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Imprinting of High Sensitivity to a High-Cholesterol Diet by Nutrition in Early Life

R. POLEDNE, J. HAJNÁ

Institute for Clinical and Experimental Medicine, Cardiac Centre, Laboratory for Atherosclerosis Research, Prague, Czech Republic

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Summary

Imprinting of an increased sensitivity to a high-fat, high cholesterol (HFHC) diet by dietary manipulation in early life was studied in two strains of rat, i.e. in Prague hereditary hypercholesterolaemic rats (PHHC) and Wistar rats, from which the PHHC strain was obtained by selection and inbreeding. Whereas no effect of early life nutrition on cholesterolaemia induced by HFHC diet was found in control Wistar rats, significant imprinting of increased sensitivity to the same diet was demonstrated in PHHC rats. This imprinting increased the concentration of apoB-containing lipoprotein and liver cholesterol concentration in animals fed HFHC diet for a period of two months after weaning. No effect of this imprinting on endogenous cholesterol synthesis could be demonstrated. It is concluded that imprinting of increased sensitivity to HFHC diet by dietary manipulation in early life is not a general phenomenon but depends on underlying genetic predisposition(s).

Key words

Cholesterol metabolism – Cholesterol dietary – Lipoproteins – Child nutrition – Gene expression

Introduction

It is generally believed that dietary cholesterol influences actual serum cholesterol levels by increasing the amount of cholesterol absorbed and, consequently by regulation of cholesterol synthesis and bile acid excretion. There is considerable interest in dietary cholesterol manipulation in the early life of newborns and infants which may persist as an imprinting which later modulates cholesterol metabolism in adulthood (Hahn 1989), similarly to intrauterine nutritional effects on pathology developing in later life (Barker 1995).

The previous studies in rats (Reiser and Sidelman 1972, Hahn and Koldovsky 1976) have shown that a high or low cholesterol intake before weaning can influence cholesterolaemia in later life by persistent changes in hydroxymethylglutaryl CoA reductase. A change in lipoprotein fractions of adult baboons was imprinted by breast or formula feeding (Mott *et al.* 1990, 1992, 1995). These results were not confirmed by other studies (Kris-Etherton *et al.* 1979, Green *et al.* 1981). No effect of a high-cholesterol diet or cholestyramine supplementation in young rats could be demonstrated when the response of serum cholesterol was tested in later life (Beynen *et al.* 1985).

In human studies, imprinting of cholesterolaemia in children and young adults by cholesterol intake before weaning (breast versus formula feeding) is small or inconsistent (review by Hamosh and Hamosh 1988) and is presumably confused by genetic factors resulting in a high variation inside the groups. The Southampton group (Fall *et al.* 1992) reported lower plasma cholesterol and apoprotein concentrations and even lower mortality from coronary heart disease in a breast-fed group compared to a bottle-fed group.

Fax +420 2 293 894
E-mail physres@sun1.biomed.cas.cz
http://www.lf2.cuni.cz/physiolres/physiolres.htm

The imprinting effect of early dietary manipulation on the response to a high-fat, high-cholesterol diet common in industrialized countries have important implications for developmental physiology. The inconsistency of all the above studies may have been due to a different experimental design and/or differences in gene expression.

The present study was undertaken to ascertain whether a high-fat, high-cholesterol diet fed to an inbred strain of rats sensitive to dietary cholesterol (the Prague hereditary hypercholestero-laemic rat) (Poledne 1986a) in early life affects the response to this diet later in life.

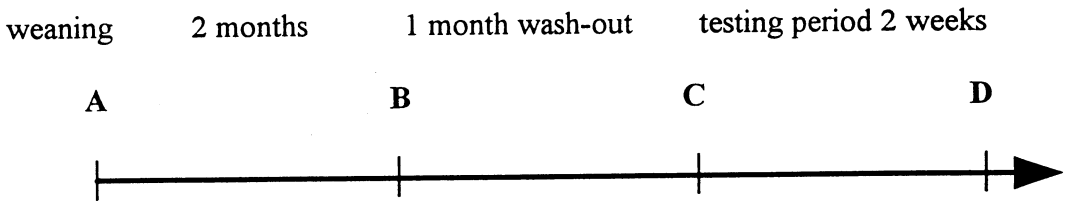
Methods

Wistar rats and Prague hereditary hypercholesterolaemic (PHHC) rats were used. The PHHC rat is an inbred line obtained in our laboratory by selection and brother-sister inbreeding (Poledne 1986a,b). The selection and development of hypercholesterolaemia in this rat strain are described elsewhere (Poledne 1986a,b). Shortly, PHHC rats display polygenous hypercholesterolaemia characterized by a moderate increase (50 %) in total serum cholesterol on a low-fat diet compared to Wistar rats from which this line was selected. PHHC rats display high sensitivity to a high-fat, high cholesterol (HFHC) diet manifested by increasing total serum cholesterol 3–5 times without bile acid supplementation and/or affecting the thyroid gland. Feeding the HFHC diet (2 % of crystalline cholesterol in 5 % lard supplementation to the control diet) increases total cholesterol in the serum and cholesterol content in the liver within several hours (Befekadu *et al.* 1992), the lipoprotein pattern being very similar to that in man but very different from all other strains of laboratory rats (Poledne 1986b).

Experimental design

Two groups of female Wistar rats (C1, C2) and two groups of female PHHC rats (PHHC 1, PHHC 2) were used immediately after weaning (at the age of 28 days), their total serum cholesterol was determined and data used for randomization. One group of Wistar rats (C2) and one group of PHHC rats (PHHC 2) were subsequently fed the HFHC diet for two months whereas the other groups remained on a control diet. At the end of a 2-month period, total serum cholesterol concentrations were determined and four animals from each group were sacrificed for analysis of liver cholesterol concentration and synthesis. In the following 4-week "wash-out" period, all animals were kept on a control lo- fat diet to remove liver cholesterol deposits in animals fed the HFHC diet. In the last part of the experiment, all four compared groups were fed the HFHC diet containing only 1% of crystalline cholesterol for two weeks and then sacrificed (Table 1).

Table 1. Experimental design



control 1 (C1)	control diet	control diet	HFHC diet (1%)
control 2 (C2)	HFHC diet (2%)	control diet	HFHC diet (1%)
PHHC 1	control diet	control diet	HFHC diet (1%)
PHHC 2	HFHC diet (2%)	control diet	HFHC diet (1%)

A, B, C, D, – blood specimens were taken at the end of each period

Analytical methods

Cholesterol concentrations in the serum were analyzed by enzymatic CHOD PAP method (Boehringer Mannheim, Germany) using a Cobas Mira autoanalyzer (Hoffmann-LaRoche, Switzerland). Plasma samples were used for lipoprotein separation by ultracentrifugation.

The livers were dissected, washed in cold saline and homogenized in methanol. Aliquots were inspected under the light microscope for routine analysis of hepatocyte size and parenchymal structure. Total lipids were extracted by Folch's method with the chloroform:methanol ratio of 2:1. An aliquot of the chloroform phase after overnight equilibration with phosphate buffer was dried under nitrogen. Sterols were purified by precipitation with digitonin and analyzed by the method of Abell *et al.* (1951). Lipoproteins were separated by sequential ultracentrifugation (100 000 x *g* for 18 hours) at densities <1.006 for VLDL and <1.0063 for IDL and LDL. HDL cholesterol was then analyzed after the last spinning of IDL and LDL. Pooled plasma samples of all the combined groups were used for analysis in triplicate in the angle rotor of Beckman's centrifuge (for details see Poledne *et al.* 1988, Poledne and Vrána 1990a).

Cholesterol synthesis in the liver was determined according to Turlay *et al.* (1981). 50 mCi of ^3H_2O were applied intraperitoneally and the animals were sacrificed after 60 min. The radioactivity of purified cholesterol (see above) was determined by liquid scintillation counting. For determination of cholesterol synthesis in absolute amounts, the specific radioactivity of whole-body water (analyzed from total serum) was used and the incorporation into the final product during 60 min. was calculated in pmol of tritiated water (Poledne 1990b).

Table 2. Total serum cholesterol (mmol/l)

Periods	A	B	C	D
Group				
Control 1 (C1)	2.03	2.11	1.97	1.92
(n=12/8)	0.21	0.13	0.12	0.39
Control 2 (C2)	2.08	2.35	2.03	1.98
(n=13/8)	0.19	0.13	0.20	0.16
PHHC 1	2.87	2.81	2.82	5.38
n=12/8)	0.12	0.13	0.27	1.37
PHHC 2	2.87	9.61*	2.60	7.71*
(n=12/8)	0.11	0.51	0.26	0.51

*Means ± S.D., n=number of animals (the first number represents the number of animals at period A and B, the second one number of rats for periods C and D). Periods A,B,C,D are described in Table 1 and Methods. *Significantly different vs preceding period (PHHC 1 vs PHHC 2).*

Results

The total serum cholesterol concentration after weaning was significantly higher in PHHC rats selected for the experiment compared to control Wistar rats ($p<0.001$) but no differences were found when comparing control groups 1 and 2 and PHHC 1 and 2 as direct results of randomization into two groups (the same number of rats of similar weight from each nest was used for groups 1 and 2) (Table 2). PHHC animals were taken from 9 litters of F_{30} generation of the inbred line and one or two young rats from each nest were randomly selected into groups PHHC 1 or 2.

Two months of feeding the HFHC diet to C2 and PHHC 2 did not alter total serum cholesterol in Wistar rats (C2 compared to C1), led to a threefold increase in PHHC 2 (compared to PHHC 1). At the end of this 2-month period, cholesterol concentration in the liver of C2 rats increased to 9 mg/g with adequate feedback regulation of endogenous cholesterol synthesis (Table 3). There was an almost

threefold decrease in cholesterol synthesis in group C2 compared to animals fed the control diet. The already high cholesterolaemia in PHHC rats was further increased three times (compared to PHHC 1) with adequate feedback regulation of cholesterol synthesis (PHHC 1 vs PHHC 2).

Total serum cholesterol dropped in both groups fed the HFHC diet (C2 and PHHC 2) after a 4-week wash-out period. No significant differences in total serum cholesterol were found when groups C1 and C2 or PHHC 1 and PHHC 2, respectively, were compared. In a separate experiment (data not shown), a decrease in cholesterol levels was found in the liver of PHHC rats after they had been switched from HFHC to the control diet. The cholesterol and triglyceride concentrations in the liver dropped to baseline within two weeks after the HFHC diet had been discontinued. Hepatocyte morphology also became normalized within this period.

After the wash-out period, all experimental groups were placed on the HFHC diet containing 1 % of crystalline cholesterol for 2 weeks. This diet had no effect on serum cholesterol in Wistar rats but significantly increased cholesterolaemia in both PHHC groups. The increase in PHHC 2 (7.71±0.51 mmol/l) was significantly higher ($p<0.01$) compared to that seen in PHHC 1 (5.38±1.67 mmol/l). This difference in the serum cholesterol runs parallel to the changes of cholesterol concentration in the liver which was higher by 50% in PHHC 2 compared to PHHC 1. The alimentary cholesterol-induced feedback of *de novo* cholesterol synthesis was demonstrated in both PHHC groups after the HFHC diet while no significant differences were found between C1 and C2 versus PHHC 1 and PHHC 2, respectively (Table 3).

Table 3. Liver cholesterol concentration and synthesis

	Period B		Period D	
Group	Concentration (mg/g)	Synthesis (pmol/g/h)	Concentration (mg/g)	Synthesis (pmol/g/h)
Control 1 (C1)	2.02	4661	6.68	1025
(n=4/8) SD	0.23	727	0.58	176
Control 2 (C2)	9.01*	1258*	7.37$^{n.s.}$	930$^{n.s.}$
(n=4/8)	0.96	727	0.55	155
PHHC 1	4.36	2878	14.29	1053
(n=4/8)	0.53	382	3.08	336
PHHC 2	37.55*	698*	20.12*	966$^{n.s.}$
(n=4/8)	4.58	1591	1.99	122

*Data are means ± S.D., n = number of animals (first number for period B, second for period D), periods B and D are described in Table 1 and the Methods. *Significantly different ($p<0.01$) when groups 1 and 2 are compared.*

Analysis of cholesterol distribution between lipoprotein fractions was performed only in PHHC 1 and PHHC 2 at the end of the experiment. No significant difference was found in the HDL fraction. On the contrary, higher levels of low density lipoproteins and the triglyceride-rich fraction (VLDL and LDL) were demonstrated in PHHC 2. The cholesterol content in all apoB-containing fractions was increased by some 50 % in PHHC 2 compared to PHHC 1 (Table 4).

Table 4. Cholesterol concentration in lipoprotein fractions

Group	VLDL+IDL	LDL	HDL
PHHC 1	2.89	1.21	1.17
PHHC 2	4.42	1.87	1.35

Pooled samples of 8 animals in triplicates. The results are expressed as mean values in mmol/l of cholesterol concentration in each fraction.

Discussion

While all clinical complications of atherosclerosis as the sequelae of long-term plaque development and superimposed thrombus formation appear in later life, the atherosclerotic process starts to evolve in childhood. Early fatty streaks can be encountered in the first years of life (Strong 1991) and intimal thickening (Stary 1989) represents the first step of atheromatous lesions developing over several decades.

It is not surprising that attention has recently been focused on the effect exerted by nutrition on the main risk factor of premature atherosclerosis – hypercholesterolaemia. Although the results emerging from various studies of the dietary influence in the preweaning period are not completely consistent (for review see Hahn 1989), in all experimental models breast feeding seems to be beneficial in terms of the long-term effect on cholesterolaemia control and adaptation of cholesterol synthesis and its degradation in adulthood (Hahn 1989, Reiser and Sidelman 1972, Mott *et al.* 1992).

The effect of nutrition after weaning on imprinting of cholesterol metabolism in adulthood has been investigated much less extensively, with very inconsistent results. Particularly controversial are the results of studies addressing the dietary effects in childhood because the acute effect of the diet overlaps with the effects of genetically mediated different individual sensitivity to the HFHC diet (Pistulková *et al.* 1994, Poledne *et al.* 1994). Thus, an experimental model is practically the only possibility of exploring the effect of diet in early life after weaning on the response to a high-cholesterol diet. Unfortunately, the most frequently used laboratory animals (mouse, rat) are not the most suitable models of hypercholesterolaemia as their sensitivity to the HFHC diet is low and their serum lipid composition is different from that of man (Poledne and Vrána 1990). The PHHC rat seems to be an exception as the above disadvantages are not inherent to this inbred line.

The differences in total cholesterol levels in the serum of PHHC and Wistar rats at the onset of the experiment were enhanced by the administration of HFHC diet in the early period after weaning. Unlike the minimal rise of cholesterolaemia in Wistar rats, cholesterol levels rose to threefold values in PHHC as a result of the HFHC diet. The feeding of this diet to C2 and PHHC 2 for two months was long enough to identify any possible adaptive changes in the control of cholesterol metabolism. This is evident not only from the increase in serum cholesterol levels but, mainly, from the highly significant rise in cholesterol levels in the liver which increased four times in the Wistar rat (C1 vs C2) and even more in PHHC (PHHC 1 vs PHHC 2). The hepatocytes responded to this period of high alimentary cholesterol intake by feedback regulation of endogenous cholesterol synthesis. The decreases found in Wistar and PHHC rats were similar.

Feedback regulation of endogenous cholesterol synthesis is shifted to a higher cholesterol concentration in hepatocytes of PHHC rats (Poledne 1986a,b) and it seems to be inadequate for high cholesterol concentration both in hepatocytes and in serum compartment. This long-lasting effect of high exogenous cholesterol availability together with high endogenous cholesterol synthesis in hepatocytes might down-regulate LDL receptor production not only during the HFHC diet but also later in life.

In the following wash-out period, cholesterolaemia returned to baseline levels both in Wistar rats and in the inbred PHHC line sensitive to exogenous cholesterol. Although the experiment was not designed to monitor the cholesterol content in the liver at the end of the wash-out period, our earlier results (Befekadu *et al.* 1992) showed that the intracellular cholesterol pool in the hepatocyte of the PHHC rat is capable of eliminating the accumulated cholesterol within 2 weeks after HFHC diet had been discontinued.

To test the effect of HFHC diet at the end of the experiment, all animals were fed a diet containing 1% of cholesterol for a period of 2 weeks. This increased intake of exogenous cholesterol did not raise cholesterolaemia in the Wistar rat, no matter whether the rats were fed in early life by the control or HFHC diet. This result corresponds to identical cholesterol levels in the liver and is in agreement with the data published previously by Kris-Etherton *et al.* (1979) and, especially by Beynen *et al.* (1985) whose study had a design similar to our experimental model. HFHC diet thus leads to no imprinting in Wistar rats and does not alter their response to such a diet in later life. There was no imprinting concerning cholesterolaemia, the liver cholesterol content, or the rate of endogenous cholesterol synthesis in the liver. By contrast, the response of PHHC rats in adulthood was markedly affected by the previous diet in early life after weaning. The markedly higher cholesterol concentration in the plasma of PHHC rats (7.71 ± 0.51 mmol/l vs. 5.38 ± 1.67 mmol/l) fed HFHC diet in early life (PHHC 2, compared to PHHC 1, which were on the control diet during that period), are consistent with the different cholesterol contents in the liver of the two compared groups. As cholesterol deposition in hepatocytes is an immediate response to increased intake of dietary cholesterol in the PHHC rat (Befekadu *et al.* 1992), the increase of cholesterolaemia in rats fed HFHC diet in early life is the consequence of an enlarged liver cholesterol pool. Endogenous cholesterol synthesis in the liver, representing the possibility of feedback regulation of intracellular cholesterol pool and, consequently, regulation of serum lipoprotein levels

was not affected by HFHC diet administration. The effect of 1 % cholesterol diet on the concentrations of individual lipoprotein fractions was analyzed only in PHHC 1 and PHHC 2. A rise in the concentrations of all lipoprotein fractions was found. The increase in apoprotein B-containing fractions (VLDL and LDL) is substantially higher in PHHC animals fed the HFHC diet in early life than in those PHHC receiving the low cholesterol diet in that period of life. This type of imprinting is in general agreement with the results of Coates *et al.* (1983) demonstrating an increased serum cholesterol concentration in rats with early exposure (including intrauterine development) to a high-fat diet. Although this effect was demonstrated in non-fasting animals cumulating predominantly chylomicrones and VLDL triglyceride rich remnants (in contrast to our fasting hereditary hypercholesterolaemic rats), the mechanism of imprinting might be similar to alimentary cholesterol exposure in our experimental design.

It can thus be concluded that control Wistar rats did not respond to the HFHC diet in early life by an altered sensitivity to this diet in adulthood. This is in agreement with the results of Beynen *et al.* (1985). An essentially different result was obtained on the inbred PHHC line highly sensitive to dietary cholesterol. It is evident from our data that a high-cholesterol diet administered in early life imprints a response to such a diet in adulthood at least in this particular model of polygenic hypercholesterolaemia.

This finding, although it can hardly be directly extrapolated to man, suggests that in individuals with a polygenic disorder as the most frequent cause of cholesterolaemia in childhood, an early change of the diet in childhood might be the reason for increased cholesterolaemia in adulthood.

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Reprint requests
Doc. R. Poledne, Institute for Clinical and Experimental Medicine, Vídeňská 800, 140 00 Prague 4, Czech Republic.