Original Article

Differences in Expression of Cholesterol 7α-Hydroxylase between PHHC and Wistar Rats

(cholesterol 7α-hydroxylase gene / gene expression / PHHC rats / hypercholesterolemia)

J. A. HUBÁČEK[1,2], D. BOBKOVÁ[1], R. BOHUSLAVOVÁ[1], R. POLEDNE[1,2]

[1]Institute for Clinical and Experimental Medicine, Prague, Czech Republic
[2]Cardiovascular Research Center, Prague, Czech Republic

Abstract. PHHC rats represent a suitable experimental model of polygenic hypercholesterolemia. It has been found that its metabolic defect is not related to alimentary cholesterol absorption and LDL clearance. We have tested possible changes in cholesterol clearance from the liver to bile acids by analysis of the expression of the cholesterol 7α-hydroxylase (*cyp7A1*) gene in PHHC (N = 20) and Wistar (controls) (N = 19) male rats. The animals were fed standard laboratory diet (CD) or control diet containing 5% fat and 2% cholesterol (HCD) for two weeks. SSCP and RT-PCR were used for mutation analysis and study of gene expression, respectively. Although the basal cholesterolemia in PHHC was similar to controls (1.80 ± 0.48 and 1.52 ± 0.39 mmol/l, respectively), it rose in rats fed on HCD to 9.81 ± 1.65 mmol/l in PHHC rats and only to 2.19 ± 0.41 mmol/l in controls. Similarly to the basal cholesterol concentration, the gene expression of *cyp7A*1 in the liver of rats on CD was the same in both compared groups on the control diet. In controls on HCD, *cyp7A1* gene expression increased almost 4-fold immediately on the first day and achieved up to ~ 20-multiple basal expression in the end of the feeding period. Compared to the controls, after switching to HCD the *cyp7A1* gene expression in PHHC rats did not change dramatically. These results suggest that the *cyp7A1* gene plays an important role in development of hypercholesterolemia in PHHC rats.

Introduction

Hypercholesterolemia represents an important risk factor of cardiovascular diseases. High plasma concentration of cholesterol (especially the LDL fraction) is associated with increased cardiovascular morbidity and mortality. Although higher cholesterol synthesis was supposed to be the main reason of hypercholesterolemia, it has been found only in a very limited number of patients with cardiovascular disease. Some studies have suggested that these patients rather have problems with subnormal bile acid secretion.

The final plasma cholesterol concentration results from combination of daily cholesterol intake from food (~0.5 g), cholesterol synthesis in the body (~0.5 g), and cholesterol excretion (~1 g). The only pathway of cholesterol elimination from the body is represented by its conversion to bile acids in the liver (Russel, 2003). The rate-limiting enzyme in the metabolic pathway of bile acid production is cholesterol 7α-hydroxylase (*CYP7A1*).

Hypercholesterolemia was analysed on many experimental models. One of the suitable models for study of polygenic hypercholesterolemia is represented by the Prague hereditary hypercholesterolemic (PHHC) rat (Poledne, 1986; Befekadu et al., 1992). This model is characterized by hypercholesterolemia induced by atherosclerotic diet. The lipoprotein disorder state was generated by selective inbreeding repeated over many generations (Poledne, 1986), in which cholesterol concentration after cholesterol diet feeding was used as a selection index. Hypercholesterolemia was first observed in the 8th generation and continued with increasing in next generations. The genes responsible for polygenic hypercholesterolemia in PHHS rats have not been identified yet.

The aim of our study was to assess the link between the expression and possible promoter variants of one of many candidate genes, the *cyp7A1* gene, and hypercholesterolemia in PHHC and control rats.

Material and Methods

Animals and diets

The experiment was carried out in PHHC (N = 24) (Wistar-derived) (Befekadu et al., 1992) male 3-month-old rats received from in-house breeding. Age-matched Wistar (N = 24) male rats were used as a control. All animals were given standard laboratory diet (chow without any thyroid toxic or hepatotoxic factors) or 2% cholesterol diet (chow containing 5% fat and 2% cholesterol) for 14 days. Eight animals of each group were sacrificed by rapid decapitation under deep anaesthesia (pentobarbital) after zero, one, or 14 days on diets, their blood was collected directly from opened carotid arteries for lipid analysis, and liver tissue from four animals in each group was used for analysis of gene expression. The experimental protocol and all animal care were approved by the ethics committee of the institute and were in compliance with the State Veterinary Administration.

Lipid and plasma lipoprotein analysis

Serum was harvested by whole blood centrifugation for 10 min at 16 000 g (Microcentrifuge Boeco U32-R). Pooled serum of all animals in the group used for expression analyses was used for lipoprotein isolation. Lipoprotein fractions were isolated by sequential ultracentrifugation (VLDL [d = 1.006 g/ml] for 18 h, IDL [d = 1.019 g/ml] for 18 h, LDL [d = 1.063 g/ml] for 18 h, HDL [d = 1.210 g/ml] for 20 h) (50.3 rotor, Beckmann, Foster City, CA) at 39 000 rpm and at 8°C (Havel et al., 1955).

Fast-performance liquid chromatography (FPLC, Bio-Sys 510, Beckmann) was used for analysis of the lipoprotein spectrum. The column matrix was equilibrated at 0.3 ml/min with running buffer (50 mM PBS, pH 7.4 containing 0.15 M NaCl, 0.001 M EDTA, and 0.02% sodium azide). Two hundred μl of serum were loaded on a Superose (6HR 10/30, Amersham Pharmacia Biotech, Herts, United Kingdom) column and collected into 21 0.6-ml fractions (Innis-Whitehouse et al., 1998).

The cholesterol and triglyceride contents in the whole serum and in each fraction after ultracentrifugation separation were analysed using colorimetric enzymatic assay kits (Boehringer Mannheim Biochemicals, Ingelheim, Germany) using a Cobas Mira autoanalyser (Hoffman La Roche, Basel, Switzerland).

RNA extraction, reverse transcription and analysis

Total RNA was isolated from 30 mg liver tissue using a Nucleospin RNA II kit (Chemos, Praha, CR). The purity of the RNA samples was assessed by $A_{260/280}$ spectrophotometric measurements. After treatment with 10 U of deoxyribonuclease I (Fermentas, Glen Burnie, MD), 1 μg of total RNA was reversely transcribed into single-stranded complementary DNA (cDNA) using a First Strand cDNA Synthesis Kit (Fermentas) according to the protocol provided by the manufacturer. Two and half μl of the cDNA product were used as the template to amplify specific fragments of the *cyp7A1* gene (250 bp) and *β-actin* gene (as a housekeeping gene) (300 bp) in a total volume of 20 μl reaction mixture. The PCR conditions were follows:

a) for the rat *cyp7A1* gene: 2 μl PCR buffer (10x concentrated), 1 μl MgCl$_2$ (25 mmol/l), 2.5 μl DMSO, 0.5 μl Syber green, 0.5 μl dNTP (10 mM/l of each), 10.7 μl H$_2$O, 0.5 U ABgene polymerase and 0.1 μl (50 pmol/l) of each primer (5'- TCT ACG TAT GTT TCT CAA TGA CAC GCT - 3' and 5'-CTC ATT CAG TTG CAC TTG ATC CAA GTA - 3'). PCR was carried out with an initial denaturation at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, annealing at 62°C for 25 s, at 72°C for 25 s, with a final extension at 72°C for 3 min.

b) for the rat *β-actin* gene: 2 μl PCR buffer (10x concentrated), 1 μl MgCl$_2$ (25 mmol/l), 2.5 μl DMSO, 0.5 μl Syber green (FMC Bioproducts, Rockland, ME), 0.5 μl dNTP (10 mM/l of each), 10.35 μl H$_2$O, 1.25 U Taq polymerase and 0.2 μl (50 pmol/l) of primers 5'- TCC ATC ACA ATG CCA GTG GTA CGA CCA G and 5'-TAG GCA CCA GGG TGT GAT GGT GGG TAT G). PCR conditions were as follows – initial denaturation at 95°C for 5 min, followed by 40 cycles at 94°C for 20 s, 65°C for 20 s, 72°C for 30 s, with a final extension at 72°C for 3 min.

Two negative controls in duplicates (PCR mixture without cDNA and PCR mixture with mRNA of each sample) were included in all PCR runs. PCR was performed in a Rotorgene RG 3000 cycler (Corbett Research, Sydney, Australia). To control the purity, all PCR products were separated by electrophoresis using 2% agarose gels stained with ethidium bromide and visualized under UV light.

Relative mRNA expression was determined by the ΔΔCT method (Livak and Smittgen, 2001). PCR determinations for each gene in each sample were performed in triplicate. The CT (cycle threshold) is the cycle at which the PCR product crosses the detection threshold, usually at mid-log stage of PCR amplification. The difference between the target gene (*CYP7A1*) CT value and the housekeeping gene (*β-actin*) CT value is ΔCT:

$$\Delta C_T = C_{T(CYP7A1)} - C_{T(\beta\text{-}actin)}$$

Relative mRNA expression, assuming 100% PCR efficiency, is exponential and defined by the formula: mRNA = $2^{-\Delta\Delta CT}$

$$\Delta\Delta C_T = \Delta C_{T(CYP7A1 - X^{th}\ day\ on\ cholesterol\ diet)} - \Delta C_{T(CYP7A1 - on\ chow)}$$

Mutation analysis

Genomic DNAs were isolated from liver tissue by the standard method (Miller et al., 1988). One μl of genom-

ic DNA was used for PCR analysis in total volume 25 μl of the reaction mixture containing: 2.5 μl buffer, 1.5 μl MgCl$_2$, 0.5 μl dNTP (10 mM of each), 18.4 μl H$_2$O, 0.5 U Taq polymerase and 0.5 μl (50 pmol/l) of each primer. For promoter screening, two pairs of primers were used : 5'- AGC TTA TCG AGT ATT GCA GCT CTC -3' and 5'- AAG CAA ACA CTA TTT GTC CAC CTA -3' or 5'- AGT TCA AGG CCG GGT AAT GCT ATT -3' and 5'- AAG CAA GGC TGT CCC GAA ACA GTG GG -3'. The overlapping products covered the rat *cyp7A1* promoter region between the position -242 and position +14 (calculated according to the GenBank, accession code AH002160). PCR reactions were carried out in a DNA engine DYAD, MJ Research (Watertown, MA) with an initial denaturation at 96°C for 3 min, followed by 35 cycles at 95°C for 15 s, 66°C for 30 s, 72°C for 30 s, with a final extension at 72°C for 3 min. To control the purity, all the amplified fragments were separated by electrophoresis on 2% agarose gels stained with ethidium bromide and visualized under UV light.

The single-stranded conformation polymorphism (SSCP) method was used for rapid analysis of point mutations in the promoter (Orita et al., 1989). Twenty μl of each PCR product were mixed with 30 μl of the buffer (95% formamide, 20 mM/l EDTA, 0.05% bromphenol blue), denatured at 95°C for 8 min and transferred into an ice-cold water bath for 3 min. Forty-five μl of each PCR product were separated on 10% polyacrylamide gel with 10% glycerol under non-denaturing conditions in 0.5x TBE buffer. Electrophoresis was performed at 300 V for 3 h. DNA bands were visualized by silver staining as described earlier (Bassam et al., 1991).

Statistical analysis

Unpaired Student's *t*-test was used to test the differences between all groups. Data are presented as means (SD). Differences are considered to be statistically significant if P < 0.05 (for lipid parameters) or < 0.005 (for expression analysis).

Results

Lipoprotein concentrations and lipoprotein profile

Total cholesterol concentration of PHHC rats on chow diet was only slightly higher in comparison to controls (Table 1). The difference in total cholesterol concentration at baseline was mainly due to the HDL fraction, whereas other fractions (VLDL, IDL and LDL) did not differ between the compared groups (Table 2). The value of the cholesterol/triglyceride ratio in the VLDL fraction, 0.3 in both groups of animals, was consistent with human data (Kovar and Havel, 2002). The feeding cholesterol diet led to an increase of cholesterol concentration in PHHC rats by 185% and in control animals by 55% from the first day on diet. After 14 days on cholesterol diet, there was a 450% increase in cholesterol concentration in PHHC rats but only a 45% increase in controls (Table 1). In PHHC rats on cholesterol diet, the most pronounced changes were found in VLDL and IDL fractions (Table 2). As a result, after feeding PHHC rats with cholesterol diet, the pattern of different lipoprotein fractions was substantially changed and more similar to a pattern in primates (with majority of cholesterol carried in apolipoprotein B-containing fractions – VLDL, IDL and LDL). Compared to PHHC rats, there were only small changes after 14 days on cholesterol diet in lipoprotein concentrations in the controls. The cholesterol/triglyceride ratio rose significantly in VLDL, IDL and LDL fractions in PHHC rats but stayed without significant changes in controls. It documents that in comparison to controls, where no substantial changes of lipid composition appeared, all lipoprotein fractions in PHHC rats were enriched in cholesterol.

The findings of changes in lipoprotein concentrations, assessed by ultracentrifugation, were confirmed by FPLC. The lipoprotein profile of PHHC rats did not differ from controls (Fig. 1) on the chow diet and almost 80 % of total cholesterol was carried in the HDL fraction. In PHHC, feeding cholesterol diet was followed by redistribution of total cholesterol among the individual lipoprotein fractions, and an increase in VLDL, IDL, and LDL fractions. Consistent with data from ultracentrifugation, cholesterol diet led to only small changes in cholesterol concentrations of lipoprotein fractions in controls (Fig. 1).

Nucleic acid analysis

Two fragments of ~150 bp of the *cyp7A1* basal promoter were screened for mutations by PCR/SSCP of three PHHC rats and three controls. Compared to con-

Table 1. Serum cholesterol concentration in PHHC and control rats on chow and cholesterol diet

	N	Chow diet cholesterol	N	HCD – 1st day cholesterol		N	HCD – 14th day cholesterol	
PHHC	8	1.80 (0.48)	8	5.17 (0.35)	**, ##	8	9.81 (1.65)	**
controls	8	1.52 (0.39)	9	2.37 (0.50)	*	7	2.19 (0.41)	*

**P < 0.001, *P < 0.05 as significant differences between chow and cholesterol diet using *t*-test
##P < 0.001 as a significant difference between 1st day and 14th day on cholesterol diet using *t*-test.
Results shown are means (SD) of serum concentration (in mmol/l), N (number of animals).

Table 2. Lipoprotein concentration in PHHC and control rats on chow and cholesterol diet

| | chow (mmol/l) | | HCD – 1st day (mmol/l) | | HCD – 14th day (mmol/l) | |
	TC	TG	TC	TG	TC	TG
			PHHC			
VLDL	0.20	0.64	1.29	1.19	2.51	1.46
IDL	0.16	0.02	0.87	0.07	3.70	0.15
LDL	0.39	0.10	1.03	0.08	1.48	0.08
HDL	1.56	0.15	1.24	0.07	0.98	0.07
			Controls			
VLDL	0.13	0.43	0.59	1.35	0.72	1.51
IDL	0.17	0.02	0.17	0.06	0.17	0.04
LDL	0.21	0.08	0.54	0.09	0.30	0.06
HDL	0.74	0.07	1.00	0.21	1.11	0.15

TC (cholesterol), TG (triglyceride). Pooled serum of four animals used for the expression analysis was used for lipoprotein isolation using ultracentrifugation.
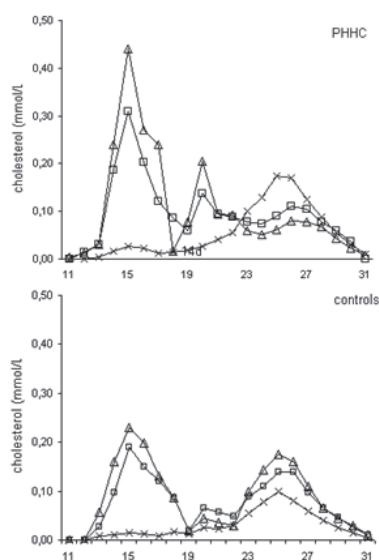

Fig. 1. FPLC profile of serum lipoproteins from PHHC (1a) and control (1b) rats on chow and cholesterol diet. The 200 μl of pooled serum were loaded on a Superose column and collected into 21 0.6-ml fractions. Fractions 14–17 represent VLDL and IDL, fractions 18–23 LDL and fractions 25–31 HDL. Cross represents chow; square represents cholesterol diet – 1st day; triangle represents cholesterol diet – 14th day.

Fig. 2. Relative *cyp7A1* gene expression in PHHC and control rats on chow and cholesterol diet. Values are presented as multiples of gene expression in PHHC rats on chow. Each group consisted of four animals.

trols, no differences were found in promoter sequences of PHHC rats (data not shown).

The *cyp7A1* gene expression in rats on chow diet was comparable in both groups of animals (Fig. 2). In controls, the *cyp7A1* gene expression increased ~ four times already on the first day on high-cholesterol/high-fat diet. The increase in the PHHC rats was not at the same level (3-fold), but this difference is barely physiologically important. After 14 days on HCD, the *cyp7A1* gene expression furthe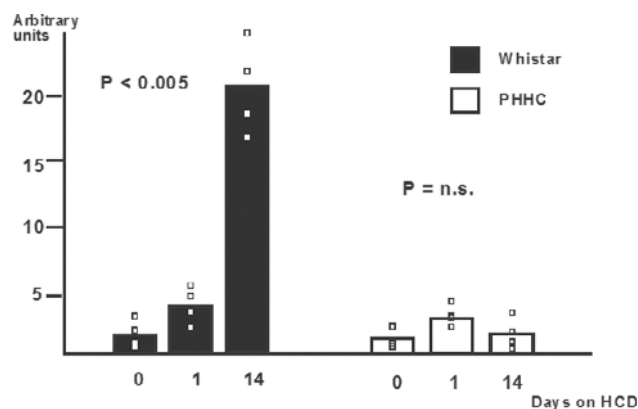r rose and it achieved ~20-multiple of the expression seen in rats on chow diet. This shows an excellent adaptation of control animals to very high alimentary intake of cholesterol. The cholesterol elimination pathway was dramatically stimulated. On the contrary, the *cyp7A1* gene expression in PHHC rats stayed without marked changes during the entire study period, and was not stimulated by feeding the animals with high-cholesterol/high-fat diet for a longer period (Fig. 2).

Discussion

We have demonstrated that *cyp7A1* mRNA expression in the liver is induced by feeding the Wistar rats high-cholesterol/high-fat diet. In contrast, under the same conditions, the PHHC rats did not exhibit enhanced expression of *cyp7A1* mRNA and simultaneously, their plasma cholesterol levels increased threefold in comparison to control Wistar rats.

CYP7A1 is the first and rate-limiting enzyme in bile acid synthesis. Conversion of cholesterol to bile acid is the only pathway responsible for the elimination of cholesterol and keeping the cholesterol homeostasis in the body. It has been found that the increased delivery of cholesterol to the liver leads to increased bile acid synthesis, and thus, presumably, to the enhanced expression/activity of CYP7A1.

In the human *CYP7A1* gene, the A-203C (previously referred as position -204) variant has been identified. Less common C-203C homozygotes have significantly elevated levels of LDL-cholesterol both in males and females (Wang et al., 1998). It has been reported that A-203>C polymorphism in children has an impact on plasma LDL/cholesterol in high cholesterolemic individuals only (Hubacek et al., 2003a). This variant also affects dietary responsiveness of total and LDL cholesterol concentrations in humans (reviewed by Hubacek and Bobkova, 2006) to dietary changes under different study protocols. Consistently, A-203A homozygotes are dietary resistant in comparison to the C-203C homozygotes, who respond well to dietary changes (Hubacek et al, 2003b; Hofman et al., 2004; Kovar et al., 2004) and will have better profit from dietary intervention.

We have screened a part of the *cyp7A1* rat gene for mutations that could possibly affect the lack of up-regulation of the *cyp7A1* expression. We cannot exclude the possibility that the mutation outside the tested promoter region could be responsible for different *cyp7A1* expression between PHHC and control animals. However, both bile acid response basic transcription elements (nucleotides -148 – -129 and -81 – -51) detected in the promoter are located within the tested region (Chiang and Stroup, 1994, Stroup et al. 1997).

As we have detected strong changes in *cyp7A1* expression under different dietary conditions, we supposed that a mutation in the promoter of the *cyp7A1* gene could be responsible for impaired regulation of *cyp7A1* expression in PHHC rats. We have screened the regulatory part of the gene by SSCP, but we have failed to detect any mutation that could be responsible for this resistance to up-regulation. It is also possible that in these animals, some transcription factors could be mutated, causing the inability of PHHC to up-regulate the *cyp7A1* expression and eliminate the abundant access of dietary cholesterol.

The CYP7A1 mRNA level is affected through farnesoid X receptor (FXR, inducing the CYP7A1 expression via binding with the RXRα) and the liver X receptor (LXRα, inhibiting CYP71A via induction of SHP) (Chen et al., 2001, Gupta et al., 2002) by many stimuli such as cholesterol, bile acids, cytokines, steroid hormones, or thyroid hormone, some of which are species-specific. The FXR/LXR heterodimer, the most powerful regulator, is able, via linkage to the CYP-7A1 promoter,

to influence its expression in rats and mice. Thus, genes for these receptors could be responsible for abnormal regulation of *cyp7A1* in PHHC rats.

Some results supporting our findings have already been published.

Hoekstra et al. (2005) have fed Wistar rats cholesterol/cholic acid diets and chow diets for two weeks. Feeding the diet enriched in cholesterol resulted in almost 10-fold increased total serum cholesterol. The expression of the *cyp7A1* gene was highly increased (82 times) in Kupfer cells, but was not changed in parenchymal and endothelial liver cells. Similarly, the expression of other genes (*BSEP*, *SHP*, *ABCA-1*) involved in cholesterol metabolism proved high response in Kupfer cells and only moderate changes in other types of liver cells. These results pointed to the possibility of strict cell-specific regulation of cholesterol catabolism, but they could be affected by the fact that the presence of cholic acid in the diet had some toxic effects (Bobkova et al., 2004)

Roberts et al. (2004) have analysed *cyp7A1* expression in female Fischer rats after feeding the animals high-fat/refined-sucrose diet for 20 months. After this period, rats exhibited almost all features of the metabolic syndrome, including development of hypercholesterolemia. Animals on this diet have developed hypercholesterolemia (~ 5.6 mmol/l vers ~ 1.5 mmol/l on chow diet) and displayed significantly lower hepatic *cyp7a1* expression and protein abundance than the animals on normal chow diet.

These results, together with ours, point to the role of relative reduction of cholesterol catabolism (caused predominantly by diminished expression of *cyp7A1* / disturbance of *cyp7A1* activation) in developing experimental dietary-induced hypercholesterolemia in different rat strains.

It is necessary to mention that also here (Roberts et al., 2004), expression of other genes involved in the regulation of cholesterol homeostasis was significantly induced/reduced and thus, similarly to humans, the genetic background of hypercholesterolemia is definitively polygenic. Also our previous results (Poledne, personal communication) show clearly that the cause of the hypercholesterolemia in PHHC rats is polygenic. So far, no gene mutation or different expression of other genes has been detected that could cause the hypercholesterolemia in PHHC rats.

The animal experiments confirmed the importance of cholesterol 7α-hydroxylase in cholesterol catabolism, similarly to some studies performed in humans.

We conclude that the loss of ability to regulate the *cyp7A1* expression is one of the causes of hypercholesterolemia development in Prague hereditary hypercholesterolemic rats.

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