

Berberine is a potent agonist of peroxisome proliferator activated receptor alpha

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1. ABSTRACT

Although berberine has hypolipidemic effects with a high affinity to nuclear proteins, the underlying molecular mechanism for this effect remains unclear. Here, we determine whether berberine is an agonist of peroxisome proliferator-activated receptor alpha (PPAR α), with a lipid-lowering effect. The cell-based reporter gene analysis showed that berberine selectively activates PPAR α (EC₅₀ =0.58 mM, E_{max} =102.4). The radioligand binding assay shows that berberine binds directly to the ligand-binding domain of PPAR α (K_i=0.73 mM) with similar affinity to fenofibrate. The mRNA and protein levels of CPT- α gene from HepG2 cells and hyperlipidemic rat liver are remarkably up-regulated by berberine, and this effect can be blocked by MK886, a non-competitive antagonist of PPAR α . A comparison assay in which berberine and fenofibrate were used to treat hyperlipidaemic rats for three months shows that these drugs produce similar lipid-lowering effects, except that berberine increases high-density lipoprotein cholesterol more effectively than fenofibrate. These findings provide the first evidence that berberine is a potent agonist of

PPAR α and seems to be superior to fenofibrate for treating hyperlipidemia.

2. INTRODUCTION

Berberine, an alkaloid isolated from the Chinese herb *Coptis chinensis*, has been widely used as a drug to treat gastrointestinal infections in China (1). Recently, berberine has been described as a new hypolipidemic drug (2). Several animal experiments and clinical trials showed that berberine considerably reduced serum triglyceride (TG), total cholesterol (TC), free fatty acid (FFA), very low density lipoprotein cholesterol (VLDL-C), and low density lipoprotein cholesterol (LDL-C), but increased high-density lipoprotein cholesterol (HDL-C), blunted apolipoprotein B (ApoB) transcription, and enhanced ApoA1 expression (3-5). However, the underlying mechanism is unclear.

It was reported that berberine might play a role in regulating lipid metabolism through a mechanism that was different from that of statins (2). It was also

reported that berberine could suppress adipocyte differentiation via decreasing CAMP-response element-binding protein (CREB) phosphorylation (6), inhibit lipid biosynthesis (7), inhibit mitochondrial respiratory chain complex I and stimulate glycolysis (8), increase LDL receptor (LDLR) expression in HepG2 cells, and promote lipid and lipoprotein metabolism (9,10). However, these reports failed to satisfactorily explain the comprehensive effects of berberine, including hypolipidemic effect (2), hypoglycemic effect (11), antioxidative and antiinflammatory effects (12), antiangiogenic and antitumor effects (13), and so on. Peroxisome proliferator-activated receptors (PPARs) are the major transcriptional regulators involving lipid metabolism-related genes (14, 15). Upon being bound by their specific agonists, PPARs activate the transcription of a series of target genes, such as carnitine palmitoyl transferase I (CPT-I) (16), acyl CoA synthetase (17) adipocyte differentiation-related protein (18), and β -ketoacyl-CoA thiolase (19), which in turn regulates lipid metabolism, including the uptake, synthesis, and oxidation of fatty acids, lipoprotein assembly, as well as lipid transport in order to maintain the balance of lipids and energy metabolism. It has been confirmed that the PPAR α agonists, such as derivatives of phenoxyacetic acids, can decrease serum TG, TC, VLDL-C, and LDL-C, increase HDL-C (20), suppress ApoC III transcription, and enhance lipoprotein lipase (LPL) and apoA1 expression (21-23).

Owing to the fact that berberine has lipid-lowering effects with high affinity to nuclear proteins, it is conceivable that berberine may function as an agonist of PPAR α . In this study, we investigated the binding and activating potency of berberine to PPAR α and analyzed the effect of berberine on the expression of CPT-I α . We also assessed the lipid-lowering effect of berberine in the hyperlipidemic rats.

3. MATERIALS AND METHODS

3.1. Materials

DEAE-52 anion exchange chromatography column was purchased from Amersham-Pharmacia. CPT-Ia antibody was obtained from Santa Cruz Company. Goat anti-rabbit IgG conjugated with HRP and other reagents generally used were provided by Beyotime Institute of Biotechnology (People's Republic of China). Rosiglitazone, arachidonic acid, and fenofibrate were obtained from Cayman (USA). (^3H)AA (250 μCi , 230 Ci/mmol) and (^3H) ROS (250 μCi , 48 Ci/mmol) were purchased from American Radiolabeled Chemicals (ARC, USA). Scintillation solution was purchased from Perkin Elmer life and analytical sciences (USA). Quenching standard source and Liquid scintillation counter (LS1801) were obtained from Beckman instruments INC.

3.2. Construction of PPAR expression vectors

Total RNA was isolated from HepG2 cells using Trizol reagent (Invitrogen,

USA). PPARs cDNAs were cloned by a RT-PCR method with the following primers: PPAR α , 5'-GCGTCGACGGCACAACCAGCACCAT-3' (forward) and 5'-CGGGAT CCTGCTCCCCCGTCTCCTTTG-3' (reverse); PPAR δ , 5'-GCGTCGACGAGGGGA GATCAGCCATG-3' (forward) and 5'-CGGGATCCTGGGTGCCGCCGTTAGTAC-3' (reverse); PPAR γ 1, 5'-CCGGTCGACTGACCA TGGTTGACACAGAGA-3' (forward) and 5'-TCCGG ATCCAAATGTTGGCAGTGG CT-3' (reverse). These cDNA and PIREs₂-EGFP plasmid were doubly excised by the Sal I and BamH I endonucleases and ligated with each other. Primer synthesis and DNA sequencing were performed by Shanghai Shenergy Biocolor Bioscience & Technology Company (People's Republic of China).

3.3. Transactivation/reporter assays

The activity of agonist for PPAR subtypes was determined by transactivation activity based on cell reporter-gene assay as previously described (24) with minor modification. Briefly, three types of expression vectors were co-transfected into CV-1 cells with LIPOFECTAMINE 2000 (Promega) according to the manufacturer's instruction: 1) pPPARs-IRES2-EGFP; 2) pTK-PPRE \times 3-Luc (kindly provided by professor T.C.He from Medical Center, University of Chicago); and 3) pRL-TK (promega, used as an internal control reporter vector). The transfected cells were cultured with or without drugs for 48 h and then the luciferase activity of the cell lysate was measured with a Gloma 20/20 Luminometer (Promega, 2030-101) using the dual luciferase assay system (Promega). Drugs were dissolved in dimethyl sulfoxide (DMSO), and the final concentration of DMSO was less than 0.1% (v/v). All points were performed in triplicate and varied in less than 10% range. The normalized luciferase activity was determined and plotted as fold-activation based on that of untreated cells.

3.4. Expression and purification of the ligand binding domain (LBD) PPAR α in *E. coli*

The experiments were performed as described in our previous study (25). Briefly, cDNA encoding the hinge and LBD of PPAR α (amino acids 167–468) were removed from the corresponding pPPAR α -IRES2-EGFP expression plasmids by cutting with BamH I and Hind III and inserted in-frame into a pMAL-p2x plasmid (New England Biolabs) that expressed fusion PPAR α receptor protein carrying a N-terminal maltose-binding protein (MBP)-tag in cultured *Escherichia coli* TB1. MBP-PPAR α LBD fusion protein were purified with an amylose-resin affinity column (New England Biolabs) and digested by the protease Factor Xa, then separated by amylose-resin affinity column and DEAE-52 anion exchange chromatography column for preparation of PPAR α LBD with high purity.

3.5. Radioligand binding assays

For saturation binding analysis or competition binding assays, 40 μ g MBP-PPAR α or 20 μ g PPAR α were incubated at 25°C for 40 min in buffer containing 10 mM Tris (pH 8.4 for MBP-PPAR α , pH 8.0 for PPAR α), 50 mM KCl, 50 μ g bovine serum albumin, and 10 mM dithiothreitol with (3 H) arachidonic acid (AA) in the presence or absence of either fenofibrate or berberine in a total volume of 0.3 ml. The reaction was terminated by bathing on ice for 5 min and adding 5 ml of ice-cold phosphate buffer diluted by five-fold with double-distilled water. Then, the mixture was filtered under gentle vacuum through a glass fiber paper that was pre-washed in 10 ml ice-cold reaction buffer containing 1 mM non-radioligand AA. Filters were washed three times with 5 ml cold phosphate buffer in 10 s. The filter was dehydrated at 80°C for 40 min and put into counting vial with 5 ml scintillation fluid for 12 h in the dark. Radioactivity was determined by liquid scintillation counting. Each assay was carried out in triplicate.

3.6. Cell culture

HepG2 and CV-1 cells were obtained from the Cell Bank of Chinese Academy of Sciences and cultured under the condition of humidified 5% CO₂ and 95% air at 37°C with Dulbecco's modified Eagle's minimal essential medium containing 10% fetal bovine serum, 100 unit penicillin G, and 100 μ g streptomycin sulfate per milliliter.

3.7. Use and care of animals

Clean grade male Wistar rats weighing 200 \pm 20 g were obtained from Animal Breeding Center of Chongqing Medical University, and were housed in a temperature-controlled (22 \pm 1)°C room with a light/dark cycle of 12 h. The animals were allowed to have freely access to the standard rat chow diet and tap water.

3.8. Establishment of hyperlipidemic model in rats

Rats were randomly divided into 5 groups (n = 8 for each group): control group, model group, high-dose (300 mg/kg) berberine group, low-dose (60 mg/kg) berberine group, and fenofibrate (30 mg/kg) positive control group. The control group was fed with regular diet while the remaining groups were fed with a high-fat diet (containing standard diet, 10% lard, 0.25% bile acid, and 2% cholesterol). All rats received intragastric administration of corresponding drugs or solvent with same volume at 6 PM per day for 12 weeks. All rats were anesthetized with sodium pentobarbital (45 mg/kg, ip.) and then blood samples were obtained for determining the TC, TG, HDL-C, and LDL-C levels directly by Automatic Biochemical Analyzer (Hitachi, Japan) and hepatic tissues were collected to measure the mRNA and protein expression of CPT-I by RT-PCR and western blot, respectively.

3.9. Reverse transcription PCR analysis

Total RNA was extracted with Trizol reagent from hepatic tissues of rats and HepG2 cells. The reverse transcription PCR (RT-PCR) was performed with the TaKaRa RNA PCR Kit (AMV) Ver. 3.0. (TaKaRa, Japan), according to the manufacturer's protocol. The primers used in this study were as follows: CPT- α , 5'-TGAACAGGTATCTACAGTCGGTG-3' (forward) and 5'-TTGCTGCCTGAATG TGAGTTGTTG-3' (reverse); β -actin, 5'-CTCGTCATACTCCTGCTTGCTG-3' (forward) and 5'-CGGGACCTGACTGATACCTC-3' (reverse). The size of the PCR products was 264 bp (CPT- α) and 546 bp (β -actin). The PCR products were observed through 1.5% agarose gel electrophoresis with staining of ethidium bromide. The relative expression amount was scored using the ratio of CPT- α to β -actin staining intensities.

3.10. Western blot analysis

To determine the CPT- α protein levels, total protein extracts from hepatic tissues of rats and HepG2 cells were separated in a SDS-10% polyacrylamide gel with 5% stacking gel in SDS-Tris-glycine running buffer and electroblotted onto polyvinylidene difluoride-plus membranes. The blots were blocked for 1 h at room temperature with 5% fat-free milk in Tris-buffered saline (TBS) plus Tween-20 (TBST), followed by overnight incubation at 4°C with CPT- α antibody (Santa Cruz) diluted by blocking buffer (1:100). Following a 5-min wash (\times 2) with TBST and a 10-min wash with TBS, blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000) for 1 h at room temperature, and the anti-PPAR α antibody was detected using an ECL detection system (Amersham Pharmacia). Following this, the membrane was stripped and reprobed with antibody against β -actin (Santa Cruz) as described above, and the density of the β -actin band was used to normalize protein loading.

3.11. Statistical analysis

All data were expressed as the mean \pm SEM. Comparisons among the control and treatment groups were made using one-way analysis of variance followed by a Student-Newman-Keuls t-test using the GraphPad Instat statistical program. $p < 0.05$ was considered significant.

4. RESULTS

4.1. Selective activation of PPAR α by berberine in transactivation reporter gene assays

Berberine activated PPAR α in a concentration-dependent manner from 10 nM to 10 μ M (Figure 1). The EC₅₀ and E_{max} values were calculated from each concentration-effect curve, which were 0.58 μ M and 102.4%, respectively. Similar results were obtained when fenofibrate was used (EC₅₀ = 0.34 μ M,

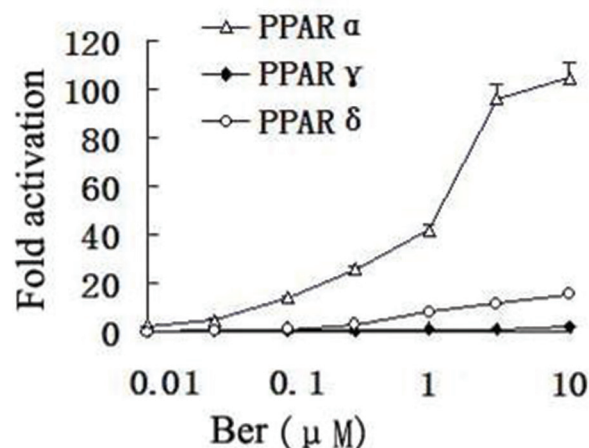


Figure 1. Concentration-activity curves of berberine (Ber) in transactivation assays for PPAR subtypes. Transactivation activity was shown as a fold activation value in each assay. The symbols and bars represent the SEM of triplicate assays.

Emax = 135.0). Berberine also activated PPAR δ slightly (EC₅₀ = 14 μ M, Emax = 18.8) but failed to activate PPAR γ (EC₅₀ > 100 μ M). The differences of more than two orders of EC₅₀ values between PPAR α and PPAR γ or PPAR δ revealed that berberine was a high selectivity of PPAR α .

4.2. Binding of berberine to PPAR α

To determine whether MBP could hinder the binding of ligand to PPAR α in the fusion proteins, purified MBP-PPAR α LBD, PPAR α LBD or control protein containing MBP were tested with (3H) AA, respectively. Saturation binding assay showed that the K_d value of (3H) AA binding to MBP-PPAR α LBD was 45 nM, which was similar to that from PPAR α LBD (data not shown). The result suggests that MBP does not affect (3H) AA to bind to PPAR α LBD in the fusion protein. In the control experiments, we failed to find specific binding of (3H) AA to MBP. The results indicate that MBP-PPAR α LBD instead of PPAR α LBD can be used for ligand binding assays.

To determine whether berberine was a specific ligand of PPAR α , its ability of competitive binding to PPAR α with various concentrations of (3H) AA was examined. The Lineweaver-Burk and Scatchard plots showed that 500 nM berberine raised the K_d value of (3H) AA binding to PPAR α from 45 nM to 83 nM, but the B_{max} value did not change (see Figure 2a, 2b). The results suggest that berberine competes to bind to PPAR α with (3H) AA. The displacement binding assays were also performed using 200 nM (3H) AA, and different concentrations of berberine and the K_i value were calculated from the competitive inhibition curves (see Figure 2c). The results showed that berberine could

bind directly to PPAR α with the K_i value of 0.73 μ M, which was similar to that of fenofibrate (K_i = 0.41 μ M).

4.3. Lipid-lowering effects of berberine involved upregulation of CPT-1 α gene expression

After rats were fed with high fat diet for 12 weeks, the levels of TC, TG and LDL-C in rat serum were significantly increased and HDL-C was decreased in experimental groups, compared to the control group, which indicate that the rat model of hyperlipidemia was successfully established (Table 1). The administration of berberine (60 mg/kg or 300 mg/kg per day for 3 months significantly decreased serum TC, TG, and LDL-C but increased HDL-C in a dose-dependent manner in hyperlipidemic rats. Similar results were observed in hyperlipidemic rats treated with fenofibrate (30 mg/kg, once a day for 3 months). It was noted that berberine led to a greater increase in HDL-C than fenofibrate did. The results indicate that berberine improved lipid metabolism in experimental hyperlipidemia, which may be superior to fenofibrate in term of upregulation of HDL-C.

Interestingly, berberine boosted both mRNA (Figure 3a) and protein (Figure 3b) expression levels of CPT-1 α in the liver of hyperlipidemic rats. CPT-1 α is a rate-limiting enzyme in the pathway of mitochondrial fatty acid oxidation. The CPT1 α gene expression is regulated by PPAR α . We determined the effects of berberine on CPT-1 α gene expression in cultured HepG2 cells. It was shown that berberine significantly up-regulated the expression of CPT-1 α mRNA in both concentration-dependent manner (1- 30 μ M, Figure 4a) and time-dependent manner (6-72 h, Figure 4b).

In order to determine if the up-regulation of CPT-1 α expression was mediated by PPAR α activation, the effects of varying concentrations of berberine on PPAR α expression was determined with or without MK886 (10 μ M), a noncompetitive inhibitor of PPAR α (26). It was shown that berberine (30 μ M) did not change the level of PPAR α expression (data not shown). On the other hand, pre-incubation with MK886 (10 μ M) blocked berberine- and fenofibrate-induced CPT-1 α expression at both the mRNA (Figure 4c) and protein levels (Figure 4d). These results demonstrated for the first time that berberine-induced up-regulation of CPT-1 α was probably mediated by activation of constitutive PPAR α .

5. DISCUSSION

In 2004, Kong W identified that berberine was a new cholesterol-lowering drug and found that berberine worked through a different mechanism from statins (2). Here, we showed for the first time that berberine was a potent and selective PPAR α agonist and could modify lipid metabolism disorders by activating PPAR α

Berberine is one PPAR α agonist

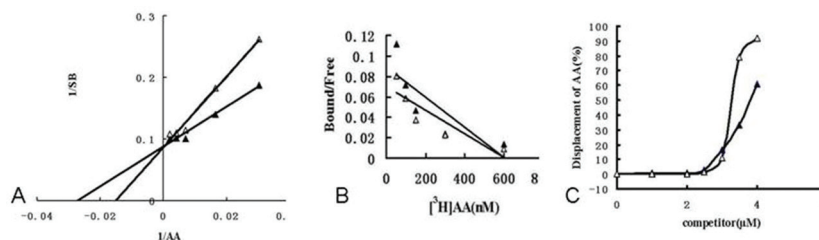


Figure 2. Binding of berberine to PPARalpha in radioactive binding assay. (A) Competition binding assays were performed with MBP-PPARalpha and an increasing concentration (up to 640nM) of (³H) AA in the absence (▲) or presence (Δ) of 500 nM berberine as competitor. Specific binding (SB) data were analyzed by Lineweaver-Burk plot; (B) Specific binding data of (A) in competitive binding assays were analyzed by Scatchard; (C) Displacement binding assays were performed using 200 nM (³H)AA in the presence of increasing concentrations (up to 5μM) of berberine (Δ) or fenofibrate (▲) as competitor.

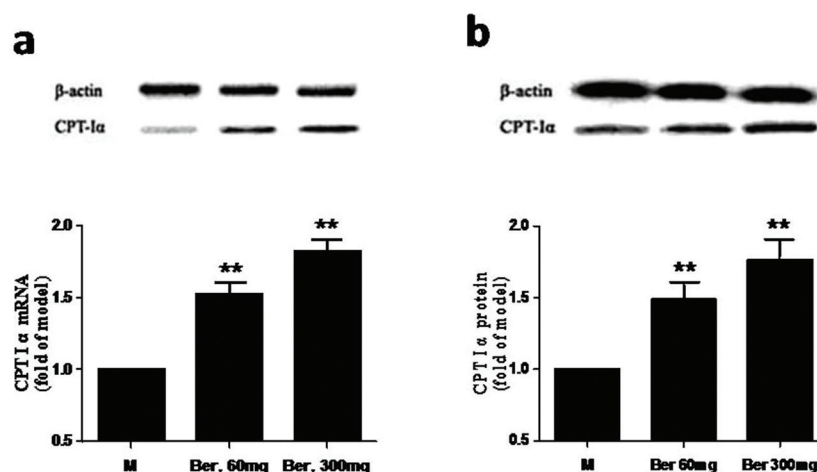


Figure 3. Up-regulation of CPT I alpha expression by berberine (Ber) in liver of hyperlipidemic rats. 12 wk after the drug treatment, three animals from each group were killed and expressions of CPT-I alpha mRNA (a) and protein (b) from the rat livers were determined by RT-PCR and western blot method, respectively. The western blot membranes were stripped and reprobed sequentially with antibodies that recognized CPT-Ialpha and β-actin. The histogram showed (lower panel) the mean of three separate experiments with the data presented as the relative expression quantity scored by the ratio of CPT-Ialpha to β-actin staining intensities according to the corresponding bands of electrophoresis (upper panel). **: p<0.001vs M (model).

Table 1. Effects of Berberine on Serum TC, TG, LDL-C, and HDL-C Levels in high fat diet-induced hyperlipidemic rats

Group	TC (mM)	TG (mM)	LDL-C(mM)	HDL-C (mM)
Control	1.85±0.17	1.05±0.17	1.08±0.22	0.65±0.07
Model	9.64±0.83 ²	1.48±0.12 ²	8.62±0.82 ²	0.45±0.19 ¹
Berberine (60 mg/kg/day)	7.06±0.99 ³	0.22±0.03 ³	5.88±0.36 ³	1.14±0.21 ³
Berberine (300 mg/kg/day)	6.03±0.68 ³	0.18±0.02 ³	4.94±0.67 ³	1.31±0.16 ³
Fenofibrate (30 mg/kg/day)	5.53±0.81 ³	0.16±0.04 ³	5.07±0.78 ³	0.99±0.15 ³

¹: p<0.05 vs control, ²: p<0.01 vs control, ³: p<0.001 vs model, Each value is mean±SEM of 8 rats

which regulated expression of lipid metabolism-related genes, such as CPT-I alpha.

Berberine, a natural isoquinoline alkaloid, widely exists in the roots, rhizome, and stem bark of *Hydrastis canadensis* (goldenseal), *Cortex phellodendri* (Huangbai), and *Rhizoma coptidis* (Huanglian) (27). These medicinal plants have been widely used as traditional medicines for

treating diarrhea and gastrointestinal disorders for more than 3000 years in many countries of north and south-east Asia. Berberine that was considered as a major ingredient of these plants was described as a cheap and effective lipid-lowering drug with relatively low toxicity to man. In this study, we found also that berberine significantly lowered serum TC, TG, and LDL-C, but increased HDL-C in hyperlipidemic rats fed with high fat food.

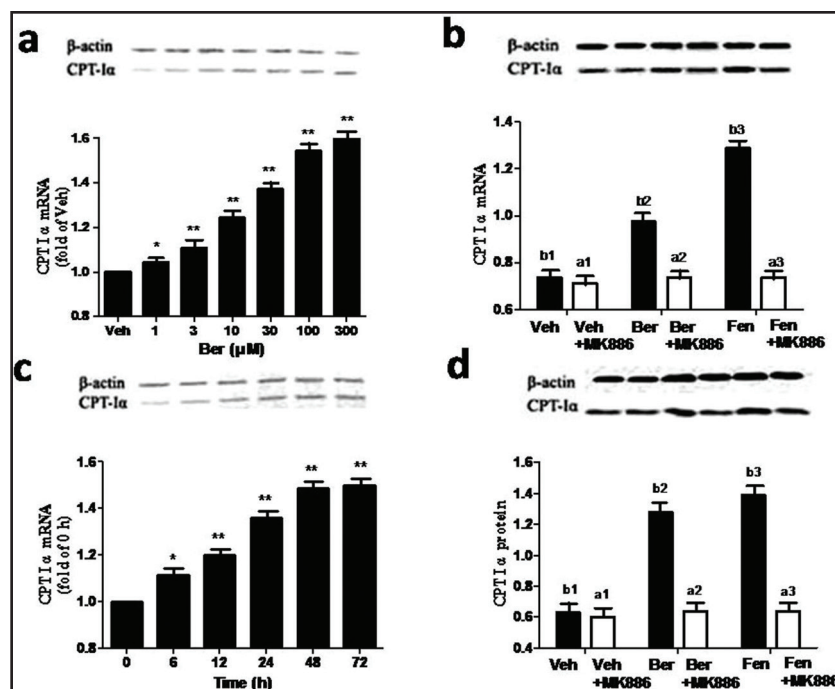


Figure 4. Up-regulation of CPT I alpha expression by berberine (Ber) in HepG2 cells. (a and b): Ber enhanced CPT-I alpha mRNA expressions from HepG2 cells in a dose- and time-dependent manner. *: $p < 0.05$ and **: $p < 0.001$ vs Veh (dimethyl sulfoxide) in a or 0 h in b; (c and d): The promotion of CPT-I alpha mRNA (c) and protein (d) level by Ber (30 μM, 72h) and fenofibrate (Fen, 30 μM, 72h) could be completely blocked by MK-886 (10 μM). b1: $p > 0.05$, b2: $p < 0.01$, and b3: $p < 0.01$ vs a1, a2, and a3, respectively.

Much effort has been spent on exploring the lipid-lowering mechanism of berberine, but the exact mechanism remains to be elucidated. Considering the high affinity of berberine to nuclear proteins (28, 29) and similarity of the lipid-lowering effects of berberine and PPAR α agonists, it is reasonable to hypothesize that berberine is a natural agonist for PPAR α . To prove this hypothesis, we performed two important experiments. First, we used the trans-activation reporter gene assays based on the level of cells to assess if berberine could activate PPARs. The results showed that berberine strongly activated PPAR α in a concentration-dependent manner. The differences of more than two orders in EC₅₀ values caused by berberine between PPAR α (0.58 μM) and PPAR γ (>100 μM) or PPAR δ (14 μM) revealed the subtype selectivity of berberine to PPAR α . Second, the radioligand binding assays were used to determine the affinity of candidate ligand binding to specific receptor. The results showed that berberine could bind directly to PPAR α with the K_i value of 0.73 μM that was similar to that of fenofibrate (0.41 μM), which indicated that berberine was a selective and potent ligand of PPAR α .

PPAR α regulates the expression of key proteins involved in all stages of lipid metabolism (30). CPT-I is the key regulatory enzyme of long-chain fatty acid β -oxidation (31) and is regulated by the PPAR α (32). If berberine is an agonist of PPAR α , it shall regulate

the expression of CPT-I. Therefore, we investigated the effects of berberine on CPT-I alpha gene expression. The results from both *in vitro* and *in vivo* experiments showed that berberine and fenofibrate potentially enhanced mRNA and protein expressions of CPT-I alpha gene in HepG2 cells and in liver of hyperlipidemic rats. MK-886, a noncompetitive inhibitor of PPAR α , blocked the effects of berberine or fenofibrate. These results further confirmed that berberine was an agonist of PPAR α .

Based on the experimental results (berberine activated PPAR α , bound to PPAR α , up-regulated CPT-I alpha expression, as well as modulated lipid metabolism), we conclude that that berberine is a selective agonist of PPAR α . In addition, this study has demonstrated that both berberine and fenofibrate display nearly the same pharmacological efficacy and potency because their EC₅₀, E_{max}, and K_i values are similar in our experiments. Comparison between berberine and fenofibrate after 3 months of drug therapy in hyperlipidaemia demonstrated that both drugs reduced serum levels of TC, TG and LDL-C to a similar degree. Notably, it seemed that berberine increased HDL-C more effectively than fenofibrate did. Considering that fibrates like fenofibrate display a series of serious side reactions, such as myopathy, acute pancreatitis, cholesterol gallstone, and so on during the period of the chronic treatment with them (33-36), it is reasonable that berberine may have a widely potential application

in treatment of diseases associated with metabolic syndromes, compared with fenobrate.

6. CONCLUSION

The results of this study demonstrated that berberine was a selective and potent agonist of PPAR α . It could attenuate dyslipidemia by up-regulating the expression of CPT-1 α . The present findings represent an important progress in understanding the lipid-lowering mechanisms of berberine and lay a solid foundation for extensive clinical applications of berberine.

7. ACKNOWLEDGEMENTS

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Abbreviations: PPAR α : peroxisome proliferator-activated receptor alpha; PPARs: peroxisome proliferator-activated receptors; CPT-I: carnitine palmitoyl transferase I; VLDL-C: very low density lipoprotein cholesterol; LDL-C: low density lipoprotein cholesterol; LDL: low density lipoprotein; LDLR: low density lipoprotein receptor; HDL-C: high-density lipoprotein cholesterol; ApoB: apolipoprotein B; ApoA I: apolipoprotein A-I; ApoCIII: apolipoprotein A-III; LPL: lipoprotein lipase

Key Words: Berberine; hPPAR α ; Agonist; CPT-I α ; Fenofibrate

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