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Berberine inhibits dyslipidemia in C57BL/6 mice with lipopolysaccharide induced inflammation

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Abstract:
Background: Inhibiting the action of proprotein convertase subtilisin/kexin type 9 (PCSK9) on the low-density lipoprotein receptor (LDLR) has emerged as a novel therapeutic target for hypercholesterolemia. Here we investigated the effect of berberine, natural plant extracts, on PCSK9-LDLR pathway in C57BL/6 mice with lipopolysaccharide (LPS) induced inflammation.
Methods: Forty female mice were divided into four groups (n =10): control, LPS (5 mg/kg), LPS + berberine 10 (5 mg/kg LPS plus 10 mg/kg berberine), and LPS + berberine 30 (5 mg/kg LPS plus 30 mg/kg berberine). Changes in the levels of blood lipids [total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C)]; pro-inflammatory cytokines [interferon-γ (IFNγ), tumor necrosis factor α (TNFα), and interleukin-1α (IL-1α)], 8-iso-prostane, hepatic expressions of PCSK9 and LDLR were determined.
Results: Berberine pretreatment reduced the expression of hepatic PCSK9, decreased the plasma TC, TG, LDL-C, IFNγ, TNFα, IL-1α, and 8-isoprostane concentrations; increased HDL-C level and LDLR expression in mice.
Conclusion: The present results suggest that berberine inhibits dyslipidemia in C57BL/6 mice with LPS induced inflammation through regulating PCSK9-LDLR pathway.

Key words:
berberine, proprotein convertase subtilisin/kexin type 9, low-density lipoprotein receptor

Abbreviations: HDL-C – high-density lipoprotein cholesterol, IFNγ – interferon-γ, IL-1α – interleukin-1α, LDL-C – low-density lipoprotein cholesterol, LDLR – low-density lipoprotein receptor, LPS – lipopolysaccharide, PCSK9 – proprotein convertase subtilisin/kexin type 9, TC – total cholesterol, TG – triglyceride, TNFα – tumor necrosis factor α

Introduction

Elevated plasma low-density lipoprotein cholesterol (LDL-C) is an important risk factor for cardiovascular disease [16]. Plasma LDL-C is controlled through its

uptake into cells upon binding the LDL receptor (LDLR). Proprotein convertase subtilisin kexin 9 (PCSK9), also called neural apoptosis-regulated convertase 1 (NARC-1) or proprotein convertase 9, is a member of the proteinase K subfamily of subtilisin-related serine endoproteases. PCSK9 binds to the LDLR in the liver and accelerates its degradation, leading to elevated plasma LDL-C [10]. Studies have reported that inactivation of PCSK9 in mice reduces plasma cholesterol levels primarily by increasing hepatic expression of LDLR protein and thereby promoting clearance of circulating LDL-C [10, 24]. Moreover, the loss of a functional PCSK9 in human is not associated with clear deleterious effect; this protease has emerged as an attractive target for lowering plasma LDL-C levels either alone or in combination with statins [20]. Thus, regulation of PCSK9-LDLR expression is becoming a novel therapeutic target for dyslipidemia.

During the past few years, it has become clear that the major classes of usually prescribed lipid-lowering drugs elevate serum PCSK9 levels. These studies most likely illuminate why these medications are not more effective in reducing LDL-C and imply that efforts should be made toward the development of new LDL-C lowering agents that either do not elevate circulating PCSK9 levels or reduce PCSK9 expression or suppress PCSK9 expression [7, 8, 15]. In 2008, Cameron et al. reported that berberine decreased PCSK9 mRNA and protein expressions in a time- and dose-dependent manner, probably because of a decreased transcription of the PCSK9 gene in *in vitro* study on HepG2 cells [6]; however, the regulatory effect of berberine on PCSK9 *in vivo* has not yet been defined. Berberine, a quaternary ammonium salt, is present in the roots, rhizomes, stems, and bark of such plants as *Coptis chinensis*, Chinese Goldthread, Huang-Lian, Huang-Lien, Oregon grape, Barberry, Tree Turmeric, Goldenseal, Amur Cork Tree, Huang Bai, Huang Po, Po Mu and *Tinospora cordifolia*. Some experiments have shown that berberine shows antioxidant effects [25, 31]. In 2009, oxidant stress was reported to stimulate the expression of LDLR [23]. Based on antioxidant property of berberine, we postulated that berberine could regulate PCSK9-LDLR pathway to improve dyslipidemia.

Inflammation induces remarkable changes in lipid and lipoprotein metabolism [14, 22]. Recent studies have shown that inflammation induced by lipopolysaccharide (LPS) stimulates the expression of PCSK9 [11]. The objective of this study was conducted to examine how berberine affects dyslipidemia. In the present study, therefore, we tested whether berberine could regulate the PCSK9–LDLR pathway to improve the dyslipidemia caused by LPS-induced inflammation in C57BL/6 mice.

Materials and Methods

Reagents

Berberine (purity: 98.0%) (Fig. 1) was obtained from Yong Jia Biotechnology Co., Ltd. (Shanghai, China). Antibody was purchased from Santa Cruz Biotechnology (USA). Other reagents were bought from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China).

Experimental animals

Female C57BL/6J mice were purchased from Laboratory Animal Center, College of Veterinary Medicine, Hunan Agricultural University (Changsha, China). Mice were housed 10 per cage in a controlled temperature animal facility with a 12 h light : 12 h dark cycle and free access to water and chow at the Animal Center of College of Veterinary Medicine. All mice received human care in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. All experiments were performed according to protocols approved by the Animal Studies Subcommittee of the Hunan Agricultural University.

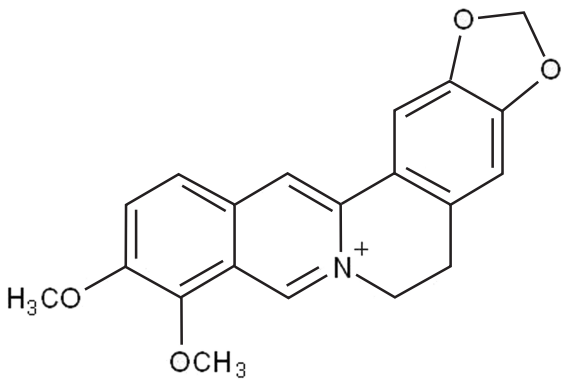

Fig. 1. Chemical structure of berberine

Experimental protocols

After acclimatization for one week, forty mice, weighed 20–22 g, were randomly divided into four groups (n = 10): control, LPS (5 mg/kg), LPS + berberine 10 (5 mg/kg LPS plus 10 mg/kg berberine), and LPS + berberine 30 (5 mg/kg LPS plus 30 mg/kg berberine). In two groups of berberine-pretreated mice, berberine (10 or 30 mg/kg per day) was given intragastrically. The pretreatment lasted four weeks. Then, animals were fed with the high-cholesterol diet for seven days. LPS-administrated mice were injected by LPS (5 mg/kg body weight, intraperitoneally) and high-cholesterol food was removed after injection. The dose of LPS was previously shown to induce the acute phase response in mice, but was far below the lethal dose [11]. Animals were supplied with normal mouse chow in the control group. At the indicated time after treatment, blood was collected from the carotid artery of mice under anesthesia (xylazine: 100 mg/kg b.w.; ketamine: 23 mg/kg b.w.; and acepromazine: 3.0 mg/kg b.w., *ip*). Liver was snap-frozen in liquid nitrogen, placed in storage tubes in a dry ice bath at the end of experiment, and then stored at –70°C until RNA extraction.

Measurement of plasma metabolic parameters

Fasting blood samples collected from the artery were added into precooled tubes containing EDTA (final concentration 4 mmol/l) and centrifuged at 2,500 × g for 20 min at 4°C. Plasma 8-isoprostane level was measured in mice using an 8-isoprostane competitive enzyme immunoassay (EIA; Caymen Chemicals, Ann Arbor, MI, USA), following the protocol provided for determination of total (free and esterified) 8-isoprostane using a previously described method [27]. Plasma interferon-γ (IFNγ), tumor necrosis factor α (TNFα) and interleukin-1α (IL-1α) levels were determined by enzyme-linked immunosorbent assay according to the manufacturers' instructions as previously reported [4, 26]. Plasma total cholesterol (TC), triglyceride (TG), LDL-C, and high-density lipoprotein cholesterol (HDL-C) levels were measured by the enzymatic method (bioMerieux, Lyon, France) using an automated analyzer (Type 7170A, Hitachi) according to the manufacturer's recommendations.

Analysis of mRNA expressions of PCSK9 and LDLR

Real-time PCR was performed to quantify PCSK9 and LDLR mRNA. Briefly, total RNA was isolated from liver by using the Tripure™ isolation reagent (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer. 36B4 mRNA was used as the invariant control for all experiments. The sequences of primers were as follows: LDLR: forward, 5'-AGGCTGTGGGCTCCATAGG-3', reverse, 5'-TGCGGTCCAGGGTCATCT-3'; PCSK9: forward, 5'-TTGCAGCAGCTGGGAACTT-3', reverse, 5'-CCGACTGTGATGACCTCTGGA-3'; 36B4: forward, 5'-GCGACCTGGAAGTCCAACTAC-3', reverse, 5'- ATCTGCTGCATCTGCTTGG-3' [11, 17].

Analysis of hepatic protein expressions of LDLR

The isolated liver was snap-frozen in liquid nitrogen, and then homogenized in ice-cold RIPA buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 15.5 mM Triton X-100, 12.1 mM sodium deoxycholate, 3.5 mM SDS, pH 7.4). The homogenate was centrifuged at 6000 × g for 15 min, and the supernatant was retained. The supernatant was stored at –70°C until use. Equal concentrations of protein were separated on a 12% SDS-PAGE and the separated proteins electrophoretically transferred to PVDF membranes. The western blot analyses of LDLR protein expressions were performed as described previously [11, 13].

Statistical analyses

Results are expressed as the means ± SD. The significance of differences was evaluated by ANOVA and Student's *t*-test for unpaired data. The significance level was chosen as $p < 0.05$.

Results

Plasma 8-isoprostane production

Plasma 8-isoprostane generation was higher in the LPS-injected mice than in control mice. Berberine (10 or 30 mg/kg) pretreatment prevented the elevation of 8-isoprostane generation in mice ($p < 0.05$ or $p < 0.01$; Fig. 2).

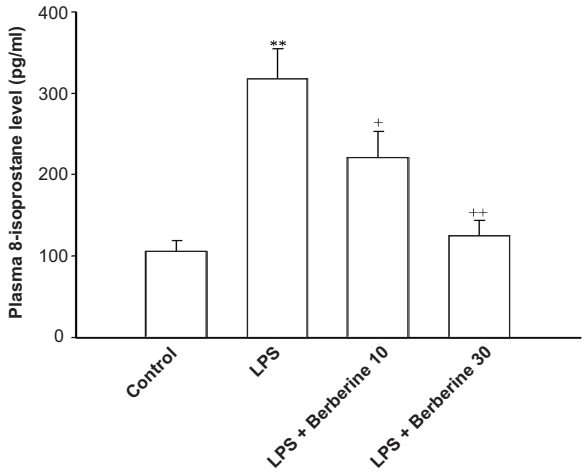

Fig. 2. Plasma 8-isoprostane level. Berberine 10: 10 mg/kg of berberine; Berberine 30: 30 mg/kg of berberine. LPS – lipopolysaccharide. Values are the mean ± SD, n = 10. Compared with control, ** $p < 0.01$; compared with LPS group, $^{+}$ $p < 0.05$ or $^{++}$ $p < 0.01$

Hepatic LDLR expression

In the liver, LPS-injected mice showed a decrease in the mRNA and protein expressions of LDLR compared with that of control group ($p < 0.01$). Pretreatment with berberine (10 or 30 mg/kg) increased hepatic mRNA and protein expressions of LDLR in mice ($p < 0.05$ or $p < 0.01$) (Fig. 3).

Hepatic PCSK9 expression

As evident in Figure 4, hepatic PCSK9 expression was clearly augmented in LPS-injected mice ($p < 0.01$), but berberine (10 or 30 mg/kg) pretreatment prevented a clear increase in PCSK9 expression ($p < 0.05$ or $p < 0.01$).

Plasma pro-inflammatory cytokines concentrations

In LPS-injected mice, plasma IFNγ, IL-1α, and TNFα levels were significantly increased. In berberine (10 or 30 mg/kg)-pretreated mice, plasma IFNγ, IL-1α, and TNFα levels were markedly decreased compared with those in the LPS group ($p < 0.05$ or $p < 0.01$) (Tab. 1).

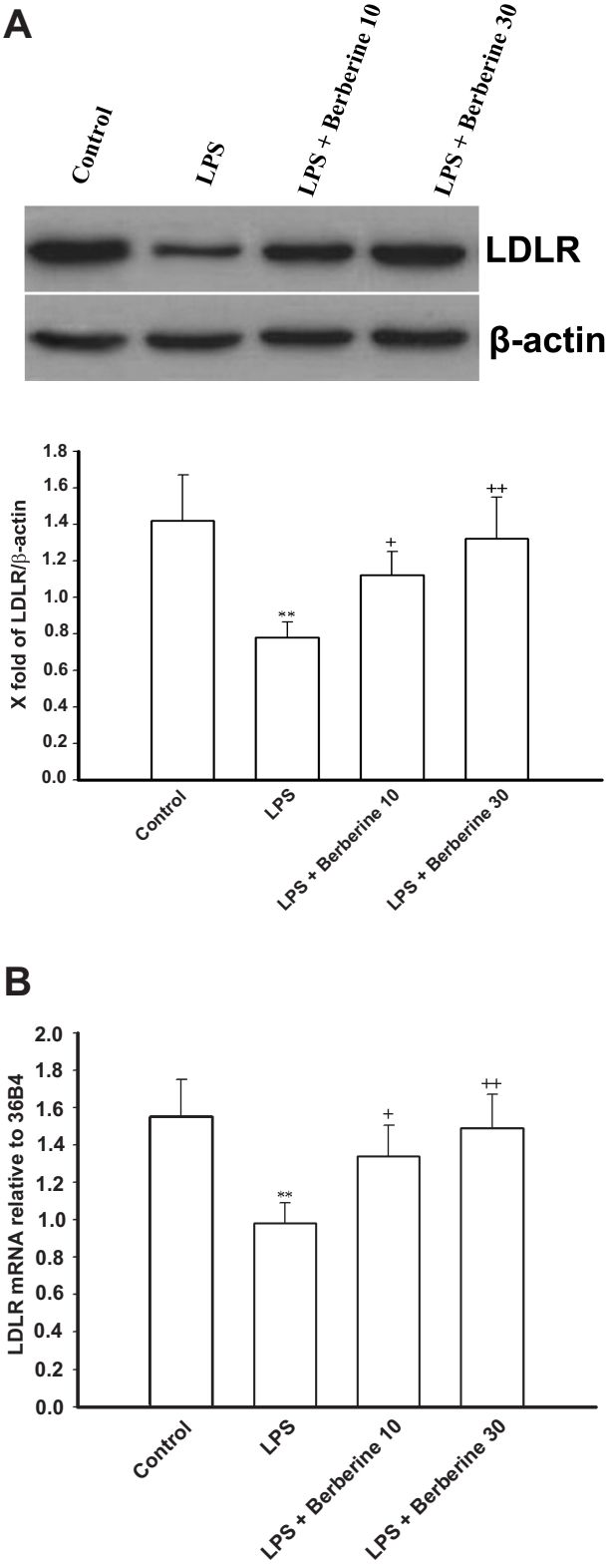

Fig. 3. LDLR expression. (**A**) LDLR protein expression. (**B**) LDLR mRNA expression. Berberine 10: 10 mg/kg of berberine; Berberine 30: 30 mg/kg of berberine. LPS – lipopolysaccharide, LDLR – low-density lipoprotein receptor. Values are the mean ± SD, n = 10. Compared with control, ** $p < 0.01$; compared with LPS group, $^{+}$ $p < 0.05$ or $^{++}$ $p < 0.01$

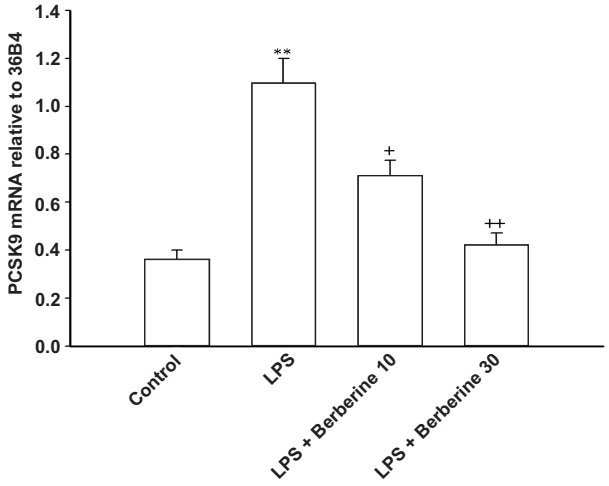

Fig. 4. PCSK9 mRNA expression. Berberine 10: 10 mg/kg of berberine; Berberine 30: 30 mg/kg of berberine. LPS – lipopolysaccharide, PCSK9 – proprotein convertase subtilisin/kexin type 9. Values are the mean ± SD, n = 10. Compared with control, ** $p < 0.01$; compared with LPS group, $^{+}$ $p < 0.05$ or $^{++}$ $p < 0.01$

Plasma lipid levels

LPS-injected mice had a reduced concentration of plasma HDL-C, an elevated level of plasma LDL-C, TG and TC ($p < 0.01$). However, pretreatment with berberine (10 or 30 mg/kg) increased plasma HDL-C level, decreased plasma LDL-C, TG and TC concentrations ($p < 0.01$) (Tab. 2).

Discussion

PCSK9 is an enzyme which, in humans, is encoded by the PCSK9 gene with orthologs found across many species [27]. The major physiologic function of PCSK9 is to mediate the degradation of LDLR. PCSK9 also plays a pivotal role in controlling the levels of LDL particles

Tab. 1. Pro-inflammatory cytokines concentrations

	Control	LPS	LPS + Berberine 10	LPS + Berberine 30
Plasma IFNγ (ng/ml)	11.07 ± 1.62	24.22 ± 3.08**	14.52 ± 2.11^{+}	12.62 ± 1.76^{++}
Plasma TNFα (ng/ml)	0.86 ± 0.18	1.59 ± 0.23**	1.20 ± 0.19^{+}	0.96 ± 0.11^{++}
Plasma IL-1α (ng/ml)	32.93 ± 4.55	86.63 ± 7.26**	49.98 ± 5.93^{+}	36.72 ± 3.68^{++}

Berberine 10: 10 mg/kg of berberine; Berberine 30: 30 mg/kg of berberine. LPS – lipopolysaccharide, IFNγ – interferon-γ, TG – triglycerides, TNFα – tumor necrosis factor α, IL-1α – interleukin-1α. Values are the mean ± SD, n = 10. Compared with control, ** $p < 0.01$; compared with LPS group, $^{+}$ $p < 0.05$ or $^{++}$ $p < 0.01$

Tab. 2. Lipid levels

	Control	LPS	LPS + Berberine 10	LPS + Berberine 30
Plasma TG (mmol/l)	1.81 ± 0.26	3.62 ± 0.33**	2.28 ± 0.32^{+}	1.90 ± 0.21^{++}
Plasma TC (mmol/l)	2.52 ± 0.55	6.66 ± 0.69**	3.82 ± 0.39^{+}	2.89 ± 0.35^{++}
Plasma LDL-C (mmol/l)	1.25 ± 0.18	3.42 ± 0.37**	1.79 ± 0.29^{+}	1.32 ± 0.16^{+}
Plasma HDL-C (mmol/l)	1.75 ± 0.22	0.86 ± 0.09**	1.19 ± 0.02^{+}	1.46 ± 0.02^{++}

Berberine 10: 10 mg/kg of berberine; Berberine 30: 30 mg/kg of berberine. LPS – lipopolysaccharide, TC – total cholesterol, TG – triglycerides, HDL-C – high-density lipoprotein cholesterol, LDL-C – low-density lipoprotein cholesterol. Values are the mean ± SD, n = 10. Compared with control, ** $p < 0.01$; compared with LPS group, $^{+}$ $p < 0.05$ or $^{++}$ $p < 0.01$

that circulate in the blood stream. PCSK9 stimulates LDLR degradation by joining to the epidermal growth factor-like repeat domains of the LDLR. Reduced LDLR levels lead to reduced metabolism of LDL, which could result in hypercholesterolemia [27]. Mutations in PCSK9 gene have been linked to an uncommon form of autosomal dominant familial hypercholesterolemia and hypocholesterolemia. Various gain-of-function and loss-of-function mutations in the PCSK9 gene, which take place naturally, have been identified and associated with hypercholesterolemia and hypocholesterolemia, respectively [5, 9, 20]. The mutations appear to give rise to the disease by elevating the protease activity of PCSK9, decreasing LDLR levels and thereby hindering the uptake of cholesterol into the cells [9]. Several variants of PCSK9 have also been found to markedly decrease circulating cholesterol. Some variants, which only lower cholesterol by 15% in whites, have been shown to cause a concurrent decrease coronary heart disease in 50% of cases, having implications for public health [20, 21, 28]. Furthermore, blocking of the PCSK-binding to the LDLR can significantly reduce plasma LDL levels [28]. Therefore, inhibition of PCSK9 function may be a means of lowering cholesterol levels.

Previous studies showed that inflammation stimulates noticeable changes in lipid and lipoprotein metabolism [14]. LPS administration decreases HDL levels while increasing VLDL and LDL levels in rodents [12]. Studies have suggested that LPS administration stimulate the expression of PCSK9 and reduce LDLR protein levels in the liver, which could result in the reduced clearance of circulating LDL and account for the increase in serum LDL levels [11, 18, 19]. Our results showed that LPS treatment increased the expression of hepatic PCSK9, the plasma concentrations of TC, TG, LDL-C, IFNγ, TNFα, and IL-1α; decreased HDL-C level and LDLR expression in mice, and the effect of LPS was prevented by berberine.

Many studies have reported the *Coptis chinensis* can regulate carbohydrate and lipid metabolism, endothelial function and the cardiovascular system [1, 25, 29, 30]. As a natural alkaloid, berberine can be isolated from the roots, bark, stems, and rhizomes of *Coptis chinensis* [3]. Berberine was shown to lower elevated plasma TC, TG LDL-C, and aterogenic apolipoproteins B [27], but the mechanism of action is not related to statins [31]. Berberine reduces LDL-C by up regulating LDLR mRNA expression posttranscriptionally while down regulating the transcription of PCSK9, a natural inhibitor of LDLR *in vitro* [6], and increasing the expression of LDLR in the liver through the extracellular signal-regulated kinase signaling pathway [5]. In contrast, statins prevent cholesterol synthesis in the liver by inhibiting 3-hydroxy-3-methylglutaryl-coenzyme A-reductase. This explains why berberine does not cause side effects typical for statins [24]. In other studies, berberine and other stanols synergistically inhibited cholesterol absorption in hamsters [29]. In the present study, berberine pretreatment inhibited the expression of hepatic PCSK9 and increased LDLR expression concomitantly with the decreased plasma concentrations of LDL-C, TG and TC and the increased HDL-C plasma level in mice with LPS induced inflammation.

It is notable that berberine pretreatment inhibited the expression of hepatic PCSK9, increased the expression of LDLR, and decreased the plasma 8-isoprostane concentration. Paim et al. previously suggested that hypercholesterolemic LDLR knockout (K/O) mice mitochondria had a lower antioxidant capacity [23]. Furthermore, well-known antioxidants – salicylates have the ability to suppress lectin-like oxidized LDLR-1 [2]. Based on its antioxidant properties, it is probable that berberine regulates PCSK9 and LDLR gene expresssions by inhibiting lipid peroxidation, which in turn lead to inhibition of dyslipidemia of mice with LPS induced inflammation.

Conclusion

Our study shows that berberine inhibits dyslipidemia in C57BL/6 mice with LPS induced inflammation through regulating PCSK9-LDLR pathway.

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