Attenuation of Acid-induced Esophagitis in VR-1 Deficient Mice

Kazunori Fujino¹, Sebastian G. de la Fuente¹, Yogi Takami¹, Toku Takahashi¹,
Christopher R. Mantyh¹

Department of Surgery¹,

Duke University Medical Center, Durham, NC 27710, USA
Veterans Administration Hospital, Durham, NC

Corresponding author:

Christopher R. Mantyh, MD
Duke University Medical Center
Box 3117
Durham, NC 27710
USA
Email: manty001@mc.duke.edu

Key words: Receptor; Substance P; Neurogenic inflammation; Esophagitis; Vanilloid
receptor subtype-1; Capsaicin.
Abstract:

BACKGROUND AND AIMS: Activation of the vanilloid receptor subtype-1 (VR-1) results in release of pro-inflammatory peptides, which initiate an inflammatory cascade known as neurogenic inflammation. We investigated its role in an acute model of surgically-induced esophagitis. METHODS: Esophagitis was induced by pyloric ligation in wild-type, and VR-1 deficient mice. A subset of animals were administered the VR-1 antagonist capsazepine, famotidine, or omeprazole 1 hour before surgery. Five hours after surgery, myeloperoxidase activity (MPO), histologic damage scores, intragastric pH, and immunocytochemical analysis of substance P (SP) receptor endocytosis were determined. RESULTS: Esophagitis-induced knockout mice exhibited significantly lower levels of MPO activity, histologic damage scores, and SP receptor endocytosis than wild-type mice. Inflammatory parameters were significantly reduced by acid inhibition and capsazepine in wild-type mice. CONCLUSIONS: We conclude that acute acid-induced esophagitis is reduced in animals lacking VR-1. This suggests that acid-induced esophagitis may act through VR-1 and that inhibition of the receptor may reduce inflammation.
Gastroesophageal reflux disease (GERD) is a common disorder affecting an estimated 5% to 7% of the global population, [1] and refers to the backflow of gastric or biliary contents into the esophagus resulting in inflammation and tissue damage of the esophagus. Continuous exposure to these compounds may result in pre-cancerous conditions such as Barrett’s esophagus or invasive subsequent cancer. Although symptomatic relief is achieved in a number of patients who are treated with therapies aimed to correct the altered anatomic or physiologic conditions, none of these correct the underlying inflammation.

Previous studies have investigated the mechanisms of neurogenic inflammation in the pancreas [2] and the intestine; [3] [4] [5] however, there has been little research focused on the association between the enteric nervous system and esophageal diseases. Neurogenic inflammation refers to the stimulation of primary sensory neurons, which in turn conveys nociceptive information to the spinal cord and exacerbates the inflammatory and immune responses in the peripheral tissues via an axon reflex.[6] [7] This neurogenic response is characterized by plasma extravasation, vasodilatation, neutrophil infiltration, and activation of immune cells.[8] The neurogenic arc is initially triggered by an unknown mechanism, which results in release of specific neuropeptides from sensory neurons that act on their respective receptors. Substance P (SP), an 11 amino acid peptide member of the tachykinin family [9] has been extensively studied in its role in neurogenic inflammation. SP is distributed throughout the gastrointestinal tract and in the peripheral and central nervous system where it mediates sensory and motor physiologic functions. Although a specific axon reflex has not been conclusively demonstrated in the intestine, pro-inflammatory neuropeptides such as SP
have been implicated in a variety of pathological conditions associated with chronic inflammation and pain. [10] [11] Specific to the esophagus, radioimmunoassay studies have found that the gastroesophageal junction region contains a relatively high number of SP-containing neurons [12] and SP receptors are found in the circular muscle and enteric plexus of the esophagus. *In-vitro* studies of muscle strips obtained from patients with Barrett’s disease have shown that SP receptors are also present in the smooth muscle of these patients. [13] These findings are consistent with the proposal that SP acts as a neurotransmitter in mediating certain responses to chemonociceptive stimuli in the region [14] and influencing the tone of the LES pressure. [15] [16]

Recent observations in animal models of dermatitis, [17] pancreatitis, [2] and acute and chronic colitis [3] [4] [5] have suggested that SP is released upon stimulation of the vanilloid receptor subtype-1 (VR-1), also known as TRPV1 (transient receptor potential vanilloid subtype-1), which is present only in primary sensory neurons. VR-1 is a non-selective cation channel that is activated by heat, protons, and the exogenous ligand capsaicin. Recently, endogenous ligands such as anandamide (ANA) and leukotriene B4 (LTB4) have also been shown to stimulate VR-1 *in-vitro* [18] [19] [20] and *in-vivo*. [21] Although the role of these compounds in the esophagus is not completely understood thus far, these findings are of interest since early experiments have demonstrated the importance of lipoxygenase metabolites in animal esophagitis models. [22] With this background, the present study was designed to test the hypothesis that VR-1 is responsible, at least in part, of the initial inflammatory process associate with acute esophagitis. Using both wild-type and mice deficient in VR-1, we surgically constructed a model of acute esophagitis by pyloric ligation as previously described [25] and
examined the effect of either inhibition of VR-1, genetic deletion of VR-1, or blockade of acid secretion using standard pharmacologic agents. To further investigate the association between VR-1 stimulation and SP release, we performed immunocytochemical analysis of substance P (SP)-receptor (neurokinin-1, NK-1) endocytosis in esophageal myenteric plexus neurons.

**Materials and Methods**

**Animals**

These studies were conducted at the Durham Veterans Affairs Medical Center, Durham, North Carolina, United States. All aspects of the research were reviewed and approved by the institution’s animal care and use committee. Male 8 – 12 weeks C57BL/6 wild-type (VR-1 +/+) and VR-1 deficient (VR-1 -/-) mice were purchased from an authorized vendor (Charles Rivers Laboratories, Raleigh, NC) or obtained by Dr. Sidney Simon and housed under standard laboratory conditions until used. Mice were genotyped using standard polymerase chain reaction (PCR) technique. Genotypic screening of offspring was done as previously described by PCR using tail clip DNA. Briefly, tails were cut into 0.4-0.6-cm pieces and DNA isolated using a QIAamp kit from Qiagen (Valencia, California). Samples were incubated in 180 µl of buffer ATL and 20 µl Proteinase K, vortexed, and incubated at 55 °C until the tissue was completely lysed. After lysis was completed, a 400 µl buffer AL-ethanol mixture was added to the sample and mixed vigorously by vortexing. The mixture was then pipetted into a DNeasy mini column setting in a 2 ml collection tube and centrifuged at 6000x g (8000 rpm) for 1 minute. 500 µl of buffer AW1 and 500 µl of buffer AW2 was mixed with the sample and
centrifuged for 3 minutes at full speed to dry the DNeasy membrane. The DNeasy mini column was placed in a clean 2 ml microcentrifuge tube and 200 µl of buffer AE was pipetted directly onto the DNeasy membrane. The final mixture was incubate at room temperature for 1 minute, and then centrifuged for 1 minute at 6000x g. PCR amplification was carried out with primers (0.5µl), 10 nM dNTPs (0.5 µl), MgCl₂ (1.5 µl) and Taq polymerase (0.1 U) for 34 cycles with at an annealing of temperatures of 94 °C for 30 seconds, 55 °C for 30 seconds and 72 °C for 1 minute. Primers for wild-type mice were CGA GGA TGG GAA TAA CTC ACT, Mw 7435,8 µg/µmole and GGA TGA TGA TGA AGA CGA CCT TGA AGT, Mw 7466,8 µg/µmole from Invitrogen (Carlsbad, California). Primers for knockout screen consisted of AAT GGG CAG GTA GCC GGA TCA AGC G, Mw 7782,0 µg/µmole and AAC AGA CAA TCG GCT GCT CTG ATG C, Mw 7652,0 µg/µmole from Invitrogen. The PCR products were then separated on an agarose gel and products were visualized with ethidium bromide.

Induction of acid reflux esophagitis

Animals were deprived from food but not water 18 hours prior to the studies. On the day of the surgery, animals were weighed and anesthesia was induced by injecting an intraperitoneal (i.p.) solution containing 90 mg/kg ketamine: 10 mg/kg xylazine. Each procedure started once the animal exhibited no response to a toe pinch. Wild-type and VR-1 deficient animals received either an acid-inducing esophagitis (n = 6 each genotype) procedure or a sham operation (n = 6 each genotype). The abdominal area was clipped of fur with an electric razor and prepped with three washes of betadine. A small upper midline incision was made and both the pylorus and the junction between the forestomach and corpus were ligated as previous reported.[28] The non-glandular corpus
region of the mouse stomach is achlorhydric with a pH between 6-7, therefore to increase the acid contact the forestomach was ligated with an additional suture. For the sham operation, the animals had an identical procedure except that the ligatures were loosely applied around the pylorus and corpus respectively, and not tied. The animals were kept under anesthesia for 5 hours, then the entire esophagus and stomach were removed and fixed in paraformaldehyde for further examination. The animals were then euthanized by cervical dislocation. Because of our provocative results from these experiments, we elected to further study the effects of anti-secretatory agents and a VR-1 antagonist on the two sets of mice. Thus, a subset of wild-type and VR-1 (-/-) animals were randomized to receive either capsazepine [(100 µmol/kg, n = 6), Tocris, Ellisville, MO], famotidine [(10 mg/kg, n = 6), Sigma St. Louis, MO], or omeprazole [(400 µmol/kg, n = 6), Sigma St. Louis, MO]. Administration of drugs was performed in all cases subcutaneously 1 hour prior to the operation. The dose of each drug was extrapolated from other mouse studies where acid secretion [23] [24] or VR-1 inhibition [21] were dose-dependently studied.

**Histologic examination**

The removed distal esophagus and proximal stomachs were paraffin-embedded and subsequently cut into 5 µm sections. The sections were H&E stained and graded according to a previously validated scale (Table 1). [26]
Table 1  Details of histological scoring system.

<table>
<thead>
<tr>
<th>Score</th>
<th>Surface Ulceration</th>
<th>Acute inflammatory infiltrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>Present</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>Absent</td>
<td></td>
</tr>
</tbody>
</table>

**Lamina propria**

<table>
<thead>
<tr>
<th>Score</th>
<th>Lamina propria</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No polymorphs</td>
</tr>
<tr>
<td>1</td>
<td>Mild infiltrate</td>
</tr>
<tr>
<td>2</td>
<td>Moderate infiltrate</td>
</tr>
<tr>
<td>3</td>
<td>Severe infiltrate</td>
</tr>
</tbody>
</table>

**Submucosa**

<table>
<thead>
<tr>
<th>Score</th>
<th>Submucosa</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No polymorphs</td>
</tr>
<tr>
<td>1</td>
<td>Mild infiltrate</td>
</tr>
<tr>
<td>2</td>
<td>Moderate infiltrate</td>
</tr>
<tr>
<td>3</td>
<td>Severe infiltrate</td>
</tr>
</tbody>
</table>

The scale takes into account the presence of mucosal injury as well as infiltration of inflammatory cells within the lamina propria or submucosa. All specimens were scored by observers unaware of the treatment groups.

*Intragastic pH measurement*
The intragastric content was extracted from the removed specimens and centrifuged at 4°C for 2 minutes. The intragastric pH was measured using a Corning 430 pH meter (Corning, NY).

**Myeloperoxidase (MPO) activity**

Segments of the distal esophagus were removed immediately after euthanasia and stored frozen at –80°C for MPO activity measurement as previously described. [27] The specimens were weighed, placed in a plastic tube on ice, and 0.5% of hexadecyltrimethylammonium bromide (HTAB) in 50 mM KH₂PO₄ (pH 6) (HTAB buffer) was added to each sample. Samples were homogenized on ice using Polytron tissue homogenizer for 15 seconds followed by three cycles of freeze/thawing. All samples were fortified with additional HTAB buffer to equal 1 ml HTAB/50 mg wet weight. The samples were then vortexed and 500 µl of each was transferred to microfuge tubes. The tubes were centrifuged in an Eppendorf microfuge [12,000x g] at 4°C for 2 minutes and the absorbance of each supernatant was read at 460 nm at 0, 30, and 60 seconds after addition of 2.9 ml of 0.167 mg/ml O-dianisidine dihydrochloride to 0.1 ml supernatant. One unit of MPO activity was defined as degradation of 1 mol of peroxide per minute at 25°C; the results are expressed in units per gram of protein.

**Immunocytochemical analysis of SP release and quantification of neurokinin 1 (NK-1) receptor endocytosis**

with capsazepine, and VR-1 (-/-) mice were fixed overnight in ice-cold freshly
depolymerized paraformaldehyde (4% in PBS) at 4°C and then placed in ice-cold PBS-
30% sucrose for 24 h. The tissue was then embedded in Tissue Tek OCT (Sakura,
Torrance, CA), frozen, sectioned at 20 µm, mounted on Superfrost Plus glass slides
(Fisher, Pittsburgh, PA), and dried with desiccant at room temperature for 4 h. After being
washed, the sections were stained overnight at room temperature using a rabbit antiserum
(no. 11886-5 from Steven Vigna) specific for the COOH-terminal 15 amino acids of the
rat NK-1 receptor (SPR_{393-407}) at a dilution of 1:3,000. The sections were then washed and
incubated with cyanine 3-conjugated donkey anti-rabbit IgG secondary antibody (Jackson
ImmunoResearch, West Grove, PA) at a dilution of 1:600 for 3 h at room temperature.
The sections were washed and coverslipped using one drop of Aquamount (Lerner
Laboratories, Pittsburgh, PA).

Immunostained sections were analyzed using a Zeiss LSM-410 inverted
krypton-argon confocal laser scanning system coupled to a Zeiss Axiovert
100 microscope. Optical sections (0.5 µm) of 512 × 512 pixels were obtained and
processed using Adobe PhotoDeluxe. Quantification of NK-1 receptor endocytosis was
performed by analyzing 10 NK-1 receptor-immunoreactive esophageal myenteric plexus
neurons per mouse and determining the number of these cells containing >50 NK-1
receptor-immunoreactive endosomes. Cytoplasmic endosomes were distinguished from
plasma membrane-associated NK-1 receptor immunoreactivity by ensuring that the
nucleus of the cell was in the same optical section as the NK-1 receptor-immunoreactive
endosomes.
Statistical analysis

Data is presented as mean ± SEM except indicated. Statistical analyses were performed using analysis of variance (ANOVA) followed by the Dunnett’s or Tukey-Kramer posttests as indicated. Statistical analysis was done using GraphPad Instat version 3.00 for Windows 95 (GraphPad Software, San Diego, CA). Significance was assumed to occur at $p < 0.05$.

Results:

The acid-induction model used produced significant levels of inflammation characterized by increased MPO activity, pathologic scores, and histology compatible with esophagitis. Both wild-type and VR-1 deficient mice exposed to the acid-induced operation exhibited significantly higher levels of MPO activity compared to those within the sham operation group (Figure 1). The same figure illustrates the significantly higher MPO activity in VR-1 (+/+ ) as compared to VR-1 (-/-) mice in acid-exposed animals. Similar to MPO activity, pathologic scores were significantly increased by the acid-induced operation in both wild type and knockout mice (Figure 2), however VR-1 (-/-) mice demonstrated significantly less pathologic damage than VR-1 (+/+ ) animals. Microscopic architecture was maintained and only few inflammatory cells were observed in the lamina propia and mucosa of animals in the sham-operated group (Figure 3 A, B) in both VR-1 (+/+ ) and (-/-) mice. Conversely, VR-1 (+/+ ) mice exposed to acid by the surgical ligation technique exhibited consistent erosions with infiltration of inflammatory cells, and disorganized architecture (Figure 3 C). However, VR-1 (-/-) mice exposed to
the acid-induction operation showed histologic patterns similar to those seen in the sham-operated group, with minimal to no inflammatory changes (Figure 3 D).

To test the effect of acid suppression in this model of acute esophagitis, a subset of wild type and VR-1 deficient mice were pre-treated with famotidine, a histamine antagonist, or omeprazole, a proton pump inhibitor. Additionally, pre-treatment with capsazepine, a VR-1 antagonist was compared to animals in which antisecretory therapy was administered. Figure 1 illustrates MPO activity in sham-operated mice and mice instrumented with an acid-induction operation treated with capsazepine, famotidine, or omeprazole. MPO activity was significantly increased in wild-type mice exposed to acid; MPO levels were significantly reduced by pharmacologic inhibition of acid with famotidine or omeprazole as well as the VR-1 antagonist capsazepine. The inhibition in MPO activity levels observed after treatment with capsazepine was statistically similar to that present in the omeprazole-treated animals. Similar to MPO, the pathologic damage score was also significantly reduced with famotidine, omeprazole, or capsazepine pre-treatment in wild type animals (Figure 2). There was no statistical difference in the pathological score between VR-1 (-/-) animals and wild type animals treated with omeprazole, famotidine, or capsazepine.

Intragastric pH was also measured in all groups tested (Table 2).
Table 2: Intragastric pH in mice with either a sham operation or esophagitis. A subset of mice was administered famotidine, omeprazole or capsazepine. Note that sham-operated mice did not have a significant reduction in intra-gastric pH, while both (+/+ ) and (-/-) mice with esophagitis had a significant reduction in pH. Note the increased in pH levels following antisecretory and VR-1 antagonism therapy. Values are expressed as mean ± SD. * p< 0.01 compare to VR-1 (+/+).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Operation</th>
<th>Drug</th>
<th>Intragastric pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>VR-1 (+/+)</td>
<td>Sham</td>
<td></td>
<td>6.12 ± 0.89</td>
</tr>
<tr>
<td>VR-1 (-/-)</td>
<td>Sham</td>
<td></td>
<td>5.98 ± 0.95</td>
</tr>
<tr>
<td>VR-1 (+/+)</td>
<td>Esophagitis</td>
<td></td>
<td>2.15 ± 0.43</td>
</tr>
<tr>
<td>VR-1 (-/-)</td>
<td>Esophagitis</td>
<td></td>
<td>2.28 ± 0.33</td>
</tr>
<tr>
<td>VR-1 (+/+)</td>
<td>Esophagitis</td>
<td>famotidine</td>
<td>4.23 ± 1.53*</td>
</tr>
<tr>
<td>VR-1 (+/+)</td>
<td>Esophagitis</td>
<td>omeprazole</td>
<td>5.81 ± 0.35*</td>
</tr>
<tr>
<td>VR-1 (+/+)</td>
<td>Esophagitis</td>
<td>capsazepine</td>
<td>4.33 ± 0.80*</td>
</tr>
</tbody>
</table>

Intragastric pH was significantly lower in both VR-1 (+/+ ) and VR-1 (-/-) mice undergoing surgically-induced esophagitis. However, wild type mice pre-treated with famotidine, omeprazole, or capsazepine had significantly elevated pH levels in the esophagitis model. Capsazepine pre-treatment resulted in intragastric pH levels comparable to those of antisecretory therapy.

*Confocal microscopy and SP release*
Confocal microscopy of the myenteric plexuses of the lower esophagus demonstrated normal distribution of the NK-1 receptor localized in the plasma membrane of neurons in animals in the sham-operated group (Figure 4 A). However, in wild type mice exposed to acid, the NK-1 receptor was observed in multiple intracellular cytoplasmatic endosomes indicating SP release and NK-1 receptor binding and subsequent internalization (Figure 4 B). We have previously demonstrated that this represents accurate substance P release and receptor activation. [7] Capsazepine pre-treatment inhibited SP release and subsequent endocytosis, as seen by the majority of NK-1 receptors found on the plasma membrane (Figure 4 C). Quantification of internalization was performed by using a previously established method of internalized NK-1 receptor positive endosomes (Figure 5). Minimal endocytosis was found in sham-operated animals, however a significant elevation in NK-1 endocytosis was seen in surgically-induced esophagitis. However, VR-1 (-/-) animals and VR-1 (+/+) animals with esophagitis pre-treated with famotidine, omeprazole, and capsazepine demonstrated a significant reduction in NK-1 endocytosis in comparison to sham-operated wild type animals.

**Discussion:**

In this report we investigated the role of VR-1 in an acute model of esophagitis. Our results indicate that acid-induced esophagitis is significantly attenuated in animals lacking VR-1 or by pharmacologically antagonizing the receptor in wild type mice. The magnitude of the reduction in inflammation was similar to those produced by standard antisecretory therapy. Additionally, reduction of SP release and subsequent NK-1
receptor activation (as measured by NK-1 receptor endocytosis) mirrored the decrease in inflammation indexes in mice either deficient in VR-1 or wild type mice provided anti-secretory drugs. These results strongly suggest that in this model of esophagitis, acid exposure stimulates VR-1, which subsequently releases SP to initiate an inflammatory cascade.

VR-1 is a non-selective cation channel with six-transmembrane domains that is found on primary sensory neurons and provides sensory information of chemical, physical, and inflammatory input. [28] The receptor has been localized using immunohistochemistry in numerous locations throughout the gastrointestinal tract, including the esophagus [26] stomach, small intestine, and colon.[29] VR-1 immunoreactivity is found predominantly in nerve fibers of the myenteric plexus, with lesser staining in the lamina propria and nerve endings that innervate the mucosa. [30] Interestingly, such nerve endings seem not to penetrate the entire extent of the mucosa suggesting that the luminal stimulants that act on VR-1 either pass across the mucosa or enhance the production of secondary mediators to operate on the receptor. Recent studies indicate that VR-1-containing primary sensory neurons are present in the lower portions of the esophagus [31] and that the number of immunoreactive fibers increase in patients with esophagitis.[26]

Stimulation of VR-1-containing primary sensory neurons release neuropeptides including SP that convey nociceptive information to the spinal cord and also modulate inflammation in the peripheral tissues via an axon reflex. Known agonists of VR-1 include heat, acid, and capsaicin. Additionally, a variety of endogenous ligands have emerged as potential agonists of VR-1. Work from our group and others have
demonstrated that inhibition of VR-1 using selective antagonists, or surgical sensory denervation attenuates several animal models of intestinal inflammation. [5] [9] Other investigators have found similar findings in models of pancreatitis, [2] and cystitis. [32] In addition to the reduction of inflammatory parameters to basal levels in these models, sensory nerve ablation or inhibition of VR-1 using selective VR-1 antagonists also significantly decreased NK-1 receptor internalization indicating a diminution of SP release. This suggested that in these models, sensory nerves containing VR-1 are stimulated and results in SP release and subsequent inflammation.

In this study we intended to exam the effect of endogenous acid, a known agonist of VR-1, in mice either deficient in VR-1 or mice provided an antagonist to VR-1 prior to acid exposure. In this model, ligation of the pylorus and forestomach results in a rapid infusion of acid into the esophagus (as demonstrated by the significant reduction in intra-gastric pH [Table 2]) and resulted in a robust inflammatory response with a significant increase in MPO activity and pathological damage. Mice genetically deficient in VR-1 [VR-1 (-/-)] demonstrated a significant reduction in MPO and pathologic damage than wild-type animals (Figures 1-2). This reduction in MPO and pathologic scoring in the VR-1 (-/-) mice was similar to mice that were pre-operatively treated with standard anti-secretagory agents, famotidine and omeprazole, indicating that acid was the causative agent in initiating inflammation. Intragastric pH was elevated in these animals, strongly suggesting that inhibition of acid production prevented inflammation. Furthermore, pre-treatment with capsazepine, an antagonist of VR-1, in VR-1 (+/+) animals, demonstrated a nearly identical inhibition of inflammation as famotidine and omeprazole. Interestingly, capsazepine pre-treatment resulted in an increase in intra-gastric pH. To
date, there is no reported effect of capsazepine on acid secretion. The cause of this is not clear, however several reports indicate that capsazepine may inhibit nicotinic acetylcholine receptors and non-specifically block voltage-activated calcium channels. 

We also examined the effects of genetic deletion or antagonism of VR-1 on SP receptor internalization in acid-exposed mice. Quantification of SP-receptor using confocal microscopy has been shown to correspond to SP release from sensory neurons and has been shown to directly correlate with inflammation. Similar to previous work, SP release (as measured by SP-receptor internalization) mirrored the inflammatory parameters of MPO and pathologic damage (Figures 1 and 2). Wild type mice and mice pre-treated with famotidine, omeprazole, and capsazepine demonstrated a significant reduction in SP receptor internalization, suggesting that without acid, VR-1 is not stimulated to release SP. Furthermore, VR-1 deficient animals with acid exposure (pH 2.15) demonstrated a significant reduction in NK-1 endocytosis, indicating that without VR-1, acid cannot stimulate VR-1 to release SP.

Clinically, these results may provide insight into the pathophysiology of the presence of chronic cough and asthma in patients suffering from reflux disease. Previously, this association has been explained by several physiopathologic mechanisms including 1) a vagal-esophageal-bronchial reflex, 2) sensitive bronchial reactivity, and 3) microaspiration of gastric contents (reviewed by Harding, [35]). Hamamoto et al. [36] proposed an alternative pathway in which C-fibers contained in the vagal nerve elicit tachykinins release that contract the bronchial smooth muscle, increase bronchial secretion, [37] and increase vascular permeability. [38] [39] The use of specific
tachykinin receptor antagonists has been used to prevent bronchoconstriction and airway edema in different animal models of acid-induced reflux disease. [40] [41] [42] To date, the involvement of VR-1 on this esophageal-pulmonary circuit is unknown, however the availability of potent VR-1 antagonists and animals deficient in VR-1 may clarify the role of the receptor in this inflammatory pathway.

Although these results are provocative, direct clinical correlation with GERD patients must be tempered on the basis of two arguments. First, the animal model used in our experiments represents an acute (five hours) esophagitis model that might not reflect the chronic process present in the majority of patients presenting with severe esophagitis. Secondarily, we used a model in which only acid was refluxated into the esophagus. Although acid is a primary factor in the inflammatory process associated to esophagitis, the role of biliary components in esophagitis has been emphasized by many authors. [39] Further studies are warranted to determine the effect of VR-1 inhibition in chronic acid exposure or alkaline reflux. Finally, the effects of genetic deletion of VR-1 or receptor antagonism do not to completely inhibit the effects of acid-induced inflammation. As demonstrated in figures 1, 2, and 5, the VR-1 (-/-) animals exposed to acid were found to have a significant reduction in inflammatory parameters and NK-1 endocytosis, when compared to wild type animals, however the levels did not return to sham-operated levels, indicating that other inflammatory processes are occurring.

In conclusion, this study presents direct evidence that acid-induced esophagitis is attenuated in mice deficient in VR-1. The reduction in inflammatory indices and pathological scoring mirrored the effects of standard antisecretory therapies. Additionally, SP release (and receptor endocytosis) was also reduced in VR-1 (-/-) mice
and wild type mice given capsazepine, indicating that acid stimulates VR-1 containing sensory neurons which in turn release SP. These results suggest a link between VR-1 and acid-induced esophagitis that might help elucidate the physiopathology of esophagitis and provide a novel therapeutic target for patients with gastroesophageal reflux disease.
Licence for Publication

"The Corresponding Author has the right to grant on behalf of all authors and does grant on behalf of all authors, an exclusive licence (or non exclusive for government employees) on a worldwide basis to the BMJ Publishing Group Ltd and its Licensees to permit this article (if accepted) to be published in Gut editions and any other BMJPG products to exploit all subsidiary rights, as set out in our licence (http://gut.bmjournals.com/misc/ifora/licenceform.shtml)."

Competing Interest

Please list Competing Interests if they exist if not please include the following statement; Competing Interest: None declared.
References:


32. Szallasi A, Conte B, Goso C, Blumberg PM, Manzini S. Characterization of a peripheral vanilloid (capsaicin) receptor in the urinary bladder of the rat. Life Sciences 1993; 52: 221-6.


**Legend**

Figure 1: MPO activity in sham operated and acid-induced animals given famotidine, omeprazole, and capsazepine one hour prior to surgery. MPO activity was significantly increased in both VR-1 (+/+) and VR-1 (-/-) acid-exposed groups compared to sham operated animals (* p < 0.05). VR-1 (-/-) mice exhibit lower levels of MPO compared to VR-1 (+/+)[† p < 0.05]. MPO activity was also significantly reduced by famotidine, omeprazole and capsazepine [‡ p < 0.05]. Data is presented as mean ± SEM.

Figure 2: Pathologic scores in sham operated and acid-induced animals given famotidine, omeprazole, and capsazepine one hour prior to surgery. Scores were significantly increased in acid induced operated animals compared to sham-operated mice (* p < 0.05). In acid-induced operated mice, VR-1 (+/+) scores were significantly higher than VR-1 (-/-) mice, famotidine treated animals, omeprazole treated animals, and capsazepine treated mice [† p < 0.05]. Data is presented as mean ± SEM.

Figure 3: H&E preparations of lower esophageal section (70X). Panel A shows normal histology in wild type animals exposed to a sham operation. The findings observed in the wild type animals were similar to those in the VR-1 (-/-) also subject to the same procedure (Panel B). When acidic reflux was stimulated in wild type mice, erosion, disorganized histoarchitecture, and infiltration of inflammatory cells were noted (Panel C). These changes however, were absent in the VR-1 (-/-) mice (Panel D), in which the microscopic morphology was maintained. All photomicrographs were at 70X.
Figure 4: Confocal microscopy of sham-operated (Panel A), acid-exposed (Panel B), and acid-exposed plus capsazepine treated mice (Panel C). Endosomal internalization of NK-1R was observed in myenteric neurons of acid-exposed animals, while sham-operated and capzasepine-treated wild-type mice had the receptors confined to the cell membrane (white arrows). The dark inner area of panel B (double arrow) represents the nucleus. Internalization of NK-1R was observed in myenteric neurons of acid-exposed animals. This phenomenon was inhibited by pretreatment with capsazepine (1000X).

Figure 5: Immunocytochemical analysis of SP release and quantification of neurokinin 1(NK-1) receptor endocytosis. Quantification of SP release in sham operated and acid-exposed animals. Exposure to acid produced a significant release of SP (*p < 0.05). SP release was reduced in VR-1 (-/-), VR-1 (+/+) plus famotidine-treated animals, VR-1 +/- plus omeprazole-treated animals, and VR-1 (+/+ plus capsazepine-treated animals [†p < 0.05]. Data is presented as mean ± SEM.
Figure 1:
Figure 2:
Figure 3:

A: VR-1 (+/+) sham operated
B: VR-1 (-/-) sham-operated
C: VR-1 (+/+) Acid reflux (+)
D: VR-1 (-/-) Acid reflux (+)
A: VR-1 (+/+) sham-operated  B: VR-1 (+/+) acid reflux (+)  C: VR-1 (+/+) + capsazepine

Figure 4:
Figure 5:
Attenuation of acid-induced oesophagitis in VR-1 deficient mice

Kazunori Fujino, Sebastian G. de la Fuente, Yogi Takami, Toku Takahashi and Christopher R. Mantyh

Gut published online August 9, 2005

Updated information and services can be found at:
http://gut.bmj.com/content/early/2005/08/09/gut.2005.066795.citation

These include:

Email alerting service
Receive free email alerts when new articles cite this article. Sign up in the box at the top right corner of the online article.

Notes

To request permissions go to:
http://group.bmj.com/group/rights-licensing/permissions

To order reprints go to:
http://journals.bmj.com/cgi/reprintform

To subscribe to BMJ go to:
http://group.bmj.com/subscribe/