Endogenously Decreasing Tissue n-6/n-3 Fatty Acid Ratio Reduces Atherosclerotic Lesions in Apolipoprotein E–Deficient Mice by Inhibiting Systemic and Vascular Inflammation

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Objective—To use the fat-1 transgenic mouse model to determine the role of tissue n-6/n-3 fatty acid ratio in atherosclerotic plaque formation. Although it has been suggested that a low ratio of n-6/n-3 polyunsaturated fatty acids (PUFAs) is more desirable in reducing the risk of atherosclerotic cardiovascular disease, the role of tissue n-6/n-3 fatty acid ratio in atherosclerosis has not been sufficiently tested in a well-controlled experimental system. The fat-1 transgenic mouse model, expressing an n-3 fatty acid desaturase, is capable of producing n-3 PUFAs from n-6 PUFAs and thereby has a ratio of n-6/n-3 fatty acids close to 1:1 in tissues and organs.

Methods and Results—To generate apolipoprotein E–deficient plus fat-1 transgenic mice (apoE−/−/fat-1), we crossed heterozygous fat-1 mice with apoE−/− mice. After 14 weeks of a Western-type diet rich in n-6 PUFAs, the apoE−/−/fat-1 mice showed a lower ratio of n-6/n-3 fatty acids than the apoE−/− mice in all organs and tissues tested. The aortic lesion area in apoE−/−/fat-1 mice was significantly reduced when compared with that of apoE−/− littermates (7.14±0.54% versus 13.49±1.61%). There were no differences in plasma cholesterol or high- and low-density lipoprotein levels between the 2 groups, except for a higher triglyceride level in the apoE−/−/fat-1 mice. A significant reduction of interleukin 6 and prostaglandin E2 in both plasma and aorta culture medium was observed in apoE−/−/fat-1 mice. RT-PCR analysis also indicated that the expression of intercellular adhesion molecule-1, monocyte chemoattractant protein-1, interleukin 6, and cyclooxygenase-2 was lower in the aortas and the circulating monocytes from apoE−/−/fat-1 mice. In addition, the expression of nuclear factor κB/p65 in the aorta and the recruitment of macrophages into atherosclerotic plaques were reduced in apoE−/−/fat-1 mice, compared with apoE−/− mice.

Conclusion—To our knowledge, this is the first study to provide direct evidence for the role of tissue n-6/n-3 ratio in atherosclerosis using the fat-1 transgenic mouse model. Our findings demonstrate that a decreased n-6/n-3 fatty acid ratio reduces atherosclerotic lesions in apoE−/− mice. This protective effect may be attributed to the antiinflammatory properties of n-3 fatty acids, rather than their lipid-lowering effect. (Arterioscler Thromb Vasc Biol. 2010;30:2487-2494.)

Key Words: atherosclerosis ■ fat-1 transgenic mice ■ n-3 fatty acids ■ n-6/n-3 fatty acid ratio ■ inflammation

Acumulating epidemiological and experimental evidence has demonstrated that dietary intake of long-chain n-3 polyunsaturated fatty acids (PUFAs), particularly those derived from fish oil, reduces the risk of cardiovascular events, including atherosclerosis.1–5 The beneficial effects of n-3 PUFAs in atherosclerotic cardiovascular disease are associated with the reduction of endothelial adhesion molecules,3 the stabilization of atherosclerotic lesions,4 the modulation of oxidative stress in macrophages,6 and improvement in the function of endothelium7–7 and macrophages.8 However, several studies on animals and humans have shown somewhat contradictory results,9–11 possibly because they did not account for the tissue background level of n-6 PUFAs, a potential confounding factor.

PUFAs are important precursors of eicosanoids that serve as signaling molecules.12 The eicosanoids derived from n-6 and n-3 PUFAs are functionally distinct, and some even have opposing physiological functions.12,13 For example, n-6–derived eicosanoids, such as prostaglandin E2 (PGE2)14 and leukotriene B4,15 promote inflammation and exacerbate the development of atherosclerosis, whereas the metabolites from n-3 PUFAs, such as eicosapentaenoic acid (EPA)–derived resolvins and docosahexaenoic acid (DHA)–derived protectins, have antiinflammatory properties and protect against atherosclerosis.16–18 According to recent findings,19,20 the ratio of n-6/n-3 fatty acids in today’s Western diet ranges from 10:1 to 30:1, indicating that modern Western diets are
deficient in n-3 fatty acids compared with the diet on which humans evolved and on which their genetic patterns were established (n-6/n-3 ratio, approximately 1:1). The excessive n-6 fatty acids and high n-6/n-3 ratio result in an overproduction of n-6–derived eicosanoids,21,22 which may contribute to the pathogenesis of modern diseases, including cardiovascular diseases.20 Thus, lowering the tissue ratio of n-6/n-3 fatty acids could be a feasible approach to controlling the development of atherosclerosis. Emerging evidence indicates that the efficacy of n-3 PUFAs in protection against atherosclerosis depends not only on the amount of n-3 PUFAs but also on the background level of n-6 PUFAs.23 Recent findings20,24,25 suggest that a low ratio of n-6/n-3 fatty acids (close to 1:1) is desirable in reducing the risk of many chronic diseases that are highly prevalent in Western societies, including atherosclerotic cardiovascular disease and inflammatory diseases. However, the role of the n-6/n-3 fatty acid ratio in the development of atherosclerosis remains to be established in well-qualified in vivo models.

The transgenic fat-1 mouse model, generated by our laboratory, was engineered to express a fat-1 gene from Caenorhabditis elegans that is absent in most animals, including mammals.26,27 This gene encodes an n-3 fatty acid desaturase that catalyzes conversion of n-6 to n-3 fatty acids. When both fat-1 transgenic mice and wild-type littermates are fed an identical diet high in n-6 but deficient in n-3 fatty acids, the fat-1 transgenic mice show a high level of n-3 fatty acids and a lower ratio of n-6/n-3 fatty acids in their organs and tissues relative to those of the wild-type littermates.26,27 Thus, use of the fat-1 transgenic mouse allows us to create 2 different fatty acid profiles (high versus low n-6/n-3 ratio) with a single diet, thereby eliminating the confounding factors of using different diets in a comparative study. The fat-1 transgenic mouse is an ideal animal model for elucidating the role of the n-6/n-3 fatty acid ratio in atherosclerosis.

The apolipoprotein E–deficient (apoE−/−) mouse is a well-characterized model that spontaneously develops atherosclerotic lesions with a high consistency when fed a Western-type diet.28 In the present study, the apoE−/−/fat-1 mice were generated by crossing apoE−/− mice with fat-1 transgenic mice. The development of atherosclerosis and the status of systemic and vascular inflammation were compared between apoE−/−/fat-1 mice (low n-6/n-3 ratio) and apoE−/− mice (high n-6/n-3 ratio).

Methods

Animals and Diet

Fat-1 transgenic mice with a genetic background of C57BL/6 were backcrossed for more than 10 generations. The heterozygous fat-1 mice (fat-1+/−), which exhibit a significant phenotype, were crossed with apoE−/− mice (Jackson Laboratory, Bar Harbor, Me); and the offspring were further mated to obtain the compound apoE−/−/fat-1 mice and pure apoE−/− colonies. The apoE genotype and the fat-1 phenotype of each animal were characterized using isolated genomic DNA29 and analysis of total lipids26 from mouse tails, respectively, after weaning and again after euthanization. Specific pathogen-free animals were maintained under barrier conditions. At the age of 6 weeks, all animals were fed for 14 weeks with an identical Western-type diet (consisting of 21% fat and 1.25% cholesterol), which is rich in n-6 fatty acids and deficient in n-3 fatty acids (Research Diets Inc, New Brunswick, NJ). The body weight and food intake of the animals were recorded weekly.

Determination of n-6/n-3 Fatty Acid Ratio

After the 14-week feeding, mice were euthanized by isoflurane inhalation (Abbott Laboratories, Chicago, Ill). Blood was immediately collected by cardiac puncture from the right ventricle. Thereafter, plasma was separated and stored at −80°C, and the remaining blood cells were replenished by an equal volume of Hank’s solution. Then, the circulating monocytes were separated using Histopaque-1083 (Sigma-Aldrich, St Louis, Mo). The main organs, including brain, skeletal muscle, spleen, kidney, heart, liver, and lung, were also harvested. Fatty acid profiles of plasma, monocytes, and main organs were analyzed by using gas chromatography, as previously described.30 In brief, tissue samples were ground to powder under liquid nitrogen and subjected to fatty acid methylation by 14% boron trifluoride–methanol reagent at 100°C for 1 hour. Fatty acid methyl esters were analyzed by gas chromatography equipped with a flame ionization detector (model HP6890N; Agilent, Palo Alto, Calif). Peaks of fatty acids were identified by comparing the relative retention times with their commercial standards (Nu-Chek Prep, Elysian, Minn). The n-6/n-3 fatty acid ratio was given as follows: (18:2 n-6+20:2 n-6+20:3 n-6+20:4 n-6+22:4 n-6+22:5 n-6):(18:3 n-3+20:5 n-3+22:5 n-3+22:6 n-3).

Assessments of Atherosclerotic Lesions

The extent of atherosclerosis was assessed in the aorta using an en face method.31,32 Briefly, after perfusion with cold PBS (pH, 7.4), the entire aorta was rapidly dissected from the proximal ascending aorta to the iliac bifurcation under a dissecting microscope. The dissected aorta was placed in PBS, and fat and connective tissue adhering to the adventitia were removed as much as possible. The vessel was fixed with 4% paraformaldehyde overnight at 4°C and then opened longitudinally and pinned onto black wax plates using microneedles (Fine Science Tools, Foster City, Calif). Lipid-rich intraluminal lesions were stained with Sudan IV (Sigma-Aldrich). Images were recorded using a digital camera (Coolpix 990; Nikon Corp, Tokyo, Japan), and lesion areas were analyzed using computer software (Image-Pro Plus; Media Cybernetics, Silver Spring, Md).

As a second assessment of atherosclerosis, the cross-sectional lesions in the aortic sinus were analyzed, as previously described.33 Hearts with the aortic sinus were collected and embedded in optimal cutting temperature and then frozen at −80°C. The aortic sinus was sectioned serially (10-μm intervals), using a cryostat (model CM1900; Leica, Richmond Hill, ON), from the appearance of the aortic valve to the ascending aorta until the valve cusps were no longer visible. After staining by oil red O (Sigma-Aldrich), the atherosclerotic area in the aortic sinus was measured from the images recorded by a charge-coupled device camera (QImaging, Surrey, BC, Canada). The percentages of stenosis and intrusion were calculated as follows: lesion area/(lesion area + lumen area)×100 and lesion area/(lesion area + media area)×100, respectively.

Plasma Analysis

The analysis of plasma total cholesterol, triglyceride (TG), and high- and low-density lipoproteins was performed at the Core Laboratory at Massachusetts General Hospital, Boston. The levels of cytokines (tumor necrosis factor [TNF] α and interleukin [IL] 6) and the inflammatory lipid mediator (PGElα) in the plasma were determined using commercial ELISA kits (BD Biosciences, San Jose, Calif) and enzyme immunoassay kits (Cayman, Ann Arbor, Mich), respectively, following their manufacturer protocols.

Ex Vivo Aorta Organ Culture

The aortic arch segment of apoE−/−/fat-1 and apoE−/− mice was dissected and washed with PBS several times. Thereafter, aorta samples were cut into 2-mm rings and placed into 1 mL of serum-free medium (Ultraculture; Lonza, Walkersville, Md). Tissue
was incubated for 24 hours at 37°C in a humidified atmosphere containing 5% CO₂. The culture supernatant was then removed and frozen at −80°C before analysis. The genomic DNA of the remaining aorta was isolated using a DNA miniprep kit (Sigma-Aldrich) and quantified by UV spectrophotometer. The levels of TNF-α, IL-6, and PGE₂ in the culture medium were assayed as described in the “Methods” section. Their values were normalized to genomic DNA content.

### Immunofluorescent Staining

Cryostat sections of the aortic sinus were fixed in 4% paraformaldehyde for 15 minutes and washed with PBS. After blocking the endogenous peroxidase with 5% goat serum in PBS (containing 0.3% Triton X-100) and washing with PBS, the sections were incubated with 1:100 rabbit anti–mouse nuclear factor (NF) κB/p65 (Cell Signaling, Danvers, Mass) and rabbit anti–mouse F4/80 (Cell Signaling, Danvers, Mass) antibodies overnight at 4°C and then exposed to 1:100 IgG (Alexa Fluor 488 conjugate anti–rabbit IgG; Cell Signaling) for 1 hour at room temperature in the dark. To quench the autofluorescence caused by the internal elastic lamina, the labeling sections were incubated with 0.5% pontamine sky blue (Sigma-Aldrich) in PBS for 10 minutes, followed by a 5-minute wash before mounting.

### Real-Time RT-PCR Analysis

Total RNA was isolated from both circulating monocytes and the entire aorta using reagent (Trizol; Invitrogen, Carlsbad, Calif). First-strand cDNA was synthesized from 1 μg of total RNA using a random primer and Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Promega, Madison, Wis). The sequences of PCR primers were obtained from the Primer bank (http://pga.mgh.harvard.edu/primerbank) and Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Promega, Madison, Wis). PCR reactions were performed in duplicate using a commercially available system (SYBR green PCR Master Mix in a StepOnePlus Real-Time PCR System; Applied Biosystems, Carlsbad, Calif) and normalized to 18S content.

### Statistical Analysis

Results were expressed as the mean±SEM. Data were analyzed by ANOVA and, subsequently, the unpaired t test for between-group comparisons. *P*<0.05 was considered statistically significant.

### Results

#### Food Intake, Body Weight, and Plasma Lipids

From the age of 6 weeks, all animals received a specially formulated Western-type diet for 14 weeks. There were no significant differences in food intake and body weight between the apoE⁻⁻/fat⁻⁻ and the apoE⁻⁻ mice throughout the feeding period. The plasma TG level in apoE⁻⁻/fat⁻⁻ mice was significantly higher than that in apoE⁻⁻ mice (123.3±21.1 versus 81.8±3.8 mg/dL), but no statistical differences for plasma cholesterol and high- and low-density lipoprotein levels were observed between the 2 groups (supplemental Table II).

#### Fatty Acid Profiles

Both apoE⁻⁻/fat⁻⁻ and apoE⁻⁻ littersmates born to the same mother were maintained on a specially designed Western-type diet rich in n-6 but deficient in n-3 fatty acids. Because the *fat⁻⁻* gene encodes an n-3 fatty acid desaturase enzyme that converts n-6 to n-3 fatty acids, the apoE⁻⁻/fat⁻⁻ mice had significantly higher amounts of long-chain n-3 PUFAs, such as EPA, DHA, and docosapentaenoic acid (DPA); and
much lower amounts of n-6 PUFAs, such as arachidonic acid, n-6 DPA, and docosatetraenoic acid, compared with apoE−/− mice (Figure 1A and Table). Also, we observed that the plasma, circulating monocytes, and aorta from the apoE−/−fat-1 mice exhibited higher total n-3 fatty acids but lower total n-6 fatty acids without a change in the total amount of PUFAs, saturated fatty acids, and monounsaturated fatty acids (P > 0.05) (Table). This enrichment of n-3 fatty acids at the expense of n-6 in apoE−/−fat-1 mice resulted in a significantly lower ratio of n-6/n-3 fatty acids and arachidonic acid/(EPA + DPA + DHA) in all organs and tissues tested (Figure 1B and C). In particular, the ratios of arachidonic acid/(EPA + DPA + DHA) in the plasma, aorta, and circulating monocytes were 13.6, 10.31, and 18.0, respectively, in apoE−/− mice; whereas the respective ratios were 1.5, 2.2, and 1.1 in apoE−/−fat-1 mice, showing that although both groups were fed the same diet, their body fatty acid profiles were quite different.

Atherosclerotic Lesions
At the end of feeding, apoE−/− mice developed severe atherosclerotic lesions, as measured by the en face method. Sudan IV stains of the entire aortas from the mice indicated that the total lesion areas of prominent lipid-rich atheromas were dramatically reduced in apoE−/−fat-1 mice (Figure 2A). Quantitative analysis revealed that the percentage of Sudan IV–stained area of the entire aorta in apoE−/−fat-1 mice was significantly lower than that in apoE−/− mice, by 46.7% (Figure 2B). The atherosclerotic plaques in the segments of the aortic arch and the thoracic aorta were also significantly less in apoE−/−fat-1 mice (17.47 ± 2.08% and 0.97 ± 0.17%, respectively), compared with apoE−/− mice (30.74 ± 2.77% and 4.34 ± 0.96%, respectively). However, there was no significant difference in the abdominal aorta between the 2 groups (Figure 2C).

The crosssectional histological features of the aortic sinus from apoE−/− and apoE−/−fat-1 mice stained with oil red O (lipid-containing lesions) and subsequent hematoxylin (nucleus) are shown in supplemental Figure A and B. The largest lesions were found in the proximal aorta near the aortic valve. The atherosclerotic plaques of the aortic sinus were more prominent in apoE−/− mice than in apoE−/−fat-1 mice. Because of variation in the size of the aortas, the percentages of stenosis and intrusion were calculated to quantify the extent of atherosclerosis in the aortic sinus (supplemental Figure, C and D). The stenosis and intrusion in the aortic sinus were significantly reduced in apoE−/−fat-1 mice (30.34 ± 1.98% and 47.57 ± 2.58%, respectively), compared with apoE−/− littermates (39.67 ± 1.83% and 59.54 ± 1.52%, respectively).

Production of Cytokines and Inflammatory Lipid Mediators
To obtain insight into the underlying mechanism by which the decreased tissue ratio of n-6/n-3 fatty acids reduced the development of atherosclerotic lesions, the status of systemic
monocytes (Figure 3C) and atherosclerotic aorta (Figure 3D). The mRNA levels of the intercellular adhesion molecule-1 (ICAM-1), cytokines (TNF-α and IL-6), chemokine (monocyte chemoattractant protein-1 [MCP-1]), and cyclooxygenase-2 (COX-2), a key enzyme in the production of PGE₂, in the circulating monocytes were decreased significantly in apoE⁻/⁻/fat-1 mice; and vascular cell adhesion molecule-1 mRNA expression was also lower but not statistically different. In the atherosclerotic aorta, the mRNA expression of all genes tested was lower in apoE⁻/⁻/fat-1 mice compared with their apoE⁻/⁻ littermates, although the changes in vascular cell adhesion molecule-1 and TNF-α expression did not reach statistical significance.

**Nuclear Factor κB Expression and Macrophage Accumulation**

We subsequently examined the expression of NF-κB, a pivotal transcription factor that regulates the transcription of many inflammatory genes in atherosclerosis, such as cytokines, chemokines, and adhesion molecules, by using immunofluorescent staining. The expression of NF-κB/p65, the most abundant complex of the NF-κB family, was lower in the plaque of apoE⁻/⁻/fat-1 mice compared with their apoE⁻/⁻ counterparts (Figure 4A).

Increasing expression of chemokines and adhesion molecules results in the migration of monocytes to the site where the monocytes will differentiate into macrophages, causing lesions to develop. To examine macrophage accumulation in atherosclerosis, the specific monocyte/macrophage marker F4/80 in plaque was stained and evaluated. As shown in Figure 4B, a weaker F4/80 staining was observed in the plaque of apoE⁻/⁻/fat-1 mice, indicating a decreased accumulation of F4/80-positive macrophage in atherosclerotic lesions.

**Discussion**

We used the fat-1 transgenic model to investigate the role of tissue n-6/n-3 fatty acid ratio in the development of atherosclerosis. Our results demonstrate that a decreased tissue n-6/n-3 fatty acid ratio notably reduced the atherosclerotic lesions in the aorta of apoE⁻/⁻ mice fed a Western-type diet. This protection against atherosclerosis correlated with reduced expression of proinflammatory cytokines, chemokines, and adhesion molecules in aortas and circulating monocytes; and decreased NF-κB expression and recruitment of macrophages into atherosclerotic plaques. These findings suggest that a low tissue ratio of n-6/n-3 fatty acids is protective against atherosclerosis in apoE⁻/⁻ mice by reducing inflammation both systemically and within the arterial wall, independent of a lipid-lowering effect.

Although a number of previous studies have examined the preventive effect of dietary long-chain n-3 PUFAs against the formation of atherosclerotic lesions in apoE-deficient mice, the outcomes were inconsistent or conflicting. These studies focused on the preventive effect of n-3 PUFAs against atherosclerosis, whereas the potential effects of the background n-6 PUFA levels were not addressed. Furthermore, dietary supplementation, a traditional approach to modifying tissue nutrient composition, was used in these
studies. However, the inevitable differences between diets can act as confounding factors that may contribute to inconsistent results and make the results of these studies difficult to compare. The problems described could easily be resolved by using the fat-1 transgenic model, as presented in this study. This genetic approach to modifying fatty acid composition by endogenously converting n-6 to n-3 fatty acids not only effectively increases the absolute amounts of n-3 fatty acids but also significantly decreases the levels of n-6 fatty acids, leading to a low n-6/n-3 fatty acid ratio in body tissues without changing the tissue mass of total PUFAs (Table). More importantly, this animal model allows us to generate 2 different fatty acid profiles between 2 experimental groups while feeding them the same high n-6 diet. Thus, many variables that may arise from differing diets and feeding procedures, such as impurities, unwanted components of oils used, flavor, oxidation, diet freshness, and storage, can be avoided. As a well-controlled system, the fat-1 transgenic mouse was chosen to be the model of a low n-6/n-3 fatty acid ratio in the present study.

The apoE-deficient mice, generated in 1992, are perhaps the most popular model for atherosclerotic studies because of their propensity to spontaneously develop atherosclerotic lesions, highly characteristic in appearance and distribution of those observed in humans. ApoE acts as a ligand for low-density lipoprotein receptors, low-density lipoprotein receptor–related protein, and cell surface proteoglycans; thereby, it has an important role in the clearance of remnants of TG-rich lipoproteins. Genetic deficiency of apoE in murine results in major defects in TG and very-low-density lipoprotein clearance from blood circulation. Long-chain n-3 fatty acids or fish oils are well known to be efficient TG-lowering agents in both human and animal models and can reduce TG synthesis in apoE/H/H mice. However, in the present study, the heightened n-3 fatty acid tissue status significantly increased the plasma TG concentration in apoE/H/H mice; no differences in plasma cholesterol or high- and low-density lipoprotein levels were observed between the fat-1 and control groups. These observations in apoE/H/H mice are consistent with the results obtained in previous reports. These results suggest that the decreased TG synthesis caused by n-3 fatty acids is not sufficient in lowering the circulating TG concentrations and that apoE is necessary for n-3 fatty acids to reduce TG levels in mice. Although a lipid-lowering effect has been thought to be one of the major antiatherosclerotic mechanisms, it seems that the antiatherosclerotic effect of n-3 fatty acids observed in our study is independent of the lipid-lowering effect.

It has become well established that inflammatory events, both systemically and in the arterial wall, play a pivotal role in the development of atherosclerosis. Inflammation is known to play a central role in the initiation and progression of atherosclerosis, and it is now recognized that inflammation is a key contributor to the development of atherosclerotic lesions. Therefore, the identification of novel strategies to attenuate inflammation in atherosclerosis is critical for the development of new therapeutic approaches to prevent and treat cardiovascular disease.

![Figure 3](http://atvb.ahajournals.org/)

**Figure 3.** The production and expression of inflammation-related factors. A and B, The levels of cytokines (TNF-α and IL-6) and the inflammatory lipid mediator (PGE2) in the plasma (A, n=10) and ex vivo culture medium of aorta (B, n=4) were examined. The TNF-α level in the plasma of all animals was less than the limit of detection. The levels of TNF-α, IL-6, and PGE2 in culture medium were normalized to the genomic DNA content. C and D, The expression of inflammation-related genes in circulating monocytes (C) and entire aorta tissue (D) in apoE/H and apoE/H/fat-1 mice was measured using real-time RT-PCR. Data are given as the mean±SEM. *P<0.05 and **P<0.01 vs the apoE/H group.
sites is regulated by cell adhesion molecules, such as ICAM-1, IL-6, TNF-α. The cytokines produced by various cell types, including infiltrated macrophages, endothelial cells, and smooth muscle cells, are critically important in the progression of atherosclerosis. The present study clearly demonstrates that a decreased ratio of n-6/n-3 fatty acids has an inhibitory effect on inflammation in the local arterial wall, as evidenced by the reduced expression of inflammation-related genes, including ICAM-1, MCP-1, IL-6, and COX-2, less macrophage infiltration; and lower production of IL-6, TNF-α, and PGE2. Given the fact that NF-κB controls the transcription of many genes with an established role in atherosclerosis, such as cytokines, chemokines, adhesion molecules, and macrophage infiltration, the most plausible underlying mechanism for the anti-inflammatory effect of a low n-6/n-3 fatty acid ratio may be the inhibition of NF-κB expression, as demonstrated by an immunohistochemical assay. This result was in agreement with previous reports. Altogether, a decreased tissue n-6/n-3 fatty acid ratio appears to inhibit vascular inflammation via multiple mechanisms during atherosclerosis. The observation that the conversion of n-6 PUFAs to n-3 PUFAs leads to a reduction in vascular inflammation and plaque formation in apoE−/− mice supports the notion that the effects of n-6 and n-3 PUFAs in atherosclerosis are differential or opposing.

In conclusion, to our knowledge, this is the first study to provide direct evidence for the role of tissue n-6/n-3 ratio in atherosclerosis using the fat-1 transgenic mice model. The decreased n-6/n-3 fatty acid ratio reduced atherosclerotic lesions in apoE−/− mice, likely because of the anti-inflammatory effects of n-3 fatty acids rather than a lipid-lowering effect. Our findings provide new insights into the inflammatory effects of n-3 fatty acids rather than a lipid-lowering effect. Our findings provide new insights into the inflammatory effects of n-3 fatty acids rather than a lipid-lowering effect.

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Disclosures
None.

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Figure 4. A and B, Representative light and immunofluorescent staining of NF-κB/p65 (A) and macrophage marker F4/80 (B) in the frozen section of the proximal aorta isolated from apoE−/− and apoE−/−/fat-1 mice. To quench the autofluorescence caused by the internal elastic lamina, the labeling sections were incubated with 0.5% pontamine sky blue in PBS for 10 minutes, followed by a 5-minute wash before mounting (original magnification ×200).

during all phases of atherosclerosis, from fatty streak formation to plaque destabilization and, subsequently, atherothrombosis. The systemic inflammatory disorders are characterized by activation of leukocytes and increased concentrations of cytokines and other inflammatory lipid mediators. This may result in endothelial dysfunction and secondary dyslipidemia and, subsequently, accelerate the atherosclerotic process. Our data revealed that a decreased tissue ratio of n-6/n-3 fatty acids remarkably reduced both IL-6 and PGE2 levels in plasma and downregulated several inflammatory genes in circulating monocytes, including ICAM-1, IL-6, TNF-α, MCP-1, and COX-2, suggesting that a decreased n-6/n-3 fatty acid ratio can reduce systemic inflammation in the atherosclerotic process.

Circulating monocyte activation and transendothelial migration, with subsequent differentiation into macrophages, are important events in the initial pathogenesis of atherosclerosis. The recruitment of monocytes to lesion-prone arterial sites is regulated by cell adhesion molecules, such as ICAM-1 and vascular cell adhesion molecule-1, which are expressed on the surface of endothelial cells, and chemotactic proteins, including MCP-1. The cytokines produced by various cell types, including infiltrated macrophages, endothelial cells, and smooth muscle cells, are critically important in the progression of atherosclerosis. The present study clearly demonstrates that a decreased ratio of n-6/n-3 fatty acids has an inhibitory effect on inflammation in the local arterial wall, as evidenced by the reduced expression of inflammation-related genes, including ICAM-1, MCP-1, IL-6, and COX-2, less macrophage infiltration; and lower production of IL-6, TNF-α, and PGE2. Given the fact that NF-κB controls the transcription of many genes with an established role in atherosclerosis, such as cytokines, chemokines, adhesion molecules, and macrophage infiltration, the most plausible underlying mechanism for the anti-inflammatory effect of a low n-6/n-3 fatty acid ratio may be the inhibition of NF-κB expression, as demonstrated by an immunohistochemical assay. This result was in agreement with previous reports. Altogether, a decreased tissue n-6/n-3 fatty acid ratio appears to inhibit vascular inflammation via multiple mechanisms during atherosclerosis. The observation that the conversion of n-6 PUFAs to n-3 PUFAs leads to a reduction in vascular inflammation and plaque formation in apoE−/− mice supports the notion that the effects of n-6 and n-3 PUFAs in atherosclerosis are differential or opposing.

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Endogenously Decreasing Tissue n-6/n-3 Fatty Acid Ratio Reduces Atherosclerotic Lesions in Apolipoprotein E–Deficient Mice by Inhibiting Systemic and Vascular Inflammation

Jian-Bo Wan, Li-Li Huang, Rong Rong, Rui Tan, Jingdong Wang and Jing X. Kang

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### Supplement Materials

#### Table I. Primer sequences used in the study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequences</th>
<th>Sizes of products (bp)</th>
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<tbody>
<tr>
<td>18S</td>
<td>Forward: CTGCCGTCTGAGTGATATCGC</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>Reverse: GCTGGGGGCTGAGAAAGTG</td>
<td></td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Forward: GTGATGCTAGGTATCCATCCA</td>
<td>213</td>
</tr>
<tr>
<td></td>
<td>Reverse: CACAGTTCTCAAAAGCAGCG</td>
<td></td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Forward: AGTTGGGGATCCTGGTTTGCTCTC</td>
<td>112</td>
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<tr>
<td></td>
<td>Reverse: CCCCTCATCCTTAACCACCC</td>
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</tr>
<tr>
<td>MCP-1</td>
<td>Forward: TTTAAAACCTGGATCGGAACCAA</td>
<td>121</td>
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<tr>
<td></td>
<td>Reverse: GCATTAGCTTCAGATTACCGGT</td>
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<tr>
<td>TNF-α</td>
<td>Forward: CCCTCACACTCAGATCTTTCT</td>
<td>61</td>
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<tr>
<td></td>
<td>Reverse: GCTACGACGTGGGCTACAG</td>
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<td>IL-6</td>
<td>Forward: TAGTCTTTCTACCCCAATTTCC</td>
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<tr>
<td></td>
<td>Reverse: TTGGTCCTTACCCACTCTTTC</td>
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<tr>
<td>COX-2</td>
<td>Forward: TGAGCAACTATTCCAAACCAGC</td>
<td>74</td>
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<tr>
<td></td>
<td>Reverse: GCACGTAHTCCTCGATCATATC</td>
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#### Table II. Food intake, body weight gain and plasma lipid profiles in apoE−/− and apoE−/−/fat-1 mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>Food intake (g/animal/day)</th>
<th>Body weight gain (g)</th>
<th>Cholesterol (mg/dl)</th>
<th>Triglycerides (mg/dl)</th>
<th>HDL (mg/dl)</th>
<th>LDL (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>apoE−/−</td>
<td>5.45±0.08</td>
<td>14.2±1.1</td>
<td>613.2±120.6</td>
<td>81.8±3.8</td>
<td>84.0±7.0</td>
<td>510.2±112.6</td>
</tr>
<tr>
<td>apoE−/−/fat-1</td>
<td>5.58±0.03</td>
<td>15.8±1.2</td>
<td>681.0±150.1</td>
<td>123.3±21.1*</td>
<td>79.7±8.2</td>
<td>576.9±138.6</td>
</tr>
</tbody>
</table>
Supplemental Figure

Figure I. Representative cross sections of aortic sinus from apoE<sup>−/−</sup> (A) and apoE<sup>−/−</sup>/fat-1 (B) mice after oil red O staining. Data of atherosclerotic lesions was expressed as percentage of stenosis (C) and intrusion (D), which were calculated as lesion area/(lesion area + lumen area)×100 and lesion area/(lesion area + media area)×100, respectively. Values are mean±SEM (n=4-5), *P < 0.05 and **P < 0.01 vs apoE<sup>−/−</sup> group (Magnification, ×40).