Understanding the role of dietary components on atherosclerosis using genetic engineered mouse models

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1. ABSTRACT

The generation by genetic engineering of two murine models to investigate atherosclerosis, such as the apoE- and LDLr-deficient mice, is providing an extraordinaire knowledge of the effect of different nutrients on this complex disease. The present revision provides a comprehensive overview of the advances in this field that point to a remarkable complexity. While some controversies over puzzling results could be explained invoking potential nutrient interactions or different food sources of nutrients, it also appears that other factors such as sex, genetic background or immunological status are emerging as generators of differential responses to nutrients during the atherosclerotic process.

2. INTRODUCTION

Atherothrombosis is a complex disease modulated either at its origin and/or progression by genetic and environmental influences. It can be regarded as a ‘lifestyle related disease’ in which diet is one of the main risk factors (1).

A hallmark in the elucidation of nutritional factors affecting atherogenesis has been the development by genetic engineering of murine models of atherosclerosis. Until then, the extent of diet-induced atherosclerotic lesions in the mouse model, although effectively used, was small and limited to the early fatty-streak stage. In addition, the diets used in these pioneering studies were often toxic
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Further compounding the interpretation of the data (2, 3). The first line of atherosclerosis-prone gene targeted animal models, namely apolipoprotein E- knockout mice (apoE-KO), was generated by homologous recombination in ES cells in 1992. This model is particularly popular since it develops extensive atherosclerotic lesions on a standard chow diet (4, 5). Moreover, when fed a hypercholesterolemic diet for fourteen weeks it develops massive xanthomatous lesions (6). The fact that this model has been thoroughly characterized and that lesions could progress into fibrous plaques, with some evidence of plaque rupture (7, 8), further underline the relevance of this mouse as an excellent model for atherosclerosis. The creation of apoE- knockout mice has changed the face of atherosclerosis research (9, 10). In 2000, Osada et al reviewed the contribution of this model to delimitate the role of nutritional components on atherosclerosis (11). As envisioned then, this field has experienced a dramatic expansion and, over this period, a travel companion has emerged in the LDL receptor - deficient (LDLr − KO) mouse. This latter model has elevated LDL levels, but no spontaneous lesions or only very small ones on the chow diet. However, gross atheromata do form when fed a western-type diet (12). It should be stressed that these models may not be directly comparable when subjected to the same experimental design due to intrinsic differences in the underlying cause of the dyslipidemia (deficient clearance of both chylomicron, and VLDL remnants in the ApoE-KO mouse model, versus deficient clearance of LDL in the LDLr-KO mouse model), and to the rate of atherosclerosis progression (which is faster in the ApoE-KO mouse model). This review will examine our present understanding of the nutritional regulation of the atherosclerotic process using these two animal models.

3. INFLUENCE OF THE CHOLESTEROL CONTENT IN DIETS

In pioneering studies of atherosclerosis using natural mice fed chow-based diets supplemented with cholesterol, saturated fat, and sodium cholate to overcome species resistance to lesion development, they were able to induce lesions only up to the small early fatty-streak stage (2). Initially, this diet was also used to feed engineered mice but, due to its high hepatotoxicity, new experiments were undertaken to select the required ingredients and to remove the toxic ones. Thus, cholate was found to be dispensable for the induction of aortic lesions in LDLr-KO mice fed for 12 weeks an AIN-76A-based diet containing 40 % of calories as fat supplemented with variable amounts of cholesterol (0.5 to 1.25%, by weight) when compared to a control AIN-76A diet containing only 10% of the calories as fat (4.3%, w/w) (13). More recently, it has been proved that the amount of cholesterol present in semisynthetic AIN-76 diet containing 4.3% (w/w) fat selectively modified the lesion location in the LDL receptor-deficient mouse (C57BL/6J background). Thus, increasing dietary cholesterol up to 0.02% (w/w) was sufficient to induce hypercholesterolemia and atherosclerosis at the aortic root and the brachiocephalic artery but did not produce significant lesions in the aorta measured by the en face method. Increasing dietary cholesterol between 0.15% and 0.50% doubled plasma cholesterol levels and resulted in the appearance of en face lesions with a significant increase in the area of atherosclerotic lesions at the aortic root and brachiocephalic arteries (14). Moreover, in apoE-deficient mice Fan and coworkers had emphasized the importance of dietary cholesterol intake for arteriosclerosis progression. Upon feeding cholesterol-free diets containing 10% (w/w) of fat for fifteen weeks, they reported the absence of any major lesions in the thoracic aortas of apoE-KO mice (15).

Dietary cholesterol also appears to enhance the side effects of other compounds. In this regard, in LDLr-KO mice fed high fat diets containing 8 mg of 13-hydroxylinoleic acid (13-HODE) with or without cholesterol, the aortic lesion only increased in those animals receiving the diet supplemented with cholesterol. Based on this observation Khan-Merchant et al. suggest that dietary cholesterol must be present to promote the atherogenic effects of 13-HODE (16). Alternatively, cholate appears to suppress the atheroprotective effects of other components. Thus, the administration of cholesteryl together with extra virgin olive oil neutralizes the beneficial effect of this oil in retarding atherosclerosis in apo E- KO mice (17).

The possible contribution of oxidative modification of cholesterol to the development of atherosclerosis has also been studied in apolipoprotein E-deficient mice. The administration of diets containing oxysterols (0.2 g /kg) revealed that, despite aortic accumulation of some of the oxidized metabolites of cholesterol (such as cholest-5-en-3beta-ol-7-one and cholestan-3beta, 5alpha, 6beta-triol), no significant increase in aortic lesion by administration of these compounds was observed (18).

In summary, current data appear to indicate that, in genetically engineered mice: i) dietary cholesterol induces arteriosclerosis depending on the amount consumed, ii) dietary cholesterol may interfere with the atheroprotective effects of other nutrients and iii) no increased atherogenic risk appears to derive from the oxidation products of cholesterol when added as dietary components.

4. INFLUENCE OF THE CHOLATE CONTENT IN DIETS

The use of the biliary salt, cholate, as an enhancer of atheroerclerosis development in early studies of this field in wild-type C56BL/6J mice has been described in the previous section (2). The fact that its administration appeared harmful for the liver due to its lithogenic properties has precluded its inclusion in Western-like diets specially designed to feed mice on the genetic background - C56BL/6J- that is more lithogenic-prone and atherosclerosis susceptible than any other strains (13, 19). However, a new perspective of the role of this agent has emerged from the work of Vergnes et al (20) where it has been shown that the dietary administration of cholate in combination with fat or cholesterol increased the activation of hepatic stellate cells and fibrosis in wild-type C56BL/6J
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mice but not in the atherosclerosis-resistant sub strain C57BL/6ByJ. The latter finding together with the cholate-induced increase in hepatic levels of transporters ABCG5 and ABCG8 (21) and the atheroprotective role of overexpressing these transporters (22) point out an interesting role of this agent per se in the atherosclerosis field. Furthermore, administration of 0.5 %, w/w cholate in combination with 1% cholesterol for 21 days has been shown to enhance the hypercholesterolemic effect and hepatic damage of cholesterol administration in apo E-KO mice (23) but no atherosclerosis data was shown in this work. Altogether this information is suggesting an independent role of cholate in atherosclerosis that needs to be addressed.

5. INFLUENCE OF QUALITY AND QUANTITY OF DIETARY FAT

This is one of the aspects more widely investigated and, as a consequence, becoming more complex. The effects on atherosclerosis development exerted by the administration of variable amounts of dietary fats, with different compositions, may be complex by itself, but in addition we must also consider the potential interactions with factors of diverse nature such as the underlying genetic deficiency, sex, immunological status, genetic background, etc.

5.1. MUFA and PUFA containing diets versus SFA

In LDLr-KO mice, diets containing 0.2% cholesterol enriched in 20% (w/w) saturated (SFA) or polyunsaturated fatty acids (PUFA safflower oil) decreased atherosclerosis to a similar extent when compared to animals fed diets enriched with monounsaturated fatty acids (MUFA high oleic content). The latter induced an increase in lesion that correlated positively with VLDL-cholesterol levels and was independent of sex (24).

In contrast, in the same animal model, George et al have shown that administration of 10% (w/w) n-6 (PUFA) coming from safflower oil induced a significant decrease in atherosclerosis when compared to animals fed an equivalent SFA-rich diet. In the group fed the n-6 polyunsaturated diet a decrease in LDL plasma levels was observed, but the decrease in atherosclerosis was more pronounced than the reduction in LDL levels (25). The inconsistencies between these studies could be due to differences either in the source of safflower oil or in the percentages of fat supplied in each study. In this regard, experiments conducted in our laboratory with apoE deficient mice appear to lend support for the second possibility. Thus, moderate fat intake (10%, w/w) of olive oil or palm oil, in the presence of very low (0.03%) dietary cholesterol, was accompanied by a decrease in atherosclerotic lesion in females associated with an increase in plasma apoA-I (26). However, a higher supply (20%, w/w) of the same olive oil not only did not add further benefit compared to the 10% intake, but atherosclerotic lesion size was similar to animals fed the carbohydrate diet (17). Our results are in agreement with those of other authors that did not find differences in ApoE-KO mice between carbohydrates and high fat (20%, w/w) diets regardless of saturation (24).

Although the reports from LDLr-KO mice suggest that MUFA containing diets are more atherogenic than PUFA or SFA containing diets (24), experiments carried out in our lab with apoE-deficient mice support a far more complex scenario. Thus, in the presence of very low (0.03%) dietary cholesterol, moderate fat intake (10%, w/w) of either olive oil II (70% MUFA, 18% SFA) or palm oil (40% MUFA, 40% SFA) was accompanied by a decrease in atherosclerotic lesion in females compared to chow fed animals. However, no significant decrease in female atherosclerosis was observed when the diets contained similar amounts of oils with high SFA (coconut) or high PUFA (sunflower I) content. Moreover, administration of sunflower oil II (70% MUFA, 13% SFA), with a fatty acid composition equivalent to that of olive oil II (70% MUFA, 18% SFA), did not show any protective effect over atherosclerosis in females (26). These results point out to other oil components such as phytosterols, triterpenes, polyphenols or vitamins, besides fatty acid composition, that may be important modulators of the effects mediated by fat intake upon arteriosclerosis development. In addition, as mentioned above, amount of fat, and dietary cholesterol play significant roles on the atherosclerotic effect of olive oil (17). These facts combined with the use of two different animal models indicate that further experiments are required to clarify this issue.

5.2. Source of PUFA may influence development of atherosclerosis

Oils containing polyunsaturated fatty acids are being compared between themselves regarding atherosclerosis development. In this sense, in the apoE deficient mice, aortic atherosclerotic lesions were significantly reduced in those consuming a 20% (w/w) fish oil diet [42% PUFA;1(omega -6):3(omega -3)] compared to those fed a high corn oil diet (53% PUFA all omega -6). The protective role of fish oil in the development of atherosclerosis may result from the induction of antioxidant enzyme activities (27). These differences do not exist between diets containing DHA (PUFA omega-3) or safflower oil that showed similar extent of lesion area in Apo E deficient mice (28). Likewise, in other study, a high-linoleic safflower oil was also more efficient reducing atherosclerosis compared to (alpha-linolenic containing) walnut oil (29). Dietary intake of another PUFA-containing oil, flaxseed, can be also beneficial in reducing atherothrombosis (30). All these studies are indicating that not all PUFA-containing oils show similar potency to control atherosclerosis development.

On the other hand, configuration of PUFA may add a new level of complexity to this field. Thus, consumption of gamma-linolenic acid (PUFA omega-6) coming from primrose oil in male apoE knockout mice significantly reduced aortic vessel wall medial layer thickness with a parallel effect on the number of proliferating aortic smooth muscle cells (15). Similarly, conjugated linoleic acid isomers have recently been shown to posse very different abilities in retarding or promoting atherosclerosis development in apo E-KO mice (31).
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5.3. Other factors

In addition to source of PUFA oil, sex is influencing the dietary response. Only male apoE-knockout mice consuming 10% (wt/wt) sunflower oils showed lesion reduction associated with the decrease of triglycerides in triglyceride-rich lipoproteins, while only female mice reduced lesion size upon consuming equivalent diets containing oleic acid-enriched palm or olive oils (26).

The dietary response may also be modulated by the underlying genotype. Thus, in ApoE-KO mice, the genetic disruption of the angiotensin II type IA receptor led to inhibition of vascular oxidative stress resulting in a decreased atherosclerosis when fed an atherogenic diet (32). On the other hand, overexpression of human apoA-I in apoE knockout mice on a high-fat diet reduced plaque lipid and increased the proportion of plaque occupied by collagen and smooth muscle cells (33). However, no differences in aortic cholesterol deposition were observed between Apo A-I/LDLr-double knockout mice and LDLr-deficient mice despite the fact that this diet induced more dyslipidemia in the LDLr-deficient mice (34). Another example of a modified response to an atherogenic diet elicited by different genetic backgrounds was observed in heterozygous lipoprotein lipase lipase deficiency in the LDLr-KO mice, where this diet induced more dyslipidemia but no more atherosclerosis (35). Likewise, in the beta3-integrin-deficient mice crossbred with either apolipoprotein E or LDLr-deficient mice, a Western-type diet caused greater aortic atherosclerosis than in littermates lacking only one of the genes. In this study, the lack of beta3-integrin enhanced the susceptibility of aorta to diet induced inflammation. These results suggest that beta3-integrin can reduce atherosclerosis in these animals by limiting the inflammatory response induced by hypercholesterolemia (36).

The complex interaction of diets with immune modulation in atherogenesis has been also reinforced by the experiments of oral tolerance with the HSP65 protein in LDLr-deficient mice despite the fact that this diet induced more dyslipidemia in the LDLr-deficient mice (34). Another example of a modified response to an atherogenic diet elicited by different genetic backgrounds was observed in heterozygous lipoprotein lipase lipase deficiency in the LDLr-KO mice, where this diet induced more dyslipidemia but no more atherosclerosis (35). Likewise, in the beta3-integrin-deficient mice crossbred with either apolipoprotein E or LDLr-deficient mice, a Western-type diet caused greater aortic atherosclerosis than in littermates lacking only one of the genes. In this study, the lack of beta3-integrin enhanced the susceptibility of aorta to diet induced inflammation. These results suggest that beta3-integrin can reduce atherosclerosis in these animals by limiting the inflammatory response induced by hypercholesterolemia (36).

5.4. Functional genomics

In an attempt to better understand these complex interactions, high throughput gene expression through chips has been introduced and applied to analyze hepatic transcriptomes in several circumstances. Thus, in mice lacking both the low density lipoprotein (LDL) receptor and Apobec1 genes, the latter being responsible for apoB mRNA editing, it has been observed that a fat-enriched diet up-regulated stress-responsive proteins and those involved in calcium signaling and bone formation, providing new insights into the significance of calcification in atherogenesis (39). When this technology has been applied to aorta from apoE-deficient mice at different time intervals after initiation of an atherogenic diet, varying patterns of gene expression have been observed. This indicates that the atherogenic process is highly dynamic, and that a detailed evaluation of gene expression during disease progression would be required for understanding atherosclerosis and implementing preventive interventions (40). Applying this technology to apoE-deficient mice fed either normal chow or the Western diet, it has been observed that the Western diet induces differential expression of 311 genes in aortic tissue. Among those genes there are candidate mediators of arterial wall injury, some of them coding for proteins involved in cell adhesion, complement cascade and histocompatibility antigens, flavin-containing monoxygenases, cytokines and collagen (41). These studies open new opportunities to unveil how diets enriched in cholesterol and fat initiate the atherosclerotic process.

6. INFLUENCE OF PROTEIN COMPOSITION

The effects of dietary soy protein isolate (SPI) were evaluated in LDL receptor deficient mice overproducing apolipoprotein B and in apoE deficient mice. In both models, atherosclerosis was inhibited by SPI independently of isoflavone content and sex compared with animals fed a casein/lactalbumin diet. The inhibitory effects of soy on atherosclerosis were not related to plasma lipoprotein cholesterol concentrations either, suggesting the existence of lipoprotein-independent pathways by which dietary soy protein isolate inhibits atherosclerosis (42). Other authors have confirmed the atheroprotective effect of soy protein and proved to be unrelated to its high L-arginine and low L-methionine contents (43). More recently, Adams et al have proved that a diet rich in beta-conglycinin, a major soy storage protein, has atheroprotective effects that greatly exceed those of isoflavone-containing soy protein isolate and are also independent of plasma lipoproteins (44). Although Adams and coworkers have speculated that atheroprotective bioactive peptides, which potentially would reach the circulation, could be generated during digestive tract breakdown of SPI (44), nothing is known about the mechanisms by which the quality of dietary protein might influence atherosclerosis other than the relative abundance of proatherogenic (L-methionine) or atheroprotective (L-arginine) amino acids. In addition, other components such as isoflavones modulate the global action of dietary soy protein in vivo.

The effects of diets rich in soy protein that were either isoflavone depleted (0.04 mg/g protein isolate) or isoflavone-replete, (1.72 mg/g protein isolate) were studied in apolipoprotein E-deficient mice crossed with estrogen receptor-alpha- and -beta-deficient mice to produce double-knockout mice. Atherosclerosis was reduced by isoflavone in apolipoprotein E-deficient mice lacking estrogen receptor beta, but was unaffected in estrogen receptor-alpha-deficient mice. These results indicate a role for estrogen receptor-alpha-dependent processes in mediating the atheroprotective effects of dietary soy isoflavones (45).

The influence of other sources of dietary protein has also been tested in apolipoprotein E-deficient mice,
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where substituting 50% of casein intake by gelatin induced a decrease in HDL cholesterol and an acceleration of atherogenesis (46).

6.1. L-arginine

This amino acid serves as a substrate for the formation of nitric oxide (NO) by the NO synthase enzymes (NOS). Dietary supplementation of L-arginine prevents xanthoma formation and reduces atherosclerosis in LDL receptor knockout mice fed a (1.25%) high-cholesterol diet. The abrogation of the beneficial effects of L-arginine by N-omega-nitro-L-arginine suggests that the atheroprotective actions of L-arginine are mediated by NOS through the restoration of NO released and improvement in endothelial function (47). This beneficial effect of (25 g/L) L-arginine supplementation on lesion formation was not observed in the western-type diet-fed apoE deficient mice. Furthermore, L-arginine supplementation abolishes the protective effect of inducible NO synthase (iNOS) gene deficiency in apoE/iNOS double-deficient mice by mechanisms that involve lipid oxidation, peroxynitrite formation, and NOS uncoupling (48). These findings indicate a complicate response to this amino acid administration in relationship to the genetic makeup of the individual.

6.2. L-homocysteine

Hyperhomocysteinemia (HHC) is an independent risk factor for cardiovascular disease (1). To prove a role of homocysteine in atherosclerosis disease, several approaches have been used. In this regard, administration of a diet enriched in methionine but depleted of folate, vitamins B6 and B12 induced hyperhomocysteinemia in apoE-KO mice, and resulted in accelerated atherosclerosis manifested by an increased atherosclerotic lesion area and complexity. However, accelerated arteriosclerosis was neither observed when mice were fed a diet exclusively deprived of folate and vitamins B6 and B12, nor when the methionine enriched diet was supplemented with those vitamins. These findings implicate HHC in atherosclerotic plaque progression and stability, and suggest that dietary enrichment in vitamins essential for the metabolism of homocysteine may impart protective effects in the vasculature (49). These results were confirmed and extended by supplementing a Western diet with (11-33 g/kg food) methionine or (0.9-1.8 g L drinking water) homocysteine to this animal model, and assaying arteriosclerosis either at 3 months (short term) or at 12 months (long term) after initiation of the study. At both time points HHC increased plaque collagen deposition, while HHC-induced atherosclerosis was only evident in the short term study. Therefore, although HHC appears to be atherogenic, it affects more the development of early lesions than the progression of established plaques (50). In another study, the enhanced development of atherosclerosis in apoE deficient mice was induced by dietary methionine and homocysteine, but not by homocysteine alone (51). The atherogenicity of hyperhomocysteinemia has also been observed in apolipoprotein E and cystathionine beta-synthase double knock-out mice where an enhanced uptake of modified LDL by macrophages was observed and proposed as an atherogenic mechanism (52).

Whether homocysteine per se or a coincident metabolic abnormality causes vascular disease is still an open question. Thus, apolipoprotein E-deficient mice fed methionine-rich diets had significant atheromatous pathology in the aortic arch even with normal plasma homocysteine levels, whereas mice fed B vitamin-deficient diets developed severe hyperhomocysteinemia without any increase in vascular pathology (53), and supplementation with B vitamins appeared to confer homocysteine-independent protection against atherosclerosis (51). These findings suggest that increased methionine intake may be atherogenic in susceptible mice. Although homocysteine may contribute to the effect of methionine, high plasma homocysteine was not independently atherogenic in this model suggesting that some product of excess methionine metabolism rather than high plasma homocysteine per se may underlie the association of homocysteine with vascular disease (53). Even though the results presented by Troen et al constitute a strong argument in favor of a potential role, direct or indirect, for excess methionine intake in atherosclerosis, the fact that HHC, induced by homocysteine supplementation or genetic manipulation, also increases arteriosclerosis (50-52) indicates that, at least in some settings, HHC could be a causal (more than a casual) companion to arteriosclerosis.

6.3. L-taurine

Administration of this amino acid for three months to apolipoprotein-E-deficient mice fed regular chow decreased atherosclerotic lesions associated with a decrease in oxidative stress, and despite a significant increase in serum LDL and VLDL cholesterol levels(54).

7. ANTIOXIDANTS

The emerging dogma that low density lipoprotein (LDL) oxidation plays a central role in early atherogenesis is based on three lines of experimental evidence: (i) lipid peroxidation products and oxidized LDLs are present in atherosclerotic lesions (55); (ii) oxidized LDL has an array of potentially proatherogenic properties in vitro, including uptake by macrophages via a number of distinct "scavenger" receptors; and (iii) cross-cultural dietary comparisons suggest an inverse correlation between coronary mortality and antioxidant vitamin intake. A corollary to the oxidation hypothesis of atherosclerosis is that the consumption of antioxidants, as potential antiatherogenic agents, will stop and/or retard the development of atherosclerosis. However, the literature is divided in support of this conclusion (56).

7.1. Vitamin E

In several animal models, a number of different antioxidant drugs have been shown to retard atherosclerosis, but the effect of vitamin E supplementation on atherosclerosis is still controversial, and at least, dependent on two aspects: the dose and putative interactions with other circumstances.

Several studies have analyzed the effect of vitamin supplementation on atherosclerosis at different doses. Thus, supplementation of vitamin E (a high dose of
1333 mg/kg diet for three months) reduced progression of established atherosclerosis and concomitantly suppressed isoprostane production, intercellular adhesion molecule-1 (ICAM-1) and monocyte chemoattractant protein-1 (MCP-1) levels, while increasing nitric oxide production in LDLr-deficient mice (57). Similarly, in apolipoprotein E knockout mice, a lower dose of alpha-tocopherol (800 mg/kg) during sixteen weeks, also confirmed these observations reducing the area of the aortic lesion, without modifying serum cholesterol or lipoprotein profile (58). These authors, in agreement with the results observed in LDLr-deficient mice, also appreciated a decreased expression of MCP-1 at the aortic lesion, decreased circulating isoprostane levels and reduced titers of autoantibodies against oxidized cardiolipin (59). In apo E-deficient mice, a moderate dose of 40 or 400 mg/kg of alpha-tocopherol acetate supplemented to atherogenic diets reduced atherogenic lipoproteins, but showed controversial results on atherogenesis. Thus, while in one study, using a 40 mg/kg dose, vitamin E resulted in the expected antioxidant effects without substantial attenuation of aortic atherosclerosis (60), in other (400 mg/kg vs a control group of 40 mg/kg) just slowed the process (61). All these studies clearly indicate the existence of dose dependence, and the potential existence of a threshold level, for implementation of the atheroprotective effects of vitamin E.

In relationship with interactions, positive and negative modulators of vitamin E action have been found. As an example of the former, the anti-atherogenic action of vitamin E was potentiated by a simultaneous administration of 0.5% (w/w) ubiquinone-10 (CoQ10) (62), an antioxidant compound that, administered at a dose of 1% (w/w) in the diet, significantly decreased atherosclerotic lesion in apolipoprotein E-deficient mice fed a high-fat diet (63). In the latter study the atheroprotective effects of CoQ10 could not be separated from the potential effect of a concomitant decrease in total plasma cholesterol (effected through VLDL), while the data reported by Thomas et al, supplementing only 0.5% (w/w) of CoQ10, obtained a decrease in atherogenesis without decreasing total cholesterol plasma levels, indicating that the atheroprotective effect of CoQ10 might be direct, or indirect but not effected through modification of plasma lipoprotein levels.

Positive modulators of the effects of antioxidant vitamins over atherogenesis are also known. For example, moderate exercise and dietetic supplementation with (6%) L-arginine synergistically reduced atherosclerosis when the atherogenic diet supplemented to LDLr-KO mice was supplemented with antioxidant vitamins (0.05% vitamin C, and 1% vitamin E) (64).

On the other hand, negative interactions have also been observed. Thus, when vitamin E supplementation was administered to acute exercising LDL receptor-deficient mice no reduction of atherosclerotic lesions were observed, suggesting that vitamin E supplementation could be ineffective in exercisers by inhibiting antioxidant enzyme buildup in the arterial wall (65).

7.2. Vitamin C

The efficacy of vitamin C reducing atherosclerosis is also controversial. Thus, addition of vitamin C (up to 33 lg/l) to the water administered to L-gulonolactone-gamma-oxidase/ApoE double knockout mice fed an atherogenic diet, failed to demonstrate significant decreases in atheroma development compared to mice fed the same atherogenic diet without vitamin C. However, in the latter group the chronic vitamin C deficiency, although did not induce an earlier initiation or progression of atherosclerotic plaques, resulted, as could be anticipated, in severely compromised collagen deposition and a lesion morphology reminiscent of that present in potentially vulnerable plaques (66). In contrast, other researchers have reported that long term (twenty eight weeks) dietetic supplementation with 1% vitamin C reduced lesion area by 32% in apoE deficient mice (67). The potential atheroprotective effects of vitamin C treatment could be mediated by the maintenance of tetrahydrobiopterin levels (67), or through reduction of aortic VEGF and VEGFR-2 expression, as appeared to be the case in apoE-KO treated with both vitamin C and E for four weeks (68).

7.3. Other compounds

Other antioxidants agents are present in aliments and, perhaps only apparently, seem to produce contradictory results. For example, red wine administration containing 6% (w/w) polyphenols (equivalent to a reported daily intake of 5.9 µg per mouse of trans-resveratrol, one of the major polyphenolic compounds in red wine) did not reduce mature atherosclerosis or changed the content of collagen in plaques in apolipoprotein E-deficient mice fed chow diets, despite a concomitant increase in HDL cholesterol (69). However, in the presence of a high fat diet, trans-resveratrol (9.6 and 96 mg/kg diet, equivalent to a per mouse daily intake of 29 and 290 µg), significantly decreased the formation of atheroma in the aorta of apolipoprotein E and low-density lipoprotein receptor deficient mice (70). Although both studies may not be directly comparable due to substantial differences in experimental design, it is also possible that they indicate the existence of a threshold for trans-resveratrol action in atherogenesis. Another antioxidant agent, boldine, an alkaloid of Peumus boldus at doses of 1 or 5 mg per day, induced a reduction (22% and 44% respectively) in lesion formation in LDLr-KO mice fed an atherogenic diet (71).

8. INFLUENCE OF ENERGY INTAKE

Restriction of caloric intake to 60% of ad libitum fed ApoE deficient mice resulted in a decrease of more than 30% in atherosclerotic lesion size at the aortic root. In addition, these lesions represented early stages of atherosclerosis when compared to ad libitum fed animals, as judged from a reduction in the presence of acellular areas, fibrous caps and calcification. Energy restriction significantly decreased the levels of lipid hydroperoxides and the production of superoxide and hydrogen peroxide in the aorta. These observations suggest that a reduction of oxidative stress in the arterial wall may be involved in the anti-atherogenic effect of energy restriction (72).
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9. INFLUENCE OF DIETARY MINERAL CONTENT

9.1. Iron

In apoE-deficient mice, dietary iron intake restriction leads to a decrease in lesion area (>25%), a reduction in iron plaque deposition and a greater resistance of plasma lipoproteins to copper-induced oxidation (73). Using the same animal model, Lee et al (74) further extended their observations, and found that dietary iron restriction induced lower matrix degradation capacity, and increased plaque stability in these mice. In contrast, a high intake of iron has been shown to increase serum and tissue levels of iron but were not atherogenic, challenging the hypothesis that elevated levels of tissue iron promote LDL oxidation and oxidative stress in vivo (75).

9.2. Magnesium

Magnesium fortification of drinking water (50 g of Mg sulfate per liter) was capable of inhibiting atherogenesis in male LDL-receptor-deficient mice fed a cholic acid containing atherogenic diet, but this effect was not observed in females (76). When the atherogenic diet was formulated without cholate, the effect of Mg fortification was also observed in females. These results demonstrate the importance of nutritional interactions, and the existence of different susceptibilities related to sex (77). Indeed, the mechanisms by which Mg exerts its action may vary in a sex-dependent way. Thus, while no differences in LDL oxidation were related to Mg, only females receiving Mg showed decreased levels of anti-oxidized LDL antibodies. No such effect could be observed in males, where the protective effects of Mg appeared associated instead with an increased liver content of retinol and retinyl palmitate (78).

Sex-specific responses to magnesium supplementation (50 g/L) were also observed in apoE-deficient mice receiving a low-fat diet. Although significant decreases in cholesterol and triglycerides plasma levels were effected by Mg administration in both sexes, only females receiving the Mg supplement showed a significant reduction in the size of arteriosclerotic plaques, without showing any changes in plaque macrophage density (79).

9.3. Sodium

Low sodium chloride diets, as compared with a normal salt diet, showed enhanced aortic wall lipid storage in LDL receptor knockout mice. Although a low salt diet was associated with increased plasma levels of total cholesterol, triglycerides and non-esterified fatty acids, only the latter appeared to be significantly correlated with aortic lipid deposition. No similar effect was observed in apolipoprotein E-KO mice where the underlying hypercholesterolemia could have masked any proatherogenic effect induced by the low salt intake (80).

10. INFLUENCE OF ALCOHOLIC BEVERAGES

Epidemiological studies have shown that moderate consumption of ethanol is associated with reduced cardiovascular events in humans. To verify the causality of this association and study the potential mechanisms implicated, ethanol administration at different concentrations (0-5%, w/v) was tested in LDLr-KO mice fed cholate-containing atherogenic liquid diets. In this model, alcohol-feeding retarded atherosclerosis in a dose-dependent manner. Although this effect was associated with an increase in plasma levels of apoA-I, it was concluded that the atheroprotective effect of ethanol could not be entirely attributable to such mechanism (81). However, when the effect of ethanol on atherosclerosis was tested in ApoE-KO or LDLr-KO mice on low-fat diets or high-fat diets without cholate, no effect on lesion size or plaque collagen content was observed despite the raised HDL cholesterol levels (69, 82). In line with these findings, it was observed that moderate beer consumption did not change the development of early or mature atherosclerosis in ApoE-KO mice (83). In contrast, a 25-50% reduction in lesion size was observed in both strains (LDLr- and ApoE-deficient mice) when fed a high-fat cholate-containing diet. Since cholate increased and ethanol reduced aortic expression levels of NF-kB, it was suggested that ethanol-inhibition of cholate-induced atherosclerosis could be effected via an anti-inflammatory mechanism (82).

11. OBESITY AS A PREDISPOSING FACTOR FORATHEROSCLEROSIS

The relationship between obesity and atherosclerosis has been explored using different strategies to determine whether obesity itself or dyslipidemia associated with obesity enhanced atherosclerosis. Thus, the obese leptin-LDLr double knockout mouse exhibited a striking hypertriglyceridemia and hypercholesterolemia that resulted in extensive atherosclerotic lesions throughout the aorta by six months (84).

Low density lipoprotein receptor deficient mice fed a high fat diabetogenic diet (high sucrose) became more obese and developed severe dyslipidemia, and consequently, atherosclerotic aortic sinus lesions were increased 3.7 fold over LDLr-KO mice fed a chow diet. However, this diet was ineffective to increase atherosclerosis in C57BL/6 or in apoE deficient mice. In the latter animal model this is likely due to a failure of the high fat diabetogenic diet to induce dyslipidemia beyond that present in chow fed animals (85). These results appear to indicate that promotion of atherosclerosis by obesity requires the concomitant presence of dyslipidemia. It should be noticed, however, that feeding a normal chow diet in apo E-KO mice resulted in atherosclerotic aortic sinus lesions that were increased 7 fold over those present in LDLr-KO with the same alimentary regimen. Thus, the apparent failure of obesity to promote atherosclerosis in apo E deficient mice could actually reflect an elevated rate for atherosclerosis development in this model per se which the high fat diabetogenic diet was unable to overcome.

12. INSULIN RESISTANCE AND DEVELOPMENT OFATHEROSCLEROSIS

The role of insulin resistance in atherogenesis is a matter for open discussion. Traditionally, fructose-containing diets have been proposed to induce insulin
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Resistance (IR). However, in LDL receptor-deficient mice, fructose-enriched diets (60% of total calories) failed to induce IR, although an increased aortic lesion area was observed when compared with animals fed a Western diet. Unexpectedly, in the latter group of animals elevated glucose and insulin plasma levels were detected, indicating the development of IR in these animals. From this study it appears that IR does not increase atherosclerosis development (86). In diabetic and non-diabetic apolipoprotein E-deficient mice, an advanced glycation end products-restricted diet decreased atherosclerosis (87). These results are indicative of a potential role for diet-derived glycation products in diabetes-accelerated atherosclerosis.

13. INFLUENCE OF PHYTOSTEROLS

Phytosterols are compounds with estrogenic effects; several studies have been addressed to study their properties in relationship with atherosclerosis. To evaluate their ability to regress atherosclerotic lesions in male apo E-deficient mice, atherosclerosis was induced by feeding a particular Western-type diet containing only 9% (w/w) fat and 0.15% (w/w) cholesterol for eighteen weeks. Then, they were divided into two groups and fed a different regression-diet for twenty five weeks. During this “regression” period, control animals received a chow diet containing 4.5% fat (w/w) without cholesterol, while the experimental group received the same diet supplemented with 2% (w/w) of a phytosterol mixture. The phytosterol-treated group showed a trend to retard the plaque growth that was not statistically significant (88). This was somehow surprising since, in the same mouse model, it had been shown that addition of the phytosterol mixture (2%, w/w) to a diet containing 9% (w/w) fat for twenty weeks (89) or to a 0.2% (w/w) cholesterol for fourteen weeks (90) decreased arteriosclerotic lesion area. The antiatherogenic effects of this mixture were associated with a slight increase in HDL cholesterol concentration along with a statistically significant decrease in hepatic lipase activity and a non significant decrease in plasma fibrinogen concentrations (89). Using a different phytosterol mixture, a significant decrease in atherosclerosis lesion development was also observed in apo E deficient mice fed a Western diet (20%, w/w fat, and 0.08% w/w cholesterol) supplemented with 2% (w/w) phytosterols during four weeks (91). In the latter study, no statistically significant decreases in lesion area were observed between the Western diet supplemented with the phytosterol mixture at either 0.5% or 1% (w/w). Thus, the use of phytosterols prevents the development of atherosclerosis. However, their potential benefit may be transformed in harmful accumulation of plant sterols, hypercholesterolemia and atherosclerosis as it is the case for individuals with sitosterolemia. The latter condition is caused by mutations of the transporters ABCG5 and ABCG8 that difficult their normal role of limiting intestinal absorption of sterols (92, 93). In this way, the overexpression of these genes in LDLr-KO mice has been found to prevent diet-induced atherosclerosis (22). Surprisingly, mice lacking ABCG5 and ABCG8 do not develop aortic lesions when fed chow or Western-like diets (94). Thus it will be interesting to wait for the effect of phytosterol supplementation in these animals as well as the phenotypic outcome of the cross-breeding of the later animals with apo E or LDLr-KO mice.

These data indicate that atheroprotective effects of phytosterols are likely dose dependent, and that perhaps fat diet content could modulate these effects. In addition, it seems that phytosterols are more effective in preventing than in regressing established plaques and their beneficial use would be limited or even lost in those subjects with sitosterolemia.

14. MISCELLANEOUS

The administration of eggplant extract had no effect on atherosclerosis or plasma cholesterol in LDLr-KO mice fed chow or atherogenic diets during twelve weeks despite increased oxidative stress (95).

Diet supplementation of carotenoid lutein (0.2%, w/w) resulted in a >40% decrease in lesion size both in apoE-KO and LDLr-KO mice (96). Similar results were observed in apoE-KO mice fed for twenty weeks with the dietary administration of 5% chitosan- the deacetylated form of chitin extracted from the shells of crustaceans-. In this case the decrease of atherogenesis was associated with decreased cholesterol levels (97).

15. ARTERIOSCLEROSIS MONITORING

From the present discussion it is apparent that atherosclerosis development and/or progression in animals with genetic deficiencies can be modulated by nutritional manoeuvres. We believe that some of these strategies might, when their mechanism of action becomes sufficiently understood, be extrapolated to human nutrition. However, so far our understanding of the atherosclerotic lesion has been limited to the end point static picture obtained from specimen necropsies. This situation may soon change. Thus, plaque changes can be no invasively monitored by serial in vivo magnetic resonance imaging (MRI) to study plaque characteristics in animal models in response to therapeutic interventions. Ex vivo high-resolution MRI has demonstrated sufficient sensitivity to detect changes in plaque size and lipid composition over time from aortic root and brachiocephalic arteries of apolipoprotein E knockout mice fed a Western diet (98). With this technology and transplantation of aortic arch segments from apolipoprotein E-deficient into wild-type or in another apoE-KO recipient mice, it was shown that correction of dyslipidemia in wild-type recipient mice resulted in regression in arterial wall volume, whereas in apoE-KO recipient mice, further plaque progression ensued (99). Therefore, these technologies are likely to speed-up and expand our knowledge of the atherosclerotic process in animal models. They will help us both, to visualize and quantitate the dynamics of plaque development (or regression), and to recollect data while the experiment is still in progress.

16. CONCLUSION AND PERSPECTIVES

The availability of apoE and LDL receptor knockout mice has contributed to improve our knowledge
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of the biological effects of nutrients in relationship with atherosclerosis development. However, the formidable quantity and diverse nature of the data generated is forcing us to a certain impasse before suggesting global recommendations to humans. To make them it will be necessary to consider, on one hand, differences in nutrient composition, origin and processing of foods, and on the other, the variable response which is to be expected in relationship with nutrient interactions, sex, genetic backgrounds, immunological status. . . . . We are in the middle of a “long and winding road” where a single outcome is certainly not going to solve the problem. We should determine how many outcomes are possible to help in preventing atherosclerosis development by nutritional recommendations.

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18. REFERENCES

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**Abbreviations:** ABCG5: ATP-binding cassette type G5, ABCG8: ATP-binding cassette type G8, AIN: American Institute of Nutrition, ApoE1: apolipoprotein B editing complex I, CoQ10: ubiquinone-10, DHA: docosahexaenoic
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Key Words: Apolipoprotein E, Knockout mice, Apolipoprotein E, Deficient Mice, Atherosclerosis, Diet, Ldl Receptor, Deficient Mice, LDL receptor, Knockout Mice, Review

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