Role of prostaglandin D$_2$ in mast cell activation-induced sensitization of esophageal vagal afferents

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Submitted 19 November 2012; accepted in final form 1 March 2013

Zhang S, Grabauskas G, Wu X, Joo MK, Heldsinger A, Song I, Owyang C, Yu S. Role of prostaglandin D$_2$ in mast cell activation-induced sensitization of esophageal vagal afferents. Am J Physiol Gastrointest Liver Physiol 304: G908–G916, 2013. First published March 7, 2013; doi:10.1152/ajpgi.00448.2012.—Sensitization of esophageal afferents plays an important role in esophageal nociception, but the mechanism is less clear. Our previous studies demonstrated that mast cell (MC) activation releases the preformed mediators histamine and tryptase, which play important roles in sensitization of esophageal vagal nociceptive C fibers. PGD$_2$ is a lipid mediator released by activated MCs. Whether PGD$_2$ plays a role in this sensitization process has yet to be determined. Expression of the PGD$_2$ DP$_1$ and DP$_2$ receptors in nodose ganglion neurons was determined by immunofluorescence staining, Western blotting, and RT-PCR. Extracellular recordings were performed in ex vivo esophageal-vagal preparations. Action potentials evoked by esophageal distension were compared before and after perfusion of PGD$_2$, DP$_1$, and DP$_2$ receptor agonists, and MC activation, with or without pretreatment with antagonists. The effect of PGD$_2$ on 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI)-labeled esophageal nodose neurons was determined by patch-clamp recording. Our results demonstrate that DP$_1$ and DP$_2$ receptor mRNA and protein were expressed mainly in small- and medium-diameter neurons in nodose ganglia. PGD$_2$ significantly increased esophageal distension-evoked action potential discharges in esophageal nodose C fibers. The DP$_1$ receptor agonist BW 245C mimicked this effect. PGD$_2$ directly sensitized DiI-labeled esophageal nodose neurons by decreasing the action potential threshold. Pretreatment with the DP$_1$ receptor antagonist BW A868C significantly inhibited PGD$_2$ perfusion- or MC activation-induced increases in esophageal distension-evoked action potential discharges in esophageal nodose C fibers. In conclusion, PGD$_2$ plays an important role in MC activation-induced sensitization of esophageal nodose C fibers. This adds a novel mechanism of visceral afferent sensitization.

esophagus; mast cell; PGD$_2$; vagal afferent; C fiber

ESOPHAGEAL DISCOMFORT, such as heartburn and esophageal-related chest pain, are the predominant symptoms of many esophageal disorders. However, the mechanism of esophageal nociception is not well defined. Tissue injury or inflammation in the esophagus may sensitize esophageal afferents to induce esophageal hyper-sensitivity and nociception (20). However, the mechanism whereby inflammatory mediators induce sensitization on esophageal peripheral afferents is less clear (19).

Mast cells (MCs) are found in abundance in the gastrointestinal (GI) tract. Inappropriate MC activation is a pivotal event in allergic reactions, such as food allergies, in the GI tract (30). Activation of MCs leads to production of various mediators, which may also participate in the immune response (30). They may also cause short- and long-lasting changes in the excitability and phenotype of juxtaposed nerves (4), which leads to GI sensory/motor dysfunctions (3). Our previous study provided the first direct evidence that selective activation of tissue MCs leads to a profound and long-lasting increase in the excitability of nodose C fibers in the esophagus (32). Histamine H$_1$ receptor antagonists inhibit the increased mechanoe-xcitability of esophageal nodose C fibers when given prior to, but not after, MC activation. In addition, exogenous histamine initiates, but does not maintain, the potentiation effect of MC activation on esophageal nodose C fibers (32). This indicates that although histamine is essential, additional mediators are critical in this potentiation process.

Prostaglandin D$_2$ (PGD$_2$) is an important lipid mediator that is released mainly by activated MCs. The biological effects of PGD$_2$ are usually mediated by its two G protein-coupled receptors: D-type prostanoid receptor 1 (DP$_1$) and D-type prostanoid receptor 2 (DP$_2$), also known as chemoattractant homologous receptor expressed on Th2 cells (CRTH2). PGD$_2$ has been recognized to regulate central (14, 28) and peripheral (3, 10, 15) nerve functions. These neuronal regulation effects are mediated mainly through the DP$_1$ receptor (17, 27), but the effect of PGD$_2$ on sensory afferents in the GI tract is largely unknown. The present study aims to determine the role of PGD$_2$ in MC activation-induced sensitization of esophageal afferents. We hypothesize that PGD$_2$ selectively sensitizes esophageal vagal afferent C fibers via the DP$_1$ receptor.

METHODS

Male Hartley guinea pigs (Hilltop Laboratory Animals, Scottsdale, PA; 100–300 g body wt) were used in these experiments, which were approved by the University of Michigan Committee on Use and Care of Animals.

Immunofluorescence staining. Nodose ganglia ($n = 4$) were dissected and processed for immunofluorescence double staining of PGD$_2$ DP$_1$ and DP$_2$ receptors with isoelectin B4 (IB4), as described previously (31). The primary antibodies included rabbit polyclonal anti-DP$_1$ receptor antibody (1:200 dilution; Cayman Chemical, Ann Arbor, MI) and rabbit anti-DP$_2$ receptor polyclonal antibody (1:200 dilution; Novus Biologicals, Littleton, CO). The secondary antibodies included Alexa Fluor 594-conjugated anti-rabbit antibody (1:200 dilution; Invitrogen, Carlsbad, CA). IB4 was detected by IB4-conjugated biotin (Invitrogen) and further stained by streptavidin conjugated to Alexa Fluor 488 (Invitrogen). The stained slides were briefly checked under an epifluorescence microscope, and four well-stained cross sections on each slide from each nodose ganglion were randomly selected for counting. Quantitative analysis of immunofluorescent-stained neurons on digitized images was determined by threshold intensity segmentation using iVision software (Biovision Technologies, Exton, PA); neurons were considered to be positive if fluorescence intensity was two times background fluorescence. In general, 20

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DP1 receptor-positive neurons from each section were selected. Then colocalization of DP1 or DP3 receptors with IB4 was determined. A total of 240 nodose neurons (n = 4 ganglia × 4 or 2 sections × 20 neurons = 280; 1 ganglion had only 2 sections to count) were counted for DP1 receptors and colocalization with IB4. A total of 300 nodose neurons (n = 4 ganglia × 3 or 4 sections × 20 neurons = 300; 1 ganglion had only 3 sections to count) were counted for DP3 receptors and colocalization with IB4.

Western blotting. Equal amounts of lysates (20 μg) freshly obtained from nodose ganglia (n = 4) were separated on Ready Gel 12% Tris-HCl, transferred to nitrocellulose Hybond ECL membranes, and blotted overnight with primary antibodies and then for 1 h with secondary antibodies. The membranes were exposed to enhanced chemiluminescence buffer (Pierce, Rockford, IL) for 30 s or 5 min and then high-chemiluminescence film in darkness. The resulting bands were scanned and analyzed. The primary antibodies used for Western blotting included rabbit anti-DP1 receptor polyclonal antibody (1:1,000 dilution; Cayman Chemical) and rabbit anti-DP3 receptor polyclonal antibody (1:1,000 dilution; Novus Biologicals). The secondary antibodies included goat anti-rabbit IgG-horseradish peroxidase (1:1,000 dilution; Pierce) and rabbit anti-DP2 receptor polyclonal antibody (1:1,000 dilution; Novus Biologicals). Western blot products were loaded in a 1.2% Tris-borate-EDTA-buffered agarose gel. GAPDH, served as an internal control. The PCR bands were scanned and analyzed. The primary antibodies used for Western blotting included rabbit anti-DP1 receptor polyclonal antibody (1:1,000 dilution; Cayman Chemical) and rabbit anti-DP3 receptor polyclonal antibody (1:1,000 dilution; Novus Biologicals). The secondary antibodies included goat anti-rabbit IgG-horseradish peroxidase (1:1,000 dilution; Pierce) and rabbit anti-DP2 receptor polyclonal antibody (1:1,000 dilution; Novus Biologicals). Western blot products were loaded in a 1.2% Tris-borate-EDTA-buffered agarose gel. GAPDH, served as an internal control. The PCR bands were scanned and analyzed.

RT-PCR. TRizol reagent (Invitrogen) was used to freshly extract total RNAs from nodose ganglia, the dorsal root ganglion (DRG), and the brain. Immediately thereafter, cDNAs were synthesized with reverse transcriptase (Promega, Madison, WI) using 5 μg of total RNA. The resultant cDNAs were used for PCR, which was performed with Taq DNA polymerase (Promega) through 30 cycles of denaturation (1 min at 94°C), annealing (2 min at 50°C), and extension (3 min at 72°C), with a final extension period of 10 min at 72°C. We designed two pairs of primers according to the published sequences for guinea pig DP1 receptor (accession no. XM_003471928) and DP2 receptor (accession no. NM_001172722): guanosine diphosphatase (GDP) 1 [5'-GCTGATCTCCACTCTATGGCC-3' (forward) and 5'-GAG-GTGTCCGGGTCTTCTC-3' (reverse)] for the DP1 receptor and GDP2 [5'-CAGCAACTCCAGCGGCTA-3' (forward) and 5'-GTGTT-GAGCACGCCAGCCG-3' (reverse)] for the DP2 receptor.

For RT-PCR, total RNA was isolated from each mouse, and the cDNA was synthesized using the M-MLV reverse transcriptase (Promega) using 5 μg of total RNA. The resultant cDNA was used for PCR, which was performed with Taq DNA polymerase (Promega) through 30 cycles of denaturation (1 min at 94°C), annealing (2 min at 50°C), and extension (3 min at 72°C), with a final extension period of 10 min at 72°C. We designed two pairs of primers according to the published sequences for guinea pig DP1 receptor (accession no. XM_003471928) and DP2 receptor (accession no. NM_001172722): guanosine diphosphatase (GDP) 1 [5'-GCTGATCTCCACTCTATGGCC-3' (forward) and 5'-GAG-GTGTCCGGGTCTTCTC-3' (reverse)] for the DP1 receptor and GDP2 [5'-CAGCAACTCCAGCGGCTA-3' (forward) and 5'-GTGTT-GAGCACGCCAGCCG-3' (reverse)] for the DP2 receptor.

Immunochemistry. Mouse and guinea pig DRG ganglia were isolated, and the resultant activity was displayed on an oscilloscope (mode TDS 340, Tektronix, Beaverton, OR) and a chart recorder. The data were stored and analyzed using TheNerveOff software (sampling frequency = 33 kHz; PHOCIS, Baltimore, MD). The nerve fiber was considered a C fiber if it conducted action potentials at <1 m/s. Conduction velocity was calculated by dividing the length of the approximated nerve pathway (from the recorded nodose neurons to the mechanosensitive receptive field in the esophagus) by conduction time. The peaks of action potential discharges of nodose C fibers in response to different treatments/stimuli were analyzed and compared. The compounds used in the electrophysiology experiment were as follows: ovalbumin (OVA; an antigen for MC activation), PGD2 (a MC mediator), capsaicin [a transient receptor potential (TRP) vanilloid subfamily member 1 (TRPV1) agonist, which usually activates C fibers], allyl isothiocyanate [AITC; a TRP subfamily A1 number 1 (TRPA1) agonist, which usually activates C fibers], and α,β-methylene-ATP (a P2X2/3 agonist, which usually activates Aδ fibers) (all from Sigma-Aldrich, St. Louis, MO), and the PGD2 DP1 receptor agonist BW 245C, the DP1 receptor antagonists AW 8686C, the PGD2 DP2 receptor agonist 15(R)-PGD2, and the DP2 receptor antagonist BAY-u-3405 (all from Cayman Chemical). The compounds were diluted in Krebs bicarbonate solution to final concentration on the day of use.

Patch-clamp recording. In a separate study, 1,1'-dioc-tadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes, Eugene, OR) retrograde labeling of nodose neurons from the esophagus was performed in two guinea pigs according to our previously described method (33). Dil (1 μl of 1% Dil solution diluted in 50% dimethylsulfoxide in saline) was injected into the wall of the esophagus 50 mm above the gastric-esophageal junction. Each esophagus received three injections. Four nodose ganglia (2 from each animal) were collected 10 days later and processed for whole cell patch-clamp recordings, as we previously described (12). Briefly, nodose ganglia from normal guinea pigs were minced in a 35-mm culture dish (containing Ca2+- and Mg2+-free Hanks’ balanced salt solution with penicillin and streptomycin) and then placed in a 1.5 ml centrifuge tube containing digestion buffer (dispase II and collagenase IA, 1 mg/ml; Roche Applied Science). After 60 min of incubation (37°C), cells were dispersed by gentle trituration through Pasteur pipettes and washed in DMEM. After resuspension in L15 medium (GIBCO BRL/Invitrogen) containing 10% fetal bovine serum, the cells were placed onto poly-L-lysine-coated (100 μg/ml) coverslips for 30 min and cultured in DMEM with 10% fetal calf serum at 37°C. Neurons were stuck to coverslips and maintained in culture for 24–48 h at 37°C prior to recording. Recordings were performed under an inverted microscope (Diaphot 200, Nikon) using patch pipettes with an access resistance of 3–5 MΩ and an internal solution consisting of (in mM) 130 potassium glutamate, 10 HEPES, 1 EGTA, 1 MgCl2, 1 CaCl2, 1 ATP, and 0.5 GTP, with pH adjusted to 7.3. Whole cell currents were measured using a patch-clamp amplifier (Axopatch 200B, Molecular Devices, Sunnyvale, CA), digitized (DIGIDATA 1322A, Molecular Devices), and recorded with a personal computer using pCLAMP9 software (Molecular Devices). Immunofluorescence staining of DP1 receptors and their colocalizations with Dil in dissociated nodose neurons was performed as described previously (31).

Data analysis. In our extracellular study, we analyzed only the results from capsaicin- or AITC-responsive nodose C fibers, which were confirmed by the end of each recording to indicate that the nerve terminals were exposed to chemical perfusion. We recorded afferent nerve activities from one nodose fiber (C or Aδ fiber) per animal, so the number of recorded fibers equals the number of animals used in the study. The distension-evoked nerve response was quantified as the peak frequency of the action potential discharge within a 20-s distension period. Peak frequency (Hz) of the action potential discharges is presented as mean ± SE and compared by one-way ANOVA or Student’s t-test. For all experiments, significance was defined as P < 0.05.

RESULTS

PGD2-induced mechanical hypersensitivity in esophageal nodose C fibers. To determine the effect of PGD2 on esophageal vagal afferent subtypes, we used ex vivo esophageal vagal preparations to perform extracellular recordings from nodose
gallion neurons with intact nerve endings in the esophagus. Esophageal distension-evoked action potentials were compared before and after PGD2 perfusion. According to our previous studies (32, 33), the response to the particular stimulus was considered positive when the stimulus evoked an action potential discharge with a peak frequency of 3 Hz (in the fibers with no baseline activity) or a peak frequency at least three times the frequency of baseline activity. In esophageal nodose C fibers, 30 min of perfusion with PGD2 (10 ìl) did not evoke action potential discharges (0.6 ± 0.3 and 1.6 ± 0.5 Hz at baseline and with PGD2, respectively) but substantially increased their responses to esophageal distension. The peaks of action potential discharges (with subtraction of baseline spontaneous activity and PGD2-induced activity) evoked by 20-s esophageal distensions significantly increased from 3.6 ± 0.4, 7.7 ± 0.6, and 11.9 ± 0.7 Hz to 7.9 ± 0.8, 15.4 ± 1.1, and 21 ± 1.0 Hz at distension pressures of 10, 30, and 60 mmHg, respectively (P < 0.01, n = 12; Fig. 1, A and B). The increased action potential discharges were sustained after 30 min of PGD2 washout with fresh buffer in six C fibers. In these six C fibers, the peaks of action potential discharges evoked by esophageal distensions did not significantly change before and after 30 min of PGD2 washout (from 8.0 ± 1.3, 14.7 ± 1.5, and 21.3 ± 1.4 Hz to 7.3 ± 0.7, 15.5 ± 2.1, and 23 ± 2.3 Hz at distension pressures of 10, 30, and 60 mmHg, respectively, P > 0.05, n = 6). All nodose C fibers were confirmed to respond to the TRPA1 agonist AITC (1 mM, peak = 10.8 ± 1.4, n = 12) at the end of each study, indicating that the recorded nerve terminals were exposed to perfused drug. In esophageal nodose Aδ fibers, 30 min of perfusion with PGD2 (10 ìl) neither evoked action potential discharges (3.8 ± 0.5 and 4.3 ± 0.5 Hz at baseline and with PGD2, respectively, n = 4) nor changed responses to esophageal distension. The peaks of action potential discharges (with subtraction of baseline spontaneous activity) evoked by 20-s esophageal distensions were not significantly changed from 21 ± 1.8, 25 ± 3, and 27.8 ± 3.2 Hz to 20.5 ± 1.6, 27 ± 2.7, and 27 ± 1.1 Hz at distension pressures of 10, 30, and 60 mmHg, respectively (P > 0.05, n = 4; Fig. 1C). These nodose Aδ fibers were confirmed to respond to the P2X receptor agonist α,β-methylene-ATP (30 ìM, n = 4) at the end of each study, indicating that the recorded nerve terminals were exposed to perfused drug.

These data demonstrate that PGD2 does not directly activate esophageal vagal afferents but selectively sensitizes nodose C fibers to increase their responses to esophageal distension. This suggests an important role of PGD2 in initiating mechanical hypersensitivity of esophageal vagal nociceptive afferents.

Expression of PGD2 DP1 and DP2 receptors in nodose ganglion neurons. The biological effects of PGD2 are usually mediated by two G protein-coupled receptors, designated DP1 and DP2. To determine which receptors participated in PGD2-induced mechanical hypersensitivity in esophageal nodose C fibers, we first examined their expressions in nodose neurons by immunostaining. DP1 and DP2 receptor expression was identified in nodose neurons (Fig. 2, A–H). Among 280 nodose neurons (4 nodose ganglia from 4 guinea pigs × 3–4 sections × 20 neurons), ~80% (224 of 280) of DP1 receptor-positive neurons were small- and medium-sized (<30 ìm diameter), and 51% of DP1 receptor-positive neurons were also positively labeled with IB4. Similarly, among 300 neurons, ~93% (280 of 300) of DP2 receptor expression was detected in small- and medium-diameter nodose neurons, and 60% of DP2 receptor-positive neurons were also colocalized with IB4.

Western blot analysis with anti-DP1 or anti-DP2 receptor antibodies also showed the presence of DP1 and DP2 receptors in nodose ganglia. Probing with DP1 or DP2 receptor antibodies revealed moderately immunoreactive bands in lysates from nodose and DRG ganglia. Strongly immunoreactive bands were also found in the brain, which is known to express high levels of DP1 and DP2 receptors (Fig. 2, J and K). Then, using RT-PCR, we explored DP1 and DP2 receptor mRNA expression in nodose ganglia. Two PCR products, 283 bp for DP1 receptor and 420 bp for DP2 receptor, were identified from RNA extracted from nodose ganglia and were also detected in positive (DRG and brain) controls (Fig. 2f).

Our studies consistently identified the expression of DP1 and DP2 receptors in nodose ganglia by immunostaining, Western blotting, and RT-PCR. The high expression rate (80–90%) of PGD2 receptors in small- and medium-diameter neurons is in agreement with the findings from our extracellular recordings showing that PGD2 selectively sensitizes nodose C fibers (but not Aδ fibers), which usually have small- or medium-sized cell bodies (neurons) situated in the ganglia.

DP1, but not DP2, receptor agonist, mimicked the effect of PGD2-induced mechanical hypersensitivity in esophageal nodose C fibers. To determine the roles of DP1 or DP2 receptors in PGD2-induced mechanical hypersensitivity in nodose C fibers, we tested the hypothesis that DP1 and/or DP2 receptor

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**Fig. 1.** PGD2 on esophageal nodose afferent subtypes. A: perfusion with PGD2 increased mechanoeexcitability of esophageal nodose C fibers. Action potential discharges evoked by esophageal distensions significantly increased at distension pressures of 10, 30, and 60 mmHg. Values are means ± SE (n = 12), ∗P < 0.01. B: typical traces of action potential discharges from esophageal nodose C fibers at distension pressure of 60 mmHg for 20 s before and after 30 min of perfusion with PGD2 (10 ìM). C: perfusion with PGD2 did not change mechanoeexcitability of nodose Aδ fibers. Values are means ± SE (n = 4), P > 0.05.
agonists mimicked the PGD2-induced sensitization effect. We chose the selective DP1 receptor agonist BW 245C and the DP2 receptor agonist 15(R)-PGD2 for our experiments. Perfusion with the DP1 receptor agonist BW 245C (10 μM) for 30 min did not evoke action potential discharges (1.2 ± 0.3 Hz at baseline vs. 1.2 ± 0.3 Hz with BW 245C, n = 8) in esophageal nodose C fibers. However, this was associated with a significant increase in their responses to esophageal distension. The peaks of action potential discharges (with subtraction of baseline spontaneous activity and DP1 receptor agonist-induced activity) evoked by 20-s esophageal distensions significantly increased from 6.4 ± 1.8, 12 ± 2.3, and 17 ± 3.4 Hz to 12.1 ± 3.5, 18.1 ± 3.6, and 26 ± 4.8 Hz at distension pressures of 10, 30, and 60 mmHg for 20 s, respectively (P < 0.05, n = 8; Fig. 3A). In another set of experiments, 30 min of perfusion with the DP2 receptor agonist 15(R)-PGD2 (2–6 μM) did not significantly evoke action potential discharges [1.6 ± 0.4 Hz at baseline vs. 2.6 ± 0.8 Hz with 15(R)-PGD2, n = 5]. The action potentials evoked by esophageal distension slightly increased but did not reach significance before and after perfusion with 15(R)-PGD2 (1.8 ± 0.3, 5.8 ± 1.1, and 10.5 ± 1 Hz vs. 2 ± 0.4, 5.5 ± 0.9, and 11 ± 1 Hz at distension pressures of 10, 30, and 60 mmHg, respectively, P > 0.05, n = 5; Fig. 3B). These nodose C fibers were confirmed to respond to the TRPA1 agonist AITC (1 mM) and/or the TRPV1 agonist capsaicin (1 μM) at the end of each study. The finding that the DP1 receptor agonist mimicked the effect of PGD2-induced mechanical hypersensitivity suggests that the DP1 receptor has a role in the PGD2-induced sensitization effect in esophageal vagal nodose C fibers.

**DP1 receptor antagonist, but not DP2 receptor antagonist, prevented PGD2-induced mechanical hypersensitivity in esophageal nodose C fibers.** To further confirm the involvement of DP1 and/or DP2 receptors in PGD2-induced sensitization of esophageal nodose C fibers, we tested the hypothesis that pretreatment with DP1 or DP2 receptor antagonists prevented PGD2-induced mechanical hypersensitivity in esophageal vagal nodose C fibers. First, we determined whether perfusion with the DP1 receptor antagonist BW A868C changed the mechanoexcitability of esophageal nodose C fibers. Before and after 30 min of perfusion with BW A868C (10 μM), action potentials evoked by esophageal distension did not change significantly.

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**Fig. 2. DP1 and DP2 receptor expression in nodose neurons.** A–C and E–G: immunofluorescence staining of DP1 and DP2 receptors and their colocalizations with isolectin B4 (IB4) in nodose ganglion neurons. About 80% (224 of 280) of DP1 receptor-positive neurons were small- and medium-sized (<30 μm diameter) (D), and 51% of DP1 receptor-positive neurons were also positively labeled with IB4 (C). About 93% (280 of 300) of DP2 receptor-positive neurons were small- and medium-sized (<30 μm diameter) (H), and 60% of DP2-positive neurons were also positively labeled with IB4 (G). DP1 and DP2 receptor proteins were revealed by Western blotting (J and K), and DP1 and DP2 receptor mRNA were detected by RT-PCR (J) in guinea pig nodose ganglia (NG) compared with positive controls [dorsal root ganglion (DRG) and brain].
Fig. 3. DP₁ and DP₂ receptor agonists/antagonists on esophageal nodose C fibers. A: perfusion with the DP₁ receptor agonist BW 245C in esophageal vagal preparations increased mechanoeexcitability of esophageal nodose C fibers. Action potential discharges evoked by esophageal distensions significantly increased at distension pressures of 10, 30, and 60 mmHg. Values are means ± SE (n = 8). *P < 0.05. B: perfusion with the DP₂ receptor agonist 15(R)-PGD₂ did not change mechanoeexcitability of esophageal nodose C fibers. Values are means ± SE (n = 5). P > 0.05. C: after 30 min of perfusion with and in the presence of the DP₁ receptor antagonist BW A868C, PGD₂ perfusion did not significantly change action potential discharges evoked by esophageal distension. Values are means ± SE (n = 8). P > 0.05. D: after 30 min of perfusion with and in the presence of the DP₂ antagonist BAY-u3405, esophageal distension-evoked action potential discharges significantly increased before and after PGD₂ perfusion. Values are means ± SE (n = 7); *P < 0.05.

(2.5 ± 0.3, 4.8 ± 0.5, and 8.1 ± 0.8 Hz vs. 2.9 ± 0.4, 5.0 ± 0.4, and 9.8 ± 0.9 Hz at distension pressures of 10, 30, and 60 mmHg, respectively, P > 0.05, n = 8). Next, we studied the inhibitory effect of BW A868C on PGD₂-induced mechanical hypersensitivity. After 30 min of perfusion with and in the presence of BW A868C (10 μM), 30 min of PGD₂ perfusion (10 μM) failed to induce mechanical hypersensitivity in esophageal nodose C fibers. Action potentials evoked by esophageal distensions did not significantly change from 2.9 ± 0.3, 5.0 ± 0.4, and 9.8 ± 0.9 Hz (with BW A868C) to 2.9 ± 0.4, 5.5 ± 0.6, and 9.5 ± 0.9 Hz (with BW A868C + PGD₂) at distension pressures of 10, 30, and 60 mmHg, respectively (P > 0.05, n = 8 in each paired value; Fig. 3C).

Similarly, we studied the inhibitory effect of the DP₂ receptor antagonist BAY-u3405 on PGD₂-induced mechanical hypersensitivity. Perfusion with BAY-u3405 (1 μM) for 30 min neither evoked action potentials nor changed the mechanoeexcitability of esophageal nodose C fibers (action potentials: 3.3 ± 0.7, 8.6 ± 1.7, and 12.3 ± 2.2 Hz before BAY-u3405 vs. 4.9 ± 1.0, 10.7 ± 2.5, and 15.9 ± 3.2 Hz after BAY-u3405 at distension pressures of 10, 30, and 60 mmHg, respectively, P > 0.05, n = 7). After 30 min of perfusion with and in the presence of BAY-u3405 (1 μM), 30 min of perfusion with PGD₂ (10 μM) still enhanced the mechanoeexcitability of esophageal nodose C fibers. Action potentials evoked by esophageal distensions significantly increased from 4.9 ± 1.0, 10.7 ± 2.5, and 15.9 ± 3.5 Hz (with BAY-u3405) to 7.3 ± 1.0, 16.3 ± 2.9, and 28 ± 4.5 Hz (with BAY-u3405 + PGD₂) at distension pressures of 10, 30, and 60 mmHg, respectively (P < 0.05, n = 7 in each paired value; Fig. 3D). Only nodose C fibers that were confirmed to respond to the TRPA1 agonist AITC (1 mM) and/or the TRPV1 agonist capsaicin (1 μM) at the end of each study were included in the analysis. These data support an important role of the DP₁ receptor in PGD₂-induced mechanical hypersensitivity in esophageal nodose C fibers.

15-Deoxy-Δ¹²,¹⁴-PGJ₂, a PGD₂ metabolite, did not change the mechanoeexcitability of esophageal nodose C fibers. PGD₂ is known to metabolize to 15-deoxy-Δ¹²,¹⁴-PGJ₂ (15d-PGJ₂) (20), which has been shown to induce nociception (6, 18) via direct interaction with TRPA1 on sensory neurons (26). Our previous studies demonstrated that the majority of esophageal nodose C fibers expressed TRPA1 and responded to the TRPA1 agonist AITC (31). Whether 15d-PGJ₂ displays activation/sensitization effects on esophageal nodose C fibers is unknown. Perfusion with 15d-PGJ₂ (30 μM) for 30 min slightly evoked action potential discharges over baseline activity (2.1 ± 0.4 and 4.6 ± 1.2 Hz at baseline and with 15d-PGJ₂, respectively, P < 0.05, n = 10) in esophageal nodose C fibers (Fig. 4A). However, this was not associated with a significant increase in their responses to esophageal distension. The peaks of action potential discharges (with subtraction of baseline spontaneous activity and 15d-PGJ₂-induced activity) evoked by 20-s esophageal distensions did not significantly change from 6.0 ± 1.4, 12.8 ± 3.8, and 15 ± 3.4 Hz to 6.8 ± 1.9, 10.2 ± 1.9, and 13 ± 2.8 Hz at distension pressures of 10, 30, and 60 mmHg for 20 s, respectively (P > 0.05, n = 10; Fig. 4B). The TRPA1 agonist AITC activated all these C fibers at the end of recording (2.1 ± 0.4 and 15 ± 5.1 Hz at baseline and with AITC, respectively, P < 0.01, n = 10; Fig. 4A), indicating the presence of TRPA1 in the nerve terminals. This result suggests that the PGD₂ metabolite 15d-PGJ₂ is unlikely to have participated in PGD₂-induced sensitization of esophageal nodose C fibers under the conditions in our studies.

PGD₂ directly sensitizes DiI-labeled esophageal nodose neurons. The effects of PGD₂ on the electrophysiological properties of DiI-labeled esophageal nodose ganglion neurons were studied by whole cell patch-clamp recording. Extracellu-
lar application of PGD2 (1 μM) sensitized 6 of 11 (55%) recorded DiI-labeled neurons, which were also positively labeled with DP1 receptors (Fig. 5, A–C). The current required to evoke an action potential (rheobase) decreased significantly in DiI-labeled esophageal nodose neurons before and after superfusion of PGD2 (1 μM): 78 ± 11 vs. 55 ± 8 pA (P < 0.05, n = 6; Fig. 5D). The number of spikes elicited by depolarizing current stimulation (double rheobase, 500 ms) increased significantly from esophageal retrogradely labeled nodose neurons treated with PGD2 (1 μM): 2.5 ± 0.4 vs. 4.6 ± 0.9 pA (P < 0.05, n = 6; Fig. 5E). This result not only supports our findings from extracellular recordings that PGD2 increased neuronal excitability but also suggests that such a sensitization effect is directly evoked by PGD2, rather than by other mediators/factors released after PGD2 perfusion in the esophagus.

The current-voltage (I-V) relationship generated from the current-clamp recording demonstrated that PGD2 increased the slope of the I-V relationship, and the effect reversed at −105 mV, which is close to theoretical K+ reversal potential in the conditions recorded.

Fig. 5. Effect of PGD2 on excitability of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI)-labeled esophageal nodose neurons. PGD2 superfusion directly sensitized 6 of 11 DiI-labeled esophageal nodose neurons. A: photomicrograph of an isolated esophageal nodose neuron retrogradely labeled with DiI (DiI+) and a DP1 receptor-positive neuron (DP1+). B and C: representative current-clamp traces in response to hyperpolarizing (−100 pA) and depolarizing (double rheobase) current pulses in a labeled neuron before and after superfusion with PGD2 (1 mM). D: summary data of current required to evoke action potential (rheobase) in nodose neurons before and after superfusion of PGD2. Data show significant decrease in action potential threshold (rheobase). Values are means ± SE (n = 6). *P < 0.05. E: summary data of number of spikes elicited by depolarizing current stimulation (double rheobase, 500 ms). Number of spikes from labeled neurons treated with PGD2 increased significantly (*P < 0.05). F: current-voltage (I-Vm) relationship before and after application of PGD2. Neuronal conductance decrease had a reversal potential of −105 mV, which is close to theoretical K+ reversal potential in the conditions recorded.

Fig. 4. Effect of the PGD2 metabolite 15-deoxyΔ¹²,¹⁴-PGJ₂ (15-d-PGJ₂) on esophageal nodose C fibers. Perfusion with 15-d-PGJ₂ slightly increased action potential discharges over control (P < 0.05; A) but did not change mechanoeexcitability of esophageal nodose C fibers (P > 0.05; B). Values are means ± SE (n = 10). AITC, allyl isothiocyanate.
mV, which is close to theoretical K\(^+\) reversal potential (−107 mV) in the conditions recorded (Fig. 5F). This may indicate that the effect of PGD\(_2\) is mediated by closure of K\(^+\) channel(s).

**DP\(_1\)** receptor antagonist attenuated esophageal mast cell activation-induced mechanical hypersensitivity in esophageal nodose C fibers. Our previous study showed that 30 min of perfusion with OVA induces MC activation, as indicated by increased release of histamine in the tissue and decreased staining of tryptase-positive MCs in the esophagus (32). To clarify the role of the DP\(_1\) receptor in mediating esophageal MC activation-induced sensitization of esophageal nodose C fibers, we tested the hypothesis that pretreatment with a DP\(_1\) receptor antagonist prevented esophageal MC activation-induced mechanical hypersensitivity in esophageal vagal nodose C fibers. First, we determined whether 30 min of perfusion with the DP\(_1\) receptor antagonist BW A868C changed the mechanoeexcitability in esophageal nodose C fibers from OVA-sensitized guinea pigs. Before and after 30 min of perfusion with BW A868C (10 \(\mu\)M), action potentials evoked by esophageal distension did not significantly change (4.4 ± 0.8, 13.5 ± 2.2, and 21.3 ± 3.9 Hz vs. 5.5 ± 1.0, 16.8 ± 2.9, and 24.4 ± 5.3 Hz at distension pressures of 10, 30, and 60 mmHg, respectively, \(P > 0.05, n = 7\)). Next, we studied the inhibitory effect of BW A868C on MC activation-induced mechanical hypersensitivity. After 30 min of perfusion with and in the presence of BW A868C (10 \(\mu\)M), MC activation induced by 30 min of OVA challenge (10 \(\mu\)g/ml) failed to induce mechanical hypersensitivity in esophageal nodose C fibers from OVA-sensitized guinea pigs (action potentials: 5.5 ± 1.0, 16.8 ± 2.9, and 24.4 ± 5.3 Hz vs. 6.4 ± 1.6, 16.5 ± 2.7, and 30.7 ± 4.3 Hz before and after MC activation at distension pressures of 10, 30, and 60 mmHg, respectively, \(P > 0.05, n = 7\)). In contrast, pretreatment with the DP\(_2\) receptor antagonist did not prevent mechanical hypersensitivity induced by MC activation in esophageal vagal nodose C fibers. Before and after 30 min of perfusion with BAY-u3405 (1 \(\mu\)M), action potentials evoked by esophageal distension did not significantly change (4.0 ± 1.0, 11.3 ± 2.0, and 19.9 ± 3.2 Hz vs. 4.9 ± 1.4, 12.0 ± 1.1, and 18.0 ± 2.6 Hz at distension pressures of 10, 30, and 60 mmHg, respectively, \(P > 0.05, n = 7\)). After 30 min of perfusion with and in the presence of BAY-u3405 (10 \(\mu\)M), MC activation induced by 30 min of OVA challenge (10 \(\mu\)g/ml) significantly increased mechanical excitability of esophageal nodose C fibers from OVA-sensitized guinea pigs (action potentials: 4.9 ± 1.4, 12.0 ± 1.1, and 18.0 ± 2.6 Hz vs. 7.6 ± 1.4, 19.0 ± 3.2, and 28.0 ± 4.0 Hz in the presence of BAY-u3405 before and after MC activation at distension pressures of 10, 30, and 60 mmHg, respectively, \(P < 0.05, n = 7\)). These data suggest an important role for the DP\(_1\) receptor in MC activation-induced mechanical hypersensitivity in esophageal nodose C fibers.

**Discussion**

For two decades, PGD\(_2\) has been recognized to be present in the esophagus (2). However, its physiological function and participation in esophageal disorders are largely unknown. PGD\(_2\) is mainly synthesized and released from activated MCs. It participates in MC activation-induced type I hypersensitivity, including smooth muscle contraction, vascular leak, and vasodilation. It also displays potent chemotactic effects on eosinophils, basophils, and Th2 cells. In addition, it may potentiate the inflammatory response induced by other relevant mediators. The biological effects of PGD\(_2\) are mediated by two G protein-coupled receptors, DP\(_1\) and DP\(_2\) (23). Expression of DP\(_1\) and DP\(_2\) receptors has been identified in central and peripheral neurons (7, 17, 22). DP\(_1\) receptor activation leads to G\(_s\) protein-mediated increases in intracellular cAMP, while DP\(_2\) receptor activation leads to G\(_i\) protein-mediated decreases in intracellular cAMP. These intracellular signaling changes are likely to regulate the excitability of sensory afferent. Our data in the present study demonstrate that PGD\(_2\) has different effects on esophageal vagal afferent nerve subtypes. In esophageal nodose C fibers, perfusion with PGD\(_2\) does not evoke action potential discharges but sensitizes their response to esophageal distension. This sensitization effect can be mimicked by a PGD\(_2\) DP\(_1\) receptor agonist and further prevented by pretreatment with a DP\(_1\) receptor antagonist. In contrast, in esophageal nodose A\(_\delta\) fibers, perfusion with PGD\(_2\) neither evokes action potential discharges nor sensitizes their response to esophageal distension. We also observed that PGD\(_2\) directly sensitized dissociated DiI-labeled esophageal nodose neurons. These patch-clamp data support the observation by extracellular recording that the sensitization effect in the nerve terminals...
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is directly induced by PGD2, rather than by a secondary released mediator after PGD2 perfusion in the esophageal tissue. Our findings provide the first direct evidence for a sensitization effect of PGD2 on esophageal vagal afferent subtypes. The observation that PGD2 failed to evoke action potential discharges in esophageal nodose C fibers but substantially increased their excitability to esophageal distension is similar to our previous observation of a MC activation-induced effect on esophageal vagal afferents (32). Pretreatment with the DP1 receptor antagonist BW A686C did not totally abolish, but significantly attenuated, MC activation-induced sensitization of esophageal nodose C fibers. This suggests that, in addition to histamine, PGD2 also plays an important role in MC activation-induced sensitization of esophageal afferents.

PGD2, which is involved in sleep regulation and pain perception, has been well known as the major prostanooid produced in the central nervous system (8, 29). Its effect on peripheral afferents is less clear. In cultured DRG neurons, PGD2 evoked an accumulation of cAMP (25) and increased the amplitude of TTX-resistant Na+ currents via the DP1 receptor (7). In nodose neurons, PGD2 inhibited postspike hyperpolarization, resulting in an increase in the firing frequency (5). Extracellular recording of direct-current potentials indicated that PGD2 perfusion caused a depolarization response of vagal C fibers (25). However, in another study using in vivo electrophysiological single-unit recording from rat trigeminal ganglion, PGD2 failed to sensitize meningeal nociceptors (34). In contrast, responses of spinal afferent neurons to PGD2 are different from responses of nodose and DRG neurons. In one study using a spinal-tail preparation, PGD2 had no or less effect on sensitization of spinal afferents than other prosanoid products such as PGE2 and PGI2 (21). Another studies demonstrated that PGD2 and DP1 receptor agonist application to the spinal cord did not change mechanoeexcitability of a normal knee joint but attenuated mechanohyperexcitability in an inflamed knee joint by in vivo extracellular recording from dorsal horn neurons with a peripheral nerve ending in the knee joint (27). We thought that several factors, such as the location of recorded afferent neurons, the presence of other mediators in the system, and the outcome measurements, might contribute to these different observations. Interestingly, the different responses (DRG and vagal afferents vs. spinal neuron afferent) may also suggest that the PGD2-induced sensitization effect more likely occurred in the peripheral afferent, which is strategically located near the sites of tissue injury and inflammation. Our study provides new evidence for the important role of PGD2 in sensitizing sensory afferents in the GI tract. This expands our knowledge of the mechanism of MC activation-induced esophageal afferent sensitization.

The intracellular signaling pathway and downstream ion channel involvement in PGD2-induced sensitization of sensory afferents are less unclear. Previous studies showed that PGD2 blocked a slow Ca2+-activated K+-dependent afterhyperpolarization in visceral afferent neurons (9). A recent study demonstrated that PGD2, via mainly the DP1 receptor, hyperpolarized DRG neurons, depending on action, at Na+, and influenced Na+, 1.8 channel functions (7). In the present study, perfusion with PGD2 increased the number of action potentials evoked by the positive current pulse. The I-V relationship generated from the current-clamp recording demonstrated that PGD2 increased the slope of the I-V relationship, and the effect reversed at −105 mV, which is close to the K+ reversal potential (−107 mV). This may suggest that the effect of PGD2 is mediated by closure of the K+ channel(s). The exact role of the K+ channel and the intracellular mechanism of PGD2-induced sensitization of esophageal vagal afferent deserves further exploration.

MC activation not only releases PGD2, but also histamine, and our previous study (32) demonstrated that histamine could sensitize esophageal afferents to induce mechanical hyperexcitability. This may explain why, in the present study, the PGD2, DP1 receptor antagonist does not totally abolish the PGD2-induced sensitization effect but only attenuates MC activation-induced sensitization on esophageal nodose C fibers. It is unlikely, in our ex vivo model, that the effect of PGD2 on the response of the esophageal nodose C fiber to intraluminal pressure (esophageal distension) is secondary to the effects on the stress-strain relationships caused by smooth muscle contraction. First, our previous study using the same preparation showed that methacholine, which contracts esophageal smooth muscle, did not increase the response of the esophageal nodose C fiber to esophageal distension (32). Second, PGD2 did not participate in regulation of smooth muscle contraction (intestinal peristalsis) in guinea pig GI tract (24). Our data also indicate that PGD2-induced sensitization of esophageal afferents is not secondary to the effect of the PGD2 metabolite 15d-PGJ2, which may interact with TRPA1 in nodose neurons (26, 31).

Clinical studies suggest that increased eosinophil and MC infiltration into the esophagus might contribute to abnormal esophageal sensation, such as heartburn, in patients with eosinophilic esophagitis (1, 11, 16). The present study provides the first evidence that PGD2, one of the important MC mediators, displays distinctive sensitization effects on esophageal sensory afferents, which contributes to MC activation-induced sensitization of esophageal afferents. This adds to our knowledge of the mechanisms of esophageal nociception, which may lead to development of a novel treatment approach for these common, but difficult to manage, clinical problems.

GRANTS
This study was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-087991 (S. Yu) and DK-084039 (C. Owyang).

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS
S.Z., G.G., X.W., M.K.J., A.H., I.S., and S.Y. performed the experiments; S.Z., G.G., X.W., M.K.J., A.H., I.S., and S.Y. analyzed the data; S.Z., G.G., X.W., and S.Y. interpreted the results of the experiments; C.O. and S.Y. are responsible for conception and design of the research; S.Y. prepared the figures; S.Y. drafted the manuscript; S.Y. edited and revised the manuscript; S.Y. approved the final version of the manuscript.

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