PROTEIN MODIFICATIONS AND LIPID COMPOSITION CHANGES IN RAT LENSES IN POSTNATAL DEVELOPMENT

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The aim of the investigation is to study age dynamics of posttranslational protein modification level and the changes of rat lens membranes, and the consideration of possible mechanisms of membrane effect on the composition and intensity of protein modifications in lens.

Materials and Methods. The experiments were carried out on Wistar rats of three age groups: 1, 12 and 24 months. Protein level, sulfhydryl (SH) group concentration, and protein carbonyl derivatives level were measured spectrophotometrically. The content of tryptophan, bityrosine and advanced glycation end-products (AGEs) were assessed by fluorescence intensity. Phospholipids and neutral lipids were fractionated by thin-layer chromatography. Densitometric analysis and quantitative processing of chromatograms were performed using NIH Image J software.

Results. Protein content in lens homogenate was found to increase with age, indicating the accumulation of slightly soluble protein aggregates. There was uniform decrease of SH-group concentration and protein carbonyl derivatives in homogenate. On the other hand, there was observed the accumulation of AGEs, bityrosine and tryptophan in water-soluble fraction. The main age changes of lens membrane lipid composition were the increasing ratio of sphingomyelin and neutral lipids. The changes could be caused by the growth of the proportion of mature fibers forming the nucleus of lens compared to poorly- and medium-moderately fibers and cells of epithelium. The principal component of neutral lipids was cholesterol and cholesterol esters.

Conclusion. Lens membrane enrichment by lipids characterized by relatively high "ordering" inhibits the formation of protein carbonyl derivatives, but at the same time, can disbalance intercellular communication resulting in proteolysis (and tryptophan exposure) and AGEs accumulation.

Key words: lens; phospholipids; free-radical processes; posttranslational modifications of lens proteins.

The lens function is light focusing on retina, and therefore its essential properties are maximum light transmission and minimum light scattering. In the process of differentiation of fibers from epithelial cells there is the loss of organelles and synthesis of specific proteins — crystallins [1]. In the course of differentiation, as well as with the increase of years, lens proteins undergo numerous posttranslational modifications (PTM), a part of which can result in high molecular insoluble aggregates formation causing lens opacity [2–4]. At the same time in the process of postnatal development, the changes of composition and properties of lens fiber membranes are observed. The problem of mutual influence of membranes and intracellular compartments in lens opacity development context has not been studied thoroughly so far. There are reports of membrane participation in high molecular protein aggregates formation [5], though the mechanisms of interaction of membranes with lens proteins remain unclear.

The essential characteristic determining membrane properties and functions is lipid composition. The researches on the changes of lens lipid composition in different mammals (including human) have shown high cholesterol, as well as the increase of sphingolipid number with aging and in the process of fiber maturation [6]. Lipid composition to a larger extent determines the intensity of free radical process proceeding on membranes, functional status of intercellular contacts and receptors, transport systems of ions and metabolites [7]. Thus, structure-function changes due to the effect of lens lipid composition can directly or indirectly participate in formation and accumulation of any PTM proteins.

On the other hand, the effect of reactive oxygen species (ROS) is considered as the main driving factor of numerous PTM proteins accumulation [8]. Among PTM associated with the development of lens opacity there are nonspecific proteolysis and non-enzymatic glycosylation [9, 10], though the problem of the formation mechanisms of these modifications and the participation of ROS in these mechanisms is not thoroughly studied yet. For this reason, it can be supposed that membranes play significant role in their accumulation.

The aim of the investigation was to study the relations of membrane lipid composition and the level of lens protein posttranslational modifications in postnatal development.

Materials and Methods. The experiments were carried out on Wistar rats of three age groups: 1, 12 and 24 months, the group size: 20, 16, and 20 animals, respectively. Life expectancy of animals was 30–40 months.

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When carrying out the experiments, ethical principles were kept inviolate according to European Convention for the protection of vertebrata used for experimental and other scientific purposes (the Convention was passed in Strasburg, 18.03.1986, and adopted in Strasburg, 15.06.2006).

Anesthetized animals were decapitated, with lenses being removed. Then the lenses were homogenized on ice in sterile saline solution in the ratio 1:25 (lens mass to solution volume). To obtain water-soluble fraction, homogenate was diluted in phosphate buffer (17 mmol/L KH_2PO_4, 52 mmol/L Na_2HPO_4, pH-7.4), volume ration being 1:3, then mixed, centrifuged at 3000 rpm, supernatant fluid was used for further procedures.

Protein content was determined by biuret method using "Total Protein-Vital" kit (Vital Diagnostics S.-Petersburg, Saint Petersburg, Russia) and stated in mg per wet weight. The concentration of sulfhydryl (SH) groups was measured spectrophotometrically using 5.5'-dithiobis-2-nitrobenzoic acid [11]. The degree of protein oxidative modification was determined spectrophotometrically by the level of carbonyl derivatives in the composition of protein oxidized amino-acid residues reacting with 2,4-dinitrophenylhydrazine (2,4-DNPH) with the formation of derivatives of 2,4- dinitrophenylhydrazone [12]. The level of tryptophan and bityrosine was assessed by fluorescence intensity, at excitation and emission wavelength 295 and 340 nm — for tryptophan, 325 and 415 nm — for bityrosine. The level of advanced glycation end-products (AGEs) was measured spectrofluorometrically [13]. The level of carbonyl derivatives, tryptophan, bityrosine, and AGEs was normalized to protein amount and stated in relative units. Spectral measurements were made on spectrofluorometer "Fluorat 02 Panorama" (Lumex, Saint Petersburg, Russia).

Lipid extraction and purification from lenticular homogenate were performed by Folch method [14]. For chromatographic analysis we used Sorbfil plates (Krasnodar, Russia). Solvent system for phospholipids separation was the following: chloroform : methanol : water : heptane — 65 : 25 : 4 : 9 [15]; solvent system for neutral lipid separation: hexane : diethyl ether : acetic acid — 70 : 30 : 1 [16]. The imaging of lipid zones was performed by the treatment of plates with 10% solution of phosphatomolybdic acid in ethanol with the following incubation at 120°C for 10 min. Densitometric analysis and quantitative processing of chromatograms were performed using NIH Image J software (http://rsb.info.nih.gov/ij/index.html).

The data were statistically processed using Statistica 6.0. The errors in figures correspond to standard error of mean. Intergroup differences were assessed using ANOVA analysis of variance with post-hoc Bonferroni correction. The samplings were considered to belong to different general population when $p<0.05$ [17].

Results. Protein concentration in supernatant that corresponds only to soluble fractions of lenticular proteins did not change significantly, while relative protein level in homogenate increased during ageing (Fig. 1, *a*). These data give evidence of the accumulation of slightly soluble protein aggregates. SH-groups concentration in both fractions decreased regularly with age (Fig. 1, *b*) that indicates the accumulation of covalent links with the involvement of cysteine and methionine, and gives evidence of SH-groups oxidation.

The level of carbonyl derivatives in homogenate also statistically significantly decreased with ageing, while in supernatant the decrease was observed only from 1 to 12 months, and then by the age of 24 months, carbonyl derivatives in water-soluble fraction remained at a plateau (Fig. 2). Thus, it can be supposed that SH-groups concentration reduction mainly is due to disulphide bond formation, rather than SH-groups oxidation.

The level of tryptophan, bityrosine, and AGEs in water-soluble lens fraction had the tendency for increase from one group to another (Fig. 3). Among the factors resulting in tryptophan accumulation the most probable one is proteolysis of crystallin molecules that involves the release of low- and moderate-molecular peptides, as well as the damage of tertiary structure of proteins, with tryptophan transition from a latent form into an exposed one. The studies have shown peptides and proteolysis-exposed "truncated" crystallin molecules to be the centers of high-molecular insoluble protein complexes formation [9, 18].

Bityrosine formation is referred to oxidative protein modifications, while AGE formation can proceed both with and without the participation of oxidative reactions. One of the studied mechanisms of AGE formation with the participation of ascorbic acid presupposes its oxidation to

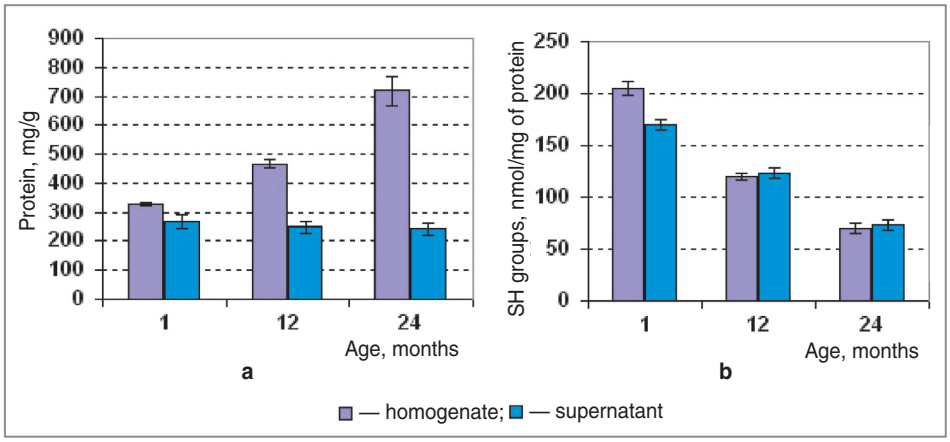

Fig. 1. The content of protein (*a*) and SH-groups (*b*) in rat lenses. All intergroup differences except protein content in supernatant are statistically significant

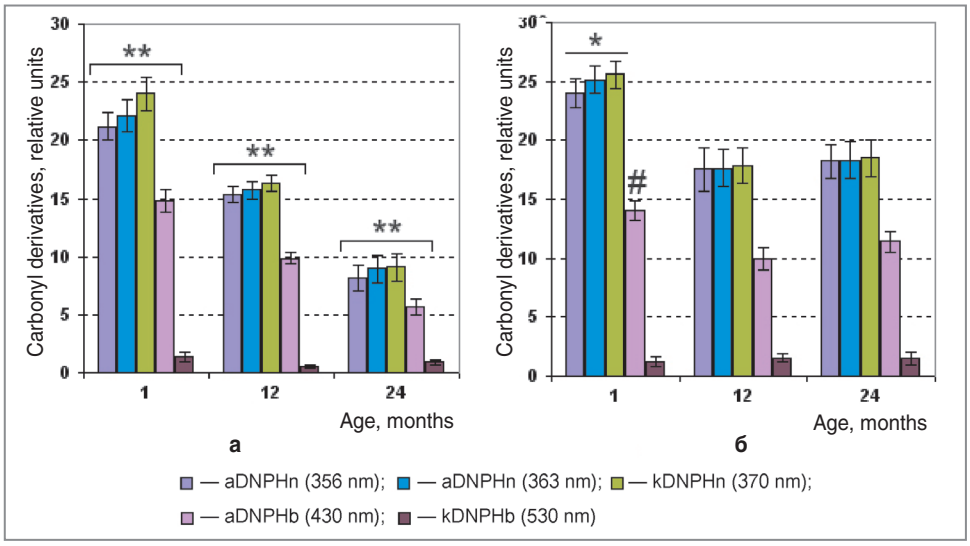

Fig. 2. Level of carbonyl derivatives in homogenate (*а*) and supernatant (*б*): aDNPH, kDNPH — ald- and ketodinitrophenylhydrazones; DNPHn, DNPHb — dinitrophenylhydrazones of neutral and basic character; ** — statistically significant differences between all groups; * — between 1-month group and other groups; # — between the groups of 1 and 12 months

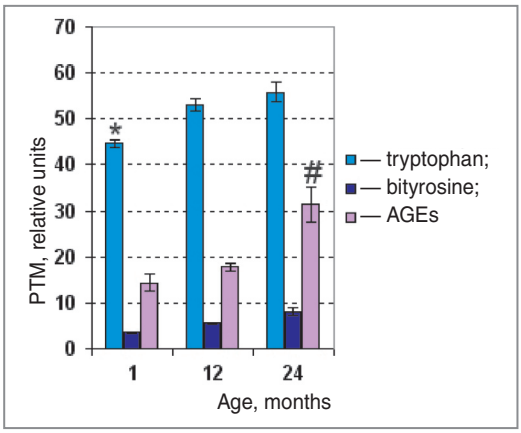

Fig. 3. Level of posttranslational modifications in water-soluble fraction of rat lenses. * — statistically significant differences between 1-month group and other groups; # — 24-months group from the others. Intergroup differences by bityrosine level are statistically significant between all groups

dehydroascorbate with the following non-oxidative stages resulting in the formation of adducts with lysine and arginine [19–21].

Figure 4, *a* shows the composition of lens phospholipids in age groups. The main feature of age changes is the increase of sphingomyelin proportion. The principal component of neutral lipids appeared to be cholesterol and its esters (Fig. 4, *b*). Molar ratio of phospholipids to neutral lipids decreased with age from 1:3 to 1:4 (Fig. 4, *c*). The increase of the proportion of sphingomyelins and neutral lipids with aging is explained by proportion increase of mature fibers constituting nucleus of lens compared to poorly or moderately differentiated epithelial fibers and cells. Such molecular composition of mature fiber membranes provides their relative physical and chemical stability, including stiffness and peroxidation resistance. Sphinomyelins and cholesterol have been shown to prevent effectively the processes of lateral oxygen transport on membranes and the oxidation of unsaturated fatty acids in phospholipids composition [22, 23]. On the other hand, sphingomyelin metabolism products including ceramides are known to inhibit proliferation [24]. Therefore, age-related increase of sphingomyelin number can be associated with the reduced number of epithelial proliferating cells and fibers of cortex lentis which are in the process of maturation.

The problem of the mechanisms of age-related accumulation of sphingomyelins and neutral lipids in lens still remains unclear. Phospholipids molecule degradation is regarded as one of the reasons [6], and supported by the tendency for the increase of diacylglycerol level and phosphatidylethanolamine proportion decrease at the age of 24 months (рис. 4 *a, b*). On the other hand, the contribution can be made by the increase of synthesis of neutral and sphingolipids by the cells of epithelium and cortex lentis, the increase being caused by the factors of humoral regulation.

The membrane properties changes caused by the increase of sphingomyelins and neutral lipids proportion can influence intercellular transport processes that, in its turn, can result in accumulation of protein modifications. Selective distribution of connexins (Cx) and aquaporins (AQP0) in lens membranes is well-known. At low cholesterol and sphingomyelin content, connexins and aquaporins are not rafting residents, though when membranes enrich with cholesterol and sphingomyelin, partial transition of AQP0 tetramers in “ordered” domain is possible [25]. The transition in oligomeric form and more “rigid” lipid environment can change the functional status of AQP0 molecules and result in dissociation of AQP0–Cx50 complexes followed by disconnection of gap junctions formed by subunits Cx50 и Cx46 [26]. The result of unbalance in gap junctions functioning in mature lens fibers is the accumulation of intracellular calcium [27]. The increased calcium level causes the activity of calpains (calcium-dependent proteases). Excessive activation of calpain 2 and Lp82 (lens-specific calpain isoforms) has been shown to result

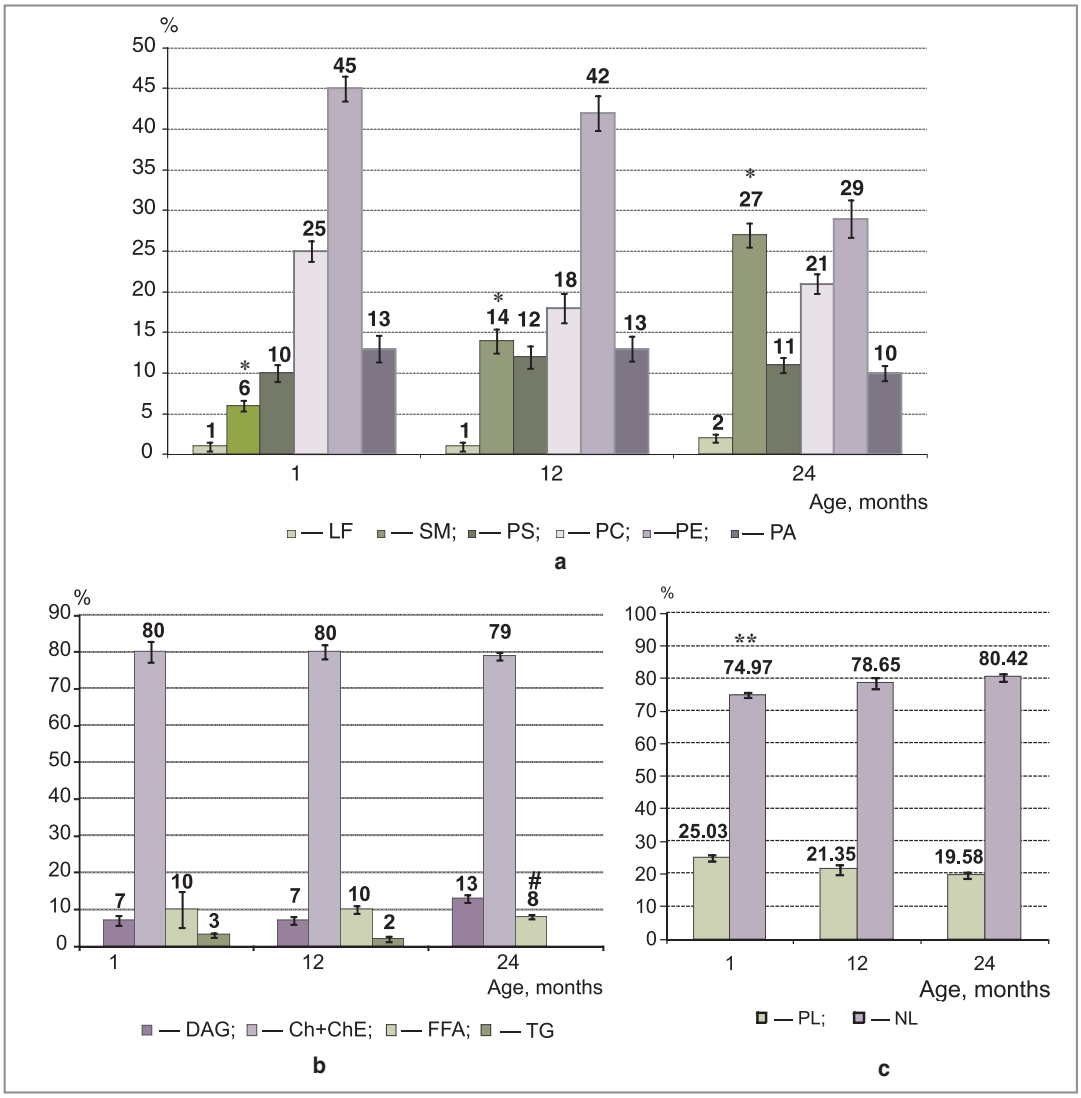

Fig. 4. Lipid composition of rat lenses: *a* — phospholipids: LF — lysoforms, SM — sphingomyelins, PS — phosphatidylserines, PC — phosphatidylcholines, PE — phosphatidylethanolamines, PA — phosphatidic acid; *b* — neutral lipids: DAG — diacylglycerols, FFA — free fatty acids, TG — triglycerides, Ch+ChE — cholesterol and cholesterol esters; *c* — mole fractions of phospholipids (PL) and neutral lipids (NL). * — statistically significant differences between all groups; # — 24-months group from other groups; ** — between the groups of 1 and 24 months

in proteolysis of crystallins and proteins of cytoskeleton and be one of the causes of cataract [28]. Thus, the observed increase of tryptophan level in groups of 12 and 24 months (See Fig. 3) can be due to the increased activation of calcium-dependent proteases in lens.

Forming intra- and intermolecular hydrogen bonds, sphingomyelin molecules effectively prevent free radical processes on membranes [29]. Based on the reports of non-covalent binding of crystallin molecules with lens membranes [30], we suggest that the enrichment of membranes with cholesterol and sphingomyelin inhibits direct oxidation of α-crystallins, and in this way decreases the level of protein carbonyl derivatives that was observed in the experiment (See Fig. 2). Despite inhibition effect of sphingomyelins and cholesterol on free-radical processes on membranes, age-related reduction of SH-groups concentration and the increase of bityrosine level can be an indicator of reactive oxygen species action in lens fibers cytosol. Since organelles (mitochondria, in particular) are lost in the course of lens fibers differentiation, the delivery (diffusion) of reactive oxygen species from overlying lens layers, epithelial cells and eye capsule fluid can be considered as the means of the accumulation of reactive oxygen species.

Thus, age-related changes of lens lipid composition, and in particular, the enrichment of membranes with sphingomyelin and neutral lipids, may have both direct and indirect effect on the character and intensity of protein modifications. Direct effect is related to inhibition of intensive protein oxidation, and primarily, α-crystallin, resulting in the formation of carbonyl derivatives. However, there are processes, in which ROS act as minor and intermediate links of protein modifications, e.g. non-enzymatic glycosylation. Indirect effect of membrane lipid composition can be the unbalance of gap junction functioning, water and ion channels that finally results in non-controlled proteolysis.

Conclusion. There was found age-related increase of neutral lipids and sphingomyelins content in lens membranes. The level of protein carbonyl derivatives in homogenate was steadily decreasing, while the reduction of carbonyl derivatives concentration in water-soluble fraction was observed from the age of 1 to 12 months, and by 24 months remaining the same. Such dynamics of carbonyl derivatives content is likely to de directly related to lipid composition changes. The level increase of tryptophan, bityrosine, and advanced glycation end products observed in the experiment can be due to the mechanisms, the role of membrane in which is worth further investigation.

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