Prostaglandin E2 mediates acid-induced heartburn in healthy volunteers

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Am J Physiol Gastrointest Liver Physiol 304: G568–G573, 2013. First published January 31, 2013; doi:10.1152/ajpgi.00276.2012.—Prostaglandin E2 (PGE2) plays a major role in pain processing and hypersensitivity. Several mechanisms have been proposed to explain the pathogenesis of heartburn in patients with gastroesophageal reflux disease (GERD) including peripheral production of inflammatory mediators and alterations in nociceptive processing leading to visceral hypersensitivity (11, 12). However, many mechanistic details are still unknown. Prostanoids, the products of arachidonic acid metabolism through the cyclooxygenase (COX) pathway, are some of the best-established inflammatory mediators, contributing to both inflammation and nociception. Several reports reveal that peripheral sensitization by inflammatory mediators cause threshold reduction of nociceptors in primary afferents, and contribute to primary hyperalgesia in human (29, 30). Among the prostanoids, prostaglandin E2 (PGE2) in particular is considered to have a major role in pain processing. In an animal model, elevation of PGE2 occurs in response to both chemical and mechanical stimuli (33), resulting in either direct activation or peripheral sensitization of nearby sensory nerve endings (18). Levels of PGE2 have been shown to increase within inflamed tissues in rodents (13, 20, 32, 35) and humans (29); peripheral administration of PGE2 reduces the threshold of nociceptive responses to a number of stimuli at the peripheral terminal (9, 16).

PGE2 exerts its biological action through binding to specific receptors, known as EP receptors. Four subtypes of PGE2 receptors, EP1, EP2, EP3, and EP4, have been identified. Among them, the EP1 receptor is considered to have a major role in pain processing. Thus pain hypersensitivity is attenuated in EP1-deficient mice (25) and after systemic or intrathecal administration of EP1 receptor antagonists (16, 19). In the rodent viscera, EP1 receptor antagonists inhibit bladder afferent nerve activity during bladder inflammation and attenuate cystitis-related bladder pain (10, 14). Furthermore, there is preclinical evidence that prior systemic treatment with an EP1 receptor antagonist attenuates acid-induced primary and secondary esophageal hyperalgesia in humans (24). It is thus accepted that PGE2, acting via the EP1 receptor, appears to have a significant role in visceral nociceptive processing at both peripheral and central levels (34). However, the role of PGE2 produced in the esophageal mucosa on specific symptom generation (heartburn) has not been systematically studied. This study aimed to investigate whether PGE2 levels are increased in the esophageal mucosa after acid infusion and whether such increases are associated with changes in esophageal sensation. Furthermore, the expression of the PGE2 receptor EP1 was investigated in normal human esophageal mucosa.

MATERIALS AND METHODS

Subjects. All experiments were performed in accordance with human ethics regulations (Hyogo College of Medicine: nos. 138, 977) with written, informed consent obtained. Fourteen healthy male volunteers (mean age 30, range 26–41 yr) were recruited for the acid-saline perfusion study; five separate healthy male volunteers (mean age 34, range 30–41 yr) were recruited for the real-time PCR (RT-PCR) and Western blotting study. None of the subjects had any previous abdominal surgery or GERD symptoms nor used proton-pump inhibitors (PPIs) or H2 antagonists before the study. Endoscopy excluded significant upper gastrointestinal pathology (esophagitis, Barrett’s esophagus, hiatus hernia, active peptic ulcer disease, esophageal/gastric cancer). Subjects were excluded whether they were taking any medications that may affect symptoms and prostaglandin generation, such as NSAIDs, COX-2 inhibitors, or prostaglandin-containing medications.

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**Trial protocol.** The trial was approved by the Ethics Committee of Hyogo College of Medicine and was conducted according to the principles governing human research in the Declaration of Helsinki (approval document nos. 138, 977).

Subjects were randomly assigned to acid infusion (n = 7, mean age 31 yr) or saline infusion (n = 7, mean age 30 yr). After an overnight fast, subjects were placed in the left lateral decubitus position and transnasal endoscopic examination (Olympus GIF-XP260N, Olympus Optical, outside diameter 5.5 mm) was performed to exclude pathology (see above) and to obtain baseline esophageal endoscopic biopsies, n = 1, 3 cm above the esophagogastric junction (EGJ) (Radial Jaw 3, Boston Scientific). After endoscopy, a 5-Fr elemental diet (ED) tube (Nippon Sherwood Medical Industries) was inserted via the nasal passage with the distal tip placed 10 cm above the EGJ, the location of which was determined during the first endoscopy. Acid or saline perfusions were performed (see Acid and saline perfusions below), and immediately thereafter the ED tube was removed and further endoscopic biopsies, n = 1, were retaken in an identical manner to the first endoscopy. Another five subjects were recruited for esophageal biopsies for the RT-PCR and Western blotting of EP1. Standard endoscopic biopsies, n = 2, 3 cm above the EGJ, were obtained.

Endoscopic esophageal mucosa samples were immediately frozen in a 1.5-ml sampling tube (ST-0150F, BIO-BIK, INA OPTIKA) with liquid nitrogen and stored in a freezer at −80°C until the measurement of PGE2 concentration and extractions of RNA and protein. Routine histological methods excluded disruption of the mucosa or infiltration of inflammatory cells in the esophageal epithelium in cryostat section and hematxylin-eosin stain (data not shown).

**Acid and saline perfusions.** These were performed by modification of a previously reported method (6, 7). The entire perfusion test was performed with the subjects in an upright sitting position. After confirmation that no subject had heartburn during the 2-min initial saline infusion (8 ml/min for 2 min), 1% hydrochloric acid (HCl 0.27 mol/l) or saline (NaCl) (as a control) was infused for 30 min into the lower esophagus via the ED tube. Infusions were delivered at a rate of 8 ml/min for 30 min with an automatic infusion pump (Terufusion System, Terumo). The sections were incubated at room temperature for 1 h in Alexa 488-conjugated donkey anti-goat IgG (1:100, Invitrogen) and Alexa 594-conjugated donkey anti-mouse IgG (1:100, Invitrogen). Negative controls were prepared as above with the primary antibody omitted. Slides were mounted in fluorescent mounting medium containing DAPI and exposed to horseradish peroxidase-labeled goat anti-rabbit IgG antibody (Vector Laboratories, PI-1000). After further rinsing, bands were detected with the Western Blotting Detection Reagents (ECL Plus; GE Healthcare UK). β-Actin was evaluated as a control.

**Protein localization by immunohistochemistry.** To confirm EP1 expression in the esophagus, surgically resected specimens of the lower esophagus were obtained from three patients undergoing total gastrectomy for gastric cancer not invading into the esophagus. Immediately after surgery, full-thickness blocks were dissected and were postfixed in 4% paraformaldehyde gel (NuPAGE Novex Bis-Tris Gels, Life Technologies) and transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P; EMD Millipore). After transfer, the PVDF membrane was immersed in blocking buffer (TBS-T containing 5% skim milk), then agitated for 2 h at room temperature. After blocking, the membranes were exposed to rabbit polyclonal anti-EP-1 antibody (1:1,000, ab95448, Abcam) and agitated overnight in a cold room. The membranes were then washed with TBS-T buffer and exposed to horseradish peroxidase-labeled goat anti-rabbit IgG antibody (Vector Laboratories, PI-1000). After further rinsing, bands were detected with the Western Blotting Detection Reagents (ECL Plus; GE Healthcare UK). β-Actin was evaluated as a control.

**Measurement of protein expression level by Western blotting.** Protein was extracted from the esophageal biopsies by homogenization. The concentration of protein in tissue extracts was measured with BCA Protein Assay Kit. Proteins were electrophoretically separated on a 10% polyacrylamide gel (NuPAGE Novex Bis-Tris Gels, Life Technologies) and transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P; EMD Millipore). After transfer, the PVDF membrane was immersed in blocking buffer (TBS-T containing 5% skim milk), then agitated for 2 h at room temperature. After blocking, the membranes were exposed to rabbit polyclonal anti-EP-1 antibody (1:1,000, ab95448, Abcam) and agitated overnight in a cold room. The membranes were then washed with TBS-T buffer and exposed to horseradish peroxidase-labeled goat anti-rabbit IgG antibody (Vector Laboratories, PI-1000). After further rinsing, bands were detected with the Western Blotting Detection Reagents (ECL Plus; GE Healthcare UK). β-Actin was evaluated as a control.

**Measurement of gene expression level by RT-PCR.** Total RNA was extracted from the lower esophageal mucosal biopsies by the acid phenol-chloroform method using TRIzol reagent (Life Technologies) and RNeasy Mini Kit (Qiagen). With the extracted total RNA as a template, reverse transcription was conducted with High Capacity RNA-to-cDNA Kit and Corbett (Cosmobio) and cDNA was prepared. Measurement of expression for EP1 mRNA was conducted by real-time PCR method with ABI PRISM 7900HT Fast Real-time PCR System (Applied Biosystems). The PCR primers employed were 5′-ACC AGA TCC TGG ACC CTT G-3′ (forward) and 5′-TAG AAG TGG CTG AGG CGG CCG-3′ (reverse) for human EP1 and 5′-CGA GAT ACC TCC AAA ATC AA-3′ (forward) and 5′-TGT GGT CAT GAG TCC TTC CA-3′ (reverse) for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping gene. Validation of the primers specific to the genes measured (EP1 and GAPDH) was checked by performing PCR with the relevant primers, conducting agarose electrophoresis with the solution after reaction, and detecting the single-target amplified fragment.

**Measurement of PGE2 concentration and gene expression level by RT-PCR.**

**Statistical analysis.** Time to first sensation and maximum heartburn severity scores were compared between saline and acid-infusion groups by nonparametric hypothesis tests (Mann-Whitney U-test using AUC data). PGE2 levels before and after perfusion test were compared between the saline and acid-infusion groups by two-way ANOVA followed by individual post hoc comparison analysis (Tukey’s test). In the acid-infusion group, changes in PGE2 were calculated as the difference between PGE2 levels in the esophageal mucosa before (baseline) and after acid infusion and used for Pearson correlation vs. AUC symptom scores. Data have been presented as means ± SE. Analyses were performed with SAS 9.2 TS2M3 (SAS Institute Japan) and its cooperative system ESSUS ver. 7.7.1 (CAC).
RESULTS

Generation of heartburn by acid or saline infusions. The time to first sensation (min) was less in the acid-infusion group compared with subjects receiving saline (saline infusion vs. acid infusion: 22.1 ± 4.1 vs. 5.4 ± 1.5, *P* < 0.05) (Fig. 1A). The symptom intensity score during infusion was also significantly greater with acid compared with saline: AUC data (saline infusion vs. acid infusion: 54.3 ± 13.1 vs. 178.5 ± 22.8, *P* < 0.01) (Fig. 1B).

Changes in PGE2 expression in the esophageal mucosa after acid or saline infusions. PGE2 levels in the esophageal mucosa were significantly increased after infusion compared with baseline in the acid but not the saline group (before acid vs. after acid: 23.2 ± 8.6 vs. 68.6 ± 18.3, *P* < 0.05; before saline vs. after saline: 9.3 ± 2.5 vs. 9.0 ± 3.2, NS; overall model *P* < 0.01; two-way ANOVA with Tukey’s post hoc test analysis) (Fig. 2A). In the acid-infusion group, symptom intensity score correlated with change in PGE2 level (delta PGE2) (*r* = 0.80, *P* = 0.029) (Fig. 2B).

Detection of EP1 mRNA and protein in the esophageal mucosa. Both EP1 transcript expression and protein were detected in esophageal mucosal biopsies from the five male healthy subjects studied (Fig. 3, A and B).

DISCUSSION

The prevalence of GERD continues to rise in Western populations (5, 22), with heartburn likely to remain the most...
common symptom. Although PPIs are rightly the main therapy, according to meta-analysis, up to 40% of GERD patient are dissatisfied with standard PPI therapy because symptoms are not fully resolved (2–4, 17). Hence, there are still unmet clinical needs in the treatment of GERD. For this reason, it remains important to investigate new mechanisms of symptom generation for heartburn. The present study has demonstrated increased PGE₂ production in the esophageal mucosa after acid but not saline perfusion in healthy volunteers. Protein expression levels were correlated with severity of heartburn, and, from a mechanistic perspective, expression of the PGE₂ receptor EP1 was confirmed in the human esophageal mucosa.

The study is acknowledged to be limited by the small number of subjects and absence of confirmation in patients with GERD. A further criticism is that although PGE₂ levels in esophageal biopsy samples were measured before and after acid or saline infusion, symptoms were measured only during infusion and not at baseline (although subjectively no subject had any heartburn at baseline). This makes interpretation of the variability of PGE₂ levels at baseline (higher in the acid-allocated group) difficult. Although this was probably accounted for by natural variation due to the small number of study subjects, it would have been desirable to observe whether this too correlated with baseline heartburn symptoms. Nevertheless, the controlled before-and-after design eliminated any gross influence of several factors affecting PGE₂ production, for example the effect of liquid infusion, tissue damage by biopsy, and the endoscope itself. Furthermore, esophageal biopsy samples were directly frozen in liquid nitrogen, thus theoretically reducing the possibility of contamination of PGE₂ from gastric or salivary secretions or their effects on the production or metabolism of the PGE₂. Another criticism is whether PGE₂ levels in the esophageal mucosa could be increased in such a short period of time (30 min) by acid infusion. Previous reports support our data; for instance, exposure of taurocholic acid or 1.0 M NaCl in the rat stomach for 30 min caused significant increases of PGE₂ in the stomach in vivo (26–28). The mechanisms by which such rapid changes occur remain unclear, and

Fig. 3. Detection of EP1 mRNA and protein in the esophageal mucosa. EP1 mRNA (181 bp) (A) and protein (42 kDa) (B) were detected in esophageal mucosal biopsies from the 5 male healthy subjects studied by RT-PCR and Western blotting. M, marker.

Fig. 4. Immunohistochemical localization of EP1 in the human esophagus. Hematoxylin and eosin staining of the epithelium (A), muscularis mucosae (E), and submucosa (I) of the human esophagus. B–D, F–H, and J–L show immunoreactivity (IR) for EP1 (green), PGP9.5 (red), and DAPI (blue). Positive EP1 staining was detected in the cytoplasmic site mostly within the upper and middle cell layer of the esophageal epithelium (DAPI staining showed that EP1 was not localized in the nucleus) (D). Some nerve fiber-like structures labeled for EP1 were detected in the muscularis mucosae (F) and submucosa (J). Double-immunofluorescent staining was performed to compare EP1 expression with PGP9.5-IR nerve fibers. PGP9.5-IR nerve fibers were demonstrated in the muscularis mucosae (G) and submucosa (K) and some colocalized with EP1 (H and L, arrowheads). Scale bar 80 μm.
further investigation, such as analysis of COX-2 in the esophageal mucosa, will be required to explore this.

PGE$_2$ is known to induce sensitization and cause pain hypersensitivity (primary hyperalgesia) at the level of peripheral nerve endings (9, 16). Furthermore, subcutaneous or systemic administration of EP1 antagonists attenuate peripheral hypersensitivity and hyperalgesia induced by PGE$_2$ (14, 21). In the viscera, previous reports have shown that PGs were synthesized in the smooth muscle and urothelium (1). Cystitis or physiological stimuli in the urinary bladder induced increased PGE$_2$ expression (23, 33). An EP1 receptor antagonist inhibited bladder afferent nerve activity during bladder inflammation and attenuated cystitis-related bladder pain in rats (10, 14). Furthermore, EP1 receptors are expressed not only in the primary afferents (19), but also in the urothelium in rats (31). The present study has adopted a similar approach with similar findings in the human lower esophagus suggesting that the PGE$_2$/EP1 mechanism is broadly similar across the visceras and conserved across species. In fact, immunohistochemical analysis showed that EP1 expression was localized both in the cytoplasm of epitheliocytes and in some nerve fibers within the submucosa. These data indicate that intraepithelial PGE$_2$ induced by acid infusion might have both indirect (within the epithelium) and direct (within the submucosa) effects on submucosal nerves of esophagus through EP1.

Further studies are clearly needed to investigate the expression of PEG2 and EP1 in the esophagus of GERD and non-erosive reflux disease patients, and to correlate such changes with GERD symptoms in the clinical context. Such studies are supported by the experimental medicine study of Sarkar et al. (24) which demonstrated the effect of an EP1 receptor antagonist in attenuating the development of primary and secondary hyperalgesia (central as well as peripheral sensitization) in a human healthy volunteer model of distal esophageal acid infusion. Given the proven algic effects of PGE$_2$ at central as well as peripheral levels (15, 19), and the potential that PGE$_2$/EP1 have effects on other nociceptive molecular pathways, e.g., TRPV1 (18), further clinical development of EP1 antagonists is merited.

In conclusion, we found that PGE$_2$ levels were increased in the esophageal mucosa after acid infusion in healthy subjects and such PGE$_2$ increases were correlated with changes in esophageal sensation (heartburn). Furthermore, EP1 receptor expression could be detected in the human esophageal mucosa and submucosal nerves. Blockade of PGE$_2$ production or use of an EP1 receptor antagonist might be a new therapeutic strategy for reducing heartburn.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES


