British Journal of Nutrition (2023), 129, 737–744 doi:10.1017/S0007114522001325
© The Author(s), 2022. Published by Cambridge University Press on behalf of The Nutrition Society

The effects of dietary linoleic acid on reducing serum cholesterol and atherosclerosis development are nullified by a high-cholesterol diet in male and female apoE-deficient mice

Xingyu Yuan, Rika Nagamine, Yasutake Tanaka, Wei-Ting Tsai, Zhe Jiang, Ai Takeyama, Katsumi Imaizumi and Masao Sato*

Laboratory of Nutrition Chemistry, Department of Bioscience and Biotechnology, Faculty of Agriculture, Graduate School, Kyushu University, 744 Motooka, Nishi-ku, Fukuoka 819-0395, Japan

(Submitted 4 February 2022 – Final revision received 23 March 2022 – Accepted 13 April 2022 – First published online 16 May 2022)

Abstract

Linoleic acid (LA) has a two-sided effect with regard to serum cholesterol-lowering and pro-inflammation, although whether this fatty acid reduces serum cholesterol and the development of atherosclerosis under high-cholesterol conditions has yet to be ascertained. In this study, we examine the effects of dietary LA on reducing serum cholesterol and atherosclerosis development under high-cholesterol conditions. Male and female apoE-deficient (ApoE$^{-/-}$) mice were fed AIN-76-based diets containing 10% SFA and 0·04 % cholesterol, 10% LA and 0·04% low cholesterol (LALC), or 10% LA and 0·1% high cholesterol (LAHC) for 9 weeks. The results revealed significant reduction in serum cholesterol levels and aortic lesions with increasing levels of pro-inflammatory biomarkers (urinary isoprostane and aortic *MCP-1* mRNA) in male and female LALC groups compared with those in the SFA groups ($P < 0·05$). Furthermore, whereas there were significant increases in the serum cholesterol levels and aortic lesions ($P < 0·05$), there was no difference in aortic *MCP-1* mRNA levels in male and female LAHC groups compared with those in the LALC groups. A high-dietary intake of cholesterol eliminated the serum cholesterol-lowering activity of LA but had no significant effect on aortic inflammation in either male or female ApoE$^{-/-}$ mice. The inhibitory effect of LA on arteriosclerosis is cancelled by a high-cholesterol diet due to a direct increase in serum cholesterol levels. Accordingly, serum cholesterol levels might represent a more prominent pathogenic factor than aortic inflammation in promoting the development of atherosclerosis.

Key words: Linoleic acid: ApoE-deficient mice: Atherosclerosis: Cholesterol: Pro-inflammation

Atherosclerosis and related complications are among the disorders contributing to the highest rates of human mortality and have become a substantial threat to human health[1]. The size of aortic lesions, accompanied by elevated levels of serum cholesterol and aortic inflammation, has been found to be a key variable in atherosclerosis[2]. Numerous clinical studies have shown that reducing serum cholesterol and aortic endothelial inflammation are salient factors in the prevention and treatment of this disease[3].

Epidemiological studies have indicated that compared with an intake of SFA, *n*-6 PUFA intake is associated with a lower risk of CVD[4]. Among the latter, linoleic acid (LA) (C18:2, *n*-6) is considered a potent dietary fatty acid that reduces plasma total cholesterol levels in humans[5]. Research has also indicated that diets extremely high in LA (16 % to 29 % of dietary fat) contribute to a significant lowering of plasma low-density lipoprotein cholesterol levels in humans by between 16 % and 22 % compared with diets high in SFA (19 % to 30 % of dietary fat) and low in LA (only 2·3 % to 4·5 % of dietary fat)[5]. In animal studies, the structured fat containing LA has been found to show a protective effect against atherosclerosis in C57BL/6J mice when fed high LA diets for 15 weeks[6]. In contrast, the findings of several studies have shown that an increased intake of LA leads to elevated levels of inflammation, which may be associated with endothelial dysfunction, given that LA is a precursor of arachidonic acid (AA) and inflammatory eicosanoids[7]. The conversion of LA to AA in healthy humans is about 1 %[8]. An excess LA intake could promote the conversion to produce a non-negligible amount of AA and sequentially intermediates of the pro-inflammatory cascade[8]. The inflammatory impact of the LA intake and conversion has been observed in experimental studies using the rat as the model[9]. *In vivo*, the pro-inflammatory

Abbreviations: AA, arachidonic acid; LA, Linoleic acid; MCP-1, Monocyte chemoattractant protein-1; PLSD, protected least significant difference; CRP, C-reactive protein; iNOS, Inducible nitric oxide synthase.

* **Corresponding author:** Masao Sato, email masaos@agr.kyushu-u.ac.jp

British Journal of Nutrition

https://doi.org/10.1017/S0007114522001325 Published online by Cambridge University Press

CrossMark

marker isoprostane is formed primarily via the non-enzymatic free radical-catalysed oxidation of AA. Turpeinen *et al.* have shown that in healthy subjects, urinary 8-iso-prostaglandin F2α (8-iso-PGF2α), a reliable biomarker of inflammation, increases with consumption of an LA-rich diet[10]. Collectively, the findings of the aforementioned studies indicate that an increased intake of LA leads to increased inflammation and oxidative stress, which may be associated with endothelial dysfunction and promotes the development of atherosclerosis in animal models.

LA is in ambivalent status on the serum cholesterol-lowering and aortic pro-inflammation. The findings of a previous study by Sato *et al.* have revealed that LA-rich safflower oil reduces the development atherosclerosis beyond its oxidative and inflammatory stress-increasing effects in apoE-deficient mice[11]. The essence of this dietary LA activity is assumed to be associated with its effect on lowering serum cholesterol levels. Consequently, dietary LA may promote arteriosclerosis when serum cholesterol levels are comparable to those of a SFA diet by controlling the cholesterol content of the LA diet. In the present study, we used apoE-deficient (ApoE$^{-/-}$) mice fed a high-cholesterol diet as an atherogenic animal model to investigate whether LA can induce the development of aortic atherosclerosis in response to the provision of a high-cholesterol LA diet.

Materials and methods

Materials

Trimethylsilylation reagent solution, a mixture of *N, O*-bis (trimethylsilyl) trifluoroacetamide and trimethylchlorosilane (99:1), was purchased from Merck-Supelco. Commercial enzyme assay kits (Cholesterol E, Free Cholesterol E and Triglyceride E test kits) were purchased from Fujifilm Wako Pure Chemical Corporation. 15-F2t-IsoPM [$^{18}O_2$], as a stable isotope labelled 15-F2t-IsoPM which is used for analysing the content of 15-F2t-IsoPM in urine by GC-MS, was kindly donated by Dr J.D. Morrow (Department of Medicine and Pharmacology, Vanderbilt University, Nashville, USA).

Animals and diets

ApoE$^{-/-}$ mice were purchased from the Jackson Laboratory. A total of 30 male and female ApoE$^{-/-}$ mice (18 males and 12 females, 7 weeks old) with initial weights of 20·5 and 19·1 g, respectively, were raised in the Animal Laboratory Building of the Department of Bioresources and Bioenvironmental Science, Faculty of Agriculture, Kyushu University. The mice were individually housed in metal cages and maintained in a temperature-controlled room (22–25°C) under a 12-h light cycle (dark period 20.00–08.00). Prior to the experimental period, the mice were given free access to a commercial non-purified diet (NMF; Oriental Yeast Co.) and deionised water for 1 week. Following this preliminary phase, the thirty male and female ApoE$^{-/-}$ mice were randomly divided into three groups (each containing six males and four females). These mice were fed one of the following three AIN-76 diets containing different fat and cholesterol contents: SFA-rich fat (50 g palm oil and 50 g lard/kg) and low cholesterol content (0·4 g/kg) (the SFA group); LA fat (100 g high-linoleic safflower seed oil/kg) and low cholesterol content (0·4 g/kg) (the LALC group); and LA fat (100 g high-linoleic safflower seed oil/kg) and high cholesterol content (1 g/kg) (the LAHC group). The overall and fatty acids compositions of the three experimental diets are listed in Tables 1 and 2, respectively. In the feeding experiment, the male and female mice were fed an experimental diet for 9 weeks, after which the animals were then sacrificed by withdrawal of aortic blood under pentobarbitone anesthesia (0·08 mg/100 g body weight), followed by immediate excision of the liver and the aorta for use in subsequent experiments. To isolate serum, blood was collected, incubated at room temperature for 30 min and centrifuged at 1750 × *g* for 10 min at 4°C. The handling and euthanasia of all animals were carried out in accordance with nationally prescribed guidelines, and all experiments were conducted following ethical approval from the Animal Care and Use Committee of Kyushu University (authorisation number: A02–005–01), as well as in accordance with law no. 105 and notification no. 6 of the Government of Japan.

Table 1. Compositions of the experimental diets

Ingredients (g/kg diet)	SFA	LALC	LAHC
Sucrose	449·6	449·6	449·0
Casein	200	200	200
Maize starch	150	150	150
Palm oil	50	–	–
Lard	50	–	–
High-linoleic-acid safflower oil	–	100	100
Cellulose	50	50	50
Mineral mix (AIN-76)	35	35	35
Vitamin mix (AIN-76)	10	10	10
DL-Methionine	3	3	3
Choline bitartrate	2	2	2
Cholesterol	0·4	0·4	1·0
Total	1000	1000	1000

LALC, LA fat and low cholesterol content; LAHC, LA fat and high cholesterol content.

Table 2. Fatty acid compositions of the experimental diets

	SFA	LALC	LAHC
		(%)	
12:0	0·1	–	–
14:0	1·2	–	–
16:0	33·0	7·2	7·2
16:1	2·6	0·2	0·2
18:0	9·7	2·6	2·6
18:1	40·9	16·1	16·1
18:2 (*n*-6)	11·4	71·6	71·6
18:3 (*n*-3)	–	0·2	0·2
20:5	–	–	–
22:6	–	–	–
Others	1·1	2·1	2·1
SFA	44·0	9·8	9·8
n-6 PUFA	11·6	71·6	71·6

LALC, LA fat and low cholesterol content; LAHC, LA fat and high cholesterol content.

Morphometric determination of atherosclerosis

ApoE$^{-/-}$ mice were perfused with 50 ml of phosphate-buffered saline (pH 7·4) via a cannula inserted into the left ventricle,

https://doi.org/10.1017/S0007114522001325 Published online by Cambridge University Press
British Journal of Nutrition

and following dissection of the aorta and its main branches from the aortic valve to the iliac bifurcation, perfusion of the heart was immediately continued with 50 mL of 10 % (v/v) neutral formalin buffer solution (pH 7·4). The heart was removed and fixed in 10 % (v/v) neutral formalin buffered solution and stored at 4°C for approximately 1 week. To determine the size and cross-sectional area of aortic lesions, hearts containing aortic roots were processed for quantitative atherosclerosis assays as previously described by Tomoyori *et al.*[12]

Analysis of serum and liver parameters

The levels of total cholesterol in serum were measured using commercial enzyme assay kits (Cholesterol E test kit), and to determine HDL cholesterol levels, HDL fractions were collected as described previously by Tomoyori *et al.*[12] and then measured using the Cholesterol E test kit. Total lipids from the liver were extracted using the method described by Folch *et al.*[13], and total liver and free cholesterol levels were measured using a method previously described by Tomoyori *et al.*[12]. Liver cholesterol ester levels were calculated as the difference between total and free cholesterol levels. Liver triacylglycerol levels were measured using a Triglyceride E test kit, liver phospholipids were chemically determined as described previously by Tomoyori *et al.*[12] and the fatty acid composition of hepatic phospholipids was determined according to the methods described by Carvajal *et al.*[14].

Determination of aortic Monocyte Chemoattractant Protein-1 (MCP-1) mRNA levels

Total RNA was isolated from the aorta using the phenol/chloroform method, as described previously by Yuan *et al.*[15]. Complementary DNA was synthesised from 1·0 μg of total RNA using a Transcriptor First Strand cDNA Synthesis Kit (Roche). Gene expression levels were analysed based on quantitative real-time reverse transcription PCR using a SYBR® Premix EX Taq II Kit (Takara) and an Mx3000P QPCR Thermal Cycler System (Agilent). mRNA levels were normalised using the *β*-actin (*Actb*) gene as an internal standard. The sequences of primers used for analyses are showed as follows: *MCP-1* (Forward primer: CACCAGCAAGATGATCCCAATG and Reverse primer: AAGGCATCACAGTCCCAGTCACAC); and *Actb* (Forward primer GGATCCCAGATCATGTTTGAGACCTTCAA and Reverse primer: GAATTCGGAGAGCATAGCCCTCGTAGATGG).

Determination of urinary isoprostaglandins

Purification and measurement of urinary 8-iso-PGF2a and 2,3-dinor-5,6-dihydro-8-iso-prostaglandin F2a (dinor) were carried out using a Shimadzu QP2020A GC/MS system (Shimadzu) as previously described by Sato *et al.*[11].

Statistical analysis

All data are presented as the means and standard error of the mean (SEM) unless otherwise indicated. Male and female groups were compared using one-way ANOVA with Fisher's protected least significant difference test (PLSD) test to identify individual differences. Analyses were performed using GraphPad 8.3 with significance set at $P < 0{\cdot}05$, and $0{\cdot}05 < P < 0{\cdot}1$ being considered indicative of a trend. In subsequent analyses, we did not distinguish between the sexes, and analysis of the correlation between related indicators was performed using a correlation set at $P < 0{\cdot}05$.

Results

Growth parameters and relative organ weights

As shown in Table 3, there were no significant differences in dietary intake, feed efficiency, liver weight or aorta weight between males and females among the three groups. In male mice, the final body weight was significantly higher in those in the LALC group than in the LAHC group, and body weight gain tended to decrease in the LAHC group ($P = 0{\cdot}052$) compared with that in the LALC group. Contrastingly, there are no significant differences in body weight gain or final body weight among female mice in the three experimental groups.

Lesion size and cross sections in the aortic root

As shown in Fig. 1, in male mice, the size and cross sections of aortic root lesions were significantly reduced in the LALC group compared with the SFA group and were significantly increased in the LAHC group compared with the LALC group. However, in female mice, there were no significant differences among

Table 3. Growth parameters assessed in the study
(Mean values with their standard errors of the mean, *n* 4 or 6)

	Male						Female					
	SFA		LALC		LAHC		SFA		LALC		LAHC	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Body weight (g)												
Initial	20·3	1·9	21·5	0·5	20·7	1·1	18·8	0·8	19·3	1·2	19·0	0·2
Final	32·4	1·7[ab]	34·6	1·6[a]	29·6	1·2[b]	27·3	0·5	28·7	1·7	25·8	0·6
Gain	12·1	1·7	13·2	1·4	8·9	1·1	8·6	1·0	9·5	1·0	6·8	0·8
Daily food intake (g/day)	4·5	0·1	4·5	0·2	4·2	0·1	5·0	0·2	4·6	0·1	4·5	0·3
Feed efficiency (%)	42·5	5·3	46·0	4·0	33·7	4·6	26·9	2·5	32·5	3·1	24·7	4·5
Liver weight (g)	1·65	0·19	1·45	0·11	1·49	0·12	1·16	0·14	1·25	0·07	1·29	0·15
Relative liver weight (g/100 g b.w*.)	5·07	0·46	4·20	0·32	4·98	0·23	4·26	0·52	4·39	0·34	4·99	0·50
Aorta (mg)	12	1	17	2	13	2	19	3	16	5	14	3

* b.w., body weight; LALC, LA fat and low cholesterol content; LAHC, LA fat and high cholesterol content.
Different superscript letters indicate a significant difference at $P < 0{\cdot}05$, as determined using Fisher's PSLD method.

British Journal of Nutrition

https://doi.org/10.1017/S0007114522001325 Published online by Cambridge University Press

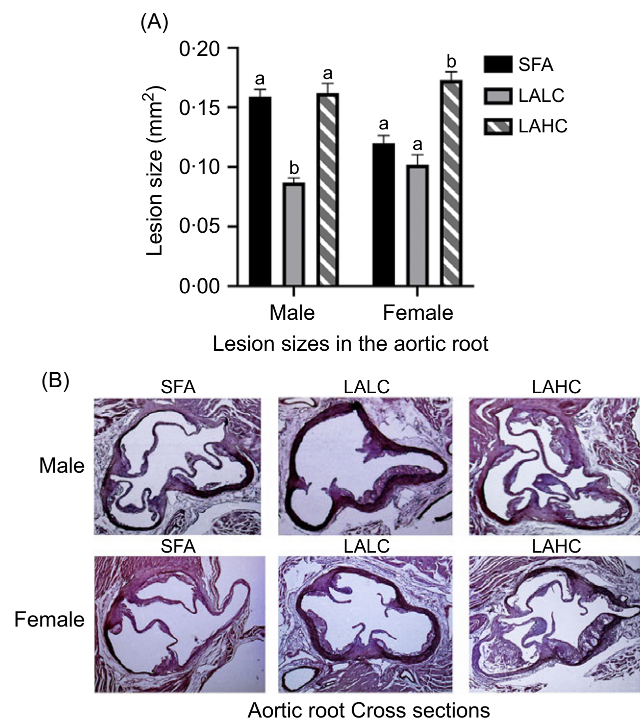

Fig. 1. Lesion sizes in the aortic root (a) and aortic root cross sections (b) from male and female ApoE-/- mice. Values are the means ± SEM (*n* 4 or 6). Different letters above bars indicate a significant difference at $P < 0{\cdot}05$, as determined using Fisher's PSLD method. LALC: LA fat and low cholesterol content; LAHC: LA fat and high cholesterol content.

females with respect to lesion size and cross section between SFA and LALC groups, although these parameters were significantly increased in the LAHC females compared with those in the LALC group.

Serum, hepatic and urinary parameters

As shown in Table 4, in male mice, serum and hepatic total cholesterol levels were significantly lower in the LALC group than in the SFA group and significantly higher in the LAHC group than in the LALC group. Hepatic cholesterol ester levels were significantly lower in the LAHC group than in the LALC group. Analogous results were obtained for the female groups with respect to serum and hepatic total cholesterol levels. Urinary 8-iso-PGF2α levels in the female LALC group were found to be significantly higher than those in the LAHC group. There was no significant difference among the three male groups with respect to urinary 8-iso-PGF2α levels. Conversely, in male mice, urinary dinor levels were significantly higher in the LALC group than those in the LAHC group, whereas no significant difference in urinary dinor levels were detected among the three female groups.

Aortic MCP-1 mRNA level

As shown in Fig. 2, in male mice, the levels of aortic *MCP-1* mRNA were significantly higher in the LALC group than in the SFA group, whereas no significant differences between the male LALC and LAHC groups were observed. In female mice, consumption of the different experimental diets had no effects on *MCP-1* mRNA levels.

Fatty acid composition of liver phospholipids

As shown in Table 5, both in male and female mice, the levels of AA (C20:4 *n*-6) were significantly higher in the LALC and LAHC groups than in the SFA group, and a similar pattern was observed regarding *n*-6 PUFA. We also found that the 20:4/18:2 ratio in the male LAHC group was significantly lower than that in the LALC group, whereas no significant difference in this ratio was detected between the female LALC and LAHC group.

Discussion

In this study, we established that compared with SFA, dietary LA intake was associated with a significant prevention of atherosclerosis development in male and female ApoE-/- mice only in the condition of the lower content (0·04 % in the diet) of dietary cholesterol. However, this positive effect of LA on atherosclerosis development was found to be negated by consumption of a diet containing a high content of cholesterol. Imaizumi reviewed the relationship between dietary components and atherosclerosis in ApoE-/- mice[16]. The analysis of fifty studies were examined that the relationship between serum cholesterol levels and aortic lesion sizes in ApoE-/- mice fed fifty different kinds of dietary components and a correlation coefficient (*r*) of 0·43 ($P < 0{\cdot}01$) was obtained for the association between serum cholesterol levels and aortic lesion sizes[16]. Notably, compared with other effective dietary supplements, supplementation with LA was found to contribute to the lowest level of serum cholesterol, although gave rise to the largest aortic lesions. We consider that, as a precursor of AA and inflammatory eicosanoids, an increase of dietary LA content leads to an increase in aortic inflammation and further promotes the development of atherosclerosis. Saini and Keum have reported that the *n*-3 and *n*-6 PUFA ratio in a diet strongly affects eicosanoid production, and a reduced consumption of *n*-3 fatty acids contributes to an enhanced synthesis of inflammatory eicosanoids from AA[17]. In the present study, the fatty acid contents of the LALC and LAHC experimental diets comprised 76% *n*-6 PUFA, which would further promote an inflammatory response. Accordingly, serum cholesterol levels and aortic root inflammation are considered to be the most important pathogenic factors contributing to the development of atherosclerosis in ApoE-/- mice.

In the present study, we observed that differences in the levels of serum cholesterol are associated with differences in the size of aortic lesions in both male and female ApoE-/- mice. Specifically, we detected significant increases in serum cholesterol levels and aortic root lesion sizes in male and female mice fed the LAHC diet compared with those fed the LALC diet (Fig. 1, Table 4). Such elevated serum cholesterol levels are considered a high-risk pathogenic factor for atherosclerosis[18]. Animal model studies have shown that a high-dietary cholesterol intake increases total serum cholesterol levels in ApoE-/- mice and ExHC rats[19,20]. Additionally, compositions of dietary fatty acids are also an important factor for serum cholesterol levels.

https://doi.org/10.1017/S0007114522001325 Published online by Cambridge University Press

https://doi.org/10.1017/S0007114522001325 Published online by Cambridge University Press

Table 4. Serum cholesterol, liver lipid and urinary isoprostane levels
(Mean values with their standard errors of the mean, *n* 4 or 6)

	Male						Female					
	SFA		LALC		LAHC		SFA		LALC		LAHC	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Serum	(mg/dl)											
Total cholesterol	799·0	27·3ᵃ	640·7	41·1ᵇ	790·4	57·7ᵃ	575·0	38·3ᵃᵇ	429·4	50·1ᵃ	667·5	55·1ᵇ
HDL-cholesterol	47·5	12·2	38·6	9·0	46·1	7·9	20·7	6·5	32·7	7·0	39·6	13·8
Liver	(mg/g liver)											
Total cholesterol	10·12	1·15ᵃ	5·59	0·43ᵇ	9·60	0·59ᵃ	8·04	0·33ᵃ	5·14	0·50ᵇ	10·38	1·10ᶜ
Free cholesterol	2·58	0·2	2·51	0·09	2·74	0·30	3·13	0·25ᵃ	1·95	0·07ᵇ	2·87	0·19ᵃ
Cholesterol ester	7·54	1·05ᵃ	3·08	0·40ᵇ	6·88	0·43ᵃ	4·91	0·31ᵃᵇ	3·19	0·57ᵃ	7·51	1·16ᵇ
Triacylglycerol	85·5	16·8	86·9	11·2	81·5	17·4	76·8	14·7	70·5	8·3	73·1	17·1
Phospholipid	27·2	2·0	30·1	0·6	30·4	1·1	29·4	0·5ᵃ	32·5	0·6ᵇ	33·0	1·3ᵇ
Urinary	(ng/mg creatinine)											
8-iso-PGF2α*	0·30	0·03	0·39	0·09	0·39	0·09	0·71	0·16ᵃᵇ	0·84	0·04ᵃ	0·54	0·06ᵇ
dinor**	3·90	0·69ᵃ	12·86	2·38ᵇ	5·01	1·77ᵃ	14·62	0·99	23·78	4·50	23·57	2·31

8-iso-PGF2α, * 8-iso-prostaglandin F2α; dinor, **2,3-dinor-5,6-dihydro-8-iso-prostaglandin F2α. LALC, LA fat and low cholesterol content; LAHC, LA fat and high cholesterol content. Different superscript letters indicate a significant difference at $P < 0·05$, as determined using Fisher's PSLD method.

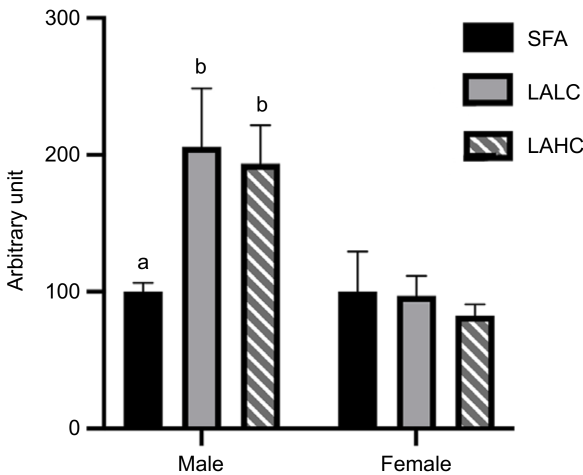

Fig. 2. Aortic *MCP-1* mRNA levels. Values are the means ± SEM (*n* 4 or 5). Different letters above bars indicate a significant difference at $P < 0·05$, as determined using Fisher's PSLD method. LALC: LA fat and low cholesterol content; LAHC: LA fat and high cholesterol content.

Generally, dietary fatty acids can both increase and decrease when dietary oils are changed. In this study, the change in oils, from palm oil and lard to high-LA safflower oil, increased LA in the diet, but at the same time it was accompanied by a decrease in SFA and oleic acid. Therefore, it is quite difficult to conclude the cause of the change in serum cholesterol levels to the change in the composition of a single fatty acid. Yu *et al.* established a multiple regression formula by meta-analysis of epidemiological studies on the effects of changes in dietary fatty acid composition on serum cholesterol levels[21]. It is not suitable to analyse the results of animal experiments with the equation established from human epidemiological studies. However, it is considered that the weighting of each fatty acids does not change significantly between species. Based on that, the effects of dietary fatty acid changes were estimated. In our research, the major changes in dietary concentrations of SFA (mainly palmitic acid (C16:0) and stearic acid (C18:0)) and MUFA (mainly oleic acid (C18:1)) caused the changes of serum cholesterol levels between SFA and LALC groups. However, considering the coefficient of ⊿PUFA in the formula of Yu is negative. LA levels were increased sevenfold in the LALC group compared with the SFA group. We conclude that the levels of LA increase also contributed to serum cholesterol decreased between SFA and LALC groups. At the same time, Keys *et al.* also reported that changes in serum cholesterol levels are affected by changes of dietary SUFA, PUFA and cholesterol levels[22]. The dietary SFA and PUFA levels are the same between LALC and LAHC groups. The serum cholesterol levels increased only due to the increase of dietary cholesterol between LALC and LAHC groups. We demonstrated that dietary cholesterol contributed directly to an increase in serum cholesterol levels and exacerbated atherosclerosis in mice. Moreover, aortic lesion size was found to be positively correlated with serum cholesterol levels (Supplemental Table). Cooper *et al.* found that conjugated LA had no effect on atherosclerosis but had adverse effects on lipoprotein and liver lipid metabolism in ApoE$^{-/-}$ mice fed a 1·25 % cholesterol diet[23]. We confirmed that dietary high-cholesterol intake had the effect of negating the serum cholesterol level-lowering effect of LA, thereby exacerbating the development of atherosclerosis.

A large number of studies have reported the increase of inflammatory biomarkers such as C-reactive protein (CRP), Tumor necrosis factor (TNF) and Inducible nitric oxide synthase (iNOS) as the development of atherosclerosis[24]. However, in the present study, we selected aortic *MCP-1* mRNA as a marker of aortic inflammatory response. *MCP-1,* the aortic levels of which are considered an index of the inflammatory response of the vascular wall, is transcriptionally regulated by *NF-kB*, a transcriptional regulator that responds to pro-inflammation[25]. Therefore, aortic *MCP-1* mRNA was considered to represent the inflammatory status in the vascular wall more accurately than other serum markers. Arterial inflammation is associated with atherosclerosis, and this association has been found to be sex-dependent[26]. In male mice, we found that male mice fed the LALC diet were characterised by elevated levels of aortic

Table 5. Fatty acid composition of hepatic phospholipids (Mean values with their standard errors of the mean, *n* 4 or 6)

	Male						Female					
	SFA		LALC		LAHC		SFA		LALC		LAHC	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
	(%)											
16:0	17·5	0·8	16·5	0·8	16·9	0·6	16·8	0·4[a]	14·8	1·0[ab]	13·5	0·8[b]
16:1	1·7	0·1[a]	0·8	0·0[b]	0·9	0·0[b]	1·2	0·1[a]	0·5	0·1[b]	0·5	0·0[b]
18:0	14·2	0·5[a]	15·8	0·3[b]	15·2	0·2[ab]	16·8	0·5	19·2	1·0	18·9	0·8
18:1(*n*-9)	14·0	0·2[a]	6·3	0·2[b]	6·7	0·2[b]	13·1	0·7[a]	5·5	0·2[b]	5·9	0·2[b]
18:1(*n*-7)	3·5	0·2[a]	1·7	0·1[b]	1·8	0·1[b]	2·9	0·2[a]	1·3	0·1[b]	1·4	0·1[b]
18:2(*n*-6)	10·0	0·4[a]	16·7	0·8[b]	19·8	0·9[c]	9·9	0·4[a]	15·4	0·4[b]	16·7	0·4[c]
20:3(*n*-6)	3·1	0·2[a]	2·2	0·1[b]	2·7	0·2[ab]	2·4	0·1[a]	1·5	0·2[b]	1·8	0·1[b]
20:4(*n*-6)	20·9	0·5[a]	26·0	0·7[b]	23·9	0·4[c]	21·7	0·4[a]	26·7	0·7[b]	26·2	0·4[b]
20:5(*n*-3)	0·2	0·1	0·3	0·1	0·2	0·1	0·1	0·1	0·1	0·1	0·2	0·1
22:4(*n*-6)	0·4	0·0[a]	0·8	0·1[b]	0·6	0·0[b]	0·2	0·1[a]	0·7	0·1[b]	0·7	0·0[b]
22:5(*n*-6)	2·6	0·5[a]	4·5	0·6[b]	2·8	0·5[a]	2·7	0·2[a]	3·8	0·3[b]	2·8	0·4[ab]
22:5(*n*-3)	0·3	0·1	0·2	0·1	0·2	0·1	0·2	0·1	0·2	0·1	0·3	0·0
22:6(*n*-3)	7·0	0·5[a]	6·2	0·4[b]	5·4	0·3[ab]	8·7	0·4	8·4	0·6	8·0	0·4
SFA	31·8	1·1	32·3	0·8	32·2	0·7	33·6	0·8	34·0	1·6	32·4	1·4
PUFA(*n*-6)	37·0	0·9[a]	50·1	0·6[b]	49·8	0·6[b]	37·0	0·1[a]	48·1	0·9[b]	48·2	0·8[b]
20:4/18:2 ratio	2·09	0·02[a]	1·53	0·05[b]	1·21	0·02[c]	2·19	0·03[a]	1·73	0·01[b]	1·60	0·03[b]

LALC, LA fat and low cholesterol content; LAHC, LA fat and high cholesterol content.
Different superscript letters indicate a significant difference at $P < 0·05$, as determined using Fisher's PSLD method.

MCP-1 mRNA, thereby indicating an aortic inflammatory response, whereas in contrast, no similar response was detected in male mice provisioned with the LAHC diet (Fig. 2). We confirmed that the higher levels of cholesterol in the LAHC diet appeared to have no significant effect on aortic inflammation in male ApoE$^{-/-}$ mice. It has been reported that endogenous AA or inflammatory eicosanoids are transported from the liver to the aorta by lipoproteins and albumin, thereby promoting the development of atherosclerosis[27]. Our observations indicated that LAHC diet not promoting inflammatory responses in the aortic root may conceivably be attributed to a reduction in liver-derived AA, and consequently, we further analysed the composition of hepatic fatty acids. The AA in hepatic phospholipids is derived from inflammatory eicosanoids and is naturally incorporated into structural phospholipids in the cell membrane[28]. In the present study, we analysed the fatty acid composition of hepatic phospholipids as a means of evaluating AA synthesis as a desaturation index, and accordingly found that consumption of the LAHC diet had the effect of significantly reducing the levels of hepatic AA and urinary dinor (an AA metabolite), and contributed to a reduction in the hepatic 20:4/18:2 ratio. LA is acted upon by desaturation enzymes (delta-6 fatty acid desaturase and delta-5 fatty acid desaturase) and is subsequently converted to AA in the liver[29]. Several studies have reported that high-cholesterol intake suppresses the action of these desaturases during the conversion of LA to AA[30]. We thus assume that the high cholesterol levels in LAHC diet inhibit the conversion of LA to AA in the livers of male mice. High level of cholesterol in this diet did not aggravate the aortic inflammatory response due to inhibition of AA synthesis in the liver, which further reduces AA transported to the aorta. In contrast to the male mice, we found that the different experimental diets had no significant effect on aortic *MCP-1* mRNA levels in female mice. Moreover, the arterial *MCP-1* mRNA levels in male mice were found to be significantly higher than those in female mice. These phenomena indicated that arterial inflammation in female mice was reduced compared with that in male mice. We believe that this reduction in arterial inflammation in females might be attributed to the action of oestrogen, which has been established to be a regulator of inflammation and exerts anti-inflammatory effects[31]. There are a few reports about using female animal models to evaluate atherosclerosis. 17β-estradiol treatment has been shown to suppress MCP-1 protein and gene expression in ovariectomised (OVX) rabbits[32], and oestrogen treatment has been found to attenuate vascular re-modelling by promoting the synthesis of *MCP-1* mRNA in endothelial cells[33]. Moreover, it has been demonstrated that oestrogen reduces *MCP-1* mRNA levels in female OVX apoE$^{-/-}$ mice, thereby preventing the development of atherosclerosis[34]. On the basis of the foregoing discussion, it is thus reasonable to assume that high-dietary cholesterol of LAHC diets does not aggravate the aortic inflammatory response in either male or female ApoE$^{-/-}$ mice, although there may be differences in the underlying mechanisms. Moreover, we established that the size of aortic lesions size shows no appreciable correlation with the levels of either aortic *MCP-1* mRNA or urinary isoprostane (Supplemental Table). We accordingly speculate that the increase in aortic lesion size and cross section are attributable to elevated levels of serum cholesterol, but not aortic inflammation. Consequently, serum cholesterol levels might be considered a more pathogenic factor than aortic inflammation in promoting atherosclerosis development.

At present, LA is still controversial in terms of cardiovascular prevention and pro-inflammation in humans. GH Johnson and K Fritsche have reported that no evidence was available from randomised, controlled intervention studies among healthy, non-infant human beings to show that addition of LA to the diet increases the concentration of inflammatory markers[35]. Unfortunately, no clinical study in which LA is the unique variable is available, which would allow to conclude on its pro-

inflammatory effects. There is also no research reported that high-dietary LA intake increases the concentration of inflammatory markers among cardiovascular patients. On the other hands, anti-effects of CVD by dietary LA were discovered with observation studies[36]. However, it has not been designed that dietary LA directly affects inflammatory status in human nutritional intervention studies. We expect that our study provide a research direction or theoretical basis for future clarification of the physiological role of LA in humans.

In conclusion, the findings of this study indicate that dietary intake of high cholesterol negates the serum cholesterol concentration-lowering effect of LA and has no significant effect on the inflammatory response on aortic root vessel walls in either male or female ApoE$^{-/-}$ mice. We believe that the inhibitory effect of LA on arteriosclerosis is cancelled by higher dietary cholesterol due to a direct increase in the level of serum cholesterol.

Acknowledgements

X.Y was supported by JST SPRING (grant number: JPMJSP2136). The authors also would like to thank Editage (www.editage.jp) for English language editing.

This research was supported in part by a Grant-in-Aid for Scientific Research (B) (Masao Sato, grant number: 11460060).

Xingyu Yuan: Investigation; Data curation; Formal analysis; Validation; Visualization; Writing—original draft; Writing—review& editing. Rika Nagamine: Data curation ; Investigation; Validation; Formal analysis. Yasutake Tanaka: Investigation; Methodology; Validation; Writing—review& editing. Wei-Ting Tsai: Writing—review& editing ; Validation. Zhe Jiang: Formal analysis; Validation. Ai Takeyama: Validation. Katsumi Imaizumi: Conceptualization; Methodology; Project administration; Resources. Masao Sato: Conceptualization; Funding acquisition; Methodology; Project administration; Resources; Supervision; Writing—review& editing

The authors declare that they have no conflicts of interest.

References

1. Neeland IJ, Ross R, Després JP, *et al.* (2019) Visceral and ectopic fat, atherosclerosis, and cardiometabolic disease: a position statement. *Lancet Diabetes Endocrinol* **7**, 715–725.
2. Markin AM, Sobenin IA, Grechko AV, *et al.* (2020) Cellular mechanisms of human atherogenesis: focus on chronification of inflammation and mitochondrial mutations. *Front Pharmacol* **11**, 642.
3. Jain S, Buttar HS, Chintameneni M, *et al.* (2018) Prevention of cardiovascular diseases with anti-inflammatory and antioxidant nutraceuticals and herbal products: an overview of pre-clinical and clinical studies. *Rec Pat Inflam All Drug Disc* **12**, 145–157.
4. Wang DD (2018) Dietary *n*-6 polyunsaturated fatty acids and cardiovascular disease: epidemiologic evidence. *Prost Leuk Ess Fatty Acids* **135**, 5–9.
5. Bowen KJ, Kris-Etherton PM, West SG, *et al.* (2019) Diets enriched with conventional or high-oleic acid canola oils lower atherogenic lipids and lipoproteins compared to a diet with a Western fatty acid profile in adults with central adiposity. *J Nutr* **149**, 471–478.
6. George J, Mulkins M, Casey S, *et al.* (2000) The effects of *n*-6 polyunsaturated fatty acid supplementation on the lipid composition and atherogenesis in mouse models of atherosclerosis. *Atherosclerosis* **150**, 285–293.
7. Lee HD, Meisel JA, de Meijer VE, *et al.* (2009) The essentiality of arachidonic acid and docosahexaenoic acid. *Prost Leuk Ess Fatty Acids* **81**, 165–170.
8. Choque B, Catheline D, Rioux V, *et al.* (2014) Linoleic acid: between doubts and certainties. *Biochimie* **96**, 14–21.
9. Marchix J, Choque B, Kouba M, *et al.* (2015) Excessive dietary linoleic acid induces proinflammatory markers in rats. *J Nutr Biochem* **26**, 1434–1441.
10. Turpeinen AM, Basu S & Mutanen M (1998) A high linoleic acid diet increases oxidative stress *in vivo* and affects nitric oxide metabolism in humans. *Prost Leuk Ess Fatty Acids* **59**, 229–233.
11. Sato M, Shibata K, Nomura R, *et al.* (2005) Linoleic acid-rich fats reduce atherosclerosis development beyond its oxidative and inflammatory stress-increasing effect in apolipoprotein E-deficient mice in comparison with saturated fatty acid-rich fats. *Br J Nutr* **94**, 896–901.
12. Tomoyori H, Kawata Y, Higuchi T, *et al.* (2004) Phytosterol oxidation products are absorbed in the intestinal lymphatics in rats but do not accelerate atherosclerosis in apolipoprotein E–deficient mice. *J Nutr* **134**, 1690–1696.
13. Folch J, Lees M & Stanley GHS (1957) A simple method for the isolation and purification of total lipids from animal tissues. *J Biol Chem* **226**, 497–509.
14. Carvajal O, Nakayama M, Kishi T, *et al.* (2000) Effect of medium-chain fatty acid positional distribution in dietary triacylglycerol on lymphatic lipid transport and chylomicron composition in rats. *Lipids* **35**, 1345–1352.
15. Yuan X, Minobe Y, Tanaka Y, *et al.* (2021) α-Globulin-rich rice cultivar, low glutelin content-1 (LGC-1), decreases serum cholesterol concentration in exogenously hypercholesterolemic rats. *J Sci Food Agric* **101**, 6417–6423.
16. Imaizumi K (2011) Diet and atherosclerosis in apolipoprotein E-deficient mice. *Biosci Biotechnol, Biochem* **75**, 1023–1035.
17. Saini RK & Keum YS (2018) *n*-3 and *n*-6 polyunsaturated fatty acids: dietary sources, metabolism, and significance – a review. *Life Sci* **203**, 255–267.
18. Douglas G & Channon KM (2014) The pathogenesis of atherosclerosis. *Medicine* **42**, 480–484.
19. Duivenvoorden I, Voshol PJ, Rensen PCN, *et al.* (2006) Dietary sphingolipids lower plasma cholesterol and triacylglycerol and prevent liver steatosis in APOE* 3 Leiden mice. *Am J Clin Nutr* **84**, 312–321.
20. Tanaka Y, Ono M, Miyago M, *et al.* (2020) Low utilization of glucose in the liver causes diet-induced hypercholesterolemia in exogenously hypercholesterolemic rats. *PLoS One* **15**, e0229669.
21. Yu S, Derr J, Etherton TD, *et al.* (1995) Plasma cholesterol-predictive equations demonstrate that stearic acid is neutral and monounsaturated fatty acids are hypocholesterolemic. *Am J Clin Nutr* **61**, 1129–1139.
22. Keys A & Parlin RW (1966) Serum cholesterol response to changes in dietary lipids. *Am J Clin Nutr* **19**, 175–181.
23. Cooper MH, Miller JR, Mitchell PL, *et al.* (2008) Conjugated linoleic acid isomers have no effect on atherosclerosis and adverse effects on lipoprotein and liver lipid metabolism in apoE-/- mice fed a high-cholesterol diet. *Atherosclerosis* **200**, 294–302.
24. Tuttolomondo A, Di Raimondo D, Pecoraro R, *et al.* (2012) Atherosclerosis as an inflammatory disease. *Curr Pharm Des* **18**, 4266–4288.
25. Panee J (2012) Monocyte Chemoattractant Protein 1 (MCP-1) in obesity and diabetes. *Cytokine* **60**, 1–12.

https://doi.org/10.1017/S0007114522001325 Published online by Cambridge University Press

26. Fiechter M, Haider A, Bengs S, *et al.* (2020) Sex-dependent association between inflammation, neural stress responses, and impaired myocardial function. *Eur J Nucl Med Mol Imag* **47**, 2010–2015.
27. Stein Y & Stein O (2003) Lipoprotein lipase and atherosclerosis. *Atherosclerosis* **170**, 1–9.
28. Hanna VS & Hafez EAA (2018) Synopsis of arachidonic acid metabolism: a review. *J Adv Res* **11**, 23–32.
29. Hastings N, Agaba M, Tocher DR, *et al.* (2001) A vertebrate fatty acid desaturase with Δ5 and Δ6 activities. *Proc Natl Acad Sci* **98**, 14304–14309.
30. Das UN (2018) Arachidonic acid in health and disease with focus on hypertension and diabetes mellitus: a review. *J Adv Res* **11**, 43–55.
31. Nilsson BO (2007) Modulation of the inflammatory response by estrogens with focus on the endothelium and its interactions with leukocytes. *Inflam Res* **56**, 269–273.
32. Pervin S, Singh R, Rosenfeld ME, *et al.* (1998) Estradiol suppresses MCP-1 expression *in vivo*: implications for atherosclerosis. *Art Thromb Vasc Biol* **18**, 1575–1582.
33. Nakagami F, Nakagami H, Osako MK, *et al.* (2010) Estrogen attenuates vascular remodeling in Lp (a) transgenic mice. *Atherosclerosis* **211**, 41–47.
34. Sato M, Sato H, Ogawa A, *et al.* (2007) Antiatherogenic effect of isoflavones in ovariectomized apolipoprotein E-deficient mice. *J Agri Food Chem* **55**, 8967–8971.
35. Johnson GH & Fritsche K (2012) Effect of dietary linoleic acid on markers of inflammation in healthy persons: a systematic review of randomized controlled trials. *J Acad Nutr Diet* **112**, 1029–1041.e15.
36. Ramkumar J & Sharma N (2017) Low dose aspirin and *n*-3 fatty acids in the pro resolving pathway of cardiovascular disorders. *Cardiol Angiol: Int* **6**, 1–12.

British Journal of Nutrition

https://doi.org/10.1017/S0007114522001325 Published online by Cambridge University Press