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Modeling the Cascade of Enzymatic Reactions in Liposomes Including Successive Free Radical Peroxidation, Reduction, and Hydrolysis of Phospholipid Polyenoic Acyls for Studying the Effect of These Processes on the Structural-Dynamic Parameters of the Membranes

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Abstract—Studies of the effect of primary products of free radical lipid peroxidation (LPO) on the structural-dynamic parameters of natural lipid–protein supramolecular complexes (biomembranes and blood serum lipoproteins) using standard inducers of radical processes *in vitro* (azo-initiators, transition metal ions, flavin oxidases, etc.) are impossible because of simultaneous production of numerous secondary LPO products that can induce structural changes. The data obtained suggest that phospholipid liposome microviscosity, as assessed by the extent of eximerization of the fluorescent probe pyrene, may significantly differ when oxidation is induced by animal C-15 lipoxygenase (yielding acylhydroperoxides only) and Fe^{2+}–ascorbate system (resulting in simultaneous accumulation of primary and secondary LPO products). It is also shown that liver glutathione S-transferase can effectively reduce hydroperoxy-acyls in phospholipid liposomes and liver microsomes without their preliminary hydrolysis with phospholipase A_2. An enzymatic system is proposed for a cascade of enzymatic reactions simulating lipohydroperoxide metabolism in living cells, including successive free radical oxidation of phosphatidylcholine polyenoic acyls, reduction of their hydroperoxy-derivatives, and hydrolysis of fatty acid residues in the course of catalysis mediated by animal C-15 lipoxygenase, glutathione S-transferase, and phospholipase A_2, respectively.

Key words: lipid–protein supramolecular complexes, structural-dynamic membrane parameters, free radical oxidation, animal C-15 lipoxygenase, glutathione S-transferase, phospholipase A_2

Free radical peroxidation of phospholipid polyenoic acyls is associated with modification of the structure and function of natural lipid–protein supramolecular complexes (such as biomembranes and blood serum lipoproteins) and, finally, may lead to their complete disintegration [1]. NADPH-dependent lipid peroxidation of liver microsome membranes is characterized by phase mode of changes in the activity of some membrane-bound enzymes [2, 3]. The activity of these enzymes at some oxidation stages may not only decrease, but increase as well [3]. The reversible oppositely directed changes in the activity of different membrane-bound enzymes at the early stages of oxidation of the same membranes may be accounted for by the structural rearrangements of phospholipid bilayer rather than direct action of produced hydroperoxides and other LPO products. For example, although enzymatic oxidation of liver microsomes yielding hydroperoxy-acyls in membrane phospholipids was not accompanied by cytochrome P-450 degradation [4], it markedly affected the activity of this microsomal enzyme [5]. To simulate free radical peroxidation in the liposomes, biomembranes, and lipoproteins, different types of inducers are usually used, i.e., azo-initiators [6], transition metal ions (Fe^{2+} or Cu^{2+}) [7, 8], or enzymatic systems generating superoxide anion radicals (xanthine oxidase [9], flavin NADPH-dependent oxygenases of liver microsomes [10] or macrophages and lymphocytes [11]). It is known that in the course of radical degradation azo-initiators produce $R^{\bullet}$-radicals [6], whereas Fe^{2+} (in the presence of reducing agent ascorbate) and flavin oxidases generate superoxide anion radical O_2^- [12, 13]. Lipid

Abbreviations: MDA) malonyl dialdehyde; LPO) lipid peroxidation; PUFA) polyunsaturated fatty acids; GSH) reduced glutathione; GSSG) oxidized glutathione.

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hydroperoxides accumulated in the course of free radical peroxidation of biomembranes and lipoproteins readily undergo oxidative destruction yielding various secondary LPO products, including aldehydes, ketones, and other carbonyl compounds, with 4-hydroxynonenal and malonyl dialdehyde (MDA) being predominant [14-16]. It should be noted that free radical peroxidation of phospholipids in biomembranes and lipoproteins is associated with cooxidation of cholesterol to form products with greater polarity [17, 18], which, apparently, also contribute to the change in the structure of these lipid–protein complexes. Thus, the systems that are usually used to induce free radical peroxidation of biomembranes and lipoproteins exclude the possibility of separate study of the modifying effect of primary and secondary LPO products. However, it is believed that the mechanisms of action of primary and secondary LPO products on biomembranes and lipoproteins are principally different. Hydroperoxy-acyls, owing to the increased polarity, should be displaced from the hydrophobic microenvironment to the aqueous phase [19], whereas MDA and other aldehydes may form intramolecular and intermolecular cross-links with free amino groups in proteins and amine-containing phospholipids [14, 15]. It was found that the cytosol of the majority of cells and animal blood serum contain enzymatic systems including Se-containing glutathione peroxidases [20-22] and non-selenic glutathione S-transferases [23-25]. These enzymes can effectively reduce different organic hydroperoxides, including hydroperoxides of polyunsaturated fatty acids (PUFAs) [20, 23] to the corresponding hydroxy-derivatives (alcohols), which prevents further oxidative degradation of lipohydroperoxides leading to production of secondary alkoxy lipid radicals ($LO^{\cdot}$) according to the reaction:

$$LOOH \rightarrow LO^{\cdot} + OH^{-}.$$

Because of the high activity of enzymatic systems of organic hydroperoxide utilization, it is unlikely that significant amounts of lipoperoxides in living cells and tissues are accumulated *in vivo* [26]. For this reason, study of the effect of the products of enzymatic oxidative metabolism of PUFAs (lipohydroperoxides and corresponding hydroxy-derivatives) on the structure and function of natural lipid–protein supramolecular complexes is an important and topical problem. Obviously, it is necessary to develop a model system that would allow controlled accumulating of solely the primary products in the course of free radical peroxidation of biomembranes and lipoproteins. In our opinion, the use of the highly specific C-15 lipoxygenase from animal cells, which is able to oxidize unsaturated acyls of phospholipids contained in native biomembranes and lipoproteins with accumulation of the corresponding hydroxy-derivatives [27-29], is the best solution to this problem. The development of a model that includes the enzymes catalyzing oxidation, reduction, and hydrolysis of polyenoic acyls of membrane phospholipids would also enable reconstruction of the coupled enzymatic system of oxidative metabolism of PUFAs in the cells.

MATERIALS AND METHODS

Phenylhydrazine hydrochloride used in the study was obtained from Fluka (Switzerland), egg phosphatidylcholine was from Merck (Germany), dilinoleoylphosphatidylcholine and amberlite IRA-400 were from Serva (Germany), DEAE Sephadex A_{50} and ampholine pH 5-8 were purchased from Pharmacia Fine Chemical (Germany). 2-Thiobarbituric acid, EDTA, GSH, GSSG, $NADP^+$, NADPH, L-isocitrate, linoleic acid, triphenylphosphine, pyrene, pig heart isocitrate dehydrogenase, soybean lipoxygenase-1, rabbit liver glutathione S-transferase, yeast glutathione reductase, phospholipase A_2 from *Vipera russelli* venom, Tris, and inorganic salts were from Sigma (USA).

The commercial preparation of egg phosphatidylcholine was preliminarily purified from the contaminating oxidation products by triple re-precipitation from acetone. Contamination with free PUFAs was removed by amberlite IRA-400 treatment (in the OH-form) as described earlier [30]. Liposomes from egg lecithin were obtained as described before [31]. Briefly, alcoholic lipid solutions were injected with the use of a microsyringe into medium containing 10 mM Tris-HCl and 0.15 M NaCl, pH 7.4, with intense stirring on a Vortex. The microsomes from the liver of puberty male Wistar rats were isolated by differential centrifuging at 105,000*g* for 60 min in a Beckman L-8M refrigerating centrifuge (USA) as described earlier [32]. NADPH-dependent oxidation of the microsomes (0.5 mg protein per ml) was conducted in medium containing 0.2 M Tris-HCl buffer (pH 7.4), 0.25 mM $NADP^+$, and NADPH-regenerating system consisting of 2.5 mM L-isocitrate, 5 mM $MgCl_2$, and pig heart isocitrate dehydrogenase (1 U/ml) without exogenous Fe^{2+} addition [32].

Experimental anemia with concomitant reticulocytosis was induced in rabbits by subcutaneous injection of the hemolytic phenylhydrazine (6.25 mg/kg) for four days. Thereafter, C-15 lipoxygenase was isolated from the lysate of reticulocyte-enriched blood cell mass by precipitation with 55% ammonium sulfate as described earlier [27, 33, 34]. The enzyme was then successively purified to homogeneous state (assessed by PAGE) using ion-exchange chromatography on DEAE-Sephadex A_{50} in NaCl linear gradient and preparative isoelectric focusing within pH range of 5-7 [27, 33, 34]. Crude preparation of rabbit reticulocyte C-15 lipoxygenase was purified in the Institute of Biochemistry (Medical Department, Alexander von Humboldt University, Germany). Commercial preparations of soybean lipoxygenase-1, rabbit liver glutathione S-transferase, and phospholipase A_2 from *Vipera russelli* venom were used without addi-

tional purification. Lipoxygenase activity was monitored spectrophotometrically by the accumulation of hydroperoxides (conjugated dienes) at 233 nm using 40 μM linoleic acid emulsion as a substrate [29, 34].

To study the effect of LPO on membrane structure, liposomal phospholipids (200 μg/ml) were oxidized for approximately 20 min in medium containing 10 mM Tris-HCl and 0.15 M NaCl, pH 7.4, at 25°C in the presence of reticulocyte C-15 lipoxygenase (180 U/ml). The reaction stopped as a result of self-inactivation of the enzyme [27, 28]. In another variant, the reaction was conducted for approximately 120 min in the presence of 5 μM Fe_2SO_4 and 0.1 mM ascorbate. In this case, the reaction was stopped by adding 1 mM EDTA. The accumulation of primary LPO products was monitored by the production of conjugated dienes at 233 nm [35]; secondary products, by the increase in absorbance at 260-280 nm and by the production of 2-thiobarbituric acid reactive substances [35] recalculated for MDA. Oxygen consumption was measured on a Beckman 0260 oxygen analyzer (USA) equipped with a Clark electrode [29]. Hydroperoxides were thermally decomposed by incubating the enzymatically-oxidized liposomes at 80°C for 10 min. To reveal the structural changes in phospholipid membranes occurred during their enzymatic (catalyzed by reticulocyte C-15 lipoxygenase) and nonenzymatic (induced by Fe^{2+}–ascorbate system) oxidation, we analyzed the changes in pyrene fluorescence parameters [36, 37]. The fluorescence spectra were recorded on an Aminco SPF-500 spectrofluorometer (France). Microviscosity of the membranes was calculated based on pyrene lateral diffusion coefficient as described in [36].

To study the effect of phospholipase A_2 and glutathione S-transferase on membrane lipohydroperoxide metabolism, in the course of free radical enzymatic oxidation we added to the incubation medium the corresponding enzymatic systems that allowed us to perform the following successive reactions:

$$\text{lecithin} + O_2 \xrightarrow{\text{reticulocyte C-15 lipoxygenase}} \text{lecithin-OOH}, \quad (1)$$

$$\text{lecithin} + H_2O \xrightarrow{\text{phospholipase } A_2} \text{lysolecithin} + \text{LH}, \quad (2)$$

$$\text{LH} + O_2 \xrightarrow[\text{soybean C-15 lipoxygenase}]{\text{reticulocyte C-15 lipoxygenase}} \text{LOOH}, \quad (3)$$

$$\text{LOOH} + 2\,\text{GSH} \xrightarrow{\text{GSH-transferase}} \text{LOH} + \text{GSSG} + H_2O. \quad (4)$$

As is seen from the schemes, we used reticulocyte C-15 lipoxygenase to oxidize polyunsaturated membrane lecithins (reaction (1)) to the corresponding hydroperoxy-derivatives (lecithin-OOH). Phospholipase A_2 catalyzed enzymatic hydrolysis of nonoxidized polyenoic lecithin β-acyls (reaction (2)) to form lysophosphatidylcholines and free polyenoic fatty acids (LH). Free PUFAs (LH) can be oxidized to the corresponding hydroperoxides (LOOH) via reticulocyte C-15 lipoxygenase catalysis and soybean C-15 lipoxygenase catalysis as well (reaction (3)). The latter, unlike animal C-15 lipoxygenase, can oxidize only free PUFAs but not polyenoic acyls of membrane phospholipids. Hydroperoxides of free PUFAs (LOOH) produced using plant or animal lipoxygenase were reduced to hydroxy acids (LOH) according to reaction (4) using rabbit liver glutathione S-transferase at coupled oxidation of reduced glutathione (GSH). To study the kinetics of enzymatic reduction of lipohydroperoxides, we used stoichiometric bioregeneration of oxidized glutathione (GSSG) in the presence of NADPH in the course of yeast glutathione reductase-mediated catalysis (reaction (5)):

$$\text{GSSG} + \text{NADPH} + H^+ \xrightarrow{\text{GSSG-reductase}} 2\,\text{GSH} + \text{NADP}^+. \quad (5)$$

When performing the experiments, we took into consideration the possibility of spontaneous oxidation of reduced glutathione by lipohydroperoxides according to reaction (6):

$$\text{LOOH} + 2\,\text{GSH} \xrightarrow{\text{nonenzymatically}} \text{LOH} + \text{GSSG} + H_2O. \quad (6)$$

In the studies of enzymatic metabolism of lipoperoxides, egg lecithin liposomes (450 μg/ml) were incubated at 25°C in 1-cm cuvette for a Hitachi-557 spectrophotometer (Japan). The incubation medium contained 50 mM potassium-sodium phosphate buffer (pH 7.4) and 50 μM $CaCl_2$. We recorded the changes in the specimen absorbance at 233 nm (control for lecithin spontaneous oxidation). After a 5-min incubation, homogeneous preparation of reticulocyte C-15 lipoxygenase (3 U/ml) or soybean lipoxygenase-1 (25 U/ml) was added to the cuvette, and the mixture was incubated for a further 5 min. Thereafter, 1 mM EDTA, 1 mM GSH, 0.2 mM NADPH, and yeast glutathione reductase (1 U/ml) were added to the same cuvette [38]. For the next 5 min, the kinetics of GSH spontaneous oxidation in the presence of enzymatically produced acylhydroperoxides (reaction (6)) was recorded by the coupled reaction (5) at 340 nm. Next, enzymatic reduction of lipoperoxides was started by the addition of rabbit liver glutathione S-transferase (1 U/ml), and the changes in absorbance at 340 nm were recorded for the next 5 min. In a parallel experiment, we added phospholipase A_2 (7 μU/ml) from *Vipera russelli* venom to the medium containing reticulocyte or soybean lipoxygenase and monitored the kinetics of oxidation of hydrolyzed PUFAs at 233 nm.

After the addition of GSH, NADPH, glutathione reductase, and glutathione S-transferase, the kinetics of reduction of free PUFA hydroperoxides was recorded at 340 nm. The rate of nonenzymatic oxidation of liposomes during 5-min incubation was negligible, and the rate of nonenzymatic reduction of hydroperoxides under the conditions described was no more than 1-3% of the rate of enzymatic reactions (Fig. 3). In some experiments, dilinoleoylphosphatidylcholine liposomes (450 μg/ml) were oxidized for 20 min with rabbit reticulocyte C-15 lipoxygenase in 50 mM sodium-potassium phosphate buffer, pH 7.4. Aliquots of oxidized liposomes were incubated for 20 min in the presence of 50 μM $CaCl_2$ and *Vipera russelli* venom phospholipase A_2 (20 μU/ml). Hydrolyzed hydroperoxyacyls were then reduced with triphenylphosphine [39]. The second portion of oxidized liposomes was preliminarily reduced for 20 min in the presence of 1 mM GSH and rabbit liver glutathione S-transferase (1 U/ml) and only then hydrolyzed with phospholipase A_2. Polar hydroperoxy- and hydroxy-derivatives of linoleic acid were isolated from the incubation medium [40, 41] and then purified and analyzed by HPLC as described before [42].

RESULTS AND DISCUSSION

Figure 1 shows the kinetics of enzymatic oxidation of egg lecithin liposomes (continuous spectrum recording). As is seen from the figure, oxidation of polyenoic acyls with reticulocyte C-15 lipoxygenase leads to accumulation in liposomes of only primary LPO products with the absorbance maximum at 233 nm, whereas secondary oxidation products absorbing at 260-280 nm [43] are completely absent. Oxidation of phospholipid polyenoic acyls is stopped approximately 20 min after the beginning of incubation as a result of inhibition of C-15 lipoxygenase by the produced lipohydroperoxides [44]. An increase in the incubation duration to 60 min did not cause any marked changes in the spectral characteristics (Fig. 1). In the second series of experiments, nonenzymatic oxidation of liposomes of similar composition was induced by Fe^{2+}–ascorbate system. After incubating the reaction mixture for approximately 120 min, the reaction was stopped by binding Fe^{2+} with an excess of chelating agent EDTA (1 mM). Note that in all series of experiments the conditions of enzymatic and nonenzymatic liposome oxidation were selected in preliminary experiments so that the amount of primary LPO products formed in the incubation medium in both cases was approximately the same (Fig. 1, Table 1). The data shown in Fig. 1 and in Table 1 indicate that in the course of enzymatic oxidation only lipohydroperoxides are produced, whereas in the course of nonenzymatic oxidation significant amounts of MDA and other secondary products with absorbance maximum of 267 nm are accumulated. We found that, in the case of enzymatic oxidation of liposomes, MDA content was not increased significantly compared to the initial level. Conversely, in the case of oxidation induced by the Fe^{2+}–ascorbate system, MDA content increased almost by an order of magnitude (Table 1). Thus, structural changes in the liposomes occurring during their enzymatic oxidation, in our experiments could only be due to the increase in the content of primary LPO products. In the case of nonenzymatic oxidation of liposomes, under conditions of simultaneous accumulation of primary and secondary LPO products, an unambiguous explanation of the observed structural changes is impossible.

Figure 2 shows the dependence of the C/F_m parameter on pyrene concentration (where C is pyrene concentration and F_m is the fluorescence intensity of pyrene monomers at 395 nm (expressed in relative units)). Straight lines obtained in this coordinate system indicate that all pyrene is contained solely in the lipid phase of membranes [45], with the slope being proportional to the lateral diffusion coefficient of pyrene in the membrane (i.e., reciprocally depending on the membrane viscosity). The data shown in Fig. 2 indicate that nonenzymatically oxidized membranes are more viscous than nonoxidized. Actually, according to the calculations, the microviscosity of nonenzymatically oxidized membranes increases by 26 ± 6%, which agrees with the previously published data [46]. Alternatively, microviscosity of enzymatically oxidized liposome phospholipids decreases by 27 ± 5% (Fig. 2). In accordance with these data, thermal destruction of lipoxygenase-produced hydroperoxides in our experiments caused an increase in membrane microviscosity by 16 ± 7% (data not shown). The polarity of the hydrophobic component of

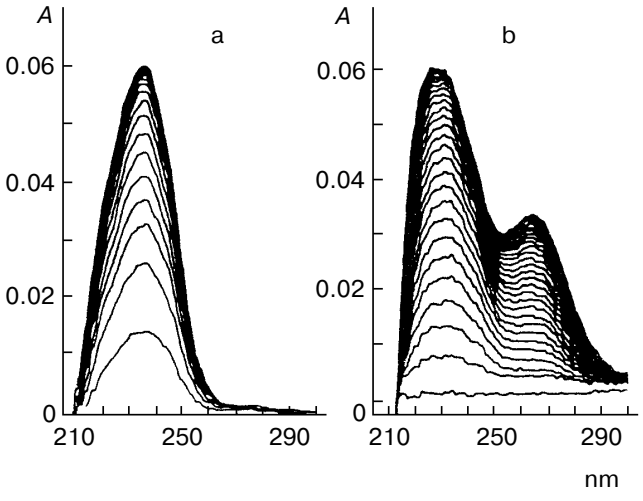

Fig. 1. UV absorbance spectra of the products of free radical peroxidation of egg lecithin liposomes generated during catalysis with rabbit reticulocyte C-15 ipoxygenase (a) and induction with Fe^{2+}–ascorbate system (b). The experimental conditions are described in "Materials and Methods". The spectra were recorded after automatic subtraction of the original spectrum at 1- (a) or 10-min (b) intervals.

Table 1. Accumulation of primary (acylhydroperoxides) and secondary (2-thiobarbituric acid reactive substances) LPO products and changes in the polarity (lipid phase dielectric permeability constant) of egg lecithin liposomal membranes in the course of their enzymatic (induced by reticulocyte C-15 lipoxygenase) and nonenzymatic (induced by Fe^{2+}–ascorbate system) free radical peroxidation (the mean values of three independent experiments are shown)

Egg phosphatidylcholine liposomes	Oxidation products, nmol/mg phospholipid		Lipid phase dielectric permeability constant (parameter characterizing membrane polarity)
	conjugated dienes (acylhydroperoxides)	MDA and other 2-thiobarbituric acid reactive substances	
Initial	1.7 ± 0.1	2.4 ± 0.03	16.4 ± 0.15
Oxidized during catalysis with reticulocyte lipoxygenase	15.3 ± 0.2*	2.9 ± 0.04	53.0 ± 0.74*
The same after thermal decomposition of lipohydroperoxides	13.9 ± 0.3*,**	9.0 ± 0.11*,**	45.3 ± 2.77*,**
Oxidized as a result of induction with the Fe^{2+}–ascorbate system	16.0 ± 0.2*	23.4 ± 0.05*,**	40.7 ± 1.49*,**

Note: Statistically significant differences ($p < 0.05$) from initial liposomes (*) and liposomes oxidized with lipoxygenase (**).

liposomal membranes was studied by analyzing the parameters of the oscillation spectrum of pyrene fluorescence, whose changes in dependence on microenvironment polarity were studied earlier [37]. The ratio between the fluorescence intensity peaks at 383 and 372 nm (F_{383}/F_{372}) was taken as a parameter of polarity. It is known that this ratio decreases as pyrene microenvironment polarity increases (i.e., upon increase in the dipole moment and/or dielectric permeability constant) and that it practically does not depend on the presence of quenchers [37]. As is seen from the data shown in Table 1, as a result of accumulation of LPO products in liposomes the F_{383}/F_{372} ratio decreases, i.e., polarity of the membrane lipid phase increases. Note that in the case of enzymatic LPO, when only hydroperoxides are accumulated, the increase in the membrane polarity is greater than that observed during nonenzymatic LPO. As expected, membrane polarity decreases as a result of thermal destruction of hydroperoxides accumulated during enzymatic hydrolysis.

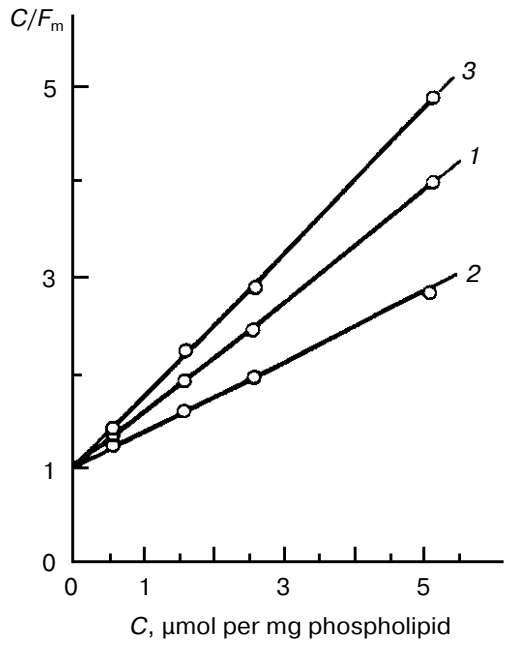

Fig. 2. Concentration self-quenching of pyrene monomer fluorescence (C is pyrene concentration, F_m is pyrene monomer fluorescence at 395 nm expressed in relative units) in initial egg lecithin liposomes (*1*), liposomes oxidized with rabbit reticulocyte C-15 lipoxygenase (*2*), and nonenzymatically (induced by Fe^{2+}–ascorbate system) oxidized liposomes (*3*).

Strictly speaking, the extent of pyrene eximerization in our experiments could depend not only on lipid viscosity, but also on quenching pyrene fluorescence by the products of lipid oxidation [46]. Nevertheless, explanation of the discovered changes by quenching pyrene fluorescence as a result of its interaction with the products of free radical peroxidation seems less probable, because the change in the membrane microviscosity caused by peroxidation was detected earlier both based on the rate of pyrene eximerization and with the use of other fluorescent probes [47]. Thus, the data suggest that enzymatic LPO is associated with a decrease in the viscosity of the hydrophobic layer of liposomal membranes and an increase in polarity. Conversely, as a result of nonenzymatic LPO, membrane microviscosity increases; however, the polarity of liposomes increases to a lesser extent than in the case of enzymatic LPO. The increase in the membrane viscosity in the course of nonenzymatic LPO is, apparently, associated with the fact that accumulation of conjugated dienes

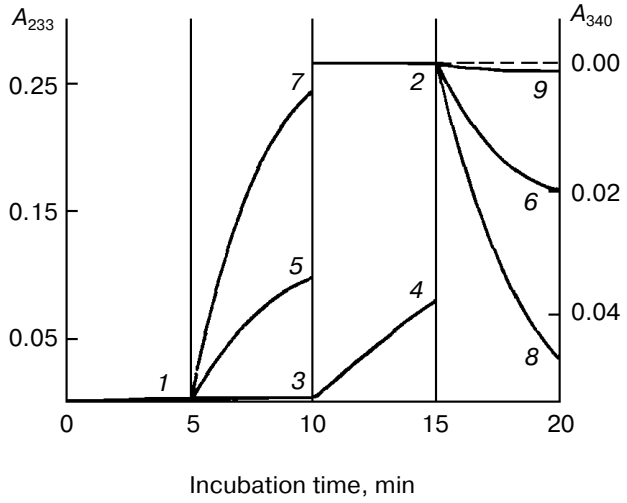

Fig. 3. Kinetics of enzymatic generation of lipohydroperoxides in egg lecithin liposomes catalyzed by animal and plant C-15 lipoxygenases (*1*, *3*, *4*, *5*, *7*) (monitored by accumulation of conjugated dienes at 233 nm) and enzymatic reduction of hydroperoxy-derivatives with rabbit liver glutathione-S-transferase (*2*, *6*, *8*, *9*) (monitored by NADPH oxidation at 340 nm in the coupled glutathione reductase-containing system): *1*) liposomes without enzymes; *2*) liposomes plus GSH-transferase; *3*) liposomes plus soybean C-15 lipoxygenase; *4*) liposomes plus soybean C-15 lipoxygenase plus phospholipase A_2; *5*) liposomes plus rabbit reticulocyte C-15 lipoxygenase; *6*) liposomes plus rabbit reticulocyte C-15 lipoxygenase plus GSH-transferase; *7*) liposomes plus rabbit reticulocyte C-15 lipoxygenase plus phospholipase A_2; *8*) liposomes plus rabbit reticulocyte C-15 lipoxygenase plus phospholipase A_2 plus GSH-transferase; *9*) nonenzymatic NADPH oxidation in the system containing liposomes plus rabbit reticulocyte C-15 lipoxygenase plus phospholipase A_2. *2*, *6*, *8*, *9*) The data were recorded in the presence of NADPH and GSSG bioregeneration system (GSH and GSSG reductase). The experimental conditions are described in "Materials and Methods".

(hydroperoxides) in phospholipid polyunsaturated acyls (with the absorbance maximum at 233 nm) occurs simultaneously upon (as seen in Fig. 1) their oxidative destruction yielding carbonyl compounds absorbing at 260-280 nm [43]. The validity of this assumption was confirmed by the results of experiments on thermal destruction of enzymatically occurring phospholipid hydroperoxy-acyls, in which the revealed structural changes were similar to those observed in the course of nonenzymatic LPO (Table 1). Nevertheless, with regard for the existence of potent systems of lipohydroperoxide utilization in living organisms [25], the probability of accumulation of considerable amounts of secondary products of hydroperoxide degradation (as it is in model systems in the course of nonenzymatic LPO) is negligible (Fig. 1, Table 1).

Therefore, the proposed model of enzymatic peroxidation of membrane lipids apparently reflects the processes occurring in the cell *in vivo* with the greatest adequacy. However, it should be kept in mind that in the course of enzymatic reactions proceeding in native cells hydroperoxy-acyls of polyenoic phospholipids may be hydrolyzed by phospholipase A_2 [47-49]. Hydroperoxy-PUFAs occurring in this case may be restored to the corresponding alcohols by cytosolic glutathione peroxidase or glutathione S-transferase [20, 22, 24, 25]. In view of this, at the next stage of the study we attempted to simulate the sequence of the processes of enzymatic oxidation, hydrolysis, and reduction of polyenoic acyls of liposomal membranes. As is seen from Fig. 3, reticulocyte C-15 lipoxygenase can oxidize polyenoic acyls of membrane phospholipids yielding corresponding hydroperoxides (conjugated dienes absorbing at 233 nm). Note that free PUFAs produced in the course of phospholipase A_2-catalyzed hydrolysis are oxidized by reticulocyte C-15 lipoxygenase much more readily (Fig. 3, Table 2). In contrast to animal C-15 lipoxygenase from rab-

Table 2. Effect of phospholipase A_2 from *Vipera russelli* venom on generation of lipoperoxides in egg lecithin liposomes during peroxidation catalyzed by rabbit reticulocyte C-15 lipoxygenase and on their subsequent utilization catalyzed by rabbit liver glutathione S-transferase (mean values of seven independent experiments are shown)

Reaction	Without phospholipase A_2	With phospholipase A_2
Generation of lipoperoxides by reticulocyte lipoxygenase (determined by accumulation of conjugated dienes at 233 nm)		
v_0, nmol·min^{-1}	0.54 ± 0.13	2.35 ± 0.96
[*C*]*, nmol·ml^{-1}	1.62 ± 0.22	5.63 ± 0.64
Reduction of lipoperoxides by liver glutathione S-transferase (determined by oxidation of NADPH at 340 nm)		
v_0, nmol·min^{-1}	0.64 ± 0.05	1.26 ± 0.05
[*C*]*, nmol·ml^{-1}	0.99 ± 0.02	2.56 ± 0.06

* The amount of enzymatically produced or reduced lipohydroperoxides during 5-min incubation.

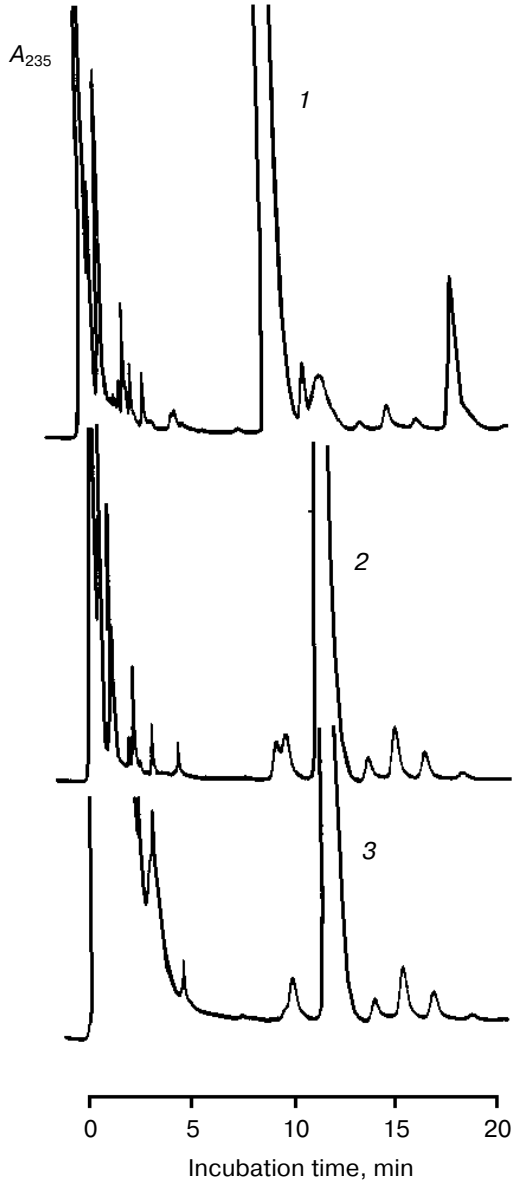

Fig. 4. Analysis of dilinoleoylphosphatidylcholine fatty acid residues using HPLC after oxidation of phospholipid liposomes with rabbit reticulocyte C-15 lipoxygenase (*1*) and after their reduction with rabbit liver glutathione S-transferase (*2*) or triphenylphosphine (*3*).

bit reticulocytes, plant C-15 lipoxygenase from soybeans can oxidize phospholipids only in micelles (i. e., in the presence of large amounts of detergents) but not in the membrane structures [25]. In the absence of detergents, this lipoxygenase can only oxidize free PUFAs produced as a result of enzymatic hydrolysis of phospholipids by phospholipase A_2 (Fig. 3). It is also known that cytosolic Se-containing glutathione peroxidase cannot directly reduce oxidized acyls of membrane phospholipids. It can reduce hydroperoxy-acyls only after their preliminary hydrolysis with phospholipase A_2 [48]. Figure 4 shows the results of HPLC analysis of hydroperoxy-derivatives occurring as a result of phospholipase hydrolysis of dilinoleoylphosphatidylcholine successively preincubated with reticulocyte C-15 lipoxygenase and liver glutathione S-transferase. As is seen from Fig. 4, we detected 13-hydroperoxylinoleate and 13-hydroxylinoleate, respectively. The product of enzymatic reduction of 13-hydroperoxylinoleate was identical (with respect to chromatographic pattern) to the products obtained by chemical reduction of this hydroperoxide with the use of triphenylphosphine (Fig. 4). Thus, we obtained decisive evidence that, in contrast to cytosolic Se-containing glutathione peroxidase non-selenic glutathione S-transferase [20, 24] is able to catalyze direct reduction of hydroperoxy-acyls of membrane phosphatidylcholines yielding corresponding hydroxy-derivatives (lecithin-OH), i.e., to catalyze the reaction:

$$\text{lecithin-OOH} + 2\,\text{GSH} \xrightarrow{\text{GSH-transferase}} \text{lecithin-OH} + \text{GSSG} + \text{H}_2\text{O}\,. \quad (7)$$

Note that the rate of nonenzymatic oxidation of glutathione with hydroxy-derivatives of phospholipids according to the reaction

$$\text{lecithin-OOH} + 2\,\text{GSH} \xrightarrow{\text{nonenzymatically}} \text{lecithin-OH} + \text{GSSG} + \text{H}_2\text{O} \quad (8)$$

which hindered our study was negligible under the conditions of our experiments (Fig. 3).

As is seen from Table 2, during 5-min incubation rabbit liver glutathione S-transferase reduced 47 ± 5% of the free

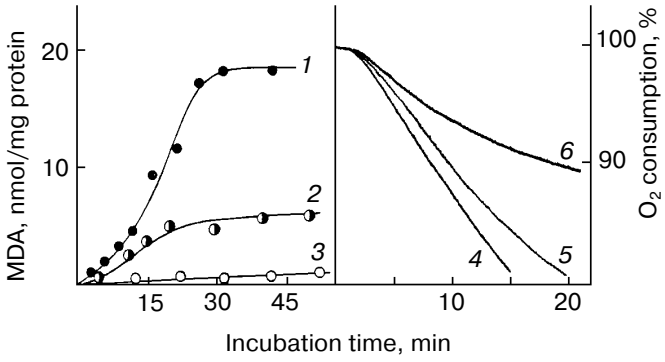

Fig. 5. Kinetics of NADPH-dependent free radical peroxidation of rat liver microsomal membranes (*1*, *4*) in the presence of 0.1 mM GSH (*2*, *5*) or 0.1 mM GSH and rabbit liver glutathione S-transferase (0.5 U/ml) (*3*, *6*) monitored by accumulation of 2-thiobarbituric acid reactive substances (MDA) (*1-3*) or by oxygen consumption (*4-6*).

PUFA hydroperoxides and 65 ± 11% lecithin hydroperoxy-acyls. This fact, on the one hand, demonstrates fairly high effectiveness of enzymatic utilization of lipid hydroperoxides. On the other hand, it is indicative of practically equal availability of free and esterified lipohydroperoxides for reduction with non-selenic glutathione S-transferase (Table 2).

In the model system used in this study, the activity of the lipoperoxide-generating enzyme was higher than the activity of the hydroperoxide-reducing enzyme. Conversely, in living cells the lipoperoxide-utilization systems are much more active than lipoperoxide-generating systems. As a result, their enzymatic reduction proceeds even more intensively, which prevents accumulation of significant amounts of lipohydroperoxides in normal cells *in vivo*. In accordance with this, in NADPH-dependent LPO of rat liver microsomes, we (Fig. 5), like other researchers [10], observed intensive production of hydroperoxy-phosphatidylcholines, which was suppressed in the presence of GSH (apparently as a result of nonenzymatic reduction of hydroperoxy-acyls according to reaction (8)) and was practically completely inhibited in the presence of GSH and rabbit liver glutathione S-transferase (Fig. 5). These findings are in good agreement with our results on rabbit liver glutathione S-transferase-catalyzed reduction of liposomal hydroperoxy-phosphatidylcholines oxidized with reticulocyte C-15 lipoxygenase (Fig. 4) and confirm the possibility of direct reduction of phospholipid hydroperoxy-acyls by this enzyme in native membranes.

Thus, our data suggest that, unlike the previously proposed mechanism [48-50], the effectiveness of enzymatic reduction of lipohydroperoxides in normal cell apparently does not depend of the action of membrane phospholipases that are activated in different pathologies, which allows us to regard non-selenic glutathione S-transferase as a key component of the reparation system of biomembranes. The ability of non-selenic glutathione S-transferase to catalyze direct reduction of membrane hydroperoxy-acyls, which was discovered in this study, allows using this enzyme in the system coupled with animal C-15 lipoxygenase and phospholipase A_2 for simulating successive enzymatic processes of free radical peroxidation, reduction, and hydrolysis of phospholipid polyenoic acyls. In an article in preparation for publication, which was based on the developed methodology, we will describe in detail the results of the study of structural-dynamic rearrangements in phospholipid membranes occurring during oxidation, reduction, and hydrolysis of unsaturated acyls.

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