Comparison of the Effects of Hange-shashin-to and Rikkunshi-to on Human Plasma Calcitonin Gene-Related Peptide and Substance P Levels

Takafumi Naitou,* Hiroki Itoh, and Masaharu Takeyama

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

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Regarding the gastroprotective function as a neural emergency system, sensory afferent neurons in the gastrointestinal mucosa regulate neuropeptide (calcitonin gene-related peptide (CGRP), substance P, etc.) levels, and those peptides play various physiological roles. To determine whether the pharmacological effects of Hange-shashin-to and Rikkunshi-to on the gastrointestinal are due to changes in gastrointestinal mucosa regulatory peptides levels, we investigated the levels of CGRP-like immunoreactive substances (IS) and substance P-IS in plasma from healthy subjects. A single oral administration of Hange-shashin-to caused significant increases in CGRP-IS (40–60 min) and substance P-IS (60–180 min) levels in the plasma compared with the levels induced by a placebo. Rikkunshi-to and a 5.0 g Pinelliae tuber extract had no significant effect on CGRP-IS and substance P-IS levels. Extract of a 2.5 g Zingiberis rhizoma significantly caused increases in CGRP-IS at 40 min and in substance P-IS at 60 min. These results, in comparison with Kampo medicines, might indicate that the pharmacological actions of Hange-shashin-to closely are related to changes in CGRP-IS and substance P-IS levels, while Zingiberis rhizoma partially might participate in those effects of Hange-shashin-to.

Key words Hange-shashin-to; Rikkunshi-to; calcitonin gene-related peptide (CGRP); substance P; Zingiberis rhizoma

Hange-shashin-to, a Chinese herbal (Kampo) medicine, is prepared from seven crude herbs, Pinelliae tuber, Scutellariae radix, Ginseng rhizoma, Atractylodis rhizoma, Glycyrrhizae radix, Zizyphi fructus, and Coptidis rhizoma. This medicine has been used for the empirical treatment of acute and chronic gastroenteric catarrh, fermentative diarrhea, and acute gastroenteritis. Hange-shashin-to has the effects of regulating the lower as well as upper gastrointestinal tract, and the mechanisms of those pharmacological effects have been partially elucidated.1–4) Hange-shashin-to has been clinically evaluated as a more effective pharmaceutical on hyper-functioning conditions.

Rikkunshi-to, with eight crude herb ingredients, Ginseng radix, Atractylodis rhizoma, Hoelen, Pinelliae tuber, Aurantii Nobilis pericarpium, Zizyphi fructus, Glycyrrhizae radix, and Zingiberis rhizoma, has been evaluated for clinical usefulness in the treatment of chronic hypofunction of the gastrointestinal tract with gastric flatulence, anorexia, nausea, and vomiting. Recently, empirical effects proved that those were based on an increased blood flow in the stomach, accelerating gastric emptying, and improving gastric mucosal damage.5–7)

One of the gastrointestinal motility regulatory factors on those empirical effects has been assumed to be the induction of changes in the levels of peptides (somatostatin, motilin, and gastrin) in plasma.8–12) On the gastroprotective function as a neural emergency system, sensory afferent neurons in the gastrointestinal mucosa regulate neuropeptides (calcitonin gene-related peptide (CGRP) and tachykinins (substance P, etc.)) levels and play various physiological roles.13,14)

CGRP possesses several potent biological activities, including vasodilation, being the most powerful vasoactive substance described to date, and it increases mucosal blood flow.15–17) CGRP is known to coexist with tachykinins, in the population of sensory neurons in man.18) Substance P is widely distributed in the central and peripheral divisions of the nervous system and in the enteroendocrine cells of gut,19) and it participates in the regulation of gastrointestinal motility and secretion,20) the hypothalmo-pituitary-adrenal axis,21) and the stimulation of salivation.22)

To determine whether the pharmacological effects of Hange-shashin-to and Rikkunshi-to on the gastrointestinal are due to changes in gastrointestinal mucosa regulatory peptides levels, we compared the plasma CGRP-like immunoreactive substances (IS) and substance P-IS levels after the administration of these Kampo medicines and related herbs.

MATERIALS AND METHODS

Materials Hange-shashin-to (EK-14, lot 011A), prepared as a 3.8 g dried powder extract of Pinelliae tuber (5.0 g), Scutellariae radix (2.5 g), Zingiberis rhizoma (2.5 g), Ginseng radix (2.5 g), Glycyrrhizae radix (2.5 g), Zizyphi fructus (2.5 g), and Coptidis rhizoma (1.0 g); and Rikkunshi-to (EK-43, lot 26L99), prepared as a 4.1 g dried powder extract of Ginseng radix (4.0 g), Atractylodis rhizoma (4.0 g), Hoelen (4.0 g), Pinelliae tuber (4.0 g), Aurantii nobilis pericarpium (2.0 g), Zizyphi fructus (2.0 g), Glycyrrhizae radix (1.0 g), and Zingiberis rhizoma (0.5 g), were kindly supplied by Kanebo (Tokyo, Japan). Zingiberis rhizoma was purchased from Sainokiyu (Osaka, Japan), and its extract contained 0.72 percent 6-gingerol as a bioactive compound. Pinelliae tuber extract were purchased from Nippon Funmatsu Yakuhin (Osaka, Japan). The placebo was the additive of the EK-14, EK-43 formulation, Zingiberis rhizoma extract, and Pinelliae tuber extract alone.

Synthetic human CGRP and its fragment (8–37), and substance P were purchased from the Peptide Institute (Osaka, Japan). Antiserum to CGRP was purchased from Biogenesis (Poole, U.K.). Antiserum to substance P (RA-08-095) was purchased from Cambridge Research Biochemicals (Cambridge, U.K.). All other reagents were of reagent grade commercially available.

Subjects Five healthy male volunteers without nasal allergy, aged 23–40 years, participated in this study. Each

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* To whom correspondence should be addressed. e-mail: naitou@hama-med.ac.jp © 2003 Pharmaceutical Society of Japan
subject received information on the study’s scientific purpose, which was approved by the Ethics Committee of Oita Medical University, and subsequently gave written informed consent. No subject received any medication for at least 1 month before the study. There were at least 3-month intervals between each study. This study was carried out in the time except February, March, and April.

**Study Schedule** Hange-shashin-to, Rikkunshi-to, Zingiberis rhizoma extract (2.5 g), Pinelliae tuber extract (5.0 g), or placebo at the same dose of 6.0 g (final weight with additive) was orally administered with water, and the five volunteers took the same test drug on the same day. All subjects ate lunch at 11:45—12:00, and the study was carried out from 14:00 until 18:00. Venous blood samples from a forearm vein were taken before and 20—240 min after the administration.

**Preparation of Plasma Extract** The blood was immediately placed in chilled tubes containing aprotinin (500 KIU/ml) and EDTA (1.2 mg/ml). After centrifugation, plasma was diluted with 4% acetic acid (pH 4.0) and loaded into Sep-Pak C18 cartridges (Millipore, MA, U.S.A.). The peptides in plasma were eluted with 70% acetonitrile in 0.5% acetic acid (pH 4.0) and lyophilized. The recovery of plasma CGRP-IS and substance P-IS was >90% with this extraction procedure.

**Enzyme Immunoassay for CGRP and Substance P** Peptide levels in plasma were measured using an enzyme immunoassay for CGRP-IS and substance P-IS as previously described. Human CGRP (8—37) and substance P were conjugated with β-d-galactosidase (Boehringer Mannheim, Mannheim, Germany) with N-(ε-maleimido-caproyloxy)-succinimide. The assay was performed using a delayed addition method, and separation of bound and free antigen was performed on an anti-rabbit IgG (55641) (ICN Pharmaceuticals, OH, U.S.A.)-coated immunoplate. The fluorescence intensity of the fluorescent product 4-methylumbelliferon was measured with an MTP-100F microplate reader (Corona Electric, Ibaraki, Japan). The enzyme immunoassays for CGRP and substance P were sensitive to detection limits of 0.084 and 0.4 fmol/well.

**Data Analysis** Plasma CGRP-IS and substance P-IS levels are expressed as mean±S.D. (pg/ml). Comparisons of mean values were made by analysis of variance and Dunnett’s test. A value of p<0.05 was regarded as significant.

**RESULTS**

**Effect of Hange-shashin-to on CGRP-IS and Substance P-IS Levels** The plasma CGRP-IS level-time profile after the administration of Hange-shashin-to is shown in Fig. 1A. CGRP-IS levels of the placebo showed a decreasing tendency from 0 to 40 min. Hange-shashin-to caused significant increases in CGRP-IS at 40 and 60 min, compared with the response of the placebo, and enhanced the secretion of CGRP-IS.

Hange-shashin-to significantly increased substance P-IS levels between 60—180 min compared with the response of the placebo (Fig. 1B). And the changes of substance P were almost twice as high levels as the placebo and showed durability compared with the CGRP-IS secretion.

**Effect of Rikkunshi-to on CGRP-IS and Substance P-IS Levels** Figures 2A and 2B show plasma CGRP-IS and substance P-IS levels after the administration of Rikkunshi-to. Rikkunshi-to had no significant effect on plasma CGRP-IS and substance P-IS levels. Plasma peptide levels in both
Rikkunshi-to and the placebo remained almost the same (CGRP-IS: about 20 pg/ml and substance P-IS: about 40 pg/ml) before and after the administration.

Effect of Herbs (Zingiberis rhizoma and Pinelliae tuber Extract) on CGRP-IS and Substance P-IS Levels

The plasma CGRP-IS levels after the administration of Zingiberis rhizoma extract are shown in Fig. 3A. Extract of Zingiberis rhizoma (2.5 g) caused significant increases in CGRP-IS at 40 min, and did not show a decreasing tendency from 0 to 40 min such as placebo.

Figure 3B shows the effects of Zingiberis rhizoma extract on plasma substance P-IS levels. Zingiberis rhizoma extract caused significant increases of substance P-IS levels at 60 min compared with the response of the placebo. And the changes of substance P showed no durability.

Figures 4A and 4B show plasma CGRP-IS and substance P-IS levels in Pinelliae tuber. A 5.0 g Pinelliae tuber had no significant effect on plasma CGRP-IS and substance P-IS levels as well as Rikkunshi-to.

DISCUSSION

Recently, some gastrointestinal motility regulatory factors relative to the pharmacological effects of Kampo medicines has been assumed to be due to changes in regulatory peptides levels. Some abnormalities of gastrointestinal function are presumed to result from changes in hormone levels. Sensory afferent neurons in the gastrointestinal mucosa regulate neuropeptide levels, which play various physiological roles in gastroprotection.

CGRP is a powerful vasoactive substance, which is released from the sensory afferent nerve endings against gastric mucosal injury (acid and the other noxious chemicals such as capsaicin, ethanol, etc.) in the stomach. CGRP increases gastric mucosal blood flow as a gastroprotective factor. In this study, Hange-shashin-to raised plasma CGRP-IS levels, but Rikkunshi-to had no effect. Hange-shashin-to might directly stimulate CGRP-containing nerves, or indirectly secrete CGRP accompanied by the stimulation of other secretion cells and some mechanisms.

The herbs contained in these Kampo medicines might have an effect on neuropeptides. Both Kampo medicines have Zingiberis rhizoma as a common ingredient, and its herb contains 6-gingerol and 6-shogaol as the main bioactive compound, both of which have vanilloid structures. Capsaicin, a vanilloid receptor agonist, stimulates capsaicin-sensitive afferent neurons, which release CGRP from their nerve endings. Hange-shashin-to has a five-fold larger amount of Zingiberis rhizoma content than Rikkunshi-to. Thus, the amount of 6-gingerol and 6-shogaol of the Zingiberis rhizoma extract in Hange-shashin-to may be greater than that of Rikkunshi-to, in estimated amount. In previous reports, Zingiberis rhizoma extract and 6-shogaol increased intestinal blood flow, and CGRP (8—37), a CGRP receptor antagonist, abolished the reaction. Furthermore, lutfudine, a histamine H2 receptor antagonist, which has vanilloid structures and is structurally related to capsaicin, changed the levels of CGRP as well as Hange-shashin-to in human plasma. In our study, Zingiberis rhizoma extract also significantly raised CGRP-IS levels, but the levels were different from changes in Hange-shashin-to in terms of degree of increase. The pattern of secretion in CGRP-IS after an administration of Zingiberis rhizoma extract showed little similarity to that of Hange-shashin-to at the increasing time. Due to this difference in degree in increase between Hange-
substance P, tachykinins, coexists with CGRP in the sensori-overflow neurons of the gastrointestinal mucosa, and is released with acetylcholine in response to depolarizing stim-
ulation in the enteric nervous system. Hange-shashin-to has been clinically shown to change the CGRP and substance P levels, while Rikkunshi-to had no effects. We hypothesized that the difference in pharmacological effect between Hange-shashin-to and Rikkunshi-to might be based on the effects of CGRP and substance P. And Zingiberis rhizoma might par-
tially participate in both peptide changes.

REFERENCES