

Fatty Acid Bile Acid Conjugates Inhibit Atherosclerosis in the C57BL/6 Mouse Model

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Key Words

Atherosclerosis · C57BL/6 mice · Cholesterol · Fatty acid bile acid conjugates

Abstract

Objective: The aim of the current research was to study whether fatty acid bile acid conjugates (FABACs) have a beneficial effect on atherosclerosis progression and blood lipid levels in mice. **Methods:** C57BL/6 female mice, fed a high-fat Paigen diet, were administered an oral dose of FABAC or DDH₂O daily. Quantification of atherosclerotic fatty-streak lesions at the aortic sinus was performed. **Results:** The FABAC-treated mice showed a significant reduction in the atherosclerotic lesion areas as compared to the control group ($p = 0.019$). A significant elevation in total cholesterol levels was observed in both the FABAC and control groups. Higher FABAC levels were measured in the high-density lipoprotein fraction as compared to the very-low-density and low-density lipoprotein fractions. **Conclusion:** Our findings demonstrate that FABACs, given orally, reduce the development of atherosclerosis in mice fed a high-fat high-cholesterol diet, despite a lack of effect on plasma lipid levels.

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Introduction

Fatty acid bile acid conjugates (FABACs) are a new family of synthetic molecules designed to solubilize cholesterol in bile and blood. In vitro, they were shown to retard and prevent cholesterol crystallization in supersaturated model lipid solutions and human bile [1]. FABACs were also shown to dissolve preexisting cholesterol crystals. In experimental animals, FABACs prevented cholesterol crystallization in gallbladder bile and dissolved preexisting crystals. In mice fed a lithogenic diet, FABACs prevented the formation of cholesterol gallstones [2] and dissolved preexisting cholesterol gallstones [3].

Atherosclerotic vascular disease is the major cause of mortality in Western societies, regardless of the recent advances in medical treatments. Atherosclerosis begins with the formation of fatty streaks, consisting of cholesterol-laden macrophages known as foam cells, beneath the endothelial cells that line the arteries. This early lesion progresses to the more advanced fibrous plaque, which is characterized by the presence of smooth muscle cells. In advanced lesions, the core becomes necrotic and lipids are released into the intima, where cholesterol crystals may form [4]. While it is a complex and multifactorial disease, there is no doubt today that elevated plasma cholesterol levels play a dominant role in atherogenesis. Low-density lipoprotein (LDL) is the major carrier of cholesterol in

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blood, and an elevated level of LDL promotes the formation of fatty streaks in animals and humans [5].

It was recently found that FABACs circulate in the peripheral blood for over 24–48 h after a single intragastric administration [1]. This is thought to be due to a slow extraction rate by the liver. The prolonged circulation in the vascular tree of these compounds, with proven cholesterol-solubilizing activity, inevitably raises the question of a potential effect in atherosclerosis. The aim of the current research was to study whether FABACs have a beneficial effect on blood lipid levels and atherosclerosis progression in mice.

Methods

FABAC. Aramchol, a conjugate of arachidic acid with cholic acid (at position 3 of the bile acid) using an NH bond, is one of the most effective FABACs in inhibiting cholesterol crystallization [1, 2] and was used in this study.

Animals and Diet. 12-week-old C57BL/6 female mice (Harlan, Israel) were randomly divided into two groups of 20 animals each. Mice were fed a Paigen diet containing 17% fat (43% saturated and 7% cholesterol; Harlan, USA). To minimize oxidation, diets were stored in the dark at 4 °C, and the mice were fed daily ad libitum. Mice were administered a daily oral dose of 150 µl DDH₂O (control group) or FABAC (150 mg/kg body weight) in a total volume of 150 µl DDH₂O (treatment group). The experiment lasted 16 weeks. Mice of both groups were healthy throughout the study and showed no differences in feeding pattern. All procedures using animals were in accordance with Sheba Medical Center guidelines.

Lipid Measurements. Total plasma cholesterol and triglyceride levels were determined after a 16- to 18-hour fast, using an automated enzymatic technique (Boehringer Mannheim, Germany), on day 0, after 8 weeks, and at the end of the study.

Lipoprotein Profile Examination. Mouse lipoproteins were separated by size exclusion chromatography using a Superose 6 column on FPLC system (Pharmacia, USA) [6]. A 200-µl aliquot of mouse serum was injected into the column and separated with buffer containing 0.15 M NaCl, 0.01 M Na₂HPO₄, 0.1 mM EDTA, pH 7.5, at a flow rate of 0.5 ml/min. Fifty fractions of 0.5 ml each were collected, with the lipoproteins being contained in tubes 18–35. Fractions 18–20 = very-low-density lipoprotein (VLDL), fractions 21–30 = LDL, fractions 31–35 = high-density lipoprotein (HDL).

FABAC Measurements. FABAC levels in each one of the FPLC fractions were determined as described previously [1]. Briefly, FABACs were extracted from the samples with chloroform:methanol (2:1, v:v). They were dissolved in methanol and measured by HPLC (Kontron, UK) using a Phenomenex Luna reverse phase C-18 column. The running phase was methanol, 100%, at a flow rate of 0.9 ml/min. FABACs were detected at 206 nm.

Lipid Analysis. Lipids were extracted from the liver according to the procedure of Leikin and Brenner [7]. The total amount was calculated after aliquot evaporation to constant weight. Neutral lipids were separated by thin-layer chromatography on silica gel plates (Merck, Germany). They were developed with a solvent system constituted by petroleum ether, ethyl ether, acetic acid 90:10:1 (v/v/v). The lipids were identified by the R_f according to known standards.

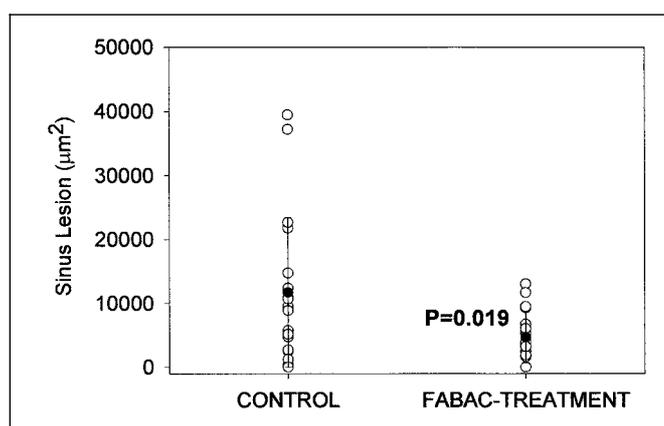


Fig. 1. The extent of atherosclerosis in control (○) and FABAC-treated (●) mice was quantified after 15 weeks of treatment. Atherosclerosis lesion area in the sinus was measured by staining lesions with oil-red O and counting by an Imagepro program.

They were detected by iodine (Sigma, Israel) vapors and quantified by densitometry in a BIS 202D Densitometer (Rhenium, Israel).

Assessment of Atherosclerosis in the Aortic Sinus. Quantification of atherosclerotic fatty-streak lesions was performed by calculating the lesion area in the aortic sinus, as previously described [8]. Briefly, the heart and the upper section of the aorta were removed from the animals and the peripheral fat was cleaned carefully. The upper section was embedded in OCT compound (Sakura, USA) and frozen. Every other section (5- to 10-µm thick) throughout the aortic root area (400 µm ~ 20 sections/mouse) was taken for analysis. The three valve cusps that form the junctions of the aorta to the heart identified the distal portion of the aortic sinus. Three sections were randomly selected, fatty-streak lesions were evaluated after staining with oil-red O. Atherosclerotic lesions were measured, using an Imagepro program (Media Cybernetics, USA), by an observer unfamiliar with the tested specimen. The average of the three sections represents the aortic lesion size for each mouse.

Statistical Analysis. Data are shown as means ± SE. Analyses of differences between groups for statistically significant differences were performed with the use of Student's t test, or the Mann-Whitney rank test. $p < 0.05$ was considered statistically significant.

Results

Effect of FABACs on the Atherosclerotic Lesion Area

The C57BL/6 mice fed an atherogenic diet and treated with FABAC showed a significant reduction in the atherosclerotic lesion area as compared to the control group, as determined by the lesion area at the aortic sinus (fig. 1). The atherosclerotic lesions in both groups are small foam cell plaques present only in the region of the aortic valve leaflets, as could be expected in this atherogenic model [9]. Daily supplementation of 150 mg FABAC/kg body

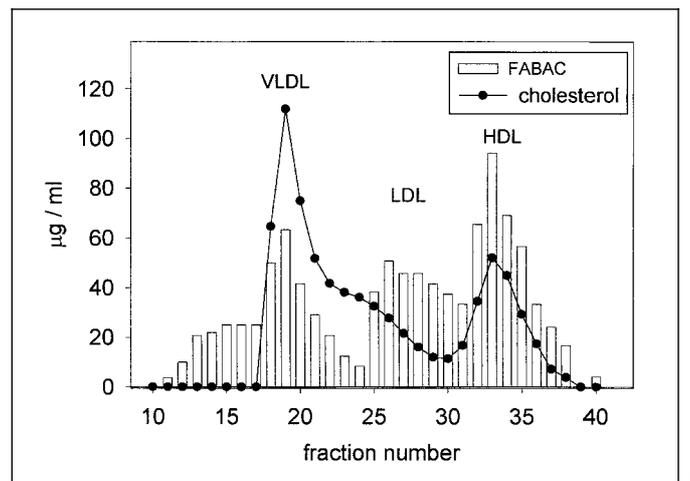
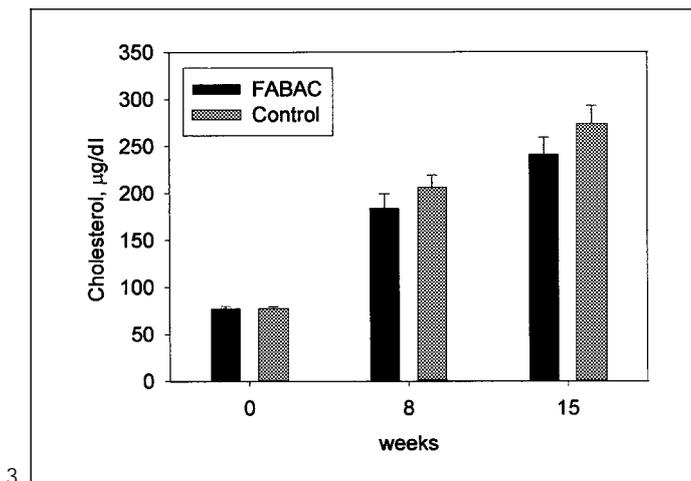
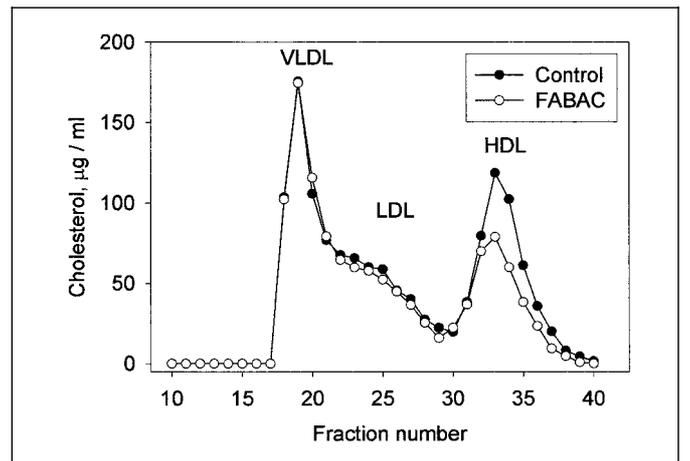
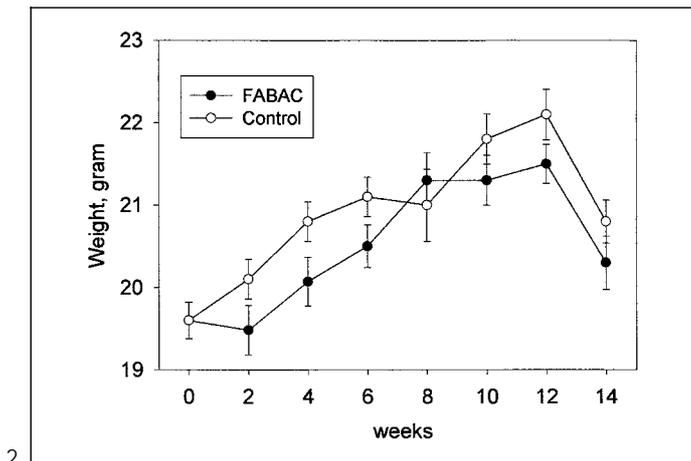


Fig. 2. Body weight, determined in control (○) and FABAC-treated (●) mice, was measured every 2 weeks. Data represent means ± SE of each group.

Fig. 3. Total plasma cholesterol levels in control and FABAC-treated mice on day 0, after 8 weeks and at the end of the study. Data represent means ± SE of each group.

Fig. 4. Distribution of serum lipoprotein cholesterol in control and FABAC-treated mice after 15 weeks on the atherogenic diet. Gel filtration chromatography profiles of plasma lipoproteins from control (○) and FABAC-treated (●) mice. Blood was obtained from fasted animals. Absorbance at 500 nm provides a measure of the relative amounts of cholesterol in each fraction.

Fig. 5. The plasma concentration of FABAC in lipoproteins was determined by HPLC analysis at the end of the experiment.

weight reduced the atherosclerotic plaque area by 60% as compared to control mice ($p = 0.019$).

Body Weight and Plasma Lipid Profile

The weight of the animals was measured every 2 weeks during the study (fig. 2). No significant differences between the control group and the FABAC-fed mice were found. A significant elevation in total cholesterol levels was observed in all groups. Analysis of serum cholesterol (fig. 3) and triglyceride levels (data not shown) at baseline, after 8 weeks and at the end of the study showed no significant differences between the control group and the

FABAC-fed mice, even though cholesterol levels tended to be lower in the FABAC-treated mice. Examination of the cholesterol and cholesterol ester levels in the liver of both groups revealed no differences (data not shown).

Plasma Lipoprotein Profile

Plasma lipoproteins were assessed by gel filtration chromatography in both groups (fig. 4). The content of VLDL cholesterol and LDL cholesterol showed no change as compared to the control group. HDL cholesterol modestly decreased in the FABAC-treated mice as compared to the controls.

FABAC Content of Lipoproteins

FABACs were found in the VLDL, LDL and HDL fractions. Higher FABAC levels were measured in the HDL fraction as compared to the VLDL and the LDL fractions (fig. 5).

Discussion

FABACs are a new family of synthetic molecules that were shown to solubilize cholesterol in bile and blood [1]. The present study demonstrates that administration of FABAC to Paigen diet-fed C57BL/6 mice inhibits atherogenesis in the aortic sinus despite the lack of a significant effect on plasma lipid levels. Atherosclerosis is a multifactorial disease in which humoral and cellular components are involved. Many studies have shown the relationship between cholesterol levels, especially high LDL levels and low HDL levels, and the development of atherosclerosis [10]. In the current study, no significant differences were observed in plasma lipid levels between the FABAC and the control groups. Moreover, the lipoprotein cholesterol profile examination revealed a slight reduction in HDL cholesterol in the FABAC-treated mice as compared to the control.

Several studies have shown that low HDL cholesterol is a risk factor for coronary heart disease [11]. However, HDL is a very heterogeneous class of lipoproteins. Differences in the stoichiometric content of proteins and lipids result in the formation of various HDL subclasses, which differ by size, density, shape and antigenicity. These parti-

cles differ in their activity to release cellular cholesterol or in their paraoxonase activity [12]. Thus, changes in HDL cholesterol may not necessarily lead to equidirectional changes in anti-atherogenic plasma activities. It is possible that this reduction in HDL cholesterol is caused by the accumulation of high levels of FABAC in the HDL fraction. It is important to note that FABAC accumulated in all lipoprotein fractions, and the effect of FABAC content on the lipoprotein structure and properties should be studied in further experiments.

The mechanism of action of the FABACs in relation to atherosclerosis does not, however, have to involve lipoproteins. In model lipid solutions, supersaturated with cholesterol, FABACs delay the onset of cholesterol crystallization and reduce the eventual crystal mass. They also dissolve preformed cholesterol crystals [1]. All this occurs without any change in the amount or proportion of the various lipids and in the complete absence of proteins or lipoproteins. In these conditions FABACs act as direct cholesterol solubilizers. The FABACs, which circulate in the vascular tree for over 48 h after a single intragastric dose, could also affect lipid fluxes from tissues to blood and bile. They may also affect nuclear receptors and/or membrane transporters in the vessel wall. All these potential modes of action need to be further explored.

In conclusion, the current study demonstrated that FABACs, given orally, reduce the development of atherosclerosis in mice fed a cholesterol-rich diet, in spite of a lack of effect on plasma lipid levels. Further studies are required to elucidate the exact mechanism of action of FABACs in atherosclerosis.

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