Research Article

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Changes in cell membranes of white blood cells treated with a common food additive E407a

Abstract

Objectives: To estimate the state of phospholipid bilayer of rats WBCs exposed to a common food additive E407a, which is used as a thickener and emulsifier, during 4 h using a fluorescent probe – ortho-hydroxy derivative of 2,5-diaryl-1,3-oxazole.

Materials and methods: Steady-state fluorescence spectroscopy: a study by the environment-sensitive fluorescent probe – 2-(2′-hydroxy-phenyl)-5-phenyl-1,3-oxazole (probe O1O).

Results: Changes are detected in the spectra of the fluorescent probe bound to rat WBCs treated with the solutions of E407a of various concentrations in comparison with the corresponding spectra of the probe incubated with the untreated leukocytes. The decrease in polarity and proton-donor ability is observed in the lipid membranes of leukocytes in the region, where the probe locates.

Conclusions: Our findings suggest a higher dehydration of leukocyte membranes of rats treated with a common food additive E407a at high concentrations and, thus, indicate that exposure to high doses of E407a leads to the increase in the lipid order (i.e. to decrease in fluidity) of the membranes of rat WBCs.

Keywords: carrageenan; cell membrane; fluorescent probe; leukocytes; processed Eucheuma seaweed.

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Anahtar Kelimeler: İrlanda yosunu; işlenmiş Eucheuma yosunu; floresan prob; hücre zar.

Sonuçlar: Bulgular ve proton verici kabiliyetinde azalma gözlenir ve��分子probita对白细胞膜的贡献。probun bulunduğunda bölgesinde lökositlerin lipid membranlarında polaritede ve proton verici kabiliyetinde azalma göstermektedir. probun bulunduğunda bölgesinde lökositlerin lipid membranlarında polaritede ve proton verici kabiliyetinde azalma göstermektedir.probun bulunduğunda bölgesinde lökositlerin lipid membranlarında polaritede ve proton verici kabiliyetinde azalma göstermektedir.

Anahtar Kelimeler: İrlanda yosunu; işlenmiş Eucheuma yosunu; floresan prob; hücre zar; lökositler.

Introduction

Carrageenans (CGNs) are currently believed to be the most widespread marine polysaccharides used in food industry [1]. Their unique rheological properties allow CGNs to occupy a niche in the market of food additives, where they are known as E407 (refined CGN) and E407a (semi-refined CGN or processed Eucheuma seaweed, PES). Both E407 and E407a are approved for use within the borders of the European Union by the European Food Safety Organization and are used as thickening and stabilizing agents for the production of processed meat, dairy products, and confectionary. CGNs are extracted from marine red algae, in which they may constitute up to 75% of dry weight, and structurally consist of sulfated-β-galactose derivatives linked with glycosidic bonds [2].

It is worth noting that application of CGNs is not limited to the food markets only. There is some evidence that CGNs show antiviral, anticancer and immunomodulatory effects and many researches have been undertaken to explore their potential as a drug [3–5]. Furthermore, these biomacromolecules can be used for drug delivery and as prebiotics [6, 7].

Despite its approval for use as a component of food by international authorities such as the Food and Drug Administration and EFSA, CGNs have been claimed to be unsafe for human consumptions [8–12]. In recent years, evidence from animal studies and cell culture experiments allows suggesting that CGN promotes intestinal inflammation and upregulates cytokines in intestinal epithelial cells [8, 12]. It is believed that CGNs are able to aggravate the already existing pathogen-induced inflammation in the gut [10]. Such hypothesis is consistent with the findings on the ability of CGN to enhance lipopolysaccharide-induced secretion of pro-inflammatory cytokines [13, 14]. However, McKim et al. claim that data on pro-inflammatory effects of CGNs and their contribution to the development of intestinal inflammation is misinterpreted due to the confusion in terms [15]. In particular, poligeenan and degraded CGN, which are products of hydrolysis of food-grade CGN with lower molecular weights, are referred to as CGNs. Their ability to induce inflammation in the gut of laboratory animals has been widely recognized and both poligeenan and degraded CGN are not approved for being used in food industry. However, the digestive fate of CGN is still under debate and more studies are required to clarify whether low-molecular-weight fragments can be produced from food-grade CGN molecules as a result of their acidic hydrolysis.

CGNs have been demonstrated to induce the generation of reactive oxygen species (ROS) by various types of cells, including neutrophils [16–18]. ROS-mediated lipid peroxidation affects cell membrane structure. It has been reported that lipid peroxidation increases the membrane viscosity and, thus, reduces the fluidity of cell membranes [19, 20]. Such changes may be detected using fluorescent probes.

The aim of our research was to assess the state of rat WBC cell membranes exposed to the solutions of PES (1, 2, and 5%) during 4 h using the fluorescent probe O10 (2′-hydroxy-phenyl)-5-phenyl-1,3-oxazole).

Methods

Study design

The study was conducted using eight female adult WAG rats weighing 160–190 g. The rats were housed in two cages (four rats in each cage) in standard laboratory conditions at 24 ± 2 °C two weeks prior to the experiment. The access to food and drinking water was free. The animals were anesthetized and sacrificed with the subsequent collection of blood into sterile EDTA VACUTAINER tubes. Two milliliters of blood collected in the VACUTAINER tubes was taken and equally distributed between four capped polysterene test tubes for each out of eight samples. Thus, four portions (500 μl each) of non-coagulated blood were prepared from each sample. Then 100 μl of phosphate buffered saline (PBS, pH 7.4; BD, USA), 1, 2 and 5% solutions of E407a, respectively, were added to each of those volumes of blood. Blood samples treated with PBS and E407a solutions were incubated for 4 h. It has been reported that the content of CGN in dairy products varies from 0.005 to 0.5% of weight [8]. Thus, for our study, we selected the concentration of CGNs within this range.

All procedures were approved by the local Bioethics Committee. The care and used of laboratory animals was performed in accordance with the EU Directive 2010/63/EU on the protection of animals used for scientific purposes, based on the Council of Europe Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS123).
PES and its solutions

Solutions with various concentrations (1, 2, and 5%) of food grade k-carrageenan-containing PES were prepared in advance 24 h prior to the experiment. PES has been reported to contain up to 15% of algal cellulose and traces of formaldehyde [21]. To prepare the solution of food additive PES, solid E407a were added to PBS (BD, USA). Then 100 μL of the corresponding stock solutions were added to 500 μL of blood. The solutions were gently vortexed prior to their addition to blood samples.

Lyse/wash protocol

WBC suspensions were obtained from the blood samples treated with PBS and solutions of PES following the lyse/wash procedure (Becton Dickinson Technical Support Protocol, 2002). According to this protocol, 100 μL of each sample was added to new 12 × 75 mm capped polystyrene test tubes. To lyse RBCs, 2 mL of 1× FACSLyse solution (Becton Dickinson, San Jose, USA) was added. The solutions were vortexed. Incubation of the solutions obtained as a result lasted for 15 min at 24 °C with no exposure to light. It was followed by centrifugation at 500 g for 5 min. The supernatant was discarded. Then 2 mL of PBS was added with the centrifugation at 500 g for 5 min. The supernatant was discarded again. The suspensions were immediately used for the incubation with the fluorescent probe O1O.

Characteristics of the fluorescent probe

The cells were fluorescently labeled by the same procedure: an aliquot of the probe stock solution in acetonitrile was added to the WBC suspensions to achieve a final probe concentration ∼5·10−6 mol/L. Lipid-to-probe molar ratio was ~200:1. Before fluorescence measurements, the cell suspensions were incubated with the probes at room temperature for 1 h. The fluorescence spectra were measured on a fluorometer “Hitachi F850” in the range of 350–630 nm, with an increment of 2 nm. The excitation wavelength was 330 nm. The excitation and emission slits were 5 nm.

In this study, we used the fluorescent probe O1O, since its fluorescence parameters depend upon the polarity and proton-donor ability of the microenvironment [22–25].

Probe O1O locates (Figure 1): 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 carbonyl groups of phospholipids [22, 23].

When the probe O1O is in the excited state, the excited state proton transfer reaction occurs [22–25] (Figure 2). In result of this reaction, the phototautomer form (T*) is formed. The photoproduct is fluorescent in significantly longer wavelengths in comparison with the initial (or so-called “normal”) form (N*) [22–25].

The presence of two-band fluorescence enables us to conduct ratiometric measurement, i.e. to use the ratio of the phototautomer form and the initial form fluorescence intensities (I_T*/I_N*) as a parameter for estimation of the physical and chemical properties of the microenvironment.

Statistical analysis

Statistical comparisons were carried out with the help of GraphPad Prism 5.0 (GraphPad software, USA). We evaluated the differences between four independent groups using non-parametric analysis of variance (Kruskal–Wallis test). Numerical values are presented as medians and interquartile ranges. p values below 0.05 were considered to be statistically significant.

Figure 1: Localization and orientation of fluorescent probe O1O in phospholipid membranes. Two molecules of phosphatidylcholine from the outer leaflet are shown to denote the localization of the probe (adapted from Posokhov and Kyrychenko [23]).

Figure 2: Scheme of excited state intramolecular proton transfer reaction in probe O1O. The upwards arrow denotes the electronic excitation and the downwards arrow designates the fluorescence. Corresponding maximum of absorption and the ranges of emission are shown in nanometers (modified from Posokhov and Kyrychenko [23]).
Results

Outcomes of fluorescence measurements are summarized in Table 1 and Figure 3. The incubation of rat leukocytes with the common food additive E407a affected the state of cell membranes.

To assess the effects mentioned above, the ratio of the fluorescence intensities of phototautomer and normal forms ($I_{T*}/I_{N*}$) of probe O1O in the leukocyte membranes of rodents was analyzed (Table 1). The $I_{T*}/I_{N*}$-ratios of probe O1O for WBC suspensions treated with 1 and 2% solutions of E407a during 4 h were higher compared with the control samples. However, in both cases, the difference was found to be statistically insignificant ($p>0.05$). Thus, at lower concentrations no changes were revealed. Meanwhile, the exposure of the leukocytes to 5% E407a solution resulted in a statistically significant increase in the $I_{T*}/I_{N*}$ ratio ($p<0.05$) (Table 1). Thus, we can observe that the state of phospholipid bilayer is affected at high concentrations of the food-grade CGN-containing food additive.

In this study, the observed increase in the fluorescence intensity ratio ($I_{T*}/I_{N*}$) points to both a decrease in polarity [22, 24, 25] and a reduction of proton-donor ability of the environment of probe O1O [23] in the membranes of rat leukocytes exposed to the 5% solution of E407a.

It is important to note that in fluorescence spectrum of probe O1O in WBCs incubated with the 5% E407a solution (Figure 3), short-wavelength shift (−22 nm) of the fluorescence maximum of the normal form (N*) and long-wavelength shift (−9 nm) of the fluorescence maximum of the phototautomer (T*) were observed in comparison with the corresponding spectrum for the control samples. Such changes were considered another demonstration that the polarity of the probe microenvironment decreased [22, 24, 25].

To sum up, only high concentrations of E407a, in contrast to its lower concentrations, could promote modifications, which were detected by measuring the fluorescence of probe O1O, in the structure of phospholipid bilayer of leukocytes incubated with this food additive.

Table 1: The ratio of the fluorescence intensities of the phototautomer and normal forms ($I_{T*}/I_{N*}$) of probe O1O in the leukocyte membranes of rats (Me [IQR]).

| Groups                  | Concentrations of E407a in the solutions used | $I_{T*}/I_{N*}$ ($I_{480}/I_{580}$) |
|-------------------------|---------------------------------------------|-----------------------------------|
| WBCs untreated with E407a (control group) | 0%                                          | 2.6 [2.4; 2.8]                    |
| Leukocytes treated with E407a | 1%                                          | 2.8 [2.3; 3.4] $p<0.05$          |
|                         | 2%                                          | 2.9 [2.7; 3.0] $p<0.05$          |
|                         | 5%                                          | 7.2 [6.8; 7.7] $p<0.05$          |

Differences were considered statistically significant at $p<0.05$.

Discussion

The abovementioned decrease in the polarity and the proton-donor ability of the microenvironment of probe O1O is indicative of dehydration [26–30] of the membrane. The discussed dehydration, in its turn, suggests the increase of the membrane lipid order [26–30].

Thus, our results provide evidence that exposure of WBCs to high doses of E407a leads to the increase in the lipid order of the leukocyte membranes.

According to the Fluid Mosaic Model, cell membranes are composed of a phospholipid bilayer, in which molecules of phospholipids are oriented in two opposite directions. The fluid state of cell membrane is of crucial importance for cellular viability, since it provides the permeability of phospholipid bilayer [31]. It is important to note that the fluid features of cell membranes is maintained by polyunsaturated fatty acids (PUFAs), while long-chain saturated fatty acids, on the contrary, diminish the fluidity and, therefore, increase the viscosity, which is in a reciprocal relationship with fluidity [32].

It has been demonstrated that when phospholipid tails in cell membranes are oxidized, they become shorter [33], which affects the fluidity of membranes, as well as their...
thickness. It is worth mentioning that ROS can initially oxidize the head groups of phospholipids and only after that fatty acid tails undergo oxidation [34]. Thus, the fluorescent probe selected by us is suitable for the detection of changes in the lipid membranes caused by ROS due to its location. We can hypothesize that the reduction of leukocyte membrane fluidity identified by the probe can be attributed to lipid peroxidation [19, 20].

The cell membrane is of huge importance in most cellular processes. Its functions are not restricted to being a physical barrier. The cell membrane also provides communication with extracellular environment. In addition, protein receptors and transporters that are crucial for determining the cell response to various challenges are embedded in the membrane. Thus, membrane packing seriously affects the features of cellular responses in normal and pathological conditions [35]. Membrane fluidity is a physical property that reflects how molecules move in the phospholipid layers. It may depend on the fatty acid composition, cholesterol percentage, and cholesterol/phospholipid ratio [36].

There is accumulating evidence that lipid order alterations in leukocytes affect their functions and, hence, features of the immune response. It has been reported that changes in membrane lipid order and fluidity reduce leukocyte motility and chemotaxis [37]. Furthermore, lipid order alterations in cell membranes of neutrophils influence their extravasation [38]. Thus, CGN-associated changes in leukocyte membranes found in this study may have negative impact on leukocyte effectiveness and modify the immune response. However, more experimental data have to be collected to confirm this hypothesis.

It is worth mentioning that structurally CGNs are reminiscent of glycosaminoglycans (GAGs). Both of them are sulfated polyanionic heteropolysaccharides [39]. Sahoo and Schwille (2013) stated that GAGs are capable of diminishing the diffusion rate of lipid in the outer leaflet of phospholipid bilayer, which resulted in an increased microviscosity, higher lipid order, and, thus, reduced hydration [40]. It is believed that the electrostatic interactions between positively charged phospholipid heads and negatively charged sulfated groups of CGNs underlie the changes in lipid dynamics. Our findings are consistent with those that occur in cell membranes under the influence of GAGs. Thus, the increased viscosity and lipid order of membranes in WBCs exposed to high concentrations of PES found in this study may be attributed to a direct interaction of negatively charged CGN characterized by the high level of sulfation and positively charged surface of outer leaflet.

**Conclusion**

The obtained results point to dehydration of membranes of rat WBCs treated with high doses of common food additive E407a and, thus, indicate that the exposure to high concentrations of E407a leads to the increase in the lipid order of the leukocyte membranes.

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**Competing interests:** Authors declare no conflict of interest.

**Ethical approval:** The study was performed in accordance with the EU Directive 2010/63/EU on the protection of animals used for scientific purposes, based on the Council of Europe Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS123).

**Çıkar Çatışması:** Yazarlar çikar çatışması bildirizler.

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