LIPID PEROXIDATION INHIBITS PHOSPHOLIPASE A_2-CATALYZED PHOSPHOLIPID HYDROLYSIS IN MICROSOMAL AND MITOCHONDRIAL MEMBRANES

P. S. Balevska, T. A. Kassabova,
É. M. Rusanov, and V. E. Kagan

UDC 612.397.2.015.14:577.152.311

KEY WORDS: lipid peroxidation; phospholipase A_2; hydrolysis of phospholipids; mitochondria; microsomes.

Two physiologically important methods of modification of the properties of the lipid bilayer of biological membranes are now known: the action of phospholipases and induction of lipid peroxidation (LPO) [1, 2]. In many pathological states (for example, in ischemic, hypoxic, and stress-induced damage) the structural and functional organization of membrane formations is disturbed by activation of both these processes [9, 13].

It was shown previously that peroxidation products of phospholipids (PL) may be the substrate for endogenous phospholipases in photoreceptor cell membranes [4]. Under real conditions *in vivo*, a very small proportion of membrane PL, not more than 1-5% of their total content in the membrane, is involved in LPO, even when it is activated sharply due to the action of extremal factors or to the development of pathological processes [2]. However, the question how induction of LPO acts on hydrolysis of the rest of the unoxidized PL in the membranes is not yet clear. Data in the literature on the problem are quite contradictory [11, 14]. Hence the importance of a study of mechanisms of the mutual effect of LPO reactions and PL hydrolysis in biomembranes.

The aim of this investigation was to study the kinetics of phospholipase A_2-catalyzed PL hydrolysis in mitochondrial and microsomal membranes from rat liver after preliminary induction of LPO in them.

EXPERIMENTAL METHOD

Experiments were carried out on male Wistar rats weighing 150-170 g. The microsomal fraction was isolated after preliminary perfusion of the liver with 1.15% KCl solution and homogenization in medium of the following composition: 1.15% KCl, 50 μM EDTA, 1% bovine serum albumin (purified beforehand from free fatty acids with the aid of activated charcoal), pH 7.4, at 4°C, by differential centrifugation (100,000g, 60 min). The residue of microsomes thus obtained was resuspended in the same medium, but without albumin and was recentrifuged under the same conditions. The microsomal fraction was kept in the form of a residue, covered by a layer of 1.15% KCl. Mitochondria were obtained by the standard procedure in 0.25 M sucrose (pH 7.4) and washed twice with the same medium. LPO was induced in suspensions of microsomes (3.0 mg/ml) and mitochondria (5.0 mg/ml) by a system of Fe^{++} + ascorbate (20 and 500 μM respectively) in 50 mM Tris-HCl buffer, pH 7.4, at 37°C. The reaction was stopped by placing the suspension in ice, after which it was centrifuged at 144,000g for 60 min to remove LPO inducers. Activity of phospholipase A_2 was recorded pH-metrically [10], using the OP-208 pH meter (Hungary) and "Servogor RE511" automatic writer (West Germany), the complete scale of which corresponded to 0.1 pH unit in medium containing 5 mM Tris-HCl, 30 mM KCl, pH 7.8, at 30°C, with the addition of 5 mM $CaCl_2$. Oleic acid ($C_{18:1}$) was used for calibration. Changes in pH during recording of phospholipase activity did not exceed 0.07 pH unit. The content of LPO products was recorded by the reaction with 2-thiobarbituric acid by the method in [6], using a coefficient of molar extinction of $\varepsilon_{535} = 1.56 \cdot 10^5\ M^{-1}\ cm^{-1}$. Lipids were extracted from the membranes with a mixture of chloroform and methanol (2:1 by volume). The UV-spectra of solutions of lipids in cyclohexane were

Institute of Physiology, Bulgarian Academy of Sciences, Sofia 1113, Bulgaria. (Presented by Academician of the Academy of Medical Sciences of the USSR T. A. Tashev.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 99, No. 2, pp. 161-164, February, 1985. Original article submitted May 29, 1984.

0007-4888/85/9902- 0187$09.50

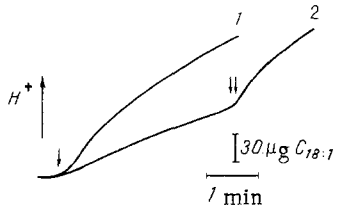

Fig. 1

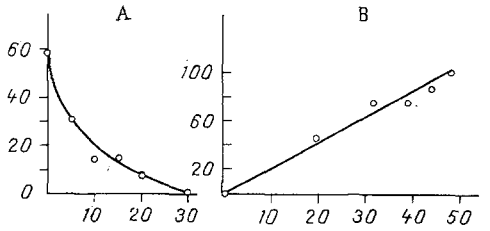

Fig. 2

Fig. 1. Changes in pH of microsomal suspension during PL hydrolysis catalyzed by phospholipase A_2 from pig pancreas. 1) Control, 2) membranes + Fe^{++} + ascorbate (20 min). Composition of reaction mixture: 5 mM Tris-HCl, 30 mM KCl, 5 mM $CaCl_2$, 0.6 mg microsomal protein/ml, pH 7.8, at 30°C; 0.14 μg phospholipase A_2 (0.083 unit/ml); total volume 5.8 ml. One arrow — phospholipase A_2; two arrows — unoxidized microsomal membranes.

Fig. 2. Effectiveness of inhibition of PL hydrolysis in microsomal membranes as a function of intensity of LPO. A: Abscissa, incubation time in presence of LPO inducers (in min); ordinate, velocity of PL hydrolysis (in μg fatty acid/min); B: abscissa, MDA concentration (in nanomoles/mg protein); ordinate, effectiveness of inhibition of PL hydrolysis (in %).

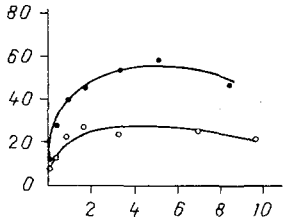

Fig. 3. Velocity of PL hydrolysis in microsomal membranes as a function of Ca^{++} concentration in incubation medium. Abscissa, Ca^{++} concentration (in mM); ordinate, velocity of PL hydrolysis, (in μg fatty acid/min).

recorded on a "Specord VSU-2P" spectrophotometer (East Germany). Protein was determined by Lowry's method.

The phospholipase A_2 was obtained from pig pancreas (from Boehringer, West Germany) and from bee venom (from Serva, West Germany), Tris was from Boehringer, 2-thiobarbituric acid was from Merck, West Germany, and EDTA and oleic acid were from Sigma, USA; the remaining reagents were of the chemically pure grade.

EXPERIMENTAL RESULT

Kinetic curves of changes in pH of the incubation medium of the microsomal suspension in the presence of phospholipase A_2, catalyzing hydrolysis of PL, with the formation of free fatty acids, are shown in Fig. 1. Under these experimental conditions the initial velocity of PL hydrolysis in microsomal membranes in the control was 58 μg fatty acid/min. Preliminary induction of LPO in the microsomes by means of a system of Fe^{++} + ascorbate led to inhibition of phospholipase A_2-catalyzed PL hydrolysis. After induction of LPO for 20 min the initial velocity of PL hydrolysis in a microsomal suspension with the same protein concentration as in the control sample was 7.5 μg fatty acid/min. The effect of inhibition of PL hydrolysis was invensified with an increase in the duration of preincubation of the microsomal membranes in the presence of LPO inducers (Fig. 2A); the degree of inhibition, moreover, was a linear function of accumulation of malonyl dialdehyde (MDA), one of the products of LPO (Fig. 2). After incubation of the microsomal suspension for 30 min in the presence of LPO inducers, leading to accumulation of 48 nanomoles MDA per milligram protein, no phospholipase A_2 activity could be recorded, i.e., the inhibitory effect reached 100%.

TABLE 1. Effect of LPO on Velocity of PL Hydrolysis in Microsomal and Mitochondrial Membranes in Presence of Phospholipase A_2 from Pig Pancreas (A) and Bee Venom (B)

Object	Experimental conditions	Phospholipase A_2 activity		MDA concentration
		A	B	
Microsomes	Control	58,3	38,8	0,4
	LPO	7,5	6,7	44,2
Mitochondria	Control	54,0	38,0	0,7
	LPO	24,2	18,8	20,4

Legend. LPO was induced for 20 min. Phospholipase activity expressed in μg fatty acid/min, MDA concentration in nanomoles/mg protein.

A similar inhibitory effect of LPO on phospholipase A_2-catalyzed PL hydrolysis also was found on mitochondrial membranes. In this case also, accumulation of LPO products led to a decrease in the velocity of free fatty acid formation during incubation of the membranes with phospholipase A_2 (Table 1). The inhibitory effect discovered was likewise independent of the source of phospholipase, for it was found whether the phospholipase A_2 used was from pig pancreas or from bee venom (Table 1).

The question arises, what is the mechanism of the inhibitory action of LPO products on phospholipase A_2-catalyzed PL hydrolysis? It can be asserted that the inhibitory effect is not based on the direct action of LPO products on the enzyme. This conclusion can be drawn from two facts: first, addition of "unoxidized" membranes to a suspension in which the velocity of hydrolysis was reduced by 60% as a result of preinduction of LPO led to an increase in the velocity of PL hydrolysis in the membrane (Fig. 2) and, second, the character of dependence of phospholipase A_2 activity on the Ca^{++} ion concentration in the incubation medium was unchanged for membranes subjected to LPO (Fig. 3).

Exhaustion of substrate for the phospholipase reaction as a result of LPO can hardly be the cause of inhibition of PL hydrolysis by phospholipase A_2. Recording UV absorption spectra of solutions of lipids extracted from control and LPO-treated membranes showed that the content of unconjugated dienes (with maximum of absorption at 210 nm), even after induction of LPO for 30 min, when the velocity of PL hydrolysis was reduced virtually to zero, was reduced by not more than 30%. When the possible causes of inhibition of phospholipase A_2-catalyzed PL hydrolysis after induction of LPO are examined, the following circumstances must be taken into account: 1) LPO substrates are the most unsaturated fatty-acid residues of PL [12]; 2) hydroperoxides and other LPO products form "peroxide clusters" in the membrane [3, 5]; 3) the phase of unoxidized lipids in the membrane is enriched as a result of LPO with more saturated PL; 4) the velocity of the phospholipase reaction depends on the degree of unsaturation and orderly arrangement of PL in the membrane [8]. Hence it can be postulated that the possible causes of the reduction in the velocity of PL hydrolysis in the membrane after LPO induction are a decrease in the content of polyunsaturated and unoxidized PL in the membrane, and a redistribution of PL in the membrane (as a result of lateral separation of oxidized and unoxidized phases) which leads to an increase in orderliness and a decrease in accessibility of PL for phospholipase attack. Incidentally, an increase in orderliness of PL in the membrane as a result of LPO was noted previously by the use of fluorescent and spin probes [5, 7, 13].

It may be concluded by stating that independently of the concrete cause of inhibition of the phospholipase reaction after LPO, this effect itself may have far-reaching consequences, for phospholipase A_2-catalyzed PL hydrolysis is the initial stage of a cascade of reactions that leads ultimately to synthesis of prostaglandins and leukotrienes, the most important intracellular regulators.

LITERATURE CITED

1. Yu. A. Vladimirov, V. I. Olenev, T. B. Suslova, et al., in: Progress in Science and Technology. Series: Biophysics [in Russian], Vol. 5, Moscow (1975), pp. 56-117.

2. V. E. Kagan, "Mechanisms of structural and functional modification of biomembranes during lipid peroxidation," Author's Abstract of Doctoral Dissertation, Moscow (1981).
3. V. E. Kagan, O. A. Azizova, Yu. V. Arkhipenko, et al., Biofizika, No. 4, 625 (1977).
4. V. E. Kagan, A. A. Shvedova, and K. N. Novikov, Biofizika, No. 2, 279 (1978).
5. N. K. Klaan, O. A. Azizova, L. A. Sibel'dina, et al., Biokhimiya, No. 4, 626 (1983).
6. J. A. Beuge and S. D. Aust, in: Methods in Enzymology, ed. S. Fleischer and L. Packer, New York (1978), p. 506.
7. G. E. Dobretsov, T. A. Borschevskaya, V. A. Petrov, et al., FEBS Lett., 84, 125 (1977).
8. M. K. Jain and G. H. De Haas, Biochim. Biophys. Acta, 736, 157 (1983).
9. F. Z. Meerson, V. E. Kagan, Yu. P. Kozlov, et al., Basic Res. Cardiol., 77, 465 (1982).
10. J. I. Salach, P. Turini, R. Seng, et al., J. Biol. Chem., 246, 331 (1971).
11. A. Sevanian, S. F. Muakkassah-Kelly, and S. Montestruque, Arch. Biochem., 223, 441 (1983).
12. L. A. Witting, J. Am. Oil Chem. Soc., 42, 908 (1965).
13. Yu. A. Vladimirov, V. I. Olenev, T. V. Suslova, et al., Adv. Lipid Res., 17, 173 (1980).
14. M. Yasuda and T. Fujita, Jpn. J. Pharmacol., 27, 429 (1977).

CHOLESTEROL EXTRACTION FROM BIOLOGICAL MEMBRANES BY POSITIVELY CHARGED PHOSPHATIDYLCHOLINE MICELLES

E. A. Borodin, M. É. Lanio,
É. M. Khalilov, S. S. Markin,
T. I. Torkhovskaya, M. M. Rozkin,
I. M. Sapelkina, S. N. Kulakova,
M. M. Levachev, A. I. Archakov,
and Yu. M. Lopukhin

UDC 616.13-004.6-092.9-07:616-008.939.22-074

KEY WORDS: atherosclerosis; membranes; phosphatidylcholine; cholesterol.

Phospholipids (PL) have the property of extracting cholesterol (CH) from biological membranes [2, 3]. The writers have shown that CH-accepting properties *in vitro* are most marked in positively charged micelles of polyunsaturated phosphatidylcholines (PCH).

This paper describes the results of a study of the possibility of extracting CH from biological membranes *in vivo* in rabbits with experimental atherosclerosis by intravenous injection of positively charged soy PCH micelles.

EXPERIMENTAL METHOD

Experimental atherosclerosis was induced in male rabbits weighing 2.5-4 kg by feeding with CH for 3 months [4]. Half of the animals (experimental group), 2 weeks after stopping the high cholesterol diet, were given PCH intravenously at the rate of 3 or 4 injections per week for 5 weeks. The total dose of PCH given to one animal was 10 g. Animals of the second (control) group were kept under conditions of spontaneous regression of their atherosclerotic lesions. Total CH, PL, triglycerides, and CH and PL of high density lipoproteins (HDL) in the blood serum were determined on an "SMA-12/60 Technicon" automatic analyzer [4]. The methods of isolating ghost erythrocytes, extracting lipids, and determining CH and PL in lipid extracts were described previously [3]. The fatty acid composition of HDL phospholipids was studied on a "Tsvet-106" gas-liquid chromatograph in the Silar 10-ts phase, column temperature 196°C, vaporizer temperature 200°C, detector temperature 200°C. Carrier gas nitrogen, flow rate 40 ml/min. Erythrocyte ATPase activity was determined by the method in [6]. The microviscosity of HDL and the erythrocyte ghosts was investigated by determining the ratio between fluorescence of pyrene excimers and monomers F_{470}/F_{391} [2]. The intensity of fluorescence was measured on a Hitachi-2MPF spectrofluorometer. Platelet aggregation

N. I. Pirogov Second Moscow Medical Institute. Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 99, No. 2, pp. 164-166, February, 1985. Original article submitted November 12, 1983.

 0007-4888/85/9902-0190$09.50