Berberine Up-Regulates Hepatic Low-Density Lipoprotein Receptor through Ras-Independent but AMP-Activated Protein Kinase-Dependent Raf-1 Activation

Zheng Li, Jian-Dong Jiang, and Wei-Jia Kong

Abstract

Our previous studies showed that berberine (BBR) increases liver low-density lipoprotein (LDL) receptor expression in an extracellular signal-regulated kinase (ERK)-dependent manner. This study was designed to explore the upstream cellular signaling molecules recruited by BBR to activate the ERK mitogen-activated protein kinase (MAPK) cascade. Chemical inhibitors such as GW5074, manumycin A, and compound C or specific small interfering RNAs (siRNAs) were used in the blocking experiments; Western blot was used to determine the phosphorylation of kinases; real-time reverse transcriptase polymerase chain reaction (RT-PCR) was used to determine the expression level of LDL receptor mRNA. Our results indicate that BBR increases p-Raf-1 (ser338) level time and dose dependently in HL-7702 cells, but has no influence on Ras activity; the stimulating activities of BBR on Raf-1 signaling and LDL receptor expression can be blocked by GW5074 completely, but not by manumycin A, a Ras inhibitor. BBR activates hepatic Raf-1 signaling and up-regulates LDL receptor expression in a rat model of hyperlipidemia with no impact on liver Ras activity. Importantly, our results show that the stimulating activities of BBR on hepatic Raf-1 signaling and LDL receptor expression are totally blocked by compound C, a selective inhibitor of AMP-activated protein kinase (AMPK), and also by silencing its expression with siRNA. Taken together, our results demonstrate for the first time that BBR up-regulates LDL receptor expression through Ras-independent, but AMPK-dependent Raf-1 activation in liver cells. Our study will help to elucidate the molecular pharmacology of BBR and provide new scientific evidence for its clinical application.

Key words: berberine; low-density lipoprotein (LDL) receptor; Raf-1; AMP-activated protein kinase (AMPK); Ras

The low-density lipoprotein (LDL) receptor is a transmembrane glycoprotein which is mainly responsible for the clearance of atherogenic lipoproteins from blood. Defects of the human LDL receptor could cause familial hypercholesterolemia (FH), which may lead to atherosclerosis and coronary heart disease (CHD), if there are no medical interventions. For decades, pharmacological modulation of the LDL receptor was proved to be useful to treat hypercholesterolemia and related cardiovascular diseases in clinic.

The expression of the LDL receptor gene is subjected to complex regulations, which include transcriptional as well as post-transcriptional events. For example, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, or “statins” could induce the transcription of the LDL receptor gene through the sterol regulatory element-binding protein (SREBP) pathway. And our previous studies proved that natural product berberine (BBR) was able to up-regulate the LDL receptor gene at the post-transcriptional level.

BBR (molecular weight: 371.8) is a kind of isoquinoline alkaloid isolated from plants such as Rhizoma coptidis (huanglian) or Hydrastis canadensis (goldenseal) and has multiple pharmacological efficacies. In recent years, BBR was proved to have beneficial effects against metabolic disorders, both in laboratory and in clinic. The pharmacological activities of BBR on lipid and sugar metabolism are extremely complex and were critically reviewed elsewhere. For example, through mitochondrial inhibition, BBR is able to activate the cellular AMP-activated protein kinase (AMPK) pathway, which plays a pivotal role in energy metabolism and balance. The stimulating activity of BBR on AMPK could partially explain its beneficial effects on glucose and triglyceride metabolism. However, the cholesterol-lowering efficacy of BBR seems to be mainly attributable to LDL receptor up-regulation.

Our previous results showed that BBR increased LDL receptor mRNA stability and expression level in an extracellular signal-regulated kinase (ERK)-dependent manner; as BBR could activate ERK directly in liver cells and U0126, a specific inhibitor of mitogen-activated protein kinase (MAPK) or MEK) could abolish the effects of BBR on LDL receptor. These results suggest that BBR up-regulates LDL receptor through the ERK MAPK signal-transduction pathway which may include the sequential activation of three kinases: Raf-1, MEK, and ERK. The ERK MAPK cascade could be activated by various extracellular stimuli and could help to transduce signals from cell surface to nucleus. Numerous studies demonstrate that the ERK MAPK cascade has close relationship to cell proliferation, differentiation, migration and death.

Although BBR was shown to activate ERK and up-regulate LDL receptor in liver cells, the upstream signaling events leading to ERK activation remain unclear. In the present study, we explore the upstream signaling molecules of the ERK MAPK cascade after BBR administration and find that the efficacy of BBR on LDL receptor is dependent on AMPK-mediated Raf-1 activation.

The authors declare no conflict of interest.
MATERIALS AND METHODS

Chemicals and Reagents BBR (with a purity ≥98%), phorbol 12-myristate 13-acetate (PMA), GW5074, staurosporine and compound C were purchased from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.); manumycin A was purchased from Cayman Chemical Company (Ann Arbor, MI, U.S.A.). Cell culture reagents including fetal bovine serum (FBS), Dulbecco’s modified Eagle’s medium (DMEM, high glucose), and other related reagents were purchased from Gibco-Invitrogen (Grand Island, NY, U.S.A.). Antibodies against total Raf-1, phosphorylated Raf-1 (p-Raf-1) (ser338), phosphorylated MEK1/2 (p-MEK1/2), total AMPKα, phosphorylated AMPKα (p-AMPKα) and β-actin (ACTB) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, U.S.A.); peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Total RNA purification kit was obtained from Qiagen GmbH (Mettmann, Hilden, Germany), Reverse Transcription System and GoTag qPCR Master Mix were obtained from Promega (Madison, WI, U.S.A.). M-PER Mammalian Protein Extraction Reagent, Pierce BCA Protein Assay kit and Protease Inhibitor Cocktail were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, U.S.A.). Ras Activation ELISA ASSAY Kit was purchased from EMD Millipore Corporation (Billerica, MA, U.S.A.), AMPKα1/2 siRNA and siRNA Reagent System were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.).

Cell Culture HL-7702 cells, a normal human liver cell line, were obtained from the Institute of Biochemistry and Cell Biology (SIBS, CAS, Shanghai, China). Cells were cultured in DMEM (high glucose) plus 10% FBS, 1% nonessential amino acids and antibiotics in an atmosphere of 5% CO₂ at 37°C. Cells were trypsinized and let grow to about 70–80% confluency before experiment.

Blocking Experiments HL-7702 cells seeded in 6-well plates were left untreated or pretreated with indicated concentrations of GW5074, manumycin A, staurosporine, or compound C for 1 h. Cells were then kept untreated or treated with BBR as indicated, the protein expression levels of p-Raf-1 (ser338), p-MEK1/2 or p-AMPKα were analyzed by Western blot, and the LDL receptor mRNA expression levels were determined by real-time reverse transcriptase polymerase chain reaction (RT-PCR).

Western Blot After treatment, cells were harvested on ice by scraping; animal liver tissues were homogenized as described previously. Total protein contents were extracted by using the M-PER Mammalian Protein Extraction Reagent according to supplier’s protocol. Samples were subjected to 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (EMD Millipore Corporation, Billerica, MA, U.S.A.) by a Semi-Dry Transfer Cell (Bio-Rad, Hercules, CA, U.S.A.). Membranes were probed with rabbit monoclonal antibodies against p-Raf-1 (ser338), p-MEK1/2, p-AMPKα or ACTB. After washing and incubation with an anti-rabbit peroxidase-conjugated secondary antibody, signals were visualized using a Chemiluminescence Kit (EMD Millipore Corporation). The membranes were stripped, total Raf-1 or AMPKα protein levels were detected with mouse or rabbit monoclonal antibodies, respectively. Blots were scanned and quantified by using the Gel-PRO ANALYZER gel analysis and electrophoresis analysis software (Media Cybernetics, Inc. Rockville, MD), p-Raf-1 (ser338) and p-AMPKα levels were normalized to those of Raf-1 or AMPKα or ACTB and plotted as fold of untreated control, which was designated as 1.

RNA Extraction and Real-Time RT-PCR After treatment, total RNAs were extracted from harvested cells or animal liver tissues and reversely transcribed into cDNAs as described previously. Quantitative real-time PCR was performed with gene specific primers (Table 1) in a 25 µL of reaction system, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or ACTB as internal controls. The reactions were performed in an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, U.S.A.) and incubated at 95°C for 2 min, followed by 40 cycles of 95°C, 15 s plus 60°C, 1 min. The comparative threshold cycle (Ct) method was used for relative quantification of LDL receptor mRNA expression, which was plotted as fold of untreated control after normalization.

Ras Activity Assay Cellular and tissue Ras activities were determined with the Ras Activation ELISA ASSAY Kit according to manufacturer’s protocol. Briefly, cells and liver tissues were scraped or homogenized with ice-cold 1 × MgCl₂ Lysis/Wash Buffer (supplied in the kit) containing the Protease Inhibitor Cocktail and incubated on ice for 15 min. After determination of protein concentrations, samples containing 50 µg of proteins were added to strips pre-coated with Ras Binding Domain (RBD) of Raf-1 (Raf-1-RBD) at 5 µg per well. The strips were incubated at room temperature with mild agitation for 1 h and washed 3 times with Tris Buffered Saline with Tween 20 (TBST). Then, an anti-Ras mouse monoclonal antibody was added and incubated at room temperature with mild agitation for 1 h. After washing for 3 times, a goat anti-mouse peroxidase conjugated secondary antibody was added to the wells and incubated at room temperature with mild agitation for 1 h. After additional washing, Chemiluminescent substrate was added for color development and signals were read by a VICTOR™ X4 Multilabel Plate Reader (PerkinElmer, Inc., Waltham, MA, U.S.A.). Each sample was repeated for at least 3 times, Ras activity (Ras-guanosine 5′-triphosphate (GTP) binding to Raf-1-RBD) was presented as fold of untreated control, which was designated as 1.

Table 1. Primers Used in Real-Time PCR

<table>
<thead>
<tr>
<th>Species</th>
<th>Gene names</th>
<th>Primer sequences (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>LDL receptor</td>
<td>Upstream: agggcctgctgggccagtctc &lt;br&gt; Downstream: gggccgaggcagaccc</td>
</tr>
<tr>
<td></td>
<td>GAPDH</td>
<td>Upstream: gacccgagggcttgaggtg &lt;br&gt; Downstream: tgggaggtgaggcgcacagt</td>
</tr>
<tr>
<td>Rat</td>
<td>LDL receptor</td>
<td>Upstream: cacgggctgctcagctc &lt;br&gt; Downstream: tggagaggtgaggcgcacagt</td>
</tr>
<tr>
<td></td>
<td>ACTB</td>
<td>Upstream: gatgatactgctgcctcag &lt;br&gt; Downstream: tctactgctgctgcctcag</td>
</tr>
</tbody>
</table>
RNA Interference  The small interfering RNA (siRNA) transfection experiment was performed according to supplier’s protocol. Briefly, HL-7702 cells were seeded onto 6-well plates with 1×10⁵ cells per well and cultured in antibiotic-free media containing 10% FBS. For 1 well of a 6-well plate, 10 µL of AMPKα1/2 siRNA duplex or control siRNA duplex (Santa Cruz Biotechnology) was mixed with 100 µL of siRNA transfection medium to make solution A, and 6 µL of siRNA transfection reagent was mixed with 100 µL of siRNA transfection medium to make solution B. Then, solution A and solution B were mixed and incubated at room temperature for 30 min followed by addition of 800 µL of siRNA transfection medium and mixture. Cells were washed once with 1 mL of siRNA transfection medium and loaded with the master mixture for 6 h at 37°C. After transfection, siRNA containing media were discarded and replaced with regular media containing 10% FBS and antibiotics. Twenty-four hours later, cells were left untreated or treated with BBR as indicated; protein expression levels of p-Raf-1 (ser338)/Raf-1, p-AMPKα/AMPKα and ACTB were analyzed by Western blot, LDL receptor mRNA expression levels were determined by real-time RT-PCR.

Animal Experiment  The animal experiment protocol was reviewed and approved by the Research Committee of the Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences; animals were cared for according to the institutional guidelines of Chinese Academy of Medical Sciences.

Male Sprague-Dawley (SD) rats (150±10 g) were obtained from Vital River Laboratories (Beijing, China). After 3 d of accommodation, some rats were fed with regular rodent diet as normal control group (n=6), others were orally administered with a fat emulsion composed of 30% lard, 10% cholesterol, 10% Tween 80, 20% 1,2-propanediol, 3% deoxycholic acid sodium and 27% water. The dose of fat emulsion was 20 mL/kg/d and was orally administered to the animals at 8a.m. Animals fed with fat emulsion were divided into two groups with 8 each; one group was treated with saline as high fat control, while the other group was orally treated with BBR at 100 mg/kg/d. BBR and saline were administered to fat emulsion-fed rats at 4 p.m. for 3 weeks. At the end of the experiment, blood samples were collected by retro-orbital puncture after overnight fasting; sera were isolated for determination of TC and LDL-c levels. The rats were then sacrificed by cervical dislocation; their livers were harvested and frozen in liquid nitrogen for RNA and protein extraction. Liver LDL receptor mRNA expression levels, protein levels of p-Raf-1 (ser338), Raf-1, ACTB, and Ras activities were assayed as described above.

Statistical Analysis  After validation of the test for homogeneity of variance, differences between or among study groups were examined by a two-tailed unpaired Student’s t-test or by one-way analysis of variance followed by the Newman–Keuls test for multiple comparisons. A p<0.05 was considered to be statistically significant.

RESULTS

BBR Up-Regulates Hepatic LDL Receptor through Raf-1 Activation  As the up-regulating activity of BBR on LDL receptor is MEK/ERK-dependent, and Raf-1 is the kinase that phosphorylates and activates MEK in the ERK MAPK cascade, we want to know whether or not BBR can activate Raf-1 directly. HL-7702 cells, a normal human liver cell line, were treated with BBR for different time intervals or at different concentrations; stimulation of Raf-1 signaling was revealed by Western blot analysis of p-Raf-1 (ser338) levels, as phosphorylation of Raf-1 at serine 338 is a hallmark of its activation. In addition, as PMA, a kind of phorbol ester, was shown to activate Raf-1 and up-regulate LDL receptor in liver cells, we used it as a positive control in order to verify the cell responsiveness and clearly show the activity of BBR on Raf-1 signaling. As shown in Figs. 1A and B, administration of 50 ng/mL of PMA for 30 min strongly increased the level of p-Raf-1 (ser338); BBR could activate Raf-1 signaling in time (Fig. 1A) and dose (Fig. 1B) dependent manners without influencing the protein level of total Raf-1. Twenty micro mol per liter of BBR could increase p-Raf-1 (ser338) level by about 60% (p<0.05 vs untreated control) after only 5 min treatment, the level of p-Raf-1 (ser338) reached a peak at about 10–30 min and started to decline after 1 h of BBR treatment (Fig. 1A). The time-dependent kinetics of BBR on Raf-1 activation was well correlated with its kinetics on ERK activation. Furthermore, the efficacy of BBR on Raf-1 signaling was confirmed in a human hepatoma cell line, HepG2 cells (data not shown).

BBR induced Raf-1 activation could result in subsequent MEK activation in HL-7702 cells, as indicated by the increase of p-MEK1/2 protein level (Fig. 1C). The stimulating effect of BBR on Raf-1/MEK signaling could be abolished by GW5074 (Fig. 1C), a Raf-1 inhibitor. The inhibitory effect of GW5074 on Raf-1/MEK signaling was dose-dependent and became statistically significant when its concentration reached 1000 nmol/L (Fig. 1C). The stimulating activity of PMA on Raf-1 signaling could also be blocked by GW5074 at a concentration ≥1000 nmol/L (data not shown).

More importantly, we found that the up-regulating activity of BBR on LDL receptor mRNA expression could be completely abolished by GW5074 when its concentration reached 1000 nmol/L (Fig. 1D). These results suggested that BBR could activate Raf-1 signaling directly in liver cells, which was indispensable for the up-regulation of LDL receptor.

The Stimulating Effects of BBR on Raf-1 Signaling and LDL Receptor Expression Are Ras-Independent  Next, we intend to explore the upstream cellular signaling molecules recruited by BBR to activate Raf-1. As the classic Ras pathway is typically involved in Raf-1 activation, for example, by growth factors, we want to know whether or not BBR activates Raf-1 through the same manner. Firstly, the influence of BBR on cellular Ras activity was determined. HL-7702 cells were left untreated or treated with BBR for different time intervals (Fig. 2A) or at different concentrations (Fig. 2B), cellular Ras activities were determined by enzyme linked immunosorbent assay (ELISA).

As shown in Fig. 2A, 20 µmol/L of BBR treatment for up to 60 min, which could induce a dynamic increase in Raf-1 signaling (Fig. 1A), did not cause any change in cellular Ras activity. And in Fig. 2B, 30 min treatment of 5 to 20 µmol/L of BBR, which could activate Raf-1 dose-dependently (Fig. 1B), did not increase cellular Ras activity, either.

To determine whether or not BBR activates Raf-1 through Ras pathway, mammumycin A, a Ras inhibitor, was used in our experiments. As shown in Fig. 2C, 10 µmol/L of manu-
mycin A decreased the baseline Ras activity of cells (p < 0.05 vs. untreated control), which was in agree with previous reports. BBR again did not stimulate Ras activity, and when manumycin A was co-administered with BBR, cellular Ras activity was also decreased below baseline level.

Importantly, in Western blot analysis of p-Raf-1 (ser338) (Fig. 2D), 10 μmol/L of manumycin A, which could inhibit baseline Ras activity, did not have any influence on the Ras stimulating activity of BBR (p > 0.05, manumycin A + BBR vs. BBR alone). Accordingly, the up-regulating activity of BBR on LDL receptor mRNA was not blocked by manumycin A (Fig. 2E). These results suggested that BBR did not activate

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**Fig. 1.** Effects of BBR and GW5074 on Raf-1 Signaling and LDL Receptor Expression in HL-7702 Cells

(A) Time-dependent efficacy of BBR on Raf-1 signaling. HL-7702 cells were left untreated or treated with 50 ng/mL of PMA for 30 min or 20 μmol/L of BBR for the indicated time intervals, levels of p-Raf-1 (ser338), Raf-1 and ACTB were determined with Western blot. Representative blots are presented in the upper panel, the level of p-Raf-1 (ser338) was normalized to that of Raf-1 and plotted as fold of untreated control (lower panel), which was designated as 1. The quantitative data in histogram were mean ± S.D. of 3 separate experiments; * p < 0.05, ** p < 0.01, *** p < 0.001 vs. that of untreated control. (B) Dose-dependent efficacy of BBR on Raf-1 signaling. HL-7702 cells were left untreated or treated with 50 ng/mL of PMA or indicated concentrations of BBR for 30 min, levels of p-Raf-1 (ser338), Raf-1 and ACTB were determined with Western blot. Representative blots are presented in the upper panel; blots were scanned, quantified, and plotted (lower panel) as described in Fig. 1A. The quantitative data in histogram were mean ± S.D. of 3 separate experiments; * p < 0.05, ** p < 0.01, *** p < 0.001 vs. that of untreated control. (C) Inhibitory effect of GW5074 on the efficacy of BBR on Raf-1/MEK signaling. HL-7702 cells were left untreated or pretreated with GW5074 for 1 h as indicated. The cells were then kept untreated or treated with BBR for 30 min, levels of p-Raf-1 (ser338), Raf-1, p-MEK1/2 and ACTB were determined with Western blot. Representative blots are presented in upper panel, the levels of p-Raf-1 (ser338) and p-MEK1/2 were normalized to that of ACTB and plotted as fold of untreated control (lower panel), which was designated as 1. The quantitative data in histogram were mean ± S.D. of 3 separate experiments; * p < 0.05 vs. that of untreated control, # p < 0.05 vs. that of BBR alone. (D) Inhibitory effect of GW5074 on the efficacy of BBR on LDL receptor expression. HL-7702 cells were treated as described in Fig. 1C, except that the duration of BBR treatment was 16 h. The expression levels of LDL receptor mRNA were determined with real-time RT-PCR, normalized to that of GAPDH, and plotted as fold of untreated control, which was designated as 1. Values are mean ± S.D. of 5 repeated experiments; * p < 0.05 vs. that of untreated control, # p < 0.05 vs. that of BBR alone.
Ras in liver cells and that the stimulating effects of BBR on Raf-1 signaling and LDL receptor expression was Ras-independent.

To validate the above results obtained in cultured cells, we examined the impact of BBR on hepatic Raf-1 signaling and Ras activity in a hyperlipidemic rat model. Rat hyperlipidemia was induced by fat emulsion feeding; BBR at 100 mg/kg/d was orally administered to the animals together with the fat emulsion for 3 weeks, with saline as control. As shown in Table 2, serum TC and LDL-c levels of the animals increased for about 3.02 and 6.05-fold, respectively, after 3 weeks of fat emulsion feeding ($p<0.001$ vs. that of normal control group). BBR partially but statistically significantly prevented the rise of serum TC and LDL-c induced by fat emulsion feeding. Compared to that of saline group ($p<0.05$ or $p<0.01$), BBR could reduce serum TC and LDL-c levels by about 29.7% and 36.8%, respectively, after 3 weeks treatment.

Hepatic LDL receptor mRNA levels were reversely correlated with serum cholesterol levels. As shown in Table 2, fat emulsion feeding could cause more than 70% loss of hepatic LDL receptor expression ($p<0.001$ vs. that of normal control group). BBR administration significantly restored liver

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**Fig. 2. Effects of BBR and Manumycin A on Ras Activity, Raf-1 Signaling, and LDL Receptor Expression in HL-7702 Cells**

(A) HL-7702 cells were left untreated or treated with 20 µmol/L of BBR for the indicated time intervals; cellular Ras activities were determined with the Ras Activation ELISA ASSAY Kit and plotted as fold of untreated control, which was designated as 1. Values are mean±S.D. of at least 3 repeated experiments. (B) HL-7702 cells were left untreated or treated with different concentrations of BBR for 30 min; cellular Ras activities were assayed and plotted as described in Fig. 2A. Values are mean±S.D. of at least 3 repeated experiments. (C) Inhibitory effect of manumycin A on Ras activity. Cells were left untreated or treated with 10 µmol/L of manumycin A for 1 h as indicated. The cells were then kept untreated or treated with 10 µmol/L of BBR for 30 min; cellular Ras activities were assayed and plotted as described in Fig. 2A. Values are mean±S.D. of at least 3 repeated experiments, *$p<0.05$ vs. that of untreated control. (D) HL-7702 cells were treated as described in Fig. 2C; total cellular proteins were extracted for determination of p-Raf-1 (ser338), Raf-1, and ACTB by Western blot. Representative blots are presented in lower panel, the level of p-Raf-1 (ser338) was normalized to that of Raf-1 and plotted as fold of untreated control (upper panel), which was designated as 1. The quantitative data in histogram were mean±S.D. of 3 separate experiments; **$p<0.01$ vs. that of untreated control. (E) Cells were treated as described in Fig. 2C, except that the duration of BBR treatment was 16 h; total cellular RNAs were extracted for determination of LDL receptor mRNA by real-time RT-PCR. The expression levels of LDL receptor mRNA were normalized to that of GAPDH and plotted as fold of untreated control, which was designated as 1. Values are mean±S.D. of at least 3 repeated experiments; *$p<0.05$ vs. that of untreated control.
Table 2. Effects of BBR on Serum Cholesterols and Liver LDL Receptor mRNA in a Rat Model of Hyperlipidemia

<table>
<thead>
<tr>
<th>Group</th>
<th>TC (mmol/L)</th>
<th>LDL-c (mmol/L)</th>
<th>Liver LDL receptor mRNA (fold of fat emulsion+saline)</th>
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<tbody>
<tr>
<td>Normal control (n=6)</td>
<td>1.84±0.31</td>
<td>0.22±0.02</td>
<td>3.63±0.42</td>
</tr>
<tr>
<td>Fat emulsion+saline (n=8)</td>
<td>5.56±1.48***</td>
<td>1.33±0.32***</td>
<td>1±0.03***</td>
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<tr>
<td>Fat emulsion+BBR (n=8)</td>
<td>3.91±0.94*</td>
<td>0.84±0.16**</td>
<td>2.12±0.32**</td>
</tr>
</tbody>
</table>

***p<0.001 vs. that of normal control; *p<0.05, **p<0.01 vs. that of fat emulsion+saline group.

**Fig. 3.** Effects of BBR on Hepatic Raf-1 Signaling and Ras Activity in a Hyperlipidemic Rat Model

Male S.D. rats fed with fat emulsion were orally treated with saline (n=8) or 100 mg/kg/d of BBR (n=8) for 3 weeks. At the end of experiment, animals were sacrificed; their livers were harvested for Western blot analysis of p-Raf-1 (ser338), Raf-1 and ACTB levels. Representative blots of two animals in each group were presented in (A); blots were scanned and quantified for all of the samples, the level of p-Raf-1 (ser338) was normalized to that of Raf-1 and plotted as fold of saline control, which was designated as 1 (B). A portion of liver tissue from each animal was also used for Ras activity assay and plotted as fold of saline control, which was designated as 1 (B). The values in (B) are mean±S.D. of 8 animals in each group, **p<0.01 vs. that of saline control.

LDL receptor in fat emulsion-fed rats by about 2.12-fold as compared to that of saline group (p<0.01). These results concerning the cholesterol-lowering and LDL receptor-increasing activities of BBR were perfectly in accordance with our previous studies.4,15)

The animals were sacrificed after the experiment; their liver tissues were harvested for determination of Raf-1 signaling and Ras activities. As shown in Figs. 3A and B, BBR administration significantly increased the level of p-Raf-1 (ser338) in rat livers as compared to that of saline group (p<0.01). In contrast, liver Ras activity remained at baseline level after BBR treatment (Fig. 3B). These in vivo results are in agreement with those obtained in vitro, and indicate that the stimulating efficacy of BBR on Raf-1 signaling dose not require Ras activation.

**The Stimulating Effects of BBR on Raf-1 Signaling and LDL Receptor Expression Are Dependent on AMPK Activation** A previous report showed that 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), an AMPK activator, could activate Raf-1/MEK/ERK cascade and up-regulate LDL receptor in HepG2 cells.22) So, we speculate that BBR might stimulate Raf-1 through the same pathway. To prove our hypothesis, compound C, a selective inhibitor of AMPK, was used to treat HL-7702 cells before BBR administration, cellular p-AMPKα as well as p-Raf-1 levels were determined by Western blot. As shown in Fig. 4A, pretreatment with compound C significantly reduced the baseline p-AMPKα level and totally blocked the stimulating activity of BBR on AMPK signaling (p<0.001 vs. BBR alone). And interestingly, when compound C was added to cells before BBR, it completely abolished the stimulating activity of BBR on Raf-1 signaling (p<0.01 vs. BBR alone). Similar results were obtained in real-time RT-PCR assay of LDL receptor mRNA levels. As shown in Fig. 4B, the up-regulating efficacy of BBR on LDL receptor was totally blocked in the presence of compound C (p<0.01 vs. BBR alone). These results suggest that AMPK activation is upstream of Raf-1 in the signaling cascade of BBR and is essential for its activity on LDL receptor expression.

To confirm the above results obtained with compound C, AMPKα siRNA was used to knockdown its expression. As shown in Fig. 4C, the expression level of AMPKα was significantly decreased by AMPKα siRNA transfection (p<0.05 vs. control siRNA). BBR had no effect on AMPKα expression, but its activity on p-AMPKα was blocked by AMPKα siRNA (p<0.01 vs. control siRNA+BBR). And importantly, the stimulating activity of BBR on Raf-1 signaling was completely abolished by AMPKα siRNA (p<0.01 vs. control siRNA+BBR). Accordingly, the up-regulating activity of BBR on LDL receptor was blocked in the presence of AMPKα siRNA (Fig. 4D, p<0.01 vs. control siRNA+BBR).

The results in the RNA interference experiment are in accordance with those of compound C. Taken together, our results indicate that BBR activates Raf-1 and up-regulates LDL receptor in an AMPK-dependent manner.

**DISCUSSION**

The expression of the LDL receptor gene is undergone complex regulations which are related to multiple cellular signaling pathways. Here in the present study, we demonstrate for the first time that natural product BBR up-regulates hepatic LDL receptor through AMPK-dependent Raf-1 activation. Schematic diagram of the cellular pathways recruited by BBR to up-regulate LDL receptor in hepatocytes is presented in Fig. 5.

Since the activity of BBR on LDL receptor is MEK/ERK-dependent,4) it is not surprising that BBR can activate Raf-1 directly and its efficacy on LDL receptor can be blocked by GW5074. In our experiments, we found that the stimulating activities of BBR on Raf-1 signaling and LDL receptor expression could be blocked by GW5074 when its concentration reached 1000 nmol/L, which was equivalent to or less than the concentrations of GW5074 (5–10 µmol/L) reported by oth-
Cellular Raf-1 signaling could be activated through Ras-dependent as well as Ras-independent mechanisms. Growth factors typically stimulate Raf-1 through Ras activation. Upon binding to receptors on cell membrane, growth factors could induce autophosphorylation of their receptors and activate Ras through the well known Shc-Grb2-SOS pathway. Activated Ras could recruit Raf family of serine/threonine kinases to cell membrane, where they are activated through multiple mechanisms, for example, by phosphorylation.

Fig. 4. Effects of BBR, Compound C, and AMPKα1/2 siRNA on AMPK/Raf-1 Signaling and LDL Receptor Expression in HL-7702 Cells

(A) Inhibitory effects of compound C on the efficacies of BBR on AMPK/Raf-1 signaling. HL-7702 cells were left untreated or pretreated with 10 μmol/L of compound C for 1 h as indicated. The cells were then kept untreated or treated with 30 μmol/L of BBR for 30 min; levels of p-Raf-1 (ser338), Raf-1, p-AMPKα, AMPKα, and ACTB were determined with Western blot. Representative blots are presented in upper panel, the levels of p-Raf-1 (ser338) and p-AMPKα were normalized to that of Raf-1 or AMPKα and plotted as fold of untreated control (lower panel), which was designated as 1. The quantitative data in histogram were mean±S.D. of 3 separate experiments; *p<0.05, **p<0.01 vs. that of untreated control, ***p<0.001 vs. that of BBR alone. (B) Inhibitory effect of compound C on the efficacy of BBR on LDL receptor expression. Cells were treated as described in Fig. 4A, except that the duration of BBR treatment was 16 h. LDL receptor mRNA levels were determined by real-time RT-PCR, normalized to that of GAPDH, and plotted as fold of untreated control, which was designated as 1. Values are mean±S.D. of at least 3 repeated experiments; **p<0.01 vs. that of untreated control, ***p<0.01 vs. that of BBR alone. (C) Inhibitory effects of AMPKα1/2 siRNA against the efficacies of BBR on AMPK/Raf-1 signaling. HL-7702 cells were transfected with AMPKα1/2 siRNA or control siRNA. Cells were then left untreated or treated with 10 μmol/L of BBR for 30 min; levels of p-Raf-1 (ser338), Raf-1, p-AMPKα, AMPKα, and ACTB were determined with Western blot. Representative blots are presented in upper panel, the levels of p-Raf-1 (ser338), p-AMPKα, and AMPKα were normalized to that of ACTB and plotted as fold of control siRNA alone (lower panel), which was designated as 1. The quantitative data in histogram were mean±S.D. of 3 separate experiments; *p<0.05, **p<0.01 vs. that of control siRNA, ***p<0.001 vs. that of BBR+control siRNA. (D) Inhibitory effect of AMPKα1/2 siRNA against the efficacy of BBR on LDL receptor expression. Cells were treated as described in Fig. 4C, except that the duration of BBR treatment was 16 h. LDL receptor mRNA levels were determined by real-time RT-PCR, normalized to that of GAPDH, and plotted as fold of control siRNA alone, which was designated as 1. Values are mean±S.D. of at least 3 repeated experiments; **p<0.01 vs. that of control siRNA, ***p<0.001 vs. that of BBR+control siRNA.
Our results (both in vitro and in vivo) prove that BBR activates Raf-1 apparently through a Ras-independent mechanism. As BBR itself does not induce Ras activation, and manumycin A, a farnesyltransferase inhibitor, cannot block the stimulating activity of BBR on Raf-1 signaling. The Ras family of GTPase belongs to a superfamily of proto-oncogenes, whose mutations could lead to malignant change of cells. Our results show that BBR has no influence on Ras activity in normal human liver cells. And in fact, in previous reports, BBR was shown to down-regulate the expression of H-Ras and c-Ki-ras2 in tumor cells.

Some factors like PMA, vitamin D3, Angiotensin II, and mechanical strain could stimulate Raf-1 signaling through protein kinase C (PKC) activation. Especially, PMA, a carcinogenic phorbol ester, was shown to activate Raf-1/MEK/ERK and increase LDL receptor gene transcription in a PKC-dependent manner. Although our previous studies showed that BBR also could activate PKC, it stimulates Raf-1 signaling and LDL receptor expression through a pathway different from that of PMA. This is because of the following two reasons, firstly, calphostin C, a selective inhibitor of PKC, could abolish the up-regulating effect of PMA on LDL receptor, but not that of BBR; and secondly, staurosporine could block the stimulating effect of PMA on Raf-1 signaling, but not that of BBR (data not shown in this study). These data indicate that the effects of BBR on Raf-1 signaling and LDL receptor expression are independent of PKC.

Interestingly, by using a specific inhibitor or siRNA silencing, our results strongly support that BBR-induced AMPK is located upstream of Raf-1 and is essential for its activation as well as the up-regulation of LDL receptor. We noticed that in the RNA interference experiment, the stimulating activity of BBR on AMPKα was completely blocked with only partial knockdown of its expression. We speculate that this could be due to some kind of tissue-specific modulation mechanisms concerning the activity of BBR on AMPKα. As firstly, in HepG2 cells, we observed similar results by using AMPKα siRNA (data not shown). And secondly, our unpublished data showed that in skeletal muscle cells, the stimulating activity of BBR on AMPKα was only partially blocked even when its expression was silenced for more than 80%. The situation in skeletal muscle cells is opposite to that of liver cells, so, we infer that the influence of AMPKα-silencing on the pharmacological activity of BBR could be tissue-specific.

Another well known AMPK activator, AICAR, was shown to activate Raf-1 signaling and up-regulate LDL receptor in HepG2 cells. However, based on knockdown experiments, it seemed that the stimulating activity of AICAR on ERK MAPK cascade was only partially dependent on AMPK activation; in other words, AMPK-independent pathways may exist. This is different from BBR, as blocking the AMPK pathway completely abolished the activities of BBR on Raf-1 signaling and LDL receptor expression (Fig. 4).

The crosstalk between AMPK and the ERK MAPK cascade is complex. For example, and intriguingly, a previous report indicated that the stimulating activity of BBR on AMPK was relied on ERK activation and could be blocked by PD98059 in HepG2 cells. It is possible that some kind of feedback...
regulatory mechanism exist between AMPK and ERK, more research works are needed to clarify this issue. In addition, BBR was shown to activate AMPK and subsequently suppress MAPK phosphorylation in macrophages treated with proinflammatory cytokines.36) These data suggest that BBR may activate AMPK and then regulate MAPK cascade differentially in different cell types or under different pathophysiological conditions.

In addition to ERK, the other two types of MAPKs, c-Jun N-terminal kinase (JNK) and p38, could also be activated by BBR in different cell types.37-39) The JNK pathway was also shown to be involved in the up-regulating activity of BBR on LDL receptor expression.40) And interestingly, BBR was proved to activate p38 in an AMPK-dependent manner in L6 skeletal muscle cells,39) suggesting that the regulatory activity of AMPK on MAPKs is not limited to ERK. It would be meaningful to study whether or not BBR could up-regulate LDL receptor through AMPK-dependent activation of MAPKs in liver cells.

As downstream kinases in the ERK MAPK cascade, MEK/ERK are critical for the LDL receptor mRNA stabilizing effect of BBR in liver cells4,40) (Fig. 5). Our previous studies showed that U0126 could block the ameliorating activity of BBR on LDL receptor mRNA decay and then abolish its stimulating effect on LDL receptor mRNA expression.4,40) Apparently, it is important to study the influences of AMPK and Raf-1 signaling on cellular LDL receptor mRNA half-life after BBR treatment by using specific inhibitors or siRNAs. These experiments will be performed in our laboratory in near future.

Like other proteins, the LDL receptor is synthesized in the endoplasmic reticulum (ER) as a precursor, and processed in the Golgi apparatus.3) The mature form of LDL receptor is transported to cell surface. BBR increases LDL receptor mRNA expression post-transcriptional,3) so, theoretical, more LDL receptor protein precursors and mature forms could be produced after translation. Our previous studies proved that BBR could enhance LDL receptor protein expression on cell surface and stimulate LDL uptake,4) so, we also plan to explore the roles of AMPK and Raf-1 signaling on cellular LDL uptake by using fluorescence-labeled LDL.

In conclusion, our results in the present study demonstrate for the first time that natural product BBR up-regulates hepatic LDL receptor expression through Ras-independent, but AMPK-dependent Raf-1 activation. Our study throws new light on the regulatory mechanisms of LDL receptor gene expression and will provide new scientific evidences for the clinical application of BBR, which is safe and efficacious,4,14,41) to treat hypercholesterolemia.

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REFERENCES


