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

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Cheng, Ling, Weibiao Cao, Jose Behar, Piero Bianciani, and Karen M. Harnett. Inflammation induced changes in arachidonic acid metabolism in cat LES circular muscle. Am J Physiol Gastrointest Liver Physiol 288: G787–G797, 2005. First published November 18, 2004; doi:10.1152/ajpgi.00327.2004.—Myogenic lower esophageal sphincter (LES) tone is maintained by arachidonic acid metabolites, such as PGF2α, and thromboxane A2/B2. Experimental esophagitis in cat reduces LES in vivo pressure and in vitro tone. Because IL-1β may mediate esophagitis-associated reduction in ACh release in esophagus, we examined whether IL-1β may also play a role in esophagitis-induced reduction of LES tone. A cat model of experimental esophagitis was obtained by repeated esophageal perfusion with HCl (Bianciani P, Barwick K, Selling J, and McCallum R. Gastroenterology 87: 8–16, 1984 and Sohn UD, Harnett KM, Cao W, Rich H, Kim N, Behar J, and Bianciani P. J Pharmacol Exp Ther 283: 1293–1304, 1997.). LES circular muscle strips were examined in muscle chambers as previously described (Bianciani P, Billett G, Hillemeier C, Nissenshon M, Rhim BY, Sweczack S, and Behar J. Gastroenterology 103: 1199–1206, 1992). Levels of inflammatory mediators were measured. IL-1β levels were higher in esophagitis than in normal LES. IL-1β reduced normal LES tone, and the reduction was reversed by catalase, suggesting a role of H2O2. This was confirmed by IL-1β-induced production of H2O2 in normal LES and elevated H2O2 levels in esophagitis. H2O2 by itself is sufficient to explain the changes that occur in the muscle, reducing its ability to contract. H2O2 increased PGF2α in normal LES, and PGF2α levels were elevated in esophagitis LES, whereas PGF2α levels were unchanged. H2O2 also increased levels of 8-isoprostanes, stable prostaglandin-like compounds formed by free radical-induced peroxidation of arachidonic acid, and 8-isoprostane levels were elevated in esophagitis. The PGF2α analog 8-iso-PGF2α caused little contraction of LES strips but reduced PGF2α binding and contraction of normal LES. In esophagitis, PGF2α binding and contraction were reduced in LES, suggesting that isoprostanes may contribute to reduction in tone in esophagitis. The data suggest that, in esophagitis, IL-1β causes production of H2O2. H2O2 increases PGF2α, which relaxes the LES, and 8-iso-F2α, which blocks PGF2α-mediated contraction.

smooth muscle; contraction; tone; signal transduction; hydrogen peroxide; cytokines; lower esophageal sphincter

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 circular smooth muscle, resulting in maintenance of LES tone (12, 13, 17).

We have shown that, in a cat model of acute experimental esophagitis, repeated perfusion of esophagus with 0.1 N hydrochloric acid causes a reduction in resting in vivo LES pressure and in vitro spontaneous LES tone (2). In the current study, we report that acid-induced injury to the esophagus reduces tone by production of inflammation-induced AA metabolites in the LES. The circular smooth muscle layer of acute esophagitis (AE) cats contains elevated levels of inflammatory mediators, such as interleukin-1β (IL-1β), which increase the production of the ROS H2O2. H2O2 increases the production of mediators, such as interleukin-1β (IL-1β), which increase the production of 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 the 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 3- to 5-mm-wide strip at 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-PGF2α 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 for 2 h while perfused continuously with oxygenated PSS at 37°C. During this time, the tension in LES strips increased, attaining a steady level at 2 h. The PSS contained the following (in mmol/l): 116.6 NaCl, 21.9 NaHCO3, 1.2 NaH2PO4, 3.4 KCl, 1.5 CaCl2, 5.4 glucose, and 1.2 MgCl2. The solution was equilibrated with a gas mixture containing 95% O2 and 5% CO2 at 37°C, pH 7.4.

After equilibration, LES strips were stimulated with square wave pulses of supramaximal voltage, 0.2 mS, 4 Hz, 8-s trains, delivered by a stimulator (model S48; Grass Instruments) through platinum wire electrodes placed longitudinally on either side of the strip. Electrical stimulation was used to further document the nature of the strips. LES strips relaxed during the stimulus train, whereas esophageal strips contracted after the stimulus train ended.

Smooth muscle tension was recorded on a chart recorder (Grass Instruments, Quincy, MA). Passive force was obtained at the end of the experiment by completely relaxing the strips with excess EDTA until no further decrease in resting force was observed. Basal LES tone is the difference between resting and passive force. Percent increase in basal tone was defined by the ratio between the increase in force after drug administration and basal LES tone. Percent basal LES tone was calculated by the ratio between the force after using the drugs and the basal LES tone.

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-isopGF$_{2\alpha}$ (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 electrophotographically transferred to a nitrocellulose (NC) membrane (Bio-Rad, Melville, NY) at 100 volts for 1 h. Transfer of proteins to the NC membrane was confirmed by comparison of sample bands with prestained molecular weight marker (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 dihydorhodamine (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. DHR 123-loaded LES cells were incubated with HEPES buffer alone or HEPES containing IL-1β antibody (100 U/ml) for 2 h. When H$_2$O$_2$ was used, cells were incubated for 30 s in HEPES containing H$_2$O$_2$ (10$^{-5}$ M). The cells were fixed by adding acrolein for a final concentration of 1%. Confocal images of the isolated cells were acquired with a Nikon PCM 2000 (Nikon, Melville, NY) using argon (488) and green helium-neon (543) lasers. Serial optical sections were performed with Simple 32, C-imaging computer software (Compix, Cranberry Township, PA). Sections parallel to the cell longitudinal axis were collected with a Dino-Lite EZ USB microscope (Coollight, Caboolture, Queensland, Australia). The resulting images were processed and reconstructed using NIH Image shareware (National Institutes of Health, Springfield, VA).

**Measurement of H$_2$O$_2$ in smooth muscle tissue.** LES and esophageal 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). The aliquots were 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. The resulting extracts were kept at −70°C. The 8-isoprostane concentration in the sample was quantified using an 8-Isoprostane Enzyme Immunoassay kit (Cayman Chemical). This assay is based on the competition between 8-isoprostane and an 8-isoprostane-acetylcholinesterase conjugate (8-isoprostane tracer) for a limited number of 8-isoprostane-specific rabbit antiseraum binding sites. The concentration of the 8-isoprostane tracer was held constant, whereas the concentration of 8-isoprostane varied. The amount of 8-isoprostane tracer that is bound to the rabbit antisem will be inversely proportional to the concentration of 8-isoprostane in the well.

**PGF$_{2\alpha}$ measurement.** LES smooth muscle squares (150 mg) were homogenized in 2.5 ml buffer solution on ice with three 10- to 20-s bursts with a Tissue Tearer (Biospec), followed by 50 strokes with a Dounce homogenizer (Wheaton). The homogenized buffer solution was 0.1 M phosphate buffer, pH 7.4, containing 1 mM EDTA, 10 μM indomethacin (catalog no. 70270; Cayman), and 0.005% butylated hydroxytoluene (Sigma). The samples were centrifuged at 1,500 g for 15 min at 40°C. Protein content was measured in 100 μl of the supernatant.

Supernatant from each sample (2 ml) was passed through an 8-isoprostane affinity column. The affinity column was prewashed with a buffer consisting of 13.3 g K$_2$HPO$_4$, 3.22 g KH$_2$PO$_4$, 0.5 g NaN$_3$, and 29.2 g NaCl in 1.0 liter ultrapure water. The column was washed with 5 ml elution solution consisting of 95% absolute ethanol and 5% ultrapure water. The effluent was collected and brought to dryness by a stream of nitrogen. The resulting extracts were kept at −70°C.

The 8-isoprostane concentration in the sample was quantified using an 8-Isoprostane Enzyme Immunoassay kit (Cayman Chemical). This assay is based on the competition between 8-isoprostane and an 8-isoprostane-acetylcholinesterase conjugate (8-isoprostane tracer) for a limited number of 8-isoprostane-specific rabbit antiserum binding sites. The concentration of the 8-isoprostane tracer was held constant, whereas the concentration of 8-isoprostane varied. The amount of 8-isoprostane tracer that is bound to the rabbit antisem will be inversely proportional to the concentration of 8-isoprostane in the well.

**Measurement of PGE$_2$.** LES and esophageal smooth circular 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. PGE$_2$ was extracted two times with 3 volumes of ethyl acetate according to the method of Saksema and Harper (54a) as follows. Fractions of ethyl acetate were pooled, washed with 5 ml distilled water, and brought to dryness by a stream of nitrogen. The resulting extracts were kept at −70°C. Samples were redissolved in ethanol and purified by passage over a Sep-Pak C$_18$ reverse-phase cartridge (17). The PGE$_2$ concentration was quantified by using a PGE$_2$ Enzyme Immunoassay kit (Cayman Chemical).

**Measurement of PGF$_{2\alpha}$.** LES and esophageal smooth circular 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). 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 PGE$_2$ 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).

**PGF$_{2\alpha}$ receptor ligand binding assay.** LES circular smooth muscle was homogenized in Krebs buffer (pH 7.4, 20 mg tissue/1 ml buffer) 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). 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 PGE$_2$ 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).

8-Isoprostane measurement. LES smooth muscle squares (150 mg) were homogenized in 2.5 ml buffer solution on ice with three 10- to 20-s bursts with a Tissue Tearer (Biospec), followed by 50 strokes with a Dounce homogenizer (Wheaton). The homogenized buffer solution was 0.1 M phosphate buffer, pH 7.4, containing 1 mM EDTA, 10 μM indomethacin (catalog no. 70270; Cayman), and 0.005% butylated hydroxytoluene (Sigma). The samples were centrifuged at 1,500 g for 15 min at 40°C. Protein content was measured in 100 μl of the supernatant.
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 Scheffe’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...
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.

**LES tone strips**

![LES tone strips](image)

**LES Circular Muscle**

![LES Circular Muscle](image)

**Fig. 2.** IL-1$\beta$ decreases LES tone. LES circular muscle strips from control cats were mounted in muscle chambers. After equilibration for 2 h, LES strips maintained a stable resting tone. Strips were then incubated at the indicated concentration of IL-1$\beta$ until tone was stable, and a cumulative dose-response relationship was obtained. IL-1$\beta$ concentration dependently decreased LES tone ($P < 0.001$, ANOVA). The decrease in tone induced by IL-1$\beta$ was reversed by pretreatment of strips with catalase (78 U/ml, 15 min). Values are means ± SE for 3 cats.

**Fig. 3.** LES circular muscle strips were mounted in muscle chambers, stretched to 2.5 g to bring them near conditions of optimum force development, and equilibrated for 2 h while perfused continuously with oxygenated physiological salt solution (PSS) at 37°C. During this time, the tension in LES strips increased, attaining a steady level at 2 h. An acute model of experimental esophagitis was obtained by esophageal perfusion with 0.1 N HCl for 45 min on three consecutive days, with experiments carried out on the 4th day. Treatment of normal LES circular smooth muscle with IL-1$\beta$ (1,000 U/ml, 2 h) significantly increased H$_2$O$_2$ levels compared with untreated muscle. After the induction of esophagitis, H$_2$O$_2$ levels were significantly higher in LES muscle compared with normal muscle ($P < 0.01$, ANOVA) and similar to those produced after exposure to IL-1$\beta$. Values are means ± SE for 6 control and esophagitis cats and 3 IL-1$\beta$-treated cats.
compared with normal muscle (P < 0.001, ANOVA; Fig. 8). Figure 8 shows that F2 isoprostane levels increased in IL-1β- or H2O2-treated muscle to levels that were comparable to the F2 isoprostane levels in esophagitis LES.

To test for a possible interaction between PGF2α 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, PGF2α caused a concentration-dependent contraction of LES circular muscle strips. In contrast, 8-iso-PGF2α alone had little effect on tone of normal LES circular muscle, but preincubation with 8-iso-PGF2α (10⁻⁵ M) almost abolished PGF2α-induced contraction (Fig. 9). In the presence of 8-iso-PGF2α, PGF2α-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-PGF2α may selectively inhibit PGF2α binding to its receptors because of structural similarities of these two AA-derived autacoids.

Fig. 5. Confocal microscopic images of circular LES smooth muscle cells. To detect H2O2, 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 and is detected at 515 nm. Untreated, DHR 123-loaded LES cells, such as the one in the top left (normal), are barely detectable. H2O2 content increases in cells pretreated with IL-1β (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 H2O2 (10⁻³ M, 30 s), which is membrane permeable and diffuses inside the cells.

Fig. 6. IL-1β and H2O2 increase PGE2 production in LES. PGE2 levels were measured in untreated LES circular smooth muscle and in muscle treated with IL-1β (200 U/ml) or H2O2 (70 μM) for 2 h. PGE2 was purified using a PGE2 purification column and then measured by enzyme immunoassay. PGE2 production was increased after IL-1β or H2O2 treatment and after induction of experimental esophagitis (P < 0.01, ANOVA). IL-1β-induced production of PGE2 was reduced by catalase, indicating that IL-1β-induced production of PGE2 depends on formation of H2O2. Values are means ± SE for 3 cats for H2O2-treated LES and 6 cats for control and esophagitis cats.

Fig. 7. PGF2α levels were measured in LES circular smooth muscle from normal cats and in muscle from esophagitis cats. PGF2α was purified using a Sep-Pak C18 reverse-phase cartridge and then measured by enzyme immunoassay. Values are means ± SE for 3 cats.
PGF\textsubscript{2\alpha} binding was therefore examined. Figure 10 shows that PGF\textsubscript{2\alpha} binding was significantly inhibited by preincubation of normal LES circular muscle with 8-iso-PGF\textsubscript{2\alpha} (10\textsuperscript{-5} M).

To confirm a functional role of isoprostanes in reducing PGF\textsubscript{2\alpha}-mediated tone in esophagitis, we examined PGF\textsubscript{2\alpha}-induced contraction of esophagitis LES after incubation in indomethacin to eliminate contribution of endogenous prostaglandins and thromboxanes. PGF\textsubscript{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\textsubscript{2\alpha}-induced contraction was because of inhibition of PGF\textsubscript{2\alpha} binding to its receptors, PGF\textsubscript{2\alpha} binding was examined. Figure 12 shows that PGF\textsubscript{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).

**PGF$_{2a}$ binding in Esophagitis LES**

(n=3)

![Graph](image)

**Fig. 12.** PGF$_{2a}$ binding is reduced in LES circular smooth muscle from esophagitis cats. PGF$_{2a}$ binding was examined according to the method of Sharif et al. (56).

matory products, such as prostanoids, ROS (51, 67), and proinflammatory cytokines like IL-1$\beta$, which are thought to derive from inflammatory cells infiltrating acid-damaged tissue (50). Preliminary evidence suggests that proinflammatory cytokines (such as IL-1$\beta$ or IL-6) are produced in the mucosa in response to inflammation and diffuse to the circular muscle layer (18), causing overproduction of H$_2$O$_2$. H$_2$O$_2$ 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.

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-1$\beta$ and H$_2$O$_2$ in the circular smooth muscle layer of the LES. H$_2$O$_2$ may have a variety of effects on a number of cellular mechanisms in diverse cell species (40). In the LES, H$_2$O$_2$ alters AA metabolism by increasing the production of PGE$_2$, which relaxes the LES, and of 8-iso-F$_{2alpha}$, which by itself causes little or no contraction but inhibits contraction of LES in response to endogenous PGF$_{2alpha}$.

We have separately reported that the inflammatory cytokines IL-1$\beta$ 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-1$\beta$ (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-1$\beta$ may also play a role in esophagitis-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-1$\beta$ reduces tone via the production of H$_2$O$_2$. This view is supported by the findings that IL-1$\beta$ treatment of normal LES increased production of H$_2$O$_2$.

Inhibiting production of H$_2$O$_2$ with an IL-1$\beta$ antagonist or antibody would show a direct sequential link between IL-1$\beta$ and H$_2$O$_2$. This experiment was unsuccessfully attempted in a recent publication on sigmoidal muscle from ulcerative colitis patients (14), who exhibit elevated levels of IL-1$\beta$ and reduced contraction in response to agonists such as neurokinin A and caffeine, agents that act by causing Ca$^{2+}$ release from intracellular stores. In the colon, an IL-1$\beta$ antagonist, however, did not restore caffeine-induced cell shortening and Ca$^{2+}$ release. A likely reason for these results is that inflammatory mediators produced in response to IL-1$\beta$ are present in the colon and affect its motor function even after IL-1$\beta$ is neutralized. In LES circular muscle, we show that in normal tissue IL-1$\beta$, similarly to H$_2$O$_2$, causes an increase in both PGE$_2$ and isoprostanes, that IL-1$\beta$-induced production of PGE$_2$ and isoprostanes is reduced by the H$_2$O$_2$ scavenger catalase, and that catalase restores the reduction in tone induced by both IL-1$\beta$ and esophagitis. These data indicate that production of these relaxants in esophagitis and in IL-1$\beta$-treated muscle depends on formation of H$_2$O$_2$ and support a role of H$_2$O$_2$ in cytokine- and in esophagitis-induced changes.

These findings suggest that the mechanism responsible for reduction in tone in esophagitis may depend on overproduction of H$_2$O$_2$ in response to cytokines. This hypothesis is consistent with the presence of increased levels of H$_2$O$_2$ 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, H$_2$O$_2$, 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). H$_2$O$_2$ 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 H$_2$O$_2$. 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 H$_2$O$_2$ in the LES circular smooth muscle layer demonstrates that production of H$_2$O$_2$ increases significantly in esophagitis, as shown in Fig. 4. Measurement of H$_2$O$_2$ in the circular muscle layer of esophagitis LES does not distinguish between H$_2$O$_2$ produced by inflammatory cells and H$_2$O$_2$ produced by the muscle cells themselves. Figure 5, however, clearly demonstrates the presence of high levels of H$_2$O$_2$ in muscle cells after exposure of the isolated cells to IL-1$\beta$, suggesting that some inflammatory mediators may induce overproduction of H$_2$O$_2$ 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 \( \text{H}_2\text{O}_2 \) 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 \( \text{H}_2\text{O}_2 \), such as catalase. \( \text{H}_2\text{O}_2 \) diffuses across biological membranes 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 PL\( \text{A}_2 \) (13, 16) that produces AA and AA metabolites. The AA metabolites PG\( \text{F}_2\alpha \) and thromboxone \( \text{A}_2/\text{B}_2 \) bind to their respective receptors activating \( \text{G}_s \) and \( \text{G}_3 \), 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 \( \text{H}_2\text{O}_2 \) levels are elevated and LES tone is reduced after the induction of AE, we examined whether \( \text{H}_2\text{O}_2 \) reduced tone by altering AA metabolism.

A mechanism for \( \text{H}_2\text{O}_2 \)-induced reduction in tone is through production of PGE\(_2\). We show that \( \text{H}_2\text{O}_2 \) 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-1\( \beta \)-induced formation of PGE\(_2\) has been elucidated in human neuroblastoma cells in which IL-1\( \beta \) induces PGE\(_2\) synthesis through two synergistic pathways, one involving ROS, nuclear factor-\( \kappa \)B, and expression of cyclooxygenase (COX)-2 and the other involving p38 MAPK to stabilize COX-2 mRNA (28).

In contrast, levels of PGF\(_2\alpha\), which contributes to maintaining tone, were unchanged after induction of AE. The effectiveness of endogenous PGF\(_2\alpha\), 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. F2 isoprostanes are stable analogs of PGF\(_2\alpha\) 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).

F2 isoprostanes may bind with varying affinity to PGF\(_2\alpha\) 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\(_{2\alpha}\) 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\(_{2\alpha}\) and that 8-iso-PGF\(_{2\alpha}\) selectively inhibits PGF\(_{2\alpha}\)-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\(_{2\alpha}\), but contract normally to ACh. These data suggest that elevated levels of F2 isoprostanes in esophagitis LES reduce contraction by binding to PGF\(_{2\alpha}\) receptors and blocking the effect of endogenous PGF\(_{2\alpha}\), perhaps by causing receptor internalization and reducing the number of receptors available for activation by PGF\(_{2\alpha}\). This view is supported by the finding of very low initial binding in the presence of 8-iso-PGF\(_{2\alpha}\) 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\(_{2\alpha}\)-induced receptor internalization, which will prevent tone development by spontaneously produced PGF\(_{2\alpha}\).

We conclude that, in a cat model of acute esophagitis, acid-induced inflammation affects the maintenance of tone by producing elevated levels of \( \text{H}_2\text{O}_2 \) in the circular smooth muscle layer of the LES. \( \text{H}_2\text{O}_2 \) alters the AA metabolism by increasing the production of PGE\(_2\), which relaxes the LES, and of 8-iso-PGF\(_{2\alpha}\), which by itself causes little or no contraction, but inhibits LES response to PGF\(_{2\alpha}\), which otherwise contributes to maintenance of resting myogenic LES tone.

GRANTS

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REFERENCES


