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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).