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Folic acid supplementation delays atherosclerotic lesion development in apoE-deficient mice

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Abstract

Folic acid is a vitamin that when used as a dietary supplementation can improve endothelial function. To assess the effect of folic acid on the development of atherosclerosis, male apolipoprotein E-deficient mice fed a standard chow diet received either water (control group) or an aqueous solution of folic acid that provided a dose of 75 μg/kg/day, for ten weeks. At the time of sacrifice, blood was drawn and the heart removed. The study measured plasma homocysteine, lipids, lipoproteins, low-density lipoprotein (LDL) oxidation, isoprostane, paraoxonase, and apolipoproteins, and aortic atherosclerotic areas. In folic acid-treated animals, total cholesterol, mainly carried in very low-density and low-density lipoproteins, increased significantly, and homocysteine, HDL cholesterol, paraoxonase, and triglyceride levels did not change significantly. Plasma isoprostane and apolipoprotein (apo) B levels decreased. The resistance of LDL to oxidization and plasma apoA-I and apoA-IV levels increased with a concomitant decrease in the area of atherosclerotic lesions. The administration of folic acid decreased atherosclerotic lesions independently of plasma homocysteine and cholesterol levels, but was associated with plasma levels of apolipoproteins A-I, A-IV and B, and decreased oxidative stress.

Keywords: Folic acid; Homocysteine; Apolipoprotein; Atherosclerosis; ApoE-deficient mice

Folate is the generic term for compounds that exhibit vitamin activity similar to that of pteroylmonoglutamic acid (folic acid), which is a co-factor in the transfer and utilization of 1-carbon moieties. Those biochemical reactions are required for transmethylation, nucleic acid synthesis, homocysteine metabolism, and the enzymatic regeneration of tetrahydrobiopterin (an essential co-factor of nitric oxide synthase). Mounting evidence suggests that folates might play a role in the prevention of cardiovascular disease (Verhaar et al., 2002). Epidemiological studies have shown that low serum folate levels are associated with increased cardiovascular risk (Robinson et al., 1998). In addition, low folate levels might play a pathogenic role in atherosclerosis, independent of homocysteine concentrations (Durga et al., 2005). The administration of folate and vitamin B_{12} for nine weeks to patients that had coronary heart disease and hyperhomocysteinemia improved vascular endothelial function, as assessed using brachial artery flow-mediated dilatation (Chambers et al., 2000). Supplementation with Vitamin B_6, B_{12} and folate reduced carotid intima-media thickness in patients at cardiovascular risk compared to that in a healthy age-matched control group with similar plasma homocysteine concentrations (Till et al., 2005). Indeed, oral supplementation of folic acid alone or its active form equally restored endothelial function in hypercholesterolemic (Verhaar et al., 1998, 1999) and in diabetic patients (Mangoni et al., 2005). Furthermore, in patients that had coronary artery disease, a high oral dose of folate

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acutely lowered blood pressure and enhanced coronary dilatation (Tawakol et al., 2005). Those effects were independent of a reduction in homocysteine levels. The underlying mechanism of the biological action might be complex. Indeed, folates possess some anti-oxidative capacity (Verhaar et al., 1998), have an ameliorative effect on dysfunction of endothelial NO synthase (Stroes et al., 2000), and inhibit VCAM-1 expression (Li et al., 2006). All these observations suggest that folate plays a role in the control of the early stages of atherogenesis. The aim of this study was to investigate the effects of folic acid fortification on the development of atherosclerotic lesions in apoE-deficient mice, which spontaneously develop atherosclerosis that has features similar to those observed in humans (Sarria et al., 2006). To investigate the mechanisms involved and changes in atherosclerosis, levels of homocysteine, lipid, lipoproteins, and oxidative stress were analyzed.

Materials and methods

Animals

Two-month-old male homozygous apoE knock-out mice, bred at the *Unidad Mixta de Investigación*, Zaragoza, Spain, were fasted overnight before being anesthetized with isofluorane. To estimate initial plasma cholesterol and triglycerides levels, blood samples were collected using retro-orbital bleeding. Twenty mice were randomly assigned to two groups of ten mice each, and housed in sterile filter-top cages. The groups had similar plasma cholesterol and triglyceride levels. Animals had access to food and water ad libitum. The protocol was approved by the Ethical Committee for Animal Research of the University of Zaragoza.

Treatment

Mouse chow was Teklad Mouse/Rat Diet no. 2014 (Harlan Teklad, Harlan Ibérica, Barcelona, Spain). The ten mice in the control group were fed chow and water, and the ten mice in the treatment group received chow and an aqueous solution of folic acid (Aspol, Interpharma, Barcelona, Spain) that provided each mouse with a dose of 75 μg/kg/d. The aqueous folic acid solution was prepared daily. The treatment was provided for ten weeks and was well tolerated.

Biochemical determinations

After the treatment period, the mice were fasted before being sacrificed by suffocation with CO_2, and blood was drawn from their hearts. Total plasma cholesterol and triglyceride concentrations were measured in a microtiter assay, using commercial kits from Sigma Chemical Co. (Madrid, Spain). Plasma homocysteine concentrations were assayed using a time-resolved immunofluorimetric assay (IMx, Abbott Diagnostic, Madrid). Total isoprostane 8-iso-$PGF_{2\alpha}$ was measured according to the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI). Paraoxonase was assayed using arylesterase activity following the protocol previously described (Acín et al., 2005). Results were expressed as μmol phenylacetate hydrolyzed per minute per liter (IU/l). The apolipoproteins A-I, A-II, A-IV, and B were quantified using ELISA and specific polyclonal antibodies (Biodesign, Saco, ME, and Santa Cruz Biotechnology, Santa Cruz, CA), as described elsewhere (Arbonés-Mainar et al., 2006; Navarro et al., 2005). All of the samples were analyzed on the same day, and all of the assays were performed in triplicate. In all cases, the intra-assay CV was <4%.

To analyze lipoprotein profiles, 100 μl of pooled samples from within each dietary group were subjected to fast protein liquid chromatography gel filtration using a Superose 6B column (Amersham Biosciences, Barcelona, Spain) (Calleja et al., 1999), and concentrated (see Navarro et al., 2004).

Low-density lipoprotein oxidation susceptibility

LDL oxidizability was measured following the method of Spranger et al. (1998). Briefly, 60 μl of lipoprotein fraction (2.3 μg of cholesterol) was diluted with 275 μl of phosphate-buffered saline (PBS). To initiate oxidation, 3.3 μl of 1 mM $CuSO_4$ solution was added. The susceptibility of lipoproteins to oxidation was continuously monitored by following changes in absorbance at 234 nm every 10 min for 4 h, and lag phase and oxidation rate were calculated using ε_{cd}=29,500 M^{-1} cm^{-1} as the extinction coefficient of conjugated dienes.

RNA preparation and analysis

Immediately after the mice were sacrificed, their livers were removed and frozen in liquid nitrogen. RNA was isolated using Trigent reagent MRC (Cincinnati, OH, USA) according to the manufacturer's instructions. Total RNA was subjected to Northern blot analysis (see Osada and Maeda, 1998). The cDNA probes used for *Apoa1*, *Apoc3* and *Apoa4* genes were described by Acín et al. (2005) and Arbonés-Mainar et al. (2006). To normalize the amount of RNA, a mouse *β-actin* fragment (Acín et al., 2005) was used. Labelling and quantification followed the methods described by Acín et al. (2005).

Evaluation of atherosclerotic lesions

Hearts were perfused first with phosphate-buffered saline and then with phosphate-buffered formalin (4%, pH 7.4, Panreac, Barcelona, Spain) under physiological pressure. The bases of the hearts were collected and processed for aortic cross-sectional analysis (as described by Calleja et al., 1999). In brief, the base of the heart and the aortic roots were embedded in paraffin and 4-μm serial sections were stained with 0.4% orcein in acid 70% ethanol for 30 min, then with 0.5% (w/v) carmin indigo in 0.37% picric acid for 5 min (Acín et al., 2005). Images were captured and digitized using a Nikon microscope equipped with a Canon digital camera. Morphometric evaluations were based on average lesion sizes from four cross-sections and using Scion Image software (Scion Corporation, Frederick, MD, USA).

Table 1
Effects of folic acid on blood plasma parameters in apoE knockout mice

	Control ($n=10$)	Folic acid ($n=10$)
Homocysteine (μmol L^{-1})	5.9±1.5	5.8±1.3
Total cholesterol (mmol L^{-1})	10.5±2.5	13.9±3.5*
Triglycerides (mmol L^{-1})	1.5±0.4	1.4±0.3
Apo A-I (AU L^{-1})	108±15	125±9*
Apo A-II (AU L^{-1})	117±22	135±13*
Apo A-IV (AU L^{-1})	90±10	110±20*
Apo B (AU L^{-1})	117±6	104±13*
8-iso-prostaglandin $F_{2\alpha}$ (pg mL^{-1})	280±42	144±5*
Arylesterase activity × 10^3 (UI L^{-1})	91±16	94±12

Results are expressed as mean±SD. Mice were fed chow (control) or folic acid diet for 10 weeks and fasted overnight before blood collection. Statistical analyses were done according to Mann–Whitney *U*-test. *, $P<0.05$ vs. control.

Statistical analysis

Statistical comparisons were made using the Mann–Whitney *U*-test, and differences were considered significant when $P<0.05$. A multiple regression analysis was performed using Instat 3.02 software for Windows (GraphPad, San Diego, CA, USA).

Results

Effect of folic acid on blood plasma parameters

After ten weeks of the folic acid treatment, homocysteine levels remained unaltered (Table 1). Average plasma cholesterol levels were higher in the folic acid-treated group than in the control group. The folic acid supplementation did not induce significant changes in triglycerides. In the treatment group, the levels of apolipoproteins A-I, A-II, and A-IV appeared to increase significantly, but apoB levels decreased significantly.

Dietary folic acid increased VLDL and LDL cholesterol (Fig. 1A), which corroborates the increase in total cholesterol (Table 1); however, the decrease in apoB was observed in VLDL particles only (Fig. 1B). The decrease in apoB, coupled with the increase in VLDL and LDL cholesterol, indicates that apoB-containing particles carry more cholesterol because of the administration of folic acid. In folic acid-treated mice, the HDL profile shifted to the right, which suggests an increase in the population of small HDL (Fig. 1A).

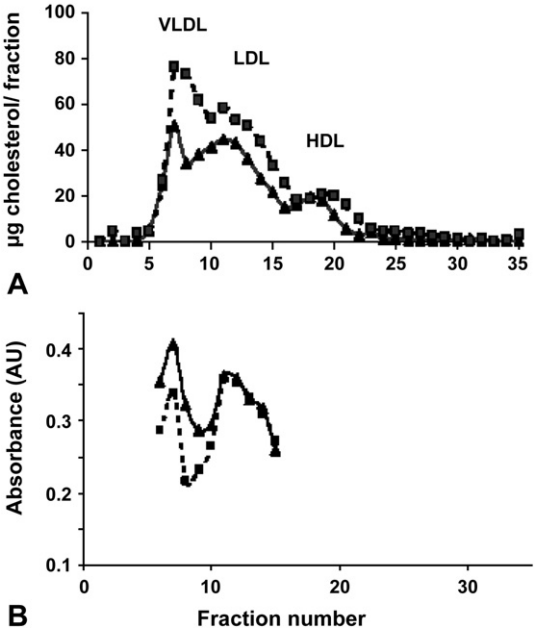

Fig. 1. Plasma lipoprotein profiles in apoE-deficient mice. Lipoproteins were fractionated using FPLC (three independent pools for each dietary condition) in control (triangles) and folic acid treatment (squares) groups. (A) Representative patterns of the cholesterol distributions are shown. The results are expressed as μg of cholesterol per fraction. (B) ApoB distribution. The results are expressed as arbitrary unit of absorbance per fraction.

Evaluation of susceptibility to oxidation and oxidative status

Fig. 2 shows the susceptibility to oxidation of LDL incubated with $CuSO_4$ expressed as the lag time of diene accumulation and the oxidation rate. LDL from folic acid-treated mice exhibited a significant increase in the lag time of diene accumulation (Fig. 2A) and a lower oxidation rate (Fig. 2B) compared to that from mice in the control group. As assessed using plasma 8-iso-prostaglandin $F_{2\alpha}$ levels, general oxidative status decreased in folic acid-treated mice (Table 1). To assess the effect of folic acid on the antioxidative mechanisms, we measured paraoxonase, an antioxidant enzyme in HDL. The

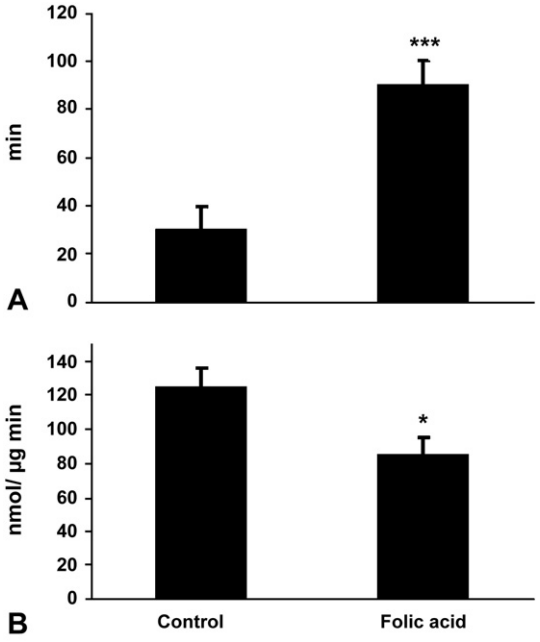

Fig. 2. Indices of oxidizability of low-density lipoproteins in apoE-deficient mice. A) Lag time of diene accumulation expressed as minutes, B) oxidation rate expressed as nmol of conjugated dienes/μg cholesterol/min. LDL from control and treatment (folic acid) groups were incubated with 10 μM $CuSO_4$ at 37 °C to induce oxidation. Absorbance at 234 nm was continuously monitored and used to calculate both indices. Data are presented as mean± SEM. *** $P<0.001$ and *$P<0.05$ vs. control LDL according to Mann–Whitney *U*-test.

administration of folic acid did not influence the arylesterase activity of paraoxonase (Table 1).

Quantification of lesion area

Fig. 3 (A and B) shows representative atherosclerotic foci of untreated and folic acid-treated mice at the end of the 10-week experiment. When the atherosclerotic lesions of the 18-week-old mice were observed at a higher magnification (Fig. 3C and D), the dramatic effect of folic acid on the characteristics of lesions was evident. The infiltration of foam cells into the intima, the alteration of elastin of media, and the presence of collagen were much less evident in the folic acid-treated mice than in the untreated mice. These differences indicate a more stable plaque after the 10-week treatment. A summary of the atherosclerotic lesion areas in all of the mice is presented in Fig. 3E. The administration of folic acid significantly reduced the amount of lesion area in apoE-deficient mice.

RNA analysis

Hepatic expressions of the apolipoprotein *a1/c3/a4* gene cluster were determined using northern blot analyses. Results in Fig. 4 are expressed as arbitrary units and refer to the level of *β-actin*. The hepatic expression of *apoa1* and *apoc3* did not differ between the two groups, but *apoa4* was induced by the folic acid supplement (Fig. 4).

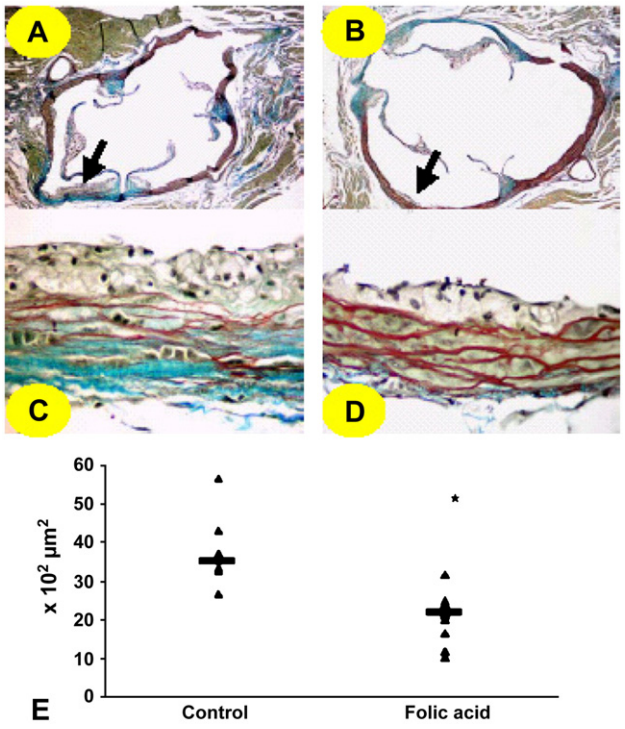

Fig. 3. Aortic atherosclerotic lesions in untreated (control) and treated (folic acid supplement) apoE knockout mice. Representative micrographs of aortic sections from control A) and folic acid treated mice B) at 40× magnification. Arrows indicate presence of atherosclerotic foci. Larger magnification (600×) of lesions from control C) and folic acid D) groups. E) Individual data of cross-sectional analysis of aortic lesion areas and mean for control ($n=9$) and folic acid groups ($n=9$). Statistical analyses were done according to Mann–Whitney *U*-test. *, $P<0.05$ vs. control.

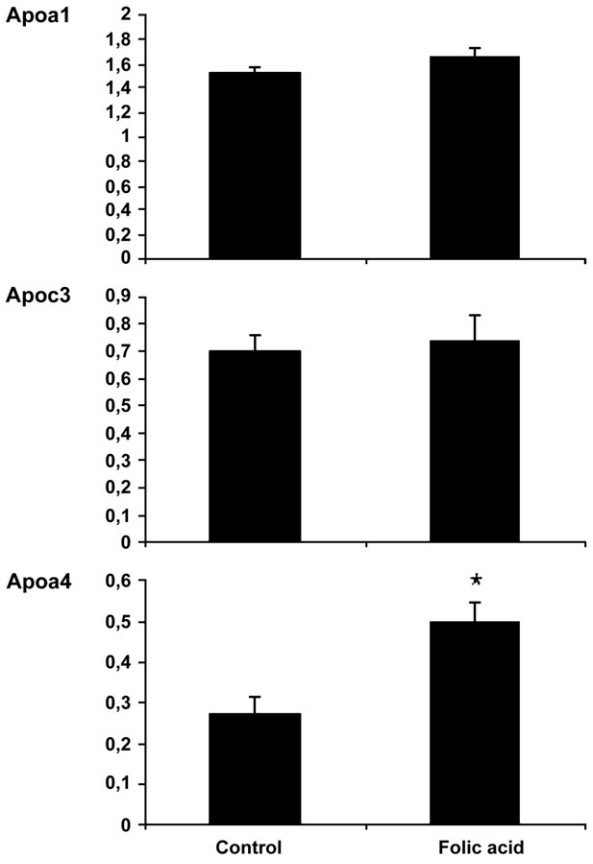

Fig. 4. Effects of folic acid on hepatic *apoa1/c3/a4* gene cluster mRNA expressions. Results are expressed as mean±SEM of arbitrary absorbance units normalized to the *β-actin* gene expression. Five micrograms of total RNA from animals was subjected to northern analysis as described in Methods. Statistical analyses were done according to Mann–Whitney U-test. *, $P<0.05$ vs. control.

Discussion

In this study, we demonstrated that an aqueous solution of folic acid decreased the area of atherosclerotic lesions in apoE knockout mice, independently of homocysteine levels. That reduction was paralleled by a decrease in the number of VLDL particles, as estimated by the apoB content, despite being enriched in cholesterol. In addition, oxidative stress decreased significantly, HDL apolipoproteins increased, and the size of HDL particles decreased. Overall, these findings show a unique picture of favorable lipoprotein metabolism, which might explain the observed patterns of atherosclerosis after folic acid treatment.

In the folic acid-treated mice, total plasma cholesterol increased, which was reflected in the plasma lipoprotein profile (Fig. 1A). The decrease in plasma apoB concentrations in treated mice (Table 1) indicates that the apoB-containing particles of folic acid-treated mice carried more cholesterol. This effect was more pronounced in VLDL than in LDL particles because, in the former fraction, there was a decrease in apoB (Fig. 1B), whereas levels of apoB in LDL particles were similar

to those in untreated mice. This metabolic setting suggests that folic acid supplements could induce the secretion of large-sized VLDL, and that some of these particles are selectively metabolized. For that reason, plasma triglycerides are kept constant and a similar number of apoB-containing particles are present in the LDL fraction. That scenario is consistent with the enhanced lipolytic action observed by Povoa et al. (1984). Large apoB-containing particles might be less atherogenic because they have greater difficult traversing the endothelial barrier (Veniant et al., 2001); however, large particles also have a spatial hindrance to entering the subendothelial space and, therefore, might be more susceptible to oxidization, particularly in apoE-deficient mice, in which the ligand of apoB48-containing particles are absent and their half-life is extended (Sarria et al., 2006). However, in our study, we estimated the general oxidative status by measuring plasma levels of 8-iso-prostaglandin $F_{2\alpha}$, a reliable marker of oxidant stress in vivo (Lawson et al., 1999), and found these levels to be decreased; moreover, LDL from folic acid-treated mice were particularly resistant to Cu^{2+}-induced oxidation (Fig. 2). The latter experiments were performed using isolated LDL, which indicates that folic acid supplements endowed these particles with some antioxidant protective mechanism. The increased plasma levels of apoA-I and apoA-IV, which have antioxidant (Bonnefont-Rousselot et al., 1999; Stan et al., 2003) and antiatherogenic (Duverger et al., 1996; Paszty et al., 1994) properties, might explain the observed atherosclerotic findings, and might neutralize the negative impact of apoA-II on atherosclerosis (Arbonés-Mainar et al., 2006). Indeed, in our study, the multiple regression analysis indicated that 63% ($P<0.01$) of the variance in the area of atherosclerotic lesions was explained by changes in the levels of apolipoproteins A-I, A-IV, and B. Thus, our data support the notion that large apoB-containing lipoproteins are less atherogenic in the presence of increased apo A-I and A-IV levels.

We found that the folic acid treatment increased apolipoprotein A-I (Table 1), which is 70% of the protein in HDL (Assman and Nofer, 2003), and shifted the HDL profile to the right (Fig. 1A), which indicates that these particles have less cholesterol. The increase in plasma apolipoprotein A-I, despite the absence of change in hepatic *apoa1* mRNA expression (Fig. 4), suggests that other post-transcriptional mechanisms, such as translation efficiency (Azrolan et al., 1995), or post-translational mechanisms, such as packaging with cholesterol and phospholipids and secretion (Zheng et al., 2005), might be influenced by folic acid, as well as a selective plasma catabolism of apoA-I containing particles. In addition, folic acid treatments influenced hepatic *apoa4* mRNA expression, which increased following treatment and might explain the increase in plasma apolipoprotein levels. This might be another mechanism in the protective action of folic acid and a new regulation of apoA-IV gene expression not associated with dietary lipid compounds (Stan et al., 2003).

Our experiment provides evidence that exogenous administration of folic acid decreased atherosclerosis in apoE-deficient mice just as it had beneficial effects on endothelial function in hypercholesterolemic (Verhaar et al., 1998, 1999) and in diabetic patients (Mangoni et al., 2005), and on blood pressure in patients that had coronary artery disease (Tawakol et al., 2005). Furthermore, our experiment demonstrated that the beneficial effect acts independently of homocysteine lowering, in agreement with reports in apoE-deficient mice fed a diet supplemented with vitamins B_{12}, B_6, and folate (Zhou et al., 2003) and in humans (Doshi et al., 2002; Durga et al., 2005; Tawakol et al., 2005). In our experiment, the folic acid intervention occurred at the early stages of lesion development (fatty streak) and folic acid was particularly efficient in retarding the recruitment of macrophages (Fig. 3C and D) pointing out a role of this compound early in the atherosclerotic process. At that time, two potential targets might be involved. On the one hand, the reduced expression in endothelial cells of adhesion molecules (Li et al., 2006) or the necessity of folate to these cells in keeping their full phenotype (Brown et al., 2006; Doshi et al., 2001) and on the other hand, the direct action of folic acid on monocytes and macrophages (Au-Yeung et al., 2006; Wang et al., 2005). In a large Canadian study on humans, folic acid fortification lowered stroke mortality (Yang et al., 2006), while folic acid supplementation had no effect in secondary prevention of cardiovascular disease (Loscalzo, 2006; Yang et al., 2006). Collectively, these observations suggest a cardiovascular benefit in fortifying food with folic acid in primary prevention.

In conclusion, a folic acid supplement decreased the atherosclerotic lesion area in apoE knockout mice, independently of homocysteine levels, concomitant with a remodelling of apoB-containing particles and high-density lipoproteins, and a general decrease in oxidative stress. Although more studies are required, the inclusion of folates in functional foods might provide an important means of reducing atherosclerotic burden.

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