Note

Consumption of Salted Pickles of Sun-dried Radish Roots (Raphanus sativus cv. YR-Hyuga-Risou) Attenuates Blood Pressure in Spontaneously Hypertensive Rats

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The effects of γ-amino butyric acid (GABA)-rich salted radish pickles on blood pressure were evaluated. Raw radish roots were dehydrated under the sun, then pickled with salt. GABA concentrations in the radish roots dramatically increased during dehydration, and decreased slightly after pickling. Male spontaneously hypertensive rats (SHR/Izm) consumed AIN-76 based diets containing 0.3% salted pickle powder or equivalent amounts of GABA (0.002%) and sodium chloride (0.055%). After two weeks, the increased systolic blood pressure observed in the control SHR group was significantly prevented in both sample groups at similar potencies, indicating that the consumption of salted pickles lowered blood pressure. Furthermore, one of the active compounds might be GABA, while sodium chloride in the salted pickles might not affect the positive effects on blood pressure observed in SHR fed salted pickles. In conclusion, the daily consumption of GABA-rich salted pickles may be ideal for lowering blood pressure.

Keywords: anti-hypertension, blood pressure, GABA, salted pickles, sun-dried radish roots, spontaneously hypertensive rat, Raphanus sativus cv. YR-Hyuga-Risou

Introduction

Globally, cardiovascular disease accounts for approximately 17 million deaths each year, representing nearly one-third of total deaths, with complications of hypertension accounting for 9.4 million of these deaths (World Health Organization, 2013). Thereby, many research groups have investigated methods for the prevention and cure of hypertension and, consequently, cardiovascular disease. γ-Amino butyric acid (GABA), a non-essential amino acid, might be a possible candidate for the prevention of hypertension. Treatment with GABA and its consumption have been shown to lower blood pressure in animal studies (Elliott and Hobbiger, 1959; Hayakawa et al., 2004; Lee and Pan, 2012). According to the report of Hayakawa et al. (Hayakawa et al., 2005), the putative mechanism of GABA-induced antihypertensive effects might be mediated by a reduction in the effects of renal sympathetic nerve activity in spontaneously hypertensive rats (SHR). Additionally, the effects of GABA manifest at low dosages; the daily consumption of 1.36 mg GABA/kg body weight significantly lowered blood pressure in SHR. According to safety tests using rodent models repeatedly treated

Abbreviations: ADI, acceptable daily intake; GABA, γ-amino butyric acid; NOAEL, no-observed-adverse-effect-level; SHR, spontaneously hypertensive rat

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with GABA for 13 weeks, its safety was supported up to a daily dose of 2,500 mg GABA/kg body weight (Takeshima et al., 2014), indicating a no-observed-adverse-effect-level (NOAEL). Acceptable Daily Intakes (ADI) represent a level of exposure ‘‘without appreciable health risk’’ for humans when consumed every day or weekly for a lifetime, and are derived by applying an uncertainty factor of 100-fold to the NOAEL (Dorne, 2010). Therefore, the ADI of GABA is calculated to be 25 mg/kg body weight for humans, indicating a higher dose than the effective amount needed for lowering blood pressure.

GABA is widely distributed in dietary plants such as eggplants, tomatoes, potatoes, and pumpkins (Ohno et al., 2007; Yoshimura et al., 2010). Eggplants and tomatoes contain about 50 mg and 180 mg of GABA per 100 g fresh weight, respectively. Interestingly, GABA amounts increase during some processes such as soaking in water, anaerobic conditions, dehydration under the sun, and enzymatic treatments including fermentation (Kato et al., 2015; Mae et al., 2012; Oda et al., 2014; Ohtsubo et al., 2000; Saikusa et al., 1994; Streeter and Thompson, 1972). For example, the amount of GABA increased remarkably during the fermentation process using a bed of rice bran, reaching approximately 0.3% (Oda et al., 2014). Furthermore, consumption of GABA-rich foods is reported to lower blood pressure in SH animals (Fayed, 2015; Lee and Pan, 2012; Liu et al., 2011; Oda et al., 2014; Oda et al., 2015; Yoshimura et al., 2010). Thus, GABA-rich foods, such as fermented pickles, might be beneficial foods for the prevention of hypertension.

Pickles are typically processed vegetables preserved in salt water or vinegar, and are usually served cold with steamed rice, meat, and salads. Salted pickles are a particularly important item in Japanese food culture; however, its total production has decreased approximately 40% over the past 30 years in Japan (The Ministry of Agriculture, 2015). One reason for this decline is consumers concern regarding the risk of developing hypertension (Ando et al., 2013). On the other hand, GABA-rich pickles, which are also rich in sodium, have been reported to lower blood pressure in two types of rats, SHR and NaCl-sensitive model rats (Oda et al., 2014; Oda et al., 2015). However, the effects of sodium in pickles on the blood pressure lowering activity exerted by GABA have not been completely elucidated. In the present study, we investigated the effects of GABA-rich salted pickles produced using sun-dried radish roots on blood pressure using SHR. Additionally, the effects of equivalent amounts of GABA and sodium contained in the salted pickles were evaluated.

Materials and Methods

Chemicals Cellulose, β-cornstarch, sucrose, vitamin mixture, and mineral mixture were purchased from Oriental Yeast Co. Ltd. (Tokyo, Japan). Casein, corn oil, DL-methionine, choline bitartrate, sodium chloride, and GABA were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other reagents were of the highest grade available.

Preparation of salted pickles of sun-dried radish roots In the present study, radishes (Raphanus sativus cv. YR-Hyuga-Risou) cultivated in a field in Miyazaki, Japan, in February 2014 were used. Parts of fresh radish roots were immediately lyophilized (raw radish roots) using a Dura-Top MP freeze dryer (FTS Systems Ltd., Warminster, PA, USA). The other parts of the fresh radish roots were placed under the sun on a scaffold for 2 weeks until dehydrated and flexible (sun-dried radish roots) (Fig. 1A). Salted pickles were made by pickling the sun-dried radish roots with salt (4.5% weight of radish) in a pickling crock. A pressing weight was placed on the top of the crock, and the radishes were pickled for 7 months at 2°C. Sun-dried radish roots and salted pickles were lyophilized using a Dura-Top MP and FD-10BU (Nihon Techno Service Co., Ltd, Ibaragi, Japan) freeze dryer, respectively. Individual lyophilized samples were ground into a powder (0.50 mm) using an Ultra Centrifugal Mill ZM200 (Retsch GmbH,
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Hean, Germany).

Analysis of GABA amounts in the radish root products  The concentrations of free amino acids, including GABA, in the three radish root powders (raw, sun-dried, and salted pickles) were analyzed according to the methods reported by Nagatomo and colleagues (Nagatomo et al., 2009). Briefly, 0.02 N hydrochloric acid (60 mL) was added to the individual radish root powders (0.5 g). Free amino acids were extracted in an ultrasonic bath for 10 min. After adjusting to 100 mL with 0.02 N hydrochloric acid, the filtrates were collected using quantitative filter paper (No. 5A, Advantec Toyo Kaisha, Ltd., Tokyo, Japan). Free amino acids in the filtrates were measured using an L-8900BF amino acid analyzer (Hitachi High-Technologies GLOBAL Co., Tokyo, Japan), and are summarized in Table 1. Following animal experiments, feeding doses of sun-dried radish roots and salted pickles were calculated on the basis of GABA concentrations in these powders and the effective dosage for anti-hypertension (daily consumption of GABA at 1.36 mg/kg body weight) evaluated using SHR (Liu et al., 2011).

Analysis of sodium chloride amounts in salted pickles  The concentrations of sodium chloride in the salted pickles were analyzed using the official method. Briefly, deionized water (70 mL) was added to the salted pickle powder (0.5 g). Sodium chloride was extracted in an ultrasonic bath for 10 min. After adjusting to 100 mL with the deionized water, the filtrates were collected using quantitative filter paper (No. 5A, Advantec Toyo Kaisha, Ltd.). The filtrate (5 mL) was added to 2% potassium chromate and titrated with 0.02 N silver nitrate until the color changed to reddish brown. Sodium chloride amounts of salted pickles were calculated using the following formula: X mL (titre) \times 1.00 \text{ (factor of silver nitrate)} \times 100 \text{ mL (adjustment amount)} \times 5 \text{ mL (filtrate volume)} \times 200 \text{ g (100/pickle weight)} \times 0.00117 \text{ g (sodium chloride weight corresponding to 1 mL silver nitrate)}.
Sodium chloride amounts were 18.4 g/100 g pickle weight.

Animals and diets  All animal maintenance and experiments were conducted according the care and use of laboratory animals policy of University of Miyazaki (Miyazaki, Japan). The experimental protocol was registered under the number 2014-003-2. Male spontaneously hypertensive rats (SHR/Izm) at 6 weeks of age were purchased from Japan SLC, Inc. (Shizuoka, Japan). Animals were housed in individual stainless-steel cages in air/temperature-controlled rooms (temperature, 23 ± 1°C; relative humidity, 55 ± 5%) with a 12-h dark/light cycle (light on time defined as Zeitgeber time (ZT) 0 and light off time was ZT12), with access to an AIN-76 based purified powder diet (American Institute of Nutrition, 1977) and deionized water ad libitum. After 4 weeks of acclimatization with the AIN-76 based diet and deionized water ad libitum, animals were submitted to the following two experiments.

Dosage  Experiment I. Eighteen rats were randomly divided into three groups. The first group consumed an AIN-76 based purified powder diet; the other two groups consumed 0.2% of raw radish root powder (0.0005% GABA) or sun-dried radish root powder (0.002% GABA) as shown in Table 2.

Experiment II. Eighteen rats were randomly divided into three groups. The first group consumed an AIN-76 based purified diet; the other two groups consumed a diet containing 0.3% of salted pickles (0.002% GABA) or a diet containing 0.002% GABA + 0.055% sodium chloride as shown in Table 2.

Measurement of systolic blood pressure  Immediately before starting the consumption of radish root-containing diets and one and two weeks after consumption, systolic blood pressure was measured in awake animals using the tail-cuff method with a blood pressure monitor (MK-2000ST, Muromachi Kikai Co. Ltd., Tokyo, Japan). Measurements were performed according to the manufacturer’s instructions under the following conditions: at ZT6; temperature 23 ± 1°C; humidity 55 ± 5%.

Assay of ACE inhibitory activity  Angiotensin-converting enzyme (ACE) inhibitory activity was determined using an ACE inhibitory assay kit (ACE Kit-WST, Dojindo Laboratories, Kumamoto, Japan). The assay was carried out according to the manufacturer’s instructions. Absorbance of the reactions was measured using Multiskan Go Advance (Thermo Fisher Scientific Inc., Waltham, MA, USA) at 450 nm. The ACE inhibitory activities of the samples were calculated using the formula given in the protocol, and indicated as IC50 values, which is the concentration of the ACE inhibitor required to reduce ACE activity to 50% under the assay condition.

Statistical analyses  Statistical analyses were conducted using Stat View for Windows (Version 5.0, SAS Institute, Cary, NC, USA). A repeated-measures ANOVA was used to assess repeated measurements of the same SHR at different points over time. Next, comparisons among the control and treated groups were carried out

<table>
<thead>
<tr>
<th>Amino acids</th>
<th>Radish roots (mg/g dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Raw</td>
</tr>
<tr>
<td>GABA</td>
<td>2.39</td>
</tr>
<tr>
<td>Glutamine</td>
<td>19.62</td>
</tr>
<tr>
<td>Proline</td>
<td>2.21</td>
</tr>
<tr>
<td>Arginine</td>
<td>4.23</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.68</td>
</tr>
<tr>
<td>Valine</td>
<td>0.91</td>
</tr>
<tr>
<td>Glutamic acid</td>
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</tr>
<tr>
<td>Threonine</td>
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</tr>
<tr>
<td>Asparagine</td>
<td>0.74</td>
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<tr>
<td>Serine</td>
<td>0.68</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.48</td>
</tr>
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</table>

GABA, γ-amino butyric acid.
Results and Discussion

Effects of sun-dried radish roots on blood pressure  In the present study, we evaluated the effects of GABA-rich salted pickles of sun-dried radish roots on blood pressure using a SHR model. The amount of GABA in raw radish roots used in this study was 2.4 mg/g dry weight (Table 1). The amount of GABA remarkably increased to 9.67 mg/g during 14 days of dehydration under the sun, similar to results reported by other research groups (Kato et al., 2015; Nagatomo et al., 2009). Next, the effects of the consumption of raw and sun-dried radish roots were evaluated. As shown in Table 2, an experimental feeding dosage of sun-dried radish root powder was established at 0.2% (equivalent to 0.002% GABA) in the diets, based on the results reported by Liu and colleagues, who reported that a daily consumption of 1.36 mg/kg body weight lowered blood pressure (Liu et al., 2011). During the 2-week experimental period, body weight gains and food intakes were similar among the control, raw, and sun-dried groups (Table 3). GABA intakes in the raw and sun-dried groups were 0.27 ± 0.01 and 1.12 ± 0.02 mg/kg body weight, respectively (Table 3). The mean systolic blood pressure of the control group remarkably increased after 2 weeks (Fig. 2A). This increase was significantly attenuated by the consumption of sun-dried radish roots, but not raw radish, indicating that the consumption of GABA-rich sun-dried radish roots (daily consumption of at least 1.12 mg/kg body weight) lowered blood pressure. Our results are similar to those observed for GABA-rich pickled vegetables such as cucumbers, eggplants, and radishes fermented in a bed composed of rice bran and white miso (Oda et al., 2014; Oda et al., 2015).

Effects of salted pickles on blood pressure  Subsequently, the sun-dried radish roots were pickled under salt-pressing for 7 months, with a GABA concentration of 6.42 mg/g dry weight, indicating that the amount of GABA decreased slightly compared to the original sun-dried radish. This might be attributable to GABA leaching into the liquid fraction from the radish roots during the salting process. Next, the effects of salted pickles were evaluated. The experimental feeding dosage of salted pickles of sun-dried radish root powder (0.3%) was calculated to be 0.002% GABA in the diets. The amount of sodium chloride in the salted pickles was ~0.18 g in 1 g powder. Thus, equivalent amounts of GABA and sodium chloride (0.002% GABA, 0.055% sodium chloride in the diets) were also used as the additive group (Table 2). During the 2-week consumption period, body weight gains and food intakes were similar among the three groups, control, salted pickles, and GABA+NaCl (Table 3). GABA intakes in the salted pickles and GABA+NaCl groups were almost the same (1.22 ± 0.05 and 1.25 ± 0.02 mg/kg body weight, respectively).
Table 3. Effects consumption of radish root on body weights and food intakes.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Body weight (g)</th>
<th>Daily intakes per 1 kg body weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Final</td>
</tr>
<tr>
<td>Experiment I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>278.4±9.3</td>
<td>301.9±8.1</td>
</tr>
<tr>
<td>Raw radish roots</td>
<td>276.4±12.3</td>
<td>311.0±7.1</td>
</tr>
<tr>
<td>Sun-dried radish roots</td>
<td>274.1±10.6</td>
<td>306.1±4.3</td>
</tr>
<tr>
<td>Experiment II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>269.5±3.1</td>
<td>308.2±4.1</td>
</tr>
<tr>
<td>Salted pickles of sun-dried</td>
<td>269.5±2.4</td>
<td>305.7±4.8</td>
</tr>
<tr>
<td>GABA + NaCl</td>
<td>269.2±2.7</td>
<td>310.4±4.5</td>
</tr>
</tbody>
</table>

Spontaneously hypertensive rats were employed in all animals on both experiments. Values are means ± SEM (n = 6). Different alphabetical superscripts indicate statistically significant differences (P < 0.05, Tukey-Kramer post hoc test).

Fig. 2. Effects of radish root consumption on systolic blood pressure in spontaneously hypertensive/Izm rats. Male rats aged 10 weeks consumed either (A) an AIN-76 based purified diets (Control, ○), a diet containing 0.2% raw radish (Raw, ■), or a diet containing 0.2% sun-dried radish (Sun-dried, ◇); or (B) Control (○), a diet containing 0.3% salted pickles of sun-dried radish (Salted pickles, ◆), or a diet containing 0.002% GABA and 0.055% sodium chloride (GABA+NaCl, △). Immediately before starting the consumption of radish root containing diets and one and two weeks after the consumptions, systolic blood pressure was measured in awake spontaneously hypertensive rats using the tail-cuff method at ZT6. Values are means ± SEM (n = 6). Different alphabetical superscripts indicate statistically significant differences (P < 0.05, Tukey-Kramer post hoc test).

Appendix Fig. 1. Effects of salted pickles for additive sodium chloride on systolic blood pressure in spontaneously hypertensive/Izm rats (SHR). Male SHR aged 10 weeks consumed (A) either an AIN-76 based purified diet (Control, ○) or a diet containing an additional 0.055% sodium chloride (NaCl, ▲), and (B) either a diet containing 5% sodium chloride (High-salted diet, □) or High-salted diet with 0.3% salted pickles of sun-dried radish (High-salted+sun-dried, ■). Immediately before starting the consumption of radish root containing diets (week 0) and individual weeks after consumptions, systolic blood pressure was measured in awake spontaneously hypertensive rats using the tail-cuff method at ZT6. Values are means ± SEM (n = 6 for A, n = 8 for B). Different alphabetical superscripts indicate statistically significant differences (P < 0.05, Tukey-Kramer post hoc test).
respectively), as shown in Table 3. After the 2-week consumption period, the increase in systolic blood pressure observed in the control group was significantly prevented at similar potencies in both the salted pickles and GABA+NaCl groups (Fig. 2B). In addition, the diet containing 0.055% sodium chloride, but not GABA, did not affect the increase in systolic blood pressure of the control group (Appendix Fig. 1A). The control diets used in the present study were AIN-76 based purified diets. These diets contained 0.259% sodium chloride in order to maintain normal rodent growth (Nutrition 1977). Therefore, the control group also consumed sodium chloride at an amount of 159.7 ± 2.1 mg/kg body weight, in addition to the salted pickles (199.1 ± 7.4 mg/kg body weight) and GABA+NaCl (196.8 ± 2.1 mg/kg body weight) groups, as shown in Table 3. These results indicated that consumption of salted pickles of sun-dried radish roots protected against the increase in blood pressure. Furthermore, consumption of 0.3% sun-dried radish root powder prevented the increase in systolic blood pressure observed in SHR fed a high sodium chloride (5%) diet (Appendix Fig. 1B). Urine excretions were reduced in the group fed a high-salt diet with radish (data not shown), indicating that the mechanism for preventing the increase in systolic blood pressure does not involve diuresis.

ACE inhibitory effects Another possible mechanism might be the inhibition of ACE activity, one of the key elements responsible for vasopressor action, because ACE inhibitors have been reported to reduce mortality in patients with hypertension (van Vark et al., 2012). Therefore, we further evaluated the ACE inhibitory effects. Among the three radish samples used in the animal study, both the salted pickles and GABA+NaCl groups (Fig. 2B). In this study, we carried out two animal experiments, Fig. 2A and 2B, using SHR. Therefore, the blood pressure of all treated groups did not exert blood pressure lowering effects. On the other hand, raw radish root exhibited relatively weak activity (1.32 mg/mL). These results indicate that ACE inhibitory activity might be one of the responsible mechanisms for preventing the increase in blood pressure. However, elucidation of the responsible mechanisms will be attempted in a future study.

Conclusions We found that the amount of GABA in the radish roots was remarkably increased during dehydration under the sun, although the amount decreased slightly following pickling using salt-preserving. In this study, we carried out two animal experiments, Fig. 2A and 2B, using SHR. Therefore, the blood pressure of all treated groups might be similarly increased compared with the controls if the treatment materials do not exert blood pressure lowering effects. On the other hand, our results clearly indicated that blood pressure decreased in the sun-dried radish, salted pickles, and GABA+NaCl groups, but not the original raw radish and NaCl groups. These findings clearly indicate that the daily consumption of GABA-rich salted pickles might be ideal for lowering blood pressure and, consequently, protecting against the development of hypertension. Furthermore, the sodium chloride in the salted pickles might not affect the blood pressure lowering effects of GABA. GABA is proposed to be one of the active compounds; however, the possibility remains that there are active compounds in addition to GABA.

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