Increased Cholesterol Absorption by Hyperlipidemia Atherosclerosis Prone (LAP) Japanese Quail

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Summary The present study describes the cholesterol absorption by hyperlipidemia atherosclerosis prone (LAP) Japanese quail to address their high susceptibility to experimental atherosclerosis. The apparent cholesterol absorption rate of LAP quail was compared with that of commercially available (CA) Japanese quail. After 14 d of cholesterol feeding by gavage, it was found that the cholesterol excretion of LAP quail was significantly lower than that of CA quail. The fecal excretion of bile acid and fat showed a similar tendency to that as shown with the case of cholesterol. The cholesterol feeding only increased the serum cholesterol level of LAP quail, and this trend holds true for the liver lipid concentration. The expression level of liver cholesterol 7 α -hydroxylase mRNA showed no difference between LAP and CA strains under the conditions of cholesterol loading. These results showed that the cholesterol absorption by LAP quail is significantly higher than that by CA quail, which may reasonably explain the higher susceptibility of this strain to experimental atherosclerosis.

Key Words cholesterol feeding, atherosclerosis, absorption, excretion

A strain of Japanese quail susceptible to experimental atherosclerosis (hyperlipidemia atherosclerosis prone: LAP) has been developed by repeated breeding of a highly susceptible line. Our previous study focused on some of the key enzymes of the cholesterol metabolism found comparable activities in LAP and commercially available (CA) quail (1). The lipoprotein and apoprotein profiles were also similar between these two strains (2–4). Thus, the reasons for the higher susceptibility of LAP strain to experimental atherosclerosis are yet to be clarified.

The LAP strain easily develops hypercholesterolemia in response to the dietary loading of cholesterol, and this fact suggests that the input-output balance under atherogenic conditions may be the factor for the increased serum cholesterol level of the LAP strain. The absorption of cholesterol by the small intestine is the first committed step to the cholesterol metabolism. The aim of this study was therefore to compare the apparent cholesterol absorption rate between LAP and CA quail. The expression of cholesterol 7 α -hydroxylase, the rate limiting enzyme of bile acid production, was studied in relation to the vehicle role of the bile acid for cholesterol absorption.

To minimize the contamination of fecal excretion by

dietary overspill, cholesterol was administered by use of gavage in this experiment. The fecal excretion was collected throughout the experiment, and the entire input-output balance of cholesterol was studied. This experimental feature allowed us, for the first time, to detect the difference in the apparent cholesterol absorption rate between LAP and CA strains.

MATERIALS AND METHODS

Animals and diets. Male normal Japanese quail (2–3 mo old) were purchased from a commercial supplier (Kyudo Co. Ltd., Kumamoto), and LAP quail (2–3 mo old) were supplied by Tetsuo Nasu, Department of Veterinary Anatomy, Miyazaki University. The animals were randomly assigned to two diet groups ($n=7$ per group), and housed individually in stainless steel cages at a constant room temperature (25°C) and with a 12 h light cycle. The quail were fed a commercial diet ad libitum. The indicated composition was 22% protein, 2.5% fat, 5% fiber, 14% ash, and 2,700 kcal/kg. The animals of the cholesterol feeding groups were fed 50 mg of cholesterol in 500 mg of corn oil by gavage at 14:00 h everyday. The control groups were only fed 500 mg of corn oil. The administration of 50 mg cholesterol and 500 mg corn oil is approximately equal to the daily ingestion of a 10 g diet containing 0.5% cholesterol and 5% corn oil.

Lipid analysis. The blood samples were drawn from

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Fig. 1. Fecal cholesterol concentration of LAP and CA quail fed control or cholesterol diets. The cholesterol concentrations in the total feces collected throughout the experiment were analyzed and expressed as excretion per day. A, cholesterol concentration of feces; B, apparent cholesterol absorption of cholesterol-fed quail (cholesterol intake-fecal cholesterol). The apparent absorption rate was 31.6% for LAP quail and 22.7% for CA quail. The upper- and lowercase letters above the right corner of the graph bar (A and a) respectively show the comparison between strains and diets (cholesterol feeding), and the letter denotes a statistically significant difference at $p < 0.05$.

quail fasted overnight at the end of the experiment, and the liver was excised and stored in a 10% formaldehyde solution. The feces were collected throughout the experiment onto tin aluminum foil, and entire specimens were lyophilized and kept for analysis at -20°C .

The fecal lipids extracted with ethanol at 70°C were analyzed enzymatically for cholesterol (5) and bile acids (6). The fecal fatty acids were measured by the method of Van de Kamer et al. (7), and were calculated back as tristearin.

Serum was obtained the centrifugation of the blood at $1,600\times g$ for 10 min. Serum lipids (cholesterol, triacylglycerol and phospholipids) were analyzed by assay kits supplied by Wako Pure Chemical Industry, Ltd. The liver lipids were extracted with chloroform-methanol (2/1, v/v). The concentrations of liver total cholesterol, free cholesterol, triacylglycerol, and phospholipids were determined chemically as described previously (8–10).

Northern blot analysis of hepatic cholesterol 7 α -hydroxylase mRNA. Total RNA was purified as described previously (2) and used for the quantitative analysis of cholesterol 7 α -hydroxylase mRNA. The liver total RNA (0.2 μg) was denatured in the presence of 6.5% formaldehyde and 50% formamide at 55°C for 15 min, and electrophoresed in a 1% agarose gel containing 6.7% formaldehyde. RNAs were transblotted to a nitrocellulose membrane filter, and cross-linked by baking at 80°C for 1 h (2).

The plasmid CpcRTMII harboring the cDNA clone of chicken cholesterol 7 α -hydroxylase was a generous gift of Dr. Furuichi, Professor of Mie University. The ^{32}P -radio-labelled DNA probe directed against the active site of cholesterol 7 α -hydroxylase was prepared by PCR reaction in the presence of ^{32}P -dCTP and the quail liver cDNA as a template. The DNA sequence of the amplicon prepared by this method was 90% homologous to that of the corresponding region of chicken cholesterol 7 α -hydroxylase.

The filter was prehybridized with a ^{32}P -labelled probe

at 68°C for 30 min in $5\times\text{SSC}$, 0.1% (w/v) SDS, 5% (w/v) dextran sulfate, and 5% (v/v) blocking reagent (Gene ImageTM random prime labelling module supplied by Amersham International plc). Hybridization was carried out for 16 h in the same solution containing probe DNA prepared as described above. The membranes were washed successively with $1\times\text{SSC}$ containing 0.1% (w/v) SDS for 15 min at 68°C , and $0.1\times\text{SSC}$ containing 0.1% (w/v) SDS for 15 min at 68°C (11).

The blot was exposed to an imaging plate (Fuji Photo Film Co., Ltd.) which integrated and converted the intensity of the radioactivity into graphic images. The spot intensity was measured by computerized analysis of the graphic images. The software used for the measurement was NIH Image (National Institutes of Health).

Statistical analysis. Data were subjected to two-way analysis of variance followed by inspection of the differences between pairs of means by the least significant difference method (12).

RESULTS

Fecal excretion of cholesterol, fats, and bile acid

Figure 1A shows the fecal excretion of cholesterol. When the animals were fed the control diet, no significant difference between the LAP (8.2 mg/d) and CA strains (9.7 mg/d) was noted in the total fecal cholesterol excretion. With the animals fed the cholesterol diet, the cholesterol excretion for the LAP strain (34.2 mg/d) was significantly lower than that for the CA strain (38.7 mg/d). The apparent cholesterol absorption for the LAP and CA strains was therefore 15.8 and 11.3 mg/d, respectively (Fig. 1B). The apparent absorption rate was 31.6% for LAP and 22.7% for CA quail. The LAP strain absorbs 4.5 mg of cholesterol more per day than CA quail, and the apparent absorption of the LAP strain was 1.4-fold higher than that of the CA strain. This is the first indication of an overt difference in the absorption rate of cholesterol between LAP and CA strains.

Table 1. Fecal bile acid and total fat concentrations of LAP and CA quail fed control or cholesterol diets.

Feces	LAP		CA	
	Chol. (–)	Chol. (+)	Chol. (–)	Chol. (+)
Bile acid (mg/d)	2.7±0.1 ^A	4.8±0.7	5.7±1.4	7.2±0.5
Total fat (mg/d)	68.5±5.3 ^A	64.0±10.6 ^A	173.1±20.9	181.5±12.9

Data are mean±SE of 7 quail. The concentrations of bile acid and total fat in the total feces collected throughout the experiment were analyzed and expressed as excretion per day. The uppercase letter in the table (A) shows the comparison between strains and denotes a statistically significant difference at $p<0.05$.

Table 2. Serum lipid concentrations of LAP and CA quail fed control or cholesterol diets.

Serum (mg/dL)	LAP		CA	
	Chol. (–)	Chol. (+)	Chol. (–)	Chol. (+)
Total chol.	219±23 ^a	1353±230 ^A	198±14	257±21
Free chol.	72.7±4.4 ^a	347±44.7 ^A	62.6±3.6	60.9±4.8
TG	109±8.0 ^A	137±14.6 ^A	75.6±7.1	73.3±7.5
PL	418±32	558±58 ^A	448±34	432±45

Data are mean±SE of 7 quail. The upper- and lowercase letters in the table (A and a) respectively show the comparison between strains and diets (cholesterol feeding), and the letter denotes a statistically significant difference at $p<0.05$.

Table 3. Liver lipid concentrations of LAP and CA quail fed control or cholesterol diets.

Liver (mg/g liver)	LAP		CA	
	Chol. (–)	Chol. (+)	Chol. (–)	Chol. (+)
Total chol.	2.99±0.11 ^a	14.7±2.29 ^A	2.81±0.08	3.04±0.20
Free chol.	2.38±0.08 ^a	4.17±0.13 ^A	2.20±0.05 ^a	2.48±0.10
TG	3.57±0.41	3.76±0.54	3.39±0.38	3.62±0.66
PL	0.39±0.03	0.42±0.02	0.39±0.03	0.43±0.06

Data are mean±SE of 7 quail. The upper- and lowercase letters in the table (A and a) respectively show the comparison between strains and diets (cholesterol feeding), and the letter denotes a statistically significant difference at $p<0.05$.

The fecal excretion of bile acid and total fat depicted similar trends as shown for cholesterol excretion, suggesting the higher capability of the LAP strain in lipid absorption (Table 1).

Serum cholesterol and lipid concentrations

Table 2 shows the lipid concentrations of serum. In the control group, the serum total and free-cholesterol level were roughly comparable between strains. As has been demonstrated, the serum total and free-cholesterol levels of cholesterol-fed LAP quail were about 5 times higher than those of the CA strain. With respect to CA quail, cholesterol feeding slightly increased the serum cholesterol concentration, but only to a statistically insignificant extent. The concentrations of serum TG and PL were increased in the LAP strain by the cholesterol feeding, but to a lesser extent compared with the case of the cholesterol level.

The profile of liver lipid concentrations manifested essentially the same tendency as shown with the serum lipid levels (Table 3).

Expression of hepatic cholesterol 7α-hydroxylase mRNA

The control level of hepatic cholesterol 7α-hydroxylase mRNA for LAP quail was 1.5-fold higher than that for CA quail ($p<0.05$) (Fig. 2). The feeding of cholesterol appeared to decrease the expression of liver cholesterol 7α-hydroxylase in LAP quail and increase that in CA quail.

DISCUSSION

We herein first demonstrated the increased cholesterol absorption of LAP quail compared to the case of CA quail. Although a similar trend in cholesterol absorption was seen with the LAP strain, no statistically significant difference was noted in the previous study, probably due to the contamination of fecal excretion by cholesterol of the dietary overspill (1). The administration of cholesterol by gavage in this study overcame the difficulty encountered in the foregoing experiment and allowed us to demonstrate the increased absorption of cholesterol by the LAP strain for the first time.

On the basis of the fecal excretion of cholesterol and

Fig. 2. Effects of cholesterol feeding on the expression of hepatic cholesterol 7 α -hydroxylase mRNA. Data are mean $\pm$ SE of 7 quail. The uppercase letter (A) denotes a statistically significant difference between strains at $p < 0.05$.

bile acid (Fig. 1 and Table 1), it is possible to estimate the total steroid (cholesterol + bile acid) retained by both LAP and CA quail during the period of cholesterol feeding. The total cholesterol retained in the body throughout 14 d of the experiment was 157.1 mg for LAP quail and 62.8 mg for CA quail. The LAP strain thus accumulated 94.3 mg of cholesterol more than the CA quail, and this was equivalent to the accumulation of 6.7 mg/d. A large part of the cholesterol accumulation was explained by the increased absorption of dietary cholesterol as described in the Results section (4.5 mg/d). The LAP strain excreted less bile acid into the feces than the CA quail did. The difference in the bile acid excretion thus proves that the rest of the cholesterol accumulated in the LAP strain. The higher capability of lipid absorption in the LAP quail therefore may reasonably explain their higher susceptibility to experimental atherosclerosis.

The total cholesterol administered was 700 mg/quail during the 14 d of this experiment. The apparent absorption rate was 31.6% for LAP quail and 22.7% for CA quail, respectively. The difference in the apparent absorption rate thus appeared to be small and much less significant in this sense. The difference was however substantial from the quantitative view, taking into consideration the limited cholesterol pool size of Japanese quail as discussed below.

The mean total volume of serum for the LAP and CA quail was estimated to be 6.7 and 7.3 mL, respectively, based on the assumption that the blood comprised 10% of the body weight and the hematocrit value (32%) of chicken. Thus, the difference in the cholesterol absorption described above was very significant to the limited serum pool size of Japanese quail. Of the total cholesterol (157.1 mg) retained by LAP quail, the serum (90.5 mg) and liver (24.9 mg) may account for 57.6 and 15.8%, respectively. (Pool size for the liver was calculated on the basis of the assumption that the liver weight is 1.7 g/100 g body weight. The liver weight of

quail is usually in a range between 1.5 to 2.0 g/100 g body weight). The rest of the cholesterol (41.7 mg) not explained by these two major pools may be retained in the extra hepatic tissues such as adipose tissues and muscles, and/or leave the body in the form of feather debris. This may inversely suggest that Japanese quail have an extra pool capable of accommodating a maximum of about 40 mg of cholesterol under atherogenic conditions. This postulate appeared to be true for the case of CA quail fed cholesterol. Of the cholesterol retained in the CA quail (62.8 mg), 39.5 mg was localized in the extra pool, and 18.1 mg and 5.2 mg respectively in the serum and liver.

The feeding of cholesterol had no effect on the expression of hepatic cholesterol 7 α -hydroxylase mRNA in LAP quail. The LAP strain appeared to lack the upward regulation of this enzyme by dietary cholesterol (13). This lack of regulation may also contribute to some extent to the higher susceptibility of this strain to atherosclerosis. It has been suggested that the activity of cholesterol 7 α -hydroxylase is considerably lower in hyper-responsive pigeons than in hyporesponsive pigeons, which reasonably explains the hypercholesterolemia of this strain (14). Thus, the reduced rate of cholesterol catabolism of the LAP strain under atherogenic conditions may similarly aggravate hypercholesterolemia.

The LAP quail are unique among the experimental animals in that they easily develop atherosclerosis due to increased cholesterol absorption. Although more cytogenetic and physiological experiments are needed before a definitive factor for the increased cholesterol absorption is known, we have found the LAP quail to be of great value in the study of the relationship between atherogenesis and cholesterol absorption.

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