1 INTRODUCTION

Hypertension is the main risk factor for cardiovascular disease (CVD), which remains a leading cause of death in most industrialized countries. The renin-angiotensin system (RAS) plays an important role in blood pressure regulation. Within the RAS, angiotensin-converting enzyme (ACE; EC 3.4.15.1) converts the inactive precursor angiotensin I to the potent hormone angiotensin II, which constricts arterial vessels and acts to raise blood pressure. Therefore, inhibition of ACE activity is a good target for antihypertensive therapy. Some reports have demonstrated that inhibition of ACE in the heart, aorta, lung and kidney is important for regulation of blood pressure.

Alpha-linolenic acid (ALA) is one of the n-3 polyunsaturated fatty acids (PUFAs) and occurs mainly in plants and vegetable oils. Several studies have shown that dietary ALA is associated with a reduced risk of CVD, including coronary heart disease and ischemic heart disease, morbidity and mortality. Furthermore, Hoffman et al. have shown that dietary linseed oil rich in ALA has a blood pressure-lowering effect in the spontaneously hypertensive rats (SHR), considered to be a good experimental model of essential hypertension in man. Some epidemiological studies have also demonstrated that dietary intake of ALA is inversely associated with blood pressure. Recently, we showed that a diet supplemented with ALA lowered systolic blood pressure (SBP) and diastolic blood pressure after 12 weeks in Japanese subjects with high-normal blood pressure and mild hypertension. However, the antihypertensive mechanism of ALA is not fully understood. In the present study, we investigated the effect of ALA-rich flaxseed oil on ACE activity and mRNA expression levels to clarify the mechanism of its antihypertensive effect in SHR.

Dietary Alpha-Linolenic Acid Inhibits Angiotensin-Converting Enzyme Activity and mRNA Expression Levels in the Aorta of Spontaneously Hypertensive Rats

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Abstract: Several studies have shown that dietary alpha-linolenic acid (ALA) is associated with a reduced risk of cardiovascular disease and has an antihypertensive effect. Blood pressure is regulated mainly by angiotensin-converting enzyme (ACE). In the present study, we investigated the effect of dietary ALA on ACE to clarify the mechanism of the antihypertensive effect in spontaneously hypertensive rats (SHR). Six-week-old SHR were fed a diet containing either 10% ALA-rich flaxseed oil or high oleic safflower oil as a control for four weeks. Systolic blood pressure (SBP) was measured by the tail cuff method once weekly. At the end of the feeding period, ACE activity was determined in the heart, aorta, lung and kidney. ACE mRNA in these organs was also measured by real-time PCR analysis. SBP in the ALA group was significantly lower than in the control group at 2, 3, and 4 weeks. The ACE activity and mRNA expression levels in the ALA group were significantly lower than in the control only in the aorta. In conclusion, the present findings suggest that the blood pressure-lowering mechanism of dietary ALA may be involved in the reduction of ACE activity and mRNA expression levels in the aorta of SHR.

Key words: alpha-linolenic acid, hypertension, angiotensin-converting enzyme, aorta, spontaneously hypertensive rats

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2 EXPERIMENTAL

2.1 Animals and diets

All animals were treated in accordance with the guidelines for the care and use of laboratory animals (Notification of the Prime Minister’s Office in Japan). The experimental plan was approved by the Laboratory Animal Care Committee of the Research Laboratory, The Nisshin OilliO Group, Ltd. The temperature of the animal room was set at 23 ± 1°C, with RH of 50 ± 5% and illumination from 0800 to 2000 h. The animals were also allowed free access to an experimental diet containing 10% high-oleic safflower oil (HOSO, The Nisshin OilliO Group, Ltd., Tokyo, Japan) before the experiment. Male SHR of the Izumo colony (SHR/Izm, Japan SLC, Inc., Hamamatsu, Shizuoka, Japan) were individually housed in stainless steel wire cages and allowed free access to sterilized water. Six-week-old SHR were randomized into two groups. Each group of rats (n = 10) was allowed free access to the experimental diet containing 10% HOSO or FSO (The Nisshin OilliO Group, Ltd., Tokyo, Japan) for four weeks. The fatty acid composition of the test lipids is shown in Table 1, and the composition of the experimental diets, based on the AIN-93G purified diet for rodents17, is shown in Table 2.

2.2 Measurement of blood pressure

SBP of conscious rats was measured once weekly using a non-invasive blood pressure monitor for mice and rats (MK-2000, Muromachi Kikai Co., Ltd., Tokyo, Japan) by the tail cuff method for four weeks18.

2.3 Preparation of tissue enzyme extract

At the end of the feeding period, all rats were killed under anesthesia. Enzyme extracts of heart, aorta, lung and kidney were prepared by the method of Masuda et al. with some modifications19. All organs were chopped into small pieces and homogenized in 50 mmol/L Tris-HCl (pH 7.9) containing 0.3 mol/L NaCl using a polytron homogenizer (PT1200, Kinematica, Luzern, Switzerland). The suspension was centrifuged at 44,000 × g for 90 min at 4°C and the supernatants were discarded. Pellets were suspended in the same buffer and centrifuged at 44,000 × g for 90 min at 4°C; the supernatants were discarded. The pellets were resuspended in the upper buffer containing 0.5% Triton X-100. After 1 h, the suspensions were centrifuged at 1000 × g for 10 min at 4°C and the supernatants were designated the detergent-solubilized ACE fraction.

2.4 Enzyme assays

ACE activity was determined using hippuryl-histidyl-leucine (HHL; Peptide Institute, Inc., Osaka, Japan) as a substrate according to the method of Cushman and Cheung with some modification20. HHL was dissolved in 0.1 mol/L sodium borate buffer (pH 8.3) containing 0.3 mol/L NaCl. The reaction was initiated by addition of 5 mmol/L HHL solution to 100 μL of the enzyme extract and the mixture was incubated for 30 min at 37°C. The reaction was terminated by addition of 250 μL of 1 mol/L HCl. The HCl was added before the enzyme in zero-time control assays. The hippuric acid released by HHL was extracted from the acidified solution into 1.5 mL ethyl acetate by vortex-mixing for 15 s. After removal of ethyl acetate by heating for 30 min at 120°C, the hippuric acid was dissolved by addition of 1mL of distilled water and the amount formed was determined from its absorbance at 228 nm. For the spectrophotometric assay, one unit of angiotensin converting enzyme (ACE) activity was defined as the amount that formed 1 μmol hippuric acid from HHL in 1 min at 37°C. The specific activity was expressed as units/mg protein.

2.5 RNA extraction and real-time PCR analysis

Total RNA was extracted from all the organs by using a RNasy Fibrous Tissue Mini Kit (Qiagen, Tokyo, Japan) according to the instruction manual. The reverse-transcriptional reaction was carried out using a 1st Strand cDNA Synthesis Kit (Super Script III Platinum Two-Step qRT-PCR Kit with SYBR Green; Invitrogen, Tokyo, Japan). Real-time PCR was performed using a LightCycler (Roche Diagnostics, Tokyo, Japan). The primers used in this study

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Table 1  Major Fatty Acid Composition of Test Lipids.

<table>
<thead>
<tr>
<th></th>
<th>Control (g/100 g)</th>
<th>ALA (g/100 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0</td>
<td>4.7</td>
<td>5.0</td>
</tr>
<tr>
<td>18:0</td>
<td>1.9</td>
<td>3.2</td>
</tr>
<tr>
<td>18:1(n-9)</td>
<td>77.1</td>
<td>17.8</td>
</tr>
<tr>
<td>18:2(n-6)</td>
<td>14.4</td>
<td>16.5</td>
</tr>
<tr>
<td>18:3(n-3)</td>
<td>0.2</td>
<td>53.9</td>
</tr>
</tbody>
</table>

ALA, alpha-linolenic acid.

Table 2  Composition of the Experimental Diets.

<table>
<thead>
<tr>
<th></th>
<th>(g/100g diet)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cornstarch</td>
<td>36.748</td>
</tr>
<tr>
<td>Casein</td>
<td>20</td>
</tr>
<tr>
<td>Alpha-corn starch</td>
<td>13.2</td>
</tr>
<tr>
<td>Sucrose</td>
<td>10</td>
</tr>
<tr>
<td>Cellulose</td>
<td>5</td>
</tr>
<tr>
<td>AIN-93 mineral mixture</td>
<td>3.5</td>
</tr>
<tr>
<td>AIN-93G vitamin mixture</td>
<td>1</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>0.3</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>0.25</td>
</tr>
<tr>
<td>Tetra-butylhydroquinone</td>
<td>0.002</td>
</tr>
<tr>
<td>Test lipid</td>
<td>10</td>
</tr>
</tbody>
</table>
were designed on-line with Primer 3 software and purchased from Invitrogen. The primer sequences were: ACE, 5′-ATTGCTTTGGGTGGAAGA-3′ (sense) and 5′-GCATCAGATGCGTGTTT (antisense); β-actin, 5′-TGCTCCGTATGCCCTCTGTG-3′ (sense) and 5′-CACG-CACGATTTCCTCT-3′ (antisense). These were based on the mRNA sequences of a database (NM_012544 for ACE, NM_031144 for β-actin). Melting curve analysis was used to determine the specific PCR products. An internal standard curve based on serial dilutions of first-strand DNA from the each organ of rats fed a commercial standard diet was used. The level of transcripts for the constitutive housekeeping gene product β-actin was measured in each sample. The data were normalized to the expression levels of β-actin, and the ACE mRNA expression level of SHR fed a control diet, which was taken as 100.

2.6 Statistical analysis
Data were expressed as mean ± SEM. The results, except for SBP, were analyzed by the F-test for equality of variance, and the significance of differences between the diet groups was determined by Student’s t-test. Two-way analysis of variance was performed for SBP, and when significant differences were confirmed, Student’s t-test was used for analysis. Differences with a value of P<0.05 were considered significant. A statistical software package (SPSS 13.0J, SPSS Japan Inc., Tokyo, Japan) was used for all of the statistical analyses.

3 RESULTS
After 4 weeks consumption of the test diets, no significant differences in initial and final body weight, or food intake, were seen between the control and ALA groups (Table 3). As shown in Fig. 1, the SBP was significantly lower in the ALA group than in the control group at 2, 3, and 4 weeks (173 ± 2 vs. 181 ± 2 mmHg, 175 ± 6 vs. 193 ± 4 mmHg, and 191 ± 3 vs. 202 ± 3 mmHg, respectively).

The ACE activity in heart extract from the ALA group tended to be lower than in that from the control group (1.5 ± 0.3 vs. 2.2 (0.4 mU/mg protein), but the difference was not significant (P = 0.14) (Fig. 2). The ACE activity in the aorta extract from the ALA group was significantly lower than in that from control group (35.1 ± 2.5 vs. 43.6 ± 2.6 mU/mg protein) (Fig. 2). However, there were no significant intra-group differences in the ACE activity of lung and kidney extracts (45.5 ± 25.6 vs. 41.9 ± 12.8 mU/mg protein and 6.7 ± 0.5 vs. 6.9 ± 0.5 mU/mg protein, respectively) (Fig. 2).

There were no significant differences in the expression levels of ACE mRNA in heart, lung and kidney between the two groups (105.7 ± 4.4 vs. 100.0 ± 5.0, 104.7 ± 3.5 vs. 100.0 ± 3.8, 91.5 ± 10.5 vs. 100.0 ± 12.3, respectively) (Fig. 3).

However, the ACE mRNA expression level in aorta from the ALA group was significantly lower than in that from control group (62.8 ± 7.0 vs. 100.0 ± 13.8) (Fig. 3). The expression level of β-actin showed no significant differences between the groups in any of the organs (data not shown).

4 DISCUSSIONS
It is now recognized that in humans ALA is an essential fatty acid that cannot be synthesized by the body and therefore must be supplied by dietary sources. ALA is abundant in certain plant foods including flaxseed, walnuts, canola, several legumes and green leafy vegetables. Several epidemiologic studies have reported that dietary ALA ingestion is associated with a reduction of CVD risk. In a prospective cohort study involving about 76,000 women, Albert et al. and Hu et al. showed that a higher intake of ALA reduced the risk of sudden cardiac death and fatal ischemic heart disease. Dolecek et al. reported...
Fig. 2  Angiotensin-Converting Enzyme Activity in SHR Fed a Control or an ALA Diet.
Values are means ± SEM, n=10. *Mean value is significantly different from those of the control group (p<0.05).

Fig. 3  Angiotensin-Converting Enzyme mRNA Expression in SHR Fed a Control or an ALA Diet.
Values are means ± SEM, n=10. *Mean value is significantly different from those of the control group (p<0.05).
significant inverse associations between ALA and mortality due to cardiovascular disease including coronary heart disease among about 12,900 men in an intervention study. Campos et al. also demonstrated that increased ALA intake was associated with a reduced risk of non-fatal acute myocardial infarction among about 3,600 subjects in a case-control study. Recently, Wang et al. and Lawes et al. reported that a decrease in blood pressure reduced the risk of CVD. Furthermore, some studies have shown that dietary ALA has a SBP-lowering effect. In the present study, SBP in the ALA group was also significantly lower than in the control group (Fig. 1), in accord with the present findings. Therefore, it has been suggested that the antihypertensive effect of dietary ALA is partly responsible for the cardioprotective effect.

The RAS plays an important role in blood pressure regulation. Some studies have provided evidence for so-called local or tissue RAS, as opposed to the circulating RAS, and shown that a variety of tissues including blood vessels, heart, lung and kidney contain all the essential components of the RAS. Furthermore, ACE, which is a component of the RAS and plays an important role in the regulation of blood pressure, is widely distributed throughout the body, particularly in cardiovascular tissues, and is predominantly localized in the luminal surface of endothelial cells, which line blood vessels. On the other hand, Kreutz et al. and Shisheva et al. have reported that plasma ACE is not involved in blood pressure regulation, suggesting that ACE present in tissues, rather than that in plasma, may have a more important role in the regulatory mechanisms related to blood pressure. To examine the underlying mechanism of the antihypertensive effect of ALA, we measured ACE activity, which plays an important role in the regulation of blood pressure in the heart, aorta, lung and kidney. Our results revealed no significant differences in the ACE activity of heart, lung and kidney extracts between the ALA and control groups, although the ACE activity in the aorta was significantly lower in the ALA group than in the control group (Fig. 2). Furthermore, when we measured the expression of ACE mRNA in these organs, although no significant intra-group differences were evident for heart, lung and kidney, the aorta in the ALA group showed significantly lower expression than aorta from the control group (Fig. 3), thus reflecting the results for ACE activity. In accord with our results, Ikemoto et al. have indicated that inhibition of ACE in the aorta coincides to a greater extent with the hypotensive effects of drugs. Murakami et al. also have demonstrated that treatment with an ACE inhibitor lowered both blood pressure and aortic ACE activity in stroke-prone SHR. Furthermore, Takai et al. have reported that treatment with ACE inhibitors significantly reduced the blood pressure in SHR, and that a significant correlation was observed between SBP and ACE activity in the aorta, but not in plasma or other tissues such as heart and brain. These results suggest that inhibition of aortic ACE might be the most important factor responsible for the antihypertensive effects of dietary ALA in hypertensive rats.

In conclusion, we have confirmed that dietary ALA exerts an antihypertensive effect in SHR, and demonstrated that ACE activity and mRNA expression are lower in SHR fed ALA than in SHR fed a control diet. These results indicate that the blood pressure-lowering mechanism of dietary ALA in SHR may involve a reduction of ACE levels in the aorta.

References
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