Roles of epidermal growth factor and Na\(^{+}/H^{+}\) exchanger-1 in esophageal epithelial defense against acid-induced injury

Yasuhiro Fujiwara,1 Kazuhide Higuchi,1 Takashi Takashima,1 Masaki Hamaguchi,1 Tsuyoshi Hayakawa,1 Kazunari Tominaga,1 Toshio Watanabe,1 Nobuhide Oshitani,1 Yutaka Shimada,2 and Tetsuo Arakawa1

1Department of Gastroenterology, Osaka City University Graduate School of Medicine, Osaka, Japan; and
2Department of Surgery and Surgical Basic Science, Kyoto University Graduate School of Medicine, Kyoto, Japan

Submitted 24 May 2005; accepted in final form 18 November 2005

Epidermal growth factor (EGF) is a multifunctional cytokine that plays critical roles in tissue repair and regeneration (33). EGF protects gastric epithelial cells against acid and promotes gastric epithelial restitution through activation of the Na\(^{+}/H^{+}\) exchanger (NHE) (11, 52). How EGF activates NHE to promote repair is not clear. Calmodulin and the protein kinase C (PKC) inhibitors significantly inhibited cytoprotection by EGF, whereas MEK, phosphatidylinositol 3-kinase, and PKA inhibitors had no effect. EGF significantly increased pH recovery after acidification, and this increase in pH recovery was significantly blocked by inhibitors of calmodulin and PKC. The Na\(^{+}/H^{+}\) exchanger (NHE) is an electroneutral transporter that mediates the ejection of intracellular hydrogen in exchange for external sodium (35, 36). When esophageal luminal acidity is pH 2.0, the microenvironment adjacent to the surface cells is reduced to pH 2.0–3.0 (33). Consequently, the gastric acid in reflux content directly affects epithelial cells and decreases the intracellular pH (pHi) of the cells.

The Na\(^{+}/H^{+}\) exchanger (NHE) is an electroneutral transporter that mediates the ejection of intracellular hydrogen in exchange for external sodium (35, 36). NHE-1 is expressed in virtually all tissues and cells, where it most likely fulfills "housekeeping" functions, including maintenance of pH (35, 36). Several studies have shown that NHE is present in rat and human esophageal epithelial cells (21, 40, 48). In humans, esophageal epithelial cells possess an H\(^{+}\)-extruding mechanism consistent with an NHE (49). Activation of NHE-1 is regulated by several agents, including growth factors such as EGF, and is associated with several intracellular signal mediators (35, 36). EGF is mainly secreted by salivary glands that produce saliva associated with esophageal defense (19). EGF stimulates activity of NHE partially through phosphorylation of the NHE cytoplasmic domain (37) or interaction with the NHE regulatory domain (51). In the gastrointestinal tract, EGF protects rat gastric epithelial cells against acid and promotes gastric epithelial restitution through activation of NHE (11, 52). However, the interactions between EGF and NHE-1 in the esophagus have not been elucidated. We examined the role of EGF and NHE-1 in esophageal epithelial defense against acid using human esophageal epithelial cell lines and a rat chronic acid reflux esophagitis model.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Address for reprint requests and other correspondence: Y. Fujiwara, Dept. of Gastroenterology, Graduate School of Medicine, Osaka City Univ., 1-4-3 Asahi-machi, Abenoku, Osaka 545-8585, Japan (e-mail: yasu@med.osaka-cu.ac.jp).
Cell culture and experimental protocol. The TE-1 human esophageal epithelial cell line was provided by the Cell Resource Center for Biochemical Research Institute of Development, Aging, and Cancer (Tohoku University, Sendai, Japan) (28). TE-1 cells were established from well-differentiated squamous cell carcinoma of the human esophagus, which have been reported to exhibit EGF-R (28). TE-1 cells were grown in RPMI-1640 medium supplemented with 10% FCS. To investigate the effect of acidified medium on cellular injury, culture medium without FCS was titrated with 0.1 M HCl to the desired pH. EGF was added to the cells 30 min before acid exposure. To examine the mechanism of cytoprotection by EGF, amilorides such as EIPA and 5-(N,N-dimethyl)amiloride (DMA), genistein, PD-98059, tyrphostin A46, W-7, BIMI, H-89, or wortmannin were added to the cells 30 min before EGF treatment. Normal human esophageal epithelial cells (HEEC) (17) were used in the pH measurement study. The cells were grown in keratinocyte serum-free medium (pH 7.4) supplemented with bovine pituitary extract and epithelial growth factor (GIBCO-BRL, Life Technologies, Rockville, MD).

Cell viability. Cell viability was assessed by the Cell Titer-Glo luminescent cell viability assay (Promega, Madison, WI). This assay is a homogenous method for determining the number of viable cells in culture based on quantity of ATP present, which signals the presence of metabolically active cells. In brief, Cell Titer reagent was added to the cells after the experiments, and samples were mixed for 2 min and allowed to incubate for 10 min at room temperature. Luminescence was measured using a microplate luminesometer (Wallac 1420, Amer sham Pharmacia Biotech), and data were expressed as a percentage of the control.

Expression of NHE-1 and EGF-R in HEEC. NHE-1 mRNA and protein were detected by RT-PCR and Western blotting as previously described (20). The sense and antisense primers for NHE-1, EGF-R, and GAPDH were S'-GACTACACACACGTGCGCACC-CC-3', S'-TCTCAGACACATCTGATG-3', and S'-TCCGAGGATGATGGGCACGACAGAAGAGAA-3' and 5'-ATGGCCAGTGTAAGGCTGA-3', respectively (7, 27). Polyclonal rabbit anti-NHE-1 antibody (Chemicon International, Temecula, CA) and polyclonal rabbit anti-EGF-R antibody (Santa Cruz Biotechnology, Santa Cruz, CA) were used for Western blot analysis.

Measurement of pH and NHE-1 activity. The pH of single cells was determined by digital fluorescence microscopy (Attofluor, Atto Bioscience, Rockville, MD) using BCECF (20). Cells were plated on glass-bottomed culture dishes (MatTek, Ashland, MA) for 10–24 h and studied in a nominally HCO₃⁻-free HEPES buffer (HBS) containing 140 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, and 10 mM glucose, with 300 mosmol/kgH₂O, pH 7.4, after adjustment with 1 M NMDG. Isosmolar Na⁺-free HEPES buffer was made by replacing Na⁺ with NMDG, and NH₄Cl solution was made isosmolar by replacing Na⁺ with NH₄⁺. Cells were loaded with BCECF-AM (5 μM) for 45 min at 37°C. The ratios of fluorescence images (emission wavelength (520 nm) excited at two wavelengths (490 and 440 nm)) were measured every 10 s for pH recovery experiments or 30 s for standard calibration with 30- to 100-ms exposures. The 490-to-440 nm fluorescence ratios were converted to pH by constructing a calibration curve using high K⁺ buffers (in mM: 150 KCl, 1 CaCl₂, 1 MgCl₂, 10 HEPES buffer) containing nigericin. For studies involving cell acidification, the HCl pulse treatments were performed by exposing the cells to 20 mM NH₄Cl buffer for 15 min (3). The recovery of pH was monitored in HBS, Na⁺-free HBS, or HBS containing NHE inhibitors such as EIPA, DMA, and HOE-642 (Aventis Pharmaceutical, Frankfurt, Germany). To determine signal pathway of NHE-1 activation by EGF, tyrphostin A46 (100 μM), BIMI (50 nM), W-7 (10 μM), H-89 (10 nM), or wortmannin (10 nM) was added to the cells before EGF treatment. Alternatively, cell acidification was achieved by exposing the cells to an HCl-acidified HBS solution (pH 3.0), monitoring the pH, and then measuring any subsequent decrease in the pH of the cells.

Chronic acid reflux esophagitis rat model. Specific pathogen-free male Wistar rats (Japan SLC, Hamamatsu, Japan) weighing ~200 g were used. Chronic acid reflux esophagitis was produced according to the method of Omura et al. (32). The duodenum near the pylorus was wrapped with a piece (approximate width, 1 mm) of 18-Fr Nélaton catheter (Sapheed; Terumo, Tokyo, Japan), and the transitional region between the forestomach and the glandular portion was ligated to enhance reflux of gastric acid into the esophagus. Rats were killed 3 wk after the operation. Sham-operated rats were used as a control group. The lesion index was defined as the total area of esophageal lesions (10). To investigate the effect of endogenous EGF on the severity of chronic esophagitis, rats underwent surgical removal of submaxillary and submandibular glands 4 wk before induction of esophagitis. In other experiments, some rats with sialoadenectomy were treated with 15 μg·kg⁻¹·day⁻¹ of human recombinant EGF in drinking water after induction of esophagitis. All experimental procedures were approved by the Animal Care Committee of Osaka City University Medical School.

Bromodeoxyuridine uptake. To assess epithelial proliferation, bromodeoxyuridine (BrdU) uptakes were performed and gauged with the BrdU labeling index (9).

RNA isolation and real-time RT-PCR. We extracted total RNA from the esophagus using ISOGEN (Nippon Genetics). The PCR primers and TaqMan probes for real-time EGF-R and NHE-1 were as follows: the sense primers for EGF-R and NHE-1 were 5'-ATCACCTGGCTTGGTGTAGCT-3' and 5'-CCCCATGTGTGATCTGTCCTCC-3', respectively, and the antisense primers were 5'-AGCAGGATGACGCAACAGTTGCTTA-3' and 5'-ATGTCATCGCCTGGACA-3', whereas TaqMan probes were 5'-CATTTGGGCTGCTCTATTA-3' and 5'-CCACATACCCGCTTCTTCAAGCT-3', respectively. We performed real-time quantitative RT-PCR analyses using an ABI PRISM 7700 sequence detection system instrument and associated software (PE Applied Biosystems). The levels of each mRNA were expressed as ratios to the mean value for normal esophageal tissue (13).

Western blot analysis. Esophageal tissues were homogenized, and total protein was separated in Tris-HCl-NaCl-EDTA buffer containing 10 μg/ml PMSF, 60 μg/ml aprotinin, and 1 mmol/l sodium orthovanadate. Target protein was separated with SuperSep 5–20% (Wako) and transferred to PVDF membrane (Nippon Genetics, Tokyo, Japan). The membrane was blocked with blocking reagent and incubated with anti-NHE-1 antibody (Alpha Diagnostic International, San Antonio, TX) at a 1:500 dilution overnight at 4°C or anti-EGF-R antibody (Santa Cruz) at a 1:50 dilution for 2 h at room temperature. After the membrane was washed with Tris-buffered saline containing 0.1% Tween 20, the membrane was incubated with anti-rabbit IgG conjugated with horseradish peroxidase.
(Amersham Pharmacia Biotech) at 1:1,000 dilution for 1 h at room temperature. Detection of target protein was achieved with an ECL Western blotting detection system (Amersham) and visualized with an LAS-1000 plus Image reader (Fuji Photo-Film).

Statistical analysis. Values are expressed as means and standard deviations (SD). Differences between the groups were compared using one-way ANOVA followed by Fisher’s paired least-significant difference test. P < 0.05 was considered significant.

RESULTS

NHE-1 and EGF-R expressions in esophageal epithelial cells. Amplifications of cDNA with the NHE-1, EGF-R, and GAPDH primers used in this study were predicted to yield fragments of 250, 512, and 371 bp in length, respectively. NHE-1 and EGF-R mRNA expression were detected in both TE-1 and HEEC cell lines (Fig. 1A). Western blot analyses using NHE-1- and EGF-R-specific antibodies detected NHE-1 and EGF-R protein expression as molecular masses of ~90 and ~170 kDa in both TE-1 and HEEC cells (Fig. 1B).

EGF prevents acid-induced cell injury. TE-1 cells were incubated with acidified medium at pH 2.0, 3.0, and 4.0 for 5–15 min. Acidified medium induced cellular injury in a pH- and time-dependent manner, whereas acidified medium at pH 4.0 did not affect the viability of TE-1 cells (Fig. 2A). EGF prevented acid-induced cellular injury resulting from 10-min incubation in acid. Open bars, vehicle-treated cells; closed bars, EGF-treated cells. Values are means and SD of each of 8 experiments. *P < 0.01 vs. control (acid alone). **P < 0.05 vs. control (acid alone).

Statistical analysis. Values are expressed as means and standard deviations (SD). Differences between the groups were compared using one-way ANOVA followed by Fisher’s paired least-significant difference test. P < 0.05 was considered significant.
used in subsequent experiments. To examine whether EGF protects TE-1 cells against acid-induced injury, EGF (100 ng/ml) was added to the cells 30 min before acid exposure, and then the cells were incubated with acidified medium (pH 3.0) for 5, 10, and 15 min. EGF significantly prevented acid-induced cell injury resulting from 10-min incubation in acid (Fig. 2B). Because the cytoprotective effect by EGF at 10-min incubation in acid was minimal, we used 15-min incubation in acidified medium (pH 3.0) subsequent experiments. When TE-1 cells were treated with various doses (0.1, 1, 10, 100 ng/ml) of EGF 30 min before acid exposure (pH 3.0 for 15 min), cytoprotective effects by EGF were observed in a dose-dependent fashion (Fig. 2C).

Effects of different agents on EGF cytoprotection against acid-induced cell injury. EIPA and DMA completely blocked EGF-induced cytoprotection against acid (Fig. 3A), suggesting that the cytoprotective effects by EGF were mediated with NHE-1. Next, we examined the intracellular signals associated with the cytoprotective effects caused by EGF against acid-induced cell injury. Genistein (tyrosine kinase inhibitor), BIMI (PKC inhibitor), and W-7 (calmodulin inhibitor) each significantly inhibited the cytoprotective effect of EGF, whereas PD-98059 (MEK1/2 inhibitor), tyrphostin A46 [specific inhibitor of EGF-R kinase/EGF-R MAPK signal transduction pathway (47)], wortmannin [phosphatidylinositol 3-kinase (PI3-kinase) inhibitor], and H-89 (PKA inhibitor) did not alter the cytoprotective effects of EGF (Fig. 3B). This result suggests that EGF prevented acid-induced cellular injury via the PKC and Ca$^{2+}$/calmodulin pathways but not via the PI3-kinase, MAPK, and PKA pathways. At the dose used in this study, the inhibitors had no effect on the viability of the TE-1 cells (data not shown).

Effect of EGF on changes in $pHi$. Figure 4A shows the effect of EGF on baseline $pHi$ of TE-1 cells. EGF (100 ng/ml) did not affect the baseline $pHi$ of TE-1 cells. Exposure of NH$_4$Cl in HBS resulted in a rapid increase in $pHi$, and the $pHi$ decreased rapidly to acidic levels after the NH$_4$Cl solution was removed. When NH$_4$Cl solution was replaced with HBS, the $pHi$ increased from acidic level toward baseline (Fig. 4B). This $pHi$ recovery was not observed when the NH$_4$Cl solution was replaced with either Na$^{+}$-free HBS or HBS containing amiloride (data not shown). Figure 4C shows $pHi$ recovery of TE-1 cells after cellular acidification on TE-1 cells using the NH$_4$Cl pulse technique and effect of EGF on $pHi$ recovery in the absence or presence of EIPA. $pHi$ recovery in EGF-treated cells was more rapid compared with that shown in controls, and this increase in $pHi$ recovery was completely blocked by treatment with EIPA. EGF significantly induced the increase in $pHi$ recovery rate of TE-1 cells compared with controls. This increase in $pHi$ recovery by EGF was blocked by treatment with anti-EGF-R antibody. Na$^{+}$-free HBS and HBS containing NHE-1 inhibitors, such as EIPA, DMA, and HOE642, completely inhibited $pHi$ recovery after cell acidification (Fig. 5A). Similarly, EGF (100 ng/ml) significantly induced the increase
in pH recovery rate of HEEC after NH4Cl pulse acidification from 0.038 ± 0.004 pH U/min in controls to 0.052 ± 0.004 pH U/min in EGF-treated cells (P < 0.001). To determine the signal pathway associated with EGF-induced increase in pH recovery after pulse acidification, tyrphostin A46, BIMI, W-7, H-89, or wortmannin was added to the cells before EGF treatment. BIMI and W-7 significantly inhibited increased pH recovery rate of TE-1 cells by EGF, whereas tyrphostin A46, H-89, and wortmannin did not affect the increase in pH recovery rate by EGF (Fig. 5B). In a separate experiment, TE-1 cells were incubated with acidified medium at pH 2.0–4.0, and changes in pH of TE-1 cells were measured. The pH of TE-1 cells gradually decreased in a pH- and time-dependent manner (data not shown). EGF (100 ng/ml) significantly inhibited decreased pH of TE-1 cells from −0.206 ± 0.015 pH U/min in controls to −0.162 ± 0.008 pH U/min (P = 0.0096) after acid exposure (Fig. 4D).

Sialoadenectomy caused increased severity of rat chronic acid reflux esophagitis without affecting epithelial proliferation. Figure 6A shows the macroscopic appearance of chronic acid reflux esophagitis in sham-operated rats (control) and rats with sialoadenectomy. Lesions of chronic esophagitis were found in the middle and the lower parts of the esophagus. The esophagitis lesion index in rats with sialoadenectomy was 2.8-fold greater than in control animals (Fig. 6B). Histologically, normal esophagus has a thin epithelial layer, whereas marked esophageal mucosal thickening and elongation of the lamina propria papillae, basal cell hyperplasia, and inflammatory cell infiltration in the submucosa were observed (Fig. 6C). A few BrdU-positive cells were found in the normal esophageal epithelium and in the background mucosa adjacent to the esophagitis lesions, whereas many BrdU-positive cells were observed in regions of basal cell hyperplasia in rat chronic esophagitis. The BrdU labeling index was significantly increased in lesions of chronic esophagitis compared with normal esophageal mucosa and background mucosa adjacent to esophageal lesions in chronic esophagitis (Fig. 6D). There was no significant difference in the BrdU labeling index in esophageal lesions between the control and sialoadenectomy groups, suggesting that sialoadenectomy had no effect on epithelial proliferation in rat chronic esophagitis.

Increased expression of EGF-R and NHE-1 in rat chronic esophagitis. Western blotting revealed that both EGF-R and NHE-1 protein expression significantly increased in esophageal lesions compared with that shown in normal esophageal tissue and background tissue adjacent to esophageal lesions (Fig. 7A). There was no significant difference in EGF-R and NHE-1 protein expression between the control and sialoadenectomy groups. Real-time RT-PCR revealed that mRNA expression of EGF-R and NHE-1 significantly increased by ~80% and 230%, respectively, compared with that in normal esophageal tissue (P < 0.01). Expressions of EGF-R and NHE-1 mRNA in esophageal lesions were significantly higher than in background tissue adjacent to esophageal lesions. There was no difference in EGF-R mRNA expression in esophageal lesions between the control and sialoadenectomy groups, whereas NHE-1 mRNA expression was significantly higher in rats with sialoadenectomy than in control rats (Fig. 7, B and C).
Effect of exogenous EGF administration on development of chronic esophagitis in rats with sialoadenectomy. Exogenous administration of EGF in drinking water significantly reduced the worsening of chronic esophagitis in rats with sialoadenectomy from 73.92 ± 24.50 to 21.30 ± 5.38 mm² (P < 0.05). There was no obvious difference in histological findings in esophageal lesions among the control, sialoadenectomy, and sialoadenectomy with exogenous EGF administration groups.

**DISCUSSION**

The present study demonstrated that EGF protects esophageal epithelial cells against acid-induced cell injury. Because NHE-1 inhibitors completely inhibited the protective effect by EGF, cytoprotection by EGF is mediated through NHE-1. The pH recovery after NH₄Cl pulse acidification was observed in Na⁺-containing medium but not in Na⁺-free medium and medium containing NHE-1 inhibitors, suggesting that NHE-1 is the main H⁺ extruder of esophageal epithelial cells. EGF significantly stimulated pH recovery after NH₄Cl pulse acidification and inhibited the decrease in pH after acid exposure. These results suggest that EGF stimulates NHE-1 activity of human esophageal epithelial cells. Because treatment with genistein, W-7, and BIMI each significantly inhibited the cytoprotective effects of EGF and increased pH recovery rate after NH₄Cl pulse acidification and tyrphostin A46, PD-98059, wortmannin, and H-89 had no effect, EGF protects esophageal epithelial cells against acid-induced cell injury through NHE-1 activation via Ca²⁺/calmodulin and the PKC pathway independently of the MEK, PI3-kinase, and PKA pathways.

NHE-1 is activated by growth factors, hormones, and osmotic stress directly or through regulation of signaling networks (36). EGF activates NHE in several types of cells such as fibroblasts (26, 37), hepatocytes (44), chondrocytic cells (23), and mesothelial cells (22). Activation of NHE-1 by receptor tyrosine kinases in response to growth factor stimulation occurs by the MAPK pathway, Ras-Raf1-MEK-ERK signaling (2), and p90 ribosomal S6 kinase (p90RSK) acts downstream of ERK to phosphorylate NHE-1 (41). Our data indicate that this classical MAPK signaling was not associated with cytoprotection by EGF through NHE-1 activation because tyrphostin A46 (specific inhibitor of EGF-R kinase/EGF-R MAPK signal transduction pathway) had no effect. Alternative
Ras-independent signaling may also have been involved in the activation of NHE-1 by receptor tyrosine kinase in response to growth factor stimulation. Several studies have shown that phospholipase C and PI3-kinase play crucial roles in the regulation of NHE-1 activity through Ras-independent signaling (6, 22, 25). Di Sario et al. (6) demonstrated that platelet-derived growth factor stimulated NHE-1 activity of rat hepatic satellite cells through the PKC pathway, independent of the PI3-kinase pathway. Similarly, our results demonstrated that cytoprotection by EGF against acid-induced cell injury through NHE-1 activation was mediated by the PKC pathway, independent of the PI3-kinase pathway, because BIMI (PKC inhibitor) but not wortmannin (PI3-kinase inhibitor) blocked the cytoprotective effect of EGF and increased pH, recovery after NH₄Cl pulse acidification. Direct regulation of NHE-1 activity is mediated by the COOH-terminal cytoplasmic domains. Binding and interaction sites for structural and regulatory proteins on the COOH-terminal cytoplasmic domain of NHE-1 include phosphatidylinositol 4,5-bisphosphate; ezrin, radixin, and moesin; calcineurin B homologous protein; calmodulin; and three serine/threonine kinases such as Rho kinase, Nck-interacting kinase (NIK), and p90RSK (36). Our data indicate that there are at least two pathways (PKC and Ca²⁺/calmodulin) involved in cytoprotection by EGF against acid through NHE-1 activation. These two pathways may function in parallel or converge to a common pathway. The role of the COOH-terminal domains with respect to NHE-1 phosphorylation in response to EGF in esophageal epithelial cells is unknown.

We used a chronic acid reflux esophagitis rat model in which histological findings are similar to those found in patients with GERD (14). Because the rat esophagus is anatomically devoid of submucosal glands, sialoadenectomy decreases the amounts of several compounds derived from saliva, especially EGF. The present study first showed that sialoadenectomy is associated with worsening severity of chronic acid reflux esophagitis in rats. Because exogenous administration of EGF in drinking water, used at a physiological dose of 15 μg·kg⁻¹·day⁻¹ (30), prevented the worsening severity of esophagitis in rats with sialoadenectomy, although EGF is also synthesized in duodenal Brunner’s glands and Paneth cells of the gastrointestinal tract (46), salivary EGF plays a significant role in the development of esophagitis in salivary contents. In addition, suppression of gastric acid secretion by EGF is not associated in this model because neither exogenous administration of EGF nor removal of salivary glands had any influence on gastric acid secretion in rats (31). Because esophageal epithelial proliferation and EGF-R expression in the lesions of esophagitis were increased in both the control and sialoadenectomy group, other growth factors apart from salivary EGF may have contributed to an increase in the epithelial proliferation of the basal layer. Several studies have shown that transforming growth factor-α (15), hepatocyte growth factor (42), and keratinocyte growth factor (1) stimulate esophageal epithelial proliferation, and our previous study showed that transforming growth factor-α mRNA expression was increased in rat chronic esophagitis with a strong expression in the superficial layer of esophageal lesions (10). Together, a deficiency in salivary EGF is associated with increasing severity of chronic esophagitis without affecting epithelial proliferation.

The mechanisms underlying the increasing severity of chronic esophagitis are unknown. Interestingly, NHE-1 mRNA expression was higher in the esophageal lesions, especially of rats with sialoadenectomy compared with rats with normal mucosa. Because the acidic reflux content directly affects esophageal epithelial cells and decreases the pH of such cells (33), chronic acid exposure may trigger NHE-1 gene expression in esophageal lesions. Upregulation of NHE-1 mRNA in the esophageal lesions of rats with sialoadenectomy, however, showed no evidence of direct NHE-1 gene induction by salivary EGF. This may be associated with the reduction of NHE-1 activity due to a deficiency in endogenous EGF, since EGF activates NHE-1 in esophageal epithelial cells in vitro. In

Fig. 7. Expression of EGF-R and NHE-1 mRNA in rat chronic esophagitis by real-time RT-PCR. A: both EGF-R and NHE-1 protein expression significantly increased in esophageal lesions compared with that in normal esophageal tissue and background tissue adjacent to esophageal lesions. There was no significant difference in EGF-R and NHE-1 protein expression between the control and SAD groups. C, control group; S, sialoadenectomy group. B: expression of EGF-R mRNA in esophageal lesions significantly increased by ~80% compared with normal esophageal tissue, but no differences in EGF-R mRNA expression were observed between the control and SAD groups. C: expression of NHE-1 mRNA increased 2.3-fold compared with control, and NHE-1 mRNA expression of esophagitis in rats with SAD was significantly higher than in controls. Open bars, control group; closed bars, SAD group. Values are means and SE of each of 10 experiments. *P < 0.01 vs. normal esophageal tissue or background mucosa adjacent to esophagitis.
addition, NHE-1 is involved in the control of cell growth and proliferation (12) and acid pulse exposure stimulates Barrett’s esophageal epithelial cells through NHE-1 (8). Increased expression of NHE-1 mRNA in chronic esophagitis may contribute to the regulation of pH, as well as the stimulation of esophageal epithelial proliferation of the basal layer.

Because salivary EGF usually affects esophageal epithelial cells from the luminal side of the esophagus and EGF-Rs are present on the basolateral side of cells of esophageal epithelial cells, it is feasible that salivary EGF actually affects esophageal epithelial cells in the basal layer by binding to EGF-R. Recently, Tobey et al. (50) demonstrated that luminal acid or acidified pepsin reduced transepithelial electrical resistance and increased shunt permeability with the dilated intercellular space of the esophageal epithelial cells without affecting macroscopic and histological epithelial injury and consequently this shunt leak allowed luminal EGF (6 kDa) to diffuse across the epithelium. Our recent study (29) showed that EGF decreased acid-induced paracellular permeability of FITC dextran using TE-1 cells cultured on a semipermeable membrane. Therefore, salivary EGF could enter esophageal epithelial cells in the basal layer in the presence of continuous acid exposure. These findings are further supported by gastric studies, which showed that EGF-Rs are localized to basolateral and luminal surface of epithelial cells at the gastric ulcer margin and EGF binds damaged basolateral gastric epithelial cells (45).

Although our results showed that sialoadenectomy is associated with the severity of esophagitis due to a deficiency in salivary EGF, other components in saliva may also affect the severity of esophagitis. Sialoadenectomy reduced the amount of glycoprotein adhering to the esophageal epithelium with increasing severity of chronic esophagitis (18) and resulted in a significant increase in permeability of the esophageal mucosa to hydrogen ions and a decrease in mucous content on the surface of the esophagus (38). Although the pre-epithelial defense of the esophagus is very poorly developed (33, 34), esophageal mucin and mucin-lipid complex in saliva may affect the results presented here.

Clinically, the role of salivary EGF in GERD is controversial (19), and it is unknown whether Séjögren syndrome is strongly associated with GERD. Although EGF is mainly produced by the salivary glands, the submucosal glands of the esophagus, the capillary endothelium of the normal esophageal papillae, and the basal mucosa can also produce EGF in humans (16). Local production of EGF in the esophagus may affect the interpretation of the contradictory results concerning salivary EGF in patients with GERD that show no clear association between Séjögren syndrome and GERD.

In conclusion, EGF protects human esophageal epithelial cells against acid-induced injury through activation of NHE-1, possibly via both the PKC and/or Ca²⁺/calmodulin pathways. Deficiency of endogenous EGF is associated with increasing severity of rat chronic acid reflux esophagitis without affecting epithelial proliferation and EGF-R expression and induced upregulation of NHE-1 mRNA of the esophageal lesions. EGF and NHE-1 play crucial roles in the esophageal epithelial defense against acid.

ACKNOWLEDGMENTS

The authors thank Junko Kawawaki for technical assistance of pHı measurement study.

GRANTS

This study was supported, in part, by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology in Japan.

REFERENCES