Original Article

Acid increases PGE_2 in the duodenal mucosa in rats

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(Received 13 May, 2021; Accepted 2 June, 2021; Released online in J-STAGE as advance publication 7 August, 2021)

Attention has recently been paid to the duodenum as the pathophysiologic center of functional dyspepsia. However, the precise mechanisms of symptom generation remain unknown. We here investigated the effect of acid on duodenal prostaglandin E_2 and localization of prostaglandin E_2 related receptors. Sprague–Dawley rats were used for this study. Hydrochloric acid was administered in the duodenum, then prostaglandin E_2 levels in the duodenum were measured using the ELISA. The expression and localization of prostaglandin receptors (EP1–4) and the mRNAs of prostaglandin synthases were investigated using *in situ* hybridization histochemistry in duodenal tissue. After acid perfusion, prostaglandin E_2 levels in the duodenum significantly increased. EP3 was expressed mainly at the myenteric plexus in the duodenal mucosa, and EP4 at both the epithelial surface and myenteric plexus. Contrary, EP2 was sparsely distributed in the villi and EP1 were not clearly seen on *in situ* hybridization histochemistry. Prostaglandin-synthetic enzymes were also distributed in the duodenal mucosa. The prostaglandin E_2 levels in the duodenum increased after acidification. Prostaglandin E_2 receptors and prostaglandin E_2-producing enzymes were both observed in rat duodenum. These observations suggest that duodenal prostaglandin E_2 possibly play a role in the symptom generation of functional dyspepsia.

***Key Words*: PGE_2, EP, functional dyspepsia, duodenal inflammation**

Functional dyspepsia (FD) is a common disease which presents with upper abdominal symptoms (e.g., abdominal pain and a heavy feeling in the stomach) despite the absence of organic disease.[1] Regarding the pathogenesis of FD, gastrointestinal hypersensitivity and motor dysfunctions have been identified as the main causes of dyspeptic symptoms. However, the exact reason behind these physiological abnormalities in patients with FD remains unclear.[2] Recent studies have revealed that patients with functional gastrointestinal disorders have mucosal microinflammation, increased permeability, and altered intestinal microbiota in the mucosa of the gastrointestinal tract.[3] Microinflammation of the duodenum in FD, which is believed to be caused by eosinophil infiltration and mast cells, may be involved in the excitability of the duodenum to the irritating stimulant.[2,4] Recent data showed that irritation of the duodenum with acid or fat caused gastric visceromotor dysfunction in healthy humans.[5–7] However, it is unclear what occurs within the duodenal mucosa in response to irritating duodenal contents. Therefore, in this study, we tried to determine the mediator is associated with duodenal activation after irritant exposure.

Prostaglandin E_2 (PGE_2), a typical inflammatory mediator, is known to be more abundant than other prostaglandins in the gastrointestinal tract. The receptors activated by PGE_2 have been pharmacologically classified into four types, EP1, EP2, EP3, and EP4.[8] PGE_2 exhibits a great impact on pain signals,[9] while EP receptors are involved in peripheral hyperalgesic responses in the somatic pain region.[10] Regarding visceral perception, we previously reported that acid stimulation caused PGE_2 production from human esophageal mucosal epithelium, which was associated with heartburn symptoms.[11] In the esophageal mucosa, EP1 receptors are abundantly located, and suppression of PGE_2 using nonsteroidal anti-inflammatory drugs or administration of EP1 receptor antagonists partially suppressed these heartburn symptoms.[12,13] This suggests that the PGE_2–EP1 pathway plays an important role in human esophageal perception to the acid. On the other hand, we recently reported that the release of PGE_2 by activating TRPA1 channels promotes rectal colon contraction,[14] while the activation of EP3 receptors has been reported to regulate gastrointestinal motility.[15] These reports suggest association of PGE_2 with gastrointestinal motility.

Thus, we speculated that PGE_2 may be one of the mediators that are activated after irritating contents pass the duodenum, causing the physiological abnormalities of the stomach. Furthermore, the distribution of PGE_2 and its relatives in the duodenum has almost never been explored. In line with this, we investigated whether acid stimulation of the duodenum in normal rats increased PGE_2 levels as well as the presence and localization of PGE_2 related receptors and its producing enzymes.

Materials and Methods

Duodenal acid infusion. This study used 7-week-old normal Sprague–Dawley (SD) rats. The abdomen was opened under adequate inhalation anesthesia. The anorectal side of the duodenum of the rats was ligated with a Kocher to avoid irritating the organ as much as possible. A 4-Fr catheter was then inserted and implanted from the gastric vestibule to the duodenal lumen, and 0.2 ml of pH 1.0 hydrochloric acid was administered from the tip of the catheter. The duodenal side was ligated using a Kocher simultaneously upon administration to create a closed space. Five minutes after the acid injection, the duodenum was excised and quickly placed in liquid nitrogen.

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doi: 10.3164/jcbn.21-59

PGE_2 concentration in the duodenum. The PGE_2 concentration was measured in the rat duodenum as described previously.[11,12] In brief, the duodenum at the site of the closed space was excised, quickly frozen with liquid nitrogen. After measuring the weight of the duodenal tissue, homogenization (300 Hz/s, 3 min) was conducted with a mixer mill (MM300; QIAGEN, Hiden, Land Nordrhein-Westfalen, Germany). The protein concentration in the supernatant was measured with the BCATM protein assay kit (Thermo Fisher Scientific Inc, MA). The level of PGE_2 was measured by enzyme-linked immunosorbent assay. To measure the amount of prostaglandin E2 (PGE_2), EIA Kit-Monoclonal (Cayman Chemical Company, Ann Arbor, MI) was used according to the methods described in the manual. The concentration was determined following absorbance measurement with a microplate reader (SPECTRA MAX 250; Molecular Devices Japan Co., Tokyo, Japan). 0.2 ml saline was added instead of hydrochloric acid (HCl) as a comparison (saline group). The normal group was defined as the one in which the duodenum was removed under laparotomy without administering anything to the duodenum.

***In situ* hybridization histochemistry.** In 7-week-old normal SD rats, the duodenum was opened under sufficient inhalational anesthesia, and, with as little irritation as possible, it was removed and promptly frozen with liquid nitrogen. The frozen duodenum was sectioned at 16 μm. The expression and distribution of mRNA encoding EP1, EP2, EP3, EP4, cyclooxygenase-1 (COX-1), cyclooxygenase-2 (COX-2), two membrane-bound PGE synthases, microsomal PGE synthase (mPGES)-1, and mPGES-2, and cytosolic prostaglandin E synthase (cPGES) in the rat duodenum, were detected using a single *in situ* hybridization histochemistry (ISHH). ISHH was performed as described previously.[14,16] For ISHH, clones (pCRII-TOPO, Invitrogen Corp., Carlsbad, CA) containing a partial sequence corresponding to the coding regions of EP1, EP2, EP3, EP4, COX-1, COX-2, mPGES-1, and mPGES-2, and cPGES were prepared (Table 1). In brief, 35S-labeled RNA probes for PGE_2 synthases and EP receptors were prepared by *in vitro* transcription using T7 or SP6 RNA polymerase. The 35S-labeled probes in the hybridization buffer were placed on the section and then incubated at 55°C overnight (>18 h). After the hybridization reaction, these sections were washed at 58°C in high-stringency buffer with 5 mM dithiothreitol (DTT) for 30 min, then treated with 1 μg/ml RNase A (Roche, Mannheim, Germany) in RNase buffer for 30 min at 37°C. Subsequently, these sections were incubated at 58°C in high-stringency buffer with 5 mM DTT for 30 min. Then, dehydrated through an ascending alcohol series, and air dried. The slides were coated with NTB emulsion (Kodak, Tokyo, Japan) and exposed for 2–4 weeks. After development in D19 and fixation in 24% sodium thiosulfate, the sections were rinsed in distilled water, stained with hematoxylin-eosin, dehydrated in a graded ethanol series, cleared in xylene and coverslipped.

Table 1. Sequence location of primers

Gene	Accession No.	Forward	Reverse
COX-1	U03388	1856–1875	2446–2427
COX-2	AF233596	1802–1821	2456–2437
mPGES-1	AB048730	25–44	453–434
mPGES-2	NM_001107832	854–873	1321–1302
cPGES	BC166579	291–310	795–776
EP1	D88751	726–745	1320–1301
EP2	U94708	453–472	874–855
EP3	X83855	562–581	963–944
EP4	D28860	192–211	719–700

Statistical analysis. PGE_2 levels before and after acid perfusions were compared between each group via two-way repeated analysis of variance followed by Bonferroni post hoc test. Data are presented as mean ± SE. Analyses were performed using JMP® 15 (SAS Institute Inc., Cary, NC). $P<0.05$ was considered to indicate statistical significance.

Results

PGE_2 concentration in the duodenum. After acid perfusion into the duodenum, PGE_2 levels (pg/mg protein) significantly increased compared to saline treatment rats (HCl, $n = 14$, 829.1 ± 76.4 vs saline, $n = 14$, 416.0 ± 71.9, $p = 0.0004$) (Fig. 1). There was no significant difference in PGE_2 levels in both rats (saline, $n = 14$, 416.0 ± 71.9 vs normal, $n = 8$, 431.2 ± 95.2, $p = 0.8994$) in the saline and normal groups.

Localization of PGE_2 synthase and EP receptors in rat duodenum. The expressions of EP1, EP2, EP3, EP4, COX-1, COX-2, mPGES-1, mPGES-2, and cPGES in the rat duodenum were investigated. Furthermore, *in situ* hybridization using the entire duodenum was performed to determine their localization. Thus, COX-1 mRNA was distributed throughout the muscular and mucosal layers of the duodenum (Fig. 2A–D), whereas COX-2 mRNA was distributed in the muscular layers, especially in the ringed muscle (Fig. 2E–G). Moreover, mPGES-1 mRNA was distributed in the submucosal tissue of the chorionic villi (Fig. 2H–J), mPGES-2 mRNA was predominantly distributed in the crypt epithelium (Fig. 2K–M), and cPGES mRNA was distributed throughout the muscular and in the crypt epithelium of the duodenum (Fig. 2N–Q). Next, EP1 mRNA-labeled cells were not clearly expressed in the duodenum (Fig. 3A–C), EP2 mRNA was mainly localized in the mucosa or tissues of the chorionic villi of the duodenum (Fig. 3D–F), EP3 mRNA was mainly localized in the myenteric plexus (Fig. 3G–I), and EP4 mRNA was in the tips of the chorionic epithelium, in the crypt epithelium and in the myenteric plexus (Fig. 3J–M).

Discussion

The duodenal mucosa is exposed to various irritants, such as acid, dietary allergens (fats and amino acids, etc.), bile acids, and intestinal bacteria. Interestingly, recent data showed that irritation of the duodenum with acid or fat causes gastric visceromotor dysfunction in healthy humans.[5–7] In patients with FD, duodenal

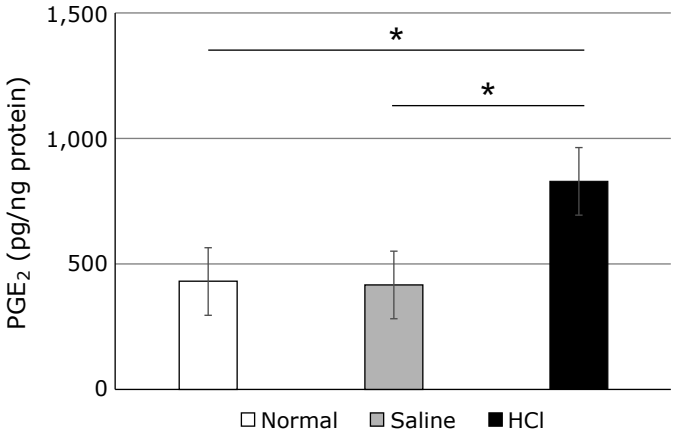

Fig. 1. Changes in PGE_2 expression after acid infusion in rat duodenum. The PGE_2 levels (pg/mg protein) in the duodenum significantly increased in both rats in the hydrochloric acid (HCl) group compared to the saline group [saline ($n = 14$) vs HCl ($n = 14$), *$p<0.005$]. Data were presented as mean ± SE.

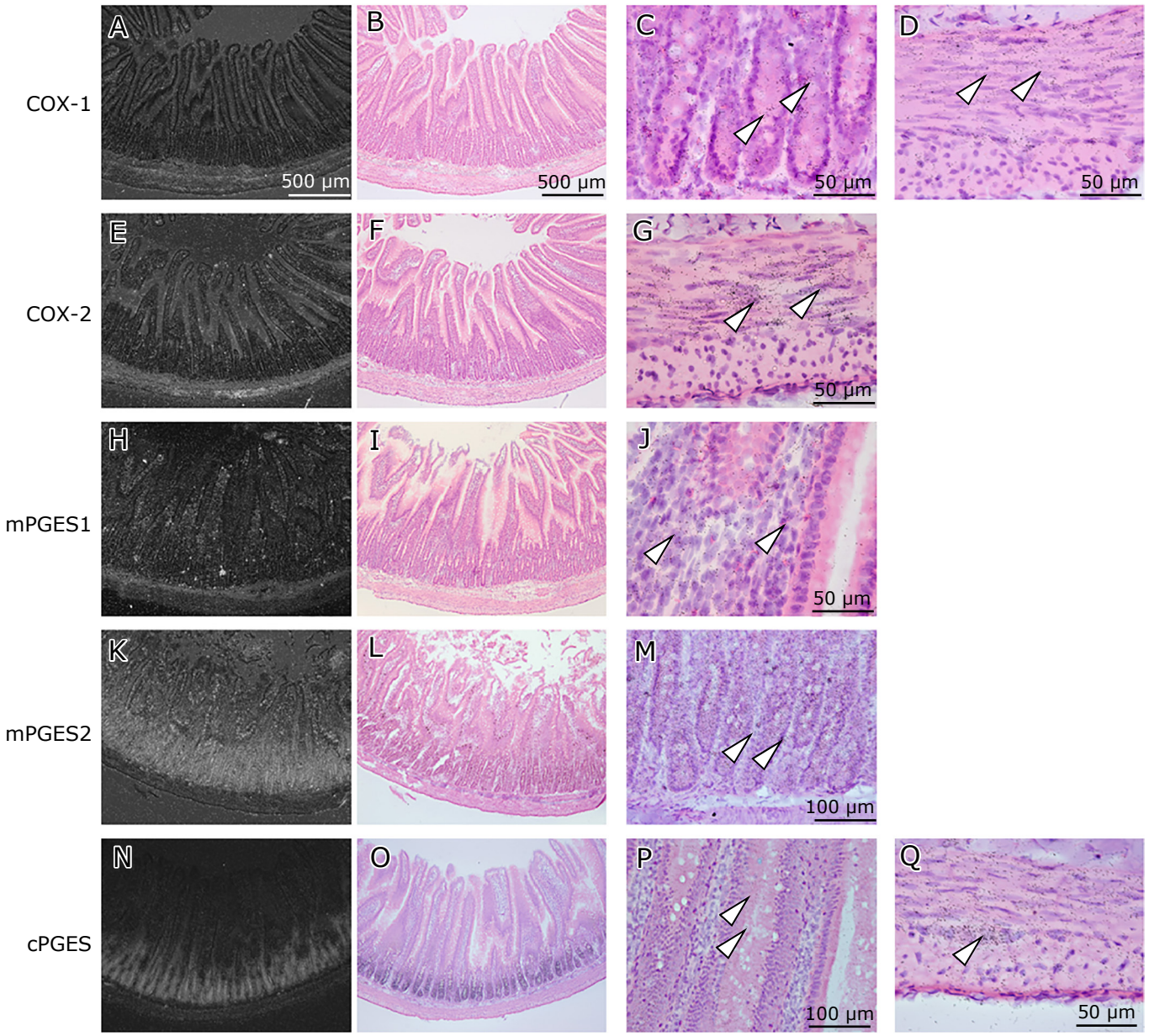

Fig. 2. COX-1, COX-2, cPGES, mPGES-1, and mPGES-2 mRNA in rat duodenal tissue. Arrowheads depict COX-1 (A–D), Cox-2 (E–G), mPges-1 (H–J), and mPGES-2 (K–M), and cPGES (N–Q) mRNA-positive cells. Dark field image (low-magnification: left) and bright field image (low-magnification: middle, high-magnification: right) showing ISHH products for EP receptors mRNA in the duodenum. Bright- and dark-field low-magnification images are the same field of view. Tissues were counterstained with hematoxylin for *in situ* hybridization.

microinflammation and mucosal permeability have been reported, which may result in hypersusceptibility to irritating duodenal contents.[17–19] Our recent study using a maternal deprivation rat model confirmed that duodenal microinflammation is closely associated with visceromotor dysfunction.[20] However, how such irritants activate the duodenum and cause visceromotor dysfunction remains unknown. Multiple speculations have been made regarding this; it is possible that irritants penetrate into the duodenal mucosa through intercellular spaces and stimulate nerve endings.[2–4] Besides the penetration theory, we hypothesized that the duodenal contents directly stimulate epithelial cells and produce the mediators from the mucosal cells, then excite the sensory nerves through the receptors on the duodenal mucosa. Prostaglandin is known to be one of the most famous and important chemical mediators and is located elsewhere in human body, and PGE_2 is believed to deeply involved in the inflammation of gastrointestinal tract. Although role of PGE_2 in the gastroduodenal mucosa have been reported, most described the effect of PGE_2 on mucosal protection or cytoprotection.[21,22] Therefore, present study focused on the relationship between FD and duodenal PGE_2.

In human esophageal mucosa, PGE_2 increment is mediated via the EP1 receptor, which plays an important role in the development of heartburn symptoms.[11–13] As shown in this study, acid increased PGE_2 levels in the duodenal mucosa, which could be mediated mainly by EP2, EP3, and EP4 receptors, but not EP1. The ISHH results in the current study showed that EP2 mRNA and EP4 mRNA were located in a relatively shallow layer, and that EP3 mRNA was located in relatively deeper layer, especially at the intermuscular plexus. In the present study, EP4 mRNA was found to be especially abundant in the epithelial surface area. PGE_2/EP4 signaling is known to affect both epithelial and

doi: 10.3164/jcbn.21-59

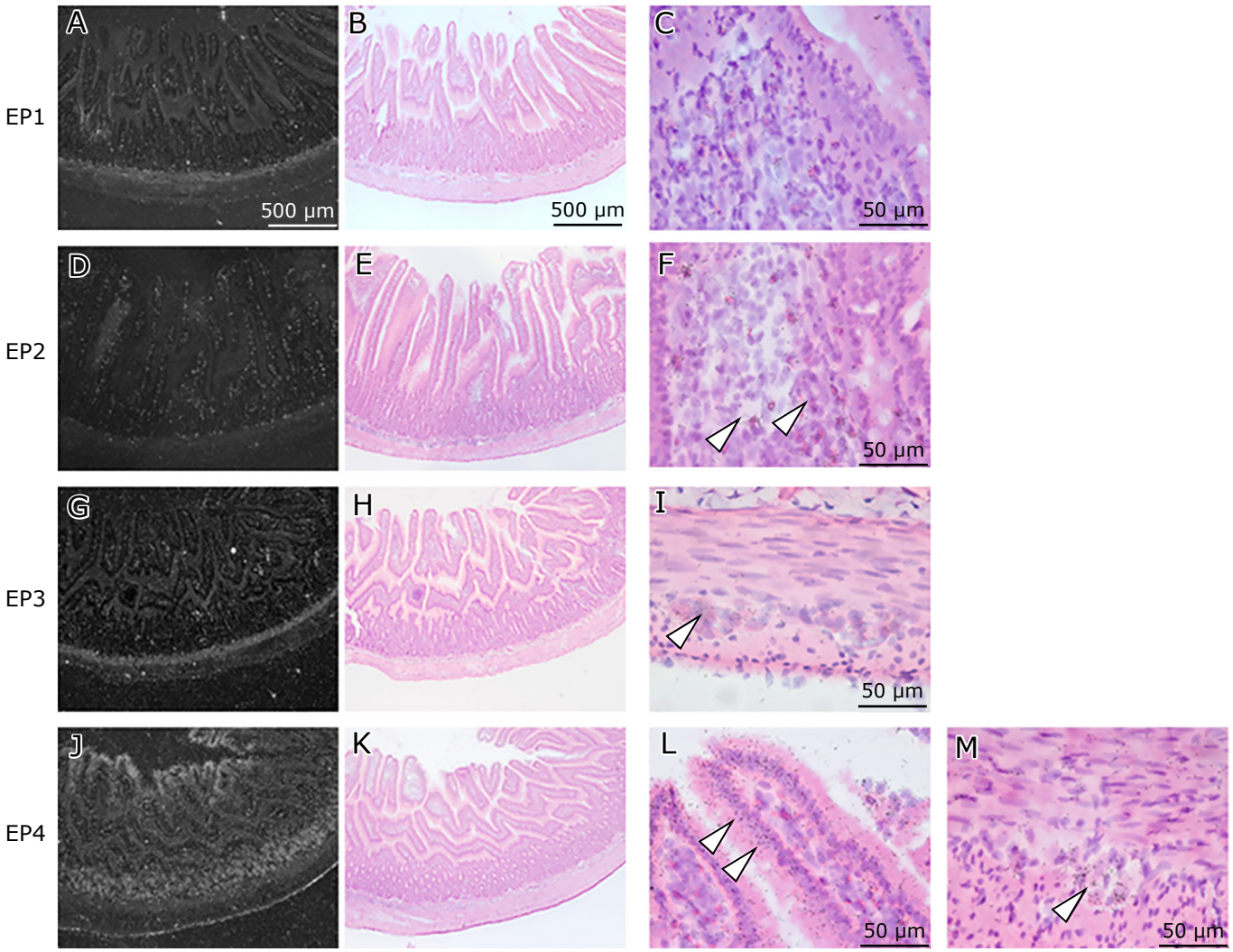

Fig. 3. EP1, EP2, EP3, and EP4 mRNA in rat duodenal tissue. Dark field image (low-magnification: left) and bright field image (low-magnification: middle, high-magnification: right) showing ISHH products for EP1 (A–C), EP2 (D–F), EP3 (G–I), and EP4 (J–M) receptors mRNA in the duodenum. Bright- and dark-field low-magnification images are the same field of view. Arrows depict mRNA-positive cells.

stromal cells, to regulate the intestinal immune system. Furthermore, the epithelial PGE_2/EP4 axis plays a crucial role in maintaining colonic homeostasis and in suppressing the development of inflammation[23,24] and PGE_2/EP2 signaling is known to be implicated in the gastric mucosal protective effect of capsaicin.[21] Thus, it is not surprising that EP2 and EP4 was mainly identified in the surface area.

Regarding the EP3 receptor, PGE_2/EP3 is reported to be associated with the regulation of gastrointestinal motility.[15] Although its exact role is still unknown, it is interesting that EP3 receptors were abundantly seen at the intermuscular plexus. Together with the findings that COX-1 mRNA, COX-2 mRNA, and PG synthases mRNA were mainly observed in the deep duodenal mucosa, the role of prostaglandin for the regulation of visceromotor function might be largely dependent on the EP3 receptor. Identifying other PGE synthases, such as mPGES-1 and mPGES-2, could confirm that the role of PGE_2 in the duodenal mucosa.

This study demonstrated the presence of PGE_2 receptors and PG synthases in the duodenum. Acid, a representative duodenal irritant, was found to increase PGE_2, which may play some roles via EP2, EP3, and EP4 receptors. Abundant EP3 receptors in the intermuscular plexus may especially imply the role of PGE_2 in the pathophysiological mechanism for dyspeptic symptom generation. The investigation of PGE_2 and its receptors in FD animal models and patients may address the question regarding the role of PGE_2 in the pathophysiology of functional dyspepsia.

In conclusion, PGE_2 levels in the duodenum increased following duodenal acidification. PGE_2 receptors (EP2, EP3, and EP4) as well as PGE_2 producing enzymes (mPGES-1, mPGES-2, cPGES, COX-1, and COX-2) were also observed in the rat duodenum. These observations suggest that duodenal PGE_2 may play roles in the symptom generation of FD.

Author Contributions

TF and SD performed experiments, analyzed data, and drafted the manuscript; TKondo designed the research, analyzed data, and drafted the manuscript; HM designed the research, analyzed data, and edited the manuscript; KK and HY perform ISH, analyzed data and revised the manuscript. YF, TKonemura, and HO analyzed data and revised the manuscript; TKono, HK, MF, TT, TO, and HF interpreted data and revised the manuscript. YD and KN edited the manuscript. All authors contributed to the interpretation of data, revised it critically and approved the final version of the manuscript.

Abbreviations

COX	cyclooxygenase
cPGES	cytosolic prostaglandin E synthase
FD	functional dyspepsia
HCl	hydrochloric acid
mPGES-1	microsomal prostaglandin E synthase-1
mPGES-2	microsomal prostaglandin E synthase-2
PGE_2	prostaglandin E_2

Grant

This work was supported by JSPS KAKENHI Grant Number 18K08019.

Ethics Approval

All procedures involving the care and use of animals were approved by the Hyogo University of Health Sciences Committee on Animal Research (No. 2018-15-2).

Conflict of Interests

The authors have no conflict of interest. YF, TK, and HO is employee of Ono Pharmaceutical Co. Ltd.

This work was partially supported by the grant by Ono Pharmaceutical Co. Ltd.

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doi: 10.3164/jcbn.21-59