In vitro model of acute esophagitis in the cat

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Cheng, Ling, Weibiao Cao, Claudio Fiocchi, Jose Behar, Piero Biancani, and Karen M. Harnett. In vitro model of acute esophagitis in the cat. Am J Physiol Gastrointest Liver Physiol 289: G860–G869, 2005. —We have shown that IL-1β and IL-6, possibly originating from the mucosa in response to injury, inhibit neurally mediated contraction of esophageal circular muscle but do not affect ACh-induced contraction, reproducing the effect of experimental esophagitis on esophageal contraction. To examine the interaction of the mucosa and circular muscle in inflammation, we examined the effect of HCl on in vitro esophageal mucosa and circular muscle. Circular muscle strips, when directly exposed to HCl, contracted normally. However, when circular muscle strips were exposed to supernatants from mucosa incubated in HCl (2–3 h, pH 5.8), contraction decreased, and the inhibition was partially reversed by an IL-6 antibody. Supernatants from the mucosa of animals with acute esophagitis (AE) similarly reduced contraction. IL-6 levels were higher in mucosal tissue from AE animals than in control mucosa and in AE mucosa supernatants than in normal mucosa supernatants. IL-6 levels increased significantly in normal mucosa and supernatants in response to HCl, suggesting increased production and release of IL-6 by the mucosa. IL-6 increased H2O2 levels in the circular muscle layer but not in mucosa. Exposure of the mucosa to HCl caused IL-1β to increase only in the mucosa and not in the supernatant. These data suggest that HCl-induced damage occurs first in the mucosa, leading to the production of IL-1β and IL-6 but not H2O2. IL-1β appears to remain in the mucosa. In contrast, IL-6 is produced and released by the mucosa, eventually resulting in the production of H2O2 by the circular muscle, with this affecting circular muscle contraction.

esophageal mucosa; inflammation; smooth muscle contraction; reactive oxygen species; cytokines

Using a well-established feline model of in vivo-induced esophagitis (3, 12, 34, 37), we previously examined changes in contractility associated with acid-induced inflammation of the esophagus and lower esophageal sphincter (LES). In this model of in vivo-induced acute esophagitis, we established that the inflammatory cytokines IL-1β and IL-6 (but not TNF-α) are elevated in the esophageal circular muscle layer. Exposure of normal esophageal circular muscle to IL-1β and IL-6 reproducibly mediates contraction of the cat esophagus. Similar to in vivo-injected acute esophagitis, IL-1β and IL-6 inhibited electrical field stimulation (EFS)-induced, neurally mediated contraction of the cat esophagus. Similar to in vivo-induced acute esophagitis, IL-1β and IL-6 inhibited ACh release from intramural cholinergic neurons but did not affect contraction in response to direct myogenic stimulation with ACh (3).

In addition, we examined human endoscopic mucosal biopsies, obtained from patients with esophagitis, and reported increased concentration of IL-6 in the inflamed tissue. IL-1β was also elevated when esophageal inflammation was severe (35).

These findings in biopsies from patients with gastroesophageal reflux disease (GERD) are consistent with data obtained in the cat model of in vivo-induced esophagitis and indicate that both acid-induced acute inflammation of the normal mucosa and chronic inflammation of the mucosa of patients with reflux esophagitis are characterized by the overproduction of the proinflammatory cytokines IL-1 and IL-6.

These cytokines are thought to derive from inflammatory cells infiltrating acid-damaged tissue (32) and may produce additional inflammatory mediators that may amplify and perpetuate tissue injury (30, 33) by acting on muscle cells and causing them to produce their own cytokines (39). This creates a vicious circle that contributes to and maintains the motility disorders found in gut inflammation (11). For these reasons, examining tissues with fully developed inflammation may provide few clues toward understanding how inflammation develops. Defining the sequential production of inflammatory mediators in reflux esophagitis and their tissues of origin seems essential to better understand the genesis of GERD pathophysiology.

To examine the interaction of the mucosa and circular muscle in originating IL-1β and IL-6 in response to inflammation, we examined the effect of HCl on the in vitro esophageal mucosa and circular muscle, separately and together, in an in vitro model of esophageal inflammation. In this model, the mucosa is freed from the circular and longitudinal muscle by sharp dissection at the level of the submucosa and tied at both ends, creating a mucosal sac, as shown in Fig. 1. The sac, with the squamous epithelium on the inside and the submucosa on the outside, is filled with either Krebs solution or HCl and kept in warm oxygenated Krebs solution. The supernatant surrounding the sac is collected after 2–3 h and applied to circular muscle strips to examine its effects on contractility or analyzed for content of inflammatory mediators. The inflammatory mediators released by the mucosa from animals with in vivo-induced esophagitis may be compared with those released by the mucosal sac preparation after 2–3 h of exposure to intraluminal HCl.

This in vitro model of esophageal inflammation has the advantage of distinguishing the sequential activation of inflammatory events in the mucosa from events occurring in the circular muscle layer. Because the model uses normal esophageal specimens, it allows us to examine not only tissue from cats but also tissue from human organ donors, a particularly attractive feature considering that human esophagitis specimens are exceedingly rare.

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METHODS

Experimental procedures were approved by the Animal Welfare Committee of Rhode Island Hospital. Adult male cats weighing between 3.5 and 5.5 kg were used in this study. After an overnight fast, animals were initially anesthetized with ketamine (Aveco; Fort Dodge, IA) and then euthanized with an overdose of phenobarbital (Schering; Kennilworth, NJ). The chest and abdomen were opened with a midline incision, exposing the esophagus and stomach. The esophagus and stomach were removed together and separated immediately above the LES. The esophagus was pinned on a wax block, and the smooth muscle layer was opened along the long axis and removed by sharp dissection at the level of the submucosa, leaving the mucosa intact as a tube. The smooth muscle layer beginning at 1 cm proximal to the LES was cut into 2-mm circular muscle strips, which were mounted in separate 1-ml muscle chambers (Mucosa tubes) and used as a control; the other part was tied at both ends of the tube. One part was filled with Krebs buffer (0.5 ml/cm tube) and used as a control; the other part was filled with the same volume of Krebs buffer equilibrated with HCl to the required pH. Both tubes were kept in Krebs buffer with 95% O2-5% CO2 at 36°C for 3 h using 1 ml Krebs buffer/100 mg mucosa. After 3 h, the supernatant outside the tube is collected and analyzed or used to incubate circular muscle strips.

To study the effect of supernatant from HCl-treated mucosa of selected cytokines on EFS-induced contraction, the strips were incubated in supernatant or in appropriate concentrations of the cytokines for 2 h before contraction in response to EFS.

**Measurements of IL-1β and IL-6.** Esophageal circular muscle (100 mg) was homogenized in 2 ml PBS (Sigma; St. Louis, MO; pH 7.4) containing 0.01 M phosphate buffer, 0.0027 M KCl, and 0.137 M NaCl. Homogenization was achieved with three 10- to 20-s bursts with a Tissue Tearer (Biospec; Racine, WI). The homogenate or supernatant was centrifuged at 2000 g and 4°C for 20 min. An aliquot of homogenate was taken for protein determination. The supernatant was frozen in liquid nitrogen for later use. The tissue concentrations of cytokines were measured using enzyme immunoassay kits from Cayman Chemical (Ann Arbor, MI) for IL-6 and R&D Systems (Minneapolis, MN) for IL-1β.

**Measurements of H2O2 in smooth muscle tissue and supernatant.** Esophageal mucosa or esophageal circular smooth muscle tissue (100 mg) were homogenized in 20 mM Tris·HCl buffer. Homogenization consisted of a 20-s burst with a Tissue Tearer (Biospec) followed by 50 strokes with a Dounce tissue grinder (Wheaton; Melville, NY). An aliquot of homogenate (100 μl) was taken for protein measurement. The homogenate or supernatant was centrifuged at 15,000 rpm (2,500 g) for 15 min at 4°C in a Beckman J2–21 centrifuge with a fixed-angle JA-20 rotor (Beckman; Palo Alto, CA), and the supernatant was collected.

**Protein determination.** The amount of protein present was determined by a colorimetric assay (Bio-Rad; Melville, NY) according to the method of Bradford (2).

**Materials.** Antibodies to IL-1β and IL-6 were purchased from R&D Systems. All other reagents were purchased from Sigma.

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Results

We have previously shown that the proinflammatory cytokines IL-1β and IL-6 are present in the esophageal muscle layer of animals with esophagitis and, when applied to normal esophageal circular smooth muscle in vitro, reproduce esophagitis-induced in vivo changes in motor function (3). To determine whether these inflammatory mediators are produced in esophageal muscle by direct exposure to HCl after breakdown of the mucosal barrier, we directly added HCl into the muscle bath, lowering the pH to 5.8, and recorded EFS-induced muscle contraction after 3 h. Figure 2A shows that direct application of HCl to in vitro circular muscle strips did not cause any change in contraction compared with muscle strips incubated in Krebs buffer for the same length of time. However, if a piece of mucosa (100 mg wet wt/ml bath volume) was added to the bath and the muscle and mucosa together were exposed to HCl at the same pH, EFS-induced contraction was almost abolished after a 3-h incubation. Shorter times were less effective. When muscle strips were incubated with the same amount of mucosa without HCl, no change in contraction occurred compared with strips incubated in Krebs buffer without the mucosa (Fig. 2B).

These data suggest that in vitro exposure of the esophageal mucosa to HCl stimulates production of inflammatory mediators, which affect muscle contraction. To examine which inflammatory mediators are produced in and released by the mucosa, we measured inflammatory mediators in the mucosa and supernatants of esophageal mucosal tube preparations (Fig. 1) filled with control Krebs solution or HCl.

pH and production of cytokines. We first examined the effect of pH on production and release of inflammatory mediators, such as IL-1β and IL-6. The mucosa tube was filled with PSS at various pHs for 3 h, and IL-1β and IL-6 concentrations were then measured in the mucosa and supernatant surrounding the mucosal sac. Figure 3 shows that in the mucosa, tissue content of IL-1β and IL-6 was highest when the sac was filled with medium at a pH between 5.8 and 4.8 and declined when the pH was lowered to 4, most likely reflecting tissue damage or necrosis. Figure 4A shows that most cells were alive after a 3-h incubation at neutral pH or at pH 5.8. When pH was lowered to 4, approximately half of the cells died within 3 h. Figure 4B shows that content of LDH in the sac supernatant (an index of cell death) did not change as pH was lowered from 7.4 to 4.9 and increased significantly when the mucosal sac was filled with Krebs solution at pH 4.0, confirming trypan blue data.

Similar to tissue content, release of IL-6 from the mucosal tube into the supernatant, as shown in Fig. 3, was highest when pH was maintained at 5.8–4.8 and decreased when pH was lowered to 4. Release of IL-1β into the surrounding medium, however, did not occur after a 3-h exposure to acidic medium and did not change from the value measured for nonacidified mucosa, regardless of pH.

In subsequent experiments, to study the effect of HCl exposure, the mucosal sac was filled with Krebs solution at pH 5.8.

Similar to Fig. 3, Fig. 5 shows that HCl-treated mucosa (pH 5.8) contained significantly (P < 0.05) higher levels of IL-6 than untreated mucosa (increasing from 443 ± 157 to 938 ± 81 pg/mg protein). We next compared these results to preparations of the esophageal mucosal tube from animals with experimental esophagitis, which was induced by repeated in vivo HCl esophageal perfusion (i.e., animals were perfused for 45 min with 0.1 N HCl for 3 days and tested on day 4) (3, 37). IL-6 levels were significantly higher in the mucosa from esophagitis animals (819 ± 81 pg/mg protein) than in control samples (pH 7.4 Krebs-treated mucosa). Similarly, IL-1β increased in HCl-treated mucosa (from 26.7 ± 9 to 498.7 ±

Statistical analysis. Data are expressed as means ± SE. Statistical differences between means were determined by Student’s t-test. Differences between multiple groups were tested using ANOVA for repeated measures and checked for significance using Scheffé’s F-test.

Fig. 2. Esophageal circular muscle strips. A: incubation of circular muscle strips without mucosa in Krebs buffer with HCl (0.01 N, 2–3 h) did not change contraction in response to electrical field stimulation (EFS; 0.5 ms, 2 Hz, 10 s). But, when mucosa and muscle were incubated together, the maximal contraction decreased from 2.9 ± 0.3 to 0.26 ± 0.06 g. B: incubation of muscle and mucosa without HCl did not change contraction. Values are means ± SE for 3 animals.
163.6 pg/mg protein) and was elevated in the mucosa from esophagitis animals compared with normal (pH 7.4) Krebs-treated mucosa. Of interest, levels of IL-6 and IL-1β were comparable between in vitro HCl-treated mucosa and mucosa from animals with esophagitis induced by HCl-treated perfusion.

Figure 6 shows that in the supernatant of HCl-treated mucosa or mucosa from esophagitis animals, IL-6 levels were significantly higher than those in normal Krebs-treated mucosa, demonstrating that after exposure to HCl or after the induction of esophagitis, the mucosa increased the production of IL-6 and released it into the supernatant. In contrast, even though these preparations had elevated levels of IL-1β, the cytokine was not released in the mucosa supernatant.

Mucosal sac supernatant and circular muscle contraction. To explain the reduction in EFS-induced contraction of circular muscle strips exposed to HCl in the presence of mucosa (shown in Fig. 2), we used supernatant of the HCl-filled mucosa tube. In muscle strips exposed to the supernatant of HCl-filled mucosa, maximal contraction decreased from 2.44 ± 0.5 to 0.39 ± 0.09 g (P < 0.02). The decrease was almost reversed by IL-6 antibody (Fig. 7A) and not by IL-1β antibody (Fig. 7B), confirming that the observed motor changes appear to be mediated largely by the release of IL-6 from the mucosa into the supernatant. The finding that IL-1β antibodies do not reverse the supernatant-induced inhibition is consistent with the lack of IL-1β release by the HCl-treated mucosa.

Similar to the supernatant of HCl-treated mucosa, the supernatant of mucosa from esophagitis animals almost abolished EFS-induced contraction of circular muscle strips (Fig. 8). This inhibition, however, was only in a small part reversed by IL-6 neutralization. This finding suggests that after 3 days of HCl perfusion, in addition to IL-6, other inflammatory mediators may also be released by the esophageal mucosa and present in the supernatant. Measurement of H$_2$O$_2$ in the mucosa supernatant (Fig. 9) indicated that the HCl-treated mucosa (3 h) did not release any more H$_2$O$_2$ than did control mucosa. After the induction of esophagitis, however, the mucosa released significantly higher levels of H$_2$O$_2$ than either control or HCl-treated mucosa.

H$_2$O$_2$ in mucosa and circular muscle. Cytokines such as IL-6 (20, 24, 40) are known to induce the production of H$_2$O$_2$, which affects a variety of mechanisms, including calcium homeostasis (16, 17, 36), lipid peroxidation (18, 19, 22, 23),...
and transcription factors (14, 27, 29). We (4, 7, 10) have reported that H$_2$O$_2$ is present in esophageal and LES muscularis propria of esophagitis animals and plays a role in esophagitis-associated motor dysfunction. We therefore examined the presence of H$_2$O$_2$ in normal esophageal muscle and mucosa layers incubated in IL-6.

Figure 10 demonstrates that IL-6 caused production of H$_2$O$_2$ when applied for 2 h to esophageal smooth muscle but not when IL-6 was applied to the mucosa. Figure 11 shows that direct application of HCl, similar to the supernatant of normal mucosa, did not increase the production of H$_2$O$_2$ in esophageal circular muscle. Application of the supernatant from HCl-treated mucosa or of the supernatant of mucosa from esophagitis animals (both containing IL-6) caused the production of H$_2$O$_2$ in the muscle, as did incubation in IL-6.

To demonstrate that levels of H$_2$O$_2$ produced in response to the supernatant of HCl-filled mucosa are sufficient to affect circular muscle contraction, circular muscle strips were incubated in that supernatant. Figure 12 shows that incubation of circular muscle strips in the supernatant of HCl-filled mucosa almost abolished contraction in response to EFS. Catalase, by itself, had no effect on EFS-induced contraction but partly restored the reduction in contraction caused by the supernatant. The remaining inhibition may be due to the presence of inflammatory mediators other than H$_2$O$_2$, for instance, IL-6 or others. Thus incubation in the supernatant results in the production of H$_2$O$_2$ at levels sufficient to affect circular muscle contraction. Because the supernatant of HCl-filled mucosa contained no excess of H$_2$O$_2$ (see Fig. 9), H$_2$O$_2$ must be produced in the muscle in response to the supernatant of HCl-treated mucosa. The amplitude of contraction recovered by exposure to catalase is consistent with H$_2$O$_2$ being largely responsible for the reduction in the response to EFS.

To confirm the source of H$_2$O$_2$, we examined H$_2$O$_2$ levels in isolated mucosa and circular smooth muscle cells using dihydrorhodamine (DHR-123) as a probe to measure intracellular H$_2$O$_2$ by confocal microscopy. DHR-123 enters the cells as a freely permeable dye that, when oxidized, is converted to rhodamine-123, which is not membrane permeable. Rhodamine-123 is a common laser dye, which is excitable at 488 and detectable at 515 nm, under confocal microscopy. Rhodamine-123 becomes localized in the cytoplasm, where some H$_2$O$_2$ is present, and in the mitochondria, where oxygen radicals are produced as part of the normal respiratory process and may be present at higher concentration than in the cytoplasm.

Figure 13 shows untreated epithelial cells, freshly isolated from the esophageal mucosa by enzymatic digestion, and epithelial cells treated with HCl or IL-6. An H$_2$O$_2$-treated epithelial cell is shown as a positive control in Fig. 13D. H$_2$O$_2$ is membrane permeable, and, in the H$_2$O$_2$-treated cell, it penetrates the cytoplasm, demonstrating the adequacy of the technique in detecting cytoplasmic H$_2$O$_2$, when present. In contrast, in control cells (Fig. 13A) or in cells treated with HCl (Fig. 13B) or with IL-6 (Fig. 13C), little H$_2$O$_2$ was present in the cells’ cytoplasmic region, demonstrating that epithelial cells initially do not produce H$_2$O$_2$ in response to HCl or IL-6.

Figure 14 shows that H$_2$O$_2$ levels are low in normal esophageal smooth muscle cells (A) and are not affected by the supernatant of Krebs-filled mucosa (B) or by HCl (C). In contrast, incubating normal smooth muscle cells with the...
supernatant of HCl-treated mucosa (Fig. 14D) or with the supernatant of mucosa from esophagitis animals (Fig. 14E) visibly increased cytoplasmic H$_2$O$_2$. Similarly, treatment of smooth muscle cells with IL-6 caused a visible increase in cytoplasmic H$_2$O$_2$ in muscle cells (Fig. 14F).

Finally, to demonstrate that exposure to the supernatant of HCl-treated mucosa reproduced esophagitis-induced changes in muscle contraction (i.e., inhibition of EFS-induced but not ACh-induced contraction), we studied ACh dose-response relationships in untreated muscle strips and in strips exposed to the supernatant of HCl-treated mucosa (Fig. 15). The supernatant did not significantly affect ACh-induced contraction of esophageal circular muscle strips.

**DISCUSSION**

We have shown that mucosal biopsies from human esophagitis patients exhibit increased concentration of the proinflammatory cytokine IL-6 in inflamed tissue (35). Similarly, in a feline model of experimental esophagitis, we have demonstrated increased levels of the cytokines IL-1β and IL-6 in the esophageal circular muscle layer. These cytokines inhibit neurally mediated contraction of esophageal circular muscle but do not affect contraction in response to direct myogenic stimulation with ACh and thus reproduce the effect of acute experimental esophagitis on esophageal contraction (3).

Cytokines are thought to derive from inflammatory cells infiltrating acid-damaged tissue (32) and may produce additional inflammatory mediators by acting on muscle cells and causing them to produce their own cytokines (39). This initiates a cycle in which inflammatory mediators released by a cell type affect other cells to create additional inflammatory mediators, which may in turn affect the original cells, further increasing the formation of inflammatory mediators and creating a self-perpetuating cycle of inflammation (11). Examining tissues with developed inflammation, as we have previously done in our model of in vivo-induced esophagitis, may provide insufficient clues toward understanding how inflammation develops. We know that in the model of esophagitis induced by repeated acid perfusion on 3 consecutive days, IL-1β, IL-6, platelet-activating factor (PAF), and H$_2$O$_2$ are all present in the circular muscle layer (3, 4, 7, 8) and contribute to esophagitis-associated dysmotility. However, any of these inflammatory products may contribute to the formation of the others. Thus defining the sequential production of inflammatory mediators in reflux esophagitis and their tissues of origin seems essential to a better understanding of the genesis of GERD pathophysiology.

To examine the interaction of the mucosa and circular muscle in originating changes in muscle contraction in response to inflammation, we examined the effect of HCl on in vitro esophageal mucosa and circular muscle.

As expected, EFS-induced contraction of circular smooth muscle in PSS was not modified by the presence of mucosal tissue in the organ bath. In addition, muscle contraction was not affected by direct exposure to HCl (at pH 5.8). When the mucosa and HCl at the same pH were incubated with circular muscle, however, contraction was almost abolished, suggesting that in the presence of acid the mucosa released inhibitory factors affecting muscle contraction.

Our in vitro mucosal sac preparation was designed to distinguish the inflammatory mediators released by the mucosal layer from those produced in the circular muscle layer in response to the mucosa. Because the mucosal sac was created by removing the muscle at the level of the submucosa, it is reasonable to assume that anything that is secreted by the mucosal sac and collected in the supernatant would have diffused to the circular muscle layer in an intact esophagus.
Fig. 13. Epithelial cells enzymatically isolated from esophageal mucosa. H$_2$O$_2$ levels were examined by confocal microscopy in isolated mucosa cells using dihydrorhodamine (DHR-123) as a probe for the measurement of intracellular H$_2$O$_2$. DHR-123 enters the cells as a freely permeable dye that, when oxidized, is converted to rhodamine-123, which is not membrane permeable. Rhodamine-123 is a common laser dye, which is excitable at 488 and detectable at 515 nm under confocal microscopy. Rhodamine-123 becomes localized in the cytoplasm, where some H$_2$O$_2$ is present, and in the mitochondria, where oxygen radicals are produced as part of the normal respiratory process and may be present at higher concentration than in the cytoplasm. A: untreated epithelial cells; B: epithelial cells treated with HCl; C: epithelial cells treated with IL-6. D: a H$_2$O$_2$-treated epithelial cell is shown as a positive control. H$_2$O$_2$ is membrane permeable, and, in the H$_2$O$_2$-treated cell, it penetrates the cytoplasm, demonstrating the adequacy of the technique in detecting cytoplasmic H$_2$O$_2$.

Fig. 14. Muscle cells enzymatically isolated from the esophageal circular muscle layer. H$_2$O$_2$ levels were examined by confocal microscopy in isolated mucosa cells using DHR-123 as a probe for the measurement of intracellular H$_2$O$_2$. H$_2$O$_2$ levels were low in normal esophageal smooth muscle cells (A) and were not affected by supernatant of normal Krebs-treated mucosa (B) or by HCl at pH 5.8 (C). In contrast, incubating normal smooth muscle cells with supernatant of HCl (pH 5.8)-filled mucosa (D) or with supernatant of mucosa from esophagitis animals (E) visibly increased cytoplasmic H$_2$O$_2$. Similarly, treatment of smooth muscle cells with IL-6 caused a visible increase in cytoplasmic H$_2$O$_2$ (F).
In vivo, the esophageal mucosa was very sensitive to the presence of HCl, producing inflammatory/inhibitory mediators at a relatively high pH (5.8). Typically, to induce experimental esophagitis, the in vivo esophagus is perfused for 45 min on 3 successive days with 0.1 N HCl. In contrast, in the in vitro mucosa, lowering the pH to 5.8 for 3 h induced maximal production of the cytokines IL-1β and IL-6. Further lowering the pH to 4 caused tissue damage, resulting in cell death and reduced production of cytokines. The in vitro mucosa may be more susceptible to damage than when in vivo, due to lack of buffering mechanisms. The in vitro mucosa has no blood flow, and neutralization of the intraluminal pH can only occur by passive diffusion of electrolytes from the medium surrounding the mucosal sac. In contrast, the in vivo mucosa may better resist a low intraluminal pH as it is continuously perfused and buffered by blood flow and exposed to saliva at high pH. In contrast to the mucosa, the circular smooth muscle, EFS-induced contraction, and production of H2O2 were not affected by acute exposure to acid at the same concentration or even at concentrations three to four times higher (L. Cheng, unpublished observations) than the ones causing release of inflammatory mediators by the mucosa.

Because IL-1β and IL-6 are present in the esophageal circular muscle layer in our model of in vivo-induced experimental esophagitis, we examined the possibility that these inflammatory mediators may be produced in the mucosa in response to HCl and may diffuse to the circular muscle layer. Using this model of in vitro-induced inflammation, we found that IL-1β and IL-6 were produced in the mucosa in response to HCl. Only IL-6, however, was collected in the supernatant outside the mucosal sac when the lumen was filled with HCl. Thus enough IL-6 is produced and released by the mucosa to diffuse past the submucosal layer into the surrounding supernatant at a concentration sufficient to affect circular muscle contraction. In contrast, IL-1β produced in the mucosa in response to HCl was not released into the supernatant and remained confined to the mucosa.

These data suggest that, in this model of in vitro inflammation, IL-6 is a major inhibitory mediator released by the mucosa in response to HCl, as the effect of exposure to the supernatant of HCl-treated mucosa on muscle contraction is inhibited to a large extent by immunoneutralization of the supernatant with IL-6 antibodies. Introducing IL-1β antibody into the supernatant did not affect the supernatant-induced inhibition (Fig. 7B), and this finding is consistent with the lack of IL-1β release by the HCl-treated mucosa.

In contrast, when circular muscle strips were incubated with the supernatant of mucosa from esophagitis animals, EFS-induced contraction was almost abolished, but the inhibition was only partly reversed by IL-6 antibodies. This finding suggests that, after 3 days of acid perfusion, the mucosa of esophagitis animals may release other inflammatory mediators in addition to IL-6. The finding of H2O2 in the supernatant of mucosa from esophagitis animals may identify one of the additional inflammatory mediators. Other inflammatory mediators or other ROS may also be present.

We have previously demonstrated that both IL-6 and IL-1β are present in the esophageal circular muscle layer of animals with in vivo-induced acute experimental esophagitis (3) and may produce H2O2, which in turn may cause the production of other inflammatory mediators (8). We therefore examined whether IL-6, released by the mucosa in response to HCl, may cause the production of H2O2 in either the mucosa or muscle. Application of HCl or IL-6 did not induce the production of H2O2 by the mucosa. Similarly, direct application of HCl did not cause the production of H2O2 by muscle. Application of IL-6 or the supernatant from HCl-treated mucosa, however, caused the production of H2O2 by muscle, and incubation in the supernatant of mucosa from animals with in vivo-induced esophagitis also caused the production of H2O2 by muscle.
H$_2$O$_2$ is one of several ROS that may be produced and is present in inflamed tissues. Other ROS include superoxide anion (•O$_2^-$), H$_2$O$_2$, and hydroxyl radical (•OH) and the reactive nitrogen species nitric oxide (NO) and peroxynitrite (ONOO$^-$.). H$_2$O$_2$ is produced mainly from dismutation of •O$_2^-$. This reaction can be spontaneous, or it can be catalyzed by superoxide dismutase (SOD), of which there are three isoforms: CuZn SOD, Mn SOD, and extracellular SOD (13). The SOD-catalyzed dismutation is favored when the concentration of •O$_2^-$ is low and when the concentration of SOD is high, which occurs under physiological conditions. H$_2$O$_2$ is lipid soluble, crosses cell membranes, and has a longer half-life than •O$_2^-$, which is unstable. H$_2$O$_2$ is physiologically produced in large amounts by cells such as granulocytes and in lower amounts by nonimmune cells (6, 38, 42). Because H$_2$O$_2$ is relatively stable, it has been widely used to assess the effects of ROS (26, 28).

We have recently demonstrated that in a human specimen with esophagitis tone of muscle strips was considerably lower (0.78 g) than in the normal LES (3.3 ± 0.2g) and was almost restored to normal (2.7 g) by the H$_2$O$_2$ scavenger catalase (10). The finding that catalase almost normalized the tone of the esopagitis specimen indicates that, among all ROS, H$_2$O$_2$ is likely to play a major role in esophagitis-associated motor dysfunction and is entirely consistent with the results shown in Fig. 12. Figure 12 shows that contraction of esophageal circular muscle in response to EFS (i.e., neural stimulation) was almost abolished by the supernatant of the HCl-filled mucosal sac and that the selective H$_2$O$_2$ scavenger catalase restored by ~70% the reduction induced by the supernatant. Taken together, these data suggest that H$_2$O$_2$ may play a major role in esophageal dysmotility.

Although superoxide may be the original ROS produced by a variety of sources, it is membrane impermeable, unstable, and short lived, and in physiological conditions, in aqueous solutions at a neutral pH, the favored reaction of superoxide anion is the dismutation reaction yielding H$_2$O$_2$.

H$_2$O$_2$ may cause lipid peroxidation and release of calcium from intracellular stores (31) and diffuses across biological membranes (41) because the molecule is not electrically charged. Thus H$_2$O$_2$ may diffuse to the nucleus, altering protein expression in the cell (15). In our model of in vivo-induced acute esophagitis, it is likely that IL-6, released by the mucosa after exposure to HCl, may cause the production of H$_2$O$_2$ by muscle. H$_2$O$_2$, in turn, may cause the production of multiple inflammatory mediators in the muscle layer, including IL-1β, which is found in the muscle layer after 3 days of repeated perfusion with HCl. The role of ROS in cytokine expression is not entirely clear, but it is generally accepted that ROS activate NF-κB and other transcription factors, resulting in upregulation of inflammatory cytokine gene expression (21, 25, 43). Once H$_2$O$_2$ is present, causing the formation of cytokines by muscle, a vicious circle may be initiated, because cytokines may cause the production of additional H$_2$O$_2$, worsening the injury. In addition, H$_2$O$_2$ may diffuse to the mucosa, causing the formation of additional inflammatory mediators by the mucosa and perhaps initiating the production of H$_2$O$_2$ in the mucosa itself, which initially does not produce H$_2$O$_2$.

It is notable that even though H$_2$O$_2$ is produced in the esophageal muscle layer, including in muscle cells, at concentrations sufficient to affect contraction, its effect is not evident in esophageal muscle cells but rather in motor neurons. As shown in Figs. 7, 12, and 15, incubation in the supernatant of HCl-treated mucosa almost abolished contraction in response to electrical (i.e., neural) stimulation but had no effect on contraction in response to direct myogenic stimulation by ACh. In this respect, the effect of incubation in the supernatant from HCl-treated mucosa is similar to that of in vivo-induced esophagitis, where we have shown that esophageal motor function is impaired (3). After in vivo induction of experimental esophagitis, the in vivo amplitude of the pressure excursion recorded during swallowing was drastically reduced compared with normal animals, and the contraction of circular muscle strips in response to EFS, which is neurally mediated, was significantly reduced. In contrast, contraction in response to the neurotransmitter ACh, which directly activates muscarinic receptors on the muscle cell membrane, was not affected, suggesting that muscle function is not directly affected by esophagitis-related inflammation. In contrast, the neurons mediating esophageal contraction are affected by in vivo-induced inflammation, and the observed inflammation-induced changes reflect the reduced release of the endogenous neurotransmitter ACh (3). We (8) have previously shown that H$_2$O$_2$, produced in response to IL-6 in esophageal circular muscle, causes the production of PGE$_2$ and PAF in the muscle layer, which in turn inhibit the release of ACh in response to electrical (i.e., neural) field stimulation. This finding may explain how, in the present model of in vitro inflammation, incubation in the supernatant of HCl-treated mucosa inhibits neural but not direct myogenic stimulation of the circular muscle.

A possible sequence of events leading to HCl-induced esophageal inflammation may be the production of cytokines or other inflammatory mediators by the mucosa, diffusion of these inflammatory mediators to circular muscle, and the production of H$_2$O$_2$ by the muscle (Figure 16). H$_2$O$_2$ may then diffuse through the muscle and mucosa, inducing the production of other inflammatory mediators and resulting in upregulation of H$_2$O$_2$-producing enzymes in the mucosa, within 3 days of the onset of inflammation. Thus the initial development of inflammation may depend on the interaction of muscle and mucosa in releasing distinct inflammatory mediators to act on both.

Because application of the supernatant of HCl-treated mucosa to circular muscle strips reproduces features of in vivo-induced esophagitis, this model of in vitro inflammation may provide a reasonably mechanistic approach to examining the interaction of muscle and mucosa in the initial development of inflammation in response to acid or other noxious elements.

GRANTS

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REFERENCES

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