New mechanisms of angiogenesis and signaling pathways of esophageal ulcer healing: the role of prostaglandin EP2 receptors, cAMP, and pCREB

Amrita Ahluwalia,* Dolgor Baatar,† Michael K. Jones, and Andrzej S. Tarnawski

Medical and Research Services, Veterans Affairs Long Beach Healthcare System (VALBHS), Long Beach, California, and the Department of Medicine/Gastroenterology, University of California, Irvine, California

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A pathway mediates the stimulatory effect of PGEs on angiogenesis. Recent studies demonstrated that esophageal ulcers express predominantly EP2 receptors and that esophageal ulceration triggers an increase in expression of the EP2 receptor, activation of CREB (the downstream target of the cAMP signaling), and enhanced VEGF gene expression. Treatment of rats with misoprostol, a PG analog, increased intracellular cAMP levels (by 163-fold), induced phosphorylation of CREB, and stimulated VEGF expression. A cAMP analog (Sp-cAMP) mimicked, whereas an inhibitor of cAMP-dependent protein kinase A (Rp-cAMP) blocked, these effects of misoprostol. These results indicate that the EP2 receptor is involved in VEGF expression and that cAMP mediates the stimulatory effect of PGEs on angiogenesis.

Angiogenesis; esophageal ulcers; cyclic AMP; prostaglandin E; misoprostol; wound healing

AN ESOPHAGEAL ULCER IS A FOCAL BREAK IN THE ESOPHAGEAL MUCOSA WITH A CLEARLY DELINEATED MARGIN AND IS HISTOLOGICALLY DEFINED AS A DEEP NECROTIC LESION PENETRATING THE ENTIRE MUCOSA AND MUSCULARIS MUCOSAE.

Esophageal ulcer healing have not been fully elucidated. Our previous studies demonstrated an important role of keratinocyte growth factor (KGF), hepatocyte growth factor (HGF), and their receptors in the esophageal ulcer healing (2–4). We also demonstrated that cyclooxygenase 2 (COX-2) is upregulated in epithelial cells lining the esophageal ulcer margin and that a selective COX-2 inhibitor significantly delays esophageal ulcer healing (2). These findings indicate a potential role for E type prostaglandins (PGE) in esophageal ulcer healing (2).

Experimental and clinical studies have demonstrated that PGEs and their synthetic analogs promote healing of gastrointestinal ulcers and dermal ulcers and wounds (1, 11, 15, 23, 24, 26, 31, 32). In general, the physiological effects of PGEs are mediated through G protein-coupled transmembrane PGE receptors, E-prostanoid (EP) receptors (14, 50). To date, four EP receptors (EP1, EP2, EP3, and EP4) have been identified (14, 50). The EP1 receptor is linked to stimulation of intracellular calcium release, whereas the EP2 receptor is coupled to a Gox (stimulatory) protein leading to increase in intracellular cAMP (20, 29, 44, 57).

However, the cellular and molecular mechanisms underlying the healing actions of PGEs and the signaling pathways mediating their effects during esophageal ulcer healing are not known. Specifically, the roles of EP receptors, cAMP, and the response element-binding protein (CREB) and exogenous PGEs in the healing of esophageal ulcers have not been explored. PGE type may exert different effects on cells depending on the type of EP receptor(s) expressed on the cells. In general, PGEs stimulate cAMP production through their interactions with two membrane-bound Gox protein-coupled EP receptors: EP2 and EP4 (14, 50). In most cells, the stimulation of EP2 receptor increases intracellular cAMP, which in turn induces phosphorylation of CREB (necessary for its activation) via activation of protein kinase A (12, 22, 37, 47). Recent studies demonstrated that PGE2 can activate (phosphorylate) CREB in some cells in vitro (9, 19, 38, 51, 56) and that inhibition of endogenous prostaglandin synthesis by nonsteroidal anti-inflammatory drugs (NSAIDs) prevents CREB activation in regenerating liver (45), suggesting that CREB may mediate some aspects of prostaglandin signaling. However, the roles of prostaglandin EP receptors, cAMP, and CREB, as well as their relation to VEGF and angiogenesis in esophageal ulcer healing, remain unknown, forming the rationale of this study.

The present study was undertaken to determine the roles of PGE type and CREB in VEGF gene activation and angiogenesis during esophageal ulcer healing. We used our established experimental esophageal ulcer model in rats to examine 1) whether ulceration alters expression of EP receptors and/or CREB activity and 2) whether a synthetic PG analog and
agonist of EP1–4 receptors), misoprostol (40), which is approved for clinical use, affects CREB activity, VEGF expression, angiogenesis, and/or esophageal ulcer healing. To gain better insight into the mechanisms and to determine human relevance, we examined expression of EP receptors and the effects of misoprostol on VEGF expression intracellular cAMP levels and CREB activity in cultured human normal esophageal epithelial (HET-1A) cells. We also studied the role of the cAMP/protein kinase A signaling pathway in CREB activation and induction of VEGF expression in these cells.

**MATERIALS AND METHODS**

**Induction of esophageal ulcers.** This study was approved by the Subcommittee for Animal Studies (Institutional Animal Care and Usage Committee) of the VA Long Beach Healthcare System. Male Sprague-Dawley rats (weighing 225–250 g) were used in the experiments. Esophageal ulcers were induced by modification of the method described by Tsuji H et al. (55) detailed in our previous studies (2–4). In brief, 100% acetic acid (30 µl) was applied to the anterior wall of intra-abdominal esophagus through a polyethylene tube (3-mm inner diameter) for 3 min. The area was then washed with isotonic saline, and the abdomen was closed. Sham-operated rats underwent similar procedures except no acetic acid was applied.

**Expression of EP receptors and CREB phosphorylation in normal and ulcerated esophageal tissue.** Rats with esophageal ulcers were euthanized 3, 6, and 9 days after ulcer induction. A 1-cm-long segment of the lower esophagus including ulcer was excised, opened, and divided longitudinally (through the center of the ulcer crater) into two parts. One part was snap-frozen in liquid nitrogen and stored at −80°C, and the other part was fixed in 10% formalin. Sham-operated rats were euthanized 3 days after sham operation, and a 1-cm-long segment of lower esophagus (normal esophageal tissue) was excised and processed as described above. In addition to determine tissue-specific distribution of EP2 and EP3 in rat esophagus, esophageal mucosa was stripped from its muscle layers by sharp dissection, and tissue samples were snap-frozen in liquid nitrogen and stored at −80°C.

**Effect of misoprostol on CREB phosphorylation in normal and ulcerated esophageal tissue.** Because our previous study demonstrated that esophageal ulcers are fully developed within 3 days after application of acetic acid (4), we examined the acute effect of misoprostol on CREB phosphorylation in rats with 3-day esophageal ulcers. Rats with ulcers and sham-operated rats were treated intragastrically with a single dose of either 50 µg/kg misoprostol [Methyl 7-(1R,2R,3R-3-hydroxy-2-[S,E-4-hydroxy-4-methyloct-1-enyl]-5-oxocyclopentyl) heptanoate; Cytotec; G.D. Searle, Chicago, IL], a synthetic PGE1 analog, or its vehicle (0.5% carboxymethyl cellulose sodium salt) and were euthanized 1 h following administration.

**Effect of misoprostol on esophageal ulcer healing.** Rats were treated intragastrically twice daily with either 50 µg/kg misoprostol or its vehicle for 3 or 6 days starting 3 days after ulcer induction. The misoprostol dose used in this study was 20 times lower than that required to significantly inhibit gastric acid output in rats (7). Therefore, any action of misoprostol on esophageal ulcer healing was entirely independent of its inhibitory effect on gastric acid secretion. Rats with esophageal ulcers were euthanized 3, 6, and 9 days after ulcer induction. Sham-operated rats were euthanized 3 days after sham operation. The esophagus was excised and opened longitudinally, and the ulcer was photographed. The ulcer area was measured using a computerized video analysis system (Image 1/FL; Universal Imaging, Westchester, PA).

**Histological assessment of angiogenesis.** To identify microvessels, enhanced polymer one-step staining (46) with mouse monoclonal anti-factor VIII-related antigen (Factor VIII RA) antibody (Dako, Carpenteria, CA), which specifically detects endothelial cells, was employed. Deparaffinized sections were incubated with Factor VIII RA antibody for 1 h at room temperature. Color detection of specific antibody binding was developed with the AEC Substrate System (Dako), and the sections were counterstained with Mayer’s hematoxylin (Dako). Factor VIII RA-positive microvessels were counted in 10 microscopic fields (>200 magnification) in granulation tissue immediately below the ulcer. The results were expressed as the number of microvessels per square millimeter of mucosal section (microvessel density). Coded mucosal sections from six rats per group were evaluated by two investigators unaware of the code, and the mean ± SD was calculated.

**Evaluation of esophageal epithelial cell proliferation.** Expression of proliferating cell nuclear antigen in formalin-fixed paraffin-embedded esophageal tissue sections was determined by the enhanced polymer one-step staining method using specific mouse monoclonal antibody (Chemicon, Temecula, CA) (46). Coded specimens were evaluated quantitatively as described previously (2). The results are expressed as the percentage of increase in the number of labeled cells in the epithelium of the ulcer margin over the number of labeled cells in the epithelium distant from the ulcer. Sections from six rats per group were evaluated, and the mean ± SD was calculated.

**Determination of VEGF mRNA expression by RT-PCR.** RNA was isolated using the RNeasy Mini Kit (QIAGEN, Valencia, CA) according to the manufacturer’s instructions. RT-PCR was performed as described previously (33). The primers for VEGF were 5′-CCTG-GTGGACATCTTTCAGGATACC-3′ (sense) and 5′-GAAGCT- CATCTCTCTATGTGCTGC-3′ (antisense). The size of the amplified fragment conserved in all of the variant-spliced forms of VEGF was 196 bp. PCR for β-actin was used as a positive control and as an internal standard. The specific primer sets for rat and human β-actin were purchased from Clontech Laboratories (Palo Alto, CA). For the quantitative assessment of the PCR products, a computerized video analysis system (Image-1/FL, Universal Imaging) was used. The results are expressed as the VEGF/β-actin ratio.

**Determination of EP1–4, phosphorylated CREB, CREB, and VEGF protein expression by Western blot analysis.** Protein extraction and Western blotting were performed as described previously (33). The membranes were incubated with rabbit polyclonal anti-EP1, anti-EP2, anti-EP3, or anti-EP4 antibodies (Cayman Chemical, Ann Arbor, MI), mouse monoclonal anti-phosphorylated CREB (pCREB) (Cytokine Signaling Technology, Beverly, MA), or anti-VEGF (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies at room temperature for 1 h. Membranes probed with pCREB were stripped and reprobed using rabbit polyclonal anti-CREB antibody (Santa Cruz Biotechnology), which recognizes both pCREB and unphosphorylated CREB. Signal of bound antibodies was visualized using enhanced chemiluminescence Western blotting detection reagents (Amersham Life Science, Arlington Heights, IL). Protein levels were measured using a computerized video analysis system (Image-1/FL, Universal Imaging).

**Determination of VEGF protein expression by immunohistochemical staining.** Formalin-fixed and paraffin-embedded tissue sections were used. The sections were incubated with mouse monoclonal anti-VEGF antibody overnight at 4°C. After being washed with PBS, the sections were incubated with fluorescein-conjugated secondary antibody. Immunofluorescence signal was evaluated under a Nikon Optiphot epifluorescence microscope with B-filter composition (Nikon, Garden City, NY).

**In vitro studies.** An immortalized HET-1A cell line (49), derived from normal human esophageal epithelium, was used. These cells were provided by Dr. Curtis C. Harris (Laboratory of Human Carcinogenesis, NCI, NIH, Bethesda, MD). The cells were maintained in keratinocyte growth medium-2 medium (Clonetics, San Diego, CA) at 37°C with 5% CO2 and 95% air in a humidified incubator. Cells were used between passages 5 and 15. Before all experiments, cells were serum starved for 24 h. Expression of EP2 and EP3 receptors in HET-1A cells were analyzed by Western blotting using specific antibodies as described above.
The concentration and sources of drugs used for in vitro studies. The final concentrations of drugs in cultured medium were as follows: 10 μmol for misoprostol (Cayman Chemical), 500 μmol for Rp-cAMP (Sigma-Aldrich, St. Louis, MO), and 100 μmol for Sp-cAMP (Sigma-Aldrich).

Effect of misoprostol on VEGF expression in HET-1A cells and on intracellular cAMP levels. HET-1A cells were treated with either misoprostol or its vehicle for 3 or 6 h. HET-1A cells were cultured in a standard 96-well plate at a cell concentration of ~10⁵ cells/ml and serum starved for 24 h. Cells were treated with misoprostol or its vehicle for 15 min. Intracellular cAMP levels were determined using a BIOTRAK cAMP enzyme immunoassay kit (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer’s instructions. cAMP data are expressed in femtomoles per well and represent the means ± SD of 12 separate experiments performed in duplicate.

Effects of Rp-cAMP and Sp-cAMP on VEGF expression in HET-1A cells. HET-1A cells were pretreated with Rp-cAMP, a membrane-permeable inhibitor of protein kinase A, or its vehicle (PBS), for 30 min. Following pretreatment, misoprostol was added for an additional 30 min or 3 h. In separate experiments, cells were treated with either Sp-cAMP, a potent and membrane-permeable cAMP analog and activator of protein kinase A, or PBS for 30 min or 3 h.

Statistical analysis. Results are expressed as the means ± SD. Student’s t-test was used to determine the statistical significance of the differences. One-way ANOVA followed by Bonferroni correction was used for multiple comparisons. Pearson product moment correlation analysis was used to determine the significance of relationships between variables. A P value of <0.05 was considered statistically significant.

RESULTS

Expression of EP receptors in normal and ulcerated rat esophagus and HET-1A cells. We examined the expression of EP receptors in normal and ulcerated rat esophagus and in vitro in cultured human esophageal epithelial cells. In normal esophageal mucosa of rats, there is a high expression level of EP2 receptor protein and a low expression level of EP3 (14.7-fold lower than EP2 levels; Fig. 1A, Mucosa). EP3 receptor protein was predominantly expressed in esophageal muscle layer, which also expressed EP2 receptors (Fig. 1A, Muscle). Human esophageal epithelial HET-1A cells expressed high levels of EP2 receptor protein and only a low level of EP3 receptor protein (8-fold lower than EP2 levels; Fig. 1A). Thus the EP2 receptor is a predominant EP receptor in normal esophageal mucosa. Three days after ulcer induction, EP2 receptor protein levels were significantly increased (1.7-fold higher) in ulcerated vs. normal esophageal tissue. EP2 receptor protein levels remained highly expressed at 6 days and slightly decreased by the 9th day after ulcer induction (Fig. 1B). EP3 receptor protein levels were not significantly affected by esophageal ulceration (Fig. 1B). Expression of EP1 and EP4 receptor proteins in esophageal mucosa was low and was not significantly affected by esophageal ulceration (data not shown).

Esophageal ulceration triggers pCREB, and misoprostol (single dose) enhances pCREB in ulcerated tissue. Because EP2 receptor signaling is mediated by cAMP/CREB pathway, we next examined the phosphorylation of CREB in normal and ulcerated rat esophagus. Three days after ulcer induction, pCREB protein levels were significantly increased in ulcerated vs. normal esophageal tissue, indicating that ulceration triggers CREB phosphorylation and thus its activation (Fig. 2A). Levels of pCREB protein remained significantly elevated 6 days and 9 days after ulcer induction (Fig. 2A). Treatment of rats with a single dose (50 μg/kg) of misoprostol at 3 days after ulcer development significantly increased pCREB levels in ulcerated, but not in normal, esophageal tissue, indicating that misoprostol enhances CREB activation induced by ulceration (Fig. 2B). Misoprostol treatment did not markedly alter total CREB protein levels in either normal or ulcerated esophageal tissue (Fig. 2B).

Esophageal ulceration triggers increase in VEGF, and misoprostol treatment further enhances VEGF expression in ulcerated tissue. Next we examined the expression of VEGF in esophageal tissue following ulceration. Three days after ulcer induction, both VEGF mRNA and VEGF protein levels were significantly increased in ulcerated vs. nonulcerated esophageal tissue (Fig. 3, A and B). Misoprostol treatment significantly increased VEGF expression, and misoprostol treatment further enhanced VEGF expression in ulcerated tissue. We examined the expression of VEGF in cultured rat esophageal epithelial cells following ulceration. Three days after ulcer induction, both VEGF mRNA and VEGF protein levels were significantly increased in ulcerated vs. nonulcerated esophageal tissue (Fig. 3, A and B). Misoprostol treatment significantly increased VEGF expression, and misoprostol treatment further enhanced VEGF expression in ulcerated tissue.

Fig. 1. Expression of E-prostanoid (EP)2 and EP3 receptors in normal and ulcerated esophageal mucosa. A: Western blot analyses of EP2 and EP3 receptor protein expression in rat esophageal mucosal and muscle layers and in human esophageal epithelial cells (HET-1A). In normal esophageal mucosa, predominantly EP2 receptor is expressed, whereas the muscle layer expresses predominantly EP3 receptor. B: top: Western blot analyses of EP2 and EP3 receptor protein expression in normal (N) and ulcerated (UL) esophageal tissue 3, 6, and 9 days after ulcer induction. Esophageal ulceration caused a significant increase in EP2 (but not EP3) receptor expression. Bottom: quantitative analysis of EP2 receptor protein expression. The results are expressed as relative density of EP2 to β-actin. NS, not significant. Values are means ± SD. For each column (n = 6).
Misoprostol accelerates esophageal ulcer healing and stimulates angiogenesis. We then examined the effect of treatment with misoprostol, a PGE1 analog, on the healing of esophageal ulcers and on angiogenesis. Misoprostol treatment significantly accelerated healing of esophageal ulcers, as reflected by almost twofold reduction in the ulcer area in rats treated with misoprostol as compared to vehicle-treated rats (Fig. 2A). Microvessel density in granulation tissue at the ulcer base strongly correlated with VEGF protein levels in ulcerated esophageal mucosa. Misoprostol treatment further increased pCREB expression by 60% and 50%, 6 and 9 days after ulcer induction, respectively, vs. vehicle-treated group (Fig. 5B). Microvessel density in granulation tissue at the ulcer base strongly and inversely correlated with ulcer area ($r = -0.961$; $P < 0.001$). Misoprostol only slightly, but not significantly, increased (vs. vehicle) epithelial cell proliferation at the ulcer margin at both 6 and 9 days after ulcer induction (Fig. 5C). Six days after ulcer induction, rats treated with vehicle, predominantly individual endothelial cells and only a few microvessels with distinct lumina were present in granulation tissue at the ulcer base. In rats treated with misoprostol, numerous microvessels with well-formed lumina were present in granulation tissue at the ulcer base 6 days after ulcer induction (Fig. 6B). Notably, microvessel density in granulation tissue at the ulcer base strongly correlated with VEGF protein levels in ulcerated esophageal tissue ($r = 0.954$; $P < 0.001$).

Misoprostol stimulates VEGF expression in HET-1A cells. Because VEGF expression was predominantly enhanced in esophageal squamous epithelium at the ulcer margin, we investigated whether misoprostol can directly activate the VEGF pathway. We then examined the expression of VEGF protein in esophageal ulcer tissues by immunohistochemical staining. In the nonulcerated esophagus of sham-operated rats, VEGF immunolocalization was detected in the cytoplasm of basal cells of the stratified squamous epithelium in the muscularis mucosae and in the submucosal blood vessels (Fig. 4A). Three days after ulcer induction, a strong cytoplasmic VEGF signal was detected in all epithelial cells, constituting the ulcer margin (Fig. 4B). VEGF signal was also detected in the stromal cells and in microvessels of granulation tissue at the ulcer base (Fig. 4B).

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gene in esophageal epithelial cells in vitro. Treatment with misoprostol significantly increased (vs. vehicle) VEGF mRNA levels in HET-1A cells within 3 h (Fig. 7A) and increased VEGF protein levels at both 3 and 6 h after treatment (Fig. 7B).

cAMP mediates misoprostol-induced pCREB and stimulation of VEGF mRNA expression in HET-1A cells. To determine whether misoprostol-induced VEGF expression is mediated by cAMP, first we determined intracellular cAMP levels in HET-1A cells following misoprostol treatment. Misoprostol dramatically increased (vs. vehicle) intracellular cAMP levels in HET-1A cells within 15 min (misoprostol = 2,346.6 ± 301.9 fmol/well vs. vehicle = 14.4 ± 10.8 fmol/well, P < 0.001). Next, we examined whether cAMP is involved in misoprostol-induced CREB activation and stimulation of VEGF mRNA expression in HET-1A cells. Similar to misoprostol, the cAMP analog Sp-cAMP induced CREB phosphorylation (Fig. 8A) and stimulated VEGF mRNA expression (Fig. 8B) in HET-1A cells within 30 min. Pretreatment with a competitive inhibitor of cAMP-dependent PKA, Rp-cAMP, significantly reduced both misoprostol-induced pCREB and stimulation of VEGF mRNA expression (Fig. 8, A and B). Rp-cAMP itself did not significantly alter basal pCREB protein and VEGF mRNA levels in HET-1A cells (data not shown).

DISCUSSION

The present study demonstrates for the first time that esophageal ulceration triggers upregulation of EP2 (but not EP3) receptor expression in the ulcerated mucosa, CREB activation, and induction of VEGF expression in esophageal epithelial cells, which represents aberrant expression of VEGF. Furthermore, an agonist of EP receptors, misoprostol, enhanced ulceration-induced CREB activation, stimulated VEGF expression and angiogenesis, and accelerated esophageal ulcer healing. Our study demonstrated that only EP2 receptors are strongly

Fig. 4. Photomicrographs of esophageal tissue. A and B: sections immunostained for VEGF. A: in normal esophagus, VEGF fluorescence signal (white) is present in cytoplasm of basal cells of esophageal epithelium (arrow heads), in endothelial cells of blood vessels (arrows), in muscularis mucosae (mm), and fibro/myo-fibroblasts (*). sm, submucosa; ep, epithelium. B: in ulcerated esophagus, VEGF fluorescence signal was significantly increased in the cytoplasm of all epithelial cells of the ulcer margin (ep). gt, granulation tissue (bars = 100 μm). C and D: esophageal sections stained with hematoxylin and eosin. C: esophageal section close to ulcer margin. D: section of the esophageal ulcer margin. A sheet of interconnected squamous epithelial cells forming ulcer margin (UM) migrated onto granulation tissue.

Fig. 5. Misoprostol accelerates esophageal ulcer healing and stimulates angiogenesis. Rats were treated intragastrically twice daily with either 50 μg/kg misoprostol or its vehicle for 3 or 6 days starting 3 days after ulcer induction. A: ulcer healing dynamics. Ulcer area was measured by a computerized video analysis of the ulcer images. The results are expressed as a percentage of ulcer area at day 3. Misoprostol treatment significantly reduced ulcer area, reflecting increased esophageal ulcer healing in rats. B: microvessel density in granulation tissue at the ulcer base. The results are expressed as the number of microvessels per square millimeter of granulation tissue section (n/mm²). Misoprostol treatment significantly increased microvessel density (reflecting angiogenesis) in granulation tissue at the esophageal ulcer base. C: proliferating cell nuclear antigen (PCNA) labeling index (LI) in the epithelium at the ulcer margin. The results are expressed as the percentage of increase in the number of labeled cells in the epithelium of the ulcer margin over the number of labeled cells in the epithelium distant from the ulcer. Misoprostol treatment slightly increased epithelial cell proliferation at the esophageal ulcer margin. NS, not significant. Values are means ± SD. For each column (n = 6).
expressed in esophageal mucosa, whereas EP3 receptors are expressed in esophageal smooth muscle layer. Esophageal ulceration upregulated expression of only EP2 receptors, indicating that these receptors are the likely targets for exogenous and endogenous PGEs during esophageal ulcer healing.

Our previous study demonstrated that esophageal ulceration significantly increases expression of Cox-2 in esophageal keratinocytes in the ulcer margin (2). That study also showed that treatment with selective Cox-2 inhibitor, celecoxib, significantly delayed esophageal ulcer healing (2). These findings indicate that Cox-2 plays an important role in esophageal ulcer healing. However, that study did not examine the role of Cox-2-generated PGEs in VEGF expression, angiogenesis, and healing of esophageal ulcers.

Esophageal ulcer healing encompasses regeneration of the epithelium accomplished by epithelial cell proliferation and migration to reestablish epithelial continuity, regeneration of mesenchymal components, and regeneration of blood microvessels though angiogenesis (52, 53). The latter process takes place in granulation tissue that develops at the ulcer base 48–72 h after ulcer development. Angiogenesis, the formation of new capillary blood vessels, enables delivery of oxygen and nutrients to the healing site, including the epithelium, and is an essential component of ulcer healing (6, 52, 54). PGEs, such as PGE1 and PGE2, have been shown to stimulate angiogenesis in various models of in vivo angiogenesis, including rabbit cornea (59), chicken embryo chorioallantoic membrane models (18), the subcutaneous matrigel plug, and subcutaneous sponge models (30, 43). VEGF is a fundamental and specific stimulator of physiological and pathological angiogenesis (17). Recent studies have demonstrated that PGE2 stimulates VEGF expression in carrageenin-induced granulation tissue in rats (21) and in rat sponge implants (36), implicating VEGF as the mediator of the stimulatory effects of PGEs on angiogenesis. In vitro studies have shown that PGE1 and PGE2 stimulate VEGF expression in osteoblasts and macrophages via increase in cAMP formation (5, 8, 25, 27, 39).

Previous studies suggested that inflammation preferentially upregulates expression of the cAMP-increasing receptors, EP2 and EP4, and not other EP receptors (16, 34). Recent studies have examined the role of PGE and EP2 receptors in inflammation (10, 58), which is also a part of ulcer healing. Our present study demonstrates that esophageal ulceration induces predominantly the expression of the EP2 receptor that is mainly expressed in esophageal mucosal epithelial cells. These results indicate that the effects of PGEs during esophageal ulcer healing are predominantly mediated via EP2 receptors. Because EP2 receptors mediate increases in cAMP production, whereas EP3 receptors antagonize cAMP production (14), preferential expression of EP2 receptor explains the finding that misoprostol dramatically increased intracellular cAMP levels in human esophageal epithelial cells.

In this study, pCREB and VEGF mRNA and protein levels were increased in ulcerated esophageal tissue, indicating that ulceration triggers CREB activation and VEGF expression. Specific EP2 agonists, such as butaprost, are not suitable for the in vivo studies. Therefore, to investigate the role of EP2 receptors in CREB activation and VEGF expression, we used misoprostol, an agonist for EP1–4 receptors (40), which is clinically used for prevention of gastric mucosal injury by NSAIDs.

In the present study, misoprostol increased CREB phosphorylation and VEGF expression in ulcerated esophageal tissue overexpressing EP2 receptors, suggesting that EP2 receptors...
mediate stimulatory effects of PGEs on CREB activity and VEGF expression during esophageal ulcer healing. This contention is further supported by our in vitro studies, which demonstrated that, similar to a cAMP analog, misoprostol induces CREB phosphorylation and stimulates VEGF mRNA expression in cultured human esophageal epithelial cells. Conversely, an inhibitor of cAMP-dependent protein kinase A inhibited these effects of misoprostol, indicating that PGs activate CREB and VEGF gene in esophageal epithelial cells via the EP2/cAMP/protein kinase A signaling pathway. In a separate study, we demonstrated in HET-1A cells cultured under baseline, serum-starved conditions that PGE analog (16,16 dimethyl PGE2) increased proliferation of these cells by 64% (P < 0.001) (our unpublished observation).

In some systems, pCREB activates transcription of several genes, including genes important for tissue injury repair (47). In regard to other tissues, inhibition of prostaglandin synthesis diminished CREB activation and cell proliferation associated with liver regeneration (45). Our present finding that a PGE1 analog activates CREB in ulcerated esophageal tissue suggests that PGs may activate transcription of genes involved in tissue repair, such as VEGF via CREB activation.

In the present study, VEGF was strongly expressed in the epithelium of the ulcer margin, suggesting that esophageal epithelial cells acquire the ability to synthesize VEGF and are the major source of VEGF during esophageal ulcer healing. This finding in respect to esophageal ulcers is corroborated by a previous study demonstrating that VEGF mRNA is expressed by dermal keratinocytes at the skin wound edge (35). Because only endothelial cells possess the VEGF receptors (17), VEGF secreted by esophageal epithelium most likely acts on the endothelial cells in granulation tissue and thus stimulates the angiogenesis in a paracrine manner. Therefore, stimulation of the VEGF expression in the esophageal epithelial cells lining ulcer margins may mediate the stimulatory effects of PGs on angiogenesis during esophageal ulcer healing.

In this study, misoprostol significantly accelerated healing of experimental esophageal ulcers; however, it only slightly (not significantly) increased epithelial cell proliferation at the ulcer margin. This is in agreement with a previous study, which showed that misoprostol reverses NSAID-induced inhibition of epithelial cell proliferation at the gastric ulcer margin but does not further stimulate epithelial proliferation that is already increased by gastric ulceration (13, 35). It should be noted that epithelial cell proliferation in the mucosa of esophageal ulcer margin is already stimulated, and therefore the small increase in proliferation caused by the addition of misoprostol is likely not discernable. However, epithelial cell migration reflected by epithelial regeneration and restoration, a major component of ulcer healing, was significantly increased by misoprostol treatment. In addition, our in vitro study demonstrated that 16,16-dimethyl PGE2 significantly increased HET-1A cell proliferation. The potential mechanisms and comediators of this misoprostol action may include KGF, HGF, and transactivation of EGF receptors, as shown in our previous studies (2–4, 41, 42). The same general principles of healing apply to esophageal, gastric, and intestinal ulcers; however, ulcer healing in these tissues has different characteristics based on anatomical differences and different luminal environments. Although healing of esophageal, gastric, and intestinal ulcers has some common features, such as formation of ulcer margin (epithelial component), formation of granulation tissue at the ulcer base, and angiogenesis (new blood vessel formation), esophageal ulcer healing has several distinct characteristics. First, the esophageal mucosa is composed of a squamous epithelium, whereas gastric and intestinal mucosa is composed of a columnar epithelium and also contains endocrine cells. Second, different growth factors regulating reepithelialization and epithelial regeneration, namely KGF, HGF, and their receptors, appear to be predominant in esophageal ulcers (2–4), whereas EGF and its receptor are predominant in gastric ulcers and intestinal ulcers (52). Third, reepithelialization and regeneration of esophageal epithelium are accomplished via simultaneous migration of several layers of interconnected squamous epithelial cells onto granulation tissue (Fig. 4D; Refs. 2–4), whereas, in
gastric and intestinal ulcers, a single layer of epithelial cells migrates onto granulation tissue (52, 53). Finally, intestinal ulcer healing involves, in addition, activation of intestinal mucosal immune cells, which is not a prominent feature of esophageal ulcer healing.

In summary, this study demonstrated that esophageal ulceration triggers upregulation of EP2 receptor expression and CREB activation. Misoprostol, a PGE1 analog and an agonist for EP1–4 receptors, activates CREB and stimulates VEGF expression in ulcerated rat esophageal tissue as well as in human esophageal epithelial cells. These results suggest that the effects of both endogenous and exogenous PGEs that promote ulcer healing are mediated by activation of CREB, resulting in stimulation of VEGF expression in esophageal epithelial cells via the EP2/cAMP/protein kinase A pathway. The release of VEGF from the regenerating epithelial cells likely activates angiogenesis in granulation tissue and thus facilitates oxygen and nutrient delivery to the healing site, resulting in accelerated ulcer healing. This paradigm may also apply, not only to the healing of esophageal ulcers, but also to the healing action of PGEs on tissue injury in general.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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