Pepsin, Mucosal Injury, and Pathophysiology of Non-acid Reflux

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KEYWORDS

• Laryngopharyngeal reflux • Gastroesophageal reflux disease
• Human immunodeficiency virus protease inhibitors • Antireflux therapeutics

KEY POINTS

- Pepsin may be the only constituent found in all refluxate and causes airway damage via distinct mechanisms during weakly versus nonacidic reflux.
- Nonacid pepsin causes inflammatory injury that can lead to fibrosis and carcinogenesis during unremitting laryngopharyngeal reflux (LPR).
- Most commercial antibodies/assays cannot discriminate between pepsin, found in refluxate, and pepsinogen, which may be synthesized at low levels in airway mucosa. Assay validation and detection limits aid interpretation of findings.
- Pepsin inhibitors attenuate peptic injury in all experimental models tested to date, including models of acid reflux.
- Several human immunodeficiency virus protease inhibitors potently inhibit pepsin and are in preclinical development for treatment of LPR.

BACKGROUND

Acid, pepsin, and bile are recognized as the primary aggressors during gastroesophageal reflux disease (GERD); the chronic gastrointestinal disorder characterized by regurgitation of gastric contents into the esophagus. It has historically been held that, because pepsin requires acid for its maturation and activity, damage caused by pepsin may be obviated by targeting acid. Proton pump inhibitors (PPIs) arose as the first-line therapy for GERD following the success of acid-suppression therapy for peptic ulcer and reflux esophagitis. With an estimated 20% of the population

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Abbreviations	
EMT	epithelial–mesenchymal transition
FDA	Food and Drug Administration
GERD	gastroesophageal reflux disease
HIF-2α	hypoxia-inducible factor-2α
HNSCC	head and neck squamous cell carcinomas
IL	interleukins
LAH	laryngeal airway hypersensitivity
LC-MS	liquid chromatography-tandem mass spectrometry
LPR	laryngopharyngeal reflux
LRP1	low-density lipoprotein receptor-related 1
MMP	matrix metalloproteinase
NLRP3	nucleotide-binding domain and leucine-rich repeat protein 3
PPI	proton pump inhibitor
RIP	regulated intramembrane proteolysis
ROS	reactive oxygen species
α2M	alpha-2 macroglobulin

suffering from GERD and/or laryngopharyngeal reflux (LPR; the backflow of gastroduodenal contents into the laryngopharynx) and a paucity of other therapeutic options for LPR, PPIs have maintained their position among top selling drug categories for >20 years. Yet, PPIs fail to resolve typical GERD symptoms in up to 40% of individuals[1] and 2 decades of clinical trials fail to demonstrate their placebo-adjusted benefit for LPR.[2,3] Potent next-generation acid suppressing medications (potassium-competitive acid blockers, PCABs) exhibit similar inadequacies for GERD and LPR symptom resolution suggesting that failure is not attributed to inadequate acid suppression.[4,5] Perhaps most disconcerting, while PPIs are the mainstay treatment for GERD, they fail to protect against its most life-threatening consequence, carcinogenesis. Widespread use of PPIs since the 1980s has not stemmed the rising incidence of reflux-attributed cancers. Instead, long-term high-adherence to PPI regimen has been found associated with esophageal adenocarcinoma and high-grade dysplasia.[6–10] The combined evidence suggests that nonacid constituents of refluxate must play a far greater role in reflux disease and its consequences than previously assumed.

Impedance-pH technology has demonstrated that while PPIs reduce the acidity of refluxate, they do not reduce its frequency, and that refluxate reaching the laryngopharynx is commonly weakly to nonacidic.[11,12] Bile is present in some but not all refluxate, and its relevance to LPR has been questioned given that unconjugated bile acids shown to cause damage at neutral-high pH are uncommon in refluxate and experimental studies employ supraphysiologic doses yielding damage uncharacteristic of LPR.[13,14] Only pepsin is present in all refluxate. The presence of pepsin in specimens of patients with airway inflammatory and neoplastic diseases and experimental evidence supporting its capacity for inflammatory airway injury, irrespective of acid, implicate pepsin as a key diagnostic and therapeutic target in LPR.[15] Given the high and increasing prevalence of reflux disease, the incredible health care burden posed by LPR, and the inadequacy of acid-suppression therapy to address GERD and LPR symptoms and related carcinogenesis, improved awareness of the implications of chronic mucosal pepsin exposure is essential to improve management of both GERD and LPR. The aim of this review is to provide the most up-to-date information regarding the pathophysiology of pepsin in LPR.

DISCUSSION

Current Evidence

Reflux disease as an inflammatory, not caustic injury

The earliest experimental models of simulated reflux indicated that pepsin is essential to reflux-attributed injury, even in the relatively injury-resistant esophagus.[16] As early as the 1960s, experiments in animal esophagi (ex vivo) demonstrated that pepsin was required for development of erosive lesions at the pH of most esophageal reflux events.[17–19] This was later corroborated in a surgical rodent model.[20] Subtler forms of damage, such as epithelial barrier dysfunction, were also shown to require pepsin or bile.[18] Although these experiments clearly demonstrated that pepsin drastically exacerbated injury during acid reflux, it was still assumed that pepsin could only cause damage under acidic conditions.

Pepsin is most enzymatically active at ~pH2, inactive at pH6.5, and stable until irreversibly denatured at pH8.0.[21] Pepsin would only be weakly active or inactive at the pH of many LPR events (pH>4) or the normal pH of the laryngopharynx (pH6.8–7.2), but would rarely encounter sufficiently high pH for its denaturation. Pepsin remains stable for at least 24 hours at pH7 and regains up to 80% of its original activity when returned to low pH.[21] In 2007, Johnston and colleagues discovered that at pH7 pepsin is endocytosed by laryngeal and hypopharyngeal epithelial cells and stored in intracellular vesicles of pH4-5 in which its enzymatic activity would be restored.[22] Its intraepithelial retention was later found to persist at least 36 hours and its endocytosis confirmed in esophageal cells as well.[23–25]

In 2009 and 2016, research performed in a surgical rodent model of GERD and GERD patients taking 2-week PPI hiatus revealed that erosive lesions and barrier disruption required weeks to develop and arose secondary to inflammation.[26,27] Contradictory to the prevailing theory at the time, injury proceeded from submucosa to luminal surface rather than the inverse: epithelial production of reactive oxygen species (ROS) led to secretion of proinflammatory cytokines, lymphocytic infiltration of submucosa then epithelia, widening of intercellular spaces, and basal cell and papillary hyperplasia, all of which preceded surface erosions. This work led to a paradigm shift: reflux disease was not a top-down caustic injury, but a cytokine-mediated inflammatory injury (**Fig. 1**).[28] While this new model originated in the esophagus, it appears aptly suited to describe mechanisms of damage incurred by weakly to nonacid pepsin during LPR.

Mechanisms of peptic injury during laryngopharyngeal reflux and its biological effects

Refluxed pepsin may be enzymatically active or inactive (but stable) across the range of pH characteristic of LPR and the mechanisms by which it produces damage differ with pH-dependent enzymatic activity. The acute inflammatory injury produced by brief pepsin exposure can contribute to fibrosis and carcinogenesis during unremitting LPR.

Acute inflammatory injury by nonacid pepsin. Pepsin endocytosis is receptor-mediated.[24] Low-density lipoprotein-related 1 (LRP1) is its putative receptor.[29] Pepsin may bind LRP-1 directly or via interaction with the protease scavenger, alpha-2 macroglobulin (α2M). Pepsin-α2M interaction can occur at pH5.5 to 7 (ie, during weakly to nonacidic reflux).[30] α2M binds proteases of all classes, entrapping them in a cage-like structure to hinder interaction with substrate and routing them for endocytosis via LRP1. The early molecular signaling events by which nonacid pepsin (endocytosed and reactivated, but sequestered in acidic intracellular vesicles) produces injury are

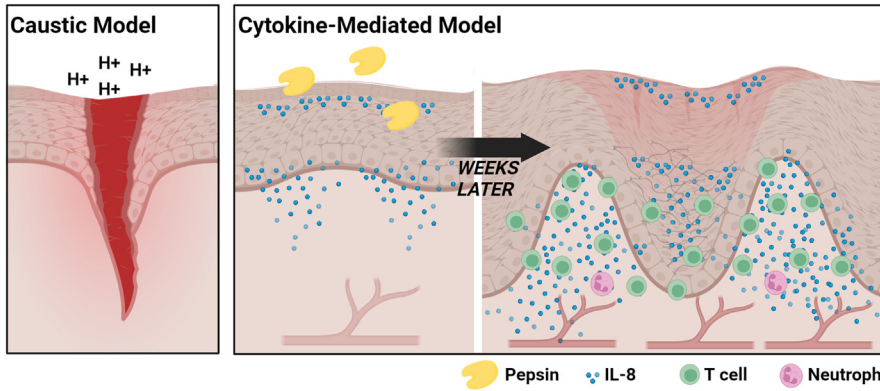

Fig. 1. Reflux Disease as An Inflammatory, Not Caustic Injury. In the Caustic Model of reflux-attributed injury, epithelial lesions are incurred by immediate chemical burn. Long incubation, large volumes, and exceedingly low pH (<pH2) are required for this effect in excised animal esophagi. The majority of reflux episodes are of insufficiently low pH to cause this type of damage, even in GERD. In the Cytokine-Mediated Model, refluxate induces intracellular ROS leading to IL1-β and IL-8 expression (~day 2–4). IL-8 recruits T-cells (~day 3) and neutrophils (~day 7) concomitant with dilated intercellular spaces, followed by basal cell and papillary hyperplasia (~weeks 2–4), all preceding surface erosions. This model is based on a surgical rat model of GERD and GERD patients taking 2-week PPI hiatus and is consistent with airway injury by nonacid pepsin. (*Created in* BioRender. Samuels, T. (2024) BioRender.com/n47g640.)

not entirely elucidated. However, experimental data clearly support airway injury by nonacid pepsin and its dependence on enzymatic reactivation.

Elevation of intracellular ROS via nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) appears to be a key initiating event of inflammatory injury during GERD. Early in the esophageal response to refluxate, plasma membrane-situated NOX enzymes synthesize ROS leading to expression of interleukins (IL) 1-β and IL-8, which in turn recruit immune cells and predict the severity and subtype of disease.[28,31–33] Likewise, pepsin in LPR patient laryngeal mucosa correlates with intracellular ROS and IL1-β.[34] A single brief exposure of laryngeal epithelial cells to nonacid pepsin induces ROS production and expression of IL1-β and IL-8, which can be inhibited by pretreatment with a NOX inhibitor.[34] IL-8 recruits T-cells and establishes a T-cell predominated response during early stages of GERD.[26] T-cell predominated response is also observed during LPR.[35] IL-8 is a biomarker of GERD severity, elevated in erosive esophagitis, and predicts symptomatic recurrence in non-erosive esophagitis.[36,37] IL-8 is a strong chemoattractant of neutrophils, which are major source of ROS during GERD and recruited subsequently to T cells.[26,38] IL-8 and intraepithelial neutrophils correlate with disease severity during erosive and non-erosive esophagitis.[39,40] Pepsin similarly promotes neutrophil invasion in the airways.[41]

In laryngeal epithelia, pepsin-induced IL1-β and IL-8 expression via ROS is mediated in part by the nucleotide-binding domain and leucine-rich repeat protein 3 (NLRP3) *inflammasome*, a multi-protein complex, which responds to ROS via caspase-mediated maturation of IL1-β precursor, pro-IL1-β.[34] The NLRP3 inflammasome has been implicated in the pathogenesis of aerodigestive tract inflammatory disorders (eg, intestinal inflammation, chronic obstructive airway disease) and carcinogenesis. NLRP3 and IL1-β independently regulate nuclear factor-kappa B (NF-κB), a major transcriptional regulator of cytokines, mucins, and other inflammatory

response proteins shown to be induced by nonacid pepsin in airway epithelial cells.[42–44] Inhibition of NLRP3 partially reduced pepsin-induced cytokine expression in laryngeal cells suggesting that other mechanisms contribute to pepsin-induced inflammation.[34] NOX-initiated and ROS-mediated depletion of prolyl hydroxylase (PHD) function promotes accumulation of hypoxia-inducible factor-2α (HIF-2α) and subsequent NF-κB/p65-dependent cytokine expression during GERD, thus HIF-2α warrants investigation in LPR.[28,45]

Inflammation is associated with nociceptor activation and pain. Accordingly, pepsin-induced ROS accumulation has been implicated in LPR symptom origination. A single 40s exposure to weakly acid pepsin (pH5) sensitized capsaicin-sensitive laryngeal afferent fibers in a rat model resulting in laryngeal airway hypersensitivity (LAH) for at least 1 hour.[46] The mechanism, which involved ROS-induced ATP release and activation of P2X receptors, may be responsible for chronic cough during GERD and LPR, including both that coincident with reflux events and sensitization to irritants absent a reflux event.

Several antioxidants have been shown to prevent pepsin-mediated damage in vitro (eg, mitochondrial dysfunction, cytokine expression, hyperproliferation, and cancer-associated gene expression) and rescue LAH in vivo.[34,46–48] Their primary mechanism of action appears to be inhibition of pepsin via denaturation rather than ROS neutralization.[47,49–52] Insufficient clinical trial data exist to validate the benefit of most formulations for LPR. Seaweed-derived alginate polysaccharides are the exception. Alginate has long been used for treatment of mild to moderate GERD. Clinical evidence supports its benefit for GERD and LPR symptoms.[53–55] In addition to its antioxidant properties and corresponding inhibition of pepsin, sodium alginate is mucoadhesive and forms a raft over the postprandial gastric acid pocket, thereby reducing the acidity (and presumably peptic activity) of refluxate.[56,57] Gaviscon (Reckitt Benckiser Group PLC, Slough, UK) may be the best studied alginate-antacids. Gaviscon Double Action Liquid reduced acid reflux events for at least 2.5 hours postprandially[57]; its topical mucoadhesive protection is likely more brief.[58]

Acute effects of weakly acidified pepsin on epithelial barrier integrity. Weakly acidified pepsin can cause cleavage of the important adhesion molecule E-cadherin, contributing to rapid loss of epithelial barrier integrity. Rather than degrading E-cadherin, weakly acidified pepsin elicits its regulated intramembrane proteolysis (RIP). RIP is an evolutionarily conserved mechanism by which transmembrane proteins are sequentially cleaved through the coordinated activity of 2 distinct enzymes, releasing peptide fragments that persist in the intracellular and extracellular space and participate in molecular signaling. Pepsin (pH4, 15 min) caused RIP of E-cadherin in laryngeal and esophageal cells in vitro, leading to dysregulation of matrix metalloproteinases (MMPs) associated with reflux-attributed cancers (**Fig. 2**).[59–61] Pharmacologic inhibition of other epithelial enzymes known to elicit RIP did not rescue the effect, suggesting a direct role for pepsin in cleavage. E-cadherin RIP has been evidenced to occur in GERD and LPR and can also be induced by acid by a slower acting MMP-dependent mechanism over 24 hours.[62–64] Several transmembrane proteins implicated in GERD are known to undergo RIP, thus further work is warranted to examine the scope of peptic RIP during LPR. Pepsin induces epithelial monolayer barrier dysfunction within 15 minutes at pH4 and can disrupt barrier function of differentiated tissue (porcine vocal fold, ex vivo) at pH3 over 1 hour.[65,66] Nonacid pepsin for up to an hour does not weaken monolayer barrier function;[41] however, chronic nonacid pepsin exposure may do so secondary to inflammation.

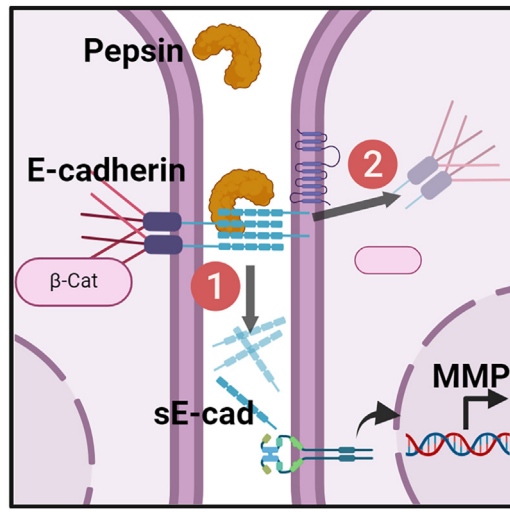

Fig. 2. Weakly acidified pepsin causes rapid regulated intramembrane proteolysis (RIP) of the adhesion molecule, E-cadherin. In laryngeal and esophageal epithelium, weakly acidified pepsin causes E-cadherin cleavage via a two-step process known as RIP: (1) initial cleavage near the plasma membrane by pepsin elicits secondary cleavage (2) by γ-secretase. N and C-terminal fragments of E-cadherin (sE-cad, CTF) persist in extracellular and intracellular space and participate in molecular signaling. sE-cad promotes EGFR signaling and transcription of MMPs, which degrade extracellular matrix. E-cadherin cleavage promotes βcatenin/Wnt signaling. (*Created in* BioRender. Samuels, T. (2024) BioRender.com/f26j458.)

Chronic nonacid pepsin exposure: fibrosis and carcinogenesis. Chronic inflammation promotes fibrosis. Pepsin is observed in clinical specimens of idiopathic subglottic stenosis and idiopathic pulmonary fibrosis.[67,68] In a rat model of aspiration injury, nonacid pepsin elicits similar histologic grade of fibrosis and TGF-β (fibrosis-associated cytokine) expression as does acidified pepsin.[69] Nonacid pepsin has been shown to promote epithelial–mesenchymal transition (EMT), a biological process involved in fibrosis and cancer progression in which epithelial cells lose polarity and adhesion and become migratory and invasive. Chronic pepsin exposure (0.1 mg/ml, 5 days) induced EMT of laryngeal squamous cell carcinoma cells as indicated by depletion of E-cadherin and elevation of mesenchymal markers (vimentin, β-catenin, snail, and slug), cell proliferation, and migration.[70] The effects were partially mediated by IL-8, as indicated by attenuation via IL-8 receptor antagonist.

Chronic inflammation contributes to tumorigenesis. Many molecular mediators of acute inflammation, including those induced by pepsin, are known to play a role. Contrary to what may be assumed, the carcinogenic potential of pepsin may be greater during weakly to nonacidic than acidic refluxate as the extremely damaging nature of acidic pepsin (pH<4) rapidly reduces laryngopharyngeal cell viability.[23,71] Meanwhile, chronic exposure to weakly to nonacid pepsin promotes hyperproliferation, cell migration, anchorage independent growth, and cancer-associated gene expression changes.[72,73] Pepsin is associated with tonsillar hypertrophy, dysplastic grading and recurrence of vocal fold leukoplakia, and laryngeal and hypopharyngeal cancer and its nodal metastasis.[25,74–76]

Unremitting high levels of ROS cause oxidative DNA damage associated with telomeric dysfunction and p53 mutation in Barrett's esophagus and its progression to esophageal adenocarninoma.[77] Bile salts and acid induce 8-OH-dG and p-H2AX,

markers of oxidative DNA damage and DNA double-strand breaks, in esophageal epithelial cells.[78,79] Similarly, pepsin levels are correlated with 8-OH-dG and p-H2AX in vocal fold polyps.[80] Chronic nonacid pepsin exposure (6 hr/d, 5 d) induced 8-OH-dG, p-H2AX and DNA fragmentation in laryngeal epithelial cells in vitro.[80]

NF-κB is a nexus of inflammatory and carcinogenic molecular signaling. NF-κB is often upregulated in head and neck squamous cell carcinomas (HNSCCs) where it promotes expression of anti-apoptotic factors (eg, BCL2, STAT3, and TNF-α) and cancer-associated cytokines IL6 and IL1β, and forms a positive feedback loop with epidermal growth factor receptor (EGFR), which is overexpressed in >80% of all HNSCCs.[81] Activation of NF-κB by weakly and nonacid pepsin (pH5-7) in airway epithelia has been widely reported.[43,71,82] Chronic exposure of human primary hypopharyngeal cells to weakly or nonacidic pepsin (15 min/day, 5 days) led to activation of NF-κB, EGFR, and STAT3 and upregulation of target genes (*EGFR, AKT1, mTOR, IL1B, TNFA, RELA[p6], BCL2, IL6,* and *STAT3*).[82] IL-1β can in turn activate JNK, which has been shown responsible for early peptic induction of anti-apoptotic heat shock protein 70 in airway epithelia.[83,84]

IL-8 is an important mediator of tumorigenesis and cancer progression during GERD.[85,86] IL-8 may similarly play a key role in pepsin-mediated tumorigenesis during LPR: pepsin and IL-8 are correlated in laryngeal cancer and an IL-8 receptor antagonist attenuated chronic pepsin-induced anchorage independent growth, cell proliferation, and migration of laryngeal epithelial cells (2 hr/d, 5 days).[70]

The mechanisms and signaling pathways implicated in airway damage by nonacid pepsin are illustrated in **Figs. 3** and **4**, respectively.

Current Controversies, Misconceptions, and Challenges

While airway mucosal injury by nonacid pepsin may no longer be considered controversial, confusion persists regarding optimal methods for its detection and their influence on interpretation of clinical and research findings. Meta-analyses conclude that the diagnostic utility of salivary pepsin for LPR is low.[87,88] Yet, there is no gold standard diagnostic for LPR to serve as reference and a valid pepsin assay and clinical sampling protocol has not been agreed upon.[89] While more sensitive than enzymatic

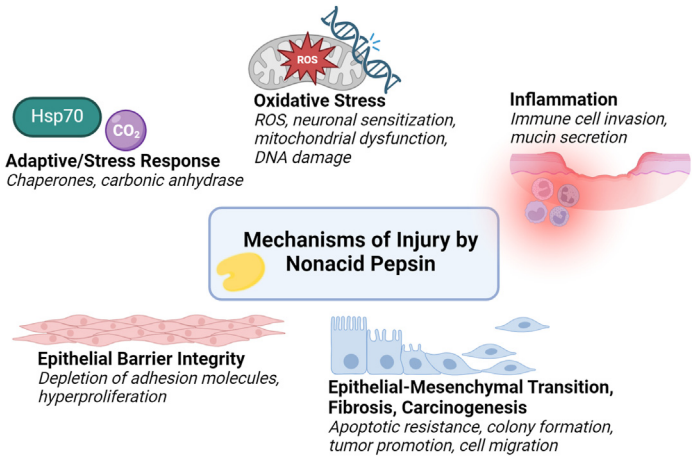

Fig. 3. Mechanisms of nonacid pepsin injury of airway epithelia. (*Created in* BioRender. Samuels, T. (2024) BioRender.com/y20f757.)

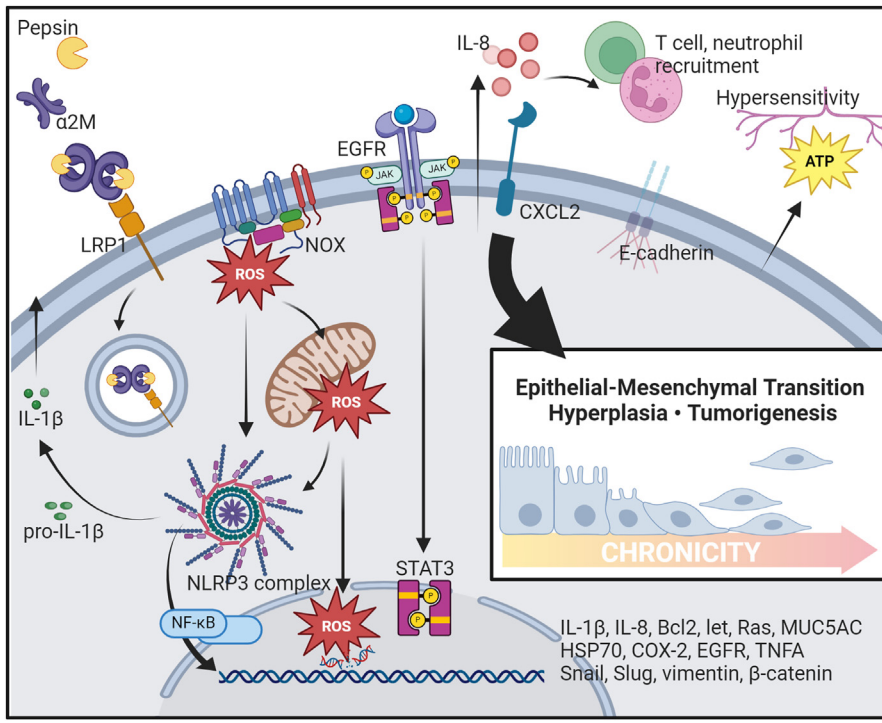

Fig. 4. Molecular Pathways Implicated in Airway Epithelia Injury by Nonacid Pepsin. Nonacid pepsin is endocytosed by aerodigestive tract epithelia, potentially via the protease scavenger α2M and its interaction with LRP-1. Nonacid pepsin induces ROS production via NADPH oxidase which exhibits crosstalk with mitochondria and can perpetuate further ROS via the mitochondrial electron transport chain. ROS induces ATP release, activating P2X receptors on capsaicin-sensitive laryngeal afferent fibers to elicit hypersensitivity. ROS also activates the NLRP3 inflammasome [NLRP3 (sensor), ASC (adaptor), pro-caspase-1 (effector)] resulting in caspase-mediated maturation of IL-1β and activation of NF-κB-mediated IL-1β and IL-8 transcription. IL-8 recruits T cells, and later neutrophils which can release further ROS and exacerbate injury. Unremitting high ROS during chronic nonacid pepsin exposure causes oxidative DNA damage. Chronic nonacid pepsin exposure also promotes activation of EGFR, STAT3 signaling and gene expression (EGFR, AKT1, mTOR, IL1B, TNFA, RELA [p65], BCL2, IL6 and STAT3). IL-8 expressed by chronic nonacid pepsin exposure promotes EMT, a process involved in fibrosis and carcinogenesis, via depletion of E-cadherin and elevation of TGF-β and mesenchymal markers (vimentin, β-catenin, snail, and slug). IL-8 mediates pepsin-induced anchorage independent growth, cell proliferation, and migration. (*Created in* BioRender. Samuels, T. (2024) BioRender.com/x49s724.)

assay, the reliability of antibody-based detection (eg, ELISA, strip test) is highly dependent upon antibody characteristics.[90] Further, a singular commercially available antibody-based salivary pepsin diagnostic device, which several authors suggest requires further validation,[29] predominates among meta-analyses, thus the reliability of that device inordinately influences study conclusions.

Assay specificity: pepsin versus pepsinogen

Pepsin is produced as the inactive pro-enzyme pepsinogen, a 373 amino acid protein, which is converted to active mature pepsin upon conformational change at pH≤5

resulting in removal of a 47 amino acid N-terminal peptide via intramolecular or intermolecular cleavage. Human pepsinogen is transcribed from *PGA3, PGA4,* and *PGA5* on chromosome 11 yielding pepsinogens A3-5 with ~99% amino acid similarity. While abundantly transcribed in the stomach, low levels of pepsinogen synthesis occur in other anatomic sites including respiratory mucosa.[90] Pepsinogen produced by chief cells in gastric crypts of the stomach is rapidly converted to mature pepsin prior to exiting the crypt (~pH3).[91] PPIs may elevate the pH of the gastric lumen above pH5 for several hours daily, which may reduce pepsinogen conversion although this has not been adequately studied. PPIs do, however, concentrate pepsin/pepsinogen in gastric juice by reducing acid secretion.[41,92] Pepsinogen produced by other anatomic regions would rarely encounter the low pH required for conversion to pepsin. Thus pepsin is abundant in refluxate and exists predominantly as pepsin, whereas very low levels pepsinogen may be locally synthesized in the upper aerodigestive tract and would exist predominantly as pepsinogen.

Designing antibodies that discriminate between pepsinogen A and pepsin is challenging as their amino acid sequences differ only by the absence of the 47 amino acid activation domains in the latter. Many commercial antibodies for pepsin have been raised against full-length pepsin or pepsinogen thus cannot discriminate between the 2. Insufficient detail is provided for most commercial ELISAs to determine their likelihood to discriminate between them. Rao and colleagues demonstrated that 3 commercial anti-pepsin antibodies and an ELISA yielded positive signal in a variety of tissues and bodily fluids that would not encounter refluxate (eg, parotid gland, kidney, seminal fluid, and urine), yet Western blot revealed that only in esophagus was the reactive band the same molecular weight as that observed in stomach, thus likely to be pepsin (refluxed and endocytosed).[90] Western blot band size and intensity was inconsistent across the antibodies, band sizes were commonly greater than that of pepsinogen (42 kDa), and liquid chromatography-tandem mass spectrometry (LC-MS) confirmed that 1 of 3 antibodies and the ELISA produced signal in samples not found to contain pepsin or pepsinogen. LC-MS confirmed pepsin/pepsinogen in tissues beyond the stomach, yet at levels several hundred times lower than in stomach. Endogenous pepsinogen production or serum pepsinogen may underlie reports of low levels of what was presumed to be pepsin detected by ELISA in tears of LPR patients.[93] Thus results must be carefully interpreted and ideally validated by methods that identify reactive proteins by band size or sequence (eg, Western blot, LC-MS). ELISA data should be carefully interpreted as the manufacturer limits for detection and quantitation under ideal conditions may not be replicated by the user. Pathologic threshold cut-off values must be considered. Thoughtful antibody design may also aid discrimination between endogenous pepsinogen and refluxed pepsin. Antibody raised to the nascent N-terminus of pepsin revealed by maturation can exhibit 100-fold preferential binding to pepsin relative to pepsinogen.[94] Commercialization of such an antibody, thoroughly validated and developed into corresponding ELISA and diagnostic tools, would be of benefit to pepsin research and reflux/aspiration diagnostics.

Detection of rare yet disease-relevant reflux events

The laryngopharynx exhibits much greater sensitivity to refluxate than the esophagus. More than a single hypopharyngeal reflux event in a 24-h recording, regardless of pH, is considered indicative of LPR.[95] Refluxed pepsin is present in saliva only transiently, thus the likelihood of false negative findings during a 24-h monitoring period is high despite multiple sampling. The 36-h retention of pepsin by airway epithelia is currently being leveraged to develop noninvasive cell-based assays (eg, brushings) of superior sensitivity and specificity relative to saliva-based tests.

Table 1
Airway/esophageal injury rescued by pepsin inhibition

Agent	Model/Injury Rescued	Citation
Pepstatin	GERD rat model (surgical). Esophageal lesions	Nagahama et al,[20] 2006
Pepstatin	Porcine laryngeal mucosa (pepsin pH4). Sep70, CAIII	Johnston et al,[21] 2007
Pepstatin, pH8	Laryngeal epithelial cells (pepsin pH7). *IL1A, CSF2, EGR1*, and Hsp70 genes (*HSPA1A, HSPA6, HSPH1).* Mitochondrial morphology and function.	Johnston et al,[24] 2010
Pepstatin	Human tonsil mucosa ex vivo (adult tonsilitis, pediatric tonsil hypertrophy; pepsin pH7). CD4-positive cells, IL-2, IFN-γ, IL-10	Kim et al,[98] 2018
Pepstatin	Laryngeal epithelial cells (pepsin pH7). IL-6 and IL-8 secretion. Vimentin, β-catenin, E-cadherin protein level.	Tan et al,[70] 2019
Pepstatin	Bronchial epithelial cells (pepsin pH3). Transepithelial neutrophil migration, HRP flux.	Hurley et al,[41] 2019
Fosamprenavir, Darunavir	LPR mouse model (topical pepsin pH7). Histologic laryngeal inflammation.	Johnston et al,[97] 2023
Amprenavir	Esophageal epithelial cells (pepsin pH4). E-cadherin cleavage, MMP gene expression.	Samuels et al,[59] 2023
Amprenavir	Laryngeal epithelial cells (pepsin pH4). E-cadherin cleavage, MMP gene expression.	Blaine-Sauer et al,[61] 2023
Darunavir	LPR mouse model (surgical). Inflammation (wet weight and myeloperoxidase activity) and mucosal integrity (transepithelial electrical resistance and fluorescein permeability).	Sales et al,[96] 2024

Emerging Therapies

Pepsin is a key therapeutic target for LPR. Pepsin is the only constituent found in all refluxate; is produced at several hundred-fold greater levels in the stomach than other anatomic regions; persists in the airways of patients with LPR; and has been evidenced to cause laryngeal inflammation, hypersensitivity, and cancer-related changes independent of gastric acid.[29] Enzymatic inactivation of pepsin via pH>8 or pharmacologic inhibitors has been found to attenuate all forms of experimentally induced peptic damage examined to date, even in models of acid reflux (eg, surgical or pH3-4; **Table 1**).[24,41,59,61,70,96–98]

While therapeutic development of the pepsin inhibitor pepstatin failed decades ago due to its peptidic nature and correspondingly poor bioavailability, human immunodeficiency virus (HIV) protease inhibitors have recently been proposed as novel pepsin-targeting therapeutics for reflux disease.[97] Oral gavage of fosamprenavir and darunavir at manufacturer-recommended doses for treatment of HIV/Acquired immunodeficiency syndrome prevented histologic inflammatory injury in an LPR mouse model (laryngeal scratch and topical pepsin). The efficacy of darunavir was confirmed in a surgical model of LPR by objective measures of inflammation (wet weight and myeloperoxidase activity) and mucosal integrity (transepithelial electrical resistance and fluorescein permeability).[96] In vitro data support the capacity of the active form of fosamprenavir (amprenavir) to inhibit peptic changes associated with epithelial barrier dysfunction and carcinogenesis in the larynx and esophagus.[60,61] Pilot epidemiologic data suggest a lower prevalence of LPR among patients taking HIV protease inhibitors relative to the general population (0.2% vs 10%–34.4%, n = 2062).[97] A sustained-release formulation of oral fosamprenavir calcium with sodium alginate was developed for use in a proof-of-concept trial for LPR. Inhalation delivery could be ideal for treatment of respiratory symptoms associated with LPR; its feasibility was supported by efficacy of aerosolized fosamprenavir in the LPR mouse model at 1/20th of the oral dose.[97] Inhaled fosamprenavir demonstrates no adverse effects in pre-Good Lab Practices toxicology assessment and optimal particle size has been determined for use in a laryngopharyngeal dry powder inhaler.[99]

Pepsin inhibitors may have additional therapeutic applications for PPI-recalcitrant GERD, peptic ulcer, and other diseases in which pepsin and/or its synergistic activity with acid appears to play a role.

Future Considerations

Pepsin presence is associated with myriad inflammatory conditions and neoplasia of the airways. Experimental evidence strongly implicates nonacid pepsin in inflammatory and carcinogenic airway injury and supports proof-of-concept that pepsin inhibitors attenuate these changes. However, without advances in LPR diagnostics, it is not yet possible to identify subjects whose symptoms are truly attributed to LPR, for whom a pepsin inhibitor may provide benefit. Further, evaluation of treatment efficacy is confounded by inadequately validated clinical outcomes measures. Given recent updates to United States Food and Drug Administration (FDA) guidelines, which simultaneously recommended the use of patient-reported outcomes measures for evaluation of therapeutic efficacy and established more rigorous standards for their use for regulatory decision-making, there are currently no clinical outcomes measures that qualify to evaluate the efficacy of novel therapeutics for LPR.[100] This represents an immense hurdle that must be overcome before scientific advancements can be translated to new therapies for this burgeoning clinical population for whom no effective medical strategy exists.

SUMMARY

An exclusively acid-targeting approach to reflux disease has proven inadequate to address reflux-attributed symptoms and carcinogenesis. While pepsin is only weakly active or inactive in the context of most LPR events, it is endocytosed and remains entrapped within airway mucosa long after a reflux event. Experimental data demonstrate that nonacid pepsin in the airways activates many of the same molecular mechanisms implicated in the pathogenesis of GERD. Pepsin elicits an inflammatory response that, when sustained, promotes fibrosis and cancer-associated changes. While its presence at high levels within refluxate and persistence in airway tissues after a reflux event makes pepsin an ideal diagnostic marker, methodologies for its detection require optimization and careful interpretation. HIV protease inhibitors, which bind and inhibit pepsin, offer a new therapeutic approach to LPR, GERD, and other diseases in which pepsin is implicated.

CLINICS CARE POINTS

- Nonacid pepsin elicits an inflammatory response that, when sustained, leads to fibrosis and cancer-associated changes airway mucosa.
- Current medical therapies for GERD (eg, PPI, PCAB) do not address nonacid pepsin.
- Acid-suppressing PPIs promote, rather than protect against reflux-attributed carcinogenesis, potentially due to continued exposure to nonacid pepsin.
- Some alginate-antacid formulations inhibit pepsin and reduce LPR symptoms. Alginates are nonsystemic and generally regarded as safe but provide temporary protection against reflux.
- Some FDA-approved HIV protease inhibitors are potent pepsin inhibitors and have demonstrated promise for preventing peptic injury in preclinical studies.

DISCLOSURES

N. Johnston is co-founder, CSO and investor in N-Zyme Biomedical and inventor on PCT/US2021/027758 Aerosolized formulations of HIV protease inhibitors for the treatment of airway reflux, PCT/US2023/071204 Sustained-release oral fosamprenavir for the treatment of reflux, PCT/US2021/027758 Methods and compositions for treatment of peptic ulcers and/or peptic ulcer disease. T.L. Samuels is an investor in N-Zyme Biomedical.

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