Lafutidine, a Protective H₂ Receptor Antagonist, Enhances Mucosal Defense in Rat Esophagus

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Original Article

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Abstract

Background Luminal acid or CO₂ induces a hyperemic response in the esophagus, via activation of acid sensors on capsaicin-sensitive afferent nerves (CSAN). Since disruption of the hyperemic response to luminal CO₂ acidifies the interstitium of the esophageal mucosa, the hyperemic response may maintain interstitial pH (pH_{int}). We hypothesized that acid-related hyperemia maintains pH_{int}, preventing acid-induced injury in the esophageal mucosa.

Methods We examined the effects of capsaicin (Cap) or lafutidine (Laf), a mucosal protective H₂ antagonist, on the regulation of pH_{int} and blood flow in rat esophagus using ratiometric microimaging and laser-Doppler measurements of the lower esophageal mucosa of living rats. The esophagus was topically superfused with pH 7.0 buffer, or a pH 1.0 or pH 1.0 + pepsin (1 mg/ml) solution with or without Laf.

Results Cap (30 or 100 µM) or Laf (0.1 or 1 mM) dose-dependently increased blood flow, accompanied by increased pH_{int}. The pH 1.0 solution increased blood flow without pH_{int} change, whereas Laf (1 mM) increased blood flow and pH_{int} during acid exposure. The effects of Laf were abolished by ablation of CSAN. Perfusion of the acidified pepsin solution gradually decreased pH_{int}, inhibited by Laf perfusion.

Conclusions Activation of CSAN by Laf with or without acid, accompanied by hyperemia, increased pH_{int}, preventing acidified pepsin-induced interstitial acidification. Stimulation of the capsaicin pathway with compounds such as Laf enhances mucosal protection from acid-related injury in the upper gastrointestinal tract.

Keywords Esophageal blood flow · Interstitial pH · Capsaicin-sensitive afferent nerves · Pepsin · Interstitial acidification

Introduction

The upper gastrointestinal mucosa responds to luminal acid by increasing HCO₃⁻ and mucus secretion, increasing mucosal blood flow, and by increasing epithelial cellular buffering in order to protect the mucosa from acid-induced injury. These mucosal defense mechanisms appear to be coordinated by the activation of submucosal acid sensors [1, 2]. The duodenal mucosa is protected from injury due to luminal acid by the ‘capsaicin pathway.’ In the duodenum, the large luminal acid load originating from the stomach is converted to the H⁺ equivalent CO₂ at the cell surface prior to absorption by the duodenal mucosa. CO₂ at the enterocyte surface is absorbed into the enterocyte, facilitated by ecto-carbonic anhydrases (CAs), followed by reconversion to H⁺ by cellular CA and transport across the enterocyte basolateral membrane into the subepithelial interstitium [1, 3]. Subepithelial H⁺ interacts with submucosal acid sensors such as transient receptor potential vanilloid 1 (TRPV1) expressed on submucosal capsaicin-sensitive afferent nerves (CSAN), releasing the vasoactive compounds...
such as calcitonin gene-related peptide (CGRP) and nitric oxide (NO), increasing mucosal blood flow [3, 4]. In contrast to the “leaky” duodenal mucosa, the esophageal mucosa, with tight intercellular junctions, responds to luminal acid with hyperemia and increased pre-epithelial layer gel thickness, but with no change of interstitial pH (pH$_{int}$) [5]. Nevertheless, CO$_2$, rather than H$^+$, traverses the epithelial layers, interacting with epithelial and neuronal CAs and acid sensors, inducing the hyperemic response via the activation of CSAN [6]. The esophageal mucosa thus shares parts of the capsaicin pathway with the duodenum as a mucosal protective mechanism from injury due to refluxed gastric acid.

The hyperemic response to luminal acid is a well-studied defense mechanism in the esophagus [7–9]. Hyperemia is believed to enhance the supply of HCO$_3^-$ to the mucosa and the removal of H$^+$ and CO$_2$ from the mucosa during acid exposure. HCO$_3^-$ delivery is an important esophageal mucosal protective factor [10]. Luminal perfusion of a high CO$_2$ solution or of a pH 1.0 acid solution increases esophageal blood flow without affecting pH$_{int}$ [6]. Disruption of CO$_2$-induced hyperemia by CA inhibition or acid sensor inhibition irreversibly lowers pH$_{int}$ implicated in mucosal injury and the generation of noxious sensations [6]. These results suggest that the hyperemic response to luminal acid or high CO$_2$ maintains pH$_{int}$ in the esophageal mucosa during acid exposure. We thus hypothesized that induction of hyperemia in the esophagus may increase pH$_{int}$, augmenting mucosal resistance to acid-induced injury. Since the hyperemic response to luminal acid is mediated via the capsaicin pathway, stimulation of acid sensors expressed on CSAN and may also protect the mucosa from injury.

Lafutidine (Laf), a histamine H$_2$ receptor antagonist, has a mucosal protective effect in addition to its antisecretory properties via indirect stimulation or sensitization of CSAN [11–15]. Laf is protective against experimental gastric lesions even in the presence of supplementary potent antisecretory therapy, suggesting that it has a direct gastrointestinal-protective effect in addition to its antisecretory properties [15]. Laf reduces esophageal injury in the gastric acid reflux model, apart from its antisecretory effect [13]. This protective effect is abolished by selectively ablating CSAN, antagonism of CGRP and inhibition of NO synthase [16, 17], suggesting that the capsaicin pathway is integral to Laf’s protective properties. We further hypothesized that Laf enhances mucosal defenses by activating or sensitizing mucosal acid-sensing mechanisms in rat esophagus.

We thus examined the effect of luminal superfusion of capsaicin or Laf on pH$_{int}$ and blood flow in rat esophagus. Furthermore, we examined the effect of Laf on acidified pepsin-induced changes in pH$_{int}$ and blood flow, a surrogate for esophageal injury. Our results showed that Laf-induced hyperemia increased pH$_{int}$ and prevented interstitial acidification due to acidified pepsin via the activation of CSAN in rat esophagus.

**Methods**

**Chemicals and Animals**

Laf was provided by Taiho Pharmaceutical Inc (Tokyo, Japan). Laf, (±)-2(furfurylsulfanyl)-N-[4-[4-(piperidinomethyl)-2-pyridyl]oxy-[(Z)-2-butenyl]acetamide (431.54 g/mol), is relatively lipophilic (log $p$ = 0.39). 5',6'-carboxyfluorescein (CF) was obtained from Molecular Probes (Eugene, OR, USA). Capsaicin (Cap), porcine pepsin A, HEPES, and other chemicals were obtained from Sigma Chemical (St. Louis, MO, USA). Krebs solution contained (in mM) 136 NaCl, 2.6 KCl, 1.8 CaCl$_2$, and 10 HEPES at pH 7.0. The pH 1.0 saline solution was made from 1 N HCl with adding NaCl to adjust isotonicity. Each solution was prewarmed to 37°C using a water bath, and temperature was maintained with a heating pad during the experiment. For stock solutions, capsaicin was dissolved in 10% Tween$^{-80}$, 10% EtOH, and 80% saline. The solution was diluted with Krebs buffer in order to ensure that final concentrations of Tween$^{-80}$, EtOH were less than 0.1%. For vehicle perfusion, Krebs solution with 0.1% solvents was used. Laf was initially dissolved in 1 N HCl and pH was adjusted to pH 7 with the addition of 1 N NaOH. Stock solution of Laf (50 mM) was diluted with pH 7.0 Krebs buffer before use.

All studies were performed with approval of the Veterans Affairs Institutional Animal Care and Use Committee (VA IACUC). Male Sprague–Dawley rats weighing 200–250 g (Harlan, San Diego, CA, USA) were fasted overnight, but had free access to water.

**Measurement of Blood Flow and Interstitial pH (pH$_{int}$)**

Esophageal blood flow and pH$_{int}$ were simultaneously measured in the lower esophagus as previously described [5, 6]. In brief, under isoflurane anesthesia (1.5–2.0%) using a rodent anesthesia inhalation system (Summit Medical Systems, Bend, OR, USA), rats were placed supine on a heating block system warmed with recirculating water (Summit Medical) to maintain body temperature at 36–37°C, as monitored by a rectal thermistor. Prewarmed saline was infused via the right femoral vein at 1.08 ml/h using a Harvard infusion pump (Harvard Apparatus, Holliston MA, USA); blood pressure was monitored via a catheter placed in the left femoral artery using a pressure transducer (Kent Scientific, Torrington, CT, USA). The lower esophageal mucosa was exposed and a concave stainless-steel disk (16 mm in diameter and 1–2 mm deep) with a 3-mm central...
aperture was fixed watertight on the mucosal surface with a silicone plastic adherent (Silly Putty, Binney & Smith, Easton, PA, USA). The serosal surface of the esophagus was supported with a right-angle laser-Doppler flow probe (R-type, Transonic, Ithaca, NY, USA) just below the chambered mucosa. A thin plastic coverslip was fixed to the disk with the silicone adherent to permit closed superfusion with solutions at a rate of 0.25 ml/min by means of a Harvard infusion pump. Two PE-50 polyethylene perfusion lines were inserted into the chamber to enable rapid changes of the perfusate.

Esophageal blood flow was measured as the voltage output of the laser-Doppler instrument (model BLF21, Transonic) and expressed relative to the stable level (the basal level) ~30 min after the perfusion was started. Blood flow was continuously recorded with a strip chart recorder and read every 5 min.

To measure pH_int, the pH-sensitive, fluorescent indicator CF (5 mg/kg) in saline was intravenously injected 5 min before the start of the experiment. Fluorescence of the microscopically observed chambered area of esophageal mucosa at 515-nm emission was recorded. Readings were taken approximately 10 s before and after each measured time point. The paired readings at 495- and 450-nm excitation needed to calculate a fluorescence ratio were thus taken at a maximum of 20 s apart. The paired images were captured every 5 min and analyzed by selecting three areas of esophageal submucosa between microvessels, which were followed throughout the experiment. In vitro calibration was accomplished using an aqueous solution containing 0.2 μM CF. In vitro calibration in solution and in excised esophageal tissue revealed that the measurable pH range using CF is between pH 6.0 and 7.5, as previously reported [5]. We have confirmed that despite a gradual decrease in the fluorescent intensity over time, the fluorescence ratio was stable during the 60-min experimental period [5, 6]. We have also shown that there is a good correlation between the measured pH_int and arterial blood pH [6], since in vivo calibration is technically difficult.

Experimental Protocol

The exposed esophageal mucosa was superfused with solutions via a mucosally placed perfusion chamber. Blood flow was stabilized during continuous perfusion of pH 7.0 Krebs buffer, after which the time was set as t = 0 min. At t = −5 min, CF was injected IV. The esophageal mucosa was then superfused with pH 7.0 Krebs buffer from t = 0 until t = 10 min (basal period); the perfusate was then changed to pH 7.0 Krebs containing vehicle, Cap (30 or 100 μM), or Laf (0.1 or 1 mM) from t = 10 until t = 20 min (challenge period). The perfusate was changed to the pH 7.0 solution from t = 20 until t = 35 min (recovery period). For acid-perfusion experiments, the mucosa was superfused with a pH 1.0 acid solution with or without pepsin (1 mg/ml) or Laf (1 mM) from t = 10 until t = 45 min (challenge period).

Ablation of CSAN was accomplished with high-dose capsaicin pre-treatment (125 mg/kg sc) as described previously [18]. Capsaicin-treated or vehicle (10% Tween®−80, 10% EtOH, and 80% saline)-treated rats were studied 10–14 days after the injections. Completeness of de-afferentation was assessed by the 0.1% NH4OH eye drop test. The esophageal mucosa of capsaicin-treated or vehicle-treated rat was superfused with an acid solution and Laf (1 mM) as described above.

Statistics

All data from six rats in each group were expressed as means ± SEM. Comparisons between groups were made by one-way ANOVA followed by Fisher’s least significant difference test. p value less than 0.05 were taken as significant.

Results

Effect of Luminal Perfusion of Cap or Laf on Esophageal pH_int and Blood Flow

We first examined the effects of the TRPV1 agonist Cap on pH_int and mucosal blood flow. Cap (30 or 100 μM) increased esophageal blood flow (Fig. 1b), consistent with prior studies [7], accompanied by the interstitial alkalinization (Fig. 1a). This result correlates the induction of hyperemia with increased pH_int. Similarly, Laf (0.1 or 1 mM) increased blood flow, accompanied by a pH_int increase (Fig. 2a, b). The effects of Laf were abolished by prior de-afferentation as a result of high-dose capsaicin pretreatment (Cap-t) (Fig. 3a, b), suggesting that Laf-induced hyperemia is mediated by the activation of CSAN.

Effect of Laf on Acid-Induced Changes in Esophageal pH_int and Blood Flow

Since Laf increased pH_int and blood flow via the activation of CSAN, we hypothesized that Laf enhances mucosal protection during acid exposure in the esophagus. We perfused Laf during the basal and also during the challenge periods in order to maximize its effects on mucosal defenses. A pH 1.0 solution had no effect on pH_int, but increased blood flow as previously reported [5, 6] (Fig. 4a, b). Laf (1 mM) pretreatment increased pH_int during coperfusion with a neutral solution. Subsequent coperfusion of Laf with a pH 1.0 solution increased pH_int more than the pH_int in the pH 1.0 group during the acid challenge period (Fig. 4a).
the Laf group, increased pH int was accompanied by increased blood flow during the basal period, followed by an enhanced hyperemic response to acid perfusion compared to the pH 1.0 group (Fig. 4b). Cap-t abolished the Laf-induced pH int increase during the basal and acid challenge periods, and further acidified pH int during the acid challenge period (Fig. 4a). This result is consistent with a previous report showing that Cap-t irreversibly lowers pH int during luminal perfusion of a high CO2 solution, consistent with mucosal injury [6]. Furthermore, Cap-t abolished the hyperemic response to acid + Laf (Fig. 4b). Cap-t also abolished the acid-induced hyperemia associated with interstitial acidification (data not shown). These results suggest that luminal acid stimulates CSAN, and that Laf enhances the hyperemic response to acid via CSAN activation or modulation, with a resultant increase of pH int.

Effect of Laf on Acidified Pepsin-Induced Changes in Esophageal pH int and Blood Flow

We further hypothesized that Laf protects the mucosa from the interstitial acidification accompanying epithelial injury or increased permeation of acid into the mucosa. We thus examined the effect of acidified pepsin on pH int and blood flow, since acidified pepsin reduces electrical resistance of rabbit esophagus in vitro and may thus also injure the rat esophagus in vivo [19]. Pepsin (1 mg/ml) was added to the pH 1.0 perfusate in order to compromise the mucosal barrier. Acidified pepsin gradually, but markedly, decreased pH int (Fig. 5a), consistent with increased H+ diffusion into the mucosa. This pH int change was reversed by Laf (1 mM) pre- and co-perfusion, consistent with mucosal protection (Fig. 5a). Perfusion of the acidified pepsin solution transiently increased blood flow as did the acid alone, followed by a second hyperemic response observed at t = 40–45 min (Fig. 5b), associated with a markedly decreased pH int (Fig. 5a). Laf increased blood flow before and after acidified pepsin superfusion, but prevented the secondary hyperemia observed in acid + pepsin group. These results suggest that Laf treatment prevents the acidified pepsin-induced mucosal injury in the esophagus by protective hyperemia and pH int increase.

Discussion

We demonstrated that the induction of hyperemia with luminal Cap or Laf increased esophageal blood flow,
accompanied by interstitial alkalinization in rat esophagus. Laf enhanced acid-induced hyperemia with increased pH int via the activation of CSAN, and prevented acidified pepsin-induced interstitial acidification. This is the first study to demonstrate that hyperemia protects the esophageal mucosa due to increased pH int and that Laf locally enhances esophageal mucosal defense mechanisms, apart from its acid antisecretory effect as an H2 receptor antagonist.

The esophageal mucosa responds to luminal acid by augmenting its intrinsic defense mechanisms such as by increasing mucosal blood flow [5, 7]. Inhibition of the hyperemic response during exposure to high luminal pCO2 lowers pH int [6], suggesting that reflex hyperemia maintains pH int homeostasis. Impairment of reflex hyperemia lowers pH int, presumably by impairing the ability of the enhanced mucosal blood flow to remove acid and CO2, and to deliver HCO3−. In multiple tissues, interstitial acidification is sensed by neuronal chemosensors, producing noxious sensations [20–22]. Thus, a clinical equivalent to the tissue acidification observed in the acid + pepsin group would likely be perceived as heartburn or a similar painful sensation. Furthermore, prolonged or marked acidification may eventually injure the affected tissue. Induction of hyperemia via the activation of CSAN increased pH int, preventing acidified pepsin-induced interstitial acidification, supporting our hypothesis that the hyperemic response maintains pH int, presumably preventing acid-related injury of the esophageal mucosa.

Activation of the capsaicin pathway can be accomplished by luminal acid perfusion or by TRPV1 agonists such as capsaicin. Capsaicin, which is the pungent component of hot peppers, is perceived as a burning or hot sensation, which limits its use as a therapeutic agent. The precise mechanism by which Laf exerts its effects is unknown; nevertheless, denervation of CSAN abrogates the protective effects of Laf, suggesting involvement of the capsaicin pathway. In vitro studies have failed to document a direct interaction between Laf and TRPV1 [12, 14], whereas Laf potentiates Cap-induced CGRP release from the gastric mucosa [14], suggesting that Laf sensitizes TRPV1 or interacts at alternate components of the capsaicin pathway. Our results demonstrated that luminal Laf perfusion increased blood flow in the absence of perfused luminal acid and that Laf enhanced acid-induced
increased acid permeability induced by acidified pepsin.

the profound interstitial acidification resulting from the
hyperemia might be due to mucosal injury resulting from
consistent with a direct acid effect, whereas the secondary
acidified pepsin superfusion. The initial hyperemia is
with our results.

in pylorus/forestomach-ligated rats [13], also consistent
prevents acid reflux-induced esophageal injury via CSAN
in all of the experiments in which it was used, consistent
with a direct effect on the mucosa, in contradistinction to
the pharmacology of clinically used oral H2 receptor
antagonists, which must be absorbed from the intestine and
transported by the circulation to the gastric mucosa. Since
Laf is soluble at strongly acidic pH, Laf in gastric juice
may reflux into the esophagus and then protect the
esophageal mucosa in humans with acid reflux. Since Laf is
not absorbed from the stomach, the gastric juice concen-
tration resulting from an orally administered clinical dose
of Laf (10 mg) may reach ~2 mM when gastric juice
volume is 10 ml, above the 0.1–1.0 mM we used in rats.

In conclusion, the induction of hyperemia via CSAN
increased pHint, preventing acid-induced interstitial acidifi-
cation in rat esophagus. Laf activated CSAN and
enhanced mucosal defense mechanisms, protecting mucosa
from acid-pepsin injury. Our results support the therapeutic
strategy in which compounds stimulating the capsaicin
pathway such as Laf enhances mucosal protection from
acid-related injury in the upper gastrointestinal tract and
thus may uniquely protect from esophageal injury.

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Fig. 5 Effect of Laf with acid-pepsin superfusion on pHint and blood
flow in rat esophagus. Pepsin (1 mg/ml) with pH 1.0 acid was
perfused with or without Laf (1 mM). Laf was perfused during the
basal and challenge periods (0–45 min) in the Laf + pH 1.0 + pepsin
group. The boxes above the graphs represent the perfusate combina-
tion for the corresponding groups. The acid-pepsin solution gradually
decreased pHint (a) accompanied by biphasic hyperemia (b). Laf pre-
 perfusion increased pHint and blood flow, and prevented acid-pepsin-
induced interstitial acidification (a). Data are expressed as mean ±
SEM (n = 6). * p < 0.05 versus pH 7.0 Krebs group, † p < 0.05
versus pH 1.0 group, ‡ p < 0.05 versus pH 1.0 + pepsin group

hyperemia, both mediated by CSAN, supporting our
hypothesis that Laf sensitizes TRPV1 or submucosal afferent nerves. Since Laf ingestion, perhaps due to its
indirect interaction with TRPV1, does not provoke a
burning sensation, it might prove useful in the therapy of
heartburn, acid-induced esophageal injury, and related
disorders.

Another finding is that the acute, local application of Laf
enhanced mucosal defenses in the esophagus, regardless
of its anti-acid secretory effect, since the perfused segment
was isolated from gastric acid. Luminal Laf increases
mucosal blood flow, mediated by CSAN, in rat stomach
[11], similar to our results. Intragastric application of Laf
prevents acid reflux-induced esophageal injury via CSAN
in pylorus/forestomach-ligated rats [13], also consistent
with our results.

We observed a biphasic hyperemic response during
acidified pepsin superfusion. The initial hyperemia is
consistent with a direct acid effect, whereas the secondary
hyperemia might be due to mucosal injury resulting from
the profound interstitial acidification resulting from the
increased acid permeability induced by acidified pepsin.

Sustained or repeated superfusion of the rabbit esophageal
mucosa over a period of days was necessary to injure the
esophagus in one study [23]. In another study, a 90-min
esophageal perfusion with a pH 1.4 solution + 0.5% pepsin
produced only edema or hyperemia in 8/9 rats [24].
Thus, macroscopic or measurable histologic injury in our
acute topical superfusion model may be difficult to pro-
duce. Nevertheless, in the rat duodenum and many other
organs, irreversible cellular acidification or tissue acidosis
usually precedes or is the cause of tissue injury [25].

Laf was topically perfused over the esophageal mucosa
in all of the experiments in which it was used, consistent
with a direct effect on the mucosa, in contradistinction to
the pharmacology of clinically used oral H2 receptor
antagonists, which must be absorbed from the intestine and
transported by the circulation to the gastric mucosa. Since
Laf is soluble at strongly acidic pH, Laf in gastric juice
may reflux into the esophagus and then protect the
esophageal mucosa in humans with acid reflux. Since Laf is
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of Laf (10 mg) may reach ~2 mM when gastric juice
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In conclusion, the induction of hyperemia via CSAN
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