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Clinical application of an enzyme immunoassay for cholecystokinin-like immunoreactive substance for determination of the human plasma levels: the effect of metoclopramide on gastrointestinal peptides and stress-related hormones

FUMIHIKO KATAGIRI,* SHIN INOUE, HIROKI ITOH and MASAHARU TAKEYAMA

Department of Clinical Pharmacy; Oita University Hospital; 1-1 Idaigaoka; Hasama-machi, Oita 879-5593, Japan

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Abstract: Metoclopramide, a prokinetic drug, is widely used to treat vomiting and nausea. Delayed gastric emptying and continual stress are considered important factors, among others, that induce nausea and vomiting. One gastrointestinal motility regulatory factor has been assumed to be the induction of changes in the levels of peptides such as gastrin, somatostatin, motilin, and cholecystokinin (CCK) in plasma. In contrast, adrenocorticotropic hormone (ACTH) and cortisol are used as indicators of stress. Here, we studied the effects of metoclopramide on human plasma gastrin-, somatostatin-, motilin-, and CCK-like immunoreactive substances (ISs) and ACTH-IS and cortisol under stress conditions using repetitive blood sampling in healthy subjects. Metoclopramide hydrochloride at a dose of 30 mg or placebo was orally administered to five healthy male volunteers. Blood samples were taken before and 20, 40, 60, 90, 120, 180, and 240 min after administration, subject to extracting procedures, and submitted to a highly sensitive enzyme immunoassay system. A single administration of metoclopramide caused significant increases in plasma somatostatin-IS levels compared with the placebo. Metoclopramide significantly decreased plasma gastrin- and suppressed ACTH-IS and cortisol levels compared with the placebo. We hypothesize that metoclopramide might have an accelerating gastric-emptying effect and a modulatory effect on the hypothalamo-pituitary-adrenal (HPA) axis and the autonomic nervous function. These effects might be beneficial in stress-related diseases, which suggest that this medicine has clinicopharmacological activities. Copyright © 2005 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: metoclopramide; adrenocorticotropic hormone; cortisol; stress; cholecystokinin; enzyme immunoassay

INTRODUCTION

Metoclopramide (Figure 1) has been confirmed to be an effective drug in treating and preventing various types of vomiting and a useful agent in esophageal reflux diseases, gastroparesis, dyspepsia, and a variety of functional gastrointestinal disorders. Of considerable importance is the recent evidence of its efficacy when administered intravenously at high doses for preventing severe vomiting associated with cisplatin. Good results have been achieved in patients not previously treated with cisplatin, but further studies are needed to determine its level of efficacy in patients who have experienced severe vomiting during the earlier course of cytotoxic therapy. Side effects consisting of mild sedation, diarrhea, and reversible extrapyramidal reactions have occurred, but are tolerated by many patients [1].

Some patients who took this medicine showed no organic disease such as peptic ulcer, reflex esophagitis, or gastric cancer but developed a condition classified as nonulcer dyspepsia (NUD) [2]. Most NUD patients tended to have depressive and psychosomatic conditions and were exposed to continual affective stress [3]. Continual stress caused abnormalities in the hypothalamo-pituitary-adrenal (HPA) axis and autonomic nervous function [4]. In general, venipuncture for blood sampling is postulated to be a stress factor that can increase circulating ACTH and cortisol levels, etc. [5,6]. Repetitive blood sampling places subjects under artificial stress, and venipuncture as a stressor is useful for evaluating the pharmacological effects of drugs [7–9].

Delayed gastric emptying is considered one of the most important factors, among others, that induce nausea and vomiting. One gastrointestinal motility regulatory factor has been assumed to be the induction of changes in the levels of peptides such as gastrin, somatostatin, motilin, and cholecystokinin (CCK) in plasma. In recent years, the pharmacological characteristics of some prokinetic medicines have been elucidated with respect to their correlation with gut-regulated hormone levels. Among these medicines, Itoh *et al.* reported that cisapride, a dopamine D_2 receptor antagonist and nonselective serotonin 5-$HT_{1,3,4}$ receptor agonist, raised plasma motilin, gastrin, and somatostatin levels [10] and mosapride, a selective

* Correspondence to: F. Katagiri, Department of Clinical Pharmacy, Oita University Hospital, Hasama-machi, Oita 879-5593, Japan;
e-mail: FKATA@med.oita-u.ac.jp

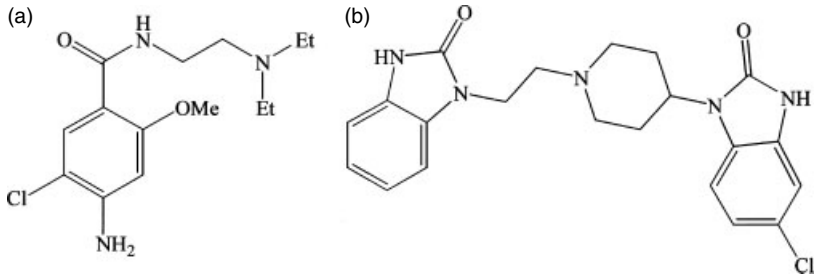

Figure 1 Structure of the dopamine D receptor antagonists, metoclopramide (a), and domperidone (b).

serotonin 5-HT_4 agonist, raised motilin and gastrin levels [11]. Furthermore, Sho-hange-ka-bukuryo-to and Nichin-to, Chinese herbal (Kampo) medicines that are used to treat nausea and vomiting, were reported to change gut-regulated peptides [12,13] and stress-related hormone levels in plasma [14].

In the present study, we use an enzyme immunoassay (EIA) and a fluorescence polarization immunoassay to examine the plasma levels of the gastrointestinal motility regulatory peptides (gastrin, somatostatin, motilin, and CCK) and stress-related hormones (ACTH and cortisol) (Figure 2). Several groups have previously used radioimmunoassays (RIAs) to detect CCK, [15–17] but, in terms of safety, sensitivity, and ease of handling, RIA methods are still less than satisfactory. Therefore, we developed a sensitive and specific double-antibody EIA for detecting CCK, using CCK-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 CCK in human plasma for clinical use.

We aimed to describe the sensitive and specific EIA for CCK and to determine the effects of metoclopramide on the plasma levels of gastrin-, somatostatin-, motilin-, CCK-, ACTH-like immunoreactive substances (ISs) and cortisol in healthy subjects.

MATERIALS AND METHODS

Materials

Metoclopramide (Primperan Tablets; Fujisawa Pharmaceuticals Co. Ltd, Osaka, Japan) and domperidone (Nauzelin tablets; Kyowa Hakko Kogyo Co. Ltd, Tokyo) were used. Lactose (Merck Hoei Co. Ltd, Osaka, Japan) was used as the placebo.

Synthetic human gastrin I, somatostatin, motilin, ACTH (1–24), CCK-33 (CCK), CCK-8 (CCK fragment (26–33)), and CCK-4 (CCK fragment (30–33), same sequence as gastrin I *C*-terminal) were purchased from the Peptide Institute

Gastrin

Pyr-Pro-Trp-Leu-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH_2

Somatostatin

Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys-OH

Motilin

H-Phe-Val-Pro-Ile-Phe-Thr-Tyr-Gly-Glu-Leu-Gln-Arg-Met-Gln-Glu-Lys-Glu-Arg-Asn-Lys-Gly-Gln-OH

Cholecystokinin (CCK)

H-Lys-Ala-Pro-Ser-Gly-Arg-Val-Ser-Met-Ile-Lys-Asn-Leu-Gln-Ser-Leu-Asp-Pro-Ser-His-Arg-Ile-Ser-Asp-Arg-Asp-Tyr-Met-Gly-Trp-Met-Asp-Phe-NH_2

SO_3H

Adrenocorticotropic Hormone (ACTH)

H-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro-Asn-Gly-Ala-Glu-Asp-Glu-Ser-Ala-Glu-Ser-Ala-Glu-Ala-Phe-Pro-Leu-Glu-Phe-OH

Cotisol

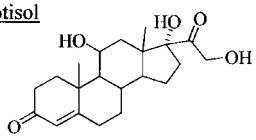

Figure 2 Structure of the gastrointestinal motility regulatory peptides (gastrin, somatostatin, motilin, and CCK) and stress-related hormones (ACTH and cortisol).

(Osaka, Japan). Fragment gastrin I (2–17) was purchased from Sigma (St Louis, MO, USA). CCK fragment (22–33) (CCK-12) was supplied by Professor H. Yajima (Kyoto University, Kyoto, Japan). Antisera to gastrin (A600/R1B) and ACTH (A516/R1H) were purchased from Biogenesis (Poole, UK), antisera to motilin (Y121) and CCK (YP030) from Yanaihara Institute (Shizuoka, Japan), and antisera to somatostatin (T-4101) from Peninsula Laboratories (San Carlos, CA, USA), and the TDx Cortisol assay kit was from Abbott Japan Co. Ltd (Tokyo, Japan). Goat affinity-purified antibody to rabbit IgGs (whole molecule) (55641) was purchased from ICN Pharmaceuticals (Aurora, OH, USA). 4-methylumbelliferyl-β-D-galactopyranoside (MUG) and *N*-(ε-maleimidocaproyloxy) (EMC)-succinimide were purchased from Sigma (St Louis, MO, USA). β-Gal and aprotinin (Trasylol) were purchased from Boerhinger Mannheim (Mannheim, Germany) and Bayer (Leverkusen, Germany), respectively. All other reagents were analytical grade reagents from commercial sources.

Subjects

Five healthy male volunteers, aged 24–31 years (median age: 29 years), weighing 55–62 kg (median: 58 kg) participated in the study. Each subject received information on the scientific purposes 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

At first, venous blood samples from a forearm vein were taken to measure CCK- and gastrin-IS levels using a plasma EIA to examine how plasma CCK-IS levels were influenced by meals. Blood samples were taken at 10:00, 11:00, 11:30, 12:30, 13:00, 14:00, 15:30, and 18:00. All subjects were allowed to have breakfast until 8:00 and all had the same lunch between 11:45 and 12:05. They were not allowed to consume any food or drink outside the above periods.

Metoclopramide hydrochloride (30 mg), domperidone (30 mg), or placebo was administered orally with 100 ml water. Each subject received these drugs at 4-week intervals. The dose of metoclopramide used in this study was the maximum daily dose used in clinical therapy. Venous blood samples (10 ml) were taken from a forearm vein before and 20, 40, 60, 90, 120, 180, and 240 min after metoclopramide administration. The study was performed from 14:00 to 16:00.

Preparation of Enzyme-labeled Antigens

Human CCK-12 was conjugated with β-Gal by EMC-succinimide according to the method of Kitagawa *et al.* [18] In brief, CCK-12 (0.10 mg) dissolved in 0.05 M phosphate buffer (pH 7.0, 0.50 ml) was mixed with EMC-succinimide (1.15 mg) in tetrahydrofuran (0.40 ml) at room temperature (20 °C) for 50 min. The EMC-CCK-12 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), which was also used to elute the column. Individual fractions (1.8 ml each) that showed an absorbance at 275 nm were collected. The purified EMC-CCK-12 fractions were combined with β-Gal (4.0 mg) by mixing at room temperature for 60 min. The β-Gal conjugates were 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 an absorbance at 275 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-, and ACTH-ISs

Plasma gastrin- [19], motilin- [20], somatostatin- [21], and ACTH-IS [22] levels were used in a highly sensitive EIA, as previously described. We applied the EIA of these peptides to that of CCK-IS in plasma. The assay was performed by a delayed addition method. Separation of bound and free antigens was performed on an anti-rabbit IgG-coated immunoplate (Nunc-Immuno Module Maxisorp F8, InterMed, Denmark) [23].

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 aprotinin. Diluted antiserum (100 µl) and the sample (100 µl of plasma extracts or standard) were mixed and incubated at 4 °C for 24 h. Diluted enzyme-labeled antigen (50 µl) was then added, and the solution was incubated at 4 °C for an additional 24 h. 100 µl 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 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 fluorescence 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) [23].

Preparation of Plasma Extracts

Blood samples were placed in chilled tubes containing aprotinin (500 KIU units/ml) and ethylenediaminetetraacetic acid (EDTA) (1.2 mg/ml). After centrifugation, the plasma was diluted fivefold with 4% acetic acid (pH 4.0) and loaded onto a C18 reversed-phase cartridge (Sep-Pak C18; Millipore Corp. Milford, MA, USA). After being washed with 4% acetic acid, the peptides in the plasma were eluted with 70% acetonitrile in 0.5% acetic acid (pH 4.0). The eluted samples were concentrated by spin-vacuum evaporation, lyophilized, and stored at −40 °C until assayed. The recovery and reproductivity of human plasma with CCK EIA were examined by adding a standard solution to hormone-free plasma [24].

Determination of Plasma Cortisol Levels

Plasma cortisol levels were measured using a fluorescence polarization immunoassay. The detection limit of cortisol was 0.64 µg/dl. This method showed minimal cross-reactivity with endogenous steroids (11-deoxycortisol (9.9%), corticosterone (6.3%) and others (<3%)) [25].

Determination of Metoclopramide Concentrations in Plasma

Plasma concentration of metoclopramide was determined by the modified method of Buss *et al.* [26] Standard metoclopramide was supplied by Fujisawa Pharmaceutical Co. Ltd. To 1 ml plasma was added 1 μg internal standard, quinidine sulphate. The sample was buffered with 0.1 ml 1 M sodium hydroxide and extracted with 5 ml chlorobutane containing 10% (v/v) acetonitrile. The mixture was shaken thoroughly for 1 min and then centrifuged for 5 min at 2500 g. The organic phase was then transferred to a conical tube and mixed for 1 min with 0.1 M hydrochloric acid. After centrifugation for a further 5 min at 2500 g, the organic phase was discarded and 20 μl acidified aqueous phase was injected into the chromatographic column. HPLC was performed using a C18 column (Cosmosil 5C18-AR; Nacalai Tesque, Kyoto, Japan) with UV detection at 275 nm, and acetonitrile containing 0.02 M potassium dihydrogen phosphate (pH 3.0) (40:60) was used as a mobile phase at a flow rate of 2.0 ml/min.

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, USA). The plasma samples (2.5 ml), purified by the Sep-Pak C18 cartridges as described above, were reconstituted to 100 μl aliquots with 0.1% trifluoroacetic acid (TFA) and passed through the column. CCK-IS was 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 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 prior to undergoing EIA.

Statistical Analysis

The results are expressed as means ± SD. Comparison of the mean values was made by a repeated measures paired *t*-test. A *P*-value of less than 0.05 indicated statistical significance.

RESULTS

Standard Curve

A typical calibration curve for the CCK-IS EIA is shown in Figure 3. When plotted as a semilogarithmic function, the linear displacement of enzyme-linked CCK-12 by CCK was noted between 10 and 625 pg/ml with antiserum YP030. The minimum amount of CCK detectable by this EIA system was 2.0 pg (0.8 pg/well), and the IC_{50} of the calibration curve was 75 pg/ml.

Specificity of Antiserum YP030

The immunospecificity of antiserum YP030 was examined by EIA using CCK-12 conjugated with β-Gal. The displacement curves (1, 10, 100, 1000, 10 000, 100 000 pg/ml) of CCK, CCK-8, CCK-4, gastrin I (CCK-related peptide), and other endogenous peptides (human motilin, vasoactive intestinal peptide (VIP), calcitonin gene-related peptide (CGRP), and substance P) are shown in Figure 3. CCK-8 exhibited cross-reactivity with synthetic CCK. Although CCK-4, motilin, VIP, CGRP, and substance P showed minimal inhibition of the binding of β-Gal-conjugated CCK-12 with CCK antiserum YP030, gastrin I exhibited little nonspecific cross-reactivity.

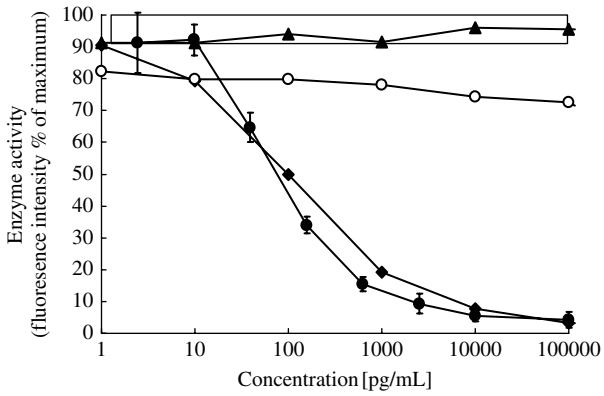

Figure 3 Inhibition curves of CCK-IS (●), CCK-8 (♦), CCK-4 (▲), gastrin I (○), and other endogenous peptides (□) in the EIA by competition between CCK-12 conjugated with β-Gal toward antiserum YP030.

Measurement of CCK-IS in Human Plasma by EIA

Human plasma extracts were subjected to reverse-phase HPLC to access the presence of CCK-IS molecular variants in human plasma. The elution profiles revealed the presence of a main immunoreactive peak (arrow) eluting at a position corresponding to standard CCK and several unknown peaks (Figure 4). The unknown peaks were found at fraction numbers 18, 37, 45, and 48. There were no peaks at the positions corresponding to standard CCK-8 and gastrin I (arrows), which exhibited cross-reactivities to synthetic CCK. The recovery rates of human plasma CCK in the proposed detectable range (10–625 pg/ml) with this EIA were 92.3 and 99.2%, respectively. The reproducibility (expressed in percentage as the coefficient of variation (CV)) for human plasma (10 pg/ml and 625 pg/ml) with this CCK EIA was 4.8 and 2.2%, respectively, for the inner assay ($n = 9$), and 8.6 and 4.6%, respectively, for the intra-assay ($n = 10$) comparisons.

Circadian Rhythms in the Daytime of Gastrin- and CCK-IS in Human Plasma

Circadian rhythms of human plasma CCK-IS levels in the daytime are shown in Figure 5 (a). The mean CCK-IS levels at each time point were 13.1 ± 3.1 pg/ml

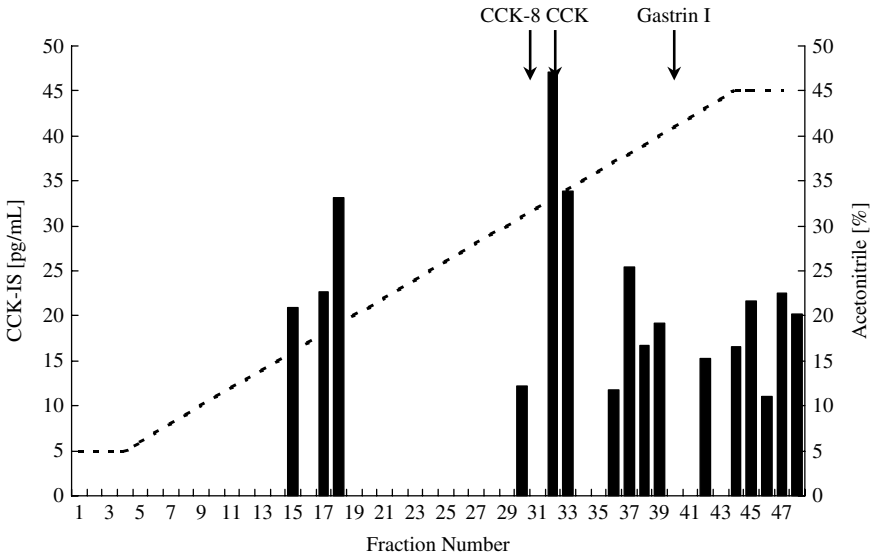

Figure 4 HPLC elution profiles of CCK-IS in human plasma. The dotted line indicates the acetonitrile gradient. Synthetic human CCK, CCK-8, and gastrin I are run in separate chromatographs under the same conditions (indicated by the arrows).

at 10:00, 6.9 ± 1.4 pg/ml at 11:00, 5.4 ± 1.0 pg/ml at 11:30, 17.5 ± 2.6 pg/ml at 12:30, 8.8 ± 1.5 pg/ml at 13:00, 13.2 ± 2.6 pg/ml at 14:00, 11.7 ± 2.7 pg/ml at 15:30, and 5.7 ± 1.2 pg/ml at 18:00; these values ranged from 4.0 to 20.2 pg/ml. The CCK-IS levels at these daytime time points revealed significant differences between data at 10:00 and 18:00, 11:00 and 12:30, 11:30 and 12:30, 11:30 and 14:00, 12:30 and 13:00, 12:30 and 18:00, and 14:00 and 18:00, respectively. Circadian rhythms of human plasma gastrin-IS levels in the daytime are shown in Figure 5 (b). The mean gastrin-IS levels at each time point were 31.2 ± 5.0 pg/ml at 10:00, 31.7 ± 7.1 pg/ml at 11:00, 28.4 ± 4.3 pg/ml at 11:30, 53.2 ± 9.7 pg/ml at 12:30, 35.6 ± 8.7 pg/ml at 13:00, 29.8 ± 3.4 pg/ml at 14:00, 28.9 ± 4.8 pg/ml at 15:30, and 29.5 ± 8.2 pg/ml at 18:00; these values ranged from 18.7 to 63.2 pg/ml. Gastrin-IS levels showed significant differences only between 12:30 and 18:00.

Plasma Concentration of Metoclopramide

The profiles of average plasma metoclopramide concentrations vs time after oral administration of 30 mg metoclopramide hydrochloride are shown in Figure 6. The metoclopramide plasma level was highest during the first 40 min (64.3 ± 11.8 ng/ml) and then decreased.

Effects of Metoclopramide and Domperidone on Plasma CCK-IS Levels

The plasma CCK-IS level-time profiles after administration of metoclopramide and domperidone are shown in Figure 7. Metoclopramide had no significant effects on plasma CCK-IS levels. However, domperidone

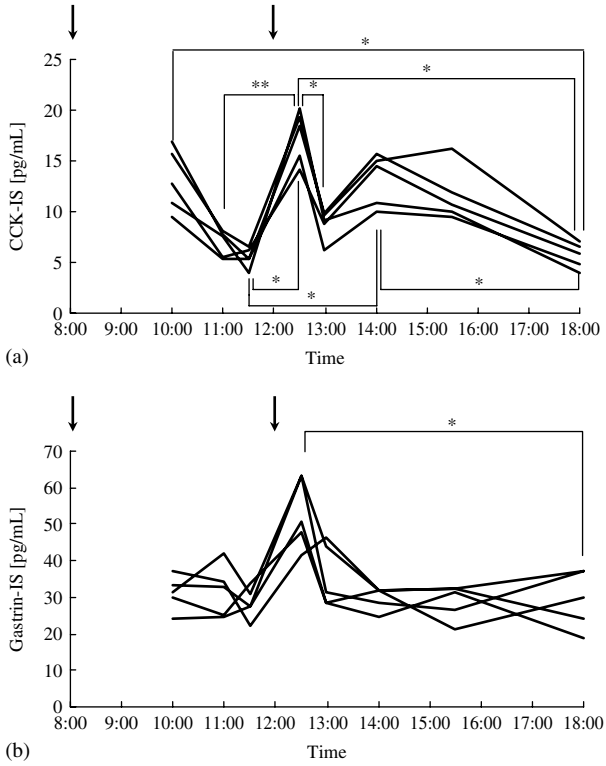

Figure 5 Circadian rhythms of plasma CCK- (a) and gastrin-IS (b) level profiles of five healthy human subjects during daytime. Times of intake of food and drink (breakfast and lunch) are indicated by arrows.

caused significant decreases in plasma CCK-IS levels between 60 and 120 min (6.4 ± 2.0 pg/ml at 60 min, 6.8 ± 0.8 pg/ml at 90 min, and 5.2 ± 0.8 pg/ml at 120 min) compared with the placebo group (11.1 ± 1.6 pg/ml at 60 min, 10.6 ± 1.1 pg/ml at 90 min, and 8.8 ± 3.4 pg/ml at 120 min).

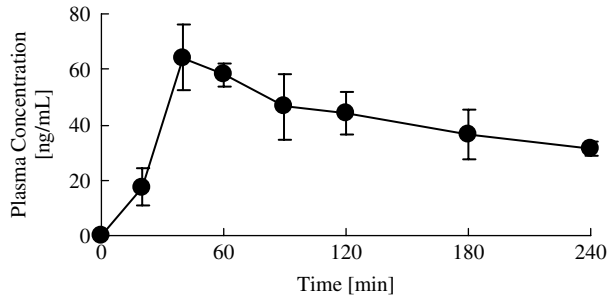

Figure 6 Plasma metoclopramide levels after oral administration of 30 mg metoclopramide hydrochloride. Each value represents the mean ± SD, $n = 5$.

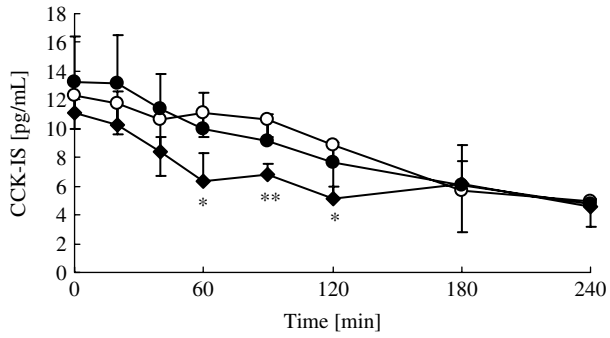

Figure 7 Effects of metoclopramide (●), domperidone (♦), or placebo (○) on plasma CCK-IS levels. Each value represents the mean ± SD, $n = 5$. *$P < 0.05$ and **$P < 0.01$ and compared significantly different from the placebo.

Effect of Metoclopramide on Plasma Somatostatin-, Gastrin-, and Motilin-IS Levels

The plasma gastrin-IS level-time profile after administration of metoclopramide is shown in Figure 8 (a). Metoclopramide caused significant decreases in gastrin-IS levels at 90 min (30.8 ± 6.0 pg/ml) compared with the response in the placebo group (42.2 ± 12.8 pg/ml). Figure 8 (b) shows plasma somatostatin-IS levels after metoclopramide administration. Metoclopramide significantly increased somatostatin-IS levels between 60 and 90 min (18.6 ± 1.7 pg/ml at 60 min and 19.8 ± 4.5 pg/ml at 90 min) compared with the response in the placebo group (12.4 ± 4.1 pg/ml at 60 min and 13.2 ± 2.7 pg/ml at 90 min). However, metoclopramide did not alter the levels of plasma motilin-IS (Figure 8 (c)).

Effects of Metoclopramide on ACTH-IS and Cortisol Levels

The plasma ACTH-IS level-time profile after a single oral administration of metoclopramide is shown in Figure 9 (a). The dotted line indicates the levels of ACTH-IS in conditions of less stress (blood sampling at 120-min intervals) (4.3 ± 2.0 pg/ml at 120 min and 5.3 ± 2.2 pg/ml at 240 min). At 120 min, repetitive sampling

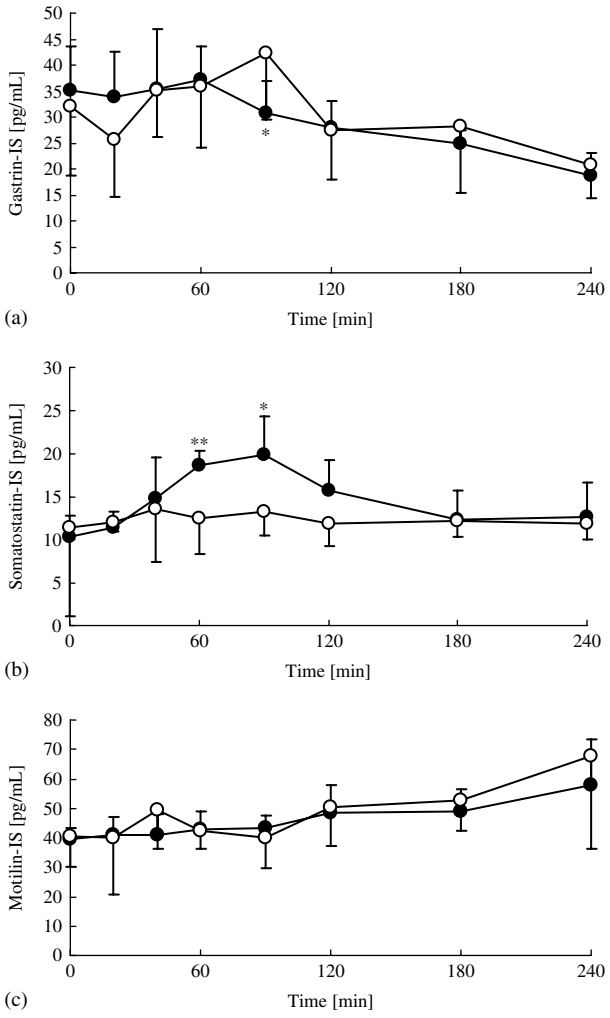

Figure 8 Effects of metoclopramide (●) or placebo (○) on plasma gastrin- (a), somatostatin- (b), and motilin-IS (c) levels. Each value represents the mean ± SD, $n = 5$. *$P < 0.05$ and **$P < 0.01$ and compared significantly different from the placebo.

(stress condition) caused significant increases in plasma ACTH-IS levels (6.3 ± 2.1 pg/ml) compared with sampling at 120-min intervals (less stress condition), which was reflected by repetitive sampling. At 60 min, metoclopramide caused a significant suppression of the increase in levels of ACTH-IS (6.4 ± 1.3 pg/ml) compared with the response in the placebo group (7.6 ± 1.4 pg/ml). Figure 9 (b) shows the plasma cortisol level-time profile after administration of metoclopramide. The dotted line indicates the levels of cortisol in conditions of less stress (blood sampling at 120-min intervals) (4.5 ± 1.5 μg/dl at 120 min and 2.9 ± 0.5 μg/dl at 240 min). There was a significant suppression of the increase compared with the placebo group at 240 min, which reflected the effects of repetitive blood sampling. Metoclopramide had a significant suppressive effect on plasma cortisol levels between 180 and 240 min (4.9 ± 1.5 μg/dl at 180 min and 5.0 ± 2.1 μg/dl at

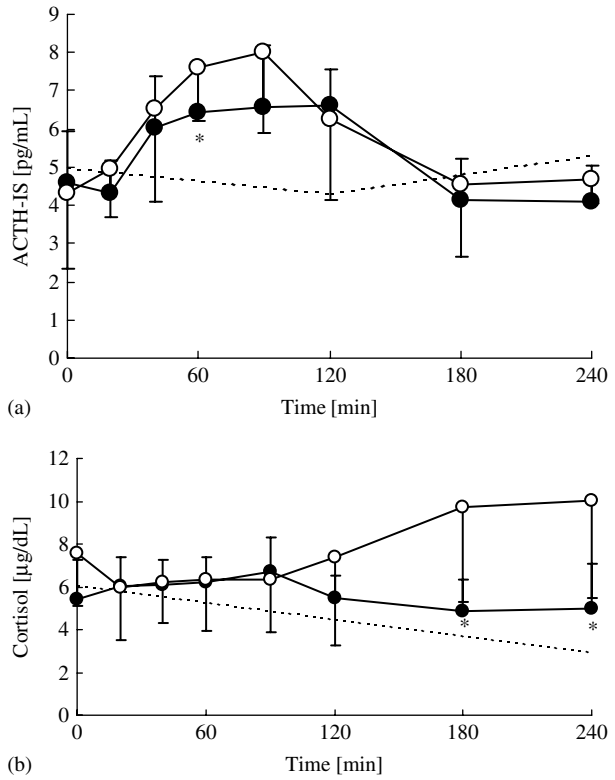

Figure 9 Effects of metoclopramide (●) or placebo (○) on plasma ACTH-IS (a) and cortisol (b) levels. Each value represents the mean ± SD, $n = 5$. *$P < 0.05$ and compared significantly different from the placebo.

240 min) compared with the placebo (9.7 ± 4.4 μg/dl at 180 min and 10.0 ± 4.5 μg/dl at 240 min).

DISCUSSION

Using β-Gal-labeled CCK-12 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 CCK in human plasma. CCK has many molecular forms (CCK-8, -33, etc.) and its *C*-terminal sequence is the same as gastrin I. Therefore, long-term specific quantitative analysis could not be established. Since the development of specific antibodies for CCK in the 1980s, RIA methods for CCK have been widely used although those methods have several disadvantages owing to the use of radioisotopes. The EIA detailed in this report retains the advantages of the RIA system while minimizing the disadvantages. The reported EIA was sensitive (2.0 pg, 0.8 pg/well) and specific for CCK, and the sharp inhibition curve obtained was linear between 10 and 625 pg/ml. The sensitivity of RIA has previously been reported as 1.0 [15] and 3.3 pM [17]. With regard to practicability, our EIA enables the simultaneous measurement of many samples (96 wells) by using an anti-rabbit IgG-coated immunoplate as the bound/free separator. The CCK antibody YP030 was found to cross-react with CCK-8 and have little cross-reactivity with only gastrin I among the other endogenous peptides. Molecular heterogeneity in human plasma was examined by HPLC. The main CCK-IS in plasma was eluted at the same elution time as the synthetic human CCK with several unknown peaks. However, CCK-IS in plasma was not eluted at the elution time of synthetic gastrin I and CCK-8. Therefore, we thought that the CCK antibody YP030 recognized the CCK *C*-terminus except for the common sequences (same as CCK-4) with gastrin I and that those unknown peaks might be because of the CCK fragments. We interpreted that our EIA was not influenced by gastrin I in plasma. We applied the novel EIA to detect CCK-IS in human plasma. The recovery (>90%) and reproducibility (CV% of inner assay and intra-assay comparisons) of this EIA with plasma samples were satisfactory. CCK was identified in 1966 as a 33 amino acid peptide; in humans, CCK-33 and CCK-8 are the major forms. A large amount of CCK-33 and a small amount of CCK-8 exist in circulatory blood, while CCK-8 mainly exists in the brain [27]. Thus, our EIA is considered useful in measuring CCKs both specifically in the whole body and in the brain.

The circadian rhythms of CCK-IS in the daytime were investigated for clinical use for human plasma. We could find significant differences in circadian rhythms for human plasma CCK-IS levels among the daytime time points ranging between 10:00 and 18:00. After a meal, CCK-IS rose significantly, then fell, and rose again 2 h after the meal. The two-phase increase of CCK-IS was reported. At the first peak, CCK-IS might stimulate pancreatic exocrine responses [28] and inhibit gastric emptying. At the second peak, CCK-IS might stimulate pancreatic digestive enzyme secretion [29] or promote peristaltic reflex [30]. The circadian rhythms of gastrin-IS, a CCK-related peptide, in the daytime were also investigated. Although the gastrin-IS levels increased significantly only after a meal, there were no significant changes among the daytime time points ranging between 10:00 and 18:00. Gastrin stimulates gastric acid secretion involving G cells and is associated with a mechanism of gastrointestinal motility involving the cholinergic nervous system [31]. An increase of gastrin-IS is necessary for digestion.

Using our EIA for CCK, we investigated the effects of the dopamine D receptor antagonists, metoclopramide, and domperidone (Figure 1) on plasma CCK-IS levels. It is well accepted that domperidone is a selective dopamine D_2 receptor antagonist, while metoclopramide is a nonselective dopamine receptor antagonist ($D_1 > D_2$). The reason is that metoclopramide passes through the blood-brain barrier and acts on central D_1 receptors, resulting in side effects encompassing extrapyramidal symptoms, but domperidone does not show such side effects when acting selectively

on peripheral D_2 receptors. Although a single administration of metoclopramide had no effects, domperidone caused a significant decrease in plasma CCK-IS levels compared with the placebo group. The secretion of CCK is stimulated by dopamine via D_2 receptors [32]. Because domperidone blocks D_2 receptors, CCK-IS levels decreased. In contrast, metoclopramide, which primarily blocks D_1 receptors, might have little effects on plasma CCK-IS levels.

Somatostatin inhibits the secretion of other hormones, including gastrin, insulin, and motilin [33]. In the gastrointestinal tract, gastric acid and pepsin secretion are inhibited by somatostatin [34]. In the interdigestive state, somatostatin induces phase-3 activities [35], and, in the digestive state, it inhibits gastric emptying [36] and slows gastrointestinal transit [37]. Motilin has a powerful fundic pouch motor-stimulating activity [38]. 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 [39]. Motilin participates in regulating gastrointestinal motility with somatostatin and stimulates gastric emptying and postprandial gastric contractions.

A single administration of metoclopramide caused a significant increase in somatostatin-IS levels. Somatostatin was secreted by direct stimulation of D cells or by an indirect pathway via CGRP [40]. In this study, we did not measure plasma CGRP concentration, but considering that the plasma metoclopramide concentration profile did not correlate with the changes in somatostatin-IS levels, we thought that the increase of somatostatin-IS levels by metoclopramide might be occurring indirectly. The significant decrease in gastrin-IS levels was thought to be due to an increase in somatostatin-IS levels. Reports on changes in gastrin-IS levels after the administration of metoclopramide offered two views: (i) that it is not altered [41] or (ii) that it decreases [42]. In the present study, because the decrease was for a short time, the results were thought to be more likely influenced by the sampling schedules. Although the somatostatin-IS levels were significantly increased, the 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. Considering that plasma motilin-IS levels were not altered, we can conclude that metoclopramide did not influence gastric emptying. However, metoclopramide was reported to accelerate gastric emptying [43]. In the digestive state, motilin promotes gastric emptying [39]; in the interdigestive state, somatostatin accelerates gastric emptying [35]. Therefore, the prokinetic effect of metoclopramide might be caused by somatostatin.

ACTH is a peptide containing 39 amino acids, and ACTH-IS is also found in tissues other than the pituitary gland (i.e. brain, adrenal gland, gastrointestinal tract, pancreas, thyroid gland, and placenta). ACTH secretion is controlled by a circadian rhythm mechanism and negative feedback from plasma cortisol and neurogenic stimulation [44]. The peptide induces secretion of glucocorticoid and rises under stress [45]. Cortisol, commonly used to indicate the level of stress, is secreted by the zona fasciculate of the adrenal cortex and its secretion is dependent on ACTH levels.

Plasma ACTH levels are regulated by the two major pathways of circadian rhythm and negative feedback. Repetitive blood sampling raised ACTH-IS levels in plasma compared with sampling at 120-min intervals in volunteers who received placebo solution. The effects of placebo on ACTH levels are assumed to result from mental and/or physiological stress in volunteers because of repetitive blood sampling. Volunteers from whom samples were taken at 120-min intervals are assumed to have been under less stress. Metoclopramide significantly suppressed the increases in ACTH-IS levels compared with the placebo-treated group. Cortisol is commonly used to indicate stress levels. Samples taken at 120-min intervals showed a decreasing trend in cortisol levels, corresponding with circadian rhythms. In general, plasma cortisol levels are high in the morning and gradually decrease from morning to afternoon [46]. Metoclopramide caused significant suppression of increases in cortisol levels compared with placebo. A previous report showed that administration of metoclopramide caused an increase in plasma cortisol levels [47]. In that report, plasma ACTH and cortisol levels of the placebo group did not rise. Those results were different from our results. We anticipate that further research to determine the best way of blood sampling that gives less stress and comparisons between stress and less stress conditions are needed.

Continual stress causes abnormalities in the HPA axis and autonomic nervous function [4]. Irritable bowel syndrome (IBS) is another disease that is assumed to be related to stress or to abnormalities in the HPA axis [48]. Most NUD or IBS patients are exposed to continual affective stress. Continual stress causes abnormalities in the HPA axis and autonomic nervous function. Many kinds of medications that regulate gastrointestinal functions have been used to treat abnormalities in the gastrointestinal system such as NUD [49]. Metoclopramide might be of good clinical use for these diseases. However, it is suspected that the type of stress caused by repetitive blood sampling is the same as that which causes the above-mentioned diseases. Therefore, investigations of the effects of metoclopramide on the HPA axis and autonomic nervous system in patients with conditions such as NUD and IBS are required.

In conclusion, we have established a sensitive and specific EIA system for measuring endogenous CCK in human plasma. Using this EIA system, we

compared the effects of the dopamine D receptor antagonists, metoclopramide, and domperidone on plasma CCK-IS levels. We revealed that a single administration of metoclopramide caused a significant increase in plasma somatostatin-IS concentration and a significant decrease in plasma gastrin-IS concentration compared with the placebo group. Metoclopramide showed modulatory effects on plasma ACTH-IS and cortisol levels. We hypothesize that metoclopramide, which is used as a prokinetic drug, could also be used to treat stress-related diseases.

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