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RADICAL-INDUCED PEROXIDATION AND FRAGMENTATION OF LIPIDS IN MODEL MEMBRANES

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In this report data permitting to estimate the interrelation of free-radical fragmentation and lipid peroxidation processes in model membranes are presented. These data were obtained in the course of radiation-induced lipid peroxidation studies in two-component liposomes, containing the easily oxidized rat liver phosphatidylcholine. As the second component peroxidation-stable saturated phospholipids, which can undergo fragmentation, were used. It is shown that intermediates and products of free-radical fragmentation of lipids can modulate the course of the peroxidation reaction of glycerophospholipids.

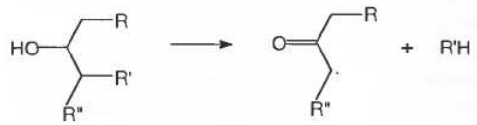
INTRODUCTION

Lipid peroxidation (LPO) and its influence on functional properties of biological membranes and the cell as a whole have been the object of numerous studies (Stark, 1991, Halliwell & Gutteridge, 1999). The LPO are known to be free radical reactions involving lipids which contain unsaturated fatty acid residues. We have demonstrated (Akhrem, Edimicheva, Zajtsev, Kisel, Timoschuk & Shadyro, 1991; Akhrem, Kisel, Shadyro & Yurkova, 1993; Edimicheva, Kisel, Shadyro, Vlasov, Yurkova; Muller, Batra, Senn, Giese, Kisel & Shadyro, 1997) that, in addition to the lipid peroxidations, interactions of lipids with free radical reaction initiators induce a fragmentation reaction which occurs in the polar part of a lipid possessing a hydroxyl group in β-position to an ester (phosphate) or amide bonding. Such lipids are phosphatidylglycerol, lysophospholipids, cardiolipin, sphingomyelin, cerebrosides, alkyl- or acylglycerols etc. So, the free radical fragmentation of phosphatidylglycerol yields phosphatidic acid and oxyacetone, while sphingomyelin and 1-palmitoyl-2-lysophosphatidylcholine undergo degradation under these conditions with the formation of fatty acid amides and palmitoxy acetone, respectively. A key step in the process is fragmentation of radicals of the initial lipids, where the unpaired electron is located on the carbon atom linked to a hydroxyl group.

In order to elucidate a possible influence of the free radical fragmentation of lipids on the LPO process, we studied some features of LPO product formation in liposomes containing phospholipids capable to undergo the free radical fragmentation. The free radical processes were initiated by γ-radiation.

EXPERIMENTAL

Rat liver phosphatidylcholine (PC) and bovine brain sphingomyelin (SM) were isolated according



Phosphatidylglycerol: R = OH; R" = H;
R' = $C_{15}H_{31}COOCH_2CH(C_{15}H_{31}COO)CH_2OP(O)(OH)O$
Sphingomyelin: R = $CH{=}CH(CH_2)_{13}CH_3$; R' = $NHCOC_{17}H_{35}$;
R" = $O(O)OPOCH_2CH_2N^+(CH_3)_3$
Lysophosphatidylcholine: R = $C_{15}H_{31}COO$; R' = $O(O)OPOCH_2CH_2N^+(CH_3)_3$; R" = H

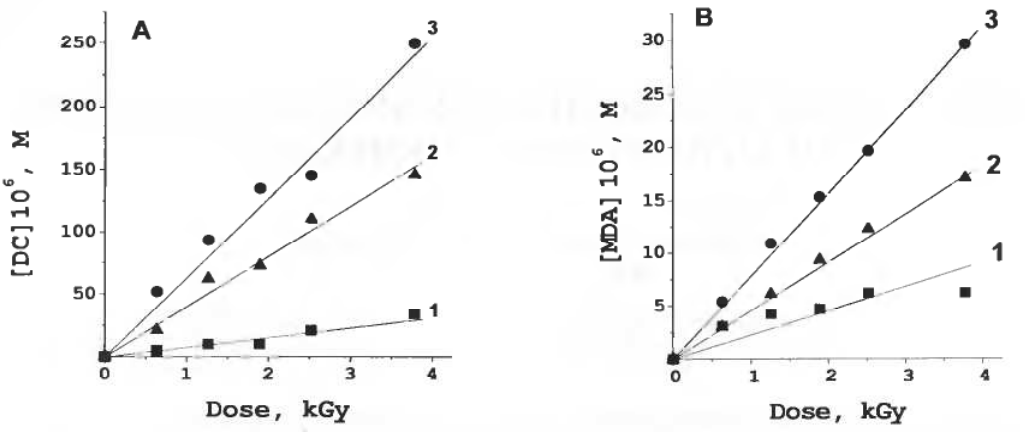


Fig. 1. The relationship between diene conjugates (A), TBA-reactive products (B) accumalation and dose absorbed during radiolysis of different liposomes: (1) phosphatidylcholine (0.02 M), (2) phosphatidylcholine (0,02 M) + 21 mol % DPPEt, (3) phosphatidylcholine (0,02 M) + 21 mol % DPPG. Each point represents a mean value of 3 (DC) or 4 (MDA) measurements.

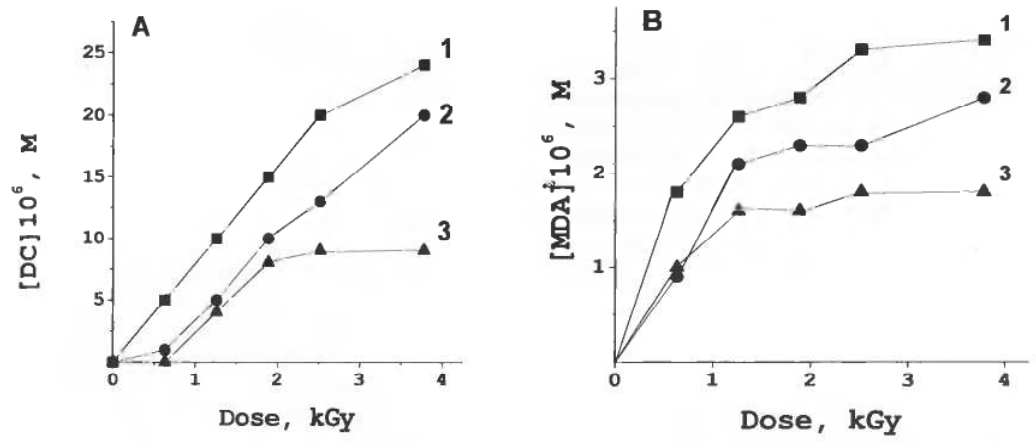


Fig. 2. The relationship between diene conjugates (A), TBA-reactive products (B) accumalation and dose absorbed during radiolysis of different liposomes: (1) phosphatidylcholine (0,02 M), (2) phosphatidylcholine (0.02 M) + 21 mol % DPPC, (3) phosphatidylcholine (0,02 M) + 21 mol % SM. Each point represents a mean value of 3 (DC) or 4 (MDA) measurements.

to the methods described by Bergelson (1980). Dipalmitoyl phosphatidylglycerol (DPPG) and dipalmitoyl phosphatidylethanol (DPPEt) were obtained from dipalmitoyl phosphatidylcholine with the aid of phospholipase D (Bergelson, 1980; Omodeo-Sale, Cestaro, Mascherpa, Monti & Masserini, 1989). Oleic and stearic acid amides were prepared by ammonolysis of the fatty acid imidazolides followed by recrystallization from ethanol. The reagents used in the study were: dipalmitoyl phosphatidylcholine (DPPC) from Sigma (USA); 1-palmitoyl-*sn*-glycero-3-phosphocholine (LPC) and palmitoxyacetone (Fluka, Switzerland); cholesterol (Merck, Germany); 2-thiobarbituric acid (Serva, Germany), and ethanol, twice distilled.

Multilamellar liposomes were prepared just before the experiment. A lipid solution in chloroform was carefully evaporated in vacuo. To the lipid film thus formed, an appropriate amount of 0.05 M phosphate buffer solution (pH 7.4) was added, the mixture was heated to 50°C under argon, and vigorously shaken until a homogeneous dispersion was formed. After cooling of the dispersion to 18°C, it was saturated with oxygen for 15 min.

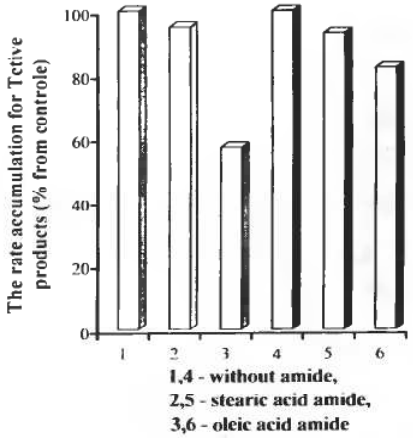


Fig. 3. The formation of TBA-reactive products in liposomes from rat liver phosphatidylcholine in the course of LPO in the absence and the presence of fatty acid amides. Phosphatidylcholine concentration was $1 \cdot 10^{-3}$ M, phosphatidylcholine : amide molar ratio was 9:1. LPO was initiated by γ-irradiation with radiation dose 0,63 kGy at a dose rate of 21 Gy/min (1-3) and by system $FeSO_4$-ATP (4-6).

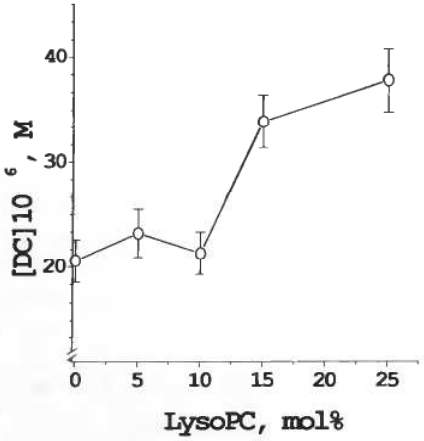


Fig. 4. The relationship between diene conjugates accumalation and lysophosphatidylcholine concentration during radiolysis of 0,01 M phosphatidylcholine with a dose of 3,3 kGy.

Irradiation of the samples obtained was performed in sealed ampoules (^{137}Cs source) with a dose rate of 0.35 Gy/s. The temperature (20 ± 1°C) was kept constant during the irradiation.

The LPO processes were followed by observing the formation of primary reaction products – diene conjugates (DC), and secondary reaction products – aldehyde type compounds which reacted with 2-thiobarbituric acid (TBA). The diene conjugates were determined spectrophotometrically. To do this, 0.1 ml of the irradiated liposomes were dissolved in 2.8 ml of ethanol, mixed thoroughly, and absorbance was measured at $\lambda = 234$ nm against a control cell containing the buffer solution. The DC concentration was calculated using molar extinction coefficient of 28000 $M^{-1}cm^{-1}$ (Raleigh, Kremers, Gaboury 1977). The TBC-reactive products were determined according to a method described by Bulge and Aust (1977). Chemical initiation of LPO was performed by adding to liposomes (prepared from PC and fatty acid amides in 0.02 M Tris-HCl buffer, pH 7.4), under stirring at 37°C, an ATP solution and a freshly prepared $FeSO_4$ solution, one after another, until final concentration 10^{-4} M of each one was reached. Within 3 min the reaction was stopped by addition of ethanol solution of butylated toluene up to concentration of 3×10^{-4} M.

RESULTS AND DISCUSSION

In our studies, liposomes of rat liver phosphatidylcholine were used as membrane models. This phospholipid contains residues of easily oxidizable arachidonic (26%), linoleic (14%) and docosahexaenic (7%) acids (Bergelson, 1980). Such fatty acid composition of the PC suggests a vigorous course of the LPO chain reaction when initiated by free radicals.

To evaluate the effects produced by variation in lipid structure on the LPO reactions. DPPG, LPC, SM[1], DPPEt and DPPC were chosen. All of them are stable towards LPO, but the first three undergo fragmentation when attacked by free radicals (Edimicheva *et al.* 1997).

Fig. 1a, 1b (curve 3) shows that the introduction of 21% of DPPG into the PC liposomes initiates substantial changes in the LPO picture of PC: the concentrations of DC and TBA-reactive products increase by a factor of more than 5.5 as compared to liposomes from PC only (curve 1). Such increase in concentration of the LPO products on introduction of DPPG into liposomes can be explained by: (i) the effect of free radical fragmentation of the latter; (ii) a possible cluster formation from the saturated DPPG and phospholipid stacking disorders at the cluster/bilayer contact sites; and, finally (iii) loosening of the bilayer due to the repulsion between negatively charged DPPG molecules.

[1] The bovine brain SM contains only saturated and monoenic fatty acid residues (Bergelson 1980).

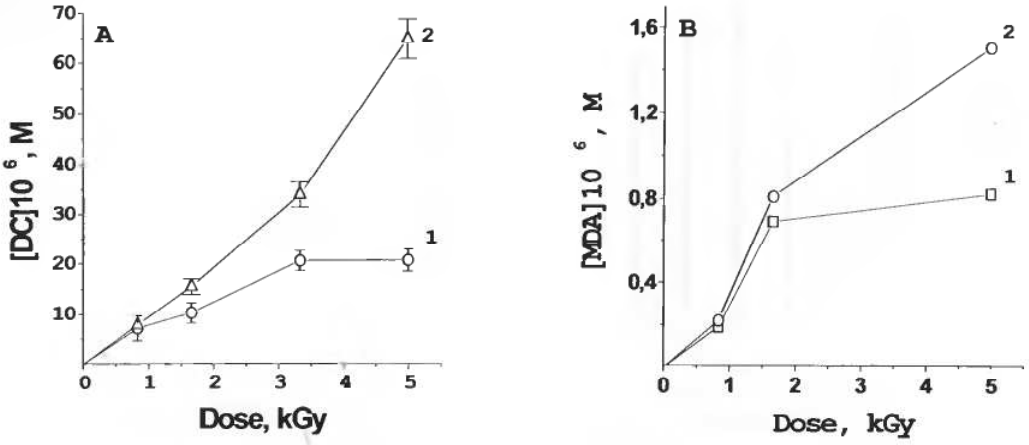


Fig. 5. The relationship between diene conjugates (A), TBA-reactive products (B) accumulation and dose absorbed during radiolysis of phosphatidylcholine liposomes (0,01 M) in the absence of lysophosphatidylcholine (1), in the presence of 15 mol % lysophosphatidylcholine (2).

The formation of defects on cluster boundary surface and the bilayer loosening can favour penetration of the OH radicals into the bedding depth of the α-methylenic groups at double bonds in fatty acid residues in the hydrophobic areas of the membrane.

To establish the cause of the LPO enhancement in liposomes containing DPPG more precisely, the accumulation of LPO products in liposomes containing 21% of DPPEt was studied. Like DPPG, the latter is a negatively charged phospholipid, and has an almost identical gel/liquid crystal transition temperature about 41°C (Omodeo-Sale *et al.* 1989). In contrast to DPPG, however, DPPEt does not contain hydroxyl groups in α-position to phosphoester bonding and hence cannot take part in the free radical fragmentation reaction. It follows from Fig. 1a, 1b (curve 2) that the introduction of DPPEt into PC liposome composition is also accompanied by an increase in DC and TBA-reactive product yields, but the accumulation of LPO products is almost twice lower in this case, as compared to DPPG alone. These differences give evidence of influence exerted by free radical fragmentation products and intermediates thereof on the course of the LPO process. This influence can be realized according to the following two most probable mechanisms.

In the first of them, as the molecular DPPG fragmentation product – phosphatidic acid (possessing a larger negative charge compared to DPPG) – is accumulated, an increase of repulsion between polar groups occurs which leads to loosening of the hydrophobic membrane layer close to the polar area. This results in facilitation of free radical access to C5 and C4 methylene groups of arachidonic and docosahaxaenic acid residues, respectively. The second mechanism involves hydrogen atom abstraction from mehtylene groups of polyenic fatty acid residues during the flip-flop, or a rapid vertical immersion of the DPPG radical intermediate into hydrophobic layer of the membrane.

The fact that dipalmitoylglycerol moiety of DPPG and DPPEt does not contribute to the LPO activation is evidenced by the data obtained for the PC peroxidation in liposomes including DPPC. The DPPC and PC have identical polar moieties. however, the introduction of DPPC into PC liposomes not only does not result in acceleration of PC oxidation but, on the contrary, even some inhibition is observed (Fig. 2a, 2b; curve 2).

SM and LPC are lipids with a structure of the polar component similar to that of PC. As stated above (see scheme), these lipids contain a free hydroxyl group and can be substrates in the free radical fragmentation reaction. The presence of SM in liposomes from PC leads to an appreciable inhibition of the LPO reactions (Fig. 2a, 2b; curve 3). With this, the concentration increase of DC and TBA-reactive products ceases at the dose rate of 1.26 kGy, and on further irradiation of the liposomes the concentration of free radical PC oxidation products remains sensibly constant. There are evidences on peroxidation-inhibiting effects of SM in some other studies (Montfoort, Bezstaroski, Groh, Metsa-Ketela 1987; Subbaiah, Subramanian, Wang 1999). Subbaiah *et al.* (1999) suggests that

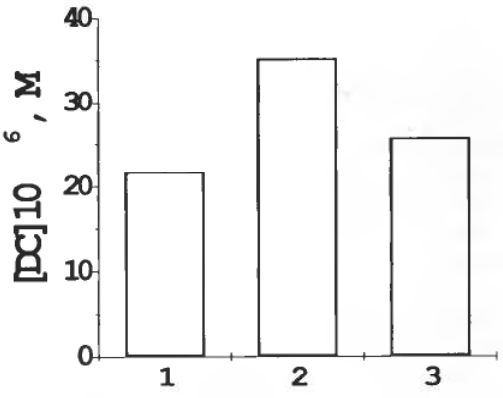


Fig. 6. Formation of diene conjugates under radiolysis of phosphatidylcholine liposomes (0.01 M) with a dose of 3,3 kGy: (1) in the absence of lysophosphatidylcholine and cholesterol, (2) in the presence of 15 mol % lysophosphatidylcholine, (3) in the presence of 15 mol % lysophosphatidylcholine and 15 mol % cholesterol.

SM protects the low density lipoproteins from LPO by modifying the fluidity of the phospholipid monolayer and thereby inhibiting the lateral propagation of the lipid peroxy radicals.

The following reasons seem to us plausible for the explanation of the LPO deactivation in the presence of SM. Firstly, the SM (liable to free-radical fragmentation) can work as a trap for free radicals shielding thereby the fatty acid residues from their attack. Secondly, the products formed as a result of SM fragmentation, among which we have identified fatty acid amides (Edimicheva *et al.*, 1997) can exert inhibiting influence on the LPO reactions.

To confirm the inhibiting action of the SM fragmentation products on the LPO process, the influence of stearic and oleic acid amides on the formation of TBA-reactive aldehydes in PC liposomes was studied on initiation of LPO by γ-radiation and by the system $FeSO_4$ – ATP. The data presented in Fig. 3 show that fatty acid amides slow down the LPO process. Mechanism of action of amides as LPO inhibitors for the time present is not clear.

While studying the LPC effects on oxidation processes in PC liposomes, the capability of lysophosphatidylcholine to cause membrane lysis at certain concentrations has been taken into account. It has been established (Morris, McNeil, Castellino, Thomas 1980; van Echteld, de Kruijff, Mandersloot, de Gier 1981) that the destruction of lipid membranes occurs only at 3540 mol.% of LPC in such systems. We have studied primary product accumulation in LPO reactions as function of LPC concentration not exceeding 25 mol.%. As it is seen in Fig. 4, the inclusion of LPC in concentrations up to 10% into PC liposomes produces virtually no effect on the concentration of diene conjugates formed as a result of the radiation-induced LPO reaction. However, on inclusion of more than 10% of LPC in the PC liposomes, a sharp increase in the DC concentration is observed. Such a change in the DC formation rate appears to be caused by vesiculation of multilamellar liposomes containing more than 10% of a lysophospholipid (Hauser 1987). It is possible that the diffusion of active radicals into the hydrophobic part of the membrane is also facilitated, which, in its turn, leads to extinguishing of the LPO chain reaction. Further, the accumulation of LPO products in liposomes from PC alone and those containing 15% LPC as function of irradiation dose have been studied. The DC concentration in liposomes containing 15 % LPC increases with the dose in a virtually linear manner (Fig. 5, 5a). A quite different picture is seen for liposomes without LPC: beginning from the dose of 3.3 kGy and more, the DC concentration remains on the same level. A similar dependence is observed also for the formation of TBA-reactive products (Fig. 5, 5b). Such influence of LPC on the LPO can be explained by either its capability to take part in the free-radical fragmentation or its effect on permeability of the lipid bilayer.

The action of LPC on barrier function of liposomes can be neutralized by the addition of cholesterol. Equimolar complexes of cholesterol in LPC are known to form bilayer structures impermeable for glycerol and glucose (Kitagawa, Inoue, Nojima 1976; Ramsammy & Brockerhoff 1982). The more cholesterol is contained in liposomes, the more LPC can be included therein without loss of barrier properties. As it follows from Fig. 6, the addition of cholesterol to liposomes containing 15% of LPC leads to an increase in stability of PC towards free radical attack. Most probably, the accumulation of LPC in membranes is accompanied by intensification of LPO due to increasing lipid layer permeability for the active radicals, which, in its turn, rises on formation of DC. In order to check the influence of LPC fragmentation on the oxidation of PC, the following experiment has been carried out. As a result of LPC fragmentation (see scheme), the formation of a lipophilic radical intermediate and a subsequent molecular product – palmitoxyacetone – takes place.

Palmitoxyacetone has been included into the composition of PC liposomes. In the dose range of 0-4 kGy, the radiation-induced peroxidationin the

PC liposomes containing 1.10^{-4} M of palmitoxyacetone proceeded virtually in the same manner as that in a control experiment. It is possible that some contribution in the development of LPO may come from a radical intermediate, due to vertical immersion into the liposomal bilayer of the membrane.

Thus, the negatively charged phospholipids with saturated fatty acid residues — DPPG, DPPEt — activate the LPO processes in rat liver liposomes, whereas neutral lipids, such as SM and DPPC, inhibit them. Both activating and inhibitory effects have their most pronounced character when phospholipids capable of undergoing free-radical fragmentation are added to the system. Most likely, the activating effect of LPC on LPO is determined by both its capability to influence permeability of the lipid layer and its disposition towards fragmentation.

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