HCl-induced inflammatory mediators in esophageal mucosa increase migration and production of H$_2$O$_2$ by peripheral blood leukocytes

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Ma J, Altomare A, de la Monte S, Tong M, Rieder F, Fiocchi C, Behar J, Shindou H, Bianconi P, Harnett KM. HCl-induced inflammatory mediators in esophageal mucosa increase migration and production of H$_2$O$_2$ by peripheral blood leukocytes. Am J Physiol Gastrointest Liver Physiol 299: G791–G798, 2010. First published July 8, 2010; doi:10.1152/ajpgi.00160.2010.—Exposure of esophageal mucosa to hydrochloric acid (HCl) is a crucial factor in the pathogenesis of reflux disease. We examined supernatant of HCl-exposed rabbit mucosa for inflammatory mediators enhancing migration of leukocytes and production of H$_2$O$_2$ as an indicator of leukocyte activation. A tubular segment of rabbit esophageal mucosa was tied at both ends to form a sac, which was filled with HCl-acidified Krebs buffer at pH 5 (or plain Krebs buffer as control) and kept oxygenated at 37°C. The medium around the sac (supernatant) was collected after 3 h. Rabbit peripheral blood leukocytes (PBL) were isolated, and sac supernatant was used to investigate PBL migration and H$_2$O$_2$ production. HCl-exposed esophageal mucosa released substance P (SP), CGRP, platelet-activating factor (PAF), and IL-8 into the supernatant. PBL migration increased in response to IL-8 or to supernatant of the HCl-filled mucosal sac. Supernatant-induced PBL migration was inhibited by IL-8 antibodies and by antagonists for PAF (CV3988) or neurokinin 1 (i.e., SP), but not by a CGRP antagonist. Supernatant of the HCl-filled mucosal sac increased H$_2$O$_2$ release by PBL that was significantly reduced by CV3988 and by a SP antagonist but was not affected by IL-8 antibodies or by a CGRP antagonist. We conclude that IL-8, PAF, and SP are important inflammatory mediators released by esophageal mucosa in response to acid that promote PBL migration. In addition, PAF and SP induce production of H$_2$O$_2$ by PBL. These findings provide a direct link between acid exposure and recruitment and activation of immune cells in esophageal mucosa.

PAF; substance P; IL-8; gastroesophageal reflux

GASTROESOPHAGEAL REFLUX DISEASE (GERD) is a common clinical condition, with ~20% of the adult Western population complaining of symptoms at least once per week (15, 34, 39). The pathophysiology of mucosal injury in GERD remains to be elucidated, but prolonged contact of mucosa with acid contributes to the reflux injury. It was thought that esophagitis may develop from a chemical injury starting at the luminal surface of the squamous epithelium, progressing through epithelium and lamina propria into the submucosa, and resulting in acid-induced death of surface cells and stimulation of a proliferative response in the basal cells (24). This view has recently been challenged; a description of the pathogenetic process has been provided (62), proposing that refluxed gastric juice does not directly damage the esophageal mucosa, but rather stimulates esophageal epithelial cells to secrete chemokines that attract and activate immune cells, causing damage to the esophageal squamous epithelial cells (62). In the present work we provide a functional study of the mediators involved.

Several studies have reported that in experimental and human reflux esophagitis proinflammatory cytokine production may underlie the development of erosive esophagitis (5, 11, 16, 27, 65), which is defined by the infiltration of neutrophils and eosinophils into the submucosa. In an animal model of acute esophagitis, Paterson et al. (49) showed that acid perfusion caused release of platelet-activating factor (PAF) from esophageal mucosa into the lumen and induced significant epithelial injury, prevented by a PAF antagonist.

Using an in vitro model of esophagitis, we have recently demonstrated that acid-induced inflammation of the esophagus begins with activation of acid-sensitive vanilloid receptors (TRPV1) in the mucosa and synthesis of the sensory neurotransmitters substance P (SP) and CGRP, and of the lipid inflammatory mediator PAF by epithelial cells (13). Release of PAF by the mucosa induces production of inflammatory mediators in the circular muscle layer, such as interleukin-6 (IL-6), H$_2$O$_2$, interleukin-1β (IL-1β), and PAF, all of which decrease muscle contraction and, possibly, initiate a self-sustaining cycle (9, 10) of motility abnormalities, leading to enhanced exposure of the mucosa to acid, with further increases in inflammation.

However, the mechanisms whereby acid exposure and PAF lead to the recruitment of immune cells in the esophageal mucosa are not known. Several factors have been suggested as being chemotactic for neutrophils and eosinophils. Expression of interleukin-8 (IL-8), a CXC chemokine with potent chemotactic activity for neutrophils, is elevated in the esophageal mucosa of esophagitis patients (26, 27), and IL-8 expression levels are decreased after lansoprazole treatment (25), suggesting a possible role of IL-8 in the pathogenesis of erosive esophagitis induced by acid reflux. SP is known to be a chemotaxtractant and activator of lymphocytes, monocytes, mast cells, and, importantly, neutrophils and eosinophils (46).

In addition, PAF may be important for the transmigration of peripheral blood leukocytes (PBL) across endothelial cells (29, 47, 64).

We propose that acid in the lumen of the esophagus activates TRPV1 receptors, causing the production and release of PAF, SP, and CGRP that attract and activate immune cells contributing to inflammation and injury of the esophageal mucosa. To
examine the effect of inflammatory mediators released by the mucosa on leukocytes we used an experimental model (11) of acid-induced inflammation that we developed, in which a tubular segment of normal esophageal mucosa is removed from the esophagus, and tied at both ends to form a mucosal sac. The sac is filled with Krebs buffer equilibrated with HCl to pH 5.0 (or with Krebs solution for control) and kept in oxygenated Krebs solution at 37°C. The medium around the HCl-filled sac (supernatant) is collected after 3-h incubation and applied to PBL to examine its effect on PBL migration and activation.

We show that the supernatant of the acid-filled mucosal sac acts as a chemoattractant for rabbit PBL and causes H2O2 release. Leukocyte migration was reduced by IL-8 antibodies, by the PAF receptor antagonist CV3988 or by an SP antagonist. H2O2 release was significantly reduced by CV3988 or by an SP antagonist, but not by IL-8 antibodies or by a CGRP antagonist. These data confirm that IL-8 is involved in GERD pathogenesis by promoting PBL migration and suggest that PAF and SP are important inflammatory mediators released from esophageal mucosa in response to acid that attract and activate PBL and, perhaps, contribute to the onset of erosive esophagitis.

METHODS

Tissue preparation. Experimental procedures were approved by the Animal Welfare Committee of Rhode Island Hospital. Adult rabbits weighing between 3.0 and 4.5 kg were used in this study. Animals were initially anesthetized with ketamine (Aveco, Fort Dodge, IA), then euthanized with an overdose of phenobarbital (Scherer, Kenilworth, NJ). The chest and abdomen were opened with a midline incision exposing the esophagus and stomach. The esophagus and stomach were removed together and separated immediately above the lower esophageal sphincter (LES). The esophagus was pinned on a wax block and the smooth muscle layer was opened along the long axis and removed by sharp microdissection at the level of the submucosa, leaving the mucosa intact as a tube, and taking care to keep the submucosa in its entirety with the mucosa preparation. This procedure has been previously described in detail (11). The esophageal mucosal tube consisted of epithelial cells, lamina propria, muscularis mucosae, and submucosa, including submucosal and lamina propria neurons (13), with the epithelial layer on the inside. The separation between mucosal sac and circular muscle strips was as close to the inner layer of the circular muscle as could be surgically achieved under dissecting microscope. The esophageal mucosa tube was divided in two parts and each part was tied at both ends. One was filled with Krebs buffer (0.5 ml/cm of tube) and used as a control; the other one was filled with the same volume of Krebs buffer equilibrated with HCl to pH 4.8–5.0. In previous work (11) we assessed epithelial cell viability after exposure of the mucosal sac preparation to acidic solutions of different pH, by examining the percentage of cells excluding Trypan blue and by measuring lactate dehydrogenase released in the supernatant as an index of cell death. Production of cytokines was highest at pH between 5.8 and 4.8 and declined when the pH was lowered to 4, most likely reflecting tissue damage or necrosis. Thus in this rabbit model we used pH 5.

Both tubes were kept in Krebs buffer with 95% O2, 5% CO2 at 37°C for 3 h, using 1 ml of Krebs buffer per 100 mg of mucosa. The pH of the supernatant remained at 7.0–7.4 and needed no adjustment. After 3 h, the supernatant surrounding the tubes was collected and analyzed. This experimental preparation has been described in detail (11).

Blood collection and isolation of leukocytes. The rabbit was tranquilized by acepromazine maleate injection (1 mg/kg weight subcutaneously, Phoenix Pharmaceutical, St. Joseph, MD). Ten ml of whole blood was collected from the central ear artery and placed in heparin-treated tubes. The heparinized whole blood was used to isolate leukocytes by density centrifugation using Percoll (Sigma-Aldrich, St. Louis, MO) according to a modification of the method developed by Harbeck (22).

Percoll was first diluted with 10× PBS (Percoll-10× PBS, 9:1 vol/vol) and then diluted again with PBS (Percoll-PBS, 4:1 vol/vol). This final Percoll solution at a ratio of 1:1 with whole blood was used to separate PBL from whole blood by centrifugation at 1,800 g for 20 min.

After centrifugation, the top layer, which consisted of serum and leukocytes, was transferred to a new tube containing two volumes of PBS. The contents were mixed, then centrifuged at 400 g for 10 min. The supernatant was discarded and the cells were resuspended and washed twice with medium. Cells used in H2O2 assay were washed with Krebs-Ringer phosphate buffer (145 mM NaCl, 5.7 mM sodium phosphate, 4.86 mM KCl, 0.54 mM CaCl2, 1.22 mM MgSO4, 5.5 mM glucose, pH 7.35), for cell migration assay the cells were washed with RPMI 1640 medium. When necessary, a red blood cell lysis buffer was used to eliminate red blood cells (eBioscience, San Diego, CA). The PBL were resuspended in their wash medium and counted.

RT-PCR. Total RNA from esophageal mucosa was isolated by RNasy Mini Kit (Qiagen, Valencia, CA). To eliminate DNA contamination, 1 μg of total RNA was treated by DNase I according to the product manual. RNA was reversely transcribed and subjected to PCR by using GeneAmp Gold RNA PCR reagent kit (Applied Biosystems, Foster City, CA).

Primers for TRPV1 mRNA were sense 5′-ATGGCCGACCTGGAGTTCCAC-3′ and antisense 5′-TTGATGATGCCCACGATGTTGTG-3′. The primers were derived from the published cDNA sequences of rabbit as described by Zhang et al. (67). We confirmed, through the BLAST database, that the primers were specific for TRPV1. Reactions were carried out in a PTC-100 Programmable Thermal Controller (MJ Research, Waltham, MA) for 1 cycle at 95°C for 10 min, followed by 40 cycles at 94°C for 20 s, 58°C for 20 s, and 72°C for 30 s; the last step was 72°C for 7 min.

Western blot analysis. Rabbit mucosa was homogenized with lysis buffer containing 50 mM Tris·HCl, pH 7.5, 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 1% (vol/vol) Triton X-100, 40 mM β-glycerophosphate, 40 mM p-nitrophenyl phosphate, 200 μM sodium orthovanadate, 100 μM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 1 μg/ml aprotinin. The homogenate was centrifuged at 10,000 g for 5 min, and the protein concentration in the supernatant was determined by using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA), based on the Bradford dye-binding method (4). The supernatant containing 80 μg protein was used for Western blot assay. The primary antibody, goat anti-TRPV1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), was diluted 1:1,000. The secondary antibody, horseradish peroxidase-conjugated donkey anti-goat antibody (Santa Cruz Biotechnology), was diluted 1:2,000. Detection was achieved with Western Lightning ECL agent (PerkinElmer, Waltham, MA). Molecular weight was estimated by comparison of sample bands with prestained molecular weight marker (Bio-Rad, Melville, NY).

Lys–PAF acetyltransferase activity. Mucosal samples were homogenized in 200 μl of ice-cold homogenization buffer containing 0.25 M sucrose, 10 mM EDTA, 5 mM mercaptoethanol, 50 mM NaF, 10−5 M phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 50 mM Tris·HCl (pH 7.4) and were homogenized by sonication (4×20 s at 1-min intervals). The homogenates were centrifuged at 4°C, 600 g for 10 min. The supernatants were collected for the lyso-PAF acetyltransferase (lyso-PAF acetyltransferase) activity assay and the protein concentration in the supernatant was measured by the Bradford method (4). The activity of the lyso-PAF acetyltransferase was measured by a method described by Nomikos et al. (44). Briefly, supernatants containing 10 μg of protein were incubated for 30 min at 37°C with 4 nmol of lyso-PAF and 40 nmol of [3H]acyetyl-CoA (100
Bq/mmol) in a final volume of 200 μl of 50 mM Tris-HCl buffer (pH 7.4) containing 0.25 mg/ml BSA and 1 mM dithiothreitol. After incubation, 1 μl of BSA 100 mg/ml were added and the reaction was stopped by addition of 64 μl of cold trichloroacetic acid solution. The reaction mixtures were kept in ice for 30 min and centrifuged at 10,000 g for 2 min. The supernatants were discarded and the pellets containing the [3H]PAF bound to the denaturated BSA were dissolved in EcoLume scintillation cocktail (MP Biomedicals, Solon, OH), and the radioactivity was determined by liquid scintillation counting. Matching controls were run in the absence of lyso-PAF to subtract the radioactivity of the endogenously produced [3H]PAF.

Measurement of PAF. PAF was extracted from esophageal sac supernatant by a modification of the method of Bligh and Dyer (3). Briefly, 1.6 ml of supernatant was transferred to a tube containing 2 ml of chloroform, 4 ml methanol for a final ratio of 0.8:1:2 H2O:chloroform-methanol (vol/vol/vol). The mixture was vortexed, then centrifuged (2,000 g, 10 min). The upper phase was discarded, keeping the lower phase. A mixture of 6.4 ml of H2O:chloroform-methanol (0.8:1:2, vol/vol/vol) was vortexed, then centrifuged (2,000 g, 10 min), and its upper phase was used to wash the lower phase of the supernatant mixture. Samples of this washed chloroform phase were dried under nitrogen and stored at −80°C. Measurement of PAF was performed within 72 h of extraction. PAF was measured by a radio-receptor binding assay method developed by Aoki and colleagues (1, 57) using PAF receptor-enriched membranes from the heart and skeletal muscle of PAF receptor transgenic mice. Dried sample residues were reconstituted in binding buffer (25 mM HEPES-NaOH, pH 7.4, 10 mM MgCl2, 0.1% BSA). Cell membranes containing PAF receptor were adjusted to 80 μg in 100 μl binding buffer. Reconstituted samples in 50 μl binding buffer were adjusted to 50 μl 40 mM [3H]WEB 2086 were added to a 96-well microplate and incubated at 25°C for 90 min. At the end of the incubation, the [3H]WEB 2086 bound to the receptor was separated from the unbound [3H]WEB 2086 by filtration through a UniFilter-96 GF/C (PerkinElmer, Shelton, CT) using a Filtermate Harvester (Packard, Meriden, CT). The filters were washed eight times with the binding buffer. The UniFilter plate was dried at 50°C. MicroScint-O scintillation cocktail (25 μl) (PerkinElmer, Shelton, CT) was added to each well and radioactivity was measured by using a TopCount-NX scintillation counter (Packard). The binding in the presence of 2 μM PAF was used as nonspecific binding and was subtracted from the values for statistical analysis.

Measurement of CGRP and SP. The mucosal sac supernatant was frozen at −80°C for later use. The concentration of CGRP and SP present in the mucosal sac supernatant was measured by use of enzyme immunoassay kits from Cayman Chemical (Ann Arbor, MI). Before measurement of CGRP and SP, the mucosal sac supernatant was diluted to 1:4 with EIA buffer provided by the immunoassay kits.

Measurement of IL-8. IL-8 levels in sac supernatant and in mucosal tissue, were measured using the KPL ELISA kit anti-mouse ABTS system (KPL, Gaithersburg, MD). Primary mouse anti-rabbit IL-8 was purchased from Abcam (Cambridge, MA). To measure tissue IL-8, esophageal mucosa (50 mg) was homogenized in 0.25 ml cell lysis buffer from the human IL-8 ELISA kit (RayBiotech, Norcross, GA) and centrifuged at 10,000 g, 4°C, for 10 min. An aliquot of the supernatant was used to quantify the protein concentration by use of a Bio-Rad protein assay kit (Bio-Rad), based on the Bradford dye-binding method (4). The supernatant was then adjusted to 10 μg/ml using the coating solution provided by the KPL ELISA kit.

To measure IL-8 levels in sac supernatant, the supernatant was diluted 1:1 using the coating solution provided by the KPL ELISA kit. Mouse anti-rabbit IL-8 monoclonal antibody (Abcam) diluted at 1:1,500 was used as primary antibody. The other steps of ELISA were performed as instructed by the product manufacturer.

Measurement of H2O2. To examine the effect of mucosal supernatant on H2O2 production, PBL were incubated for 20 min at 37°C in mucosal supernatant from sac incubated in Krebs buffer alone (control) or in Krebs buffer at pH 5. When using antagonists or antibodies, cells were pretreated with the antagonist or antibody for 30 min before exposure to mucosal supernatant. The antagonists used were CV-3988 (10−5 M) for PAF, CGRP8–37 (10−6 M) for CGRP, the neurokinin-1 receptor antagonist (10−7 M) for substance P. IL-8 antibodies (1:200) were used to immunoneutralize IL-8. H2O2 levels released from isolated leukocytes were measured using the Amplex Red hydrogen peroxide assay kit (Invitrogen, Eugene OR), using 0.5–1 × 106 cells in 20 μl of Krebs-Ringer phosphate buffer (40).

Peripheral blood leukocyte migration. Peripheral blood leukocyte migration was measured by the ATP luminescence-based motility-invasion assay developed by de la Monte et al. (14). Briefly, 100,000 cells in 100 μl RPMI-1640 cell culture medium (Invitrogen, Grand Island, NY) were seeded into the upper chamber of a dual-chamber motility system (Neuro Probe, Gaithersburg, MD) and separated from the lower chamber by a polycarbonate track-etch (PCTE) membrane with 3-μm pores (Neuro Probe). Cell migration was allowed to proceed from the upper to the lower chamber for 60 min at 37°C in a CO2 incubator. The lower chamber contained materials to be tested for their effect on PBL motility. The materials tested were IL-8 (0.5 × 10−7 M), acid-treated sac supernatant alone, or acid-treated sac supernatant with antagonists to PAF (CV-3988, 10−5 M), CGRP (CGRP8–37, 10−6 M), or SP (neurokinin-1 receptor antagonist, 10−5 M). Sac supernatant was also tested in the presence of IL-8 antibodies (1:200). When using antagonists or antibodies, cells were pretreated with the antagonist or antibody for 30 min before being placed in the upper chamber. Cells were collected from the upper chambers (nonmigrated cells), as well as under the surface of the membranes and at the bottom of the wells (migrated cells). Cells were quantified with use of an ATPL lite kit (PerkinElmer, Walthham, MA). The percentages of nonmigrated and migrated cells were calculated and used for statistical analysis.

Materials and reagents. Bio-Rad protein assay kit was purchased from Bio-Rad. SP and CGRP EIA kits, PAF C-16, and lyso-PAF were obtained from Cayman Chemical. KPL ELISA kit anti-mouse ABTS system was purchased from KPL. Amplex Red hydrogen peroxide assay kit was obtained from Invitrogen. Primary mouse anti-rabbit IL-8 was purchased from Abcam. IL-8 was purchased from Bio-Rad. IL-8 antibodies were obtained from Cayman Chemical. IL-8 was purchased from R&D Systems (Minneapolis, MN). CV-3988 was from Biomol International (Plymouth Meeting, PA). Neurokinin-1 receptor antagonist was purchased from Calbiochem (La Jolla, CA), and SP, CGRP, and CGRP antagonists (CGRP8–37) were purchased from AnaSpec (Fremont, CA). The dual-chamber motility system and PCTE membrane were from Neuro Probe. The ATPL lite kit was purchased from PerkinElmer (Waltham, MA). MicroScint-O scintillation cocktail, UniFilter-96 GF/C plates, [3H]acetyl-CoA, and [3H]WEB 2086 were purchased from PerkinElmer (Shelton, CT). EcoLume scintillation cocktail was from MP Biomedicals. The other reagents were obtained from Sigma.

RESULTS

We demonstrate that, similarly to cat esophageal mucosa (13), rabbit mucosa contains TRPV1 receptors and responds to HCl exposure (pH 5) by releasing PAF, CGRP, and SP, in addition to IL-8, into the surrounding supernatant (Figs. 1–5). First we show via Western blot and RT-PCR data supporting the presence of TRPV1 receptors in the mucosal sac (Fig. 1). Second, 3-h exposure to pH 5.0 results in increased mucosal activity of the enzyme responsible for the production of PAF through the remodeling pathway (52, 56) (Fig. 2A). Third, PAF release into the supernatant increases in response to exposure to HCl (Fig. 2B). Taken together, Fig. 2, A and B, indicates that HCl causes increased lyso-PAF acetyltransferase activity in the sac tissue resulting in release of PAF into the supernatant. In addition, HCl causes increased SP and CGRP production in the mucosa and increased SP and CGRP release into the sac.
supernatant, similar to the cat esophageal mucosa (13) (Figs. 3 and 4).

HCl-induced IL-8 production in the mucosa and supernatant is shown in Fig. 5. The figure indicates that in the presence of HCl (pH 5) rabbit mucosa significantly increased IL-8 production and release into the supernatant. This confirms that the rabbit mucosal response to acid is comparable to our previously described cat model.

IL-8 is a powerful chemokine (2, 33). We therefore examined PBL migration in response to IL-8 and to mucosal supernatant by using a migration chamber. Figure 6 shows that IL-8 (4.0 ng/ml) induced PBL migration. Supernatant of HCl-treated mucosa induced PBL migration that was comparable to that induced by IL-8. Selective antagonists were used to assess the individual contribution of inflammatory mediators present in the supernatant. The figure shows that a selective CGRP antagonist did not affect PBL migration. In contrast, a selective IL-8 antibody, a PAF receptor antagonist, and an NK-1 receptor antagonist significantly reduced PBL migration, supporting a role of IL-8, PAF, and SP as relevant chemokines.

PAF is known to be a powerful activator of PBL, particularly eosinophils (64), evoking the release of reactive oxygen species (58, 71). The contribution of the individual mediators to PBL activation was assessed by using the appropriate antagonists. H2O2 levels, taken as measure of PBL activation, increased when PBL were exposed to sac supernatant at pH 5 (Fig. 7). The increase was not affected by a CGRP antagonist or by IL-8 neutralization by IL-8 antibodies. These data suggest that neither CGRP nor IL-8 at the concentrations present in the supernatant play a role in inducing H2O2 production by PBL. In contrast, a PAF antagonist and a neurokinin-1 (NK-1) receptor antagonist (i.e., SP antagonist) significantly reduced supernatant-induced H2O2 production.

**DISCUSSION**

We have studied inflammation-induced changes in esophageal/LES circular muscle in the cat (8, 10, 12) because its muscle is smooth as it is in humans. For inflammation-induced changes in esophageal mucosa, however, the rabbit has been used extensively (6, 32, 43, 48, 63) and is thought to constitute a suitable model for the study of the human esophageal mucosa. We therefore first confirmed that rabbit esophageal mucosa, similarly to cat (13), contains TRPV1 receptors and responds to HCl by producing SP, CGRP, and PAF. In addition, the rabbit esophageal mucosa produces IL-8, a powerful chemokine that is known to be elevated in GERD and to attract leukocytes, particularly neutrophils (2, 20, 41, 66, 72).

Immune cells may be activated by numerous agents, particularly when used at pharmacological concentrations. It is therefore important to establish which inflammatory mediators are released by HCl-stimulated esophageal mucosa at in vivo concentrations sufficient to attract and activate peripheral blood leukocytes. To study the interaction of mucosa and leukocytes we adopted a relevant ex vivo sac model (11) that allows to examine the inflammatory mediators released by the tissue in a system mimicking the in vivo situation.

Peripheral blood leukocytes were used either to examine cell migration or production of H2O2 in response to the inflamma-
ory mediators or chemokines present in the supernatant of the HCl-stimulated mucosal sac. Activated polymorphonuclear leukocytes (including neutrophils, basophils, and eosinophils) have been used as the prototype of cells that vigorously produce superoxide anions (28), which, in turn, generate other reactive oxygen species (ROS) together with microbiocidal peptides and proteases. ROS comprise species such as superoxide, hydrogen peroxide (H₂O₂), nitric oxide (NO), and hydroxyl radicals (19). These highly reactive molecules are known to regulate many important cellular events, including gene expression (45), transcription factor activation (54), DNA synthesis (30), and cellular proliferation (42). The enzymes NADPH oxidase and dual oxidase generate ROS in a regulated manner, producing reactive oxygen in cells and tissues in response to growth factors, cytokines, and calcium signals (31).

Although activated PBL produce inflammatory products other than H₂O₂, we have used H₂O₂ as a model for PBL-derived ROS because H₂O₂ is physiologically produced in large amounts by cells such as granulocytes, is relatively stable, and has been widely used to assess the effects of ROS (19).

Similarly to cat esophageal mucosa (13) and the human esophageal epithelial cell line HET-1A (35), rabbit esophageal mucosa contains TRPV1 receptors, which are activated by several stimuli, including acid (7). Exposure of rabbit mucosa

Fig. 4. When mucosa was filled with HCl (pH 5) for 3 h, CGRP levels doubled in the mucosa and in the supernatant compared with control (pH 7.4) (*P < 0.05 ANOVA). Data represent means + SE of mucosal tissue from 3 animals.

Fig. 5. When the mucosal sac was filled with HCl (pH 5) for 3 h, IL-8 levels approximately doubled in the tissue and in the supernatant compared with control (pH 7.4) (*P < 0.05 ANOVA). Data represent means + SE of mucosa and supernatant from 3 animals.

Fig. 6. Peripheral blood leukocyte (PBL) migration was examined by using a dual-chamber motility system, in which the upper chamber was separated from the lower chamber by a polycarbonate track-etched membrane with 3-μm pores. Cell migration was allowed to proceed from the upper to the lower chamber for 60 min at 37°C in a CO₂ incubator. The lower chamber contained materials to be tested for their effect on PBL motility. PBL motility was measured with an ATP luminescence-based motility-invasion assay. PBL migration (60 min at 37°C) was significantly increased by IL-8 (0.5 × 10⁻⁹ M), and by the supernatant (Sup) of the HCl (pH 5, 3 h)-filled mucosal sac (*P < 0.05 ANOVA). When using antagonists (Antag) or antibodies, cells were pretreated with the antagonist or antibody for 30 min before being placed in the upper chamber. The lower chamber contained the same concentration of antagonists or antibodies as the upper chamber. The increased PBL migration was significantly reduced by IL-8 immunoneutralization by an IL-8 antibody (1:200), by a PAF receptor antagonist (CV3988, 10⁻⁵ M), and by an NK-1 receptor (NK1R) antagonist (10⁻⁵ M) (*P < 0.05 ANOVA). The increased PBL migration was not affected by a CGRP antagonist (CGRP8–37, 10⁻⁶ M). Data represent means + SE of 3 experiments.

Fig. 7. To examine the effect of mucosal supernatant on H₂O₂ production, PBL were incubated for 20 min at 37°C in mucosal supernatant from sacs incubated in Krebs buffer alone (control) or in acidified Krebs buffer (pH 5, 3 h) (supernatant). The supernatant of the acidified mucosal sac caused a 2-fold increase in H₂O₂ production by PBL. When using antagonists or antibodies, PBL were pretreated with the antagonist or antibody for 30 min before exposure to the supernatant. The increase in H₂O₂ levels was not affected by a CGRP antagonist (CGRP8–37, 10⁻⁶ M) or by IL-8 immunoneutralization by an IL-8 antibody (1:200). The increased H₂O₂ levels, however, were significantly reduced by a PAF receptor antagonist (CV3988, 10⁻⁵ M) and by an NK-1 receptor antagonist (10⁻⁵ M) (*P < 0.05 ANOVA). Data represent means + SE of 8 experiments.
to HCl causes activation of lyso-PAF acetyltransferase and production of PAF, similarly to cat and human epithelial cells, where signaling for production of PAF has been described in detail (35).

**PAF and lyso-PAF acetyltransferase.** We have shown that esophageal mucosa releases PAF when exposed to acid (9, 10, 13, 35) and demonstrated that epithelial cells are the site of PAF production (13, 35). PAF is an important inflammatory mediator that acts as a chemoattractant and activator of immune cells. PAF induces production of H$_2$O$_2$ in leukocytes (58, 71) and in esophageal circular muscle (9, 10). Other than for one experimental study in the opossum (49), and work from our laboratory (9, 10, 12, 13, 35), a role of PAF in esophageal inflammation has not been investigated to any significant extent. Because PAF is a potent phospholipid mediator of many leukocyte functions, however, its activation in esophageal epithelial cells may play an important role in the pathophysiology of inflammatory disorders in the esophagus.

The enzymatic synthesis of PAF has been characterized in endothelial cells (68, 70), in inflammatory and vascular cells (69) and, recently, in esophageal epithelial cells (35). This pathway is highly regulated and most commonly involves a two-step mechanism. Namely, the precursor of PAF, lyso-PAF, is synthesized by the action of phospholipase A$_2$ (51, 55, 61), removing arachidonic acid (AA) from a membrane phospholipid, and resulting in AA and 1-alkyl-phosphatidylcholine (lysoPAF). Lyso-PAF is converted to PAF by lyso-PAF acetyltransferase, which has been recently cloned by Shindou and coworkers (21, 56).

In HCl-stimulated rabbit esophageal mucosa PAF is produced at concentration sufficient to induce production of H$_2$O$_2$ and migration of PBL. The role of PAF in these PBL functions is demonstrated by the significant reduction in H$_2$O$_2$ production and PBL migration by the selective PAF receptor antagonist CV3988. These data support a role of PAF as a potentially important inflammatory mediator and chemokine in HCl-induced esophageal injury.

**SP and CGRP.** Similarly to the cat, 3-h exposure of rabbit esophageal mucosa to a moderately low pH (pH 5) results in increased levels of SP and CGRP in the mucosa and increased SP and CGRP release into the mucosal supernatant. Increased levels of SP and CGRP in the mucosal tissue suggest synthesis of these neurotransmitters and the presence of neural cells in this mucosal sac preparation. SP- and CGRP-containing neural cells have been demonstrated in the human esophageal submucous plexus (59, 60) and epithelium (59) and in cat submucosa (13). We have recently demonstrated SP and CGRP immunoreactivity localized in ganglion cells in the myenteric and submucosal plexus of the cat esophagus (13). Increased levels of SP and CGRP may produce symptoms of neurogenic inflammation by interacting with endothelial cells, mast cells, immune cells, and arterioles (36). Similar findings were reproduced by administration of SP or CGRP agonists and attenuated by administration of antibodies directed against these peptides or by their receptor antagonists (36).

Most of the published research on neurogenic inflammation has focused on release of SP through neural mechanisms (17, 37, 38). Our data indicate that both CGRP and SP are released by esophageal mucosa and submucosa in response to HCl, but, at the concentration present in the supernatant, CGRP does not contribute to attracting PBL or inducing H$_2$O$_2$ production. In contrast, SP is present at concentrations sufficient to act as a chemokine and as an activator of PBL, consistent with a role of SP in acid-induced inflammation (46).

SP induces release of inflammatory mediators such as cytokines, oxygen radicals, arachidonic acid derivatives, and histamine; potentiates tissue injury; and stimulates further leukocyte recruitment (46), amplifying the inflammatory response (23). SP can specifically stimulate chemotaxis of lymphocytes, monocytes, neutrophils, and fibroblasts (18, 53). In general, SP potently affects the migratory and cytotoxic functions of human leukocytes, suggesting that neurogenic stimuli may prime neutrophils for an increased inflammatory response to other mediators (50).

In the rabbit, supernatant of HCl-stimulated esophageal mucosa induces H$_2$O$_2$ production in PBL and PBL migration, and both responses are significantly inhibited by an NK1 receptor antagonist. The inhibition was comparable to that caused by a PAF antagonist, supporting a role of SP as an activator and chemokine in rabbit esophageal mucosa.

**IL-8.** IL-8 has long been established as a chemokine, with preference for neutrophils (2, 20, 72). IL-8 has been extensively studied in GERD and is expressed in high amounts in the affected mucosa of GERD patients (16, 27, 65) and in experimental models of reflux-induced inflammation (62). IL-8 levels in the esophagus correlate and increase with both endoscopic and histological disease severity (27, 65). Our data suggest that 3-h incubation at pH 5 significantly increases IL-8 release in the tissue supernatant. IL-8 (4.0 ng/ml)-induced PBL migration, as shown in Fig. 6, was of a magnitude comparable to that induced by the supernatant. Immune neutralization of IL-8 in the supernatant by IL-8 antiserum (1:200) significantly reduced supernatant-induced migration, indicating the presence of an effective IL-8 concentration in the supernatant. The same immune neutralization, however, had no effect on H$_2$O$_2$ production (Fig. 6), suggesting that supernatant IL-8 levels may be selectively effective in attracting PBL, but not in inducing H$_2$O$_2$ production.

We conclude that rabbit esophageal mucosa responds to HCl by producing several inflammatory mediators, including IL-8, CGRP, SP, and PAF. At the concentrations released into the sac supernatant, IL-8 is an effective chemokine promoting migration of PBL, but it does not induce production of H$_2$O$_2$. CGRP is a sensory neurotransmitter, perhaps mediating esophageal sensation, but ineffective in promoting PBL migration or H$_2$O$_2$ production. In contrast, SP and PAF are effective in promoting PBL migration and production of H$_2$O$_2$ and may be important mediators to induce the onset of esophageal inflammation. Thus the inflammatory mediators produced by HCl exposure may act as chemokines, as activators, or both. In our system, IL-8 acts only as a chemokine, substance P and PAF act as both chemokines and activators, and CGRP does neither. The data suggest that PBL migration and H$_2$O$_2$ production may be different functions, possibly depending on different signal transduction pathways.

These results, derived from an ex vivo model mimicking reflux-induced events in vivo, identify the type and quantity of inflammatory mediators likely produced in GERD, which may become future targets for novel therapeutic approaches. Since cellular immune infiltrate is a prerequisite for severe inflammation and tissue damage, inhibition of IL-8, SP, and PAF-
induced PBL migration and activation may provide potential targets for therapy.

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DISCLOSURES

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REFERENCES


