Berberine suppresses proinflammatory responses through AMPK activation in macrophages

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Jeong HW, Hsu KC, Lee J-W, Ham M, Huh JY, Shin HJ, Kim WS, Kim JB. Berberine suppresses proinflammatory responses through AMPK activation in macrophages. Am J Physiol Endocrinol Metab 296: E955–E964, 2009. First published February 10, 2009; doi:10.1152/ajpendo.90599.2008—Berberine (BBR) has been shown to improve several metabolic disorders, such as obesity, type 2 diabetes, and dyslipidemia, by stimulating AMP-activated protein kinase (AMPK). However, the effects of BBR on proinflammatory responses in macrophages are poorly understood. Here we show that BBR represses proinflammatory responses through AMPK activation in macrophages. In adipose tissue of obese db/db mice, BBR treatment significantly downregulated the expression of proinflammatory genes such as TNF-α, IL-1β, IL-6, monocyte chemoattractant protein-1 (MCP-1), inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2). Consistently, BBR inhibited LPS-induced expression of proinflammatory genes including IL-1β, IL-6, iNOS, MCP-1, COX-2, and matrix metalloprotease-9 in peritoneal macrophages and RAW 264.7 cells. Upon various proinflammatory signals including LPS, free fatty acids, and hydrogen peroxide, BBR suppressed the phosphorylation of MAPKs, such as p38, ERK, and JNK, and the level of reactive oxygen species in macrophages. Moreover, these inhibitory effects of BBR on proinflammatory responses were abolished by AMPK inhibition via either compound C, an AMPK inhibitor, or dominant-negative AMPK, implying that BBR would downregulate proinflammatory responses in macrophages via AMPK stimulation.

mitogen-activated protein kinase; adenosine 5′-monophosphate-activated protein kinase; reactive oxygen species

INFLAMMATION IS AN IMPORTANT RESPONSE that protects host organisms against external injuries and pathogens. Nevertheless, many recent reports (19, 42, 49) have suggested that obesity is tightly associated with a chronic and low-grade inflammatory state. In obese subjects, macrophage infiltration is increased into the adipose tissue, which contributes to developing insulin resistance (16, 43). Inflammation is also known to trigger atherosclerosis, a coronary artery disease the hallmark of which is the formation of fatty deposits inside the artery walls (17, 35, 36). Thus accumulating evidence suggests that chronic inflammatory processes would constitute a crucial part in the pathogenesis of metabolic disorders including obesity, lipid dysregulation, insulin resistance, and atherosclerosis.

Cellular events of inflammatory responses are associated with the redox balance and mitogen-activated protein kinase (MAPK) signaling pathways. In macrophages, lipopolysaccharide (LPS), a major component of bacterial cell walls, potently increases the levels of cellular reactive oxygen species (ROS) and MAPK phosphorylation, resulting in promoting proinflammatory responses (51). Consistently, specific inhibition of cellular ROS and MAPK suppresses inflammatory signaling, implying that the cellular regulator for AMPK activity might be a key factor for inflammatory responses (25, 48).

Berberine (BBR) is a major form of isoquinoline alkaloid derived from medicinal herbs such as *Hydrastis canadensis* (goldenseal), *Cortex phellodendri* (huangbai), and *Rhizoma copidis* (huanglian), showing antibacterial and antiparasitic activities (40). Recent studies have demonstrated that the beneficial effects of BBR on metabolic disorders including weight-reducing, cholesterol-lowering, anti-lipogenic, and hypoglycemic effects (23, 28, 33, 54) are associated with activation of AMP-activated protein kinase (AMPK) in peripheral tissues (3, 7, 33). Additionally, it has been also reported that BBR suppresses the expression of inflammatory molecules in several cell types (9, 22, 27, 29, 32). However, it is unclear how BBR mediates anti-inflammatory responses, especially in macrophages.

In the present study, we address the questions whether BBR indeed downregulates inflammatory responses and how BBR exhibits anti-inflammatory responses in macrophages. We demonstrate that BBR reduces the expression of proinflammatory genes upon inflammatory stimuli in an AMPK-dependent manner. In macrophages, BBR alleviated MAPK activation and ROS generation induced by various proinflammatory signals. Moreover, the inhibitory effects of BBR on proinflammatory gene expression and signaling cascades were abrogated by inhibition of AMPK activity. Together, these results suggest that BBR would exhibit advantageous effects against inflammation-related metabolic disorders through AMPK stimulation in macrophages.

MATERIALS AND METHODS

**Materials.** BBR chloride was purchased from Wako (Osaka, Japan). Metformin, free fatty acid (FFA; stearic acid), TNF-α, and LPS were purchased from Sigma (St. Louis, MO). Compound C (ComC) was purchased from Calbiochem (La Jolla, CA). The antibodies for phospho-SAPK/JNK (Thr183/Tyr185), SAPK/JNK1, phospho-p44/42 MAPK (Thr202/Tyr204), ERK, phospho-p38 MAPK (Thr180/Tyr182), and p38 MAPK were purchased from Cell Signaling (Danvers, MA). GAPDH antibody was purchased from AB Frontier (Seoul, Korea). Lipofectamine 2000 reagent and H2O2, 7′-dichlorodihydrofluorescein diacetate (DCF-DA) were purchased from Invitrogen (Carlsbad, CA).

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Animal experiments and primary cell preparation. All animal experiments were approved by the Seoul National University Animal Experiment Ethics Committee. All mice were purchased from Central Laboratory Animals (Seoul, Korea). The 8- or 18-wk-old male C57BLKS/J-Lepr/dbLepr/db mice were intraperitoneally injected with BBR (5 mg/kg) or vehicle daily for 4 wk. For peritoneal macrophage preparation, mice were stimulated by intraperitoneal injection of thioglycollate solution (3 ml per mouse) and kept in pathogen-free conditions for 3 days before peritoneal macrophage isolation. Total peritoneal macrophages were harvested by washing the peritoneal cavity with PBS containing 30 mM of EDTA (8 ml per mouse), centrifuged, and suspended in RPMI-1640 medium (Hyclone, Logan, UT) with 10% FBS (Hyclone), 100 U/ml penicillin, and 100 mg/ml streptomycin. Peritoneal macrophages were cultured in six-well plates (1 × 10^6 cells per plate) and maintained at 37°C in a 5% CO_2_-humidified air. Nonadherent cells were removed by washings with medium at 2 h after seeding. Experiments were undertaken after the cells were firmly adhered to the culture plates.

For preparation of the primary adipocytes and stromal vascular cell (SVC) fractions, mice were killed and gonadal fat pads were isolated and incubated in 30 ml of collagenase buffer (0.1 M HEPES pH 7.4, 0.125 M NaCl, 50 mM KCl, 1.3 mM CaCl_2, 0.5 M glucose, 0.45 g BSA, and 30 mg of collagenase) at 37°C for 1 h with shaking and shearing. Then, treated tissues were filtered by 100 μM pore-size-nylon mesh to remove debris and centrifuged at 2,000 rpm for 3 min. Primary adipocytes were collected from floating fraction, and SVCs were obtained in pellet. To remove red blood cells (RBC), the pellet was incubated in RBC lysis buffer (1.7 M Tris, pH 7.65, and 0.16 M NH_4Cl) for 15 min. After RBC lysis, the SVCs were washed several times with PBS.

Cell culture and dominant negative AMPKα1 transfection. Raw 264.7 macrophages and 3T3-L1 adipocytes were obtained from the American Type Culture Collection (Manassas, VA). RAW 264.7 cells were grown in DMEM (Hyclone) supplemented with 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. 3T3-L1 preadipocytes were maintained in DMEM supplemented with 10% bovine calf serum (Hyclone), 100 U/ml penicillin, and 100 mg/ml streptomycin. To differentiate 3T3-L1 preadipocytes into adipocytes, postconfluent (2 days) cells were incubated in DMEM medium supplemented with 10% FBS, 0.5 mM 3-isobutyl-1-methylxanthine (Sigma), 1 μM dexamethasone (Sigma), and 5 μg/ml of insulin (Sigma). After 48 h, the medium was replaced every other day with DMEM containing 10% FBS and 5 μg/ml of insulin.

For dominant-negative AMPKα1 (DN-AMPKα1) overexpression, RAW 264.7 macrophages were transfected with rat DN-AMPKα1 (T72A) expression vector (0.5 μg/10^5 cell) or empty vector using Lipofectamine 2000 reagent and incubated in OPTI-MEM (Invitrogen) medium. Four hours after transfection, the medium was replaced with fresh DMEM medium containing 10% FBS.

Western blot analysis. An equal amount of protein (60 μg) from each sample was separated on SDS-PAGE gels and transferred to nitrocellulose membranes. Blots were blocked with 5% nonfat milk in Tris-buffered saline with 0.1% Tween-20 (TBST, 25 mM Tris, pH 8.0, 137 mM NaCl, 2.7 mM KCl, and 0.1% Tween-20) at room temperature for 30 min, followed by overnight incubation with primary antibodies at 4°C. After being washed with TBST three times, the blots were hybridized with secondary antibodies conjugated with horseradish peroxidase (Bio-Rad, Hercules, CA) in 5% nonfat milk dissolved in TBST at room temperature for 2 h and washed three times with TBST. The membranes were then incubated with enhanced-chemiluminescence reagents and exposed to X-ray film (Fuji, Japan).

Quantitative RT-PCR analysis. Total RNA was isolated from RAW 264.7 cells, mouse peritoneal macrophages, 3T3-L1 adipocytes, mouse primary adipocytes, and SVC using TRIzol reagent (Invitrogen), and cDNA was synthesized with M-MuLV reverse transcriptase (Fermentas, Glen Burnie, MD). For quantitative RT-PCR (qRT-PCR) reactions, the amplification was performed for 40 repetitive thermal cycles with SYBR green (94°C for 30 s, 57°C for 20 s, and 72°C for 20 s), followed by a final extension at 72°C for 5 min. The primers were designed and synthesized from Bioneer (Daejeon, Korea), and the primer sequences are available upon request.

Glucose uptake assay. 3T3-L1 adipocytes were washed with PBS three times and incubated with HEPES-buffered saline (140 mM NaCl, 1 mM CaCl_2, 5 mM KCl, 2.5 mM MgSO_4, and 20 mM HEPES)
pH 7.4) containing [14C]deoxyglucose (PerkinElmer Life, Boston, MA) at 37°C for 10 min. After incubation, cells were washed with PBS another three times. Cells were lysed with 0.1% SDS solution, and extracts were obtained from centrifuged supernatant. The 14C content was measured in a scintillation counter in the presence of scintillation enhancer fluid (PerkinElmer Life).

**AMP-to-ATP ratio measurement.** RAW 264.7 cells were treated with metformin (500 μM) or BBR (5 μM) for 2 h. Adenosine nucleotide extracts were prepared as described previously (7). The amounts of AMP and ATP were determined by HPLC (TSP AS1000) with an Eclipse column (XDB-C18; 5 μM, 4.6 × 150 mm). Internal standards (0.1 nM, 1 nM, 10 nM of AMP and 1 nM, 10 nM, and 100 nM of ATP in distilled-deionized H2O) were used to calculate standard curves. ATP and AMP were eluted in a 259-nM wavelength at ~2 and 3 min, respectively.

**ROS measurement.** After each reagent (LPS, FFA, or H2O2) treatment, cells were washed with PBS two times and incubated with 5 μM of H2-DCF-DA for 30 min at 37°C in the dark. After two additional washings with PBS, the fluorescence intensity was measured with a multiplate reader (excitation wavelength: 492 nm; emission wavelength: 530 nm).

**Statistical analysis.** Results represent data from multiple, at least more than three times, independent experiments. All data points were average of at least triplicate samples. Error bars represent SD, and the P values calculated from the Student’s t-test and one-way ANOVA (followed by two-tailed unpaired Student’s t-test) <0.05 are interpreted statistically significant.

**RESULTS**

BBR suppresses the expression of proinflammatory genes in the white adipose tissues of obese mice. In obese subjects, white adipose tissue (WAT) exhibited increased macrophage infiltration (16, 43), and such infiltrated macrophages provoke low-grade chronic inflammation (16, 19, 20, 42), which is closely associated with metabolic disorders including insulin resistance. Previously, we (33) have shown that BBR-treated obese and diabetic animals improve insulin resistance and decrease body weight with a loss of excess fat mass. However, it is still unknown whether BBR also reduces proinflammatory responses concomitant with fat reduction in obese animals. To answer the question whether BBR could affect the expression of proinflammatory genes in the WAT of obese animals, we first examined the adipogenic/lipogenic gene expression in the WAT of obese db/db mice. As we and others have shown (9, 23, 33), BBR decreased expression of most adipogenic/lipogenic genes such as aP2, peroxisome proliferator-activated receptor-γ, acetyl-CoA carboxylase, and fatty acid synthase in the WAT of obese db/db mice (Supplemental Fig. 1; supplemental data for this article are available online at the Am J Physiol Endocrinol Metab website). Next, we investigated proinflammatory gene expression by qRT-PCR in the same

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**Fig. 2.** Inhibitory effects of BBR on proinflammatory gene expression in macrophages and adipocytes. A: RAW 264.7 cells were pretreated with metformin (Met; 0.5 mM), rosiglitazone (Rosi; 5 μM), or BBR (5 μM) for 2 h before LPS treatment (10 ng/ml) for 6 h. Relative mRNA level of each gene was analyzed by use of qRT-PCR and normalized by GAPDH. B: peritoneal macrophages were treated with LPS alone (10 ng/ml) or LPS with BBR (5 μM) for 24 h. After treatment, the relative mRNA level of each gene was determined by qRT-PCR and normalized by GAPDH. C: fully differentiated 3T3-L1 adipocytes were treated with Met (0.5 mM), Rosi (5 μM), or BBR (5 μM) for 2 h before TNF-α treatment (1 ng/ml) for 4 h. Expression level of each proinflammatory gene was determined by qRT-PCR and normalized by GAPDH. Each bar represents means ± SD of triplicates. Similar results were obtained from at least 4 independent experiments (A–C). #P < 0.05 vs. negative control. ##P < 0.01 vs. negative control. ###P < 0.001 vs. negative control. *P < 0.05 vs. LPS (A and B) or TNF-α (C). **P < 0.01 vs. LPS (A and B) or TNF-α (C). ***P < 0.001 vs. LPS (A and B) or TNF-α (C).
Similar results were obtained from 3 independent experiments (indicated in MATERIALS AND METHODS. Each bar represents means (DCF-DA) at 37°C for 30 min, followed by 2 additional PBS washings. Fluorescence intensity in proportion to cellular ROS level was measured at wavelength Fig. 4. Inhibitory effects of BBR on intracellular ROS formation induced by proinflammatory stimuli in macrophages. 

Fig. 3. Inhibitory effects of BBR on MAPK phosphorylation induced by various inflammatory stimuli in macrophages. A: RAW 264.7 cells were preincubated with or without BBR (5 μM) for 2 h and then treated with LPS (10 μg/ml) for indicated time points (15, 30, and 60 min). Total cell lysates were subjected to Western blot analysis as described in MATERIALS AND METHODS. After pretreatment of vehicle or BBR (5 μM) for 2 h and then treated with LPS (10 ng/ml) for 30 min, H2O2 (100 μM) for 15 min or FFA (500 μM) for 30 min. Total cell lysates were subjected to Western blot analysis as described in MATERIALS AND METHODS. Similar results were obtained from at least 3 independent experiments (A and B). Relative phosphorylation levels of MAPKs were normalized by each MAPK. The average phosphorylation of each MAPK from independent experiments was calculated and is shown in Supplemental Fig. 4, A (data from Fig. 3A) and B (data from Fig. 3B).

samples. The mRNA levels of proinflammatory genes, including TNF-α, IL-1β, IL-6, monocyte chemotactic protein-1 (MCP-1), iNOS, and cyclooxygenase-2 (COX-2), were decreased in the WAT of db/db mice treated with BBR (Fig. 1A). Since most macrophages with chronic proinflammatory properties in WAT reside in the SVC fraction of WAT, we further examined the expression of proinflammatory genes in both adipocytes and SVC fractions from the WAT of db/db mice treated with or without BBR. Consistent with an earlier study (16), high levels of proinflammatory gene expression were detected in SVCs compared with adipocytes (Fig. 1B). BBR administration clearly downregulated the expression of proinflammatory genes in both adipocytes and SVC fractions (Fig. 1B). It is of interest to note that the repressive effects of BBR on proinflammatory genes were more dramatic in SVC than in adipocytes, implying that BBR might preferentially exert its inhibitory effect on proinflammatory responses in the SVC with macrophages from WAT of obese animals.

BBR suppresses LPS- and TNF-α-induced proinflammatory gene expression in macrophages and adipocytes. To ascertain whether BBR downregulates proinflammatory gene expression in vitro, RAW 264.7 macrophages, primary mouse peritoneal macrophages, and differentiated 3T3-L1 adipocytes were treated with inflammatory stimuli and examined for the expression of proinflammatory genes in the presence or absence of BBR. In RAW264.7 cells, BBR repressed LPS-induced expression of proinflammatory genes to a similar or greater degree compared with rosiglitazone and metformin (Fig. 2A), which are well-known anti-inflammatory reagents (16, 18).

Fig. 4. Inhibitory effects of BBR on intracellular ROS formation induced by proinflammatory stimuli in macrophages. A: after incubation with BBR (5 μM) for 2 h or N-acetyl cysteine (NAC; 1 mM) for 1 h, RAW 264.7 cells were treated with LPS (1 μg/ml) for 30 min, H2O2 (100 μM) for 15 min, or FFA (stearic acid, 500 μM) for 30 min. Cells were then washed with PBS and incubated with the redox-sensitive fluorescent dye H2-DCFDA at 37°C for 30 min, followed by 2 additional PBS washings. Fluorescence intensity in proportion to cellular ROS level was measured at wavelength indicated in MATERIALS AND METHODS. Each bar represents means ± SD of triplicates. B: mouse peritoneal macrophages were incubated with BBR (5 μM) for 2 h, and cells were stimulated with LPS (10 ng/ml) for 30 min. After LPS treatment, peritoneal macrophages were then washed with PBS and incubated with the redox-sensitive fluorescent dye H2-DCFDA at 37°C for 30 min, followed by 2 additional PBS washings. Each bar represents means ± SD of triplicates. Similar results were obtained from 3 independent experiments (A and B). *P < 0.05 vs. negative control. **P < 0.01 vs. negative control. ***P < 0.005 vs. negative control. *P < 0.05 vs. LPS. **P < 0.05 vs. H2O2. ***P < 0.05 vs. FFA.
BBR also showed a dose-dependent repression in the expression of proinflammatory genes (Supplemental Fig. 2). However, a certain range of BBR concentration did not alter cell viability in RAW 264.7 cells (Supplemental Fig. 3), indicating that the suppressive property of BBR on proinflammatory gene expression may not be a result of its cytotoxicity. Furthermore, we observed that BBR potently inhibited LPS-induced proinflammatory gene expression in primary macrophages (Fig. 2B) and TNF-α-mediated proinflammatory gene expression in differentiated 3T3-L1 adipocytes (Fig. 2C). These observations evidently suggest that BBR would repress proinflammatory genes in both macrophages and adipocytes.

**BBR inhibits LPS-induced MAPK phosphorylation in RAW 264.7 cells.** MAPKs have been implicated in the signaling pathways that induce cytokine expression in response to various inflammatory stimuli (30, 31). Thus we hypothesized that BBR may downregulate the expression of proinflammatory genes by interfering with the MAPK signaling pathways upon inflammatory signals. To investigate this, we examined the effect of BBR on the levels of MAPK phosphorylation induced by LPS in RAW 264.7 cells and primary macrophages. While LPS elevated the phosphorylation levels of MAPKs including p38, ERK, and JNK, BBR treatment decreased LPS-dependent MAPK phosphorylation in both RAW 264.7 and peritoneal macrophages (Fig. 3A and B; Supplemental Fig. 4, A and B).

In obese subjects, increased FFA and ROS released from WAT are able to stimulate proinflammatory responses by activating MAPK pathways (12, 24, 34, 38, 43, 45, 46). Thus we examined whether BBR would decrease FFA- or ROS-induced phosphorylation of MAPK in macrophages. The phosphorylation levels of p38, ERK, and JNK were elevated by H2O2 (a ROS donor) or FFA treatment in macrophages (Fig. 3). In contrast, BBR suppressed the phosphorylation of these MAPKs upon H2O2 or FFA (Fig. 3A and B). Therefore, these results indicate that various proinflammatory stimuli (i.e., LPS, H2O2, and FFA) would enhance MAPK phosphorylation/activation in macrophages and that BBR might exhibit anti-inflammatory effects, at least in part, through downregulation of MAPK activation.

**BBR suppresses ROS generation and NO production in macrophages.** Since BBR has been reported to scavenge ROS (41), we raised the question whether BBR may also exhibit an antioxidant property in macrophages. To address this, we investigated whether BBR would affect LPS-, H2O2- and FFA-induced ROS generation in macrophages. Consistent with previous reports (21, 27), ROS generation was promoted approximately twofold by those stimuli in RAW 264.7 cells (Fig. 4A). In contrast, BBR substantially repressed the levels of ROS about 20–30% upon LPS, H2O2, and FFA in macrophages, although to an extent weaker than N-acetyl cysteine (NAC; Fig. 4A). Similarly, BBR decreased LPS-induced ROS generation in primary macrophages (Fig. 4B). Furthermore, BBR antagonized LPS-induced NO production in RAW 264.7 cells (Supplemental Fig. 5). These results suggest that BBR could attenuate cellular ROS and NO generation elicited by proinflammatory signals in macrophages, which appears to be associated with a decrease of proinflammatory responses in macrophages.

**BBR stimulates AMPK activation in macrophages.** Recent studies (3, 7, 33) have revealed that BBR is capable of stimulating AMPK in adipocytes, myotubes, and hepatocytes. However, it is unclear whether BBR is also able to activate AMPK in macrophages. To evaluate the effect of BBR on AMPK activation, which is directly linked with its phosphorylation state, we treated macrophages with BBR and examined the level of AMPK phosphorylation. In macrophages, BBR enhanced AMPK phosphorylation within a short-term period (<30 min) and prolonged its phosphorylated state (at least up

### Table 1. Changes of cellular AMP-to-ATP ratio by BBR in RAW 264.7 cells

<table>
<thead>
<tr>
<th>Condition</th>
<th>AMP pmol/10^5 cells</th>
<th>ATP pmol/10^5 cells</th>
<th>AMP-to-ATP Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.59±0.22</td>
<td>40.28±13.00</td>
<td>0.015±0.001</td>
</tr>
<tr>
<td>Metformin</td>
<td>2.00±0.000†</td>
<td>54.68±12.59</td>
<td>0.036±0.008*</td>
</tr>
<tr>
<td>BBR</td>
<td>6.07±0.000†</td>
<td>38.85±1.38</td>
<td>0.145±0.005†</td>
</tr>
</tbody>
</table>

RAW 264.7 cells were treated with metformin (500 μM) or BBR (5 μM) for 2 h. Cellular adenosine nucleotides were prepared and determined as described in MATERIALS AND METHODS. *P < 0.01 vs. negative control. †P < 0.001 vs. negative control.

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to 4 h; Fig. 5A; Supplemental Fig. 4C). It has been reported that BBR could inhibit mitochondrial respiratory complex I and would change the AMP-to-ATP ratio to activate AMPK in melanoma and L6 muscle cells (7, 15, 47a). To test this in macrophages, the changes of cellular AMP-to-ATP ratio were monitored in the absence or presence of BBR treatment. Similar to other cell types, BBR increased the AMP-to-ATP ratio in RAW 264.7 cells (Table 1), indicating that BBR is able...
to promote AMPK activity probably by alteration of the AMP-to-ATP ratio in macrophages.

To examine whether BBR increases AMPK phosphorylation in the presence of LPS, we cotreated macrophages with LPS and BBR. As illustrated in Fig. 5, A and B, BBR clearly promoted AMPK phosphorylation even in the presence of LPS, indicating that BBR effectively activates AMPK in macrophages regardless of inflammatory signals.

AMPK inhibition attenuates the repressive effects of BBR on inflammatory responses. Although BBR stimulated AMPK in macrophages regardless of inflammatory signals, indicating that BBR effectively activates AMPK in macrophages, it is unknown whether AMPK activation is required for the inhibitory effects of BBR on proinflammatory responses. To this end, we utilized ComC, an AMPK inhibitor, to suppress the cellular AMPK activity. In the presence of ComC, the anti-inflammatory effects of BBR were abrogated in macrophages (Fig. 6A). As another approach to dampen AMPK activity, RAW 264.7 cells were transfected with DN-AMPKα1 expression vector. While the empty vector did not affect the inhibitory effect of BBR on proinflammatory gene expression, overexpression of DN-AMPKα1 abolished the suppressive effects of BBR on LPS-induced inflammatory gene expression (Fig. 6B). We monitored the expression of DN-AMPKα1 and the attenuation of BBR-induced AMPK phosphorylation by DN-AMPKα1 (Supplemental Fig. 6). These results imply that BBR-induced AMPK phosphorylation/activity might be an important step in mediating the anti-inflammatory responses of BBR in macrophages.

To further explore whether AMPK activation is also involved in the downregulation of cellular ROS and MAPK activation by BBR, macrophages were cotreated with ComC and BBR. As shown in Fig. 7A, inhibition of AMPK by ComC alleviated the suppressive effects of BBR on MAPK phosphorylation upon LPS in RAW 264.7 cells. Similarly, pretreatment of ComC into primary peritoneal macrophages prevented the effect of BBR on MAPK regulation (Fig. 7B). Moreover, ComC treatment attenuated the inhibitory effects of BBR on ROS generation with LPS in both RAW 264.7 (Fig. 8A) and primary macrophages (Fig. 8B). Similar to ComC, DN-AMPK also blunted the suppressive effect of BBR on inflammatory responses, including MAPKs phosphorylation (Fig. 7C) and ROS production (Fig. 8C) in macrophages. Together, these results propose that BBR-dependent AMPK activation in macrophages would play a crucial role in downregulating proinflammatory responses through suppression of inflammatory signaling cascades including activation of MAPK and increase in ROS.

Conditioned media from BBR-treated macrophages improve insulin sensitivity in adipocytes. During chronic inflammatory responses, macrophages release inflammatory cytokines such as TNF-α, IL-6, and MCP-1, which mediate insulin resistance in adipocytes (2, 16, 19). To test whether BBR-dependent suppression of proinflammatory gene expression in macrophages could rescue insulin sensitivity in adipocytes, we incubated 3T3-L1 adipocytes with macrophage-conditioned media treated with BBR or rosiglitazone in the presence of LPS (Fig. 9A). Compared with the control group, conditioned media from LPS-treated macrophages downregulated insulin-stimulated glucose uptake in adipocytes (Fig. 9B, lanes 1 and 2 vs. lanes 3 and 4). As expected, pretreatment of BBR or rosiglitazone into macrophages improved insulin-stimulated glucose uptake of differentiated adipocytes in the presence of LPS (Fig. 9B, lanes 3 and 4 vs. lanes 5–8). These results imply that BBR in macrophages might alter cytokine expression and release an altered repertoire of cytokines and chemokines, leading to the improvement of insulin response in neighboring adipocytes.

DISCUSSION

Macrophages have been implicated in the development of low-grade chronic inflammation-mediated insulin resistance in obesity (16, 43). Compared with small adipocytes, hypertrophic enlarged adipocytes tend to release altered adipocytokines...
to stimulate macrophage infiltration into adipose tissue (2, 10, 43, 45). As a result, recruited macrophages secrete a number of cytokines and chemokines, including TNF-α, interleukins, and MCP-1. These cytokines provoke insulin resistance in adipocytes by activating several pathways including PKC, IKK, and JNK (11, 19). This futile cycle would further worsen insulin resistance in adipose tissue and cause systemic metabolic disorders. Thus therapies with immunosuppressive activities are expected to have beneficial outcomes in the treatment of immune diseases and metabolic disorders.

Recent studies (3, 9, 27–29, 32, 33, 52) have shown that BBR ameliorates metabolic disorders via multiple pathways including the decrease of inflammatory gene expression. However, the molecular mechanism(s) by which BBR represses proinflammatory responses in macrophages remains unclear. In this study, we demonstrated that BBR potently inhibited proinflammatory responses by decreasing cellular ROS production and MAPK activation in macrophages through AMPK activation. We observed that various inflammatory stimuli, including LPS, IFNγ, and TNF-α (5, 6). Further, activation of all the three MAPKs (p38 MAPK, ERK1/2, and SAPK/JNK) is linked with induction of iNOS expression in macrophages (5, 6). However, it is unclear which MAPK(s) critically plays a key role to turn on proinflammatory gene expression in macrophages. Interestingly, we observed that BBR repressed the phosphorylation of all the three MAPKs induced by several stimuli, suggesting a general inhibitory effect of BBR on MAPK activation through AMPK activation, leading to pleiotropic effects of BBR on anti-inflammatory responses.

In obese and diabetic subjects, increased FFA released from WAT along with elevated oxidative stress contributes to MAPK activation and inflammatory responses (11, 37). The observations that BBR repressed LPS-, H2O2-, and FFA-induced MAPK activation suggest that BBR might not only decrease acute inflammatory responses but also attenuate chronic and low-grade inflammation in WAT of obese subjects. Although it has been reported that BBR stimulates p38 to enhance glucose uptake and activates ERK to stabilize LDL receptor expression in certain cell lines (1, 7, 28, 54), we failed to observe that BBR alone increases MAPK phosphorylation in macrophages (Supplemental Fig. 7).
It has been well established that proinflammatory stimuli such as LPS and TNF-α promote an early and acute increase of cellular ROS levels (13, 21) that stimulate MAPK cascade and downstream transcriptional factors to mediate inflammatory responses (13, 21, 50). On the other hand, it is also known that inflammatory molecules such as IFN-γ and LPS rapidly activate classical MAPK kinase pathways, implying that both ROS and MAPK signaling appear to augment each other during inflammatory responses (5). Currently, it is unclear which signaling cue or cascade might act as an upstream regulator upon proinflammatory signals, although there might be a closely correlative relationship between ROS and MAPKs in inflammatory responses.

Previously, it has been shown that BBR functions as a ROS scavenger in embryoid bodies and vascular smooth muscle cells (8, 50). Consistent with this, we observed that BBR reduced ROS production in macrophages and revealed that AMPK activation is essential for the inhibitory effect of BBR on ROS generation in macrophages. Recently, it has been proposed that AMPK might reduce ROS generation in endothelial cells through the regulation of PKC and uncoupling protein-2 (4, 26, 53). However, it is unknown whether PKC and/or uncoupling protein-2 might be involved in BBR-mediated ROS scavenging in macrophages. Further studies are required to elucidate the detailed mechanism of how BBR-activated AMPK activation is able to reduce cellular ROS and MAPK activation in macrophages.

Compared with the established roles of AMPK in energy sensing and metabolic switching, little is known about the involvement of AMPK in inflammatory responses. Here we exhibited that BBR promoted AMPK phosphorylation in macrophages regardless of LPS. It is of interest to note that AMPK inhibition with either ComC or DN-AMPKαI nullified the suppressive effect of BBR on inflammatory responses, implying that AMPK activation is essential for BBR-mediated repression of proinflammatory responses in macrophages. Consistent with these findings, other AMPK activators such as metformin and 5-aminoimidazole-4-caroxamide-1-β-D-ribofuranoside have been shown to repress proinflammatory signals in endothelial and glial cells (14, 18).

Increased macrophage infiltration is one of the hallmarks in the development of chronic inflammation in obesity. When we examined the numbers of resident macrophages in WAT of obese animals with or without BBR treatment, the number of infiltrated macrophages was increased in WAT of BBR-treated db/db mice (Supplemental Fig. 8). Interestingly, BBR elevated the levels of CD206+ (mannose receptor) macrophages and stimulated the expression of several alternatively activated M2 marker genes (e.g., arginase type I and II), implying that these increased macrophages might be alternatively activated M2-type macrophages. Consistent with our data, a very recently published report (44) showed that rosiglitazone increases infiltration of alternatively activated macrophages in adipose tissue. However, BBR increased the expression of a subset of M2 marker genes such as arginase type I and II (Supplemental Fig. 8), while it also decreased the expression of anti-inflammatory genes such as IL-10 and IL1Ra in RAW 264.7 macrophages (Supplemental Fig. 2). Thus it is likely that BBR would reduce not only proinflammatory responses but also anti-inflammatory responses in macrophages.

In spite of this, it remains to be elucidated whether BBR might change the population and/or property of macrophages in WAT of obese subjects because we tested limited surface markers and marker gene expression via fluorescence-activated cell sorting and qRT-PCR analyses, respectively. Further studies should address the effects of BBR on macrophage infiltration as well as macrophage polarization in adipose tissue of obese subjects.

In conclusion, we have shown that BBR potently suppresses proinflammatory responses in macrophages by inhibition of MAPK signaling and cellular ROS through AMPK activation. Together with the fact that BBR exhibits advantageous effects on metabolic disorders, including obesity, insulin resistance, hyperlipidemia, and hypercholesterolemia, our findings further suggest that BBR might be useful as a therapeutic agent for the treatment of inflammation associated with disorders.

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GRANTS

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