BULLETIN DE L'ACADÉMIE
POLONAISE DES SCIENCES
Série des sciences biologiques
Cl. VI. Vol. XXV, No. 2, 1977
Publié en mars 1977

Catabolism of Mitochondrial Membrane Phospholipids in Conditions of Ischemia and Barbiturate Anesthesia

by

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Presented by M. MOSSAKOWSKI on October 4, 1976

Summary. The degradation process of mitochondrial membrane phospholipids from guinea pig brain was studied in conditions of ischemia and barbiturate anesthesia. Ischemia resulted in the decrease of phosphatidylethanolamine (PE) level and in the increase of the content of free fatty acids (FFA). Calcium ions were activating PE hydrolysis and FFA release and may be the factor stimulating the activity of mitochondrial phospholipase in conditions of oxygen defficiency. The *in vitro* studies demonstrate that procain and the $ATP+Mg^{2+}$ system strongly inhibit the activity of mitochondrial phospholipase, while nembutal has no effect.

Nembutal anesthesia decreases the hydrolytic changes of phospholipids, produced by ischemia. It is suggested that besides the general metabolic and physiological state of the cell, produced by anesthesia acetylcholine, which level in the brain is increased twice during barbiturate anesthesia, may be one of the factors of anesthesia responsible for the protection of brain mitochondria against ischemia. *In vitro*, acetylcholine inhibits the activity of the mitochondrial phospholipase.

The present study deals with some breakdown processes of mitochondrial phospholipids in conditions of ischemia and nembutal anesthesia. The increase in the amount of free fatty acids (FFA) and the hydrolysis of phosphatidylethanolamine (PE) were measured. An attempt was made to explain the observed changes basing on experiments performed *in vitro*.

Methods

Experiments were performed on guinea pigs weighing 180-250 g. The animals from the anesthetized group were administrated intraperitoneally 50 mg of nembutal per kg of body weight and used to the experiments 30 min after falling asleep. Postdecapitative ischemia was produced by the incubation of decapitated animal heads for 0.5-5 min at 37°C. After this period the brains were quickly removed and placed in ice-cold isolation medium (0.3 M mannitol, 0.1 mM EDTA, pH 7.4).

Mitochondria were isolated from cerebral cortex and purified on ficoll gradients, as described previously [11].

The level of free fatty acids was determined by the colorimetric method of Itaya and Ui [9], and their identification was carried out by gas chromatography. Methyl esters, obtained according to the description of McGee [5], were dissolved in chloroform and assayed on a GIDE GCHF 18.3 gas chromatograph with ionization flame detector. The column was packed with 10% PEGA

on chromosorb W. The temperature was 210°C and argon flow 40 ml/min. Erucic acid methyl ester was used as internal standard. The areas of peaks were calculated from the measurements of their height and retention time.

Lipids were extracted from mitochondria by the method of Folch and separated by thin-layer chromatography on a plate with silica gel H in the solvent chloroform: methanol: water (65:25:4). After drying, the spot containing phosphatidylethanolamine (PE) was scraped off and phosphorus was assayed by the method of Bartlett [1]. Protein was estimated by the method of Lowry.

Results

Effect of ischemia and barbiturate anesthesia on PE hydrolysis and FFA release. In conditions of ischemia the level of free fatty acids in mitochondria is markedly increased (Fig. 1). When ischemia is produced in animals submitted previously to nembutal anesthesia, the observed increase of FFA level is 5 times lower than in the case of ischemia of control animals. Anesthesia protects partly the mitochondria against the damaging action of oxygen defficiency.

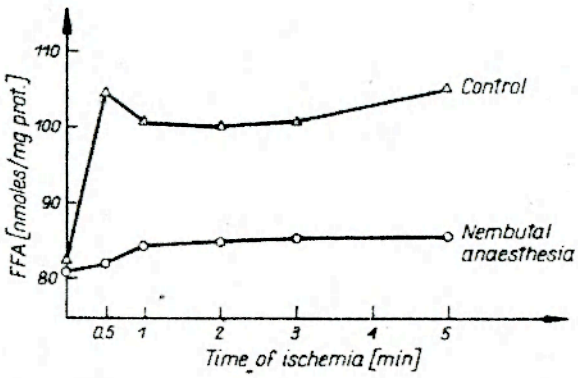

Fig. 1. Effect of ischemia on the liberation of FFA in brain mitochondrial fraction from and anesthetized guinea pigs

A similar phenomenon was observed in the study of hydrolysis of the mitochondrial phospholipids in ischemic conditions (Fig. 2). Phosphatidylethanolamine content was taken as the measure of the activity of this process, assuming that this is the main substrate for the mitochondrial phospholipase [20]. In this case, barbiturate anesthesia also showed a protective action, inhibiting the phospholipid breakdown induced by ischemia.

The plots presented in Figs. 1 and 2 indicate that the catabolic changes in brain phospholipids appear very quickly from the moment of action of the damaging stimulus—oxygen deficiency. Already in the case of 0.5 min ischemia, a large increase in the amount of free fatty acids and the decrease of phosphatidylethanolamine level were observed.

Effect of various factors on PE hydrolysis; role of calcium ions. In the attempt to elucidate which factors may be responsible for the changes in the levels of phospholipids and fatty acids, observed in ischemia and anesthesia, the hydrolysis of mitochondrial endogenous PE was investigated in different systems *in vitro*. Calcium

ions, nembutal, the local anesthetic—procain and the system ATP+Mg^{2+} responsible for the contraction of swelled mitochondria were considered.

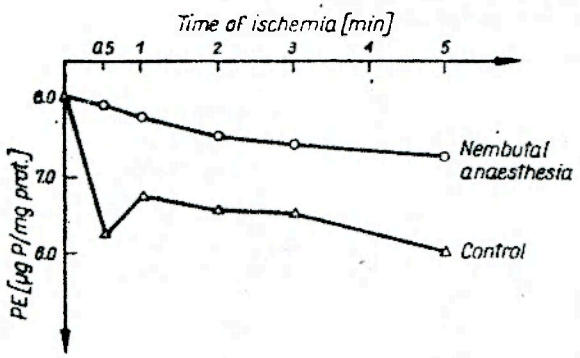

Fig. 2. Effect of ischemia on PE hydrolysis of brain mitochondrial and anaesthetized animals

As it may be seen from Fig. 3, calcium ions strongly stimulate the hydrolysis of mitochondrial PE, nembutal has no effect, maintaining hydrolysis on the level of the control group, procain strongly inhibits, while the system ATP+Mg^{2+} also decreases twice PE hydrolysis, as compared to the control.

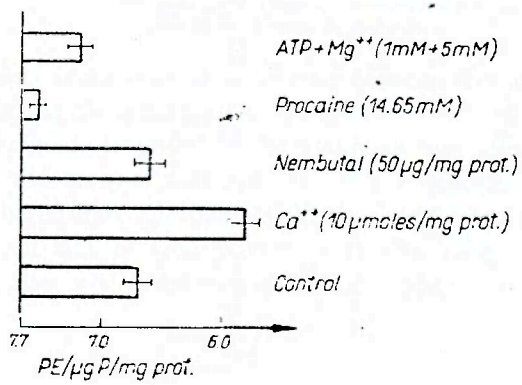

Fig. 3. Effect of various factors on mitochondrial PE hydrolysis

Incubation was carried during 60 min is 37°C in medium containing 0.1 M Tris-Cl, pH 7.4 :ATP+Mg^{2+}; procaine; nembutal; Ca^{2+} — as in shown; and 1 mg mitochondrial protein in total volume 1.0 ml

The action of calcium ions on the process of release of free-fatty acids was studied in more details, assuming their possible role in the catabolism of mitochondrial phospholipids in conditions of oxygen lack [2]. The results presented in Fig. 3 indicate that the incubation with calcium ions increases twice PE hydrolysis, as compared to the control incubation. Similarly, the level of free fatty acids is doubled after the incubation with calcium ions (Table). The analysis of fatty acids with gas chromatography shows a predominance of unsaturated acids. This could indicate that calcium ions activate mainly the mitochondrial phospholipase A_2, splitting unsaturated fatty acids at the 2nd carbon atom of phospholipid glycerol.

TABLE

Effect of Ca^{2+} on the release of FFA in mitochondrial fraction from guinea pig brain

	Incubation 60 min in 37°C	
	− Ca	+ Ca
Netto-increase of total FFA nmoles/mg protein *)	17.6±2.1 5	33.5±3.0 5
% of control value of individual FFA		
16:0	107	112
18:0	108	109
18:1	107 (3)	125 (3)
18:2	104	157
20:4	103	137
*)		
**)		

Incubation medium: 0.1 M Tris-Cl, pH 7.4; Ca^{2+} 10 µmoles/mg mitoch. prot. and 1 mg mitochondrial protein in total volume of 1.0 ml.

*) Measured by the method of Itaya and Ui

**) Measured by the gas chromatography method

Number of experiments in parentheses

Since there is an essential difference between the action of nembutal on mitochondria during the incubation *in vitro*, and the effect of nembutal anesthesia, an attempt was made to elucidate the nature of this phenomenon.

Taking into account that the level of acetylcholine is increased twice in conditions of barbiturate anesthesia [10], we investigated its effect on PE hydrolysis (Fig. 4). As it may be seen from these results, acetylcholine strongly inhibits PE hydrolysis, also in the presence of calcium ions.

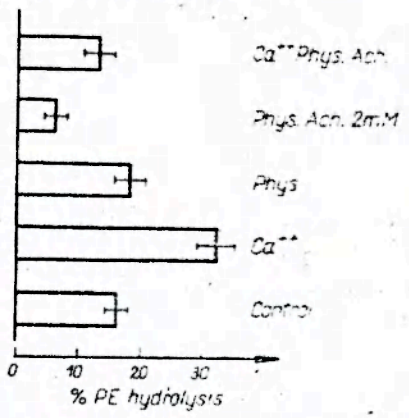

Fig. 4. Effect of Ca^{2+} and acetylcholine on mitochondrial PE hydrolysis

Incubation medium: 0.1 M Tris-Cl, pH 7.4: Ca^{2+} — 10 µmoles/mg physostygmine — 10^{-4} M; acetylcholine — 2 mM; and 1 mg mitochondrial protein in total volume 1.0 ml; 37°C; incubation time 60 min

Discussion

The results presented in this paper indicate distinctly that the conditions of oxygen defficiency result in the activation of several metabolic reactions responsible for the breakdown of mitochondrial phospholipids and for the accumulation of free fatty acids. This leads to a handicap of the mitochondrial energy metabolism, observed also in our previous works [6, 13].

Phospholipase A_2 is presumably the enzyme directly responsible for this phenomenon, since its localization on the external mitochondrial membrane renders it more susceptible to various metabolic changes occurring within the cytoplasmic system in the result of alterations of the natural environmental conditions of the cell.

In our experimental model phospholipase A_2 from brain mitochondria is stimulated by calcium ions. Cooper and Webster [4] have not observed any activating effect of calcium ions on phospholipase A_2. However, their study concerned the action of phospholipase A_2 contained in the extract of the whole brain, and the acid pH optimum of this system indicates mainly the lysosomal phospholipase [19]. On the other hand, the enzyme considered in this work is bound to the mitochondrial membrane and reacts with an endogenous membrane substrate at physiological pH.

The conditions of oxygen defficiency are favourable for the elevation of calcium ions concentration in the cytoplasm [2]. The increased permeability of cellular membranes, resulting from a depolarization of nerve cell membrane, as well as the decrease of energy-linked calcium accumulation in mitochondria [3, 11] lead presumably in ischemic conditions to the increase of the level of these ions in the extramitochondrial space. This state favours calcium activation of phospholipase localized in the external mitochondrial membrane.

When postdecapitative ischemia was produced on animals submitted previously to nembutal anesthesia the hydrolytic changes, measured by either PE decrease or FFA increase, were visibly diminished.

The protective effect of barbiturate anesthesia with regard to the energy activity of mitochondria from ischemic animals has been already observed in the previous work [13].

Michenfelder [15] and Nilsson [17] have not observed any evident protective effect of barbiturate anesthesia on ATP and lactate levels in conditions of postdecapitative anoxia or asphyxia, while during hypothermia this effect was visible. Nilsson and Sjesjo [16] have not found, however, any obvious decrease of the body temperature during barbiturate anesthesia, produced by a dose of pentobarbital similar to that used in our experiments, thus the protective effect of this anesthesia on the mitochondria of ischemic animals cannot be explained only by hypothermia, especially that in our experimental conditions ischemia was carried out at 37°.

The visible protective effect of barbiturate anesthesia with regard to ischemia, observed in the present work is not correlated with the data concerning the effect of the anesthetic itself, *in vitro*, on the rate of PE hydrolysis. This effect has not been either observed with regard to other catabolic processes induced by ischemia [14]. Thus, it may be assumed that the essential role in the protective effect of barbiturate

anesthesia is played by the general metabolic and physiological state of the cell produced by anesthesia.

In our previous paper [13] we have suggested that the doubled level of acetylcholine [10] may be one of the factors of this state. As it may be seen in Fig. 4, acetylcholine *in vitro* strongly inhibits the hydrolysis of PE, abolishing even the stimulating action of calcium ions.

It is known that phospholipids constitute the main component of biological membranes, responsible for calcium binding, and the complex of phospholipid with calcium ions, makes it more susceptible to phospholipase action [18].

From the report of Hemminki [8] it results that acetylcholine slightly (12%) increases the binding of calcium ions to plasmic membranes. In this situation, during the incubation with acetylcholine, an increased phospholipase A_2 activity with the endogenous substrate could be expected. However, since the amount of bound Ca^{2+} was calculated with respect to protein, and the membranes with different ratio lipid: protein (such as brain plasmic and myelin membranes) show a similar percent of the increase of Ca^{2+} binding under the effect of acetylcholine, it may be assumed that this increase concerns rather the glycoprotein and the protein parts of the membrane than the lipid part, and should not be of a great significance in the determination of the activity of mitochondrial phospholipase.

Gullis and Rowe [7] have observed an activating action of various transmitters on the activity of phospholipase A_2 from synaptosomal membranes, without finding their visible effect on the mitochondrial phospholipase. On the other hand, as it results from our experiments, acetylcholine inhibits the activity of phospholipase from mitochondria.

The results of our preceding work [14] suggest that the protective effect of acetylcholine in the catabolism of phospholipids may be due to its antioxidative action.

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М. Д. Маевска, Е. В. Лазаревич, Ё. Строшнайдэр, Катаболический распад фосфолипидов митохондриальных мембран в условиях ишемии и барбитурановой анестезии

Содержание. Исследовался процесс распада фосфолипидов митохондриальных мембран мозга морских свинок в условиях ишемии и нембуталовой анестезии. Обнаружено падение уровня фосфатидилэтанольамина (PE) и повышение количества свободных жирных кислот (FFA) в условиях ишемии. Ионы Ca^{+} активируют гидролиз PE и освобождение FFA и могут быть фактором возбуждающим активность митохондриального фосфолипаза в условиях кислородного голодания, Из опытов следует, что прокаин и система $ATP+Mg^{2+}$ сильно ингибируют активность митохондриального фосфолипаза, в то время как нембутал не оказывает влияния.

Нембуталовая анестезия смягчает вызванные ишемией гидролитические изменения фосфолипидов. Выдвигается предположение, что кроме вызванного анестезией общего метаболическо-физиологического состояния ткани, одним из факторов этой анестезии, ответственым за защиту митохондриев мозга против ишемии, может быть ацетилхолин, уровень которого в мозгу возрастает вдвое в условиях барбитуранового наркоза. Дело в том, что ацетилхолин (*in vitro*) тормозит активность митохондриального фосфолипаза.