Life Sciences 141 (2015) 179–187

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Experimental evidence of heparanase, Hsp70 and NF-κB gene expression on the response of anti-inflammatory drugs in TNBS-induced colonic inflammation

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ARTICLE INFO

Article history:
Received 18 June 2015
Received in revised form 25 September 2015
Accepted 30 September 2015
Available online 03 October 2015

Keywords:
Intestinal inflammation
Heparanase
Hsp70
NF-κB
Inflammatory bowel disease
Intestinal anti-inflammatory drugs

ABSTRACT

Aim: Etiopathogenesis of inflammatory bowel disease is unclear and results from a complex interplay of genetic, microbial, environmental and immune factors. Elucidating the mechanisms that drive IBD depends on the detailed characterization of human inflammatory mediators in animal models. Therefore, we studied how intestinal inflammation affects heparanase, NF-κB and Hsp70 gene expression in rats, and if current intestinal anti-inflammatory drugs (sulphasalazine, prednisolone and azathioprine) act on these expressions. Moreover, we investigated the relationships among these genes with colonic cytokines levels (IL-1β, TNF-α, IL-6, INF-γ and IL-10) and oxidative stress that have fundamental role in IBD.
Material and methods: Macroscopic parameters (diarrhea, extension of lesion, colonic weight/length ratio and damage score), biochemical markers (myeloperoxidase and alkaline phosphatase activities, and glutathione, IL-1β, TNF-α, IL-6, INF-γ and IL-10 levels), gene expressions (heparanase, NF-κB and Hsp70), and microscopic evaluations (optic, electronic scanning and transmission microscopic) were performed in rats.
Key findings: Expression of heparanase, Hsp70 and NF-κB and oxidative stress were increased by inflammatory process and differentially modulated by sulphasalazine, prednisolone and azathioprine treatments. Protective effects of drugs were also related to differential modulation of cytokine changes induced by inflammatory process, showing different mechanisms to control inflammation.
Significance: Heparanase, NF-κB and Hsp70 gene expression participate in the inflammatory response induced by TNBS and represent pharmacological targets of the intestinal anti-inflammatory drugs. In addition, current drugs used to treat IBD (sulphasalazine, prednisolone and azathioprine) differentially modulate heparanase, NF-κB and Hsp70 gene expression, cytokine production and oxidative stress.

1. Introduction

Inflammatory bowel disease (IBD) is principally categorized as either ulcerative colitis or Crohn's disease and is a chronic inflammation in the bowel. The etiopathogenesis of IBD is unclear, but is currently thought as result from an interaction of genetic, microbial, environmental and immune factors with an exaggerated inflammatory response to intestinal luminal contents, triggered by defective intestinal barrier function [1]. Currently, the mechanisms that regulate intestinal barrier integrity and the pathologic alterations during IBD development include multiple genes involved in microbe recognition, lymphocyte activation, cytokine signaling and intestinal epithelial defense mechanisms [2]. Current first-line treatment for IBD includes 5-aminosalycilates, glucocorticoids, immunosuppressants and biological therapeutics [3,4], which generally targeting events downstream of the inflammatory cascade [4,5]. However, none of them is disease specific and there is currently no cure for IBD [6,7]. Elucidating the mechanisms that drive IBD depends on the detailed characterization of human inflammatory mediators in animal models of intestinal inflammation, and the preclinical evaluation of inflammatory markers is a relevant approach to obtain new insights into this complex intestinal disease. Recently, in an elegant review about experimental models of intestinal inflammatory diseases, Lin and Hackam recorded that rodent models for the study of IBD mimic the human disease because rodent intestinal epithelium has the same cell types of humans [8]. In previous study, we demonstrated that intestinal inflammation induced by TNBS affects Mapk1, Mapk3, Mapk6 and Mapk9 gene expression in rats, while current intestinal anti-inflammatory drugs (sulphasalazine, prednisolone and azathioprine) counteracted these changes, showing that these expressions were involved in both pathogenesis of intestinal inflammation and in the intestinal anti-inflammatory response produced by these drugs

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http://dx.doi.org/10.1016/j.lfs.2015.09.023
0024-3205/

[9]. Recently, several studies have demonstrated that gene expressions of heparanase, nuclear factor kappa B (NF-κB) and heat shock protein 70 (Hsp70) are important for intestinal barrier structure and function, which are compromised in human IBD [10–14]. However, no systematic experimental studies have been performed to identify how the expression of these genes participates on the intestinal inflammatory process and affects the pharmacological response promoted by current used drugs to treat IBD. Accordingly, we studied if heparanase, NF-κB and Hsp70 gene expression participates on the intestinal inflammation in the TNBS-model of rat colitis and, to corroborate our findings, we evaluated the effects of the current drugs used to treat human IBD (sulphasalazine, prednisolone and azathioprine) on these genes and studied the relationships among these genes with cytokines and oxidative stress that have key role in maintaining cell integrity and function of gut. Our study might be useful in the understanding of the etiopathogenesis aspects of mucosal damage induced by TNBS, in providing new insights into the mode of action of intestinal anti-inflammatory drugs and in identifying potential targets as pharmacological tools for the development of novel drugs to treat human IBD.

2. Material and methods

2.1. Reagents

All chemicals, prednisolone, sulphasalazine and azathioprine were provided by Sigma (Germany), dissolved in methylcellulose (1% *w*/*v*) and prepared fresh daily for administration to the animals.

2.2. Animals

Male Wistar rats (weighing 180–200 g) obtained from the Central Animal House of the São Paulo State University (UNESP), Botucatu, São Paulo (Brazil), were housed in standard environmental conditions (21 °C, 60–70% humidity) with 12 h light/dark cycles and air filtration. Animals had free access to water and food (Biobase). Experimental protocol was approved by the Commission of Ethics in Animal Experimentation (Protocol number 042/04-CEEA), Institute of Biosciences, São Paulo State University.

2.3. Induction of colitis and assessment of the inflammatory process

Colitis was induced using the method originally described by Morris et al. [15]. Briefly, animals were fasted overnight and then anesthetized with halothane. Under anesthesia, they were administered 10 mg of trinitrobenzenesulphonic acid (TNBS) dissolved in 0.25 ml 50% ethanol (*v*/*v*) by means of a teflon cannula inserted 8 cm into the anus. During and after TNBS administration, the rats remained in a head-down position until they recovered from the anesthesia. Rats from the non-colitic (normal) group received 0.25 ml of saline instead of TNBS. Rats received 2 mg/kg prednisolone, 50 mg/kg sulphasalazine or 2 mg/kg azathioprine orally at 96, 72, 48, 24 and 2 h before colitis induction. The drugs were administered by means of an esophageal catheter (volume: 10 ml/kg). Two additional groups were included for reference: a non-colitic group that received saline intracolonically and the oral vehicle, and a colitic group that received TNBS and vehicle (10 ml/kg methylcellulose) orally. The animal body weights, the occurrence of diarrhea and the total food intakes for each group were recorded daily. Animals from all groups ($n = 8$) were killed 48 h after colitis induction with an overdose of halothane. The colonic segments were obtained after laparotomy and the eventual occurrence of adhesions between the colon and adjacent organs was noted. They were placed on an ice-cold plate, cleaned of fat and mesentery, and blotted on filter paper, and the colon was weighed and its length measured under a constant load (2 g). The colon was opened longitudinally and scored for macroscopically visible damage on a 0–10 scale by two observers unaware of the treatment, according to the criteria previously described by Bell et al. [16]. The colon was subsequently divided longitudinally into different pieces to be used for the biochemical determinations and gene expression analysis.

2.4. Biochemical assays in colonic specimens

MPO activity was measured according to the technique previously described by Krawisz et al. [17]. Total GSH content was quantified with the recycling assay [18]. ALP activity was measured spectrophotometrically using methods described by Bessey et al. [19] and Smith et al. [20].

Colonic samples for the determinations of TNF-α, IL-1β, IL-6, IL-10 and INF-γ were weighed, homogenized, minced on an ice-cold plate and resuspended in a centrifugation tube containing 10 mM/L phosphate buffered saline pH 7.4 (1:5 *w*/*v*). The tubes were placed in a shaker submerged in a 37 °C water bath for 20 min and then centrifuged at 9000 ×*g* for 5 min at 4 °C. The supernatants were frozen at −80 °C until assayed. The cytokine levels were quantified by a DuoSet ELISA Kit to measure the concentration of the natural and recombinant rat enzyme according to the manufacturer's instructions (R&D Systems, Inc., Minneapolis, Minnesota, USA).

2.5. Gene expression analysis

Colon samples (100 mg) were collected in TRIzol (Invitrogen Life Technologies), homogenized with a Polytron, and submitted to total RNA extraction and reverse transcription with SuperScript III (200 U/ml; Invitrogen) according to the manufacturer's protocol. Primers for targets and housekeeping genes were designed based on the rat sequences. Relative real-time RT-PCR analysis was performed with an ABI 7500 using Power SYBR Green PCR Master Mix (Applied Biosystems, Sao Paulo, Brazil) for all the genes. Amplification efficiencies for target and housekeeping genes were similar. The primer sequences, fragment size, annealing temperature and primer concentration for each gene are shown in Table 1. Reactions were optimized to provide maximum amplification efficiency for each gene. PCR was performed in 25 μl reaction volumes in duplicate and the specificity of each PCR product was determined by melting curve analysis. Negative controls (water replacing cDNA) were run in every plate. The relative expression of each target gene was calculated using the DDCt method with efficiency correction [21,22]. To select the most stable housekeeping gene for detailed analyses, GAPDH (glyceraldehyde-3-phosphate dehydrogenase), β-actin and HPRT amplification profiles were compared using the geNorm applet for Microsoft Excel (medgen.ugent.be/geNorm). All gene expression analysis was performed with GAPDH as the housekeeping gene for colon tissue.

2.6. Optic, scanning and transmission electron microscopy analysis

Representative whole gut specimens were taken from a region 2 cm above the inflamed region of colon and were fixed in 4% buffered formaldehyde. Cross-sections were selected and embedded in paraffin. Equivalent colonic segments were also obtained from the non-colitic group. Full-thickness sections of 6 μm were obtained and stained with hematoxylin and eosin. After staining, images were subjected to analysis and photomicrography with a Leica microscope utilizing Leica Qwin Plus version 3.3 e 3.40.

For morphological analysis by scanning and transmission electron microscopy (SEM), colon samples from healthy, control and treated animals were fixed for 24 h in 2.5% glutaraldehyde with a 0.1 M phosphate buffer, pH 7.3. In addition, colon samples were post-fixed with 1% osmium tetroxide for 2 h, dehydrated in a graded alcohol series, critical point dried, coated with gold and examined under a Fei-Quanta 200 scanning electron microscope (Phillips, Czechoslovakia).