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Research Article

HSP90α overexpression in small intestinal mucosa and high blood serum levels of HSP70 and 8-isoprostane in carrageenan-induced intestinal inflammation

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Abstract. Aims. The aim of our research was to evaluate HSP90α expression in small intestinal tissue, levels of HSP70 and 8-isoprostane in blood serum of rats with chronic carrageenan-induced enteritis. Methods. We determined immunohistochemically HSP90α expression in small intestine of ten rats with intestinal inflammation caused by the four-month-long oral administration of k-carrageenan-containing 1% processed Eucheuma seaweed solution and compared it with the small intestinal HSP90α expression in ten healthy animals. Levels of 8-isoprostane and HSP70 were determined in blood serum of animals from both groups using commercially available ELISA kits. Results. HSP90α overexpression was observed in small intestine of rats with carrageenan-induced enteritis. The disease was also associated with the elevated circulating levels of 8-isoprostane, indicating the development of oxidative stress. The level of HSP70 in blood serum was also found to be higher in animals with carrageenan-induced intestinal inflammation compared with control rats. Conclusion. Our findings suggest that HSP90α and HSP70 play an important role in the pathogenesis of chronic carrageenan-induced enteritis.

Key Words:food additive; heat shock proteins; 8-isoprostane; intestinal inflammation; oxidative stress

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Introduction

Carrageenans (CGNs) are galactans of marine origin widely used in food industry as gelling agents, thickeners, emulsifiers, and stabilizers (David et al 2015; Necas & Bartosikova 2013). They are high molecular weight highly sulfated polysaccharides that structurally resemble glycosaminoglycans and are composed of repeating galactose derivatives linked with β-1,4 and α-1,3-glycosidic bonds (Bhattacharyya et al 2013). Commercial processing of seaweeds results in the formation of six major types of CGNs: θ, ι, κ, λ, μ, and ν (David et al 2015).

The topic of CGN safety has been under debate for several decades since the appearance of the first reports concerning its toxic effects demonstrated in in vivo animal experiments (Bhattacharyya et al 2013). There is strong evidence that CGNs are able to induce intestinal inflammation as a result of their oral consumption. Numerous in vivo experiments have demonstrated the dose-dependent ability of CGNs to cause both small and large intestinal inflammation and ulcerations in various laboratory animals, including rats, mice, guinea pigs, rabbits, etc (Martino 2017; Necas & Bartosikova 2013; Tobacman 2001). It has been shown that carrageenan-induced intestinal inflammation is accompanied by oxidative stress, overproduction of pro-inflammatory cytokines, structural changes in cell membranes of enterocytes and their apoptosis (Tkachenko et al 2018; Wei et al 2016; Gubina-Vakyulyk et al 2015).

In vitro experiments using human epithelial cells also confirm CGN toxicity. According to Bhattacharyya S et al, CGNs stimulate cell death and inhibit cell proliferation of human intestinal epithelial cells in an in vitro experiment even at low doses (Bhattacharyya et al 2008). Choi HJ et al have demonstrated that exposure of intestinal epithelial cells to CGNs leads to the upregulation of NF-kappaB and early growth response gene 1 (Choi et al 2012). Borthakur A et al have proven that exposure to CGNs stimulates pro-inflammatory IL-8 synthesis in intestinal epithelial cells (Borthakur et al 2007). Thus, adverse effects of CGNs have been revealed both in vitro and in vivo studies. However, despite the strong body of evidence concerning adverse effects of CGNs, this food additive is allowed to be consumed with no concern even by infants in accordance with a WHO Joint Expert Committee on Food Additives (JECFA) 2015 report.

Despite some reports aiming at elucidating the mechanisms by which CGNs trigger inflammation, pathogenesis of carrageenan-induced intestinal inflammation is still poorly understood (Bhattacharyya et al 2008; Borthakur et al 2007). In particular, the role of heat shock proteins (HSPs) that act as chaperones and protein refolding performed by them in the development of carrageenan-induced intestinal inflammation has not been

clarified. It has been known that inflammation is associated with oxidative stress conditions. Oxidative stress activates mechanisms involved in protein quality control. This is related to the fact that almost 70% of molecules damaged inside the cells under oxidative stress are of protein nature (Dahl et al 2015). Heat shock protein 90 (HSP90) and heat shock protein 70 (HSP70) responsible for refolding of oxidatively modified proteins are important regulators of protein quality, which makes them a promising object for studying in inflammatory diseases.

The aim of our study was to evaluate HSP90α expression in small intestinal tissue, 8-isoprostane and HSP70 concentrations in blood serum of rats with chronic carrageenan-induced enteritis.

Materials and methods

1. Description of animals, groups, and disease modeling

Twenty female adult WAG rats were provided by Kharkiv National Medical University. They were randomly divided into two groups. Each group included ten animals. Rats from the experimental group consumed k-carrageenan-containing 1% processed Eucheuma seaweed (PES) solution daily orally during 4 months. In addition to carrageenan, PES contains up to 15% of algal cellulose (Cohen & Ito 2002). PES consumption by animals resulted in the development of enteritis confirmed morphologically. Control group included intact healthy rats received drinking water.

2. Immunohistochemical study

HSP90α expression was assessed immunohistochemically using samples of small intestine collected from animals of both groups. After fixation in a 10% formalin solution, samples were embedded in paraffin, and 4 μm-thick sections were obtained. Anti-HSP90α mouse monoclonal antibodies manufactured by "Thermo Fischer Scientific" (UK) were used to analyze HSP90α accumulation in small intestinal tissues. After incubation with the primary antibodies to HSP90α, microslides were treated with anti-(mouse IgG)–horseradish peroxidase conjugate. 3,3′-Diaminobenzidine (DAB) was used for visualization.

3. Determination of blood serum levels of HSP70 and 8-isoprostane

HSP70 high sensitivity ELISA kit produced by Enzo Life Sciences (USA) was used to determine blood serum HSP70 levels. Levels of 8-isoprostane in blood serum of animals were determined using commercially available ELISA kits produced by IBL-Hamburg GmbH (Hamburg, Germany). All procedures were performed in accordance with manufacturers' instructions. The Awareness Technology Stat Fax 303 Plus Microstrip Reader (USA) was used to determine the optical density of solutions. HSP70 concentrations were expressed in ng/ml, and 8-isoprostane levels were shown in ng/ml.

4. Bioethics

All experimental procedures were performed in accordance with the provisions of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes. Animals were killed under slight thiopental anesthesia.

5. Statistical analysis

Concentrations of HSP-70 and 8-isoprostane between two groups were compared using "GraphPad Prism 5" software using Mann-Whitney U test. P values below 0.05 were considered statistically significant. Numerical data are shown as median and interquartile range.

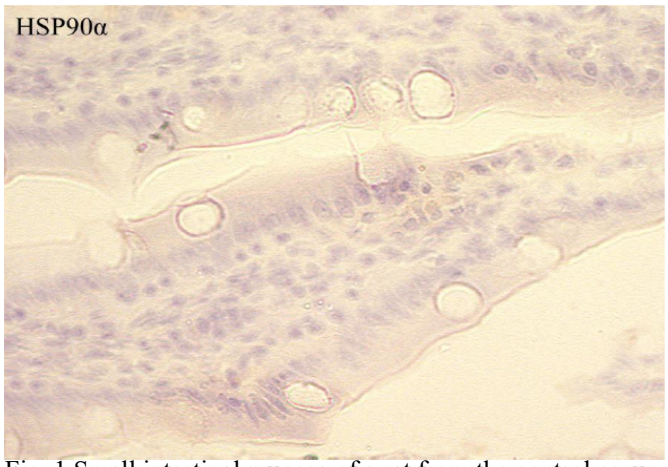

Fig. 1.Small intestinal mucosa of a rat from the control group. Epithelial layer is virtually intact. HSP90α expression is not observed. Immunohistochemical reaction with antibodies to HSP90α. x400.

Results

Examination of immunochemically stained samples of small intestine obtained from control animals allowed us to reveal that the epithelial lining of villi and glands was intact. Basement membrane was thin and not damaged. The lamina propria was insignificantly infiltrated with macrophages and lymphocytes. HSP90α labeling was virtually absent. Some villi had areas with slightly and moderately HSP90α-positive epithelial cells. Most glands lacked HSP90α-positive cells (Fig. 1).

The lamina propria of small intestinal mucosa in animals with carrageenan-induced inflammation was wider, edematous, considerably infiltrated with macrophages and lymphocytes. The number of fibrocytes was higher compared to the control group. Blood and lymphatic vessels were wider. Areas with destroyed and desquamated epithelium were found in villi. The basement membrane was not revealed in a number of places (Fig. 2 a-d).

Immunohistochemical study of HSP90α expression revealed that HSP90α-positive cells were found in small intestinal glands of rats with carrageenan-induced enteritis. Labeling intensity varied from slight and moderate to fairly strong. Similar changes in HSP90α expression were observed in the stroma. Strong HSP90α staining was revealed in the bulk of epithelial cells in the small intestinal villi of rats with enteritis (Fig. 2 a-d). Thus, HSP90α was widely overexpressed in inflammation-involved small intestinal tissues.

Analysis of the content of HSP70 in blood serum demonstrated the statistically significant ($p=0.0068$) elevation of its concentrations in rats with enteritis compared with the control group. HSP70 serum level was 2.7-fold higher than in the control group and reached 2.56 [1.39; 4.63] ng/ml, while in the control animals it was found to be 0.95 [0.50; 1.84] ng/ml (Fig. 3).

To assess the rate of oxidative stress, we determined the concentration of the widely recognized oxidative stress and lipid peroxidation marker, namely 8-isoprostane. Its blood serum concentration was revealed to be statistically significantly ($p=0.0002$) 8.6-fold higher in rats with enteritis than in the control group reaching 11.46 [10.03; 12.10] ng/ml against 1.33 [1.25; 1.57] ng/ml (Fig. 4).

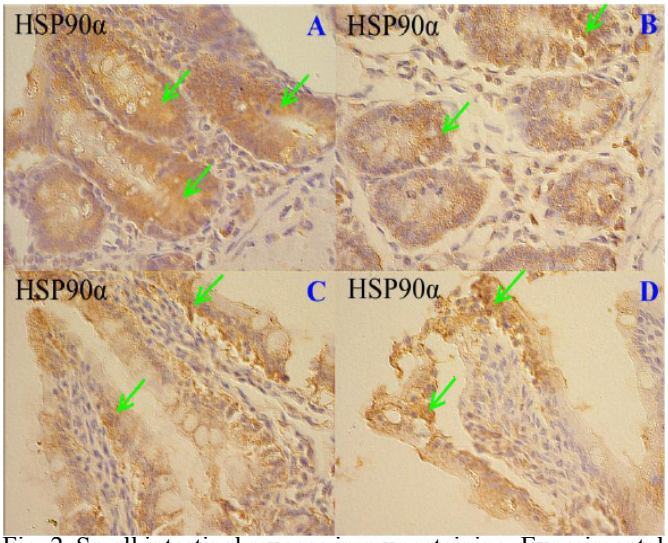

Fig. 2. Small intestinal mucosa immunostaining. Experimental group. A) HSP90α is overexpressed in intestinal glands (marked with green arrows). Immunohistochemical reaction with antibodies to HSP90α. x100. B) HSP90α-labeled cells are observed in glands (marked with green arrows). Numerous macrophages and lymphocytes are found in the edematous lamina propria. Immunohistochemical reaction with antibodies to HSP90α. x100. C). HSP90α is revealed in the epithelial cells of intestinal villi (marked with green arrows). Immunohistochemical reaction with antibodies to HSP90α. x100. D) HSP90α is strongly expressed in intestinal epithelium (marked with green arrows). Immunohistochemical reaction with antibodies to HSP90α. x100.

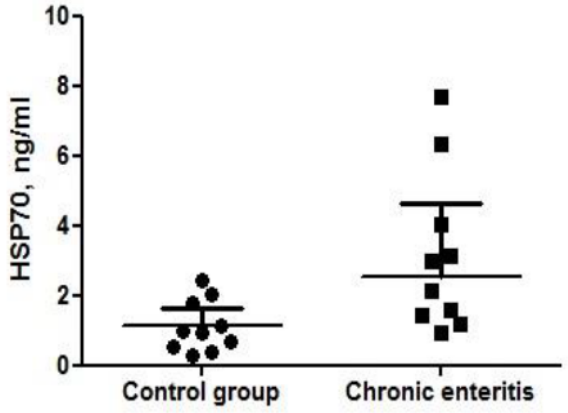

Fig. 3. The content of HSP70 in blood serum of rats with enteritis caused by the consumption of κ-carrageenan-containing 1% processed Eucheuma seaweed solution was determined. Serum levels of HSP70 were found to be statistically significantly (p=0.0068) elevated in carrageenan-induced enteritis compared with the control group.

Discussion.

HSPs are known to be highly conserved intracellular proteins involved in protein folding and maintenance of intracellular proteostasis acting as chaperones (Sevin et al 2015). They can be subdivided into two families depending on their molecular weight: low molecular weight and high molecular weight HSPs. HSP70 and HSP90 are representatives of high molecular weight chaperones, which are quite abundant inside the cells. In particular, HSP90 accounts for approximately 1% of the entire

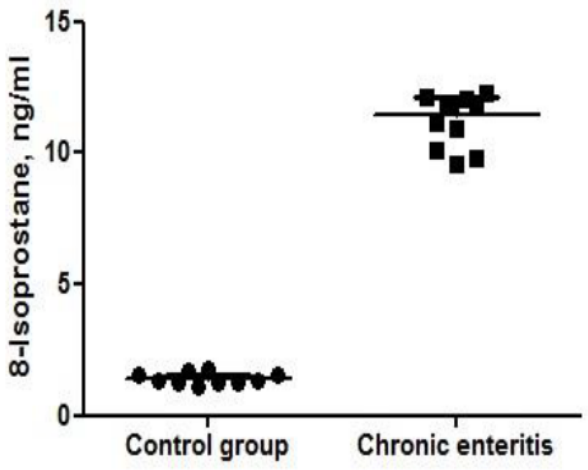

Fig. 4. Levels of 8-isoprostane in blood serum of rats with carrageenan-induced enteritis were evaluated. A statistically significant (p=0.0002) 8.6-fold increase in circulating 8-isoprostane levels in rats with carrageenan-induced enteritis was detected compared with control animals, indicating the development of oxidative stress.

pool of intracellular proteins (Lackie et al 2017). Chaperones are expressed either constitutively or in response to various stress triggers, including oxidative stress, high and low temperature, inflammation, etc (Voth & Jakob 2017). It has been reported that the HSP70/HSP90 machinery is involved in inflammation. There is strong evidence that HSP70 and HSP90 are overexpressed in inflamed tissues, including intestine (Tukaj & Węgrzyn 2017). Collins CB et al have demonstrated that experimental murine colitis is associated with the upregulation of HSP90 (Collins et al 2013).

In this study, we have revealed that chronic carrageenan-induced enteritis is accompanied by overexpression of HSP90 alpha isoform in intestinal tissue and elevated concentrations of HSP70 in blood serum. We believe that the source of circulating HSP70 is the inflamed intestine. HSP70 can be either directly released into the bloodstream from necrotic cells or secreted via a special pathway (Jenei et al 2013). Since under normal conditions HSP90α is not observed at the mucosal surface and our HSP90α immunostaining data show that in carrageenan-induced inflammation HSP90α is exactly expressed on the surface of villi, we believe that its upregulation has a compensatory character as a response to epithelial injury. Development of oxidative stress in the animals from the experimental group, evidenced by elevation of 8-isoprostane in blood serum, may contribute to the elevation of HSP70 blood serum levels and HSP90α upregulation in the small intestine, since reactive oxygen species (ROS) are able to induce synthesis of chaperones (Ikwegbue et al 2017). Thus, oxidative environment observed in carrageenan-induced enteritis results in protein oxidative modification and accumulation of misfolded proteins with the subsequent upregulation of HSP90α and HSP70. In addition, it is worth mentioning that an alpha isoform of HSP90 is inducible unlike the beta one (Beck et al 2012), which has substantiated our choice to study the expression of alpha isoform. Activation of HSP70/HSP90-based chaperone machinery in rats with carrageenan-induced intestinal inflammation may be aimed at refolding ROS-damaged proteins accumulated as a result of oxidative stress. Moreover, chaperones investigated in this study

are demonstrated to play an immunological role (Binder 2014). Thus, the release of intracellular HSP70, evidenced by the elevation of circulating HSP70 concentrations, contributes to the regulation of immune response in carrageenan-induced intestinal inflammation. There is some evidence that extracellular chaperones may provoke autoimmune processes contributing to the exacerbation of inflammation (Binder 2014). Therefore, despite cytoprotective, anti-apoptotic and anti-inflammatory effects of intracellular HSP70, its release from necrotic cells may mediate and exacerbate carrageenan-induced intestinal inflammation. We believe that HSP90α and HSP70 are supposed to be a double-edged sword in the development and progression of carrageenan-induced enteritis.

Conclusions

In this study, HSP90α overexpression in the small intestine against the background of elevated blood serum concentrations of HSP70 and 8-isoprostane was found in animals with carrageenan-induced intestinal inflammation. Our findings suggest that HSP90α and HSP70 play an important role in the pathogenesis of chronic carrageenan-induced enteritis, reinforce the link between carrageenan oral consumption and intestinal inflammation and question the safety of this food additive.

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