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THE HETEROGENEITY OF DEVELOPMENT OF LIPID PEROXIDATION PROCESSES IN BULK AND ANNULAR LIPIDS OF BIOLOGICAL MEMBRANES

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The spectral and kinetical parameters of phosphorescence of lipid peroxidation (LPO) products in human erythrocyte membranes and in bioptates from human stomach mucous shell have been investigated. Based on results of analysis of phosphoresce of LPO products *in vitro* and *in situ* the heterogeneity of development of lipid peroxidation processes in cellular membranes has been shown.

INTRODUCTION

Studying lipid peroxidation processes is an actual problem of modern biology and medicine (Vladimirov & Archakov, 1972). But, in spite of great attention to this problem, many questions remain unknown, in particular, the information about the development of LPO processes in different regions of cellular membranes. To study LPO processes in biological membranes we have used the phenomenon of phosphorescence of products of LPO in the millisecond range.

When we begun our experiment there were few papers devoted to phosphorescence of LPO products in the literature. Systematic studies of this problem were absent. We investigated phosphorescence of LPO products at –196°C in a solution (Mazhul & Shcharbin, 1997a; 1998a; 1999b), the LPO products phosphorescence in liposomes in a temperature range of 2–38°C (Mazhul & Shcharbin, 1999a), and in isolated human erythrocyte membranes in a temperature range of 2–25°C (Mazhul & Shcharbin, 1998b; 1999b; 2000). In these experiments the spectral and kinetic parameters of phosphorescence of LPO products have been investigated in detail; the nature of lipid chromophores capable to phosphorescence has been established and the dynamic mechanism of LPO products phosphorescence quenching has been determined. The obtained results allow us to propose the phosphorescent method to study possible heterogeneity of development of LPO processes in different regions of membrane lipid matrix (annular and bulk lipid regions).

This work is devoted to studying heterogeneity of development of LPO processes in different regions of membrane lipid matrix.

MATERIALS AND METHODS

Blood was obtained from healthy donors. The isolated membranes of human erythrocytes (in 0.155 M Na-phosphate buffer, pH 7.4, protein concentration 1–7 mg/ml) were prepared as described by Dodge, Mitchell and Hanahan (1963). Generally LPO products were accumulated in samples due to autooxidation while storing. The degree of lipid peroxidation was determined spectrophotometrically as the amount of thiobarbituric acid-reactive substances (TBARS) (Gavrilov, Gavrilova & Mazhul, 1987). The phosphorescence analysis of LPO products in cells *in situ* was realized while studying bioptates of human stomach mucous shell of healthy donors, patients with chronic atrophic gastritis (peaking stage) and stomach cancer (stages T1 and T2; histological form of cancer: adenocarcinoma). Cell bioptates were obtained by fibrogastroscopy. After biopsy the samples were placed in 0.155 M Na-phosphate buffer, pH 7.4. One part of bioptates was used for phosphorescent analysis, second part for determi-

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nation of LPO products content (TBARS), third part for cytomorphological analysis by standard techniques. During phosphorescence studying bioptates of approximately 2 mm^3 were placed in a quartz cuvette of optical width 1 mm in 0.155 M Na-phosphate buffer, pH 7.4. All phosphorescence measurements were made in deoxygenated media. Deoxygenation was achieved by introduction of the enzyme system composed of glucose oxidase (80 nM), catalase (16 nM) and 0.3% (w/v) glucose (Calhoun, Englander, Wright & Vanderkooi, 1988) or sodium sulfite (40 mM) (Mazhul & Shcharbin, 1997b) to the samples. The equipment for phosphorescence measurements has been described elsewhere (Mazhul & Shcharbin, 1997b). Phosphorescence decay curves were analyzed in terms of a sum of exponential components by a non-linear least-squares fitting algorithm. To analyze the accuracy of restored parameters of a phosphorescence decay curve the χ^2 criterion, the visual analysis of residual arrays (difference between measured values of a decay curve and its restored values) and the autocorrelation function of residuals were used. All the decay data presented were obtained from three or more independent measurements. During the first millisecond the emission was gated out by double shutter to exclude a contribution of fluorescence emission. The spectra were corrected for the instrumental response.

RESULTS AND DISCUSSION

Earlier we have described the room temperature (20°C) phosphorescence of suspensions of freshly prepared human erythrocyte membranes (Mazhul, Ermolaev, & Konev, 1980; Mazhul, Volkova, Shcharbin, Apanasovich & Novikov, 1995; Mazhul & Shcharbin, 1997) and the room temperature phosphorescence (RTP) of LPO products of isolated human erythrocyte membranes (Mazhul & Shcharbin, 1998c; 2000). These studies have shown that suspensions of freshly prepared membranes which contain LPO products in small amounts (0.1–0.7 nmole MDA per mg of protein), have only phosphorescence of protein nature at room temperature and the contribution of other chromophores is absent. The moderate oxidation of membranes (LPO products content of 2–7 nmole MDA per mg of protein) leads to appearance of small long-wave phosphorescence of LPO products at room temperature as in the case of liposomes. The intensity of this phosphorescence increased at cooling of the samples reaching to significant levels at 0°C. The characteristics of kinetic of LPO products phosphorescence in composition of moderately oxidized membranes were close to that of LPO products phosphorescence of liposomes (Mazhul & Shcharbin, 1998c; 1999; 2000).

While studying phosphorescent properties of oxidized membranes containing moderate amounts of LPO products (2–7 nmole MDA per mg of protein), we have found an interesting effect. Short-term (5-minute) incubation of membrane suspensions at 90–100°C resulted in sharp (2–5 times) increase of RTP intensity of lipid chromophores (Fig. 1 and 4). The same incubation of control samples (suspensions of freshly prepared erythrocyte membranes which practically did not contain LPO products), did not change the form of their RTP spectrum at excitation in a range of 280–297 nm, and at $\lambda_{ex} > 310$ nm the RTP of lipid chromophores was not registered. It is essential, that the heat treatment itself did not result in additional increase in the content of LPO products (TBARS and diene conjugates), and the effect of increase of RTP intensity of lipid chromophors did not decrease during the incubation (90–100°C, 5 min) in the presence of inhibitors of LPO processes such as α-tocopherol (5×10^{-4} M), β-ionol (7×10^{-4} M), or in oxygen-free media. The kinetic curve of phosphorescence decay of LPO products in heated (97°C, 5 min) membranes (Fig. 2) was well approximated by the sum of two exponential curves and was characterized by the following parameters: τ_1, lifetime of a fast component; τ_2, lifetime of a slow component; α_1, the contribution of a fast component; $\alpha_2 = 1 - \alpha_1$, the contribution of a slow component. After incubation the values of τ_1 and τ_2 of RTP of LPO products in membranes were significantly (approximately 3-fold) increased.

Based on the dynamic character of phosphorescence quenching of LPO products (Mazhul & Shcharbin, 1997–2000) it is possible to suppose, that the decrease of non-radiated deactivation of triplets observed after short-term incubation (97°C, 5 min) of oxidized membranes is connected with immobilization of lipid chromophores.

One of the reasons of such immobilization may be covalent linkages of LPO products (MDA and other aldehydes) with membrane proteins and formation of Schiff-like bases and similar compounds. It is concluded from the similarity of RTP spectra of lipid chromophors in heated membranes and Schiff bases, synthesized from MDA and glycine and included in a rigid matrix, i.e. film of polyvinyl alcohol (Fig. 1).

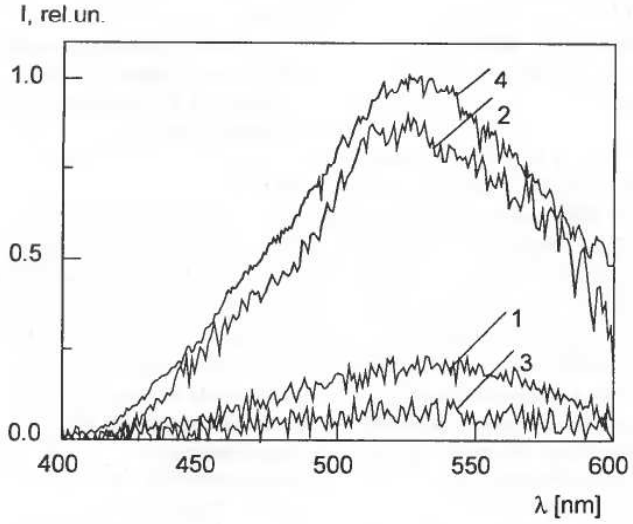

Fig. 1. Room temperature phosphorescence spectra of oxidized human erythrocyte membranes suspensions (1–3) and Shiff bases in a film of polyvinyl alcohol (4), 1 – membrane preparations before heat treatment (control); 2 – membranes after heat treatment at 97°C for 5 minutes; 3 – heated membranes solubilized by 0.6% sodium dodecyl sulfate. λ_{ex} = 360 nm; 0.155 M Na-phosphate buffer, pH 7.4; T = 20°C; protein concentration 4.5 mg per ml; the LPO products content 5.4 nmol MDA per mg of protein

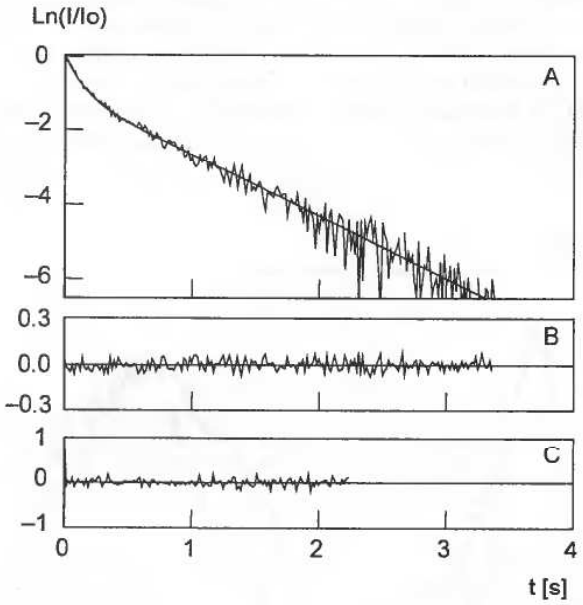

Fig. 2. Decay kinetics of room temperature phosphorescence of suspensions of oxidized human erythrocyte membranes after preliminary incubation at 97°C for 5 minutes. Experimental curve and reconstructed curve of biexponential approximation (A), average-weighted residues (B) and autocorrelation function of residues (C). λ_{ex} = 320 nm; 0.155M Na-phosphate buffer, pH 7.4; 20°C; protein concentration 4.5 mg per ml; the LPO products content 4.8 nmol MDA per mg of protein

Taking into account the ability of membrane proteins to interact with lipids via hydrophobic interactions and electrostatic forces and to form annular layers of immobilized lipid molecules, non-covalent immobilization of LPO products is also quite possible.

The disturbance of protein-lipid interactions, the reduction of rigidity of membrane protein (the

matrix for immobilized LPO products) at solubilization of heated membranes by detergent (0.6% sodium dodecyl sulfate) resulted in a sharp decrease of RTP intensity of lipid chromophores (Fig. 1).

The immobilization of LPO products by membrane proteins was verified by changes of the shape of RTP excitation and emission spectra of heated membranes. These changes indicate the effective migration of electronic excitation energy from tryptophanyls of membrane proteins (donor) to lipid chromophores (acceptor) located inside of Förster radius. The contribution of band with maximum at λ = 280 nm (corresponding to absorption of tryptophanyls of membrane proteins) increased and the contribution of bands at 310–320 nm and 340–360 nm (characteristic for LPO products) decreased in the RTP excitation spectrum (at 20°C) of preheated membranes (Fig. 3). The intensity of tryptophan phosphorescence decreased and the phosphorescence of LPO products increased in RTP emission spectra (20°C) of preheated membranes at excitation λ = 297 nm (Fig. 4) after short-term heat treatment. The measurements of RTP decay kinetics of incubated (90–100°C, 5 min) membranes show that the decrease of tryptophan phosphorescence intensity is not connected with an increase of millisecond motions of membrane proteins after the heat action. Therefore it is possible to ascribe the changes in phosphorescent properties of oxidized membranes after short-term heat treatment to suppression of the rate of collisional relaxation of excited triplet levels of immobilized LPO products by increase of migration efficiency of electronic excitation energy from membrane proteins to immobilized lipid chromophores.

An important proof of the role of membrane proteins in the immobilization of LPO products is the result of experiments with liposomes prepared from oxidized phosphatidylcholine and total lipid fraction of erythrocyte membranes. Preincubation of suspensions of such liposomes at 90–100°C for 5 minutes did not result in any changes of phosphorescence properties of LPO products included in these liposomes.

Thus, membrane proteins immobilize the LPO products. The increase of matrix rigidity of LPO products in heated membranes results in displacement of the highest temperature at which the phosphorescence is reliably registered from 26°C up to 50°C (Fig. 5, compare with (Mazhul & Shcharbin, 1998b; 2000).

The data mentioned above concern membranes with moderate contents of LPO products (2–7 nmol MDA per mg of protein). Our experiments have shown that increase of LPO products content in membranes up to 8–15 nmol MDA per mg of protein and higher without preincubation resulted in changes of phosphorescence properties of lipid

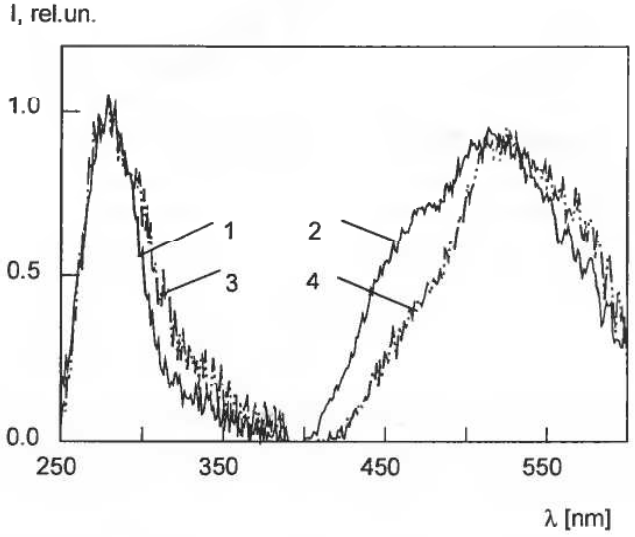

Fig. 3. Room temperature phosphorescence excitation (1, 3) and emission (2, 4) spectra of LPO products in composition of heated (97°C, 5 min) oxidized human erythrocyte membranes. 1 – λ_{em} of 520 nm; 2 – λ_{ex} of 320 nm; 3 – λ_{em} of 570 nm; 4 – λ_{ex} of 360 nm, 0.155 M Na-phosphate buffer, pH 7.4; T = 20°C; protein concentration 4.5 mg per ml; LPO product content 5.4 nmol MDA per mg of protein

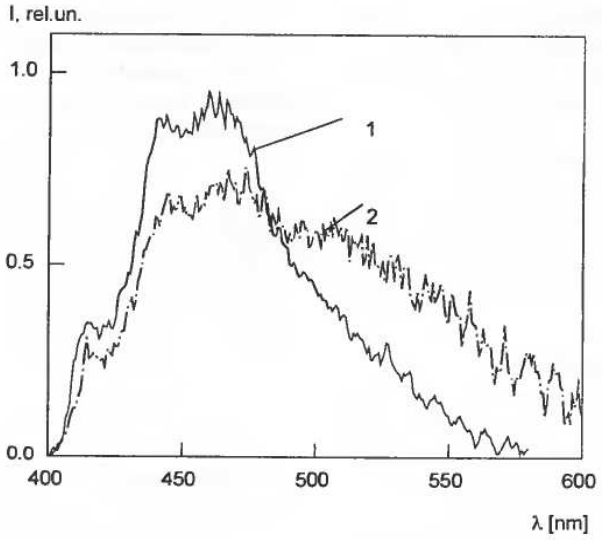

Fig. 4. Room temperature phosphorescence emission spectra oxidized human erythrocyte membranes (1) before and (2) after preincubation at 97°C for 5 min. λ_{ex} = 297 nm; 0.155 M Na-phosphate buffer, pH 7.4; T = 20°C; protein concentration 5.1 mg per ml; LPO products content 6.3 nmol MDA per mg of protein

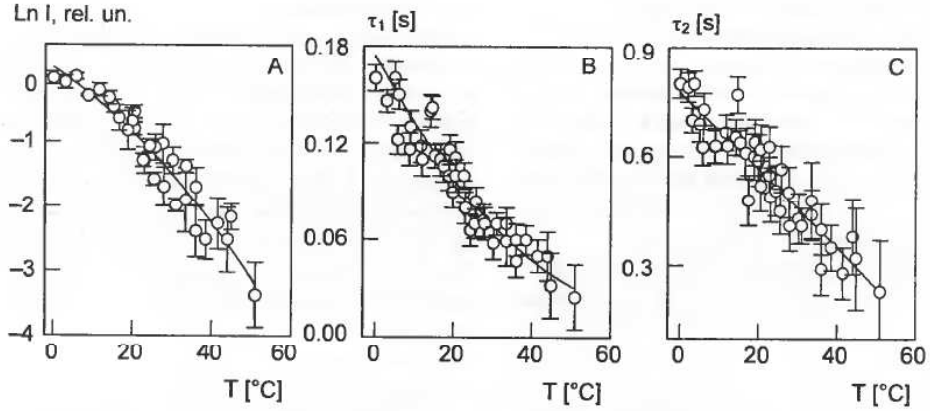

Fig. 5. Temperature dependencies of I (A), τ_1 (B) and τ_2 (C) of phosphorescence of lipid chromophores in heated (97°C, 5 min) oxidized human erythrocyte membranes. λ_{ex} = 320 nm; 0.155 M Na-phosphate buffer, pH 7.4; protein concentration 4.5 mg per ml; LPO products content 5.4 nmol MDA per mg of protein

chromophores close to the changes in the case of moderately oxidized membranes after heat treatment (90–100°C, 5 min); the values I, τ_1, τ_2 of RTP, and the shape of RTP excitation and emission spectra were changed. These results indicate that intensification of lipid peroxidation processes leads to effective immobilization of LPO products by membrane proteins already at moderate temperatures and does not require the high-temperature structural reorganization in the membrane.

Thus, the results of phosphorescence analysis indicate the heterogeneity of immobilization and allocation of LPO products in the membrane. In the case of moderate by oxidized membranes the LPO products are poorly immobilized and are separated in space from membrane proteins, being located mainly in bulk lipid zones. Owing to bimolecular deactivation of triplets in given conditions the lipid chromophors show low values of I, τ_1 and τ_2 of their RTP. The intensification of LPO processes leads to immobilization of LPO products by membrane proteins. Thus the values of I, τ_1, τ_2 of lipid chromophores increase in some cases. The nonuniformity of LPO products allocation in the

interior of the membrane may be caused by heterogeneity of chemical structure and physical-chemical properties of membrane components and their structural compartmentalization (Bergelson, Gawrisch, Ferretti & Blumenthal, 1995; Jorgensen & Mouritsen, 1995; Pedersen, Jorgensen, Baekmark & Mouristen, 1996) and to unequal localization of molecules which may influence LPO processes. For example, cholesterol is able to suppress LPO processes in the erythrocyte membrane and to modify locally the structure of lipid bilayer (Clemens & Waller, 1987).

The data presented indicate that in the case of moderate LPO processes membrane proteins are protected from massive attack by LPO products. The distribution of lipid peroxidation "centers" in zones of annular lipids can result in chemical modification and inactivation of proteins (formation of Schiff-like bases), of LPO products immobilization (increase of I, τ_1 and τ_2 of RTP) and, finally, disturbance of cells function and consequent development of pathologies.

The unique feature of the phosphorescence method is the possibility of selective studies of LPO products in membranes *in situ*, without isolation of membranes. This is due to that the fact LPO products dissolved in the cytoplasm have no room temperature phosphorescence because of effective molecular relaxation of triplet states in the millisecond range (see higher). Therefore, only lipid chromophores included in membrane structures have the ability of RTP in a cell.

The phosphorescent analysis of LPO products in cells *in situ* was realized while studying bioptates of human stomach mucous shell of healthy donors, patients with chronic atrophic gastritis (peaking stage) and stomach cancer (stages T1 and T2; histological form of cancer: adenocarcinoma). The phosphorescence of bioptates was excited by monochromatic light at $\lambda = 297$ nm.

The data obtained shows that the RTP spectrum of bioptates from healthy donors had a tryptophan nature (Fig. 6A). The band at 505–515 nm corresponding to RTP of lipid chromophors in RTP spectra of normal bioptates was practically absent. In the case of chronic atrophic gastritis of such bioptates the "lipid" band appeared at 505–515 nm in RTP spectrum, which was appreciably amplified after preincubation of samples at 97°C during 5 minutes (Fig. 6B). In the case of healthy donors such an effect was not visible (Fig. 6A). The most distinct presence of LPO products in RTP spectrum of bioptates was observed in the case of stomach cancer. As follows from Fig. 6C, the RTP spectrum of such bioptates had a sharp-cut "lipid" component already without heat treatment. This component increased after incubation of samples at 97°C for 5 minutes.

According to biochemical analysis the bioptates contained: 1.2–3.3 nmol MDA per mg of protein in control, 5.4–9.0 nmol MDA per mg of protein in chronic atrophic gastritis and 8.4–10.8 nmol MDA per mg of protein in cancer. The comparison of results of phosphorescence and biochemical analyses indicates that at moderate intensity of LPO processes (healthy donors) the LPO products in membrane locates in mobile molecular environment and their immobilization occurs only as

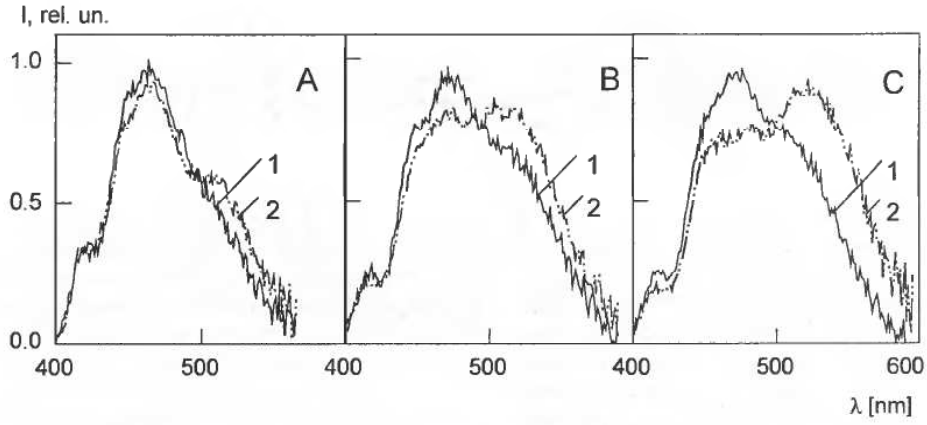

Fig. 6. Room temperature phosphorescence emission spectra of bioptates of human stomach mucous cells. A – healthy donor; B – chronic atrophic gastritis; C – adenocarcinoma, T2 stage. 1 – before heat treatment; 2 – after incubation at 97°C during 5 min. $\lambda_{ex} = 297$ nm; 0.155 M Na-phosphate buffer, pH 7.4; LPO products content (nmol MDA per mg of protein): A – 2.7; B – 8.4; C – 10.8

result of short-term heat treatment (Fig. 6A). The intensification of LPO processes in case of chronic atrophic gastritis is connected with increasing immobilization of LPO products in membranes (appearance of band at 505–515 nm in RTP spectra of non-incubated bioptates (Fig. 6B). This effect is pronounced in the case of cancer (Fig. 6C). It is important, that the results of phosphorescence analysis of LPO products in isolated human erythrocyte membranes and in bioptates of human stomach mucous cells are concordant.

CONCLUSIONS

As follows from the experiments on the millisecond RTP, LPO products are well detectable when they are immobilized and, accordingly, the rate of triplet bimolecular deactivation is decreased. The high sensitivity of intensity and lifetimes of phosphorescence of LPO products to the rigidity of microenvironment of lipid chromophores, the detected differences in the rigidity of microenvironment and in the level of immobilization between the bulk and annular lipid regions allow to monitor the accumulation of LPO products in different regions of membrane lipid matrix. Such differences in the accumulation of LPO products in bulk and annular lipid regions of erythrocyte membranes in various stages of development of LPO processes were found. Based on results of analysis of fluorescence of LPO products the heterogeneity of development of lipid peroxidation processes in cellular membranes has been shown. At the moderate level of LPO processes in membranes the lipid peroxidation centers and areas locate mainly in bulk lipid regions. Activation of lipid peroxidation processes leads to the expansion of lipid peroxidation areas and the involvement of annular lipids in the LPO processes. The LPO products, which are appearing in annular regions, attack the membrane proteins both covalently (Schiff like bases) and noncovalently (of LPO products immobilization). The enhanced stability of annular lipids to peroxidation in comparison with bulk lipids detected by phosphorescence method has important physiological significance for membrane proteins providing their protection against damage by LPO products, and constancy of chemical structure and physical-chemical properties of lipid molecules surrounding the proteins. The intensification of lipid peroxidation processes and the occurrence of LPO products in annular layers can cause chemical and structural modification of membrane proteins, damage the normal function of cells and result in pathological growth. The obtained results were verified in experiments with bioptates of human stomach mucous shell (norm, chronic atrophic gastritis and cancer). The intensification of LPO processes in cells of stomach mucous shell in case of chronic atrophic gastritis was accompanied by an increase of the level of LPO products immobilized by proteins. The maximal effect of LPO products immobilization was observed in the case of cancer.

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