Effects of Dried Bonito (Katsuobushi) and Captopril, an Angiotensin I-Converting Enzyme Inhibitor, on Rat Isolated Aorta: A Possible Mechanism of Antihypertensive Action

Kazuyo Kouno,¹,² Shin-ichi Hirano,³,⁴ Hiroshi Kuboki,³ Midori Kasai,¹ and Keiko Hatae¹

¹Graduate School of Humanities and Science, Ochanomizu University, Otsuka 2-1-1, Bunkyo-ku, Tokyo 112-8610, Japan
²Ajinomoto Foundation for Dietary Culture, Takanawa 3-13-65, Minato-ku, Tokyo 108-0074, Japan
³Environmental Research Center, Mercian Cleantech Corporation, 9-1 Johnan 4 Chome, Fujisawa 251-0057, Japan

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In order to elucidate the mechanism of the antihypertensive action of dried bonito (katsuobushi), we compared the effects of dried bonito extracts with those of captopril, an angiotensin I-converting enzyme (ACE) inhibitor, on aorta preparations isolated from rats. Dried bonito extracts (3 x 10⁻⁴ to 3 x 10⁻⁵ g/ml) more potently relaxed contractions induced by norepinephrine (10⁻⁷ M) than contractions induced by KCl (55.9 mM). Dried bonito extracts (3 x 10⁻⁵ g/ml) inhibited 10⁻⁷ M angiotensin I-induced contractions. In contrast, captopril (10⁻⁸ to 10⁻⁷ M) did not affect 10⁻⁷ M norepinephrine- or 55.9 mM KCl-induced contractions, but a higher concentration of captopril (10⁻⁶ M) markedly inhibited 10⁻⁷ M angiotensin I-induced contractions in a concentration-dependent manner. These results suggest that antihypertensive mechanism of action induced by dried bonito involves direct action on vascular smooth muscle in addition to ACE-inhibitory activity.

Key words: dried bonito (katsuobushi); captopril; rat aorta; angiotensin-I converting enzyme inhibitor; antihypertensive effect

Dried bonito muscle (katsuobushi) extracted with boiling water is used as a traditional seasoning in Japan. In the process of production, the muscle is cleared of fats, boiled, and dried. A good taste is produced by this process. In addition to the good taste, it has been reported that dried bonito extract shows various physiological functions such as antihypertensive effects in spontaneously hypertensive rats (SHR),²⁻³ and prophylactic effects on the development of cerebrovascular lesion in stroke-prone spontaneously hypertensive rats (SHRSP).⁴

Angiotensin I-converting enzyme (ACE) converts angiotensin I to angiotensin II which is known to be a strong vasopressor, and inactivates bradykinin, a vasodilator.⁵ ACE inhibitors such as captopril and enalapril show potent antihypertensive activity.⁶ It is well-known that the peptides derived from dried bonito exhibit ACE-inhibitory effect⁷ and that the mechanism of antihypertensive action of dried bonito is due to the ACE-inhibitory effects of its peptides.⁸⁻¹⁰ But, Fujita and Yoshikawa¹¹ reported that ACE-inhibitory peptides isolated from dried bonito exerted remarkably higher antihypertensive activities in vivo but weaker ones in vitro. This was ascertained by using captopril as the reference drug. The reason for this difference is unclear.

Blood pressure is regulated by the contractility of vascular smooth muscle and endothelium function.¹¹⁻¹³ Hence, first of all, we examined the effects of dried bonito extracts on the contractile response to norepinephrine or KCl in denuded aorta preparations isolated from rats, and compared them with those of captopril as a reference drug. Then, using the aorta preparations, we examined the effects of both agents on the contractile response to angiotensin I. In order to elucidate the whole action of dried bonito, we used crude extracts of it instead of digested and purified peptides as samples.

Materials and Methods

Dried bonito extracts. We asked Oshige Marine Products Co. of Makurazaki, Kagoshima, Japan to prepare arahonbushi and hongarebushi samples from the same fish. The flaked dried bonito was transported by air to our laboratory and used within a week. The extraction of flaked dried bonito was done in our laboratory. The extraction methods are described in Fig. 1. To eliminate the factor of individual differences, we prepared the samples from 4 bonitos.

Rat aortic strips. All procedures using animals were

¹ To whom correspondence should be addressed. Tel: +81-466-35-6367; Fax: +81-466-35-6445; E-mail: hirano-sn@mercian.co.jp
in accordance with the “Guidelines for Animal Experimentation of the Japanese Association for Laboratory Animal Science”, and were approved by the Animal Use and Care Committee at Mercian Cleantech Corporation. Male Wistar rats (8 to 9 weeks old, 300 to 400 g, Japan SLC, Shizuoka, Japan) were killed by a blow to the head and exsanguinated. The thoracic aorta was removed and placed in Krebs–Hensleit solution. The solution contained (mM): NaCl 118.3, KCl 4.7, CaCl$_2$ 2.0, MgSO$_4$ 1.2, NaHCO$_3$ 25.0, KH$_2$PO$_4$ 1.2, calcium EDTA 0.026, and glucose 11.1. Each thoracic aorta was carefully cleaned of surrounding connective tissue and cut into several helical strips, 2 to 3 mm wide and 8 to 10 mm long. The endothelium was removed by gently rubbing the intimal surface with a finger moistened with Krebs–Hensleit solution. Each muscle strip was attached to a

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**Dried Bonito Preparation**

Frozen Bonito (4 fish)

Thawed

Cut the head and removed the internal organs

Cut into 2 parts (A, left side and B, right side) of each fish following the backbone line

Left side (A1 to A4) Right side (B1 to B4)

Boiled

Smoked

Arahonbushi

Hongarebushi

Flaked dried bonito

**Extraction Method for Dried Bonito**

Added separately to boiling deionized water (100g/1000ml water) 10%

Boiled for 30 min

Left for 5 min

Filtered with filter paper

Lyophilized (freeze dried)

Arahonbushi extract (A1, A2, A3, A4) Hongarebushi extract (B1, B2, B3, B4)

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Fig. 1. Sample Preparation and Extraction Method of Dried Bonito. Arahonbushi and hongarebushi are dried bonito extracts. The dried flaked bonito samples were donated by Oshige Marine Products Industry Co., Makurazaki, Kagoshima, Japan.
holder under a resting tension of 0.5 g and equilibrated for 60 min in a 10 ml organ bath filled with Krebs–Hensleit solution (37 °C, pH 7.4), bubbled with 95% O2 and 5% CO2. The contractile tension of muscle was recorded isometrically with a force transducer (TB-611, Nihon Kohden, Tokyo) connected to a multipurpose polygraph (RM-6000, Nihon Kohden).

Effects on muscle contractions. In order to examine the effects on the contractile response to norepinephrine or KCl, dried bonito extracts or captopril was cumulatively applied to the organ bath during the sustained contraction induced by norepinephrine (10^-7 M) or KCl (55.9 mM).

In another series of experiments, to examine the effects on the contractile response to angiotensin I, the aorta preparations were treated with norepinephrine (10^-7 M). The preparations were then treated with either dried bonito extracts or captopril for 10 min, followed by the addition of angiotensin I (10^-7 M), and the contractions induced were recorded.

Pharmacological agents. Dried bonito extracts were prepared in the laboratory of Ochanomizu University. Captopril, (−)-norepinephrine bitartrate, and angiotensin I (human type) were obtained from Wako Pure Chemical, Tokyo. These agents were dissolved in Krebs–Hensleit solution and expressed as final concentrations in the organ baths. Dried bonito extract solutions were applied to yield a final volume of less than 4% to eliminate the direct action of contained minerals and other materials.

Statistical analysis. Numerical results are expressed as the mean ± standard errors (mean ± SE). Data were analyzed for statistical significance by Fisher’s PLSD test for multiple comparisons. A p-value less than 0.05 was considered a significant difference.

Results

Effects on norepinephrine- or KCl-induced contractions

Although neither arahonbushi (A1, A2, A3, and A4) nor hongarebushi (B1, B2, B3, and B4) relaxed the aorta precontracted with 10^-7 M norepinephrine at a concentration of 10^-4 g/ml, they produced concentration-dependent relaxations at concentrations of 3 × 10^-4 to 3 × 10^-3 g/ml. The maximum responses of arahonbushi and hongarebushi at a concentration of 3 × 10^-3 g/ml were 51.2 to 84.0% in the norepinephrine-induced contraction. With the same range of concentrations, both arahonbushi (A1, A2, A3, and A4) and hongarebushi (B1, B2, B3, and B4) were much less effective in relaxing the KCl-induced contraction, since 3 × 10^-3 g/ml of arahonbushi and hongarebushi relaxed only at 12.8 to 23.8%. There were no significant differences in the effects of 8 samples on norepinephrine- or KCl-induced contractions (Table 1).

Application of captopril at concentrations of 10^-8 to 10^-7 M had no effect on the sustained contraction induced by 10^-7 M norepinephrine. Higher concentrations of captopril (3 × 10^-7 to 10^-6 M) was slightly effective in relaxing the 10^-7 M norepinephrine-induced contraction, since 10^-6 M captopril relaxed only at 17.4%. Also, captopril slightly relaxed the KCl-induced contraction, since 10^-6 M captopril relaxed only at 7.2% (Table 2).

Effects on angiotensin I-induced contractions

A4 and B2 were selected at random since similar responses were obtained based on their effects on

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Dried bonito extracts</th>
<th>Relative relaxation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10^-4 g/ml</td>
<td>3 × 10^-4 g/ml</td>
</tr>
<tr>
<td><strong>Norepinephrine</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A1</td>
<td>0 ± 0</td>
<td>4.3 ± 2.2</td>
</tr>
<tr>
<td>A2</td>
<td>0 ± 0</td>
<td>16.0 ± 2.7</td>
</tr>
<tr>
<td>A3</td>
<td>0 ± 0</td>
<td>23.0 ± 3.8</td>
</tr>
<tr>
<td>A4</td>
<td>0 ± 0</td>
<td>15.7 ± 5.7</td>
</tr>
<tr>
<td><strong>KCl</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B1</td>
<td>0 ± 0</td>
<td>22.6 ± 11.8</td>
</tr>
<tr>
<td>B2</td>
<td>0 ± 0</td>
<td>13.6 ± 4.7</td>
</tr>
<tr>
<td>B3</td>
<td>0 ± 0</td>
<td>10.0 ± 3.4</td>
</tr>
<tr>
<td>B4</td>
<td>0 ± 0</td>
<td>20.0 ± 1.6</td>
</tr>
</tbody>
</table>

Table 1. Relaxing Effects of Dried Bonito Extracts on Norepinephrine- or KCl-Induced Contraction of Rat Isolated Aorta

Values are mean ± S.E. of 3 experiments. Relaxation is expressed as the percentage of decrease in 10^-7 M norepinephrine- or 55.9 mM KCl-induced contraction, taking basal tone as 100% and maximal contraction by each agonist as 0%.
norepinephrine- and KCl-induced contractions. Angiotensin I induced relative contractions to 56.1 ± 3.2% of 10^{-7}M norepinephrine in control preparations. At a lower concentration (10^{-3}g/ml), A4 and B2 did not inhibit angiotensin I-induced contractions, but at a higher concentration (3 × 10^{-3}g/ml) they significantly inhibited contractions (p < 0.01). Pretreatment of A4 and B2 (3 × 10^{-3}g/ml) showed 40.2 ± 3.3% and 39.7 ± 4.3% respectively to relative contractions of norepinephrine (10^{-7}M) (Fig. 2).

Captopril (10^{-8} to 10^{-6}M) significantly inhibited angiotensin I-induced contractions in a concentration-dependent manner (p < 0.01 or p < 0.001). Pretreatment of 10^{-8}M, 10^{-7}M, and 10^{-6}M captopril showed 39.9 ± 5.3%, 16.7 ± 5.1%, and 2.3 ± 1.3% respectively to relative contractions of norepinephrine (10^{-7}M) (Fig. 2).

Discussion

It has been found that norepinephrine produces vascular smooth muscle contractions that result in the activation of α-adrenergic receptors. On the other hand, KCl-induced contractions are produced by the opening of voltage-dependent calcium channels. It has been thought that both vascular smooth muscle contractions are mediated by the entry of extracellular Ca^{2+} into smooth muscle cells. In our present study, dried bonito extracts more potently relaxed norepinephrine-induced contractions than KCl-induced ones, suggesting that they markedly affect smooth muscle contractions via the activation of α-adrenergic receptors. Also, Captopril at higher concentrations slightly relaxed norepinephrine- and KCl-induced contractions. These results might explain the fact that captopril has little influence on Ca^{2+} influxes via the activations of α-adrenergic receptors and by the opening of voltage-dependent calcium channels, as another mechanism of antihypertensive action.

Commercial antihypertensive drugs such as captopril and enalapril are potent ACE inhibitors. ACE-inhibitory peptides have been isolated from thermolysin digest of dried bonito, and some of them are effective in the suppression of angiotensin I-induced hypertension in normotensive rats and in lowering the blood pressure of SHR through oral administration. Thus it is evident that the antihypertensive action mechanism of dried bonito is based on its ACE-inhibitory activities. Since angiotensin I does not have direct vascular contractile activity, it is probable that angiotensin II, transformed from angiotensin I by ACE localized in the vascular wall, contracted the aorta. Therefore, in the present study, we compared the effects of dried bonito extracts with those of captopril on the contractile response to angiotensin I. Captopril markedly inhibited angiotensin I-induced contractions in a concentration-dependent manner, suggesting that the mode of action of this drug is ACE-inhibitory activity. Dried bonito extracts at higher concentrations slightly inhibited the contractions. These results suggest that dried bonito extracts also have ACE inhibitor-activity, though weaker than that of captopril.

Many kinds of peptides are released from dried bonito after enzymatic digestion. Yokoyama et al. reported that the ACE-inhibitory activity of the thermolysin digest of dried bonito is the most potent among the various protease digests. Hence, it is probable that hydrolyzation by microbial proteases such as thermolysin is important. In the present study, however, all dried bonito extracts, arahonbushi (A1, A2, A3, and A4) and hongarebushi (B1, B2, B3, and B4) showed similar effects on norepinephrine- and KCl-induced contractions. Moreover, there were no significant differences between A4 and B2 in the inhibitory effects on angiotensin I-induced contractions. These results sug-

Table 2. Relaxing Effects of Captopril on Norepinephrine- or KCl-Induced Contraction of Rat Isolated Aorta

<table>
<thead>
<tr>
<th>Agonist</th>
<th>10^{-8}M</th>
<th>3 × 10^{-8}M</th>
<th>10^{-7}M</th>
<th>3 × 10^{-7}M</th>
<th>10^{-6}M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Norepinephrine</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>−0.2 ± 3.3</td>
<td>4.0 ± 4.0</td>
<td>17.4 ± 2.7</td>
</tr>
<tr>
<td>KCl</td>
<td>0 ± 0</td>
<td>−2.8 ± 1.8</td>
<td>0 ± 0</td>
<td>1.5 ± 1.5</td>
<td>7.2 ± 5.1</td>
</tr>
</tbody>
</table>

Values are means ± S.E. of 5 to 6 experiments.
gest that it is not always necessary to isolate the peptides from dried bonito after enzymatic digestion.

In this study, we found that the mechanism of antihypertensive action involves not only ACE-inhibitory activity, as has been believed, but also other mechanisms such as the direct action on vascular smooth muscles. We believe that this new finding might be helpful in expanding information on the role of dried bonito on the mechanism of antihypertensive action and in further related studies.

Acknowledgment

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References