Effects of Dietary Arachidonic Acid Supplementation on Age-Related Changes in Endothelium-Dependent Vascular Responses

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Summary The purpose of the present study was to examine the effects of dietary supplementation of arachidonic acid (ARA) on age-related changes in endothelium-dependent vascular responses. Young male Fisher-344 rats (2-mo-old) and aged rats of the same strain (22-mo-old) were randomly separated into a control diet group (young control, YC; old control, OC) and an ARA-containing diet group (young ARA, YA; old ARA, OA). After a 2-mo feeding period, vascular responses were evaluated using both endothelium-intact and -denuded aortic rings. Phenylephrine (α₁-adrenoceptor agonist)-induced vasoconstrictor responses in endothelium-intact rings from group OC tended to be augmented compared with those of rings from groups YC and YA, although this augmentation was significantly suppressed by dietary supplementation of ARA. There were no significant differences in vascular responses to phenylephrine in endothelium-denuded rings among groups YC, YA, OC, and OA. Acetylcholine (Ach)-induced, endothelium-dependent vasorelaxation was attenuated in groups OC and OA compared with that in groups YC and YA. ARA supplementation induced slight enhancement of Ach-induced vasorelaxation in aged rats. Ach-induced vasorelaxation correlated very well with aortic ARA concentration in aged rats, but not in young rats. There were no significant differences in endothelium-independent vasodilator responses to sodium nitroprusside in endothelium-denuded rings among groups YC, YA, OC, and OA. These findings suggest that dietary ARA supplementation improves the age-related endothelial dysfunction that leads to various cardiovascular diseases.

Key Words aging, arachidonic acid, endothelial dysfunction, acetylcholine, phenylephrine

Age is a recognized independent risk factor for the development of cardiovascular disease. There is accumulating evidence in both humans and experimental animals that aging is associated with endothelial dysfunction characterized by progressive decline in endothelium-dependent vasorelaxation in resistance and conductance arteries (1–3). Although the mechanisms underlying this age-related endothelial dysfunction are not fully understood, several factors, including attenuation of nitric oxide (NO)-mediated vasodilation, augmented production of oxygen-derived free radicals, and increased release of cyclooxygenase-derived vasoconstrictor substances, appear to contribute to this vascular change (4).

Arachidonic acid (ARA) is a major constituent of cell membranes, and in this capacity plays important roles in the maintenance of physiological function. Several studies have demonstrated that the amount of ARA in membrane phospholipids in the brain is lower in aged animals than in young animals, and that certain neural deficiencies in aged animals are closely related to this decrease in membrane ARA concentration (5, 6). It has recently been reported that dietary ARA supplementation to aged rats can alleviate age-related neural dysfunction (7, 8).

However, little is known concerning the involvement of membrane ARA in age-related cardiovascular dysfunction. Loiacono et al. (9) investigated the effect of dietary arachidonate deficiency on acetylcholine (Ach)-induced endothelium-dependent vasorelaxation in aortic rings, using weanling rats, and observed that vascular responses to Ach were not impaired in this condition. To our knowledge, however, no study has examined the effect of dietary ARA supplementation on age-related changes in vascular responses. The purpose of the present study was to evaluate whether supplementation of ARA in the diet of aged rats could improve age-related endothelial dysfunction. For this purpose, the endothelium-dependent vascular responses to several agents in male Fisher-344 rats (22-mo-old) and young rats of the same strain (2-mo-old) were compared.

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Table 1. Fatty acid compositions of the diets used in this study.

| Fatty acid (%) | Control diet | ARA-containing diet |
|----------------|--------------|---------------------|
| 16:0 Palmitic acid | 11.00 | 11.25 |
| 18:0 Stearic acid | 2.05 | 2.56 |
| 18:1 n-9 Oleic acid | 28.99 | 27.05 |
| 18:2 n-6 Linoleic acid | 55.94 | 51.51 |
| 18:3 n-3 α-Linolenic acid | 1.61 | 1.62 |
| 20:3 n-6 Dihomo-γ-linolenic acid | — | 0.24 |
| 20:4 n-6 Arachidonic acid | — | 4.28 |
| Others | 0.41 | 1.49 |
| Total | 100.00 | 100.00 |

**MATERIALS AND METHODS**

**Diet.** The experimental powder diet, a modified AIN-76A rodent diet, contained 5 g of ARA VITA 40 (ARA ca. 40% containing oil, Sunory, Osaka, Japan) and 45 g of corn oil per kg. The control powder diet contained no ARA, and instead contained additional corn oil (5 g/kg). The fatty acid compositions of the diets used in this study are shown in Table 1.

**Animal experiments.** Male Fisher 344 rats (Japan Charles River, Yokohama, Japan) were used. Rats were 18 mo old when they arrived in the laboratory, and were then given the control diet for 4 mo. At 22 mo of age, the animals were randomly separated into a control diet group (old control, OC, n=10) and an ARA-containing diet group (old ARA, OA, n=10). At the same time, young male rats (2-mo-old) of the same strain were separated into two groups in the same manner (young control, YC, n=11 and young ARA, YA, n=10). After a 2-mo-feeding period, the animals were sacrificed by exsanguination under anesthesia. The thoracic aortas were removed, fat and adherent connective tissue were excised from them, and they were used for isometric tension studies. A portion of aortic tissue was used for fatty acid analysis. During the 2-mo-feeding period, one of the 10 OC rats and two of the 10 OA rats died of natural causes. In addition, we could not determine the fatty acid concentrations in some rats of each group, because of the limited amount of aortic tissue available for fatty acid analysis.

The animals were housed in an animal room under the following conditions: temperature, 24±1°C; relative humidity, 55±5%; and 12-h light-dark cycle. The animals were given sufficient amounts of the control and ARA-containing diets once per day. Tap water was available ad libitum. The amount of food consumed by each rat was approximately 20 g per day. Since ARA VITA 40 contained ARA and its precursor, dihomo-γ-linolenic acid, 40% by weight, daily intake of ARA by each rat in the OA and YA groups was approximately 40 mg.

The protocols for and animal care methods used in the experiments were approved by the Experimental Animal Committee of Osaka University of Pharmaceutical Sciences.

**Isometric tension studies.** The thoracic aorta was isolated and cut into strips with special care taken to preserve the endothelium. The specimens were suspended in organ chambers containing 10 mL of Krebs-Ringer bicarbonate solution (118.5 m NaCl, 4.7 m KCl, 2.5 m CaCl₂, 1.2 m KH₂PO₄, 1.2 m MgSO₄, 25 m NaHCO₃, 10 m glucose) under a resting tension of 1.5 g at 37°C and aerated with 95% O₂–5% CO₂. Contraction and relaxations were measured as changes in isometric tension by a force displacement transducer (TB-612T, Nihon Kohden, Osaka, Japan) coupled to a polygraph (RM 6000, Nihon Kohden). An approximately 1.5 h equilibration period was allowed before the start of experiments. The strips were precontracted with 1-phenylephrine (10⁻⁶ M), an α₁-adrenoceptor agonist. After a plateau was attained, the strips were exposed to Ach (10⁻⁹ to 10⁻⁵ M) to construct dose-response curves with or without 30-min pretreatment with 5×10⁻⁵ m indomethacin, a cyclooxygenase inhibitor (10). In some strips, the endothelium was removed by gently rubbing the intimal surface with a cotton ball. Removal of endothelium was verified by abolition or marked suppression of the relaxation caused by 10⁻⁶ M Ach. In these strips, after precontraction with phenylephrine, endothelium-independent vasorelaxation to sodium nitroprusside (SNP, 10⁻⁹ to 10⁻⁵ M), which is known to produce NO (11), was examined in the absence or presence of indomethacin pretreatment. Vasodilator responses to Ach and SNP were expressed as percentages of the response to phenylephrine in each tissue specimen.

**Fatty acid analysis.** Lipids in the aortic tissue were extracted and purified, as described (12), revealing that the aortic tissue included phospholipids but little neutral lipids (data not shown). To quantify the small amount of fatty acids in phospholipids with precision, freeze-dried tissue was directly transmethylated without lipid separation, according to Sakuradani et al. (13). Briefly, freeze-dried tissue with an additional internal standard (pentadecanoic acid) was incubated in methanolic HCl at 50°C for 3 h for transmethylation of fatty acid residues into fatty acid methyl esters. Fatty acid methyl esters were extracted with n-hexane and analyzed by capillary gas-liquid chromatography. Analytical conditions were as follows: 1) apparatus: Agilent 6890 (Agilent, Delaware, USA); 2) column: SP-2330 (30 m×0.32 mm×0.2 μm, Supelco, Pennsylvania, USA); 3) carrier: He (30 cm/s); 4) column temperature: 180°C (2 min) followed by +2°C/min to 220°C (3 min); 5) detector: FID (flame ionization detector) 250°C.

**Statistical analysis.** All values are the mean±SE. For statistical analysis, we used one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison tests. Correlations were obtained by Pearson linear correlation analysis. Differences were considered significant at p<0.05.
RESULTS

Body weight changes

At the beginning and the end of the 2-mo feeding period, the body weights of the groups were as follows: YC (170±1 and 287±3 g, n=11), YA (170±2 and 289±4 g, n=10), OC (461±10 and 441±17 g, n=9), and OA (459±10 and 444±18 g, n=8). ARA supplementation affected body weight gain in neither young nor aged rats. As noted above, one of the 10 OC rats and two of the 10 OA rats died of natural causes, with gradual but marked body weight loss during the feeding period.

Phenylephrine-induced vasoconstriction

Figure 1 shows the maximum tension development induced by 10^-6 M phenylephrine in aortic rings from young and aged rats, which were given control or ARA-containing diet. In the presence of endothelium, the contractile responses in group OC tended to be augmented, compared with those in groups YC and YA. However, this augmentation was eliminated by dietary ARA supplementation in aged rats (OA). On the other hand, there were no significant differences among groups when responses of endothelium-denuded vessels were measured, and the tension development in these vessels was enhanced, compared with those in endothelium-intact vessels.

Ach-induced vasorelaxation

Figure 2 shows the endothelium-dependent vasorelaxation induced by Ach. In the absence of indomethacin, the addition of Ach at concentrations of 10^-9 to 10^-5 M produced dose-dependent relaxation in aortic strips obtained from YC and YA rats, although the responses to higher concentrations of Ach were slightly greater in the YA group. In contrast, in aged rats, Ach-induced relaxation was markedly attenuated at higher concentrations, and this trend was more pronounced in group OC, although the difference between groups OC and OA was not statistically significant. When indomethacin-treated rings were used, slight differences were observed between groups YC and YA, and the attenuation of Ach-induced relaxation at high concentrations in aged rats was abolished, although the responses to Ach in specimens from OC rats was still slightly less than those in specimens from OA rats.

Relationship between vascular ARA concentrations and responses to Ach

As described above, Ach-induced, endothelium-dependent vasorelaxation in the absence of indomethacin tended to be greater in ARA-containing diet-fed rats (groups YA and OA) than in the respective control rats (groups YC and OC). We therefore evaluated the relationship between the vascular responses to Ach and ARA concentrations in aortic tissues. Figure 3A indicates the vasorelaxation in response to 10^-7 M Ach in the aortic ring of each animal (indicated by arrow in Fig. 2; submaximal responses to Ach were obtained at
this concentration). Figure 3B shows the aortic ARA concentration measured in each animal, although this could not be determined for some samples, because of the limited amount of aortic tissue available for fatty acid analysis. ARA supplementation did not increase vascular concentrations of ARA in young rats. On the other hand, there was a modest decrease in ARA concentrations in OC rats, though dietary supplementation of ARA tended to reverse the decrease in level of aortic ARA. When these two parameters were examined in each animal, Ach-induced vasorelaxation was found to be positively correlated with aortic ARA concentration in all groups (Fig. 4A). Furthermore, this correlation was much stronger in aged rats (Fig. 4C), with lack of significant correlation in young rats (Fig. 4B).

**SNP-induced vasorelaxation**

Figure 5 shows the dose-response curves to $10^{-9}$ to $10^{-5}$ M SNP in endothelium-denuded aortic rings obtained from the four experimental groups. In neither the absence nor the presence of indomethacin were there significant differences in endothelium-independent vasodilator responses to SNP among groups YC, YA, OC, and OA.
YA, OC, and OA, although slightly increased responses were observed in young rats.

**DISCUSSION**

In the present study, we found that Ach-induced, endothelium-dependent vasorelaxation was strongly correlated with vascular ARA concentration in aged rats, irrespective of dietary ARA supplementation. To our knowledge, this is a first report that vascular ARA is related to endothelium-dependent vasorelaxation, and suggests that ARA supplementation improves age-related endothelial dysfunction. Only one study related to this issue, that by Loiacono et al. (9), is available. They noted that Ach-induced vasorelaxation in aortic rings from rats fed an essential fatty acid-deficient diet was not impaired. In addition, they found that phenylephrine (α-adrenoceptor agonist)-induced vasoconstrictor responses in aortic rings of these rats were markedly enhanced by endothelium removal, compared with such responses in aortic rings of normal diet-fed rats, suggesting that partial depletion of phospholipid arachidonate either augments spontaneous release of endothelium-derived relaxing factor (EDRF) or impairs EDRF-inactivating mechanisms. Our findings differ markedly from those described in this report. One reason for this is that Loiacono et al. used weanling rats (12–22 d old). In addition, effects of dietary depletion of essential fatty acids other than ARA were not examined. In our study, vessels from young animals exhibited no significant correlation between Ach-induced vasorelaxation and vascular ARA level. Dietary ARA supplementation thus appears to be efficacious only in aging animals.

Moreover, phenylephrine-induced vasoconstriction, which was enhanced in endothelium-intact vessels of aged rats, was restored by ARA supplementation. Since these alterations were not observed in endothelium-denuded vessels, it appears that both the age-related enhancement of effects of phenylephrine and its restoration by dietary ARA are endothelium-dependent events. These results, taken together with the finding that α-adrenoceptor agonist and biological amines are able to release EDRF (14, 15), suggest that dietary supplementation of ARA can reverse age-related decline in the release of EDRF.

Experimental evidence has indicated that cardiovascular risk factors such as aging, atherosclerosis, and hypertension are characterized by the presence of endothelial dysfunction, which is mainly induced by attenuation of NO-mediated vasodilation, augmented production of oxygen-derived free radicals, and increased release of cyclooxygenase-derived vasoconstrictor substances (4). On the other hand, there is accumulating evidence that dietary supplementation of fish oil containing abundant ω3 polyunsaturated fatty acids, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), reduces the incidence of coronary heart disease, atherosclerosis, and hypertension (16–18). One of the mechanisms underlying the cardio- and vaso-protective effects of these ω3 fatty acids is facilitation of endothelium-dependent vasorelaxation and/or attenuation of endothelium-dependent contraction (19, 20). In contrast to ω3 fatty acids, it has remained unclear whether dietary supplementation of ω6 fatty acids improves the endothelial dysfunction observed in cardiovascular diseases or aging. Of particular interest in the present study is the finding of a positive correlation between endothelium-dependent vasorelaxation and aortic ARA concentration in aged but not in young rats. In addition, using the same samples, we performed regression analysis between endothelium-dependent vasorelaxation and aortic ARA concentration, but found no significant correlation between these two parameters (data not shown). Further studies are needed to determine whether dietary ARA supplementation can attenuate aging-related cardiovascular disease.

In the present study, it was found that aortic ARA concentration decreased with age, and that long-term supplementation of ARA to aged rats returned ARA concentration to the same level as that in young rats. Several studies have indicated that Δ-6 desaturase.

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**Fig. 5.** Dose-response curves to SNP in the absence (A) or presence (B) of indomethacin pretreatment in aortic rings (endothelium-denuded) from YC, YA, OC, and OA rats. Values are the mean ± SE.
activities in liver microsomes decrease with aging (21, 22). It appears that the capacity of the liver to synthesize ARA decreases in aged rats, and that ARA supplementation of aged rats is of great importance in restoring endothelial function to the same level as that in young rats. Unexpectedly, in the present study, aortic ARA concentration did not differ significantly between the two ages studied, and long-term supplementation of ARA significantly increased aortic ARA concentrations in neither young nor aged rats. The reason for this is unknown. However, a study (8) using the same diets and animals as we did also found that ARA level in the brain was not significantly increased by long-term feeding of an ARA-containing diet, suggesting that the ARA profile in brain might be strongly protected against variation and does not change easily. The same might be true of vascular tissues, though further studies are needed to determine whether this is the case.

In aged rats, endothelium-dependent, Ach-induced vasorelaxation was markedly attenuated at higher concentrations, and this attenuation disappeared in the presence of indomethacin pretreatment, suggesting that cyclooxygenase metabolites are responsible for it. Relatively high concentrations of Ach are known to release endothelium-derived vasoconstrictor substances as well as EDRF (23). One possible candidate for such a substance is thromboxane A2, as previously described (24). It thus seems likely that both a decline in the release of EDRF and an increase in endothelium-derived vasoconstrictor substances are involved in the attenuation of responses to higher concentrations of Ach in the aortic tissues of aged rats. On the other hand, endothelium-dependent, Ach-induced vasorelaxation tended to be greater in OA rats than in OC rats, irrespective of indomethacin treatment. This suggests that eicosanoids and/or substances related to them do not contribute to the differences due to ARA supplementation in aged rats noted above.

The precise mechanisms by which ARA supplementation improves age-related endothelium dysfunction cannot be determined from our findings. Very recently, it was reported that long-term administration of ARA to senescent rats helped to preserve membrane fluidity and maintain hippocampal plasticity (25). Interestingly, in that study, cell membrane fluidity was detected directly, using fluorescence recovery after photobleaching (FRAP) experiments, and it was found that hippocampal membrane of senescent rats supplemented with ARA exhibited significantly better fluidity than that of rats without ARA supplementation. Thus, preservation of cell membrane fluidity and plasticity by ARA supplementation might be related to improvement of age-related vascular endothelium dysfunction.

In conclusion, dietary ARA supplementation in aged rats decreased the age-related endothelial dysfunction that leads to various cardiovascular diseases.

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