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Hypocholesterolemic effects of fatty acid bile acid conjugates (FABACs) in mice

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Abstract

Fatty acid bile acid conjugates (FABACs) prevent and dissolve cholesterol gallstones and prevent diet induced fatty liver, in mice. The present studies aimed to test their hypocholesterolemic effects in mice. Gallstone susceptible (C57L/J) mice, on high fat (HFD) or regular diet (RD), were treated with the conjugate of cholic acid with arachidic acid (FABAC; Aramchol). FABAC reduced the elevated plasma cholesterol levels induced by the HFD. In C57L/J mice, FABAC reduced plasma cholesterol by 50% ($p < 0.001$). In mice fed HFD, hepatic cholesterol synthesis was reduced, whereas CYP7A1 activity and expression were increased by FABAC. The ratio of fecal bile acids/neutral sterols was increased, as was the total fecal sterol excretion. In conclusion, FABACs markedly reduce elevated plasma cholesterol in mice by reducing the hepatic synthesis of cholesterol, in conjunction with an increase of its catabolism and excretion from the body.

Keywords: Cholesterol; Bile acid; Fatty acid; Liver; Lipoproteins; Neutral sterols; Gallstone; Mice

Fatty acid bile acid conjugates (FABACs)[2] are synthetic lipid molecules recently designed to solubilize biliary cholesterol. FABACs were shown to delay, reduce, or prevent cholesterol crystallization *in vitro* and *ex vivo* in bile model solutions and in human bile [1]. FABACs have also been shown to prevent the formation of cholesterol gallstones and to dissolve preexisting gallstones in experimental animal models [2,3]. Several FABACs have been synthesized [1,2]. The FABACs containing the longer chain fatty acids, arachidic (C-20) and stearic (C-18), were shown to be preferable in relation to cholesterol metabolism. Accordingly, Aramchol (C-20 FABAC) was used in the present studies, in which the bile acid moiety is represented by cholic acid.

In addition to the effects on gallstones, FABACs have been found to have other metabolic effects. They prevented or reduced the formation of fatty liver, induced by high fat diets in mice, hamsters and rats [4]. Aramchol increased fecal sterol output in rats [5]. Recent studies have shown that FABACs induce cholesterol and phospholipid efflux from human fibroblasts in an ABCA1-dependent and Apo AI-independent manner [6]. It was also observed that in rodents on high fat diets, FABAC therapy reduced the elevated plasma cholesterol levels.

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[2] *Abbreviations used:* FABACs, fatty acid bile acid conjugates; CSI, cholesterol saturation index; HMGCoAR, 3-hydroxy-3-methylglutharyl CoA reductase; TG, triglyceride; SEC, size exclusion chromatography; TNA, total nucleic acid; FGF15, fibroblast growth factor 15; HFD, high fat diet; RD, regular diet.

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The present studies were initiated to test and validate the hypocholesterolemic effects of Aramchol and to investigate its mechanism of action in gallstone susceptible C57L/J mice.

Materials and methods

Animals and diets

Inbred, gallstone susceptible C57L/J male mice, 4–5 weeks old, were purchased from the Jackson Laboratory (Maine, USA), and used in most experiments. Regular powdered rodent chow diet (4% fat, RD) was purchased from Koffolk (Petach Tikva, Israel). The high fat diet (HFD) was prepared as previously described [7] and consisted of 15% butter fat, 1% cholesterol, 0.5% cholic acid and 2% corn oil added (w/w) to RD.

Mice were kept on a 12 h day/night cycle and had free access to water and food. The studies were approved by the institutional committee for animal experiments at Tel-Aviv University, Tel-Aviv, Israel.

Experimental procedure

The FABAC used in the present experiments was 3β-arachidyl amido, 7α, 12α-dihydroxy, 5β-cholan, 24-oic acid, which is an amide conjugate (at position 3 of the bile acid) of arachidic and cholic acids (Aramchol; C-20 FABAC). Animals were fed RD or HFD. Aramchol was given by gavage at a dose of 25 mg/kg/day or 150 mg/kg/day (FABAC 25 or FABAC 150, respectively) suspended in 0.1 ml saline. Control animals received saline by oral gavage. After the experimental period, all animals were given ketamine anesthesia, and sacrificed. Blood was drawn from the abdominal aorta and collected in tubes with 0.01% EDTA. Plasma was separated by centrifugation and kept frozen at −20 °C for further determinations. The gallbladder was ligated, excised and inspected. Bile was aspirated with a thin (23G) needle. Thereafter, the gallbladder was opened and inspected for the presence of gallstones and crystals, using a stereoscopic light microscope and polarized light. The liver was excised and immediately frozen in liquid nitrogen for further analyses.

Design of studies

Three sets of different experiments were performed. In the first set, the molecular effects of FABAC on plasma cholesterol were studied in C57L/J after four weeks of treatment. Animals were fed RD or HFD. FABAC was administered at a dose of 25 or 150 mg/kg/day. In the second set of experiments, the molecular effects on cholesterol and bile acid metabolism of FABAC were studied in C57L/J mice. In these experiments, animals were either on RD or on HFD. FABAC was administered at a dose of 25 or 150 mg/kg/day for a period of 2 weeks. In the last set of experiments, animals were treated for a period of time ranging between 3 and 8 weeks in order to evaluate the effects of FABAC on plasma cholesterol levels.

Bile analyses

Biliary lipids were extracted by chloroform:methanol (2:1, v:v) and phase separation, according to Folch et al. [8]. Bile salts and cholesterol were quantitated enzymatically [9,10]. Phospholipids were determined by the method of Bartlett [11]. Because of minute volumes, biles were pooled and statistical significance could not be tested. Cholesterol saturation index (CSI) was calculated according to Carey's critical table [12].

Preparation of liver microsomes, enzyme assay, and cholesterol determination

Liver biopsies were homogenized with a loose-fitting Teflon pestle in nine volumes of 50 mM Tris–HCl buffer, pH 7.4, containing 0.3 M sucrose, 10 mM DTT, 10 mM EDTA, and 50 mM NaCl. The homogenate was then centrifuged at 20,000*g* for 15 min at 4 °C. The supernatant was thereafter centrifuged at l00,000*g* for 60 min. Half of the microsomal fraction was resuspended in the homogenizing medium and spun at 100,000*g* for 60 min. The 100,000*g* pellet was suspended in 20 mM imidazole buffer, pH 7.4, containing 10 mM DTT in a volume corresponding to that of the 20,000*g* supernatant, and used for the assay of the 3-hydroxyl-3-methylglutharyl CoA reductase (HMGCoAR). The other half of the original microsomal fraction was recentrifuged at 100,000*g* in the absence of DTT. The pellet was suspended in 0.1 M phosphate buffer, pH 7.4, containing 1 mM EDTA, and used for assay of cholesterol 7α-hydroxylase (CYP7A1) activity, determined as the formation of 7α-hydroxycholesterol (pmoles/mg protein/min) from endogenous microsomal cholesterol using isotope dilution-mass spectrometry [13]. Microsomal HMGCoAR activity was assayed by determining the conversion of HMG CoA to mevalonate, and expressed as pmoles formed per mg protein per min [14]. The enzyme assays were carried out in duplicate. The concentrations of free and total cholesterol in the liver homogenates and the microsomal fractions were determined by isotope dilution-mass spectrometry after addition of deuterium-labeled cholesterol as an internal standard, as previously described [15,16]. Esterified cholesterol levels were calculated as the difference between the total and free cholesterol.

Chemical analysis of plasma

In the first set of experiments, an Advia 1650 autoanalyzer (Bayer, Japan) was used for cholesterol determinations. In the second set of experiments, total cholesterol and triglycerides were determined in pooled plasma using a Monarch automated analyzer (ILS Laboratories Scandinavia AB, Sollentuna, Sweden). Since the kit for the determination of triglycerides detects free glycerol, serum triglyceride (TG) values were corrected with regard to their respective glycerol content. Size-fractionation of lipoproteins and their cholesterol and triglyceride content was performed on 10 μl of pooled plasma from each group by size exclusion chromatography (SEC) using a 300 × 3.2 nm Superose 6B column (Amersham Pharmacia Biotech AB, Uppsala, Sweden) at a flow rate of 40 μl/min as described in detail by Parini et al. [17].

Unesterified lathosterol was determined by isotope dilution-mass spectrometry after the addition of a deuterium-labeled internal standard [18].

Total nucleic acid (TNA) preparation and analysis of mRNA levels

Frozen liver specimens (0.4 g) from each animal were homogenized in 4 ml of SET buffer [1% (w/v) sodium dodecyl sulphate, 10 mmol/L EDTA, and 20 mmol/L Tris–HCl, pH 7.5] with a Polytron (Kinematica, type PT 10/35, Kriens, Lucerne, Switzerland). The samples were subsequently sonicated on ice by two 5 s pulses, in a Branson B 15 Sonifier, and digested with proteinase K (200 μg/ml) for 45 min at 45 °C. TNA was precipitated with ethanol after phenol–chloroform extraction, and the pellet was suspended in 300 μL of 20% SET buffer. LDL-receptor mRNA, CYP7A mRNA, and HMGCoAR mRNA were quantitated by a solution hybridization titration assay, as previously described [19]. The slopes of the linear hybridization signals were calculated by the method of least squares, and compared with the slope generated by synthetic mRNA standards. Data are expressed as Arbitrary Units, calculated from moles mRNA/μg TNA.

Analysis of fecal sterols

Feces were collected during the last 72 h of the experimental period. Fecal lipids were analyzed by GLC according to Batta et al. [20]. In brief, 75 mg of lyophilized feces were butylated by addition of 1 ml *n*-butyl alcohol and 0.1 ml 6 N HCL. Nor-cholic acid and 5α-cholestane were added as internal standards. The mixture was incubated for 4 h at 60 °C and thereafter the solvents evaporated at 60 °C under air stream. Samples were silylated with 1 ml Sil-Prep kit (Alltech, Alltech Ill, USA) at 55 °C for 30 min and thereafter resuspended in 1 ml hexane, centrifuged, and