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Effects of Ischemia and Exogenous Fatty Acids on the Energy Metabolism in Brain Mitochondria

by

J. W. ŁAZAREWICZ, J. STROSZNAJDER and A. GROMEK

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Summary. The following effects of 5-min postdecaptive ischemia on the metabolic properties of guinea pig brain mitochondria have been observed: 1) remarkable inhibition of mitochondria respiratory activity in state 3; 2) oxidative phosphorylation uncoupling together with a decrease in ADP/O ratio and RC index; 3) increase in the Mg^{++}-stimulated ATPase with a simultaneous decrease of ATPase activity dependent on DNP. A twofold increase in the free fatty acids content of these mitochondria has been noticed, and the effect of exogenous oleate on the metabolic properties of brain mitochondria has been shown to be similar to that on liver mitochondria. Oleate inhibits respiratory activity of brain mitochondria, uncouples oxidative phosphorylation, stimulates ATPase activity dependent on Mg^{++} and at the same time inhibits ATPase induced by DNP. It also inhibits ATP-Pi exchange and produces swelling of brain mitochondria. The obtained results suggested that free fatty acids liberated in the brain during ischemia may — through their inhibitory activity on energy metabolism of mitochondria — become one of the factors of the development of irreversible brain damages in ischemia.

Material and methods

Animals. Male guinea pigs, weighing 150—250 g, obtained from the Laboratory Animals Breeding Centre in Łomna, fed *ad libitum*, were used.

Preparation of mitochondria. Brain mitochondrial fraction was prepared according to the method of Ozawa [11] slightly modified. Guinea pigs were decapitated, the intact brains were removed within 20 sec (except when ischemia was produced) and immersed in ice-cold isolation medium (0.3 M mannitol : 0.1 mM EDTA, pH 7.4). After chilling the cerebral heimspheres of 4 animals were removed and manually homogenized together with 40 ml of ice-cold isolation medium with a glass-teflon homogenizer (clearance 0.1 mm), by 15 up and down strokes. A further 20 ml of ice-cold isolation medium were added and the total homogenate centrifuged for 8 min at 600 *g*. The supernatant was carefully decanted and then centrifuged at 10,000 *g* for 10 min. The sediment was resuspended in 40 ml of isolation medium and centrifuged at 5000 *g* for 10 min. The supernatant from this spin was decanted and the slight fluffy layer removed from the pellet. The mitochondrial pellet was resuspended in a small volume of isolation medium and made up to a concentration of 10 mg of protein/ml. All preparations were performed at 0—4°C. MSE Magnum refrigerated centrifuge was used. When the effect of exogenously added oleate on mitochondrial activity was investigated, 0.4-% bovine serum albumin was added to the isolation medium in order to get rid of endogenous free fatty acids, liberated during the preparation procedure.

Incubation conditions. Incubation medium for oxidative phosphorylation [11] contained 0.3 mannitol, 10 mM KCl, 10 mM Tris-Cl, pH 7.4, 5 mM K-phosphate buffer pH 7.4, 0.2 mM EDTA, 5 mM glutamate, 333 μM ADP and 1 mg mitochondrial protein in a total volume of 1.5 ml. Temperature 25°. O_2 was measured polarographically; ADP/O ratio was calculated by the Chance method [4] and respiratory control (RC) index was assayed as the ratio of respiration rate in the presence of excess substrate and ADP to the repiration rate after ADP has been exhausted (state 3 and 4, respectively).

Incubation medium for ATPase activity contained 75 mM KCl, 0.1 M mannitol, 50 mM Tris-Cl pH 7.4, 1 mM EDTA, 2 mM ATP and 0.5 mg mitochondrial protein in a total volume of 1.5 ml 2.4.-dinitrophenol and $MgCl_2$ were added, as shown, to the medium in the concentration of 0.1 mM and 3 mM, respectively. Temperature 25°, incubation time 15 min.

Incubation medium for ATP-Pi exchange contained 0.1 M KCl, 12.5 mM mannitol, 1.5 mM EDTA, 75 mM Tris-Cl pH 7.4, 5 mM ATP, 3 mM KH_2PO_4 (containing ^{32}P 5×10^6 cpm/sample), 0.5 mg mitochondrial protein in a total volume of 1 ml. Temperature 25°, incubation time 15 min. ^{32}P ATP formation was determined following the procedure of Wadkins and Lehninger [14]. Corrections for ATPase activity were considered.

Incubation medium for mitochondrial swelling contained 0.15 M KCl, 20 mM Tris-Cl, pH 7.4, and 0.5 mg mitochondrial protein in a total volume of 3 ml. Incubation was started by the addition of the mitochondrial suspension and was carried out at 25° for 40 min in the thermostatized glass cells. Absorbancy was measured at 520 mμ with a SP-500 Unicam spectrophotometer.

Methods of estimation. The protein was determined according to Lowry [8]. The inorganic phosphate was estimated by the method of Fiske and Subbarow [6]. Free fatty acids from the freshly prepared mitochondrial fraction were extracted by the method of Dole [5] and estimated according to Duncombe's colorimetric method modified as in [7].

Chemicals. Mannitol, ADP, bovine serum albumin , Tris, $MgCl_2$ were obtained from Sigma, glutamate and EDTA were BDH products. All other reagents of the highest purity available, were obtained from Polskie Odczynniki Chemiczne, Gliwice. ^{32}P as a K-orthophosphate was the product of the Institute of Nuclear Research in Świerk.

Results and discussion

Considering the exceptional susceptibility of the brain to hypoxia, brain ischemia as a consequence of cardiac arrest seems to be one of the most serious pathologies of the central nervous system encountered in the clinic. Disturbances of brain energy metabolism with a sharp decrease in high energy compounds and accumulation of such cellular metabolites as lactic and pyruvic acids are the earliest biochemical changes accompanying brain ischemia [9]. Electrical activity on EEG declines simultaneously. 3—4 min is the maximum period of arrest which can be tolerated with full recovery of the brain function. A longer period of ischemia is followed by neurological complications and irreversible cerebral damage. Since oxidative phosphorylation is practically an exclusive source of ATP in the brain, proper functioning of that process in mitochondria is indispensable for supporting the physiological activity of the brain. In relation to these facts it seemed to be logical to take an interest in the phosphorylating activity of ischemic brain mitochondria searching for the mechanisms of rapid development of irreversible pathological changes in ischemic brain cells.

The Table as well as Figs. 1—4 show the metabolic properties of isolated control brain mitochondrial fraction. The mitochondrial fraction which shows a considerable activity respiratory and control — when incubated in the fairly simple medium

TABLE

Effect of ischemia on metabolic properties of brain mitochondria

		Control		Ischemia	
		— albumin	+ albumin	— albumin	+ albumin
O_2-uptake ngatom/mg protein/min	state 3	62.1±9.4 (7)	57.6±10.00 (7)	28.9±10.8 (6)	30.3±19.5 (6)
	state 4	17.7±4.5 (7)	11.3±3.8 (7)	15.1±5.5 (6)	9.3±3.5 (6)
RC		3.6±0.9 (7)	5.4±1.1 (7)	1.9±0.2 (6)	3.3±1.3 (6)
ADP/O		1.9±0.2 (7)	2.2±0.3 (7)	1.2±0.3 (6)	1.8±0.2 (6)
ATPase activity μmoles Pi/mg protein/min	endogenous	0.26±0.18 (5)		0.34±0.21 (5)	
	DNP	1.37±0.34 (5)		0.31±0.24 (5)	
	Mg^{++}	1.57±0.44 (5)		2.03±0.44 (5)	
FFA content nmoles/mg protein		23.4±4.5 (8)		40.1±8.3 (8)	

Ischemia was produced by incubation of decapitated heads for 5 min at 37° before extirpation of the brain. Bovine serum albumin was added as shown only to the oxidative phosphorylation medium at a level of 4.0 mg/ml. 5 mM glutamate was the respiratory substrate. Free fatty acids were extracted from the freshly prepared mitochondrial fraction. All other conditions as described under Material and methods.

Values represent ±S.D. Number of experiments is given in parenthesis.

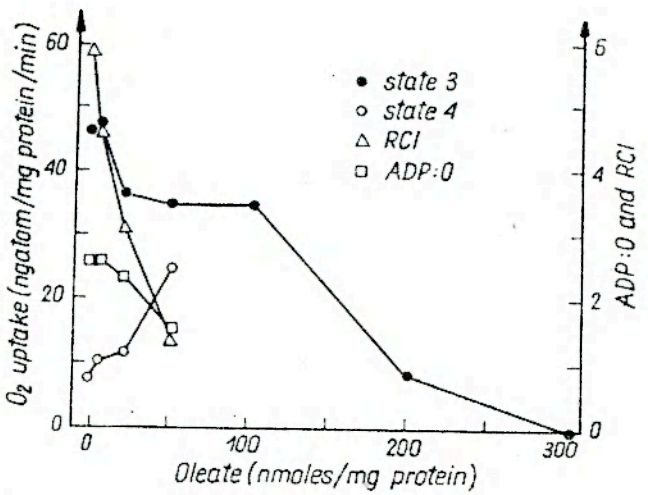

Fig. 1. Effect of oleate on oxidative phosphorylation and on glutamate oxidation by brain mitochondria

In Figs. 1—3 each point represents mean for 4 experiments

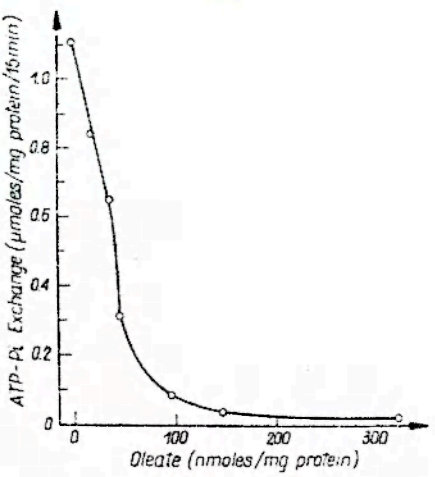

Fig. 2. Effect of oleate on the ATP-Pi exchange reaction in brain mitochondria

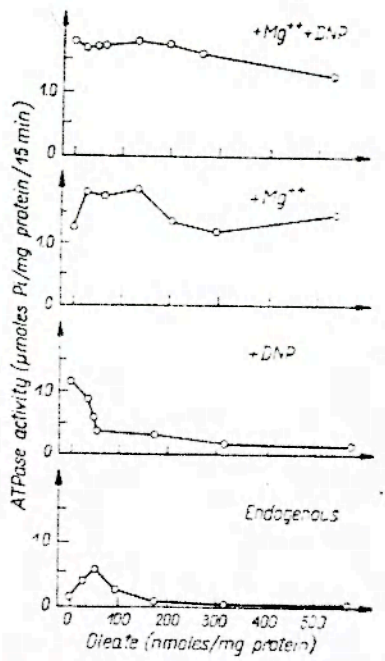

Fig. 3. Effect of oleate on the ATPase activity in brain mitochondria

without albumin, with glutamate as a substrate — needs however the presence of 0.4-% albumin in the incubation medium to exhibit acceptable values of ADP/O ratio and high RC index (Table). Also the use of the isolation medium containing 0.4-% albumin during the preparation procedure makes it possible to obtain tightly coupled mitochondrial fraction with a high RC index (Fig. 1). These results are consistent with the results obtained by Ozawa [11, 12] that brain mitochondria are extremely labile, and *in vitro* — as well as during the preparation — they lose their ability to perform oxidative phosphorylation [9] as a symptom of ageing (10 times faster than liver mitochondria). The endogenous uncoupling factor released in mitochondria during their ageing [16] has been identified as free long chain fatty acids. Bovine serum albumin strongly absorbing free fatty acids restores mitochondrial respiratory control.

It is noteworthy that Mg^{++}-induced ATPase activity in brain mitochondria [18]. On the contrary ATP-Pi exchange reaction (Fig. 3) reaches values comparable with that of liver mitochondria [18].

Ischemic brain mitochondria (isolated from the brain 5 min after decapitation) show an evident decrease in both ADP/O ratio and RC index, an inhibition of the oxygen uptake in the metabolic state 3 (Table). In the presence of 0.4% bovine serum albumin in the incubation medium, only partial restoration of ADP/O ratio and RC index of ischemic brain mitochondria can be observed. Under these conditions respiratory activity in metabolic state 4 slightly decreases but respiration in the state 3 remains inhibited. Inhibition of DNP-induced ATPase and activation of Mg^{++}-induced ATPase in ischemic brain mitochondria can also be noticed. These data confirm the previous observations [11].

An almost twofold increase in the free fatty acids content of brain mitochondria occurs following ischemia (Table), the content reaching values of about 40 nmoles/mg of mitochondrial protein. Nonestrified fatty acids estimated in this work were at a lower level than shown in [3]. This inconsistency seems to be the result of differences in the method of free fatty acids determination used in the above-mentioned works.

The pattern of observed disturbances in the respiratory and phosphorylating activity of ischemic brain mitochondria as well as the specific displacement in the ATPase activity resembles the known inhibitory effect of free unsaturated fatty acidl on the mitochondrial metabolic activity [18]. Both, the enlargement of the poos of free fatty acids in ischemic brain mitochondria and the specific effect of albumin on the intensity of the observed metabolic changes are indicative of the participation of endogenous free fatty acids in the development of metabolic disorders in ischemic brain mitochondria. The fact that inhibition of respiratory and phosphorylating activity in ischemic brain mitochondria was only partially abolished by bovine serum albumin may be explained by the insufficient concentration of albumin or by the strong absorption of fatty acids by mitochondria [18]. It may also be due to the fact that inhibitors other than fatty acids are involved or that the damage of mitochondrial structure and function is irreversible.

As it was recently reported by Bazan [3], the mature brain, unlike other tissues is capable of rapidly enlarging its free fatty acids pool within the first 4 min of complete

ischemia. The observed production of mainly unsaturated long chain free fatty acids may be due both to phospholipids breakdown and to the lack of activity of reacylating enzyme system [3]. Beyond any doubt, long chain fatty acids play a unique role in the energy metabolism. They are good respiratory substrates, but on the other hand they are well-known inhibitors of liver mitochondria activity as they uncouple oxidative phosphorylation, inhibit oxidative processes, adenine nucleotides translocation and ATP-Pi exchange; they influence ATPase activity, produce swelling and damage of mitochondrial membranes [18]. They also inhibit glycolysis [10] and protein synthesis [1].

The above mentioned results and data showed the need to examine more closely the effect of exogenous fatty acids on the metabolic properties of brain mitochondria.

Fig. 1 shows the effect of oleate on oxidative phosphorylation of brain mitochondria. It can be seen that oleate inhibits the glutamate oxidation in state 3, but stimulates oxidation in state 4. It reduces ADP/O ratio and RC index. That inhibitory and uncoupling effect, strongly dependent on the oleate concentration is similar to the effect of oleate on liver mitochondria [18], and is generally consistent with the data found on rat brain mitochondria by Scholefield [13]. It is also compatible with the above mentioned [Table] effect of ischemia on oxidative phosphorylation of brain mitochondria.

The effect of oleate on the ATP-Pi exchange reaction is shown in Fig. 2. Oleate blocks the ATP-Pi exchange in brain mitochondria to the extent like in liver mitochondrial fraction [18]. At very low levels (2—5 nmoles/mg protein) oleate stimulates exchange reaction in liver mitochondria [18], however no attempts were undertaken in this work to reproduct this effect on brain mitochondria. Inhibition of the ATP-Pi exchange in rat brain mitochondria observed by Ahmed and Scholefield [2] has been less pronounced than in the present work.

Fig. 3 shows the effect of oleate on the ATPase activity in brain mitochondria. This effect differs in certain points from that on liver mitochondria. Strong inhibition of DNP-activated and endogenous ATPase, which has been observed in rat liver mitochondria [18], is apparently due to the inhibition of ATP translocation into the mitochondrion and,— when higher amounts of oleate are used — to the release of intramitochondrial magnesium from mitochondria [18]. That explanation could be adapted to the phenomena observed in brain mitochondria. Mg^{++}-dependent ATPase, very active in control, is stimulated by small amounts of oleate and behaves unspecifically when higher amounts of oleate are used. Mg^{++}+DNP-stimulated ATPase is practically not affected by oleate. This picture, unlike that of liver mitochondria is rather difficult to explain. It may be due to specific differences between brain and liver mitochondria as well as to the impurities present in our crude mitochondrial fraction. It is seen however that the effects of oleate on ATPase activity are compatible with changes of ATPase activity in ischemic brain mitochondria. When the amount of oleate is about 40 nmoles/mg protein, inhibition of DNP-activated ATPase and stimulation of Mg^{++}-induced ATPase is seen. The same effect was observed on ischemic brain mitochondria (Table).

Fatty acids are powerful swelling-producing agents for mitochondria [19]. Swelling of mitochondria can be reversed by the addition of ATP in the presence of Mg^{++}. In order to test the swelling effect of oleate on brain mitochondria 30 and 6000 nmoles of oleate/mg protein were used (Fig. 4). It was shown that the rates of both swelling and contraction depends on oleate concentration. A similar phenomenon has been observed for liver mitochondria [17] and it is not surprising, since it was shown that free fatty acids are directly involved in the mechanism of mitochondrial contraction [15].

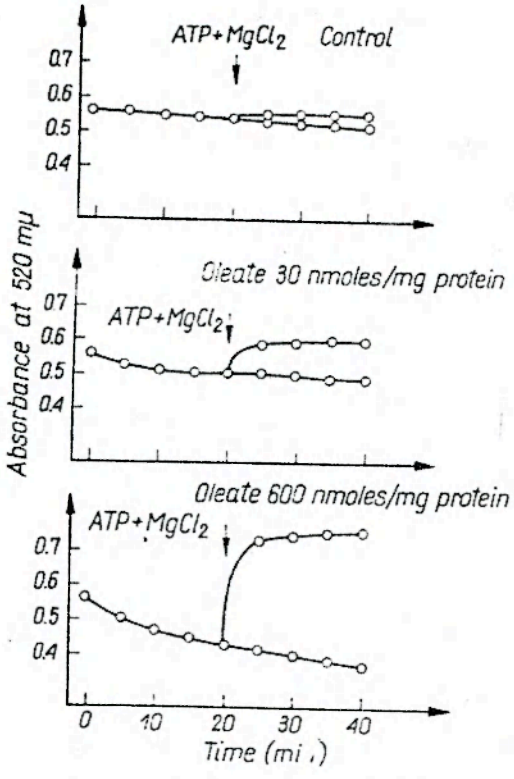

Fig. 4. Effect of oleate on the swelling of brain mitochondria

Sodium oleate if added was present in the medium from the beginning of the experiment. Swelling — downwards, shrinkage after the addition of 5 mM ATP — 3 mM $MgCl_2$ as indicated by the arrows — upwards. Other experimental conditions as under Material and methods

Judging by the above mentioned data serious metabolic disorders observed in brain mitochondria after 5-min ischemia (Table), could be at least in part explained by the effect of endogenous free fatty acids liberated during ischemia. The enlargement of the pool of free fatty acids in ischemic brain mitochondrial fraction (Table), and, on the other hand, the inhibitory and uncoupling effect of exogenous oleate on brain mitochondria, observed in our experiments (Fig. 1—4) comparable with changes noticed in postischemic mitochondria seem to support that opinion. In that case free fatty acids released in the brain during ischemia may become one of the factors involved in the development of irreversible pathological changes in

ischemic brain cells, since they inhibit not only oxidative phosphorylation but also other important metabolic pathways in the cell.

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LABORATORY OF NEUROCHEMISTRY, EXPERIMENTAL AND CLINICAL MEDICAL RESEARCH CENTRE, POLISH ACADEMY OF SCIENCES, WARSAW, DWORKOWA 3
(ZESPÓŁ NEUROCHEMII, CENTRUM MEDYCYNY DOŚWIADCZALNEJ I KLINICZNEJ, PAN, WARSZAWA)

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Я. В. Лазаревич, И. Строшнайдэр, А. Громэк, Влияние ишемии и экзогенных жирных кислот на энергетический метаболизм в митохондриях

Содержание. Исследовано влияние постдекапитативной ишемии на метаболические свойства митохондриальных препаратов мозга морской свинки. Ишемия вызывает следующие изменения: 1) понижение дыхательной активности митохондриальных препаратов мозга в присутствии АДФ; 2) расщепление окислительного фосфорилирования при одновременном понижении соотношения АДФ/О и дыхательного коэффициента; 3) увеличение Mg^{++} АТФ-азной активности и уменьшение активности АДФ—АТФ-азы; 4) двукратное увеличение содержания свободных жирных кислот. Влияние экзогенной олеиновой кислоты на биохимические свойства митохондриальных препаратов мозга сводилось к упомянутым выше изменениям, возникшим во время ишемии. Олеиновая кислота вызывает ингибицию процесса обмена АТФ—неорганический фосфат и приводит к набуханию митоходриальных препаратов мозга. Возможно, что освобождаемые жирные кислоты являются одним из факторов, ответственных за развитие необратимых биохимических и морфологических повреждений в мозге во время постдекапитативной ишемии.