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Oxidative destruction of human RPE melanosomes induced by superoxide radicals leads to the formation of reactive aldehydes and ketones

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Abstract: Oxidative destruction of the natural pigment melanin leads to a decrease in its concentration in the cell, a decrease of antioxidant properties and the accumulation of products that exhibit prooxidant activity. This work shows for the first time that oxidative destruction of melanosomes from human retinal pigment epithelium (RPE) cells induced by superoxide radicals results in the formation of toxic carbonyl compounds, namely aldehydes and ketones. Analysis of the products of oxidative degradation of melanosomes was performed by absorption and fluorescence spectroscopy and time-of-flight secondary ion mass spectrometry (ToF-SIMS). It has been shown the water-soluble products of oxidative destruction of RPE melanosomes induce proteins modification with the formation of fluorescent Schiff bases. It is assumed that carbonyl products of oxidative destruction of melanosomes can have a toxic effect on RPE cells, which is important for understanding the mechanisms of development of retina senile degenerative diseases.

Keywords: Retinal pigment epithelium, melanosomes, superoxide, carbonyl stress

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Окислительная деструкция меланосом из клеток рпэ человека, индуцированная супероксидными радикалами, приводит к образованию реакционноспособных альдегидов и кетонов

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Аннотация. В работе впервые показано, что в результате окислительной деструкции меланосом из клеток ретинального пигментного эпителия (РПЭ) глаза человека, вызванной супероксидными радикалами, образуются токсичные карбонильные соединения, в частности альдегиды и кетоны. Показано, что водорастворимые продукты окислительной деструкции РПЭ меланосом индуцируют модификацию белков с образованием флуоресцентных оснований Шиффа.

Ключевые слова: ретинальный пигментный эпителий, меланосомы, супероксид, карбонильный стресс

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Introduction

Melanosomes contained in RPE cells of the eye perform the function of screening photoreceptor cells from excessive illumination and the function of antioxidant protection of the cell from free radical oxidation caused by irradiation and reactive oxygen species [1–4]. Aging leads to a significant decrease in the number of melanosomes in RPE cells. While in the age group below 20 years melanosomes occupy up to 8% of the RPE cell volume, in the age group 40–90 years this volume gradually decreases to 3.5% [5]. At the same time the total concentration of melanin in the RPE cell decreases by 2.5 times [6]. It is assumed the content of melanin decreases due to its photooxidative and/or oxidative destruction [6, 7]. Oxidative destruction of RPE melanosomes leads on the one hand to a decrease in their antioxidant activity and on the other hand, to the appearance of prooxidant properties and an increase in photoreactivity [8–11]. In other words the oxidative destruction of melanin in RPE melanosomes causes the degradation of native organelles, significant decrease of their antioxidant activity and the resulting destruction products can exhibit prooxidant activity. All this leads to a decrease in the antioxidant status of RPE cells. It is known that oxidative destruction of melanin can be caused by hydrogen peroxide and UV irradiation [12, 13], as well as by the action of superoxide radicals [7]. In this case a mixture of water-soluble fluorescent products is formed. The aim of this work was to study the nature and physicochemical properties of water-soluble products formed during the oxidative degradation of RPE melanosomes induced by potassium superoxide. The results obtained indicate that during the oxidative destruction of melanosomes toxic carbonyls are formed that can damage cell structures in the dark.

Materials and methods

Obtaining water-soluble degradation products of melanosomes. Melanosomes were isolated from RPE cells of human donor eyes obtained from the eye tissue bank of the S.N. Fedorov NMRC "MNTK Eye Microsurgery", according to the standard method [7, 14]. Oxidative degradation of melanosomes was carried out by incubating a suspension of melanosomes in 0.1 M K-phosphate buffer, pH 7.4, ($1.0x10^8$ – $5.0x10^8$ granules/mL) with dry potassium superoxide (3–8 mg) at room temperature for 1–2 hours. At the end of the reaction, the water-soluble degradation products of melanosomes were separated by centrifugation in a Beckman Allegra 64R centrifuge at 10,000xg for 20 min.

Measurement of melanosome degradation products. RPE melanosome degradation products obtained in the reaction with superoxide radicals were registered by the accumulation of low molecular weight fluorescent compounds, which were measured at an exciting light wavelength of 450–470 nm [7]. The emission intensity was measured on a Shimadzu RF5301PC Spectro fluorophotometer (Japan). Water-soluble degradation products had an emission maximum in the

region of 520–525 nm. Melanosome samples not treated with potassium superoxide were used as controls. A calibration dependence curve of the emission intensity at 520–525 nm on the concentration of synthetic DOPA-melanin completely oxidized by KO_2 was used to estimate the concentration of melanosome destruction products.

Determination of the concentration of products that react with thiobarbituric acid (TBA-reactive products). Water-soluble degradation products of RPE melanosomes were evaluated for the content of reactive carbonyls reacting with thiobarbituric acid [15]. The concentration of TBA-reactive products was determined spectrophotometrically at a wavelength of 532 nm [16] on a Shimadzu UV-1700 spectrophotometer (Japan). The initial samples of melanosomes not oxidized with superoxide served as the control.

Analysis of melanosome degradation products by time-of-flight secondary ion mass spectrometry. Mass spectrometry experiments were performed on the TOF.SIMS.5 instrument (ION-TOF, Germany) equipped with 30 keV Bi_3^+ cluster beam. At least 10 measurements for each sample were recorded in both positive and negative ion modes. An electron flood gun was activated to avoid charging effect during analysis. Due to high energy of bismuth projectiles organic species are regularly fragmented and therefore presented on mass spectrum as a set of characteristic ions.

Measurement of protein modification by water-soluble degradation products of RPE melanosomes. The process of protein modification was assessed by the formation of fluorescent Schiff bases in the reaction between free amino groups of proteins and aldehydes. Modification of proteins (bovine serum albumin BSA or hemoglobin from human erythrocytes) with water-soluble products of melanosomes oxidative degradation was carried out by incubation at 37°C for 24-48 hours, followed by dialysis to remove low molecular weight unreacted substances. The incubation medium contained: 0.1 M sterile potassium phosphate buffer, pH 7.4; 2-4 mg/ml BSA (or 2-3 mg/ml hemoglobin), 3-4 mM sodium azide and 0.4-0.7 ml supernatants of oxidized and non-oxidized (control) RPE melanosomes. Samples containing only proteins without supernatants and supernatants without added proteins were used as additional control samples. The content of modified proteins was estimated by fluorescence spectrum at the emission maximum at 440–455 nm (an excitation wavelength of 365 nm) after dialysis.

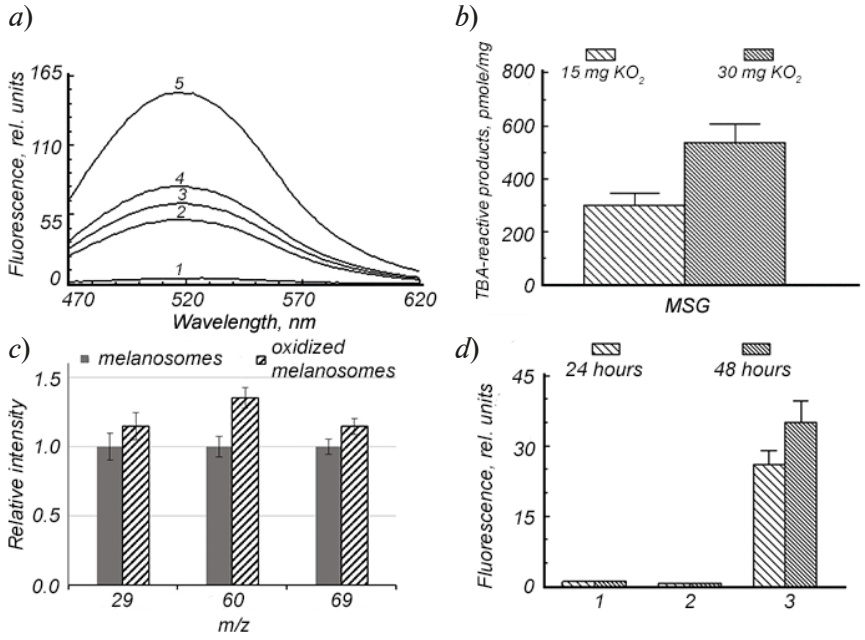

Fig. 1. Melanin suspension fluorescence spectra at different incubation times with KO_2 (curves *1–5* correspond incubation during 0 min, 30 min, 60 min, 90 min and 180 min, respectively) (*a*); excitation wavelength was 450 nm; content of TBA-reactive products in melanin for different amounts of added KO_2 (*b*); carbonyl ion signals in melanin samples under the destructive effect of KO_2 (*c*); the corresponding chemical formulas are given in the text; integrated fluorescence signal from albumin and modified albumin (*d*)

Results and discussion

Fig. 1,*a* shows that, under normal physiological conditions, potassium superoxide causes the destruction of melanin with the formation of fluorescent degradation products with an emission maximum at 520–525 nm. Time-of-flight mass spectrometry was performed to determine the presence of aldehydes in water-soluble fractions of melanosomes in human eye RPE cells. As can be seen from the diagram (Fig. 1,*c*), there is a significant increase in carbonyl ions ($m/z = 29 - CHO^+$, $m/z = 60 - C_2H_4O_2^+$, $m/z = 69 - C_4H_7O^+$) after exposure to superoxide. The ion with $m/z = 60$ shows the largest increase (about 1.5 times). This indicate the presence of aldehydes in the water-soluble degradation products formed during the oxidation of melanosomes by superoxide radicals. These results are confirmed by experiments on the content of carbonyl compounds that react with thiobarbituric acid (TBA-reactive products) (Fig. 1,*b*). One can see that the supernatants of oxidized samples contain significantly more TBA-reactive products than the supernatants of control samples and higher dose of added KO_2 formed more TBA-reactive products.

Obtained results indicate that the oxidation of melanosomes with potassium superoxide leads to the formation of carbonyl products that are readily soluble in the aqueous phase. The degree of melanin destruction depends on the melanin/superoxide ratio. Increased superoxide concentration results in increased accumulation of TBA-reactive products (Fig. 1,*b*). Calculations show that one RPE cell with a volume of $2x10^{-9}$ ml contains about 10–20 picograms of melanin and with its complete destruction up to 0.3 nmol of TBA-reactive products can be accumulated. This amount is quite a lot for the manifestation of the toxic effect of carbonyls. It was also found that the aldehydes and ketones contained in the water-soluble fraction obtained as a result of the oxidative degradation of melanosomes are quite stable, since the content of TBA-reactive products in them practically did not change. The appearance of such long-lived and active chemicals during oxidative destruction of RPE melanosomes may contribute to the development of various eye pathologies.

To the best of our knowledge, it was shown for the first time that water-soluble products of oxidative degradation of RPE melanosomes showed the ability to modify proteins. Thus, incubation of BSA at 37 °C for 24 and 48 h in the presence of water-soluble melanosome degradation products leads to a significant increase in the intensity of albumin fluorescence (Fig. 1d, bars 3). On the contrary, only a slight increase in the fluorescence intensity is observed in the control samples (Fig. 1,*d*, bars 1 and 2). This indicates the formation of Schiff bases during the reaction between the amino groups of albumin and aldehydes contained in water-soluble fractions of oxidized melanosomes. The process of BSA modification depended on the incubation time and was absent in the case of BSA incubation with water-soluble products of unoxidized melanin.

Obtained results indicate the toxicity of the products of oxidative degradation of RPE melanosomes. The accumulation of carbonyl products in the cell can lead to an increase in carbonyl stress, the development of inflammatory processes and be of great importance in the pathogenesis of senile eye pathologies. Previously we have shown that during the oxidative destruction of RPE human melanosomes the polymer structure of melanin is destroyed, which is accompanied by a decrease in the concentration of paramagnetic centers and the accumulation of water-soluble fluorescent products of melanin oxidation [7]. This work presented a detailed study of the melanin oxidative destruction induced by the action of superoxide radicals. The oxidative destruction of melanosomes leads to the formation of TBA-reactive products containing highly active carbonyl compounds. Water-soluble products formed during the oxidation of melanosomes cause the modification of bovine serum albumin with the formation of fluorescent Schiff bases.

Conclusion

It is assumed that one of the main mechanisms of age-related melanin concentration decrease in human RPE cells is its destruction in melanolipofuscin granules. Melanin in the melanolipofuscin granule is in close contact with lipofuscin and lipofuscin fluorophores are capable of forming reactive oxygen species, superoxide radicals, under the action of light [17]. Hence, the age-related decrease of melanosomes amount [5] and melanin concentration [6] in the RPE cell and the formation of reactive aldehydes and ketones, that are toxic to the cell, can be explained by the action of reactive oxygen species formed during photoinduced oxygen reduction by lipofuscin fluorophores [7].

The accumulation of carbonyl products in the cell as a result of the melanin oxidative breakdown can lead to the development of carbonyl stress, inflammatory processes and therefore this process is of great importance for understanding the pathogenesis of senile eye pathologies. In addition, our results suggest that under oxygen stress caused by various reasons and accompanied by the generation of superoxide radicals one of the mechanisms of the toxic effect on RPE cells may be connected with accumulation of highly reactive aldehydes and ketones formed during the destruction of melanosomes by superoxide radicals.

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