

The pathophysiological roles of COX-1 and COX-2 in the intestinal smooth muscle contractility under the anaphylactic condition

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ABSTRACT

Various inflammatory mediators released from antigen-activated mast cells are considered to play a key role in the pathogenesis of food allergy. The aim of the present study was to determine the mechanisms underlying the antigen-induced anaphylactic responses in the rat colons. Wistar rats were sensitized by intraperitoneal injection of ovalbumin (OVA). The contractilities of isolated proximal colons of the sensitized rats were studied in the organ bath. OVA challenges of sensitized tissues induced prolonged contractile responses. The antigen-induced contractions were greatly reduced by mast cell stabilizer doxantrazole (10 μ M). However, the contractions were resistant to histamine H1 receptor antagonist and prostaglandin D₂ receptor antagonist. In contrast, non-selective cyclooxygenase (COX) inhibitor indomethacin (1 μ M) significantly reduced the contractions by 61.0%. Furthermore, selective COX-1 inhibitor FR122047 (10 μ M) as well as selective COX-2 inhibitor NS-398 (10 μ M) significantly inhibited the contractions by 50.1% and 50.3%, respectively. Nevertheless, the transcript levels of COX-2 as well as COX-1 were not up-regulated by OVA in the proximal colons of the sensitized rats. The present results indicate that de novo arachidonic acid metabolites synthesis by constitutive COX-1 as well as constitutive COX-2 within mast cells contribute to the altered smooth muscle contractilities in the colons under the anaphylactic condition.

Food allergy is adverse immunological response to proteins in food. Clinical manifestations of food allergy are revealed as disturbed gastrointestinal motility (*eg.* abdominal cramping, emesis and diarrhea). It is well known that intestinal anaphylaxis induces intestinal inflammation and then results in functional changes of the smooth muscle cells (10), which may be attributed to activation of mast cells, eosinophils and other immune cells in the mucosal immune sys-

tem of the intestine (2, 10). However, the mechanisms underlying the altered smooth muscle contractilities during the allergic inflammation have yet to be fully understood.

Mast cells are important effector cells in allergic and inflammatory reactions (1). Under allergic conditions, mast cells are able to produce and release a great variety of substances such as histamine, cytokines and arachidonic acid metabolites (prostaglandins, leukotrienes, thromboxanes) which are the key factors in the development and regulation of the inflammatory responses (2). Mast cell activation gives rise to cyclooxygenase (COX)-mediated conversion of arachidonic acid to prostaglandins (4, 8) which act as mediators in inflammatory responses and also regulate the contractile activities of digestive smooth

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muscles (3).

There are two isoforms of COX enzymes throughout the gastrointestinal tract. COX-1 is constitutively expressed in the digestive tract; COX-2 was initially regarded as an inducible form that has been highly expressed at sites of inflammation, but subsequently constitutive expression of COX-2 in normal gastrointestinal tissues has been demonstrated (7). COX-2 as well as COX-1 is constitutively and considerably expressed in unstimulated mast cells (5, 6). COX is well-known to play a pivotal role in the pathophysiology of gut inflammatory and neoplastic disorders (12, 14) and it is now well recognized that chronic inflammation is a risk factor for most type of cancers (13). COX-2 has been implicated in the growth and progression of a variety of human cancers, whereas COX-1 expression in all carcinoma tissues is associated with enhanced expression of COX-2 mRNA and protein (11). Thus, it is important to reveal the precise role of COX under anaphylactic conditions for investigating a strong link between chronic inflammation and cancer.

The present study was designed to determine the mechanisms underlying the specific antigen-induced responses in the colons of the sensitized rats.

MATERIALS AND METHODS

Animals and induction of intestinal anaphylaxis. All experiments were approved by the Animal Experiments Committee of University of Toyama. Adult male Wistar rats (250 ~ 350 g) were purchased from Japan SLC, Inc. (Shizuoka, Japan). All rats were housed in the experimental animal facility at University of Toyama. Rats were sensitized once with 50 µg of albumin from chicken egg white (OVA fraction V; Sigma, St Louis, MO, USA) in the presence of 13 mg of aluminum hydroxide gel (Sigma) as an adjuvant by intraperitoneal injection. Two weeks after systemic sensitization, the excised proximal colons (~2 cm in length) were opened along both the mesenteric borders and anti-the mesenteric borders and were divided into two preparations. The preparations were suspended in the longitudinal direction in organ baths (25 mL volume) filled with Tyrode's solution (pH 7.4) aerated with 100% O₂. Tyrode's solution had the following ionic composition (in mM): 136 NaCl, 5.4 KCl, 1.0 MgCl₂, 0.33 NaH₂PO₄, 1.8 CaCl₂, 10.0 glucose and 5.0 HEPES. The preparations were prestretched to a resting tension of ~0.5 g. Mechanical force was recorded in the longitudinal direction with an auxotonic force displacement transducer (UL-20GR; Minebea, Naga-

no, Japan). Drugs were added to the organ baths at the final concentrations specified in the text and figures. The divided preparations of the proximal colon from the same animal were mounted in the organ baths and paired with respect to responses to the antigen, OVA (1 mg/mL). For each pair, one preparation was pretreated with a test drug; the other received a vehicle because repeated applications of OVA produce marked desensitization. All test drugs and vehicle were applied to the baths 30 min before the application of OVA. At the end of each experiment, bethanechol (Sigma, 1 mM) was applied to record a maximum contractile response of the preparation. The relaxation response to OVA was expressed in the magnitude as a % of the maximum response to bethanechol in the same preparation.

Expression of COX-1 and COX-2 mRNA. Five minutes after the applications of OVA in the organ baths, the proximal colons from the sensitized rats and the non-sensitized rats were immediately frozen in liquid nitrogen. Total RNAs were extracted from the proximal colons by Sepasol Super (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's instruction. Reverse transcription was performed using the Exscript RT reagent kit (Takara Bio, Shiga, Japan) and random primers, followed by real-time PCR. Real-time PCR amplification of COX-1, COX-2, and GAPDH was performed using SYBR Premix Ex Taq (Takara). The following primer pairs were used: COX-1, forward: 5'-GCCGAGGATGTCATCAAGGA-3'; reverse: 5'-GAACTCTAAAGCATCGATGTCACCA-3'; COX-2, forward: 5'-GGAGCATCCTGAGTGGGATGA-3'; reverse: 5'-AAGCAGGTCTGGGTGCGAACTTG-3'; GAPDH forward: 5'-GGCACAGTCAAGGCTGAGAATG-3'; reverse: 5'-ATGGTGGTGAAGACGCCAGTA-3'. Real-time PCR was carried out using the Mx3000p (Stratagene, La Jolla, Calif., USA). The PCR reaction condition was 10 sec at 95°C, followed by 40 cycles of 5 sec at 95°C and 20 sec at 60°C. Target mRNA was normalized to GAPDH mRNA as an internal control in each sample. Results were expressed as relative ratio to the average of the non-sensitized rats group.

Chemicals. All drugs except for bethanechol chloride and OVA used in the pharmacological studies were dissolved in dimethyl sulfoxide at concentrations of 10 mM as a stock solution. Other chemicals were dissolved in Tyrode's solution. The maximal final concentrations of dimethyl sulfoxide were 0.1%, which have no effects on the responses of the prep-

arations.

Statistical analyses. Data are expressed as the mean $\pm$ S.E. N indicates a number of rats. Statistical comparisons were made with Student's paired t-test. Probability values (P) of <0.05 were considered as statistically significant.

RESULTS AND DISCUSSION

Antigen-induced response in the colon of the sensitized rat

The antigen OVA (1 mg/mL) challenges of non-sensitized preparations induced no changes in tension, whereas OVA challenges of sensitized preparations induced prolonged contractile responses associated with suppressed spontaneous contractions (Fig. 1). The magnitude of the contractile responses was $88.8 \pm 4.2\%$ ($n = 39$) of the responses to bethanechol. The antigen-induced contractions were specific to the sensitizing antigens because responses to bovine serum albumin were never present in the sensitized preparations.

Involvement of mast cells in antigen-induced contractile response

We next investigated the role of mast cells in the antigen-induced contractile responses in the proximal colons of the sensitized rats. The OVA-induced contractions ($98.0 \pm 11.9\%$ of the responses to bethanechol, $n = 6$) were greatly reduced by the mast cell stabilizer doxantrazole (Sigma, $10 \mu\text{M}$;

$22.3 \pm 9.5\%$ of the responses to bethanechol, $n = 6$), which is in good agreement with the previous report in the distal colons of sensitized rats (10). Furthermore, the role of mast cell activation in the antigen-induced contractions was evaluated in Ws/Ws rats (white spotting in the skin) that have small deletions of the c-kit genes, and are genetically deficient in mast cells (14). Ws/Ws rats and sibling wild-type rats $+/+$ ($250 \sim 350$ g, Japan SLC, Inc.) were sensitized with OVA similarly to Wistar rats. In the colons from sensitized $+/+$ control rats, the contractile responses to OVA were reproduced ($60.4 \pm 2.4\%$ of the responses to bethanechol, $n = 3$). The contractile responses to OVA were also apparent in the colons from the sensitized Ws/Ws rats, but the magnitude of the increase in the responses ($40.6 \pm 5.6\%$ of the responses to bethanechol, $n = 4$) was significantly ($P < 0.05$) less than that in the colons from the sensitized $+/+$ control rats. Therefore, these data suggest that mast cells infiltrated into the sensitized colon are likely the major participants in the anaphylaxis-induced contractile responses.

Involvement of mast cell-associated mediators in antigen-induced contractile response

Mast cells contain, or elaborate upon appropriate stimulations, a variety of biologically active mediators, including histamine, platelet-activating factor, prostaglandins and leukotrienes that have many different potential effects in inflammation, and have organ function at sites of mast cell activation (15). In particular, histamine and prostaglandins are considered to be the most important mast cell-derived mediators and are also able to induce contractions when applied directly to the rat proximal colons. COX metabolites of arachidonic acid are synthesized de novo in mast cells and the major product appeared to be prostaglandin D_2 . Thus, we addressed the effects of specific antagonists of the mediators on the OVA-induced contractile responses in the sensitized rat colons. However, neither H1 histamine receptor antagonist mepyramine (Sigma, mepyramine $10 \mu\text{M}$; $84.6 \pm 4.6\%$ of the responses to bethanechol: vehicle; $82.2 \pm 21.5\%$ of the responses to bethanechol, $n = 4$) nor prostaglandin D_2 receptor antagonist BW A868C (Sigma, BW A868C $10 \mu\text{M}$; $74.1 \pm 19.6\%$ of the responses to bethanechol: vehicle; $88.2 \pm 9.5\%$ of the responses to bethanechol, $n = 5$) affected the OVA-induced contractile responses in the colons of the sensitized rats, suggesting that it is unlikely that histamine and prostaglandin D_2 released from mast cells mediate the contractile response to the antigen challenges.

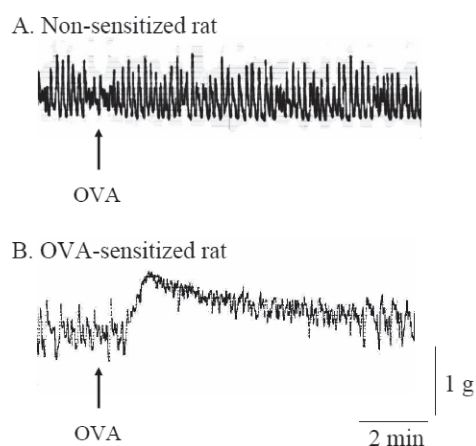


Fig. 1 Typical tracings of smooth muscle contractilities in the rat proximal colons. The antigen OVA (1 mg/mL) challenge of non-sensitized preparation induced no change in tension (A), whereas OVA challenge of sensitized preparation induced prolonged contractile response associated with suppressed spontaneous contractions (B).

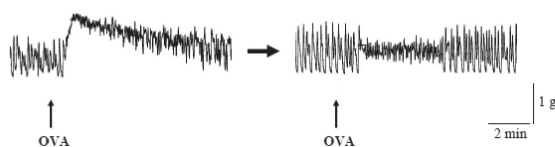
We then examined the effect of potent non-selective COX inhibitor indomethacin (Sigma) on the OVA-induced contractile responses in the colons of the sensitized rats. In contrast, indomethacin (1 μ M) significantly inhibited the contractile responses (indomethacin; $33.9 \pm 7.2\%$ of the responses to bethanechol: vehicle; $86.9 \pm 5.2\%$ of the responses to bethanechol, $P < 0.01$, $n = 8$), whereas it remains to be determined which COX isozymes is responsible for mast cells-mediated contractile responses to the antigen challenges under the anaphylactic conditions. Next, in order to investigate the role of COX isozymes, relatively selective COX-1 inhibitor piroxicam (Sigma), selective COX-1 inhibitor FR122047 (Sigma, 10 μ M) and selective COX-2 inhibitor NS-398 (Sigma, 10 μ M) were used. Interestingly, not only COX-2 inhibitor (NS-398; $39.6 \pm 11.3\%$ of the responses to bethanechol: vehicle; $79.7 \pm 14.4\%$ of the responses to bethanechol, $P < 0.05$, $n = 5$, Fig. 2) but also COX-1 inhibitors (piroxicam; $35.5 \pm 9.2\%$ of the responses to bethanechol: vehicle; 84.6

$\pm 11.4\%$ of the responses to bethanechol, $P < 0.01$, $n = 5$, FR122047; $48.9 \pm 5.3\%$ of the responses to bethanechol: vehicle; $98.0 \pm 11.9\%$ of the responses to bethanechol, $P < 0.01$, $n = 6$, Fig. 3) significantly reduced the contractile responses to the OVA challenges. Our findings suggest that mast cell activation leads to the generation of COX products, which in turn mediate the OVA-induced contractile responses in the colons of the sensitized rats and that COX-1 as well as COX-2 is involved in the development of the contractile responses under the anaphylactic conditions.

Expression levels of COX-1 and COX-2 mRNAs at antigen-induced contractile response

To further examine the role of COX isoforms at the OVA-induced contractile responses in the colons of the sensitized rats, we undertook real time PCR analysis to determine changes in transcript levels of COX-1 and COX-2 between non-OVA-sensitized normal colons and OVA-sensitized anaphylactic colons when applied OVA to the colons. OVA-induced contractile responses were observed in the colons of

A.



B.

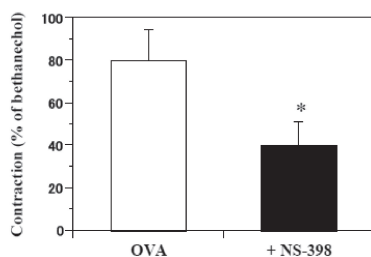
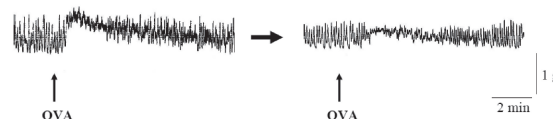


Fig. 2 Effect of selective COX-2 inhibitor NS-398 on the OVA-induced contractile response in the colon of the sensitized rat. (A) shows typical tracings of smooth muscle contractilities in the proximal colon of the sensitized rat. Left tracing shows OVA-induced prolonged contractile response associated with suppressed spontaneous contractions. Right tracing show OVA-induced contraction in the presence of NS-398 (10 μ M). (B) shows that in the NS-398-pretreated preparation (+ NS-398), the contractile responses to OVA were much smaller than those to OVA in the vehicle-pretreated preparations (OVA). Data are presented as the mean $\pm$ S.E. for five preparations. * $P < 0.05$.

A.



B.

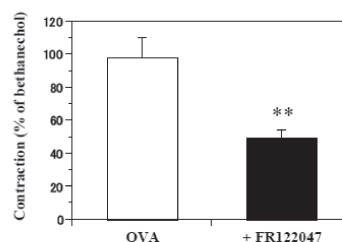


Fig. 3 Effect of selective COX-1 inhibitor FR122047 on the OVA-induced contractile response in the colon of the sensitized rat. (A) shows typical tracings of smooth muscle contractilities in the proximal colon of the sensitized rat. Left tracing shows OVA-induced prolonged contractile response associated with suppressed spontaneous contractions. Right tracing show OVA-induced contraction in the presence of FR122047 (10 μ M). (B) shows that in the FR122047-pretreated preparation (+ FR122047), the contractile responses to OVA were much smaller than those to OVA in the vehicle-pretreated preparations (OVA). Data are presented as the mean $\pm$ S.E. for five preparations. ** $P < 0.01$.

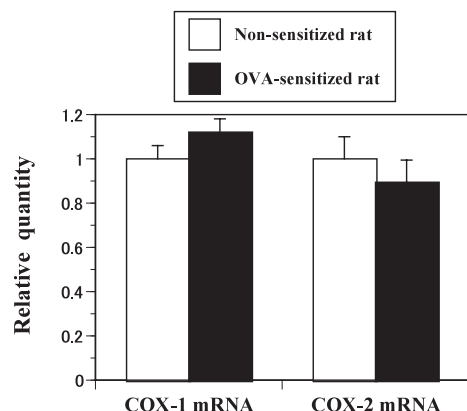


Fig. 4 Expression levels of COX-1 and COX-2 mRNAs at OVA-induced contraction in the proximal colon of the sensitized rat. Note that the transcript levels of both COX isoforms in the OVA-sensitized colons were almost the same as those in the non-sensitized colons. Data are presented as the mean $\pm$ S.E. for five preparations.

the sensitized rats, but not in the colons of the non-sensitized rats, whereas the transcript levels of COX-2 in the sensitized colons were almost the same as those in the non-sensitized colons ($n = 5$, Fig. 3). Similarly, there was no difference in the transcript levels of COX-1 between the non-sensitized colons and the sensitized colons ($n = 5$, Fig. 4). These findings suggest that mRNAs of both COX isoforms are expressed constitutively in the rat colons, but the transcriptions of both COX isoforms are not upregulated under the anaphylactic conditions.

In conclusion, the present results indicate that de novo arachidonic acid metabolites synthesis by constitutive COX-1 as well as constitutive COX-2 within mast cells contribute to the altered smooth muscle contractilities in the colons under the anaphylactic conditions. We have already reported as to intestinal anaphylaxis-induced abnormalities of water transport that allergic diarrhea is associated with a downregulation in aquaporins 4 and 8 expression in the mouse model of food allergy (16). With regard to intestinal anaphylaxis-induced alterations in intestinal motility, the present findings can provide a basis to interpret pathophysiological mechanisms underlying the major intestinal allergic symptom diarrhea induced by ingestion of food allergens.

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