Dai-kenchu-to Raises Levels of Calcitonin Gene-Related Peptide and Substance P in Human Plasma

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Sensory afferent neurons in the gastrointestinal mucosa regulate neuropeptides [calcitonin gene-related peptide (CGRP), substance P, etc.], which play various physiologic roles and are gastroprotective. To determine whether the pharmacologic effects of Dai-kenchu-to (DKCT) on the gastrointestinal mucosa are due to changes in gastrointestinal mucosa regulatory peptide levels, we examined the effects of the DKCT on the levels of CGRP-like immunoreactive substances (IS) and substance P-IS in plasma taken from five healthy subjects. A single oral administration of DKCT 7.5 g caused significant increases in plasma CGRP-IS at 40 min, and in substance P-IS levels at 20 and 60 min, compared with a placebo group. The present study may indicate that the pharmacologic action of DKCT is closely related to changes in CGRP- and substance P-IS levels.

Key words 
Dai-kenchu-to; calcitonin gene-related peptide (CGRP); substance P; intestinal blood flow

Dai-kenchu-to (DKCT, Da-Jian-Zhong-Tang), a traditional Chinese (Kampo) herbal medicine, is prepared from three different herbs: ninjin (Ginseng radix), sanshou (Zanthoxyli fructus), and kankyou (Zingiberis siccatum rhizoma). DKCT has been used for abdominal obstruction, including bowel obstructions and a feeling of coldness in the abdomen. DKCT has recently been evaluated for its clinical usefulness in the treatment and prevention of uncomplicated postoperative adhesive intestinal obstruction and its clinical efficacy is well reported.1 It has been reported that DKCT is related to both increments in gastrointestinal motility and intestinal blood flow. Nagano et al. confirmed that DKCT causes significant increases in the level of motilin, a powerful inducer of gastrointestinal motor activity, in human plasma.2 The results indicate that the gastrointestinal motor activity of DKCT is closely related to changes in motilin in human plasma. Nagano et al. also reported that DKCT causes increases in plasma vasoactive intestinal polypeptide (VIP) which has a vasodilating effect.

Sensory afferent neurons in the gastrointestinal mucosa regulate neuropeptide [calcitonin gene-related peptide (CGRP) and tachykinins (substance P)] levels and play various physiologic roles. CGRP has several potent biological activities, including vasodilation. It is the most powerful vasoactive substance known and it increases mucosal blood flow.3) CGRP is known to coexist with tachykinins in the population of sensory neurons in humans.5) Substance P is widely distributed in the central and peripheral nervous system and in the enterocoidine cells of the gut and it participates in the regulation of gastrointestinal motility, secretion, and blood flow.6) Recently, it has been reported that DKCT increases intestinal blood flow, which is mainly mediated by CGRP, in rats.9) However, the effects of DKCT on plasma CGRP and substance P levels in humans have not been described previously. Therefore we examined the plasma levels of CGRP and substance P in humans after administration of DKCT.

MATERIALS AND METHODS

Materials DKCT (TJ-100), prepared as a dried powder extract of Ginseng radix (3.0 g), Zanthoxyli fructus (2.0 g) and Zingiberis siccatum rhizoma (5.0 g) were purchased from Tsumura Co. Ltd. (Tokyo, Japan). A mixture of glucose and maltose (Summalto, Hayashibara Co. Ltd., Okayama, Japan) was used as a placebo.

Synthetic human CGRP and its fragment (8-37), substance P, were purchased from the Peptide Institute (Osaka, Japan). Antisera to CGRP were purchased from Biogenesis (Poole, U.K.), and substance P (RA-08-095) from Cambridge Res- search Biochemicals (Cambridge, U.K.). All other reagents were of analytical reagent grade from commercial sources.

Subjects Five healthy male volunteers (nonsmokers), aged 25—27 (median 26) years and weighing 55—68 (median 62) kg, participated in this study. Each subject received information about the scientific purpose of the study, which was approved by the Ethics Committee of Oita Medical University, and gave informed consent. No subject received any medication for 1 month preceding the test and no stimulator of gastrointestinal motility was administered to any subject during the study.

Study Schedule DKCT or placebo was orally administered as single dose of 7.5 g with water. Venous blood samples (10 ml) from a forearm vein were taken for enzyme immunoassay (EIA) of the levels of CGRP and substance P. Samples were taken 20, 40, 60, 90, 120, 180, and 240 min after administration of the drugs. All subjects ate lunch (11:30—12:00) and the study was carried out from 14:00 (2 h after lunch) to 18:00.

Preparation of Plasma Extracts The blood samples were collected in a chilled tube containing aprotinin (500 KIU/ml) and ethylenediaminetetraacetic acid (EDTA) (1.2 mg/ml). After centrifugation, the plasma were diluted with 4% acetic acid (pH 4.0), loaded onto Sep-Pak C18 cartridges (Millipore Corp., Milford, 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, and reconstituted to 100 µl with the assay buffer and subjected to EIA. For the EIA system, plasma samples were concentrated five-fold with Sep-Pak C18 cartridges. The recovery of plasma CGRP-IS and substance P-IS was >90% with this extraction procedure.

EIA of CGRP and Substance P EIs for CGRP-IS10) and substance P-IS11) were performed using a delayed-addi-
tion method. Human CGRP (8-37) and substance P were conjugated with β-D-galactosidase (Boehringer Mannheim, Mannheim, Germany) with N-(ε-maleimidocaproyloxy)-suc-cinimide. The assay was performed using a delayed-addition method, and separation of bound and free antigen was performed on anti-rabbit IgG (55641) (ICN Pharmaceuticals, OH, U.S.A.)-coated immunoplates. The fluorescence intensity of the fluorescent product 4-methylumbelliferone was measured with an MTP-100F microplate reader (Corona Electric, Ibaraki, Japan). The enzyme immunoassays for CGRP and substance P was sensitive to detection limits of 0.08 and 0.40 fmol/well, respectively.

**Data Analysis** All values are expressed as mean±S.D. Comparisons of plasma peptide levels among blood sampling times were made with the Mann–Whitney U-test. Values of \(p <0.01\) and \(p<0.05\) were regarded as statistically significant.

**RESULTS AND DISCUSSION**

Postoperative ileus involves an inhibition of bowel transit caused by an impairment of motility. However, maintaining or increasing blood flow is thought to be a central element in protecting the gastrointestinal tract from endogenous and exogenous injurious factors. Although the underlying mechanisms of DKCT remain unclear, several reports have shown that DKCT improves to gastrointestinal motility and increases intestinal blood flow. In this study, CGRP and substance P levels were examined to study the effects of DKCT.

The profiles of average plasma CGRP-IS levels against time in five healthy subjects after a single oral administration of DKCT or placebo are shown in Fig. 1. DKCT caused significant increases in CGRP-IS at 40 min (95.1 pg/ml) compared with the response in the placebo group (about 31.0 pg/ml) (Table 1). Figure 2 shows plasma substance P-IS levels after administration of DKCT in five healthy subjects. DKCT significantly increased substance P-IS levels at 20 and 60 min (57.5±31.6 pg/ml at 20 min, 52.9±9.4 pg/ml at 60 min), compared with placebo (about 29.6 pg/ml) (Table 1).

CGRP is a powerful vasoactive substance, which is released from the sensory afferent nerve endings in response to gastric mucosal injury in the stomach. CGRP increases gastric mucosal blood flow as a gastroprotective factor. In addition, CGRP has a potent effect on gastrointestinal motility and secretion. A role for CGRP in the intestine has not been shown. However, there is a report that CGRP effects on submucosal blood vessels in the rat intestine are involved in the regulation of intestinal mucosal blood flow. Therefore CGRP is thought to be the predominant mediator in increasing blood flow. In our results, DKCT significantly raised plasma CGRP-IS levels after administration to healthy subjects. DKCT may directly stimulate CGRP-containing nerves or indirectly secrete CGRP accompanied by the stimulation of other secretory cells and mechanisms.

Substance P coexists with CGRP in the sensory afferent neurons of the gastrointestinal mucosa and is released with acetylcholine in response to depolarizing stimulation in the enteric nervous system. In the intestine, substance P controls motility and secretion. Previous studies have shown that substance P neurons project orad into the myenteric plexus and circular muscle layer and may be involved in the regulation of the ascending contraction component of the peristaltic reflex. Interestingly, VIP neurons project caudad into the myenteric plexus and circular muscle layer and may be involved in the regulation of the descending relaxation component of the peristaltic reflex. In our results, DKCT raised plasma substance P-IS levels after administration in healthy subjects. Also, Nagano et al. reported that DKCT causes an increase in plasma VIP-IS after a single oral administration in healthy humans. Increased VIP-IS may improve a feeling of coldness in the abdomen. Accordingly, the action by which DKCT improves gastrointestinal motility may be closely related to changes in VIP- and substance P-IS in plasma. Furthermore, Schmidt et
al. have recently reported that substance P increased mucosal blood flow of the small bowel blood dose dependently when infused intravenously into healthy subjects under fasting conditions.23) Tachykinergic effects on blood flow seem to be primarily mediated by neurokinin 1 (NK1) receptors. In inflammatory bowel disease, a 1000-fold increase in the number of NK1 receptors on small bowel blood vessels has been described.24,25) Therefore substance P may not only play a role in the mediation of peristaltic contractions, but also a pathophysiological role in the increased blood flow in the small intestine.8) However, since there are no findings that the increase in intestinal blood flow by DKCT is related to substance P and that it participates in the physiologic regulation of mucosal blood flow in the gastrointestinal tract, further studies are needed.

Several neurochemical and pharmacological bases for DKCT effects on CGRP and substance P are supported by the present study. DKCT has Z. rhizoma as one of its ingredients. This herb contains 6-gingerol and 6-shogaol as its main bioactive compounds. These compounds have vanilloid entents. This herb contains 6-gingerol and 6-shogaol as its main bioactive compounds. These compounds have vanilloid receptors. Capsaicin, a vanilloid receptor agonist, stimulates structures and act as capsaicin-like stimulators of vanilloid bioactive compounds. These compounds have vanilloid receptors. Capsaicin, a vanilloid receptor agonist, stimulates capsaicin-sensitive afferent neurons, which releases CGRP and substance P from their nerve endings.26—29) In previous reports, Zingiberis rhizoma extract and 6-shogaol produced an increase in intestinal blood flow, and the CGRP receptor antagonist CGRP (8-37) completely abolished the reaction.9) Satoh et al. reported that the substance P antagonist sapan-tide tended to inhibit the contraction by hydroxyl β-sanshoohl, an ingredient of Zanthoxyli fructus (a constituent herb of DKCT) in longitudinal muscle of guinea pig ileum.26) Bartho et al. reported that the contractile response of the guinea pig ileum to capsaicin was abolished by substance P antagonists (D-Pro2, D-Trp7, 9).30) Naito et al. also reported that plasma CGRP and substance P levels significantly increased after administration of Zingiberis rhizoma extract to healthy subjects.31) Therefore these results suggest that hydroxyl β-sanshoohl and 6-shogaol are the predominant ingredients that stimulate CGRP and substance P release from nerve endings.

We conclude that DKCT improves the action of the ileus, gastrointestinal motility, and intestinal blood flow by significantly increasing CGRP- and substance P-IS levels in plasma. Our results indicate that DKCT is closely related to changes in CGRP-IS and substance P-IS levels in plasma.

REFERENCES