Disodium Ascorbyl Phytostanyl Phosphate Reduces Plasma Cholesterol Concentrations and Atherosclerotic Lesion Formation in Apolipoprotein E–Deficient Mice

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Disodium ascorbyl phytostanyl phosphate (FM-VP4) consists of ascorbic acid covalently bound to phytostanols by a phosphodiester linkage and is derived as the disodium salt. The purpose of this study was to evaluate the lipid-lowering and antiatherosclerotic properties of FM-VP4 following administration to apolipoprotein E (ApoE)-deficient mice. Four-week-old male C57BL/6J mice with a homozygous deletion of the *ApoE* gene (apolipoprotein E knock-out) were administered 0 (control), 0.1%, 0.5%, 1.0%, and 2.0% (wt/vol) FM-VP4 in their drinking water or 2.0% FM-VP4 (wt/wt) in their diet for 12 consecutive weeks. All animals received a standard mouse chow diet consisting of 9.0% (wt/wt) fat and 0.2% (wt/wt) cholesterol. Plasma cholesterol and triglyceride levels were determined at baseline and at 4-week intervals (4, 8, and 12 weeks) throughout the term of the study. At the end of the study, mice were killed using CO_2 gas, and blood was taken from the heart. The heart and aorta were removed and sections of the aortic roots were stained with oil red O (ORO) and Movat's stain. The lesions found in this area were measured using a computer-assisted image analysis. Consumption of FM-VP4 by either food or drinking water routes was associated with an approximately 75% reduction in total plasma cholesterol levels and a 75% decrease in aortic atherosclerotic lesion area in ApoE-deficient mice over 12 weeks compared to controls. A trend in decreasing plasma triglyceride levels was also observed. Taken together these data suggest that FM-VP4 has both lipid-lowering and antiatherosclerotic properties following 12-week administration to ApoE-deficient mice.

ELEVATED PLASMA cholesterol levels have been associated with increased risk of atherosclerosis and coronary artery disease.[1-3] Furthermore, the decrease in low-density lipoprotein (LDL) cholesterol levels within hypercholesterolemic patients has been shown to significantly decrease the number of myocardial events in both primary and secondary prevention studies.[4-6] The current major strategy for cholesterol lowering in humans involves the use of inhibitors of hydroxymethyl glutaryl coenzyme A (HMG CoA) reductase known as statins.[7] However, recent safety issues related to some third-generation statins and the observation that not all individuals respond similarly to statins[8-10] has prompted a revised interest in hypolipidemic agents whose mechanism of action is distinct from these compounds.

It is well established that certain plant sterols and stanols, called phytosterols and phytostanols, can effectively reduce plasma cholesterol levels both in animal models and in humans.[11-15] Furthermore, phytosterol mixtures have been investigated for their effects on the development of atherosclerotic lesions.[16-19] The hypolipidemic, and potentially antiatherosclerotic, properties of unesterified phytosterols described above appear to be dependent on the biological availability in the gut since phytosterols are thought to inhibit uptake by competing with dietary and biliary cholesterol for absorption by the enterocyte.[20,21] However, studies to confirm this hypothesis remain to be undertaken.

Disodium ascorbyl phytostanyl phosphate (FM-VP4) is a water-soluble analog of phytostanols in which ascorbate is covalently bound to the sn-3 position of the phytostanols via a phosphodiester linkage. Previously, we have demonstrated that this compound is capable of significantly decreasing both total and LDL cholesterol levels in the fat-fed gerbil.[22,23] Recent studies from our laboratory have indicated that when rats were coadministered a formula containing FM-VP4 plus both radiolabeled and unlabeled cholesterol incorporated into a lipid emulsion (Intralipid, Baxter Inc, IL), the area under the blood [^{3}H]cholesterol concentration versus time curve and the maximum plasma concentration of [^{3}H]cholesterol were decreased in a dose-dependent manner.[20] Although these studies have clearly demonstrated the hypolipidemic effects of FM-VP4 in experimental animals, the relationship of this finding to the potential development of atherosclerosis is not known. Thus, the purpose of this study was to evaluate the lipid-lowering and antiatherosclerotic properties of FM-VP4 in apolipoprotein E (ApoE)-deficient mice over a 12-week period and to determine the dose-response relationship of these observations in ApoE-deficient mice. This mouse model of atherosclerosis has been used extensively, since mice develop severe hypercholesterolemia and atherosclerotic lesions similar in distribution and appearance to those observed in humans.[24-27]

MATERIALS AND METHODS

Chemicals

FM-VP4 (Lot no. 81699 BRI FM-VP4-01) was prepared by the chemistry group of Forbes Medi-Tech Inc Research Laboratories, Vancouver, Canada. FM-VP4 is a semisynthetic esterified phytostanols derivative, produced as the disodium salt. The 2 major components of FM-VP4 are disodium ascorbyl campestanyl phosphate and disodium

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ascorbyl sitostanyl phosphate. The powdered active ingredient was stored at 4°C and to date has been demonstrated to be stable for up to 12 months under these conditions. Cholesterol was purchased from Sigma-Aldrich (St Louis, MO) and 9% (wt/wt) PicoLab mouse chow was purchased from Jamieson's Pet Food Distributor, Delta, Canada.

Animal Model

Four-week-old male C57BL/6J mice with homozygous deletion of the A*poE* gene (apolipoprotein E knock-out) were purchased from the Jackson Laboratory, Bar Harbor, ME. These animals exhibit severe hypercholesterolemia following the consumption of a high-cholesterol diet.[16,18,28]

Experimental Design

Dose response effect of FM-VP4. This study involved 5 groups of 8 animals each. Each group consumed a control diet of regular mouse chow containing 9% (wt/wt) fat and 0.2% (wt/wt) cholesterol and tap water containing 0, 0.1%, 0.5%, 1.0%, and 2.0% (wt/vol) FM-VP4, respectively. Mice were administered 5, 25, 50, and 100 mg, respectively, in drinking water daily. Allocation of treatment to each group was randomly determined before the start of the study. Homogeneity of groups was validated on the criteria of body weight, plasma cholesterol, and plasma triglyceride on the day of randomization. Animals were weighed weekly and blood drawn from the nonfasting animals at 4, 8, and 12 weeks. FM-VP4 at 2% in food or drinking water as well as lower concentrations in water did not show any side effects. FM-VP4 is well tolerated even at a high daily dose (100 mg/d) without producing diarrhea or other gastrointestinal intolerance signs.

Effects of ascorbic acid and unesterified stanols. Since FM-VP4 comprises vitamin C (ascorbic acid) and phytostanyl moieties, covalently linked by a phosphodiester bridge, it is possible that the hypolipidemic effects might be due to the ability of unesterified stanols to inhibit cholesterol absorption, or the combined effect of free ascorbate and unesterified stanols following cleavage of FM-VP4 into its component parts by digestive lipases.[20,21] Thus, we have compared the effects of FM-VP4 with equivalent amounts of ascorbic acid or phytostanols given individually or together in the diet. After a 10-day adaptation period, 48 male apoE-KO mice were divided into 6 groups of 8 mice each and had similar initial mean plasma cholesterol levels and body weight. For 12 weeks, the animals were fed PicoLab mouse diet containing 0.2% wt/wt cholesterol with the following supplements: (1) without additional supplements (control); (2) with 2% wt/wt FM-VP4; (3) without additional supplements but with 2% wt/vol FM-VP4 in the drinking water; (4) with 0.7% vitamin C (ascorbic acid); (5) with 1.3% FM-3P4 (parent phytostanols); or (6) with 0.7% vitamin C and 1.3% FM-3P4. The dosages used in groups 4, 5, and 6 were designed to provide equivalent amounts of ascorbate and phytostanols to that provided by 2% FM VP4 in the diet.

Diet Preparation and Animal Care

Diet preparation was as previously published.[16,18,28] The Animal Care Committee of the University of British Columbia approved the study. The concentration of FM-VP4 in food was confirmed at the beginning of study. A 15- to 20-g quantity of the control food was collected in glass containers, labeled accordingly, and stored at 4°C. Since the test substance in water was prepared every week in house, there was no indication for further assessment. Body weight was measured every week for the entire duration of the study. Considering that the amount of food and water intake is 5 g and 5 mL, respectively, mice were administered about 100 mg FM-VP4 each day, equivalent to 2% of the diet.

Collection of Blood and Tissues

Preparation and analysis of plasma. Blood was sampled from the tail vein and collected in EDTA tubes at each time point throughout the study. Blood cells were pelleted by centrifugation and plasma was harvested. Plasma cholesterol and triglyceride levels were determined at baseline and at 4-week intervals until the end of the study using enzymatic kits (Boehringer Mannheim, Germany) and methods as previously described.[22,23]

Quantitative analysis of atherosclerotic lesions. At the end of each study, the mice were killed using CO_2 gas, and blood was taken from the right ventricle. The hearts of the animals were perfused slowly with 1 mL 10% buffered formalin solution through the left ventricule. The heart and aorta were removed and placed in 10% buffered formalin. Tissues surrounding the aorta including all fat were trimmed, hearts frozen in liquid nitrogen, and the aorta cut transversely at the aortic root, in 10 μmol/L sections.[16] Slides were stained with oil red O (ORO), Movat's pantachrome, and hematoxylin-eosin. The lesional area was measured using a computer-aided image analysis system (Image-Pro Plus, Spot Diagnostic Instruments, San Diego, CA) with a magnification factor of 6x in a blinded fashion. Each slide had 4 sections and the measurements were done twice; the means were subjected to statistical analysis.

Statistical Analysis

Results were expressed as mean ± SD. Statistical analyses were conducted using a 2-tailed Student's *t* test, and assuming unequal variance was used to assess the differences between the FM-VP4 treatment groups and the untreated control group. A *P* value of less than .05 indicated a significant difference between groups.

RESULTS

Dose Response Effect of FM-VP4

Body weight increased by the same amount over the experimental period in all treated and control groups as a result of similar food and water consumption throughout the duration of the study (at baseline the body weight was 18.2 g to 20.0 g and after 12 weeks was 25.5 g to 28 g).

Plasma total cholesterol levels. The effects of FM-VP4 on plasma total cholesterol levels are shown in Fig 1A. Control ApoE-deficient mice exhibited high plasma cholesterol levels compared to baseline. The total plasma cholesterol levels in mice administered FM-VP4 0.5%, 1%, and 2% (wt/wt) in their diet were significantly reduced at 4, 8, and 12 weeks compared to nontreated controls ($P < .005$).

Plasma triglycerides. The effects of FM-VP4 on plasma triglyceride levels are shown in Fig 1B. Total plasma triglyceride levels in mice administered FM-VP4 2% (wt/wt) in their diet were significantly reduced at 4 and 8 weeks compared to controls ($P < .05$). No significant differences between all other FM-VP4 treatment groups and controls were observed.

Atherosclerotic lesions. The extent of aortic atherosclerotic lesions detected in control and FM-VP4–treated mice at the end of the study is shown in Fig 2A and B. A reduction of atherosclerotic lesions by 27% in the FM-VP4 0.1% group ($P < .005$) and a reduction of atherosclerotic lesions by 80% in the FM-VP4 0.5%, 1%, and 2% treatment groups ($P < .0005$) was observed compared to controls.

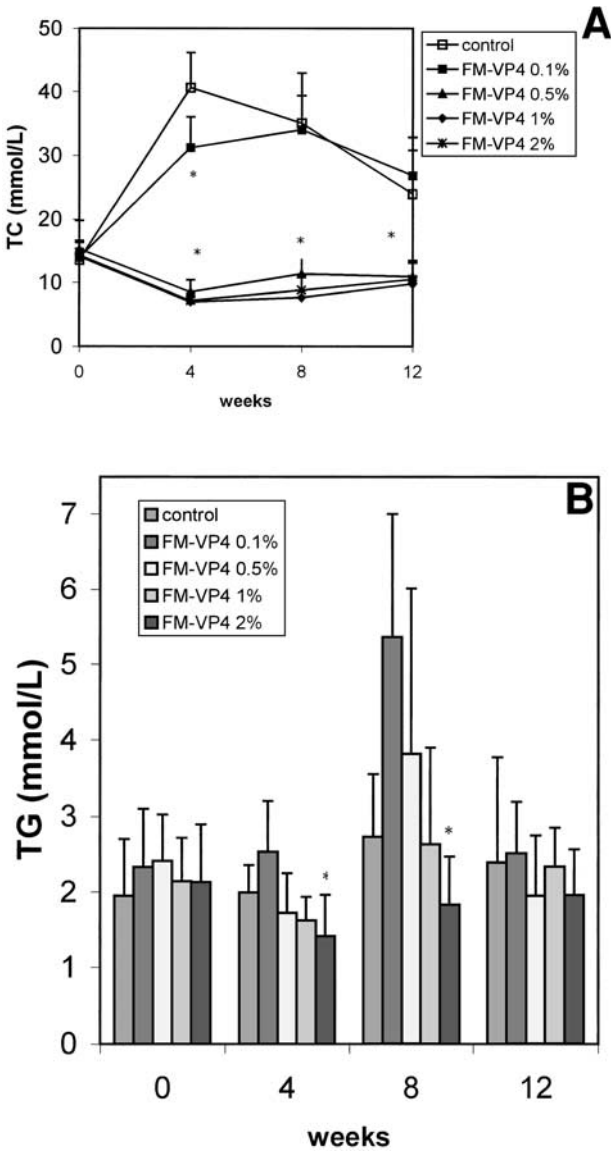

Fig 1. (A) Effects of FM-VP4 on plasma total cholesterol. *P < 0.005 for water-solubilized FM-VP4 *v* control. Total plasma cholesterol levels in mice administered FM-VP4 0.5%, 1%, and 2% (wt/wt) in their diet were significantly reduced at 4, 8, and 12 weeks compared to controls. (B) Effects of FM-VP4 on total triglyceride levels in cholesterol-fed (0.2% wt/wt) apoE-KO mice *P < .05 for water-solubilized FM-VP4 *v* control. Total plasma triglyceride levels in mice administered FM-VP4 2% (wt/wt) in their diet were significantly reduced at 4 and 8 weeks compared to controls. No significant differences between all other FM-VP4 treatment groups and control were observed. N = 8 for each treatment group

Effects of Ascorbic Acid and Unesterified Stanols

Food consumption did not change whether FM-VP4 was added to the diet or water over the 12-week span. Body weight increased similarly in both groups (21.7 to 23 g at baseline and 25.4 to 27.5 g after 12 weeks).

Plasma total cholesterol levels. The effects of FM-VP4 on plasma total cholesterol levels are shown in Fig 3. Control ApoE-deficient mice exhibited very high total plasma cholesterol levels after 12 weeks (30.7 ± 3.9 mmol/L), 1.7 times higher than baseline cholesterol levels (18.1 ± 3.8 mmol/L; $P < .05$). The total plasma cholesterol levels in mice administered dietary or aqueous FM-VP4 were significantly reduced at 4, 8, and 12 weeks ($P < .00001$) compared to the control mice. The most dramatic FM-VP4–induced decrease in total plasma cholesterol levels occurred within the first 4 weeks of the study, when cholesterol levels were reduced from a baseline level of 17 mmol/L to 10 mmol/L. After 12 weeks, plasma cholesterol levels were further decreased to 5.9 and 8.1 mmol/dL in mice administered dietary and aqueous FM-VP4, respectively. For both FM-VP4 formulations, the percent reduction in plasma cholesterol concentrations was consistently close to 75% compared to control mice at weeks 4, 8, and 12 (−80.8% for FM-VP4 in diet, −73.6% for FM-VP4 in water; $P < .00001$). Both parent compounds (FCP-3P4 and ascorbate alone, as well as the combination of FCP-3P4 and ascorbate) were tested. The parent phytostanol compound FCP-3P4 alone or in combination with ascorbate also significantly decreased total plasma cholesterol levels by 24% and 29%, respectively, compared to control mice ($P < .05$). Ascorbate alone increased total cholesterol level in this animal model by 8.5%, but it did not reach statistical significance. In addition, FM-VP4 significantly decreased total plasma cholesterol levels compared to FCP-3P4 alone or in combination with ascorbate ($P < .05$). Taken together these results indicate that FM-VP4 is a chemical entity that remarkably differs in potency compared to parent components.

Plasma triglycerides. The effects of FM-VP4 on plasma triglyceride levels are illustrated in Fig 4. Control mice exhibited no significant reduction in total plasma triglyceride levels from the start of the study (1.6 ± 0.6 mmol/L) and week 12 (1.3 ± 0.6 mmol/L). Dietary and aqueous FM-VP4 had significant ($P < .005$) triglyceride-lowering effects after 4 weeks of administration to ApoE-deficient mice compared to controls. After 8 weeks of FM-VP4 administration, however, significantly reduced ($P < .0005$) plasma triglyceride levels were detectable only in mice administered FM-VP4 in food compared to the control group. A triglyceride-lowering effect of both FM-VP4 formulations was not detectable after 12 weeks of treatment compared to the control group. FCP-3P4 with and without ascorbic acid and ascorbic acid alone had no effect on plasma triglyceride levels.

Atherosclerotic lesions. The extent of aortic atherosclerotic lesions detected in control and FM-VP4-treated mice at the end of the study is shown in Figs 5 and 6. Lesion areas were measured in square millimeters and are presented as the percent of the total area studied. In control mice, the atherosclerotic lesion area covered 33.9% ± 11.5% of the aortic lumen. In contrast, the atherosclerotic lesion area of the aortic lumen in mice administered dietary and aqueous FM-VP4 was significantly decreased compared to control to 11.9% ± 2.5% and 8.6% ± 1.6% of the aortic lumen ($P < .0001$), respectively. Administration of the parent compound FCP-3P4 with or without ascorbate resulted in a significant decrease in atherosclerotic lesion size by 30% ($P < .05$) compared to control. In addition, FM-VP4 treatment significantly decreased atherosclerotic lesion area ($P < .05$) compared to FCP-3P4 with or

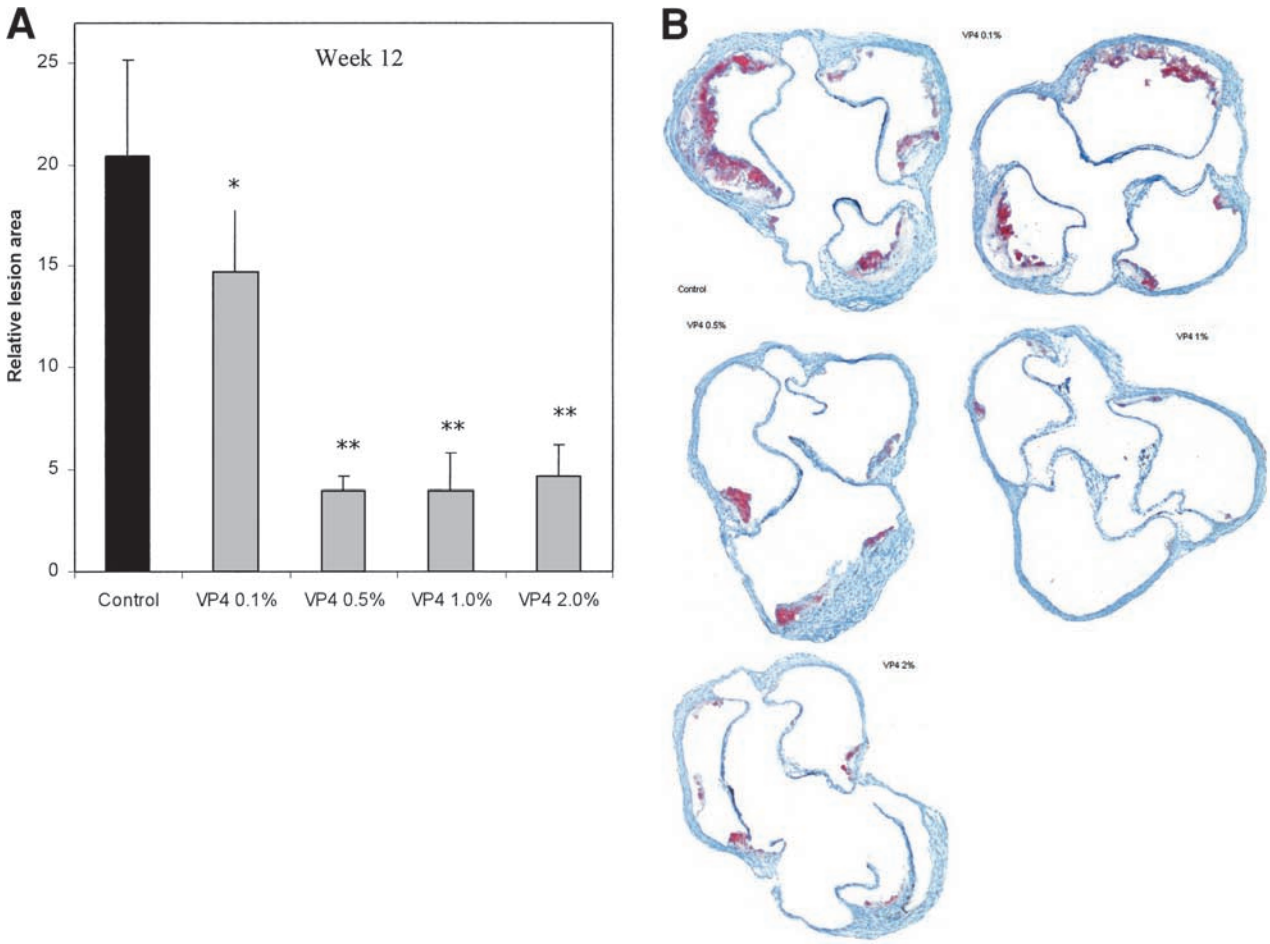

Fig 2. (A) Change in atherosclerotic lesion area in apoE-KO mice on a cholesterol-containing diet (0.2%) with up to 2% FM-VP4 coadministered in the diet or drinking water compared to mice not treated with FM-VP4 (control). *P < .005 for dietary and water-solubilized FM-VP4 *v* control and **P <.0005 for dietary and water-solubilized FM-VP4 *v* control. A reduction of atherosclerotic lesions by 27% in the FM-VP4 0.1% group and reductions of atherosclerotic lesions by 80% in the FM-VP4 0.5%, 1%, and 2% treatment groups were observed compared to controls. N = 8 for each treatment group. (B) Dose-response relationship between FM-VP4 (control, 0.1%, 0.5%, 1.0%, and 2.0% wt/wt) and lesion development. Representative photomicrographs of aortic roots of one mouse in each treatment group. A reduction of atherosclerotic lesions by 27% in FM-VP4 0.1% group and reductions of atherosclerotic lesions by 80% in the FM-VP4 0.5%, 1% and 2% treatment groups were observed compared to controls. N = 8 for each treatment group.

without ascorbic acid. Ascorbic acid added separately to the diet formulation did not positively influence the prevention of atherosclerotic plaque formation.

DISCUSSION

The purpose of this study was to evaluate the lipid-lowering and antiatherosclerotic properties of FM-VP4 following administration to ApoE-deficient mice. Administration of FM-VP4 by either food or drinking water was associated with an approximately 75% reduction in total plasma cholesterol levels and a 75% decrease in aortic atherosclerotic lesion area over the 12-week study period compared to controls with no apparent side effects. A trend in decreasing plasma triglyceride levels was also observed. These observations were specific to FM-VP4 and could not be explained by the presence of related stanols or ascorbate.

It is well established that ApoE-deficient mice are hyper responsive to dietary cholesterol and the consumption of a high-fat/cholesterol diet would result in hypercholesterolemia and accelerated development of atherosclerosis in these animals.[24,27] Initial acute studies by our group reported that "tall oil"–derived phytosterols (2% wt/wt) significantly lowered plasma cholesterol concentration by 33% and reduced the atherosclerotic lesion area in ApoE-deficient mice fed a cholesterol-enriched diet for 18 weeks compared to untreated controls.[16] A recent study by Volger et al reported that plant sterols (1% wt/wt in diet) derived from vegetable oil (sitostanol 65.7%, campestanol 30.1%), wood (sitostanol 87.6%, campestanol 9.5%), or a mixture of vegetable oil and wood (sitostanol

73%, campestanol 24.7%) significantly reduced plasma cholesterol concentration (42% to 51%) and atherosclerotic lesion area (78% to 97%) in ApoE*3-Leiden transgenic mice compared to untreated controls.[19] However, 38 weeks of treatment were required to obtain these findings.

In the current study, we observed that FM-VP4 was effective at a concentration of 0.5% wt/wt in decreasing plasma cholesterol levels by approximately 75% (Fig 1A) and atherosclerotic lesion area by 75% compared to controls (Fig 2A and B) following only 4 week of administration. Furthermore, at all concentrations tested we observed that the FM-VP4–induced reduction in cholesterol was associated with a marked decrease in atherosclerotic lesion area. Lipid composition and cholesterol clefts were less apparent in aortic sections from both treated groups (Fig 6A and C) as compared to lipid-rich lesion in controls (Fig 6B). Taken together these findings suggest that FM-VP4 appears to be more potent than the unesterified sterols and stanols previously reported in the literature[16,19] and from our preliminary studies with unesterified sterols (FCP-3P4) and ascorbic acid alone (Figs 3 through 5). The mechanism(s) by which these findings occur cannot be defined by this study. However, we have observed increased cholesterol excretion in the feces of animals administered FM-VP4 (data not shown) suggesting that prevention of cholesterol gastrointestinal absorption as a potential mechanism. Studies to further investigate the mechanism(s) of action are ongoing.

An interesting observation was that at 0.1% (wt/wt) FM-VP4 did not alter plasma cholesterol and triglyceride levels (Fig 1A and B); however, a significant reduction in the size of atherosclerotic lesions was observed (Fig 2A and B). This leads to the hypothesis that the antiatherosclerotic effect of FM-VP4 may be independent of the lipid-lowering effect.

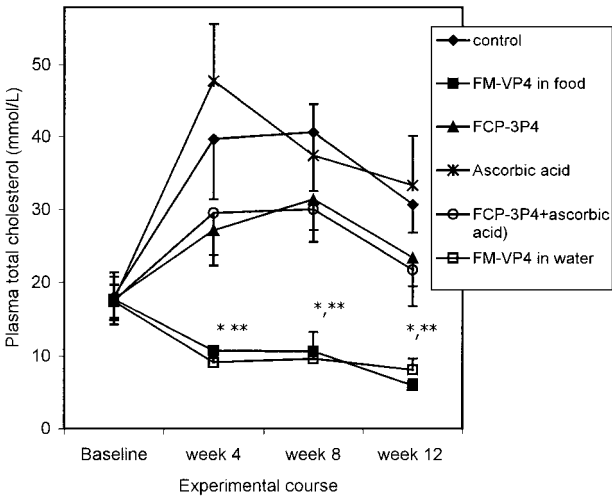

Fig 3. Effects of FM-VP4 on plasma total cholesterol levels in cholesterol-fed (0.2% wt/wt) apoE-KO mice. *$P < .00001$ for dietary or water-solubilized FM-VP4 *v* control. **$P < .05$ for dietary or water-solubilized FM-VP4 *v* FCP-3P4 or FCP-3P4 + ascorbic acid. Total plasma cholesterol levels in mice administered dietary or aqueous FM-VP4 were significantly reduced at 4, 8, and 12 weeks compared to FCP-3P4 with or without ascorbic acid and control. N = 8 for each treatment group

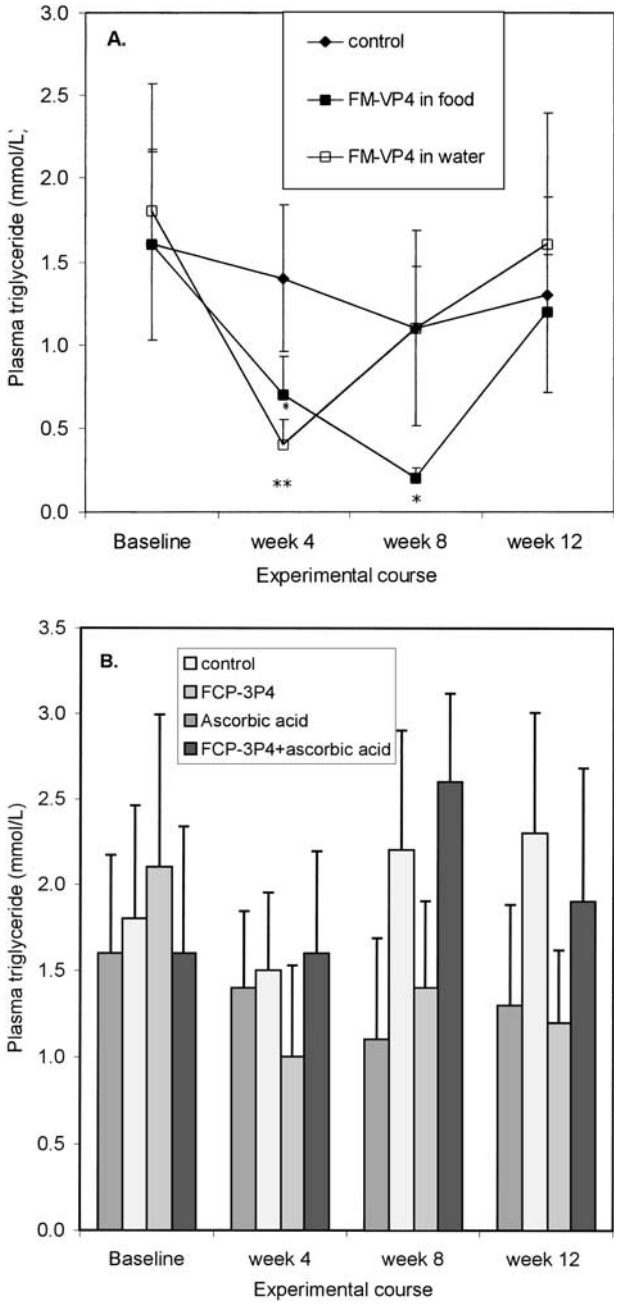

Fig 4. Effects on plasma triglyceride levels in cholesterol-fed (0.2% wt/wt) apoE-KO mice. (A) Administration of FM-VP4 (0.2% wt/wt) in their diet (food or water). (B) Administration of FCP-3P4 (unesterified phytostanols) and ascorbic acid in their diet. *$P < .005$ for dietary FM-VP4 *v* control. **$P < .0005$ for dietary and water-solubilized FM-VP4 *v* control. Dietary and aqueous FM-VP4 had significant triglyceride-lowering effects after 4 weeks of administration compared to control. Only dietary FM-VP4 significantly decreased plasma triglyceride levels compared to controls after 8 weeks of administration. FCP-3P4 with or without ascorbic acid and ascorbic acid alone did not significantly alter triglyceride levels at all time points measured compared to control. N = 8 for each treatment group

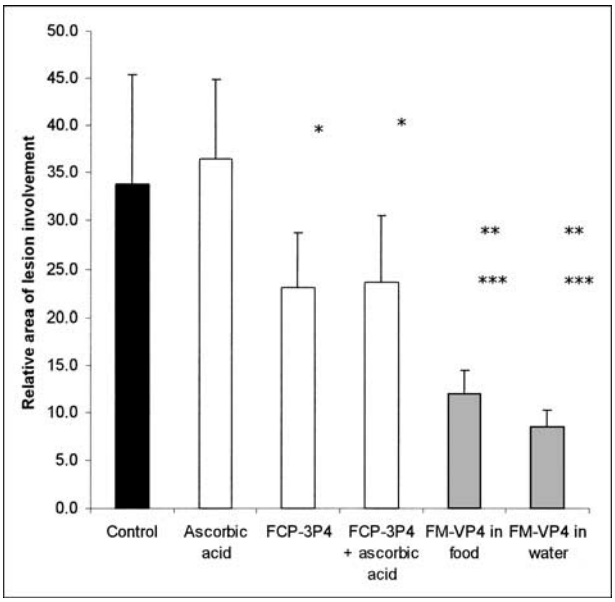

Fig 5. Change in atherosclerotic lesion area in apoE-KO mice on a cholesterol-containing diet (0.2%) with 2% FM-VP4 coadministered in the diet or drinking water for 12 weeks compared to mice not treated with FM-VP4 (control). *$P < .05$ for FCP-3P4 ± ascorbic acid *v* control. **$P < .0001$ for FM-VP4 in food and water *v* control. ***$P < .05$ for FM-VP4 *v* FCP-3P4 ± ascorbic acid *v* control. Administration of dietary and aqueous FM-VP4 resulted in a significant decrease in atherosclerotic lesion size compared to FCP-3P4 with or without ascorbic acid and control. Administration of FCP-3P4 with or without ascorbic acid resulted in a significant decrease in atherosclerotic lesion size compared to control. Ascorbic acid alone did not alter atherosclerotic lesion size. N = 8 for each treatment group

Recent studies have reported the ability of other compounds to delay and/or prevent the development of atherosclerosis independent of changes in serum cholesterol levels. Nagate et al reported that dietary taurine prevented the development of atherosclerosis, independent of serum cholesterol levels in ApoE-deficient mice[29] and Watanabe heritable hyperlipidemic (WHHL) rabbits.[30] They speculated that the antioxidant action of taurine might be the mechanism involved in preventing and/or delaying the onset of atherosclerosis in these animal models. Sumi et al[31] reported that fluvastatin, an HMG-CoA reductase inhibitor, delayed the onset of atherosclerosis in rabbits through the improvement of nitric oxide bioavailability suggesting its effects may be due to nonlipid factors. Bracht et al recently reported that the dihydropyridine calcium antagonist isradipine has antiatherosclerotic effects and improves endothelium-mediated nitric oxide–dependent vasodilation in hypercholesterolemic patients independent of changes in lipids or blood pressure.[32] Taken together these studies suggest that there are a number of different mechanisms involved in the delay and/or prevention of atherosclerosis other than just lipid lowering. Determining if FM-VP4 exhibits these potential mechanisms merits investigation.

In conclusion, this study describes the cholesterol-lowering and antiatherogenic effects of a water-soluble analogue of plant stanols (disodium ascorbyl phytostanyl phosphates; FM-VP4). Consumption of this drug in the diet reduced plasma cholesterol levels by approximately 75% and atherosclerotic lesion area by 75% in cholesterol-fed ApoE-deficient mice compared to controls. Studies to investigate the mechanism(s) of action underlying to explain these findings are currently ongoing.

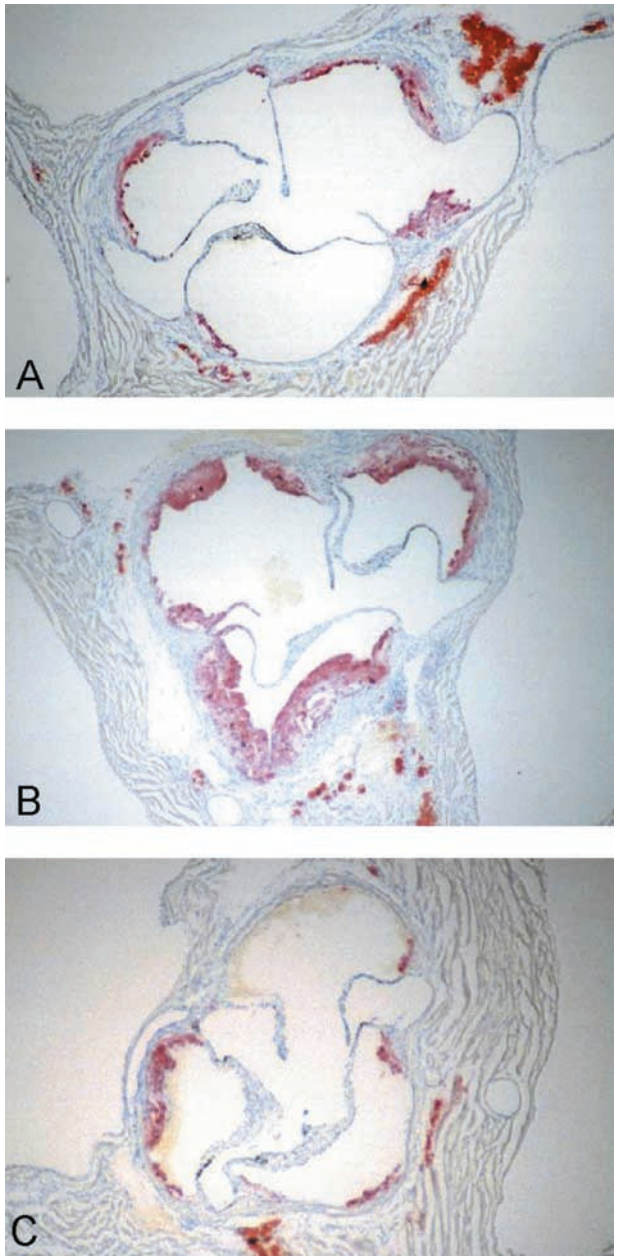

Fig 6. Representative photomicrographs of aortic roots of one mouse in each treatment group over 12 weeks; FM-VP4 2% dietary treatment (A), control (B), and FM-VP4 2% water treatment (C). Severity and lipid component of the lesions are significantly reduced in both treatment groups (A & C) as compared to controls (B). ORO; original magnification × 10.

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