Biochemical and Biophysical Research Communications 463 (2015) 48–53

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Spontaneous and diet-aggravated hemolysis and its correction by probucol in SR-BI knockout mice with LDL-R deficiency

Jiawei Liao [a], Mingming Gao [a], Mengyu Wang [a], Xin Guo [a], Wei Huang [a, *], George Liu [a, b, *]

[a] Institute of Cardiovascular Sciences and Key Laboratory of Molecular Cardiovascular Sciences, Ministry of Education, Peking University Health Science Center, Beijing 100191, China
[b] Center for Molecular and Translational Medicine, Peking University Health Science Center, Beijing 100191, China

ARTICLE INFO

Article history:
Received 25 April 2015
Accepted 6 May 2015
Available online 15 May 2015

Keywords:
Hemolysis
SR-BI
Free cholesterol
Probucol

ABSTRACT

Background: High density lipoprotein receptor SR-BI plays a vital role in cholesterol homeostasis. Depletion of SR-BI causes plasma free cholesterol (FC) accumulation, which disrupts erythrocytes membrane and might induce hemolytic anemia. Here we explored the effects of hypercholesteremia, induced by depletion of low density lipoprotein receptor (LDL-R) and high fat diet (HFD) feeding, on plasma FC and possible hemolysis in SR-BI knockout (KO) mice, and the therapeutic effects of a lipid-lowering drug probucol.

Methods and results: To determine the effects of LDL-R depletion, SR-BI KO mice were cross-bred with LDL-R KO mice to generate SR-BI/LDL-R double KO (dKO) mice. Compared to control wild type (WT), SR-BI KO and LDL-R KO mice fed normal chow diet (NCD), dKO mice fed NCD had increased plasma FC and developed macrocytic anemia, splenomegaly, jaundice and renal tubular hemosiderin deposition, indicating spontaneous hemolysis. To determine the effects of HFD feeding and probucol therapy, dKO and LDL-R KO mice were fed HFD containing 0.5% cholesterol and 20% fat with or without 1% probucol. HFD further increased plasma FC and aggravated hemolysis while probucol almost normalized plasma FC and corrected hemolysis in dKO mice.

Conclusion: We demonstrated that in SR-BI KO mice, hypercholesteremia due to LDL-R deficiency significantly increased plasma FC and induced spontaneous hemolysis, which could be further exacerbated by HFD feeding. Probucol almost normalized plasma FC and corrected diet-aggravated hemolysis in SR-BI KO mice with LDL-R deficiency.

1. Introduction

Scavenger receptor class B type I (SR-BI) is the major high density lipoprotein (HDL) receptor in mammalians. Mainly expressed in the liver and steroidogenic organs such as the adrenal glands, the ovaries and the testis, it mediates the selective uptake of cholesterol esters (CEs) in the HDLs, thus playing a vital role in reverse cholesterol transport (RCT) and global cholesterol homeostasis. Defect in SR-BI causes disrupted RCT and thus cholesterol enrichment, mainly as the form of free cholesterol (FC), in the HDLs, contributing to HDL dysfunctions [1–3].

Circulating in the bloodstream, the erythrocytes exchange lipids directly with lipoproteins via the erythrocyte membrane in nonspecific mechanisms [4]. Abnormalities in lipoproteins might affect the lipids content and thus the properties of the erythrocyte membrane. A previous study has already demonstrated that due to increased FC in the HDLs, cholesterol accumulated in the erythrocyte membrane of SR-BI knockout (KO) mice, leading to decreased membrane deformability and osmotic fragility [5]. The changes in the erythrocyte membrane might cause accelerated erythrocytes degradation and induce hemolysis. Here we explored the effects of hypercholesteremia, induced by depletion of low density lipoprotein receptor (LDL-R) and high fat diet (HFD) feeding, on the plasma FC and possible hemolysis in SR-BI KO mice, and the therapeutic effects of a lipid-lowering drug probucol.

* Corresponding authors. Institute of Cardiovascular Sciences and Key Laboratory of Molecular Cardiovascular Sciences, Ministry of Education, Peking University Health Science Center, Beijing 100191, China.
E-mail addresses: huangwei@bjmu.edu.cn (W. Huang), georgeliu@bjmu.edu.cn (G. Liu).

http://dx.doi.org/10.1016/j.bbrc.2015.05.015
0006-291X/

2. Material and methods

2.1. Animals and diets

To determine the effects of hypercholesterolemia induced by LDL-R deficiency in SR-BI KO mice, SR-BI/LDL-R double KO (dKO) mice and control wild type (WT), SR-BI KO and LDL-R KO mice were fed normal chow diet (NCD) all the way until there were 5 months old; To determine the effects of HFD feeding and probucol therapy, SR-BI/LDL-R dKO and control LDL-R KO mice of 10–12 weeks old were fed a HFD containing 0.5% cholesterol (AMRESCO, USA) and 20% fat with or without 1% probucol (Natural-Med, USA) for 12 weeks. WT, SR-BI KO and LDL-R KO mice were supplied by Peking University Experimental Animal Center while SR-BI/LDL-R dKO mice were generated by cross-breeding SR-BI KO with LDL-R KO mice. All the mice included in the experiments were females. The housing, care and all the experimental procedures were conducted following the regulations of the National Institute of Health and approved by Animal Care Committee at Peking University.

2.2. Plasma lipids analysis

After the mice were fasted for 4 h, blood samples were drawn into tubes coated with heparin by retro-orbital venous plexus puncture and centrifuged at 4000 rpm for 10 min to collect the plasma supernatant. The plasma total cholesterol (TC) and FC were measured using commercial kits (Applygen, China).

2.3. Erythrocyte parameters analysis

Blood samples were drawn by retro-orbital venous plexus puncture and collected in tubes with no anticoagulant. The bloods (20 μl) were then immediately diluted and blended in specialized dilution buffer (Nihon Kohden, Japan). Erythrocyte parameters were measured with an automated blood count analyzer (MEK-6318K; Nihon Kohden, Japan).

2.4. Organ gravimetric and histological analysis

Mice were weighed, euthanized and flushed with 20 ml 0.01M phosphate buffer solution through the left ventricle. The spleens and kidneys were weighed, fixed in 4% paraformaldehyde solution for 4 h and then kept in 20% sucrose solution for overnight. Embedded in paraffin, the kidneys were cross-sectioned at 2 μm. Renal tubular hemosiderin deposition were visualized by Perl's Prussian blue staining.

2.5. Statistical analysis

Data were presented as mean ± SEM. Statistical significance was evaluated by one-way ANOVA and P value <0.05 was regarded as significant.

3. Results

3.1. LDL-R deficiency significantly increased plasma FC and led to spontaneous hemolysis in SR-BI KO mice

On NCD feeding, SR-BI depletion led to a roughly 1-fold and 4-fold increase of plasma TC and FC respectively, resulting in a roughly 1.5-fold increase of plasma FC/TC in WT mice (Table 1). A previous study has demonstrated that the increased plasma FC in SR-BI KO mice led to cholesterol accumulation in the membrane of the erythrocytes, resulting in decreased membrane deformability and osmotic fragility and thus increased degradation of the erythrocytes [5]. Here we showed mice depleted of SR-BI had slightly decreased erythrocyte count and hemoglobin (HGB), and slightly enlarged mean corpuscular volume (MCV) and decreased mean corpuscular hemoglobin concentration (MCHC) (Table 1), indicating no significant anemia in our female SR-BI KO mice. No significant enlargement of the spleen (Fig. 1A–B), no jaundice (Fig. 1C) and renal tubular hemosiderin deposition visualized by Perl's Prussian blue staining (Fig. 1D) were also observed in SR-BI KO mice. LDL-R depletion led to a roughly 1-fold increase of plasma TC and FC in both WT and SR-BI KO mice, however, as the increase of plasma TC and FC was proportional, there was no significant change of plasma FC/TC (Table 1). While hypercholesteremia due to LDL-R depletion failed to induce any significant change of the erythrocytes in WT mice, it caused a significant 26.77% decrease of erythrocyte count and 11.59% decrease of HGB together with a significant 26.76% increase of MCV and a slightly decrease of MCHC in SR-BI KO mice (Table 1). LDL-R depletion also led to a roughly 3-fold enlargement of the spleen (Fig. 1A–B) and the onset of jaundice (Fig. 1C) and renal tubular hemosiderin deposition (Fig. 1D) in SR-BI KO mice. When compared to WT mice, combined depletion of SR-BI and LDL-R led to a 33.46% decrease of erythrocyte count and 18.67% decrease of HGB together with a 31.51% increase of MCV and a 6.63% decrease of MCHC (Table 1), indicating the spontaneous onset of macrocytic anemia in SR-BI/LDL-R dKO mice. Macrocytic anemia,

Table 1
Analysis of the plasma cholesterol and erythrocytes in mice on NCD.

	WT (n = 6)	SR-BI KO (n = 5)	LDL-R KO (n = 11)	SR-BI/LDL-R dKO (n = 10)
Plasma lipids				
TC(mg/dl)	95.23 ± 4.642	199.9 ± 4.453,*	222.1 ± 9.577,###	406.8 ± 14.41, $$$; &&&
FC(mg/dl)	21.33 ± 2.233	95.35 ± 5.486,**	47.27 ± 2.484	211.8 ± 9.056, $$$; &&&
TC/FC	0.2220 ± 0.0162	0.4798 ± 0.0361,***	0.2134 ± 0.0082	0.5220 ± 0.0170, &&&
Erythocytes				
RBC Count(× 10^{12}/L)	8.978 ± 0.2990	8.158 ± 0.4530	8.825 ± 0.1700	5.974 ± 0.3680, $$$; &&&
HGB(g/L)	150.2 ± 3.219	138.0 ± 3.240	151.1 ± 1.654	122.0 ± 5.774, &&&
MCV(fL)	44.30 ± 0.2530	45.96 ± 0.8948	44.12 ± 0.1689	58.26 ± 1.640, $$$; &&&
MCHC(g/L)	380.2 ± 18.11	370.4 ± 6.990	360.7 ± 2.115	355.0 ± 6.493

Data were presented as mean ± SEM. RBC: red blood cell.
*: SR-BI KO vs WT.
#: LDL-R KO vs WT.
$: SR-BI/LDL-R dKO vs SR-BI KO.
&: SR-BI/LDL-R dKO vs WT.

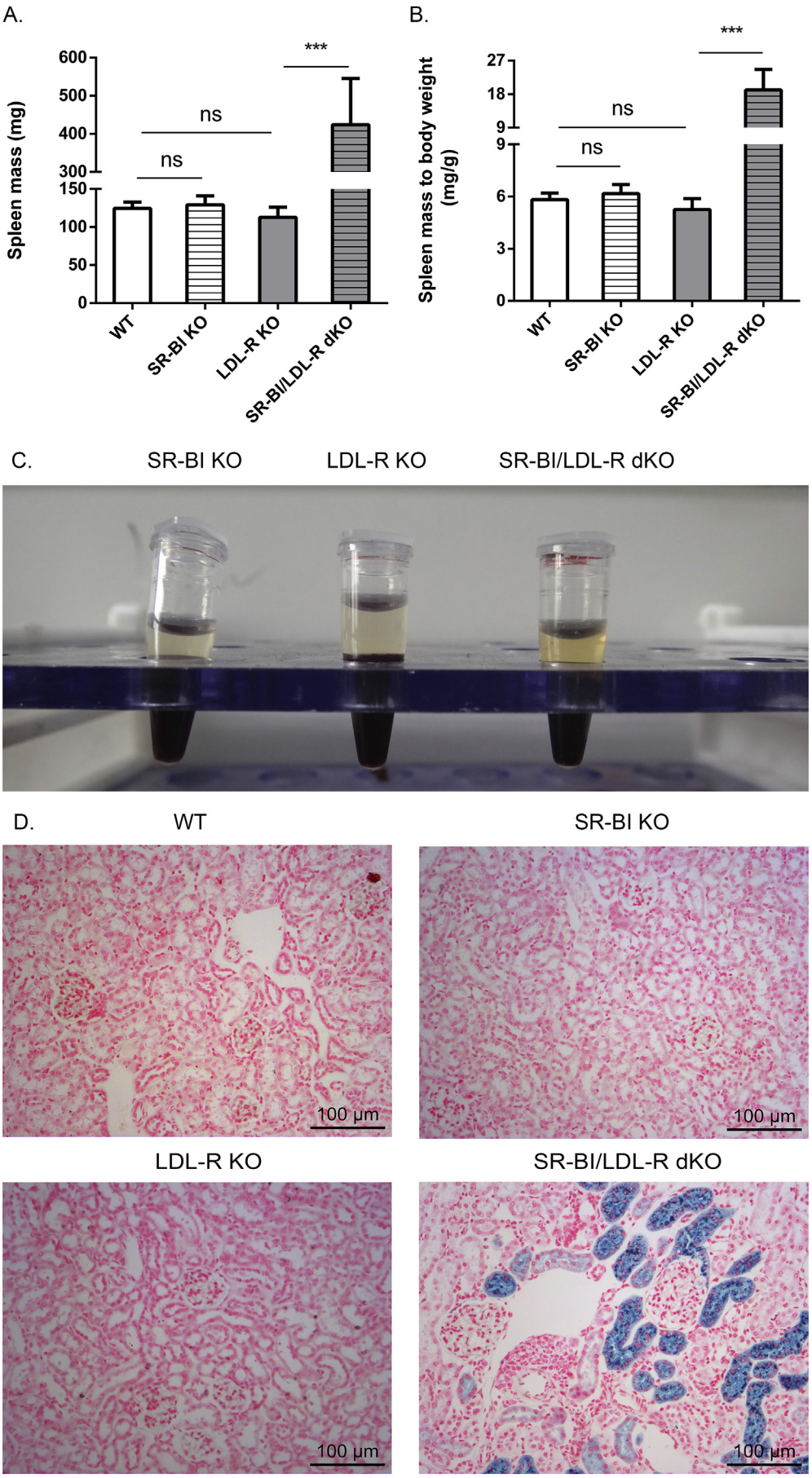

Fig. 1. Analysis of the spleen mass, appearance of the centrifuged plasma and renal Perl's staining in mice on NCD. A: Comparation of spleen mass; B: Comparation of the ratio of spleen mass to body weight; C: Appearance of the centrifuged plasma; D: Renal Perl's staining.

Table 2
Analysis of the plasma cholesterol and erythrocytes in mice on HFD with or without probucol addition.

	HFD		HFD + P	
	LDL-R KO (n = 8)	SR-BI/LDL-R dKO (n = 7)	LDL-R KO (n = 9)	SR-BI/LDL-R dKO (n = 7)
Plasma lipids				
TC(mg/dl)	816.9 ± 43.27	793.2 ± 56.71	427.4 ± 41.81	417.6 ± 43.77,$^{\$\$\$}$
FC(mg/dl)	258.5 ± 14.74	522.9 ± 42.00,***	117.5 ± 15.74	149.2 ± 21.68,$^{\$\$\$}$
TC/FC	0.3200 ± 0.0191	0.6571 ± 0.0199,***	0.2700 ± 0.0163	0.3486 ± 0.0196,$^{\#;\$\$\$}$
Erythocytes				
RBC Count($\times 10^{12}$/L)	9.205 ± 0.1372	5.079 ± 0.1114,***	7.323 ± 0.1862	8.031 ± 0.1625,$^{\#;\$\$\$}$
HGB(g/L)	135.4 ± 2.471	101.7 ± 1.107,***	130.0 ± 2.953	130.1 ± 2.262,$^{\$\$\$}$
MCV(fL)	48.49 ± 0.2546	69.26 ± 1.358,***	53.00 ± 0.3416	50.10 ± 0.8580,$^{\$\$\$}$
MCHC(g/L)	303.3 ± 3.233	290.0 ± 3.162,	335.0 ± 2.198	324.0 ± 5.090,$^{\$\$\$}$

Data were presented as mean ± SEM. RBC: red blood cell; HFD + P: HFD with probucol addition.
*: SR-BI/LDL-R dKO on HFD vs LDL-R KO on HFD.
$^{\#}$: SR-BI/LDL-R dKO on HFD + P vs LDL-R KO on HFD + P.
$^{\$}$: SR-BI/LDL-R dKO on HFD vs SR-BI/LDL-R dKO on HFD + P.

splenomegaly, jaundice and renal tubular hemosiderin deposition combined indicated the spontaneous development of hemolysis in SR-BI/LDL-R dKO mice.

3.2. HFD further increased plasma FC and aggravated hemolysis while probucol therapy decreased plasma FC and corrected hemolysis in SR-BI/LDL-R dKO mice

On HFD feeding for 12 weeks, the plasma TC and FC in SR-BI/LDL-R dKO mice underwent a further proportional 1-fold increase, compared to SR-BI/LDL-R dKO mice fed NCD (Tables 1 and 2). Besides, the erythrocyte count and HGB further increased 14.98% and 16.64% while the MCV further increased 18.88% and the MCHC further decreased 18.31% (Tables 1 and 2), together with a further enlargement of the spleen (Fig. 1 A–B and Fig. 2A–B) and exacerbation of the jaundice (Fig. 1C and Fig. 2C) and renal tubular hemosiderin deposition (Fig. 1D and Fig. 2D) in SR-BI/LDL-R dKO mice fed HFD, compared to those fed NCD.

Even on HFD feeding, probucol therapy almost reduced the plasma TC and FC levels in SR-BI/LDL-R dKO mice to the levels in LDL-R KO mice, although the plasma FC/TC was still slightly higher than that in LDL-R KO mice (Table 2). In addition to the plasma cholesterol metabolism, probucol therapy almost normalized all the main erythrocyte parameters as compared to those in LDL-R KO mice (Table 2). Probucol therapy also corrected splenomegaly (Fig. 2A–B), jaundice (Fig. 2C) and renal tubular hemosiderin deposition (Fig. 2D) in SR-BI/LDL-R dKO mice.

Together, HFD further increased plasma FC and aggravated hemolysis while probucol therapy decreased plasma FC and corrected hemolysis in SR-BI/LDL-R dKO mice.

4. Discussion

In this study, we showed that hypercholesterolemia due to LDL-R deficiency significantly increased plasma FC and led to spontaneous hemolysis featuring macrocytic anemia, splenomegaly, jaundice and renal tubular hemosiderin deposition in SR-BI KO mice. HFD feeding further increased plasma FC and exacerbated hemolysis while the lipid-lowering drug probucol almost normalized plasma FC and corrected hemolysis, in SR-BI KO mice with LDL-R deficiency. In fact, spontaneous hemolysis was also seen in SR-BI KO mice with hyperlipidemic apoE deficiency, which could also been corrected by probucol via its FC-lowering effect (data not shown).

FC in the plasma is mainly distributed in HDL particles. Exported from peripheral cells including endothelial cells, macrophages, smooth muscle cells, erythrocytes and platelets et al., FC was then accepted by lipid-free apolipoprotein (apo) AI to form immature preβ-HDL [6]. Lecithin: cholesterol acyltransferase (LCAT) in the surface of HDL catalyzes the esterification of FC to form CE, which is then packed into the core of the HDL particles [7]. Only after the esterification of FC and translocation of CE, preβ-HDL finally transforms into mature α-HDL. Defect in LCAT disrupts the esterification of FC, resulting in FC accumulation in the plasma and immaturation of HDLs [7]. Previous studies have demonstrated that mice with SR-BI depletion displayed significantly reduced LCAT activity, which resulted in the observed plasma FC accumulation [8,9]. Here we showed hypercholesteremia induced by LDL-R deficiency and HFD feeding significantly increased plasma FC, however, whether and how the protein expression and/or enzymatic activity of LCAT is modulated by hypercholesteremia is unknown.

Probucol is a lipid-lowering drug capable of reducing plasma TC. The mechanisms for its lipid-lowering effects include inhibition of cholesterol synthesis via competitive inhibition of 3-hydroxy-3-methyl glutaryl coenzyme A reductase (HMGCR) [10], increasing LDL degradation [11–13], inhibition of cellular cholesterol efflux via inhibition of both synthesis of apoAI [14] and cholesterol efflux mediated by ATP-binding cassette transporter A1 (ABCA1) [15], increasing the activity of cholesteryl ester transfer protein (CETP) and liver uptake of HDL cholesterol via up-regulation of SR-BI expression [16] et al. In addition to reducing plasma TC, probucol also inhibits FC accumulation in the HDLs, which has been currently exclusive in SR-BI deficient mice, whose liver selective uptake of HDL-CE and LCAT-mediated esterification of FC are largely disturbed [6]. In this study, we demonstrated that by inhibition of plasma FC accumulation, probucol almost normalized macrocytic anemia, splenomegaly, jaundice and renal tubular hemosiderin accumulation in SR-BI/LDL-R dKO mice on HFD diet as compared to LDL-R KO mice on the same diet, indicating that probucol prevented diet-aggravated hemolysis in SR-BI KO mice with LDL-R depletion. However, how probucol inhibits plasma FC accumulation is still unknown.

In conclusion, we demonstrated that in SR-BI KO mice, hypercholesteremia due to LDL-R deficiency significantly increased plasma FC and induced spontaneous hemolysis, which could be further exacerbated by HFD feeding. Probucol almost normalized

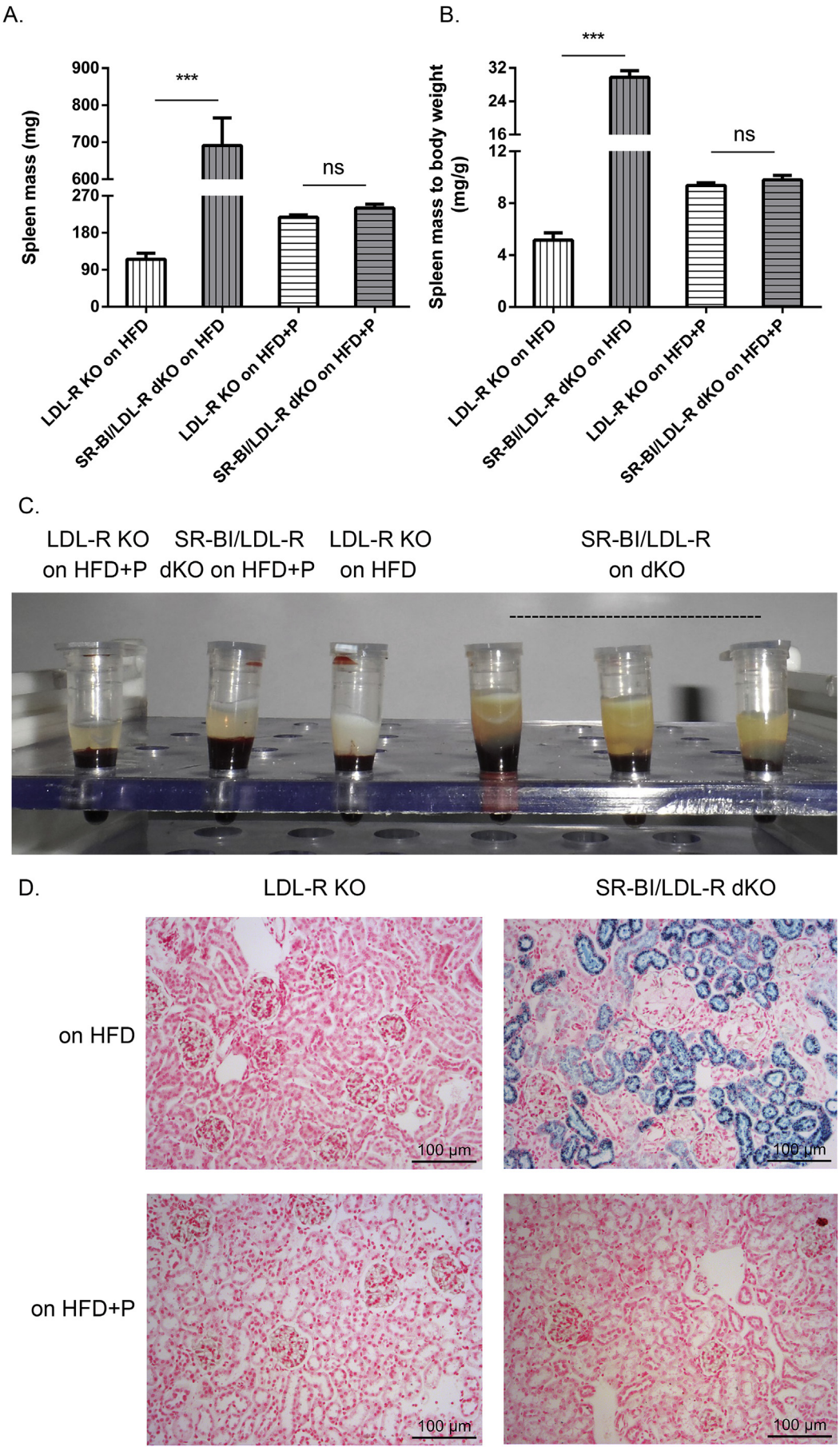

Fig. 2. Analysis of the spleen mass, appearance of the centrifuged plasma and renal Perl's staining in mice on HFD with or without probucol addition. A: Comparation of spleen mass; B: Comparation of the ratio of spleen mass to body weight; C: Appearance of the centrifuged plasma; D: Renal Perl's staining.

plasma FC and corrected diet-aggravated hemolysis in SR-BI KO mice with LDL-R deficiency.

Conflicts of interest

None declared.

Acknowledgments

This work is supported by a collaborative grant from Center for Molecular and Translational Medicine to George Liu (BMU20140475). We gratefully thanked Natural-Med Inc. for kindly provision of probucol.

Abbreviations

ABCA1	ATP-binding cassette transporter A1
apo	apolipoprotein
CE	cholesterol ester
CETP	cholesteryl ester transfer protein
dKO	double knockout
FC	free cholesterol
FC/TC	FC to TC ratio
HDL	high density lipoprotein
HFD	high fat diet
HFD + P	high fat diet with probucol
HGB	hemoglobin
HMGCR	3-hydroxy-3-methyl glutaryl coenzyme A reductase
KO	knockout
LCAT	lecithin: cholesterol acyltransferase
LDL	low density lipoprotein
MCV	mean corpuscular volume
MCHC	mean corpuscular hemoglobin concentration
NCD	normal chow diet
RBC	red blood cell
RCT	reverse cholesterol transport
SR-BI	scavenger receptor class B type I
TC	total cholesterol

References

[1] M. Hoekstra, T.J. Van Berkel, M. Van Eck, Scavenger receptor BI: a multi-purpose player in cholesterol and steroid metabolism, World J. Gastroenterol. 16 (2010) 5916–5924.
[2] W.J. Shen, J. Hu, Z. Hu, F.B. Kraemer, S. Azhar, Scavenger receptor class B type I (SR-BI): a versatile receptor with multiple functions and actions, Metabolism 63 (2014) 875–886.
[3] G. Valacchi, C. Sticozzi, Y. Lim, A. Pecorelli, Scavenger receptor class B type I: a multifunctional receptor, Ann. N. Y. Acad. Sci. 1229 (2011) E1–E7.
[4] H. Czarnecka, S. Yokoyama, Regulation of cellular cholesterol efflux by lecithin:cholesterol acyltransferase reaction through nonspecific lipid exchange, J. Biol. Chem. 271 (1996) 2023–2028.
[5] I. Meurs, M. Hoekstra, E.J. van Wanrooij, R.B. Hildebrand, J. Kuiper, F. Kuipers, M.R. Hardeman, T.J. Van Berkel, M. Van Eck, HDL cholesterol levels are an important factor for determining the lifespan of erythrocytes, Exp. Hematol. 33 (2005) 1309–1319.
[6] A.R. Tall, An overview of reverse cholesterol transport, Eur. Heart J. 19 (Suppl. A) (1998) A31–A35.
[7] R. Saeedi, M. Li, J. Frohlich, A review on lecithin:cholesterol acyltransferase deficiency, Clin. Biochem. 48 (2015) 472–475.
[8] K. Ma, T. Forte, J.D. Otvos, L. Chan, Differential additive effects of endothelial lipase and scavenger receptor-class B type I on high-density lipoprotein metabolism in knockout mouse models, Arterioscler. Thromb. Vasc. Biol. 25 (2005) 149–154.
[9] M. El Bouhassani, S. Gilibert, M. Moreau, F. Saint-Charles, M. Treguier, F. Poti, M.J. Chapman, W. Le Goff, P. Lesnik, T. Huby, Cholesteryl ester transfer protein expression partially attenuates the adverse effects of SR-BI receptor deficiency on cholesterol metabolism and atherosclerosis, J. Biol. Chem. 286 (2011) 17227–17238.
[10] J.L. Witztum, D. Simmons, D. Steinberg, W.F. Beltz, R. Weinreb, S.G. Young, P. Lester, N. Kelly, J. Juliano, Intensive combination drug therapy of familial hypercholesterolemia with lovastatin, probucol, and colestipol hydrochloride, Circulation 79 (1989) 16–28.
[11] Y.A. Kesaniemi, S.M. Grundy, Influence of probucol on cholesterol and lipoprotein metabolism in man, J. Lipid Res. 25 (1984) 780–790.
[12] M. Naruszewicz, T.E. Carew, R.C. Pittman, J.L. Witztum, D. Steinberg, A novel mechanism by which probucol lowers low density lipoprotein levels demonstrated in the LDL receptor-deficient rabbit, J. Lipid Res. 25 (1984) 1206–1213.
[13] P.J. Nestel, T. Billington, Effects of probucol on low density lipoprotein removal and high density lipoprotein synthesis, Atherosclerosis 38 (1981) 203–209.
[14] M. Tsujita, S. Yokoyama, Selective inhibition of free apolipoprotein-mediated cellular lipid efflux by probucol, Biochemistry 35 (1996) 13011–13020.
[15] E. Favari, I. Zanotti, F. Zimetti, N. Ronda, F. Bernini, G.H. Rothblat, Probucol inhibits ABCA1-mediated cellular lipid efflux, Arterioscler. Thromb. Vasc. Biol. 24 (2004) 2345–2350.
[16] G. Franceschini, M. Sirtori, V. Vaccarino, G. Gianfranceschi, L. Rezzonico, G. Chiesa, C.R. Sirtori, Mechanisms of HDL reduction after probucol. Changes in HDL subfractions and increased reverse cholesteryl ester transfer, Arteriosclerosis 9 (1989) 462–469.