Research in Veterinary Science 96 (2014) 464–471

Contents lists available at ScienceDirect

Research in Veterinary Science

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

Proteinase-activated receptor 2 expression in the intestinal tract of the horse

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ARTICLE INFO

Article history:
Received 20 November 2013
Accepted 1 March 2014

Keywords:
Horse
Proteinase-activated receptor 2
Jejunum
Ileum
Pelvic flexure

ABSTRACT

Proteinase-activated receptor 2 (PAR_2) is a G-protein-coupled receptor for trypsin and mast cell tryptase; it is highly expressed at the intestinal level with multiple functions, such as epithelial permeability and intestinal motility. Many proteases activate PAR_2 during tissue damage, suggesting a role of the inflammatory response receptors. The aim of the study was to evaluate the distribution and expression of PAR_2 in the jejunum, the ileum and the pelvic flexure, using samples collected from healthy adult horses after slaughter. Proteinase-activated receptor 2 immunoreactivity (PAR_2-IR) was observed in the enterocytes, intestinal glands, the smooth muscle of the *muscularis mucosae*, and the longitudinal and circular muscle layers; there were no differences in the distribution of PAR_2-IR in the different sections of the intestinal tract. The protein expression level showed that the relative amount of the PAR_2 content in the mucosa of the intestinal tract decreased from the small to the large intestine while the PAR_2 mRNA analysed showed similar values. This study provides relevant findings concerning the distribution of the PAR_2 in the intestines of healthy horses and represents the starting point for evaluating the role of the PAR_2 during strangulative intestinal disease and consequent systemic intestinal reperfusion/injury complications in horses in order to identify and employ antagonist PAR_2 molecules.

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1. Introduction

Proteinase-activated receptors (PARs) constitute a family of G protein-coupled receptors (GPCRs) at the cell surface. The activation of PARs by proteinases, especially serine proteases, is characterised by irreversible proteolytic cleavage of the N-terminal domain of the receptor after which the receptor cannot be reactivated by proteolysis. After proteolytic activation, the receptor is internalised by a clathrin-mediated mechanism and is then targeted to lysosomes (Bohm et al., 1996a). Four members of this receptor family (PAR_1, PAR_2, PAR_3, PAR_4) have previously been described (Ossovskaya and Bunnett, 2004). Proteinase-activated receptor 2 (PAR_2), the GPCR for trypsin and mast cell tryptase, is highly expressed in endothelial cells, colonic myocytes, enterocytes, enteric neurons, terminal mesenteric sensory nerves and immune cells at the intestinal level (Cenac et al., 2002; Ossovskaya and Bunnett, 2004; Vergnolle, 2000; Vergnolle et al., 2001). Tryptase, the most abundant protease of the mast cells, has been shown to activate PAR_2 on epithelial as well as endothelial cells and neurons (Cenac et al., 2002; Ossovskaya and Bunnett, 2004; Vergnolle, 2000; Vergnolle et al., 2001), suggesting a role of this receptor when the mast cells are involved, e.g., during inflammation, hypersensitivity reaction and pain (Ossovskaya and Bunnett, 2004).

In recent years, an important inflammatory and/or anti-inflammatory role of PAR_2 has been hypothesised in mice (Hyun et al., 2008) and rodents (Cattaruzza et al., 2006; Cenac et al., 2002; Gobbetti et al., 2012) during intestinal ischemia–reperfusion injury (IRI).

In the mouse model, subjected to experimentally induced inflammatory intestinal damage, luminal proteinase-activated PAR_2 increased the paracellular permeability of the colon and induced bacterial translocation into the peritoneal organs (Cattaruzza et al., 2006; Cenac et al., 2002). Loss of mucosal integrity is implicated in the pathogenesis of organ dysfunction syndrome. Moreover, the alterations of gastrointestinal motility, which can be partly due to structural changes and neuronal plasticity occurring within the enteric nervous system (ENS), may also contribute to the development of bacterial overgrowth with subsequent bacterial translocation and endotoxemia (Cattaruzza et al., 2006; Cenac et al., 2002).

In equine medicine, the signs of colic are frequently present and may be associated with severe gastrointestinal diseases (Ihler et al., 2004). Colic and consequent endotoxemia are considered to

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http://dx.doi.org/10.1016/j.rvsc.2014.03.006
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be the primary causes of death in horses (Sutton et al., 2009). Endotoxemia and the serial events of the reduction of tissue perfusion, inflammation, disseminated intravascular coagulation and multiple organ dysfunction may produce a fatal outcome (Cesarini et al., 2010; Graham et al., 2011; Groover et al., 2006). Moreover, intestinal ischemia and reperfusion induce acute inflammatory response; in horses, they are associated with the enhanced generation and release of proteinases from mast cells and neutrophils (Grosche et al., 2011a, 2011b; Moore et al., 1995; Wong et al., 2012), and coagulation cascade activation (Monreal and Cesarini, 2009) in addition to the digestive and bacterial proteinases normally present in the lumen.

In the gastrointestinal tract (GIT) of horses, PAR_2 distribution and expression have not yet been described either under physiological or under pathophysiological conditions. Thus, in order to further expand knowledge regarding the role of PAR_2 in the equine model, the aim of the present study was to evaluate the distribution and expression of PAR_2 in the small (jejunum and ileum) and large (pelvic flexure) intestines, using samples collected from healthy adult horses after slaughter at an abattoir. Thus, immunofluorescence and gene and protein expression studies were carried out to evaluate PAR_2 abundance in the different sections of the GIT.

2. Materials and methods

2.1. Animals

The study was conducted from October 2012 to April 2013. A total of 30 adult horses sent to the slaughterhouse were evaluated. The subjects were selected and included in the study based on history, and physical and clinicopathological examination. No clinical or clinicopathological abnormalities were present at admission. Moreover, the inclusion criteria for this study were the absence of pathological lesions estimated by means of gross and histological evaluation of the jejunum, the ileum and the pelvic flexure.

The care and handling of the animals were in accordance with the provisions of European Economic Community Council Directive 86/609, adopted by the Italian Government (D.L. 27/01/1992 no. 116).

2.2. Sample collection

The blood samples undergoing clinicopathological evaluation were collected at exsanguination at the time of jugular vein sticking. Tubes with K_3EDTA anticoagulant, citrate and clot activator were used. The blood samples were processed and analysed within one hour from collection or were stored at –80 °C. Samples with gross hemolysis were excluded from the analysis.

The samples of the intestines were taken after the evisceration stages of the slaughter process. In all horses, gross GIT internal and external examinations were performed to ensure the absence of macroscopic intestinal lesions. The stomach-gut package of each horse was collected in a plastic bag.

In a separate room within the slaughterhouse, a part of the jejunum, the ileum, and the pelvic flexure were dissected and, after several washings with phosphate buffered saline (PBS) (Gibco-Invitrogen, Paisley, UK), the intestinal mucosa was scraped using two glass slides; the samples were then frozen in liquid nitrogen and stored at –80 °C until RNA and protein extraction. Full thickness bioptic samples were also collected at slaughter from 3 gut segments. The tissue samples were rinsed in PBS and immediately placed in 10% neutral buffered formalin and 4% paraformaldehyde.

2.3. Clinicopathological evaluation

A complete blood count including blood smear examination was carried out using an automated haematology system (ADVIA 2120, Siemens Healthcare Diagnostics, Tarrytown NY, USA). A chemistry profile, including glucose, creatinine, urea, aspartate transaminase, creatine kinase, lactate dehydrogenase, uric acid, total bilirubin, bile acids, γ-glutamyltransferase, total proteins, albumin, albumin to globulin ratio, total calcium, phosphorus, sodium, potassium, chloride and fibrinogen was carried out using an automated chemical analyser (AU 400, Olympus/Beckman Coulter, Brea CA, USA).

2.4. Morphological examinations

2.4.1. Histology

From the samples stored in formalin, 5 μm paraffin-embedded sections were obtained and stained with haematoxylin and eosin to confirm the absence of histopathological lesions. Tissue viability was determined by evaluating the morphological features using the scoring system described for horses by Van Hoogmoed et al. (2000) on the basis of the grading of the following parameters: intestinal crypt ratio, percentage loss of luminal epithelium, percentage loss of glandular epithelium, haemorrhage and edema score. The samples of the jejunum and the ileum were also evaluated using an inflammation score based on the semiquantitative estimation of the quantity of inflammatory cells present in the mucosa and submucosa (Packer et al., 2005). The only cases included in the study were those in which all sections collected from the three intestinal tracts were within the previously reported ranges of normality (Packer et al., 2005; Van Hoogmoed et al., 2000).

2.4.2. Immunohistochemistry

The tissue was fixed for 48 hours in 4% paraformaldehyde in 0.1 M PBS, pH 7.4, at 4 °C. To obtain frozen tissue, small longitudinal portions (1 × 0.3 cm) of cranial intestine were washed in PBS and stored at 4 °C in PBS containing 30% sucrose and sodium azide (0.1%). On the following day, the tissues were placed in a mixture of PBS–30% sucrose azide and Optimal Cutting Temperature (OCT) compound (Sakura Finetek Europe, NL) at a ratio of 1:1 for an additional 24 hours before being embedded in 100% OCT in Cryomold® (Sakura Finetek, Zoeterwoude, NL, Europe). The sections were prepared by freezing tissues in isopentane cooled in liquid nitrogen. Serial longitudinal sections (14–16 μm thickness) of tissues were cut on a cryostat (Leica, Wetzlar, Germany) and mounted on polysine-coated slides. The sections were stored at –80 °C until the histological and/or immunohistochemical experiments were begun.

2.4.2.1. Single immunofluorescence experiments. The cryostat sections were rehydrated in PBS and were then processed for immunostaining. To block non-specific binding, the sections were incubated in a solution containing 10% normal goat serum (Colorado Serum Co., Denver, CO, #CS 0922) and 0.5% Triton X-100 (Merck, Darmstadt) in PBS for 1 h at room temperature (RT). Thereafter, the sections were incubated in primary antibody rabbit anti-PAR_2 (1:200, sc-5597, Santa Cruz Biotechnology, Santa Cruz, Dallas, TX, USA) diluted in antibody diluent (1.8% NaCl in 0.01 M sodium phosphate buffer containing 0.1% sodium azide) for 24 h at +4 °C. After washing in PBS (3 × 10 min), the sections were incubated in secondary antibody Alexa Fluor 594 goat anti-Rabbit (1:400, A-11037, Molecular Probes, Leiden, the Netherlands) diluted in PBS for 1 h at RT. After washing in PBS (3 × 10 min), the slides were coverslipped with buffered glycerol (pH 8.6). Ten percent normal

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