Gastroprotective and vasodilatory effects of epidermal growth factor: the role of sensory afferent neurons

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Matsumoto, Yoji, Kohki Kanamoto, Keishi Kawakubo, Hitoshi Aomi, Takayuki Matsumoto, Setsuro Ibayashi, and Masatoshi Fujishima. Gastroprotective and vasodilatory effects of epidermal growth factor: the role of sensory afferent neurons. Am J Physiol Gastrointest Liver Physiol 280: G897–G903, 2001.—Epidermal growth factor (EGF) has been shown to exert gastric hyperemic and gastroprotective effects via capsaicin-sensitive afferent neurons, including the release of calcitonin gene-related peptide (CGRP). We examined the protective and vasodilatory effects of EGF on the gastric mucosa and its interaction with sensory nerves, CGRP, and nitric oxide (NO) in anesthetized rats. Intragastric EGF (10 or 30 μg) significantly reduced gastric mucosal lesions induced by intragastric 60% ethanol (50.6% by 10 μg EGF and 70.0% by 30 μg EGF). The protective effect of EGF was significantly inhibited by pretreatment with capsaicin desensitization, human CGRP1 antagonist hCGRP-(8–37), or Nω-nitro-l-arginine methyl ester (l-NAME). Intravital microscopy showed that topically applied EGF (10–1,000 μg/ml) dilated the gastric mucosal microvessels dose dependently and that this vasodilatory effect was significantly inhibited by equivalent pretreatments. These findings suggest that EGF plays a protective role against ethanol-induced gastric mucosal injury, possibly by dilating the gastric mucosal arterioles via capsaicin-sensitive afferent neurons involving CGRP and NO mechanisms.

The increase of the gastric mucosal blood flow have been suggested to be the possible mechanisms (12, 16). Recent studies have shown that capsaicin-sensitive afferent neurons, as well as prostaglandins, play an important role in the gastric mucosal defensive mechanisms in rats. The stimulation of these neurons by intragastric capsaicin alters the gastric mucosal blood flow (27, 28), motility (33), and acid and HCO3 secretion (27, 32) and thus reduces gastric mucosal damage (11, 17, 38, 41). EGF increases gastric mucosal blood flow and induces gastric mucosal protection, possibly via capsaicin-sensitive afferent neurons (16). However, the precise mechanisms of the gastric hyperemic and protective effects of EGF are still not fully understood. The aims of the present experiments were to elucidate 1) whether intragastric EGF protects the gastric mucosa against ethanol injury, 2) whether topically applied EGF dilates the gastric mucosal microvessels, and, if so, 3) what are the mechanisms involved.

MATERIALS AND METHODS

Animal preparation. The experiments were reviewed by the Committee on the Ethics of Animal Experiments at the Graduate School of Medical Sciences, Kyushu University and were done according to the Guidelines for Animal Experiments of the Graduate School of Medical Sciences, Kyushu University and the law (no. 105) and notification (no. 6) of the Japanese Government.

Male Wistar rats (conventional, 250 g) were fasted for 24 h. Free access to tap water was allowed before experiments. After anesthetization with intraperitoneal urethane (1.25 g/kg), the rectal temperature was continuously monitored and maintained between 37 and 38°C with a heating lamp. Systemic blood pressure was monitored via a catheter inserted in the left femoral artery. To avoid dehydration, saline was continuously infused at a rate of 1.5 ml/h via a catheter inserted in the left femoral vein.

Experiment I. The possible protective effect of intragastric EGF on the gastric mucosa of urethane-anesthetized rats was examined. EGF was dissolved in 0.01 M PBS at the appropriate doses. Gastric mucosal injury was induced by the intragastric application of 60% ethanol (5 ml/kg) through a...
plastic cannula intubated orally. Sixty minutes after anesthesia, 10 or 30 μg of EGF in 1 ml PBS or vehicle were orally intubated. Fifteen minutes later, the ethanol was applied topically. The stomach was removed 60 min thereafter and fixed in 0.5% formalin for 30 min. Then the stomach was cut along the greater curvature and photographed. The percentage of injured corpus mucosa was calculated by computerized image analysis (NIH Image, v. 1.61).

The effect of pretreatment with sensory desensitization by capsaicin, human calcitonin gene-related peptide (CGRP)-1 antagonist hCGRP-(8–37), or nitric oxide (NO) synthase inhibitor N^6-nitro-L-arginine methyl ester (L-NAME) was investigated in animals treated with 30 μg EGF or vehicle (n = 5/group). Capsaicin-sensitive afferent neurons were desensitized through the systemic and functional ablation by capsaicin. As previously described (42), capsaicin was injected subcutaneously in three consecutive doses of 25, 50, and 50 mg/kg (total of 125 mg/kg) during the 2-wk period before the experiment. Sensory desensitization was confirmed by instilling a drop of capsaicin solution (0.1 mg/kg) into an eye of each rat. The instant responses with wiping movements toward the eyes was regarded as inadequate desensitization. The capsaicin-pretreated rats with a negative wiping movement test were regarded as functionally ablated and used for the experiment. Either hCGRP-(8–37) (100 nmol/kg) or L-NAME (10 mg/kg) was bolus injected intravenously 10 min before the intragastric administration of EGF or vehicle. L-NAME was given either alone or in combination with L-arginine (300 mg/kg iv) as a substrate for NO synthase.

**Intravital microscopy.** Intravital microscopy was applied by the method reported by Ohono et al. (29), with a slight modification. Briefly, the stomach was exposed through a ventral midline abdominal incision and cut along the greater curvature. After the anterior wall was resected with an electric cautery scalpel (B-3396; Summit Medical, Tokyo, Japan), the posterior wall of the glandular stomach was fixed in a plastic chamber with the mucosal surface facing the bottom and superfused with modified Krebs buffer (3) (in g/l: 8.0 NaCl, 0.20 KCl, 0.265 CaCl_2·2H_2O, and 2.25 Tris; pH adjusted to 7.40 with HCl) warmed at 37°C. A small window (3 mm diameter) was made by a partial removal of the serosa, the smooth muscle, and the submucosa using microsurgical scissors. Minimal bleeding during the procedure was controlled with bipolar coagulator (MICRO-3E; Mizuho Ikakogyo, Tokyo, Japan). Microvasculature in the basal part of the gastric mucosa was observed through the window by transillumination under a stereomicroscope with a long working-distance objective lens (BX50–33; Olympus, Tokyo, Japan). The images were displayed on a video monitor (PVM-20550M; Sony, Tokyo, Japan) via a charge-coupled device (CCD) camera (DXC-108; Sony) connected to the microscope.

**Experiment II.** The peripheral effect of EGF on gastric mucosal microcirculation was investigated in urethane-anesthetized rats. The diameter of gastric mucosal microvessels was measured using the intravitral microscopic technique. After surgery, a resting period of at least 15 min was allowed to stabilize the preparation. Arterioles with inner diameters of 25–50 μm and 30–60-μm venules in the basal part of the gastric mucosa were examined. A series of increasing concentrations of EGF (0, 10, 20, 40, 100, and 1,000 μg/ml) was topically applied on the gastric wall (20 μl) through the window every 9 min (n = 6/group). Changes in the diameter of the arteriole and the venule were recorded on videotape for 3 min. The videotape was played back, and then the maximal diameter of the vessel was directly measured on the video monitor. The superfusion of the buffer was stopped 15 s before the topical application of EGF and then was resumed for 6 min until the next application to wash out the preceding compound.

The effect of pretreatment with sensory desensitization by capsaicin, hCGRP-(8–37), or L-NAME was tested (n = 6/group). For sensory desensitization, capsaicin at 5 mM was superfused for 10 min after the confirmation of arteriolar dilatation by topical capsaicin at a concentration of 160 μM and then by modified Krebs solution for 60 min. The arteriolar dilatation reached a maximum within 1 min after initiation and then remained at that level for at least 10 min. The arteriolar diameter gradually returned to the basal value within 60 min after the removal of capsaicin. In a preliminary experiment, capsaicin desensitization was confirmed by the second topical capsaicin application 70 min later at a concentration of 160 μM, which has been demonstrated to induce a maximal response (40). Arteriolar dilatation by the second application of capsaicin was <10%, and therefore the capsaicin-sensitive afferent neurons were considered to be desensitized. Either hCGRP-(8–37) (100 nmol/kg) or L-NAME (10 mg/kg) was bolus injected intravenously 10 min before the topical EGF application (100 μg/ml). L-NAME was given either alone or in combination with L-arginine (300 mg/kg iv).

**Chemicals and treatments.** The following chemicals were used: EGF (kindly provided by Dr. B. Nakajima, Hitachi Chemical, Japan), capsaicin (Wako Chemical, Osaka, Japan), hCGRP-(8–37), L-NAME, L-arginine (Sigma, St. Louis, MO), and ethanol (Wako Chemical, Osaka, Japan). EGF was dissolved in 0.01 M PBS (Sigma) in experiment I and in modified Krebs buffer in experiment II. Capsaicin was dissolved in a solvent composed of 10% ethanol, 10% Tween 80 (Sigma), and 80% vol/vol normal saline (0.15 N NaCl). hCGRP-(8–37), L-NAME, and L-arginine were dissolved in saline containing 0.1% BSA. Ethanol was diluted in distilled water. All chemicals were freshly prepared just before the experiments.

**Statistics.** Values are expressed as means ± SE. Student's t-test was used for comparisons of two groups. Significance of differences was determined with a one-way ANOVA followed by Fisher's protected least significant difference (PLSD) for the comparison of multiple groups. A two-factor repeated-measures ANOVA followed by Fisher's PLSD was used for the data of serial measurements. P values <0.05 were considered statistically significant.

**RESULTS**

**Experiment I.** Gastric mucosal lesions 60 min after ethanol injection occupied 24.3 ± 2.6% of the glandular area in vehicle-treated rats. The intragastric application of EGF (10 or 30 μg) significantly reduced the gastric mucosal lesions (12.0 ± 3.5% in the 10 μg group, P < 0.01, and 7.3 ± 1.3% in the 30 μg group, P < 0.001, Fig. 1).

**Pretreatment with either capsaicin desensitization, hCGRP-(8–37), or L-NAME slightly, but not significantly, increased the gastric mucosal lesions (33.9 ± 5.1%, 31.1 ± 6.7%, and 38.2 ± 6.6%, respectively) in the vehicle-treated rats. All of these pretreatments inhibited the protective effect of intragastric EGF (30 μg) against ethanol-induced mucosal lesion (38.6 ± 3.9% in the capsaicin desensitization group, 33.0 ± 3.7% in the hCGRP-(8–37) group, and 36.9 ± 5.1% in the L-NAME group, n = 5, Fig. 2). There was no significant difference in the gastric mucosal lesions between the vehicle-treated and EGF-treated rats with...
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Fig. 1. The effect of intragastric epidermal growth factor (EGF) on macroscopic gastric mucosal damage induced by 60% ethanol (n = 5/group). Lesion index (%erosions in glandular stomach) was significantly lower in the groups treated by EGF. *P < 0.05 and **P < 0.001 vs. vehicle-treated group. Error bars represent SE.

Table 1. arterial diameters before and after EGF administration

<table>
<thead>
<tr>
<th>Dose of EGF (µg)</th>
<th>Vehicle</th>
<th>10</th>
<th>30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal arterioles</td>
<td>34.5 ± 2.5 µm</td>
<td>32.0 ± 2.0 µm</td>
<td>30.5 ± 1.5 µm</td>
</tr>
<tr>
<td>Venules</td>
<td>42.5 ± 2.4 µm</td>
<td>40.0 ± 1.9 µm</td>
<td>37.5 ± 1.4 µm</td>
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**DISCUSSION**

Our results showed that in urethan-anesthetized rats 1) intragastric EGF prevents ethanol-induced gastric mucosal injury and topically applied EGF dilates the arterioles but not the venules in the basal part of gastric mucosa in a dose-dependent manner and 2) these effects of EGF are mediated through the capsaicin-sensitive afferent neurons via CGRP- and NO-dependent mechanisms. Because EGF was applied to the serosal side of the glandular stomach in the second experiment, the effect of EGF on the arteriole observed in the experiment may be slightly different from that under physiological conditions. However, our observations suggest that EGF does dilate arterioles in damaged gastric mucosa that lacks an epithelial layer (i.e., gastric ulcer and erosion).

The EGF receptor (EGF-R) has been shown to belong to the type 1 tyrosine kinase receptor family and to be located in the gastric tissue of both rodents and humans (31, 34). At the acute and healing stage of gastric mucosal damage, EGF-R has been shown to be overexpressed in the epithelia (19, 35). It has also been confirmed in rats that the main source of EGF in the gastric contents is the submandibular glands (20, 22), and that growth factor exists at a concentration of 19.6 µg/l in the rat (14). Furthermore, EGF in the salivary glands (9) and in the gastric juice (23) increases by 2.5-fold within 15 min after ethanol injection (data not shown)

**Fig. 2.** The effect of capsaicin desensitization, human calcitonin gene-related peptide 1 antagonist hCGRP-(8–37), or N-nitro-l-arginine methyl ester (L-NAME) on the protective effect of intragastric EGF (30 µg, n = 5/group). Capsaicin desensitization or pretreatment with hCGRP-(8–37) (100 nmol/kg iv) or L-NAME (30 mg/kg iv) significantly inhibited the protective effect of EGF. Concomitant treatment with l-arginine restored the L-NAME-inhibited inhibition induced by L-NAME. *P < 0.001 vs. L-NAME-pretreated group. Error bars represent SE.
severalfold that of the basal value under conditions of gastric mucosal damage induced by various stimuli. Whereas we applied EGF to rats at extremely high concentrations compared with those in physiological conditions, a similar preventive effect of large amounts of EGF against mucosal injury has been shown in other experiments (12, 16).

Intragastric EGF protects the gastric mucosa against various stimuli such as stress, ethanol, hypertonic saline, and aspirin (12, 16, 21, 24, 26). Although parenteral EGF has been shown to decrease gastric acid secretion (25), intragastric EGF revealed a protective effect against aspirin- and stress-induced mucosal damage without reducing acid secretion (24). It thus seems likely that acid suppression alone is not the significant mechanism of the protective effect of EGF in our experiments. The trophic action to the gastric mucosa characterized by increase in DNA, RNA, protein, and mucus secretion (15, 18) has been confirmed, but this effect seems to be unrelated to the preventive effect of intragastric EGF. Cytoprotection through the stimulation of prostaglandin production by EGF (5, 8) has been suggested to be another mechanism for the preventive effect. However, the role of prostaglandin synthesis in the protective effect of EGF still remains controversial because intragastric EGF exhibited a preventive effect even against aspirin-induced mucosal injury of the rat stomach without affecting prostaglandin production (26).

It has been shown in several experiments that intragastric EGF increases the mucosal blood flow of the
stomach. Hui et al. (12) demonstrated that intragastric EGF increased the blood flow of the rat gastric mucosa after topical ethanol treatment, and it also dose-dependently reduced the degree of mucosal damage. Although Hui et al. (12) measured the mucosal blood flow by laser-Doppler flowmetry, we directly observed the arterioles in the gastric mucosa induced by intragastric EGF by using intravital microscopy. Whereas an increase in the gastric mucosal blood flow has been shown in rats treated by subcutaneous EGF (16), the vasodilatory action of EGF seems to be attributed to an extremely topical response, because the arterioles dilated even after the removal of submucosal tissue in our experiment.

Recently, the interaction of EGF and NO in the gastric protection has been investigated in animal experiments (1, 36, 37). Tripp and Tepperman (37) reported in sialoadenectomized rats that subcutaneous EGF did not influence NO synthase activity in ethanol-treated gastric lesions, whereas EGF reduced ethanol-induced mucosal lesions. However, Brzozowski et al. (1) reported that in stress-induced mucosal blood flow has been shown in rats treated by subcutaneous EGF (16), the vasodilatory action of EGF seems to be attributed to an extremely topical response, because the arterioles dilated even after the removal of submucosal tissue in our experiment.

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It has been established in both rodents and humans that capsaicin plays a protective role against gastric mucosal injury and that the capsaicin-sensitive afferent neurons play a major role in the regulation of the gastric mucosal blood flow (10, 27, 28). The release of CGRP from stimulated capsaicin-sensitive neurons and subsequent increase in endothelial NO have been shown to result in vasodilatation and increase in blood flow (4, 10, 13, 40). Our results strongly suggested that EGF, as well as capsaicin, dilated the arteriole through capsaicin-sensitive afferent neurons. Kang et al. (16) have also reported that the EGF-induced increase in the blood flow of the rat gastric mucosa was inhibited by either capsaicin desensitization or hCGRP-(8–37). These findings suggest that the protective effect of EGF against gastric mucosal injury is due partly to mucosal hyperemia through the stimulation of capsaicin-sensitive afferent neurons.

The precise mechanism of stimulation of capsaicin-sensitive neurons by EGF remains unclear. On the sensory afferent neuron, a receptor, which is sensitive to capsaicin, protons, and noxious heat, has recently been cloned and referred to as vanilloid receptor subtype 1 (2). EGF may directly stimulate the vanilloid receptor subtype 1. The stimulation of a capsaicin-sensitive afferent neuron through mast cells may be another explanation, because substances released from these cells have been shown to stimulate the sensory neurons in the rat gastric mucosa (39). However, the EGF receptors on mast cells remain to be determined.

In past experiments, capsaicin desensitization for sensory neurons was completed by systemic administration of high dose capsaicin (42), as in our first experiment. The procedure of desensitization seems to induce a systemic functional depletion of capsaicin-sensitive neurons. In experiment II, however, we intentionally applied a high dose of capsaicin directly on the gastric wall, and a substantial desensitization could thus be achieved. The method of desensitization coupled with intravital microscopy may be a model for investigating the role of capsaicin-sensitive afferent neurons in the regulation of mucosal blood flow.
In conclusion, intragastric EGF plays a protective role against gastric mucosal injury induced by ethanol, and the effect may be attributable to hyperemia through stimulation of capsaicin-sensitive afferent neurons and subsequent CGRP- and NO-dependent mechanisms. It is presumed that the dilatation of the arterioles may thus be an essential event in the protective effect of EGF against gastric mucosal injury.

REFERENCES


