

Berberine-induced activation of 5'-adenosine monophosphate-activated protein kinase and glucose transport in rat skeletal muscles

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Abstract

Berberine (BBR) is the main alkaloid of *Coptis chinensis*, which has been used as a folk medicine to treat diabetes mellitus in Asian countries. We explored the possibility that 5'-adenosine monophosphate-activated protein kinase (AMPK) is involved in metabolic enhancement by BBR in skeletal muscle, the important tissue for glucose metabolism. Isolated rat epitrochlearis and soleus muscles were incubated in a buffer containing BBR, and activation of AMPK and related events were examined. In response to BBR treatment, the Thr¹⁷² phosphorylation of the catalytic α -subunit of AMPK, an essential step for full kinase activation, increased in a dose- and time-dependent manner. Ser⁷⁹ phosphorylation of acetyl-coenzyme A carboxylase, an intracellular substrate of AMPK, increased correspondingly. Analysis of isoform-specific AMPK activity revealed that BBR activated both the $\alpha 1$ and $\alpha 2$ isoforms of the catalytic subunit. This increase in enzyme activity was associated with an increased rate of 3-O-methyl-D-glucose transport in the absence of insulin and with phosphorylation of AS160, a signaling intermediary leading to glucose transporter 4 translocation. The intracellular energy status estimated from the phosphocreatine concentration was decreased by BBR. These results suggest that BBR acutely stimulates both AMPK $\alpha 1$ and AMPK $\alpha 2$ and insulin-independent glucose transport in skeletal muscle with a reduction of the intracellular energy status.

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1. Introduction

Skeletal muscle plays an important role in glucose metabolism and homeostasis in humans. Skeletal muscle is the principal site of glucose transport, the rate-limiting step in glucose utilization under physiologic conditions [1,2]. Similar to insulin stimulation, exercise increases the rate of glucose transport into contracting skeletal muscle by the translocation of glucose transporter 4 (GLUT4) to the surface membranes. This phenomenon is considered to be responsible for the acute hypoglycemic effect of exercise, with glucose in the blood being taken up by contracting skeletal muscles. Indeed, the exercise-stimulated GLUT4 translocation is not impaired in insulin-resistant conditions such as type 2 diabetes mellitus and obesity [3]. Thus, the insulin-independent mechanisms of exercise have been widely used to decrease blood glucose in patients with type 2 diabetes mellitus.

It has been demonstrated that exercise and insulin use different signaling mechanisms in skeletal muscle, and 5'-adenosine monophosphate-activated protein kinase (AMPK) has been identified as part of the mechanisms leading to the acute enhancement of glucose transport in contracting muscles (reviewed by Kahn et al [4], Hardie et al [5], and Fuji et al [6]). AMPK is a member of a metabolite-sensing protein kinase family and acts as an energy-sensing and signaling molecule in muscle cells by monitoring cellular energy levels, such as the AMP to adenosine triphosphate (ATP) ratio. Skeletal muscle AMPK is also implicated in a variety of antidiabetic properties of exercise, including GLUT4 expression [7,8], glycogen regulation [9,10], fatty acid oxidation [11,12], mitochondrial biogenesis [13,14], and enhanced insulin sensitivity [8,15,16]. In addition, skeletal muscle AMPK mediates part of the action of adipokines, including leptin and adiponectin, in glucose and lipid homeostasis, and the antidiabetic effect of metformin (MET) (reviewed by Kahn et al [4] and Hardie et al [5]). Thus, through these effects in skeletal muscle, AMPK serves as a metabolic activator that may reduce the risk for type 2 diabetes mellitus.

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Recent reports show that berberine (BBR), a natural plant alkaloid, stimulates glucose transport and activates AMPK in L6 cultured myocytes [17,18]. BBR decreases intracellular energy status, represented by the AMP/ATP ratio [17]; and it has been suggested that BBR and its more biologically available derivative dihydroberberine inhibit cellular respiration by inhibiting complex 1 of the mitochondrial respiratory chain in L6 myotubes [19]. BBR is isolated from medicinal plants, such as *Coptis chinensis* (family Ranunculaceae), a Chinese herb that has been used for thousands of years in Asia to treat diabetes; and BBR treatment has beneficial effects on hyperglycemia in patients with type 2 diabetes mellitus [20,21]. Considering that BBR and exercise can exert similar effects in stimulating AMPK accompanied with energy deprivation, skeletal muscle might be the principal site of action for the antidiabetic effect of BBR. However, to our knowledge, no study has examined the direct effects of BBR on AMPK activity in skeletal muscle tissue. AMPK is a heterotrimeric kinase consisting of a catalytic α -subunit and 2 regulatory subunits, β and γ . Two distinct α -isoforms (AMPK α 1 and α 2) exist in skeletal muscle. It is notable that AMPK α 1 is the predominant α -isoform and AMPK α 2 activity is undetectable in L6 cells [22].

The purpose of this study was to elucidate whether BBR acts on skeletal muscle to stimulate AMPK α 1 and α 2 and leads to metabolic enhancement, including glucose transport. We examined the short-term effects of BBR on both fast-glycolytic epitrochlearis and slow-oxidative soleus muscles using an isolated rat skeletal muscle preparation.

2. Materials and methods

2.1. Experimental animals and materials

Male Wistar rats aged 5 weeks and weighing 100 to 120 g were obtained from Shimizu Breeding Laboratories (Kyoto, Japan). The animals were housed in an animal room maintained at 23 °C with a 12:12-hour light/dark cycle and fed a standard laboratory diet and water ad libitum. All protocols for animal use and euthanasia were reviewed and approved by the Kyoto University Graduate School of Human and Environmental Studies and Kyoto University Radioisotope Research Center in Japan. The rats were randomly assigned to the experimental groups. BBR chloride was purchased from Sigma (Saint Louis, MO).

2.2. Muscle incubation

Two muscles, epitrochlearis and soleus, were used owing to their specific fiber type composition. The epitrochlearis is composed predominantly of fast-twitch glycolytic fibers (60%–65% fast-twitch white, 20% fast-twitch red, 15% slow-twitch red) [23], and the soleus is composed primarily of slow-twitch oxidative fibers (0% fast-twitch white, 13% fast-twitch red, 87% slow-twitch red) [24]. Muscles were

treated as described previously [25,26] with modifications. The rats were killed by cervical dislocation without anesthesia, and the muscles were rapidly and gently removed. Both ends of each muscle were tied with sutures, and the muscle was mounted on an incubation apparatus with the resting tension set to 0.5 g. To recover from AMPK activation induced during the isolating procedure, the muscles were preincubated in 7 mL of Krebs-Ringer bicarbonate buffer (KRB): 117 mmol/L NaCl, 4.7 mmol/L KCl, 2.5 mmol/L CaCl₂, 1.2 mmol/L KH₂PO₄, 1.2 mmol/L MgSO₄, and 24.6 mmol/L NaHCO₃ containing 2 mmol/L of pyruvate (KRBP) for 30 minutes.

The muscle was then incubated in 7 mL of fresh buffer containing various concentrations (0–0.6 mmol/L) of BBR for 30 minutes. The muscles were also incubated with 2 mmol/L of 5-aminoimidazole-4-carboxamide-1- α -D-ribonucleoside (AICAR) for 30 minutes, 0.5 mmol/L 2,4-dinitrophenol (DNP) for 15 minutes, or 1 μ mol/L insulin for 30 minutes to elicit maximal activation of AMPK (AICAR, DNP) or glucose transport (insulin) in isolated rat muscle [25,27]. The muscles were also incubated with 2 mmol/L MET for 30 minutes [28]. To measure the time course of changes in AMPK phosphorylation, the muscles were preincubated for 30 minutes and then incubated in the presence of BBR (0.3 mmol/L) for 0, 15, 30, 45, or 60 minutes. The buffer was bubbled with gas continuously using a mixture of 95% O₂–5% CO₂ and maintained at 37°C. The muscle was then either used fresh to measure glucose uptake (see 3-O-methyl-D-glucose [3MG] transport) or frozen immediately in liquid nitrogen for the subsequent measurement of isoform-specific AMPK activity, ATP, and phosphocreatine (PCr) concentrations, and Western blot analysis.

2.3. Western blot analysis

Sample preparation and Western blot analysis for detection of phosphorylated AMPK α , total AMPK α , and phosphorylated acetyl-coenzyme A carboxylase (ACC) were performed as described previously [26]. The muscle was homogenized in ice-cold lysis buffer (1:40 wt/vol) containing 20 mmol/L Tris-HCl (pH 7.4), 1% Triton X, 50 mmol/L NaCl, 250 mmol/L sucrose, 50 mmol/L NaF, 5 mmol/L sodium pyrophosphate, 2 mmol/L dithiothreitol, 4 mg/L leupeptin, 50 mg/L trypsin inhibitor, 0.1 mmol/L benzamidine, and 0.5 mmol/L phenylmethylsulfonyl fluoride (buffer A) and centrifuged at 16 000g for 30 minutes at 4°C. The supernatant (10 μ g of protein) was mixed with Laemmli sample buffer containing mercaptoethanol and boiled, and the denatured proteins were separated on either a 10% polyacrylamide gel for AMPK and Akt or a 6% gel for ACC.

The muscle for Western blot analysis of phosphorylated p38 mitogen-activated protein kinase (MAPK) and phosphorylated p44/42 MAPK was homogenized in ice-cold lysis buffer (1:40 wt/vol) containing 20 mmol/L HEPES (pH 7.4), 1.5% Triton X-100, 50 mmol/L NaF, 5 mmol/L Na₄P₂O₇, 50 mmol/L β -glycerophosphate (disodium-5-hydrate), 250 mmol/L sucrose, 5 mmol/L EDTA-Na and

EGTA-Na, 50 mg/L soybean trypsin inhibitor, 2 mmol/L dithiothreitol, 4 mg/L leupeptin, 25 mg/L aprotinin, 0.1 mmol/L benzamidine, 1 mmol/L Na_3VO_4 , and 0.5 mmol/L phenylmethylsulfonyl fluoride and centrifuged at 16 000g for 30 minutes at 4°C. The supernatant (10 μg of protein) was mixed with the loading buffer containing mercaptoethanol and boiled, and the denatured proteins were separated on a 10% polyacrylamide gel.

The proteins were then transferred to polyvinylidene difluoride membranes at 100 V for 1 hour. The membrane was blocked overnight at 4°C with Block Ace (Yukijirushi Nyugyo, Tokyo, Japan) and then incubated with AMPK α (#2532; Cell Signaling Technology, Beverly, MA), phosphospecific AMPK α Thr¹⁷² (#2531, Cell Signaling Technology), phosphospecific ACC Ser⁷⁹ (#07-303; Upstate Biotechnology, Lake Placid, NY), phosphospecific Akt Ser⁴⁷³ (#9271, Cell Signaling Technology), phospho-p38 MAPK Thr¹⁸⁰/Tyr¹⁸² (#9211, Cell Signaling Technology), or phospho-p44/42 MAPK Thr²⁰²/Tyr²⁰⁴ (#9101, Cell Signaling Technology).

To immunoprecipitate the Akt substrate of 160 kd (AS160), an aliquot of the supernatant of buffer A was prepared as described above (300 μg of protein) and incubated with anti-AS160 (Rab-GAP) (#07-741, Upstate Biotechnology) and Protein A Sepharose CL4B beads (Amersham Biosciences, Uppsala, Sweden) overnight at 4°C with end-over-end rotation. The immunoprecipitate was washed 3 times in buffer A and then centrifuged at 4000g for 30 seconds at 4°C. The supernatant was removed, and the beads were mixed with the loading buffer and boiled. The denatured proteins were then separated on a 10% polyacrylamide gel. The proteins were then transferred to polyvinylidene difluoride membranes at 100 V for 1 hour. The membrane was blocked with Block Ace (Yukijirushi Nyugyo) overnight at 4°C and then incubated with anti-AS160 (Rab-GAP) (#30544, Upstate Biotechnology) or antiphospho-(Ser/Thr) Akt substrate (#9611, Cell Signaling Technology) as described previously [29].

The membrane was washed, reacted with anti-rabbit immunoglobulin G coupled to peroxidase, and developed with enhanced chemiluminescence reagents according to the manufacturer's instructions (Amersham Biosciences, Buckinghamshire, United Kingdom). The signals on the blot were detected and quantified using a Lumino LAS-1000 System image analyzer (Fuji Photo Film, Tokyo, Japan). The mean intensity of basal samples in each membrane was used as a reference for controlling gel-to-gel variation. Equal protein loading and transfer were confirmed by Coomassie brilliant blue staining of the membranes.

2.4. Isoform-specific AMPK activity assay

The AMPK activity assay was performed as we described previously [26,27]. Muscle was homogenized in buffer A as described in the Western blot analysis subsection, and the resultant supernatant liquid (100 μg of protein) was immunoprecipitated with isoform-specific polyclonal anti-

bodies directed against the $\alpha 1$ or $\alpha 2$ catalytic subunit of AMPK [26] and Protein A Sepharose CL4B beads. The immunoprecipitate was washed twice in both buffer A and wash buffer (240 mmol/L HEPES, 480 mmol/L NaCl). The kinase reaction was performed in 40 mmol/L HEPES (pH 7.0), 0.1 mmol/L SAMS peptide [26,27], 0.2 mmol/L AMP, 80 mmol/L NaCl, 0.8 mmol/L dithiothreitol, 5 mmol/L MgCl_2 , and 0.2 mmol/L ATP (2 μCi of [γ -³²P] ATP; PerkinElmer, Wellesley, MA) in a final volume of 40 μL for 20 minutes at 30°C. At the end of the reaction, a 15- μL aliquot was removed and spotted onto Whatman P81 paper (Whatman International, Maidstone, United Kingdom). The filter paper was washed at least 6 times in 1% phosphoric acid and once in acetone. ³²P incorporation was quantified using a scintillation counter, and kinase activity was expressed as picomoles of incorporated ATP per milligram of immunoprecipitated protein per minute.

2.5. ATP and PCr assay

The ATP and PCr contents were measured fluorometrically in perchloric acid extracts of epitrochlearis and soleus muscles according to the method of Lowry and Passonneau [30]. In brief, each frozen muscle was homogenized in 0.2 mol/L HClO_4 (3:25 wt/vol) in an ethanol–dry ice bath (–20°C to –30°C) and centrifuged at 16 000g for 2 minutes at –9°C. The supernatant of the homogenate was neutralized with a solution of 2 mol/L KOH, 0.4 mol/L KCl, and 0.4 mol/L imidazole; centrifuged at 16 000g for 2 minutes at –9 °C; and then subjected to enzymatic analysis [30]. The ATP and PCr contents were expressed as nanomoles per milligram wet weight of muscle.

2.6. 3MG transport

The 3MG transport assay was performed as we described previously [25,26]. The muscle was transferred to 2 mL of KRB containing 1 mmol/L of 3-*O*-[methyl-³H]-D-glucose (1.5 $\mu\text{Ci}/\text{mL}$) (American Radiolabeled Chemicals, St Louis, MO) and 7 mmol/L D-[¹⁴C]-mannitol (0.3 $\mu\text{Ci}/\text{mL}$) (American Radiolabeled Chemicals) at 30°C and incubated for 10 minutes. The muscle was blotted onto filter paper, trimmed, frozen in liquid nitrogen, and stored at –80°C. Each frozen muscle was weighed and incubated in 300 μL of 1 mol/L NaOH at 80°C for 10 minutes. The digest was neutralized with 300 μL of 1 mol/L HCl, and the particulates were precipitated by centrifugation at 20 000g for 2 minutes. The radioactivity in aliquots of the digested protein was measured using liquid scintillation counting for dual labels, and the extracellular and intracellular spaces were calculated [31].

2.7. Statistical analysis

The data are expressed as means \pm SE. The mean values were compared using a 1-way analysis of variance followed by a post hoc comparison with Fisher protected least significant difference method. Two mean values were

compared using Student *t* test. Differences between the groups were considered significant if $P < .05$.

3. Results

3.1. BBR increased phosphorylation of AMPK α Thr¹⁷² in skeletal muscles

The Thr¹⁷² residue in both the $\alpha 1$ and $\alpha 2$ catalytic subunits is the primary site responsible for AMPK activation. To determine whether BBR activates AMPK, we measured the degree of phosphorylation of AMPK α Thr¹⁷² using Western blot analysis with a phosphospecific antibody. Compared with the basal condition, both epitrochlearis (Fig. 1A) and soleus (Fig. 1B) muscles stimulated with BBR (0.3 mmol/L, 30 minutes) exhibited increased phosphorylation of Thr¹⁷² without any change in the total amount of AMPK α . Acetyl-coenzyme A carboxylase is a downstream target of AMPK in skeletal muscle, and the phosphorylation of the Ser⁷⁹ site of ACC reflects the total AMPK activity [32,33]. The marked phosphorylation of ACC paralleled the increase in AMPK phosphorylation in epitrochlearis (Fig. 1A) and soleus (Fig. 1B) muscles. The AMPK activator AICAR also caused a strong phosphorylation of AMPK and ACC in epitrochlearis (Fig. 1A), but not in soleus (Fig. 1B).

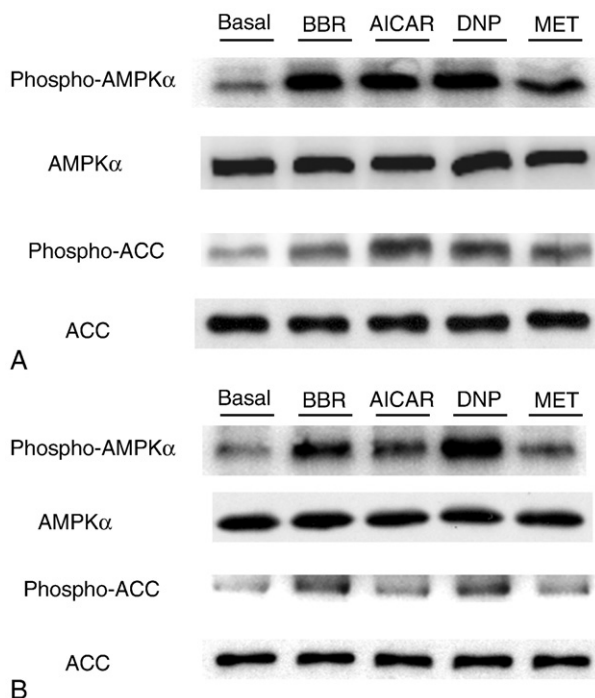


Fig. 1. BBR increases the phosphorylation of AMPK α and ACC in rat skeletal muscle. Isolated epitrochlearis (A) and soleus (B) muscles were incubated in the absence (basal) or presence of BBR (0.3 mmol/L), AICAR (2 mmol/L), or MET (2 mmol/L) for 30 minutes or in the presence of DNP (0.5 mmol/L) for 15 minutes. The tissue lysate was subjected to Western blot analysis with antibodies to phosphorylated AMPK α , AMPK α , phosphorylated ACC, or ACC. Representative immunoblots are shown.

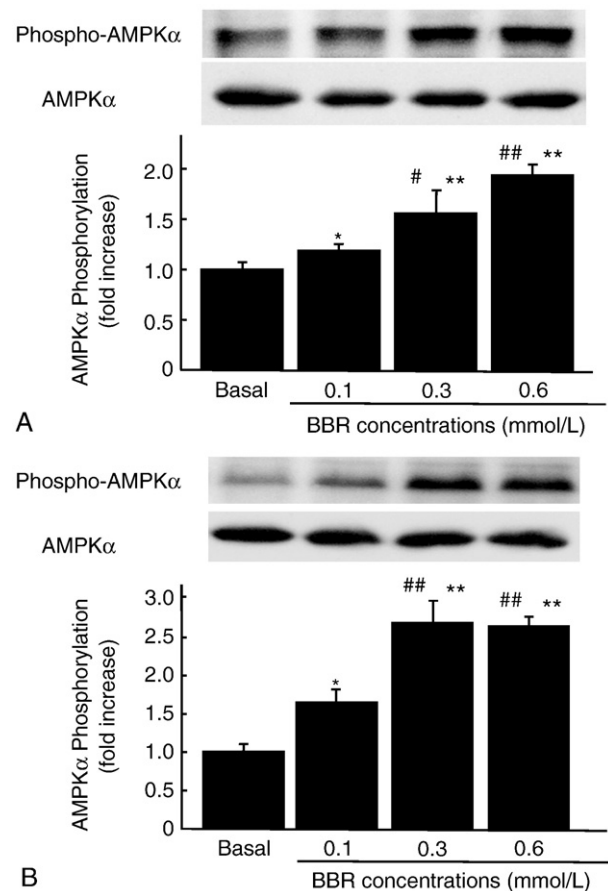


Fig. 2. BBR increases phosphorylation of AMPK α in a dose-dependent manner in rat skeletal muscles. Isolated epitrochlearis (A) and soleus (B) muscles were incubated in the presence of 0 (basal), 0.1, 0.3, or 0.6 mmol/L of BBR for 30 minutes. The tissue lysate was subjected to Western blot analysis with antibodies to phosphorylated AMPK α or AMPK α . Fold increases are expressed relative to the level in muscles of the basal group. The values are means \pm SE. The number of muscles in each group is as follows: basal (8), BBR 0.1 mmol/L (6), BBR 0.3 mmol/L (8), and BBR 0.6 mmol/L (6). * $P < .05$ and ** $P < .01$ vs basal; # $P < .05$ and ### $P < .01$ vs BBR 0.1 mmol/L.

AICAR is taken up into skeletal muscle and metabolized by adenosine kinase to form ZMP, a monophosphorylated derivative that mimics the effects of AMP on AMPK [5]. The pharmacologic inhibitor of oxidative phosphorylation, DNP, which induces a marked reduction of muscle ATP and PCr levels [27], also caused significant phosphorylation of AMPK and ACC in both muscle types (Fig. 1A and B). Metformin elicited moderate phosphorylation of AMPK and ACC in both muscles (Fig. 1A and B).

To determine the dose and time dependency of the AMPK phosphorylation, muscles were stimulated with BBR at various concentrations and for various times. The dose-response study showed a significant phosphorylation of 1.2-, 1.6-, and 2.0-fold in epitrochlearis muscle (Fig. 2A) and 1.6-, 2.7-, and 2.6-fold in soleus muscle (Fig. 2B) at concentrations of 0.1, 0.3, and 0.6 mmol/L of BBR, respectively. The time course study revealed that 0.3 mmol/L of BBR induced

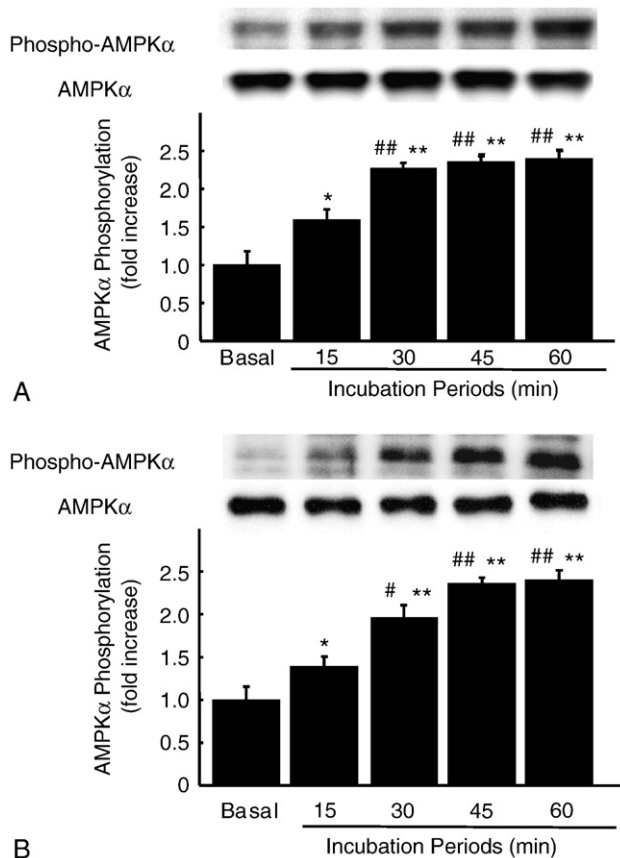


Fig. 3. BBR increases phosphorylation of AMPK α in a time-dependent manner in rat skeletal muscles. Isolated epitrochlearis (A) and soleus (B) muscles were incubated in the presence of BBR (0.3 mmol/L) for 0 (basal), 15, 30, 45, or 60 minutes. The tissue lysate was subjected to Western blot analysis with antibodies to phosphorylated AMPK α or AMPK α . Fold increases are expressed relative to the level in muscles of the basal group. The values are means \pm SE. The number of muscles in each group is as follows: basal (8), BBR 15 minutes (6), BBR 30 minutes (8), BBR 45 minutes (6), and BBR 60 minutes (6). * P < .05 and ** P < .01 vs basal; # P < .05 and ## P < .01 vs BBR 15 minutes.

acute phosphorylation of AMPK in both epitrochlearis (Fig. 3A) and soleus (Fig. 3B) muscles after an incubation period as short as 15 minutes.

3.2. BBR activated both AMPK α 1 and α 2 in skeletal muscles

To identify which catalytic subunit is activated by BBR, isoform-specific AMPK activity was measured in anti- α 1 and anti- α 2 immunoprecipitates of the muscle after treatment with BBR. BBR stimulation (0.3 mmol/L, 30 minutes) significantly increased the activity of both AMPK α 1 and AMPK α 2 in epitrochlearis muscle: the activity of AMPK α 1 = 14.0 ± 1.1 pmol/(mg min) in the basal condition and 50.7 ± 2.9 pmol/(mg min) after incubation with BBR, and the activity of AMPK α 2 = 12.2 ± 1.3 pmol/(mg min) in the basal condition and 30.4 ± 2.5 pmol/(mg min) after incubation with BBR (Fig. 4A). In soleus muscle, the activity of

AMPK α 1 was 54.3 ± 7.8 pmol/(mg min) in the basal condition and 104.0 ± 5.0 pmol/(mg min) after incubation with BBR; and the activity of AMPK α 2 was 17.1 ± 1.5 pmol/(mg min) in the basal condition and 29.3 ± 1.9 pmol/(mg min) after incubation with BBR (Fig. 4B).

3.3. BBR stimulated glucose transport in skeletal muscles in the absence of insulin

We investigated whether the activation of AMPK in skeletal muscle by BBR is associated with enhanced glucose transport. In the absence of insulin, incubation with BBR (0.3 mmol/L, 30 minutes) increased the rate of 3MG transport 1.7-fold in epitrochlearis muscle (Fig. 5A) and 1.4-fold in soleus muscle (Fig. 5B) compared with the basal conditions. AICAR significantly increased 3MG transport activity in epitrochlearis muscle (Fig. 5A), but not in soleus (Fig. 5B). Akt is a key molecule in the insulin-stimulated signaling pathway leading to enhanced glucose transport and metabolism. Recent studies suggest that AS160 is a downstream nexus for both Akt-mediated and AMPK-

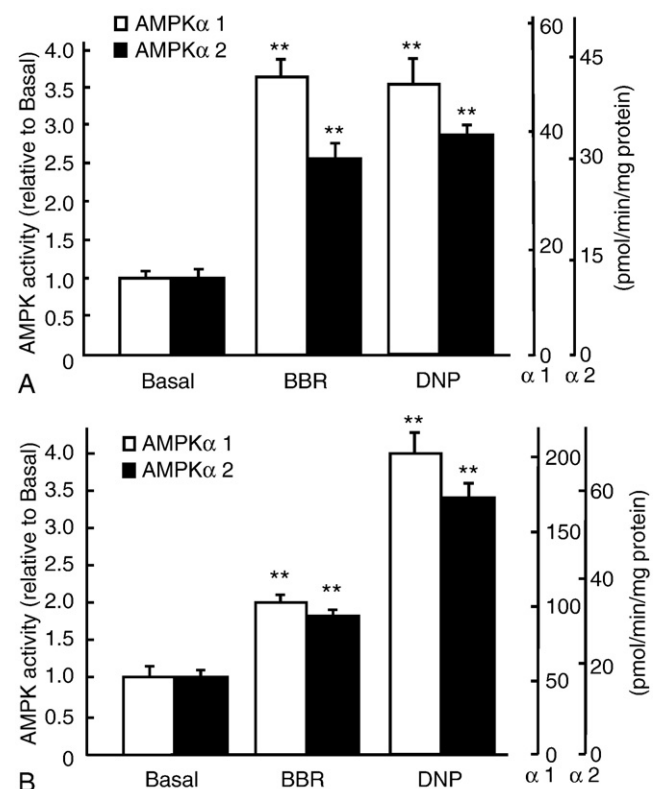


Fig. 4. BBR activates both AMPK α 1 and α 2 activities in rat skeletal muscles. Isolated epitrochlearis (A) and soleus (B) muscles were incubated in the absence (basal) or presence of BBR (0.3 mmol/L) for 30 minutes or in the presence of DNP (0.5 mmol/L) for 15 minutes. The isoform-specific AMPK activity was determined in the anti-AMPK α 1 and anti-AMPK α 2 immunoprecipitates. Fold increases are expressed relative to the level in muscles of the basal group. The values are means \pm SE. The number of muscles in each group is as follows: basal (7), BBR (7), and DNP (4). ** P < .01 vs basal.

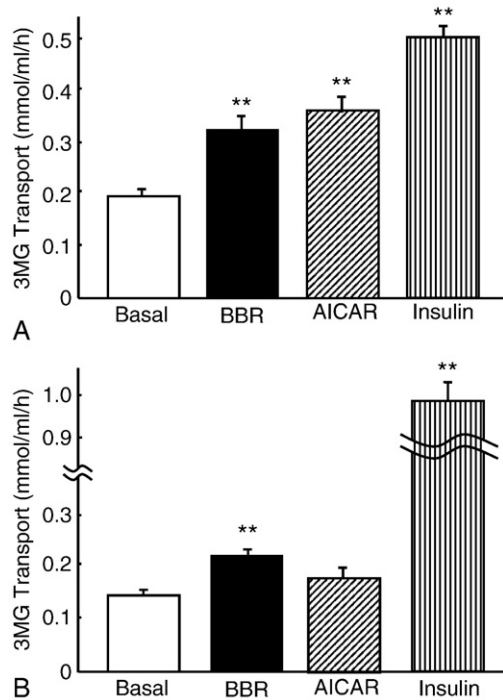


Fig. 5. BBR increases 3MG transport in rat skeletal muscles. Isolated epitrochlearis (A) and soleus (B) muscles were incubated in the absence (basal) or presence of BBR (0.3 mmol/L), AICAR (2 mmol/L), or insulin (1 μ mol/L) for 30 minutes; and then 3MG transport activity was determined. The values are means \pm SE. The number of muscles in each group is as follows: basal (7), BBR (7), AICAR (4), and insulin (4). ** $P < .01$ vs basal.

mediated glucose transport in skeletal muscle [34,35] and that AS160 is an intracellular substrate of AMPK in skeletal muscle [35]. In our study, BBR significantly increased phosphorylation of AS160 (Fig. 6) without changing the phosphorylation status of Akt (Fig. 7). In addition, BBR

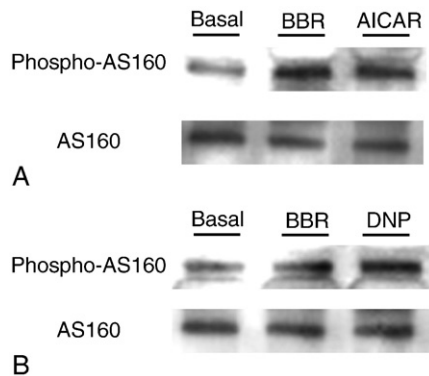


Fig. 6. BBR increases phosphorylation of AS160 in rat skeletal muscles. Isolated epitrochlearis (A) muscle was incubated in the absence (basal) or presence of BBR (0.3 mmol/L) or AICAR (2 mmol/L) for 30 minutes. Isolated soleus (B) muscle was incubated in the absence (basal) or presence of BBR (0.3 mmol/L) for 30 minutes or DNP (0.5 mmol/L) for 15 minutes. The immunoprecipitate with anti-AS160 (Rab-GAP) was subjected to Western blot analysis with antiphospho-(Ser/Thr) Akt substrate or anti-AS160 (Rab-GAP). Representative immunoblots are shown.

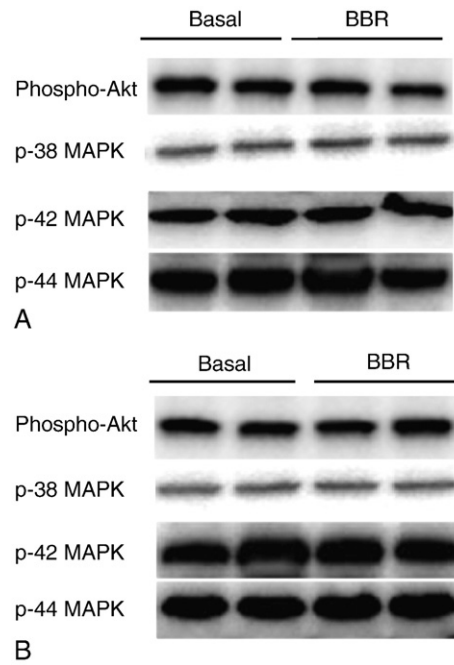


Fig. 7. BBR does not increase phosphorylation of Akt, p38 MAPK, or p42/44 MAPK in rat skeletal muscles. Isolated epitrochlearis (A) and soleus (B) muscles were incubated in the absence (basal) or presence of BBR (0.3 mmol/L) for 30 minutes. The tissue lysate was subjected to Western blot analysis with antibodies to phosphorylated Akt, p38 MAPK, or p44/42 MAPK. Representative immunoblots are shown.

treatment had no effect on the phosphorylation status of p38 or p44/42 MAPK (Fig. 7).

3.4. BBR decreased energy status in skeletal muscles

AMPK is activated in response to energy-consuming stress, such as muscle contraction, hypoxia, and inhibition of oxidative phosphorylation [27]. To clarify whether BBR increases AMPK activity by energy deprivation, we measured the muscle content of ATP and PCr. The decrease in ATP content was not statistically significant, but PCr content after the stimulation of BBR (0.3 mmol/L, 30 minutes) was significantly lower than that of the basal samples in both epitrochlearis and soleus muscles (Table 1).

Table 1
BBR decreases PCr levels in rat skeletal muscles

		Basal	BBR
Epitrochlearis	ATP	5.7 \pm 0.5	5.2 \pm 0.4
	PCr	19.7 \pm 0.7	12.9 \pm 1.6*
Soleus	ATP	2.5 \pm 0.3	2.1 \pm 0.3
	PCr	9.8 \pm 1.2	5.5 \pm 0.5*

Isolated epitrochlearis and soleus muscles were incubated in the absence (basal) or presence of BBR (0.3 mmol/L) for 30 minutes, and the intracellular ATP and PCr contents (in nanomoles per milligram wet weight of muscle) were measured. The values are means \pm SE. The number of muscles in each group is as follows: epitrochlearis—ATP (8), PCr (8); soleus—ATP (6), PCr (8).

* $P < .01$ vs basal.

4. Discussion

Our data show 3 novel findings relating to the metabolic effect of BBR on skeletal muscle. First, BBR had the ability to increase AMPK α Thr¹⁷² phosphorylation accompanied with both AMPK α 1 and α 2 activities (Figs. 1–4). The increased phosphorylation of endogenous substrate AS160 (Fig. 6) and enhanced 3MG transport (Fig. 5) as well as ACC phosphorylation (Fig. 1) are indicative of a substantial increase in AMPK activity *in vivo*. Second, these effects were observed in both fast-glycolytic epitrochlearis and slow-oxidative soleus muscles, suggesting that the stimulatory effect of BBR on AMPK is not specific to a particular muscle type. Third, BBR-stimulated AMPK activation was associated with a reduction in the fuel status of skeletal muscle (Table 1), as with contraction-stimulated AMPK activation.

We used an isolated muscle preparation incubated *in vitro* to eliminate systemic confounders, such as circulatory, humoral, and neuronal factors. In particular, we considered the possibility that oral administration of BBR, which acts as an α -galactosidase inhibitor [36,37], could evoke indirect metabolic changes in skeletal muscle by suppressing glucose absorption in intestines. In addition, because AMPK α 1 is activated immediately as a postmortem artifact during the dissection procedure [38], we exposed muscles to BBR after a preincubation period sufficient to stabilize AMPK α 1 activity. Thus, our method made it possible to evaluate the direct effect of BBR on muscle tissue and both AMPK α 1 and AMPK α 2 activities.

Incubation of epitrochlearis and soleus muscles with BBR (≥ 0.1 mmol/L, ≥ 15 minutes) increased AMPK phosphorylation in dose- and time-dependent manners, with peak levels of phosphorylation at greater than or equal to 0.3 mmol/L and greater than or equal to 30 minutes (Figs. 2 and 3). We have recently reported that incubation of rat epitrochlearis and soleus muscles with caffeine (≥ 3 mmol/L, ≥ 15 minutes) increases AMPK phosphorylation in dose- and time-dependent manners [39]. We have also shown that activation of AMPK α 1 and α 2 by caffeine is associated with a reduction in PCr content and an increased rate of 3MG transport in the absence of insulin [39]. These results suggest that BBR has similar actions to caffeine by acutely stimulating both AMPK α isoforms and insulin-independent glucose transport with a reduction of the intracellular energy status. It is noteworthy that consumption of beverages containing caffeine including coffee and green tea is associated with a reduced risk of type 2 diabetes mellitus [40,41].

We examined the effects of BBR in fast-glycolytic and slow-oxidative muscles because activation of AMPK and its effects may vary by muscle type. In the studies using rat skeletal muscle, repeated AICAR administration elicited the greatest increases in GLUT4 and glycogen content in fast-twitch muscles [42–44]. Daily subcutaneous injections of AICAR for 4 weeks increased GLUT4 and glycogen content

in the red and white quadriceps, but not in the soleus muscle in rats [43]. Furthermore, AICAR exposure stimulated glucose transport in rat white muscles, but had no effect in soleus muscle [45,46]. We also found significant increases in AMPK phosphorylation and 3MG transport activity by AICAR in epitrochlearis muscle, but not in soleus (Figs. 1 and 5). In contrast, incubation with BBR markedly increased AMPK phosphorylation and 3MG transport in both epitrochlearis and soleus muscles (Figs. 1 and 5). Considering that caffeine stimulates AMPK activity with a reduction of the intracellular energy status in both incubated epitrochlearis and soleus muscles [39], energy deprivation *per se* may be important in activating AMPK in rat soleus. Because of the non-muscle type-specific stimulation by BBR, we believe that most skeletal muscles respond to BBR.

The finding that BBR increased AMPK α Thr¹⁷² phosphorylation provides evidence that BBR induces covalent modification via upstream kinases. The LKB1 complex is constitutively active, and the binding of AMP to AMPK facilitates the phosphorylation of AMPK by the LKB1 complex [47,48]. Thus, LKB1 is believed to be a crucial AMPK kinase in the response to energy deprivation in skeletal muscle. In the present study, AMPK activation was accompanied by a decrease in PCr content (Table 1), raising the possibility that LKB1 is involved in AMPK activation by BBR.

Although we did not examine the long-term effect of BBR on muscle metabolism, Kim et al [49] have recently shown that intraperitoneal administration of BBR once a day for 3 weeks increases AMPK and ACC phosphorylation in gastrocnemius muscle of the *db/db* mouse (they did not examine the acute effect of BBR). In our earlier study, repeated activation of AMPK by intraperitoneal injection of AICAR, 3 times a day for 7 days, increased GLUT4 protein and insulin-stimulated 2-deoxyglucose transport activity in mouse soleus muscle [8]. Similarly, in ZDF rats, which are characterized by a progressive β -cell dysfunction and a leptin receptor defect, AICAR treatment over a period of 8 weeks increased whole-body insulin sensitivity, which was associated with the level of muscle GLUT4 expression [50]. These data lead us to speculate that repeated activation of AMPK by BBR may achieve long-term metabolic benefits in skeletal muscle and protect against the development of diabetes. In this context, further studies are needed to clarify whether oral administration of BBR at a physiologic dose (1.0–1.5 g daily) [20,21] results in AMPK activation in human skeletal muscle.

Cheng et al [17] have shown that BBR stimulates glucose transport and promotes AMPK phosphorylation and p38 MAPK phosphorylation, and that the BBR-stimulated glucose transport is partially inhibited by an AMPK inhibitor compound C or p38 MAPK inhibitor SB202190 in L6 myotubes. In contrast, Zhou et al [51] have reported that, in 3T3-L1 adipocytes, BBR-stimulated glucose transport is not affected by SB203580 but is partially inhibited by PD98059, a p44/42 MAPK kinase (MEK) inhibitor that leads to

inactivation of p44/42 MAPK. In the present study, BBR stimulation (0.3 mmol/L, 30 minutes) did not induce an increase in p38 MAPK phosphorylation or p44/42 MAPK phosphorylation, although the BBR stimulation significantly increased the rate of 3MG transport in skeletal muscles. Our data suggest that p38 and p44/42 MAPK signaling does not play a major role in activating glucose transport in intact skeletal muscle.

In summary, we demonstrated for the first time that BBR increases both AMPK α 1 and α 2 activities in fast- and slow-twitch skeletal muscles, and that this activation is accompanied by insulin-independent glucose transport and a reduction of muscle energy status. We propose that, BBR can activate muscle glucose metabolism, at least in part, by stimulating AMPK.

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