Inflammation induced changes in arachidonic acid metabolism in cat LES circular muscle

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The pathophysiology of GERD has been intensely investigated, and its multifactorial nature is well recognized. The current belief is that GERD results from impaired esophageal defenses because of defective motor function responsible for antireflux and luminal clearance. At times, particularly after meals (26, 36, 55), reflux of gastric contents may occur as a result of transient relaxation of the LES (TLESR) unrelated to swallowing or to secondary peristalsis (26, 27, 44). In the early stages of GERD, as in patients without endoscopic evidence of reflux esophagitis, TLESR accounts for the largest proportion of reflux episodes (25, 27, 44). TLESR-unrelated reflux, however, increases with progressive severity of the disease. Impairment of LES tone and esophageal contraction become more prevalent and important in the pathogenesis of GERD as the severity of the disease increases (27, 39).

Impairment of these motor functions coupled with inflammation-associated decrease in tissue resistance (54) may increase the likelihood of further reflux episodes and of impaired acid clearance, aggravating their damage. The spiral of damage leading to worse damage may contribute to permanent impairment of LES tone and of esophageal peristalsis.

Considerable attention has been paid to the role of injurious agents in the gastric refluxate, such as hydrochloric acid, bile salts, pepsin, and trypsin and to the mechanisms of esophageal epithelium’s failure to resist chemical aggression (54); however, so far, little attention has been given to esophageal inflammation itself, a process that is probably responsible for the high rate of relapse observed in humans after cessation of medical therapy. This lack of interest is surprising, considering that many substances considered critical to reflux esophagitis are classical inflammatory products, such as prostanoids and reactive oxygen species (ROS; see Refs. 51, 66, 67). These products are thought to derive from inflammatory cells infiltrating acid-damaged tissue (50). Furthermore, some of the immune cell-derived cytokines act upon muscle cells and cause them to produce their own cytokines (60), perhaps creating a vicious circle that contributes to and maintains the motility disorders found in gut inflammation (22). Examination of the relationship between inflammatory mechanisms and mechanisms responsible for LES tone should help in understanding the pathophysiological events associated with esophageal disease.

A significant component of LES tone is thought to be myogenic and not affected by neural antagonists, including TTX (1, 21, 30). Functionally, this muscle is specialized, with muscle strips from this region developing higher basal and active forces than esophageal strips (6, 20, 21).

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Myogenic LES tone is mediated by the spontaneous activity of a group I phospholipase A2 (PLA2) and production of arachidonic acid (AA), which is metabolized to prostaglandins and thromboxanes. The AA metabolites PGF2α and thromboxane A2/B2 maintain activation of G proteins, which activate phospholipases, producing second messengers and resulting in protein kinase C (PKC)-dependent sustained contraction of LES. This metabolic activity can be prevented by the use of PGF2 analogs, such as 8-iso-PGF2α, which itself causes little or no contraction but inhibits contraction of LES in response to PGF2α. The mechanisms for cytokine-induced production of H2O2 in the LES have not been examined in any detail, but it is entirely possible that phenotypic changes, resulting in increased generation of H2O2, may continue to prevent normalization of muscle contraction, even as the mucosa heals.

METHODS

Esophagitis model. 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, they were anesthetized with ketamine hydrochloride (10 mg/kg; Aveco, Fort Dodge, IA), and maintenance doses of ketamine (2.5–5 mg/kg) were administered as needed. To determine LES position, esophageal pressure was measured by a repeated-station pull-through technique, 1–2 mm at a time, with a multilumen catheter having three proximal openings 3 cm apart. The animals were kept on a slant board at a 15° angle during the perfusion to avoid aspiration. A distal perfused Dent sleeve was used to monitor sphincter pressure after the location of the high-pressure zone was established with the perfused side openings, and the three proximal openings measured the amplitude of contraction in the esophageal body. LES pressure was monitored for 15–20 min and recorded before acid perfusion. After a stable LES pressure was recorded and before acid perfusion, the cat received 0.02 mg/kg atropine to prevent excessive secretion and 1.0 mg/kg xylazine im for anesthesia. After a 5- to 10-min period to allow the additional anesthetic to take effect, with the sleeve placed across the LES, the most proximal opening, which was 9 cm proximal to the LES, was used to perfuse 0.1 N HCl in the esophagus at a rate of 1 ml/min for 30–45 min. After perfusion, the cat was injected subcutaneously with 100 ml saline to prevent dehydration and received 0.01 mg/kg buprenorphine subcutaneously to maintain analgesia. After animals were returned to their cages, they were monitored for signs of discomfort and maintained on broth and a soft diet. The animals appeared alert and comfortable and ate normally.

In each experiment, the following two groups of animals were examined: the first group consisted of normal, untreated animals, whereas the second group (animals with esophagitis), after initial measurement of LES pressure, had their esophagus perfused with 0.1 N HCl over three consecutive days. This protocol has been shown to produce inflammatory changes in the esophageal mucosa and concurrent reduction in LES in vivo resting pressure and in vitro spontaneous tone, whereas esophageal perfusion with distilled water had no effect on mucosa appearance or LES resting pressure.

Preparation of LES circular smooth muscle strips and tissue squares. Animals were initially anesthetized with ketamine. LES resting pressure was measured as described, and then the animals were killed with an overdose of phenobarbital (Schering, Kenilworth, NJ). The chest and abdomen were opened with a midline incision, exposing the esophagus and stomach. The esophagus and LES were isolated and excised as previously described (5, 6). The esophagus and stomach were removed together and pinned on a wax block at their in vivo dimensions and orientation. The esophagus and stomach were opened along the lesser curvature. The high-pressure zone is characterized by a visible thickening of the circular muscle layer in correspondence of the squamo-columnar junction and immediately proximal to the sling fibers of the stomach. We have shown previously that a 5- to 8-mm band of tissue, coinciding with the thickened area, constitutes the LES and has distinct characteristics when examined in vivo in the organ bath. The mucosa and submucosal connective tissue were removed by sharp dissection, and the LES was excised. A 5- to 7-mm-wide strip of the junction of LES and esophagus was discarded to avoid overlap. Two-millimeter-wide LES strips were obtained by cutting the tissue with blades held in a metal block at a fixed 2-mm distance to ensure uniform width for all strips. The strips were mounted in separate 1-ml muscle chambers and equilibrated for 2 h with continuous perfusion of oxygenated physiological salt solution (PSS), as previously described in detail (4, 5, 16, 17). During this time, the tension in LES strips increased, attaining a steady level at 2 h (6).

To prepare LES muscle for enzymatic digestion, the circular muscle layer was cut into 0.5-mm-thick slices with a Stadie Riggs tissue slicer (Thomas Scientific Apparatus, Philadelphia, PA), and tissue squares were made by cutting two times with a 2-mm blade block, the second cut at right angles to the first. This circular smooth muscle tissue was also used for Western blot analysis, measurement of H2O2, PGF2α, and 8-iso-F2α levels, and for PGF2α receptor binding.

Measurements of in vitro LES tone. Circular muscle strips (2 mm wide) were mounted in separate 1-ml muscle chambers as previously described in detail (3). They were initially stretched to 2.5 g to bring them near conditions of optimum force development and equilibrated overnight fast, they were anesthetized with ketamine hydrochloride weighing between 3.5 and 5.5 kg were used in this study. After an overnight fast, they were anesthetized with ketamine hydrochloride (10 mg/kg; Aveco, Fort Dodge, IA), and maintenance doses of ketamine (2.5–5 mg/kg) were administered as needed. To determine LES position, esophageal pressure was measured by a repeated-station pull-through technique, 1–2 mm at a time, with a multilumen catheter having three proximal openings 3 cm apart. The animals were kept on a slant board at a 15° angle during the perfusion to avoid aspiration. A distal perfused Dent sleeve was used to monitor sphincter pressure after the location of the high-pressure zone was established with the perfused side openings, and the three proximal openings measured the amplitude of contraction in the esophageal body. LES pressure was monitored for 15–20 min and recorded before acid perfusion. After a stable LES pressure was recorded and before acid perfusion, the cat received 0.02 mg/kg atropine to prevent excessive secretion and 1.0 mg/kg xylazine im for anesthesia. After a 5- to 10-min period to allow the additional anesthetic to take effect, with the sleeve placed across the LES, the most proximal opening, which was 9 cm proximal to the LES, was used to perfuse 0.1 N HCl in the esophagus at a rate of 1 ml/min for 30–45 min. After perfusion, the cat was injected subcutaneously with 100 ml saline to prevent dehydration and received 0.01 mg/kg buprenorphine subcutaneously to maintain analgesia. After animals were returned to their cages, they were monitored for signs of discomfort and maintained on broth and a soft diet. The animals appeared alert and comfortable and ate normally.

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Effect of agonist and antagonists on LES tone. To examine the effect of exogenous prostaglandins and thromboxanes, LES strips were incubated in indomethacin (10−4 M, 30 min) to eliminate production of endogenous prostaglandin and thromboxane. We have previously shown that indomethacin causes a 90% reduction in LES tone (17). After tone had stabilized, cumulative dose responses were
obtained for PGF\(_{2\alpha}\). These autacoids have been shown to participate in maintenance of resting LES tone (16, 17). To examine the effect of isoprostanes on LES response to PGF\(_{2\alpha}\), cumulative concentration-
response relationships were obtained before and after incubation in 8-isop-PGF\(_{2\alpha}\) (10\(^{-5}\) M) for 1.5 h.

**IL-1β Western blot.** Esophageal and LES circular muscle (100 mg) was homogenized in 2 ml PBS (pH 7.4; Sigma, St. Louis, MO) containing: 0.01 M phosphate buffer, 0.0027 M KCl, and 0.137 M NaCl. The suspension was centrifuged at 2,000 g for 20 min. The supernatant was frozen in liquid nitrogen for later use. The supernatant was mixed with SDS loading buffer (Sigma) containing 0.125 M Tris•HCl (pH 6.8), 20% glycerol, 10% 2 mercaptoethanol, 0.04% bromphenol blue, and 10% glycerol and was boiled for 5 min. Prestained molecular weight marker was prepared in the same manner. After these supernatants were boiled, samples were subjected to SDS-PAGE (90 volts, 2 h) using 15% SDS gel. The separated proteins were electrophoretically transferred to a nitrocellulose (NC) membrane (Bio-Rad, Hercules, CA) for 2 h. The transferred proteins were blocked with 5% donkey serum in PBS for 2 h followed by three rinses in serum-free buffer. Samples were incubated with anti-rat IL-1β antibody (1:500; R&D Systems, Minneapolis, MN) for 2 h with shaking followed by three washes with antibody-free PBS with 0.5% Tween. This was followed by a 60-min incubation in horseradish peroxidase-conjugated goat anti-rabbit antibody (1:5,000; Amersham, Arlington Heights, IL). Detection was achieved with an enhanced chemiluminescence agent (Amersham). Molecular weight was estimated by comparison of sample bands with prestained molecular weight marker (Amersham).

**Measurement of H\(_2\)O\(_2\) in smooth muscle cells.** Cells were loaded with dihydrorhodamine (DHR 123; 20 µg/ml, 30 min, 37°C), a probe for measurement of intracellular H\(_2\)O\(_2\) (Cayman Chemical, Ann Arbor, MI). DHR 123 is a nonfluorescent permeable dye that enters the cell, is converted to rhodamine 123, and subsequently localizes in the mitochondria. The oxidation product, rhodamine 123, is excitable at 488 and emits at 515 nm.

**Measurement of H\(_2\)O\(_2\) in smooth muscle tissue.** Esophageal and LES circular smooth muscle (100 mg) was homogenized in PBS buffer. Homogenization consisted of a 20-s burst with a Tissue Tearer (Biospec) followed by 50 strokes with a Dounce tissue grinder (Wheaton). An aliquot of homogenate was taken for protein measurement. The homogenate was centrifuged at 15,000 rpm at 4°C for 15 min. Each sample supernatant (100 µl) was used for protein measurement. Supernatant (2 ml) was used for PGF\(_{2\alpha}\) purification by Affinity column (Cayman Chemical). The resulting extracts were kept at −70°C. The PGF\(_{2\alpha}\) concentration was quantified by using a PGF\(_{2\alpha}\) Competitive Enzyme Immunoassay kit (Cayman Chemical).

**Measurement of H\(_2\)O\(_2\) in smooth muscle tissue**. LES and esophageal circular smooth muscle tissue (100 mg) were homogenized in PGE\(_2\) homogenization buffer [0.1 M phosphate buffer (pH 7.4) containing 1 mM EDTA and 20 µg/ml indomethacin] at 4°C. Homogenization consisted of 10- to 20-s bursts with a Tissue Tearer (Biospec) followed by 40–60 strokes with a Dounce tissue grinder (Wheaton). An aliquot of homogenate was taken for protein measurement. The homogenate was centrifuged at 15,000 rpm at 4°C for 15 min. Each sample supernatant (100 µl) was used for protein measurement. Supernatant (2 ml) was used for PGF\(_{2\alpha}\) purification by Affinity column (Cayman Chemical). The resulting extracts were kept at −70°C. The PGE\(_2\) concentration was quantified by using a PGE\(_2\) Competitive Enzyme Immunoassay kit (Cayman Chemical).
120 min. The reaction was stopped by the addition of 0.9 ml ice-cold stop-buffer, and the tubes were left on ice. A 0.1-ml volume from each tube was counted in a γ-counter to obtain total activity. To separate bound from free radioligand, 300 μl of each sample were filtered utilizing a vacuum filtering manifold (Millipore, Bedford, MA; see Ref. 68). The filters were washed three times with 1 ml ice-cold incubation medium without BSA. The radioactivity on the filter was counted in a gamma scintillation counter. The nonspecific binding was determined with 1 μM unlabeled PGF2α.

Scatchard analysis. We used the ligand-fitting program, based on the displacement curve binding data, to obtain the maximum specific binding capacity and affinity of PGF2α receptors.

Protein determination. The homogenates of LES and esophageal tissues were solubilized by adding 6 ml of 0.1 N NaOH and heating the sample at 80°C for 30 min. The amount of protein present was determined by colorimetric analysis (Bio-Rad) according to the method of Bradford (9).

Materials. The 8-isoprostane affinity column, 8-isoprostane enzyme immunoassay (EIA) kit, PGE2 affinity column, PGE2 EIA kit-monoclonal, 8-isoprostane EIA kit, and 8-iso-PGF2α, were purchased from Cayman Chemical. [5,6,8,9,11,12,14,15(n)-3H] PGF2α was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Bioxytech H2O2-560 quantitative H2O2 assay was purchased from Oxis International. PGE2 and PGF2α were purchased from Biomol (Plymouth Meeting, PA). Human IL-1β was purchased from Pierce Endogen (Rockford, IL).

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.

RESULTS

LES tone in esophagitis. We confirmed, as previously shown (2), that induction of experimental esophagitis in the cat by esophageal perfusion with HCl results in a significant reduction of in vivo resting LES pressure and of in vitro spontaneous LES tone. LES tone decreased from 4.73 ± 0.4 g in control strips to 0.61 ± 0.1 g after induction of AE (P < 0.001, unpaired t-test).

IL-1β and H2O2 in esophagitis. To establish a possible role of IL-1β in esophagitis, we used Western blot analysis to measure IL-1β in LES circular smooth muscle from normal cats and after the induction of experimental esophagitis. Figure 1 shows that IL-1β levels were 15-fold higher in LES circular muscle from esophagitis cats when compared with normal cats (P < 0.001, unpaired t-test). We therefore examined the effect of IL-1β on in vitro tone of LES muscle strips. IL-1β concentration-dependently reduced LES resting tone in normal LES strips (P < 0.001, ANOVA), and the reduction was reversed or reduced by the H2O2 scavenger catalase (78 U/ml; Fig. 2).

Similarly, incubation of LES strips from AE cats with catalase (1,000 U/ml, 30 min) significantly increased tone from 0.61 ± 0.1 g to 2.94 ± 0.6 g (Fig. 3, P < 0.05, unpaired t-test). These data suggest that IL-1β reduces tone via the production of H2O2. This view is supported by the findings that H2O2 levels are significantly elevated in esophagitis LES circular smooth muscle, and IL-1β treatment of normal LES significantly increased production of H2O2 (P < 0.01, ANOVA; Fig. 4).

Acute treatment of normal LES circular smooth muscle with IL-1β caused an increase in H2O2 levels from 2.0 ± 0.2 nmol/mg in untreated muscle to 5.1 ± 1.2 nmol/mg after IL-1β (1,000 U/ml, 2 h) that was comparable with the H2O2 levels (4.3 ± 0.6 nmol/mg protein) found in esophagitis muscle.

To confirm that IL-1β and esophagitis enhance production of H2O2 in LES muscle cells, we examined H2O2 levels in isolated circular smooth muscle cells from normal and esophagitis LES. DHR 123 was used as a probe for measurement of intracellular H2O2 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 that is excitable at 488 nm and detectable at 515 nm under confocal microscopy. Rhodamine 123 becomes localized in the cytoplasm, where some H2O2 is present, and in the mitochondria, where oxygen radicals are produced as part of the normal respiratory process and are present at a higher concentration than in the cytoplasm. Figure 5 shows H2O2 levels in normal LES cell before and after exposure to IL-1β and H2O2 and after induction of esophagitis. Figure 5 shows elevated H2O2 levels in the muscle cells after exposure to IL-1β and H2O2 and after induction of

Cat LES Circular Smooth Muscle
IL-1β Western Blot

17kDa

Control Negative Positive Normal Esophagitis

Density

0 0.01 0.02 0.03 0.04 0.05 0.06 0.07

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esophagitis. It is noteworthy that, because H$_2$O$_2$ is membrane permeable, H$_2$O$_2$ levels are elevated everywhere in the cell, including in the nucleus.

Because H$_2$O$_2$ is known to alter membrane phospholipids (10, 65), we examined its effect on production of prostaglandins in normal LES circular muscle.

**H$_2$O$_2$ prostaglandins and isoprostanes in esophagitis.** We have previously shown that resting or basal LES tone depends on AA production by a secreted PLA$_2$ and production of the AA metabolites PGF$_{2\alpha}$ and thromboxane A$_2$, which contract LES circular smooth muscle. Incubation of normal LES tissues with IL-1$\beta$ (200 U/ml) or with H$_2$O$_2$ (70 $\mu$M) for 2 h (Fig. 6) significantly increased PGE$_2$ levels ($P < 0.01$, ANOVA) in treated muscle, and IL-1$\beta$-induced production of PGE$_2$ was reduced by catalase, indicating that the production of PGE$_2$ depends on formation of H$_2$O$_2$. Increases in PGE$_2$ by lower (30 –50 $\mu$M) H$_2$O$_2$ concentrations were not statistically significant. PGE$_2$ has been previously shown to decrease LES tone (24, 29, 31), and this was confirmed in the present investigation. PGE$_2$ dose dependently reduced LES tone (from 5% at 10$^{-7}$ to 50% at 10$^{-5}$). After induction of AE, PGE$_2$ levels were significantly elevated compared with normal LES ($P < 0.01$, ANOVA), as shown in Fig. 6, but PGF$_{2\alpha}$ levels did not change (Fig. 7), suggesting that altered prostaglandin production in esophagitis may result in excess production of the relaxant prostaglandin PGE$_2$, which is not balanced by a corresponding increase in production of the contractile prostaglandin PGF$_{2\alpha}$.

Lipid peroxidation of membrane phospholipids by H$_2$O$_2$ may increase the production of isoprostanes (48), which are stable prostaglandin analogs formed in situ on the cell membrane by free radical-induced peroxidation of AA. Incubation of normal LES circular muscle with IL-1$\beta$ (200 U/ml) or H$_2$O$_2$ (30 –70 $\mu$M) for 2 h caused significant increases in F$_2$ isoprostane levels ($P < 0.01$, ANOVA; Fig. 8), and IL-1$\beta$-induced production of F$_2$ isoprostane was reduced by catalase, indicating that the production of F$_2$ isoprostane depends on formation of H$_2$O$_2$. In addition, F$_2$ isoprostane levels were elevated in LES circular muscle from esophagitis animals.
compared with normal muscle ($P < 0.001$, ANOVA; Fig. 8). Figure 8 shows that F2 isoprostane levels increased in IL-1$\beta$- or H$_2$O$_2$-treated muscle to levels that were comparable to the F2 isoprostane levels in esophagitis LES.

To test for a possible interaction between PGF$_{2\alpha}$ and F2 isoprostane affecting LES tone, we examined LES circular muscle strips incubated with indomethacin (10$^{-5}$ M) to eliminate any contribution of endogenous prostaglandins and thromboxanes. Indomethacin abolished LES tone and, as previously reported (16) and shown in Fig. 9, PGF$_{2\alpha}$ caused a concentration-dependent contraction of LES circular muscle strips. In contrast, 8-iso-PGF$_{2\alpha}$ alone had little effect on tone of normal LES circular muscle, but preincubation with 8-iso-PGF$_{2\alpha}$ (10$^{-5}$ M) almost abolished PGF$_{2\alpha}$-induced contraction (Fig. 9). In the presence of 8-iso-PGF$_{2\alpha}$, PGF$_{2\alpha}$-induced contraction was significantly lower at each concentration examined ($P < 0.001$, ANOVA), but the isoprostane did not affect ACh-induced contraction. These data suggest that 8-iso-PGF$_{2\alpha}$ may selectively inhibit PGF$_{2\alpha}$ binding to its receptors because of structural similarities of these two AA-derived autacoids.

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Fig. 5. Confocal microscopic images of circular LES smooth muscle cells. To detect H$_2$O$_2$, cells were loaded with dihydrorhodamine (DHR 123), a nonfluorescent membrane-permeable dye that enters the cell and, upon oxidation, is converted to rhodamine 123. The oxidation product is excitable at 488 nm and is detected at 515 nm. Untreated, DHR 123-loaded LES cells, such as the one in the top left (normal), are barely detectable. H$_2$O$_2$ content increases in cells pretreated with IL-1$\beta$ (100 U/ml for 2 h; top right) and in cells isolated from esophagitis animals (bottom left). Bottom right: positive control showing cells incubated in a high concentration of H$_2$O$_2$ (10$^{-5}$ M, 30 s), which is membrane permeable and diffuses inside the cells.

Fig. 6. IL-1$\beta$ and H$_2$O$_2$ increase PGE$_2$ production in LES. PGE$_2$ levels were measured in untreated LES circular smooth muscle and in muscle treated with IL-1$\beta$ (200 U/ml) or H$_2$O$_2$ (70 $\mu$M) for 2 h. PGE$_2$ was purified using a PGE$_2$ purification column and then measured by enzyme immunoassay. PGE$_2$ production was increased after IL-1$\beta$ or H$_2$O$_2$ treatment and after induction of experimental esophagitis ($P < 0.01$, ANOVA). IL-1$\beta$-induced production of PGE$_2$ was reduced by catalase, indicating that IL-1$\beta$-induced production of PGE$_2$ depends on formation of H$_2$O$_2$. Values are means ± SE for 3 cats for H$_2$O$_2$-treated LES and 6 cats for control and esophagitis cats.

Fig. 7. PGF$_{2\alpha}$ levels were measured in LES circular smooth muscle from normal cats and in muscle from esophagitis cats. PGF$_{2\alpha}$ was purified using a Sep-Pak C$_{18}$ reverse-phase cartridge and then measured by enzyme immunoassay. Values are means ± SE for 3 cats.
PGF$_{2\alpha}$ binding was therefore examined. Figure 10 shows that PGF$_{2\alpha}$ binding was significantly inhibited by preincubation of normal LES circular muscle with 8-iso-PGF$_{2\alpha}$ (10$^{-5}$ M).

To confirm a functional role of isoprostanes in reducing PGF$_{2\alpha}$-mediated tone in esophagitis, we examined PGF$_{2\alpha}$-induced contraction of esophagitis LES after incubation in indomethacin to eliminate contribution of endogenous prostaglandins and thromboxanes. PGF$_{2\alpha}$-induced contraction was almost abolished in esophagitis LES ($P<0.001$, ANOVA), but ACh-induced contraction was unchanged (Fig. 11) and similar to ACh-induced contraction of normal LES. To confirm that the reduction in PGF$_{2\alpha}$-induced contraction was because of inhibition of PGF$_{2\alpha}$ binding to its receptors, PGF$_{2\alpha}$ binding was examined. Figure 12 shows that PGF$_{2\alpha}$ binding was significantly inhibited in esophagitis LES compared with normal LES circular muscle.

DISCUSSION

Reflux-related inflammation affects esophageal and LES smooth muscle function, but little attention has been given to inflammation and inflammatory mediators and to inflammation-induced mechanisms responsible for impairment of esophageal and LES smooth muscle function. Many substances considered critical to reflux esophagitis are classical inflam-
Sharif et al. (56).

inhibits contraction of LES in response to endogenous PGF2α increasing the production of PGE2, which relaxes the LES, and smooth muscle layer of the LES. H2O2 may have a variety of mechanisms responsible for release of excitatory neurotransmitters (12). Namely, incubation of normal esophageal circular muscle with IL-1β releases the LES, and may be responsible for delayed recovery of muscle function, even as the mucosa is healing.

In a cat model of acute esophagitis, we show that inflammation induced by acid affects the maintenance of tone by producing elevated levels of IL-1β or IL-6 are produced in the mucosa in response to inflammation and diffuse to the circular muscle layer (18), causing overproduction of H2O2. H2O2 is known to cause lipid peroxidation, release of calcium from intracellular stores, and, in addition, may diffuse to the nucleus, altering protein expression in the cell. Overproduction of oxygen radicals may be a feature of chronic inflammation, since it occurs in esophagitis (19) or ulcerative colitis (15, 64), and may be responsible for delayed recovery of muscle function, even as the mucosa is healing.

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We have separately reported that the inflammatory cytokines IL-1β and IL-6 may mediate some of the changes in esophageal contraction in esophagitis by altering cholinergic neural mechanisms responsible for release of excitatory neurotransmitters (12). Namely, incubation of normal esophageal circular muscle with IL-1β (or IL-6) caused a significant reduction in contraction in response to electrical stimulation by inhibiting release of the excitatory neurotransmitter ACh in response to electrical stimulation but had no effect on ACh-induced contraction. This alteration in esophageal contractility is similar to the changes observed in our model of acute experimental esophagitis, where contraction in response to electrical stimulation is reduced, but ACh-induced contraction is unaltered (12).

IL-1β may also play a role in esagitis-induced motor dysfunction of LES because it was present in elevated concentrations in esophagitis LES compared with normal LES, and it produced a concentration-dependent reduction in tone of in vitro LES circular muscle strips. This reduction was reversed by catalase, suggesting that IL-1β reduces tone via the production of H2O2. This view is supported by the findings that IL-1β treatment of normal LES increased production of H2O2.

Inhibiting production of H2O2 with an IL-1β antagonist or antibody would show a direct sequential link between IL-1β and H2O2. This experiment was unsuccessfully attempted in a recent publication on sigmoid circular muscle from ulcerative colitis patients (14), who exhibit elevated levels of IL-1β and reduced contraction in response to agonists such as neurokinin A and caffeine, agents that act by causing Ca2+ release from intracellular stores. In the colon, an IL-1 antagonist, however, did not restore caffeine-induced cell shortening and Ca2+ release. A likely reason for these results is that inflammatory mediators produced in response to IL-1β are present in the colon and affect its motor function even after IL-1β is neutralized. In LES circular muscle, we show that in normal tissue IL-1β, similarly to H2O2, causes an increase in both PGE2 and 8-iso-prostaglandins, that IL-1β-induced production of H2O2 and isoprostanes is reduced by the H2O2 scavenger catalase, and that catalase restores the reduction in tone induced by both IL-1β and esophagitis. These data indicate that production of these relaxants in esophagitis and in IL-1β-treated muscle depends on formation of H2O2 and support a role of H2O2 in cytokine- and esophagitis-induced changes.

These findings suggest that the mechanism responsible for reduction in tone in esophagitis may depend on overproduction of H2O2 in response to cytokines. This hypothesis is consistent with the presence of increased levels of H2O2 in esophagitis LES when compared with control LES smooth muscle and with the finding that the reduced in vitro tone in esophagitis LES muscle strips is reversed by catalase.

ROS, such as radical anions, singlet oxygen, H2O2, hydroxyl radical, and hypochlorous acid, are normally produced as byproducts of oxidative metabolism. However, their production is enhanced during inflammation, aging, radiation exposure, endotoxic shock, and ischemia-reperfusion, perhaps by upregulation of cytosolic enzymes similar to those present in neutrophils (7, 8, 11, 32, 34, 58, 59). H2O2 is physiologically produced in large amounts by cells such as granulocytes and has been widely used to assess the effects of ROS (40).

Acid-induced injury to esophageal epithelial cells causes an inflammatory response with influx of neutrophils, polymorphonuclear leukocytes, and macrophages and, possibly, activation of resident macrophages. Activated neutrophils and polymorphonuclear leukocytes bind to target cells and release granule enzymes (elastase and others), superoxide anion, and H2O2 (62, 63). In addition, ROS production may depend on the interaction of immune cells with target cells, causing production of some inflammatory mediators by the target cells themselves. Our measurement of H2O2 in the LES circular smooth muscle layer demonstrates that production of H2O2 increases significantly in esophagitis, as shown in Fig. 4. Measurement of H2O2 in the circular muscle layer of esophagitis LES does not distinguish between H2O2 produced by inflammatory cells and H2O2 produced by the muscle cells themselves. Figure 5, however, clearly demonstrates the presence of high levels of H2O2 in muscle cells after exposure of the isolated cells to IL-1β, suggesting that some inflammatory mediators may induce overproduction of H2O2 by the muscle cells themselves.
This or a related mechanism may explain why LES and esophageal motor dysfunction may persist even after inflammation is reduced by neutralizing or eliminating acid production. It is possible that muscle cell phenotype may be changed in inflammation, resulting in continued overproduction of H$_2$O$_2$ by LES muscle, which may contribute to reducing LES tone, even in the absence of inflammatory cell infiltrate in proximity of the circular muscle layer.

The mechanisms of ROS action at the cellular level are not well understood, but pharmacological identification of the ROS effects may be achieved by examining the modulatory effects of specific scavengers for H$_2$O$_2$, such as catalase. H$_2$O$_2$ diffuses across biological membranes (61) and may be neutralized by extracellular scavengers, such as catalase, that do not cross the cell membrane.

We have previously reported that LES tone depends on the activity of a group I PLA$_2$ (13, 16) that produces AA and AA metabolites. The AA metabolites PGF$_2$α and thromboxane A$_2$/B$_2$ bind to their respective receptors activating G$_q$ and Gi$_3$, which are coupled to phosphatidylycholine-specific phospholipase C and phosphatidylinositol-specific phospholipase C. Activation of these phospholipases results in the generation of second messengers that activate PKC and maintain a sustained PKC-dependent contraction of LES circular muscle. Because H$_2$O$_2$ levels are elevated and LES tone is reduced after the induction of AE, we examined whether H$_2$O$_2$ reduced tone by altering AA metabolism.

A mechanism for H$_2$O$_2$-induced reduction in tone is through production of PGE$_2$. We show that H$_2$O$_2$ increases production of PGE$_2$ in normal LES circular smooth muscle and that PGE$_2$ levels are significantly higher in esophagitis than in normal LES. PGE$_2$ is known to relax LES smooth muscle and reduce LES tone (23), and these results have been confirmed in our study. A similar pathway for IL-1β-induced formation of PGE$_2$ has been elucidated in human neuroblastoma cells in which IL-1β induces PGE$_2$ synthesis through two synergistic pathways, one involving ROS, nuclear factor-κB, and expression of cycloxygenase (COX)-2 and the other involving p38 MAPK to stabilize COX-2 mRNA (28).

In contrast, levels of PGF$_2$α, which contributes to maintaining tone, were unchanged after induction of AE. The effectiveness of endogenous PGF$_2$α, however, may be reduced by the presence of elevated levels of isoprostanes found in AE LES circular smooth muscle.

ROS have high affinity for lipids, causing lipid peroxidation (10, 65). Lipid peroxidation alters membrane function by modifying phospholipid pools or by altering membrane-bound enzymes (33, 35, 37, 38). Free radical-induced peroxidation of AA (45–47) results in formation of isoprostanes, which are stable prostaglandin-like compounds that are formed enzymatically (53) and nonenzymatically in vivo. F$_2$ isoprostanes are stable analogs of PGF$_2$α that are formed in situ on the cell membrane. Unlike primary prostaglandins, which act locally and are rapidly metabolized to inactive compounds, isoprostanes may be cleaved from peroxidized membrane phospholipids by phospholipases and continue to circulate (52) until they are finally excreted in urine (45). Measurement of isoprostanes in affected tissues and in urine is considered a reliable index of oxidative stress in vivo (48, 49).

F$_2$ isoprostanes may bind with varying affinity to PGF$_2$α receptors (41) and act as weak agonists (42) and may bind to specific isoprostane receptors (69). Because isoprostanes are not metabolized, prolonged exposure to isoprostanes may eventually cause desensitization or sequestration of PGF$_2$α receptors (41, 42); thus, they are not expected to work as competitive antagonists.

We report that normal LES muscle strips do not contract in response to 8-iso-PGF$_2$α and that 8-iso-PGF$_2$α selectively inhibits PGF$_2$α-induced contraction of normal LES strips, without affecting ACh-induced contraction. Similarly, LES muscle strips from esophagitis cats do not contract in response to PGF$_2$α but contract normally to ACh. These data suggest that elevated levels of F$_2$ isoprostanes in esophagitis LES reduce contraction by binding to PGF$_2$α receptors and blocking the effect of endogenous PGF$_2$α, perhaps by causing receptor internalization and reducing the number of receptors available for activation by PGF$_2$α. This view is supported by the finding of very low initial binding in the presence of 8-iso-PGF$_2$α and in esophagitis samples, which clearly suggests that a reduced number of receptors may be present in both of these conditions. Thus a mechanism responsible for reduced tone in esophagitis may be 8-iso-PGF$_2$α-induced receptor internalization, which will prevent tone development by spontaneously produced PGF$_2$α.

We conclude that, in a cat model of acute esophagitis, acid-induced inflammation affects the maintenance of tone by producing elevated levels of H$_2$O$_2$ in the circular smooth muscle layer of the LES. H$_2$O$_2$ alters the AA metabolism by increasing the production of PGE$_2$, which relaxes the LES, and of 8-iso-PGF$_2$α, which by itself causes little or no contraction, but inhibits LES response to PGF$_2$α, which otherwise contributes to maintenance of resting myogenic LES tone.

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**REFERENCES**


