Platelet-activating factor and prostaglandin E2 impair esophageal ACh release in experimental esophagitis

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IT IS KNOWN THAT cytokines are involved in most aspects of inflammation in organs and systems, including intestinal inflammation. IL-1β, IL-6, and TNF-α are the major proinflammatory cytokines involved in the inflammatory cascade. Enhanced production of IL-1 has been demonstrated in animal models of intestinal inflammation (20), in the colonic mucosa, and in monocytes isolated from colonic mucosa of ulcerative colitis patients. Circulating levels of IL-6 are high in patients with active Crohn’s disease and consistently elevated in inflammatory bowel disease, where its sources are primarily macrophages and epithelial cells (27)

Very little is known about cytokine profiles in esophagitis. We and others have recently reported that in human esophagitis, endoscopic mucosal biopsies contained elevated levels of IL-6, and patients with reflux esophagitis showed increased concentration of this proinflammatory cytokine in the inflamed tissue compared with controls (58). IL-1β was detected in patients with more severe inflammation, whereas TNF-α was present sporadically and in low concentrations.

We have shown that induction of acute experimental esophagitis in the cat by repeated daily perfusion of 0.1 N HCl (45 min for 3 days) causes a dramatic decrease in amplitude of peristaltic contractions in the body of the esophagus in response to swallowing and in the amplitude of contractions in response to EFS (12). In contrast, the response to direct myogenic stimulation by acetylcholine was not affected, suggesting that inflammation associated with esophagitis affects release of neurotransmitters responsible for esophageal contraction, without directly affecting the myogenic contractile machinery. This possibility was confirmed by direct measurement of ACh release in response to EFS. ACh release was reduced almost to basal levels, i.e., to the levels measured in the absence of EFS, confirming that in this model of experimental esophagitis, ACh release is almost abolished (12)

Incubation of in vitro circular muscle strips with the proinflammatory cytokine IL-1β or IL-6 reproduced the changes in contraction observed with esophagitis and, similar to esophagitis, inhibited release of ACh. The finding that IL-1β and IL-6 were elevated in the esophageal circular muscle from esophagitis animals supports the possibility that these cytokines may be part of the inflammatory mediators responsible for esophagitis-associated esophageal motor dysfunction (12).

We therefore investigated whether IL-1β and IL-6 induce production of other inflammatory mediators to inhibit neurally mediated (but not myogenic) contraction of esophageal circular smooth muscle.

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), and maintenance doses of ketamine (2.5–5 mg/kg) were administered as needed. To determine lower esophageal sphincter (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. A distal perfused Dentsleeve 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. 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. The animals were kept on a slant board at a 15° angle during the perfusion to avoid aspiration. Before acid perfusion, the cat received 0.02 mg/kg atropine to prevent excessive secretion and 1.0 mg/kg im xylazine for anesthesia. After a 5- to 10-min period to allow the additional anesthetic to take effect, 0.1 N HCl was perfused into the esophagus at a rate of 1 ml/min for 30 to 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 the animals were returned to their cages, they were monitored for signs of discomfort and were maintained on broth and a soft diet. The animals appeared alert and comfortable and ate normally.

In each experiment, 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 3 consecutive days, and the esophagus was tested on the fourth day. 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 in sham-treated animals that had esophageal perfusion with distilled water, no effect on mucosa appearance or LES resting pressure was noted (6, 25).

A group of six animals, esophageal pressures were measured at 3 cm proximally to the LES during six successive swallows induced by introduction of 2 ml of water in the proximal esophagus. The pressure measurements were obtained on day 1, before beginning the acid perfusion, and on day 4, before euthanizing the animals.

Preparation of esophageal circular smooth muscle strips and tissue. Animals were initially anesthetized with ketamine (Avecgo, Fort Dodge, LA), then euthanized with an overdose of phenobarbital (Schering, Kenmilworth, 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 in detail (14). Tissues were removed by introduction of 2 ml of water in the proximal esophagus. The pressure measurements were obtained on day 1, before beginning the acid perfusion, and on day 4, before euthanizing the animals.

To prepare esophageal muscle for PAF, PGE2, and H2O2 measurement, the mucosa and submucosal connective tissue were removed by sharp dissection, and the esophagus was excised beginning at 1 cm proximal to the thickened area and extending proximally to the smooth-striated muscle junction, which was visible to the naked eye. Two-millimeter esophageal circular muscle strips were mounted in separate 1 ml muscle chambers as previously described (14). Mounted strips were incubated for 1 h at 37°C in Krebs buffer, before dissection. 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 squamocolumnar 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 esophagus was excised beginning at 1 cm proximal to the thickened area and extending proximally to the smooth-striated muscle junction, which was visible to the naked eye. Two-millimeter esophageal circular muscle 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 (14).

To prepare esophageal muscle for PAF, PGE2, and H2O2 measurement, 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 twice with a 2-mm blade block, the second cut at right angles to the first.

Measurements of muscle strip contraction. Full-thickness esophageal circular muscle strips 2 mm wide were cut with a blade block with blades held at a fixed (2 mm) distance. Strips were mounted in separate 1 ml muscle chambers as previously described in detail (14). They were initially stretched to 2.5 g to bring them near conditions of optimum force development and equilibrated for 2 h while being perfused continuously with oxygenated PSS at 37°C. The PSS contained the following (in mM): 116.6 NaCl, 21.9 NaHCO3, 1.2 NaH2PO4, 3.4 KCl, 2.5 CaCl2, 5.4 glucose, and 1.2 MgCl2. The solution was equilibrated with a gas mixture containing 95% O2-5% CO2 at 37°C, pH 7.4.

After equilibration, esophageal muscle strips were stimulated with square-wave pulses of supramaximal voltage, 0.2 ms, 2–10 Hz, 8-s trains, delivered by a stimulator (Grass Instruments, model S48) through platinum wire electrodes placed longitudinally on either side of the strip. Muscle strips responded with a variable contraction (“on” contraction) at the beginning of the stimulus and a more reliable and larger contraction after the end of the stimulus (“off” contraction). All data reported are off contractions. We have reported previously (5) and more recently (12) that the off contraction is abolished by atropine, and thus it must be a contraction in response to release of Ach.

After the series of electrical stimulations ended, the strips were equilibrated for 30 min, then cumulative dose responses were obtained for Ach (10–7 to 10–4 M). It was previously shown (5) that bethanecol-induced contraction of cat esophageal circular muscle was not affected by large doses of TTX (3 × 10–3), supporting the view that Ach-induced contraction is myogenic.

To study the effect of IL-1β and IL-6 on contraction in response to EFS and Ach, after frequency-response and dose-response relationships in control conditions were obtained, the strips were incubated in the cytokines for 2 h, then contractions in response to EFS and Ach were obtained again. The concentration and incubation times for IL-1β (100 μU/ml) and IL-6 (ng/ml) used in these experiments have been previously shown to mimic esphagitis-induced changes in esophageal smooth muscle contraction, i.e., they inhibited EFS-induced but not Ach-induced contraction. PAF and PGE2 were dissolved in ethanol. When they were added to the muscle chamber, the highest concentration of ethanol in the chamber was 10–3 M and had no effect on esophageal smooth muscle contraction.

ACh release. The release of Ach was measured using a well-established technique in which Ach stores in a circular smooth muscle preparation are previously labeled with [3H]choline (18). This technique has been used extensively to examine myenteric or submucosal plexus function of several species (62, 65). Muscle strips were mounted in 1 ml muscle chambers as previously described (14). Mounted strips were incubated for 1 h at 37°C in Krebs buffer containing 0.2 μM [3H]choline (80 Ci/mm; New England Nuclear, Boston, MA) and 50 μM phystostigmine. The strips were washed by changing the solution every 3 min for 1 h. After 1 h, the basal tritium release approached a plateau level. After incubation in [3H]choline, the strips were washed three times with 1 ml Krebs containing 50 μM phystostigmine to inhibit Ach breakdown and 10 μM hemicholinium to inhibit choline uptake. At this time, a 3-ml sample, resulting from washing the 1 ml chamber three times, was collected, pooled, and used to measure basal release. To measure EFS-induced Ach, release strips were stimulated with the appropriate frequency for 30 s. After 30 s, strips were washed three times with 1 ml of Krebs containing 50 μM phystostigmine and 10 μM hemicholinium, and the 3-ml sample was collected for radioactivity measurement. Phystostigmine and other cholinesterase inhibitors may have variable effects on Ach release, either enhancing (24, 66) or reducing it (4, 21, 22, 26, 37) depending on the type and function of the muscarinic receptors present in the tissue. Nevertheless, a cholinesterase inhibitor is necessary to prevent the enzymatic breakdown of Ach. Strips were allowed to rest 30 min before the next stimulation. Frequencies tested included 0.5, 1, 2, and 5 Hz. Under these experimental conditions, Collins et al. (18) reported that 90% of the radioactivity in the superfusate was [3H]Ach, as measured by high-performance liquid chromatography (HPLC). The finding of a linear relationship between force developed and released counts per minute, as shown in Fig. 10, indicates that counts per minute are a measure of Ach release in our preparation.

Measurement of H2O2 in smooth muscle tissue. Esophageal circular smooth muscle (100 mg) was homogenized in PBS buffer. Homogenization consists of a 20-s burst with a Tissue Tearer (Biospec, Racine, WI) followed by 50 strokes with a Dounce tissue grinder.
resulting extracts were kept at cation by affinity column (Cayman Chemical, Ann Arbor, MI). The thromboxane B2 was measured using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Melville, NY). Thromboxane B2 was measured by using a PGE2 competitive enzyme immunoassay kit (Cayman Chemical) or 10

The mixture was centrifuged again, and the two supernatants were left at room temperature for 1 h, then centrifuged (5,000 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.

H2O2 content is measured by BIOXYTECH H2O2–560 quantititative hydrogen peroxide assay kit (OXIS International, Portland, OR). This assay is based on the oxidation of ferrous ions (Fe2+) to ferric ions (Fe3+) by hydrogen peroxide under acidic conditions. The ferric ion binds with the indicator dye xylene orange [3,3’-bis[N,N-di(carboxymethyl)-aminomethyl]-o-cresolsulfone-phthalein sodium salt] to form a stable colored complex that can be measured at 560 nm.

**Measurement of PGE2 in smooth muscle tissue.** Esophageal circular smooth muscle tissue (100 mg) was homogenized in PGE2 homogenization buffer [0.1 M phosphate buffer (pH 7.4) containing 1 mM EDTA, 20 μg/ml indomethacin] at 4°C. Homogenization consisted of 10- to 20-s bursts with a Tissue Teaser (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. One hundred microliters of each sample supernatant were used for protein measurement. Two milliliters of supernatant were used for PGE2 purification by affinity column (Cayman Chemical, Ann Arbor, MI). The resulting extracts were kept at −70°C. The PGE2 concentration was quantified by using a PGE2 competitive enzyme immunoassay kit (Cayman Chemical).

**Measurement of thromboxane B2 in smooth muscle tissue.** Thromboxane A2 is an unstable arachidonate metabolite with a half-life of 30 s. It rapidly decays nonenzymatically to the stable thromboxane B2 (53). We therefore measured thromboxane B2 as an index of formation of thromboxane A2. Normal esophageal circular muscle tissue was incubated in IL-6 (2 U/ml, 2.5 h), with or without 10−7 M NS-398 [cyclooxygenase (COX)-2 inhibitor] or 10−6 M SC-560 (COX-1 inhibitor). The solution was equilibrated with 95% O2–5% CO2 at 37°C, pH 7.4 for 2.5 h, then the supernatant and tissue were collected, immediately frozen in liquid nitrogen, and kept at −70°C until measurements were obtained. The measurements were normalized per protein content of the tissue. Protein content in each sample was measured by using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Melville, NY). Thromboxane B2 was measured using the EIA Kit (catalog no. 519031) from Cayman Chemical.

**Measurement of PAF in smooth muscle tissue.** PAF was extracted from esophageal circular smooth muscle by a modification of the method of Bligh and Dyer (9). One hundred micrograms of esophageal tissue squares were homogenized in 2 ml of methanol. One milliliter of homogenate was transferred to a tube containing 0.5 ml chloroform, 1 ml methanol, 0.4 ml H2O for a final ratio of 1:2:0.8 chloroform/methanol/H2O solution. The mixture was vortexed, left at room temperature for 1 h, then centrifuged (5,000 g for 5 min). The supernatant was transferred to another glass tube, and the pellet was reextracted with 3.8 ml of the chloroform/methanol/H2O solution. The supernatant was transferred to another glass tube, and the pellet was reextracted with 3.8 ml of the chloroform/methanol/H2O solution. The mixture was centrifuged again, and the two supernatants were combined. Two milliliters of chloroform and 2 ml 1 M NaCl were added, and the phases were separated by centrifugation (5,000 g for 5 min). The upper phase was aspirated and discarded, and the lower phase was washed once with 4 ml 1 M NaCl/methanol (9:1 vol/vol). Samples of this washed chloroform phase were dried under nitrogen and stored at −20°C. Measurement of PAF was performed within 72 h of extraction.

**Measurement of tissue levels of PAF were made using the PAF-3H-labeled scintillation proximity assay system (TRK 990; Amersham International, Amersham, UK). Scintillation proximity assay is a sensitive assay system that uses microscopic beads containing scintillant that emits light when radiolabeled molecules of interest bind to the surface of the bead.**

**Protein determination.** The homogenates of esophageal tissues were solubilized by addition of 6 ml 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).

**Materials and reagents.** Human interleukin IL-1β and IL-6 were purchased from Pierce Endogen (Rockford, IL). The agents used were collagenase type II and soybean trypsin inhibitor from Worthington Biochemicals (Freehold, NJ), sodium dodecylsulfate from Bio-Rad, polyacrylamide from BDH Chemicals (Poole, England), and acetic acid from Malinkrodt Specialty Chemicals (Paris, KY). Acetylcholine, physostigmine, hemicholinium, saponin, BME amino acid supplement, creatine phosphate, creatine phosphokinase, ATP, antimycin A, and other reagents were purchased from Sigma (St. Louis, MO).

**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**

**IL-1β and IL-6 cause production of PGE2 and PAF through H2O2-dependent mechanisms.** We have previously reported (12) that experimental esophagitis significantly reduced EFS-induced (but not Ach-induced) contraction in cat esophageal circular smooth muscle strips (P < 0.001, ANOVA). Incubation of normal esophageal strips with IL-1β or IL-6 (but not TNF-α) replicated these esophagitis-induced changes in esophageal motor function, as shown in Fig. 1. The proinflammatory cytokines IL-1β and IL-6 (but not TNF-α) are present in esophagitis at higher levels than in normal esophageal circular muscle and have been shown to affect production of inflammatory lipid mediators, such as PGE2 and PAF (11, 33), in several organ systems. We therefore investigated whether esophagitis or the associated increase in the levels of inflammatory cytokines IL-1β and IL-6 affect production of PGE2 or PAF and whether these lipid mediators participate in esophagitis-induced motor dysfunction.

Figure 2 shows that circular muscle content of PAF and of PGE2 were significantly elevated in esophagitis specimens compared with normals (P < 0.05, ANOVA). Similarly, the levels of PAF and of PGE2 increased significantly in normal esophageal circular muscle incubated in IL-1β (P < 0.05, ANOVA) and in normal esophageal circular muscle incubated in IL-6 (P < 0.05, ANOVA).

We have shown in LES that the esophagitis-induced decrease in resting tone was reduced by the H2O2 scavenger catalase, suggesting involvement of H2O2, and IL-1β and IL-6 significantly increased H2O2 levels in esophagitis (16). We therefore tested the effect of catalase on PGE2 and PAF production in response to IL-1β and IL-6. Figures 3 and 4 show that IL-1β- and IL-6-induced production of PGE2 and PAF were both significantly reduced by catalase (P < 0.05, ANOVA), suggesting involvement of H2O2. This possibility was investigated by examining production of PGE2 and PAF in response to exogenously applied H2O2.

Figure 5 shows that relatively short-term exposure (2 h) to both IL-1β and IL-6 significantly increased H2O2 levels in normal esophageal circular muscle (P < 0.01, ANOVA). We have previously shown that IL-1β (100 U/ml) or IL-6 (1 ng/ml) reproduces the motor dysfunction found in esophagitis. At these concentrations, IL-1β and IL-6 increased H2O2 to levels comparable with those induced by experimental esophagitis.

Figure 6 shows that H2O2, in turn, causes a significant increase in the levels of PGE2 and PAF in circular muscle of the esophagus (P < 0.01, ANOVA).
The data presented so far suggest therefore that exposure of circular muscle to inflammatory cytokines, such as IL-1β and IL-6, causes production of H₂O₂ in the circular muscle layer, which, in turn, causes an increase in the levels of PGE₂ and PAF, which may ultimately be responsible for reduction of contraction in response to EFS, without reducing the response to direct myogenic stimulation with ACh. If H₂O₂ is central to this effect, exposure of the circular muscle to H₂O₂ should produce the same effect as exposure to IL-1β or IL-6 or esophagitis. Figure 7 shows that 2-h incubation of circular muscle strips in H₂O₂ (10⁻⁴ M) caused a statistically significant decrease in contraction in response to EFS (P < 0.05, ANOVA) but did not significantly reduce contraction in response to direct stimulation with ACh.

PGE₂ and PAF affect ACh release without affecting myogenic contractile mechanisms. To demonstrate that PGE₂ and PAF, produced in response to H₂O₂, reproduce the effect of esophagitis, IL-1β or IL-6, or H₂O₂, circular muscle strips were incubated with PGE₂ or PAF for 2 h. Esophageal muscle strips have little or no tone. Adding PAF did not cause any contraction, and relaxation could not occur because resting force could not get any lower. When electrical stimulation is applied, as shown in Fig. 8A, PAF causes a reduction in the force developed. Both PGE₂ and PAF significantly reduced the contraction in response to EFS (P < 0.001, ANOVA) when used alone, and in combination they abolished EFS-induced contraction (Fig. 8A) without affecting ACh-induced contraction (Fig. 8B).

The data suggest that esophagitis and esophagitis-associated PGE₂ and PAF affect neural release of ACh, without affecting smooth muscle contractile mechanisms.

To confirm that ACh release in response to neural stimulation was damaged, we examined ACh release in response to EFS in normal and esophagitis specimens and in normal muscle strips incubated in IL-1β or IL-6, suggesting that esophageal circular muscle is not damaged by acid perfusion but that the release of excitatory neurotransmitters may be affected. Some of these data have been previously reported (12). The experiments have been repeated in the present study and confirm the previous data. They are shown here for the readers' convenience. Values are means ± SE for 3 cats.

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significant reduction in ACh release \((P < 0.001, \text{ANOVA})\) that was comparable with the reduction induced by IL-1\(\beta\) or IL-6 and only slightly less pronounced than the reduction caused by esophagitis.

To demonstrate that the released radioactivity was due to ACh release, we compared forces and counts per minute obtained at the same parameter of EFS, as shown in Fig. 10. We have previously shown that EFS-induced esophageal contraction is nearly abolished by atropine and thus almost entirely dependent on neural release of ACh \((5, 12)\). The finding of a linear relationship \((r = 0.98)\) between contraction and released counts per minute indicates that the counts per minute measured represent ACh release.

To show that the reduction in contraction is due to cytokine-induced formation of PAF and PGE\(_2\), we examined EFS-induced contraction in the presence of the PAF receptor antagonist CV3988 and in the presence of the prostaglandin synthesis inhibitor indomethacin (Fig. 11). As shown in Fig. 1, IL-1\(\beta\) or IL-6 almost abolished EFS-induced contraction. The reduction by IL-1\(\beta\), however, was partially blocked by CV3988 \((P < 0.001, \text{ANOVA})\) or by indomethacin \((P < 0.001, \text{ANOVA})\) when used alone and was completely reversed when CV3988 and indomethacin were used in combination (Fig. 11). This finding suggests that production of PAF and PGE\(_2\), formed in response to IL-1\(\beta\), may fully account for the substantial reduction in ACh release and contraction occurring in our model of experimental esophagitis.

Figure 12 shows that the IL-6-induced reduction in contraction in response to EFS was partially reversed by the PAF antagonist CV3988 \((P < 0.001, \text{ANOVA})\) but was not affected by the COX inhibitor indomethacin. This was surprising because, as shown in Figs. 2 and 3, IL-6 increases PGE\(_2\) and PGE\(_2\) clearly inhibits EFS-induced ACh-dependent contraction. We therefore examined whether IL-6 may induce formation of other arachidonic acid (AA) metabolites that would counterbalance the effect of PGE\(_2\). If that were the case, indomethacin, by inhibiting both, would have no effect. We therefore measured thromboxane formation in response to IL-6. Because thromboxane A\(_2\) is an unstable arachidonate metabolite that rapidly decays to the stable thromboxane B\(_2\) \((53)\), we measured thromboxane B\(_2\) as an index of formation of thromboxane A\(_2\).

Figure 13A shows that IL-6 caused formation of thromboxane A\(_2\)/B\(_2\) as well as PGE\(_2\). Thromboxane A\(_2\)/B\(_2\) formation was inhibited by the COX-1 inhibitor SC-560, whereas PGE\(_2\)
formation was blocked by the COX-2 inhibitor NS-398. Figure 13B shows that IL-6 inhibited the contraction in response to EFS and that the inhibition was partly reversed by the TXA2 receptor agonist U46619 and by the COX-2 inhibitor NS398. Taken together, Fig. 13, A and B, confirm that IL-6 causes formation of PGE2, which reduces contraction in response to EFS and of thromboxane A2/B2, which increase it.

DISCUSSION

ACh is proven to be a neurotransmitter in cat esophageal circular muscle, as atropine nearly abolishes contraction of in vitro circular muscle strips in response to EFS (5, 12). Induction of experimental esophagitis by perfusion with HCl reduced EFS- but not ACh-induced contraction of esophageal circular muscle (12), suggesting that esophagitis impairs neurotransmitter release. IL-1β and IL-6 are produced in esophagitis and reproduce these changes in normal esophageal muscle (12). These inflammation-induced changes are similar to those reported by Collins and colleagues (18, 19, 28) in Trichinella spiralis-infected rodents. Trichinella spiralis infection induced an acute inflammatory response in intestinal muscle of rats and mice resulting in suppression of neurotransmitter release, such as ACh and norepinephrine, by enteric

Fig. 7. Normal esophageal muscle strips were incubated for 2 h in H2O2 (10-4 M) and contraction in response to EFS or ACh was determined. H2O2 significantly decreased contraction in response to EFS (P < 0.05, ANOVA) but did not significantly reduce contraction in response to direct stimulation with ACh. Values are means ± SE for 3 cats.

Fig. 8. A: normal esophageal muscle strips were incubated for 2 h in PGE2 (10-5 M) and PAF (10-5 M) alone or in combination, and contraction in response to EFS was measured. Both PGE2 and PAF significantly reduced the contraction in response to EFS (P < 0.001, ANOVA) when used alone, and in combination, they abolished EFS-induced contraction. Values are means ± SE for 3 cats. B: ACh-induced contraction was determined in normal ESO muscle strips, in ESO strips from esophagitis cats, and in normal ESO strips incubated for 2 h in PGE2 (10-5 M) or PAF (10-5 M). ACh-induced contraction of esophageal strips was unaltered by incubation of experimental esophagitis or by incubation of strips in PGE2 or PAF. Values are means ± SE for 3 cats.

Fig. 9. The release of ACh was measured using an established technique in which ACh stores in a circular smooth muscle preparation are prelabeled with [3H]choline (18). [3H]ACh release from normal strips, measured in the supernatant, increased with frequency of EFS, compared with levels in the absence of EFS (Basal). EFS-induced release of ACh was significantly reduced (P < 0.01, ANOVA) in muscle strips from esophagitis animals and from normal muscle strips incubated for 2 h in PGE2 (10-5 M), PAF (10-5 M), IL-1β (100 U/ml), and IL-6 (1 ng/ml). All experiments were performed in the presence of physostigmine (50 μM) to prevent ACh breakdown. Some of these data have been previously reported (12). The experiments have been repeated in the present study and confirm the previous data. They are shown here for the readers’ convenience. Values are means ± SE for 3 cats.

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nerves. The proinflammatory cytokines IL-1β and TNF-α released from macrophages were reported as potential mediators of the functional alterations because 1) administration of exogenous IL-1β and TNF-α to normal control tissue mimicked the impairment of neurotransmitter release from longitudinal muscle-myenteric plexus observed during Trichinella spiralis infection; 2) increased mRNA expression of these cytokines was present in the longitudinal muscle-myenteric plexus of infected animals; and 3) macrophage depletion prevented the suppression in [3H]ACh release (28, 35, 44). Trichinella-induced inflammation, however, increased the amplitude of intestinal smooth muscle contraction (8, 51, 70, 71), whereas HCl-induced inflammation of the esophagus did not affect the circular muscle response to ACh but reduced release of ACh from intramural neurons, decreasing the amplitude of neurally mediated contraction.

Unlike this well-explored model of Trichinella-induced inflammation and in agreement with human esophagitis (58), our esophagitis cat model does not involve TNF-α, but only IL-1β and IL-6. We therefore examined the role of IL-1β and IL-6 in this motor dysfunction.

Inflammation and inflammatory mediators such as IL-1 (3) cause activation of inflammatory phospholipase A2 (PLA2) and production of reactive oxygen species (ROS). ROS and PLA2 may both cleave phosphatidylcholine into AA and lysophosphatidylcholine (PAF) precursor (46). The AA liberated is converted to PGE2, possibly by COX-2, which is induced by inflammatory stimuli. The remaining lysophosphatidylcholine may be reincorporated into the cell phospholipid pool or, by enzymatic or nonenzymatic processes, may be cleaved further to produce PAF or PAF-like lipids.

We show that these products of PLA2-dependent phosphatidylcholine metabolism, i.e., PGE2 and PAF (or PAF-like lipids) are the major mediators in esophageal injury associated with esophagitis. These lipids may be formed enzymatically or their analogs may be produced nonenzymatically through lipid peroxidation.

Enzymatic and nonenzymatic production of PGE2. IL-1β induces expression of sPLA2, COX-2, and PGE2 synthesis (34, 41, 43), sometimes resulting in synthesis of IL-6 (2, 32, 40, 42, 54, 55, 69, 73). In addition IL-1β and IL-6 may induce production of ROS (47, 67, 72a). ROS have high affinity for lipids, causing lipid peroxidation. Free radical-induced peroxidation of AA (49) results in formation of isoprostanes, which are stable prostaglandin-like compounds that are formed enzymatically and nonenzymatically in vivo.

Many of the effects of 8-isoprostanes are mediated through prostaglandin receptors; for instance, 8-iso-PGE2 has been re-
reported to inhibit ACh release in guinea pig tracheal muscle by acting at prostaglandin E3 (EP3) receptor subtypes (17).

**Enzymatic and nonenzymatic production of PAF.** After PLA2 cleaves phosphatidylcholine into AA and lyso-PAF, PAF biosynthesis may occur via a “remodeling pathway,” in which 1-0-alkyl-2-lyso-glycerol-3-phosphocholine/acyetyl CoA acetyltransferase catalyzes the acetylation of inactive lyso-PAF into bioactive PAF (57). This pathway is activated when a variety of cells (1) are challenged with IL-1β.

The finding that the H2O2 scavenger catalase significantly reduces PGE2 and PAF formation in response to IL-1β supports a role of H2O2 in the production of these lipid mediators. In this pathway, IL-1β binds to IL-1β receptors, a subfamily of IL-1β-Toll-like receptors (45), resulting in activation of NADPH oxidase, a cytosolic enzyme similar to neutrophil NADPH oxidase that produces H2O2 independently of NADPH and of the mitochondrial respiratory process (39, 48, 68).

Unlike IL-1β, IL-6 signaling is mediated by a cell surface signaling assembly composed of IL-6, the IL-6α receptor, and the shared signaling receptor gp130. IL-6 is first engaged by IL-6Rα and then presented to gp130 in the proper geometry to facilitate a cooperative transition into a high-affinity signaling complex (31). gp130 is a shared cell-surface signaling receptor for at least 10 different hematopoietic cytokines (10). IL-6-type cytokines, via the signal transducer gp130, activate the JAK/STAT and MAPK cascades resulting in gene expression.

Cytokine-induced production of PGE2 and PAF may depend on formation of H2O2 as an inflammatory intermediate (39), may be H2O2 independent and associated with enzymatic action of PLA2, or may be any combination of the two. The finding that H2O2 does not cause complete abolition of contraction in response to EFS, as shown in Fig. 7, and that the H2O2 scavenger catalase does not completely abolish PAF and PGE2 production in response to IL-1β and IL-6, as shown in Figs. 3 and 4, suggests that H2O2 may be partly responsible for the formation of PAF and PGE2.

The synthesis of PAF is carefully controlled, but the formation of potent PAF mimetics after oxidation of phosphatidylcholines (60, 63) is unregulated. Because the structure of these bioactive lipid products differs from PAF whose sn-2 residue is exclusively derived from acetyl CoA, these phospholipids are termed “PAF-like.” Precise quantitation of PAF-like lipids is difficult because oxidation of phosphatidylcholines generates a large number of modified phospholipid products, some of which do not stimulate the PAF receptor whereas others activate PAF receptor in a variety of experimental preparations (30, 60, 63), and their biological activity is completely blocked by specific PAF receptor antagonists (30). Because of their heterogeneity, we cannot separate the effects of enzymatically produced PAF from those caused by oxidatively produced PAF-like lipids.

**PGE2 and PAF (and H2O2) inhibit ACh release.** PAF-induced neurotoxicity has been demonstrated in human and rat culture systems (29). Consistent with our findings, PAF has been shown to inhibit neurotransmitter release from rat brain slices and was selective for K+ depolarization-induced ACh release but did not affect release of other neurotransmitters (72).

In addition, PAF increased the release of PGE2 from astrocyte-enriched cortical cell cultures, and this effect was mimicked by the nonhydrolyzable PAF analog methylcarbamyl-PAF, by the PAF intermediate lyso-PAF, and by AA, supporting the view that intracellular PAF may be a physiological stimulus of PGE2 production (64).

PGE2 (23, 36, 52, 56, 59, 74) and the structurally related E-ring 8-isoprostanes, resulting from lipid peroxidation, have been shown to inhibit release of ACh from cholinergic neurons in airway smooth muscle preparations, whereas the opposite effect has been reported in guinea pig ileal preparations (15, 38, 50). The difference in the effects of PGE2 may be related to different prostaglandin E receptor subtypes present in the different preparations. For instance, PGE2 contracts gall bladder smooth muscle by acting on EP3 receptors but relaxes LES smooth muscle. In this respect, esophageal smooth muscle is different from ileal muscles and similar to airway muscle. In guinea pig trachea, the isoprostanes 8-iso-PGE1 and 8-iso-PGE2 and the selective EP3-receptor agonist sulprostone suppressed EFS-evoked ACh release, and the inhibition was re-
duced by a highly selective EP3-receptor antagonist (17). Consistently with previously reported data (61), these findings suggest that PGE2 and the E-ring 8-isoprostanes inhibit EFS-evoked ACh release from cholinergic nerves innervating guinea pig trachea by interacting with prejunctional prostanoid receptors of the EP3 subtype.

Our data demonstrate that PAF and PGE2 affect ACh release, without interfering with ACh-activated contractile mechanisms and reproduce the effects of acid-induced esophagitis on esophageal contraction. PAF and PGE2 in combination almost abolish EFS-induced contraction. The finding that the response to ACh is not affected by esophagitis (or PAF, PGE2, IL-1β, or IL-6) excludes the possibility that inhibitory products that act directly on muscle (such as, for instance, NO) may contribute to the reduced contraction observed in esophagitis muscle strips. Conversely, the prostaglandin synthesis inhibitor indomethacin and the PAF antagonist CV3988, when used in combination, abolish IL-1β-induced inhibition of contraction in response to EFS. These findings support the possibility that IL-1β-induced formation of PAF and PGE2 are the mediators of esophagitis-associated dysmotility.

Similar to IL-1β, IL-6-induced inhibition of contraction in response to EFS, was reduced by the PAF antagonist CV3988. IL-6-induced inhibition, however, was not affected by indomethacin, although IL-6 caused production of PGE2, as shown in Figs. 2 and 3. We first thought that inclusion of nonenzymatically produced 8-Iso-PGE2 in our measurements of PGE2 might provide an explanation for these findings, because 8-Iso-PGE2 may be indistinguishable from PGE2 under assay conditions. If 8-Iso-PGE2 were formed through H2O2-dependent lipid peroxidation, indomethacin would have little or no effect. We therefore measured thromboxane formation in response to IL-6. The IL-6-induced inhibitor that, together with PAF, causes complete inhibition of EFS-induced contraction is presently unknown.

It should be noted that inflammation affects different contractile mechanisms in esophageal and LES circular muscle layers. PGE2 and PAF affect ACh release from neurons without affecting esophageal muscle contraction in response to ACh. Thus, in the body of the esophagus, these inflammatory mediators have an effect on neurons and no effect on muscle. This is different from LES muscle, where PAF and PGE2 reduce myogenic tone.

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**REFERENCES**


