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The cholesterol lowering effect of steroid sequestrants is modulated by large intestine fermentations

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The cholesterol lowering effect of steroid sequestring compounds, such as cholestyramine or β-cyclodextrin, has been examined to assess the respective importance of bile acids excretion and the fermentation process. In contrast to cholestyramine, β-cyclodextrin is metabolized by the large intestine microflora yielding short chain fatty acids (SCFA), especially propionic acid which is absorbed in the portal vein and metabolized by the liver. β-cyclodextrin was less potent than cholestyramine at elevating the fecal excretion of bile acids and depressing soluble bile acids in the large intestine but only the former compound was definitely hypocholesterolemic. Changes in circulating lipoproteins (depressed HDL1 and apoE abundance) were observed only in the β-cyclodextrin–fed group. Cholestyramine was more potent than β-cyclodextrin to induce the activity of hepatic HMG CoA reductase or cholesterol 7α-hydroxylase, whereas that of fatty acid synthase (FAS) was depressed only in the β-cyclodextrin group. It appears that fermentable bile acid sequestrants are the most effective at depressing plasma cholesterol, probably in relation to the capacity of fermentation end-products to counteract the up-regulation of bile acids and cholesterol biosynthesis. (J. Nutr. Biochem. 6:158–162, 1995.)

Keywords: cholesterol; bile acids; lipoproteins; cholestyramine, β-cyclodextrin; large intestine microflora

Introduction

The hepatic conversion of cholesterol into bile acids is the prevailing pathway for the elimination of cholesterol from the mammalian body. A major part of bile acids secreted into the intestine is reabsorbed (chiefly in the ileum and to a lesser extent in the large intestine); thus, only a small percentage is excreted in the feces.[1] Increased fecal loss of bile acids may be achieved through several ways leading to partial interruption of their enterohepatic circulation. Cholestyramine, an insoluble anion-exchange resin that binds bile acids in the intestine, has been used to increase bile acid excretion.[2,3] Some soluble fermentable polysaccharides of plant origin have received considerable interest concerning their antiatherogenic potential.[4,5] One major mechanism for their hypocholesterolemic action lies in the increase of solution viscosity of the intestinal contents or in the binding of bile acids, hence a reduced ileal reabsorption.[6] Cyclic molecules of bacterial origin, like cyclodextrins, are able to encapsulate various organic compounds, especially sterols and bile acids.[7] These carbohydrates are broken down by microorganisms in the large intestine, yielding short chain fatty acids (SCFA) which are present at concentrations in the range of 100 mM. The subsequent absorption of SCFA in the portal vein and their metabolism in the liver is another process whereby complex carbohydrates may affect cholesterol metabolism.[8,9]

Thus, the purpose of this study was to investigate the relative importance of bile acid excretion and of the end products of cecal fermentations on the regulation of cholesterolemia. The use of cholestyramine, a nonmetabolizable bile acid sequestring agent, and β-cyclodextrin, a potent complexing molecule for neutral and acidic steroids which is extensively fermented to SCFA by cecal bacteria, have been chosen to differentially manipulate bile acid excretion and liver lipid metabolism.

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Methods and materials

Animals and diets

Male Wistar rats (IFFA-CREDO, L'Arbresle, France) weighing 150 to 160 g were fed for 4 weeks semipurified diets that contained (as g/100 g of dry weight): casein (L. François, Paris, France), 15; peanut oil, 5; mineral and vitamin mixes (Usine d'Alimentation Rationnelle, Villemoisson/Orge, France), 6 and 1, respectively. In the control diet, the carbohydrate supply was: wheat starch (L. François), 73. In the experimental groups, a part of wheat starch were replaced by β-cyclodextrin (4.5 g) or by cholestyramine (1 g). β-cyclodextrin was purchased from Roquettes (Lestrem, France) and cholestyramine from Sigma (St. Louis, MO USA). Rats were housed two per cage and maintained at 22°C with the dark period from 20:00 hr to 8:00 hr. Food and water were allowed ad libitum during the dark period. During the last week of experiment, the feces were collected daily and stored at −20°C until analysis. At the end of the experimental period, the body weights of rats from the three experimental groups were not statistically different (335 ± 11, 313 ± 13 and 327 ± 12 g, for controls, β-cyclodextrin and cholestyramine groups, respectively).

Sampling and analytical procedures

Rats were used at the end of the dark period, namely at a time at which cecal fermentations are still very active. They were anesthetized with sodium pentobarbital (40 mg/kg) and maintained on a hot plate at 37°C. Blood was withdrawn into heparinized syringes from abdominal aorta. After centrifugation at 10,000*g* for 2 min, plasma samples were separated and kept at 4°C until analysis. A portion of liver was freezed-clamped and stored at −80°C for the measurement of fatty acid synthase (FAS) activity. In parallel, 2 g of liver were quickly homogenized for microsome purification as previously described.[10] The microsomal preparation was stored at −80°C until measurement of enzyme activities. The protein content of the preparation was determined using the Pierce BCA Reagent kit (Interchim, Montluçon, France).

The cecum with content was removed and weighed, then approximately 1 g of cecal contents were transferred into microfuge tubes that were immediately frozen and stored at −20°C. SCFA were measured by gas-liquid chromatography in aliquots of supernatant of cecal contents after ethanolic extraction.[11] Bile acid analysis were performed on cecal supernatants (soluble) or after extraction from untreated cecal samples or feces (total) by 10 vol of ethanolic KOH 0.5 M, using the reaction catalyzed by the 3α-hydroxysteroid dehydrogenase (EC 1.1.1.50; Sigma).

Plasma lipoproteins were separated by density gradient ultracentrifugation[12] using pooled samples. The gradient was then fractionated (into 24 × 500 μL fractions) and kept at 4°C for lipid and protein analysis. Fractions 10 to 15 were precipitated with trichloroacetic acid, washed with acetone, and solubilized as described by Rudling et al.[13] Samples were separated by SDS-PAGE on 4 to 20% gradient gels electrophoresis (Bio-Rad, Paris, France) and calibrated with low molecular mass standards (Pharmacia Fine Chemicals, Piscataway, NJ USA). Gels were stained with Coomassie blue (R250). Triglycerides (Biotrol, Paris, France), total cholesterol, and phospholipids (BioMérieux, Charbonnières-les-bains, France) were determined in plasma and lipoprotein fractions by enzymatic procedures. The activity of HMG CoA reductase was measured on liver microsomal fractions as described by Wilce and Kroone.[14] Labeled mevalonolactone was separated from unreacted HMG CoA by column chromatography using AG1-X8 resin (200 to 400 mesh, formate form) (Bio-Rad, Paris, France). Fatty acid synthetase (FAS) activity was determined according to the method of Hsu et al.,[15] and expressed in μmol of [2-^{14}C]malonyl CoA incorporated into fatty acids/min/mg of protein. Cholesterol 7α-hydroxylase was assayed as described by Chiang[16] using 20α-hydroxycholesterol as internal standard. Their 3-keto derivatives were analyzed by reversed-phase HPLC and detected at 240 nm.

Data analysis

Values are given as the means ± SEM and, where appropriate, the significance of the differences between mean values was determined by analysis of variance (ANOVA) and multiple range comparisons by Fisher's PLSD procedures (StatView 512+, Brain Power, Calabasas, CA USA).

Results

As shown in *Table 1*, the cecal SCFA pool in rats fed β-cyclodextrin was much higher than in controls, together with a very high proportion of propionate (46% of the total pool) in accordance with the fermentable characteristics of this oligosaccharide.[17] The presence of cholestyramine in the cecum had no noticeable effect on the SCFA pattern observed in controls (namely a high proportion of acetate).

The cecal pool of bile acids was significantly enlarged in rats fed the diets containing bile acid sequestrants, 1% cholestyramine being 2 fold more potent than 4.5% β-cyclodextrin in this respect. This reflects the fact that while cholestyramine caused an enhancement of both cecal volume and bile acid concentration, β-cyclodextrin led to an enlargement of the cecal volume without increasing bile acid concentration in this organ (data not shown). The drastic reduction of the proportion of soluble bile acids in the cecum of rats fed cholestyramine (0.6% of total bile acids) reflects the potency of this resin to bind them. β-cyclodextrin was extensively broken down by the cecal microflora (as reflected by the higher occurrence of SCFA), thus releasing the bound bile acids in the lumen. The observed reduction of soluble bile acids with β-cyclodextrin, compared with controls, could be the result of acidic pH conditions (6.49 vs. about 7.25 for the control and cholestyramine groups) and, possibly, of the presence of still intact cyclodextrin molecules. The fecal excretion of steroids was markedly enhanced in the two experimental groups; bile acids excretion was particularly responsive to the diet con-

Table 1 Changes in the cecal pools of SCFA and bile acids and in the fecal excretion of steroids

Diet	Control	β-Cyclodextrin	Cholestyramine
Cecal pools (μmol/cecum)			
SCFAs	166 ± 19	268 ± 46*	165 ± 3
(% Ac/Pr/Bu)	(62/28/10)	(47/46/7)	(57/33/10)
Bile acids	6.3 ± 0.8	12.0 ± 1.1*	25.1 ± 2.1‡
(% solubility)	(41 ± 6)	(20 ± 4)	(0.6 ± 0.2)
Fecal excretion (μmol/day)			
Cholesterol	8.6 ± 0.8	12.8 ± 1.3*	19.8 ± 1.1‡
Bile acids	15.0 ± 0.8	37.9 ± 3.2†	70.6 ± 6.3‡

Values are the means ± SEM, $n = 10$.
*$P < 0.05$, †$P < 0.01$, ‡$P < 0.001$ versus control.

ditions (2.5 and 4.7 fold enhanced by β-cyclodextrin and cholestyramine, respectively).

As shown in *Table 2*, cholestyramine failed to lower plasma cholesterol whereas β-cyclodextrin was effective. There was also a significant reduction of plasma triglycerides with this oligosaccharide. Analysis of the lipoprotein profile (*Figure 1*) shows that the hypocholesterolemic effect of β-cyclodextrin corresponds to a depressed concentration of cholesterol in the fractions with a density ranging from 1.040 to 1.080 (referred to as HDL1 in the rat). Protein and phospholipids followed the same pattern as cholesterol (data not shown). Fractions showing the greatest modifications (fractions 10 to 15, corresponding to a large part of HDL1) were subjected to electrophoresis in order to examine their apolipoprotein content. As shown in *Figure 2*, there was a marked reduction of the apoE aboundance in the β-cyclodextrin group, whereas Apo A-I appeared affected to a lesser extent. Electrophoretic analysis of the same fractions in cholestyramine-treated rats showed no modification of their apolipoprotein composition, in keeping with the fact that the lipoprotein profile was not distinguishable from the profile of control rats (see Figure 1).

The activity of hepatic enzymes of lipid metabolism has been determined in parallel to lipoprotein characterization (Table 2). Both microsomal HMG CoA reductase and cholesterol 7α-hydroxylase activities were induced by β-cyclodextrin (+116 and +76%, respectively) as well as, to a larger extent, by cholestyramine (about +350% for both enzymes). In contrast, cytosolic fatty acid synthetase activity was unchanged in rats fed cholestyramine and it was even significantly depressed (−40%) in those adapted to β-cyclodextrin.

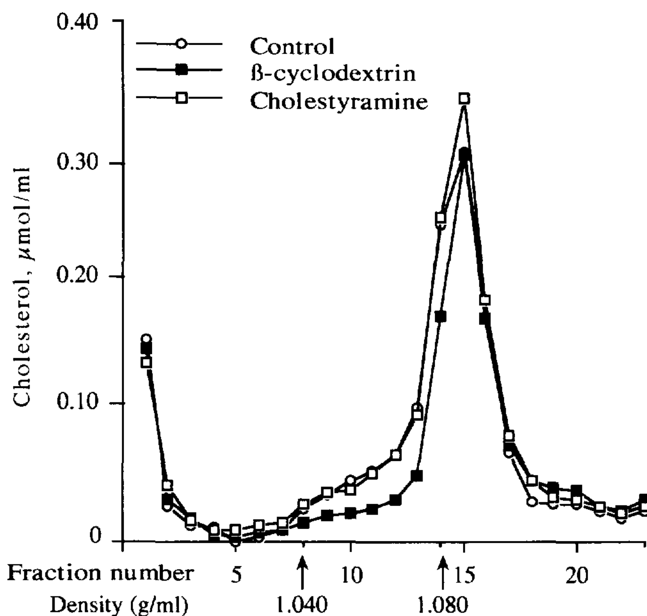

Figure 1 Cholesterol distribution in lipoprotein fractions separated by density gradient ultracentrifugation of pooled plasmas from control and β-cyclodextrin- or cholestyramine-fed rats. The LDL, HDL1, and HDL2 corresponded to the fractions within a density range of 1.006 to 1.040, 1.040 to 1.080, and 1.080 to 1.16, respectively.

Discussion

Bile acid binding to cholestyramine in the intestinal lumen led to a considerable rise in the fecal excretion of steroids, essentially as bile acids. These losses were efficiently counteracted by homeostatic processes (a compensatory rise in cholesterol and bile acid synthesis); as a result, no noticeable cholesterol-lowering effect was observed. In contrast, a significant hypocholesterolemic effect of cholestyramine has been reported with high fat diets supplemented with cholesterol, which could reflect some adsorption of cholesterol on the resin due to hydrophobic interaction with its apolar core.[18]

Although β-cyclodextrin was less potent at enhancing bile acids excretion than cholestyramine, a significant reduction of plasma cholesterol was obtained only with this oligosaccharide. This result shows that, in the absence of fermentations in the large intestine, a high rate of bile acids excretion is not always sufficient to induce a cholesterol-lowering effect. A high rate of organic acid production leads to acidic colonic pH, which in turn could depress the solubility of bile acids, hence their reabsorption. However, our results show that the impact of β-cyclodextrin on bile acid solubility was much less pronounced than that of cholestyramine. Thus, a striking feature from this study is that both active fermentations and enhanced fecal excretion of bile acids result in a maximal lowering of plasma cholesterol. This is illustrated by the fact that some readily fermentable carbohydrates with low bile acid binding capacities (i.e., inulin) have practically no hypocholesterolemic effect.[17]

Utilization of β-cyclodextrin at a moderate level of 4.5% was unlikely to substantially alter the nature of energetic fuels available for liver metabolism, and the replacement of glucose by SCFA should be limited in such conditions.[19] The putative role of fermentation end products (especially propionate) on the activities of key enzymes of cholesterol metabolism is still disputed.[8,20,21] This hypothesis has been further examined in a variety of models: by supplementation of the diet with propionate,[20,22] or by intracecal infusion of propionate,[23] or in vitro on isolated hepatocytes.[21,24] These investigations have not always been conclusive as to the actual role of propionate in the effect of fibers on lipid metabolism. It is noteworthy that in the present study a potent effect on plasma cholesterol was seen in the presence of high propionic acid fermentations in the large intestine (β-cyclodextrin diet).

In rats, the hypocholesterolemic effect of fiber is accompanied by a characteristic modification of the lipoprotein profile, namely a decreased abundance of the HDL1 component along with a fall of plasma apoE concentration.[10,25] The effect of β-cyclodextrin could reflect an enhanced uptake of apoE-containing particles by the liver via the apoB/E receptor. A coordinate regulation of HMGCoA reductase and the apo B/E receptor has been shown at the transcription level,[26,27] but other studies support the view that hepatic sterol synthesis and receptor-mediated uptake of LDL particles could be independently regulated in rats.[28] Furthermore, a more recent study strongly suggests that hepatic LDL receptors may be induced independently of the mRNA level.[12] Our results are in accordance with this latter hy-

Table 2 Changes in plasma lipids concentrations and in the activity of liver enzymes of lipid metabolism

Diet	Control	β-Cyclodextrin	Cholestyramine
Plasma lipids (mmol/L)			
Cholesterol	1.55 ± 0.08	1.32 ± 0.05*	1.63 ± 0.05
Triglycerides	1.20 ± 0.08	0.94 ± 0.05*	1.15 ± 0.09
Liver enzymes			
HMGCoA reductase (pmol/min/mg protein)	41 ± 7	88 ± 11*	195 ± 19†
Cholesterol 7α hydroxylase (pmol/min/mg protein)	18 ± 3	33 ± 6*	84 ± 9†
Fatty acid synthase (nmol/min/mg protein)	1.12 ± 0.06	0.81 ± 0.05†	1.10 ± 0.07

Values are the means ± SEM, $n = 10$. *$P < 0.05$, †$P < 0.01$ versus control.

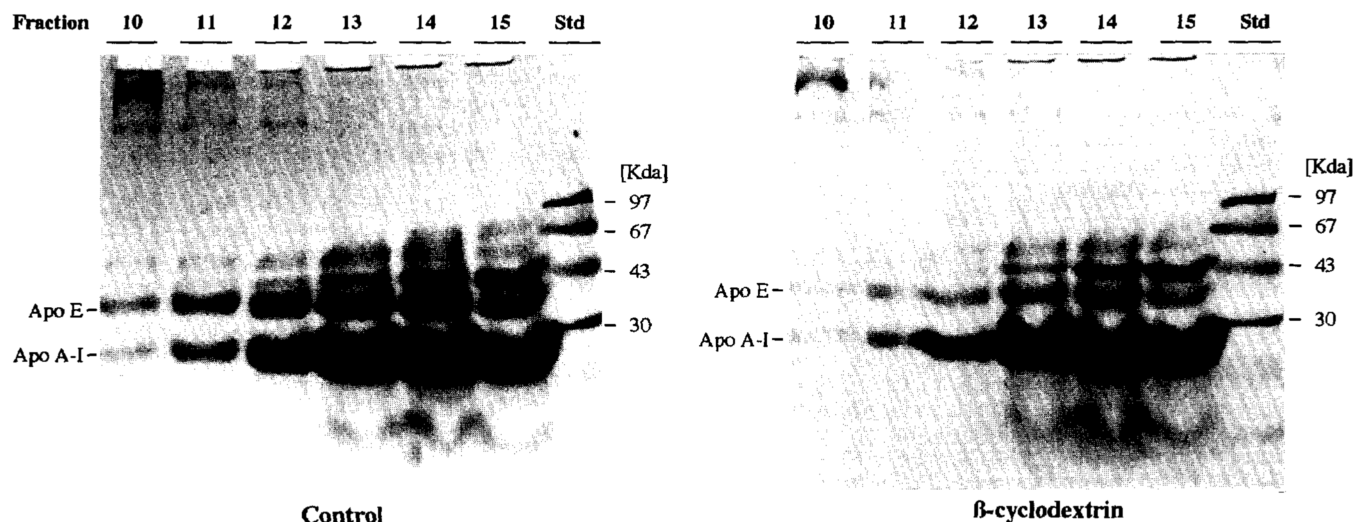

Figure 2 Coomassie-stained SDS-PAGE gradient gels (4 to 20%) of apolipoproteins from the 10 to 15 fractions isolated by ultracentrifugation (see Figure 1). Samples were calibrated with low molecular mass standards (Pharmacia Fine Chemicals, Piscataway, NJ USA) containing the following size markers: phosphorylase b (97 kD), albumin (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), trypsin inhibitor (20.1 kD), and α-lactalbumin (14.4 kD).

pothesis and with data showing no effect of cholestyramine treatment upon LDL clearance from the plasma, although hepatic sterol synthesis was actually enhanced.[28] Since HMGCoA reductase may be negatively regulated at both a transcriptional and a post-transcriptional level by bile acids,[29] the lower induction of HMG CoA reductase and cholesterol 7α-hydroxylase observed in the liver of rats fed β-cyclodextrin (compared with cholestyramine) could also be the result of a higher rate of bile acids reabsorption from the large intestine.

The cholesterol lowering effect of β-cyclodextrin could also be connected to a reduced secretion of VLDL triglyceride, in keeping with the lower activity of fatty acid synthase (FAS) in the liver (as well as that of other enzymes involved in fatty acid synthesis)[30] and the depressed plasma triglycerides of animals fed this compound. According to Nishina et al.,[21] propionate is particularly effective to inhibit the hepatic lipogenic activity in acute conditions. FAS activity was not affected by cholestyramine, in keeping with data showing that this compound is very effective at accelerating cholesterogenesis but not fatty acid synthesis;[31] furthermore, the low percentage of cholestyramine used in this experiment did not affect plasma triglycerides.

In conclusion, a moderate induction of cholesterol biosynthesis seems frequently connected to the cholesterol-lowering effect of fibers or related compounds (in contrast to drugs such as lovastatin). In these conditions, the decrease of HDL1 may reflect a compensatory increase of the hepatic uptake of cholesterol-containing particles. It appears that the effectiveness of bile acid sequestrants on plasma cholesterol depends on their capacity to limit the counterregulation processes modulating bile acids and cholesterol biosynthesis. Factors liable to counteract the induction of the cholesterol 7α-hydroxylase or HMG CoA reductase have been identified, such as bile acids (particularly the apolar species) and, possibly, SCFA. However, further investigations are required to elucidate, for example, the role of the composition (apolar/polar) of bile acids absorbed from the large intestine, the actual mechanisms for down-regulation of the key enzymes of cholesterol metabolism, as well as the physiological importance of SCFA in the control of lipid metabolism.

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