PIBB 生物化学与生物物理进展
Progress in Biochemistry and Biophysics
2015, 42(9): 866~876
www.pibb.ac.cn

Probucol Inhibits Atherosclerosis by Regulating ABCA1, SR-BⅠ, ABCG5 and ABCG8 Expression and Anti-inflammatory Effects in Hypercholesterolemic Rabbits*

CUI Li-Bao[1,2)**], YU Xiao-Hua[1)**], JIANG Chong-Hui[2)], TANG Ya-Ling[1)], OUYANG Xin-Ping[1)], HE Ping-Ping[1)], LÜ Yun-Cheng[1)], YAO Feng[1)], ZHANG Min[1)], TANG Chao-Ke[1)***]

([1)] *Institute of Cardiovascular Research, Key Laboratory for Atherosclerology of Hunan Province, Life Science Research Center, Hunan Province Cooperative Innovation Center for Molecular Target New Drug Study, University of South China, Hengyang* 421001, China;
[2)] *Department of Pharmacy, Central Hospital of Hengyang, Hengyang* 421001, China)

Abstract Probucol is a potent hypolipidemic drug that decreases plasma high-density lipoprotein cholesterol (HDL-C) levels but attenuates atherosclerosis. However, the detailed mechanisms are not fully understood. The aim of this study was to explore the molecular mechanisms of the HDL-C-lowering and antiatherogenic effects of probucol. New Zealand white rabbits were randomly divided into normal diet group, normal diet+probucol group, high fat diet (HFD) group and HFD+probucol (HFD+P) group. After 7 weeks of treatments, the extent of the atherosclerotic lesions, hepatic lipid accumulation and plasma levels of triglycerides, total cholesterol, low-density lipoprotein cholesterol and HDL-C were significantly reduced in HFD+P group as compared to HFD group. Probucol effectively inhibited down-regulation of hepatic scavenger receptor class B type Ⅰ (SR-BⅠ) expression, and ATP-binding cassette (ABC) transporters G5 (ABCG5) and G8 (ABCG8) expression in the liver and small intestine induced by HFD but further promoted HFD-induced reduction in hepatic ABC transporter A1 (ABCA1) expression. In addition, probucol also significantly prevented HFD-induced increases of tumor necrosis factor-α, interleukin-1, interleukin-6 and monocyte chemotactic protein-1 levels in the aortic arch and plasma. Thus, our data provide strong evidence that probucol alleviates atherosclerosis through regulating ABCA1, SR-BⅠ, ABCG5 and ABCG8 expression and inhibiting the secretion of proinflammatory cytokines in hypercholesterolemic rabbits.

Key words probucol, atherosclerosis, HDL-C, ABCA1, SR-BⅠ, ABCG5, ABCG8, inflammation
DOI: 10.16476/j.pibb.2015.0124

Atherosclerotic cardiovascular disease is one of the major causes of death in the world. Atherosclerosis is characterized by increased circulating cholesterol levels and inflammation in the arterial wall[1]. Numerous clinical and epidemiological studies have found that high-density lipoprotein (HDL) levels are inversely correlated with the risks of atherosclerotic cardiovascular disease[2-3]. The antiatherogenic role of HDL is primarily a result of its involvement in reverse cholesterol transport (RCT) that removes excess cholesterol from peripheral tissues and delivers it to the liver for the final excretion[4]. Of note, plasma levels of HDL do not always reflect the dynamic process of RCT from macrophage to bile and feces and the incidence of atherosclerotic cardiovascular disease. For example, probucol is a lipophilic antioxidative agent that has been reported to reduce plasma levels of low-density lipoprotein cholesterol (LDL-C) and HDL cholesterol (HDL-C) but significantly suppresses the development of atherosclerosis in hypercholesterolemic rabbits[5].

*This work was supported by grants from The National Natural Sciences Foundation of China (81070220, 81170278).
**These authors contributed equally to this work.
***Corresponding author.
Tel: 86-734-8281853, E-mail: tangchaoke@qq.com
Received: April 24, 2015 Accepted: August 5, 2015

Scavenger receptor class B type Ⅰ (SR-BⅠ) and ATP-binding cassette (ABC) transporter A1 (ABCA1) are key proteins involved in RCT[6]. SR-BⅠ is highly expressed in hepatocytes, where it allows the incorporation of cholesteryl ester (CE) from circulating HDL into cells through a process called selective cholesterol uptake. Several studies have demonstrated that liver-specific overexpression of murine SR-BⅠ decreases HDL-C levels, increases hepatic uptake of HDL-C and promotes RCT [7-8], suggesting that SR-BⅠ mediates HDL metabolism. On the other hand, deletion of hepatic SR-BⅠ expression leads to accelerated atherosclerosis and increased HDL-C levels in apolipoprotein E (apoE) knockout mice[9]. ABCA1 is also expressed in the liver, and promotes cholesterol efflux from hepatocytes to apolipoprotein A-Ⅰ (apoA-Ⅰ) to form HDL particles. Hepatic overexpression of human ABCA1 remarkably raises plasma HDL-C levels in mice [10], whereas specific knockout of hepatic ABCA1 reduces plasma HDL-C by approximately 80% in mice [11]. Thus, hepatic ABCA1 and SR-BⅠ may be major determinants of plasma HDL-C levels.

In addition to SR-BⅠ and ABCA1, ABC transporters G5 (ABCG5) and G8 (ABCG8) play an important role in the process of RCT. ABCG5 and ABCG8 are half ABC transporter and form heterodimers in the endoplasmic reticulum and then traffic to the apical membranes of hepatocytes and enterocytes, where they facilitate biliary secretion and limit intestinal absorption of cholesterol[12]. Mutations in either of the two genes cause the autosomal recessive disease sitosterolemia, which is characterized by accumulation of plant sterols in plasma, accelerated atherosclerosis and premature coronary artery disease[13]. Conversely, transgenic mice overexpressing ABCG5 and ABCG8 markedly reduce plasma LDL-C levels and increase biliary cholesterol levels and fecal cholesterol excretion rates, thereby significantly reducing atherosclerotic leisons[14].

Probucol is a potent hypolipidemic drug with anti-oxidant and anti-inflammatory properties. Paradoxically, probucol decreases plasma HDL-C levels but attenuates atherosclerosis in animal models and reduces xanthomas in humans [15]. Moreover, several studies have demonstrated that the HDL-C-lowering effects of probucol are associated with upregulation of SR-BⅠ expression and downregulation of ABCA1 expression in the liver tissues [16-17]. However, the molecular mechanisms by which probucol reduces HDL-C levels yet inhibits atherogenesis are not fully understood. Thus, the present study was designed to observe the effects of probucol on plasma HDL-C levels and atherosclerosis in hypercholesterolemic rabbits, and further explore the involved mechanisms. We demonstrated that probucol downregulated hepatic ABCA1 expression, upregulated the expression of hepatic SR-BⅠ, ABCG5 and ABCG8 in the liver and small intestinal. We also found that probucol inhibited the secretion of proinflammatory cytokine in hypercholesterolemic rabbits. These results may provide a reasonable explanation for the beneficial effects of probucol on atherosclerosis despite its HDL-C-lowering properties.

1 Materials and methods

1.1 Drug and reagents

Probucol was purchased from Qilu Pharmaceutical Co., Ltd(Jinan, China). Anti-ABCA1, SR-BⅠ, ABCG5 and ABCG8 polyclonal antibodies were provided by Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA). TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), BCA Protein Assay Reagent (Pierce Chemical, Rockford, IL, USA), ReverAid™ First Strand cDNA Synthesis Kit (#k1622) (Fermentas, Burlington, Ontario, Canada), DyNAmo™ SYBR® Green qPCR Kits (Finnzymes, Espoo, Finland), and immobilon-P transfer membranes (Millipore, Boston, MA, USA) were obtained as indicated. All other chemicals were from Sigma (St Louis, MO, USA).

1.2 Animals, diet and experimental protocol

Thirty-six male New Zealand white rabbits, weighing 1.2～1.5 kg, were purchased from the Animal Department of University of South China and individually housed in air-conditioned room. After 7 days of adaptation, they were randomly divided into four groups of nine rabbits each: normal diet (ND) group was fed a standard rabbit chow; ND+probucol (ND+P) group received the same standard rabbit chow plus probucol (50 mg•kg^{-1}•d^{-1}); high fat diet (HFD) group was fed a HFD containing 1% cholesterol, 5% lard and 0.47% gravy salt; HFD+probucol (HFD+P) group was treated with the same HFD plus probucol (50 mg•kg^{-1}•d^{-1}). Each rabbit consumed about 120 g of food daily with free access to water. After 7 weeks, blood samples were obtained from the ear middle

artery and centrifugated to get plasma. Immediately, all rabbits were weighed and sacrificed by a 25 mg/kg pentobarbital. Liver was removed and weighed. The ratio of liver weight to body weight was calculated. Aorta and small intestine were also collected for further analyses. The experiments were approved by the Animal Care and Use Committee of University of South China and were performed in compliance with the "Guide for the Care and Use of Laboratory Animals" published by the US National Institues of Health (NIH Publication No. 85-23, revised 1996).

1.3 Detection of plasma lipids

Plasma concentrations of triglycerides (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C) and HDL-C were detected using an automated biochemical analyzer (Type CX717, Hitachi, Japan) at week 0 (baseline) and at the end of treatment period.

1.4 Hematoxylin and eosin staining

Aortic samples were fixed in 10% neutral formalin solution, and then embedded in paraffin. The paraffin blocks were sectioned at 5 μm and stained with hematoxylin and eosin (HE) as previously reported [18]. The thickness of intimal was observed under an optical microscopy.

1.5 Analysis of atherosclerotic lesions

For en face analysis, aortas from experimental groups were opened longitudinally from the heart to the iliac arteries, and then lesions were stained with Sudan Ⅳ as we described earlier [19]. En face aortic lesion area was digitized using a Nikon S6 digital camera, analyzed by Image-Pro Plus image analysis software (Media Cybernetics) and expressed as the percentage of the total aortic surface area covered by lesions.

1.6 Oil red O staining of liver tissues

Liver samples were washed in cold PBS and fixed overnight at 4℃ with a 10% neutral formalin solution in PBS. Samples were embedded in optimal cutting temperature compound (Fisher, Tustin, CA) and stored at −70 ℃. Five-μm thick sections were cut by a cryotome. Sections were stained with oil red O and hematoxylin. After washing with copious amounts of distilled water, images were photographed using a microscope at 200× magnification. Forty fields in three individual sections were randomly selected, and the Oil Red O-stained area and the total tissue area were analyzed by an ImageJ 1.43 analyzing system. The ratio of the Oil Red O-stained area to the total tissue area was calculated.

1.7 RNA isolation and real-time quantitative PCR analysis

Total RNA from liver and small intestine tissues was extracted by using TRIzol reagent in accordance with the manufacturer's instructions. The first strand cDNA synthesis containing 1 μg of total RNA was primed with oligo (dT). real-time quantitative PCR, using SYBR Green detection chemistry, was performed on Roche light Cycler Run 5.32 real-time PCR System. Specific primers for ABCA1, SR-B Ⅰ, ABCG5, ABCG8 and β-actin were designed as follows: ABCA1, forward primer, 5′ AGGAGGTGATGTTTCGAC 3′ and reverse primer, 5′ AG CTCCATGGACTTGTTGA 3′; SR-B Ⅰ, forward primer, 5′ CAGTGGGCATTGTGTCCTGTC 3′ and reverse primer, 5′ GGCTCAGTGCAGGCTGATGTC 3′; ABCG5, forward primer, 5′ CTCAGGTCCCTTGGTTTG 3′ and reverse primer, 5′ TGCCCACTTCTCACTTTG 3′; and β-actin, forward primer, 5′ CCATCATCTTGCAGGAGCG 3′ and reverse primer, 5′ CTGGCAGTGAGCTATACTCG 3′. Melt curve analyses of all real-time PCR products were performed and shown to produce a single DNA duplex. Quantitative measurements were determined using the $\Delta\Delta C_t$ method and expression of β-actin was used as the internal control.

1.8 Protein extraction and Western blot analysis

Western blot assay was performed as previously described [20]. In brief, liver and small intestine tissue samples were lysed with lysis buffer. Supernatants were used as sample proteins. Protein concentrations were determined by BCA Protein Assay according to the protocol from the manufacturer. Same amounts of proteins (20 μg) were applied to 10% SDS gels and then transferred to nitrocellulose membranes (Millipore). Membranes were incubated with various primary antibodies indicated at a dilution of 1∶2 000 at 4℃ overnight. After washing, the membranes were incubated with the corresponding secondary antibodies at room temperature for 2 h. The proteins were visualized using a chemiluminescence method (ECL Plus Western blotting Detection System; Amerisham Biosciences, Foster City, CA, USA).

1.9 Measurement of proinflammatory cytokine levels in the aortic arch and plasma

The aortic arch tissues were homogenized in 1 ml

of phosphate buffered saline (PBS) with 2 mmol/L phenylmethylsulfonyl fluoride(PMSF) and centrifuged. The contents of tumor necrosis factor-α (TNF-α), interleukin (IL)-1, IL-6 and monocyte chemotactic protein-1 (MCP-1) in the supernatants were measured by enzyme-linked immunesorbent assay (ELISA) using standard kits (Nanjing Jiancheng Bio-engineering Institute, Nanjing, China) according to the manufacturer's instruction. Similarly, plasma levels of these cytokines were determined by ELISA kits from the same company.

1.10 Statistical analysis

Data are expressed as means ± SD. Results were analyzed by one-way ANOVA and Student's *t* test, using SPSS 13.0 software. Statistical significance was obtained when *P* values were less than 0.05.

2 Results

2.1 Probucol decreases plasma lipid levels in hypercholesterolemic rabbits

At the end of experiment, all rabbits were alive. As shown in Table 1, plasma levels of TC, TG, HDL-C and LDL-C were markedly increased in HFD group as compared to ND group, suggesting that a hypercholesterolemia model was successfully established. Treatment of HFD group with probucol for 7 weeks (HFD +P group) significantly reduced plasma levels of TC, TG, HDL-C and LDL-C when compared to HFD only group. However, 7-week treatment of probucol in ND group had no significantly effect. These findings suggest that probucol prevents HFD-induced increases in plasma levels of TC, TG, HDL-C and LDL-C.

Table 1 Effects of probucol on plasma levels of TC, TG, HDL-C and LDL-C

Groups	*n*	TC	TG	HDL-C	LDL-C
ND	9	3.15±0.83	1.83±0.13	0.75±0.19	2.87±1.75
ND+P	9	3.03±0.65	1.86±0.15	0.70±0.16	2.68±1.04
HFD	9	22.68±1.18**	2.93±0.11**	6.92±1.04**	17.19±0.73**
HFD+P	9	14.39±1.21$^{\Delta\Delta}$	2.01±0.13$^{\Delta\Delta}$	3.21±0.27$^{\Delta\Delta}$	13.29±1.28$^{\Delta}$

**P<0.01 *vs* ND group. $^{\Delta}P$ < 0.05, $^{\Delta\Delta}P$ < 0.01 *vs* HFD group.

2.2 Probucol inhibits atherosclerosis in hypercholesterolemic rabbits

To determine whether probucol exerts preventive action on atherosclerosis, we detected the effects of probucol on aortic intima thickness by HE staining. As illustrated in Figure 1a, the intimal structure in the ND and ND +P groups was well defined and intact with even thicknesses. After 7 weeks of HFD, the intima was obviously thickened, while administration of probucol (HFD +P group) significantly attenuated this damage. Next, we examined atherosclerotic lesion area by en face analysis, and found that rabbits in HFD group developed severe lesions throughout the aortic arch, thoracic aorta and abdominal aorta (Figure 1b). Probucol treatment (HFD +P group) led to a 56.4% reduction in aortic atherosclerotic lesion area compared with HFD group (Figure 1c). No atherosclerotic lesions were observed in either ND or

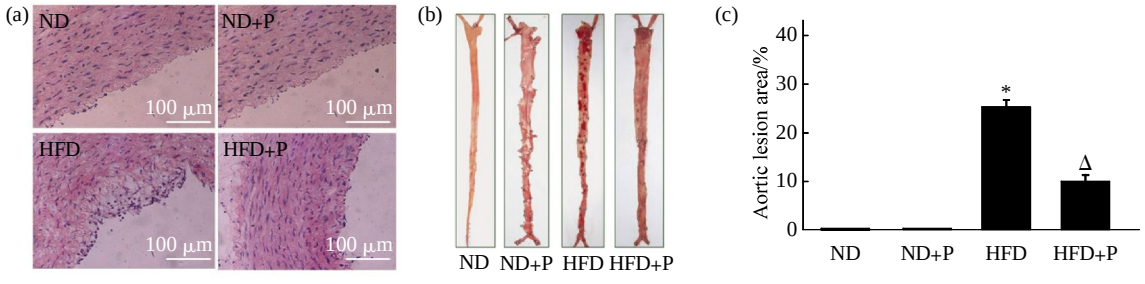

Fig. 1 Probucol prevents atherosclerosis in hypercholesterolemic rabbits

(a) Representative HE staining of aortas. (b) Representative Sudan Ⅳ-stained aortas. En face aortic preparations show distribution of sudanophilic (red) atherosclerotic lesion areas in aortas. (c) Quantification of atherosclerotic lesion. Aortic surface covered by Sudan Ⅳ-stained lesions was quantified and expressed as a percent of total aortic area. All values are presented as mean ± SD (n=9 in each group). *P < 0.05 *vs* ND group. $^{\Delta}P$ < 0.05 *vs* HFD group.

ND +P groups. Together, these data suggest that probucol significantly attenuates atherosclerosis in hypercholesterolemic rabbits.

2.3 Probucol reduces HFD-induced accumulation of hepatic lipids

The ratio of liver weight to body weight is a good indicator to evaluate hepatic lipid accumulation[21]. As depicted in Figure 2a, the ratio of liver weight to body weight was significantly increased in HFD group when compared to NFD group, which was significantly suppressed by probucol treatment (HFD +P group), revealing a protective role of probucol in controlling lipid accumulation within the liver. In contrast, no difference was found between the ND and ND +P groups. To further confirm the effects of probucol on lipid accumulation in the liver, hepatic sections were stained with Oil Red O. As expected, Oil Red O staining area was significantly higher in HFD group than that in ND group, and the HFD-induced effect was inhibited by probucol treatment (Figure 2b, c). There was no statistically significant difference in hepatic Oil Red O staining area between the ND and ND +P groups. Thus, these results demonstrated that probucol contributes to reducing HFD-induced accumulation of liver lipids in rabbits.

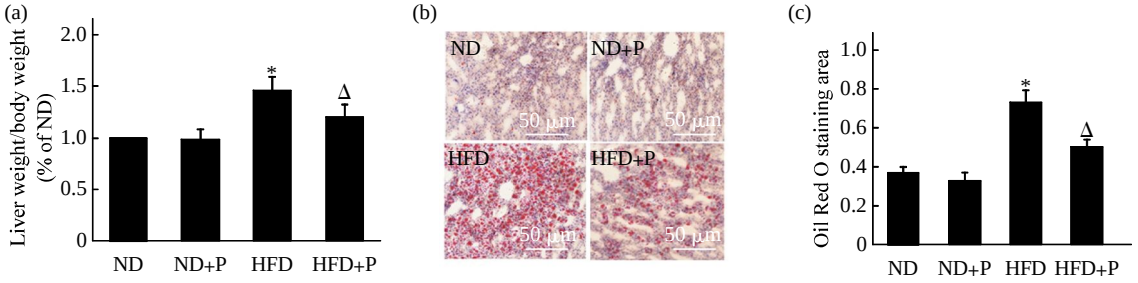

Fig. 2 Effects of probucol on the accumulation of hepatic lipids in hypercholesterolemic rabbits

(a) The ratio of liver weight to body weight. (b) Representative Oil Red O staining of liver tissues. (c) Quantification of Oil Red O staining area. Oil Red O staining area of hepatic tissue sections was quantified and expressed as a percent of the total tissue area. Data are mean ± SD (n=9 in each group). *P < 0.05 *vs* ND group. $^{\Delta}P$ < 0.05 *vs* HFD group.

2.4 Probucol downregulates ABCA1 expression but upregulates SR-B Ⅰ expression in the liver

HDL-derived CE in plasma is taken up by the liver through hepatic SR-B Ⅰ. After hydrolysis, the hepatic CE is converted to free cholesterol, which has several potential fates including resecretion back into the plasma *via* ABCA1. To investigated the possible underlying mechanism of probucol-induced HDL-C-lowering effect, we examined if probucol affected the expression of hepatic ABCA1 and SR-B Ⅰ using real-time quantitative PCR and Western blot analyses. As shown in Figure 3, probucol had no significant effect on the expressions of ABCA1 and SR-B Ⅰ in the ND group. Treatment of rabbits with HFD for 7 weeks strikingly caused a decrease in mRNA and protein levels of hepatic ABCA1 and SR-B Ⅰ when compared with ND group. Co-treatment with probucol (HFD + P group) further downregulated ABCA1 expression in HFD group (Figure 3a, b), but abolished HFD-induced lowering effect on SR-B Ⅰ expression (Figure 3c, d).

2.5 Probucol increases the expression of ABCG5 and ABCG8 in the liver and small intestine

ABCG5 and ABCG8 have been shown to play a critical role in promoting biliary cholesterol secretion in the liver and limiting intestinal absorption of cholesterol[22]. To determine whether the ABCG5 and ABCG8 expression in the liver and small intestine was affected by probucol, real-time quantitative PCR and Western blot analyses were performed. As depicted in Figure 4, the mRNA and protein expression levels of ABCG5 and ABCG8 in the liver and small intestine were significantly decreased in HFD group as compared to ND group, while these decreases were effectively blocked by a 7-week treatment of probucol (HFD +P group). Like the expression of ABCA1 and SR-B Ⅰ, the mRNA and protein levels of ABCG5 and ABCG8 were not influenced by probucol in ND group. Thus, probucol upregulates the expression of hepatic and small intestinal ABCG5 and ABCG8 in hypercholesterolemic rabbits.

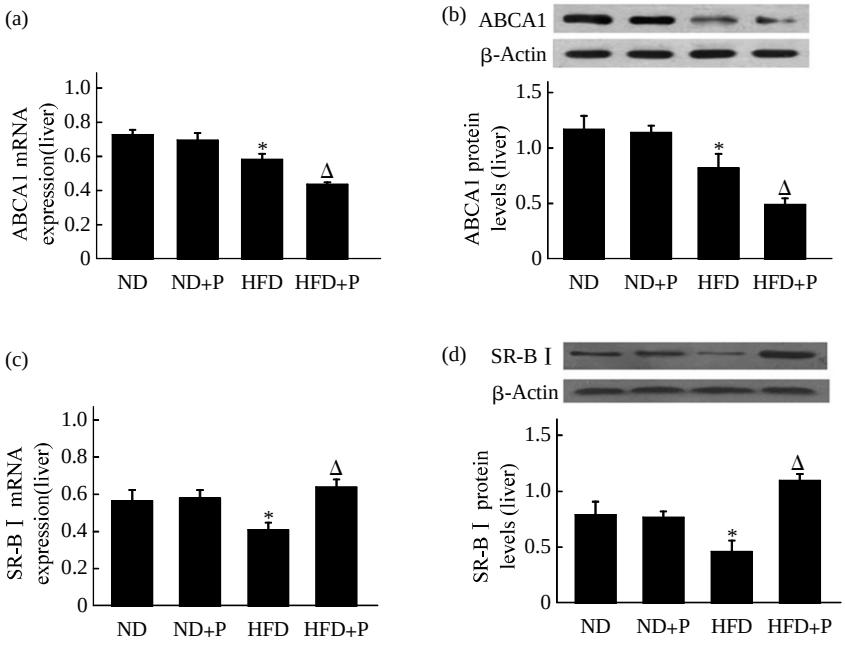

Fig. 3 Effects of probucol on ABCA1 and SR-B Ⅰ expression induced by HFD in the liver

Rabbits (n=9 in each group) were fed the experimental diets for 7 weeks. The expression of mRNA for hepatic ABCA1 (a) and SR-B Ⅰ (c) was determined by real-time quantitative PCR. Protein levels of hepatic ABCA1 (b) and SR-B Ⅰ (d) was examined by Western blot analysis. In all blots, staining for β-actin was used as a loading control. Data represent the mean ± SD. *P < 0.05 *vs* ND group. $^{\Delta}P$ < 0.05 *vs* HFD group.

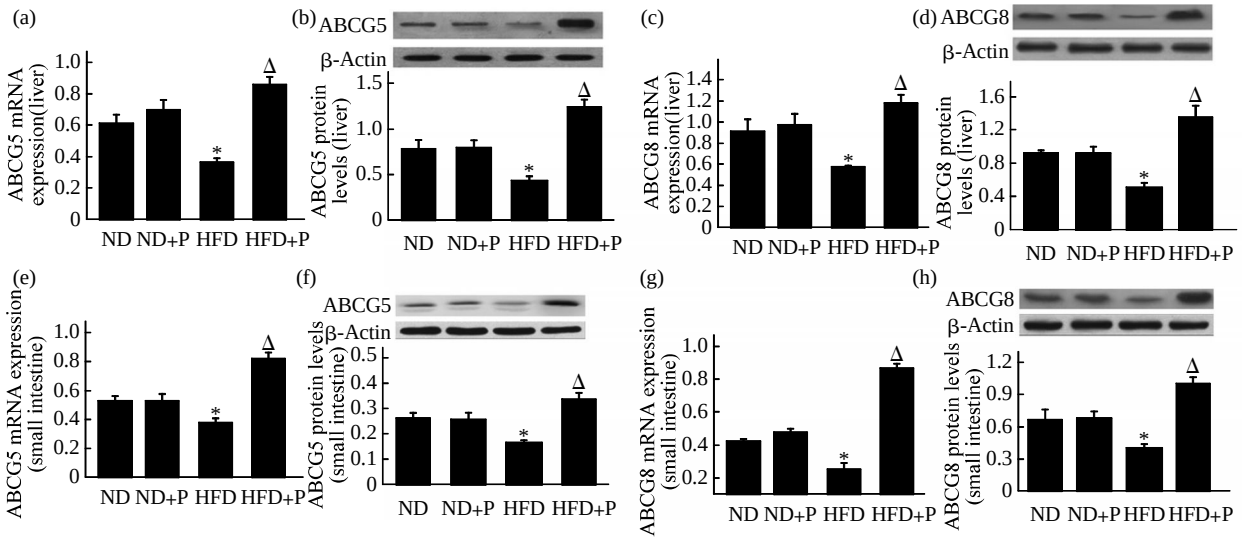

Fig. 4 Probucol blocks HFD-mediated down-regulation of ABCG5 and ABCG8 expression in the liver and small intestine

Rabbits (n=9 in each group) were fed the experimental diets for 7 weeks. Total RNA was extracted from the liver and small intestine, and real-time quantitative PCR was performed to determine the expression of ABCG5 (a, e) and ABCG8 (c, g) mRNA. The levels of ABCG5 (b, f) and ABCG8 (d, h) expression in the liver and small intestine were evaluated by Western blot using the anti-ABCG5 and anti-ABCG8 antibodies, respectively. In all blots, staining for β-actin was used as a loading control. Data are mean±SD. *P < 0.05 *vs* ND group. $^{\Delta}P$ < 0.05 *vs* HFD group.

2.6 Probucol suppresses inflammatory response

It is well known that proinflammatory cytokines including TNF-α, IL-1, IL-6 and MCP-1 are involved in the progression of atherosclerosis[23-24]. We therefore examined the effects of probucol on secretion of the four cytokines by ELISA. Compared with ND group, the TNF-α, IL-1, IL-6 and MCP-1 contents in the aortic arch were markedly increased in HFD group, and these increases were inhibited by probucol treatment (Figure 5). However, probucol had no

influence on these indicators in rabbits fed ND. In addition, a similar change of plasma TNF-α, IL-1, IL-6 and MCP-1 levels was seen (Figure 6). These findings suggest that the anti-inflammatory property of probucol also contributes to its antiatherogenic effects.

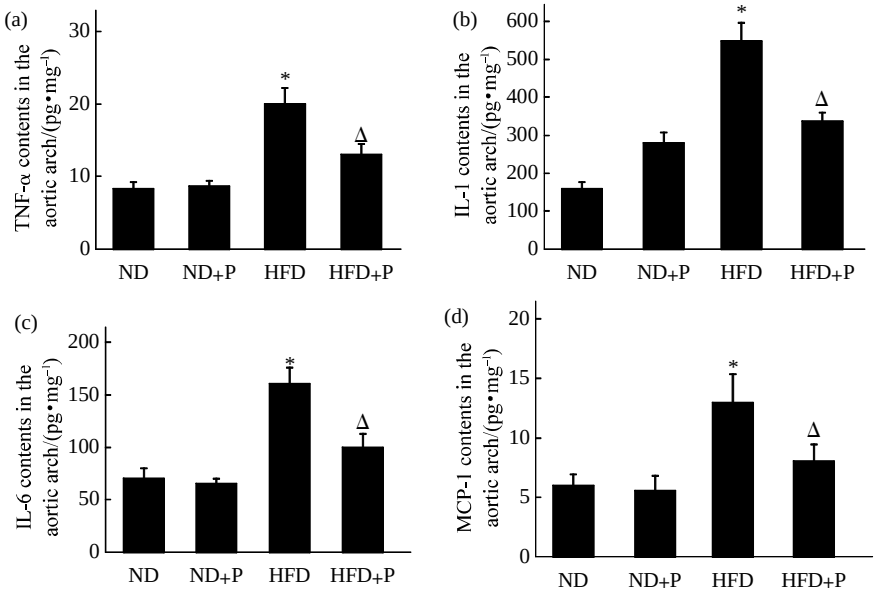

Fig. 5　Probucol attenuates TNF-α, IL-1, IL-6 and MCP-1 contents in the aortic arch

Rabbits (n=9 in each group) were fed the experimental diets for 7 weeks. Following sacrifice, the aortic arch tissues were isolated, homogenized and centrifuged. The supernatants then underwent ELISA to detect the contents of TNF-α (a), IL-1 (b), IL-6 (c) and MCP-1 (d). Data are mean ± SD. *P < 0.05 vs ND group. $^{\Delta}P$ < 0.05 vs HFD group.

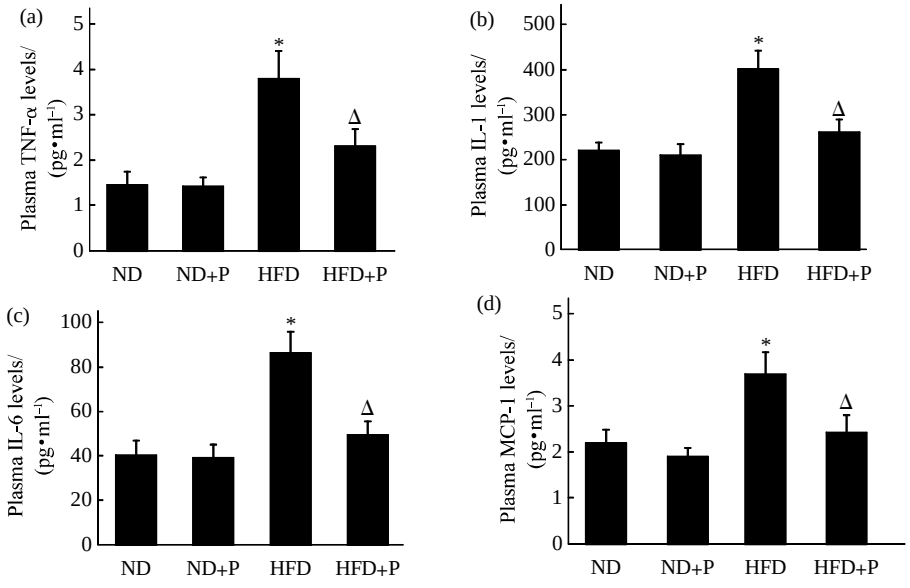

Fig. 6　Probucol decreases plasma levels of TNF-α, IL-1, IL-6 and MCP-1

Rabbits (n=9 in each group) were fed the experimental diets for 7 weeks, and plasma levels of TNF-α(a), IL-1(b), IL-6 (c) and MCP-1 (d) were detected by ELISA as described in the **Materials and Methods** section. Data are mean ± SD. *P < 0.05 vs ND group. $^{\Delta}P$ < 0.05 vs HFD group.

3　Discussion

In the present study, we showed that New Zealand white rabbits fed HFD for 7 weeks exhibited increased plasma levels of TC, TG, HDL-C and LDL-C as well as severe atherosclerosis, which were significantly blocked by administration of probucol. These findings confirm the HDL-C-lowering but

antiatherogenic effects of probucol, and are consistent with previous reports[5].

RCT is believed to be crucial for protecting against atherosclerosis. The process of RCT can be divided into three stages: cholesterol efflux from macrophages to plasma HDL acceptors, transport of plasma HDL-C to the liver, and HDL-derived cholesterol excretion from the liver to bile. Given the fact that probucol decreases plasma levels of HDL-C but reduces atherosclerosis, we hypothesized that the second or third stage of RCT might be increased in probucol-treated animals. To investigate the possibility, we analyzed the accumulation of hepatic lipids in rabbits. As anticipated, we found that probucol treatment significantly reduced the ratio of liver weight to body weight and hepatic Oil Red O staining area in rabbits fed a HFD, suggesting that probucol raises the ability of the liver to dispose cholesterol.

SR-B Ⅰ is highly expressed in the liver. Hepatic SR-B Ⅰ promotes selective uptake of HDL-C by the liver and plays a key role in the second stage of RCT. Hepatic SR-B Ⅰ expression is a positive regulator of the rate of RCT from macrophage to liver, bile, and feces [25]. SR-B Ⅰ deficiency greatly reduces the clearance of HDL-C from blood [26]. Rinninger and colleagues reported that probucol enhances selective uptake of HDL-C by the liver through an SR-B Ⅰ-dependent mechanism *in vitro* [27]. Here, our data showed that probucol significantly prevented HFD-induced down-regulation of hepatic SR-B Ⅰ expression. This is consistent with a previous report that probucol upregulates hepatic SR-B Ⅰ expression and increases the selective uptake of HDL-C by hepatocytes in hypercholesterolemic rabbits [16]. One possible mechanism of the effect of probucol on hepatic SR-B Ⅰ expression is that it can stabilize SR-B Ⅰ protein [28]. Together, these results indicate that the increased hepatic SR-B Ⅰ expression may lead to enhanced uptake of HDL-C by the liver, thereby accelerating catabolism of HDL-C and reducing plasma HDL-C levels. An enhancement of plasma HDL-C clearance via the hepatic SR-B Ⅰ pathway may in part be responsible for the HDL-C-lowering and antiatherogenic properties of probucol.

HDL-C taken up by the liver, once hydrolyzed in the liver, is a substrate for efflux back into the plasma *via* hepatic ABCA1. Recent studies have revealed that probucol inhibits ABCA1-mediated cholesterol efflux from fibroblasts, macrophages and HepG2 cells *in vitro* [29–31]. In this report, we found that the levels of hepatic ABCA1 mRNA and protein were significantly decreased in HFD group as compared to ND group, whereas probucol treatment further enhanced these decreases. It has been reported that probucol diminishes plasma HDL-C levels, suppresses HDL-derived cholesterol efflux from the liver to plasma and augments macrophage RCT by inhibiting hepatic ABCA1 expression in SR-B Ⅰ knockout mice [17]. Favari *et al* have demonstrated that probucol has an inhibitory effect on ABCA1 translocation to the plasma membrane and reduces the binding of HDL-derived cholesterol to apoA-Ⅰ in J774 macrophages [30]. Therefore, it is possible that the reduced hepatic ABCA1 expression lead to increasing hepatic disposal of HDL-derived cholesterol, which may partly contribute to the HDL-C-lowering action of probucol.

In addition to hepatic SR-B Ⅰ and ABCA1, the heterodimer of ABCG5 and ABCG8 involves in the process of RCT. ABCG5 and ABCG8 are predominantly expressed in the liver and small intestine [32], and promote biliary cholesterol secretion in the liver and prevent intestinal cholesterol absorption [33–34]. However, it is unclear whether the antiatherogenic effect of probucol is associated with regulation of ABCG5 and ABCG8 expression in the liver and small intestine. We observed that probucol upregulated the expression hepatic and small intestinal ABCG5 and ABCG8 in rabbits fed HFD. Thus, these data reveal that the increased expression of hepatic and small intestinal ABCG5 and ABCG8 may facilitate biliary and intestinal sterol excretion, thereby contributing to the role of probucol in attenuating atherosclerosis.

TNF-α, IL-1, IL-6 and MCP-1, as master proinflammatory cytokines, play a crucial role in the development of atherosclerosis. Elevated levels of these four proinflammatory cytokines in the plasma and atherosclerotic plaques have been reported in patients with coronary heart disease and in atherosclerotic animal models [35–38]. Probucol is a hypolipidemic drug with an anti-inflammatory function. Recent studies have shown that this drug can inhibit the secretion of TNF-α, IL-1, IL-6 and MCP-1 [39–40]. In the current study, our results revealed that the levels of TNF-α, IL-1, IL-6 and MCP-1 in the plasma and aortic arch were significantly increased in HFD group as compared to ND group, indicating the involvement of

these proinflammatory cytokines in the pathogenesis of atherosclerosis. However, these increases were partially reversed in the presence of probucol. Thus, inhibition of proinflammatory cytokine secretion may also be one of the mechanisms underlying the antiatherogenic potential of probucol.

In summary, our results demonstrate that probucol decreases plasma HDL-C levels but inhibits atherosclerosis in hypercholesterolemic rabbits, which may be related to the upregulation of hepatic SR-BⅠ expression and ABCG5 and ABCG8 expression in the liver and small intestine as well as the downregulation of hepatic ABCA1 expression. Probucol also reduces plasma levels of TNF-α, IL-1, IL-6 and MCP-1, which may be essential for its antiatherogenic effects. To our knowledge, the findings of this study for the first time completely explain the antiatherogenic but HDL-C-lowering properties of probucol, and may help clinicians further apply this drug to decrease the risks of atherosclerotic cardiovascular disease.

References

[1] Yu X H, Zhang J, Zheng X L, *et al*. Interferon-gamma in foam cell formation and progression of atherosclerosis. Clin Chim Acta, 2015, **441**: 33-43

[2] Eren E, Yilmaz N, Aydin O. High density lipoprotein and it's dysfunction. Open Biochem J, 2012, **6**: 78-93

[3] Uehara Y, Saku K. High-density lipoprotein and atherosclerosis: roles of lipid transporters. World J Cardiol, 2014, **6** (10): 1049-1059

[4] Levinson S S, Wagner S G. Implications of reverse cholesterol transport: recent studies. Clin Chim Acta, 2015, **439**: 154-161

[5] Kita T, Nagano Y, Yokode M, *et al*. Probucol prevents the progression of atherosclerosis in Watanabe heritable hyperlipidemic rabbit, an animal model for familial hypercholesterolemia. Proc Natl Acad Sci USA, 1987, **84**(16): 5928-5931

[6] Yu X H, Fu Y C, Zhang D W, *et al*. Foam cells in atherosclerosis. Clin Chim Acta, 2013, **424**: 245-252

[7] Wang N, Arai T, Ji Y, *et al*. Liver-specific overexpression of scavenger receptor BI decreases levels of very low density lipoprotein ApoB, low density lipoprotein ApoB, and high density lipoprotein in transgenic mice. J Biol Chem, 1998, **273** (49): 32920-32926

[8] Ueda Y, Gong E, Royer L, *et al*. Relationship between expression levels and atherogenesis in scavenger receptor class B, type Ⅰ transgenics. J Biol Chem, 2000, **275**(27): 20368-20373

[9] Braun A, Trigatti B L, Post M J, *et al*. Loss of SR-BI expression leads to the early onset of occlusive atherosclerotic coronary artery disease, spontaneous myocardial infarctions, severe cardiac dysfunction, and premature death in apolipoprotein E-deficient mice. Circ Res, 2002, **90**(3): 270-276

[10] Vaisman B L, Lambert G, Amar M, *et al*. ABCA1 overexpression leads to hyperalphalipoproteinemia and increased biliary cholesterol excretion in transgenic mice. J Clin Invest, 2001, **108**(2): 303-309

[11] Timmins J M, Lee J Y, Boudyguina E, *et al*. Targeted inactivation of hepatic Abca1 causes profound hypoalphalipoproteinemia and kidney hypercatabolism of apoA-Ⅰ. J Clin Invest, 2005, **115**(5): 1333-1342

[12] Brown J M, Yu L. Opposing gatekeepers of apical sterol transport: niemann-pick C1-like 1 (NPC1L1) and ATP-binding cassette transporters G5 and G8 (ABCG5/ABCG8). Immunol Endocr Metab Agents Med Chem, 2009, **9**(1): 18-29

[13] Klett E L, Lu K, Kosters A, *et al*. A mouse model of sitosterolemia: absence of Abcg8/sterolin-2 results in failure to secrete biliary cholesterol. BMC Med, 2004, **2**: 5

[14] Wilund K R, Yu L, Xu F, *et al*. High-level expression of ABCG5 and ABCG8 attenuates diet-induced hypercholesterolemia and atherosclerosis in Ldlr$^{-/-}$ mice. J Lipid Res, 2004, **45**(8): 1429-1436

[15] Yamamoto A, Matsuzawa Y, Yokoyama S, *et al*. Effects of probucol on xanthomata regression in familial hypercholesterolemia. Am J Cardiol, 1986, **57**(16): 29H-35H

[16] Hong S C, Zhao S P, Wu Z H. Effect of probucol on HDL metabolism and class B type Ⅰ scavenger receptor (SR-BI) expression in the liver of hypercholesterolemic rabbits. Int J Cardiol, 2007, **115**(1): 29-35

[17] Yamamoto S, Tanigawa H, Li X, *et al*. Pharmacologic suppression of hepatic ATP-binding cassette transporter 1 activity in mice reduces high-density lipoprotein cholesterol levels but promotes reverse cholesterol transport. Circulation, 2011, **124** (12): 1382-1390

[18] Liu D, He Z, Wu L, *et al*. Effects of induction/inhibition of endogenous heme oxygenase-1 on lipid metabolism, endothelial function, and atherosclerosis in rabbits on a high fat diet. J Pharmacol Sci, 2012, **118**(1): 14-24

[19] Dai X Y, Ou X, Hao X R, *et al*. The effect of T0901317 on ATP-binding cassette transporter A1 and Niemann-Pick type C1 in apoE$^{-/-}$ mice. J Cardiovasc Pharmacol, 2008, **51**(5): 467-475

[20] Yu X H, Jiang H L, Chen W J, *et al*. Interleukin-18 and interleukin-12 together downregulate ATP-binding cassette transporter A1 expression through the interleukin-18R/nuclear factor-kappaB signaling pathway in THP-1 macrophage-derived foam cells. Circ J, 2012, **76**(7): 1780-1791

[21] Koh S L, Ager E, Malcontenti-Wilson C, *et al*. Blockade of the renin-angiotensin system improves the early stages of liver regeneration and liver function. J Surg Res, 2013, **179**(1): 66-71

[22] Duan L P, Wang H H, Ohashi A, *et al*. Role of intestinal sterol transporters Abcg5, Abcg8, and Npc1l1 in cholesterol absorption in mice: gender and age effects. Am J Physiol Gastrointest Liver Physiol, 2006, **290**(2): G269-276

[23] Tian G P, Chen W J, He P P, *et al*. MicroRNA-467b targets LPL gene in RAW 264.7 macrophages and attenuates lipid accumulation and proinflammatory cytokine secretion. Biochimie, 2012, **94**(12):

2749−2755

[24] Yin K, Chen W J, Zhou Z G, *et al*. Apolipoprotein A-I inhibits CD40 proinflammatory signaling *via* ATP-binding cassette transporter A1-mediated modulation of lipid raft in macrophages. J Atheroscler Thromb, 2012, **19**(9): 823−836

[25] Zhang Y, Da Silva J R, Reilly M, *et al*. Hepatic expression of scavenger receptor class B type Ⅰ (SR-BI) is a positive regulator of macrophage reverse cholesterol transport *in vivo*. J Clin Invest, 2005, **115**(10): 2870−2874

[26] Liadaki K N, Liu T, Xu S, *et al*. Binding of high density lipoprotein (HDL) and discoidal reconstituted HDL to the HDL receptor scavenger receptor class B type Ⅰ. Effect of lipid association and APOA-Ⅰ mutations on receptor binding. J Biol Chem, 2000, **275**(28): 21262−21271

[27] Rinninger F, Wang N, Ramakrishnan R, *et al*. Probucol enhances selective uptake of HDL-associated cholesteryl esters *in vitro* by a scavenger receptor B-I-dependent mechanism. Arterioscler Thromb Vasc Biol, 1999, **19**(5): 1325−1332

[28] Hirano K, Ikegami C, Tsujii K, *et al*. Probucol enhances the expression of human hepatic scavenger receptor class B type Ⅰ, possibly through a species-specific mechanism. Arterioscler Thromb Vasc Biol, 2005, **25**(11): 2422−2427

[29] Wu C A, Tsujita M, Hayashi M, *et al*. Probucol inactivates ABCA1 in the plasma membrane with respect to its mediation of apolipoprotein binding and high density lipoprotein assembly and to its proteolytic degradation. J Biol Chem, 2004, **279**(29): 30168−30174

[30] Favari E, Zanotti I, Zimetti F, *et al*. Probucol inhibits ABCA1-mediated cellular lipid efflux. Arterioscler Thromb Vasc Biol, 2004, **24**(12): 2345−2350

[31] Tsujita M, Wu C A, Abe-Dohmae S, *et al*. On the hepatic mechanism of HDL assembly by the ABCA1/apoA-Ⅰ pathway. J Lipid Res, 2005, **46**(1): 154−162

[32] Dieter M Z, Maher J M, Cheng X, *et al*. Expression and regulation of the sterol half-transporter genes ABCG5 and ABCG8 in rats. Comp Biochem Physiol C Toxicol Pharmacol, 2004, **139**(4): 209−218

[33] Plosch T, Kosters A, Groen AK, *et al*. The ABC of hepatic and intestinal cholesterol transport. Handb Exp Pharmacol, 2005, **170**(5): 465−482

[34] Yu L, Li-Hawkins J, Hammer R E, *et al*. Overexpression of ABCG5 and ABCG8 promotes biliary cholesterol secretion and reduces fractional absorption of dietary cholesterol. J Clin Invest, 2002, **110**(5): 671−680

[35] Zakynthinos E, Pappa N. Inflammatory biomarkers in coronary artery disease. J Cardiol, 2009, **53**(3): 317−333

[36] Sertic J, Slavicek J, Bozina N, *et al*. Cytokines and growth factors in mostly atherosclerotic patients on hemodialysis determined by biochip array technology. Clin Chem Lab Med, 2007, **45** (10): 1347−1352

[37] Zhao Z, Song G, Tian H, *et al*. Triacetyl-3-hydroxyphenyladenosine, a derivative of cordycepin, attenuates atherosclerosis in apolipoprotein E-knockout mice. Exp Biol Med (Maywood), 2012, **237**(11): 1262−1272

[38] Minatti J, Wazlawik E, Hort M A, *et al*. Green tea extract reverses endothelial dysfunction and reduces atherosclerosis progression in homozygous knockout low-density lipoprotein receptor mice. Nutr Res, 2012, **32**(9): 684−693

[39] Oh G T, Choi J H, Hong J J, *et al*. Dietary hematein ameliorates fatty streak lesions in the rabbit by the possible mechanism of reducing VCAM-1 and MCP-1 expression. Atherosclerosis, 2001, **159**(1): 17−26

[40] Meng C Q, Somers P K, Hoong L K, *et al*. Discovery of novel phenolic antioxidants as inhibitors of vascular cell adhesion molecule-1 expression for use in chronic inflammatory diseases. J Med Chem, 2004, **47**(25): 6420−6432

普罗布考通过调控 ABCA1、SR-BⅠ、ABCG5、ABCG8 表达及抗炎作用抑制高胆固醇血症兔动脉粥样硬化 *

崔立宝[1, 2)**]　于小华[1)**]　蒋崇辉[2)]　唐雅玲[1)]　欧阳新平[1)]　何平平[1)]
吕运成[1)]　姚　峰[1)]　张　敏[1)]　唐朝克[1)***]

([1)] 南华大学心血管研究所，湖南省动脉硬化学重点实验室，南华大学生命科学研究中心，
湖南省分子靶标新药研究协同创新中心，衡阳 421001；
[2)] 衡阳市中心医院药剂科，衡阳 421001)

摘要　作为一种有效的降脂药物，普罗布考能够降低血浆高密度脂蛋白胆固醇(HDL-C)水平并抑制动脉粥样硬化，但其机制尚未完全阐明．本研究的目的旨在进一步阐明普罗布考降脂及抗动脉粥样硬化的机理．将新西兰白兔随机分为 4 组：正常饮食组、正常饮食+普罗布考组、高脂饮食组(HFD 组)、高脂饮食+普罗布考组(HFD+P 组)．结果显示，处理 7 周后，与 HFD 组比较，HFD+P 组动脉粥样硬化病变程度、肝脏脂质蓄积明显减轻，血浆甘油三脂、总胆固醇、低密度脂蛋白胆固醇及 HDL-C 水平降低，肝脏中清道夫受体 -BⅠ(SR-BⅠ)以及肝脏与小肠中三磷酸腺苷结合盒转运体(ABC)G5(ABCG5)、ABCG8 表达上调，肝脏中 ABCA1 表达下调，主动脉弓与血浆肿瘤坏死因子 α、白介素 1、白介素 6、单核趋化蛋白 1 水平降低．这些结果表明普罗布考的抗动脉粥样硬化作用可能与其调控 ABCA1、SR-BⅠ、ABCG5、ABCG8 表达及抑制促炎介质的分泌有关．

关键词　普罗布考，动脉粥样硬化，高密度脂蛋白胆固醇，三磷酸腺苷结合盒转运体 A1, 清道夫受体 -BⅠ，三磷酸腺苷结合盒转运体 G5，三磷酸腺苷结合盒转运体 G8，炎症
学科分类号　R363　　**DOI**: 10.16476/j.pibb.2015.0124

* 国家自然科学基金资助项目(81070220, 81170278).
** 共同第一作者.
*** 通讯联系人.
Tel: 0734-8281853, E-mail: tangchaoke@qq.com
收稿日期：2015-04-24，接受日期：2015-08-05