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Research Article

A study of erythrocyte membranes in carrageenan-induced gastroenterocolitis by method of fluorescent probes

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Abstract. Objective. The aim of our research was to investigate erythrocyte membranes of rats with carrageenan-induced gastroenterocolitis using fluorescent probes (ortho-hydroxy derivatives of 2,5-diaryloxazole).Materials and methods. Fluorescence of O1O (2 - (2'-OH-phenyl)-5-phenyl-1,3-oxazole) and PH7 (2-(2'-OH-phenyl)-phenanthro[9,10]-1,3-oxazole) probes in suspensions of erythrocytes of rats with chronic carrageenan-induced gastroenterocolitis was determined and compared to the control group consisted of intact animals.Results. It has been found that the hydration of erythrocyte membranes increases in rats with carrageenan-induced gastroenterocolitis. The disease is associated with the changes in the polar regions of the membranes, while no changes in the hydrophobic areas of the erythrocyte membranes are observed. Conclusion. The detected changes allow us to make a conclusion that the fluidity of the erythrocyte membranes of the rats with chronic carrageenan-induced gastroenterocolitis increases.

Key Words: erythrocytes, biomembrane, carrageenan-induced gastroenterocolitis, fluorescent probes.

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Introduction

Carrageenans are sulfated heteropolysaccharides extracted from red algae, which are industrially used as thickeners and emulsifiers (E407) in food industry. Three types of carrageenans (λ, κ, and ι) are of great commercial importance. They differ from each other in the number of sulfate groups and in the content of 3,6-anhydrogalactose (Chan et al 2017; Barth et al 2016; Necas & Bartosikova 2013; Yang et al 2012).

The use of carrageenans as food additives has been approved by the Food and Drug Administration (FDA) and the Joint Expert Committee on Food Additives (JECFA). However, it has been reported that the prolonged ingestion of carrageenans is able to induce intestinal inflammation in laboratory animals (Martino et al 2017; Barth et al 2016). Carrageenan-induced intestinal inflammation is characterized by the development of oxidative stress, formation of proinflammatory cytokines, activation of apoptosis and proliferation of intestinal epithelium, and damage to membranes of enterocytes (Gubina-Vakyulyk et al 2015; Bhattacharyya et al 2013; Posokhov et al 2013; Zhukov & Tkachenko 2013).

Since monosaccharide residues in carrageenan are linked with α-1,3 and β-1,4-glycosidic bonds which are not subject to human digestive enzymes, it is not digested and absorbed. Thus, its action should be limited to the gastrointestinal tract. To verify this hypothesis, we have decided to study the changes in erythrocyte membranes in rats with chronic experimental carrageenan-induced intestinal inflammation.

The purpose of the research was to investigate erythrocyte membranes of rats with chronic carrageenan-induced gastroenterocolitis by means of fluorescent probes (ortho-hydroxy derivatives of 2,5-diaryloxazole), which non-covalently bind to cell membranes of experimental animals and have a quick response to changes in their microenvironment.

Materials and methods

1. Experiment design and induction of carrageenan-induced gastroenterocolitis.

The female WAG rats weighing 150-180 g were used for the experiment. Laboratory animals were randomly divided into 2 groups. Group 1 consisted of 10 intact animals. Group 2 included 10 experimental animals with chronic carrageenan-induced gastroenterocolitis. It was caused by the free access of animals to 1% solution of lambda-carrageenan in drinking water. A month after the beginning of the experiment, animals were killed by decapitation.

2. Description of fluorescent probes and staining of cells.

The suspension of red blood cells (RBCs) was obtained from blood of animals using physiological solution. Fluorescent probes were dissolved in acetonitrile to the initial concentration of $1*10^{-4}$ mol / l. Twenty μl of each corresponding probe was added to the suspension of erythrocytes. The final concentration

Table 1. Fluorescence intensity of probes O1O and PH7 in erythrocyte membranes of animals with carrageenan-induced gastroenterocolitis

Sample	Fluorescence intensity, a.u.					
	Probe O1O			Probe PH7		
	375 nm	470 nm	I_{470}/I_{375}	425 nm	485 nm	I_{485}/I_{425}
Control (n=10)	12.7±0.7	156.5±7.8	12.3±0.6	42.6±2.1	65.2±3.3	1.5±0.1
Gastroenterocolitis (n=10)	12.9±0.3 p>0.05	115.2±5.8 p<0.001	8.9±0.5 p<0.01	42.9±2.1 p>0.05	64.8±3.3 p>0.05	1.5±0.1 p>0.05

of each probe in the suspensions was $1*10^{-6}$ mol / l. Thus, the molar ratio of lipid / probe was 1000:1. We measured the fluorescence of O1O (2 - (2'-OH-phenyl)-5-phenyl-1,3-oxazole) and PH7 (2-(2'-OH-phenyl)-phenanthro[9,10]-1,3-oxazole) probes in suspensions of RBCs of rats with chronic carrageenan-induced gastroenterocolitis. Measurement of the fluorescence was performed by spectrofluorometer "Hitachi F4010" after 1 hour after the addition of probes to the erythrocyte suspensions. The fluorescence spectra of probes were measured in the range of 340-600 nm with monochromator slit width of excitation and fluorescence 5 and 5 nm, respectively, and the excitation wavelength of 330 nm. The erythrocytes of intact healthy animals were used as control samples. The fluorescent probes successfully used for studies of biological membranes (Posokhov 2012; Posokhov 2011; Posokhov et al 2001): 2-(2'-OH-phenyl)-5-phenyl-1,3-oxazole (probe O1O) and 2-(2'-OH-phenyl)-phenanthro [9,10]-1,3-oxazole (probe PH7) were used for investigation of RBC membranes. The choice of O1O, PH7 (ortho-hydroxy 2,5-diaryl-1,3-oxazole) fluorescent probes for our research was explained by the fact that the fluorescent characteristics of the probes depend on the physico-chemical properties of their microenvironment: the proton-donor ability, the polarity and viscosity of the microenvironment (Posokhov 2012; Doroshenko et al 2002; Doroshenko et al 2000; Doroshenko & Posokhov 1999; Doroshenko et al 1997).

3. Bioethics

All experimental procedures were performed in accordance with the provisions of the European Convection for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Strasbourg, 1986). The experiment was approved by the Committee of Ethics and Bioethics of Kharkiv National Medical University. Humans were not involved in the current research.

4. Statistical analysis.

Experimental data were processed using the "GraphPad Prism 5" software. We used Student's t test to compare the numerical values of two groups of normally distributed variables. Differences between groups were considered statistically significant at $p < 0.05$.

Results

A noticeable decrease in the intensity of the long-wavelength fluorescence band (470 nm) was observed for probe O1O in erythrocyte membranes during the development of the carrageenan-induced gastroenterocolitis, while, the intensity of the short-wavelength fluorescence band (375 nm) of probe O1O was almost unchanged (see Table 1, Figure 1). Thus, the intensity ratio of the long-wavelength and short-wavelength fluorescence bands I_{470}/I_{375} of probe O1O decreases in case of rats with the carrageenan-induced gastroenterocolitis (see Table 1).

At the same time, no significant changes were observed in the fluorescence parameters of probe PH7 in case of animals with gastroenterocolitis (see Table 1).

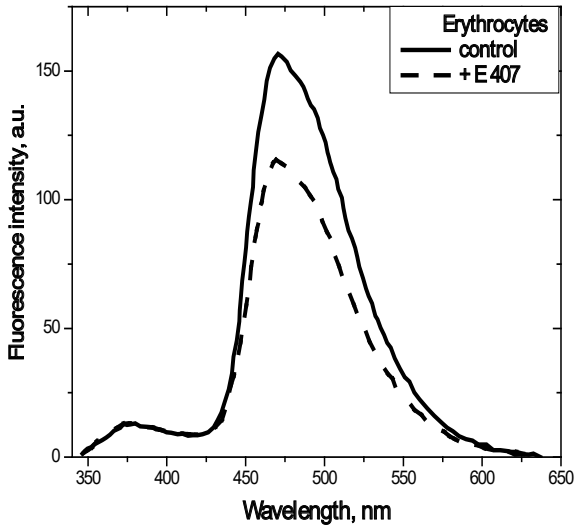

Fig. 1. The fluorescence spectra of the probe O1O in erythrocyte suspensions: (a) control (solid line), (b) the animals with carrageenan-induced gastroenterocolitis (dashed line)

Discussion

It has been known that in the excited state the ortho-hydroxy 2,5-diaryl-1,3-oxazole is characterized by excited state proton transfer (ESIPT) reaction (see Figure 2): hydroxyl group in the ortho-position of the lateral benzene ring acts as protonodonor and the nitrogen atom of oxazole ring acts as proton acceptor (Doroshenko et al 2002; Doroshenko et al 2000; Doroshenko & Posokhov 1999; Doroshenko et al 1997). The result of the ESIPT reaction is the formation of phototautomeric form (T*), fluorescent in significantly longer wavelengths in comparison with the initial form (N*) (Doroshenko et al 2002; Doroshenko et al 2000; Doroshenko & Posokhov 1999; Doroshenko et al 1997). The presence of two-band fluorescence allows to perform ratiometric measurement, i.e. to use the ratio of phototautomeric form and the initial form fluorescence intensities (I_{T*}/I_{N*}) as a parameter for evaluation of the physical and chemical properties of the microenvironment. The use of ratiometric fluorescent probes allows to exclude the measurement error associated

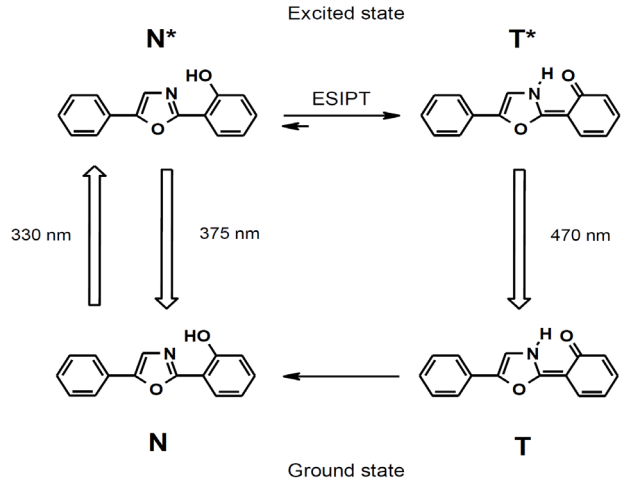

Fig. 2. Scheme of excited state proton transfer (ESIPT) reaction in 2 - (2'-OH-phenyl)-5-phenyl-1,3-oxazole (probe O1O). Upwards arrow shows the electronic excitation and downwards arrow represents the light emission (fluorescence). Corresponding maxima of fluorescence are measured in nanometers.

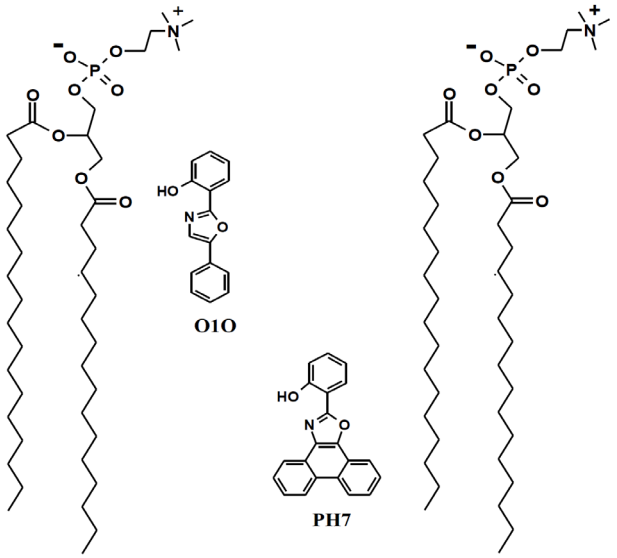

Fig. 3. Expected location and orientation of fluorescent probes O1O and PH7 based on their fluorescence properties in lipid membranes (Posokhov 2012; Posokhov 2011; Posokhov et al 2001). and on the basis of their structural similarity with fluorescent probes with known localization in lipid membranes (Dobretsov 1989). Two molecules of phosphatidylcholine from the outer monolayer are shown to denote the localization of the probes.

with the deviation of the fluorescent probe concentration (e.g., uneven distribution of the fluorescent probe in various membranes) and the measurement error associated with a deviation of fluorescence technique settings (deviation of the intensity of the exciting source, a change in focus, changes in the sensitivity of the photodetector, etc.) (Shapiro 1995).

Compounds that differ in their lipophilicity (Posokhov 2012; Posokhov 2011; Posokhov et al 2001) were selected for the present study. It is expected that the regions of localization of selected probes in the membrane are different and correspond to the lipophilicity of the probes (see Figure 3) (Posokhov 2012; Posokhov 2011; Posokhov et al 2001). Expected location and orientation of O1O and PH7 in lipid membranes is based on their fluorescence properties in lipid membranes (Posokhov 2012; Posokhov 2011; Posokhov et al 2001) and on the basis of their structural similarity with fluorescent probes with known localization in lipid membranes (Dobretsov 1989). The location of the probes in lipid membranes: probe O1O is located in the area of glycerol backbones of phospholipids (closer to the center of the lipid bilayer), in the area of carbonyl groups of phospholipids and in the area of hydrocarbon chains of phospholipids (near the area of the carbonyl groups of phospholipids); probe PH7 is located in the area of hydrocarbon chains of phospholipids (near the center of the bilayer) (see Figure 3).

The decrease in the intensity of the tautomeric form fluorescence band (I_{T^*}) and the decrease in the ratio I_{T^*}/I_{N^*}, observed for probe O1O in case of the disease (see Figure 1, Table 1), indicate an increase in polarity and proton-donor ability of the microenvironment of probe O1O in the erythrocyte membranes of rats with gastroenterocolitis. Such increase in polarity and proton-donor ability of the microenvironment of probe O1O indicates an increase in hydration of the probe microenvironment in the erythrocyte membranes of the experimental group.

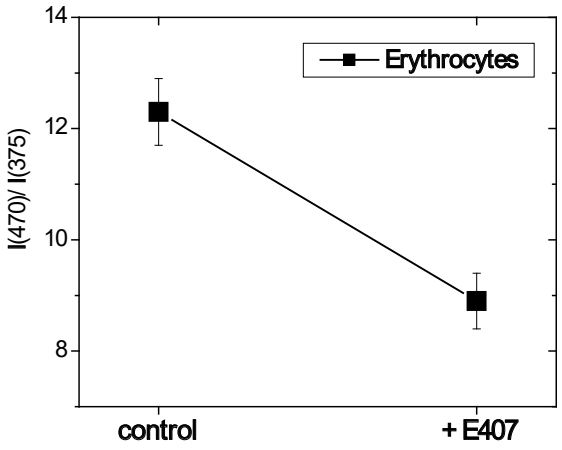

Fig.4. The ratio of the tautomeric and the initial form intensities I_{T^*}/I_{N^*} for O1O probe in the erythrocyte membranes.

The discussed increase in hydration points to the decrease in the membrane viscosity (or points to the increase in fluidity of the lipid bilayer) (Ho et al 1995; Ho & Stubbs 1995).

Thus, it was shown that fluorescent probe O1O (2-(2'-OH-phenyl)-5-phenyl-1,3-oxazole) might be used as an indicator for estimation of the nature and types of changes in the structure of erythrocyte membranes of rats during the development of carrageenan-induced gastroenterocolitis. It was found that during the gastroenterocolitis the changes in the membranes of erythrocytes occur in the regions of probe O1O localization, i.e. in quite polar regions of the membrane: presumably, in the area of glycerol backbones of phospholipids and in the area of the carbonyl groups of phospholipids. On the other hand, no changes occur in the regions of probe PH7 membrane localization, i.e. in a more hydrophobic region of the erythrocyte membranes:

presumably, in the area of hydrocarbon chains of the phospholipids near the center of the bilayer.

Conclusions

The following changes occur in the erythrocyte membranes of experimental animals with carrageenan-induced gastroenterocolitis: the increase in the membrane hydration and the decrease in the viscosity of the lipid bilayer (i.e. the increase in the membrane fluidity).

The use of fluorescent probes (ortho-hydroxy oxazole derivatives) allows to assess the type and nature of the damaging effect of carrageenan on the structural components of membranes. Thus, the mentioned method, using ortho-hydroxy oxazole derivatives as fluorescent probes, may be suggested for application in the practical medicine for the study of the negative effects similar to carrageenan food additives on the human body.

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