Biochemical and Biophysical Research Communications 424 (2012) 100–104

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Gambogic acid activates AMP-activated protein kinase in mammalian cells

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ARTICLE INFO

Article history:
Received 29 May 2012
Available online 22 June 2012

Keywords:
AMPK
Gambogic acid
Reactive oxygen species

ABSTRACT

AMP-activated protein kinase (AMPK) plays a key role in maintaining intracellular and whole-body energy homeostasis. Activation of AMPK has been shown to ameliorate the symptoms of metabolic diseases, such as type 2 diabetes and obesity. Here we show that gambogic acid (GB), a known antitumor agent, activates AMPK by increasing the phosphorylation of AMPKα and its downstream substrate ACC in various cell lines. Further study revealed that GB stimulated AMPK activity independent of upstream kinases. Moreover, the AMPK inhibitor, compound C, has no effects on the GB-induced AMPK activation. We also found that GB promptly increased intracellular ROS level, and antioxidants attenuated the ROS production. Interestingly, only the thiol antioxidants significantly abolished GB-enhanced AMPK activation. In addition, analysis of binding and dissociation kinetics indicated that GB bound to the AMPKα subunit. Collectively, these results suggest that GB may be a novel direct activator of AMPK.

1. Introduction

AMP-activated protein kinase (AMPK) is a serine/threonine kinase that functions as a sensor of AMP/ATP and ADP/ATP levels to maintain energy balance at both the cellular and the whole-body levels, therefore, AMPK is a potential target for drug design against metabolic syndrome, obesity and type 2 diabetes [1–3]. In addition, recent reports have suggested that AMPK activators may also potentially function as anticancer drugs [3–5]. The AMPK activator, metformin, has been the subject of many recent studies in various tumor models, starting from efforts to establish its inhibitory effects on cell lines and animal models, ultimately leading to human clinical trials.

Gambogic acid (GB) is a polyprenylated xanthone isolated from the resin of gamboges, a brownish to orange resin exuded from *Garcinia hanburryi* in southeast Asia [6]. It has been demonstrated that GB has anticancer effects and inhibits the growth of several types of human cancer cells in vitro and in vivo. Many proteins have been revealed in the action of GB including Hsp90, p53/Mdm2 complex, bcl-2 family, topoisomerase II, transferrin receptor and stathmin [7]. More recently, it was reported that GB covalently binds to the IKKβ protein thus mediating the suppression of the NF-κB activity in macrophages [8].

During our screening for AMPK activators, GB was serendipitously identified as a AMPK activator. In the present study, we report for the first time that GB activates the AMPK signaling pathway by directly interacting with AMPK.

2. Materials and methods

2.1. Materials

Gambogic acid, 5-amino-4-imidazolecarboxamide riboside (AICAR), STO-609, compound C and anti-β-actin antibody were purchased from Sigma (St. Louis, MO). Anti-AMPKα, anti-ACC, anti-phospho-AMPKα (Thr-172) and anti-phospho-ACC (Ser-79) antibodies were purchased from Cell Signaling Technology (USA).

2.2. Cells and cell cultures

The primary mouse hepatocyte cell line AML12 (a gift from Dr. Qiao Wu, Xiamen university, China) was maintained in the growth medium [a mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium (1:1) with 0.005 mg/ml insulin, 0.005 mg/ml transferrin, 5 ng/ml selenium, 40 ng/ml dexamethasone, and 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA)]. HeLa cells were cultured in DMEM medium with 10% heat-inactivated fetal calf serum (Invitrogen). Cultures were maintained in a humidified incubator at 37 °C in an atmosphere of 5% CO_2.

2.3. Quantitative analysis of intracellular ROS level

Production of ROS was measured by flow cytometry as described previously [9]. Briefly, after being treated with GB for

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http://dx.doi.org/10.1016/j.bbrc.2012.06.078

0.5 or 1 h, cells were collected and resuspended in PBS supplemented with 10 μM 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA, Invitrogen, Carlsbad, CA) or a DMSO vehicle. After incubation for 0.5 h at room temperature, fluorescence was measured by flow cytometry on a FACScan flow cytometer. Average ROS production (relative to level of vehicle-treated controls) was calculated from four individual wells in at least three independent plates.

2.4. Western blot analysis

Lysate proteins (20 μg) from treated cells were subjected to Western blot analysis following the protocols as described previously [10]. The amount of protein and the extent of phosphorylation were estimated using the following primary antibodies: anti-phospho-AMPKα-Thr-172 antibody (1:1000 dilution), anti-AMPKα antibody (1:1000 dilution), anti-phospho-ACC-Ser-79 antibody (1:1000 dilution) and anti-ACC antibody (1:1000 dilution). The total protein content of each sample was quantified using β-actin (1:5000 dilution).

2.5. Expression and purification of recombinant proteins

The expression and purification of the recombinant human AMPKα1 subunit was carried out as described previously [11]. Briefly, recombinant proteins were induced with 0.1 mM isopropyl β-D-thiogalactopyranoside, and cultures were grown at 16 °C overnight. Then cells were harvested, washed, resuspended in lysis buffer [15% sucrose (w/v), 50 mM sodium phosphate, pH 7.5, 100 mM NaCl, 10 mM imidazole, and 1 mM β-mercaptoethanol] with 1% Triton X-100, and sonicated on ice. After centrifugation, the supernatant was loaded onto nickel-nitrilotriacetic acid-agarose. After washing with lysis buffer containing 50 mM imidazole, the proteins were eluted with lysis buffer containing 250 mM imidazole and dialyzed with buffer [50 mM Tris–HCl (pH 7.5), 1 mM β-mercaptoethanol, 1 mM EDTA] at 4 °C to remove imidazole and then stored at −70 °C until use.

2.6. Fluorescent measurements

Fluorescence intensities were recorded in the range of 290–500 nm at 303 K using a Cary Eclipse (Varian). The experiments were performed in 2 ml of a 5 μM proteins solution (10 mM PBS buffer, pH 7.6) with successive additions of GB solution (in 0.2% DMSO) from 0.5 to 50 μM [12]. All tests were repeated in triplicate.

2.7. ForteBio Octet Red system assay

The interaction between AMPK and GB was measured tested by the ForteBio Octet Red System (ForteBio Inc., American). The biotinylated protein targets (e.g., AMPKα subunit) were immobilized onto Super Streptavidin Biosensors, and the association and dissociation of the molecule GB was monitored in parallel to minimize time [13]. All tests were repeated in triplicate.

3. Results

3.1. GB activates AMPK in various cells

To investigate whether AMPK is activated by GB in primary rat hepatocyte AML12, the phosphorylation of AMPK and its downstream target, ACC, an enzyme in the fatty acid synthesis pathway, was used as indicators of AMPK activation. AICAR was used as a positive control in this experiment. An 1 h treatment of AML12 with GB dose-dependently increased AMPK and ACC phosphorylation, as detected by Western blot analysis using phospho-specific antibodies targeted to Thr-172 of the AMPKα subunit and Ser-79 of ACC (Fig. 1A). The phosphorylation of AMPK and ACC reached a maximal level by the treatment of 2 μM GB, which was greatly

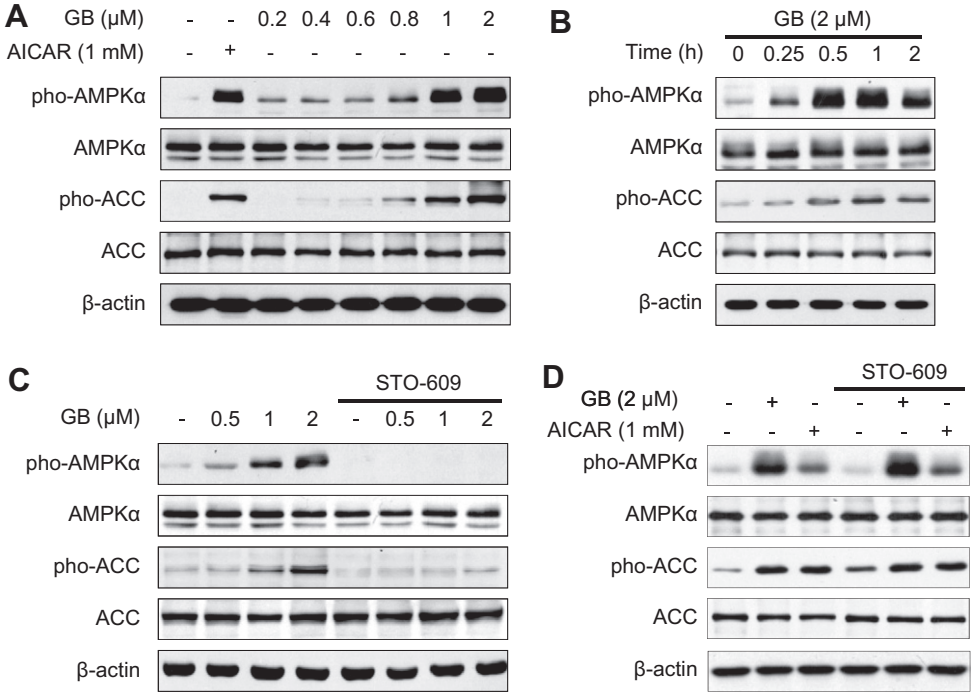

Fig. 1. GB activated the intracellular AMPK. (A) GB stimulates AMPK and ACC phosphorylation in AML12 cells does-dependently after incubation for 1 h. AICAR was used as a positive control. (B) GB stimulates AMPK and ACC phosphorylation in AML12 cells time-dependently. (C) GB stimulates AMPK and ACC phosphorylation in HeLa cells after incubation for 1 h, and these effects were blocked by 10 μg/ml CaMKKβ inhibitor STO-609 pretreatment for 1 h. (D) Effects of STO-609 on GB-induced AMPK activation. The AML12 cells were pretreated with or without 10 μg/ml STO-609 for 1 h prior to the 1 h treatment of GB.

higher than that induced by 1 mM AICAR. The immunoblot result showed that GB greatly increased the phosphorylation of AMPK and ACC in a time-dependent manner (Fig. 1B), which reached a maximal level by the treatment of 2 μM GB for 30 min. No commensurate changes were observed in the overall levels of AMPK and ACC proteins after treatments with GB (Fig. 1).

To further examine the broad-spectrum AMPK activation by GB, we examined the phosphorylation status of AMPK and ACC in other four cell lines, including human breast carcinoma cell line MDA-MB-435, human hepatoma cell line HepG2, mouse embryo fibroblast cell line NIH/3T3 and mouse preadipocyte cell line 3T3-L1. In these cells exposed for 1 h to varying concentrations of GB, the phosphorylation of AMPK and ACC was significantly stimulated, but the expression of endogenous AMPK and ACC protein were not changed, respectively (Supplemental Fig. S1).

There are two kinases, LKB1 and CaMKKβ, identified as AMPK upstream kinases in cells, and the major AMPK upstream kinase in AML12 cells is LKB1 [14]. To investigate whether GB activates AMPK through LKB1 activation, we chose HeLa cells which is deficient in LKB1 for the analysis of AMPK activation. We found that GB can also stimulate the phosphorylation of AMPK and ACC in HeLa cells after GB treatment for 1 h (Fig. 1C), which suggests that GB activated AMPK independent of LKB1. To further determine if CaMKKβ is required for GB-enhanced AMPK activation, STO-609 (10 μg/ml), a specific CaMKKβ inhibitor, was preincubated with HeLa cells prior to GB treatment. STO-609 at the stated concentrations significantly inhibited the basal or GB-enhanced phosphorylation of the phosphorylation of AMPKα and ACC in HeLa cells (Fig. 1C). However, STO-609 has no effect on the GB-mediated AMPK activation in AML12 cells (Fig. 1D). These data indicated that GB activates the cellular AMPK pathway independent of AMPK upstream kinases activation.

3.2. GB-induced AMPK activation is independent of ROS generation

Previous studies have shown that reactive oxygen species (ROS) plays an important role in the AMPK activation [15–18]. To investigate whether endogenous ROS was also involved in AMPK activation caused by GB, the generation of intracellular ROS was monitored using the fluorescent probe H_2DCFDA. As shown in Fig. 2A, exposure of AML12 cells to GB resulted in a rapid transient increase in ROS levels. ROS generation was significantly increased as early as 30 min after treatment with GB, and continued to rise when incubation prolonged, indicating that GB triggered intracellular ROS generation.

We next evaluated if antioxidants altered GB-induced AMPK activation in AML12 cells. Preincubation of AML12 cells with *N*-Acetyl-L-cysteine (NAC), glutathione (GSH) and DTT significantly attenuated GB-enhanced phosphorylation of AMPK and ACC. In contrast, Vitamin C (VC), a non-thiol antioxidant, had no effects on GB-enhanced AMPK activation in AML12 cells (Fig. 2C).

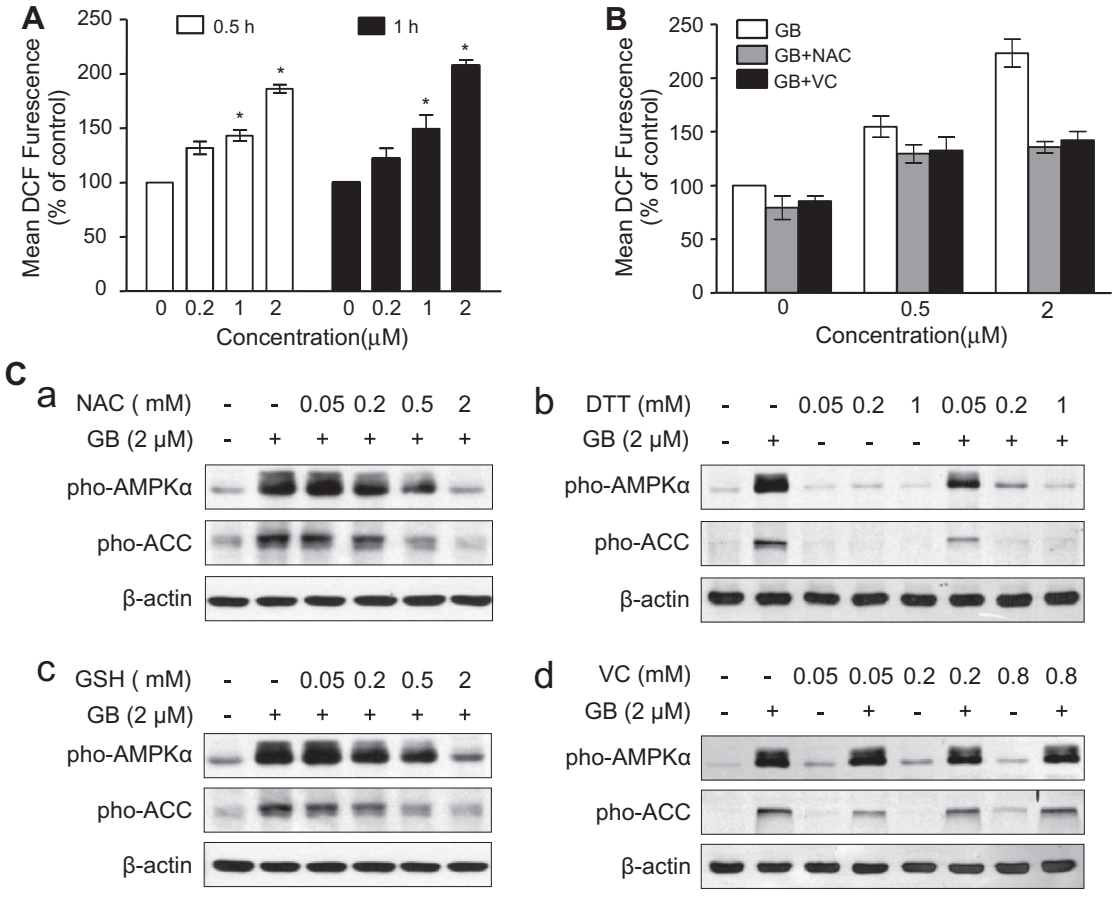

Fig. 2. ROS is not involved in GB-induced AMPK activation. (A) Effects of GB on the ROS level in AML12 cells. AML12 cells were treated with different concentrations of GB for 0.5 h or 1 h, and then the ROS level was determined by measuring DCF fluorescence intensity as described in the materials and methods. The data that are shown are the mean ± SD ($n = 3$). *$p < 0.05$ vs. the control. (B) Effects of NAC and VC on the GB-stimulated ROS production. AML12 cells were pretreated with NAC (2 mM) or vitamin C (VC, 0.5 mM) for 0.5 h prior to the 1 h treatment of MED, and then the ROS level was determined by measuring DCF fluorescence intensity. The data that are shown are the mean ± SD ($n = 3$). (C) Effects of ROS scavengers on the GB-induced AMPK activation. AML12 cells were pretreated with NAC (a), DTT (b), GSH (c) and vitamin C (VC, d) for 0.5 h prior to the 1 h treatment of GB separately, and the phosphorylation of AMPKα at Thr172 and ACC at Ser79 were analyzed by Western blotting using relevant antibodies.

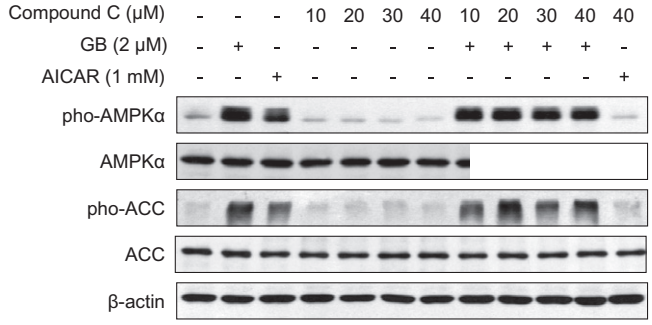

Fig. 3. Effects of compound C on the GB-induced AMPK activation in AML12 cells. AML12 cells were pretreated with compound C for 1 h prior to the 1 h treatment of GB. The phosphorylation of AMPKα at Thr172; ACC at Ser79; total AMPKα and ACC were determined by Western blotting. β-Actin was used for normalization and verification of protein loading.

To further confirm if ROS is responsible for GB-induced AMPK activation, AML12 cells were pretreated for 0.5 h with NAC (2 mM), or VC (400 μM) prior to the stimulation of GB respectively. Similarly to NAC, VC also inhibited GB-induced ROS generation (Fig. 2B). Overall these results indicate that ROS is not involved in GB-induced AMPK activation.

3.3. Compound C does not inhibit GB-induced AMPK activation in AML12 cells

Compound C, a cell-permeable pyrrazolopyrimidine compound, selectively inhibits AMPK activity by acting as a competitive inhibitor of ATP binding to the catalytic α subunit of AMPK [19]. The phosphorylations of AMPK and ACC in AML12 cells were determined by Western blotting after pretreatment with compound C prior to treatment with 2 μM GB for 1 h (Fig. 3). Interestingly, compound C has no effect on the GB-mediated AMPK activation in AML12 cells, even at the highest concentration tested (40 μM, a concentration of compound C that has usually been used). However, under the same conditions, compound C significantly inhibits the phosphorylations of AMPK and ACC induced by 1 mM AICAR. These data indicated that the mechanism by which GB activates AMPK is not the same as the mechanism by which AICAR enhances AMPK activation.

3.4. GB interacts physically with AMPKα subunit

GB-induced AMPK activation was eliminated by the thiol antioxidants (NAC, GSH and DTT) (Fig. 2C), suggesting that a covalent link was likely formed between GB and the thiol antioxidants. In addition, we observed a slight mobility change of the phosphorylated form of AMPKα subunit on the electrophoresis gel after GB treatment (Fig. 1). This mobility change, however, was not observed for the unphosphorylated form of AMPKα subunit. Taken together, we speculate that GB may directly bind to AMPKα subunit.

To test the hypothesis, the binding of GB to the AMPK α subunit was firstly characterized by fluorescence quenching. His-tagged AMPKα subunit displayed the maximal fluorescence at 327 nm. When AMPKα subunit was incubated with increasing concentrations of GB, the fluorescence intensity gradually decreased (Fig. 4A), indicating that GB interacts with AMPKα subunit. We also used the ForteBio Octet Red system to determine the binding affinities of GB to AMPKα subunit (Fig. 4B). The two calculated dissociation constants (Kd) were within the same range as that we obtained from quenching experiments (6.56 versus 7.81 μM, respectively), verifying the binding of GB to the AMPKα subunit.

4. Discussion

AMPK is composed of a catalytic subunit (α subunit) and two regulatory subunits (β and γ subunits) [11]. In order for the kinase activity of AMPK to be induced, phosphorylation of residue Thr172 located in the α subunit of the complex is required [20]. The phosphorylation status of Thr172 is determined by the relative rates of phosphorylation (catalyzed by upstream AMPK kinases) and dephosphorylation (catalyzed by AMPK phosphatases) in response to changes in AMP/ATP and ADP/ATP ratio. Under lowered intracellular ATP levels, AMP or ADP can directly bind to the γ regulatory subunits of AMPK, leading to a conformational change that promotes AMPK phosphorylation and also protects AMPK from dephosphorylation [21]. AICAR, due to its intracellular conversion to ZMP that binds to the γ subunit and mimics the effect of AMP, has been widely used to stimulate AMPK.

To data, there are only two small molecular AMPK activators A769662 and PT1 that directly activate AMPK through interaction with the glycogen binding domain of the β subunit and antagoniz-

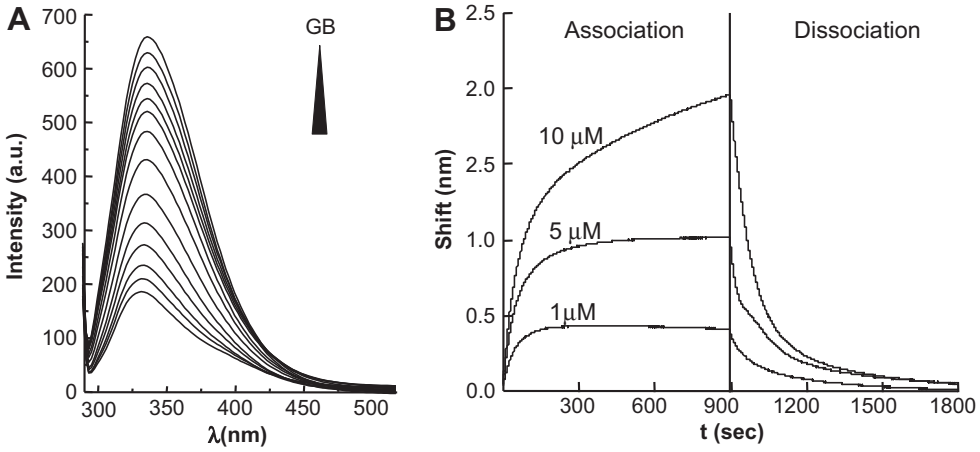

Fig. 4. GB physically binds to the AMPK α subunit. (A) GB quenched the intrinsic fluorescence of the AMPK α subunit in a concentration-dependent manner. The vertical and horizontal axes represent the fluorescent intensity and emission wavelength, respectively. The excitation wavelength is 280 nm, whereas the AMPK α subunit emission peak is at 327 nm. (B) The vertical and horizontal axes represent the light shift distance (nm) and association/dissociation time (s), respectively. The dissociation constant Kd (6.56 μM) of GB is calculated from the association and dissociation curve of GB with AMPKα subunit.

ing the autoinhibition in α subunit, respectively. The effects of A769662 and PT1 were blocked by compound C [22,23]. In this study, we found that GB promptly activated AMPK by increasing the phosphorylation of AMPKα and its downstream substrate ACC in various cell lines (Fig. 1A and B). Further study revealed GB stimulated AMPK activity independent of upstream kinases (Fig. 1C and D). In addition, our data showed that AMPK activation by GB is independent of ROS generation (Fig. 2) and cannot be blocked by compound C (Fig. 3). Therefore, our data suggest that GB may be a novel direct activator of AMPK.

It was reported that compound C inhibits the adenosine transporter, the primary transporter for the uptake of AICAR into cells [24]. However, we found that AICAR-enhanced AMPK activity was significantly inhibited regardless of the pretreatment or post-treatment with compound C (data not shown). Recently, the crystal structure of the human AMPKα2 subunit in complex with compound C has revealed that compound C could reduce the phosphorylation of Thr172 by AMPK kinases and/or promote the dephosphorylation of Thr172 by protein phosphatases [25]. The observation that compound C did not affect GB-mediated AMPK activation strongly suggests that GB stimulates AMPK activity in a manner that differs from AMP, A769662 and PT1 (Fig. 3).

It is known that the electrophilic α,β-unsaturated group in bioactive compounds selectively reacts with protein thiols, by which these compounds regulate the activity of proteins [26,27]. In addition, GB has been demonstrated as an inhibitor of IKK that covalently reacted with IKK by Michael addition. We speculated that GB may also directly bind to AMPK by the same reaction mechanism, leading to a conformational change that promotes AMPK phosphorylation and/or protects AMPK from dephosphorylation. The fact that GB-induced AMPK activation was attenuated by the thiol antioxidants (NAC, GSH and DTT) treatment supports this hypothesis (Fig. 2C). Moreover, we also observed that GB directly interact with AMPKα subunit through the fluorescence quenching and ForteBio Octet Red System (Fig. 4). In fact, GB was observed to bind to AMPKα subunit and the heterotrimer (α1β1γ1) with similar dissociation constants (Kd), but not to γ subunit (data not shown). However, the detailed mechanism by which GB interacts with AMPKα subunit remains to be understood.

In summary, our findings provide the first evidence that GB activated the AMPK signaling pathway by directly interacting with AMPK, and future studies should explore the effects of GB as a novel direct activator of AMPK in more detail.

Acknowledgments

This work was supported by 973 Program (2010CB833802) and NSFC (90913024).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.06.078.

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