Effects of an ace-inhibitory agent, katsuobushi oligopeptide, in the spontaneously hypertensive rat and in borderline and mildly hypertensive subjects

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Abstract

It has been previously documented that the thermolysin digest of “Katsuobushi,” or dried bonito, a traditional Japanese food, potently inhibits the angiotensin I-converting enzyme (ACE). This hydrolyzate “Katsuobushi oligopeptide (KO)” has antihypertensive effects in hypertensive subjects at a dose of 3 g/day. In order to reduce the amount of KO required to produce an effect, we obtained a stronger KO (S-type KO) by ultra-filtration. The S-type KO had two-fold higher ACE-inhibitory activity, and also showed two-fold higher antihypertensive activity in the spontaneously hypertensive rat after oral administration, compared with the original KO. In a human study, the antihypertensive effects of 1.5 g/day of S-type KO were monitored against a placebo in a double-blind, randomized, cross-over study in 61 borderline and mildly hypertensive subjects. S-type KO showed antihypertensive activity without any side-effects. Thus, the ACE-inhibitory S-type KO demonstrated more potent antihypertensive effects than the original formulation. © 2001 Elsevier Science Inc. All rights reserved.

Keywords: Angiotensin I-converting enzyme inhibitor; Peptide; spontaneously hypertensive rat; Bonito; Borderline hypertensive human; Antihypertensive activity

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Abbreviations: KO: katsuobushi oligopeptide; ACE: angiotensin I-converting enzyme; SHR: spontaneously hypertensive rat; IC_{50}: 50% inhibitory concentration; LKPNM: Leu-Lys-Pro-Asn-Met; SBP: systolic blood pressure; DBP: diastolic blood pressure; HHL: hippuryl-histidyl-leucine.
1. Introduction

The angiotensin I-converting enzyme (ACE) catalyzes the formation of angiotensin II, a strong vasopressor, from angiotensin I, and inactivates bradykinin, which possesses hypotensive activity [1]. ACE inhibitors, such as the commercially available antihypertensive drugs captopril and enalapril, block both of these actions, which contributes to their potent antihypertensive activity in the spontaneously hypertensive rat (SHR) and in hypertensive patients [2]. Recently, ACE-inhibitory peptides derived from casein [3–6], fish muscle [7,8], and other proteins [6,9] were isolated. The present authors have recently shown that dried bonito (katsuobushi), a traditional Japanese seasoning made of bonito muscle, also has potent ACE-inhibitory activity when digested by thermolysin, and eight ACE-inhibitory peptides have been isolated from it [10]. Of these peptides, Leu-Lys-Pro-Asn-Met (LKPNM) showed long-lasting and dose-dependent antihypertensive activity after oral administration in the SHR. LKPNM (IC50 = 2.4 μmol/L) is hydrolyzed into Leu-Lys-Pro (LKP; IC50 = 0.32 μmol/L) by ACE itself, resulting in a peptide with 8-fold higher activity [11]. Thus, LKPNM is a typical ACE-inhibitory peptide derived from thermolysin-digested katsuobushi (Katsuobushi oligopeptide, KO); the LKPNM content in KO is 0.17% (w/w) [12]. KO exerts an antihypertensive activity after oral administration both in the SHR [11,12] and in borderline and mildly hypertensive subjects [13,14], and a “Peptide soup” containing KO has been approved as a “Foods for Specified Health Use” in Japan. However, in order to effectively lower blood pressure, an excessive amount of KO is needed. In this paper, we describe the enrichment of ACE-inhibitory peptides from KO, and the increased antihypertensive activity of this strong-type KO (S-type KO) in SHR and in humans.

2. Materials and methods

2.1. Ace-inhibitory activity

ACE-inhibitory activities were measured using hippuryl-histidyl-leucine (HHL; Peptide Institute Inc., Osaka, Japan) as a substrate and extracts of rabbit lung acetone powder according to the method of Cushman and Cheung [15], with the minor modification of Yamamoto et al. [16]. The phosphate buffer used in the original method was not suitable for our assay, and was therefore changed to borate buffer. Each assay mixture contained the following components at the indicated final concentration: borate buffer (pH 8.3), 100 mmol/L; sodium chloride, 400 mmol/L; HHL, 5 mmol/L; and ACE (Sigma Chemical Co., St. Louis, MO., USA) 3 mU per 250 μl of assay volume (1 unit of ACE activity is defined as the amount catalyzing the formation of 1 μmol of hippuric acid from HHL in 1 min at 37°C). This assay mixture was incubated at 37°C for 30 min. The IC50 value is the concentration of peptide that resulted in 50% ACE inhibition in the reaction mixture.
2.2. Preparation of original and S-type KO

KO was prepared as previously reported [10]. Briefly, 100 g of katsuobushi (dried bonito) was ground and suspended in 900 mL of water, boiled for 10 min, and digested with thermolysin (Sigma Chemical Co., St. Louis, MO., USA) at 70°C for 3 h at pH 7.0. The solution was then boiled for 10 min to inactivate the protease. This was followed by centrifugation at 2,050 x g for 10 min at room temperature, and filtration of the supernatant. This hydrolyzate, the original KO, was used previously in animal and human studies. The IC50 value of this original KO was 58.3 μg/mL, and it contained 0.17% (w/w) LKPNM [12,14]. For further purification and concentration of the active ACE-inhibitory peptide from the original KO, substances with a molecular weight in excess of MW 3,000 were removed using a cross-flow ultra filtration module (SEP-1013, Asahikasei Industry Co. Ltd., Tokyo, Japan). The filtrate, S-type KO, showed more potent ACE-inhibitory activity (IC50 = 31.5 μg/mL), and contained 0.34% (w/w) of LKPNM. The S-type KO was used in the present study.

2.3. Antihypertensive activity after oral administration in SHR

SHR were obtained from Charles River Japan, Inc. (Kanagawa, Japan). SHR were fed with MF chow (Oriental Yeast Co. Tokyo, Japan) consisting of 54.0% carbohydrates, 23.8% protein, 5.1% fat, 3.2% fiber, 6.1% minerals and 0.24% sodium. Each type of KO was dissolved in saline and 125–1,000 mg/kg was administered via a metal gastric tube. Blood pressure was measured by the tail cuff method using a UR-5000 (Ueda Seisakusho, Kyoto, Japan) before [0], and 2, 4, 6 and 8 hr after the administration of each type of KO. The systolic blood pressure of the SHR registered 241.3 ± 1.9 mm Hg before administration with KO. Animals were treated according to the guidelines for care and use of laboratory animals (NRC 1985).

2.4. Human study of S-type KO

A comparative double-blind study was conducted between October 1998 and April 1999 at a hospital in Osaka, Japan. Sixty-five borderline (high normal) and mildly hypertensive subjects were enrolled in the study, all of whom had blood pressure exceeding the standard of JNC VI (systolic blood pressure [SBP] registered more than 130 mm Hg [mean 149.4 ± 1.0 mm Hg] and diastolic blood pressure [DBP] levels were in excess of 85 mm Hg [mean 93.5 ± 1.3 mm Hg]) [17]. All subjects gave informed consent for the study after having being briefed on its nature, purpose, and possible side-effects. The investigation was approved by the Institutional Review Board in September 1998. Exclusion criteria were known secondary causes of hypertension, such as diabetic or renal dysfunction, or serious hypertension (SBP>170 mm Hg or DBP>110 mm Hg). Those who abused alcohol or were being treated for serious cardiac, renal, or hepatic diseases were also excluded. All subjects were asked to maintain their normal physical activities and food intake throughout the investigation, and were asked to keep their sodium chloride intake below 8 g/day using the Food-Calorie Conversion Table [18]. Blood pressure was measured every day during the
2-week period that preceded the start of the test, and the averaged value was taken to be the baseline value. Subjects were randomly assigned to one of two groups. Subjects in one group received 10 tablets every day that resulted in a total daily dose of 1.5 g of S-type KO (5 mg/day as LKPNM). Each tablet (250 mg) contained S-type KO (60%) and diluent bulk increasers, including cornstarch (20%), cellulose (15%) polysaccharide (0.5%), lubricating and glossing agents (3%) and others (1.5%). Subjects in the other group received 10 placebo tablets, which contained katsuobushi powder (60%) instead of S-type KO. Katsuobushi does not have any ACE-inhibiting activity or LKPNM before its digestion with thermolysin, so the placebo tablets also did not contain any LKPNM, and did not show any ACE-inhibitory activity. The S-type KO and placebo tablets contained 0.1% sodium. The test schedule (Table 1) contained an observation period followed by a crossover test: for the first 5 weeks, one group received the drug and the other the placebo, and for the second 5 weeks the treatments for each group were reversed. Blood pressure was measured every day between 8:00 and 9:00 a.m. by using a sphygmomanometer on the left arm; the measurements were made with the subject in a sitting position, after the subject had rested for 5 minutes. Compliance and subjective and objective symptoms were monitored by distributing a questionnaire or by consulting the subjects.

2.5. Analytical procedure

The evaluation of the effect was conducted based on “Guidelines for the clinical evaluation method of antihypertensive agents” (dated May 21, 1989, No.1443 of the official bulletin of the Pharmaceutical and Supply Bureau).” Blood pressure decrease was scored into five classes. SBP and DBP in the last week of each test period were compared with the baseline values of individual subjects, and compared across groups. The statistical significance of differences before and after treatment for each group was assessed using the Mann-Whitney U test (StatView, Abacus Concepts, Inc, Berkeley, CA USA). Results were expressed as the mean ± S.E.

3. Results

3.1. Comparison of the antihypertensive activities of original KO and S-type KO in SHR

The ACE-inhibitory peptide content of KO was enriched by ultra-filtration to yield strong-type (S-type) KO, which has almost twice the ACE-inhibitory activity of original KO.
In the SHR, oral administration of only a half-dose of the S-type KO was needed to give the same antihypertensive activity as a full dose of the original KO. As shown in Fig. 1, both types lowered SBP in a dose-dependent fashion following oral administration. The minimum effective dose of the S-type KO was 125 mg/kg, as compared to 250 mg/kg for the original KO, while the reduction of SBP by 10 mm Hg required an oral dose of 290 mg/kg of the S-type KO and 680 mg/kg of the original KO. Thus, S-type KO has approximately 2-fold higher activity than original KO in vivo.

3.2. Long-term ingestion in humans

3.2.1. Antihypertensive effect

The original KO effectively lowered blood pressure in borderline and mildly hypertensive subjects after long-term ingestion at a dose of 3 g/day [12,14]. As mentioned above, the S-type KO showed 2-fold higher activity than the original KO. We therefore studied whether long-term ingestion of half as much of the S-type KO (1.5 g/day) would also show antihypertensive activity in borderline and mildly hypertensive subjects. Sixty-five subjects were originally enrolled in the study, but one female and three male subjects were unable to pursue the study to the end. The reasons for non-compliance were either a failure to appear for visits during the treatment period for personal reasons not related to health conditions (2 males and one female were transferred to different work sites by their companies) or the failure to keep taking the tablets (one male). The data from these subjects were excluded from the analysis. All other subjects completed the study. Thirty subjects received the S-type KO in the first test period, and the other 31 subjects received the placebo. The baseline data
of SBP, DBP, and body mass index (BMI) for each group during the observation period are shown in Table 2. The baseline SBP of the drug-first group was 149.5 ± 1.6 mm Hg and that of the placebo-first group was 149.3 ± 1.3 mm Hg, not a significant difference. There were also no differences in SBP and DBP with gender (male: 149.8 ± 1.5 mm Hg and 93.1 ± 3.7 mm Hg, female; 148.9 ± 2.0 mm Hg and 92.4 ± 1.9 mm Hg), nor were any of the other baseline values significantly different between the groups.

The changes in SBP and DBP following the 2 treatment periods are shown in Fig. 2. In the first 5-week period, the group that received S-type KO showed a clear decrease in blood pressure. The SBP decreased by 11.7 ± 1.3 mm Hg (p < 0.01 compared with the placebo group) and the DBP by 6.9 ± 1.0 mm Hg (p < 0.01). The group that received the placebo showed a temporary blood pressure decrease in the second week, but this low value quickly returned to the baseline level. After the crossover, the group that had previously received the placebo and failed to show any significant decrease in blood pressure, now started to show a blood pressure decrease following the administration of the S-type KO: the SBP decreased by 9.4 ± 1.2 mm Hg (p < 0.01 compared with the placebo group) and the DBP decreased by 4.6 ± 0.8 mm Hg (p < 0.01). In the group that first took the S-type KO, no subject showed an abnormal blood pressure elevation (rebound effect) when switched to the placebo, but the blood pressure values began to return back to the baseline level during the course of placebo ingestion (Fig. 2).

### 3.3. Judgement of within-subject blood pressure reduction

The blood pressure decrease in each subject was evaluated by comparing the value obtained during the last week of the S-type KO ingestion period with the baseline value. As a result of this evaluation, the number of subjects who had significantly decreased blood pressure was 2 out of 30 (6.7%) in the first S-type KO ingestion group and one out of 31 (3.2%) in the latter ingestion group (Table 3). The positive effective ratio (ratio of subjects who were judged to have significant or moderate blood pressure decrease) was 63.4% (19 among 30) in the first group and 61.3% (19 among 31) in the latter group. The total effective ratio was 62.3% (38 among 61) overall (Table 3).

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>First ingestion group (Mean ± S.E.)</th>
<th>Latter ingestion group (Mean ± S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>61.6 ± 1.4</td>
<td>61.1 ± 1.8</td>
</tr>
<tr>
<td>SBP (mm Hg)</td>
<td>149.5 ± 1.7</td>
<td>149.3 ± 1.3</td>
</tr>
<tr>
<td>DBP (mm Hg)</td>
<td>93.6 ± 1.8</td>
<td>92.7 ± 1.7</td>
</tr>
<tr>
<td>Heart rate (beat/min)</td>
<td>70.6 ± 1.7</td>
<td>69.9 ± 1.6</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.3 ± 0.5</td>
<td>23.7 ± 0.6</td>
</tr>
<tr>
<td>Gender: Male</td>
<td>18 (60.0%)</td>
<td>18 (58.1%)</td>
</tr>
<tr>
<td>Female</td>
<td>12 (40.0%)</td>
<td>13 (41.9%)</td>
</tr>
</tbody>
</table>
3.4. Side effects

According to the questionnaires given before and after the entire test period, no subject experienced any abnormal symptoms. No subject presented with any significant change in
body mass index after the test (the mean BMI of KO-first group was 23.3 ± 0.5 kg/m² before and 23.1 ± 0.6 kg/m² after; that of the placebo-first group was 23.7 ± 0.6 kg/m² before and 23.5 ± 0.5 kg/m² after). This was also the case for the heart rate, which remained essentially unchanged. The mean heart rates of the KO-first group and the placebo-first group were 70.6 ± 1.7 beats/min and 69.9 ± 1.6 beats/min, respectively, before treatment, and 70.1 ± 1.5 and 70.7 ± 1.3 beats/min, respectively, after treatment.

4. Discussion

In a previous paper, we reported that the original KO effectively lowered blood pressure with no side effects in more than 60% of borderline hypertensive subjects, when the subjects were given a dose of 3 g/day [11,14]. Thus, the original KO showed more potent antihypertensive activity compared to ACE-inhibitory substances derived from other foods [3–7, 8,9]. However, the amount of the original KO that was needed to effectively lower blood pressure was prohibitively large. The original KO contained high molecular weight substances that do not participate in the inhibition of ACE or in antihypertensive activity, such as proteins or polypeptides that are further indigestible by thermolysin. We therefore tried to eliminate these substances. S-type KO was obtained by ultra-filtration, and had twice the ACE-inhibitory activity of the original KO in vitro. In this study, the S-type KO showed a similar increase in potency in vivo. In fact, S-type KO significantly lowered blood pressure in borderline and mildly hypertensive subjects at a dose of 1.5 g/day, a dose that was two-fold lower than the original KO dose, and an amount that can reasonably be taken as a daily dietary supplement. With regard to the action of S-type KO on anti-hypertensive activity and various biochemical indices, additional and larger clinical trials are in progress.

In the first stage of the human study, the group that received the S-type KO displayed a significant decrease in blood pressure. Although the placebo group also showed a decrease in systolic blood pressure, this decrease was only temporary, indicating that it was due to the placebo effect. In the second stage of the crossover study, the group that experienced the temporary placebo effect in the first stage showed a significant decrease in blood pressure.

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Judgement of effectiveness after the ingestion of S-type KO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>First Ingestion Group</td>
</tr>
<tr>
<td>No. (%) of Subjects</td>
<td>No. (%) of Subjects</td>
</tr>
<tr>
<td>(1) Increase (SBPₐ &gt; 10 or DBPₗ &gt; 5)</td>
<td>0 (0.0%)</td>
</tr>
<tr>
<td>(2) No Change (SBPₐ ± 9 or DBPₗ ± 4)</td>
<td>11 (36.6%)</td>
</tr>
<tr>
<td>(3) Moderate Decrease (-19 &lt; SBPₐ &lt; -10 or -9 &lt; DBPₗ &lt; -5)</td>
<td>17 (56.7%)</td>
</tr>
<tr>
<td>(4) Significant Decrease (-20 &gt; SBPₐ or -10 &gt; DBPₗ)</td>
<td>2 (6.7%)</td>
</tr>
<tr>
<td>(5) Positive Effect (((3) + (4)))</td>
<td>19 (63.4%)</td>
</tr>
</tbody>
</table>

ₐ(base line SBP (mm Hg))—(last week of test period SBP (mm Hg)).
ₐ(base line DBP (mm Hg))—(last week of test period DBP (mm Hg)).
when administered S-type KO. In the group that received S-type KO in the first stage, blood pressure returned slowly to the original baseline level on ingestion of the placebo, but did not return completely to this level probably because of the placebo effect. SBP and DBP did not decrease below the normal healthy level after the ingestion of S-type KO (data not shown).

Some antihypertensive drugs are known to produce a rebound phenomenon (an abnormal elevation of the blood pressure) after the termination of drug administration. In this study of S-type KO, as had been shown before in the clinical tests of the original KO, no subject showed any rebound effect or abnormally high blood pressure after the termination of drug administration. Moreover, no subject presented with any side effect such as a dry cough, which often appears with ACE-inhibitory drugs, and no changes in heart rate or in subjective or objective symptoms were observed during the test period. Thus, S-type KO may be considered to be a relatively mild ACE-inhibitor for the maintenance of blood pressure within the normal range.

We have isolated many kinds of ACE-inhibitory peptides derived from food proteins or their protease hydrolyzates [10,17]. However, some of them failed to show any antihypertensive activity in SHR after oral administration. In fact, when katsuobushi was digested with intestinal proteases rather than thermolysin, the digests did not show any antihypertensive activity, nor does katsuobushi itself. When we investigated this discrepancy, the stability of ACE itself proved to be important for the antihypertensive activity of orally-administered ACE-inhibitory peptides [11]. Of the eight ACE-inhibitory peptides isolated from KO that lowered blood pressure after oral administration in the SHR, LKPNM had a unique profile [11,17]. LKPNM (IC$_{50}$ = 2.4 $\mu$mol/L) was found to be hydrolyzed by ACE to produce LKP (IC$_{50}$ = 0.32 $\mu$mol/L), which had 8-fold higher ACE-inhibitory activity compared to LKPNM. This suggests that LKPNM is a prodrug type of ACE-inhibitory peptide. After oral administration in SHR, the antihypertensive activity of LKPNM showed maximal effect at 6 hrs, while LKP showed maximal effect at 4 hrs. LKPNM also showed a longer-lasting effect compared to LKP.

LKPNM is a typical ACE-inhibitory peptide derived from KO, whose LKPNM content is 0.17% (w/w) [12,14]. The other peptides isolated from KO are also classified as prodrug-type ACE-inhibitory peptides, (Ile-Trp-His-His-Thr [IC$_{50}$ = 5.8 $\mu$mol/L] and Ile-Val-Gly-Arg-Pro-Arg-His-Gln-Gly [IC$_{50}$ = 2.4 $\mu$mol/L]) [11,19]. These also show long-lasting antihypertensive activity after oral administration in SHR. S-type KO, with an LKPNM content of 0.34% (w/w), showed two-fold greater antihypertensive activity as compared to the original KO both in vitro and in vivo.

KO, which is derived from the traditional Japanese food “Katsuobushi,” or dried bonito, is expected to be welcomed by the public for use as a dietary supplement. Moreover, the amount of the S-type KO that significantly decreases blood pressure by strongly inhibiting ACE activity is not excessive. Thus, it should be more suitable than original KO as a dietary supplement. As it has proven effective in improving blood pressure control without any problematic side effects, KO represents a food supplement that can be employed extensively in borderline and mildly hypertensive subjects.
References