Transient Control of Serum Cholesterol Homeostasis in Adult ExHC (Exogenous Hypercholesterolemic) Rats by Dietary Cholesterol during Weanling Period

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Summary Rats hyper-responsive to a diet containing cholesterol plus cholic acid (exogenous hypercholesterolemic (ExHC) rats) were used to assess if cholesterol feeding at weanling period influences later serum cholesterol homeostasis. Diets containing cholesterol plus cholic acid (atherogenic diet) in early life, when compared to non-atherogenic diet, caused a transient suppression of serum cholesterol elevation in very-low- and low-density lipoprotein fractions during refeeding of the atherogenic diet in later life. Such an effect was not observed when ExHC rats were early given a diet supplemented with cholesterol or cholic acid alone, nor when ordinary Sprague-Dawley rats were given atherogenic diet. Early atherogenic diet caused an increased secretion of cholesterol as very-low-density lipoprotein from the perfused livers of adult ExHC rats. Neither the activity of hepatic cholesterol-7α-hydroxylase of fecal steroid excretion in later life was influenced by the early dietary manipulation. Therefore, the present results show the deferred effect of early dietary manipulation on later serum cholesterol metabolism in ExHC rats, but the underlying mechanism(s) remains to be determined.

Key Words imprinting effect, cholesterol homeostasis, hypercholesterolemic rat, dietary cholesterol, serum lipoproteins, weanling, liver perfusion

Some studies show that feeding a high-cholesterol diet to experimental animals in early life can protect against cholesterol challenge in adult life without developing a significant increase in the serum cholesterol level (1-3), but other studies refute such an effect (4-6). Although in most of these studies the response was evaluated by the concentration of serum cholesterol, rats used widely in the previous experiments may not be an appropriate animal model to assess such a deferred effect on cholesterol metabolism in later life because of their serum cholesterol levels.
being less susceptible to dietary cholesterol. Furthermore, a wide variability in individual cholesterolemic response among rats offered regimens designed to induce hypercholesterolemia has been seen: some animals tend to be exceptionally hypercholesterolemic while others are remarkably refractory (7).

In the present experiment, we used ExHC (exogenous hypercholesterolemic) rats as an animal model to study whether early feeding of a diet enriched with cholesterol plus cholic acid can deferred effect toward cholesterol challenge in adult life, since they are established as a strain highly susceptible to dietary cholesterol (7–9). The following responses, such as levels of cholesterol in serum lipoproteins, hepatic cholesterol 7α-hydroxylase activity, fecal steroid excretion, and secretion of lipoproteins by the perfused livers, were measured. The results show deferred effects of a simultaneous dose of cholesterol and cholic acid at weanling period on cholesterol metabolism in adult ExHC rats.

MATERIALS AND METHODS

Animals, diets, and experimental design. Age-matched ExHC rats (Takatsuki substrain) (7–9) were obtained from Takeda Chemical Industries, Ltd., Osaka, and bred in our animal facilities. They were fed commercial nonpurified diet (NMF, Oriental Yeast Co., Tokyo) ad libitum and had free access to drinking water. The animals were exposed to a 12-h light cycle (0600–1800) and an ambient temperature of 22–25°C. The pregnant ExHC rats were allowed to complete their gestation and newborn rats, 8 to 10 pups, were nursed by their respective dams until 17 days of age. At 18 days of age, neonatal male rats were weaned and the following diets were given for 18 days (phase I) as summarized in Table 1. The basal diet was composed of commercial nonpurified diet (NMF) containing 10% olive oil. The experimental group was given the basal diet supplemented with 2% cholesterol and 0.4% sodium cholate (atherogenic diet). The rats were than given the commercial nonpurified diet for 28 days (phase II), and were subsequently switched to the atherogenic diet for 63 days as a cholesterol challenge (phase III). Rats were again returned to the commercial diet for 56 days as a regression period (phase IV). During each phase, blood samples were obtained periodically from the tail vein. Rats were sacrificed by withdrawing blood from abdominal aorta under light diethyl ether anesthesia between 0900 and 1100 h. In some experiments, male pups of

Table 1. Experimental design.

| Group         | Phase I (days 18–35) | Phase II (days 36–63) | Phase III (days 64–126) | Phase IV (days 127–182) |
|---------------|----------------------|-----------------------|-------------------------|-------------------------|
| Control       | Basal                | NMF                   | Atherogenic             | NMF                     |
| Experimental  | Atherogenic          | NMF                   | Atherogenic             | NMF                     |

NMF, nonpurified diet.
Sprague-Dawley rats (Seiwa Experimental Animals Co., Fukuoka) were used.

Liver perfusion. Liver perfusion was carried out as described previously (10). The perfusion medium consisted of Krebs-Henseleit buffer containing 0.15% glucose, 25% bovine erythrocytes, and antibiotics (penicillin G, 750 units; streptomycin sulfate, 0.75 mg). After a 20-min equilibration, the initial medium was discarded and the liver was recirculated again with fresh medium for 135 min. Perfusate lipoprotein fractions, $d<1.006\text{g/ml}$ ($105,000 \times g$ for 18 h) and $1.006<d<1.063\text{g/ml}$ ($105,000 \times g$ for 18 h) were prepared by sequential ultracentrifugation according to the method of Havel et al. (11).

Hepatic cholesterol 7α-hydroxylase. Liver microsomes were prepared (12) and the cholesterol 7α-hydroxylase activity was assayed by the method of Van Cantfort et al. (13) using $[4-{^14}C]\text{cholesterol}$ (57.5 mCi/mmol, 0.5 µCi for each measurement) as a substrate. The hydroxylase activity was expressed as pmole of 7α-hydroxy cholesterol formed per mg of microsomal protein per min.

Chemical analyses. Feces were collected for two days and lyophilized. The fecal acidic and neutral steroids were quantitated by gas-liquid chromatography on 3% AN-600 (14) and 3% OV-17 (15) columns with nordeoxycholic acid and 5α-cholestane as an internal standard, respectively. Discontinuous polyacrylamide gel (3% and 10%) electrophoresis containing sodium dodecyl sulfate was performed (16). The protein was stained with Coomassic brilliant blue R-250 and the relative concentration of each apoprotein was determined (17). Blood serum and liver lipids were extracted (18) and used for the measurement of cholesterol, phospholipid, and triacylglycerol as previously described (19). Cholesterol in periodical blood samples from the tail vein was assayed using a commercially available kit (Wako Pure Chemical Industries, Osaka). The protein content was measured by the method of Lowry et al. (20).

Statistical analysis. Values were expressed as means±SE. The Student’s $t$-test was used to test the statistical significance of difference (21).

RESULTS

There were no significant differences in the body weight gain (day 18, 37–38 g; day 35, 119–126 g) and food intake (7–8 g/day) during phase I. The values for the subsequent periods were also comparable between these two groups (data not shown).

Changes of serum cholesterol level

The changes of serum cholesterol level are shown in Fig. 1. Simultaneous supplementation of cholesterol and cholic acid (atherogenic diet) to ExHC rat pups caused a marked increase in the serum cholesterol level at the end of phase I when compared to the basal diet. At the end of phase II, the serum cholesterol level of the experimental group decrease to the level of control group. When cholesterol challenge was carried out with the control and experimental groups (phase III),
the serum cholesterol level increase sharply in both control and experimental groups, but the extent was significantly small in the experimental group when compared to the control group until day 25 of phase III (88 days of age). When rats were fasted overnight at days 7–8 of phase III, the serum cholesterol level was still significantly lower in the experimental group when compared to the control group (377 ± 26 vs. 558 ± 40 mg/dl, p < 0.05). Such a different response of serum cholesterol level to the cholesterol challenge was observed as early as at the first day phase III (346 ± 15 and 278 ± 12 mg/dl for control and experimental groups, respectively, p < 0.05). This deferred effect, however, disappeared at the end of the phase III (day 62 of phase III). The slope of the serum cholesterol level in the regression period (phase IV) was indistinguishable between the groups.

In accordance with the previous reports (8, 9), long-term feeding of atherogenic diet caused an increased deposition of cholesterol in the aorta (end of phase III) and the withdrawal of the dietary cholesterol diminished the aortic depositions (phase IV) (data not shown). Early cholesterol manipulation, however, did not influence the aortic cholesterol deposition.

Effect of dietary components and susceptibility to dietary cholesterol on later serum cholesterol level

In order to assess if the transient suppression of serum cholesterol elevation at phase III as shown in Fig. 1 was attributable to an individual component
Fig. 2. Response of serum cholesterol level in adult ExHC or Sprague-Dawley (SD) rats to early supplementation of cholesterol and cholic acid. The basal diet supplemented with either cholesterol or cholic acid was given to the ExHC rat pups during phase I. Atherogenic diet was given to the Sprague-Dawley rat pups during phase I. A, B, C: control, cholesterol-, or cholic acid-supplemented ExHC rats, respectively. D, E: control and cholesterol plus cholic acid-supplemented Sprague-Dawley rats, respectively. Each bar shows means±SE for 6 rats per groups in each strain. * Significantly different from the respective control group at p<0.05.

(cholesterol or cholic acid), or to both, in the weanling diet, each of the two individual components was supplemented to the basal diet (Fig. 2). Either supplementation of cholesterol (B) or cholic acid (C) increased the serum cholesterol to the similar extent during phase I (35 Days) when compared to the control group (A), but the extent was less when compared to when the atherogenic diet (cholesterol plus cholic acid) was given (Fig. 1).

The cholesterol level at day 7 of phase III (70 Days) was similar among the groups (A, B, and C in Fig. 2).

Although simultaneous supplementation of cholesterol and cholic acid to Sprague-Dawley rats increased serum cholesterol levels at the end of phase I (35 Days) when compared to the control group (D and E in Fig. 2), there were no differences in the serum cholesterol level at day 7 of phase III (70 Days) between the control and experimental groups.

Serum and lipoprotein lipids

Table 2 shows the concentration of lipids in serum lipoprotein fractions at day 7 of phase III. Serum lipoprotein fractions were separated at d=1.006 g/ml, since in the preliminary experiment it was observed that the cholesterol in d>1.063 g/ml fraction was only composed of less than 7% of that in d>1.006 g/ml fraction when ExHC rats were given atherogenic diet for one week. All the lipids (triacylglycerol, phospholipid, and cholesterol) were significantly lower in d<1.006 g/ml fraction in the experimental group when compared to the control group. The cholesterol in...
Table 2. Lipids in serum lipoprotein fractions at density less than 1.006 g/ml and density greater than 1.006 g/ml at day 7 of phase III.

| Group           | Triacylglycerol (mg/dl) | Phospholipid (mg/dl) | Cholesterol (mg/dl) |
|-----------------|-------------------------|----------------------|---------------------|
| Whole serum     |                         |                      |                     |
| Control         | 177 ± 17.8              | 237 ± 20.2           | 784 ± 71.3          |
| Experimental    | 128 ± 7.89*             | 184 ± 10.2*          | 503 ± 51.7*         |
| $d<1.006$ g/ml  |                         |                      |                     |
| Control         | 99.8 ± 7.42             | 43.8 ± 4.24          | 221 ± 24.9          |
| Experimental    | 73.4 ± 2.35*            | 26.2 ± 3.45*         | 147 ± 13.7*         |
| $d>1.006$ g/ml  |                         |                      |                     |
| Control         | 61.6 ± 4.94             | 194 ± 16.9           | 563 ± 51.4          |
| Experimental    | 54.6 ± 6.42             | 155 ± 11.8           | 324 ± 37.3*         |

Values are means ± SE for 6 rats per group. *Significantly different from the control group. Proteins at $d<1.006$ g/ml were 41.1 ± 3.2 and 32.3 ± 2.1 mg/ml for control and experimental groups, respectively, ($p<0.05$).

$d>1.006$ g/ml fraction was also significantly lower in the experimental group, suggesting that very-low- and low-density lipoprotein fractions carried less cholesterol in the experimental group.

**Secretion of lipoproteins from the perfused livers**

The effect of early dietary manipulation on the secretion of cholesterol and triacylglycerol by the perfused livers at day 5 of phase III is shown in Fig. 3. The...
serum cholesterol was significantly lower in the experimental than in the control group (403 ± 29 vs. 562 ± 33 mg/dl, p < 0.05). There were no significant differences in the bile flow (0.87 ± 0.07 vs. 0.79 ± 0.02 ml/h) and biliary cholesterol (20.5 ± 2.0 vs. 21.1 ± 1.9 μg/h). Secretion of the perfusate triacylglycerol was significantly greater in the control group while the difference for cholesterol was not as evident as triacylglycerol. Accumulations of cholesterol and triacylglycerol in the perfusate lipoprotein fractions (d < 1.006 g/ml and 1.006 < d < 1.063 g/ml) are shown in Table 3. The accumulation of cholesterol in the d < 1.006 g/ml fraction was significantly greater in the experimental than in the control group and vice versa in the 1.006 < d < 1.063 g/ml fraction. The accumulation of triacylglycerol in d < 1.006 g/ml tended to be less in the experimental group when compared to the control group and the triacylglycerol in 1.006 < d < 1.063 g/ml fraction was significantly lower in

Table 3. Accumulation of lipids in liver perfusate lipoprotein fractions in rats at day 5 of phase III.

| Lipoproteins | Triacylglycerol (μg/ml perfusate) | Cholesterol (μg/ml perfusate) |
|--------------|----------------------------------|------------------------------|
| d < 1.006 g/ml |                                  |                              |
| Control      | 80.4 ± 6.2                       | 27.3 ± 1.1                   |
| Experimental | 68.0 ± 4.0                       | 38.4 ± 2.1*                  |
| 1.006 < d < 1.063 g/ml |                          |                              |
| Control      | 10.4 ± 2.0                       | 40.4 ± 4.2                   |
| Experimental | 5.6 ± 0.7*                       | 26.5 ± 3.0*                  |

Liver perfusion was carried out for 135 min. Values are means ± SE for 5 rats per group. *Significantly different from the control rats.

Table 4. Relative percentage of apolipoproteins (apo) in serum and perfusate density less than 1.006 g/ml fraction at day 7 of phase III.

| Group     | apo Bₜ | apo B₁ | apo E (%) | apo A-I | apo Cs |
|-----------|--------|-------|-----------|--------|-------|
| Serum     |        |       |           |        |       |
| Control   | 4.4 ± 0.5 | 6.5 ± 0.5 | 34.2 ± 1.5 | 33.0 ± 1.5 | 14.4 ± 0.5 |
| Experimental | 5.2 ± 0.1 | 7.5 ± 0.1 | 29.2 ± 0.3* | 31.9 ± 1.2 | 15.9 ± 0.4 |
| Perfusate |        |       |           |        |       |
| Control   | 11.2 ± 1.8 | 12.0 ± 0.5 | 32.1 ± 2.7 | 11.3 ± 0.9 | 21.4 ± 1.3 |
| Experimental | 7.9 ± 0.5 | 9.7 ± 0.8 | 40.1 ± 1.9* | 15.0 ± 1.5 | 19.8 ± 2.0 |

Values are means ± SE for 5–6 rats per group. apo Bₜ: high-molecular-weight apo B; apo B₁: low-molecular-weight apo B. *Significantly different from control group at p < 0.05.

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Table 5. Hepatic and fecal lipids and the activity of cholesterol 7α-hydroxylase at the end of phase II and at day 7 of phase III.

| Parameters                      | End of phase II | Day 7 of phase III |
|---------------------------------|-----------------|--------------------|
|                                 | Control         | Experimental       | Control         | Experimental       |
| Hepatic lipids (mg/g liver)     |                 |                    |                 |                    |
| Triacylglycerol                 | 16.1 ± 1.1      | 15.2 ± 1.3         | 28.2 ± 2.1      | 34.3 ± 3.3*        |
| Phospholipid                    | 31.2 ± 1.8      | 31.5 ± 1.6         | 30.3 ± 2.5      | 31.1 ± 1.8         |
| Cholesterol                     | 2.92 ± 0.22     | 3.60 ± 0.33        | 53.4 ± 4.5      | 51.8 ± 3.1         |
| Fecal sterols (mg/day)          |                 |                    |                 |                    |
| Neutral sterols                 | 27.3 ± 1.7      | 29.5 ± 2.5         | 403 ± 12.2      | 396 ± 17.4         |
| Acidic sterols                  | 10.1 ± 1.1      | 12.2 ± 1.3         | 66.3 ± 4.3      | 62.5 ± 2.4         |
| Microsomal cholesterol (µg/mg protein) | 9.4 ± 0.3   | 10.0 ± 0.5         | 18.4 ± 0.9      | 18.2 ± 0.3         |
| 7α-Hydroxylase (pmol/min/mg protein) | 1.8 ± 0.1      | 2.4 ± 0.2*         | 18.6 ± 1.6      | 22.1 ± 2.0         |

Values are means ± SE for 6 to 7 rats per group. *Significantly different from the control group at p<0.05.

the experimental group.

Distribution of apolipoproteins in the serum and perfusate d<1.006 g/ml fraction are shown in Table 4. The relative distribution of the serum apo E was slightly, but significantly, lower in the experimental than in the control group. The relative distribution of the perfusate apo E, however, was significantly greater in the experimental group.

Hepatic and fecal lipids and cholesterol 7α-hydroxylase activity and fecal steroid excretion

Table 5 shows the hepatic and fecal lipids and the activity of hepatic microsomal cholesterol 7α-hydroxylase and microsomal cholesterol at the end of phase II and at day 7 of phase III. At the end of phase II, these were no marked differences in the parameters between the groups, except for slightly but significantly higher activity of cholesterol 7α-hydroxylase in the experimental group. Upon feeding atherogenic diet at phase III, all of the parameters except for hepatic phospholipid increased. The deferred effect of early cholesterol feeding on the above parameters at day 7 of phase III, except for slight increase in hepatic triacylglycerol, was not observed.

DISCUSSION

Conflicting results regarding the effect of nutritional modulation early in

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life on cholesterol metabolism in later life may be attributable to the differences in the experimental conditions such as the species and strains of the animals, experimental design, and diet composition (3-6, 22-27). Furthermore, the variability in the susceptibility to dietary cholesterol may modify the deferred effects of early dietary manipulation on homeostasis of later cholesterol metabolism, since diet may act at the modus operandi for revealing any purported inherent defect(s). In fact, when rats that are relatively refractory to hypercholesterolemia were compared to hyper-responders, significant differences in protein synthesis in vivo were observed after short-term cholesterol feeding (28). In the present experiment, ExHC rats, which were induced from the Sprague-Dawley strain (7-9), were selected as a model animal since their serum lipids and lipoproteins are constantly sensitive to exogenous cholesterol plus cholic acid without using hypothyroid drugs (7-9). The mechanism of such a genetic effect is not clear at present, but catabolic defect(s) of hepatic and intestinal lipoproteins rather than intestinal absorption of dietary cholesterol appears to be responsible for the dietary-induced hypercholesterolemia in ExHC rats, since plasmic catabolism of [125I]β-VLDL prepared from ExHC rats was markedly slow when compared to that from Sprague-Dawley strain (unpublished observation), and the response of β-VLDL prepared from ExHC rats to rat peritoneal macrophage differed from that of normal Sprague-Dawley rats (29).

Using this strain genetically prone to hypercholesterolemia, we presented the evidence that marked modulation of cholesterol metabolism early in life by the atherogenic diet resulted in a lower concentration of the cholesterol in very-low- and low-density lipoprotein fractions during the early period of phase III when the atherogenic diet was loaded again. Such a deferred effect of early atherogenic diet was not found for Sprague-Dawley rats. The deferred effect on serum cholesterol level was not prolonged for a longer period (at the end of phase III or phase IV). Such a transient, but repeatedly observed, effect on later serum cholesterol level was not found when weanling ExHC rats were given either cholesterol or cholic acid alone. Therefore, drastic alterations of cholesterol metabolism in early life observed as a high serum cholesterol level (cf. Figs. 1 and 2) are definitely involved in an imprinting effect of cholesterol metabolism in later life.

In order to examine the mechanism(s) for transient homeostasis of serum cholesterol metabolism at early phase III, the excretion of steroids and the secretion of cholesterol from the perfused liver were examined. As shown in Table 5, fecal steroid excretion and the activity of hepatic cholesterol 7α-hydroxylase appeared not to be influenced by the early dietary manipulation, implying that the fecal excretion of bile acids and neutral steroids may not be primarily responsible for the decreased serum cholesterol level at early phase III. As shown in Fig. 3, triacylglycerol secretion from the perfused liver was less in the experimental group, but the cholesterol secretion was similar between the groups. The perfusate \( d < 1.006 \text{g/ml} \) fraction in the experimental group contained higher amounts of cholesterol and apo E when compared to the control group (Table 4). This contrasted to the serum counterparts since the levels of cholesterol and apo E in the serum...
$d < 1.006 \text{g/ml}$ fraction were lower in the experimental group. Therefore, the deferred effect of early dietary manipulation on the later serum cholesterol metabolism cannot be simply explained by the secretory mechanism. However, it is interesting to note that the cholesterol accumulated in the perfusate $1.006 < d < 1.063 \text{g/ml}$ fraction, so-called low-density lipoprotein fraction, was 1.6-fold greater in the control than the experimental group. A half-life (5 min) in the blood stream of the cholesterol in the very-low-density lipoprotein is much shorter than that (8.5 h) in the low-density lipoprotein when these lipoproteins were infused into the normocholesterolemic rats (30, 31). Further study is necessary to clarify the underlying mechanism(s).

In summary, the present results showed that ExHC rats can be a suitable animal model for examining the underlying mechanism(s) of the deferred effect of early cholesterol feeding on the regulation of serum cholesterol metabolism in later life.

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