was terminated by the addition of chloroform/methanol (2:1, v/v). The lipids were separated by t.l.c. with, as solvent system, chloroform/methanol/water/acetic acid (70:30:4:2, by vol.). Enzyme activity was calculated from the radioactivity associated with the fatty acid fraction.

Partial purification of phospholipase A from rat cytosol

Rat heart cytosol (20 ml) was applied to a column (1 cm × 21 cm) of CM-cellulose equilibrated with 50 mM-Hepes buffer/5 mM-EDTA, pH 7.4. The column was washed with 120 ml of the same buffer, followed by the application of a linear gradient of 0–0.5 M-NaCl in 200 ml of the same buffer. Each fraction was assayed for phospholipase A activity. Most phospholipase A (containing both phospholipase A₁ and A₂ activities) was eluted from the column at 0.1 M-NaCl. The fractions containing high enzyme activity were pooled and used for kinetics studies.

Other determinations

Plasma pyruvate kinase activity [9] and lyso phosphatidylcholine:acyl-CoA acyltransferase activities from the subcellular fractions of the rat heart were determined by procedures described in earlier studies [13]. Serum tocopherol was determined by h.p.l.c. [14]. The lipid phosphorus of the phospholipid fractions isolated by t.l.c. was determined by the procedure of Bartlett [15]. Protein was determined by the method of Lowry et al. [16], with bovine serum albumin as standard.

Analysis of data

Radioactivity was determined with an LKB liquid-scintillation counter. Counting efficiency was calculated by the channel-ratio calibration method. Student’s t test was used for statistical analysis. The level of significance was set at P < 0.05.

RESULTS

Vitamin E status of animals

The plasma pyruvate kinase activity, an indicator of myopathy, and plasma tocopherol levels were used to verify the vitamin E status of the rats. Pyruvate kinase activities in the vitamin E-deficient rats were markedly elevated in comparison with rats fed with 50 or 5000 p.p.m. dietary vitamin E. Plasma tocopherol levels, quantified by h.p.l.c., were found to be significantly different in all three groups of rats. The differences are in accordance with the level of vitamin E present in the diets (Table 1) [6]. There were no significant differences in body weight or heart weight among these three groups of rats.
Table 1. Effect of dietary vitamin E on plasma pyruvate kinase activity and tocopherol content

Plasma pyruvate kinase activity and tocopherol content were determined as described in the Materials and methods section. The results are expressed as means ± s.d. (n). Means not sharing a common superscript letter are significantly different (P < 0.05).

<table>
<thead>
<tr>
<th>Dietary vitamin E (p.p.m.) . . .</th>
<th>0</th>
<th>50</th>
<th>5000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvate kinase activity (units/ml)</td>
<td>2.27 ± 0.41 (7)*</td>
<td>0.35 ± 0.12 (7)b</td>
<td>0.10 ± 0.02 (6)a</td>
</tr>
<tr>
<td>[Tocopherol] (mg/dl)</td>
<td>0.10 ± 0.01 (7)*</td>
<td>1.24 ± 0.25 (7)b</td>
<td>2.65 ± 0.19 (6)a</td>
</tr>
</tbody>
</table>

Table 2. Phospholipid composition in the hearts of rats fed different levels of vitamin E

Lipids were extracted from the hearts of the rats, and phospholipid classes were separated from the lipid extracts by t.l.c. Only the phospholipid classes containing more than 0.1 μmol of phospholipid/g wet weight are given. The results are expressed as means ± s.d. calculated from four separate experiments. Means not sharing a common superscript letter are significantly different (P < 0.05).

<table>
<thead>
<tr>
<th>Phospholipid(s)</th>
<th>Dietary vitamin E (p.p.m.) . . .</th>
<th>Composition (μmol/g of heart)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>9.76 ± 0.35</td>
<td>10.08 ± 0.24</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>12.04 ± 1.00</td>
<td>12.66 ± 0.88</td>
</tr>
<tr>
<td>Phosphatidylserine and</td>
<td>1.29 ± 0.23</td>
<td>1.23 ± 0.12</td>
</tr>
<tr>
<td>phosphatidylinositol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphatidic acid and</td>
<td>3.56 ± 1.21</td>
<td>3.87 ± 1.74</td>
</tr>
<tr>
<td>cardiolipin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>0.69 ± 0.05</td>
<td>0.65 ± 0.03</td>
</tr>
<tr>
<td>Lysophosphatidylcholine</td>
<td>0.26 ± 0.06</td>
<td>0.12 ± 0.02</td>
</tr>
</tbody>
</table>

Table 3. Effect of dietary vitamin E on phospholipase A activity in the subcellular fractions of rat heart

Phospholipase A (phospholipase A₁ and A₂) activity in rat heart subcellular fractions was assayed using rat heart phosphatidyl[Me-3H]choline as substrate. Enzyme activity was calculated from the radioactivity associated with lysophosphatidylcholine after the reaction. The results are expressed as means ± s.d. (n). Means not sharing a common superscript letter are significantly different (P < 0.05).

<table>
<thead>
<tr>
<th>Subcellular fraction</th>
<th>Dietary vitamin E (p.p.m.) . . .</th>
<th>Enzyme activity (nmol/h per mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>Mitochondrial</td>
<td>8.63 ± 1.27 (7)*</td>
<td>6.99 ± 0.73 (7)b</td>
</tr>
<tr>
<td>Microsomal</td>
<td>9.07 ± 1.11 (7)*</td>
<td>7.93 ± 0.81 (7)b</td>
</tr>
<tr>
<td>Cytosolic</td>
<td>5.79 ± 0.94 (7)*</td>
<td>4.44 ± 1.17 (7)b</td>
</tr>
</tbody>
</table>

Effect of vitamin E on phospholipid composition in the rat hearts

Rat hearts were homogenized in 20 mM-Tris/HCl/0.25 M-sucrose, pH 7.5, and lipids were extracted from a portion of the homogenate by the procedure described by Folch [17]. The phospholipid classes were separated by t.l.c., and the amount of lipid phosphorus in each fraction was determined. As depicted in Table 2, no significant difference in major cardiac phospholipids was detected between the animal groups. However, levels of lysophosphatidylcholine were significantly elevated in the heart of the rats fed the vitamin E-deficient diet.

Effect of vitamin E on rat heart phospholipase A

Since phospholipase A (both phospholipase A₁ and A₂) is the major enzyme responsible for the generation of lysophosphatidylcholine, its activity in the subcellular fractions of the rat heart among the three animal groups was measured. Phospholipase A activity was determined with phosphatidyl[Me-3H]choline as substrate, enzyme activity being estimated by the amount of radioactivity from the lysophosphatidylcholine fraction after the reaction (Table 3). Enzyme activities were significantly elevated (P < 0.05) in all subcellular fractions of vitamin E-deficient-rat hearts when compared with those of
Enzyme activities were assayed with 1-stearoyl-2\([^{14}C]\)arachidonylglycerophosphocholine as substrate. After incubation, phospholipase A₁ activity was estimated from the radioactivity present in the lysophosphatidylcholine fraction, whereas phospholipase A₂ activity was determined from the radioactivity associated with the released free arachidonate. Values are means±s.d. from three separate samples in each group, and each sample was determined in duplicate. Phospholipase A₁ activity (3.92 nmol/h per mg) and phospholipase A₂ activity (6.10 nmol/h per mg) in the 50 p.p.m.-vitamin E-supplemented group are expressed as 100%. The activities of phospholipase A₁ or A₂ are significantly different between all groups \((P < 0.05)\).

Effect of exogenous vitamin E on the activity of phospholipase A

The direct effect of vitamin E on phospholipase A activity in the rat heart was investigated. Different amounts of \(\alpha\)-DL-tocopherol were added to the subcellular fractions before enzyme assays. Addition of vitamin E markedly lowered phospholipase A activity in all rat heart subcellular fractions (Fig. 2). Over the range of vitamin E tested (0.025–1.0 mm), the magnitude of inhibition was similar among various cardiac subcellular fractions.

In order to understand better the mechanism of inhibition by vitamin E, rat heart cytosolic phospholipase A was partially purified by column chromatography, and the fractions that contained high phospholipase A activities devoid of lysophospholipase activity were pooled and used for kinetic studies. Fig. 3 shows that partially purified phospholipase A activities were also inhibited by added tocopherol in a dose-dependent manner. The double-reciprocal plot clearly indicates that the nature of inhibition was essentially non-competitive.

Effect of vitamin E on lysophospholipase and lysophosphatidylcholine:acyl-CoA acyltransferase activities

The significant increase in lysophospholipid from the vitamin E-deficient rat hearts depicted in Table 2 could be due to an increase in endogenous phospholipase A activities, or, conversely, decreases in lysophospholipase or lysophosphatidylcholine:acyl-CoA acyltransferase activity. Hence the activities of these two major enzymes responsible for the metabolism of lysophosphatidylcholine in the heart subcellular fractions of the three

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**Fig. 1. Phospholipase A₁ and A₂ activities in the microsomal fractions from the heart of rats fed three levels of dietary vitamin E**

**Fig. 2. Inhibition in vitro of phospholipase A activity by \(\alpha\)-DL-tocopherol**

The subcellular fractions from normal rat hearts were preincubated with \(\alpha\)-DL-tocopherol for 10 min at 37 °C before assay. The reaction was initiated by the addition of phosphatidyl[\(\text{Me-}^{3}\text{H}\)]choline as substrate. Enzyme activity was calculated from the amount of radioactivity associated with lysophosphatidylcholine fraction after the reaction. Symbols used: \(\bullet\), cytosol; \(\bigcirc\), mitochondria; \(\Delta\), microsomal fraction. Values depicted are means ± s.d. for three separate samples, each determined in duplicate.
animal groups were determined. As depicted in Table 4, there is no significant difference in the activities of cardiac lysophospholipase or acyltransferase in the subcellular fractions among the three animal groups. Our results thus indicate that dietary vitamin E has no effect on the enzymes of lysophospholipid metabolism.

DISCUSSION

Although the inhibition of phospholipase A activity by vitamin E has been reported in the liver and platelets [5,6], this is the first study to show that this key enzyme of phosphatidylcholine catabolism in the rat heart is also inhibited by vitamin E. It is clear from the present study that the vitamin E status in the animal is an important biochemical factor for the maintenance of lysophosphatidylcholine levels in the heart. Since the vitamin E content in the cardiac tissue has recently been shown to be directly proportional to dietary vitamin E intake [18], our results indicate that there is a reverse relationship between phospholipase A activity in the rat heart and the tocopherol content of the tissue. The other lysophosphatidylcholine metabolic enzymes in the heart are not affected by the vitamin E status of the animal.

It is noteworthy that phosphatidylcholine isolated from the heart was used as substrate for the assay of phospholipase A activity. Cardiac phospholipase A was found to display acyl-group-specificity towards certain phosphatidylcholine species [11]. The problems and limitations of using synthetic substrates (with defined acyl groups) for the studies of phospholipase A in mammalian tissues have been well-documented [11,19]. We consider that the use of a natural substrate (with mixed acyl groups) would provide us with a better assessment of enzyme activity. Owing to the difficulties in obtaining uniform labelling of the different acyl groups in the natural substrate, the radioactive label was placed at the base group. The use of phosphatidyl[Me-3H]choline as substrate allowed us to assay for the sum of phospholipase A1 and A2 activities simultaneously [11] by monitoring the formation of lysophosphatidyl[Me-3H]choline. In order to discriminate the effect of vitamin E between phospholipase A1 and phospholipase A2, it was necessary to use a phospholipid substrate containing a labelled acyl group [11,19]. The utilization of 1-stearoyl-2-[14]C-arachidonoylphosphatidylcholine as substrate permitted us to assay for phospholipase A1 and A2 activities simultaneously by monitoring the formation of labelled lysophosphatidylcholine and labelled fatty acid fractions respectively. The sum of phospholipase A1 and A2 activities obtained from this assay (10.02 nmol/h per mg of microsomal protein; Fig. 1) was not the same as that obtained with phosphatidyl[Me-3H]choline (7.93 nmol/h per mg of microsomal protein; Table 3). Such a discrepancy was observed in our previous studies [11] and probably resulted from the specificity of the enzyme towards phosphatidylcholine with an arachidonoyl group [11] as well as the difference in the dispersion of substrates. Since lysophospholipase activity is present in the cytosolic and microsomal fractions [11,20], the addition of unlabelled lysophosphatidylcholine in the assay mixture was shown to be effective in the inhibition of the hydrolysis of the labelled lysophosphatidylcholine formed in the reaction under normal assay conditions [11]. However, the presence of high concentrations of lysophosphatidylcholine was

Table 4. Effect of dietary vitamin E on rat heart lysophospholipase and lysophosphatidylcholine:acyl-CoA acyltransferase activities

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Subcellular fraction</th>
<th>Dietary vitamin E (p.p.m.)</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lysophospholipase (nmol/h per mg of protein)</td>
<td>Microsomal</td>
<td>30.00 ± 5.04</td>
<td>31.50 ± 3.40</td>
</tr>
<tr>
<td>Lysophosphatidylcholine:acyl-CoA transferase (nmol/min per mg of protein)</td>
<td>Cytosolic</td>
<td>51.54 ± 3.13</td>
<td>50.82 ± 2.55</td>
</tr>
<tr>
<td></td>
<td>Microsomal</td>
<td>10.1 ± 2.9</td>
<td>13.4 ± 2.3</td>
</tr>
</tbody>
</table>

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mechanism may result from the elevated levels of lysosphatidylcholine and other lysophospholipids. In the heart of the vitamin E-deficient animal may be one of the biochemical factors preceding the cardiac dysfunctions observed in previous studies [7].

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REFERENCES


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