HCl-induced and ATP-dependent upregulation of TRPV1 receptor expression and cytokine production by human esophageal epithelial cells

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Running head: ATP and Chemokines in esophageal epithelium

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Abstract

The pathogenesis of gastroesophageal reflux disease (GERD) remains elusive, but recent evidence suggests that early secretion of inflammatory cytokines and chemokines by the mucosa leads to influx of immune cells followed by tissue damage. We have previously shown that exposure of esophageal mucosa to HCl causes adenosine triphosphate (ATP) release resulting in activation of Lyso-PAF acetyltranferase (Lyso-PAF AT), the enzyme responsible for the production of platelet activating factor (PAF). In addition, HCl causes release of interleukin (IL)-8 from esophageal mucosa. We now demonstrate that esophageal epithelial cells secrete pro-inflammatory mediators in response to HCl, and that this response is ATP-mediated.

Monolayers of the human esophageal epithelial cell line HET-1A were exposed to acidified cell culture medium (pH 5) for 12 minutes, a total of 7 times in 48h, to simulate the recurrent acid exposure clinically occurring in GERD. HCl upregulated mRNA and protein expression for the acid sensing receptor TRPV1, Lyso-PAF AT, IL-8, eotaxin-1, -2, and -3, macrophage inflammatory protein-1α (MIP-1α) and monocyte chemoattractant protein-1 (MCP-1). The chemokine profile secreted by HET-1A in response to repeated HCl exposure parallels similar findings in erosive esophagitis patients. In HET-1A cells the TRPV1 agonist capsaicin reproduced these findings for mRNA of the inflammatory mediators tested (Lyso-PAF AT, IL-8 and eotaxin-1). These effects were blocked by the TRPV1 antagonists iodoresiniferatoxin (IRTX) and by JNJ-17203212. These effects were imitated by direct application of ATP and blocked by the non-selective ATP antagonist suramin.

We conclude that HCl/TRPV-induced ATP release upregulated secretion of various chemoattractants by esophageal epithelial cells. These chemoattractants are selective for leukocyte subsets involved acute inflammatory responses and allergic inflammation. The data support the validity of HET-1A cells as a model of the response of the human esophageal mucosa in GERD.

Key Words: Gastro-esophageal reflux, chemokines, TRPV1, ATP, esophageal epithelium
Introduction

Several studies using animal models and human tissue samples suggest that pro-inflammatory cytokine production may underlie the development of erosive esophagitis in gastroesophageal reflux disease (GERD) (8, 11, 15, 22, 56). The secretion of these mediators is believed to precede the infiltration of neutrophils and eosinophils into the mucosa and submucosa (4, 18, 21, 50), ultimately leading to tissue damage and ulcerations. This viewpoint is supported by endoscopically obtained esophageal biopsies from patients with erosive esophagitis showing infiltration of immune cells. Esophageal biopsies primarily contain epithelial cells, the major component of the esophageal mucosa, suggesting that chemoattractants may be present in and derived from epithelial cells. Enhanced expression of chemokines such as interleukin 8 (IL-8), a potent chemoattractant for neutrophils, has been detected in esophageal biopsy samples of erosive esophagitis patients, with IL-8 levels being associated with the endoscopic severity of erosive esophagitis (22). Thus, production of chemokines by esophageal epithelial cells may be an important step in initiating the inflammatory process. However, the specific mediators responsible for infiltration of immune cells (4, 18) in erosive esophagitis have not yet been defined.

Given the nature of the inflammatory infiltrate in GERD possible candidates for chemoattractants released by epithelial cells are IL-5, IL-8, RANTES (or CCL-5), eotaxin-1, eotaxin-2, eotaxin-3, monocyte chemoattractant protein-1 (MCP-1 or CCL-2), macrophage inflammatory protein 1α (MIP-1α or CCL3). These act primarily as chemotactic factors for eosinophils, monocytes and other immune cells (29, 57). PAF is a potent chemoattractant for eosinophils and selectively induces the migration of eosinophils over that of neutrophils (24, 49, 55).

We have previously shown that the esophageal mucosa contains acid sensing transient receptor potential cation channel, subfamily vanilloid member 1 (TRPV1) receptors and, when exposed to HCl, releases substance P (SP), CGRP, PAF and IL-8 (32). We have further shown that HCl-induced activation of TRPV1 causes adenosine triphosphate (ATP) release from esophageal epithelial cells that in turn causes releases of CGRP and SP from esophageal submucosal neurons and activation of Lyso-PAF acetyltranferase (Lyso-PAF AT), the enzyme responsible for the production of PAF in epithelial cells (33). Repeated application of HCl or of ATP causes upregulation of Lyso-PAF AT in epithelial cells (33). These data point to ATP as a critical inducer for release of inflammatory mediators, such as PAF and possibly for cytokines or chemoattractants, such as IL-8.
In the present investigation we performed a systematic screening of possible chemoattractants or cytokines released by TRPV1 activation in the human esophageal epithelial cell line HET-1A and examined the role of ATP in mediating the release of these mediators as well as their upregulation by repeated TRPV1 stimulation. After repeated acid exposure, mimicking reflux episodes in GERD patients (2, 43, 47), HET-1A cells upregulate mRNA and protein for the TRPV1 receptor itself, and increase expression for Lyso-PAF AT as well as selected cytokines and chemokines known to function as chemoattractants for neutrophils, eosinophils and monocytes. The cytokine increase was inhibited by an ATP receptor antagonist. The HCL-induced cytokine increases observed in HET-1A cells were similar to those observed in human esophageal biopsies from patients with erosive esophagitis. Taken together, these data indicate that acid-induced changes in the esophageal mucosa may begin with epithelial cells responding to acid by releasing ATP. ATP, in turn, induces upregulation of TRPV1 as well as secretion of multiple cytokines with preference for mediators that promote epithelial infiltration by eosinophils, neutrophils and monocytes.
Methods

HET-1A Cell Culture

Human esophageal squamous HET-1A cells (ATCC, Manassas, VA) were cultured at 37 °C in a 5% CO₂-humidified atmosphere in bronchial epithelial cell medium (BEGM BulletKit, Lonza, Walkersville, MD), containing basal medium (BEBM) plus additives (BEGM SingleQuots, Lonza, Walkersville, MD) in wells pre-coated with a mixture of 0.01 mg/ml fibronectin and 0.03 mg/ml vitrogen 100 (Cohesion, Palo Alto, CA). HET-1A was originally obtained from normal human esophageal autopsy tissue. It has been shown to retain epithelial morphology, it stains positively for cytokeratins and has remained non-tumorigenic (51).

Experimental procedure

HET-1A cells were exposed to BEBM pH 5 with 1.2mM Ca²⁺ for 12 minute episodes a total of 7 times in 48h. 2N HCl was added to BEBM media to bring the solution to pH 5.0. Cells were exposed for 12 minutes to acidified media at 8:30 AM, 11:30 AM and 2:30 PM each on day 1 and day 2. On day 3 final exposure took place at 8:30 AM for 12 minutes. One hour after non-acidified culture media was re-added the cells were harvested. Gene expression (mRNA) was determined by real-time PCR and protein expression by multiplex ELISA cytokine assay (Eve Technologies Corporation Calgary, Alberta, Canada). To confirm involvement of vanilloid receptors in acid-induced mRNA changes cells were pretreated with the TRPV1 receptor antagonist iodoresiniferatoxin (IRTX) (Sigma-Aldrich, St. Louis, MO) at 3×10⁻⁶ M or with JNJ17203212 (Tocris Bioscience Minneapolis, MN) at 10⁻⁶ M for 20 minutes before each exposure to acid.

To confirm involvement of the TRPV-1 receptor in these acid-induced changes, we used the selective TRPV1 agonist, capsaicin (10⁻⁵ M) (Sigma-Aldrich, St. Louis, MO) with the same repeated exposure protocol and measured mRNA for Lyso-PAF AT, IL-8 and eotaxin-1 to compare to acid-induced changes. To confirm involvement of ATP in these acid-induced changes we used the non-selective purinergic receptor antagonist suramin (10⁻⁶ M) (Sigma-Aldrich, St. Louis, MO) for 20 minutes before each exposure to acid or direct exposure to the ATP analog ATP-γS (10⁻⁴M) (Sigma-Aldrich, St. Louis, MO) with the
same repeated exposure protocol and measured mRNA for Lyso-PAF AT, IL-8, eotaxins, MCP-1 and MIP-1α to compare to acid-induced changes.

Real-time PCR

Total RNA derived from HET-1A cells was isolated by RNeasy Mini Kit (Qiagen, Valencia, CA). Two µg of total RNA were treated by DNAse I to eliminate DNA contamination. The sample was reversely transcribed and subjected to real-time PCR using GeneAmp Gold RNA PCR Reagent Kit and Power SYBR Green PCR Master Mix Kit (both Applied Biosystems, Foster City, CA). Primers used are shown in table 1.

Western Blot Analysis

Cells, incubated in 1.2 mM Ca\(^{2+}\) BEBM basal medium, were acid-treated, as described in the experimental procedures. Cells were then lysed in Triton X lysis buffer containing 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 50 mM NaF, 5mM EDTA, 1% (v/v) Triton X-100, 40mM β-glycerol phosphate, 40 mM p-nitrophenyl phosphate, 200 µM sodium orthovanadate, 100µM phenylmethylsulfonyl fluoride, 1µg/ml leupeptin, 1 µg/ml pepstatin A, and 1 µg/ml aprotinin. The homogenate was centrifuged at 10,000 g for 5 minutes, and the protein concentration in the supernatant was determined using the Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA), based on the Bradford dye-binding method (7). The supernatant containing 80µg protein was used for Western blot assay. The primary antibody for LysoPAF acetylCo-A transferase (Lyso-PAF AT; Novus Biologicals, Littleton, CO) was diluted 1:1500. The secondary antibody, horseradish peroxidase-conjugated anti-mouse antibody (Cell Signaling Technology, Danvers, MA), was diluted 1:2000. Detection was achieved with Western Lightning ECL agent (Perkin Elmer, Waltham, MA). Molecular weight was estimated by comparison of sample bands with prestained molecular weight marker (Bio-Rad, Melville, NY).

Multiplex ELISA Assay

The cell culture medium (from control and acid-treated HET-1A) was collected and sent to Eve Technologies Corporation (Calgary, Alberta, Canada) for multiplex ELISA 65 cytokine assay including
Eotaxin-1, Eotaxin-2, Eotaxin-3, IL-8, MCP-1, MIP-1α or and RANTES. The complete panel of cytokines examined is shown in Table 2.

**Human data**

A total of 25 consecutive patients (mean age 63 years, range 47–76) attending the out-patient units of Campus Bio Medico University of Rome for recurrent typical GERD symptoms (heartburn and/or acid regurgitation), lasting more than 6 months, and with evidence of erosive esophagitis (EE) at endoscopy (Grade A in 10, Grade B in 4, Grade C in 1, according to the Los Angeles classification), were invited to take part in the study. Exclusion criteria were: presence of Barrett’s esophagus, peptic ulcer disease, history of gastrointestinal (GI) cancer, and GI tract surgery (with the exception of appendectomy). Patients on Proton Pump Inhibitors, H2-antagonists or prokinetic drugs underwent a 3-week pharmacological wash-out before upper endoscopy. Seventeen asymptomatic, hospital staff volunteers, with no history of GERD, underwent the same protocol, thus representing a healthy control group.

After an overnight fast, patients and controls underwent upper endoscopy, performed by the same operator (AA). A combination of midazolam and propofol was used for sedation. The distal portion of the esophagus was carefully evaluated in order to determine the presence of mucosal injury. A total of four biopsies from each individual, two for routine histological evaluation and two for real-time PCR, were taken at 5 cm above the squamo-columnar junction in normal appearing mucosa from erosive esophagitis (EE) patients and control subjects.

**Results:**

**TRPV1 receptors in esophageal epithelium**

To verify the presence of acid sensing receptors on HET-1A cells we measured mRNA levels for TRPV1 to TRPV6 (figure 1). TRPV1 mRNA expression was higher than TRPV2-TRPV6 (p<0.05 ANOVA), suggesting that TRPV1 is the dominant transient receptor potential channel vanilloid subfamily member transducing the effects of H⁺ in epithelial cells.

To simulate the recurrent epithelial exposure to acid in GERD (2, 43, 47) HET-1A cells were exposed for 7 times during a 48 h period to pH5 with varying length of each exposure period (2 to 16 minutes). TRPV1, IL-8 and Eotaxin-1 mRNA increased upon incubation with acidified media, with a significantly higher mRNA expression with longer exposure episodes (Figure 2). For instance, when the cells were exposed 7 times to pH5 for 2 minutes exposure episodes, their mRNA levels did not increase with respect...
to non exposed cells. The maximum mRNA increase occurred for repeated exposure episodes of 12 minutes. Increasing the length of the exposure episodes to 16 minutes did not measurably increase mRNA for TRPV1, IL-8 or eotaxin-1. The maximally effective 12 minute exposure setting was therefore used for the subsequent experiments. This acid exposure protocol induced <2% cell death over the treatment period as measured by Trypan blue exclusion with HET-1A monolayers remaining intact when examined microscopically. We have previously demonstrated that in vitro exposure of esophageal mucosa to a more acidic pH, for example pH 4, causes significant cell death (11), most likely because the preparation is not buffered by continuous blood perfusion, as it occurs in vivo.

Figure 3 shows that the selected acid exposure protocol increases TRPV1 mRNA and protein, similar to the TRPV1 increase previously reported for EE patients (18).

**HCl-induced inflammatory mediators and cytokines**

Consistently with previously reported data (33), the selected acid exposure protocol caused an increase in Lyso-PAF AT mRNA and protein (figure 4), likely reflected by increased PAF production in response to HCl-induced stimulation (32). A similar increase in Lyso-PAF AT mRNA was noted in esophageal biopsies from EE patients. Figure 5 shows a similar increase in IL-8 mRNA and protein in HET-1A cells and increased IL-8 mRNA in esophageal biopsies from EE patients, consistent with data previously demonstrated in rabbit mucosa (32).

A multiplex Elisa for 65 cytokines (table 2) was used to explore the changed cytokines or chemokines on protein level in response to repeated HCl exposure.

HCl also caused an increase in mRNA for eotaxin-1, -2 and -3 (figure 6-8) that was associated with a corresponding increase in protein secretion. A similar increase in mRNA was observed in esophageal biopsies from EE patients.

A comparable HCl-induced increase in MCP-1 and MIP-1α mRNA and protein expression in HET-1A cells is shown in figures 9-10. A similar mRNA increase was observed in esophageal biopsies from EE patients.

**Role of TRPV1 and ATP in upregulation of inflammatory mediators/cytokines**

To investigate the mechanisms underlying the HCl-induced mediator secretion by HET-1A we performed experiments exploring the role of TRPV-1 and examining a possible role of ATP in this process. After HCl exposure mRNA expression by HET-1A for Lyso-PAF AT, IL-8, eotaxin-1, -2 and -3,
MCP-1 and MIP-1α was significantly greater than controls, as previously shown (figure 11, p<0.05). Pre-
incubation with the TRPV1 receptor antagonists IRTX and JNJ-17203212 inhibited the increase in mRNA
for Lyso-PAF AT, IL- 8, Eotaxin 1-2,-3, MCP-1, and MIP-1α (p<0.05) The values after TRPV1 blockade
were not significantly different from control values, supporting a role of TRPV1 in mediating the HCl-
induced mRNA increase. Similarly, preincubation with the non-selective ATP antagonist suramin
inhibited the increase in mRNA for Lyso-PAF AT, IL- 8, Eotaxin-1, -2, -3, MCP-1, and MIP-1α (p<0.05).
The values after suramin exposure were not significantly different from control values, supporting a role of
ATP in mediating the HCl-induced, TRPV1-mediated mRNA increase.

To confirm the involvement of the TRPV-1 receptor and ATP in the observed HCl-induced changes
HET-1A cells were treated with the selective TRPV1 agonist capsaicin using the same repeated exposure
protocol as for HCl, and mRNA was measured for Lyso-PAF AT, IL-8 and eotaxin-1 (figure 12).  Repeated capsaicin exposure increased Lyso-PAF AT, IL-8 and eotaxin-1 mRNA in comparable levels to
those induced by HCl exposure. As expected, the capsaicin-induced mRNA increase was significantly
(p<0.05) reduced by the TRPV1 antagonists IRTX, and JNJ-17203212 similarly to antagonism for the
HCl mediated response. The capsaicin-induced mRNA increase was also blocked by the ATP antagonist
suramin (p<0.05), indicating a role of ATP in TRPV1-induced mRNA upregulation.

Direct and repeated exposure of the epithelial cells to the non-hydrolysable ATP analog ATPγS using
the above protocol reproduced the HCl- and TRPV1-induced upregulation of Lyso-PAF AT, IL-8,
Eotaxin-1, -2, -3, MCP-1, and MIP-1α further supporting a role of ATP in the upregulation (Figure 13).

It has been suggested that IRTX may act as a TRPV1 agonist at 10^-7-10^-6 M concentrations evoking a
hypothermic response similar to that evoked by capsaicin. To exclude this possibility in our system, HET-
1A cells were exposed for 5 min to IRTX (10^-7 - 10^-5 M). In addition cell were exposed for 5 min to HCl
(pH 5.0) as positive control. ATP released by HET-1Acells in response to these stimuli was measured
using the ATP light Luminescence ATP Detection Assay System from PerkinElmer (Waltham, MA). The
medium was used to measure ATP, according to the manufacturer’s instructions.

Figure 14 indicates that, whereas HCl induced significant release of ATP from epithelial cells, IRTX
did not cause ATP release from these cells, excluding the possibility that the inhibitory role of IRTX in
this system may be due to receptor desensitization.
While in many chronic inflammatory processes the initiating event of inflammation remains elusive, in esophagitis a likely trigger is the effect of the gastro-esophageal refluxate, containing HCl, enzymes such as pepsin, and sometimes bile acids, on the esophageal epithelium. We have focused our investigations on the mechanisms of HCl-induced inflammation in the esophageal mucosa. We previously reported that circular muscle contraction is not affected by exposure to low pH, but is significantly reduced when the muscle is exposed to mucosa derived mediators after preincubating the mucosa with HCl (11). However, the relevance of HCl-induced esophageal mucosa-derived mediators might extend beyond influencing muscle contraction and may directly contribute to recruitment of the immune cell infiltrate seen in GERD and subsequent inflammatory damage. Epithelial cells constitute the first barrier encountered by acid reflux. Production of inflammatory mediators and cytokines by these cells may therefore be the first step in the inflammatory process, contributing to induction of esophagitis.

The human esophageal epithelial cell line HET-1A was originally obtained from normal human esophageal autopsy tissue. It retains epithelial morphology and cytokeratin expression, and has remained non-tumorigenic (51). HET-1A cells have been used to examine signaling pathways and transcriptional regulation of cytokine expression (44), to characterize the role of fibroblast growth factor in normal esophageal epithelium and in eosinophilic esophagitis (39) as well as to examine expression of mucin genes in the esophageal mucosa (54). It is a relevant and well established tool to examine esophageal epithelial cell response to bile acids (30, 40, 41) and acid-induced activation of TRPV1 (34).

We have previously shown that HCl-induced activation of TRPV1 causes release of ATP in HET-1A cells, that in turn activates Lyso-PAF AT, inducing production of the inflammatory mediator PAF, and that repeated exposure to low pH or ATP enhanced the expression of Lyso-PAF AT mRNA and protein (33). Exposure of esophageal epithelium to HCl also causes production and release of the cytokine IL-8 at concentrations sufficient to promote directed migration of leukocytes (32).

To extend the above findings we undertook a systematic investigation of cytokines and chemokines released by esophageal epithelial cells in response to HCl-induced activation of TRPV1 receptors. We used the selective TRPV1 agonist capsaicin to confirm the role of TRPV1 in mediator secretion, and confirmed that the regulation is mediated by TRPV1-induced ATP release.
To simulate recurrent exposure to acid, as it clinically is observed in GERD patients, HET-1A cells were exposed to acidified media, multiple times over two days. The selected protocol (seven episodes of 12 minutes exposure to pH 5 over two days) caused the maximal increase in mRNA for TRPV1, IL-8 and eotaxin 1, that was not increased by extending the duration of low pH exposure to 16 minutes. Thus HCl exposure episodes of 12 minutes may achieve maximal effects for release of inflammatory mediators/cytokines without damaging the cells and closely reproduces mRNA changes observed in human biopsies from erosive esophagitis patients (figures 4-10).

HCl-induced upregulation of TRPV1

HET-1A cells contain mRNA for several vanilloid receptors, with TRPV1 mRNA levels being substantially higher than those of TRPV2-TRPV6. We show that the selected HCl exposure protocol caused mRNA and protein upregulation of TRPV1. This is in agreement with the previously found TRPV1 upregulation in esophageal biopsies from EE patients, when compared to controls (18).

HCl-induced upregulation of Lyso-PAF–AT

In cat (9, 12), rabbit (32), and in human esophageal mucosa (9, 10) exposure to acid results in formation of PAF. PAF is an important chemoattractant and activator of immune cells (32), particularly eosinophils (55). In this study we confirmed that HET-1A cells repeatedly exposed to acidified media increase Lyso-PAF AT mRNA and protein presumably reflecting increased synthesis of PAF, as previously demonstrated (33). A similar increase in Lyso-PAF AT mRNA was observed in esophageal biopsies from EE patients.

HCl-induced upregulation of other chemoattractants in epithelial cells mediated by TRPV1 receptors.

We have recently shown that acid exposure of esophageal mucosa causes release of IL-8 in concentrations sufficient to stimulate directed peripheral blood leukocyte migration in an experimental model of acid-induced inflammation (32), confirming the role of acid-induced release of this cytokine in immune cell recruitment. We now demonstrate that repeated acid exposure increased IL-8 mRNA and protein in HET-1A cells. The increase in IL-8 mRNA in HET-1A cells was similar to the increase in IL-8 previously demonstrated in rabbit mucosa (32) and similar to the increase observed in esophageal biopsies.
from EE patients. These results are consistent with data by Souza et al in several esophageal epithelial cell lines (50).

We next performed a screening approach using a multiplex Elisa for 65 cytokines demonstrating upregulation of selected mediators that induce infiltration of eosinophils, neutrophils and monocytes. These were: IL-8, a CXC chemokine with potent chemotactic activity for neutrophils; eotaxin-1, eotaxin-2 and eotaxin-3, members of CC chemokines that promote the recruitment of inflammatory cells, particularly eosinophils (45); and MCP-1, MIP-1\(\alpha\) that are involved primarily in recruitment of monocytes and macrophages (3, 38). These findings were confirmed at the mRNA level using quantitative PCR.

To date, there are three members of the eotaxin family, eotaxin-1 (CCL11), eotaxin-2 (CCL24), and eotaxin-3 (CCL26), whose genes are located in different chromosomal positions but all act on the same chemokine receptor, CCR3, that is highly expressed on eosinophils (1, 25, 26, 42). Thus, eotaxins promote eosinophil recruitment and activation, enhanced adhesion, superoxide generation and degranulation (17, 23, 57). An increase in eotaxin levels was also observed in esophageal biopsies from EE patients and is consistent with recruitment of eosinophils in esophageal inflammation during acid reflux injury (6, 31, 35, 36).

In addition, the data show an increase in MCP-1 and MIP-1\(\alpha\) mRNA and protein expression after acid exposure. A similar mRNA increase was found in esophageal biopsies from EE patients. MCP-1, MIP-1\(\alpha\) are involved primarily in recruitment of monocytes and macrophages (3, 38). The presence of mononuclear cells in the esophageal lamina propria, but not within the epithelium, is indicative of erosive esophagitis (16, 20, 27). Production of these chemoattractants by esophageal epithelial cells after acid exposure may promote monocyte recruitment, even if the presence of monocytes is difficult to assess in esophageal biopsies of erosive esophagitis patients because few biopsies are sufficiently deep to contain a significant amount of lamina propria (27).

The TRPV1 antagonists IRTX and JNJ-17203212 significantly inhibited HCl-induced increases in mRNA levels for the investigated mediators, supporting a role of TRPV1. IRTX is a classic TRPV1 antagonist, but may also have other effects (14), including acting as an agonist at high doses (48). In the current study, however, IRTX completely inhibited the effect of the selective TRPV1 agonist capsaicin, and showed no evidence of acting as an agonist (figure 14). In any case the more recently developed antagonist JNJ-17203212, (52) a “second generation” TRPV1 antagonist (5) was used for comparison, and had exactly the same effect as IRTX. The critical involvement of TRPV1 in HCl-induced mRNA
upregulation of Lyso-PAF AT and selected cytokines is confirmed by the finding of a comparable mRNA upregulation by the selective TRPV1 agonist capsaicin.

Similarly, the ATP antagonist suramin significantly inhibited HCl- or capsaicin-induced increases in mRNA levels for the cytokines examined, supporting a role of ATP in the upregulation. For all cytokines there were no significant differences in mRNA levels between control values and values after exposure to HCl+TRPV1 antagonists or HCl+suramin. Repeated direct exposure to ATP over two days results in an increase in mRNA for Lyso-PAF AT and all relevant cytokines. Interaction between TRPV1 and ATP has been demonstrated in several experimental preparations, with TRPV1 inducing ATP release (33, 46) and ATP, in turn, potentiating TRPV1-mediated signaling (28, 53).

These results using HET-1A cells are consistent with data obtained in esophageal biopsies from patients with erosive esophagitis, that had significantly elevated mRNA for LysoPAF-AT, IL-8, eotaxin-1, and -2, MIP-1α, MCP-1, when compared to biopsies from healthy controls.

The current study shows that a highly selective spectrum of mediators is induced by exposure of human esophageal epithelial cells to low pH, as it occurs in patients with GERD. Among the significantly elevated cytokines there was a remarkable dominance for mediators that induce infiltration of eosinophils, neutrophils and monocytes. This suggests that acid exposure is not an indiscriminate inducer of mediator release that leads to non-specific recruitment of inflammatory cells. HCl can be considered a highly selective inducer of epithelial cell-released mediators recruiting restricted leukocyte subsets involved in acute inflammatory responses (neutrophils and monocytes) and allergic inflammation (eotaxins). This observation may help clarify, at least in part, the poorly understood clinical overlap between GERD and eosinophilic esophagitis: in fact, esophageal eosinophils at levels consistent with eosinophilic esophagitis can be found in up to 30% of patients with reflux esophagitis (13). In addition, our findings may also help explain why suppression of acid production can be beneficial in both eosinophilic esophagitis and GERD patients (37) (19). Both diseases likely begin at the epithelial level and the acidic refluxate may be seen as a regulator of a highly selective set of mediators by esophageal epithelial cells that determine the fate of the inflammatory process.

In summary, this study suggests that exposure to acid activates TRPV1 receptors in esophageal epithelial cells, causing release of ATP. ATP then causes upregulation of the TRPV1 receptors as well as PAF, IL-8, eotaxins, MCP-1 and MIP-1α that may contribute to inflammation and injury of the esophageal mucosa. Parallel findings in HET-1A cells treated with this HCl-exposure protocol and
esophageal biopsies from EE patients provides a simple and powerful experimental model to examine inflammation-related changes in esophageal epithelium.

Acknowledgments: Supported by NIDDK RO1 57030
### Table I

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### Table II

**Human 65-Plex Cytokine/Chemokine Panel**

- 6Ckine, BCA-1, CTACK, EGF, ENA-78, Eotaxin-1, Eotaxin-2, Eotaxin-3, FGF-2, Fit-3L, Fractalkine, G-CSF, GM-CSF, GRO, I-309, IFNα2, IFNγ, IL-1α, IL-1β, IL-1ra, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-16, IL-17, IL-20, IL-21, IL-23, IL-28α, IL-33, IP-10, LIF, MCP-1, MCP-2, MCP-3, MCP-4, MDC, MIP-1α, MIP-1β, MIP-1d, PDGF-AA, PDGF-AB/BB, RANTES, SDF-1 α+β, sCD40K, SCF, sIL-2Rα, TARC, TGFα, TNFα, TNFβ, TPO, TRAIL, TSLP, VEGF.


Figure Legends.

Figure 1
HET-1A cells contain mRNA for several vanilloid receptors, with TRPV1 mRNA levels being greater than those of TRPV2 - TRPV6 (p<0.05 ANOVA). Data represent mean ± SEM of three different sets of data from three separate experiments.

Figure 2
HET-1A cells were exposed 7 times in 48 hrs to pH 5 with exposure episodes of varying length. mRNA levels, obtained by real time PCR, are shown as a function of the length of individual exposures to pH5. The maximum mRNA increase occurred for repeated exposure episodes of 12 minutes. Increasing the exposure times to 16 minutes did not measurably increase mRNA. For simplicity data are shown only for TRPV1, IL-8 and Eotaxin-1. Values are mean ± SEM, N=3 for three different sets of data from three separate experiments. * denotes significant differences from control values (p<0.05)

Figure 3
Real time PCR and Western blot for TRPV1. Human esophageal squamous epithelial cells (HET-1A) were cultured in bronchial epithelial cell medium. HET-1A cells were treated with the selected HCl exposure protocol (pH 5 for 12 minutes, 7 times in 48h), then used to determine mRNA by real-time PCR (left panel) or used for Western Blot (right panel) to determine protein expression for TRPV1. Relative mRNA expression was calculated with respect to mRNA from untreated cells. The results of Western blot are shown as the relative optical density (OD) normalized to GAPDH. The exposure protocol caused a significant increase in mRNA and protein expression for TRPV1 (* p<0.01). Values are mean ± SEM, N=3 for three different sets of data from three separate experiments.

Figure 4
Real time PCR and Western blot for LysoPAF acetylCo-A transferase (LysoPAF-AT).
Human esophageal squamous epithelial (HET-1A) cells were treated with the selected HCl exposure protocol, then used to determine mRNA by real-time PCR (left panel) or used for Western Blot (center panel) to determine protein expression for LysoPAF-AT (the enzyme responsible for production of PAF). Relative mRNA expression was calculated with respect to mRNA from untreated cells. Repeated exposure episodes at pH 5 over two days caused a significant increase in mRNA and protein expression for LysoPAF-AT (* p<0.01). The results of Western blot are shown as the relative optical density (OD) normalized to GAPDH. Values are mean ± SEM, N=3 for three different sets of data from three separate experiments. The right panel shows real-time PCR for human biopsies taken from patients with erosive esophagitis or from normal controls. Increased LysoPAF-AT mRNA in esophagitis patients parallels the increase in HET-1A cells after exposure to HCl.

Figure 5

Real time PCR and ELISA for IL-8. Human esophageal squamous epithelial (HET-1A) cells were treated with the selected HCl exposure protocol, then used to determine RNA by real-time PCR (left panel) or used for ELISA (center panel) to determine IL-8 protein expression. Relative mRNA expression was calculated with respect to mRNA from untreated cells. Repeated exposure episodes to pH 5 over two days caused a significant increase in mRNA and protein expression for IL-8 (* p<0.02). Values are mean ± SEM, N=3 for three different sets of data from three separate experiments. The right panel shows real-time PCR for human biopsies taken from patients with erosive esophagitis or from normal controls. Increased IL-8 mRNA in esophagitis patients parallels the increase in HET-1A cells after exposure to HCl.

Figure 6

Real time PCR and ELISA for Eotaxin-1. Human esophageal squamous epithelial cells (HET-1A) cells were treated with the selected HCl exposure protocol, then used to determine mRNA by real-time PCR (left panel) or used for ELISA to determine Eotaxin-1 protein expression (center panel). Repeated exposure to pH 5 over two days caused significant increase in mRNA and protein expression for Eotaxin-
1 (* p<0.05). Relative mRNA expression was calculated with respect to mRNA from untreated cells. Values are mean ± SEM, N=3 for three different sets of data from three separate experiments. The right panel shows real-time PCR for human biopsies taken from patients with erosive esophagitis or from normal controls. Increased Eotaxin-1 mRNA in esophagitis patients parallels the increase in HET-1A cells after exposure to HCl.

Figure 7

Real time PCR and ELISA for Eotaxin-2. Human esophageal squamous epithelial cells (HET-1A) cells were treated with the selected HCl exposure protocol, then used to determine mRNA by real-time PCR (left panel) or used for ELISA to determine Eotaxin-2 protein expression (center panel). Repeated exposure to pH 5 over two days caused significant increase in mRNA and protein expression for Eotaxin-2 (* p<0.02). Relative mRNA expression was calculated with respect to mRNA from untreated cells. Values are mean ± SEM, N=3 for three different sets of data from three separate experiments. The right panel shows real-time PCR for human biopsies taken from patients with erosive esophagitis or from normal controls. Increased Eotaxin-2 mRNA in esophagitis patients parallels the increase in HET-1A cells after exposure to HCl.

Figure 8

Real time PCR and ELISA for Eotaxin-3. Human esophageal squamous epithelial cells (HET-1A) cells were treated with the selected HCl exposure protocol, then used to determine mRNA by real-time PCR (left panel) or used for ELISA to determine Eotaxin-3 protein expression (center panel). Repeated exposure to pH 5 over two days caused significant increase in mRNA and protein expression for Eotaxin-3 (* p<0.01). Relative mRNA expression was calculated with respect to mRNA from untreated cells. Values are mean ± SEM, N=3 for three different sets of data from three separate experiments. The right panel shows real-time PCR for human biopsies taken from patients with erosive esophagitis or from normal controls. Increased Eotaxin-3 mRNA in esophagitis patients parallels the increase in HET-1A cells after exposure to HCl.
Figure 9

Real time PCR and ELISA for MCP-1. Human esophageal squamous epithelial cells (HET-1A) were treated with the selected HCl exposure protocol, then used to determine RNA by real-time PCR (left panel) or used for ELISA to determine MCP-1 protein expression (center panel). Repeated exposure episodes to pH 5 over two days caused a significant increase in mRNA and protein expression for MCP-1 (* p<0.001). Relative mRNA expression was calculated with respect to mRNA from untreated cells. Values are mean ± SEM, N=3 for three different sets of data from three separate experiments. The right panel shows real-time PCR for human biopsies taken from patients with erosive esophagitis or from normal controls. Increased MCP-1 mRNA in esophagitis patients parallels the increase in HET-1A cells after exposure to HCl.

Figure 10

Real time PCR and ELISA for MIP-1α. Human esophageal squamous epithelial cells (HET-1A) were treated with the selected HCl exposure protocol, then used to determine RNA by real-time PCR (left panel) or used for ELISA to determine MIP-1α protein expression (center panel). Repeated exposure episodes to pH 5 over two days caused a significant increase in mRNA and protein expression for MIP-1α (* p<0.05). Relative mRNA expression was calculated with respect to mRNA from untreated cells. Values are mean ± SEM, N=3 for three different sets of data from three separate experiments. The right panel shows real-time PCR for human biopsies taken from patients with erosive esophagitis or from normal controls. Increased MIP-1α mRNA in esophagitis patients parallels the increase in HET-1A cells after exposure to HCl.

Figure 11

The figure examines the role of TRPV1 and ATP in HCl-induced upregulation of mRNA for LysoPAF-AT, IL-8, Eotaxin-1, 2, 3, MCP-1 and MIP-1α. HET-1A cells were treated with the selected HCl exposure protocol. To examine the role of TRPV1 or ATP in the upregulation some cells were pre-treated with either the TRPV1 antagonists IRTX (3×10⁻⁶ M)
or JNJ-17203212 (10^{-6} \text{ M}) or with the nonselective purinergic receptor antagonist suramin (10^{-4} \text{ M}) for 20 minutes before each exposure to acid. Real-time PCR was used for mRNA determination. Relative mRNA expression was calculated with respect to mRNA values after acid exposure shown as diagonally striped bars and reported as 1. Initial mRNA values, before exposure to HCl are shown as white bars. For LysoPAF-AT, and all of the cytokines/chemokines the initial mRNA values were significantly lower (*p<0.05) that the values after acid exposure. Relative mRNA expression in epithelial cells pre-treated with IRTX is shown as dotted bars. Relative mRNA expression in epithelial cells pre-treated with JNJ-17203212 is shown as crossed bars Relative mRNA expression in cells pre-treated with suramin is shown as vertically striped bars. There was no significant difference between initial mRNA values and values after TRPV1 antagonists or after suramin pretreatment, indicating that TRPV1 antagonists and suramin inhibit the HCl-induced increase in mRNA. None of the antagonists affected cell viability as assessed by trypan blue exclusion (>98%). Values are mean ± SEM, N=3 for three different sets of data from three separate experiments. * denotes significance (p<0.05) compared to the values after acid exposure.

The selective TRPV1 agonist capsaicin was used to confirm TRPV1-mediated upregulation of mRNA for LysoPAF-AT, IL-8 and Eotaxin-1. HET-1A cells were exposed to culture medium containing capsaicin (10^{-5} \text{ M}) for 12 minute episodes, 7 times in 48h. Capsaicin-induced TRPV1 activation produced a similar mRNA upregulation as HCl exposure (as shown in figures 4-6). mRNA values after repeated capsaicin exposure were not significantly different from those induced by HCl exposure, and significantly greater (*p<0.001) than control values. As expected pretreatment with IRTX (3×10^{-6} \text{ M}) or JNJ-17203212 (10^{-6} \text{ M}) significantly reduced the capsaicin mediated mRNA increase (# p<0.001) confirming that the antagonists properly inhibit TRPV1 activation. Inhibition by the non-selective ATP antagonist suramin (10^{-4} \text{ M}) supports a role of ATP in mediating mRNA upregulation.

ATP-induced upregulation of LysoPAF-AT, IL-8, Eotaxin-1, 2, 3, MCP-1, and MIP-1α mRNA.
HET-1A cells were exposed to the ATP analog ATP-γS (10^{-4}M) for 12 minute episodes, 7 times in 48h, then used to determine mRNA by real-time PCR. Relative mRNA expression was calculated with respect to mRNA from untreated cells. Repeated exposure episodes to ATP over two days caused a significant increase (*p<0.01) in mRNA for LysoPAF-AT, IL-8, Eotaxin-1, 2, 3, MCP-1, and MIP-1α. Values are mean ± SEM, N=3 for three different sets of data from three separate experiments.

HCl (pH 5, 5 min) induced significant release of ATP from epithelial cells (* p<0.001) when compared to untreated cells (control). IRTX (10^{-7}M to 10^{-5}M) did not stimulate ATP release demonstrating that the concentrations of IRTX used in the present study do not activate TRPV1 receptors in HET-1A cells. Values represent mean ± SEM, N=3 for three different sets of data from three separate experiments.
TRPV

Real Time PCR

Relative Expression

TRPV1  TRPV2  TRPV3  TRPV4  TRPV5  TRPV6
Real Time PCR

TRPV1

IL-8

Eotaxin-1

Relative expression

Control 2 5 8 12 16

minutes at pH 5

Control 2 5 8 12 16

minutes at pH 5

Control 2 5 8 12 16

minutes at pH 5
TRPV1
HET-1A cells, pH5

Real Time PCR

Western Blot

Relative expression

Control     pH 5.0

Relative OD

Control     pH5
Lyso-PAF AT

HET-1A

Human Biopsies

Real Time PCR (relative expression)

Control  pH 5

n=3  *

Western Blot (relative OD)

Control  pH 5

*  *

Real Time PCR (relative expression)

Control  Esophagitis

n=17  n=15 *

n=17  n=15

Lyso-PAF AT

GAPDH
IL-8

HET-1A

Human Biopsies

Real Time PCR
(relative expression)

Multiplex ELISA
(pg/ml)

Real Time PCR
(relative expression)

n=3
n=3
n=16
n=15

Control  pH 5  Control  pH 5  Control  pH 5

*  *  *
Eotaxin-1

HET-1A

Real Time PCR (relative expression)

n=3

Control pH 5

Multiplex ELISA (pg/ml)

n=3

Control pH 5

Human Biopsies

Real Time PCR (relative expression)

n=17 n=15

Control Esophagitis
Eotaxin-2

HET-1A

Human Biopsies

Real Time PCR (relative expression)

Multiplex ELISA (pg/ml)

Real Time PCR (relative expression)

Control pH 5

Control pH 5

Control Esophagitis

n=3

n=3

n=17

n=15
Eotaxin-3

**HET-1A**

- Real Time PCR (relative expression): n=3
- Multiplex ELISA (pg/ml): n=3

**Human Biopsies**

- Real Time PCR (relative expression): n=17, n=15

* Compared to control
MCP-1
(CCL-2)

HET-1A Human Biopsies

Real Time PCR (relative expression)

Control  pH 5

n=3

Multiplex ELISA (pg/ml)

Control  pH 5

n=3

Real Time PCR (relative expression)

Control  Esophagitis

n=17  n=15

* indicates statistical significance.
MIP-1α
(CCL-3)

HET-1A

Human Biopsies

Real Time PCR
(relative expression)

Multiplex ELISA
(pg/ml)

Real Time PCR
(relative expression)

Control  pH 5

Control  pH 5

Control  Esophagitis

n=3  n=3  n=3

0

0.5

1.0

1.5

n=14  n=13

0

2

4

6

8

*
Inhibition of HCL-induced mRNA upregulation by IRTX, JNJ-17203212 and Suramin

- Control
- pH5
- pH5 + IRTX
- pH5 + JNJ-17203212
- pH5 + Suramin

![Bar chart showing relative expression of various cytokines and chemokines under different conditions.](chart.png)
Inhibition of capsaicin-induced mRNA upregulation by IRTX, JNJ-17203212 and Suramin
**Lyso-PAF AT**

- Control: 1
- ATP: * (increased)

**IL-8**

- Control: 1
- ATP: * (increased)

**EOT-1**

- Control: 1
- ATP: * (increased)

**EOT-2**

- Control: 1
- ATP: * (increased)

**EOT-3**

- Control: 1
- ATP: * (increased)

**MCP-1**

- Control: 1
- ATP: * (increased)

**MIP-1α**

- Control: 1
- ATP: * (increased)
HCL but not IRTX stimulates ATP release from HET-1A cells.