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Long-term downregulation of protease-activated receptor-2 expression in distal colon in rats following bacillary dysentery

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ABSTRACT

The aim of this study was to determine changes of PAR-2 expression in distal colon and the sensitivity of colonic muscle to SLIGRL-NH_2, the PAR-2-activating peptide (PAR-2-AP) following bacillary dysentery. *Shigella flexneri* was administrated intragastrically in healthy male rats to induce bacillary dysentery. The effect of SLIGRL-NH_2 on the motility of colonic muscle strips were examined. The expression of PAR-2 was determined by immunohistochemistry and Western blotting. Intragastric administration of *S. flexneri* induced acute inflammation at the mucosa of the distal colon at 4–11 days, and the inflammation disappeared 18 days later. PAR-2-AP-induced TTX insensitive relaxation of the colonic muscle strips. This inhibitory effect on colonic circular muscle strips was reduced on days 11–35, but the carbachol-induced contraction did not change. PAR-2 was located at the colon smooth muscles cells and myenteric nerve plexus. The amount of PAR-2 expression in distal colon was down regulated on days 11–35. These data indicated that bacillary dysentery exerted a long-term downregulation on the expression of PAR-2 in distal colon. This might be the reason of the low sensitivity of the colon circular muscle strips to the PAR-2-AP-induced relaxation following intragastric administration of *S. flexneri*.

1. Introduction

Protease-activated receptors (PARs) belong to a subfamily of seven transmembrane G-protein coupled receptors. Four members of this subfamily, including PAR-1, PAR-2, PAR-3 and PAR-4, have been cloned. They are proteolytically cleaved by proteases at specific site within the extracellular NH_2 terminus, unmasking a new amino terminus that serves as a 'tethered ligand' to binds to the conserved regions in the second extracellular loop of PARs, and resulting in the initiation of multiple signaling cascades [1]. A synthetic peptide, called activating peptides (AP), mimics the tethered ligand domain and directly activates intact PARs except for PAR-3 [2–4].

Among the members of PARs family, PAR-2 is activated by trypsin, mast cell tryptase, factor Xa, acrosin, gingipain and neuronal serine proteinases [5], is widespread in gastrointestinal tract [1]. After activated by endogenous or exogenous agonist, PAR-2 increased the secretion of Cl^- in cultured intestinal and pancreatic duct epithelial cells, increased the exocrine secretion of salivary, pancreatic, gastric mucosa [6–10]. It is widely recognized that PAR-2 also modulates the gastrointestinal motility, although the results reported were different because of the difference of the species and animal model used by different research groups [11–21].

As we know, digestive tract is exposed to a variety of endogenous and exogenous proteases, such as digestive enzymes, proteinases from pathogens or inflammatory cells. All these proteases are potential activators of PAR-2 and might modulate the function of gastrointestinal tract, especially during some pathological conditions, like intestinal infection and inflammation. So it is possible that PAR-2 is probably contributed to the disorder of gastrointestinal tract during these pathological states.

Although it is well known that the gastrointestinal motility is altered in animal models of colitis and in patients with inflammatory bowel disease (IBD) such as Crohn's disease and ulcerative colitis [22–25], the pathophysiological mechanisms underlying these disturbances remain unclear. Numerous reports have found that these disturbances had close relationship with inflammation. Bossone et al reported that both of the frequency and amplitude of colonic spontaneous contraction increased 4 h and 28 days following intrarectal administration of trinitrobenzenesulfonic acid (TNBS) in rat distal colon [26]. Contractility is variably changed in different region of gut, in contrast to the enhanced contractile responses found in small intestine in human Crohn's disease specimens, colon contractility appears to be impaired in the inflamed colon, moreover, decrease in transit is also been observed [27,28]. However, little is

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known about the pathogenesis of dysmotility in post infectious irritable bowel syndrome (PI-IBS) patients, even though approximately 1/10 of IBS cases describe a post infectious onset and bacillary dysentery is a causative factor in PI-IBS [29,30]. Although it is well known that PAR-2 is involved in the hypersensitivity of IBS patients [31], litter is know about its effect on the dysmotility following gastrointestinal inflammation. We hypothesized that expression of PAR-2 might be downregulated and that is one of the reasons of the dysmotility of the colon following inflammation. In order to test this hypothesis, the response of colonic smooth muscle to an agonist of PAR-2, PAR-2-activating peptide (PAR-2-AP) in rats following *Shigella flexneri* infection at different time was detected. The expression of PAR-2 is detected by Western blot. The location of PAR-2 on rat distal colon is examined by immunohistochemisty.

2. Materials and methods

2.1. Bacteria preparation

Clinical isolates of *S. flexneri* were obtained from Clinical Laboratory, Qi Lu hospital, Ji Nan, China. The strains were routinely grown in blood plate at 37 °C, overnight.

2.2. Intestinal infection and tissue preparation

All experimental procedures were approved by the Committee on Animal Research and Ethics of Shandong University School of Medicine. Healthy male Wistar rats (200–220 g, provided by Animal Center of Shandong University) were paired according to the weight and then divided into control and *S. flexneri*-treated (SF-treated) groups at random. After an overnight fast (water *ad libitum*), rats received intragastric administration of inocula of 10^8 colony forming units (CFU) in 1 ml of sterile normal saline. The rats in control group were treated with 1 ml sterile normal saline. Rats were raised individually in cage which was exposed to ultra-violet for 20 min for sterilization before and after the administration. Padding was treated with autoclaving before threw away. On days 4, 7, 11, 18, 25 and 35 following the *S. flexneri* treatment, rats were sacrificed by cervical dislocation and distal (1 cm from rectum, 2–3 cm length) colon was excised. The colonic tissue isolated from control or SF-treated rats was prepared for Myeloperoxidase (MPO) assay, Western blot or tension recording.

2.3. MPO assay and histological analysis

Myeloperoxidase (MPO) activity was measured as an index of inflammation [32]. The colonic tissue removed from the distal colon was homogenized, and the homogenate was used to measure MPO activity by using a kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). One MPO activity unit was defined as per gram tissue decomposes 1 μM H_2O_2 in reaction system at 37 °C.

Full-thickness paraffin-embedded sections (4 μm) of distal colon from control and SF-treated Wistar rat were stained with Hematoxylin and Eosin (H&E) for evaluation of the degree of inflammation.

2.4. Immunohistochemistry

Expression of PAR-2 was examined in rat distal colon tissue frozen sections (4 μm) by a two-step IHC detection reagent kit PV 6003 (ZSGB-BIO, Beijing, China). Briefly, the sections were washed three times in phosphate-buffered saline (PBS) and incubated with 3% hydrogen peroxide for 10 min to block the activity of endogenous peroxidase. The sections were then washed with PBS again and treated with 5% normal rabbit non-immune serum for 15 min followed by incubation with primary goat polyclonal antibody for PAR-2 (1:50 dilution, sc-8205, Santa Cruz) overnight at 4 °C. The sections were rinsed and incubated with HRP conjugated rabbit anti goat secondary antibody (ZSGB-BIO, Beijing, China) for 30 min at room temperature followed by washing with PBS. The reaction products were visualized with a 3,3′-diaminobenzidine tetrahydrochloride substrate kit (ZSGB-BIO, Beijing, China). For the sections of negative controls, the primary antibody was replaced with PBS.

2.5. Recording of the tension of colonic muscle strips

The muscle tension was recorded using a method previously described [33]. Briefly, a segment (2–3 cm length) of distal colon (1 cm from rectum) was immediately removed from control and SF-treated rats. The segment was opened along the mesenteric border and pinned flat in a dish filled with oxygenated Krebs solution. Full-thickness muscle strips (4 mm wide, 10 mm long) were prepared. Muscle strips parallel to the long axis of the circular muscle layer of colon were designated circular muscle (CM), muscle strips parallel to the long axis of the longitudinal muscle layer of colon were designated longitudinal muscle (LM). Strips were suspended in a organ bath which was filled with 5 ml Krebs solution, continuously perfused with 95% O_2 and 5% CO_2 and maintained at 37 °C. One end of the strip was tied with silk thread to an external isometric force transducer (JH-2B, Instrument Company of Chengdu, Chengdu, China) for recording the muscle mechanical activity. Another end was tied to a hook at the bottom of the chamber. After equilibration for at least 45 min with flushing every 15 min under a preload of 1 g, spontaneous contraction of colonic strips was recorded using a polygraph system (SMUP-PC, Fudan University, Shanghai, China). The mechanical response to the PAR-2-AP, SLIGRL-NH_2, carbachol, and PAR-2 reverse peptide (PAR-2-RP), LRGILS-NH_2 were examined. In some experiments, muscle strips were pretreated with tetrodotoxin (TTX, 10 μM) for 30 min before administration of PAR-2-AP or PAR-2-RP.

2.6. Western blot analysis

Tissue from distal colon was homogenized, centrifuged at 12,000 *g* for 10 min at 4 °C, and the supernatant was used for protein quantitative analysis by using Protein Quantitative Analysis Kit (k3001-BCA; Shenergy Biocolor, Shanghai, China). Total proteins (approximately 12.5 μg) were fractionated on a 5% to 10% gradient sodium dodecyl sulfatepolyacrylamide gel. They were transferred to 0.45 μm Polyvinylidene-Fluoride (PVDF) membranes. Membranes were blocked in blocking buffer (5% non-fat dry milk, TTBS) for 90 min at room temperature, incubated with primary goat anti-PAR-2 antibody (1:2000 dilution, sc 8205; Santa Cruz, CA, USA) and β-actin primary antibody (1:4000 dilution, sc-1616-R; Santa Cruz, CA, USA) respectively at 4 °C overnight. After washing 5 min for three times, the PVDF membranes were incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibodies respectively (1:80,000 dilution, ZB-2306; 1:30,000 dilution, ZB2301; ZSGB-BIO, Beijing, China). Finally, immunoreactive proteins were detected by ECL plus (Millipore, Bedford, MA, USA). Quantification of each band was performed using Scion Image densitometry analysis software (Scion Corporation, Maryland, U.S.A.).

2.7. Chemicals

PAR-2-AP (SLIGRL-NH_2) and PAR-2-RP (LRGILS-NH_2) were purchased from CL BIO-SCIENTIFIC CO., LTD (Xi an, China). Carbachol and TTX were purchased from Sigma-Aldrich Corp (St Louis, MO, USA). The Krebs solution was composed of the following reagents (mM): NaCl 120.6, KCl 5.9, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgCl_2$ 1.2, $NaHCO_3$ 15.4 and glucose 11.5.