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## Sterol balance and cholesterol absorption in inbred strains of rabbits hypo- or hyperresponsive to dietary cholesterol

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### Summary

In 2 inbred strains of rabbits with high or low response of plasma cholesterol to dietary cholesterol, excretion of steroids in the feces and efficiency of cholesterol absorption were determined. Rates of whole-body cholesterol synthesis, measured as fecal excretion of bile acids and neutral steroids minus cholesterol intake, were similar in hypo- and hyperresponders fed a low-cholesterol (8  $\mu\text{mol}/100\text{ g}$ ) diet. Transfer of the rabbits to a high-cholesterol (182  $\mu\text{mol}/100\text{ g}$ ) diet caused an increase in fecal bile acid excretion in hypo- but not in hyperresponders. Dietary cholesterol did not affect neutral steroid excretion in either rabbit strain. Hyperresponders tended to accumulate more cholesterol in their body than did hyporesponders. After the rabbits were switched back from the high- to the low-cholesterol diet, rates of whole-body cholesterol synthesis were significantly higher in the hypo- than in the hyperresponders. With the use of the simultaneous oral administration of [<sup>3</sup>H]cholesterol and  $\beta$ -[<sup>14</sup>C]sitosterol, hyperresponders were found to absorb significantly higher percentages of cholesterol than hyporesponders. It is concluded that the differences in stimulation of bile acid excretion after cholesterol feeding and the efficiency of cholesterol absorption are important determinants of the phenomenon of hypo- and hyperresponsiveness in the 2 inbred rabbit strains.

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Key words: Dietary cholesterol; Inbred rabbits; Plasma cholesterol; Excretion of steroids; Cholesterol absorption

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## Introduction

An increased intake of cholesterol elicits marked differences in cholesterolemic response between individuals of the same species. Individuals showing only small changes in the concentration of plasma cholesterol (hyporesponders) can be discriminated from those showing high degrees of hypercholesterolemia (hyperresponders). This phenomenon has been well established in various animal species and in man [1].

As to the underlying mechanisms of hypo- and hyperresponsiveness, important observations have been made. Hyperresponsive monkeys absorb higher percentages of dietary cholesterol than hyporesponders [2,3]. On low-cholesterol diets, hyperresponsive monkeys [2-4] and man [5] have lower rates of whole-body cholesterol synthesis than hyporesponders. Possibly, hyperresponders compensate not as well as hyporesponders for increased intakes of cholesterol by feedback inhibition of cholesterol synthesis [4,6]. Hyperresponding squirrel monkeys enhance their fecal bile acid excretion after cholesterol feeding more slowly and to a lesser extent than their hypo-responsive counterparts [2].

Although important data have been collected, the metabolic basis for human hypo- and hyperresponsiveness is not yet complete [1]. Progress is hampered by the fact that invasive studies cannot be performed in humans. The availability of inbred strains of rabbits with defined but different cholesterolemic responses to changes in diet [7,8] may be of great importance in this respect. In these animals the response to dietary cholesterol is highly reproducible between experiments. The inbred rabbits can be used to unravel the genetic basis of hypo- and hyperresponsiveness. In addition, the rabbits can be kept in the laboratory more easily than monkeys. This study was carried out in an attempt to find out whether there are similarities between the phenomenon of hypo- and hyperresponsiveness in monkeys and man and that in inbred strains of rabbits. For this purpose we have studied cholesterol absorption, fecal excretion of bile acids and neutral steroids, and cholesterol synthesis in inbred strains of hypo- and hyperresponsive rabbits. Part of this work has appeared in a preliminary form [9].

## Materials and methods

### *Animals and housing*

We used 6 adult male rabbits of each of the 2 inbred strains. The animals were 1-3.5 years old. The strains were IIIVO/JU and AX/JU and originated from the Jackson Laboratory colony, Bar Harbor, ME, U.S.A. [10]. The IIIVO/JU strain has previously been shown to be hypo-responsive, the AX/JU strain to be hyperresponsive to dietary cholesterol [7].

The animals were maintained at the Department of Laboratory Animal Science and kept individually in cages with wire-mesh bases constructed of galvanized steel, in a room with controlled lighting (light, 05.00-19.00 h), temperature (16-19°C) and relative humidity (55-65%). The rabbits were fed 100 g daily of commercial pellets (LK-04®, Hope Farms, Woerden, The Netherlands). Throughout the experiment the hypo-responsive rabbits weighed about 3.5 kg, and the hyperresponders about 3.0 kg.

### *Experimental design*

On day 0 of the experiment, all animals were transferred from the commercial diet to the low-cholesterol, semipurified diet. On day 41 cholesterol was added to the diet and on day 111 it was removed again. The rabbits were fed 75 g of the diets per day and had free access to water. Daily food intake was recorded throughout the entire experiment. On days 33, 95 and 188, after an 18 h fast, each rabbit ingested a mixture of [<sup>3</sup>H]cholesterol and β-[<sup>14</sup>C]sitosterol which had been added to a cube of whole wheat bread. Feces of individual rabbits were collected daily during 6 periods: days 34-38, 42-46, 96-100, 112-116, 152-156 and 189-193. Below the wire-mesh base of each cage a tray was placed. Feces were in the form of dry balls and could be separated easily from spilled feed. The rabbits were allowed to practice cecotrophy. Plasma total cholesterol and body weights were determined regularly.

### *Semipurified diets*

The composition of the low-cholesterol semipurified diet (g/100 g diet) was as follows: soy isolate, 20.8; methionine, 0.2; corn starch, 17; dextrose, 21; sawdust, 18; coconut fat, 9; corn oil,

1; molasses, 5; dicalcium phosphate, 2.9; sodium chloride, 0.6; potassium bicarbonate, 1.8; magnesium carbonate, 0.3; magnesium oxide, 0.2; vitamin premix, 1.2; mineral premix, 1. The compositions of the vitamin and mineral premixes have been described elsewhere [8]. Cholesterol (0.08%, w/w) was added at the expense of the sawdust component. Upon analysis, the low- and high-cholesterol semipurified diets were found to contain on average 8 and 182  $\mu\text{mol}$  cholesterol/100 g of diet, respectively.

#### *Administration of radioactive sterols*

[1 $\alpha$ ,2 $\alpha$ (n)- $^3\text{H}$ ]Cholesterol and  $\beta$ -[4- $^{14}\text{C}$ ]sitosterol were obtained from Amersham International plc, Amersham, Bucks, U.K. The isotopes were at least 97% pure, according to the manufacturer. To measure cholesterol absorption [11,12], [ $^3\text{H}$ ]cholesterol (7.4–11.9  $\mu\text{Ci}$ ) and  $\beta$ -[ $^{14}\text{C}$ ]sitosterol (0.75–1.22  $\mu\text{Ci}$ ) were given orally to the rabbits. For this purpose, 150  $\mu\text{l}$  [ $^3\text{H}$ ]cholesterol in toluene (1 mCi/ml) and 750  $\mu\text{l}$   $\beta$ -[ $^{14}\text{C}$ ]sitosterol solution (20  $\mu\text{Ci}/\text{ml}$  toluene/ethanol, 9:1, v/v) were combined and evaporated under nitrogen at 30°C. The residue was dissolved in 250  $\mu\text{l}$  96% (v/v) ethanol and mixed with 3 ml corn oil, containing 15 mg cholesterol and 15 mg  $\beta$ -sitosterol (Sigma Chemical Company Ltd., Poole, U.K.); 200  $\mu\text{l}$  of this mixture was added to a cube of bread.

#### *Analytical methods*

Samples of blood were taken from the marginal ear vein of the rabbits into heparinized tubes. Sampling and measurement of the body weights were performed between 07.00 and 10.00 h after the removal of any remaining food, at 16.00 h the previous day. Total cholesterol in plasma was measured enzymatically using a test-combination (Monotest<sup>®</sup>) supplied by Boehringer-Mannheim GmbH (F.R.G.).

Steroids were analysed by gas-liquid chromatography [13] in samples of feces pooled per rabbit and per collection period. 5 $\alpha$ -Cholestane was used as internal standard for neutral steroid determination, and 23-nordeoxycholic acid for that of bile acids. The combined within- and between-run coefficients of variation for the contents of neutral steroids and total bile acids in a control

pool of rabbit feces were 6.2% and 9.0% (n = 4). Feces samples were also extracted [14] for determination of radioactivity in neutral steroids and bile acids fractions.

Radioactivity in samples of administered doses and fecal extracts were counted with Instagel<sup>®</sup> scintillation liquid (Packard Instrument Company Inc., IL, U.S.A.) in a Philips LSC PW 4700 scintillation counter (Philips, Eindhoven, The Netherlands). Appropriate corrections for quenching were made for both single and dual labelled samples.

#### *Calculation of sterol balance and cholesterol absorption*

For the calculation of cholesterol balance the following equation was used:

Cholesterol balance = (cholesterol intake) – (excretion of neutral steroids plus bile acids).

Negative values refer to net whole-body cholesterol synthesis. Positive values should be interpreted as accumulation of cholesterol in the body.

Percentage of dietary cholesterol absorbed was calculated by the following formula [11,12]:

Cholesterol absorption (%)

$$= \left\{ 1 - \frac{(^3\text{H in FNS})}{(^{14}\text{C in FNS})} \times \frac{(^{14}\text{C in OD})}{(^3\text{H in OD})} \right\} \times 100\%$$

where FNS = fecal neutral steroids, and OD = oral dose. Radioactivities are expressed in dpm.

#### *Statistics*

Comparisons between the hypo- and hyperresponsive strains were evaluated by Student's two-tailed *t*-test.

#### **Results**

Body weights of the rabbits did not change throughout the experiment. Feed intake was on average 13% lower in the hyper- than in the hypo-responders (data not shown). At day 155 one rabbit of the hyporesponsive strain was struck by a luxation in the lumbosacral region and had to be killed. Feeding the semipurified diet containing 182  $\mu\text{mol}/100$  g of cholesterol caused a more

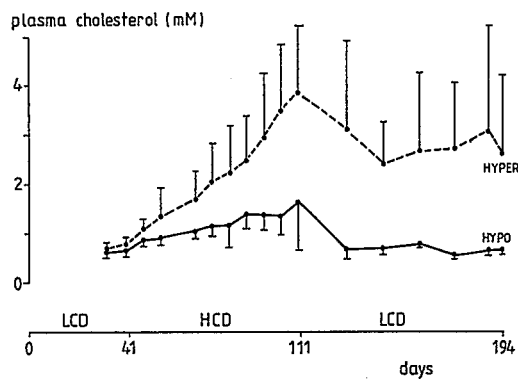


Fig. 1. Plasma cholesterol concentrations in hypo- and hyper-responsive rabbits. LCD = low-cholesterol diet; HCD = high-cholesterol diet. Results, expressed as means  $\pm$  SD for 5 or 6 animals per strain.

pronounced increase of plasma cholesterol in the hyper-responsive rabbits than in the hyporesponders (Fig. 1). After they had been switched back to the low-cholesterol semipurified diet, plasma cholesterol concentrations of the hyporesponders returned to baseline levels, whereas plasma cholesterol remained increased in the hyper-responsive rabbits.

Figure 2 illustrates the calculated percentages of cholesterol absorption during 5 consecutive days after oral administration of [ $^3\text{H}$ ]cholesterol and  $\beta$ -[ $^{14}\text{C}$ ]sitosterol. Calculated efficiencies of cholesterol absorption varied during the course of fecal collection after administration of both labels.

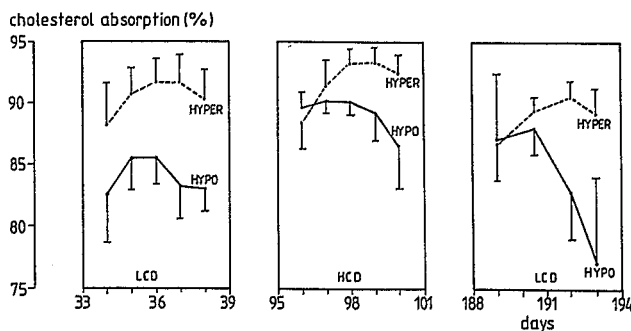


Fig. 2. Calculated percentages of cholesterol absorption, based on oral administration of [ $^3\text{H}$ ]cholesterol and  $\beta$ -[ $^{14}\text{C}$ ]sitosterol, in hypo- and hyper-responsive rabbits. LCD = low-cholesterol diet; HCD = high-cholesterol diet. Values, shown as mean  $\pm$  SD for 5 or 6 animals. Labels were given on days 33, 95 and 188 of the experiment.

TABLE 1

PERCENTAGE CHOLESTEROL ABSORPTION IN HYPO- AND HYPERRESPONSIVE RABBITS FED A LOW- ( $8 \mu\text{mol}/100 \text{g}$ ) AND A HIGH-CHOLESTEROL ( $182 \mu\text{mol}/100 \text{g}$ ) SEMIPURIFIED DIET

Diet	Period (days)	Cholesterol absorption (%)	
		Hypo-responders	Hyper-responders
LCD <sup>a</sup>	34-38	85.5 $\pm$ 2.3	91.6 $\pm$ 2.1 *
HCD	96-100	90.1 $\pm$ 0.8	93.2 $\pm$ 1.1 *
LCD	189-193	88.0 $\pm$ 2.1	90.5 $\pm$ 1.3 *

<sup>a</sup> LCD = low-cholesterol diet; HCD = high-cholesterol diet. Results, expressed as means  $\pm$  SD for 5 or 6 animals. Significant strain difference: \*  $P < 0.05$ .

Nevertheless, cholesterol absorption was almost always more effective in the hyper- than in the hyporesponders. In the hyporesponders, 'maximum' cholesterol absorption was reached at 2-3 days after label administration, whereas in the hyperresponders there tended to be a delay of 1 day. The maximum value is the best estimate of cholesterol absorption [12]. For both strains 'maximum' cholesterol absorption was calculated, and Table 1 shows that the hyperresponders absorbed significantly higher percentages of dietary cholesterol than did the hyporesponders. The amount of dietary cholesterol neither affected cholesterol absorption nor the strain difference in percentage cholesterol absorption.

Recoveries of orally given  $\beta$ -[ $^{14}\text{C}$ ]sitosterol in the neutral steroid fraction of 5-day pools of feces were on average 63% of the dose, and did not differ between both strains. Percentages recovery of orally administered [ $^3\text{H}$ ]cholesterol were also similar in the hypo- and hyper-responsive rabbits, the mean value being 7% of the dose. Recoveries of both labels were not significantly affected by the amount of cholesterol in the diet.

Fecal excretion rates of neutral steroids and bile acids in the 2 rabbit strains were similar during days 34-38 and 42-46 when the low- and high-cholesterol diets were fed successively (Table 2). Subsequently (days 96-100), fecal excretion of bile acids, but not of neutral steroids, increased in the hyporesponders. As a result, total excretion of steroids with feces was increased in hyporesponders. After switching the animals back to the

TABLE 2

CHOLESTEROL INTAKE AND STEROID EXCRETION IN HYPO- AND HYPERRESPONSIVE RABBITS FED A LOW- (8  $\mu\text{mol}/100\text{ g}$ ) AND A HIGH-CHOLESTEROL (182  $\mu\text{mol}/100\text{ g}$ ) SEMIPURIFIED DIET <sup>a</sup>

	Steroid intake, excretion or balance ( $\mu\text{mol}/\text{kg}$ body wt/day)					
	LCD (days 34–38)	HCD (days 42–46)	HCD (days 96–100)	LCD (days 112–116)	LCD (days 152–156)	LCD (days 189–193)
Cholesterol intake						
Hyporesponders	2 $\pm$ 0	40 $\pm$ 3	40 $\pm$ 4	2 $\pm$ 0	2 $\pm$ 0	2 $\pm$ 0
Hyperresponders	2 $\pm$ 0	41 $\pm$ 11	42 $\pm$ 4	2 $\pm$ 0	2 $\pm$ 0	2 $\pm$ 0
Fecal neutral steroids						
Hyporesponders	12 $\pm$ 2	13 $\pm$ 1	12 $\pm$ 2	12 $\pm$ 2	12 $\pm$ 1	11 $\pm$ 2
Hyperresponders	8 $\pm$ 2 *	10 $\pm$ 3	11 $\pm$ 3	11 $\pm$ 2	11 $\pm$ 3	11 $\pm$ 2
Fecal bile acids						
Hyporesponders	24 $\pm$ 1	29 $\pm$ 3	38 $\pm$ 5	40 $\pm$ 8	31 $\pm$ 3	24 $\pm$ 4
Hyperresponders	25 $\pm$ 3	26 $\pm$ 9	25 $\pm$ 11 *	23 $\pm$ 12 *	15 $\pm$ 4 *	13 $\pm$ 5 *
Total steroid excretion						
Hyporesponders	36 $\pm$ 2	42 $\pm$ 2	50 $\pm$ 6	52 $\pm$ 9	43 $\pm$ 2	34 $\pm$ 4
Hyperresponders	34 $\pm$ 4	37 $\pm$ 11	37 $\pm$ 12 *	33 $\pm$ 12 *	26 $\pm$ 5 *	24 $\pm$ 6 *
Cholesterol balance						
Hyporesponders	-34 $\pm$ 2	-1 $\pm$ 2	-10 $\pm$ 3	-50 $\pm$ 8	-41 $\pm$ 2	-32 $\pm$ 4
Hyperresponders	-32 $\pm$ 3	4 $\pm$ 4 *	5 $\pm$ 9 *	-32 $\pm$ 12 *	-24 $\pm$ 5 *	-22 $\pm$ 6 *

<sup>a</sup> See footnote to Table 1.

low-cholesterol diet (day 111), rates of bile acid excretion dropped gradually in both strains, but the strain difference persisted (Table 2). Similarly to plasma cholesterol levels (Fig. 1), rates of fecal excretion of bile acids in hyporesponders returned to initial values (days 34–38) at the end of the experiment (days 189–193). In hyperresponders however, bile acid excretion at the end of the experiment was markedly lower than at the beginning. Dietary cholesterol intake did not differ between the strains.

On the low-cholesterol diet (days 34–38) cholesterol balances were similar in the hypo- and hyperresponders. Immediately after the transfer of the rabbits from the low- to the high-cholesterol diet, cholesterol synthesis was markedly suppressed (Table 2). In the hyperresponders the cholesterol balance tended to be positive, suggesting that there was cholesterol accumulation in the body. When the rabbits were switched from the high- to the low-cholesterol diet, cholesterol balances became more negative, indicating that cholesterol synthesis was activated. The hypo-responsive rabbits now displayed significantly higher

rates of cholesterol synthesis than the hyperresponders.

### Discussion

In earlier investigations [7,8] the 2 rabbit strains have been shown to differ markedly in their cholesterolemic response to diets containing cholesterol concentrations as high as 0.5% (w/w). On such diets there is massive accumulation of cholesterol [9]. Therefore, in this study the high-cholesterol semipurified diet was formulated to contain only 0.08% (w/w) cholesterol (182  $\mu\text{mol}/100\text{ g}$ ). On the basis of our earlier work [9] this would result in a slightly negative sterol balance, and thus no extreme cholesterol accumulation in the body. Indeed, this prediction was borne out (Table 2). Figure 1 shows that after feeding the diet containing 0.08% of cholesterol, the hyperresponders still showed a more pronounced increase in plasma cholesterol than did the hyporesponders.

Higher rates of cholesterol synthesis in hypo-

sponders fed low-cholesterol diets, when compared with hyperresponders, have been reported for monkeys [2-4] and man [5]. On this point our results with hyper- and hyporesponsive rabbits fed low-cholesterol diets are not clear-cut. After transferring the rabbits from the commercial to the low-cholesterol diet, there was no difference in cholesterol balance between the hypo- and hyperresponders (Table 2, days 34-38). However, when the rabbits were switched from the high- to the low-cholesterol, semipurified diet, cholesterol balance was significantly more negative in the hypo- than hyperresponders. Thus hyporesponders synthesized more cholesterol than hyperresponders. This observation should be interpreted with caution as the rabbits may not have been in a steady-state of cholesterol metabolism. In the hyperresponders plasma cholesterol concentrations were still increased, and so possibly also body stores of cholesterol. On the high-cholesterol diet the hyperresponders rather than the hyporesponders may have accumulated some cholesterol because the cholesterol balance tended to be positive. Increased body stores, especially in the liver, will depress cholesterol synthesis [1]. Thus a difference in body cholesterol concentrations in the hypo- and hyperresponders could explain the difference in cholesterol balance after transfer from the high- to the low-cholesterol diet.

Bile acid excretion in the hyporesponders was increased after cholesterol feeding (Table 2). No such effect was seen in the hyperresponders. The hyperresponsive rabbits did show a decrease of bile acid excretion after changing from the high- to the low-cholesterol, semipurified diet (Table 2), which may relate to the observation that plasma cholesterol concentrations did not return to baseline values (Fig. 1).

Thus cholesterol-induced stimulation of bile acid excretion or lack of it, may explain, at least partly, the phenomenon of hypo- and hyperresponsiveness. The finding that the hyporesponsive rabbits accelerated bile acid excretion after cholesterol feeding agrees with observations in hyporesponsive squirrel monkeys [2]. In contrast, fecal excretion of neutral steroids was similar in hypo- and hyperresponders fed the low-cholesterol diets (Table 2). This suggests that the excretion of neutral steroids does not play a major role in

determining the response of plasma cholesterol to dietary cholesterol.

The percentage cholesterol absorption was measured using the [ $^3\text{H}$ ]cholesterol/ $\beta$ -[ $^{14}\text{C}$ ]sitosterol method [11,12]. The efficiency of cholesterol absorption agrees with values found by others in rabbits fed semipurified diets [15]. Table 1 documents that the hyperresponsive rabbits more effectively absorbed cholesterol than did the hyporesponders. Hyperresponsive monkeys have also been shown to absorb higher percentages of dietary cholesterol than their hyporesponsive counterparts [2,3]. Although the difference in percentage cholesterol absorption between hypo- and hyperresponders is small, it may have a significant impact on cholesterol metabolism. This relates to the fact that there is an enterohepatic cycle of cholesterol through which this sterol may circulate up to 7 times per day [16]. It could be argued that the difference in cholesterol absorption between hypo- and hyperresponsive rabbits is artifactual because the strains differ in colonic bacterial activity. Differences in colonic bacteria might be associated with differential breakdown of the sterol moiety of cholesterol and/or  $\beta$ -sitosterol [15], which in turn would affect the outcome of calculated cholesterol absorption. This can be ruled out because the recovery in feces of orally administered labelled  $\beta$ -sitosterol was almost identical in the 2 rabbit strains.

It may be concluded that the limited stimulation of bile acid excretion after cholesterol feeding and the higher percentage of cholesterol absorption in the hyperresponsive rabbits are factors which mutually determine hyperresponsiveness to dietary cholesterol. Possibly, the higher efficiency of cholesterol absorption is the primary factor. The higher efficiency of cholesterol absorption could be associated with enhanced reabsorption of bile acids, which results in less loss of bile acids with the feces. In any event, the higher efficiency of cholesterol absorption will lead to increased fluxes of cholesterol into the liver in hyperresponders, when compared with hyporesponders. The liver of hyperresponsive rabbits does not react by sufficiently stimulating bile acid excretion. The resulting increased liver cholesterol pools in hyperresponders after cholesterol feeding, cause increased rates of cholesterol release into plasma

when compared with hyporesponders. Cholesterol output with apoprotein B-containing lipoproteins by perfused rabbit livers has been shown to be increased after cholesterol feeding [7]. Stimulation of very low density lipoprotein (VLDL) production may lead to an increase in plasma cholesterol. This effect may be more pronounced in hyperthan in hyporesponders. Indeed, the increase of cholesterol in VLDL, which probably represents  $\beta$ -VLDL particles, is significantly higher in hyperthan in hyporesponders [18].

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