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EXPERIMENTAL MEDICINE

Peroxidation of Fatty Acids in Microsomes Prepared from Normal and Ischemic Guinea Pig Brains

by

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Summary. Peroxidation of unsaturated fatty acids in membrane phospholipids occurs more rapidly in microsomes isolated from brains subjected to 5 min ischemia than in control microsomes. The activation by ischemia was found for both enzymic and nonenzymic peroxidation of unsaturated fatty acids. Increased cleavage of the membrane-bound polyunsaturated fatty acids was accompanied by a simultaneous increase in the metabolic products of these reactions: conjugated double bonds, peroxides and malondialdehyde. The increased activity of both peroxidation processes during ischemia may account for the structural and functional changes in the microsomal membranes that result from the ischemia.

In the living organism, peroxidation of fatty acids takes place by enzymic and non-enzymic reactions which have been characterized by Hochstein *et al.* [5, 6].

In the present study we have altered the oxido-reductive balance by ischemia and found an activation of both types of peroxidation.

Materials and methods

Preparation of microsomal fractions. Guinea pigs of 150—200 g were obtained from the Laboratory Animals Breeding Centre in Łomna for use in these experiments. After decapitation the brains were removed within 20 sec and immersed in ice-cold 0.32 M sucrose buffered with 0.1 M tris-HCl at pH 7.4. With some animals, complete ischemia was obtained by the incubation of decapitated animal heads for 5 min at 37°C. After fragmentation the tissues were homogenized manually in 9 volumes of the same sucrose solution in glass-Teflon homogenizer (clearance 0.1 mm) by 15 up and down strokes.

The 10% homogenate was centrifuged at 1000 *g* for 10 min. The supernate was carefully decanted and centrifuged at 10,000 *g* for 10 min. This supernatant was centrifuged at 10,000 *g* for 30 min. The resulting supernatant fraction was centrifuged at 105.000 g for 30 min to obtain the microsomal pellet which was suspended in a known volume of 0.1 M tris-HCl, pH 7.4, for protein determination [7] and use in the experiments. The entire procedure was carried out at temperature of 0—4° C utilizing MSE Magnum and Janetzki Vac-60 centrifuges.

Incubation systems. The incubation system used for enzymic lipid peroxidation experiments contained in final concentrations: 0.1 M tris-HCl, pH 7.4; 4 mM ADP; 0.012 mM $FeSO_4$; 50 mM

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nicotinamide; 0.3 mM NADP, 9 mM glucose-6-phosphate; 1—2 μg of glucose-6-phosphate dehydrogenase and 1 mg of microsomal protein in a volume of 1 ml. Incubation system used for non-enzymic lipid peroxidation experiments contained in final concentrations: 0.1 M tris-HCl, pH 7.4; 0.012 mM $FeSO_4$; 0.3 mM NADP; 0.8 mM ascorbic acid and 1 mg of microsomal protein in volume of 1 ml. Incubation were stopped by the addition of 19 volumes of chloroform-methanol (2:1) Folch *et al.* [4]. After shaking the mixture was kept for 30 min at room temperature before the addition of 0.2 volume of 0.57% NaCl. After 6 h the mixture was centrifuged and the upper aqueous phase was discarded. The lower chloroform phase was washed 3 times with the upper phase solvents [4] taken to dryness with a rotary evaporator, redissolved in chloroform and stored under nitrogen. Oxygen uptake was measured polarographically with a Clark electrode as described by Chance *et al.* [2].

Other enzyme assays. Of the total homogenate activity, the microsomal fraction contained 60% of the NADPH: cytochrom c reductase, 0.9% of cytochrom c oxidase, and 7.6% of the acid phosphatase.

The enzyme were assayed by the methods of Mackler [8], Yonetani [17] and Zgirski [18], respectively.

Lipid determinations. Fatty acid compositions were determined by gas chromatography. Hydrolysis of the mixtures was carried out in metanolic sodium hydroxide. Fatty acid methyl esters were formed by reaction with diazomethane, dissolved in chloroform and separated with a Pye Unicam S-104 gas chromatograph equipped with a flame ionization detector. The column packed with 10% PEGA on Chromosorb-W was at 210°. Argon flow was 40 ml per min. Methyl erucate was added to the methyl esters as an internal standard and peak areas were calculated from measurements of height and peak width. Other assay methods were described by Ernster *et al.* [3] for malondialdehyde, Bunyan *et al.* [1] for hydroperoxides and Placer [15] for conjugated dienes.

Reagents. We obtained Tris, $MgCl_2$, ADP, NADP, glucose-6-phosphate and thiobarbituric acid from Sigma; glucose-6-phosphate dehydrogenase from Koch-Light; L-ascorbic acid from BDH and all other reagents from Polskie Odczynniki Chemiczne, Gliwice.

Results

The amounts of linoleate, arachidonate and docosahexaenoate were substantially decreased after both types of incubation and with both control and ischemic microsomes (Table I). For incubations with NADPH, the loss of polyunsaturated fatty acids from ischemic microsomes was greater that the loss from control microsomes but ischemic and control microsomes had nearly identical polyunsaturated fatty acid contents before incubation. Thus enzymic peroxidation of acyl groups at 2 position of phosphoglycerides is more active in the microsomal fraction with an altered oxygen balance due to ischemia. The content of polyunsaturated fatty acids also decreased during nonenzymic lipid peroxidation linked with ascorbic acid oxidation but in these conditions the decreases were similar for control and ischemic microsomes.

The next phase of this study was a kinetic analysis of enzymic process of polyunsaturated fatty acid oxidation in microsomal fractions. The formation of unsaturated fatty acid peroxides in microsomal lipids is dependent on NADPH (Fig. 1) and is much more extensive in ischemic than in control microsomes. The oxidation of fatty acid peroxides yields aldehydes and ketones. The malondialdehyde content was much higher in experimental systems containing NADPH, especially in the ischemic microsomal fraction (Fig. 2).

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TABLE I

Content of polyunsaturated fatty acids in microsomal lipids from guinea pig brain

Experiment	Fatty Acid							
	18:2		20:4		22:6		Total	
	nmols/mg protein							
Control	8.2		66.6		107.9		182.7	
+ NADPH	5.5	(2.7)	41.5	(25.1)	56.8	(51.1)	103.8	(78.9)
Ischemia	8.2		67.9		113.5		189.6	
+ NADPH	3.8	(4.4)	29.1	(38.8)	49.9	(63.6)	82.8	(106.8)
Control								
+ ascorbic acid	6.6	(1.6)	49.4	(17.2)	84.8	(23.1)	140.8	(41.9)
Ischemia								
+ ascorbic acid	7.1	(1.1)	52.9	(15.0)	72.7	(40.8)	132.7	(56.9)

Incubation mixture: For enzymic lipid peroxidation experiments: 0.1 M tris-HCl pH 7,4; 4 mM ADP; 0.012 mM $FeSO_4$; 50 mM nicotinamide, 0.3 mM NADP, 9 mM glucose-6-phosphate; 1—2 μg of glucose-6-phosphate dehydrogenase. For nonenzymic lipid peroxidation experiments: 0.1 M tris-HCl ph 7.4; 0.012 mM $FeSO_4$; 4 mM ADP and 0.8 mM ascorbic acid. Both incubation mixtures contained 1 mg microsomal protein in a final volume of 1 ml. Incubations were carried out at 37° C for 60 min. Values are the means from experiments with two preparations of microsomes. Data in parentheses are the rate of disappearance of polyunsaturated fatty acids (nmoles/mg protein/h).

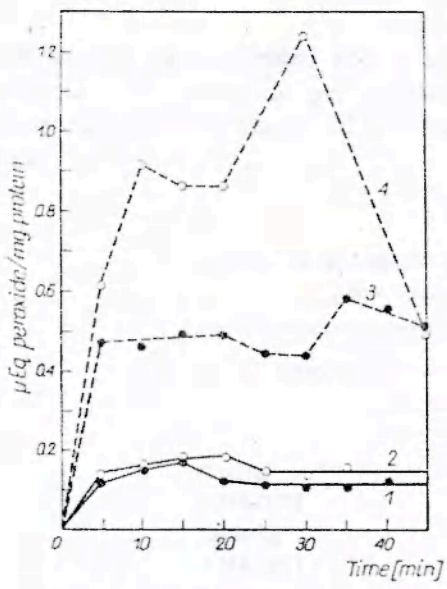

Fig. 1. Peroxide formation during the enzymic oxidation of NADPH by brain microsomes
1 — control, 2 — +NADPH, 3 — ischemia, 4 — ischemia +NADPH

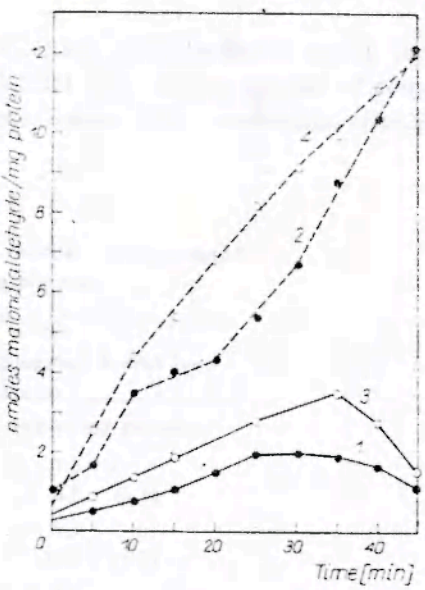

Fig. 2. Time course of malondialdehyde formation during the enzymic oxidation of NADPH by brain microsomes
1 — control, 2 — +NADPH, 3 — ischemia, 4 — ischemia +NADPH

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The increase of the absorbance at 260—270 nm indicates an increase in the content of carbonyl groups during the enzymic oxidation of unsaturated fatty acids (Fig. 3). The absorbance at 233—234 nm due to conjugated dienes also increased.

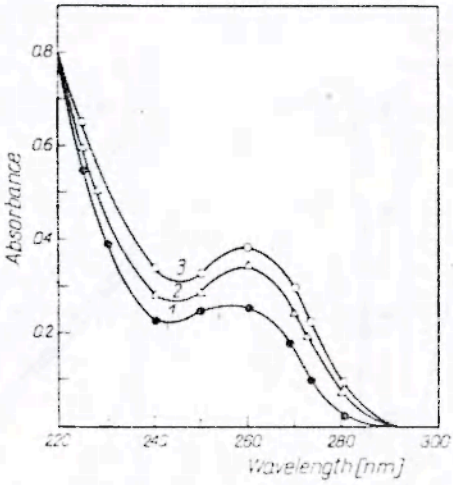

Fig. 3. Absorption spectrum of lipid from microsomes
1 — control, 2 — control + NADPH, 3 — ischemia + NADPH

The amount of conjugated double bonds increased in microsomes during their enzymic and nonenzymic peroxidation (Table II). The highest content of conjugated double bonds was found in ischemic microsomes after incubations of either type.

TABLE II

Content of conjugated double bonds in microsomal lipids from guinea pig brain

Experiment	Conjugated double bonds	
	nmoles/mg protein	
Control	7.3	
+NADPH	8.8	(1.5)
Ischemia	9.3	
+NADPH	10.6	(1.3)
Control	7.3	
+ascorbic acid	10.5	(3.2)
Ischemia	9.3	
+ascorbic acid	10.7	(1.4)

The incubation conditions as described in Table I. Data in parentheses are the rate of increase of conjugated double bonds (nmols/mg protein/h)

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The uptake of oxygen by control and ischemic microsomes was stimulated by NADPH (Fig. 4) and by ascorbic acid (Fig. 5). In both cases the oxygen uptake was greater with ischemic microsomes than with control microsomes, particularly with ascorbic acid.

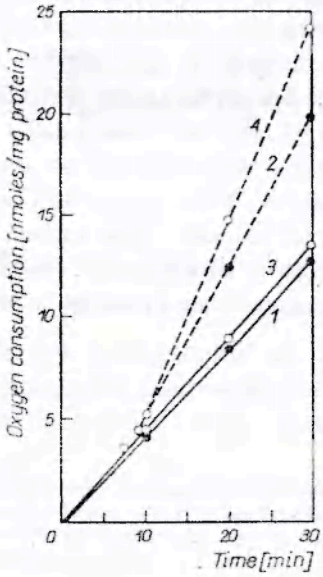

Fig. 4. The oxygen uptake by the microsomal fraction of the brain during the action of NADPH oxidase

1 — control, 2 — +NADPH, 3 — ischemia, 4 — ischemia + NADPH

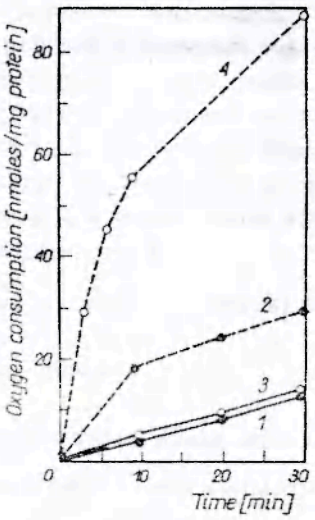

Fig. 5. The oxygen uptake by the microsomal fraction of the brain during the oxidation of the ascorbic acid

1 — control, 2 — +ascorbic acid, 3 — ischemia, 4 — ischemia + ascorbic acid

Discussion

Phosphoglycerides are important components of membranes. Often these phosphoglycerides contain a polyunsaturated fatty acids at 2 position which is susceptible to peroxide oxidation [9, 10, 16]. Normally fatty acid peroxidation is controlled by intracellular antioxidants and by the stable oxygen content. When the oxido-reductive balance is altered in pathologic conditions, changes in peroxide oxidation process may be observed. These undoubtedly influence the structure and function of cellular membranes [12, 14].

In the present study we have examined enzymic and non-enzymic peroxide oxidation of unsaturated fatty acids from microsomal phospholipids. Incubations of microsomes under conditions favoring peroxidation caused losses of polyunsaturated fatty acids which were accompanied by simultaneous increases in oxygen consumption and amounts of conjugated double bonds. In the incubation with NADPH, we also found an early but distinct increase in the amounts of lipid peroxides and a marked continuing increase in the content of malondialdehyde.

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Enzymic peroxidation was more extensive than non-enzymic peroxidation as judged by the loss of polyunsaturated fatty acids from both control and ischemic microsomes. However, the largest increase in content of conjugated double bonds was found after nonenzymic incubation. Polyunsaturated fatty acids were lost at a faster rate from ischemic microsomes than from control microsomes during enzymic and nonenzymic peroxidation. During the first 30 min of enzymic peroxidation lipid peroxide and malondialdehyde were formed and oxygen was taken up at a faster rate in ischemic microsomes than in control microsomes. During non-enzymic peroxidation, the oxygen uptake by ischemic microsomes was nearly three times greater than that by the control microsomes. All of these results make us conclude that the ischemia activated both the enzymic and nonenzymic peroxidation. The activation of peroxidation reactions of membrane phospholipid fatty acids may be responsible for the first important changes in the structure of cellular membranes in conditions of oxygen deficiency, being in a close relation with the change of structure and function of a number enzymes [11, 13].

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REFERENCES

[1] J. Bunyan, E. A. Murrell, J. Green, A. T. Diplock, Brit. J. Nutr., 21 (1967), 475.
[2] B. Chance, G. R. Williams, Nature, 175 (1955), 11.
[3] L. Ernster, K. Nordenbrand, in: *Methods in enzymology*, vol 10, ed. by S. P. Colowick, N. O. Kaplan, R. W. Estabrook, M. E. Pullman, New York, 1967, p. 574.
[4] J. Folch, M. Lees, G. H. Sloane-Stanley, J. Biol. Chem., 226 (1957), 497.
[5] P. Hochstein, L. Ernster, Biochem. Biophys. Res. Communs., 12 (1963), 388.
[6] P. Hochstein, K. Nordenbrand, L. Ernster, ibid., 14 (1964), 323.
[7] O. H. Lowry, N. J. Rosenbrough, A. L. Farr, R. J. Randall, J. Biol. Chem., 193 (1951), 265.
[8] B. Mackler, in: *Methods in enzymology*, vol. 10, ed. by S. P. Colowick, N. O. Kaplan, R. W. Estabrook, M. E. Pullman, New York, 1967, p. 551.
[9] H. E. May, P. B. McCay, J. Biol Chem., 243 (1968), 2288.
[10] — , — , ibid., 2296.
[11] R. C. McKnight F. E. Hunter, Jr., ibid., 241 (1966), 2757.
[12] C. E. Mengel, Amer. J. Med. Sci., 255 (1968), 341.
[13] V. M. Mishin, V. V. Yakhovich, Biofizika, 19 (1974), 83.
[14] L. Packer, D. W. Deamer, R. L. Heath, Adv. Gerontol Res., vol. 2, ed. by B. L. Strehler, Acad. Press, New York—London, 1967, p. 77.
[15] Z. Placer, Nahrung, 12 (1968), 679.
[16] B. K. Tam, P. B. McCay, J. Biol. Chem., 243 (1970), 2295.
[17] T. Yonetani, in: *Methods in enzymology*, vol. 10, S. P. Colowick, N. O. Kaplan, R. W. Estabrook, M. E. Pullman, New York, 1967, p. 332.
[18] A. Zgirski, Pol. Tyg. Lekarski, 5 (1965), 171.

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Е. Строшнайдэр, З. Домбровецки, **Свободнорадикальное окисление жирных кислот в микросомах изолированных из нормальных и ишемических мозгов морских свинок**

Содержание. В настоящий работе исследовались процессы энзиматического и неэнзиматического свободнорадикального окисления ненасыщенных жирных кислот фосфолипидов микросомальной фракции из мозгов морских свинок подвергнутых до этого 5 минутной подекапитационной ишемии. Обнаружено, что понижению содержания многоненасыщенных жирных кислот в микросомальных липидах сопутсвует увеличение потребления кислорода, увеличение количества неспаренных двойных связей, временное значительное увеличение количества гидроперекисей и накопление малонового диальдегида. Активация процессов свободнорадикального окисления может быть ответственна за первые существенные изменения структуры клеточных мембран в условиях нарушенного кислородного равновесия.

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