IN VITRO STUDIES ON THE ACYLATION OF 1-*O*-ALKENYL GLYCERO-3-PHOSPHORYLETHANOLAMINE BY RAT BRAIN PREPARATIONS

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1. Introduction

Studies on the elucidation of plasmalogen biosynthesis carried out in a number of laboratories on a variety of tissues [1–10] and our unpublished *in vivo* experiments with 15 day old rat brain have indicated that hexadecanol serves as a better precursor for the ether linked long chain moieties of ethanolamine phospholipids. These studies further suggested that 1-*O*-alkyl lipids serve as precursors for the 1-*O*-alkenyl lipids. Based on the available data, the formation of ethanolamine plasmalogen has been proposed to proceed via the following sequence of reactions [11–13]: Dihydroxyacetone phosphate + alcohol → 1-*O*-alkyl dihydroxyacetone phosphate $\xrightarrow{\text{NADPH}}$ 1-*O*-alkyl glycerophosphate → 1-*O*-alkyl 2-acyl glycerophosphate → 1-*O*-alkyl 2-acyl glycerol + CDP ethanolamine → 1-*O*-alkyl 2-acyl GPE → 1-*O*-alkenyl 2-acyl GPE*. However, the exact stage at which biodehydrogenation takes place is not established and several possibilities exist. *In vivo*, [3, 7, 10, 13–15] as well as *in vitro* experiments [16–18] indicated that intact 1-*O*-alkyl 2-acyl GPE is desaturated to 1-*O*-alkenyl 2-acyl GPE. On the other hand, *in vivo* studies of Debuch and her colleagues [19, 20] with myelinating rat brain suggested that the plasmalogen formation involves dehydrogenation of the ether bond to form an enol–ether bond mainly at the stage of lysophosphatides followed by acylation of lysoplasmalogen and this possibility has also been considered by Paltauf [13]. Supporting this pathway, the acylation of 1-*O*-alkenyl GPE giving rise to ethanolamine plasmalogens by 16 day old rat brain cell free preparations is reported here.

* *Abbreviations*:
GPE: glycerophosphorylethanolamine; GPC: glycerophosphorylcholine; DTE: dithioerythritol; TLC: thin-layer chromatography.

2. Methods

2.1. Preparation of ^{32}P-labelled 1-alkenyl GPE

[^{32}P]Orthophosphate in saline was injected intracerebrally to 15 day old rats (approx. 300 μCi/animal). The rats were sacrificed 24 hr after injection, brains removed and the total lipids were extracted [21]. The lipids were freed from proteolipid protein [22] and ethanolamine phospholipids were separated by preparative TLC on silica gel C (ACME Synthetic Chemicals, Pocna) using chloroform:methanol:water (65:25:3, v/v/v) as solvent. 1-Alkenyl GPE was prepared as described by Waku and Lands [23] starting with 80 μmoles of ethanolamine phospholipid. The crude 1-alkenyl GPE was chromatographed on a 5 g column of silicic acid and eluted with 30 ml each of chloroform, chloroform:methanol (4:1, v/v), chloroform:methanol (3:2, v/v), and chloroform:methanol (2:3, v/v). 10 ml fractions were collected. 1-Alkenyl GPE was eluted mostly with chloroform:methanol (3:2, v/v) and with the first fraction of chloroform:methanol (2:3, v/v). The fractions containing 1-alkenyl GPE were pooled. This gave a single spot on TLC and it was further characterised by chromatography on TLC using chloroform:methanol:water (65:25:3, v/v/v), exposure to HCl fumes and chromatography in the

second direction with chloroform:methanol:ammonia (100:50:12, v/v/v) [24]. This procedure indicated that 92% of the total counts and 95% of lipid phosphorus were present in 1-alkenyl GPE. Thus the preparation contained about 5–8% of 1-alkyl GPE.

2.2. Enzyme assay

Sixteen day old rats were killed by decapitation and brain homogenates were prepared in 0.25 M sucrose containing 10^{-3} M EDTA (pH 7.1) using 4 ml/g tissue. The mitochondrial and microsomal fractions were prepared by centrifugation at 22,000 *g* for 30 min and 100,000 *g* for 30 min, respectively, as described by Ansell and Spanner [25]. The brain preparations were incubated with 1-alkenyl GPE in a total volume of 1.2 ml as described in the tables and the reaction was terminated by the addition of 3 ml of methanol. The lipids were extracted from the assay mixture by the method of Bligh and Dyer [26]. The lipid extract was resolved by one-dimensional TLC using chloroform: methanol:water (65:25:3, v/v/v) and counts in ethanolamine phospholipid spot were determined with a Beckman LS 100 liquid scintillation counter. The radioactivity in the ethanolamine phospholipid spot was taken as a measure of the enzyme activity. The radioactivity in the upper aqueous methanol phase of the lipid extracts of enzyme digests was taken as a measure of the lysoplasmalogenase activity. The values expressed in tables were corrected for zero min controls.

Phosphorus was estimated by the method of Bartlett [27] and protein by Lowry et al. [28].

3. Results and discussion

Acylation of 1-alkenyl GPC with long chain fatty acid thioesters was demonstrated in human erythrocytes and rabbit muscle [23]. Similar acyltransferase reactions were also shown to occur with both 1-alkenyl- or 1-alkyl GPC in rabbit sarcoplasmic reticulum and Ehrlich Ascites tumor cells [29, 30] and to a small extent in intestinal mucosa [31] but no appreciable enzymatic activity could be observed in brain and heart homogenates [23]. In contrast, acylation of ether linked lysoethanolamine phosphatides has not been report-

Table 1
Acylation of ^{32}P-labelled 1-alkenyl GPE by rat brain preparations.

Incubation mixture	Experiment 1		Experiment 2		
	Protein (mg)	1-alkenyl 2-acyl GPE formed (nmoles/mg/hr)	Protein (mg)	1-alkenyl 2-acyl GPE formed (nmoles/mg/hr)	Lysoplasmalogenase activity (nmoles/mg/hr)
1. *Homogenate*					
Complete	1.81	3.2	1.88	3.5	6.5
−ATP, −CoA		1.6		1.4	3.6
2. *Mitochondria*					
Complete	0.44	8.8	0.63	7.5	9.7
−ATP, −CoA		4.3		4.4	6.7
3. *Microsomes*					
Complete	1.09	4.8	1.11	5.4	9.6
−ATP, −CoA		3.1			
4. *Supernatant*					
Complete			2.23	0.7	2.6
−ATP, −CoA				0.5	1.9

Complete system consisted of ^{32}P-labelled 1-alkenyl GPE (109.2 nmoles; 13,436 cpm) dissolved in 0.1 ml of ethanol and dispersed by the addition of 0.1 ml of Tris buffer (0.2 M; pH 7.1); other additions were 0.1 ml of ATP-Mg^{2+} (8 μmoles); 0.1 ml CoA (0.2 μmoles); 0.8 ml of the enzyme preparation. The final volume was adjusted to 1.2 ml with 0.25 M sucrose-EDTA solution. Incubation was carried out at 37° for 45 min. The values given for the ethanolamine plasmalogen formed were after correcting for zero minute control values.

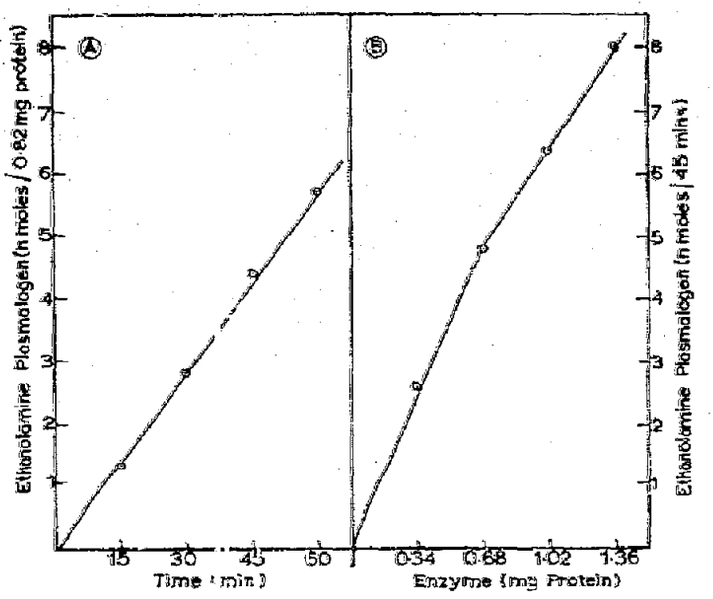

Fig. 1. A) Effect of time on the formation of ethanolamine plasmalogen. 122.5 nmoles, 19,647 cpm of ^{32}P-labelled 1-alkenyl GPE, 2 mg of Tween-80, 0.5 ml of mitochondrial preparation (0.82 mg of protein), 0.3 ml of Tris buffer (0.2 M, pH 7.1), 0.1 ml of ATP-Mg^{2+} (8 μmoles, 0.1 ml of CoA (0.2 μmoles) were incubated in a total volume of 1.2 ml at 37°. B) Effect of protein concentration on the formation of ethanolamine plasmalogen. Conditions were similar to A except the protein concentration was varied and incubated for 45 min. The results are expressed in terms of nmoles formed/45 min.

ed and experiments with rabbit muscle were not definitive [23]. One of the reasons for the inability to observe acylation of 1-alkenyl GPE may be that the acyltransferase is specific towards unsaturated long chain fatty acids such as $C_{20:4}$, $C_{22:4}$ and $C_{22:6}$ which normally occupy position 2 of the glycerol moiety in these lipids in brain. Indeed, Waku and Nakazawa [29] have observed acyltransferase activity towards 1-alkenyl and 1-alkyl GPC only with linoleyl CoA in rabbit sarcoplasmic reticulum. In the experiments reported here, when ^{32}P-labelled 1-alkenyl GPE was incubated with rat brain preparations, the formation of ethanolamine plasmalogen has been observed indicating the acylation of 1-alkenyl GPE.

From table 1 it may be seen that brain homogenate exhibits enzyme activity which is found mainly associated with mitochondrial and microsomal fractions. The activity is enhanced when CoA and ATP were added and the fatty acids are presumably of endogenous origin. The formation of ethanolamine plasmalogen was found to be proportional to time as well as protein concentrations when mitochondrial pellet was used as enzyme source (fig. 1), while boiled enzyme showed very little activity (table 2). It has been observed that during the course of incubation, a part of the ^{32}P radioactivity (approx. 10–12% of the substrate used) appeared in the water soluble form, the remainder being recovered as unreacted substrate or as product. The radioactivity in the water phase is presumed to be GPE formed due to the hydrolysis of vinyl ether bond. The presence of ethanolamine lysoplasmalogenase has been demonstrated earlier [32]. When the ethanolamine plasmalogen formed enzymatically was analysed by two-dimensional TLC after exposure to HCl fumes (see Methods), more than 90% of the incorporated radioactivity was recovered as 2-monoacyl GPE. This indicates that the primary product is 1-alkenyl 2-acyl GPE. Addition of dithioerythritol has no effect on the enzymic reaction (table 2), but Tween-80 slightly activated ethanolamine plasmalogen formation, while Triton-X 100 was found to be highly inhibitory.

Table 2

Effect of dithioerythritol, Tween-80 and Triton-X 100 on the acylation of ^{32}P-labelled 1-alkenyl GPE by the mitochondrial and microsomal preparation of rat brain.

Incubation mixture	1-alkenyl 2-acyl GPE formed (nmoles/mg/hr)	
	Mitochondria	Microsomes
Complete	8.0	5.4
+ DTE (1 μmole)	6.7	5.4
+ Tween-80 (2 mg)	10.8	7.5
+ Triton-X 100 (2 mg)	0.0	1.8
Boiled enzyme	1.06	0.52

Complete system consisted of ^{32}P-labelled 1-alkenyl GPE (109.6 nmoles, 13,436 cpm) dissolved in 0.1 ml of ethanol and dispersed by the addition of 0.3 ml of Tris buffer (0.2 M; pH 7.1), other additions were 0.1 ml of ATP-Mg^{2+} (8 μmoles), 0.1 ml of CoA (0.2 μmoles) and 0.5 ml of the mitochondrial (0.42 mg) or microsomal (1.1 mg) enzyme preparation. The final volume of the assay mixture being 1.2 ml. Incubation was carried out at 37° for 45 min. Wherever Tween-80 or Triton-X 100 was used ethanol was omitted. The values reported were corrected for zero min controls.

These results suggest that acylation of 1-alkenyl GPE occurs in 16 day old rat brain and support the hypothesis [19, 20] that this reaction may be involved in the biosynthesis of ethanolamine plasmalogen.

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