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Comparative effect of berberine and its derivative 8-cetylberberine on attenuating atherosclerosis in ApoE$^{-/-}$ mice

Min Feng [a,b], Zongyao Zou [a,c], Xia Zhou [a,c], Yinran Hu [a], Hang Ma [a], Yubo Xiao [a], Xuegang Li [a,c,*], Xiaoli Ye [c,**]

[a] School of Pharmaceutical Sciences, Southwest University, Chongqing, 400715, PR China
[b] Chongqing Key Lab of Catalysis & Functional Organic Molecules, College of Environment and Resource, Chongqing Technology and Business University, Chongqing 400067, PR China
[c] Engineer Research Center of Chongqing Pharmaceutical Process and Quality Control, Chongqing, 401122, PR China

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ABSTRACT

Berberine (BBR), one of the main bioactive compounds in *Rhizoma coptidis*, has multiple pharmacological activities. It has been reported that 8-cetylberberine (8-BBR-C16) has increased anti-microbial property in vivo and a higher bioavailability in hamsters. Therefore, in the present study, we used apolipoprotein E-deficient mice (ApoE$^{-/-}$) as an atherosclerosis model to investigate the anti-atherosclerosis effects of 8-BBR-C16. After 12 weeks of treatment, the atherosclerotic plaque area of the aorta, serum lipid profile, the plasma redox state and the expression of inflammatory cytokines in ApoE$^{-/-}$ mice were determined. Both BBR and 8-BBR-C16 significantly decreased the atherosclerotic plaque area by suppressing inflammatory and oxidative markers in ApoE$^{-/-}$ mice. Treatment with BBR or 8-BBR-C16, decreased serum levels of IL-1β and TNF-α as well as mRNA levels of NF-κBp65, i-NOS, ICAM-1, IL-6 in the aorta. In addition, the expression of NF-κB p65 protein decreased in the nucleus, whereas IκBα levels increased in the cytosol. The anti-inflammatory and anti-oxidative effect of BBR and 8-BBR-C16 attributed to inhibition of the translocation of NF-κB to the nucleus. Since the dosage of BBR used was 10 fold higher than that of 8-cetylberberine, we conclude that 8-BBR-C16 is more efficient in treating atherosclerosis in ApoE$^{-/-}$ mice.

1. Introduction

Atherosclerosis (AS) is a condition affecting arterial blood vessels due to a chronic inflammatory response in the artery wall, which results in artery wall thickening [1,2]. Both oxidative stress and lipid deposition play key roles in the formation of atherosclerotic lesions. Oxidized low density lipoprotein (ox-LDL) stimulates endothelial cell injury, monocyte adhesion, and platelet aggregation and inhibits apoptosis and endothelial nitric oxide synthase (eNOS) expression/activity, all of which promote the process of atherosclerosis [3]. Experimental, clinical and population studies demonstrated active roles of oxidative stress and inflammation in the development of cardiovascular diseases. Hulsmans et al. have presented evidence of a vicious circle between inflammation and oxidative stress in AS [4]. Many studies have demonstrated that an association exists between circulating pro-inflammatory molecules such as interleukin-6 (IL-6) [5], Interleukin-1β (IL-1β) [6], tumor necrosis factor-alpha (TNF-α) [7], intracellular adhesion molecule-1(ICAM-1) [8]and atherosclerosis. Therefore, normalizing the levels of these inflammatory markers in patients may play a vital role in the prevention and/or treatment of AS.

Berberine (BBR) (Fig. 1A) is an isoquinoline alkaloid isolated from the herb *Rhizoma coptidis* (HuangLian). Recent studies have demonstrated that BBR has multiple pharmacological activities. BBR was capable of suppressing inflammatory agents-induced cytokine production in human lung cells by inhibiting phosphorylation and subsequent degradation of the inhibitory I-κB [9]. Reports have shown that BBR can attenuate lipopolysaccharide-induced IL-1β expression and prevent cartilage degradation in a rat model of osteoarthritis by acting on the NF-κB pathway [10]. BBR also prevents hyperglycemia-induced endothelial injury and dysfunction [11]. By up-regulation of autophagy via the AMPK/mTOR signaling pathway, BBR can also alleviate ox-LDL-induced inflammatory factors [12]. The above studies suggest that BBR may have potential clinical benefits and application prospects for the treatment of cardiovascular diseases such as AS.

Because the bioavailability of BBR is low, we synthesized a variety of BBR analogs to increase in vivo efficacy [13]. Among them, we found that the 8-cetylberberine(8-BBR-C16, Fig. 1B) exhibits increased antibacterial activities as compared to BBR, especially against Gram-positive bacteria [14] and the antimicrobial activity of BBR derivatives increased as the length of aliphatic chain was elongated [15]. The bioavailability of 8-BBR-C16 is higher than that of BBR, as demonstrated

* Correspondence to: X. Li, School of Pharmaceutical Sciences, Southwest University, Chongqing 400715, PR China.
** Corresponding author.
E-mail addresses: Xuegangli@swu.edu.cn (X. Li), yexiaoli@swu.edu.cn (X. Ye).

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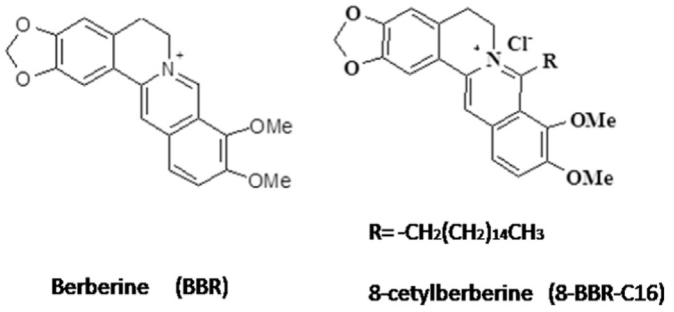

Fig. 1. Structure of berberine (BBR) (A) and its derivative 8-cetylberberine (8-BBR-C16) (B).

by a higher concentration in various organs and the extended retention time [16]. Pharmacological characterization of 8-BBR-C16 has not been extensively performed in our prior studies. Considering the prominent role of inflammation and oxidative stress in the development of AS, one can hypothesize that BBR and 8-BBR-C16 may have beneficial effects for the treatment of AS. In this study, a well-defined classical atherosclerotic model, ApoE$^{-/-}$ mice were used to determine the effects of BBR and 8-BBR-C16 on cardiovascular diseases and in particular AS. Our results indicate that BBR and 8-BBR-C16 significantly decreased the atherosclerotic plaque area by suppressing inflammatory and oxidative markers in ApoE$^{-/-}$ mice.

2. Materials and methods

2.1. Materials and reagents

BBR was extracted from *Rhizoma coptidis* according to previous methods, and its structure was confirmed by using its physical-chemical and spectral properties [17]. 8-BBR-C16 was synthesized, purified (>98%) and analyzed by HPLC [15]. All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified. ELISA kits to determine mouse TNF-α and IL-1β were purchased from Abcam (Cambridge, UK). Cytosolic and nucleic protein kits and RIPA lysis buffer were from Dingguo Changsheng biotechnology Co. Ltd. (Beijing, PR China).

2.2. Animal treatment

All animal experiments were performed in accordance with the National Research Council's guidelines. This study was conducted in conformity with the policies and procedures of the Institutional Animal Care and Use Committee of Southwest University (Permit Number: 12-1036). Six week old ApoE$^{-/-}$ mice on a C57BL/6J background and C57BL/6J wild type mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (SCXK 2013-0008). All animals were maintained in an air-conditioned environment with a controlled temperature at 22 ± 2 °C and 50–60% relative humidity under a 12:12 h light/dark cycle. After an adaptation period of a week, 36 ApoE$^{-/-}$ mice were randomly divided into 3 groups: (1) AS model group: Western-type (WT) diet (1.25% cholesterol, 21% fat) + saline, (2) BBR group: WT diet + BBR (150 mg/kg/d), (3) 8-BBR-C16 group: WT diet + 8-cetylberberine (15 mg/kg/d). As a baseline control group, 12 C57BL/6J wild type mice received 12 weeks of normal chow containing a similar volume of saline as used in the normal control (NC) group. Every morning at 10:00, each group was administered 0.4 mL corresponding drugs or saline by gavage. According to our previous study, BBR at a dosage of 150 mg/kg/day is the optimal concentration to exert its antihyperlipidemic activity in mice [14,16], and considering the severe hyperlipidemia that is found in ApoE$^{-/-}$ mice as well as the significantly better bioavailability of 8-BBR-C16 [18], we decided to use 150 mg/kg/d of BBR and 15 mg/kg/d of 8-BBR-C16 as dosage for our studies.

After 12 weeks of treatment, mice were sacrificed using 0.3% sodium pentobarbital (60 mg/kg, ip). Mice were perfused with phosphate buffered saline (PBS) after blood samples were collected from the heart. Aortas were rapidly dissected from the aortic root to the iliac bifurcation and adventitial fat and connective tissue were removed as much as possible. Aorta's were put in a tissue-freezing medium, snap-frozen in liquid nitrogen and stored at −80 °C for further analysis.

2.3. Serum lipids analysis

Serum was prepared from each blood sample by centrifugation at 3000 rpm for 15 min. Serum levels of total cholesterol (TC), triglycerides (TG), low-density lipoprotein cholesterol (LDL-C) were determined using an auto-analyzer (Hitachi 917,Tokyo, Japan)and according to the manufacturer's instructions.

2.4. Plasma anti-oxidation

Plasma levels of total antioxidant capability (T-AOC), catalase (CAT), superoxide dismutase (SOD) and glutathione (GSH) were determined by commercially available ELISA kits according to the manufacturer's instructions.

2.5. Lipid staining by oil red O

Oil red O staining was performed to evaluate lipid and plaque accumulation in the aorta. Atherosclerotic lesions were quantified on cross-sections of the aorta beginning at the level of the aortic sinus and as described previously [19,20]. Bright field images were digitally acquired using a Nikon 80i microscope with NISelements3.2 software (Nikon, Tokyo, Japan). Quantitative analysis of positive staining areas was done using Image-Pro Plus 6.0 software (NIH Image, USA) according to a modified method described by Stevens et al. [21].

2.6. Hematoxylin–eosin staining

For histological analysis, six hearts from each group were removed, fixed overnight in 10% neutral buffered formalin at 4 °C, embedded in paraffin and sectioned at 6-μm thickness using a Leica RM2265microtome (Germany). Sections were stained with hematoxylin- eosin (H&E). Bright field images for H&E staining as well as for measurement of the average thickness of the vessel wall were digitally acquired using a Nikon 80i microscope with NISelements3.2 software [22].

2.7. Serum inflammatory cytokines

ELISA kits were used to determine serum levels of TNF-α and IL-1β following the manufacturer's instructions. Optical density (OD) values were measured at 450 nm using an ELISA auto-analyzer and concentrations of TNF-α and IL-1β were calculated based on the standard curves.

2.8. RNA extract, cDNA synthesis and qRT-PCR

SYBR green quantitative real-time polymerase chain reaction (qRT-PCR) was used to detect mRNA levels of NF-κBp65, i-NOS, ICAM-1 and IL-6. The TRIzol method was used to extract total RNA from mouse aortic tissue. RNA yield and purity was confirmed by measuring the ratio of the absorbance at 260 nm and 280 nm. cDNA was synthesized using the SuperScript III First-Strand Synthesis System. The qRT-PCR reaction, containing target genes and SYBR Green PCR master mix, was performed on a Bio-Rad CFX connect real-time system (Bio-Rad, USA). Primer sequences are shown in Table 1.