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Effect of High-fat Diet on Cholesterol Metabolism in Rats and Its Association with Na^+/K^+-ATPase/Src/pERK Signaling Pathway*

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Summary: Abnormal cholesterol metabolism is associated with an elevated risk of developing atherosclerosis, hypertension, and diabetes etc. Na^+/K^+-ATPase was found to regulate cholesterol synthesis, distribution and trafficking. This study aimed to examine the effect of high-fat diet on cholesterol metabolism in rats and the role of Na^+/K^+-ATPase/Src/ERK signaling pathway in the process. Forty male SD rats were evenly divided into high-fat diet group and control group at random. Animals in the former group were fed on high-fat diet for 12 weeks, and those fed on basic diet served as control. Blood lipids, including total cholesterol (TC), triglyceride (TG), high density lipoprotein-cholesterol (HDL-C), and low density lipoprotein-cholesteral (LDL-C) levels, were detected at 3, 6 and 12 weeks. The ratio of cholesterol content in cytoplasm to that in cell membrane was detected in liver tissues. RT-PCR and Western blotting were used to measure the expression of lipid metabolism-associated genes (HMG-CoA reductase and SREBP-2) after 12-week high-fat diet. Na^+/K^+-ATPase/Src/ERK signaling pathway-related components (Na^+/K^+-ATPase α1, Src-PY418 and pERK1/2) were also measured by Western blotting. The results showed that the serum TC, TG, and LDL-C levels were significantly higher in high-fat diet group than those in control group, while the HDL-C level was significantly lower in high-fat diet group at 6 weeks (P<0.01). High-fat diet led to an increase in the cholesterol content in the cytoplasm and cell membrane. The ratio of cholesterol content in cytoplasm to that in cell membrane was elevated over time. The expression of HMG-CoA reductase and SREBP-2 was significantly suppressed at mRNA and protein levels after 12-week high-fat diet (P<0.05). Moreover, high-fat diet promoted the expression of Na^+/K^+-ATPase α1 but suppressed the phosphorylation of Src-PY418 and ERK1/2 at 12 weeks (P＜0.05). It was concluded that high-fat diet regulates cholesterol metabolism, and Na^+/K^+-ATPase signaling pathway is involved in the process possibly by regulating the expression of lipid metabolism-associated proteins HMG-CoA reductase and SREBP-2.
Key words: Na^+/K^+-ATPase; cholesterol; high-fat diet; lipid metabolism-associated genes; Na^+/K^+-ATPase /Src/pERK signaling pathway

Cholesterol is indispensable to human cells and organs as it not only participates in the formation of cell membrane but also serves as a precursor for the synthesis of bile acid and vitamin D[1]. Cholesterol balance is finely maintained to keep the normal activity in the organisms[2]. Na^+/K^+-ATPase was first discovered by Skou JC in 1957 and it is the key structure that maintains the transmembrane ion gradient in most organisms and all mammals[3]. It is also involved in many non-ion pump functions such as tumor cell proliferation, regulation of hypertension, and Ca^{2+} metabolism[4]. Recently, research on synthesis, distribution, and transportation of cholesterol reveals that Na^+/K^+-ATPase plays a regulatory role in cholesterol metabolism[5].

About 50% of the Na^+/K^+-ATPases and their binding partners such as Src and epidermal growth factor receptor were found in caveolae, which are composed of cholesterol, sphingolipids, sphingomyelin, and proteins[6]. It has been reported that approximately half of the cholesterol in cells is distributed in caveolae, which facilitates endocytosis, cholesterol homeostasis, protein transportation, etc[7]. Moreover, homo-/hetero-oligomeric signaling proteins are found to be enriched in the caveolae[8]. Our previous study showed that caveolae localized Na^+/K^+-ATPase and promoted its signaling functions. In addition, change of Na^+/K^+-ATPase expression in the caveolae could affect the synthesis and distribution of cholesterol in the cell membrane[9].

It was previously shown that digitalis glycosides treatment or reduction of cellular cholesterol can activate Src and extracellular signal-regulated kinase (ERK), which further trigger series of signaling pathways by induction of tyrosine phosphorylation on other proteins[10].

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The present study was to test if Na^+/K^+-ATPase and its signaling pathway are involved in regulating the expression and distribution of lipid metabolism-associated proteins such as 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase and sterol regulatory element-binding protein 2 (SREBP2).

1 MATERIALS AND METHODS

1.1 Experimental Animals and Grouping

In total, 40 specific pathogen-free (SPF) grade male Sprague-Dawley (SD) rats, weighing around 100 g, were provided by the Experimental Animal Center at Tongji Medical College, Huazhong University of Science and Technology, China. The rats were accommodated in the SPF grade feeding environment for a week. Later 20 rats were randomly selected to receive high-fat diet (yolk 10%, lard 10%, choline 0.5%, basic formula 79.5%) and the remaining rats were fed on basic formula diet (corn flour 30%, flour 30%, wheat bran 35%, fishmeal 2%, bone meal 2%, salt 1%) supplemented with cod liver oil (100 g/10 kg basic formula) for 12 weeks. The rats were fasted for 12 h before they were sacrificed. Blood samples were collected from the abdominal aorta, and were centrifuged to isolate serum and plasma. Serum and plasma samples were stored at –20°C before use. About 10–20 mg liver tissue was homogenized in the presence of 1.5 mL TRIzol (Invitrogen, USA). The homogenate was placed at room temperature for 5 min to allow complete cell lysis. Tissue RNA was then extracted according to the manufacturer's manual. All the procedures on animals were in accordance with the guidelines for the care and use of laboratory animals at Tongji Medical College.

1.2 Measurement of Blood Lipids

The fully automatic biochemical analyzer (Hitachi 760; Hitachi, Japan) was used to analyze the content of serum total cholesterol (TC), triglyceride (TG), high density lipoprotein-cholesterol (HDL-C), and low density lipoprotein-cholesterol (LDL-C). All processes were performed according to the manufacturer's manual.

1.3 Isolation of Cytoplasm and Cell Membrane and Measurement of Cholesterol

Liver tissues, weighing 0.5 g, were washed with ice-cold PBS and scraped in 2 mL of sucrose buffer A (30 mmol/L histidine, 250 mmol/L sucrose, 1 mmol/L EDTA Na, pH 7.4). The tissues were homogenized by a Polytron tissue grinder (three 6-second bursts) and subjected to sonication (three 40-second bursts). Then, the cell lysates were transferred to 15 mL Falcon centrifuge tubes and subjected to centrifugation at 1000 r/min for 5 min to remove cell debris. The supernatants were transferred to ultracentrifuge tubes and were centrifuged at 100 000 × g in a Beckman type 65 rotor for 1 h. The supernatant (cytosol fraction) was collected in the Eppendorf tubes. The pellet (membrane fraction) was resuspended in 250 μL buffer A. Cholesterol content in both fractions was measured using Amplex Red Cholesterol Assay Kit according to the manufacturer's instructions, and the absorbance (A) value was detected by a microplate spectrofluorometer (excitation: 560 nm; cut off: 570 nm; emission: 590 nm).

1.4 Real-time Polymerase Chain Reaction (RT-PCR) Analysis

The 20-μL reaction system was composed of 2× Trans Start qPCR supermix 10 μL, cDNA 1 μL, forward and reverse primers 2 μL (10 pmol), and ddH_2O. The reaction cycle was 95°C preheating for 3 min, then 45 cycles with 95°C for 15 s, 58°C for 20 s, and 72°C for 30 s. The reaction was performed in Bio-Rad iQ5 fluorescent quantitative PCR machine and the results were analyzed using Bio-Rad iQ5 PCR software. The real-time primers were synthesized by Sangon Biotech (Shanghai, China) and the sequences were as follows: HMG-CoA reductase: forward primers: 5'-CCTGCGTGTCCCTGGTCCTA-3'; reverse primers: 5'-CTTTGGGTTACTGGGTTTGG-3'; SREBP-2: forward primers: 5'-GGCTCTGGCCGCAATGTA-3'; reverse primers: 5'-TGACCGAGGAGCGTGAGT-3'; β-actin: forward primers: 5'-TCCATCATGAAGTGTGACGT-3'; reverse primers: 5'-CTCAGGAGGAGCAATGATCT-3'.

1.5 Detection of the Expression of Na^+/K^+-ATPase α1, Src-PY418 and pERK1/2 Proteins by Western Blotting

The liver tissue homogenate was centrifuged and the supernatant was collected. The protein concentrations were measured using BCA method and were adjusted to the same level. The samples were denatured in the presence of a loading buffer at 100°C for 5 min. The proteins were separated using 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and were transferred to the nitrocellulose membrane. The membrane was blocked for 2 h at room temperature with 5% nonfat milk and incubated with primary antibody at 4°C overnight. Later the membrane was washed and incubated with secondary antibody followed by imaging analysis.

1.6 Statistical analysis

Data were analyzed using SPSS 13.0 software. Normal distribution test was performed in all data and the variables were presented as $\bar{x}\pm s$. Comparisons between groups were performed using one-way analysis of variance. A value of $P<0.05$ was considered statistically significant.

2 RESULTS

2.1 Comparison of Blood Lipid Indices between Groups

There were no significant differences in the serum TC, TG, HDL-C, and LDL-C levels between the high-fat diet and basic formula (control) groups by the third week. The serum TC, TG, and LDL-C levels were significantly higher in the high-fat diet group than those in the control group at 6 weeks ($P<0.05$), while the HDL-C level was significantly lower in the high-fat diet group ($P<0.05$). Such differences were even more significant at the end of the 12th week ($P<0.01$) (table 1).

Table 1 Comparison of blood lipid indices between groups

Groups	Time (weeks)	Serum lipid level ($\bar{x}$±s, n=40) TC	TG	HDL-C	LDL-C
Control					
	3	1.49±0.33	0.51±0.17	0.91±0.24	0.29±0.21
	6	1.58±0.17	0.56±0.19	0.95±0.11	0.36±0.16
	12	1.63±0.18	0.58±0.21	0.93±0.19	0.38±0.17
High-fat diet					
	3	1.52±0.17	0.53±0.09	0.89±0.16	0.31±0.18
	6	1.91±0.14*	0.77±0.17*	0.77±0.18*	0.49±0.19*
	12	2.18±0.16**	0.92±0.17**	0.75±0.13**	0.65±0.15**

*P<0.05, **P<0.01 *vs.* control group at the same time points

2.2 Comparison of Cholesterol Content in the Cytoplasm and Cell Membrane between Groups

High-fat diet led to an increase in the cholesterol content in the cytoplasm and cell membrane. The ratio of cholesterol content in the cytoplasm to that in the cell membrane was elevated over time. At the end of the sixth week, the ratio of cholesterol content in the cytoplasm to that in the cell membrane was 1.18±0.13, which was increased to 1.47±0.19 at the end of the 12th week (fig. 1).

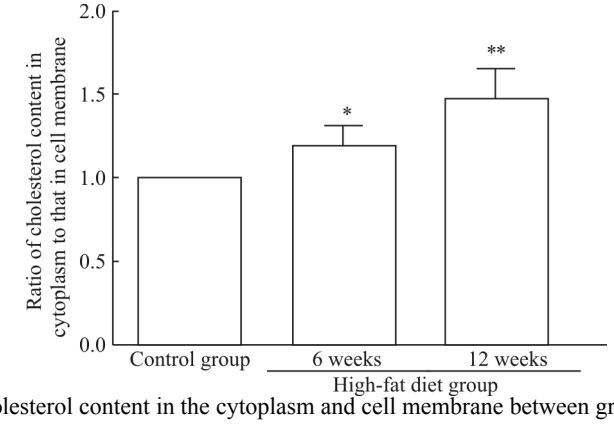

Fig. 1 Comparison of cholesterol content in the cytoplasm and cell membrane between groups

2.3 Comparison of Lipid Metabolism-associated Genes between groups

After the rats were fed on the high-fat diet for 12 weeks, the expression of lipid metabolism-associated genes such as HMG-CoA reductase and SREBP-2 were tested using RT-PCR. The results showed that both gene expression levels were significantly suppressed and were only 0.52±0.11 and 0.61±0.07 fold of the expression in the control group, respectively (fig. 2A). Western blotting indicated that the protein levels in the high-fat diet group were significantly lower than those in the control group as well (P<0.05) (fig. 2B).

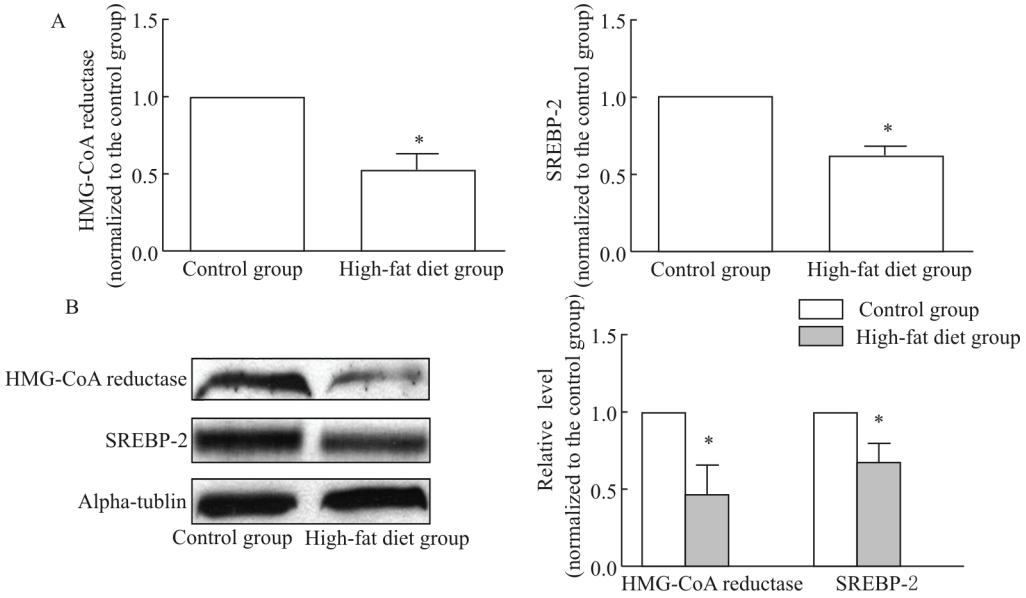

Fig. 2 Comparison of lipid metabolism-associated gene expressions between groups

A: RT-PCR analysis of the expression of HMG-CoA reductase and SREBP-2; B: Western blot analysis of the expression of HMG-CoA reductase and SREBP-2

2.4 Comparison of Na^+/K^+-ATPase/Src/ERK Signaling Pathway-related Protein Expression

The 12-week high-fat diet promoted the expression of Na^+/K^+-ATPase α1, which was 1.29±0.17 fold of the expression in the control group (P<0.05). Meanwhile, the high-fat diet suppressed the expression of Src-PY418 and pERK1/2, which was only 0.73±0.08 and 0.69±0.11 fold of the expression in the control group, respectively. The differences were significant between the two groups (P<0.05) (fig. 3).

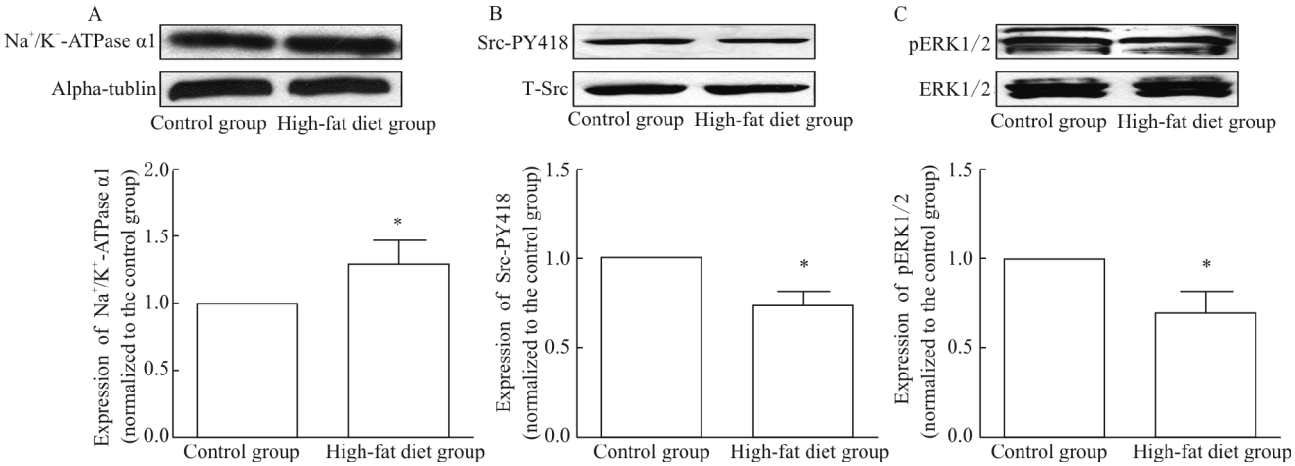

Fig. 3 High fat diet increases the expression of Na^+/K^+-ATPase α1 and suppresses phosphorylation of Src and ERK
A–C: expression of Na^+/K^+-ATPase α1 (A), Src-PY418 (B) and pERK1/2 (C) after 12 weeks of high-fat feeding; $^*P$<0.05 *vs*. control group

3 DISCUSSION

Our study demonstrated that the serum TC, TG, and LDL-C levels were significantly increased and serum HDL-C levels significantly decreased in male SD rats fed on high-fat diet for 12 weeks. However, the 3-week high-fat diet did not lead to significant changes in the serum lipid metabolism indices, suggesting that the lipid metabolism could be self-regulated and the effect of excessive amount of fat taken was not significant within a certain period of time. Nonetheless, with the continuation of high-fat diet, such self-regulation gradually failed over time, and the differences in TC, TG, and related lipoproteins became apparent between the high diet group and basic formula diet group.

HMG-CoA reductase is the rate-limiting enzyme of cholesterol synthesis in hepatocytes[11]. Inhibition of HMG-CoA reductase prevents the synthesis of cholesterol. LDL is converted from very low-density lipoprotein (VLDL). Its main function is to deliver cholesterol to the whole body[12]. Each lipoprotein carries a certain amount of cholesterol and LDL carries the most. LDL binds to low-density lipoprotein receptor (LDLR) on the cell surface and is cleared by receptor-mediated endocytosis. Goldstein *et al* identified a 10-bp DNA element in the promoter of HMG-CoA reductase and LRLR genes[13]. It is an essential element in sterol-mediated transcriptional regulation and was named SRE-1. Later, a specific binding protein of SRE-1, SREBP, with a molecular weight of 68 kD was identified. It was confirmed that SREBP potentiated the promoter activity of LDLR and HMG-CoA reductase[14]. Currently, there are three identified SREBPs: SREBP-1a, SREBP-1c, and SREBP-2[15]. The present study showed that after being fed with high-fat diet for 12 weeks, the rats had lower levels of HMG-CoA reductase and SREBP-2 transcription in hepatocytes. It also proved that when cells were exposed to high concentrations of cholesterol, the transcription of HMG-CoA reductase and SREBP-2 was reduced. On the other hand, when the sterol concentration in the culture media decreased or the competitive HMG-CoA reductase inhibitor was present, the transcription of these genes increased[16].

Research has suggested that a large amount of Na^+/K^+-ATPase and cholesterol is localized in the caveolae[9]. It was further speculated that Na^+/K^+-ATPase might sense the cholesterol level in cell membrane by directly binding with cholesterol and therefore regulate the synthesis and distribution of cholesterol via signaling transduction[17]. Our previous study also confirmed that Na^+/K^+-ATPase was able to regulate the distribution of cholesterol[17]. Knockout of Na^+/K^+-ATPase could alter the distribution of cholesterol in the cell membrane and facilitate the transportation of cholesterol from the cell membrane to the organelles, which led to an increase in the ratio of cholesterol content in the cytoplasm and in the cell membrane. Restoration of Na^+/K^+-ATPase could rescue the distribution of cholesterol in the cell; consistent results were obtained in both *in vitro* cell cultures and *in vivo* animal experiments[17]. It was also validated that in the presence of exogenous glycosides, Na^+/K^+-ATPase could activate Src and ERK and further trigger a series of signaling pathways by the induction of tyrosine phosphorylation on other proteins. Knockdown of Na^+/K^+-ATPase not only activated Src/ERK signaling pathway but also induced SREBP2, therefore regulating the synthesis of cholesterol[18].

The present study showed that after being on high-fat diet for a period of time, the rats had altered cholesterol content and distribution in hepatocytes. Moreover, the expression of regulatory proteins of cholesterol such as SREBP2 and HMG-CoA reductase was reduced. Additionally, upregulation of Na^+/K^+-ATPase inhibited the Na^+/K^+-ATPases/Src/ERK signaling pathway. Based on these findings, it was further speculated that Na^+/K^+-ATPase might maintain cholesterol homeo-

stasis by regulating the synthesis and distribution of cholesterol via the Na^+/K^+-ATPases/Src/ERK signaling pathway. By manipulating the expression of Na^+/K^+-ATPases/Src/ERK signaling pathway-related proteins to activate or inhibit the pathway, future studies will examine the changes in cholesterol metabolism and further discover the relationship between Na^+/K^+-ATPases, its associated pathways, and cholesterol metabolism, and the underlying molecular mechanisms.

Cholesterol is an important substance for human tissues, whose metabolic disorder can cause atherosclerosis, hypertension, diabetes etc[19]. Na^+/K^+-ATPase is significantly reduced in those cholesterol metabolic diseases[20]. Our study further demonstrated the important role of the Na^+/K^+-ATPase in regulation of cholesterol metabolism and provides experimental basis for development of new therapeutic targets for metabolic deseases.

Conflict of Interest Statement

The authors declare that there is no conflict of interest with any financial organization or corporation or individual that can inappropriately influence this work.

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