PAR-2 activation enhances weak acid-induced ATP release through TRPV1 and ASIC sensitization in human esophageal epithelial cells

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Wu L, Oshima T, Shan J, Sei H, Tomita T, Ohda Y, Fukui H, Watari J, Miwa H. PAR-2 activation enhances weak acid-induced ATP release through TRPV1 and ASIC sensitization in human esophageal epithelial cells. Am J Physiol Gastrointest Liver Physiol 309: G695–G702, 2015. First published August 20, 2015; doi:10.1152/ajpgi.00162.2015.—Esophageal visceral hypersensitivity has been proposed to be the pathogenesis of heartburn sensation in nonerosive reflux disease. Protease-activated receptor-2 (PAR-2) is expressed in human esophageal epithelial cells and is believed to play a role in inflammation and sensation. PAR-2 activation may modulate these responses through adenosine triphosphate (ATP) release, which is involved in transduction of sensation and pain. The transient receptor potential vanilloid receptor 1 (TRPV1) and acid-sensing ion channels (ASICs) are both acid-sensitive nociceptors. However, the interaction among these molecules and the mechanisms of heartburn sensation are still not clear. We therefore examined whether ATP release in human esophageal epithelial cells in response to acid is modulated by TRPV1 and ASICs and whether PAR-2 activation influences the sensitivity of TRPV1 and ASICs. Weak acid (pH 5) stimulated the release of ATP from primary human esophageal epithelial cells (HEECs). This effect was significantly reduced after pretreatment with 5-iodoresiniferatoxin (IRTX), a TRPV1-specific antagonist, or with amiloride, a nonselective ASIC blocker. TRPV1 and ASIC3 small interfering RNA (siRNA) transfection also decreased weak acid-induced ATP release. Pretreatment of HEECs with trypsin, tryptase, or a PAR-2 agonist enhanced weak acid-induced ATP release. Tryptsin treatment led to the phosphorylation of TRPV1. Acid-induced ATP release enhancement by trypsin was partially blocked by IRTX, amiloride, or a PAR-2 antagonist. Conversely, acid-induced ATP release was augmented by PAR-2 activation through TRPV1 and ASICs. These findings suggested that the pathophysiology of heartburn sensation or esophageal hypersensitivity may be associated with the activation of PAR-2, TRPV1, and ASICs.

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Adenosine triphosphate (ATP) is a neurotransmitter in the central and peripheral nervous systems that is also involved in peripheral inflammation and transmission of pain (4, 5). In the esophagus, the esophageal epithelial cell is a source of ATP, and released ATP can induce the secretion of platelet-activating factor (PAF), IL-8, eotaxins, monocyte chemoattractant protein-1 (MCP-1), and macrophage inflammatory protein-1α (MIP-1α) that contribute to inflammation and injury of the esophageal mucosa (22). ATP can also mediate the release of neurotransmitters such as substance P and calcitonin gene-related peptide that may contribute to transmission of the sensation of pain (23). In the light of the critical role of ATP release in the pathogenesis of GERD, the mechanism of ATP release needs to be investigated.

Protease-activated receptor-2 (PAR-2) belongs to a family of four G protein-coupled receptors and is involved in inflammatory and neuroinflammatory epithelial responses in the gut (29). PAR-2 can be activated by mast cell tryptase and by pancreatic and extra-pancreatic trypsin that widely exist throughout the gastrointestinal tract in physiological or pathophysiological conditions (27). Our previous study showed that PAR-2 is also expressed in human esophageal epithelial cells (33).

Weak acid can activate transient receptor potential vanilloid receptor 1 (TRPV1), which is abundant in some nonneuronal tissues and cells and is also expressed on human esophageal epithelial cells (22). Weak acid is also known to activate the acid-sensing ion channels (ASICs), which belong to the voltage-insensitive, amiloride-sensitive degenerin/epithelial Na+ channel superfamily (41). ASIC1, ASIC2, and ASIC3 are expressed on rat esophageal epithelial mucosa (1), and ASIC3 is expressed on human esophageal mucosa (42).

Although previous reports showed that ATP can be released through TRPV1 activation from immortalized human esophageal epithelial cells, these cells are far different from normal esophageal squamous epithelial cells (16, 22, 23, 38). Furthermore, potential interaction between acid-induced ATP release via TRPV1 and ASICs and PAR-2 activation has never been examined and remains to be elucidated. Such interactions may contribute to the development of symptoms and the pathogenesis of GERD. To further address the roles of TRPV1, ASICs, and PAR-2 in acid-induced ATP release in the esophagus, in the present study we used a normal primary human esophageal epithelial cell (HEEC) model to more reliably imitate the conditions of a normal esophagus. We examined whether weak acid-induced ATP release from esophageal epithelial cells is modulated by TRPV1 and ASICs and whether activation of PAR-2 modulates the sensitivity of TRPV1 and ASICs.
MATERIALS AND METHODS

Cell culture. HEECs were purchased from ScienCell Research Laboratories (Carlsbad, CA). The cells were cultured in epithelial cell medium-2 (EpiCM-2; ScienCell Research Laboratories) without antibiotics at 37°C in a balanced air humidified incubator under an atmosphere of 5% CO₂. EpiCM-2 medium was serum free. The medium was HEPES and bicarbonate buffered and had a pH of 7.4. Cells that had reached ~80% confluence were harvested using a 0.05% trypsin-EDTA solution and were stored frozen in Cell Banker-2 solution (serum-free type; Nippon Zenyaku Kogyo, Fukushima, Japan). HEECs at passages 3 to 7 were cultured at a density of 1 × 10⁴/well in a 96-well plate (Thermo Fisher Scientific, Waltham, MA) and used for this study.

Measurement of ATP release. After HEECs were stabilized in the medium at least for 24 h before the experiments, the time was set as t = 0. For control condition, the medium was changed into 100 μl fresh EpiCM-2 (pH 7.4) from t = 0 min until t = 5 min. For acid challenge conditions, the solution was change to 100 μl fresh EpiCM-2 (pH 5), the challenge period is from t = 0 min until t = 5 min. A small amount of hydrochloric acid (5 mol/l) was directly added to EpiCM-2 to make the solution to pH 5.

After a 5-min acid stimulation, ATP release by HEECs in the supernatant on the control and acid challenge conditions was measured using an ATP Bioluminescence Assay Kit CLS II (Roche, Mannheim, Germany) according to the manufacturer’s instructions. Finally, chemiluminescence was detected using an ImageQuant Imager 350 system (GE Healthcare Life Sciences, Tokyo, Japan). Membranes were stripped (10% SDS, 2-mercaptoethanol, PBS) for 1 h at 40°C. Stripped membrane were reblocked for 1 h in 5% milk-PBS and then reprobed overnight with anti-VR1 (1 μg/ml, Abcam) or ACCN3 (1 μg/ml; GenWay Biotech) antibody at 4°C. The results of a typical experiment are shown. The quantity of phosphoserine was normalized relative to TRPV1 or ASIC3 protein levels and was calculated as the fold increase over the control by using ImageJ software (Bio-Arts, Fukuoka, Japan).

Small interfering RNA knockdown experiments. For small interfering RNA (siRNA) silencing of human TRPV1 or ASIC3, ON-TARGET plus SMART pool and nontargeting pool control siRNA were purchased from Dharmacon (Lafayette, CO). HEECs were transfected with 25 nM siRNA using the DharmaFECT transfection reagent 1 (Dharmacon) as previously described (34). Control siRNA groups were treated with the transfection reagents and nonspecific control siRNA. Assays were performed 24 h after transfection.

Quantitative RT-PCR. Total mRNA was extracted using the RNeasy Micro Kit (Qiagen, Hilden, Germany). Contaminating DNA was cleared by RNase-Free DNase treatment (Qiagen). cDNA was synthesized using High-capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA). PCR was carried out using a PCR master mix in a 7900HT Fast Real-Time PCR System (Applied Biosystems). Taqman probes and primers for ASIC3 (Hs00245097_s1) and TRPV1 (Hs0018912_s1) were assay-on-demand gene expression products obtained from Applied Biosystems. The GAPDH gene was used as an endogenous control (Hs02758991_g1; Applied Biosystems). Thermal cycler conditions were as follows: 2 min at 50°C, then 10 min at 95°C, followed by 40 cycles of 15 s denaturing at 95°C and 1 min annealing/extension at 60°C. Amplification data were analyzed using Applied Biosystems Sequence Detection Software version 2.2 (Applied Biosystems). The ΔACT method recommended by the manufacturer was used to compare relative expression levels.

Statistical analysis. All values are presented as the means ± SD. Data were analyzed using paired t-tests for two groups and one-way ANOVA followed by Tukey’s multiple comparisons test. Significance was accepted at P < 0.05.

RESULTS

Weak acid induced ATP release through TRPV1 and ASICs. Exposure of esophageal mucosa to weakly acidified medium (pH 5, 5 min) induced significant release of ATP from HEECs compared with control (Fig. 1A). The ATP amount in the medium without cells was repeatedly measured and the level of ATP decreased in a similar speed in control and acidified medium (data not shown). To show the data simply, released
ATP in the medium was express as percent control in this study. The EpiCM-2 medium did not contain ATP.

Although weak acid was focused on this study, acidified medium (pH 2 and 3, 5 min) also induced significant release of ATP from HEECs compared with control. The degree of ATP release was different and pH 3 had strongest effect for the release of ATP (Fig. 1B).

Acid-induced ATP release was transient and the level of ATP decreased time dependently when the cells were incubated for longer time (Fig. 1C).

Capsaicin induced ATP release through TRPV1. Exposure of esophageal mucosa to capsaicin (0.1–10 μM, 5 min) induced significant release of ATP from HEECs compared with control. However, the values were smaller than that of pH 5 stimulation and were not concentration dependent (Fig. 2A). Capsaicin (1 μM)-induced ATP release was abolished by pretreatment with the TRPV1 antagonist IRTX (10⁻⁵ M, 30 min) or with the ASIC3 nonselective blocker amiloride (Amil; 0.3 mM, 30 min) before acid-induced ATP release (n = 9). Each value represents the means ± SD. *P < 0.05, **P < 0.01, ***P < 0.001 vs. control (pH 7.4); ns, not significant vs. control (pH 7.4).

TRPV1 and ASIC3 siRNA transfection reduced weak acid-induced ATP release. To confirm that weak acid-induced ATP release occurred through TRPV1 and ASIC3 in HEECs, the effect of TRPV1 and ASIC3-knockdown on this ATP release was performed. Before this analysis, the efficacy of siRNA knockdown of TRPV1 and ASIC3 in HEECs was confirmed by examination of TRPV1 and ASIC3 mRNA levels, TRPV1 and ASIC3 siRNA treatment significantly decreased the mRNA level of TRPV1 and ASIC3 in HEECs (Fig. 3A). Following knockdown of TRPV1 and ASIC3, the HEECs were stimulated with weak acid (pH 5). The acid-induced ATP release was significantly lower in TRPV1 and ASIC3 siRNA transfected cells than in control siRNA transfected cells (Fig. 3B).

Activated PAR-2 enhanced acid-induced ATP release. To determine the role of PAR-2 activation in the acid-induced
ATP release, the HEECs were pretreated with the PAR-2 activators trypsin (4 μg/ml, 60 min; Fig. 4A), SLIGLV-NH2 (100 μM, 30 min; Fig. 4B), or tryptase (500 ng/ml, 60 min; Fig. 4C) before stimulation with weak acid (pH 5). However, pretreatment with these agents significantly enhanced acid-induced ATP release, indicating the increased sensitivity of TRPV1 and ASICs to acid by PAR-2 activation. LDH release and ATP release from HEECs was unaffected by pretreatment chemicals compared with control (data not shown).

Trypsin induced serine phosphorylation of TRPV1. To assess the effect of PAR-2 activation on the activation of TRPV1, HEECs were exposed to trypsin (4 μg/ml, 10–60 min) and the serine phosphorylation of TRPV1 was examined by immunoprecipitation with TRPV1 antibody and probing of the immunoprecipitate with an anti-phosphoserine antibody by Western blotting. Trypsin treatment induced phosphorylation of TRPV1 in HEECs whereas control medium had no effect (Fig. 5A). Phosphorylation levels were normalized to TRPV1 protein and were calculated as the fold increase over the control. The level of TRPV1 protein in each group was not statistically different. (Fig. 5A).

We hypothesized that PAR-2 activation might also modify the serine phosphorylation ofASIC3. However, the phosphoserine level of ASIC3, which was normalized to the ASIC3 protein level, was not altered by trypsin (Fig. 5B).

Effect of IRTX, amiloride, or a PAR-2 antagonist on trypsin enhanced weak acid-induced ATP release. To confirm the involvement of TRPV1, ASICs, and PAR-2 in the trypsin-induced enhancement of ATP release induced by acid, the effect of their specific inhibitors was assayed. IRTX (10^-5 M, 45 min) and amiloride (0.3 mM, 45 min) partially but significantly blocked the trypsin-induced enhancement of ATP release induced by acid, whereas the vehicle (DMSO) had no effect (Fig. 6A). The potentiating effect of trypsin on acid-induced ATP release was also significantly blocked by pretreatment with a PAR-2 antagonist (FSLLRY-NH2, 50 μM; Fig. 6B). These data imply that PAR-2 activation enhances weak acid-induced ATP release through TRPV1 and ASICs sensitization. LDH release and ATP release from HEECs was unaffected by pretreatment chemicals compared with control (data not shown).

TRPV1 and ASIC3 siRNA transfection reduced trypsin-enhanced weak acid-induced ATP release. To further confirm that trypsin-induced enhancement of acid-induced ATP release occurred through TRPV1 and ASIC3 in HEECs, double knockdown of TRPV1 and ASIC3-siRNA was performed. TRPV1 siRNA and ASIC3 siRNA treatments partially but significantly blocked the trypsin-induced enhancement of acid-induced ATP release. Double knockdown of TRPV1 and ASIC3 tended to
In this study, the activation of PAR-2 significantly enhanced weak acid-induced ATP release through the nociceptors TRPV1 and ASIC3 in HEECs. We have thus identified a previously unrecognized nociceptive pathway that may introduce a new therapeutic strategy for GERD. ATP was identified as a neurotransmitter in the late 1970s (7). In the enteric sensory system, keratinocytes, and bladder epithelial cells, ATP release has been shown to be involved in transduction of sensation to the central nervous system (3, 4, 14). Since transmission of acid stimuli in the esophageal lumen to epithelial cells and neurons via nociceptors is one of the mechanisms underlying sensation transmission in the esophagus, the peripheral release of ATP plays an important role in this process (23).

In the present study, we originally focused on weak acid because in clinical conditions, PPI-refractory GERD/NERD is an important issue and some of the patients still aware weak acid (pH more than 4). The cell viability was not affected after
was a more potent stimulus than capsaicin. ASIC3 is known to play a role in the transmission of nociceptive information (10, 19, 39). Studies of ASIC3 knockout mice indicated that ASIC3 is required for normal esophageal acid sensation (6) and that ASIC3 but not ASIC1 is involved in primary or chronic hyperalgesia (36, 40). Since the human esophagus has been shown to express TRPV1 and ASIC3 (33, 42), TRPV1 and ASIC3 were knocked down in this study and the knockdown of each target partially blocked weak acid-induced ATP release. The combined data suggest that TRPV1 and ASIC3 function as acid sensors not only in neurons but also at the level of epithelial cells.

Although ASIC3 mRNA was not upregulated in NERD patients (42) and amiloride had no significant effect on acid-induced heartburn frequency or severity in NERD (8), our study still suggests the possibility that ASIC3 might modulate ATP release and be a target of treatment. Thus, in the previous clinical study, amiloride perfusion might not have been sufficient to inhibit esophageal ASICs because of suboptimal absorption or suboptimal contact time of the drug with ASICs. Further studies regarding the blocking of ASICs, including the choosing of appropriate blocking methods and appropriate doses and times, may still be worthwhile for evaluating the efficacy of this treatment.

The present study showed for the first time that PAR-2 activation enhances weak acid-induced ATP release in human esophageal epithelial cells through TRPV1 and ASIC3. PAR-2 is known to be expressed in HEECs and its activation has been shown to be associated with proinflammatory roles (33). The present data are consistent with previous studies that showed that PAR-2 activation can sensitize TRPV1 and exaggerate citric acid- and resiniferatoxin-induced cough (15), can en-

Fig. 6. Effect of IRTX, amiloride or a PAR-2 antagonist on trypsin enhanced weak acid-induced ATP release in HEECs. A: HEECs were pretreated with or without DMSO, IRTX (10^{-3} M, 45 min), or amiloride (0.3 mM, 45 min) and were then incubated with or without trypsin (4 μg/ml, 60 min) before weak acid (pH 5, 5 min) or no acid stimulation as indicated. ATP release was then measured as described in the Fig. 1 legend. Each value represents means ± SD (n = 9). B: HEECs were pretreated without or with trypsin or with trypsin plus the PAR-2 antagonist [FSLLRY-NH_{2} (50 μM)] for 60 min before weak acid (pH 5, 5 min) or no acid stimulation as indicated. ATP release was then measured as described in the Fig. 1 legend. Each value represents means ± SD (n = 9). *P < 0.05. ****P < 0.0001.

pH 5 stimulation. To identify esophageal epithelial cells as a source of ATP, primary HEECs were exposed to weak acid and acid-induced ATP release was demonstrated. This release was shown to be mediated via both TRPV1 and ASICs. These data are consistent with previous studies that showed the involvement of esophageal and bladder TRPV1 and bladder ASIC activation in ATP release (23, 30, 31). In our data, the level of acid-induced ATP was decreased time dependently even in control condition. Mizumori et al. (25) proved that ENTPDase and intestinal alkaline phosphatase are involved in the degradation of ATP in rat duodenum. These may explain the transient ATP release after stimulation and gradual degradation. Capsaicin is known to induce ATP release from rat bladder mucosal strips (31). Here, we have shown that capsaicin can also induce ATP release from HEECs through TRPV1. Acid
hance TRPV1 Ca$^{2+}$ responses in HEK-TPRV1 cells and DRG neurons (2), and can potentiate both acid-evoked ASIC- and TPRV1-like whole cell inward currents in rat pulmonary sensory neurons (17). Indeed in the present study, IRTX and amiloride not only blocked direct acid-induced ATP release but also inhibited trypsin-induced enhancement of weak acid-induced ATP release. Recent studies have indicated that PAR-2 antagonists can also inhibit colonic inflammation and mast cell infiltration (28). Therefore, PAR-2 is also an attractive target for the development of novel therapeutics for the regulation of esophageal symptoms.

TRPV1 is regulated in part by its phosphorylation state. Dephosphorylation of TRPV1 by calcineurin, a Ca$^{2+}$- and calmodulin-dependent kinase, can desensitize capsaicin responsiveness (12), whereas phosphorylation of TRPV1 potentiates the capsaicin-evoked currents (26). Phosphorylation of TRPV1 is related to its functional activation (2, 21). Since our results showed that trypsin induced the serine phosphorylation of TRPV1, it is likely that the trypsin-induced phosphorylation of TRPV1 led to enhancement of ATP release. TRPV1 in the human esophageal epithelial cells of the present study was detected at a molecular size of 60 kDa. The size of TRPV1 in rat esophageal mucosa is also 60 kDa, whereas that in rat DRG is 95 kDa (1), suggesting the presence of a possible epithelial splice variant (13, 18, 32) or a posttranscriptional modification (20).

Since amiloride can partially block the potentiating effect of weak acid-induced ATP release, ASICs may also be involved in the sensitization. Phosphorylation of ASIC3 may relate with its functional activation (11). Here, we hypothesized that PAR-2 activation might modify the phosphorylation of ASIC3. However, trypsin could not induce the serine phosphorylation of ASIC3 in this study. This result may be due to the possibility that the phosphorylation sites of ASIC3 might be different and/or multiple. Further investigations are warranted to reveal the sensitization mechanisms of ASIC3.

In conclusion, weak acid-induced ATP release from HEECs occurs through TRPV1 and ASIC3. The activation of PAR-2 mediates the sensitization of TRPV1 and ASIC3 and enhances weak acid-induced ATP release. These findings suggest that the pathogenesis of heartburn sensation, esophageal visceral hypersensitivity, or cytokine-mediated injury induced by acid is associated with the activation of PAR-2, TRPV1, and ASICs in NERD. These molecules can therefore be considered as targets in a new treatment strategy for GERD including NERD.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

L.W. and T.O. performed experiments; L.W., J.S., H.S., T.T., and Y.O. analyzed data; L.W. prepared figures; L.W., T.O., J.S., H.S., T.T., Y.O., H.F., J.W., and H.M. approved final version of manuscript; T.O. and H.M. conception and design of research; T.O., H.F., and J.W. interpreted results of experiments; T.O., H.F., J.W., and H.M. edited and revised manuscript.

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