

Epidermal Growth Factor Protects Rat Epithelial Cells Against Acid-Induced Damage Through the Activation of Na⁺/H⁺ Exchangers

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Accepted for publication August 28, 1998 This paper is available online at <http://www.jpvet.org>

ABSTRACT

We examined the effect of epidermal growth factor (EGF) on acid-induced cell damage in rat gastric epithelial cells (RGM1) and investigated the mechanisms of this effect. Cells were incubated with EGF for 5, 15, 30, and 60 min, and then immersed in an acidified medium (pH 4.0) for 30 min to induce cell damage. EGF prevented cell damage in a concentration- and time-dependent manner. EGF reduced the effects of the acidified medium on the cells, preventing the reduction of intracellular pH. Replacement of Na⁺ with K⁺ in the acidified medium canceled the effect of EGF. Indomethacin and W-7 (a Ca-

calmodulin inhibitor) did not alter the protective effect of EGF. In contrast, genistein (a tyrosine kinase inhibitor), amiloride (a Na⁺/H⁺ exchangers I and II inhibitor), and wortmannin (a phosphatidylinositol 3-kinase inhibitor) significantly decreased the effect of EGF. Expression of Na⁺/H⁺ exchangers type I and type II was confirmed by reverse transcription-polymerase chain reaction. Our results demonstrate that EGF prevents acid-induced cell damage, most likely through the activation of a Na⁺/H⁺ exchanger II via phosphatidylinositol 3-kinase.

Epidermal growth factor (EGF) is known to play an important role in the pathophysiology of the stomach (Szabo et al., 1995; Playford et al., 1996). In vivo studies have shown that EGF promotes the healing of gastric ulcers (Dembinski et al., 1982; Kuwahara et al., 1989). Through stimulation of DNA synthesis, EGF can prevent acidified aspirin-induced gastric mucosal injury (Konturek et al., 1981). In in vitro studies, EGF has been shown to prevent gastric epithelial cell damage induced by noxious agents (Hiraishi et al., 1984; Ishikawa et al., 1992). In addition, several studies have demonstrated that EGF promotes Na⁺ absorption in the intestine (Donowitz et al., 1994; Khurana et al., 1996). These reports suggest that the process of Na⁺ absorption is partially due to the activation of Na⁺/H⁺ exchangers on the brush border membrane. Recently, several types of Na⁺/H⁺ exchangers (NHE1, NHE2, NHE3, and NHE4) were cloned (Orlowski et al., 1992; Collins et al., 1993) and the expression of Na⁺/H⁺ exchangers was demonstrated in the stomach as well as in the intestine (Bookstein et al., 1994; Ghishan et al., 1995). In this study, we examined the effect of EGF on acid-induced cell damage using a rat gastric epithelial cell line (RGM1) (Hassan et al., 1996; Furukawa et al., 1997). The mechanism

of the protective effect of EGF was elucidated by focusing on Na⁺/H⁺ exchangers.

Materials and Methods

Chemicals. Human recombinant EGF was provided by Otsuka Pharmaceuticals (Tokushima, Japan). Indomethacin, 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), and amiloride were purchased from Sigma Chemical Co. (St. Louis, MO); bovine serum albumin fraction V (BSA) and genistein were purchased from Nacalai Tesque (Kyoto, Japan); and W-7 was purchased from Seikagaku Industries (Tokyo, Japan). Wortmannin was purchased from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA) and 3'-O-acetyl-2',7'-bis(carboxyethyl)-4 or 5-carboxyfluorescein, diacetoxymethyl ester (BCECF-AM) was purchased from Dojindo (Kumamoto, Japan). TRIzol was purchased from GIBCO BRL, Life Technologies, Inc. (Rockville, MD).

Cell Culture. RGM1 cells, passaged in Ham's F12/Dulbecco's-MEM supplemented with 20% fetal bovine serum (FBS), were cultured at 2 × 10⁴ cells/well in 96-well flat-bottomed plates (CORNING Costar, Corning, NY). The cells were maintained at 37°C under 5% CO₂ in air for 24 h. Cells that reached confluency were used for the experiments.

Induction of Cell Damage. The test solution containing confluent cells was aspirated and the cells were washed with a normal buffer. Cells were then placed in one of two acidified mediums (pH 4.0): either phosphate-buffered saline (PBS, comprised of the following: 136.9 mmol/l NaCl, 2.68 mmol/l KCl, 8.09 mmol/l Na₂HPO₄, 1.47

Received for publication February 2, 1998.

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ABBREVIATIONS: BSA, bovine serum albumin fraction V; EGF, epidermal growth factor; PI-3 kinase; phosphatidylinositol 3-kinase; RT-PCR, reverse transcription-polymerase chain reaction.

mmol/l KH₂PO₄, 0.9 mmol/l CaCl₂, and 0.49 mmol/l MgCl₂) or Na⁺-free PBS (Na⁺-free PBS, comprised of the following: 139.58 mmol/l KCl, 8.09 mmol/l K₂HPO₄, 1.47 mmol/l KH₂PO₄, 0.9 mmol/l CaCl₂, and 0.49 mmol/l MgCl₂). The effect of Na⁺ concentration was examined by serial replacement of Na⁺ with K⁺ (solutions A to E), as shown in Table 1. Thirty minutes later, the cells were washed twice with normal buffer and subjected to a viability assay.

Estimation of Cell Viability. A colorimetric assay was performed using MTT in 50 μ l of culture medium. In brief, 10 μ l of MTT solution (5 mg/ml in PBS) was first added to the culture medium. The cells were then incubated at 37°C under 5% CO₂ in air for 4 h. Subsequently, 150 μ l of 0.04 mol/l HCl in isopropanol was added and the mixture was left at room temperature for 18 h. Color change was measured with a microplate reader (Molecular Devices, Menlo Park, CA) at 595 nm and 650 nm.

Measurement of Intracellular pH (pHi). Changes in the pHi of RGM1 cells were detected with an image analyzer (Attofluor Ratio-Vision, Karl Zeiss Co., Ltd., Obernkochen, Germany). RGM1 cells cultured in a glass-bottomed culture dish (MatTek Co., Ashland, MA) were incubated with 0.3 μ M BCECF-AM for 30 min in a respiratory buffer comprised of the following: 1 mmol/l CaCl₂, 1.2 mmol/l MgSO₄, 114 mmol/l NaCl, 5.4 mmol/l KCl, 10 mmol/l glucose, 1 mmol/l NaH₂PO₄, 5 mmol/l Na₂HPO₄, pH 7.4, and 1 mg/ml BSA at 37°C. Cells were washed three times with this warmed respiratory buffer. Changes in pHi were then examined for 30 min under mediums of different pH compositions. Approximately 12 to 30 cells, in focus under a microscope, were selected as samples for the determination of pHi. The data was analyzed using AttoGraph software (Toyko, Japan).

Treatment with Various Agents. EGF was dissolved either in a normal buffer or a Na⁺-free buffer. Buffer compositions were as follows. Normal buffer (pH 7.4): 114 mmol/l NaCl, 5.4 mmol/l KCl, 10 mmol/l glucose, 1 mmol/l NaH₂PO₄, 1 mmol/l CaCl₂, 1.2 mmol/l MgSO₄, along with 2.5 mg/ml BSA, and 1% FBS. Na⁺-free buffer (pH 7.4): 119.4 mmol/l KCl, 10 mmol/l glucose, 1 mmol/l KH₂PO₄, 5 mmol/l K₂HPO₄, 1 mmol/l CaCl₂, 1.2 mmol/l MgSO₄, along with 2.5 mg/ml BSA and Na⁺-removed 1% FBS. Cells were incubated at 37°C for 5, 15, 30, and 60 min with EGF dissolved in one of the buffers. To further elucidate the mechanism of EGF, the cells were pretreated with one of the following agents for the indicated lengths of time, before the application of a 10-ng/ml EGF treatment for 30 min: genistein (a tyrosine kinase inhibitor, 10⁻⁷ to 10⁻⁵ mol/l for 30 min), W-7 (a Ca-calmodulin inhibitor, 10⁻⁷ to 10⁻⁵ mol/l for 30 min), wortmannin [a phosphatidylinositol 3-kinase (PI-3) inhibitor, 10⁻⁹ to 10⁻⁷ mol/l for 30 min], and indomethacin (a cyclooxygenase inhibitor, 10⁻⁵ mol/l for 4 h). Amiloride (Na⁺/H⁺ exchangers I and II inhibitor, 10⁻⁹ to 10⁻⁷ M) was not pretreated, and the mixture of this agent and normal acidified PBS was used for the induction of cell damage.

RNA Extraction and Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Total RNAs from RGM1 cells and rat kidney were extracted by TRIzol. cDNA was synthesized from RNA samples in 15 μ l of reaction buffer containing 50 mM Tris-HCl, 75 mM KCl, and 3 mM MgCl₂ at 37°C for 60 min. The RNA samples contained 200 U Moloney murine leukemia virus reverse transcriptase, 20 pmol of downstream primer, 4 μ g of yeast tRNA, 2.5 mM each deoxyribonucleotide, 10 mM dithiothreitol, and 2 U of ribonu-

lease inhibitor. For the PCR reaction, 10 μ l of cDNA was supplemented with 5 μ l of 10 times PCR buffer, 5 μ l of a 25-mM MgCl₂ solution, 10 pmol of each primer (shown in Table 2), 1 μ l of a 25-mM deoxyribonucleotide solution, and 1.25 U *Taq* polymerase, for a final total volume of 15 μ l. Denaturation of the samples at 94°C (4 min) was followed by 30 cycles consisting of denaturation at 94°C (1 min), annealing at 60°C (1 min), and extension at 72°C (1 min). PCR was completed by a final extension step for 10 min at 72°C. DNA was resuspended and the PCR products were size fractionated on 2% agarose gels stained with ethidium bromide. Oligonucleotide primers for PCR of NHE1, NHE2, NHE3, NHE4, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and size of PCR products were shown in Table 2.

Statistical Analysis. Data are means \pm S.E. for six cultures. Statistical significance was evaluated using the Dunnett's multiple comparison test or Student's *t* test; a *P* value of <0.05 was regarded as significant.

Results

Time Course of Effect of EGF on Acid-Induced Cell Damage. Viability of the cells incubated with EGF in the normal buffer remained unchanged in all groups (data not shown). Cell damage, induced by the acidified medium, was inhibited by EGF in both time- and concentration-dependent manners (Fig. 1). After more than 30 min, EGF at 1 and 10 ng/ml significantly inhibited the cell damage. At 30 min, inhibition amounted to 40.3% and 63.7% with 1 and 10 ng/ml EGF, respectively, when compared with acid damage to untreated cells. Due to the marked inhibitory result, we selected 10 ng/ml EGF at 30 min as the standard conditions for evaluating the effects of several agents on the protective effect of EGF.

Effect of EGF on pHi. Although RGM1 cells were exposed to the pH 7.4 medium, the pHi did not change throughout 30 min of observation. However, in the case of the pH 4.0 medium, the pHi abruptly decreased, then leveled off, reaching pH 6.3 by *t* = 30 min. When cells were pretreated with 10 ng/ml EGF before acidification, the acid-induced decrease in pHi was markedly attenuated (Fig. 2).

Effect of EGF Pretreatment With or Without Na⁺ Medium. When cells were incubated with EGF in a Na⁺-free buffer at pH 7.4 for 30 min, cell viability did not change compared with the cells in the normal buffer (data not shown). When the cells were placed in an acidified medium, the protective effect of EGF was observed in both groups in a concentration-dependent manner. The degree of the protective effect of EGF was not influenced by the different buffers. (Fig. 3).

TABLE 2
Oligonucleotide primers for RT-PCR

		Position	RT-PCR Product
NHE1	TCTGCCGTCTCAACTGTCTCTA	2568	422
	CCCTTCAACTCCTCATTACCA	2968	
NHE2	GCAGATGGTAATAGCAGCGA	2254	310
	CCTTGGTGGGGGCTTGGGTG	2546	
NHE3	GGAACAGAGGCGGAGGAGCAT	1885	321
	GAAGTGTGTGCCAGATTCTC	2186	
NHE4	GGCTGGGATTGAAGATGTATGT	1972	501
	GCTGGCTGAGGATTGCTGTAA	2451	
GAPDH	CCAGTATGATTCTACCCACGGCAA	165	625
	ATACTTGGCAGGTTTCTCCAGCGC	766	

TABLE 1
Ion compositions of buffers used in Fig. 4A

(mmol/l)	PBS	Solution				
		A	B	C	D	E
Na ⁺	153.0	15.3	1.53	0.153	0.0153	0
K ⁺	4.1	141.9	155.7	157.1	157.2	157.2
Cl ⁻	142.4	142.4	142.4	142.4	142.4	142.4
PO ₄ ³⁻	9.5	9.5	9.5	9.5	9.5	9.5
Ca ⁴⁺	0.9	0.9	0.9	0.9	0.9	0.9
Mg ⁺⁺	0.5	0.5	0.5	0.5	0.5	0.5

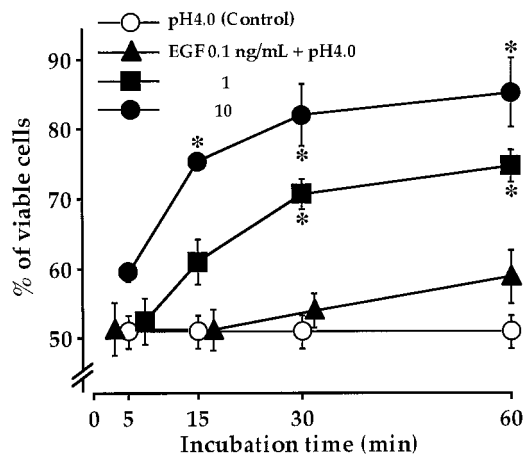


Fig. 1. Time course of effect of EGF on acid-induced cell damage to RGM1. Note that EGF prevented the cell damage in time- and concentration-dependent manner. Data are means \pm S.E. for six cultures. * indicate significant ($P < .05$) differences from control (pH 4.0) as determined by analysis of Dunnett's multiple comparison test.

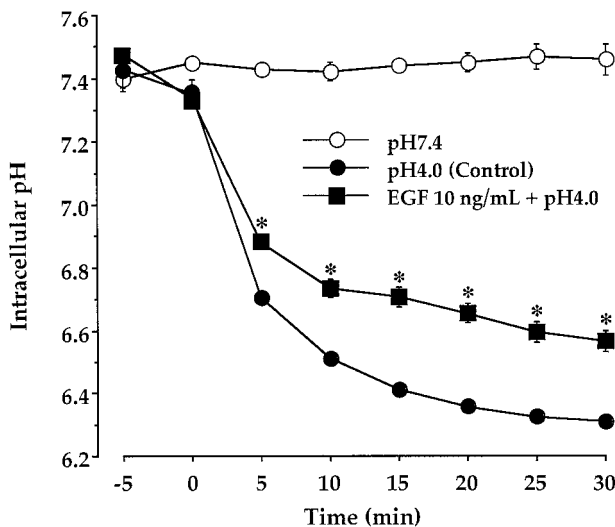


Fig. 2. Time course of the effect of EGF (10 ng/ml) on the reduction of pHi caused by acid (pH 4.0). Cells cultured in a glass-bottomed culture dish were incubated with BCECF-AM for 30 min, treated with 10 ng/ml EGF for 30 min, and subjected to the pH 4.0 medium. The changes in pHi were monitored by an image analyzer. * indicate significant ($P < .05$) differences from control (pH 4.0) as determined by analysis of Student's t test.

Effect of Na^+ Concentration in Acidified Medium on Protective Effect of EGF. After EGF-pretreated cells were placed in a Na^+ -free acidified medium for 30 min, a reduction in cell viability was observed. It was noteworthy that under such conditions, the protective effect of EGF did not manifest itself even at a concentration of 10 ng/ml (Fig. 4A). The critical Na^+ concentration for the protective effect of 10 ng/ml EGF was less than 1.53 mM (Fig. 4B, solution B). At concentrations 1.53 mM and above, EGF's protective activity remained unaffected, whereas lower concentrations markedly diminished the influence of EGF.

Effects of Different Agents on EGF Cytoprotection. Pretreatment of the cells with indomethacin (10^{-5} mol/l) for 4 h with subsequent EGF treatment did not alter cell viability (data not shown). When cells were subjected to the acidified medium (pH 4.0), EGF prevented cell damage in a concentration-dependent manner in both untreated and in-

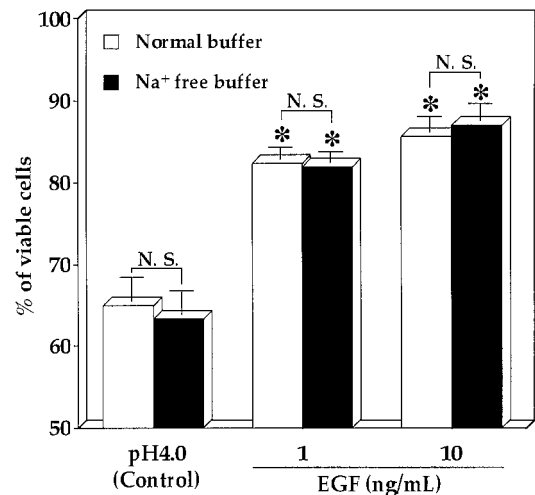


Fig. 3. Effect of a Na^+ -free buffer on the protective effect of EGF. Cells were incubated with EGF in a normal respiratory or Na^+ -free respiratory buffer for 30 min and then subjected to pH 4.0 acidic medium. Cell viability was determined by the MTT assay. Data are means \pm S.E. for six cultures. * indicate significant ($P < .05$) differences from controls as determined by analysis of Dunnett's multiple comparison test.

domethacin-treated groups. There was no significant difference between the two groups regarding the protective effect of EGF (Fig. 5A). Genistein decreased the protective effect of EGF in a concentration-dependent manner (Fig. 5B). At 10^{-5} mol/l, genistein nearly eliminated the effect of EGF.

The addition of amiloride to the acidified medium significantly lowered the protective effect of EGF (Fig. 6A). Furthermore, wortmannin pretreatment also decreased the effect of EGF (Fig. 6B); at 10^{-7} mol/l wortmannin, EGF's influence is nullified. However, the Ca-calmodulin inhibitor W-7 at 10^{-7} to 10^{-5} mol/l did not affect the protective effect of EGF (Fig. 6C). All agents used in this study neither affected the cell viability at pH 7.4 nor cell damage at pH 4.0 (data not shown).

RT-PCR Analysis of Na^+/H^+ Exchangers in Rat Kidney and RGM1 Cells. In the rat kidney, four Na^+/H^+ exchanger isoforms designated NHE1, NHE2, NHE3, and NHE4 were expressed (Fig. 7A). In contrast, only expression of NHE1 and NHE2 were confirmed in RGM1 cells (Fig. 7B).

Discussion

The results clearly indicate that EGF prevents acid-induced cell damage in RGM1 cells in a dose-related manner. Our results are consistent with those of Hiraishi et al. (1984) and Ishikawa et al. (1992) who reported the protective effect of EGF on gastric epithelial cell damage induced by ethanol or a high concentration of indomethacin, respectively. However, they did not discuss the mechanisms of the protective effect of EGF on cell damage induced by such noxious agents. Our cell damage model used in the present study is different from theirs with respect to the cells and agents used. Furthermore, an acid that is used as a necrotizing agent is not foreign to the gastrointestinal tract. This model allows mimicry of the environment of the gastrointestinal tract and will be useful for studying the acid resistance of gastrointestinal epithelial cells.

Previously, Konturek et al. (1981) reported that continuous i.v. infusion of EGF (1–10 $\mu\text{g}/\text{kg}/\text{h}$) for 3 h significantly in-

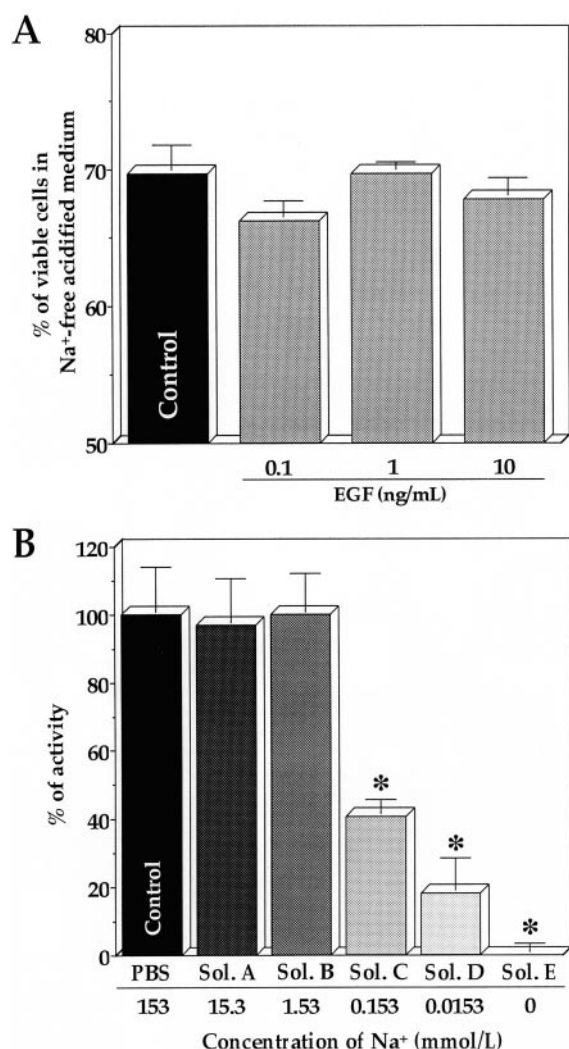


Fig. 4. A, effect of Na⁺ on the inhibitory effect of EGF. Cells were treated with EGF for 30 min and then cell damage was induced with Na⁺-free PBS at pH 4.0. Cell viability was determined by the MTT assay. Data are means \pm S.E. for six cultures. B, influence of Na⁺ concentration of acidified PBS on the inhibitory effect of EGF. Cells were treated with 10 ng/ml EGF for 30 min. Cell damage was then induced with pH 4.0 PBS and several concentrations of Na⁺. Cell viability was determined by the MTT assay. Data are means \pm S.E. for six cultures. * indicate significant ($P < .05$) differences from control as determined by analysis of Dunnett's multiple comparison test.

hibited HCl and aspirin-induced gastric lesions in rats. They suggested that the mechanism of the inhibitory effect of EGF was related to enhanced cell proliferation in the gastric mucosa in response to the agent. In fact, they demonstrated that DNA synthesis in the rat gastric mucosa was markedly stimulated by EGF at the doses used. It is thought that mitogenic activity is one principal factor of the protective effect of EGF.

In gastric mucosal cells, EGF is known to stimulate the production of prostaglandins (Chiba et al., 1982; Nakano et al., 1995). Moreover, prostaglandins are known to inhibit cell damage caused by several noxious agents (Terano et al., 1984, 1987; Arakawa et al., 1996). Mechanisms of the cytoprotective effect of prostaglandins is still unclear. However, in the present study, pretreatment of the cells with indomethacin for 4 h before EGF treatment did not alter the protective effect of EGF. Accordingly, it appears that EGF is not mediated by endogenous prostaglandins.

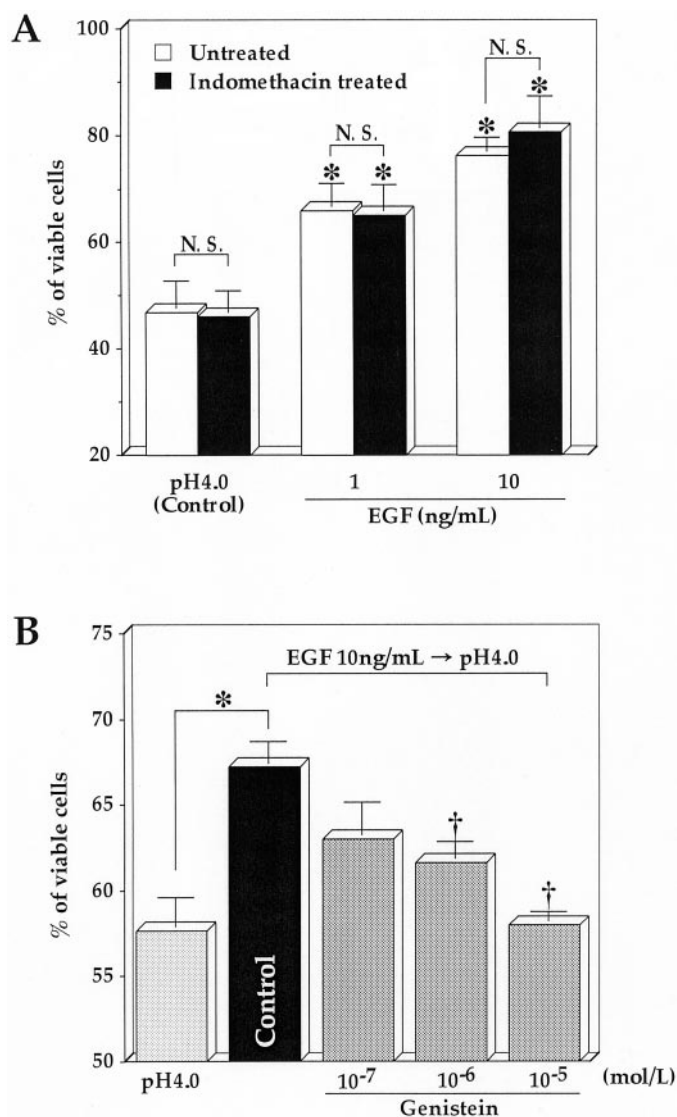


Fig. 5. Effects of indomethacin (A) and genistein (B) on the inhibitory effect of EGF. Cells were treated with each drug for 4 h (indomethacin) or 30 min (genistein) before the addition of EGF. Cell viability was determined by the MTT assay. Data are means \pm S.E. for six cultures. In A, * indicates significant ($P < .05$) differences from controls as determined by analysis of Dunnett's multiple comparison test. In B, * indicates significant ($P < .05$) differences from acid alone (pH 4.0) as determined by analysis of Student's *t* test, whereas [†] indicate significant ($P < .05$) differences from control as determined by analysis of Dunnett's multiple comparison test.

The method of determining Na⁺/H⁺ exchange rates by measuring a range of pHi values has been well established (Donowitz et al., 1994). The recovery rate of pHi to neutral is then measured as the activity of Na⁺/H⁺ exchangers. Therefore, the amount of pHi change induced by acid, as used in the present study, is used to measure Na⁺/H⁺ exchanger activity. The fact that EGF partially prevented intracellular acidification in the present study may suggest that EGF activates Na⁺/H⁺ exchangers. Moreover, our additional findings showed that the protective effect of EGF was completely canceled when Na⁺ was removed from the acidic medium. This strengthens our argument that the protective effect of EGF is related to Na⁺/H⁺ exchanger activities.

Numerous researchers have shown that Na⁺/H⁺ exchangers are important for enhanced Na⁺ absorption by means of

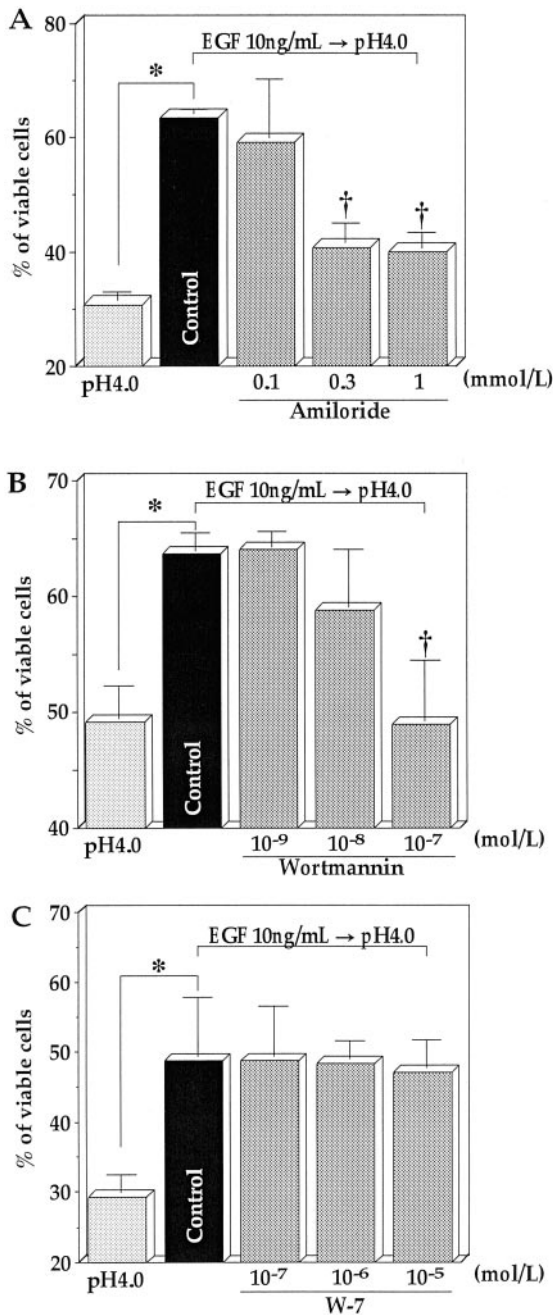


Fig. 6. Effects of amiloride (A), wortmannin (B), and W-7 (C) on the inhibitory effect of EGF. Cells were treated with wortmannin or W-7 30 min before EGF. In the case of amiloride, the drug was added to the acidified medium and then the mixture was used for the induction of cell damage. Cell viability was determined by the MTT assay. Data are means \pm S.E. for six cultures. * indicate significant ($P < .05$) differences from acid alone (pH 4.0) as determined by analysis of Student's *t* test, whereas † indicate significant ($P < .05$) differences from controls as determined by analysis of Dunnett's multiple comparison test.

EGF in the intestine in both in vitro and in vivo studies (Donowitz et al., 1994; Khurana et al., 1996; Maher et al., 1996). Furthermore, these Na⁺/H⁺ exchangers are expressed in epithelial cells located in the stomach as well as the intestine (Ghishan et al., 1995; Noël et al., 1995). Accordingly, we examined the influence of Na⁺ on the protective effect of EGF. We first examined the effects of EGF pre-treated with either Na⁺-containing solution or Na⁺-free so-

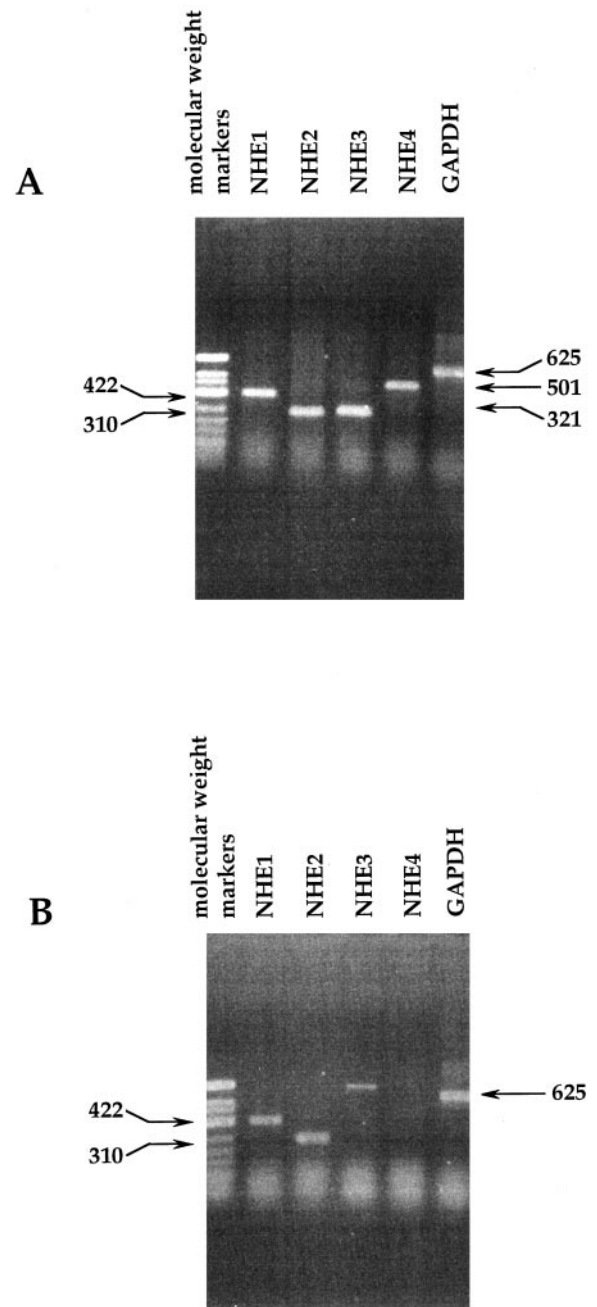


Fig. 7. RT-PCR products from rat kidney (A) and RGM1 cells (B) in ethidium bromide-stained agarose gel for NHE1, NHE2, NHE3, NHE4, and GAPDH. Far left lane, molecular weight markers (Φ X174 digested with *Hinf* I).

lution for 30 min, and subjected to cell damage test using Na⁺ containing pH 4.0 PBS. However, the protective effects of EGF were similarly observed in both solutions. Next, the influence of Na⁺ in the acidified medium was examined. The protective effect of EGF was completely diminished when Na⁺ in the acidified medium was eliminated. These results suggest that the activation of Na⁺/H⁺ exchangers in response to acid treatment, but not during EGF treatment, is involved with the protective effect of EGF in acid-induced cell damage.

Diuretic amiloride and some of its derivatives are useful compounds for characterizing Na⁺/H⁺ exchanger isoforms

because of their high sensitivity to the proteins (Tse et al., 1994; Noël et al., 1995). Clark et al. (1991) and Chambrey et al. (1997) determined the amiloride sensitivity of Na⁺/H⁺ exchangers (NHE1, NHE2, NHE3, and NHE4) in different tissues and cells. They concluded that NHE1 and NHE2 were sensitive to amiloride and amiloride derivatives, whereas NHE3 and NHE4 were resistant to amiloride derivatives. In this study, we confirmed that the protective effect of EGF is attenuated by the addition of amiloride in acidified medium in a concentration-dependent manner, suggesting that amiloride-sensitive Na⁺/H⁺ exchanger isoforms such as NHE1 or NHE2 (but not NHE3 or NHE4) are involved in the protective effect of EGF. Previously, we thought that the protective effect of EGF is mainly mediated by NHE3, because hexamethylene amiloride, a potent amiloride derivative, was not effective to the protective effect of EGF after concomitant treatment of EGF and hexamethylene amiloride of the cells before the acid treatment (Furukawa et al., 1997). However, in our current study, the action of EGF was only diminished when amiloride was added directly to the acidified solution. We think that, in our previous report, most of hexamethylene amiloride was washed out and could not act against the Na⁺/H⁺ exchangers. However, in this study, when amiloride was added to the acidified solution, the result indicated that the protective effect of EGF is related to amiloride-sensitive proteins. Moreover, we demonstrated Na⁺/H⁺ exchanger isoform expression by RT-PCR and confirmed that NHE1 and NHE2 are expressed in RGM1 cells. These results further indicate that the protective effect of EGF is related to the activation of NHE1 and/or NHE2.

Previous studies demonstrated that the activity of each Na⁺/H⁺ exchanger isoform is regulated by Ca⁺⁺ in different ways (Wakabayashi et al., 1992; Levine et al., 1995). In particular, NHE1 is known to be a Ca-calmodulin binding protein (Bertrand et al., 1994) that is activated by influxed Ca⁺⁺ through several ligands (Aviv et al., 1996). In addition, Takaichi et al. (1993) reported that a Ca-calmodulin inhibitor attenuates the activity of NHE1. If NHE1 was the major Na⁺/H⁺ exchanger with respect to the protective effect of EGF in our experiments, the protective effect of EGF should have been diminished by W-7, a Ca-calmodulin inhibitor. However, we confirmed that W-7 did not affect the protective effect of EGF. Accordingly, we conclude that NHE2 is the major Na⁺/H⁺ exchanger in the protective effect of EGF.

Wortmannin inhibits Na⁺ absorption via an EGF-activated Na⁺/H⁺ exchanger in intestinal cells, suggesting that PI-3 kinase is involved in Na⁺/H⁺ exchanger activation (Donowitz et al., 1994). The regulatory mechanisms of Na⁺/H⁺ exchanger by PI-3 kinase are not fully understood at present. Because Na⁺/H⁺ exchangers have been shown to contain proline-rich sequences, it is possible that PI-3 kinase associates directly through the Src homology 3 (SH3) group, furthermore, D-3 polyphosphoinositides themselves act as second messengers. Another possibility is that PI-3 kinase activation may be an intermediate step but not the final step of activation of Na⁺/H⁺ exchanger and it is likely that several steps downstream from PI-3 kinase activation are necessary for activation of Na⁺/H⁺ exchanger.

A noteworthy finding was wortmannin inhibition of the protective effect of EGF in a concentration-dependent manner. Accordingly, it seems that PI-3 kinase activated by EGF

is involved in the positive regulation of EGF's protective effect.

In conclusion, our results strongly suggest that EGF exhibits a protective effect against acid-induced damage to gastric epithelia because of the prevention of excessive acidification through the expulsion of influxed H⁺ via NHE2.

Acknowledgments

The authors wish to thank Dr. H. Matsui (Department of Gastroenterology, Institute of Clinical Medicine, Tsukuba University) for impartation of RGM1 cells and M. Okamoto, T. Kimura, M. Shimose, and A. Hasegawa for their technical assistance and A. Ho (Department of Neuroscience, Brown University, Providence, RI) for his critical reading of the manuscript.

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