Effects of Some Kampo Medicines on Plasma Levels of Neuropeptide Y under Venipuncture Stress

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Some traditional Chinese herbal (Kampo) medicines have recently been evaluated for their clinical usefulness in stress and depression. These medicines have modulatory effects on the hypothalamic-pituitary-adrenal axis and sympathetic nervous system (SNs). We examined the effects of Rikkunshi-to, Hange-shashin-to, Hange-koboku-to, and Ninjin-to on plasma levels of neuropeptide Y (NPY), which is the representative neurotransmitter of the SNS, under venipuncture stress. Rikkunshi-to and Hange-shashin-to suppressed increases in plasma NPY-immunoreactive substance levels compared with the response to a placebo. In this study, Rikkunshi-to and Hange-shashin-to altered plasma levels of NPY under venipuncture stress. These effects might be beneficial in stress-related diseases and our results suggest that these medicines have clinical pharmacologic activity.

Key words: neuropeptide Y; enzyme immunoassay; sympathetic nervous system; venipuncture stress

Traditional Chinese herbal (Kampo) medicines (Ninjin-to, Hange-shashin-to, Hange-koboku-to, Rikkunshi-to, etc.) have frequently been used in the empirical treatment of chronic hypofunction of the gastrointestinal system. Some Kampo medicines such as Rikkunshi-to have recently been evaluated for their clinical usefulness in stress and depression.1–3) These medicines have modulatory effects on the hypothalamic-pituitary-adrenal (HPA) axis and regulate adrenocorticotropic hormone (ACTH) and glucocorticosteroid (cortisol) levels in plasma to normal ranges.4,5) Some abnormalities of the gastrointestinal function are presumed to result from changes in hormone levels of the HPA axis. In addition to the HPA axis, the symapathetic nervous system (SNS) is also activated by various stresses.6,7) Neuropeptide Y (NPY) and no-radrenaline are coreleased from the sympathetic nerve terminals by activation of the SNS and are released into plasma during intensive sympathetic activation.8) During stress, activation of the SNS is associated with the HPA axis, and stress-dependent NPY release causes abnormalities in HPA axis activity.9) In depressive disorder patients, increases in plasma NPY levels have been found.9,10)

NPY, a 36-amino acid COOH-terminally amidated peptide, was originally isolated from porcine brain by Tatemoto et al.,11,12) and found to have a structural similarity to peptide YY (PYY) and pancreatic polypeptide, which are found in the gastrointestinal tract and pancreas, respectively. NPY is widely distributed in central and peripheral neurons.13–15) NPY is a sympathetic neurotransmitter and neuromodulator intrinsically involved in the stress response of the body.16) Since norepinephrine is rapidly oxidized, the more stable NPY is often used as an indicator of the activity of the SNS in response to stressors.17)

Venipuncture for blood sampling is postulated to be a stressor is useful for the evaluation of the pharmacologic effects of drugs.18,19) Naito et al. have already reported that some Kampo medicines (Rikkunshi-to, Hange-shashin-to, and Hange-koboku-to) modulate plasma ACTH and cortisol levels, which are often used as an indicator of stress, under venipuncture stress.20) However, it has not been reported previously whether these Kampo medicines affect plasma NPY levels.

A radioimmunoassay (RIA) of NPY was reported for the first time by Allen et al.21) However, in terms of safety, sensitivity, and ease of handling, RIA methods are still less than satisfactory. In this report, we applied a sensitive and specific double-antibody enzyme immunoassay (EIA) for detecting NPY, using NPY-linked β-d-galactosidase (D-galactosidase) as a marker antigen, a secondary antibody-coated immunoplate, and 4-methylumbelliferyl-β-d-galactoside as a fluorgenic substrate. We examined the effects of four Kampo medicines (Rikkunshi-to, Hange-Shashin-to, Hange-koboku-to, and Ninjin-to) on plasma NPY levels under venipuncture stress.

MATERIALS AND METHODS

Materials Rikkunshi-to (EK-43, lot 26L99), prepared as a 4.1-g dried powder extract of Ginseng Radix (4.0 g), Atractylodis Rhizoma (3.0 g), Pinelliae Tuber (3.0 g), and Aurantii Fructus (2.0 g), was kindly supplied by Kanebo Co., Ltd. (Tokyo, Japan). The placebo was excipient alone (crystalline cellulose and lactose) for the above formulations.

Synthetic human NPY (1–36), porcine NPY (13–36), N-ethylmaleimidoacapoxyoxy)succinimide (EMC-succinimide), and 4-methylumbelliferyl-β-d-galactosidase (gal from Escherichia coli) (MUG) were purchased from Sigma Chemical Co. Synthetic human calcitonin gene-related peptide receptor was kindly supplied by Kanebo Co., Ltd. (Tokyo, Japan). The placebo was excipient alone (crystalline cellulose and lactose) for the above formulations.
(CGRP), substance P (SP), cholecystokinin (CCK), somatostatin (SS), porcine motilin, vasoactive intestinal peptide (VIP), and PYY were purchased from the Peptide Institute Inc. (Osaka, Japan). Rabbit antisera to NPY (A602/R2R) were purchased from Biogenesis (Poole, U.K.). Goat affinity-purified antibodies to rabbit IgG (whole molecule) were purchased from ICN Pharmaceuticals, Inc. (Aurora, OH, U.S.A.). β-D-Galactosidase and aprotinin (Trasylol) were purchased from Boehringer Mannheim (Mannheim, Germany) and Bayer (Leverkusen, Germany), respectively. All other chemicals were of analytical reagent grade.

**Subjects** Five healthy male volunteers (nonsmokers), aged 23—40 years were studied. They comprised the subjects from a previously reported study. Each volunteer received information about the scientific purpose of the study, which was approved by the Ethics Committee of Oita Medical University, and gave written informed consent for participation. No volunteer had received any medication for at least 1 month before the study, and there were at least 3-month intervals between other studies. The volunteers had participated in more than three similar studies.

**Study Schedule** All volunteers ate lunch at 11:45—12:00, and the study was carried out from 14:00 until 18:00. Each subject was orally administered Rikkunshi-to, Hange-shashin-to, Hange-koboku-to, or Ninjin-to at a dose of 6.0 g at 14:00. The same dose of placebo was also given to the volunteers. Each subject received these medicines at intervals of 3 months. The dose of medicines was the maximum daily dose in clinical therapy. Blood samples (10 ml) were immediately taken before and 20, 40, 60, 90, 120, 180, and 240 min after administration of the medicines (eight times) from a forearm vein, using standard venipuncture with a 21-gauge needle. For blood sampling with minimal disturbance, sampling was performed at 14:00, 14:40, 16:00, and 18:00 (0, 40, 120, and 240 min, respectively) 3 months after the studies of all test medicines. All blood samples were collected in chilled tubes containing aprotinin (500 KIU/ml) and ethylenediaminetetraacetic acid (EDTA) (1.2 mg/ml). They were promptly centrifuged at 3000 g for 15 min at 4°C, and then the plasma was frozen at −40°C until the assay was performed.

**Preparation of Enzyme-Labeled Antigen** Human NPY (1—36) was conjugated with β-D-galactosidase by N-(ε-maleimidocaproyloxy) (EMC)-succinimide according to the methods of Kitagawa et al. NPY was dissolved in phosphate buffer 0.05 M (pH 7.0) and mixed with EMC-succinimide in tetrahydrofuran at 20°C for 40 min. The EMC-NPY thus obtained was purified via separation on a Sephadex G-25 column (1.5×50.0 cm) preequilibrated with phosphate buffer 0.05 M, pH 7.0, to elute the column. Individual fractions (1.8 ml each) that showed absorbance at 275 nm were collected. The purified EMC-NPY fractions were combined with β-D-galactosidase by mixing at 20°C for 60 min. The β-D-galactosidase conjugate was then applied to a Sephacryl S-300 column. The fractions containing β-D-galactosidase activity were collected and stored at 4°C after the addition of bovine serum albumin (BSA) and sodium azide.

**Preparation of Plasma Extracts** After centrifugation, the plasma samples were diluted with 4% acetic acid (pH 4.0) and loaded onto Sep-Pak C18 cartridges (Millipore Corp., Milford, MA, U.S.A.), and washed with 4% acetic acid. The peptides in the plasma were eluted with 70% acetonitrile in 0.5% acetic acid (pH 4.0), lyophilized, reconstituted to 100 μl with the assay buffer, and subjected to EIA. For the NPY EIA system, plasma samples were concentrated 5-fold with Sep-Pak C18 cartridges. The recovery and reproducibility for human plasma with this NPY EIA were examined by adding standard solution to hormone-free plasma.

**Assay Procedure for NPY-Immunoreactive Substance** The assay was performed using a delayed-addition method. Separation of bound and free antigen was performed on an anti-rabbit IgG-coated immuno probes (Nunc-Immu Module Maxisorp F8, InterMed, Denmark). Test tubes containing 0.1 ml of NPY antisera and sample (or standard) were mixed and incubated at 4°C for 24 h. The EIA assay buffer consisted of phosphate buffer 0.05 M (pH 7.0) containing 0.5% BSA, MgCl2 1 mM, and 250 kallikrein inhibitor units/ml aprotinin. Diluted enzyme-labeled antigen (50 μl) was then added, and the solution was incubated at 4°C for an additional 24 h. The antigen-antibody solution (100 μl) for each sample was added to the secondary antibody-coated immunoplate. The plate was incubated at 4°C overnight, washed with phosphate buffer 0.01 M (pH 7.0), containing NaCl 0.15 M, and 0.05% Tween 20, and then 200 μl of MUG 0.1 mM in phosphate buffer 0.05 M (pH 7.0) containing MgCl2 1 mM was added to each well. The plate was incubated at 37°C for 180 min, and then the fluorescence intensity (λE max 360 nm, λEmax 450 nm) of the fluorescent product, 4-methylumbelliferone, was measured with an MTP-100F microplate reader (Corona Electric, Ibaraki, Japan).

**HPLC of Plasma Extracts** HPLC was performed using a reverse-phase C18 column (Cosmosil SC18, Nacalai Tesque, Kyoto, Japan). The plasma samples (1.0 ml), purified on the Sep-Pak C18 cartridges as described above, were reconstituted in the first composed mobile phase and passed through the column. NPY-like immunoreactive substances (IS) were eluted with a linear gradient of acetonitrile (from 5% to 50% over 45 min) in 0.1% TFA. Synthetic NPY was applied (10 μg) to the column under the same conditions. The flow rate was 1.0 ml/min and the fraction size was 1.0 ml. Eluted fractions were then concentrated by spin-vacuum evaporation, lyophilized, and stored (−40°C) until EIA.

**Data Analysis** NPY-IS levels in plasma are expressed as mean±S.D. (pg/ml). Comparisons of plasma peptide levels among blood sampling times were made using one-way analysis of variance and the Mann-Whitney U-test and a value of p<0.01 or p<0.05 was considered to represent a statistically significant difference.

**RESULTS**

**EIA of NPY in Human Plasma** Typical calibration curves for the NPY (1—36)-EIA are shown in Fig. 1. When plotted as a semilogarithmic function, a linear displacement of enzyme-linked NPY (1—36) by synthetic NPY was noted between 35 and 4000 pg/ml. The minimum amount of NPY (1—36) detectable in this EIA system was 3.5 pg (1.4 pg/well) with antiserum A602/R2R, and the IC50 of the calibration curve was 640 pg/ml. The immunospecificity of the antiserum A602/R2R was examined in EIA using NPY (1—36)-β-D-galactosidase. The displacement curves of NPY (1—36) and other endogenous
peptides are shown in Fig. 1. SP, SS, CGRP, VIP, CCK, motilin, and PYY, which has known homology of 50—70% with PYY,11) minimally inhibited the binding of NPY (1—36)-β-gal with the NPY-antibody. The carboxyl-terminal NPY fragment (13—36) amide exhibited little cross-reactivity. Thus NPY antiserum A602/R2R, which recognizes NPY outside of fragment (13—36), can distinguish NPY from other endogenous peptides.

**Measurements of NPY-IS in Human Plasma in EIA**

Human plasma extracts were subjected to reverse-phase HPLC to assess the presence of NPY-IS molecular variants in human plasma. The elution profiles revealed the presence of the main immunoreactive peak (arrow in Fig. 2), eluting at a position corresponding to standard NPY, and several unknown peaks (Fig. 2). The unknown immunoreactive peaks were found at around fraction number 37—40. When 800 pg/ml of synthetic NPY was added to hormone-free plasma prepared by the method of Tai and Chey,26) the recovery of NPY by the extraction procedure was 95.9 ± 10.0%.

The reproducibility (C.V.%) for human plasma with this NPY was 5.5% (n = 5) for interassay, and 3.5% (n = 8) for intraassay comparisons. The levels of NPY in human plasma from 5 healthy volunteers 2 h after lunch were 128.2 ± 17.9 pg/ml (data not shown).

**Effects of Rikkunshi-to, Hange-shashin-to, Hange-koboku-to, and Ninjin-to on Plasma NPY-IS Levels**

The plasma NPY-IS level-time samples when blood was taken at 0, 20, 40, 60, 90, 120, 180, and 240 min after administration of placebo are shown in Fig. 3. Plasma NPY-IS showed significant increases at 40 min (370.1 ± 132.7 pg/ml, p < 0.05 compared with the dotted line). Plasma NPY-IS level-time profiles when blood was sampled at 0, 40, 120, and 240 min are also shown in Fig. 3 (dotted line). The levels of NPY-IS in samples at these timepoints showed suppression of increases compared with placebo (118.7 ± 29.3 at 0 min, 106.0 ± 26.7 at 40 min, 135.4 ± 13.9 at 120 min, 76.0 ± 19.4 pg/ml at 240 min, respectively), which reflected the effects of repetitive blood sampling. Figure 3A shows the profiles of plasma NPY-IS levels against time after the administration of Rikkunshi-to. Rikkunshi-to significantly suppressed increases in NPY-IS at 40 min (151.7 ± 60.6 pg/ml), compared with the response to the placebo. Hange-shashin-to significantly suppressed increases in NPY-IS at 40 min (128.7 ± 69.3 pg/ml) (Fig. 3B). Hange-koboku-to and Ninjin-to had no significant effects on plasma NPY-IS levels (Figs. 3C, D).
DISCUSSION

Some Kampo medicines have modulatory effects on the HPA axis and SNS. Some abnormalities of gastrointestinal function are caused by the obstruction of the HPA axis and SNS and by changes in hormone levels. Some Kampo medicines (Rikkunshi-to, Hange-shashin-to, Hange-koboku-to) have already been reported to regulate ACTH and cortisol levels under stress. The HPA axis activity is controlled by the SNS in the brain, where NPY acts on the HPA axis as a neurotransmitter.

Using β-gal-labeled NPY (1—36) as a marker antigen, an anti-rabbit IgG-coated immunoplate as a bound/free (B/F) separator, and MUG as a fluorogenic substrate, we developed a highly sensitive and specific EIA for the quantitation of NPY (1—36). Since 1984, RIA methods for NPY have developed and a number of RIA kits have been on the market, although these methods have several disadvantages due to the use of radioisotopes. The EIA detailed in this report retains the advantages of previous RIA systems while minimizing the disadvantages. This EIA is highly sensitive and specific for NPY (1—36). The sensitivity of this EIA is equal to that of RIA methods and is sufficient to detect plasma NPY levels.

This EIA is sensitive (3.5 pg, 1.4 pg/well) and specific (amino-terminal region) for NPY, and the sharp standard inhibition curve obtained was linear between 35 and 4000 pg/ml. The sensitivity of other immunoassays was reported to be <9.4 pg (RIA) and 10 pg (EIA) With regard to performance, our EIA enables the measurement of many samples (96 wells) at the same time using an anti-rabbit IgG-coated immunoplate as the bound/free separator. The NPY antibody A602/R2R was found to have no-cross-reactivity with the structurally related peptide PYY. We applied our EIA to the detection of plasma NPY-IS levels. The detectable plasma levels of previously reported EIA and RIA methods were 130.4 ± 62.6 pg/ml and 223.6 ± 14.7 pg/ml, respectively. The levels of NPY in human plasma from 5 healthy volunteers using our EIA were nearly equivalent to those reported by Onouha and Alpar. The recovery and reproducibility (C.V. % of interassay and intraassay comparisons) of this EIA with the plasma samples were satisfactory.

The molecular heterogeneity in human plasma was examined using HPLC. The main NPY-IS in plasma was eluted at the same elution time as synthetic human NPY, with several unknown peaks. These unknown peaks may be fragments of NPY containing the amino-terminal region. Our proposed NPY-EIA recognized the amino-terminal region specifically, and may be useful to clinical evaluations.

Repetitive blood sampling raised plasma NPY-IS levels compared with sampling at 0, 40, 120, and 240 min in volunteers who received placebo. Volunteers in whom samples were taken at 0, 40, 120, and 240 min were assumed to be under less stress. In general, venipuncture (pain stress) for blood sampling is postulated to be a stress factor that can stimulate the SNS and produce a release of catecholamines into plasma. The use of venipuncture permitted examination of the kinetics of catecholamine responses to a series of stressors. It has not been reported previously whether venipuncture produces a transitory peak in plasma NPY levels. In our studies, blood sampling at 20-min intervals signif-
icantly increased plasma NPY levels 40 min after the administration of placebo, but blood sampling at 40-min intervals had no effect. Plasma ACTH and cortisol levels were markedly altered by venipuncture stress, and there was a significant positive correlation between the serum cortisol and NPY levels in healthy volunteers. Thus it was thought that venipuncture might be an intense stressor that stimulates the HPA axis and SNS, and that the blood collection interval is a critical factor to examine the stress response to venipuncture. The effects of placebo on NPY are assumed to result from venipuncture stress in volunteers due to repetitive blood sampling.

NPY is a representative neurotransmitter of the SNS and is often used as an indicator of SNS activity. Under stress, NPY is associated with HPA axis activity and the modulation of immunofunction. The stress-dependent NPY release causes abnormalities in the SNS and HPA axis and is associated with depressive disorders. Plasma NPY levels in patients with depression are increased compared with those in healthy individuals and are positively related to plasma cortisol levels under various stresses.10) In our studies, Rikkunshi-to and Hange-shashin-to suppressed the increases in NPY-ISH levels compared with placebo, but Hange-koboku-to and Ninjin-to had no effects. The reason why those latter two medicines had no effects on plasma NPY-ISH levels is unknown. In the four Kampo medicines used this study, Pinelliae Tuber, Zingiberis Rhizoma, and Ginseng Radix are included in the two that suppressed increases in plasma NPY levels and also act on the central nervous system. Katagiri et al. reported that the administration of Pinelliae Tuber or Zingiberis Rhizoma suppresses increases in ACTH and cortisol levels in human plasma under venipuncture stress. Ginseng Radix is also known to be useful for treatment of stress-related disorders like peptic ulcer, depression, and anxiety disorder. Central administration of Ginseng saponin affected NPY mRNA expression in the hypothalamic paraventricular nucleus. Thus Pinelliae Tuber, Zingiberis Rhizoma, and Ginseng Radix, or their combinations, might affect plasma NPY levels. Further studies are needed to elucidate their activities.

Rikkunshi-to and Hange-shashin-to have been used empirically to treat abnormalities of the gastrointestinal system. Based on the empirical results, the effects are assumed to be due to changes in the levels of gut-motor regulatory hormones (i.e., SS, gastrin, motilin, and VIP). On the other hand, these Kampo medicines regulate plasma ACTH and cortisol levels in the HPA axis. In addition to these actions, Rikkunshi-to and Hange-shashin-to were found to affect plasma NPY levels in this study. Thus these effects might reflect pharmacologic activities of Kampo medicines.

In this study, plasma NPY levels were altered by administration of Rikkunshi-to and Hange-shashin-to. Although it is necessary to examine the effects of these Kampo medicines in patients with stress-related disorders, these effects might be beneficial in stress-related disorders and the pharmacologic activities of these medicines should be investigated clinically.

REFERENCES