ORIGINAL ARTICLE

A study of enterocyte membranes during activation of apoptotic processes in chronic carrageenan–induced gastroenterocolitis

Anton Tkachenko[1], Dmytro Marakushyn[2], Iuliia Kalashnyk[3], Yevgen Korniyenko[4], Anatolii Onishchenko[1], Tatyana Gorbach[1], Oksana Nakonechna[1], Yevgen Posokhov[5], Alexander Tsygankov[5]

[1]Department of Biochemistry, Kharkiv National Medical University, [2]Department of Physiology, Kharkiv National Medical University, [3]Department of Otorhinolaryngology[3], Kharkiv National Medical University, [4]Department of Human and Animal Physiology, V. N. Karazin Kharkiv National University, [5]Department of Organic Chemistry, Biochemistry and Microbiology, The National Technical University "Kharkiv Polytechnic Institute"; Kharkiv, Ukraine

ABSTRACT

Aim To investigate the lipid membranes of rat enterocytes in chronic carrageenan-induced gastroenterocolitis accompanied by the activation of apoptotic processes.

Methods Steady-state fluorescence spectroscopy: a study by fluorescent probes – by ortho-hydroxy derivatives of 2,5-diaryl-1,3-oxazole. Activity of apoptosis signal-regulating kinase 1 and *poly (ADP-ribose) polymerase* in small intestinal homogenates, blood serum levels of matrix metalloproteinase-2 and caspase-3 and the level of DNA fragmentation in small intestinal homogenates were determined.

Results Biochemical analysis revealed that an activation of apoptotic processes occurred in the intestinal epithelium of rats during chronic carrageenan-induced gastroenterocolitis. The fluorescence probes showed that activation of apoptotic processes in carrageenan-induced gastroenterocolitis was accompanied by changes in polar regions of rat enterocyte membranes, while no changes were revealed in more hydrophobic regions of the membranes.

Conclusion The increase in hydration of membranes was attributed to the activation of the apoptosis of enterocytes. It has been shown that a fluorescent probe (2-(2′-hydroxyphenyl)-5-phenyl-1,3-oxazole) can be used for the detection of apoptosis of enterocytes.

Key words: apoptosis, caspases, food additives, rats, inflammation

Corresponding author:
Anton Tkachenko
Department of Biochemistry,
Kharkiv National Medical University
Nauky av. 4, 61022 Kharkiv, Ukraine
Phone: +380 50 109 45 54;
Fax: +380 57 700 41 32;
E-mail: antontkachenko555@gmail.com
ORCHID ID: http://www.orcid.org/0000-0002-1029-1636

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INTRODUCTION

Carrageenan is a high molecular weight anionic polymer, which is obtained by extraction from red seaweeds (1). Carrageenan acts as a natural gelling agent and a thickener in dairy, confectionery and meat products. Food carrageenan (food additive E407) has an average molecular weight of over 100 kDa with a small percentage of smaller fragments. It has been reported that carrageenans are widely used to induce inflammation such as peritonitis, pleurisy, arthritis and carrageenan-induced paw edema (2,3). There is evidence that carrageenans also contribute to tumour development (4). Moreover, the oral intake of carrageenans by laboratory animals may lead to intestinal inflammation. It has been reported that carrageenan is able to induce colitis in mice (5).

In this connection, the question about the safety of carrageenan as a food additive arises (6). The clinical study of the impact of the systematic use of carrageenan on the body is problematic, so there is an urgent need to study the impact of carrageenan on metabolic parameters in experimental models (2). A model of moderate chronic carrageenan-induced gastroenterocolitis without necrotizing process was designed through the use of lower doses of this food additive on the base of the model of necrotizing carrageenan-induced gastroenterocolitis (7).

In our previous publications (6-8) we have shown that the development of chronic carrageenan-induced gastroenterocolitis in rats leads to activation of apoptotic processes. This was confirmed by the activation of apoptosis signal-regulating kinase 1 (ASK-1) (8,9), inactivation of *poly (ADP-ribose) polymerase* (PARP) (6, 8) and an increase in DNA fragmentation level (8,9) in small intestinal homogenates; activation of metalloproteinase-2 (MMP-2) (10) and activation of caspase-3 (10) in blood serum.

However, the study of changes in the membranes of enterocytes during activation of apoptotic processes in chronic carrageenan–induced gastroenterocolitis has not been conducted.

The aim of this research was to study the lipid membranes of rat enterocytes in chronic carrageenan-induced gastroenterocolitis accompanied by the activation of apoptotic processes.

MATERIALS AND METHODS

Study design

The experiment was carried out on 20 adult female WAG rats, which were kept under standard vivarium conditions. Laboratory animals were divided into 2 groups: group 1 consisted of animals with experimental chronic carrageenan-induced gastroenterocolitis, and group 2 was the control and consisted of intact animals. Chronic carrageenan-induced gastroenterocolitis was evoked by free access of animals to the 1% carrageenan solution in drinking water.

The following biochemical parameters after carrageenan intake were measured: apoptosis signal-regulating kinase 1 (ASK-1), *poly (ADP-ribose) polymerase* (PARP) and DNA fragmentation in small intestinal homogenates, as well as matrix metalloproteinase-2 (MMP-2) and caspase-3 in blood serum (Table 1).

Table 1. Parameters of apoptosis in chronic carrageenan-induced gastroenterocolitis in blood serum and small intestinal homogenates

Parameter	Median (interquartile 25%; 75%)		p
	Control	Gastroenterocolitis	
Apoptosis signal-regulating kinase 1 (ASK-1) activity in small intestinal homogenate (units/min. mg of protein)	1.79 (1.72;1.80)	4.45 (4.27; 4.59)	p<0.001
Level of matrix metalloproteinase-2 (MMP-2) in blood serum (ng/mL)	7.29 (6.87;7.72)	11.31 (10.73;12.37)	p<0.01
Poly (ADP-ribose) polymerase (PARP) activity in small intestinal homogenate (mmol/mg of protein)	1.39 (1.30;1.42)	0.47 (0.42; 0.50)	p<0.001
Concentrations of caspase-3 in blood serum (ng/mL)	0.93 (0.65;1.14)	34.66 (30.40;38.20)	p<0.0001
DNA fragmentation in small intestinal homogenate (%)	15.5 (15.0;16.7)	23.8 (23.0; 24.7)	p<0.001

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 (11).

Methods

A month after the beginning of carrageenan intake the animals were derived from the experiment by decapitation. The intestine was removed in the cold immediately after the decapitation of rats.

Perfusion of intestine was performed using chilled physiological solution. The epithelial cells were detached from the internal surface of the intestine by scraping with anatomical knife. A suspension of epithelial cells in Tris-HCl buffer (pH 7.4) was prepared. The cell suspension, corresponding to 56-57%, was used for the study.

To investigate the lipid membranes of rat enterocytes, we used fluorescent probes - ortho-hydroxy derivatives of oxazole, whose molecules non-covalently bind to the membranes of cells and respond to changes in the microenvironment (12-14): 2-(2'-hydroxyphenyl)-5-phenyl-1,3-oxazole (probe 1) and 2-(2'-hydroxyphenyl)phenanthrene (10,11)-1,3-oxazole (probe 2).

The choice of fluorescent probes 1 and 2 (ortho-hydroxy derivatives of 2,5-diaryl-1,3-oxazole) for the studies of rat enterocyte membranes is due to the fact that the fluorescence characteristics of these fluorescent probes depend on the physicochemical properties of their microenvironment: on proton-donor ability, polarity and viscosity of the medium (15-18).

Fluorescence of the probes was measured in physiological solutions, containing: the enterocytes of rats with chronic carrageenan-induced gastroenterocolitis (experimental group) and the enterocytes of intact healthy animals (control).

For all the fluorescence measurements, the cells were fluorescently labelled using the same procedure. Since the solubility of the probes is limited in water, the 0.2 mM stock solution of the probes in acetonitrile was used. An aliquot of 0.2 mM stock solution of the probe in acetonitrile was added to physiological solution with enterocytes (i.e. acetonitrile final concentration was ≤ 0.5% vol.) to achieve final probe concentration of 1 μM (such concentration of the probe corresponded to lipid-to-probe molar ratio 1000:1). The cell suspension was then incubated with the probes in a dark at room temperature for 1 hour before fluorescence measurements.

Fluorescence spectra were recorded on a Hitachi F850 steady-state fluorescence spectrometer at room temperature. The slits on excitation and emission monochromators were 5 nm. The measurements were made in a 10 mm×10 mm cuvette. An excitation wavelength was 330 nm. Emission was recorded in the range of 340 – 600 nm, with an increment of 1 nm. Data were collected with a 1 s interval.

The accuracy of the fluorescence intensity measurements of the samples was 5%.

Statistical analysis

Mann-Whitney U test was used to compare the numerical values of two groups. Differences between groups were considered statistically significant at $p<0.05$.

RESULTS

Biochemical parameters whose changes were observed after carrageenan intake were ASK-1, PARP and DNA fragmentation in small intestinal homogenates and MMP-2, caspase-3 in blood serum. Activation of apoptotic processes in the intestinal epithelium of rats during chronic carrageenan-induced gastroenterocolitis was judged by the activation of apoptosis signal-regulating kinase 1 (ASK-1): the activation from 1.79 [1.72; 1.80] (control) to 4.45 [4.27; 4.59] units/min. mg of protein (gastroenterocolitis); inactivation of *poly (ADP-ribose) polymerase* (PARP): from 1.39 [1.30; 1.42] (control) to 0.47 [0.42; 0.50] μmol/mg of protein (gastroenterocolitis); activation of caspase-3 from 0.93 [0.65; 1.14] (control) to 34.66 [30.40; 38.20] ng/mL (gastroenterocolitis); increase in the level of DNA fragmentation: from 15.5 [15.0; 16.7] % (control) to 23.8 [23.0; 24.7] % (gastroenterocolitis) (Table 1).

A marked decrease in the intensity of the long-wavelength fluorescence band (470 nm) and a slight increase in the intensity of the short-wavelength fluorescence band (390 nm) of probe 1 were observed in the case of enterocytes from the animals with carrageenan-induced gastroenterocolitis in condition of activation of apoptotic processes (Table 2, Figure 1). Thus, the fluorescence intensity ratio I_{470}/I_{390} of probe 1 was reduced in case of activation of apoptotic processes (Table 2).

Table 2. Fluorescence intensity of probes 1 and 2 in enterocyte membranes of rats

Sample	Fluorescence intensity*					
	Probe 1			Probe 2		
	390 nm	470nm	I_{470}/I_{390}	425 nm	485 nm	I_{485}/I_{425}
Control (n=10)	3.7±0.2	21.0±1.1	5.7±0.3	56±3	112±6	2.0±0.1
Gastroenterocolitis (n=10)	4.0±0.2	14.1±0.7	3.5±0.2	54±3	114±6	2.1±0.1

*arbitrary units

At the same time, no significant changes in the fluorescence parameters of probe 2 were observed in the case of the enterocyte membranes of rats with

carrageenan-induced gastroenterocolitis in condition of activation of apoptotic processes (Table 2, Figure 1).

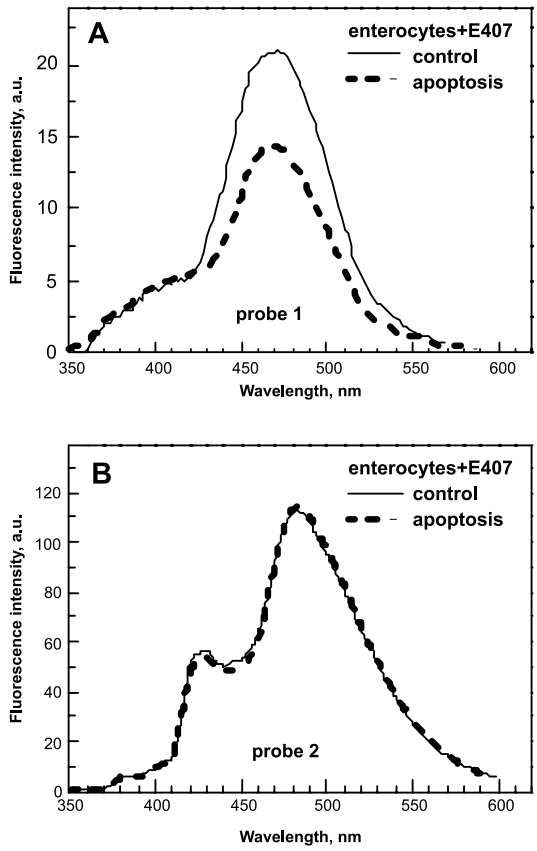

Figure 1. Fluorescence spectrum of probes 1 (panel A) and 2 (panel B) were recorded for: (I) the probes bound to enterocyte membranes from the control group of rats (solid line); (II) the probes bound to enterocyte membranes of animals with carrageenan-induced gastroenterocolitis in condition of activation of apoptotic processes (dashed line)

DISCUSSION

It is well-known that during apoptotic processes some biochemical parameters are changed (19). Biochemical parameters whose changes were observed after carrageenan intake in the present study were ASK-1, PARP and DNA fragmentation in small intestinal homogenates and MMP-2, caspase-3 in blood serum (8-10). Our results are consistent with numerous researches that confirm the activation of apoptosis in inflammatory intestinal diseases, e.g. inflammatory bowel disease (20). In particular, there is strong evidence that inflammatory bowel disease is associated with the activation of apoptosis in the intestinal mucosa of patients (21).

Probes 1 and 2 are capable of isomerization in the excited state (Figure 2), forming normal (N*) and phototautomer (T*) species (12-16). This reaction is known as an excited state intramolecular proton transfer (ESIPT) (15,16).

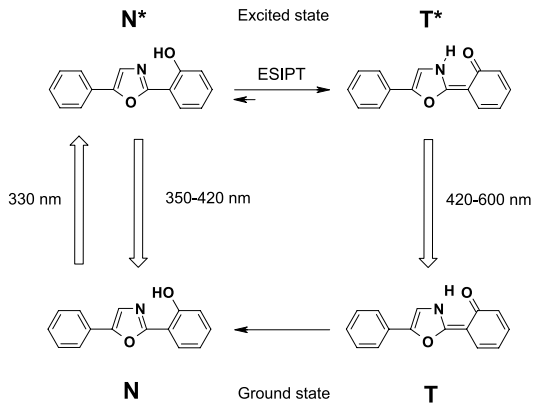

Figure 2. Scheme of excited state intramolecular proton transfer (ESIPT) in 2-(2′-hydroxyphenyl) -5-phenyl-1,3-oxazole (probe 1). The upwards arrow shows the electronic excitation and the downwards arrow represents the emission of light (fluorescence). Corresponding maximum of absorption and the ranges of emission are measured in nanometres

The presence of dual fluorescence allows us to perform ratiometric measurements, i.e. to use the ratio of fluorescence intensities of the phototautomer form (I_{T^*}) and of the normal form (I_{N^*}) as a parameter to investigate the physicochemical properties of the microenvironment (12-14).

Usage of ratiometric fluorescent probes eliminates not only the measurement error caused by the deviation of the fluorescent probe concentration (e.g., uneven content of fluorescent probe in various membranes), but also the measurement errors due to deviation in configuration and adjustment of equipment for measurements of fluorescence (e.g. deviation in the intensity of the source of excitation light, changes in focusing, changes in the sensitivity of the photodetector, etc.) (17).

The fluorescent probes (i.e. probes 1 and 2), which differ in lipophilicity (12-14), were chosen for the present study. It is expected that the regions of location of the selected probes in the membrane are different and correspond to lipophilicity of the probes (Figure 3). The expected location and orientation of probes 1 and 2 are based on their fluorescence properties in lipid membranes (12-14) and on their structural similarity to the fluorescent probes with known location in lipid membranes (17): probe 1 located in the area of glycerol residues of the phospholipids (closer to the centre of the lipid bilayer), in the area of carbonyl groups of the phospholipids and in the area of fatty acid chains of the phospho-

lipids (closer to the carbonyl groups); probe 2 - in the area of fatty acid chains of the phospholipids (near the centre of the bilayer) and in the centre of membrane lipid bilayer (Figure 3). According to some authors (18,19,22), apoptosis is accompanied by the following changes in the cell membrane: (a) lost of lipid asymmetry (18,19). Charged lipids (phosphatidylserine (PS), phosphatidylethanolamine (PE)) appear in the outer layer of the membrane (18,19); (b) the negative charge on the external lipid layer of the membrane is increased (18,19), the lipid ordering is reduced (18,19), the activation of the oxidation of lipids is observed (22). All such changes should lead to an increase in polarity and to increase in hydration of the membrane (23).

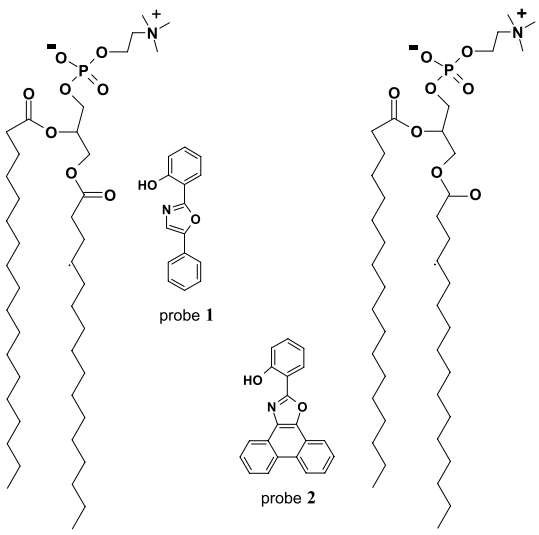

Figure 3. Expected location and orientation of fluorescent probes 1 and 2. Two molecules of phosphatidylcholine from the outer monolayer are shown to denote the location of the probes

Based on the properties of the probes 1 and 2 (12-16), one can expect that the growth in polarity and in hydration of the membrane will result in: an increase in the relative fluorescence intensity of the normal form N*; a decrease of the relative fluorescence intensity of phototautomer form T*; a decrease of the ratio of fluorescence intensities of the phototautomer and normal forms (I_{T*}/ I_{N*}).

As it was expected, the decrease in fluorescence intensity of the band of phototautomer form (I_{T*}) and the increase in the ratio I_{T*}/I_{N*} were observed for probe 1 in case of the disease. The mentioned changes in the fluorescent parameters of probe 1 indicate an increase in polarity and proton-donor ability of the microenvironment of the probe (14) in enterocyte membranes of rats with carrageenan-induced gastroenterocolitis during activation of apoptotic processes. Such an increase in polarity and proton-donor ability of the microenvironment of probe 1 indicates an increase in hydration of the probe microenvironment (14) in the enterocyte membranes of the experimental group of animals. The observed increase in hydration of the area of location of probe 1, (i.e. the polar regions of the membrane) indicates that apoptosis is activated (18) in enterocytes during the course of chronic carrageenan-induced gastroenterocolitis.

The absence of changes in the area where probe 2 is located (i.e. in more hydrophobic regions of the lipid bilayer), is explained by the fact that, apparently, no significant changes in hydration occur in hydrophobic regions of the lipid bilayer during the activation of apoptosis of enterocytes.

Thus, in condition of activation of apoptotic processes in the course of chronic carrageenan-induced gastroenterocolitis, an increase in hydration of rat enterocyte membrane is observed in the area where probe 1 is located, i.e. in rather polar regions of the membrane - presumably in the area of glycerol residues of phospholipids (closer to the centre of the lipid bilayer), and in the area of the carbonyl groups of phospholipids and fatty acid chains of the phospholipids (closer to the area of the carbonyl groups). The observed changes in the hydration of the polar regions of the membrane are attributed to the activation of apoptosis of enterocytes in the course of chronic gastroenterocolitis. At the same time, in condition of activation of apoptotic processes, no changes are noticed in the area, where probe 2 is located, i.e. in more hydrophobic regions of enterocyte membrane: presumably in the area of fatty acid chains of the phospholipids (near the center of the bilayer) and in the centre of membrane lipid bilayer.

In conclusion, an increase in hydration of the polar regions of enterocyte membranes occurs in rats with chronic carrageenan-induced gastroenterocolitis. The increase in hydration has been attributed to the activation of the apoptosis of enterocytes. A fluorescent probe (2-(2′-hydroxyphenyl)-5-phenyl-1,3-oxazole) could be used for the detection of apoptosis of enterocytes.

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TRANSPARENCY DECLARATION

Conflicts of interest**:** none to declare

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