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Ecabet sodium raises plasma levels of calcitonin gene-related peptide and substance P in healthy humans

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Abstract

Ecabet sodium (ecabet), a cytoprotective drug, produces an increase in mucosal blood flow. One of the gastrointestinal motility regulatory factors has been assumed to be the induction of changes in the levels of peptides (gastrin, somatostatin and motilin) in plasma. On the other hand, recently, capsaicin-sensitive afferent nerves were shown to play an important role in gastric mucosal defensive mechanism. Capsaicin stimulates afferent nerves and enhances the release of calcitonin gene-related peptide (CGRP) and substance P in the stomach. We studied the effect of ecabet on human plasma gastrin-, somatostatin-, motilin-, CGRP- and substance P-like immunoreactive substance (IS) in healthy subjects. Ecabet sodium at a dose of 3.0 g, or placebo, was orally administered in five healthy males. The blood samples were taken before and at 20, 40, 60, 90, 120, 180 and 240 min after administration, subjected to extracting procedures, and submitted to a highly sensitive enzyme immunoassay system. Single administration of ecabet caused significant ($P < 0.05$) increases in plasma CGRP-, substance P- and somatostatin-IS concentration compared with placebo. Ecabet significantly decreased plasma gastrin-IS levels compared with placebo. In this study, we hypothesized that ecabet might stimulate capsaicin-sensitive afferent nerves indirectly and improve mucosal blood flow; this might be a key mechanism underlying its gastroprotective action.

Introduction

Apart from the significant contribution of *Helicobacter pylori*, the pathophysiology of acid-peptic disease may be thought of as an imbalance between aggressive factors (acid and pepsin) and local mucosal defences (the secretion of bicarbonate, mucus and prostaglandins). Although the treatment of peptic ulcer is often directed at the reduction of aggressive factors, it can be directed at strengthening mucosal defences of the stomach and the duodenum with so-called cytoprotective agents.

Ecabet sodium (ecabet) is a cytoprotective agent that protects gastric mucosa against gastric acid (Ito et al 1993a, b). The medicine does not transfer into circulating blood, its cytoprotective effect is caused by direct action on the gastric mucosa (Azuma et al 1992) and there is a report that ecabet improves gastric mucosal microcirculation (Onoda et al 1990).

One of the factors that regulate gastrointestinal motility has been assumed to be the induction of changes in the levels of peptides (gastrin, somatotatin and motilin) in plasma. On the gastroprotective function as a neural emergency system, sensory afferent neurons in the gastrointestinal mucosa regulate neuropeptides (calcitonin gene-related peptide (CGRP)) and levels of tachykinins (substance P, etc.)) and play various physiological roles (Holzer 1998).

In recent years, some anti-ulcer medicines have been elucidated pharmacologically from the viewpoint of gut-regulated hormone levels. Among the medicines, H_2-receptor antagonists change gut-regulated hormones levels in healthy human plasma (Takeyama et al 1998, Itoh et al 2002) and cetraxate hydrochloride, a cytoprotective drug, raises plasma CGRP and substance P levels (Katagiri et al 2004). These results indicate new pharmacological effects of the medicines.

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Thus, we examined the plasma levels of the gastrointestinal motility regulatory (gastrin, somatostatin and motilin) and cytoprotective peptides (CGRP and substance P) using an enzyme immunoassay (EIA). Radioimmunoassay (RIA) to detect somatostatin has been developed by several groups (Saito 1980). However, in terms of safety, sensitivity and ease of handling, RIA methods are still less than satisfactory. We developed a sensitive and specific double-antibody EIA for detecting somatostatin, using somatostatin-linked β-D-galactosidase (β-Gal) as a marker antigen, a secondary antibody-coated immunoplate and 4-methylumbelliferyl-β-D-galactopyranoside as a fluorogenic substrate, and applied this proposed EIA to the measurement of somatostatin in human plasma for clinical use.

The purpose of this study was to describe the sensitive and specific EIA for somatostatin and to determine the effects of ecabet on plasma levels of gastrin-, somatostatin-, motilin-, CGRP- and substance P-like immunoreactive substance (IS) in healthy subjects.

Materials and Methods

Materials

Ecabet (Gastrom granules; Tanabe Seiyaku Co. Ltd, Osaka, Japan) was used. Lactose (Merck hoei Co. Ltd, Osaka, Japan) was used as placebo.

Synthetic human gastrin I (G17), somatostatin, motilin, CGRP and its fragment (8-37) and substance P were purchased from the Peptide Institute (Osaka, Japan). Fragment gastrin I (2-17) was purchased from Sigma Chemical (St Louis, MO). Antisera to gastrin (A600/R1B) and CGRP (CA1132) were purchased from Biogenesis (Poole, UK), substance P (RA-08-095) from Cambridge Research Biochemicals (Cambridge, UK), motilin (Y121) from Yanaihara Institute (Shizuoka, Japan) and somatostatin (T-4101) from Peninsula Laboratories (San Carlos, CA). Goat affinity-purified antibody to rabbit IgG (whole molecule) (55641) was purchased from ICN Pharmaceuticals (Aurora, OH). 4-Methylumbelliferyl-β-D-galactopyranoside (MUG) and *N*-(ϵ-maleimidocaproyloxy) (EMC)-succinimide were purchased from Sigma (St Louis, MO). β-Gal and aprotinin (Trasylol) were purchased from Boehringer Mannheim (Mannheim, Germany) and Bayer (Leverkusen, Germany), respectively. All other reagents were analytical reagent grade from commercial sources.

Subjects

Five healthy male subjects, aged 24–31 years (median 29 years), 55–62 kg (median 58 kg), participated in the study. Each subject received information on the scientific purpose of the study and gave written informed consent. The study was approved by the ethical committee of Oita Medical University. The subjects did not receive any medication a month before, and during, the study and fasted for 2 h before the study commenced and during the experiments.

Study schedule

Initially, venous blood samples from a median cubital vein were taken for measurement of the somatostatin level in the plasma EIA to examine how plasma somatostatin-IS levels were influenced by meals. Blood samples were taken at 1000, 1100, 1130, 1230, 1300, 1400, 1530 and 1800 h. All subjects ate breakfast before 0800 h and had the same lunch at 1155–1215 h, and did not take food or drink outside the above periods.

Ecabet (3.0 g) or placebo was administered orally with 100 mL water. Each subject was administered these drugs at four-week intervals. The dose of ecabet in this study was the maximum daily dose used in clinical therapy. Venous blood samples (10 mL) were taken from a medium cubital vein before and at 20, 40, 60, 90, 120, 180 and 240 min after drug administration. The study was carried out from 1400 to 1600 h.

Preparation of enzyme-labelled antigens

Human somatostatin was conjugated with β-Gal by EMC-succinimide according to the method of Kitagawa et al (1981). Somatostatin (0.20 mg) dissolved in 0.05 M phosphate buffer (pH 7.0, 0.40 mL) was mixed with EMC-succinimide (0.40 mg) in tetrahydrofuran (0.04 mL) at room temperature (24°C) for 60 min. The EMC-somatostatin obtained was purified by separation through a Sephadex G-25 column (1.5 × 50 cm) pre-equilibrated with 0.05 M phosphate buffer (pH 7.0), to elute the column. Individual fractions (1.8 mL each) that showed absorbance at 280 nm were collected. The purified EMC-somatostatin fractions were combined with β-Gal (3.5 mg) by mixing at room temperature for 60 min. The β-Gal conjugate was applied to a Sephacryl S-300 column (1.5 × 52 cm) and eluted with 0.05 M phosphate buffer (pH 7.0), containing 1 mM $MgCl_2$. Individual fractions (1.8 mL each) that showed absorbance at 280 nm were collected. The fractions containing β-Gal activity were collected and stored at 4°C after the addition of 0.2% bovine serum albumin (BSA) and 0.1% sodium azide.

EIA procedure for gastrin, somatostatin, motilin, CGRP and substance P

Plasma gastrin- (Takeyama et al 1993), motilin- (Naito et al 2002), CGRP- (Nagano et al 1998) and substance P-IS (Takeyama et al 1990) levels were measured using a highly sensitive EIA as previously described. We applied the EIA of these peptides to that of somatostatin-IS in plasma. The assay was performed by a delayed addition method. Separation of bound and free antigen was performed on an anti-rabbit IgG-coated immunoplate (Nunc-Immuno Module Maxisorp F8; InterMed, Denmark) (Takeyama et al 1989).

The assay buffer consisted of 0.05 M phosphate buffer (pH 7.0) containing 0.5% BSA, 1 mM $MgCl_2$ and 250 KIU mL^{-1} aprotinin. Diluted antiserum (100 μL) and sample (100 μL of plasma extracts or standard) were mixed and incubated at 4°C for 24 h. Diluted enzyme-labelled antigen (50 μL) was then added, and the solution was incubated at 4°C for an additional 24 h. One-hundred

microlitres of the antigen–antibody solution for each sample was added to the secondary antibody-coated immunoplate. The plate was incubated at 4°C overnight, washed with 0.01 M phosphate buffer (pH 7.0) containing 0.15 M NaCl and 0.05% Tween 20, and then 200 mL of 0.1 mM MUG in 0.05 M phosphate buffer (pH 7.0) containing 1 mM $MgCl_2$ was added to each well. The plate was incubated at 37°C for 180 min, and then the fluorescent intensity (λ_{Ex} 360 nm, λ_{Em} 450 nm) of the fluorescent product, 4-methylumbelliferon, was measured with an MTP-100F microplate reader (Corona Electric, Ibaraki, Japan).

Preparation of plasma extracts

The blood samples were placed in chilled tubes containing aprotinin (500 KIU mL^{-1}) and ethylenediaminetetraacetic acid (EDTA) (1.2 mg mL^{-1}). After centrifugation, the plasma was diluted five fold with 4% acetic acid (pH 4.0), and loaded onto C18 reversed-phase cartridge (Sep-Pak C18; Millipore Corp., Milford, MA). After washing with 4% acetic acid, the peptides in the plasma were eluted with 70% acetonitrile in 0.5% acetic acid (pH 4.0). Elutes were concentrated by spin-vacuum evaporation, lyophilized and stored at −40°C until assayed. The recovery and reproductivity for human plasma with somatostatin EIA were examined by adding standard solution to hormone-free plasma (Tai & Chey 1978).

HPLC of plasma extracts

HPLC was performed using a reversed-phase C18 packed column (Cosmosil 5C18; Nacalai Tesque, Kyoto, Japan). The HPLC consisted of a model 600E pump system (Millipore Corp., Milford, MA). The plasma samples (2.5 mL), purified by the Sep-Pak C18 cartridges as described above, were reconstituted to 100 μL with 0.1% trifluoroacetic acid (TFA) and passed through the column. Somatostatin-IS were eluted with a linear gradient of acetonitrile (from 5% to 45% over 40 min) in 0.1% TFA. The flow rate was 1.0 mL min^{-1} and the fraction size was 1.0 mL. Eluted fractions were concentrated by spin-vacuum evaporation, lyophilized, and reconstituted to 100 μL with an assay buffer before undergoing EIA.

Statistical analysis

Result are expressed as mean ± s.d. Comparison of mean values was made by repeated measures one-way analysis of variance and paired *t*-test. $P < 0.05$ indicated statistical significance.

Results

Standard curve

A typical calibration curve for the somatostatin-IS EIA was prepared. When plotted as a semi-logarithmic function, a linear displacement of enzyme-linked somatostatin by somatostatin was noted between 40 and 625 pg mL^{-1} with antiserum T-4101. The minimum amount of somatostatin detectable by this EIA system was 8.0 pg (3.2 pg/well), and the IC50 of the calibration curve was 100 pg mL^{-1}.

Specificity of antiserum T-4101

The immunospecificity of the antiserum T-4101 was examined by EIA using somatostatin conjugated with β-Gal. The displacement curves (1, 10, 100, 1000, 10 000, 100 000 pg mL^{-1}) of somatostatin and other endogenous peptides (human gastrin I, motilin, vasoactive intestinal peptide (VIP), CGRP, substance P, cholecystokinin (CCK), neuropeptide Y (NPY), galanin and secretin) were investigated. Gastrin I, motilin, VIP, CGRP, substance P, CCK, NPY, galanin and secretin showed minimal inhibition of the binding of β-Gal-conjugated somatostatin with the somatostatin antiserum T-4101. Thus, somatostatin antiserum T-4101, which recognizes somatostatin, can distinguish somatostatin from other endogenous peptides.

Measurement of somatostatin-IS in human plasma by EIA

Human plasma extracts were subjected to reverse-phase HPLC to access the presence of somatostatin-IS molecular variants in human plasma. The elution profiles revealed the presence of a main immunoreactive peak, eluting at a position corresponding to standard somatostatin, and several unknown peaks. The recovery rates of human plasma somatostatin in the proposed detectable range (40 pg mL^{-1} and 625 pg mL^{-1}) with this EIA were 93.1% and 95.5%, respectively. The reproducibility (CV%) for human plasma (40 pg mL^{-1} and 625 pg mL^{-1}) for this somatostatin EIA was 6.9% and 2.0% for inter-assay (n = 9), and 8.6% and 5.1% for intra-assay (n = 10) comparisons.

Circadian rhythms in the daytime of somatostatin-IS in human plasma

The circadian rhythms of the human plasma somatostatin-IS levels in the daytime are shown in Figure 1. The mean somatostatin-IS levels at each time point were 12.2 ± 1.7 pg mL^{-1} at 1000 h, 10.6 ± 2.3 pg mL^{-1} at 1100 h, 11.2 ± 0.9 pg mL^{-1} at 1130 h, 14.5 ± 3.2 pg mL^{-1} at 1230 h, 14.0 ± 3.3 pg mL^{-1} at 1300 h, 11.3 ± 0.8 pg mL^{-1} at 1400 h, 12.1 ± 0.9 pg mL^{-1} at 1530 h, and 12.0 ± 1.9 pg mL^{-1} at 1800 h, and these values showed a range of 8.0–18.0 pg mL^{-1}. The somatostatin-IS levels at these daytime points (1000, 1100, 1130, 1230, 1300, 1330, 1400, 1530 and 1800 h) showed no significant differences ($P = 0.050$).

Effect of ecabet on plasma somatostatin-, gastrin-, motilin-, CGRP- and substance P-IS levels

The plasma CGRP-IS level–time profile after the administration of ecabet is shown in Figure 2A. Ecabet caused significant increase in CGRP-IS between 40 and 180 min (10.2 ± 2.8 pg mL^{-1} at 40 min, 12.5 ± 1.5 pg mL^{-1} at

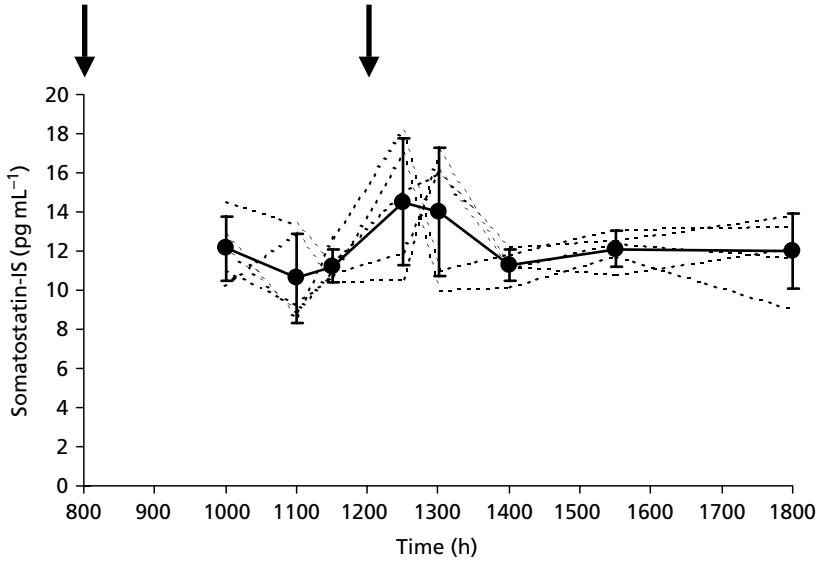

Figure 1 Circadian rhythms of human plasma somatostatin-IS levels in the daytime. Time of taking food and drink (breakfast and lunch) is indicated by the arrow.

60 min, 12.0 ± 2.0 pg mL^{-1} at 90 min, 11.1 ± 1.7 pg mL^{-1} at 120 min and $7.3 \pm$ pg mL^{-1} at 180 min), compared with the response of the placebo group (6.2 ± 1.2 pg mL^{-1} at 40 min, 5.2 ± 1.1 pg mL^{-1} at 60 min, 5.7 ± 1.4 pg mL^{-1} at 90 min, 5.4 ± 0.5 pg mL^{-1} at 120 min and 5.0 ± 0.2 pg mL^{-1} at 180 min). Figure 2B shows plasma substance P-IS levels after the administration of ecabet. Ecabet significantly increased substance P-IS levels between 40 and 120 min (31.0 ± 8.3 pg mL^{-1} at 40 min, 28.2 ± 3.8 pg mL^{-1} at 60 min, 29.5 ± 5.2 pg mL^{-1} at 90 min and 28.1 ± 7.7 pg mL^{-1} at 120 min) compared with the response of the placebo (20.4 ± 5.7 pg mL^{-1} at 40 min, 21.6 ± 2.2 pg mL^{-1} at 60 min, 23.3 ± 2.8 pg mL^{-1} at 90 min and 16.6 ± 5.6 pg mL^{-1} at 120 min). The plasma somatostatin-IS levels were significantly increased at 90–120 min (16.6 ± 2.4 pg mL^{-1} at 90 min and 16.5 ± 3.9 pg mL^{-1} at 120 min) compared with placebo (13.2 ± 2.7 pg mL^{-1} at 90 min and 11.8 ± 2.5 pg mL^{-1} at 120 min) (Figure 2C). At 40 min and 90–120 min, the plasma gastrin-IS levels (25.3 ± 5.9 pg mL^{-1} at 40 min, 21.4 ± 10.4 pg mL^{-1} at 90 min and 16.7 ± 3.1 pg mL^{-1} at 120 min) were significantly suppressed compared with placebo (35.2 ± 9.1 pg mL^{-1} at 40 min, 42.2 ± 12.8 pg mL^{-1} at 90 min and 27.4 ± 9.4 pg mL^{-1} at 120 min) (Figure 2D). But the medicine did not alter levels of motilin-IS (Figure 2E).

Discussion

Using β-Gal-labelled somatostatin as a marker antigen, an anti-rabbit IgG-coated immunoplate as a bound/free separator and MUG as a fluorogenic substrate, we have developed a sensitive and specific EIA for the quantification of somatostatin in human plasma. Since 1985, RIA methods developed for somatostatin have been widely used, although the methods have several disadvantages due to the use of radioisotopes. The EIA detailed in this report retains the advantages of the RIA system while minimizing the disadvantages. This EIA was sensitive (8.0 pg, 3.2 pg/well) and specific for somatostatin, and the sharp inhibition curve obtained was linear between 40 and 625 pg mL^{-1}. The sensitivity of RIA has previously been reported as 1.9 pg/tube (Katakami et al 2002). With regard to the operation, our EIA enables the measurement of many samples (96 wells) at the same time by using an anti-rabbit IgG-coated immunoplate as the bound/free separator. The somatostatin antibody T-4101 was found to have no cross-reactivity with the other endogenous peptides. We applied the EIA to detect somatostatin-IS in human plasma. The recovery (> 90%) and reproducibility (CV% of inter-assay and intra-assay comparisons) of this EIA with plasma samples were satisfactory. The molecular heterogeneity in human plasma was examined by HPLC. The main somatostatin-IS in plasma was eluted at the same elution time as synthetic human somatostatin with several unknown peaks. These unknown peaks might be fragments of somatostatin.

The circadian rhythms of somatostatin in the daytime were investigated for application to clinical use in human plasma. We could not find significant circadian rhythms for human plasma somatostatin levels among the daytime time points (1000–1800 h), but it is not denied that the somatostatin levels in human plasma might be influenced by meals. After the lunch, the plasma somatostatin levels were increased from 1230 to 1300 h and then the plasma somatostatin levels were stable. Therefore, in this study, we investigate the effect of ecabet on plasma peptides between 1400 and 1800 h.

CGRP is a powerful vasoactive substance, which is released from the sensory afferent nerve endings against gastric mucosal injury in the stomach. CGRP increases gastric mucosal flow as a gastroprotective factor (Holzer 1998). Substance P coexists with CGRP in the sensory

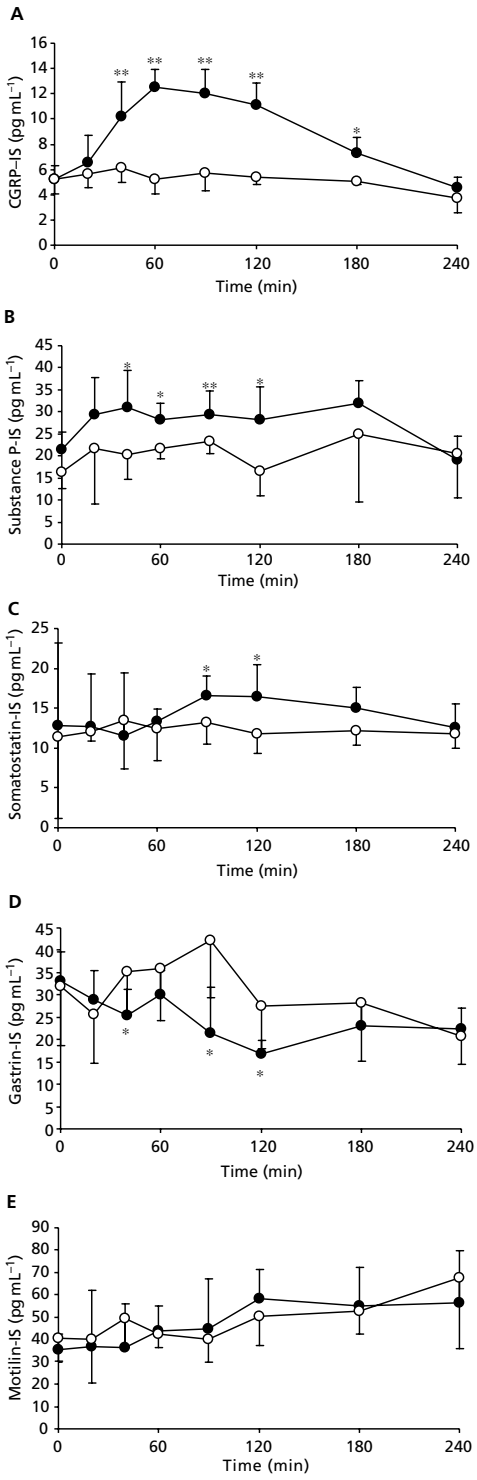

Figure 2 Effect of ecabet 3.0 g (●) or placebo (○) on plasma CGRP- (A), substance P- (B), somatostatin- (C), gastrin- (D) and motilin-IS (E) levels in healthy subjects. Each value represents the mean ± s.d., n = 5. *$P < 0.05$ and **$P < 0.01$, compared with placebo.

afferent neurons of the gastrointestinal mucosa, and is released with acetylcholine in response to depolarizing stimuli in the enteric nerve system (Hellström et al 1991). In this study, ecabet raised plasma CGRP- and substance P-IS levels. It is known that ecabet increases the mucosal prostaglandin (PG) E_2 and PGI_2 in human stomach (Kohli et al 1990). Although a deficiency of cytoprotective PG is known to be responsible for the mucosal lesions caused by non-steroidal anti-inflammatory drugs, the possibility that chemosensitive afferent neurons monitor noxious tissue challenge and in turn activate mechanisms of protection was not considered until recently. Capsaicin, the pungent ingredient in red peppers such as paprika or chilli, stimulates capsaicin-sensitive afferent neurons, which release CGRP and substance P from their nerve endings (Chen et al 1992). The results of several neurochemical and pharmacological studies support the hypothesis that capsaicin-sensitive afferents afford gastric mucosal protection via release of CGRP and substance P from their peripheral nerve endings. PGE_2 and PGI_2 have a cytoprotective effect via capsaicin-sensitive afferent nerves (Arai et al 2003; Takeuchi et al 2003). Furthermore, Kinoshita et al (1995) reported that the cytoprotective effect of ecabet was due to both endogenous PGs and capsaicin-sensitive sensory nerves.

Somatostatin inhibits the secretion of other hormones, including gastrin, insulin, and motilin (Ling et al 1973). In the gastrointestinal tract, gastric acid and pepsin secretion, and gastric emptying are inhibited by somatostatin (Schrumpf 1978). In the interdigestive state somatostatin induces phase-3 activity (Hostein 1984) and in the digestive state it inhibits gastric emptying (Seal et al 1982) and slows gastrointestinal transit (Johansson et al 1978). Motilin has a powerful fundic pouch motor-stimulating activity (Brown & Parkes 1967). It plays an important physiological role in intestinal contractility and is one of the most important factors controlling the regular occurrence of phase-3 contractions of the migrating motor complex (MMC) (Itoh 1976). Motilin participates in regulating gastrointestinal motility with somatostatin, and stimulates gastric emptying and postprandial gastric contraction. Gastrin stimulates gastric acid secretion involving G cells, and it is associated with a mechanism of gastrointestinal motility involving the cholinergic nervous system (Szelenyi 1980). Somatostatin inhibits the secretion of gastrin and motilin. In this study, although somatostatin-IS levels were significantly increased, gastrin-IS levels were inhibited but motilin-IS levels were not. Kawashima et al (2002) reported that CGRP increased somatostatin secretion and decreased gastric acid secretion via somatostatin-induced reduction of gastrin and histamine. In our result, plasma somatostatin-IS levels were significantly increased, followed by plasma CGRP-IS levels. Furthermore, after the increase of somatostatin-IS levels, gastrin-IS levels decreased. The result was supported by Kawashima's report (Kawashima et al 2002). Furthermore, there were no previous reports of increase in plasma CGRP- and somatostatin-IS after administration of ecabet, but there was a report of the inhibition of gastrin-IS levels after administration of the medicine (Funakoshi et al 1996). Considering plasma motilin-IS levels did not alter, we thought that the medicine did not influence gastric emptying. Although somatostatin-IS

levels were significantly increased, motilin-IS levels did not decrease. This implies that the intercellular communication between somatostatin and motilin is paracrine and somatostatin might not inhibit all pathways of motilin release.

Ecabet was clinically used as enemas, which were prepared to suspend ecabet granules in saline, to treat inflammatory bowel disease, such as ulcerative colitis and Crohn's disease (Kono et al 2001). CGRP plays important role in improvement of intestinal blood flow (Murata et al 2002). Furthermore, in Japan, a clinical trial on ecabet enema is on-going.

In conclusion, we have established a sensitive and specific EIA system for endogenous somatostatin in human plasma. Using this EIA system, we have revealed that single administration of ecabet caused significant increases in plasma somatostatin-, CGRP- and substance P-IS concentration, and significant inhibition of plasma gastrin-IS concentration compared with placebo. We hypothesized that ecabet might indirectly stimulate capsaicin-sensitive afferent nerves and increase the mucosal blood flow; in result, it shows a gastroduodeno-protective action without delaying gastric emptying.

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