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

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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

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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 gastro-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, (\pm)-2-(furfurylsulfinyl)-*N*-[4-[4-(piperidino-methyl)-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, and 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% NH_4OH 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 \pm 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). In

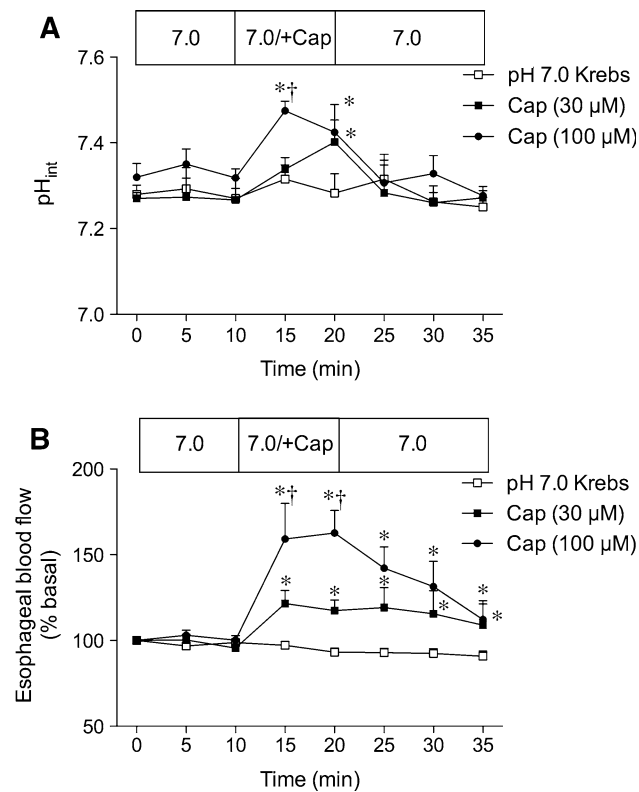


Fig. 1 Effect of luminal capsaicin (Cap) on interstitial pH (pH_{int}) and blood flow in rat esophagus. pH_{int} (a) and blood flow (b) were monitored during the luminal perfusion of Cap (30 and 100 μ M) in rat esophagus under in vivo microscopy. Cap increased the blood flow, accompanied by pH_{int} increase. Data are expressed as mean \pm SEM ($n = 6$). * $p < 0.05$ versus pH 7.0 Krebs group, † $p < 0.05$ versus Cap (30 μ M) group

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 CO_2 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

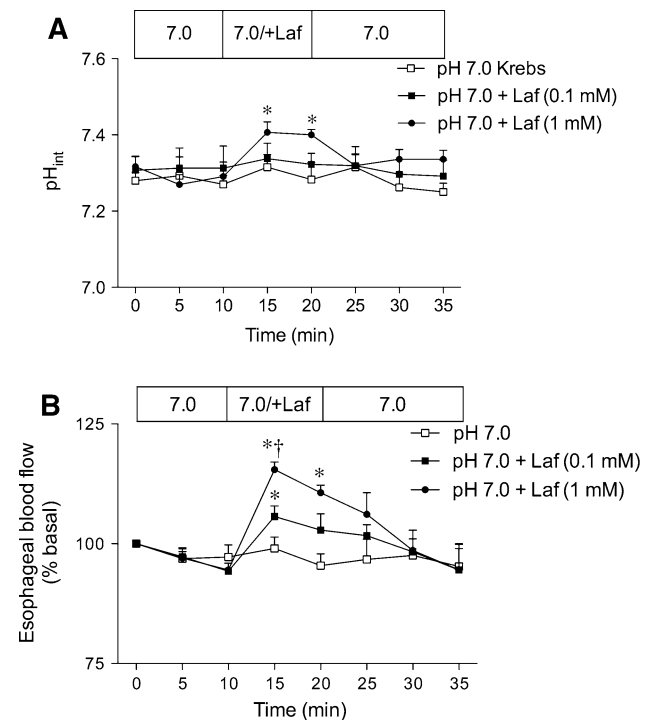


Fig. 2 Effect of luminal lafutidine (Laf) on pH_{int} and blood flow. Perfusion of Laf increased esophageal blood flow (b), accompanied by pH_{int} increase (a). Data are expressed as mean \pm SEM ($n = 6$). * $p < 0.05$ versus pH 7.0 Krebs group, † $p < 0.05$ versus pH 7.0 + Laf (0.1 mM) group

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,

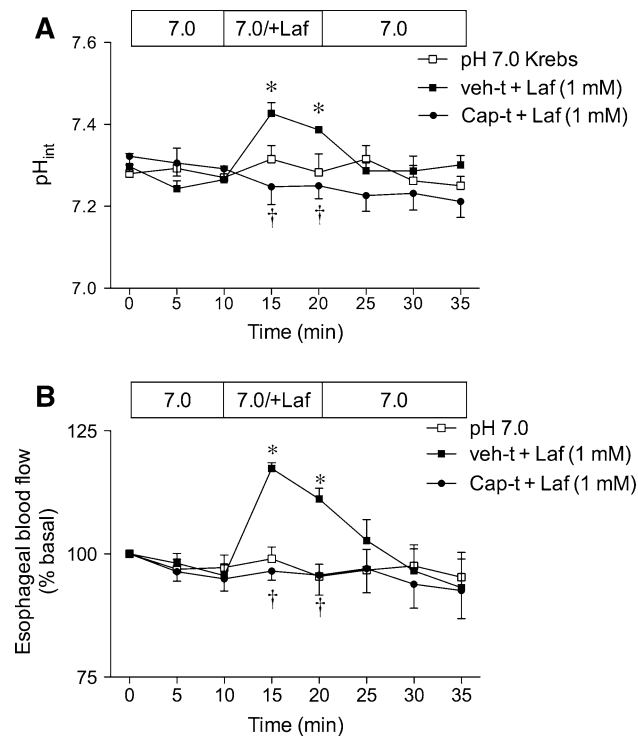


Fig. 3 Effect of capsaicin pretreatment (Cap-t) on Laf-induced changes in pH_{int} and blood flow in rat esophagus. Animals were pretreated with vehicle (veh-t) or high-dose capsaicin 10–14 days before the experiments. Cap-t abolished the Laf-induced pH_{int} increase (a) and hyperemia (b). Data are expressed as mean \pm SEM ($n = 6$). * $p < 0.05$ versus pH 7.0 Krebs group, † $p < 0.05$ versus veh-t + Laf group

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 H_2 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 pCO_2 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 CO_2 , and to deliver HCO_3^- . 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

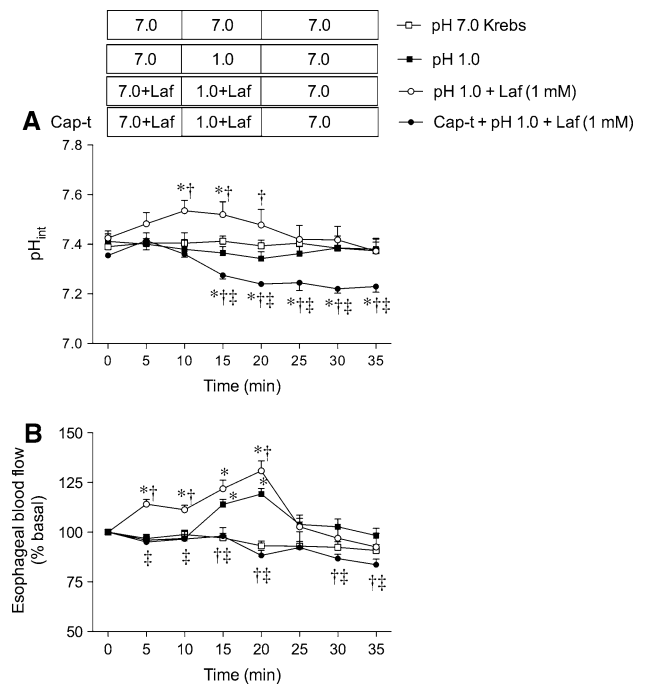


Fig. 4 Effect of Laf with acid on pH_{int} and blood flow in rat esophagus. A pH 1.0 solution was perfused with or without Laf (1 mM). Laf was perfused during the basal and challenge periods (0–20 min) in the pH 1.0 + Laf and in the Cap-t (capsaicin pretreated) + pH 1.0 + Laf groups. The boxes above the graphs represent the perfusate combination for the corresponding groups. Pretreatment with Laf increased pH_{int} (a) and blood flow (b), and enhanced acid-induced hyperemia. Cap-t abolished the effects of Laf perfusion and further decreased pH_{int} . Data are expressed as mean \pm 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 + Laf group

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

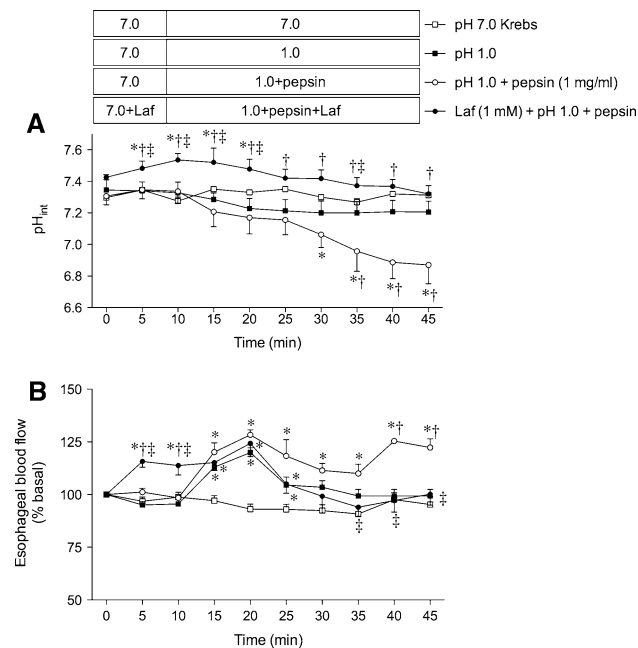


Fig. 5 Effect of Laf with acid-pepsin superfusion on pH_{int} and blood flow in rat esophagus. Pepsin (1 mg/ml) with pH 1.0 acid was perfused with or without Laf (1 mM). Laf was perfused during the basal and challenge periods (0–45 min) in the Laf + pH 1.0 + pepsin group. The boxes above the graphs represent the perfusate combination for the corresponding groups. The acid-pepsin solution gradually decreased pH_{int} (a) accompanied by biphasic hyperemia (b). Laf pre-perfusion increased pH_{int} and blood flow, and prevented acid-pepsin-induced interstitial acidification (a). Data are expressed as mean \pm 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 produce. 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 H_2 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 concentration 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 pH_{int} , preventing acid-induced interstitial acidification 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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