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EXPERIMENTAL MEDICINE

Enzymic Synthesis of Ethanolamine Plasmalogens in the Microsomal Fraction of Rat Brain under Oxygen Deficiency

by

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Summary. The conversion of O-/-1-alkyl-sn-glycero-3-phosphoryl/-ethanolamine to ethanolamine plasmalogen by rat brain microsomes under oxygen deficiency is decreased by about 60%. Acylation of 1-alkyl-sn-glycero-3-phosphorylethanolamine to 1-alkyl-2-acyl-sn-glycero-3-phosphorylethanolamine is reduced by about 72%. Inhibition of plasmalogene biosynthesis in CNS under hypoxia and ischemia may be one of the factors responsible for the changes of structure and function in microsomal membranes.

Under oxygen deficiency, phospholipid level in the microsomal fraction from brains decreases. The synthesis of ethanolamine and choline phospholipids particularly of ether type is inhibited markedly as it was demonstrated in previous investigations [15].

In the present study we have examined the last step of the synthesis of ethanolamine plasmalogens (1-alk-1′-enyl-2-acyl-sn-glycero-3-phosphorylethanolamine) in the microsomal fraction obtained from hypoxic and ischemic brains. The process of enzymic dehydrogenation of 1-alkyl-sn-glycero-3-phosphorylethanolamine to ethanolamine plasmalogen and the acylation reaction of 1-alkyl-sn-glycero-3-phosphorylethanolamine to 1-alkyl-2-acyl-sn-glycero--3-phosphorylethanolamine were studied.

Materials and methods

Wistar rats, weighing ±200 g were used to the experiments. The animals were decapitated and the isolated brains were homogenized in a teflon homogenizer with 10 volumes of 0.32 M sucrose, by 10 up and down strokes at 350 rpm. The total homogenate was centrifuged for 10 min at 1,000 *g*. The supernatant was carefully decanted and centrifuged for 20 min at 18,000 *g*. The resulting supernatant was recentrifuged as above. The microsomal fraction was obtained by the centrifugation of the supernatant for 60 min at 105,000 *g*. The pellet was suspended in 0.1 M buffer Tris-HCl pH 8.0, and after protein estimation, was used to the experiments. The whole procedure was carried out at 0-4°C. MSE centrifuges: Magnum and Super Speed 65 were used.

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In a similar manner was obtained the microsomal fraction from brains of rats submitted previously to hypoxia and ischemia. Hypoxia was carried out by keeping the animals for 2-3 min periods in a chamber saturated with nitrogen with subsequent reanimations, the whole procedure lasting for 30 min. In the case of ischemia, the decapitated head of the animal was incubated at 37°C for 5 min. The substrate, [^{14}C] 1-alkyl-sn-glycero-3-phosphorylethanolamine, was obtained by the following procedure: [^{14}C] sodium acetate in aqueous solution was administrated intracerebrally to 9 16-day-old rats. After 24 h the animals were decapitated and the lipids were extracted according to Folch [5]. Then, using column chromatography on Florisil 100-200 mesh [6], the phosphatidylethanolamine fraction was isolated and subsequently hydrogenated in the presence of platinum oxide, as catalyzer. The hydrogenated compound was gently hydrolyzed according to Dawson [2]. Alkyl-sn-glycero-3-phosphorylethanolamine was obtained after separation on a silic acid column [6]. The purity of the substrate was checked using thin layer chromatography.

Protein was estimated according to Lowry *et al.* [9].

Phosphorus of phospholipids was assayed by the method of Bartlett [1], and plasmalogens according to Feulgen *et al.* [4]. The incubation mixture contained in a final volume of 0.3 ml: 10 mM ATP, 0.1 mM CoA, 4 mM $MgCL_2$, 2 mM NADP, 6.7 mM glucose-6-phosphate, 5 units of glucose-6-phosphate dehydrogenase, 0.1 M Tris-HCl buffer, pH 7.6, 0.006% Tween 20, as well as the microsomal protein and the substrate in concentrations as given in the tables. The lipid substrate was suspended before use in 0.1 M Tris-HCl buffer, pH 7.6 and 0.06% Tween 20, and sonicated for 2 min on a MSE Ultrasonic Disintegrator. The incubation was carried out in oxygen atmosphere at 37° for 30 min. The reaction was stopped adding 19 volumes of the mixture chloroform - methanol 2:1 v/v.

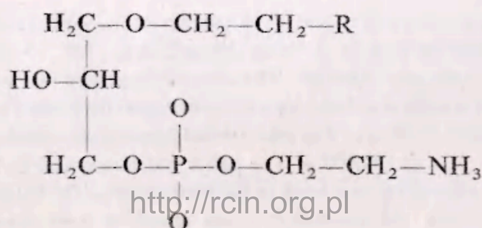
Extraction and analysis of phospholipids. Lipids were extracted according to Folch [5] and separated using two-dimensional thin-layer chromatography [14] on silica gel Kiselgel H. The plate was developed in two directions using as first solvent chloroform-methanol-water, 65:25:4 (v/v/v) and as the second: petroleum ether-ethyl ether, 80:20 v/v. After the first development the plates were dried and exposed to HCl fumes for 5 min. After the elimination of HCl vapours, the plate was developed in the second direction.

The radioactivity was determined in spots of free aldehydes (using 15 ml of Bray scintillations liquid) on an Isocap 300 Nuclear Chicago scintillation counter.

Reagents. ^{14}C sodium acetate — Institute of Nuclear Research, Świerk, Poland; Florisil — Koch-Light Lab. Ltd; silic acid Malinckrodt — Fa. C. Roth, Karlsruhe; Kiselgel G and H — E. Merck A. G. Darmstadt; ATP, CoA, NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase-BDH; Tween 20 — Atlas Goldschmidt GmBH, Essen, Germany

Results

In the studies on plasmalogen dehydrogenase from microsomal fraction of adult rat brains, in conditions of normoxia and oxygen deficiency, ^{14}C labelled O-/-1-alkyl-sn-glycero-3-phosphoryl/ethanolamine was used as substrate.



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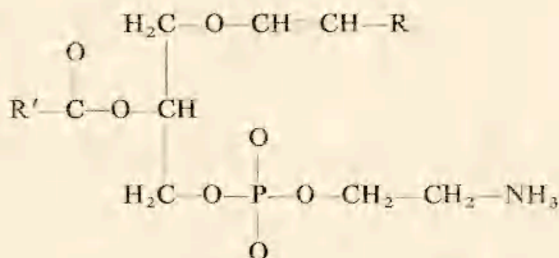
This precursor of plasmalogen synthesis is converted to ethanolamine plasmalogen by enzyme system from brain microsomes.



As it results from Table I about 10% of the added substrate is converted to 1-alk-1'-enyl-2-acyl-GPE. The reaction is stimulated by ATP, CoA, Mg^{2+} and NADPH. For the total activity the enzyme requires the presence of an unindentified factor from soluble fraction, as well as oxygen. The reaction is inhibited in 30% by sodium cyanide.

In similar experimental conditions, in the case of microsomes from hypoxic and ischemic brains an about 60% inhibition of plasmalogen synthesis was observed. The added substrate is converted to ethanolamine plasmalogens only in 4.09% under hypoxia and 4.04% under ischemia. The effect of the particular cofactors on the reaction of plasmalogen synthesis in conditions of hypoxia and ischemia is less distinct than in the control, and this concerns NADPH, CoA as well as the unidentified factor present in cytosol fraction. The same experimental conditions were applied to study the acylation process of 1-alkyl-sn-glycero-3-phosphorylethanolamine to 1-alkyl-2-acyl-sn-glycero-3-phosphorylethanolamine. The acylation reaction is much more active than the reaction of plasmalogen dehydrogenase (Figure). In conditions of oxygen deficiency a marked decrease of the formation of 1-alkyl-2-acyl-GPE is observed, amounting to 7.16 nmoles/mg protein in ischemia and 7.56 nmoles/mg protein in hypoxia, as compared to 25.17 nmoles/mg protein in control conditions (Table II). The reaction is dependent on ATP, Mg^{2+} and CoA. In the absence of these cofactors the acylation of 1-alkyl-sn-glycero-3-phosphorylethanolamine occurs only in 28.5%, as compared to 100% in the complete incubation mixture in the case of control microsomes. In the system containing microsomes from brains of animals submitted to 5-min postdecapitative ischemia or hypoxia acylation amounts respectively to 55.7% and 64.0% of the activity observed in the incubation system containing all reaction cofactors.

Discussion

The obtained results confirm the observations of several investigators [3, 6, 7, 10] that 1-alkyl-sn-glycero-3-phosphorylethanolamine is the precursor in plasmalogen synthesis. The studies *in vitro* and *in vivo* demonstrate that alkyl-GPE is more actively converted into plasmalogens than 1-alkyl-2-acyl-sn-glycero-3-phosphorylethanol-

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TABLE I

Biosynthesis of ethanolamine plasmalogens in rat brain microsomes under hypoxia and ischemia

System	Normoxia			Hypoxia			Ischemia		
	Spec. act. nmole/mg prot./h	Plasmalogen synthesized relative to Exp. 1 %	Net con-version %	Spec. act. nmole/mg prot./h	Plasmalogen synthesized relative to Exp. 1 %	Net con-version %	Spec. act. nmole/mg prot./h	Plasmalogen synthesized relative to Exp. 1 %	Net con-version %
Complete	3.31	—	10.31	1.34	—	4.09	1.28	—	4.04
Complete — CoA	1.89	57.3	5.91	1.14	85.0	3.48	1.26	98.8	3.99
Complete — NADPH	1.33	40.2	4.15	1.25	93.4	3.82	0.89	69.4	2.81
Complete — ATP	1.93	58.3	6.02	0.88	65.5	2.68	1.06	82.7	3.34
Complete — Mg^{++}	1.94	58.7	6.06	0.82	61.2	2.50	—	—	—
Complete — cytosol	2.17	65.7	6.78	1.18	88.3	3.61	—	—	—
Complete + 10^{-3}M KCN	2.08	62.8	6.48	1.13	84.3	3.45	0.96	75.6	3.06
Complete − O_2 + air	2.09	63.3	6.53	—	—	—	—	—	—

Complete system: 75 mM Tris-HCl pH 7.6; 4 mM $MgCl_2$; 0.01% Tween 20; 10 mM ATP; 2 mM NADP; 6.7 mM glucoso-6-phosphate; 5 glucoso-6-phosphate dehydrogenase; 0.1 mM CoA; 20 nmoles of ^{14}C alkyl-GPE (1×10^4dpm) microsomal protein from normal brain 623,2 μg, microsomal protein from hypoxic brain 608.0 μg from ischemic brain 0.6 μg. Incubation was for 30 min at 37°C in a final volume of 0.30 ml under oxygen atmosphere. Each value represents the mean from two experiments.

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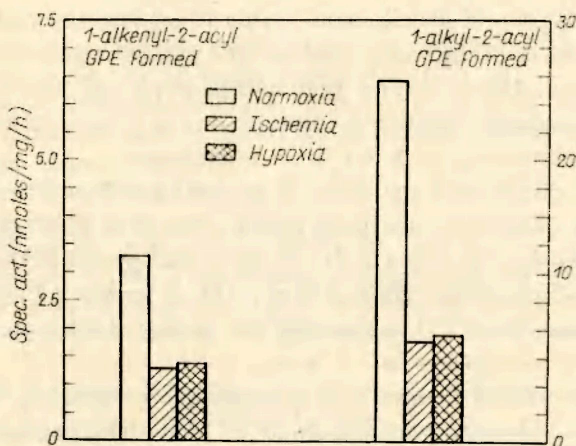

TABLE II

Acylation of ^{14}C-labelled 0-(1-alkyl-sn-glycerol-3-phosphoryl) ethanolamine by rat brain microsomes

Incubation mixture	Normoxia		Ischemia		Hypoxia	
	1-alkyl-2-acyl GPE formed	1-alkyl-2-acyl GPE formed relative to complete system	1-alkyl-2-acyl GPE formed	1-alkyl-2-acyl GPE formed relative to complete system	1-alkyl-2-acyl GPE formed	1-alkyl-2-acyl GPE formed relative to complete system
	nmoles/mg	%	nmoles/mg	%	nmoles/mg	%
Complete system	25.17	100.0	7.16	100.0	7.56	100.0
-ATP, -Mg, -CoA	7.18	28.5	3.99	55.7	4.84	64.0

Complete system consisted: 20 nmoles of ^{14} C alkyl-GPE 1×10^4 dpm, microsomal protein from normal brain 0.6 mg from ischemic hypoxic brain 608.0 mg 10 mM ATP, 4 mM $MgCl_2$, 0.1 mM CoA, buffer Tris HCl pH 7.6. Incubation was for 30 min at 37°C in final volume 0.3 ml. Each value represents the mean from two experiments.

amine. In the case of *in vivo* studies, of a considerable importance may be the easier penetration of the substrate through the cellular membranes. In the studies *in vitro*, with the use of the microsomal fraction, a better binding of this substrate by microsomal membranes may be expected, thus increasing the availability of the substrat for the enzyme, as it is suggested by Paltauf [11, 12]. These data justified the choic of alkyl-GPE as substrate for the studies of plasmalogen dehydrogenase activity in conditions of CNS oxygen deficiency. However, a report should be mentioned [17] in which the authors point to the presence of lysophospholipase D activity in rat brain. This enzyme very actively hydrolyzes 1-alkyl-sn-glycero-3-phosphorylethanolamine and its participation in the effect observed in this work is possible. Plasmalogen dehydrogenase requires for its total activity oxygen and the presence of reaction cofactors, as it is shown in results. In this aspect our results confirm

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the earlier observations of Paltauf and Wykle *et al.* [13, 16] which have demonstrated the dependence of the reaction on reduced pyridine nucleotides, ATP, Mg^{++}. On the other hand, Horrocks and Radomińska-Pyrek [8] have observed the inhibitory effect of reduced pyridine nucleotides on the process of enzymic synthesis of ethanolamine plasmalogens in rat brain microsomes.

An important question in the study of plasmalogen biosynthesis is to identify the level at which occurs the dehydrogenation of alkyl to alk-1'-enyl moiety. As it results from the work of Wykle *et al.* [16] this process appears at the level of 1-alkyl-2-acyl-sn-glycero-3-phosphorylethanolamine. This is supported also by the results obtained in previous work [7], concerning the *in vivo* studies on the biosynthesis of ethanolamine plasmalogens in rat brain.

Basing on the present results, it is not possible to conclude directly whether 1-alkyl-sn-glycero-3-phosphorylethanolamine is first dehydrogenated to 1-alk-1'-enyl-GPE and then acylated to 1-alk-1'-enyl-2-acyl-GPE or does the process of acylation occur before dehydrogenation. However, since the activity of acyltransferase (Acyl CoA : 1-alkyl-glycero-3-phosphorylethanolamine acyltransferase) considerably exceeds the activity of dehydrogenase, it could be supposed that the acylation process may precede plasmalogen dehydrogenase reaction.

In the attempt to explain the reasons for the decrease of plasmalogen biosynthesis in the microsomes obtained from brains of animals subjected to hypoxia or ischemia, several factors may be suggested: lack of oxygen, increase of lysophospholipase D and plasmalogenase activities as well as some possible changes of enzyme structure. The distinct inhibition of ethanolamine plasmalogen synthesis in conditions of oxygen deficiency may be one of the factors considerably altering the quantitative and qualitative composition of microsomal membrane phospholipids. Such changes may then importantly influence the structure and function of membranes, as well as the activity of several enzymes. Plasmalogens, being competitive inhibitors of phospholipase, can regulate the activity of these enzymes. The decrease of their level during hypoxia of CNS may remain in a direct relation with the rise of phospholipase activities in these conditions. The activity of plasmalogen dehydrogenase may seriously affect the level of ethanolamine plasmalogens in CNS under the conditions of normoxia, hypoxia and other pathological states. In order to provide a comprehensive study in the field of plasmalogen metabolism in the CNS further studies are intended to deal with the activity of ethanolamine plasmalogenase, an enzyme responsible for the hydrolysis of plasmalogen.

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Е. Строшнайдэр, З. Домбровецки, **Ферментный синтез этаноламиновых плазмологенов в микросомальной фракции мозга крысы при кослородном голодании**

Содержание. В условиях кислородного голодания превращение 0-/-1-алкил-sn-глицеро-3-фосфорил-этаноламина в этаноламиновый плазмалоген понижается примерно на 60%.

Ацилирование 1-алкил-sn-глицеро-3-фосфорилэтанолоамина уменьшается примерно на 72%. Торможение биосинтеза плазмалогенов в центральной нервной системе в условиях гипоксии и ишемии может быть одинм из факторов, ответственных за изменения структуры и функции микросомальных мембран.

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