Proinflammatory Cytokines Suppress the Expression Level of Protease-Activated Receptor-2 through the Induction of iNOS in Rat Colon

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(Received 17 July 2009/Accepted 19 August 2009)

ABSTRACT. Protease-activated receptor (PAR)-2 plays important roles in intestinal inflammatory responses and also contributes to intestinal digestive motility. In the distal colon of a rat experimental colitis model, expression level of PAR-2 mRNA was decreased, and relaxation through PAR-2 activation was attenuated. This study shows the effects of proinflammatory cytokines on changes to PAR-2 in rat colonic smooth muscle using an organ culture method. Colonic inflammation was induced in rats by administering dextran sodium sulphate in drinking water. Organ culture of distal colonic smooth muscle layer of normal rat was performed for up to 3 days. In the experimental colitis rat, mRNA expression levels of proinflammatory cytokines such as IL-1β and TNF-α increased with inflammation. After the incubation with IL-1β and TNF-α for 3 days, trypsin (PAR-2 agonist)-induced relaxation was attenuated, simultaneous with suppression of PAR-2 mRNA expression. Conversely, in this preparation, mRNA expression levels of iNOS were significantly increased. When 1-NNMMA was added to the medium with IL-1β and TNF-α, changes to PAR-2 by these cytokine recovered. Moreover, when samples were cultured with NOC-18 (slow-releasing NO donor) for 3 days, relaxation induced by trypsin and expression of PAR-2 mRNA were attenuated. These results suggest that suppression of PAR-2 expression under inflammatory conditions is at least partially induced by NO produced in the colonic muscularis externa by protease-activated cytokines.

KEY WORDS: colonic smooth muscle, inflammatory bowel disease, motility disorder, protease-activated receptor-2.

Inflammation of the bowel is associated with decreased motility, and this abnormality may lead to diarrhea or constipation characteristic of inflammatory bowel disease (IBD) [8, 21, 34]. Contraction of intestinal smooth muscle is reportedly decreased in IBD patients or animal models of intestinal inflammation [1, 2, 13, 28]. Some of the molecular mechanisms responsible for contractility disorders in the inflamed gut have recently been clarified. These include increased activity of ATP-sensitive K+-channels and myosin light chain phosphatase [1, 24], and decreased activity of smooth muscle L-type Ca2+ channels [13, 28]. In the regulation of digestive motility, mechanisms of relaxation are as important as those of contraction. In IBD patients and animal models, relaxation mechanisms have been suggested to be damaged by the impairment of CGRP and NK-1 receptor-mediated relaxation and the cGMP-relaxation pathway [30, 31].

The protease-activated receptor (PAR) family is a unique family of G-protein-coupled seven-transmembrane domain receptors activated by proteolytic cleavage of the extracellular N-terminal domain, resulting in the generation of a new tethered ligand activating the receptor itself [9, 11, 22, 32]. All of the identified members of the PAR family, PAR-1, -2, -3 and -4, are activated by endogenous proteinases. Among the PAR family, the function of PAR-2 has been widely studied, and this receptor is known to be involved in inflammation, allergies, haemorrhaging, exocrine activities and intestinal ion transport [12, 20, 25]. We have already reported that stimulation of PAR-2 with trypsin induces relaxation in rat colon by the activation of small conductance K+-channels, and we suggested that the PAR-2-mediated relaxation system in colonic smooth muscle is inhibited by the attenuation of PAR-2 mRNA expression in an experimental colitis rat model. However, we did not analyse the mechanisms of PAR mRNA downregulation, although we speculated that an excess of protease-mediated inflammatory responses would be counterbalanced by downregulation of PAR-2 [26].

Proinflammatory cytokines such as interleukin (IL)-1β and tumour necrosis factor (TNF)-α are known to be likely to play important roles in the pathogenesis of IBD. Elevated IL-1β and TNF-α levels have been measured in both the mucosa and the muscle layer during acute and chronic intestinal inflammation. These cytokines secondarily stimulate the production of many other inflammatory cytokines, such as macrophage infiltrating factor, IL-6, IL-12 and IL-18. IL-1β and TNF-α thus play a crucial role in the initiation and amplification of inflammatory reactions.

Proinflammatory cytokines also drive inducible NO synthase (iNOS) gene expression under inflammatory conditions, increasing the release of NO [3, 10, 23]. NO is therefore one of the mediator of pathophysiological processes in the inflamed gastrointestinal tract, including mucosal protection, regulation of blood flow, regulation of motility, and tissue damage [10, 15].

Based on these results, the present study focused on the
proinflammatory cytokines and the mediators induced by cytokines, to assess possible mechanisms underlying the downregulation of PAR-2-mediated relaxation and PAR-2 mRNA expression in colonic smooth muscle using a rat model of dextran sodium sulphate (DSS)-induced colitis.

MATERIALS AND METHODS

Induction of colitis and tissue preparation: Colonic inflammation was induced in male Sprague Dawley rats (7 to 12 weeks old) by administering 5% (wt/vol) DSS (molecular weight, 36,000–50,000) in drinking water for 0, 5 or 7 days (DSS-treated rats), as previously reported [26]. Procedures and care were approved by the Animal Care and Use Committee of Yamaguchi University. Rats were anaesthetized using ether, stunned by a blow on the head and exsanguinated. The distal colon was then excised. Colonic tissue isolated from control or DSS-treated rats was cut open along the mesenteric attachment, and the mucosal layer was removed. The remaining muscle layers prepared for mechanical experiments were placed in tris (hydroxymethyl) aminomethane (Tris)-buffered solution (NaCl, 123.7 mM; KCl, 2.7 mM; MgCl_2, 1.0 mM; CaCl_2 1.8 mM; glucose, 5.5 mM; Tris, 25 mM; and ethylenediaminetetraacetic acid (EDTA), 0.01 mM; pH 7.4 at 37°C).

Organ culture procedure: Organ culture was performed as previously described [24]. Briefly, a segment of the distal colon dissected from male normal rat was placed in sterile Hanks’ balanced salt solution, after which the smooth muscle were measured isometrically under a resting tension of 10 mN and recorded. After equilibration for 15 min in a bath, each strip was repeatedly exposed to 65.4 mM KCl until responses stabilised. For experiments examining the inhibitory effects of trypsin on carbachol-induced contraction, areas under the curve (AUCs) were compared in the presence and absence of trypsin.

Reverse transcriptase polymerase chain reaction: Total RNA was extracted from rat colonic smooth muscle strips using TRIzol reagent (Invitrogen). RNA concentration was adjusted to 1 μg/μl with RNase-free distilled water. Reverse transcriptase polymerase chain reaction (RT-PCR) was performed using an Access Quick RT-PCR System (Promega, Tokyo, Japan), which combined cDNA synthesis and PCR in a single reaction. For single-step RT-PCR, reverse transcription (48°C for 50 min) was followed by initial denaturation at 94°C for 2 min. Two cycling profiles were used. The first comprised denaturing at 94°C for 1 min, annealing at 55°C for 30 s, and extension at 72°C for 1 min for IL-1β, TNF-α, PAR-2 and β-actin. The second comprised denaturing at 94°C for 40 s; annealing at 55°C for 1 min; and extension at 72°C for 90 s for iNOS. Both profiles used a total of 27 cycles, followed by a final extension step of 5 min at 72°C. The following oligonucleotide primers were designed [10, 26]: PAR-2, 5’-CACCATGAAGGAGAAG TCT-3’ (sense) and 5’-GGGACACCGTGCAGCAGGT-3’ (antisense); IL-1β, 5’-TCTAGATGCTTCCAAGG-3’ (sense), 5’-GTGCTGATGTACCAAGTTG-3’ (antisense); TNF-α, 5’-AAATGCGTCCTCCTCTCATA-3’ (sense), 5’-AGCCTTTGTCCTTGAAGAGA-3’ (antisense); iNOS, 5’-CTTACCTACTGGGAGAACCTGGG-3’ (sense) and 5’-GGAGAGCTGATGGAGATGTCG-3’ (antisense); β-actin, 5’-GGTGGGCGCCTAGGCACCA-3’ (sense) and 5’-GGACTGCTATTGTTCCAGG-3’ (antisense). After PCR products in each cycle were electrophoresed on 2% agarose gel, the products were stained using ethidium bromide and visualized using an ultraviolet transilluminator (UVP, Cambridge, UK). Quantification of each band was performed using Scion Image densitometry analysis software (Scion Corporation, Frederick, MD, U.S.A.). Each PCR signal was normalised to the PCR signal generated by primers for β-actin.

Chemicals: Chemicals used were as follows: carbachol, IL-1β and TNF-α (Sigma, Tokyo, Japan); EDTA and NOC-18 (Dojindo Laboratories, Tokyo, Japan); and l-NMMA (Nω-Monomethyl-L-arginine), trypsin, DSS and Tris (Wako Pure Chemical, Osaka, Japan).

Statistical analyses: Results are expressed as means ± standard error of the mean (SEM). Statistical evaluations of data were performed using paired or unpaired Student’s t-tests for comparisons between 2 groups and one-way analysis of variance followed by the Tukey test for comparisons among ≥3 groups using Prism software (Graph Pad Software, San Diego, CA, U.S.A.). Values of P<0.05 were considered statistically significant.

RESULTS

Levels of mRNA expression for proinflammatory cytokines and iNOS: Expression of proinflammatory cytokines (IL-1β and TNF-α) and iNOS mRNA in rat colonic muscularis externa was assessed in a semi-quantitative manner using RT-PCR (Fig. 1). All of these mRNA levels were significantly upregulated in smooth muscle layers of rat colon treated with DSS for 5 or 7 days as compared to control rat colon at a constant level of housekeeping gene β-actin. We have previously reported that expression levels of β-actin mRNA in DSS-treated rat colon are no different from those in control rat colon [26]. Suppression of PAR-2 agonist-induced relaxation in
organ cultured tissue: We have previously reported that trypsin-induced relaxation in rat colonic smooth muscle is attenuated by the induction of inflammation [26]. To determine the direct effects of proinflammatory cytokines on PAR-2-mediated relaxation, we adapted an organ culture method. Chronic treatment with IL-1β has also been reported to suppress the contraction of rat ileal smooth muscle induced by carbachol in a time-dependent manner in organ culture system [24]. In studies of mechanical responses, adopting the same degree of pre-contractions is important for comparing PAR-2-induced relaxation in control and cytokine-treated rat colon.

As a preliminary experiment, we examined the effect of the organ culture system on relaxation induced by 1 μM of trypsin. Trypsin-induced relaxation of 0.3 μM carbachol-induced contractions was well maintained in the tissue cultured in culture medium for 3 days (Fig. 2A). In the presence of 10 ng/ml of IL-1β, relaxation elicited by trypsin was suppressed in a time-dependent manner (Fig. 2C). In the presence of 10 ng/ml of TNF-α, trypsin-induced relaxation was inhibited, but to lesser extent than with than IL-1β (Fig. 2C). We also tested the synergistic effect of IL-1β and TNF-α, with simultaneous application of these cytokines causing much higher suppression of trypsin-induced relaxation in colonic smooth muscle tissue, particularly in tissue cultured for 3 days (Fig. 2D).

Expression of PAR-2 mRNA detected by RT-PCR in organ cultured tissue: Expression of PAR-2 mRNA in rat colonic muscularis externa was assessed in a semi-quantitative manner using RT-PCR (Fig. 3). In organ cultured rat colon without cytokines for 3 days (Day 3), expression level of PAR-2 mRNA was no different from intact rat colon (Day 0). Expression level of PAR-2 mRNA was significantly suppressed in rat colon treated with IL-1β for 3 days, as determined by RT-PCR (Fig. 3).
but was not significantly affected by TNF-α for 3 days. Simultaneous application of IL-1β and TNF-α for 3 days decreased expression levels of PAR-2 mRNA more strongly, whereas expression level of β-actin mRNA was no different from that in control rat colon (data not shown). IL-1β with TNF-α had the strongest effect on inhibition of trypsin-induced relaxation and attenuation of PAR-2 mRNA expression, so simultaneous application of these cytokines was applied in the following experiments.

**Effects of NO on trypsin-induced relaxation and expression of PAR-2 mRNA in tissue cultured with IL-1β and TNF-α:** DSS-treated rat colon has already been shown to display increased expression of iNOS mRNA (Fig. 1C). We therefore examined the changes of these mRNA expressions in rat colonic smooth muscle cultured with IL-1β (10 ng/ml) and TNF-α (10 ng/ml) for 3 days. Expressions of iNOS mRNA were significantly increased in colonic smooth muscle exposed to IL-1β and TNF-α (Fig. 4). In smooth muscle cells, iNOS is well known to produce NO. To clarify the participation of NO in the inhibitory effect of IL-1β and TNF-α on trypsin-induced relaxation and PAR-2 mRNA expression level, rat colonic smooth muscle preparations were cultured with cytokines in the presence of non-selective NOS inhibitor, l-NMMA (200 μM) for 3 days. l-NMMA significantly suppressed the inhibitory effects of cytokines on trypsin-induced relaxation (Fig. 5A) and recovered expression levels of PAR-2 mRNA in cultured rat colon (Fig. 5B). Next, a NO-releasing compound, NOC-18 (300 μM), was applied to the cultured medium for 3 days to evaluate the direct effect of NO. NOC-18 significantly inhibited trypsin-induced relaxation (Fig. 6A). NOC-18 also significantly suppressed expression of PAR-2 mRNA (Fig. 6B).

**DISCUSSION**

Inflammatory factors such as cytokines, chemokines, NO, reactive oxygen species and prostaglandins may play various roles in intestinal dysmotility in the inflamed bowel. The present study examined time-dependent changes in colonic muscularis inflammation by measuring levels of the proinflammatory cytokines IL-1β and TNF-α in a rat model of DSS-induced colitis (Fig. 1A, B). Levels of many types of cytokines, including proinflammatory cytokines, are reportedly changed in IBD model animals [6, 7, 14]. Recently, smooth muscle contraction has also been reported to be inhibited in the inflamed proximal colon of rats, with motility disorder prevented by pretreatment with NF-κB inhibitors, suggesting that proinflammatory cytokines may play crucial roles in the intestinal dysmotility of colitis model animals [13]. To clarify the influence of proinflammatory cytokines, an organ culture method was adapted for the present experiments. Treatment with IL-1β for more than 1 day in organ cultured tissue significantly suppressed trypsin-induced relaxation of carbachol-induced contraction (Fig. 2B). In addition, this suppression was accompanied by reduced PAR-2 mRNA expression level (Fig. 3). TNF-α also reduced trypsin-induced relaxation and PAR-2 mRNA expression. Simultaneous application of IL-1β and TNF-α induced synergistic inhibitions (Figs. 2D, 3). These results suggest that proinflammatory cytokines induce attenuation of PAR-2 mRNA in DSS-treated colitis model rats.

In the inflammatory condition, NO also plays an essential role in intestinal motility [10, 27]. In DSS-treated rat colonic muscularis externa, expression levels of iNOS was increased as for IL-1β and TNF-α mRNA (Fig. 1). iNOS is induced in inflamed human colonic tissues such as ulcerative colitis [29]. In the rat ileal smooth muscle layer, exposure to LPS (lipopolysaccharide) increases expression of iNOS mRNA and results in the production of NO [10]. To confirm the possibility that proinflammatory cytokines induce expression of iNOS mRNA in rat colonic muscle, IL-1β and TNF-α were added to the organ culture system for 3 days. In the presence of IL-1β and TNF-α, iNOS mRNA...
incubation with IL-1α NO produced from colonic smooth muscularis externa by [10]. The present experiment did not measure the level of the activity in IBD [29]. Production of NO by lipopolysaccharide in intestinal mucosa is known to be associated with disease [16]. Under inflammatory conditions, the activity of smooth muscle L-type Ca2+ channels is suppressed [1, 13, 28], the cGMP-relaxation pathway is damaged [31] and non-adrenergic non-cholinergic nerves are impaired [17]. These results suggest that, in the present experiment, l-NMMA could restore the expression of PAR-2 mRNA, but the relaxing effect of thrombin is only partially retrieved due to damage to the relaxing mechanism itself.

Increased NO production along with expression of iNOS in intestinal mucosa is known to be associated with disease activity in IBD [29]. Production of NO by lipopolysaccharide has also been shown to result in intestinal dysmotility [10]. The present experiment did not measure the level of NO produced from colonic smooth muscularis externa by incubation with IL-1β and TNF-α. To evaluate the direct effects of NO, colonic smooth muscle was incubated with NOC-18 for 3 days. As a result, trypsin-induced relaxation and expression of PAR-2 mRNA were suppressed (Fig. 6). In cachexia (the progressive destruction of muscular mass), iNOS induced the loss of MyoD mRNA by NO production, resulting in muscle wasting for both in vitro and in vivo experiments [5]. Another report suggested that TNF-α induces expression of iNOS, leading to the production and release of NO and subsequent oxidative stress in the skeletal muscles of cachectic animals [4, 35]. NO has also been reported to act at the transcriptional level to suppress gene expression in rat mesangial cells under inflammatory conditions [33]. These results suggest that, in the presence of inflammation, proinflammatory cytokines increase the expression of iNOS, leading to the production and release of NO. Thus, oxidative stress in colonic muscularis externa mediated PAR-2 mRNA decay and the suppression of trypsin-induced relaxation.

In summary, these results suggest that the suppression of PAR-2 expression caused by proinflammatory cytokines under inflammatory conditions such as ulcerative colitis was at least partially induced by NO produced in colonic muscularis externa.

ACKNOWLEDGMENT. This work was supported by a Grant-in-Aid for Scientific Research from the Japanese Ministry of Education, Culture, Sports, Science and Technology.

REFERENCES


