Atherosclerosis is a multifactorial, long-lasting process in humans. Accordingly, animal models in which more rapid changes occur can be useful for the study of this process. Among such models are apolipoprotein E-deficient (apoE−/−) mice, which give insight into the human process. ApoE−/− mice show impaired clearing of plasma lipoproteins and develop atherosclerosis in a short time, and hence they are an excellent model in which to assess the impact of dietary factors. This review considers lipid metabolism and inflammation as well as nutritional constituents affecting atherosclerosis, with reference to apoE−/− mice, and discusses the mechanisms through which they act.

Key words: apolipoprotein E-deficient; diet; cholesterol; inflammation; atherosclerosis

Despite significant advances in treatment and in the understanding of its biology, coronary atherosclerosis remains the leading cause of morbidity and mortality for men and women in industrialized societies. Hypercholesterolemia is a well-established risk factor for atherosclerosis and its pathologic complications. In recent years, there is increasing evidence of a contribution of systemic and local inflammatory processes to atherosclerosis, indicating that chronic inflammation is a necessary condition of the progression of atherosclerosis.

Identification of the atherogenic mechanisms in humans is hindered by the complexity and chronicity of the disease process. Hence there has been a reliance on animal models of the disease to identify pathogenetic steps and causality. Mouse models in particular have proved useful to study atherosclerotic lesion development, and a number of recent reviews have discussed extensively the various mouse models available.

Among the most widely used mouse models are apolipoprotein E-deficient (ApoE−/−) mice, in which targeted deletion of the apoE gene leads to severe hypercholesterolemia and spontaneous atherosclerosis. After a brief overview of apoE−/− mice in atherosclerosis, including an assessment of the underlying mechanisms, I review studies of the response of apoE−/− mice on an atherogenic diet to dietary components focusing on treatment of risk factors hyperlipidemia, hypertension, inflammatory status, and oxidative stress.

I. Underlying Mechanisms of Atherosclerosis in Apolipoprotein E-Deficient Mice

1. The role of apoE in lipid metabolism and arterial lesion development

ApoE is synthesized in the liver and in macrophages, and plays a role in a number of important antiatherogenic functions. As a constituent of plasma lipoproteins, it serves as a ligand for cell-surface lipoprotein receptors such as low density lipoprotein (LDL)-receptor (r) and LDLr related proteins, promoting the uptake of atherogenic particles such as chylomicrons and very low density lipoprotein (VLDL) remnants from the circulation.

ApoE−/− mice were created simultaneously from C57BL/6J mice by two separate groups, through gene inactivation by targeting. The number of cumulative citations in relation to apoE−/− is 1,590 at present (October 2010), and is gradually increasing, as shown in Fig. 1. The citations include drugs, gene manipulations, and others in addition to diet and food. The number of citations in relation to diet and food is 113 at present (7.1% against total citations).

On a chow diet, apoE−/− mice in comparison with C57BL/6J mice demonstrated a total cholesterol level of >500 mg/dl, mostly in VLDL and chylomicron remnant fractions, and developed lesions throughout the macrovasculature (Fig. 2). Over time, these lesions become complex, progressing well beyond the fatty streak. The Western diet accelerates the development of lesions at all stages, from the foam cell lesion to the fibrous plaque. The lesions of animals fed the Western diet are more lipid-rich than those of animals fed chow. Two quantitation methods are used extensively to measure plaques. The first measures plaque cross sectional area in slices taken at the level of the aortic sinus in Apolipoprotein E-Deficient Mice

Diet and Atherosclerosis in Apolipoprotein E-Deficient Mice

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Fig. 1. Numbers of References per Year in Relation to apoE−/− Mice Obtained from PubMed between 1992 and 2010.

The numbers of references were obtained from PubMed by combining one key word, “apoE−/− mice,” with another key word, “atherosclerosis,” that appeared in the abstract and title.

sinus (Fig. 2, right).14) The second en face method involves pinning out the aorta and quantifying the lesion area as a percentage of the total surface area.15) In addition, the cholesterol ester content in the aorta is often used as an index of lesion development.16,17) It was found recently that in older apoE−/− mice, brachiocephalic arterial plaques demonstrate features likely to be the murine parallel of those in vulnerable human plaques, including the formation of an acellular necrotic core, erosion of the necrotic mass through to the lumen, and intraplaque hemorrhage.17)

2. Inflammation

The initial monocyte-endothelial interactions are triggered by local expression of cellular adhesion molecules by the endothelium, including vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1), and proinflammatory cytokines and chemokines such as tumor necrosis factor-α (TNFα), interleukin-6 (IL-6), and monocyte chemoattractant protein-1 (MCP-1). Studies using experimental animals and humans have found increased expression of adhesion molecules and MCP-1 in developing and established atherosclerotic lesions.18,19) Similarly, our results indicate that genetic deficiencies in adhesion molecules, MCP-1, or TNFα demonstrate features likely to be the murine parallel of those in vulnerable human plaques, including the formation of an acellular necrotic core, erosion of the necrotic mass through to the lumen, and intraplaque hemorrhage.17)

3. Serum lipoproteins

LDL retention in atherosclerosis-susceptible sites results from increased flux into the arterial wall and from reduced lipoprotein efflux. LDL uptake via LDLr is a major pathway, and is critical for cholesterol delivery to many cell types,20,21) but cholesterol accumulation in the arterial wall with limited LDLr expression indicates that cholesterol delivery is mediated by mechanisms independent of the LDLr. Moreover, cell-surface proteoglycans that are ubiquitously expressed in cells have been found to mediate LDL uptake by a low-affinity, high-capacity process.20) This process has generally been studied by evaluating apoB uptake, an indicator of LDL cholesterol uptake.

Pathological arterial lipid accumulation, an important contributor to atherogenesis, is linked to a number of pathways involving CD36 (fatty acid translocase) and other scavenger receptors that bind to normal or modified LDL.22–25) There are two main classes of scavenger receptors, scavenger receptor class A (SR-A) and class B, which includes CD36, better known as scavenger receptor class B type I (SR-BI). SR-A is a macrophage integrated membrane protein the deletion of which in apoE−/− mice significantly reduced atherosclerosis despite increased serum cholesterol.26) In apoE−/− mice, disruption of CD36 led to a dramatic decrease in the atherosclerotic lesion area despite increased severity of the proatherogenic lipid profile.35)

LDL cholesteryl esters can be delivered independently to cells without concomitant uptake of the whole LDL particle, a process called selective uptake.20) LDL-selective uptake is mediated via SR-BI mediated pathways, and is markedly increased by increased lipoprotein lipase levels.37)

4. Reverse cholesterol transport

Two adenosine triphosphate-binding cassette (ABC) transporters, ABCA1 and ABCG1, facilitate the efflux of cellular phospholipids and cholesterol to acceptors, such as apoA-I and apoE. Deletion of Abca1 and Abcg1 in mice leads to additive defects in macrophage cholesterol efflux and reverse cholesterol transport and accelerates atherosclerosis given a susceptible hypercholesterolemic background.38) Human ABCA1 overexpressing bacterial
transgenic mice were generated and crossed with apoE−/− mice. The mice demonstrated reduced lesion areas and increased cholesterol efflux from peritoneal macrophages. More recently, Yvan-Charvet et al. indicated that ABCA1, ABCG1, and high density lipoprotein (HDL) inhibit the proliferation of hematopoietic stem and multipotential progenitor cells, and connected the expansion of these populations with leukocytosis and accelerated atherosclerosis.

These findings suggest that suppression of bone marrow myeloid proliferation, leukocytosis, and monocytosis might represent a previously unidentified anti-atherogenic effect of HDL.

ApoA-I, the main apolipoprotein in HDL, is a key acceptor of cholesterol from macrophages, via ABCA1-mediated transport. ApoE−/− mice overexpressing human apoA-I show decreased lesion size with increasing HDL formation.

5. Hypertension

Hypertension increases the rate of atherosclerotic plaque development in models of atherosclerosis. Hypertension can occur in two forms, at elevated and at normal plasma angiotensin (Ang) II levels. Ang II, which is generated from Ang I by the angiotensin-converting enzyme (ACE), is the principal effector of the renin-angiotensin system, and modulates blood pressure. To study the contribution of Ang II to the renin-angiotensin system, and modulates blood pressure. To study the contribution of Ang II to atherosclerotic lesion development, Mazzolai et al. generated hypertensive hypercholesterolemic apoE−/− mice with normal with endogenously increased Ang II production. They found that these two forms of hypertension led to increases in lesion extension similar to those in normotensive mice, and that the atherosclerotic plaques of hypertensive animals with high Ang II were more advanced. Most of the known effects of Ang II are related to Ang II type 1 (AT1) receptor activation, which is perhaps involved in the multifactorial pathogenesis of atherosclerosis. In fact, genetic disruption of the AT1 receptor in apoE−/− mice leads to inhibition of atherosclerotic lesion formation, irrespective of blood pressure or plasma cholesterol levels. Furthermore, inhibition of Ang II action on the arterial wall by blocking of its production with ACE inhibitors (e.g., Captopril and others), or by blocking of binding to its receptors on cells with AT1 receptor antagonists (e.g., Candesartan and others) has been found to attenuate atherosclerosis.

6. Nuclear hormone receptors

The nuclear hormone receptor superfamily of ligand-activated transcription factors regulates gene expression in such diverse processes as metabolism, development, and reproduction. The subfamilies known as peroxisome proliferator-activated receptors (PPARs) and liver-X-receptors (LXRs) have emerged as dominant regulators of processes that influence cardiovascular risk, including atherosclerosis. PPARs and LXRs regulate lipid homeostasis and inflammation in macrophages, endothelial cells, and smooth muscle cells within the vessel wall. A diet that specifically activates these receptors might therefore retard the development of atherosclerosis at several levels.

PPARα: Studies of the role of PPARα in mouse models of atherosclerosis have yielded complex results. Tordjman et al. observed that PPARα−/−/apoE−/− mice had fewer en face atherosclerotic lesions than control ApoE−/−/− mice, suggesting a proatherogenic role of PPARα. In another study, Fu et al. found that treatment of apoE−/− mice with a PPARα agonist, ciprofibrate, aggravated hyperlipidemia and increased atherosclerosis. On the other hand, Calkin et al. found that gemfibrozil, an agonist for PPARα, decreased atherosclerotic lesion areas in apoE−/− mice in association with a reduction in LDL cholesterol. These discrepancies among mouse atherosclerosis find-
ings might be attributable to the animal models used, the type of agonist, the diet, and the duration of experimental treatment.\textsuperscript{43}

**PPARγ:** Chen et al. discovered an antiatherogenic action of troglitazone, PPARγ agonist, in apoE−/− mice.\textsuperscript{47}\textsuperscript{7} Studies of PPARγ are in general agreement that activation of this receptor is beneficial in reducing atherosclerosis in LDLr deficient (LDLr−/−) mice.\textsuperscript{48}

However, the extent of atherosclerosis, as defined by the extent of lipid-stained lesions in the root of the ascending aorta in the apoE−/− mice, was unaffected by treatment with rosiglitazone, a PPARγ agonist.\textsuperscript{49}

**Dual PPARα/γ agonist:** Claudel et al. found that a PPARα/γ coagonist, GW2331, decreased atherosclerosis by 32% in apoE−/− mice.\textsuperscript{50} Co-agonists were expected to integrate the actions of fibrates (regulating lipoprotein metabolism and anti-inflammatory action) and thiazolidinediones (TZDs, PPARγ agonists) (regulating insulin resistance and blood glucose levels, and anti-inflammatory action), thereby addressing several of the risk factors for cardiovascular disease. However, non-TZD PPARα/γ co-agonist 3q caused increased atherosclerosis in apoE−/− mice, despite decreased plasma cholesterol, possibly as a result of a concomitant decrease in HDL and an increase in aortic expression of plasma cholesterol, possibly as a result of a concomitant atherosclerosis in apoE−/− mice, without the development of hepatic steatosis or hypertriglyceridemia.\textsuperscript{55}

### II. Diet and Atherosclerosis in Apolipoprotein E-Deficient Mice

#### 1. Fat and fatty acids

**Saturated fatty acids:** In humans, saturated fatty acids increase the levels of LDL- and VLDL-cholesterol, and current recommendations include decreasing their intake as part of a heart-healthy diet.\textsuperscript{50}\textsuperscript{6} Merkel et al. used two mouse models, LDLr−/− and apoE−/− mice, to measure the effects of four isocaloric diets enriched with either saturated fatty acid, monounsaturated fatty acid, polyunsaturated fatty acid, or carbohydrate on atherosclerotic lesion area.\textsuperscript{57} They used coconut oil (71% saturated fatty acids and 19% monounsaturated fatty acid) as a primary source of dietary fats for the saturated fatty acid diet. In the LDLr−/− mice, as compared with the saturated fatty acid diet, the mono-unsaturated fatty acid and carbohydrate diets significantly increased atherosclerosis, but the polyunsaturated fatty acid diet had no effect.

In contrast, in apoE−/− mice, there were no significant dietary effects on atherosclerosis. These results are surprising, and suggest that depending on the underlying genotype, dietary monounsaturated fatty acid and carbohydrates can actually increase atherosclerosis susceptibility.

In contrast to the results of Merkel et al., Seo et al. reported an unfavorable effect of saturated fatty acids on atherosclerosis in mice.\textsuperscript{59} ApoE−/− mice were fed a chow or a saturated fat-rich (SAT) diet containing 0.2% wt/wt cholesterol, and were injected with double radio-labeled or fluorescent-labeled human LDL to trace independently LDL-cholesterol ester (CE) core and whole-particle uptake, respectively.\textsuperscript{58} The SAT diet consisted of 21% fat (wt/wt; 71% saturated fat from coconut oil, 19% monounsaturated fat from olive oil, and 9% polyunsaturated fat from safflower and corn oil). The results indicate that a SAT diet increased the contributions of selective uptake to total arterial LDL-CE delivery in apoE−/− mice and increases the degree of atherosclerosis.

**N-6 unsaturated fatty acids:** In a pooled analysis of individual-level data from 11 prospective cohort studies in the United States, Europe, and Israel that included 344,696 participants, each 5% increment of energy of polyunsaturated fat consumption in place of saturated fat was associated with a 13% lower risk of coronary heart disease (CHD).\textsuperscript{59} In contrast, each 5% increment of energy of carbohydrate consumption in place of saturated fat was associated with a 7% higher risk of CHD, and 5% increments of energy of monounsaturated fat consumption in place of saturated fat was not significantly associated with CHD risk. The relative benefit of replacing saturated fatty acid with polyunsaturated fatty acid such as linoleic acid is still being debated, because a linoleic acid-enriched diet increases oxidative and inflammatory stress, although it is associated with reductions in serum cholesterol levels.

We attempted to evaluate the effect of dietary supplementation with linoleic acid-rich fat (100 g of containing diet to apoE−/− mice, and found that DMHCA reduced atherosclerosis in male and female apoE−/− mice, without the development of hepatic steatosis or hypertriglyceridemia.\textsuperscript{55}
high-linoleic safflower-seed oil (kg of diet) as compared with a saturated fatty acid-rich fat (50 g of palm oil and 50 g of lard/kg of diet) on atherosclerotic lesion areas, serum cholesterol levels, oxidative stress (urinary isoprostanes) and inflammatory stress (expression of aortic MCP-1) in apoE−/− mice.60) As compared with the saturated-fat diet, the polyunsaturated-fat diet lowered atherosclerosis after 9 weeks, reduced serum total cholesterol levels, and increased HDL-cholesterol levels. The polyunsaturated-fat fed mice showed increased expression of aortic MCP-1 mRNA levels and urinary excretion of an isoprostane (2,3-dinor-5,6-dihydro-8-isoprostaglandin F2α). These results suggest that in apoE−/− mice, the cholesterol traffic between the serum and the arterial wall is the primary determinant of the atherosclerosis process (Sat vs. Linoleic acid in Fig. 3). Furthermore, our unpublished observations indicate that raising the serum cholesterol concentration by adding cholesterol to the linoleic acid-rich diet resulted in an increase in the serum cholesterol concentration and an increase in the lesion area, indicating disappearing advantage of the linoleic acid rich-diet for atherosclerosis. Calleja et al. also reported that male apoE−/− mice fed a chow-based diet containing 10% sunflower-seed oil (56% linoleic acid) had fewer lesions than did those fed 10% palm oil.61) Furthermore, George et al. found that dietary enrichment with polyunsaturated n-6 fatty acid decreased the serum concentration of LDL and was associated with a reduction in the extent of atherosclerotic plaque development in LDLr−/− mice.62)

Fan et al. evaluated the antiatherogenic effects of dietary γ-linolenic acid (primrose oil) in apoE−/− mice.63) Five-week-old male mice were fed cholesterol-free diets containing 10 g/100 g lipid as corn oil, and primrose oil (10 mol/100 mol γ-linolenic acid) for 15 weeks. Subsequently, the diets were supplemented with cholesterol (1.25 g/100 g) and sodium cholate (0.5 g/100 g) for an additional 10 and 16 weeks. The plasma cholesterol levels generally did not differ between the groups. A parallel γ-linolenic acid-dependent suppression of the number of proliferating aortic smooth muscle cells and atherosclerotic lesion size was also observed. These results indicate that dietary γ-linolenic acid can suppress smooth-muscle cell proliferation in vivo and can retard the development of diet-induced atherosclerosis in apoE−/− mice.

N-3 unsaturated fatty acid: Consumption of fish oil and n-3 fatty acids is associated with beneficial modifications of plasma lipid levels,64) but direct evidence of the anti-atherogenic properties of fish oil has not been documented in apoE−/− mice. We fed apoE−/− mice an atherogenic diet with either 1% ethyl ester docosahexaenoic acid or safflower oil as a source of linoleic acid for 8 weeks.65) Dietary fats led to no significant effect on lesion size in the aortic arch and the thoracic plus abdominal aorta. Xu et al.66) also investigated the impact of fish oil consumption on the quality and quantity of lipoprotein fatty acids and its influence on atherosclerosis in apoE−/− mice. Male apoE−/− mice were treated with 1% dietary fish oil for 14 weeks. High amounts of n-3 fatty acids (eicosapentaenoic and docosahexaenoic acid) were found in plasma lipid fractions. These changes were accompanied by significant increases in plasma triglyceride levels, but these changes did not affect atherogenesis. Similar results showing no beneficial effect of fish oil or docosahexaenoic acid on atherosclerosis in apoE−/− mice have been reported by Mavrommatis et al.67) In contrast to these reports, a diet containing 5% fish oil, as compared to 5% corn oil, significantly reduced atherosclerosis at the aortic root and in the aorta (Corn vs. Fish oil in Fig. 3).68) A decrease in the surface area of atherosclerotic lesions at the aorta and decreases in P-selectin, VCAM-1, and ICAM-1 expression were observed in fish oil-fed mice as compared to corn oil-fed mice. Differences in the percentages of fish oil and individual long-chain n-3 polyunsaturated fatty acid, age at the initiation of the dietary regimens and duration, and the absence or presence of cholesterol in the diet make studies with fish oil diets in apoE−/− mice difficult to compare.

Epidemiological studies have suggested that ingesting α-linolenic acid (18:3, n-3) might be beneficial in protecting humans from cardiac disease.69) Since walnut oil is a good source of α-linolenic acid (13.7%) as well as linoleic acid (61.2% 18:2, n-6), we compared the effects of walnut oil and high-linoleic safflower oil (0.5% 18:3 and 73.2% 18:2) in male and female apoE−/− mice over 9 weeks.70) Female mice fed the walnut diet had a greater lesion area in the aortic root than did those on the high-linoleic safflower oil diet. This suggests that the unfavorable effects of the walnut diet on apoE−/− mice may be attributable to α-linolenic acid.

Trans fatty acids: In a meta-analysis of prospective cohort studies, each 2% increment of calories from trans fat was associated with a 23% higher risk of CHD.71) As far as we know, no report has been published on the role of trans fats in the development of atherosclerosis in apoE−/− mice.

Oxidized fatty acids: The typical Western diet contains large quantities of polyunsaturated fatty acids that are heated or processed to varying degrees, resulting in oxidized lipids.72) Khan-Merchant et al. evaluated the effects of 11–13 weeks of consumption of a well-defined dietary oxidized fatty acid, 13-hydroxylinoleic acid (13-HODE), on atherosclerotic lesion development and plasma cholesterol concentrations in LDLr−/− mice.73) This study was unique in that heated oils were not used as the source of oxidized lipids. The researchers enzymatically oxidized linoleic acid to form 13-hydroxyperoxylinoleic acid, which was then converted into a more stable form, 13- HODE. Oxidized fatty acid consumption increased the aortic lesion areas. It also tended to increase plasma total cholesterol, LDL cholesterol, and oxidative stress, as indicated by higher levels of auto-antibodies to oxidatively modified proteins. Penumetcha et al. found that dietary oxidized fatty acids can be absorbed by the intestine and incorporated into lipoproteins, and might create oxidative stress and exacerbate atherogenesis.74)

2. Sterols
Cholesterol: Nakashima et al. maintained apoE−/− mice on chow, which contained 4.5% fat by weight (0.02% cholesterol), or a Western diet, which contained 21% fat by weight (0.15% by weight cholesterol and 19.5% by weight casein without sodium cholate).13) The
plasma cholesterol levels of the apoE−/− mice fed a chow diet ranged from 360 to 885 mg/dL with a mean of 606 mg/dL, whereas feeding a Western-type diet to the apoE−/− mice resulted in much higher levels of cholesterol, ranging from 1,085 to 4,402 mg/dL. The mice fed the Western diet generally had more advanced lesions and higher levels of serum cholesterol than those fed the chow diet (X axis and Y axis in Fig. 3; 2.7 and 5.0 respectively).

Oxysterol: When exposed to heat, air, light, or oxidizing agents, cholesterol undergoes spontaneous oxidation, forming oxidation products. Food processing, especially heat treatment and drying, induces lipid oxidation in foods, including dairy products, eggs, meat, and fish.75 Oxidized cholesterol is also present in bakery products, some of the major ingredients, eggs and butter, contain large amounts of oxidized cholesterol. There are conflicting reports as to whether diet-derived cholesterol-oxidation products initiate early atherosclerotic lesions and accelerate the development of advanced lesions, as reviews by Brown and Jessup.76

Staprans et al. found increased levels of oxidized cholesterol in apoE−/− mice after ingestion of diets containing oxidized cholesterol, indicating that in apoE−/− mice, dietary oxidized cholesterol is absorbed.77 In the serum of those mice, 7-ketocholesterol, 7β-hydroxycholesterol, 7β-epoxycholesterol, and 7α-hydroxycholesterol were present in the serum of the mice that fed the control diet, which contained no oxidized cholesterol. The mice that were fed the oxidized cholesterol diet showed a 4-fold increase in serum concentrations of 7-ketocholesterol and a 100% increase in 7β-hydroxycholesterol, the two main oxidized cholesterol components identified in the diet. In these experiments, oxidized cholesterol was measured by gas liquid chromatography. Feeding of an oxidized cholesterol diet resulted in a 32% increase in fatty-streak lesions. Our results78 were different from those of Staprans et al.77 We fed apoE−/− mice a purified diet or the same purified diet containing 0.2 g cholesterol or oxycholesterol/kg. The dietary oxycholesterol had no significant effect on serum lipid levels. Although all of the diet-derived oxycholesterol accumulated in the serum, GC-MS analyses showed that only cholest-5-en-3β-ol-7-one and cholestan-3β, 5α, 6β-triol accumulated significantly in the aorta. The oxycholesterol diet did not result in elevation of the aortic cholesterol level or the lesion volume in the aortic valve. The discrepancy between the two groups remains to be resolved.

Plant sterol: Dietary plant sterols are known to reduce intestinal cholesterol absorption, and thereby they reduce plasma total and LDL-cholesterol levels in both humans79 and laboratory animals.80 Moghadasian et al. have found that dietary phytosterols significantly reduce plasma cholesterol concentrations, and have determined the extent of atherosclerotic lesions in apoE−/− mice regardless of presence or absence of dietary cholesterol.81,82 It is not likely that the anti-atherogenic action of phytosterols is to be attributed to hepatic LDLr, since LDLr could not be upregulated in the absence of apoE.83 Among many anti-atherogenic drugs, probucol is distinguished due to its strong cholesterol-lowering and antioxidant activities, making it attractive for the prevention of CHD. On the contrary, probucol para-doxically promotes atherogenesis in apoE−/− mice despite its strong LDL-cholesterol-lowering and antioxidant activities.84 Hence Yeganeh et al. investigated to determine whether dietary phytosterols reduce probucol-induced atherosclerosis in apoE−/− mice.85 Male apoE−/− mice were fed an atherogenic diet supplemented with phytosterols or probucol or combination of them for 14 weeks. Single therapy with phytosterols and with probucol resulted in a 25% reduction in the plasma total cholesterol concentration as compared to the control group. The effects of the combination therapy were more profound (60% reduction). While phytosterols reduced atherosclerosis 60%, probucol caused an increase of 150% in atherogenesis. The addition of phytosterols to probucol substantially reduced the pro-atherogenic effects of probucol. This was associated with an improved HDL concentration.

Furthermore, diets enriched with phytosterols have yielded immune modulatory effects in both humans and experimental animals.86 Accordingly, Nashed et al. investigated to determine whether the anti-atherogenic effects of phytosterols are associated with reductions in pro-inflammatory cytokine production, as well as the effect of this diet on global immunocompetence.87 ApoE−/− mice were fed a cholesterol-supplemented diet in the presence and the absence of 2% phytosterols for 14 weeks and then immunized with ovalbumin. The phytosterol-enriched diets were associated with higher anti-inflammatory (IL-10) and lower pro-inflammatory cytokines (IL-6, TNF-α). The development of cytokine and chemokine responses to ovalbumin was as strong as in the phytosterol-treated mice, or even stronger, relative to the controls. Thus the desirable suppression of pro-inflammatory cytokine production associated with the inhibition of atherogenesis did not impair the capacity to mount responses to foreign antigens.

Oxysterol: Phytosterol oxidation products (oxyphytosterols) are formed during the processing and storage of foods,88 but it is unknown whether oxyphytosterols affect human health. To address this issue, we prepared oxides of β-sitosterol and campesterol, evaluated their lymphatic absorption in rats, and examined the effect of an oxyphytosterol diet on atherosclerosis in apoE−/− mice.89 The lymphatic absorption of cholesterol and six oxyphytosterols of β-sitosterol or campesterol was assessed in thoracic duct-cannulated rats fed an AIN-93G-based diet containing 2.5 g of cholesterol, oxyphytosterols, or intact phytosterols per kg. The lymphatic recovery of oxy-campesterols and oxy-sitosterols was higher than for campesterol or β-sitosterol, but lower than for cholesterol. Independently apoE−/− mice were fed an AIN-93G-based diet containing 0.2 g of oxyphytosterols or intact phytosterols per kg for 9 weeks.80 Diet-derived oxyphytosterols accumulated in the serum, liver, and aorta. Furthermore, the oxyphytosterol diet increased oxycholesterol in the serum as compared to the phytosterol diet. In particular, the oxycholesterol and the oxyphytosterol diet resulted in elevations in liver 4β-hydroxycholesterol,90 which is known to be one of the strongest ligands for LXR,91 but there was no significant difference between the two groups in serum and aortic cholesterol concentrations, the lesion area in the aortic root, or the 8-iso-prostaglandin F2α concen-
tration in the urine (F2α is an isoprostane). Thus, exogenous oxyphytosterols are well-absorbed and accumulate in the body, but do not promote the development of atherosclerosis in apoE−/− mice.

3. Proteins, peptides, and amino acids

Soy and rice protein: We and others have found that apoE−/− mice fed soy protein isolate (SPI) have less atherosclerosis than those for whom casein is the major protein source.92,93 These experiments used 10% olive oil as the source of fat in the diet with and without the addition of 1% cholesterol and 0.25% cholate. Whether cholesterol or cholate was present, SPI led to lower atherosclerosis in various aortic segments, despite similar plasma cholesterol levels (Casein vs. SPI, Fig. 3). When mice were fed a diet containing isoflavone-free SPI or casein plus isoflavone-rich fraction for 9 weeks, there were no differences in thoracic aorta lesion area between the two groups. Soy protein has less l-methionine, a precursor of homocysteine, which leads to increased plasma homocysteine levels and less significant differences in the mice fed the two different diets. This indicates that the anti-atherogenic effect of native SPI cannot be explained by its effect on serum lipids or homocysteine, and suggests that both the protein component and the isoflavones contribute to the anti-atherogenic effect of native SPI.

Soy and rice proteins are known to be rich in l-arginine as compared with animal proteins such as casein. It has been reported that this amino acid is beneficially involved in atherosclerosis in experimental animals such as rabbits, pigs, and LDLr-deficient mice.95 In fact, dietary supplementation with l-arginine prevented atherosclerosis development, probably through conversion to NO, which has a variety of beneficial effects on the cardiovascular system (Casein vs. Casein + Arg, Fig. 3).96 Hence it is to be expected that a rice protein isolate (RPI)- and SPI-containing diet would result in a decrease in lesion formation in apoE−/− mice as compared with those fed a casein-containing diet. In fact, we found that the en face lesion area in the aorta and the lesion size in the aortic root in mice fed the casein-based diet were greater than those in the SPI or RPI groups.97 The plant protein groups had an increased concentration of serum l-arginine and NO metabolites (NO2− plus NO3−) as compared with the casein group. In an independent experiment, the l-arginine and l-methionine contents were adjusted to be 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. There were no significant differences in the en face lesion area or in lesion size as between the casein group and the l-arginine-supplemented group, although the serum l-arginine and NO metabolite concentrations in the supplemented group were higher than those in the casein group. There were again no significant effects of l-methionine supplementation on lesion formation. These results demonstrate the anti-atherogenic potential of SPI- and RPI-derived proteins, but their l-arginine and l-methionine contents were not sufficient to explain the underlying mechanisms. In contrast, Chen et al. found adverse effects of supplemental l-arginine in atherosclerosis in apoE−/−/iNOS mice, raising the possibility that l-arginine supplementation paradoxically contributes to, rather than reducing, lesion formation by a mechanism that involves lipid oxidation, peroxynitrite formation, and NOS uncoupling.98

Recently, Nagarajan et al. addressed the mechanisms contributing to the atheroprotective effects of a soy-based diet using apoE−/− mice fed SPI with and without phytochemicals (SPI+ and SPI− respectively) or casein.99 Reduced atherosclerotic lesions were observed in aortic sinus and en face analyses of the descending aorta in the SPI+ and SPI− fed apoE−/− mice as compared with the casein-fed mice. The mice fed SPI+ showed 20% fewer lesions than the mice fed SPI−, suggesting that phytochemical components contributed to the atheroprotective effect.100 Plasma lipid profiles did not differ among the three groups, indicating that alternative mechanisms might have contributed to the atheroprotective effect of the soy-based diets. PCR analyses of the proximal aorta showed reduced expression of MCP-1 in the mice fed both soy-based diets as compared with the casein-fed mice. These findings suggest that the reduction in atherosclerotic lesions observed in the mice fed the soy-based diet is mediated in part by inhibition of MCP-1 which might result in reduced monocye migration, an early event in atherogenesis.

Burris et al. recently addressed the mechanisms contributing to the atheroprotective effects of a rice-based diet using apoE−/− mice fed RPI or casein.101 Reduced atherosclerotic lesions were observed in aortic sinus and en face analyses of the descending aorta in RPI-fed apoE−/− mice as compared to casein-fed mice. Plasma total- and HDL-cholesterol levels were not different among the two groups. Plasma oxidized LDL and anti-oxidized LDL IgG levels were significantly decreased in the RPI-fed as compared to the casein-fed mice. The plasma and aortic tissue glutathione (GSH) levels and GSH:oxidized form of GSH (GSSG) ratio were higher in the RPI-fed mice than in casein-fed ones. RPI feeding increased mRNA and the protein expression of superoxide dismutase, and also the mRNA expression of catalase, glutathione peroxidase, and glutathione reductase, key antioxidant enzymes implicated in the inhibition of oxidative stress which leads to atherosclerosis. This suggests that the reduction in atherosclerotic lesions observed in mice fed the rice-based diet is mediated in part by inhibition of oxidative stress and subsequent oxidized LDL generation, which can result in reduced foam cell formation, an early event in atherogenesis.

Soy and rice globulin: β-Conglycinin (7S globulin), a major soy storage protein accounting for about 30% of soy protein, stimulates the expression of LDLr and the degradation of LDL by hepatocytes in vitro.102,103 Adams et al. have found that consumption of concentrated β-conglycinin has an inhibitory effect on atherosclerosis in male and ovariectomized female apoE−/− mice that greatly exceeds the effect of isoflavon-containing SPI, glycinin (11S globulin, another major soy storage protein), and soy protein devoid of β-conglycinin.104 These effects were unrelated to variations in the isoflavone content of the protein source and of influences on plasma lipoproteins. Furthermore,
our preliminary experiments indicated that intragastric administration of rice globulin for 9 weeks resulted in greater decreases in atherosclerosis in the sinus than did casein treatment in apoE<sup>−/−</sup> mice (unpublished observation). These findings confirm that the anti-atherogenic action of soy and rice is to be attributed to the protein components.

**Betaine:** Betaine serves as a methyl donor in a reaction converting homocysteine to methionine. Four groups of apoE<sup>−/−</sup> mice were fed AIN-93G diets supplemented with 0, 1, 2, and 4 g betaine/100 g diet. Higher doses of betaine were correlated with a smaller atherosclerotic lesion area. Betaine supplementation also reduced aortic expression of TNF, but not MCP-1, in a dose-dependent way in four groups of apoE<sup>−/−</sup> mice, and the atherosclerotic lesion area was positively associated with the aortic TNF level. ApostE<sup>−/−</sup> mice receiving betaine supplementation had higher concentrations of serum total cholesterol. These data suggest that despite exacerbating hyperlipidemia in apoE<sup>−/−</sup> mice, betaine can exert an anti-atherogenic effect by inhibiting the aortic inflammatory response mediated by TNF.

**Peptides:** Few studies regarding anti-atherosclerotic small peptides, except for the tetrapeptide of Lys-Arg-TNF-inhibiting the aortic inflammatory response mediated by apoE<sup>−/−</sup> mice, have been reported. The conclusion is that the anti-atherosclerotic action of Lys-Arg-Glu-Ser was positively associated with the aortic TNF level. ApostE<sup>−/−</sup> mice receiving betaine supplementation had higher concentrations of serum total cholesterol. The study involved a 9 weeks of successive administration of Try-His at doses of 0, 10, or 100 mg/kg per day. En face analyses provided direct evidence that the atherosclerotic lesion area was significantly reduced, by 27 and 38%. For Try-His doses at 10 and 100 mg/kg per day respectively, as compared with the control group. Total serum cholesterol and HDL-cholesterol did not differ between the tested groups. In sum the peptide can exert the beneficial action by alternative mechanisms, not by the regulation of lipid metabolism. The endothelial-independent vasodilating action of Try-His<sup>109</sup> might also provide alternative mechanisms by which the proliferation or migration of vascular smooth muscle cells can be inhibited through suppression of extracellular Ca<sup>2+</sup> influx as in the case of Val-Tyr.<sup>109</sup>

4. **Vitamins**

A potential place for oxidative stress in atherosclerosis led to the notion that it might be prevented by antioxidant treatment, and especially by antioxidant vitamins such as α-tocopherol and ascorbic acid. However, recent large clinical trials have generally not supported the notion that these antioxidant vitamins can slow the progression of established atherosclerosis.<sup>110,111</sup> Whether antioxidant vitamins can prevent or slow early atherosclerosis may be a more relevant question that is not readily answered in humans, given the prolonged asymptomatic period of the disease. Studies using animal models of atherosclerosis might provide support for such clinical trials.

**Tocopherol:** Previous studies show inhibition of early lesion initiation and progression in apoE<sup>−/−</sup> mice with supplementation with antioxidants (α-Toc, Fig. 3).<sup>112,113</sup> However, animal studies with α-tocopherol have been inconclusive.<sup>114</sup> In apoE<sup>−/−</sup> mice, 0.05 g/100 g of α-tocopherol had no effect on the spontaneous formation of atherosclerotic lesions,<sup>115</sup> whereas about 0.2 g/100 g of vitamin E reduced the progression of atherosclerotic lesions.<sup>112,113</sup> None of the supplements affected plasma cholesterol concentrations.

Black et al.<sup>116</sup> evaluated the effects of a vitamin E homolog in female heterozygous apoE<sup>−/−</sup> mice, which develop atherosclerotic lesions only when they are fed a diet high in saturated fat and cholesterol.<sup>117</sup> The mice were fed a nonpurified control diet (5.3 g/100 g of triglyceride and 0.2 g/100 g of cholesterol), an atherogenic diet alone (15.8 g/100 g of triglyceride, 1.25 g/100 g of cholesterol, and 0.5 g/100 g of Na cholate) or the atherogenic diet supplemented with 0.5 g/100 g of (++)-α-tocopherol (mixed isomers); 0.5 g/100 g of palm tocopherols (palm-E containing 33% α-tocopherol, 16.1% α-tocotrienol, 2.3% β-tocotrienol, 32.2% γ-tocotrienol, 16.1% δ-tocotrienol); or 1.5 g/100 g of palm-E. The mice fed the atherogenic diet had large atherosclerotic lesions at the level of the aortic valve. With supplements of 0.5 g/100 g of palm-E and 1.5 g/100 g of palm-E, the sizes of the lesions were 92 and 98% smaller respectively. The 0.5 g/100 g of α-tocopherol supplements had no effect. Furthermore, Qureshi et al. compared the effects of α-tocopherol and a tocotrienol-rich fraction of rice bran on the pathogenesis of atherosclerotic lesions in apoE<sup>−/−</sup> mice.<sup>118</sup> When a high-fat diet was supplemented with the tocotrienol-rich fraction and fed to mice for 24 weeks, the atherosclerotic lesion size was reduced 23% as compared with the group fed control diet. Alpha-tocopherol supplementation resulted in only a small reduction, indicating the superior efficacy of tocotrienols as compared to α-tocopherol. The decrease in atherosclerotic lesions in the tocotrienol group appears to be due in part to serum LDL cholesterol reduction (α-Toc + Tot, Fig. 3).

**Vitamin C:** It has been found that vitamin C reduces vascular oxidative stress and increases NO-mediated endothelium-dependent relaxation in a mouse model in which gulonolactone oxidase was deleted by targeted gene disruption.<sup>119</sup> Twenty-six to twenty-eight weeks of diet supplementation with vitamin C (1%/kg of chow) significantly increased the circulating levels of vitamin C in apoE<sup>−/−</sup> mice. Aortic lesion areas were reduced by 51% after treatment of apoE<sup>−/−</sup> mice with vitamin C.<sup>120</sup> Long-term treatment with vitamin C restored endothelial nitric oxide synthase (eNOS) enzymatic activity in the aortas of apoE<sup>−/−</sup> mice (vitamin C, Fig. 3). Treatment with vitamin C had no effect on the plasma lipid profile. Combined deficiencies of vitamin E and C are reported to worsen early atherosclerosis in apoE<sup>−/−</sup> mice,<sup>121</sup> but deficiencies to the extent induced in these studies (15% to 28% of normal) are uncommon in humans.

**Coenzyme Q10:** Thomas et al. tested whether vitamin E plus ubiquinone-10 (CoQ10) co-supplementation is more anti-atherogenic than either antioxidant alone by the use of apoE<sup>−/−</sup> mice fed a high-fat diet with 0.2% vitamin E, 0.5% CoQ10, or 0.2% vitamin E plus 0.5%
CoQ10 for 24 weeks. Compared with controls, vitamin E plus CoQ10 supplementation decreased atherosclerosis at the aortic root and arch and the descending thoracic aorta to an extent that increased with increasing distance from the aortic root. CoQ10 significantly inhibited atherosclerosis at the aortic root and arch, whereas vitamin E decreased the disease at the aortic root only. Thus, in apoE−/− mice, vitamin E plus CoQ10 supplements were more anti-atherogenic than CoQ10 or vitamin E supplements alone, and disease inhibition is associated with a decrease in aortic lipid hydroperoxides.

It has been found that exposure to sidestream cigarette smoke (SSCS) enhances atherosclerotic lesion formation in apoE-deficient mice. Gairola et al. tried to determine whether CoQ10 protects against SSCS-mediated atherosclerosis. Female apoE−/− mice were fed a saturated fat-enriched diet alone, or one supplemented with 1% wt/wt CoQ10. The mice in each diet group were exposed to SSCS for 4 h/d, 5 d/week in a whole-body exposure chamber. The mice were euthanized after 6 or 15 weeks of SSCS exposure. Dietary supplementation with CoQ10 significantly reduced atherosclerotic lesions in the control group. As reported above, exposure to SSCS increased the sizes of the lesions in the apoE−/− mice at both time points, but dietary supplementation with CoQ10 had no effect on atherosclerotic lesions augmented by SSCS exposure. Thus, it is possible that the acceleration of atherosclerosis due to smoke exposure involves mechanisms other than or in addition to oxidative stress that are not mitigated by CoQ10 supplementation.

α-Lipoic acid: Four-week-old female apoE−/− mice were fed for 10 weeks on a Western chow diet containing 15% fat and 0.125% cholesterol without and with 0.2% wt/wt R,S-α-lipoic acid, or on a normal chow diet containing 4% fat without and with 0.2% wt/wt R-α-lipoic acid respectively. Supplementation with α-lipoic acid significantly reduced atherosclerotic lesion formation in the aortic sinus. This anti-atherogenic effect of α-lipoic acid was associated with almost 40% less body weight and lower serum and VLDL levels of triglycerides, but not of cholesterol. In addition, α-lipoic acid supplementation reduced the aortic expression of adhesion molecules and pro-inflammatory cytokines and the aortic macrophage accumulation. Thus, the inhibition appears to be due to the anti-obesity, anti-hypertriglyceridemic, and anti-inflammatory effects of α-lipoic acid. The finding that α-lipoic acid supplementation lowers serum triglycerides has important implications for the prevention and treatment of cardiovascular disease, because hypertriglyceridemia is an independent predictor of myocardial infarction and stroke.

6. Flavonoids and polyphenol

Isoflavones: As described in the section “Soy and rice protein,” soy isoflavones inhibit the initiation and progression of early atherosclerotic lesions in young apoE−/− mice. That soy isoflavones might be functioning as phytoestrogens is suggested by the requirement of the presence of estrogen receptor-α for manifestation of its atheroprotective effect. The term “phytoestrogen” is commonly applied to the soy isoflavones genistein, daidzein, and glycitein. While initially the function of phytoestrogens was cholesterol reduction, a number of more recent reports definitely conclude that dietary isoflavones make no contribution to the hypcholesterolemic action, including a clinical study performed on pure genistein.

The consumption of isoflavone-containing foods such as soybean and soybean products has been reported to have beneficial effects on the cardiovascular system in post-menopausal women. Bourassa et al. studied the effects of exogenous estrogen on atherosclerotic lesions in ovariectomized apoE−/− mice. Hormone replacement with subdermal 17-β-estradiol pellets releasing 6, 14, or 28 μg/d significantly decreased atherosclerotic lesion areas in ovariectomized female mice. We examined the mechanism underlying the beneficial effects of isoflavones in apoE−/− mice subjected to ovarian resection. Compared with sham-operated mice, the ovariectomized mice had larger arterial lesion areas in the aortic root. Feeding the ovariectomized mice an isoflavone-containing diet (0.055 mg/kg of total isoflavones/cal of diet) reduced the sizes of these lesions more than did feeding them an isoflavone-free diet. Neither ovariectomy nor diet had a significant effect on the concentration of cholesterol in serum or the urinary.
levels of isoprostanes. The ovariectomized mice showed a greater increase in mRNA abundance for MCP-1 in the aorta than did the sham-operated mice. The isoflavone-containing diet lowered the MCP-1 expression more than did the isoflavone-free diet. This suggests that dietary isoflavones have an antiatherogenic effect by preventing the activation of macrophages due to the removal of ovaries. 

Catechins: Catechins are widely distributed flavonoids present in various fruits and beverages (green tea, red wine, fruit juice), and in chocolate.\textsuperscript{136} Although few studies have evaluated the anti-atherosclerotic effect of pure catechin in an apoE\textsuperscript{−/−} mice model, all studies done showed inhibition of the development of atherosclerotic lesions.\textsuperscript{137–139} Auclair et al. recently investigated the anti-atherosclerotic effects of catechin supplementation of the diet of apoE\textsuperscript{−/−} mice at a low nutritional level (0.02% in diet) to explore the mechanisms of action by a transcriptomic approach.\textsuperscript{139} Catechin supplementation for 6 weeks reduced the mean atherosclerotic lesion area by 32%, but had no effect on total cholesterol or triacylglycerol levels in the plasma. Plasma antioxidant capacity and inflammatory status (serum amyloid A) were unchanged. The expression of 450 genes in the aorta was significantly modified by catechin supplementation. Some of the most significantly downregulated genes included genes coding for adhesion molecules such as CD34 and P-selectin.

Catechins, flavonoids, and proanthocyanidins: Proanthocyanidins rich extract (PSE) is a mixture of flavonoids that are mainly compounds of proanthocyanidin containing the polyhydroxy flavan-3-ol unit in di- and tetra form.\textsuperscript{142} It has many physiological effects, including antioxidant and anti-inflammatory. We investigated to determine whether pine bark extract has an anti-atherogenic effect in apoE\textsuperscript{−/−} mice.\textsuperscript{143} Male and female mice were fed a diet based on the AIN-76 formula, and that was supplemented with 2% pine bark extract. The lesion area of the valve and the levels of serum cholesterol in the male mice decreased on the pine-bark extract diet. The pine-bark extract diet, however, had no significant effect on the levels of urinary isoprostanes. These results indicate that unlike the apple polyphenol extract,\textsuperscript{140} dietary pine bark extract can have beneficial effects on atherosclerosis development in male apoE\textsuperscript{−/−} mice by lowering the serum cholesterol level.

Anthocyanin-rich extract: Xia et al. found that supplementation with anthocyanin-rich extract prepared from a black rice-pigment fraction of the diet significantly inhibited atherosclerotic plaque formation in apoE\textsuperscript{−/−} mice.\textsuperscript{144} This observation corresponded with significantly lower total serum cholesterol and higher HDL cholesterol concentrations in the group fed than in the control group. The anthocyanin-rich extract from black rice was identified as containing cyaniding-3-glucoside and peonidin-3-glucoside.\textsuperscript{145}

Beneficial effects of berries have been reported in relation to CHD. Consumption of blueberries was associated with a reduced risk of CHD in a cohort of post-menopausal women in the Iowa Women’s Health Study.\textsuperscript{146} The protective effectiveness of blueberries against atherosclerosis has been confirmed in apoE\textsuperscript{−/−} mice.\textsuperscript{147} Most of the protective effects of blueberries are ascribed to their high content of phytochemicals, in particular, anthocyanins.\textsuperscript{148} Bilberry (Vaccinium myrtillus L.) is one of the richest dietary sources of anthocyanins, with an anthocyanin glycoside content of about 300–600 mg/100 g of fresh weight.\textsuperscript{149} Mauray et al. evaluated the effects of a bilberry extract, one rich in anthocyanins, on the development of atherosclerosis in apoE\textsuperscript{−/−} mice.\textsuperscript{150} The mice received for 16 weeks a diet supplemented with 0.02% bilberry extracts (around 0.01% anthocyanin glycosides). Supplementation of the diet with bilberry extracts led to a significant inhibition of plaque development, whereas no effect on oxidative stress parameters or lipid profiles was observed, suggesting the involvement of other mechanisms of action.

### III. Conclusions

I conclude that a wealth of published materials has proved that the apoE\textsuperscript{−/−} mouse is an excellent experimental model for studying the histopathological development of atherosclerosis and dietary therapies to retard the formation of atherosclerotic lesions. In fact, daily dietary components, such as lipids, peptides and amino acids, carbohydrates, vitamins, minerals, and polyphenols, are intimately involved in the aggravation and alleviation of atherosclerosis. These dietary components are involved in atherosclerotic development in apoE\textsuperscript{−/−} mice through mechanisms of cholesterol metabolism, inflammatory reactions, and blood pressure.
The present review confirms that cholesterol metabolism is one of the important underlying mechanisms. The correlation coefficient $r$ between serum cholesterol and lesion size is 0.43 (Fig. 3).

Although the etiology of atherosclerosis in humans is complex and still not fully resolved, the study of apoE$^{-/-}$ mice has given a major boost to experimental atherosclerosis research, especially as it permits ongoing studies of means of preventing and retarding the development of atherosclerosis.

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