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Hesperidin Lowers Activities of 3-Hydroxy-3-methylglutaryl-CoA Reductase and Acyl-CoA:Cholesterol Acyltransferase in Rats Fed High-Cholesterol Diet

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Abstract

In this study, a potential mechanism through which the hesperidin might work on the effect was examined *in vivo*. Male rats were fed a high cholesterol synthetic diet (1%, wt/wt) with hesperidin (0.1%, wt/wt) for 42 days. Activity of hepatic HMG-CoA reductase was significantly lowered by the hesperidin supplement compared to the control. Hesperidin did not significantly alter plasma or hepatic lipids, but tended to lower those lipid levels. Hesperidin also subsequently reduced the fecal neutral sterols compared to the control (254.3 mg/d vs. 521.9 mg/d). The inhibition of HMG-CoA reductase resulting from the hesperidin supplementation could count for the reduction in fecal neutral sterols that appears to compensate for the decreased cholesterol biosynthesis. The dose of hesperidin in a high cholesterol diet should apparently be more than 0.1% to exhibit the hypocholesterolemic response in these rats. It remains to be determined whether the observed alterations in cholesterol metabolism are specific to the rat or also could be applied to the humans.

Key words: high-cholesterol diet, hesperidin, HMG-CoA reductase, ACAT, fecal neutral sterols

INTRODUCTION

It is now well established that hypercholesterolemia is a risk factor for the development of coronary heart disease (1). Any excess cholesterol needs to be removed to such an amount consistent with maintenance of the normal body function.

In the regulation of cholesterol metabolism, two key enzymes are involved, 3-hydroxy-3-methylglutaryl-coenzyme A (EC 1.1.1.34) (HMG-CoA) reductase and acyl coenzyme A:cholesterol O-acyltransferase (EC 2.3.1.26) (ACAT). The rate-limiting step in cholesterol synthesis is conversion of HMG-CoA into mevalonate by HMG-CoA reductase. Inhibition of HMG-CoA reductase results in the decrease of cholesterol synthesis and its inhibitors have been repeatedly shown to be very effective in lowering serum cholesterol in most animal species including human (2-4). The inhibitors are now widely used in hypocholesterolemic drugs (5,6). The ACAT, another key enzyme catalyzing the intracellular esterification of cholesterol, has been shown to be involved in the cholesterol absorption, the secretion of hepatic very low density lipoprotein-cholesterol and the cholesterol accumulation in the arterial wall (7). For these reasons, ACAT inhibitors have been used in test drugs as cholesterol lowering agents as well as antiatherosclerotic agents. Treatment of selective ACAT inhibitors has led variable degrees of reduction in plasma cholesterol in different animal species (8-10).

Some bioflavonoids have shown to be associated with a prevention of chronic diseases such as cancer and hyperlipidemia (11,12). Recently among naturally occurring flavonoids, hesperidin was pharmacologically evaluated as a potential anti-inflammatory agent (13), and a cholesterol-lowering agent by improving the cholesterol metabolism in the diet-induced hypercholesterolemic rats (14). A significant decrease in the plasma cholesterol was previously observed in rats fed 1% high-cholesterol diet with 0.1% hesperetin (wt/wt) (15), which is aglycone compound of hesperidin. The present study was designed to examine the effects of a low dose hesperidin (0.1%, wt/wt) on the cholesterol metabolism in rats fed a high-cholesterol diet.

METHODS AND MATERIALS

Animals and diets

Twenty male Sprague-Dawley weighing between 90 and 100 g were purchased from Daehan Laboratory animal research center (Chungbuk, Korea). The animals were individually housed in stainless steel cages in a room with controlled temperature (20~23°C) and lighting (alternating 12 hours period of light and dark) and fed a commercial chow diet for six days after arrival. Rats were randomly divided into 2 groups (n=10) and fed a high-cholesterol diet (1%, wt/wt) supplemented with or without hesperidin (0.1%, wt/wt, Sigma

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Chemical Co.) for 6 weeks. The diet compositions are shown in Table 1; the control diet was based on AIN-76 semi-synthetic diet (16,17). The animals were given free access to food and distilled water during the entire experimental period. Every day for the last 5 d feces was collected using metabolic cages and analyzed for fecal neutral sterols. The food consumption and weight gains were measured every third day. At the end of the experimental period, animals were anesthetized with Ketamine-HCl following a 12 h fast. Blood samples were taken from the inferior vena cava for the determination of plasma lipid. The livers were removed and rinsed with physiological saline. Plasma, livers and feces were stored at -60°C prior to use analysis.

Plasma and hepatic lipid analyses

Plasma cholesterol and HDL-cholesterol concentrations were determined using a commercial kit (Sigma Chemical Co., St. Louis, MO) based on a modification of the cholesterol oxidase method of Allain et al. (18). The HDL-fractions were separated using a Sigma kit based on the heparin-manganese precipitation procedure (19). The plasma triglyceride concentrations were measured enzymatically using a kit from Sigma Chemical Co., a modification of the lipase-glycerol phosphate oxidase method (20). The hepatic lipids were extracted using the procedure developed by Folch et al. (21). The dried lipid residues were dissolved in 1 ml of ethanol for cholesterol and triglycerides assays. Triton X-100 and sodium cholate solutions (in distilled H_2O) were added to 200 µl of the dissolved lipid solution to produce final concentrations of 5 g/L and 3 mmol/L, respectively. The hepatic cholesterol and triglycerides were analyzed with the same enzymatic kit used in the plasma analysis.

Table 1. Composition of experimental diets containing high cholesterol (%)

Ingredients \ Dietary groups	Control (n=10)	Hesperidin (n=10)
Casein	20	20
D,L-methionine	0.3	0.3
Corn starch	15	15
Sucrose	49	48.9
Cellulose powder	5	5
Mineral mixture[1)]	3.5	3.5
Vitamin mixture[2)]	1	1
Choline bitartrate	0.2	0.2
Corn oil	5	5
Cholesterol	1	1
Hesperidin		0.1
Total	100	100

[1)]AIN-76 mineral mixture

[2)]AIN-76 vitamin mixture contained (in g/kg mixture) : thiamin HCl, 0.6; riboflavin, 0.6; pryidoxine HCl, 0.7; nicotinic acid, 3.0; D-calcium panthothenate, 1.6; folate, 0.2; D-biotin, 0.02; cyanocobalamin (vitamin B_{12}), 0.001; retinyl palmitate premix, 0.8; DL-alpha tocopheryl acetate, premix, 20; cholecalciferol (vitamin D_3), 0.0025; menaquinone (vitamin K), 0.05; antioxidant, 0.01; sucrose, finely powdered, 972.42.

Fecal neutral sterols

The fecal neutral sterols were determined by a simplified micro-method developed by Czubayko et al. (22). A gas-liquid chromatograph was carried out with a Hewlett Packard gas chromatograph (Model 5809) equipped with a hydrogen flame ionization detector and using a Sac-5 capillary column (30 m $\times$0.25 mm ID, 0.25 m film, Supelco Inc., Bellefonate, PA). Helium was used as a carrier. Temperatures were set at 230°C for the column (isothermal) and 280°C for the injector/detector temperature. The internal standard used was 5α-cholestane (Supelco Inc.). The daily neutral sterol excretion was calculated from the amount of cholesterol, coprostanol, and coprostanone in each fecal sample.

HMG-CoA reductase and ACAT activities

Microsomes were prepared according to Hulcher and Oleson (23) with a slight modification. Two grams of the liver tissues were homogenized in 4 ml of an ice-cold buffer (pH 7.0) containing 0.1 mol/L triethanolamine, 0.02 mol/L EDTA and 2 mmol/L dithiothreitol, pH 7.0. The homogenates were centrifuged twice at both 10,000$\times$g and 12,000$\times$g for 10 min at 4°C. Then, the supernatants were ultracentrifuged twice at 100,000$\times$g for 60 min at 4°C. The resulting microsomal pellets were redissolved in 1 ml of a homogenation buffer for protein determination (24) and finally analyzed for HMG-CoA reductase and ACAT activities.

The HMG-CoA reductase activities were determined as described by Shapiro et al. (25) with a slight modification of using freshly prepared hepatic microsomes. The incubation mixtures (120 µl) containing microsome (100~150 g) and 500 nmol of NADPH (dissolved in a reaction buffer containing 0.1 mol/L triethanolamine and 10 mmol/L EDTA) were preincubated at 37°C for 5 min. Then, 50 nmol of [^{14}C]-HMG-CoA (specific activity; 2.1420 GBq/mmole, NEN™ Life Science Products, Inc. Boston) was added and incubated for 15 min at 37°C. The reaction was terminated by the addition of 30 µl of 6 mol/L HCl and the resultant reaction mixture was further incubated at 37°C for 15 min to convert the mevalonate into mevalonolactone. The incubation mixture was centrifuged at 10,000$\times$g for 5 min and the supernatant was spotted on a Silica Gel 60 F_{254} TLC plate with a mevalonolactone standard. The plate was developed in bezene-acetone (1:1, v/v), and air-dried. Finally, the region Rf 0.3~0.6 was removed by scraping with a clean razor blade and its [^{14}C] radioactivity was determined using a liquid scintillation counter (Packard Tricarb 1600TR, Packard Instrument Company, Meriden, Connecticut). The results were expressed as picomole mevalonate synthesized · min^{-1} · mg microsomal $protein^{-1}$.

The ACAT activities were determined using freshly prepared hepatic microsomes according to the method developed by Erickson et al. (26) as modified by Gillies et al. (27). To prepare the cholesterol substrate, 6 mg of cholesterol and 600 mg of Tyloxapol (Triton WR-1339, Sigma, USA) were each dissolved in 6 ml of acetone, mixed well, and completely dried under a N_2 gas. The dried substrate was then redissolved

in 20 ml of distilled water to give a final concentration of 300 μg cholesterol/mL. Then, reaction mixture containing 20 μl of a cholesterol solution (6 g cholesterol), 20 μl of a 1 mol/L potassium-phosphate buffer (pH 7.4), 5 μl of 0.6 mM bovine serum albumin, 50~100 μg of microsomal fraction, and distilled water (up to 180 μl) was preincubated at 37°C for 30 min. The reaction was then initiated by adding 5 nmoles of [^{14}C]-Oleoyl CoA (specific activity; 2.0202 GBq/mmole, NEN^{TM} Life Science Products, Inc., Boston) to give a final volume of 200 μl; the reaction time was 30 min at 37°C. The reaction was stopped by adding 500 μl of an isopropanol-heptane mixture (4:1, v/v), 300 μl of heptane and 200 μl of 0.1 mol/L potassium phophate (pH 7.4) and the reaction mixture was allowed to stand at room temperature for 2 min. Finally, 200 μl amount of aliquot from the supernatant was subjected to scintillation counting. The ACAT activity was expressed as picomole of cholesteryl oleate synthesized·min^{-1}·mg microsomal protein^{-1}.

Statistical analysis

All data were presented as the mean±SE. Significant differences among the groups were determined by Student's t-test using standard social science statistical packages.

RESULTS

Body weights, organ weights and food intakes were not altered by 0.1% hesperidin supplementation as shown in Table 2. The supplementation of 0.1% hesperidin did not lower the levels of plasma and hepatic lipids significantly (Table 3). However, it tended to lower the plasma and hepatic lipids. The ratios of HDL-to total-cholesterol in two groups were almost identical (15.7% vs. 15.0%).

However, hesperidin supplementation caused 24% reduction (1896.5 vs. 2487.9 pmole·min^{-1}·mg microsomal protein^{-1}) in the HMG-CoA reductase activity compared to control (Fig. 1). There was no significant difference between these groups in the ACAT activities. These suggest that the hesperidin might inhibit the regulation of cholesterol biosynthesis. The hesperidin-supplementation resulted in distinct changes in the fecal neutral sterols. Although the amount of cholesterol intake was about same level in both groups according to their food intake (Table 2), over two-fold as much neutral sterol was excreted in control group compared to that of hesperidin-supplemented group (521.8 vs. 254.4 mg/day) (Fig. 2). Major neutral sterol was cholesterol followed by coprostanone and coprostanol in that order. Cholesterol excretion was the largest since all animals were on the high-cholesterol diet during the experimental period. Excretions of the cholesterol and coprostanone were significantly lower in hesperidin-supplemented group than in control.

Table 2. Effects of 0.1% hesperidin supplementation on food intake, weight gain and organ weights in high cholesterol-fed rats

	Control	Hesperidin
Food intake (g/day)	21.8±0.22[1)]	21.9±0.19[NS2)]
Weight gain (g/day)	5.7±0.13	5.4±0.11[NS]
Organ weight (g)		
Liver	12.36±0.33	12.95±0.41[NS]
Heart	1.15±0.04	1.06±0.03[NS]
Kidney	2.64±0.08	2.50±0.07[NS]

[1)]Mean±S.E.
[2)]Not significantly different ($p<0.05$) from control group.

Table 3. Effects of 0.1% hesperidin supplementation on the plasma and hepatic lipids in high cholesterol-fed rats

Lipids conc. \ Groups		Control	Hesperidin
Plasma	Total-cholesterol (mmol/L)	3.8±0.3[1)]	3.4±0.2[NS2)]
	HDL-cholesterol (mmol/L)	0.57±0.01	0.48±0.04[NS]
	HDL-C/Total-C (%)	15.7±1.6	15.0±1.5[NS]
	Triglyceride (mmol/L)	1.12±0.07	1.05±0.08[NS]
	Athero. index[3)]	5.7±1.1	6.1±1.2[NS]
Liver	Cholesterol (mg/g)	70.3±1.5	61.2±4.8[NS]
	Triglyceride (mg/g)	84.1±2.3	76.1±3.3[NS]

[1)]Mean±S.E.
[2)]Not significantly different ($p<0.05$) from control group.
[3)]Athero. index (Atherogenic index): (Total cholesterol - HDL-cholesterol)/HDL-cholesterol

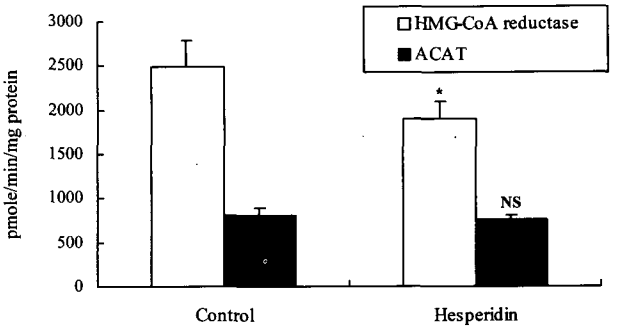

Fig. 1. Effect of 0.1% hesperidin supplementation on hepatic HMG-CoA reductase and ACAT activities in high cholesterol-fed rats. Mean±S.E., NS: No significance
*Means are significantly different ($p<0.05$) from control group.

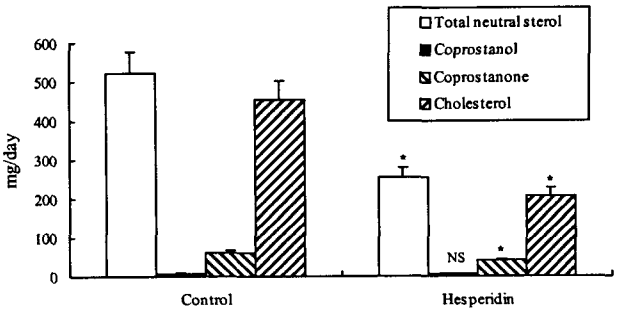

Fig. 2. Effect of 0.1% hesperidin supplementation on excretion neutral sterols in high cholesterol-fed rats.
Mean±S.E., NS: No significance
*Means are significantly different ($p<0.05$) from control group.

DISCUSSION

A few reports on the effects of hesperidin in cholesterol metabolism have been reported. Kawaguchi et al. (28) showed that 10% hesperidin diet inhibits the pancreatic lipase and lowers the concentrations of plasma triglycerides in rats compared to control, and thereafter Monforte et al. (29) reported a hypolipidemic activity of hesperidin in hypercholesterolemic rats. However, the hypocholesterolemic effect of 0.1% hesperidin was not significant when in the high cholesterol diet from this study.

HMG-CoA reductase inhibitors are well-established drugs for the treatment of hypercholesterolemia. The cholesterol lowering effect of HMG-CoA reductase inhibitors is attributed to increase in VLDL catabolism (30) as well as increases in specific receptor-mediated uptake in the liver (31,32) in various animal species. When tested *in vitro*, hesperidin did not inhibit the activities of either HMG-CoA reductase or ACAT (14). As shown in the test with cholesterol-fed rats, hesperidin is a potent agent for the inhibition of HMG-CoA reductase. However, well-known HMG-CoA reductase inhibitor drugs do not have a hypocholesterolemic action in rodents (4,33), yet do in hamsters, rabbits (32) or humans (3). This might be due to differences in lipoprotein metabolism among animal species (34). Dosages of HMG-CoA reductase inhibitors must be very high to exert a hypocholesterolemic response in rats (2,35). In present study, the hesperidin-supplementation with a low dosage (0.1%, wt/wt) may not be sufficient to exert its hypolipidemic effect when used with the high cholesterol diet in rats. When the same level (0.1%, wt/wt) of hesperetin, deglycosylated form of hesperedin, was supplemented with high-cholesterol (1%) laboratory chow diet in our previous study, it significantly lowered plasma cholesterol as well as HMG-CoA reductase activity in rats (15). Bioavailability of hesperidin was evaluated to be relatively low, less than 25% (36). Hesperidin may undergo some structural changes to be an active form in intestines or other organs after digestion. The sugar moiety of hesperidin may be presumably cleaved by glycosidases forming a derivative, hesperetin. This hypothetical action is partly supported by the presence of hesperetin in urine and plasma after the hesperidin administration (36).

This study identified decreased fecal neutral sterols in animals supplemented with hesperidin. Cholesterol biosynthesis was concomitantly reduced by the hesperidin as indicated by the decreased HMG-CoA reductase activities. The decreased amount of fecal neutral sterols suggests that the absorption of dietary cholesterol might be increased by the hesperidin supplement. Since the cholesterol intake was about the same for all groups, the supplementation of hesperidin seemed to promote an efficient utilization of dietary cholesterol, i.e. a possible increase of cholesterol uptake by tissues. In order for the plasma cholesterol to be unchanged or decreased with an elevated level of exogenous cholesterol, the endogenous synthesis of cholesterol would be suppressed to such a degree that suppressed synthesis can override the increased amount of cholesterol by the intestinal absorption. Thus, it seems that hesperidin exhibits a very unique mode in the regulation of cholesterol metabolism in rats fed a high-cholesterol diet.

Our results suggest the hesperidin reduce the cholesterol biosynthesis by an inhibition of hepatic HMG-CoA reductase, resulting to an increased absorption of dietary cholesterol in the intestine, which may subsequently result to a simultaneous decrease in the fecal neutral sterols in the hesperidin-supplemented animals. It therefore seems plausible that HMG-CoA reductase activity is inhibited first in animals fed hesperidin-supplemented high-cholesterol diet. This then led to an efficient utilization of exogenous cholesterol, an increased absorption of cholesterol, and a decrease in fecal neutral sterols.

Another assumption remained to be proved is a possible increase of the hepatic cholesterol uptake by an increase in the lipoprotein receptor activities due to the inhibition of hepatic HMG-CoA reductase. The inhibition of HMG-CoA reductase was reported to induce a decrease in the rate of cholesterol biosynthesis and is followed by an increase in low density lipoprotein receptors, enhancing the cellular uptake of cholesterol and lowering plasmas low density lipoprotein cholesterol (37). This possibility warrants further evaluation.

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