

Anti-atherogenic effect of soya and rice-protein isolate, compared with casein, in apolipoprotein E-deficient mice

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(Received 18 June 2002 – Revised 20 January 2003 – Accepted 31 January 2003)

Our objective was to determine whether dietary plant proteins such as soya-protein isolate (SPI) and rice-protein isolate (RPI) compared with animal proteins, such as casein, could afford beneficial effects on atherosclerosis development in apolipoprotein E-deficient mice. In experiment 1, male and female mice were fed on a purified diet containing either casein, SPI or RPI for 9 weeks. The *en face* lesion area in the aorta ($P < 0.05$) and the lesion size in the aortic root ($P < 0.05$) in mice fed the casein-based diet were greater than those in the SPI or RPI groups. The plant protein groups had an increased concentration of serum L-arginine ($P < 0.05$) and NO metabolites (NO_2 plus NO_3) ($P < 0.05$) than did the casein group. The inhibitory effect of the plant proteins on the lesion formations was unrelated to gender and total serum cholesterol. In experiment 2, the L-arginine and L-methionine contents were the same in the L-arginine-supplemented casein-based and SPI-based diets, and between the L-methionine-supplemented SPI-based and the casein-based diets. Male mice were fed on the diets for 15 weeks. There were no significant differences in the *en face* lesion area and the lesion size between the casein group and the L-arginine-supplemented group, although the serum L-arginine ($P < 0.05$) and NO_2 plus NO_3 ($P < 0.05$) concentrations in the supplemented group were higher than those in the casein group. There were no significant effects of L-methionine supplementation on the lesion formations. In experiment 3, male mice were given the casein-based diet or the L-arginine-supplemented casein-based diet together with water or water containing an NO synthesis inhibitor for 9 weeks. When given the casein-based diet, the inhibitor drinking, compared with water drinking, resulted in a reduction of the serum NO_2 plus NO_3 concentration ($P < 0.01$) and an increase in the *en face* lesion area ($P < 0.05$) and the lesion size ($P < 0.01$). When given the L-arginine-supplemented diet, the inhibitor drinking, compared with water drinking, resulted in no increase in the lesion area and size. These results demonstrate anti-atherogenic potentials of SPI- as well as RPI-derived proteins, but their L-arginine and L-methionine contents were not sufficient enough to explain the underlying mechanism(s).

Apolipoprotein-E-deficient mice: L-Arginine: Atherosclerosis: Soya protein: Rice protein

Soya and rice proteins are known to be rich in L-arginine and poor in L-methionine compared with animal proteins such as casein. It has recently been reported that these amino acids are differently involved in atherosclerosis in experimental animals such as rabbits, pigs, and LDL receptor-deficient mice (Cooke & Tsao, 1997). In fact, dietary supplementation of L-arginine prevents atherosclerosis development, probably through the conversion to NO, which has a variety of beneficial effects on the cardiovascular system (Cooke *et al.* 1992; Böger *et al.* 1996; Aji *et al.* 1997). However, there is a report showing that L-arginine treatment does not improve lesion formations in apolipoprotein (apo) E-deficient mice, an animal model of human atherosclerosis (Nakashima *et al.* 1994), fed a Western-type diet (Kuhlencordt *et al.* 2001). In contrast, supplementation of L-methionine to a non-purified diet has been reported to induce typical arteriosclerotic lesions in rabbits (Toborek *et al.* 1995; Hofmann *et al.* 2001), possibly

through conversion of L-methionine to homocysteine (Zhou *et al.* 2001). We reported that the *en face* lesion area in the aorta and the lesion size in the aortic root in apo E-deficient mice fed a diet containing soya protein were smaller than those in the casein-fed groups (Ni *et al.* 1998). Therefore, if these two amino acids were involved in the development of atherosclerosis, it would be expected that a rice protein-containing diet would result in a decrease in lesion formations in experimental animals including apo E-deficient mice compared with those fed a casein-containing diet.

In the present study, the aim was to show that apo E-deficient mice fed a diet containing soya or rice protein had a lowered extent of atherosclerotic lesion formations in the aorta and in the aortic root than did those fed a casein-containing diet. Then, an L-arginine-supplemented casein-based diet or an L-methionine-supplemented soya protein-based diet was fed to apo E-deficient mice,

Abbreviations: apo, apolipoprotein; RPI, rice-protein isolate; SPI, soya-protein isolate; L-NAME, N^ω-nitro-L-argininemethylester.

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to examine if the beneficial effect of the plant proteins could be attributed to these amino acids. Finally, a role of NO synthesis in the development of atherosclerosis was examined by treating apo E-deficient mice with an inhibitor of NO synthesis.

Materials and methods

Animals and diets

C57BL/6J apo E-deficient mice engineered at the University of North Carolina (Chapel Hill, NC, USA) (Zhang *et al.* 1992) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) in 1994, and maintained at the Laboratory of Animal Experiments in Kyushu University Faculty of Medicine (Fukuoka, Japan). They were housed in plastic cages (one mouse per cage, 120 mm×170 mm×240 mm) containing paper chips (ALPHA-dri; Shepherd Specialty Papers, Inc., Kalamazoo, MI, USA), kept in a temperature-controlled room at 23–25°C with a 12 h light–dark cycle (light on 08.00–20.00 hours) and given free access to diet and non-ionized water throughout the experimental period. The diet and water were changed every other day and body weight and food intake were recorded simultaneously.

The composition of a basal diet, based on the AIN-93G formulation (Reeves *et al.* 1993), has been described previously (Ni *et al.* 1998; Ando *et al.* 2002), except that, as a source of dietary fat, olive oil (100 g/kg diet) was used, instead of soyabean oil. The basal diet contained (g/kg): casein 200.0; olive oil 100.0; α -maize starch 132.0; sucrose 100.0; cellulose 50.0; vitamin mixture (AIN-93G) 10.0; mineral mixture (AIN-93G) 35.0; L-cysteine 3.0; choline bitartrate 2.5; *tert*-butylhydroquinone 0.014; maize starch to 1000 g. Diets were packed in a pouch containing an O₂ absorbent, flushed with N₂ and stored at 4°C. The diets were freshly prepared every week, and changed every 2 d, with any remaining diet being discarded.

In the first experiment, fifteen female and fifteen male apo E-deficient mice, 12–13 weeks old, were divided into three groups each and fed for 9 weeks the basal diet containing (per kg diet) 200.0 g casein (142 mg N/g protein; Wako Pure Chemicals, Osaka, Japan), 211.0 g soya-protein isolate (SPI, 133 mg N/g protein, Fujipro R; Fuji Oil, Osaka, Japan) or 213.0 g rice-protein isolate (RPI, 132 mg N/g protein), as a source of dietary protein. The RPI was prepared according to the method described by Morita & Kiriya (1993). In brief, 5 kg rice grits of *Oryza sativa* L. Japonica (cv. Todoroki) (Shimada Chemical Co., Niigata, Japan) was digested with 10 litres of distilled water containing 60 ml of a heat stable α -amylase (Termamyl 120 L; Novo Laboratories, Sapporo, Japan) at 97°C for 2 h, and the residue was washed with boiling water followed by ethanol. The N content (Miller & Houghton, 1945) and amino acid composition (Chang *et al.* 1986) of the three proteins were determined. The proportion of L-arginine and L-methionine (mol %) was 3.5 and 2.8 for casein, 7.4 and 1.4 for SPI and 8.1 and 2.2 for RPI, respectively. The amino acid compositions of casein, SPI and RPI were similar to those reported elsewhere (Morita *et al.* 1997).

In the second experiment, twenty male apo E-deficient mice, 9–10 weeks old, were divided into four groups and fed the casein-based diet, the casein-based diet with supplementation of L-arginine (6.9 g/kg diet), the SPI-based diet and the SPI-based diet with supplementation of L-methionine (2.4 g/kg diet) for 15 weeks. Thus, the L-arginine and L-methionine contents were the same in the L-arginine-supplemented casein-based and SPI-based diets, and between the L-methionine-supplemented SPI-based and the casein-based diets.

In the third experiment, twenty-nine male apo E-deficient mice, 11–12 weeks old, were divided into four groups and fed the casein-based diet or the casein-based diet with supplementation of L-arginine, as described in the second experiment, and given either with non-ionized water or non-ionized water containing 400 mg N^ω-nitro-L-argininemethylester (L-NAME)/l (Sigma, Tokyo, Japan), an NO synthase inhibitor (Aji *et al.* 1997), for 9 weeks.

These experiments were carried out under the guidelines for Animal Experiments in the Faculty of Agriculture and the Graduate Course, Kyushu University, Fukuoka, Japan and the Law (no. 105) and Notification (no. 6) of the Government of Japan. Animals were killed by withdrawing blood from the left ventricle under anaesthesia with intraperitoneal injection of Somnopentyl (50 mg/kg body weight, pentobarbital sodium; Kyoritsu Shoji, Tokyo, Japan).

Tissue preparation and morphometric determination of atherosclerosis

Animals were killed by withdrawing blood, and the circulatory system was perfused with 50 ml PBS, pH 7.4, via a cannula inserted into the left ventricle, allowing unrestricted efflux from an incision in the vena cava. Perfusion was continued with 50 ml 10% (v/v) neutral formalin buffer solution, pH 7.4. The heart and the aorta were removed and fixed in 10% neutral formalin-buffered solution. The percentage of the aortic surface covered by lesions was determined using an *en face* preparation as described previously (Ni *et al.* 1998). To determine the cross-sectional lesion area, hearts containing aortic roots were processed for the quantitative atherosclerosis assay using a modification of the method described by Paigen *et al.* (1987). Briefly, the heart was cut along a plane between the tip of two atria and the top half was embedded in paraffin. Serial sections (10 μ m thick) were prepared from the ascending aorta to the aortic sinus until the aortic tissue was invisible. The sections were mounted on glass slides and stained with elastic Van Gieson and haematoxylin as described previously (Ni *et al.* 1998). Five sections of each heart were selected for intimal area determination. The first and most distal sections to the heart were taken where the aortic valve was just visible, from this section, moving to the base of the heart, the 15th, 30th, 45th and 60th sections were also photographed along with the first one, using a video camera mounted on an Olympus IX70 light microscope (Olympus Optical, Tokyo, Japan). The intimal area was measured by NIH image/68k 1.57 software (National Institute of Health,

Bethesda, MD, USA) on a Macintosh computer. The mean intimal area was calculated for each animal and subsequently for each group.

Analysis of serum free amino acid, lipid and nitrite plus nitrate

Serum free amino acid concentrations were determined as described previously (Ikeda *et al.* 1994). Serum lipid concentrations were determined by commercially available kits (Cholesterol C Test, Triglyceride G Test and Phospholipid B Test; Wako Pure Chemicals, Osaka, Japan). In order to determine the serum nitrite plus nitrate (NO₂ plus NO₃) (final metabolites of NO) concentration, 100 µl serum was mixed with 40 µl 35% (w/v) sulfosalicylic acid and centrifuged at 3000 rpm at room temperature for 15 min to precipitate the lipoproteins and haemoglobin as described previously (Wishnok *et al.* 1996). The NO₂ plus NO₃ concentrations in the supernatant fraction were measured by an NO₂/NO₃ Assay Kit-C (Wako Pure Chemicals, Osaka, Japan) according to the manufacturer's instructions.

Statistical analysis

Data are expressed as means with their standard errors and were analysed by two- or one-way ANOVA followed by a *post-hoc* test (Fisher's protected least significant difference test) in the first and the second experiment, and by Student's *t* test in the third experiment. In all cases, the effects were considered statistically significant at $P < 0.05$.

Results

Effect of dietary protein

There was a difference in the initial mean body weight between females; (25.5 (SE 1.2), 24.8 (SE 0.7) and 24.8 (SE 0.8) g for the casein, SPI and RPI groups, respectively) and males (29.5 (SE 1.9), 29.4 (SE 1.7) and 30.8 (SE 1.7) g for the casein, SPI and RPI groups, respectively); $P < 0.01$. Final mean body weight in females, (29.4 (SE 2.6), 27.5 (SE 1.0) and 28.3 (SE 1.8) g for the casein, SPI and RPI groups, respectively) was smaller than that in males, (39.5 (SE 3.5), 37.0 (SE 1.8) and 38.0 (SE 2.9) g for the casein, SPI and RPI groups, respectively); $P < 0.01$, but there was no significant dietary effect. Gender and type of dietary protein had no significant effect on the mean daily food intake: 4.17 (SE 0.15), 4.59 (SE 0.17) and 4.45 (SE 0.19) g for the casein-, SPI- and RPI-fed females, respectively; and 4.38 (SE 0.14), 4.73 (SE 0.16) and 4.40 (SE 0.10) g for the casein-, SPI- and RPI-fed males, respectively.

Table 1 shows the degree of atherosclerotic lesions, and concentrations of serum L-arginine and L-methionine, NO₂ plus NO₃ and lipids in the apo E-deficient female and male mice fed a diet containing different proteins. Two-way ANOVA revealed a main effect of protein on the *en face* lesion area in the aorta ($P < 0.01$), the lesion size in the aortic root ($P < 0.05$) and the serum NO₂ plus NO₃ concentration ($P < 0.05$); there was no main effect of gender.

Table 1. Effect of dietary casein (CAS) soya-protein isolate (SPI) and rice-protein isolate (RPI) on atherosclerotic lesions and serum lipids, nitrite plus nitrate, and L-arginine and L-methionine concentrations in female or male apolipoprotein E-deficient mice*
(Mean values with their standard errors for five mice per gender in each group)

	Female						Male						ANOVA		
	CAS		SPI		RPI		CAS		SPI		RPI		Protein	Gender	Interaction
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE			
<i>En face</i> lesion area (%)	18.0 ^a	4.1	7.70 ^b	1.98	10.5 ^{ab}	1.0	15.5 ^{ac}	2.7	10.0 ^{bc}	1.6	10.5 ^{bc}	1.6	0.01	NS	NS
Lesion size in aortic root (mm ²)	0.243 ^a	0.030	0.172 ^{ab}	0.024	0.164 ^b	0.021	0.224 ^{ab}	0.017	0.162 ^b	0.028	0.192 ^{ab}	0.019	0.05	NS	NS
Arginine (µmol/l)	n.d.		n.d.		n.d.		42.0 ^a	1.4	86.1 ^b	1.2	61.8 ^c	2.7			
Methionine (µmol/l)	n.d.		n.d.		n.d.		34.7 ^a	0.6	40.0 ^b	0.6	41.8 ^b	1.5			
NO ₂ plus NO ₃ (µmol/l)	24.6 ^a	4.2	34.1 ^{ab}	5.0	37.6 ^b	2.9	25.5 ^a	3.3	34.8 ^{ab}	4.1	39.9 ^a	2.9	0.05	NS	NS
Chol (mmol/l)	14.3 ^a	1.1	14.9 ^a	1.1	14.7 ^a	1.5	24.0 ^b	4.7	27.7 ^b	1.8	26.9 ^b	3.8	NS	0.01	NS
TG (mmol/l)	1.51 ^a	0.46	1.23 ^a	0.31	0.77 ^a	0.19	2.21 ^a	0.72	2.10 ^a	0.56	2.22 ^a	0.38	NS	0.05	NS
PL (mmol/l)	3.39 ^a	0.38	3.43 ^a	0.33	3.50 ^a	0.39	5.51 ^{ab}	1.10	6.63 ^b	0.68	5.92 ^b	1.00	NS	0.01	NS

Chol, cholesterol; TG, triacylglycerols; PL, phospholipids; n. d., not determined.
^{abc}Mean values within the same row with unlike superscript letters were significantly different ($P < 0.05$) (Fisher's protected least significant difference test).
^{*}For details of diets and procedures, see p. 14.

Accordingly, the data were summarized in Fig. 1, stratified by diet but not gender. In contrast, there was a significant main effect of gender on the concentrations of serum cholesterol ($P < 0.01$), triacylglycerols ($P < 0.05$) and phospholipids ($P < 0.01$); the concentrations of these lipids were lower in females than in males. Dietary protein had no significant effect on the serum lipids. Reflecting the dietary content of L-arginine, the serum L-arginine concentration in male apo E-deficient mice was higher when plant proteins were given than when casein was consumed ($P < 0.05$). The concentration of L-methionine in the casein group was lower than that in the plant protein groups ($P < 0.05$), although the dietary proportion of L-methionine in casein was approximately 2-fold as high as in SPI.

As shown in Fig. 1, the *en face* lesion area in the casein group was significantly greater than in the SPI ($P < 0.01$) and the RPI ($P < 0.01$) groups. The lesion size in the casein group was also significantly greater than in the SPI ($P < 0.01$) and the RPI ($P < 0.05$) groups. The serum NO₂ plus NO₃ concentration in the casein group was significantly lower than in the SPI ($P < 0.05$) and the RPI ($P < 0.01$) groups.

Effect of supplementation of amino acids

There were no differences in the initial mean body weight among the groups (26.5 (SE 0.3), 25.3 (SE 0.6), 25.6 (SE 0.7)

and 26.1 (SE 0.8) g for the casein, casein plus L-arginine, SPI, and SPI plus L-methionine groups, respectively) and the final mean body weight (34.2 (SE 1.9), 34.1 (SE 1.8), 32.0 (SE 1.7) and 35.6 (SE 1.1) g for the casein, casein plus L-arginine, SPI, and SPI plus L-methionine groups, respectively). Mean daily food intake in both groups of mice fed the casein-based diet (3.80 (SE 0.15) and 3.61 (SE 0.06) g for the casein and casein plus L-arginine groups, respectively) was lower than that in both groups of mice fed the SPI-based diet (4.24 (SE 0.04) and 4.34 (SE 0.05) g for the SPI and SPI plus L-methionine group, respectively); $P < 0.01$.

Table 2 shows the degree of atherosclerotic lesions, and concentrations of the serum L-arginine, L-methionine, NO₂ plus NO₃ and lipids in male apo E-deficient mice fed a diet with or without supplementation of amino acids. There was no significant difference in the *en face* lesion area in the aorta among the four dietary groups. These results are in contrast to those in experiment 1 where there was a significant difference in the lesion area between the casein and the SPI groups. It is probable that the feeding period influenced the results. The lesion size in the aortic root in mice fed the SPI diet and SPI plus L-methionine diet was lower than that in the casein group ($P < 0.05$). There was no difference in the lesion size between the casein and the casein plus L-arginine groups ($P = 0.08$), and between the SPI and the SPI plus L-methionine

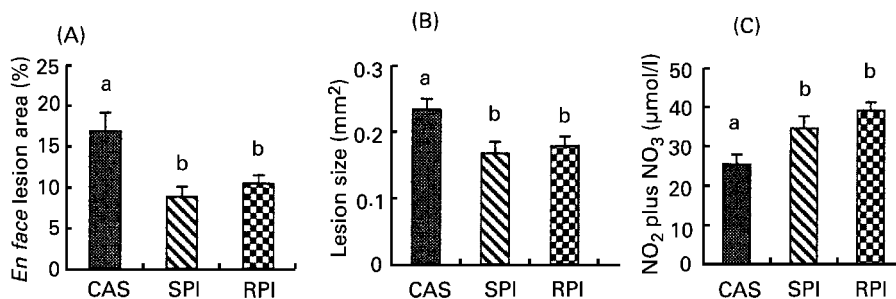


Fig. 1. *En face* lesion area in the aorta (A), lesion size in the aortic root (B) and NO metabolites in the serum of mice fed a diet containing casein (CAS), soya-protein isolate (SPI) and rice-protein isolate (RPI). Mean values for ten mice per group are shown, with their standard errors represented by vertical bars. ^{abc}Mean values with unlike letters were significantly different ($P < 0.05$).

Table 2. Effect of supplementation of L-arginine and L-methionine to casein (CAS)- and soya-protein isolate (SPI)-based diets, respectively, on atherosclerotic lesions and serum lipids, nitrite plus nitrate, and L-arginine and L-methionine concentrations in male apolipoprotein E-deficient mice*

(Mean values with their standard errors for five male mice in each group)

	CAS		CAS+Arginine		SPI		SPI+Methionine	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
<i>En face</i> lesion area (%)	19.0 ^a	1.6	17.2 ^a	1.7	21.2 ^a	2.1	20.1 ^a	1.6
Lesion size in aortic root (mm ²)	0.536 ^a	0.040	0.442 ^{ab}	0.030	0.361 ^b	0.030	0.377 ^b	0.040
Arginine (µmol/l)	40.0 ^a	2.7	67.5 ^b	4.3	88.7 ^b	4.3	88.5 ^b	13.3
Methionine (µmol/l)	27.7 ^a	3.5	22.0 ^a	2.0	21.3 ^a	3.3	22.5 ^a	2.6
NO ₂ plus NO ₃ (µmol/l)	11.9 ^a	3.3	33.6 ^b	5.1	39.6 ^b	11.5	40.9 ^b	6.7
Chol (mmol/l)	18.2 ^a	0.9	21.0 ^{ac}	2.6	27.7 ^{bc}	3.1	31.5 ^b	2.5
TG (mmol/l)	1.08 ^a	0.34	1.46 ^a	0.38	3.12 ^{ab}	1.41	4.91 ^b	1.27
PL (mmol/l)	5.68 ^a	0.86	6.99 ^a	1.13	9.07 ^{ab}	1.49	10.9 ^b	1.2

Chol, cholesterol; TG, triacylglycerols; PL, phospholipids.

^{abc}Mean values within the same row with unlike superscript letters were significantly different ($P < 0.05$) (Fisher's protected least significant difference test).

* For details of diets and procedures, see p. 14.

groups, respectively. There was no difference in the lesion area between the SPI and the SPI plus L-methionine groups. The serum L-arginine concentration in the SPI, SPI plus L-methionine and casein plus L-arginine groups was higher than that in the casein group ($P < 0.05$). The serum L-methionine concentration was not affected by the dietary protein or amino acid supplementation. The serum NO₂ plus NO₃ concentration in the SPI, SPI plus L-methionine, and the casein plus L-arginine groups was higher than that in the casein group ($P < 0.05$). Supplementation of L-methionine had no effect on the serum NO₂ plus NO₃ concentration. The amino acid supplementation had no significant effect on the serum lipids. The SPI-based diet, in comparison with the casein-based diet, tended to increase the serum lipid concentration.

Effect of nitric oxide synthesis inhibitor

There were no differences in the initial mean body weight among the groups (28.8 (SE 0.5), 28.9 (SE 0.6), 29.4 (SE 0.7) and 29.4 (SE 0.6) g for the casein, casein plus L-NAME, casein plus L-arginine, and casein plus L-arginine plus L-NAME groups, respectively), and the final mean body weight (38.1 (SE 2.0), 41.8 (SE 1.4), 39.1 (SE 1.4) and 41.4 (SE 1.6) g for the casein, casein plus L-NAME, casein plus L-arginine, and casein plus L-arginine plus L-NAME groups, respectively). There were no differences in mean daily food intake among the groups (4.54 (SE 0.18), 4.48 (SE 0.11), 4.74 (SE 0.08) and 4.41 (SE 0.12) g for the casein, casein plus L-NAME, casein plus L-arginine, and casein plus L-arginine plus L-NAME groups, respectively). Mean daily water intake was also similar among the groups (3.30 (SE 0.18), 3.18 (SE 0.15), 3.46 (SE 0.23) and 3.67 (SE 0.33) ml for the casein, casein plus L-NAME, casein plus L-arginine, and casein plus L-arginine plus L-NAME groups, respectively).

Table 3 shows the degree of atherosclerotic lesions, and concentrations of the serum L-arginine, L-methionine, NO₂ plus NO₃ and lipids in male apo E-deficient mice fed casein or casein plus L-arginine and given water with

or without supplementation of L-NAME. Two-way ANOVA revealed a main effect of L-NAME on the *en face* lesion area in the aorta ($P < 0.01$). The lesion area in the casein plus L-NAME group was significantly greater than in the casein group ($P < 0.05$), but there was no significant difference in the lesion area between the casein plus L-arginine and the casein plus L-arginine plus L-NAME groups ($P = 0.08$). There was an interactive effect of L-arginine supplementation and L-NAME treatment on the lesion size in the aortic root ($P < 0.05$). The lesion size in the casein plus L-NAME group was the highest among groups ($P < 0.01$), but there was no significant difference in the lesion size between the casein plus L-arginine and the casein plus L-arginine plus L-NAME groups. As expected, there was a supplementary L-arginine effect on the serum L-arginine concentration ($P < 0.01$). Two-way ANOVA revealed a main effect of L-NAME on the serum NO₂ plus NO₃ concentration ($P < 0.01$). Although there was an L-arginine effect on NO₂ plus NO₃ ($P < 0.05$), the mean values between the casein and the casein plus L-arginine ($P = 0.071$) and those between casein plus L-arginine and casein plus L-arginine plus L-NAME ($P = 0.16$) were not significant. There were main effects of L-NAME treatment ($P < 0.01$) and L-arginine supplementation ($P < 0.05$) on the serum cholesterol concentration. The serum cholesterol concentration in the casein plus L-arginine plus L-NAME group was higher than that in the casein group ($P < 0.05$), but there were no significant differences between the casein plus L-arginine plus L-NAME and the casein plus L-NAME groups ($P = 0.089$) and between the casein plus L-NAME plus L-arginine and the casein plus L-arginine groups ($P < 0.058$).

Discussion

The first experiment showed that both RPI and SPI, in comparison with casein, were effective to the same extent to lower atherosclerotic lesion formations in the aorta (*en face* lesion area) and aortic root (lesion size)

Table 3. Effect of nitric oxide synthase inhibitor on atherosclerotic lesions and serum lipids, nitrite plus nitrate, and L-arginine and L-methionine (Met) concentrations in male apolipoprotein E-deficient mice*

(Mean values with their standard errors for seven male mice for CAS, CAS+Arg, and CAS+Arg+L-NAME groups, respectively, and eight male mice for CAS+L-NAME group)

	CAS		CAS+L-NAME		CAS+Arg		CAS+Arg+L-NAME		ANOVA		
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Arg	L-NAME	Int
<i>En face</i> lesion area (%)	8.04 ^a	1.00	14.0 ^b	2.0	9.05 ^{ac}	1.60	13.6 ^{bc}	2.1	NS	0.01	NS
Lesion size in aortic root (mm ²)	0.188 ^a	0.021	0.312 ^b	0.023	0.15 ^a	0.017	0.173 ^a	0.013	0.01	0.01	0.05
Arg (μmol/l)	39.1 ^a	11.1	48.6 ^a	3.4	70.5 ^b	6.9	69.5 ^b	5.1	0.01	NS	NS
Met (μmol/l)	48.6 ^a	0.8	48.4 ^a	0.6	50.8 ^a	1.5	48.5 ^a	3.4	NS	NS	NS
NO ₂ plus NO ₃ (μmol/l)	22.8 ^a	2.6	3.56 ^b	0.76	31.0 ^a	5.0	9.53 ^b	2.37	0.05	0.01	NS
Chol (mmol/l)	16.5 ^a	2.4	20.6 ^{ab}	2.0	19.9 ^{ab}	0.7	25.3 ^b	2.1	0.05	0.01	NS
TG (mmol/l)	2.17 ^a	0.50	2.23 ^a	0.19	1.77 ^a	0.13	2.43 ^a	0.35	NS	NS	NS
PL (mmol/l)	5.86 ^a	0.81	6.38 ^a	0.50	5.74 ^a	0.27	7.08 ^a	0.54	NS	NS	NS

CAS, casein; Arg, arginine; L-NAME, N^ω-nitro-L-argininemethylester; Int, interaction; Chol, cholesterol; TG, triacylglycerols; PL, phospholipids.

^{abc}Mean values within the same row with unlike superscript letters were significantly different ($P < 0.05$) (Fisher's protected least significant difference test).

* For details of diets and procedures, see p. 14.

in apo E-deficient mice. This is the first report showing an anti-atherogenic effect of RPI in experimental animals. Since the RPI and SPI preparations were relatively rich in protein (830 and 870 g/kg for SPI and RPI, respectively), it is probable that the anti-atherogenic effect of the plant-protein preparations can be attributed to the protein rather than other ingredients such as isoflavones in the SPI, although isoflavones have been shown to exert anti-atherogenic effects in experimental animals (Kirk *et al.* 1998; Yamakoshi *et al.* 2000; Adams *et al.* 2002). In fact, our RPI preparations obviously contained no isoflavones. Furthermore, an SPI preparation that was exhaustively washed with ethanol in order to remove as much isoflavones as possible still maintained an ability in apo E-deficient mice to lower the atherosclerotic lesion area in the aorta compared with those fed a diet containing casein (Ni *et al.* 1998).

Plant proteins derived from soya and rice are rich in L-arginine and poor in L-methionine, in comparison with casein. These differences in amino acid composition between plant and animal proteins might be involved in the extent of the lesion formations. In fact, L-arginine supplementation results in lowering the development of atherosclerotic lesions (Cooke *et al.* 1992; Böger *et al.* 1996; Aji *et al.* 1997), whereas L-methionine supplementation aggravates the lesion (Toborek *et al.* 1995; Zhou *et al.* 2001). In experiment 1, the male mice fed the SPI and RPI, compared with those fed the casein, had an increased concentration of serum L-arginine. However, the casein group had a lower concentration of serum L-methionine than did the plant-protein groups. Therefore, it was assumed that the difference in the L-arginine content rather than the L-methionine content in the diet affected the lesion formations in the mice fed the plant proteins and casein, respectively. L-Arginine is the precursor of NO, which exerts beneficial effects on the atherosclerotic lesion formations through mediating endothelium-dependent vasodilator responses of the coronary and peripheral blood vessels (Böger *et al.* 1995, 1996). Since serum determination of NO metabolites has been suggested to be one of the useful indicators for systemic NO production rates from L-arginine in various physiological and pathophysiological conditions (Bode-Böger *et al.* 1994), we measured the serum NO₂ plus NO₃ concentration. In experiment 1, the SPI- and RPI-fed mice had an increased concentration of serum NO₂ plus NO₃ than did the casein group. Therefore, it was suggested that the plant protein consumption, in comparison with casein, facilitated production of NO in the vascular system, due to its high L-arginine content, thereby contributing to lowering the lesion formations.

To examine further a role of L-arginine and L-methionine in the lesion formations, in experiment 2, L-arginine was supplemented to the casein-based diet, or L-methionine to the SPI-based diet; in this way, the L-arginine and L-methionine contents were equivalent to those in SPI- and casein-based diets, respectively. Supplementary L-methionine to the SPI diet resulted in neither an elevation of the serum L-methionine nor aggravation of the lesion formations. The L-arginine supplemented group, compared with the casein group, had increased concentrations of

serum L-arginine and NO₂ plus NO₃ to an extent that was in the same range as that in the SPI-based diet-fed groups. The results are in agreement with those who found that the production of NO and its metabolites by vascular rings was increased above control in rabbits fed L-arginine (Minor *et al.* 1990; Tsao *et al.* 1994; Candipan *et al.* 1996). However, in contrast to the SPI-based diet groups, the L-arginine-supplemented casein-based diet group, compared with the casein group, had no significant prevention from the lesion size in the aortic root. Our results are in agreement with those by Kuhlencordt *et al.* (2001), who reported that L-arginine supplementation resulted in no significant effect on the lesion area in apo E-deficient mice assayed by an *en face* method. However, our results are in contrast to Aji *et al.* (1997) and Böger *et al.* (1995), who reported that L-arginine supplementation reduced the lesion formations in LDL-receptor knockout mice and hypercholesterolaemic rabbits, respectively. Therefore, the results obtained by Kuhlencordt *et al.* (2001) and by us indicate that the L-arginine supplementation may not be sufficient enough to lower the lesion formations in apo E-deficient mice. Alternatively, these results suggest that, anti-atherogenic potentials of SPI and RPI in experiment 1 should be ascribed to not only an increased NO formation due to high L-arginine content in the plant proteins but also an unclarified factor belonging to the plant proteins. In this context, it remains a possibility that amino acids other than L-arginine and L-methionine in the plant proteins might contribute to reducing the lesion formations, because both SPI and RPI, compared with casein, contain in common an increased proportion of glycine (41, 42 and 16 g/kg for SPI, RPI and casein), and cysteine (12, 22 and 3.0 g/kg for SPI, RPI and casein). It also remains to be determined if an L-arginine-containing peptide present in the SPI and RPI preparations, compared with the free form of L-arginine, might exert an effect that is effective in lowering the lesion formations.

Experiment 3 was carried out to confirm a role of NO formation from L-arginine in lowering the atherosclerotic lesion formations of apo E-deficient mice. For this purpose, the mice were fed a diet containing casein or the casein-based diet supplemented with L-arginine, and given water or water containing an NO synthesis inhibitor, L-NAME (Aji *et al.* 1997). When the mice were fed the casein-based diet without supplementation of L-arginine, L-NAME treatment, compared with no L-NAME, resulted in a prominent reduction in the concentration of serum NO metabolites and an increased lesion area in the aorta and lesion size in the aortic root. These results confirm that an efficient utilization of L-arginine for NO synthesis is a prerequisite for preventing an increase of the lesion formations in apo E-deficient mice. It is, however, interesting to note that L-NAME-treated mice that were given the casein-based diet supplemented with L-arginine had lowered lesion size in the aortic root than did L-NAME-treated mice that were given the diet without L-arginine supplementation, although those mice had a decreased concentration of the serum NO metabolites. These results suggest that an increase of dietary L-arginine could exert a beneficial effect on the lesion formations through a mechanism

such as becoming a precursor of NO (Böger *et al.* 1995, 1996) as well as an antioxidant (Böger *et al.* 1998). However, our results are not in agreement with Aji *et al.* (1997), who showed that treating LDL-receptor knockout mice with a high-cholesterol diet and L-NAME abolished the beneficial effect of L-arginine supplementation on the lesion size in the aortic root. The discrepancy between our results and those of Aji *et al.* (1997) might be due to the animals and diets.

Dietary soya protein, compared with animal proteins, has long been known to possess a hypocholesterolaemic effect in a large number of clinical and experimental studies (Beynen, 1992; Anderson *et al.* 1995; Potter, 1996). Furthermore, SPI and RPI, compared with casein, are hypocholesterolaemic in the rat (Morita *et al.* 1997). In the present study, however, the SPI- or RPI-based diets, compared with the casein-based diet, did not lower the serum total cholesterol concentration in apo E-deficient mice. In addition, supplementation of L-arginine or L-methionine did not alter the intrinsic effect of SPI and casein on the serum cholesterol concentrations. Furthermore, female mice had significantly lower serum cholesterol concentration than did male mice, but there was no significant difference in the lesion formations between gender. Therefore, it was unlikely that the serum cholesterol level was relevant to the different response of arterial lesion formations to dietary protein source.

Both experiments 1 and 2 showed anomalous results. Namely, the SPI group had a lowered lesion size in the aortic root than did the casein group in both experiments, but there was no significant difference in the lesion area in the aorta between the groups in experiment 2. Furthermore, dietary proteins resulted in no significant effect on the serum cholesterol concentration in experiment 1, while it was approximately 50% higher in the SPI group than in the casein group in experiment 2. This might contribute to the differences in atherosclerosis between the two experiments. Again, this might be attributed to the different length of the experiments. It also seems that experiment 3 was confounded by serum cholesterol. The addition of L-NAME increased the serum cholesterol, which may, at least in part, be responsible for the increase in atherosclerosis.

In summary, the present study showed that dietary SPI and RPI, in comparison with dietary casein, led to reduced lesion formations that appeared to be preceded by an increase in the concentration of the serum L-arginine and NO metabolites. Dietary L-arginine supplementation resulted in an increased concentration of the serum NO metabolites, but this was not accompanied with the lowering of lesion formations. Therefore, anti-atherogenic potentials of the plant proteins include not only an increase in the formation of NO from L-arginine but also an uncharacterized factor intrinsic to the SPI and RPI.

Acknowledgements

This research was supported by Grant-in-Aid for Scientific Research (B) (2) (11460060 and 13460059).

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