Oxidative stress-induced posttranslational modification of TRPV1 expressed in esophageal epithelial cells

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Kishimoto E, Naito Y, Handa O, Okada H, Mizushima K, Hirai Y, Nakabe N, Uchiyama K, Ishikawa T, Takagi T, Yagi N, Kokura S, Yoshida N, Yoshikawa T. Oxidative stress-induced posttranslational modification of TRPV1 expressed in esophageal epithelial cells. Am J Physiol Gastrointest Liver Physiol 301: G230–G238, 2011. First published June 2, 2011; doi:10.1152/ajpgi.00436.2009.—Human esophageal epithelium is continuously exposed to physical stimuli or to gastric acid that sometimes causes inflammation of the mucosa. Transient receptor potential vanilloid 1 (TRPV1) is a nociceptive, Ca2+ selective ion channel activated by capsaicin, heat, and protons. It has been reported that activation of TRPV1 in expressed esophageal mucosa is involved in gastroesophageal reflex disease (GERD) or in nonerosive GERD symptoms. In this study, we examined the expression and function of TRPV1 in the human esophageal epithelial cell line Het1A, focusing in particular on the role of oxidative stress. Interleukin-8 (IL-8) secreted by Het1A cells upon stimulation by capsaicin or acid with/without 4-hydroxy-2-nonenal (HNE) was measured by ELISA. Following capsaicin stimulation, the intracellular production of reactive oxygen species (ROS) was determined using a redox-sensitive fluorogenic probe, and ROS- and HNE-modified proteins were determined by Western blotting using biotinylated cysteine and anti-HNE antibody, respectively. HNE modification of TRPV1 proteins was further investigated by immunoprecipitation after treatment with synthetic HNE. Capsaicin and acid induced IL-8 production in Het1A cells, and this production was diminished by antagonists of TRPV1. Capsaicin also significantly increased the production of intracellular ROS and ROS- or HNE-modified proteins in Het1A cells. Moreover, IL-8 production in capsaicin-stimulated Het1A cells was enhanced by synthetic HNE treatment. Immunoprecipitation studies revealed that TRPV1 was modified by HNE in synthetic HNE-stimulated Het1A cells. We concluded that TRPV1 functions in chemokine production in esophageal epithelial cells, and this function may be regulated by ROS via posttranslational modification of TRPV1.

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**THE CLONED TRANSIENT RECEPTOR POTENTIAL VANILLOID subtype 1 (TRPV1) functions as a nonspecific cation channel that plays an important role in thermal nociception and inflammatory hyperalgesia (9). TRPV1 is activated by capsaicin (the active ingredient of chili peppers), heat (>43°C), protons (pH < 5.9), and endogenous lipids (7, 22), resulting in the mediation of calcium entry and the enhancement of cytosolic calcium concentration (45). TRPV1 has been reported to be primarily expressed in peripheral afferent sensory neurons type C and in Aδ fibers originating from dorsal root ganglia (8). However, recent studies have also demonstrated that TRPV1 is expressed in some nonneuronal tissues and cells (2, 6, 25, 35, 41), where it functions to regulate inflammatory cytokine production following exposure to TRPV1 agonists (12, 21, 29, 33, 48). Although TRPV1 has been considered as a molecular sensor of chemical and physical stimuli in nonneuronal cells, the precise function of this protein is not yet clear.

Human esophageal epithelium is continuously exposed to physical stimuli or gastric acid that sometimes causes inflammation of the mucosa. Acute or chronic exposure of the human esophagus to acid can lead to hypersensitivity of the mucosa. Gastroesophageal reflex disease (GERD), which is induced by reflux of the gastric and duodenal contents into the esophagus, has recently come to be recognized as a serious clinical problem that manifests as reflux symptoms, hurt burn, and chest pain. The occurrence of GERD is related to relaxation of the lower esophageal sphincter (LES) and to increased gastric acid secretion. Moreover, inflammatory cytokines, leukocytes, and oxidative stress have been demonstrated to be involved in the hypersensitivity of reflux esophagitis (30, 43, 44, 46). Interestingly, TRPV1 expression has been detected in sensory nerve fibers of esophageal mucosa from patients with GERD or nonerosive reflux disease (5, 28). This finding might suggest that esophageal hypersensitivity is also associated with acid-induced activation of TRPV1 (3, 12) because TRPV1 is believed to be a mediator of neurogenic inflammation. However, it remains unknown whether TRPV1 is expressed in human esophageal epithelial cells or whether TRPV1 mediates inflammation of the esophagus.

In this study, we investigated the expression and function of TRPV1 in the human esophageal epithelial cell line Het1A, with a specific focus on the role of oxidative stress.

**MATERIALS AND METHODS**

**Reagents.** Capsaicin, capsazepine, and ruthenium red were obtained from Sigma-Aldrich (St. Louis, MO). Stock concentrations of capsaicin (100 mM), capsazepine (100 mM), and ruthenium red (10 mM) were dissolved in ethanol, dimethyl sulfoxide, and distilled water and were diluted in media to the appropriate concentration. Redox Sensor Red CC-1 was purchased from Molecular Probes (Eugene, OR). Biotinylated cysteine was prepared as previously described (15, 20). 4-Hydroxy-2-nonenal (HNE) was a generous gift from Dr. Uchida, Graduate School of Bioagricultural Sciences, Nagoya University (Nagoya, Japan). Polyclonal rabbit and goat anti-TRPV1
antibody was from Santa Cruz Biotechnology (Santa Cruz, CA) and Osenses (Flagstaff Hill, Australia), polyclonal rabbit anti-HNE antibody was provided by Dr. Uchida, and the monoclonal mouse anti-HNE antibody was from Japan Institute for the Control of Aging (Shizuoka, Japan). The polyclonal anti-HNE antibody detects HNE-modified amino acids, such as histidine, lysine, and cysteine, but not HNE itself, and the monoclonal anti-HNE antibody detects HNE-modified histidine. IL-8 ELISA kits were from R&D Systems (Minneapolis, MN).

Cell culture. Het1A (a human esophageal epithelial cell line) cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and were cultured in Bronchial Epithelial Cell Medium (BEGM BulletKit from Clonetics, Walkersville, MD). PC12 (a rat pheochromocytoma 12 line) cells were purchased from Riken Cell Bank (Tsukuba, Japan) and were cultured in DMEM (Gibco, Grand Island, NY) supplemented with 5% FBS and 5% horse serum. HaCaT (a human keratinocyte cell line) cells were kindly supplied by Dr. Kato (the Department of Dermatology of Kyoto Prefectural University of Medicine, Japan) and cultured in DMEM supplemented with 5% FBS. All cells were cultured in the appropriate medium at 37°C in a humidified atmosphere of 5% CO₂ and were passaged at confluence every 3–5 days.

Western blot analysis. Cells were washed twice with ice-cold PBS and harvested in lysis buffer (Celllytic from Sigma-Aldrich) for 20 min on ice. The protein content of samples was measured using Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein were loaded in each lane together with loading buffer (Invitrogen, Tokyo, Japan). Before gel loading, samples were boiled at 70°C for 10 min. The samples were electrophoresed on a 4–12% SDS-polyacrylamide gel and transferred to nitrocellulose membranes (Invitrogen). The membranes were blocked for 20 min with a blocking buffer and then incubated overnight at 4°C with the anti-TRPV1 (1:1,000) or anti-HNE (1:2,000) antibody in PBS. Afterward, the membranes were washed and incubated for 1 h with a horseradish peroxidase-conjugated secondary antibody (GE Healthcare Bio-Sciences, Tokyo, Japan). Immune complexes were visualized using a Western blotting analysis system with a commercial kit (ECL plus, GE Healthcare Bio-Sciences) according to the manufacturer’s recommendations.

Immunocytochemistry. For the detection of TRPV1 expressed in Het1A and HaCaT cells, the cells were plated on glass chamber slides and cultured for 3 days, following which they were fixed with 4% paraformaldehyde for 20 min. The cells were then washed with ice-cold PBS and treated with an anti-TRPV1 antibody (1:100) overnight at 4°C, followed by incubation with a FITC-conjugated secondary antibody (1:200, Invitrogen). Stained cells were viewed with a laser-scanning confocal microscope (FV1000; Olympus, Tokyo, Japan). Images were taken from four random fields in three independent experiments.

For the detection of HNE-modified TRPV1 in Het1A cells, Het1A cells were cultured on chamber slides for 3 days and then incubated with 1 μM of HNE diluted with serum-free medium for 24 h. The cells were then fixed with 4% paraformaldehyde for 20 min and incubated with a mouse anti-HNE antibody (1:50) and a rabbit anti-TRPV1 antibody (1:100) overnight at 4°C. Afterward samples were stained with AlexaFluoro 488-labeled goat anti-mouse IgG antibody (1:200) and AlexaFluoro 647-labeled goat anti-rabbit IgG antibody (Invitrogen, Carlsbad, CA) (1:200), respectively. Stained cells were observed using a confocal laser-scanning fluorescent microscope FV10i (Olympus). Images were taken from four random fields in three independent experiments.

Immunofluorescent detection of capsaicin-induced oxidative stress. Capsaicin-induced oxidative stress in Het1A cells was detected by trafficking of 2,3,4,5,6-pentafluorodihydrotetra-methylrosamine (Redox Sensor Red CC-1; Molecular Probes, Eugene, OR) as reported in our previous study (27) using a laser-scanning confocal microscope (FV10i). The Redox Sensor Red CC-1 has been reported to be oxidized in the presence of O₂⁻ and H₂O₂ (10, 24). Het1A cells, cultured on 35-mm dishes or 96-well culture plates for 3 days, were incubated with capsaicin (50 μM) for 60 min. In some experiments, the cells were pretreated with capsazeine (0.5 μM) for 30 min before capsaicin stimulation. These cells were then loaded with Redox Sensor Red CC-1 (1 μM) at 37°C for 15 min. The cells in culture dishes were visualized using the FV10, and the relative fluorescent intensity was measured by a microplate reader (SpectraMax M2; Molecular Devices, Tokyo, Japan). Images were taken from four random fields in three independent experiments.

Detection of S-oxidized proteins using biotinylated cysteine in Het1A cells. Het1A cells that had been plated and cultured on 60-mm dishes for 3 days were incubated with 100 μM biotinylated cysteine for 30 min. The cells were then washed twice with PBS and incubated with 50 μM capsaicin for 24 h. These cells were harvested in lysis buffer and centrifuged at 12,000 g for 15 min at 4°C. Samples containing 20 μg of protein, with or without added diethiothreitol (DTT), were boiled with SDS sample buffer for 10 min at 70°C, were electrophoresed on 4–12% SDS-polyacrylamide gels, and were then transferred to nitrocellulose membranes (Invitrogen). After the membrane was washed and blocked, biotinylated proteins were detected using horseradish peroxidase-conjugated Avidin (Streptavidin, Invitrogen) and an ECL kit.

Immunoprecipitation for detection of HNE-modified TRPV1 in Het1A cells. TRPV1 antibody affinity columns were prepared using anti-rabbit TRPV1 antibody bound to protein A. Het1A cells that had been plated and cultured on 10-mm dishes for 3 days were treated with 10 μM of HNE for 20 min and harvested in lysis buffer. The lysates were centrifuged at 12,000 g at 4°C for 15 min to remove cellular debris. Supernatants were incubated with the TRPV1 affinity column at 4°C overnight. The columns were washed several times with PBS, and the bound TRPV1 was eluted in 50 μl of elution buffer (Invitrogen) at 70°C for 10 min. These samples were then analyzed by Western blotting.

Determination of IL-8 production. Cellular expression of IL-8 was measured using a human IL-8 ELISA system (R&D Systems) according to the manufacturer’s protocol. Briefly, Het1A cells were plated in 96-well culture plates. Before stimulation with capsaicin for 24 h or acidified medium (pH 4.5) prepared by 1N HCl for 20 min, some of the cells were pretreated with ruthenium red, capsaquin, or HNE for 30 min. The supernatants were harvested and centrifuged at 400 g for 5 min to remove cell debris. The samples were then added to IL-8 antibody-coated 96-well plates. After the addition of biotinylated anti-IL-8 and streptavidin-peroxidase, tetramethylbenzidine was used as a stabilized chromogen. The absorbance of each well was read at 450 nm using a microplate reader (MPR-A4i; Tosoh, Tokyo, Japan). Concentrations of IL-8 were determined from a standard curve of recombinant human IL-8.

Tissue preparation. To further confirm the expression of TRPV1 in esophageal epithelial cells, we used human esophagus biopsy specimens, rat esophageal tissues, and rat peripheral nerve fiber (as a positive control).

Human esophagus biopsy specimens were obtained from two healthy volunteers, who gave informed, written consent. Esophageal tissues were obtained from biopsies taken from the normal mucosa at 1 cm above the esophagogastric junction using gastroendoscopy. These biopsy specimens were fixed as previously described (47).

Adult male Wistar rats (250–350 g) were obtained from SHI-MIZU Laboratory Supplies (Kyoto, Japan). They were anesthetized with diethyl ether and then perfused with 100 ml of physiological saline and 80 ml of 4% paraformaldehyde or 0.5% gluteraldehyde/4% paraformaldehyde in 0.1 M PBS through the heart. The peripheral nerve fiber at the C1–5 segmental level and the entire esophagus were removed and fixed in the same fixative for 3 h at 4°C. After being rinsed in PBS, these tissues were immersed overnight in 30% buffered sucrose. Subsequently, they were frozen.
and cut into 10-μm thick sections on cryostat. All protocols were approved by Animal Care and Use Committee of Kyoto Prefectural University of Medicine.

Immunohistochemical analysis. Acetone-fixed frozen sections of the biopsy specimens or animal tissues were dried in air for 60 min. After they were washed in PBS, the sections were blocked with blocking solution (1% BSA/PBS) for 30 min. They were incubated overnight at 4°C with a mixture of rabbit anti-TRPV1 serum (1:200, Osenses). The sections were then incubated for 1 h at room temperature with an Alexa Fluor 594-conjugated chicken anti-rabbit IgG (1:1,000; Molecular Probes). After the sections were washed, the slides were treated with DAPI (Southern Biotech, Birmingham, AL). Fluorescent signals were detected using a confocal laser-scanning fluorescent microscopy FV10i (Olympus).

Statistical analysis. Data were analyzed for statistical significance using ANOVA and paired t-tests. Statistical probability of P < 0.05 was considered significant. All data are expressed as means ± SE.

RESULTS

Het1A cells express TRPV1 on their cell membranes. To detect the expression of TRPV1 in the human esophageal epithelial cell line Het1A, immunocytochemistry (Fig. 1A) and Western blot analysis (Fig. 1B) were performed. Following immunocytochemistry, TRPV1 was predominantly stained on the apical side of the membrane of Het1A cells, as has previously been reported for PC12 (32) and HaCat keratinocytes (6, 35). TRPV1 protein was expressed at a molecular weight of 95 kDa in Het1A cells by Western blot analysis as well as that in PC12 cells and HaCat cells (positive control).

TRPV1 is also expressed in human and rat esophageal mucosa. Immunohistochemical examination was performed to show the presence of TRPV1 in the esophageal mucosa of human and rat as well as in the peripheral nerve fiber of rats (Fig. 1C). TRPV1 was stained in peripheral nerve fiber (Fig. 1C, a) and esophageal mucosa (Fig. 1C, b) of rats, as has previously been reported (1, 11). TRPV1 was also located in biopsy specimens of human normal esophageal mucosa (Fig. 1C, c).

TRPV1 activation induces IL-8 production in Het1A cells. Previous studies have shown that treatment of cells with capsaicin or with other TRPV1 agonists induces IL-8 secretion from some epithelial cells (33, 35). We therefore determined whether TRPV1 activation by capsaicin induces IL-8 production from human esophageal epithelial cells. Het1A cells were treated with capsaicin (1–100 μM, for 24 h), and IL-8 production was determined by ELISA. IL-8 secretion from Het1A cells was significantly increased by stimulation with capsaicin in a dose-dependent manner (Fig. 2A). Furthermore, IL-8 production was markedly suppressed by pretreatment with ruthenium red (Fig. 2B) and capsazepine (Fig. 2C), antagonists of TRPV1, indicating that TRPV1 does play a role in this effect.

TRPV1 is activated with not only capsaicin but also acid. We examined that Het1A cells were incubated with acidified medium (pH 4.5) for 20 min. IL-8 production was significantly increased by stimulation with acid that was also suppressed by treatment with capsazepine (Fig. 2D), supporting the presence of acid-sensitive TRPV1 in these cells.

Capsaicin stimulation resulted in increased intracellular ROS levels and in the production of ROS- and HNE-modified proteins in Het1A cells. To determine whether oxidative stress is modulated by capsaicin stimulation of Het1A cells, the redox-sensitive fluorogenic dye, Redox Sensor Red CC-1, was utilized. This Redox Sensor was oxidized in the presence of capsaicin (Fig. 3A), suggesting that intracellular reactive oxygen species (ROS) is produced in capsaicin-stimulated Het1A cells.

Because ROS has been reported to modify intracellular molecules such as proteins and lipids, we first assessed the effect of capsaicin stimulation of Het1A cells on the production of ROS-modified proteins. Biotinylated cysteine can be used as a molecular probe for the detection of proteins modified by ROS through cysteine-targeted oxidation, and this oxidation effect is reversible by the application of chemical-reducing agents such as DTT (15, 20). Het1A cells were therefore pretreated with biotinylated cysteine (100 μM) before stimulation with capsaicin (50 μM), and S-oxidized proteins were analyzed by Western blot analysis of biotinylated cysteine-labeled proteins. The expression of unknown S-oxidized proteins was increased in capsaicin-stimulated cells compared with the control group (Fig. 3B, left). Furthermore, DTT treatment of these samples abolished this S-oxidation (Fig. 3B, right). Oxidation-specific bands were identified as bands that disappeared following the addition of DTT, whereas nonspecific bands were insensitive to reduction.

It has been shown that HNE is produced in response to oxidative insults and mediates the effects of ROS as a second messenger. Stimulation of Het1A cells with capsaicin resulted in the induction of several HNE-modified proteins in a dose-dependent manner, as assessed by Western blot using a polyclonal anti-HNE antibody (Fig. 3C). As described in MATERIALS AND METHODS, this polyclonal anti-HNE antibody detects HNE-modified amino acids such as histidine, lysine, and cysteine, but not HNE itself. HNE-modified proteins were not detected in control cells (lane 1) or in cells treated or cotreated with capsazepine (lanes 2 and 3, respectively), an antagonist of TRPV1, suggesting that capsaicin induced HNE through activation of TRPV1 on Het1A cells.

HNE treatment induces TRPV1 modification. To investigate whether capsaicin-induced HNE production affects the function of TRPV1 in Het1A cells, capsaicin-induced IL-8 production was assessed in the presence or absence of synthetic HNE (Fig. 4). Following preincubation with HNE (1 μM) for 30 min, Het1A cells were cotreated with HNE and capsaicin (10 or 100 μM) for 24 h. Subsequent IL-8 production was determined by ELISA. This experiment indicated that 100 μM, but not 10 μM, of capsaicin significantly enhanced IL-8 production from Het1A cells even in the absence of HNE. However, although a low concentration of HNE (1 μM) alone did not induce IL-8 production in Het1A cells, HNE did significantly enhance IL-8 production when the cells were cotreated with capsaicin. This result suggests that oxidative stress might enhance capsaicin-induced cytokine production from Het1A cells even at a very low concentration. We therefore hypothesized that capsaicin-induced ROS production might, in turn, affect TRPV1 function, thereby forming a vicious circle of IL-8 production. To determine whether TRPV1 is modified with HNE in Het1A cells, the TRPV1 protein was immunoprecipitated from synthetic HNE-treated cells using an anti-TRPV1 antibody and immunoblotted with a monoclonal anti-HNE and an anti-TRPV1 antibody. As shown in Fig. 5A, the TRPV1 protein was modified by HNE in synthetic HNE-treated cells but not in control cells. As described in MATERIALS AND METHODS, this monoclonal anti-HNE antibody detects...
HNE-modified amino acids such as histidine but does not detect HNE itself.

Moreover, double-immunofluorescence staining was performed (Fig. 5B). HNE-modified TRPV1 was more strongly stained in HNE-treated cells than in control cells, and merged images revealed the colocalization of HNE-modified protein and TRPV1 in HNE-treated cells. Although two immunolabels colocalizing is diffusely stained, combined with the immunoprecipitation results, these data raise the possibility that TRPV1 in Het1A cells is modified by synthetic HNE.

**DISCUSSION**

TRPV1 is a calcium-permeable nonselective cation channel that is activated by capsaicin, heat, protons, and endogenous...
lipids. The expression of TRPV1 in sensory neurons is well known (9, 38). Recently, the presence of functional TRPV1 receptors in nonneuronal cells such as keratinocytes of the epidermis (35), human hair follicles (6), human bronchial epithelial cells (41), the bladder urothelium (2), and rat gastric epithelial cells (25) has been reported. All of these findings illustrate the fact that TRPV1 is widely expressed in both neuronal and nonneuronal cells and is involved in diverse physiological functions as a molecular sensor of potentially noxious stimuli.

In the esophagus, acute acid exposure leads to hypersensitivity of the human esophagus toward mechanical and chemical stimuli. The existence of TRPV1 in the vagal and spinal afferents in the gastrointestinal tract has recently been documented (31), and TRPV1 is receiving increasing attention for its possible role in reflux-induced esophagitis (3). Esophagitis leads to overexpression of TRPV1-positive nerve fibers in esophageal mucosa, and increased TRPV1 expression may mediate the heartburn of erosive esophagitis (28). Moreover, even in patients with nonerosive esophagitis, increased acid exposure is associated with an increase in TRPV1-expressing nerve fibers in the esophagus (5). All of these findings indicate that TRPV1 expressed in the primary afferents of esophagus may be involved in hypersensitivity to acid. It has recently been reported that TRPV1 is expressed not only on nerves but also on epithelial cells of cat esophageal mucosa and that HCl-activated epithelial TRPV1 initiates the induction of esophagitis (12). In the present study, we found that TRPV1 is also expressed in the human esophageal epithelial cell line Het1A and human esophageal epithelial cells as well as in rat esophageal mucosa and peripheral nerve fiber and that activation of TRPV1 by capsaicin and acid resulted in significant IL-8 production from Het1A cells. This production was markedly suppressed by pretreatment with antagonists of TRPV1, suggesting that the activation of TRPV1 on esophageal epithelial cells might be involved in the development of esophageal inflammation via cytokine production. These results are supported by previously reported findings that TRPV1 mediates inflammatory responses in various cells that are stimulated by certain types of particulate materials (21, 29, 33, 48). Other studies have shown that proinflammatory cytokines are important mediators of esophagitis associated with gastroesophageal reflux (34). Thus these data raise the possibility that TRPV1, when expressed not only on nerves but also on human esophageal epithelial cells, plays an important role on inflammatory responses at the cellular level against noxious stimuli.

In the present study, we also found that activation of TRPV1 by capsaicin resulted in marked production of intracellular ROS in Het1A cells. This phenomenon has also been reported in cultured dorsal root ganglion neurons (26) and in synoviocytes (19). We further investigated the effect of the ROS produced by capsaicin on intracellular proteins in Het1A cells.
because it is well known that, when ROS is generated in cells by several pathways, it modulates cellular functions that regulate many cellular metabolic activities through the modification of intracellular molecules (17, 23). Our study shows that the increase in ROS-modified and HNE-modified proteins in Het1A cells following treatment with capsaicin was blocked by a TRPV1 antagonist. This result suggested that capsaicin might specifically induce ROS and HNE production in Het1A cells through a TRPV1-dependent pathway and that the ROS and HNE produced might further modify intracellular molecules.
including TRPV1 itself. Interestingly, it has been previously reported that oxidative stress and oxidative DNA damage can be induced in esophageal tissues and cells by short exposures to bile acids and low pH (14, 16). In this study, we examined whether TRPV1 activation by not only capsaicin but also acid induces IL-8 production in human esophageal epithelial cells.

Fig. 4. Synthetic HNE significantly enhanced capsaicin-induced IL-8 production in Het1A cells. IL-8 production in Het1A cells treated with the indicated concentrations of capsaicin and/or HNE, or with vehicle alone, was evaluated using an ELISA. Data are expressed as the mean ± SE of 3 separate experiments. *P < 0.05 vs. capsaicin treatment.

Fig. 5. Synthetic HNE modified TRPV1 in Het1A cells. A: Het1A cells were incubated with or without (control) synthetic HNE (10 μM) for 20 min. Cell lysates were centrifuged, and the supernatants were immunoprecipitated with a polyclonal anti-TRPV1 antibody (Anti-TRPV1-IP). Immunoprecipitates were analyzed by Western blot analysis using a monoclonal anti-HNE and an anti-TRPV1 antibody. B: Het1A cells were incubated with (d, e, and f) or without (a, b, and c) synthetic HNE (1 μM) for 20 min. The cells were costained with a monoclonal anti-HNE antibody (a and d) and an anti-TRPV1 antibody (b and e). The anti-HNE and anti-TRPV1 antibodies were detected using AlexaFluoro 488-labeled goat anti-mouse IgG antibody and AlexaFluoro 647-labeled goat anti-rabbit IgG antibody, respectively. The merged images (yellow color) show the colocalization of TRPV1 with HNE-modified proteins (c and f).
expressed on Het1A cells. Because the monoclonal anti-HNE antibody used in this study recognizes HNE-modified amino acid residues such as histidine, HNE might bind to extracellular or intracellular sites of TRPV1 and modify the function of TRPV1.

In conclusion, although low concentration of HNE (1 μM) alone did not induce IL-8 production from Het1A cells, this concentration of HNE significantly enhanced IL-8 production in capsaicin-pretreated Het1A cells (even at 10 μM capsaicin). This result suggests that a low level of oxidative stress can enhance capsaicin-induced cytokine production from Het1A cells even at a low concentration of capsaicin. We therefore hypothesize that capsaicin-induced ROS might, in turn, modify amino acid residues of TRPV1 and affect its function, thereby forming a vicious circle of IL-8 production and subsequent inflammation. In contrast to our findings, it has been previously reported that HNE diminished IL-8 release and induced apoptotic cell death in vascular endothelial cells (18). In our study, a low concentration of synthetic HNE did not induce apoptosis (data not shown) or affect IL-8 production. Therefore, the difference between these two studies may depend on the concentration of HNE used or the type of cell employed, as different cells may show different sensitivities to HNE. The precise function of TRPV1 on esophageal epithelial cells should be further elucidated.

In conclusion, capsaicin-activated TRPV1-induced ROS might directly modify amino acid residues of TRPV1 and affect its function to form a circle of IL-8 production and subsequent inflammation of the esophagus. Although it remains to be investigated whether oxidative stress-induced modification of TRPV1 raises the hypersensitivity of the esophagus, defining these mechanisms may help in the development of therapies for GERD.

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

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