SMALL MOLECULE ANTAGONIZES AUTOINHIBITION AND ACTIVATES AMP-ACTIVATED PROTEIN KINASE IN CELLS

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AMP-activated protein kinase (AMPK) serves as an energy sensor and is considered a promising drug target for treatment of type II diabetes and obesity. Previous report has shown that mammalian AMPK α1 catalytic subunit including autoinhibitory domain, was inactive. To test the hypothesis that small molecules can activate AMPK through antagonizing the autoinhibition in α subunits, we screened a chemical library with inactive human α1394 (α1, residues 1–394), and found a novel small-molecule activator, PT1, which dose-dependently activated AMPK α1394, α1335, α2398, and even heterotrimer α1β1γ1. Based on PT1-docked AMPK α1 subunit structure model and different mutations, we found PT1 might interact with Glu-96 and Lys-156 residues near the autoinhibitory domain and directly relieve autoinhibition. Further studies using L6 myotubes, showed that the phosphorylation of AMPK and its downstream substrate, acetyl-CoA carboxylase (ACC), were dose-dependently and time-dependently increased by PT1 without an increase in cellular AMP:ATP ratio. Moreover, in Hela cells deficient with LKB1, PT1 enhanced AMPK phosphorylation, and which can be inhibited by the CaMKKs inhibitor STO-609, and AMPK inhibitor compound C. PT1 also lowered hepatic lipid content in a dose-dependent manner through AMPK activation in HepG2 cells, and this effect was diminished by compound C. Taken together, these data indicate that this small-molecule activator may directly activate AMPK via antagonizing the autoinhibition in vitro and in cells. This compound highlights the effort to discover novel AMPK activators and can be a useful tool for elucidating the mechanism responsible for conformational change and autoinhibitory regulation of AMPK.

The AMP-activated protein kinase (AMPK) is a highly conserved serine/threonine protein kinase that is widely expressed in higher eukaryotes, yeast, and plants and plays a unique and central role in the responses of cells to metabolic stresses such as nutrient starvation, heat shock,
ischemia/hypoxia, and vigorous muscular exercise by depleting cellular ATP and elevating AMP levels (1, 2). Once activated, AMPK prevents depletion of ATP by increasing the rate of ATP generation, triggering changes in the rates of glucose transport, fatty acid oxidation, lipogenesis, sterol synthesis, and gluconeogenesis through direct regulation of key metabolic enzymes and transcriptional control of specific genes (1-4). There is mounting evidence of the involvement of AMPK in human physiological and pathological processes, especially type 2 diabetes and obesity. Previous studies indicate that several of the beneficial effects of rosiglitazone and metformin, two widely used antidiabetic drugs, are mediated by indirect activation of AMPK, suggesting the potential role of the AMPK pathway in the treatment of type 2 diabetes (5-7). Two adipocyte-derived hormones, leptin and adiponectin stimulate fatty acid oxidation and glucose uptake in peripheral tissues such as skeletal muscle and liver, which are also induced by AMPK activation. (8-13) Furthermore, total AMPK α2 knockout mice displayed impaired glucose tolerance, reduced insulin-stimulated whole-body glucose utilization and skeletal muscle glycogen synthesis (14), and increased body weight and fat mass as compared with the wild-type mice following high-fat diet (15). Therefore, AMPK is considered as a promising target of drugs for treatment of type II diabetes and obesity (16, 17).

AMPK is a heterotrimer consisting of a catalytic α subunit (63 kDa) and two noncatalytic subunits, β (30 kDa) and γ (37-63 kDa) (3). Each subunit has multiple isoforms (α1, α2, β1, β2, γ1, γ2, γ3), the expression of which varies between tissues and subcellular locations (1-3, 18-21). The heterotrimeric complex is required for maximum enzymatic activity (22, 23). AMPK is allosterically stimulated by AMP and is phosphorylated by physiological upstream AMPK kinase (AMPKK) (3, 4), recently identified as LKB1 and calcium/calmodulin-dependent protein kinase kinase beta (CaMKKβ) that make AMPK phosphorylated and activated in vitro and in vivo (24-31). This cascade is activated in a sensitive manner by the increasing cellular AMP:ATP ratio (32). The major regulatory site is Thr-172, phosphorylation of which is essential for AMPK activity (33-35).

The mammalian AMPK α1 subunit consists of a constitutively active catalytic domain, α1312 (residues 1–312), when Thr-172 is phosphorylated, and a C-terminal domain (residues 313–548), which is responsible for the binding of β and γ subunits. Binding of the C-terminal domain with the β and γ subunits induces a conformational change in the α1 subunit, which results in Thr-172 phosphorylation by AMPKK and protects α subunits from degradation. AMPK α1 subunit exhibits little activity in the absence of regulatory β/γ subunits, and truncation of the α1 subunit from residues 1–548 to 1–392 results in loss of β/γ binding and catalytic activity (22, 25, 35). We recently reported that the autoinhibitory domain (AID) of the AMPK α1 subunit is located between residues 313 and 335 (36) and influences exposure of the catalytic cleft or the Thr-172-activating phosphorylation site or both.

5-amino-imidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) was first reported for regulation of cellular metabolism (37), and is a well-known, cell permeable activator (38) of AMPK (39,40). AICAR mediated AMPK activation through being converted into ZMP by an adenosine kinase inside cell, which acts as an AMP analogue to activate AMPK and LKB1 without affecting the cellular AMP:ATP ratio (38). Very recently, Cool and coworkers have firstly reported a small-molecule AMPK activator, A-769662, which stimulated partially purified rat
liver AMPK with EC\textsubscript{50} of 0.8 μM, and had potential metabolic effects \textit{in vivo} (41).

In this study, we identified a novel small-molecule AMPK activator, PT1, which directly activated the inactive forms of α\textsubscript{135}, α\textsubscript{1394}, homologous α\textsubscript{2398} (α2, residues 1–398) in a dose-dependent manner. According to PT1-docked human AMPK α1 subunit structure model, PT1 may interact with Glu-96 and Lys-156 residues near the autoinhibitory domain in α1 subunit and directly relieve the autoinhibition. Further study revealed that PT1 also activated native AMPK in L6 myotubes or Hela cells deficient in LKB1 by increasing the phosphorylation of AMPK α Thr-172 and its downstream substrate, acetyl-CoA carboxylase (ACC) without an increase in the AMP:ATP ratio. Moreover, PT1 treatment lowers hepatic lipid content in a dose-dependent manner in HepG2 cells. It suggested that a novel small-molecule activator can directly activate AMPK through conformational change to stimulate AMPK functions.

MATERIALS AND METHODS

Materials

The expression plasmid pET28b was obtained from Novagen (Milwaukee, WI, USA). The recombinant plasmid, pMAL-CaMKKβ, was kindly donated by Dr. Charles R. Mena of Duke University Medical Center. The SAMS peptide (HMRSAMSGLHLVKRR, a synthetic substrate of AMPK) was synthesized by SynPep Inc. (Dublin, CA, USA). All restriction enzymes were obtained from Takara (Dalian, China). The expression plasmid pET28b was obtained from Novagen (Milwaukee, WI, USA). The recombinant plasmid, pMAL-CaMKKβ, was kindly donated by Dr. Charles R. Mena of Duke University Medical Center. The SAMS peptide (HMRSAMSGLHLVKRR, a synthetic substrate of AMPK) was synthesized by SynPep Inc. (Dublin, CA, USA). All restriction enzymes were obtained from Takara (Dalian, China). The Escherichia coli strain BL21-Codon-Plus (DE3)-RIL and the QuikChange site-directed mutagenesis kit were purchased from Stratagene (La Jolla, CA, USA). Ni-NTA agarose beads were purchased from Qiagen (Basel, Switzerland). Chelating-Sepharose column, Scintillants [γ-\textsuperscript{33}P] ATP, Western blotting detection kits (enhanced chemi-luminescence, ECL) and Hyperfilm were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Anti-phospho-AMPK-α-Thr-172 antibody and anti-phospho-ACC-Ser-79 antibody were obtained from Cell Signaling Technology (Beverly, MA, USA). P30 filter paper was purchased from Wallac (Turku, Finland) and liquid scintillator (OptiPhase SuperMix) was obtained from PerkinElmer Life Sciences (Boston, MA, USA). Protein purification was carried out using an AKTA FPLC system from Amersham Biosciences (Piscataway, NJ, USA).

Liquid scintillation counting was performed using a Wallac MicroBeta TriLux (Wallac Oy, Turku, Finland). L6 cells, Hela cells and HepG2 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Inhibitor STO-609, Compound C, and PD98059 were from Calbiochem (La Jolla, CA, USA). AICAR and metformin were from Sigma (St. Louis, MO, USA). Triacylglycerol and Cholesterol Reagents kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Dulbecco’s modified Eagle’s medium (DMEM), α-MEM and fetal bovine serum (FBS) were purchased from Invitrogen. All other reagents were purchased from Sigma (St. Louis, MO, USA).

Recombinant plasmid construction

The coding sequences of human AMPK α1 or α2 subunit truncations and mutants were amplified from cDNA of human AMPK α1 or α2 subunit and cloned into pET28b vector. All mutants of AMPK-α1(1-394) were prepared using the QuikChange site-directed mutagenesis kit for generating the single point mutants from the plasmid pET28b/AMPK-α1(1-394). All recombinant plasmids containing wild-type truncations and mutations were verified by DNA sequencing, and transformed into \textit{Escherichia coli} strain BL21 Codon Plus (DE3)-RIL for
Expression and purification of recombinant proteins

The expression and purification of the recombinant human AMPK α1 or α2 subunit truncations, mutations, heterotrimer (α1β1γ1) and the recombinant calcium/calmodulin-dependent protein kinase kinase beta (CaMKKβ) were carried out as described previously (36). Briefly, recombinant proteins were induced with 0.1 mM IPTG and cultures were grown at 22°C overnight. Then cells were harvested, washed, resuspended in lysis buffer (15% sucrose (w/v), 50 mM Na-phosphate, pH 7.5, 100 mM NaCl, 10 mM imidazole, and 1 mM β-mercaptoethanol) with 1% Triton X-100, and sonicated on ice. Following centrifugation, the supernatant was loaded onto Ni-NTA agarose. After washed 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.

Phosphorylation and activation of recombinant AMPK proteins

Recombinant AMPK proteins were fully phosphorylated by incubation with CaMKKβ (200 nM) at 30°C for 4 h in 20 mM Tris-HCl, pH 7.5, 1 mM DTT, 5 mM MgCl₂, 200 μM ATP, as described previously (36). Analyses of the activities of AMPK proteins were carried out in a typical assay conditions of 50 μL reaction mixture containing 20 mM Tris-HCl, pH 7.5, 1 mM DTT, 5 mM MgCl₂, 50 mM NaCl, 50 μM ATP (0.4 μCi [γ-32P] ATP per reaction), and 50 μM SAMS peptide. The reaction was initiated by addition of AMPK proteins (100 nM), incubated at 30°C for 10 min, and terminated by addition of 50 μL 1% H₃PO₄. Particulate matter was then transferred to P30 filter paper and washed three times with 0.1% H₃PO₄. Radioactivity that had been incorporated in the AMPK proteins was determined by liquid scintillation counting in a Wallac Microbeta plate counter. Background radioactivity estimated from reactions conducted without enzymes was subtracted from sample radioactivities. All reactions were repeated in three independent experiments.

Discovery of a Novel Small-molecule Activator and Its Effects on AMPK Activation

Until now, there have been no reports of small-molecule activators that can be used for studying the autoregulatory function of the AMPK α catalytic subunit in the absence of regulatory subunits. We speculate that small molecules might activate AMPK through inducing a conformational change, which antagonizes the autoinhibition. Using the inactive α1394 form (100 nM), we randomly screened 3,600 compounds using a diverse compound library under typical assay conditions containing 2 μL compound (40 μg/mL) dissolved in dimethyl sulfoxide (DMSO), and discovered a novel small-molecule activator, PT1. The effects of PT1 on α1 truncations (100 nM) and α2398 (400 nM) were studied by varying the concentration of PT1 from 0 μM to 60 μM. The EC₅₀ (concentration of the compound at which enzymatic activity equals 50% of maximum activity) was calculated from a nonlinear regression curve fitted using GraphPad software (San Diego, California).

Homology Modeling and Molecular Docking

Homology modeling, molecular dynamics simulations, and energy minimization were calculated by using the molecular operating environment (MOE) 2006.08 (42) on a HP xw8200/Linux workstation. The molecular
docking simulation was made by using the Moloc molecular modeling package (43) on a HP NC6220 laptop running Windows XP SP 2. Energy minimization was calculated again with the Moloc package by using the MAB force field.

Homology modeling - The protein sequence of human AMPK α1 was retrieved from the ExPASy server (Q13131, 550aa) (44). Based on the sequence and structure analysis as well as our previous work (36), the templates proteins were determined to be the activated AURORA-A (PDB entry 1OL5) and MARK2 (PDB entry 1Y8G), which were used to build up the kinase domain (KD, residues M1-C312) and autoinhibitory domain (AID, residues L313-N332) of human AMPK α1, respectively. The chain alignment was performed with the MOE-Align module, which implements a modified version of the alignment algorithm originally introduced by Needleman and Wunsch (45), using the Blosum50 matrix with a penalty gap of 3 and a penalty for extending a gap of 1 and combining with manual examination. The construction of the three-dimensional model was carried out with the MOE-Homology module (46, 47) by means of calculation of 20 intermediate models that were coarsely minimized by using 1OL5 and 1Y8G crystal structure as templates, and the final model was taken as the Cartesian average of all the intermediate models, and it was further refined by molecular dynamic simulations and energy minimizations, during which only the side chains of all residues were allowed to move. The molecular dynamics simulations were set to 5ps of heating to 300K, 10ps of equilibrium at 300K, and 5 ps of cooling to 0K. Energy minimization were made in 100 steps of steepest decent with an RMS gradient test of 100, and 500 truncated Newton steps with an RMS gradient test of 0.01. The Amber94 force field was used. The polypeptide backbone and side chains were then evaluated by MOE-Ramachandran plots (48), a program used to check the stereochemical quality of protein structures (dihedrals, bond angles, etc). This model was used to identify the active site and for docking of activator with the kinase.

Active site identification of human AMPK α1 - The active site of modeled human AMPK α1 was identified using the Alpha Site Finder in MOE, a methodology based upon Alpha Shapes which are a generalization of convex hulls developed by Edelsbrunner (49). In brief, a collection of 3D points is triangulated using a modified Delaunay triangulation. For each resulting simplex there is an associated sphere i.e. alpha sphere. These spheres have different radii including infinite radii (corresponding to the planes of the convex hull of the point set). The collection of alpha spheres is pruned by eliminating those that correspond to inaccessible regions of the receptor as well as those that are too exposed to solvent. In addition, only the small alpha spheres are retained since these correspond to locations of tight atomic packing in the receptor. Next, each alpha sphere is classified as either "hydrophobic" or "hydrophilic" depending on whether the sphere is in a good hydrogen bonding spot in the receptor. Hydrophilic spheres not close to a hydrophobic sphere are eliminated (since these generally correspond to water sites). Finally, the alpha spheres are clustered using a single-linkage clustering algorithm to produce a collection of sites. Each site consists of one or more alpha spheres at least one of which is hydrophobic.

Molecular docking studies - Initial geometric optimizations of the activator, here is PT1, was carried out using the MAB all atom force field of Moloc (43) with a final root-mean square gradient of 0.1 kcal/mol/Å. And the optimization parameters and thresholds were adopted with their default values. Mdck of Moloc was used for batch run docking human AMPK α1 60 times. The force entry used by Mdck was generated based on the
active site determined by Alpha Site Finder as described above. An ensemble of conformers of PT1 was generated by a systematic search as implemented in the Mdck with the dihedral variation to be 1, force margin to be 1.5. The resulting complexes were visually inspected in order to discard the unacceptable ones. All the complexes were finally minimized by using the MAB force field again with all the residues of the active site fixed during the optimization. The estimated binding energy was calculated by the score function in the Moloc implementation which is an empirical formula of the Boehm type (50).

**Cell Culture and PT1 Treatment in L6 Myotubes, Hela Cells and HepG2 Cells**

L6 myoblasts were routinely maintained in α-MEM supplemented with 10% fetal bovine serum (FBS), 4.5 g/L glucose, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified incubator in an atmosphere of 5% CO₂ at 37°C. For L6 myoblasts differentiation in six-well plates, the concentration of FBS was decreased from 10% to 2% over five days. Cells were deprived of serum for 3 h prior to the subsequent compound incubation, which lasted for 1 h. PT1 was prepared as 40 mM stock solutions in DMSO, and diluted in medium before application to the cells. Before western blotting analysis, medium was aspirated, and the cells were rinsed with PBS and lysed using SDS-PAGE loading buffer.

Hela cells were maintained in HG-DMEM medium in six-well plates supplemented with 10% FBS and antibiotics. Before compounds treatments for 6 h, Hela cells were starved overnight in free of serum, and then specific AMPK inhibitor (40 μM Compound C) or specific CaMKKβ inhibitor (10 μg/mL STO-609) was added, and cells were incubated for a further 30 min. Before western blotting analysis, medium was aspirated, and the cells were rinsed with PBS and lysed using SDS-PAGE loading buffer.

Human hepatoma HepG2 cells were cultured in DMEM containing 10% FBS, antibiotics, and 5.5 mM D-glucose. For experiments, HepG2 cells were incubated in complete medium with 10% FBS in 100 mm diameter dishes, grown to 70% confluence, and maintained in serum-free DMEM overnight. Cells were treated with compounds as indicated in figure legends, using metformin as a positive control. Before activator PT1 or metformin treatment, 40 μM of compound C was added and cells were incubated for another 60 min.

**Immunoblot**

Samples were subjected to 8% SDS-PAGE and proteins were transferred onto PVDF membranes. The amount of protein and the extent of phosphorylation were estimated using the following primary antibodies: anti-phospho-AMPK-α-Thr-172 antibody (1:1,000 dilution), anti-AMPK-α antibody (1:1,000 dilution), anti-phospho-ACC-Ser-79 antibody (1:1,000 dilution), anti-ACC antibody (1:1,000 dilution), and anti-β-actin antibody (1:5,000 dilution). The total protein content of each sample was quantified using β-actin.

**Other Methods**

The cellular adenine nucleotides were analyzed and detected as we previously described (51). Preparation of the cell lysates and determination of triacylglycerol and total cholesterol content in cell lysates were performed as described previously (52). Triacylglycerol and total cholesterol content were determined in cell lysates using a colorimetric assay and expressed as μg of lipid per mg cellular protein.

**Statistical Analysis**

Results are expressed as the means ± the
standard error of the mean (SEM). Significance was analyzed using a two-tailed unpaired Student \( t \) test. \( P < 0.05 \) was considered significant.

RESULTS

Discovery of a novel small-molecule activator \( \text{PT1} \) for \( \text{AMPK} \) \( \alpha \) subunit- Until now, there have been no reports of small-molecule activators that can be used for studying the autoregulatory function of the \( \text{AMPK} \) \( \alpha \) catalytic subunit in the absence of regulatory subunits. We considered it worthwhile to search for small molecules that might affect the conformation of \( \text{AMPK} \) \( \alpha \) subunit and thus antagonize the autoinhibition. Using inactive form human \( \text{AMPK} \) \( \alpha_{194} \), we carried out a random screening of a small organic-compound library for low molecular-weight activators. The 3,600 compounds were initially screened at concentrations of 40 \( \mu \text{g/mL} \) using typical assay protocols. One hit (PT1) was identified (Fig. 1, A), and its activation effect was confirmed by an \( \text{EC}_{50} \) of about 8 \( \mu \text{M} \). Various concentrations of PT1 did not induce significant change in the activity of \( \alpha_{1312} \) but increased \( \alpha_{1394} \) activity in a dose-dependent manner (Fig. 1, B). \( \alpha_{1394} \) activity reached a maximum at 20 \( \mu \text{M} \) PT1, which is about eight-fold higher than that obtained in the absence of PT1.

As the autoinhibitory mechanism of the \( \text{AMPK} \) \( \alpha \) subunit is similar to that of the \( \text{AMPK} \) \( \alpha 1 \) subunit, the effect of PT1 on inactive \( \text{AMPK} \) \( \alpha 2 \) subunit \( \alpha_{2398} \) was studied. As expected, PT1 activated \( \alpha_{2398} \) in a dose-dependent manner (\( \text{EC}_{50} \approx 12 \mu \text{M} \)). \( \alpha_{2398} \) activity was maximal at 30 \( \mu \text{M} \) PT1, which is more than four-fold higher than that recorded in the absence of PT1 (Fig. 1, C). Also, we investigated whether PT1 can directly activate \( \text{AMPK} \) heterotrimer in vitro for \( \text{AMPK} \) exists as three subunits heterotrimer in eukaryotic cell. Using recombinant \( \text{AMPK}(\alpha1\beta1\gamma1) \) heterotrimer purified from bacterial cells, we found that PT1 did stimulate \( \text{AMPK}(\alpha1\beta1\gamma1) \) heterotrimer activity in a dose-dependent manner (\( \text{EC}_{50} \approx 0.3 \mu \text{M} \)). \( \text{AMPK}(\alpha1\beta1\gamma1) \) activity reached a maximum at 5 \( \mu \text{M} \) PT1, which is approximate 1.5-fold of that obtained in the absence of PT1 (Fig. 1, D). However, PT1 could not stimulate the activities of \( \text{AMPK} \)-related protein kinases such as human MARK2, BRSK1, NUAK2, and MELK, which indicates that PT1 has a strong selectivity for \( \text{AMPK} \) \( \alpha \) catalytic subunits activation (data not shown).

PT1 stimulates truncated \( \text{AMPK} \) \( \alpha 1 \) subunit proteins including autoinhibitory domain of residues 313-335- We have previously reported that autoinhibitory domain of human \( \text{AMPK} \) \( \alpha 1 \) subunit was located between residues 313 and 335 (36). Whether PT1 stimulates \( \text{AMPK} \) \( \alpha \) subunit through relieving the autoinhibitory conformation by interaction with autoinhibitory domain, we investigated the effects of variety concentrations of PT1 on five truncated \( \alpha \) subunit proteins \( \alpha_{1335}, \alpha_{1341}, \alpha_{1351}, \alpha_{1367}, \alpha_{1377}, \) each of which includes autoinhibitory domain of residues 313-335. We found that PT1 stimulates these truncations in a dose-dependent manner with similar \( \text{EC}_{50} \) of about 8 \( \mu \text{M} \) (Fig. 2). Since \( \alpha_{1312} \) activity can’t be stimulated by PT1, it is suggestive that the autoinhibitory conformation of \( \text{AMPK} \) \( \alpha 1 \) catalytic subunit can be regulated by PT1 interaction with kinase domain or autoinhibitory domain.

Construction of PT1 docking model in human \( \text{AMPK} \) \( \alpha 1 \) subunit structure- In order to determine which domain or which residues in \( \alpha \) subunit to interact with PT1, we constructed a PT1-docked \( \text{AMPK} \) \( \alpha 1 \) subunit structural model, and the binding site for activator in the current study was estimated to lie in a cleft formed between the AID domain and N-lobe of \( \text{AMPK} \) \( \alpha 1 \) after comparing the modeled protein structure to the computational results of Alpha Site Finder. Docking of the activator, PT1, was performed using Mdkc of
Moloc. The algorithm exhaustively searches the entire rotational and translational space of the ligand with respect to the receptor. The flexibility of the ligand is given by dihedral angle variation, while the protein flexibility is not considered. Various solutions are evaluated by a Boehm type empirical score function. The final solution was determined based on the lowest binding energy together with shape complement. As shown in Figure 3, PT1 was inserted on the interface between the kinase domain and the autoinhibitory domain of α1 subunit. Due to the residues surrounding PT1 should influence on the binding of PT1 to AMPK α1 and the subsequent activation efficacy, around 14 residues based on the interaction model were chosen to conduct the site-directed mutagenesis experiment to investigate their role consequently. It was found that the region supposed to interact with PT1 is very sensitive to maintain the autoinhibition of α1 subunit, since most mutants increased the basal activity of α1. Although 20 μM of PT1 still stimulated mutants of α1 subunit except for E96A and K156A, the ratios of α1 enzymatic activity induced by PT1 were all decreased in mutants compared with wild type. Among them, E96 and K156 are the most significant, since PT1 eventually lost the ability to activate α1 when they were mutated to Ala (Fig. 4). However, when residue Glu-96 was mutated to Aspartic acid, or when residue Lys-156 was mutated to Arginine in α1, the E96D or K156R mutant was stimulated by PT1 in a dose-dependent manner (Fig. 4, C), similar as wild type of α1. Since Aspartic acid or Arginine has similar polarity and similar molecule size with Glutamic acid or Lysine respectively, it is deliberately inferred that activator PT1 with carboxyl group has strong polar interaction such as H-bond with similar polar residues such as Glutamine acid or Lysine (Fig. 3). The experimental phenomenon that PT1 removed carboxyl acid was lack of activation efficacy could partly support this point (data not shown).

**AMPK activation by PT1 in L6 myotubes**

Although PT1 dose-dependently activated AMPK α1394 and AMPK α2398, which has an autoinhibitory domain in the C-terminus and is originally inactive, it was unclear whether it could activate the full-length α subunit or the holoenzyme α1/β1/γ1 heterotrimer. To investigate whether AMPK is activated by PT1 in L6 cells, the phosphorylations of AMPK and its downstream target, ACC, an enzyme in the fatty acid synthesis pathway, were used as indicators of AMPK activation. AICAR was used as a positive control in this experiment. The L6 myotubes were treated with 80 μM PT1 for 0.5 h, 1 h, 2 h, 3 h, and 6 h. The immunoblot result showed that PT1 stimulated the phosphorylation of Thr-172 of the AMPK α subunit and the phosphorylation of Ser-79 of ACC in a time-dependent manner, and its effects reached a maximal level when at 2 h of treatment time (Fig. 5, A). Then we investigate the dose-reponse of PT1 on AMPK activity in L6 myotubes. The cells were treated with 10 μM, 20 μM, 40 μM, 80 μM PT1, 1 mM AICAR or DMSO for 1 h. we found that PT1 greatly increased the phosphorylations of AMPK and ACC in a dose-dependent manner (Fig. 5, B). The phosphorylations of AMPK and ACC reached to a maximal level by treatment of 40 μM PT1, which were greatly higher than that induced by 1 mM AICAR.

Several reported compounds induced an increase in cellular AMP:ATP ratio to activate AMPK (53). To investigate whether PT1 stimulates AMPK activity by an increase in AMP:ATP ratio in L6 cells, the L6 myotubes were treated by 40 μM PT1 for 2 h, and 100 μM of compound PD98059 was used as a positive control, which has been reported to activate AMPK by increasing the cellular AMP:ATP ratio (53). As
shown in Fig. 5, compared to control, PT1 didn’t induce an increase in AMP:ATP ratio ($p = 0.699$), but PD98059 can significantly increase cellular AMP:ATP ratio ($p = 0.004$), which means PT1 stimulates cellular AMPK activity without an increase in AMP:ATP ratio.

**AMPK activation by PT1 in Hela cells independent of LKB1** - There are two kinases LKB1 and CaMKKβ identified as AMPK upstream kinases in cells, and the major AMPK upstream kinase in L6 cells is LKB1. To investigate whether AMPK is activated by PT1 independent of LKB1, we chose Hela cells deficient of LKB1 to be treated by PT1. We found that PT1 can also stimulate the phosphorylations of AMPK and ACC in Hela cells after PT1 treatment for 6 h (Fig. 6). For Hela cells only express CaMKKβ as AMPK upstream kinase, we used specific CaMKKβ inhibitor STO-609 and specific AMPK inhibitor Compound C to determine PT1’s effects on AMPK phosphorylation and ACC phosphorylation. After 10 μg/mL STO-609 or 40 μM Compound C pretreatment in Hela cells as described in “Materials and Methods”, the phosphorylations of AMPK and ACC induced by 40 μM PT1 were significantly inhibited, respectively (Fig. 6, A). Moreover, the cellular AMP:ATP ratio was not changed after 40 μM PT1 treatment for 6 h, compared to control of DMSO treatment (Fig. 6, B). These data demonstrate that PT1 is a novel direct activator of AMPK and the cellular AMPK pathway independent of LKB1.

**PT1 stimulates AMPK and ACC phosphorylation, decreases lipid content, and these effects are diminished by pretreatment of compound C in HepG2 cells** - To test whether PT1 has certain cellular function in lipid metabolism, We then determine the effect of PT1 treatment in human hepatoma HepG2 cells on lipid accumulation. We first examined the phosphorylation state of AMPK and ACC. In HepG2 cells exposed to increasing concentration of PT1 for 24 h, the phosphorylation of AMPK and ACC was significantly stimulated, but the expression of endogenous AMPKα and ACC protein were not changed, respectively (Fig. 7, A). As a positive control, 2 mM metformin also caused a significant increase in the phosphorylation of AMPK and ACC, without an increase in total endogenous AMPKα and ACC protein. To determine whether PT1 has a cellular lipid-lowering effect, intracellular levels of triacylglycerol and cholesterol in HepG2 cells exposed to PT1 for 24 h also were measured, using metformin as a positive control. Increasing concentrations of PT1 (5 μM, 10 μM, 20 μM, 40 μM, 80 μM) decreased intracellular triacylglycerol and cholesterol content in a dose-dependent manner (Fig. 7, B), which were in concert with these changes in phosphorylation of AMPK and ACC. Concentration of PT1 above 20 μM lowers intracellular lipid content more than that of 2 mM metformin.

To determine whether AMPK activity is required for PT1 to lower lipid, HepG2 cells were pretreated with AMPK inhibitor compound C (40 μM), followed by incubation with or without PT1 (40 μM) for 24 h, using metformin (2 mM) as a positive control. The ability of PT1 or metformin to stimulate the phosphorylation of ACC was diminished by compound C (Fig. 8, A). Moreover, compound C, at least in part, blocked the inhibitory effect of PT1 or metformin on triglyceride in HepG2 cells (Fig. 8, B), which indicates that AMPK may mediate the effect of PT1 on cellular lipid content.

**DISCUSSION**

AMPK is a heterotrimeric complex comprising a catalytic subunit α and two regulatory subunits β and γ. The mammalian AMPK α1 subunit consists
of a constitutively active catalytic domain, α1312 (residues 1–312), when Thr-172 is phosphorylated, and a C-terminal domain (residues 313–548), which is responsible for the binding of β and γ subunits. AMPK α catalytic subunit exhibits little activity in the absence of regulatory β/γ subunits. Binding of the C-terminal domain with the β and γ subunits induces a conformational change in α subunit, which results in an increase in AMPK activity. In addition to regulatory subunits β and γ, NAD was recently shown to have an activation effect on AMPK (54). However, the details of these mechanisms remain unclear because of a lack of information about the three-dimensional structure of AMPK α catalytic subunit. It is possible that conformational change is a common requirement for AMPK activation. Until now, there have been no reports of small-molecule activators that can be used for studying the autoregulatory function of the AMPK α catalytic subunit in the absence of regulatory subunits. We considered it worthwhile to search for small molecules that might affect the conformation of AMPK α subunit and thus antagonize the autoinhibition.

AICAR, an activator of AMPK in vitro and in vivo, has been described previously (38). However, AICAR was incapable of direct activation of AMPK in an in vitro enzymatic assay, and it is unclear whether the various metabolic effects by AICAR treatment are mediated primarily through AMPK activation. Recently, it has been reported that compound D942, a furancarboxylic acid derivative, increases glucose uptake in L6 myocytes by AMPK activation and is a potent AMPK activator. D942 does not activate AMPK directly at the molecular level in vitro, but binds specifically to mitochondrial complex I and inhibits its activity, resulting in an increase in the ratio of AMP to ATP (55). Recently, Abbott laboratory has firstly reported a small molecule AMPK activator, A-769662, which stimulated partially purified rat liver AMPK with EC50 of 0.8 μM, and had potential metabolic effects in vivo, but it didn’t show whether these effects at large dose of this activator were related on AMPK activation (41). Their research results suggested that activator A-769662 stimulates AMPK with an activation mechanism which is different from that of AMP, and the other two papers published very recently, have indicated that A-769662 allosterically activate AMPK probably through interaction with the glycogen binding domain of the β subunit, but it has no direct effect on the activity of the α subunit kinase domain, with or without autoinhibitory domain (56, 57). As regulatory subunits β and γ can antagonize the autoinhibitory conformation of the α1 subunit, we hypothesized that a small-molecule activator that induced a similar conformational change would activate AMPK.

AMPK activity was determined by incorporation of 33P-phosphate into the SAMS peptide. It has been previously reported that CaMKKβ phosphorylated and activated AMPK in vitro, and AMPK activity was increased with the presence of AMP (24). Both LKB1 and CaMKKβ function as upstream kinase of AMPK in different tissues and cell lines respectively (26-31), suggesting different roles for each in the regulation of AMPK activity in vivo. Since CaMKKβ is an efficient AMPK kinase in vitro (24, 25, 29-31), we used rat CaMKKβ as one upstream kinase of AMPK to assess AMPK activity in all enzyme assay experiments. In addition, AMP did not appear to allosterically regulate the activities of truncated human AMPK α1 proteins, even in the presence of compound PT1 (data not shown), so all kinases assay were performed without the addition of AMP. As expected, human AMPK α1312 without the autoinhibitory sequence (residues 313–394) was phosphorylated by CaMKKβ and
was active; human AMPKα1394 was also phosphorylated by CaMKKβ but remained inactive, which is consistent with a previous report on mammalian AMPKα1 (25, 36).

Through screening with inactive form human AMPKα1394, we for the first time discovered a novel small-molecule activator PT1 to antagonize the autoinhibitory conformation of AMPKα catalytic subunit. PT1 activated α1394 and α2398, which were originally inactive, but did not increase the activity of AMPKα1 truncation without autoinhibitory sequence, α1312 that was already constitutively active (Fig. 1). It is likely that the activation of α1394 by PT1 is mediated through conformational change, possibly resulting in dissociation of the autoinhibitory domain from the AMPK kinase domain. Interestingly, we found that PT1 can directly stimulate AMPK(α1β1γ1) heterotrimer activity in vitro dose-dependently with EC50 of 0.3 μM (Fig. 1). It is possible that AMPK heterotrimer may have basal activity without stimulus, for the AMPKα catalytic subunit binding with β/γ regulatory subunits may still have partly open conformation. However, once binding with PT1, the properly catalytic conformation of AMPKα catalytic subunit which also binds with β/γ regulatory subunits might be further opened, and then AMPK will exhibit higher kinase activity. It has been demonstrated by Abbott laboratory that one of AMPK activators, A-769662 stimulated AMPK probably through interaction with the glycogen binding domain of the β subunit, but the AMPKα catalytic subunit wasn’t involved in the activation effect (56, 57). However, in this paper, our study for the first time provides strong biochemical evidence that PT1 might have direct activation effect on activity of AMPK through interaction with several key residues in the AMPKα catalytic subunit.

To better understand the molecular mechanism of PT1-activated AMPKα subunit, we established a docking model of PT1 in human AMPKα1 (1-335) subunit. Previously we have constructed a structural model for human AMPKα1 (1-335) subunit, in which the autoinhibitory domain bound to the small lobe of the kinase domain, on the opposite face to the substrate-binding groove, and it demonstrated that some mutation of critical residues of the autoinhibitory domain in AMPKα1 subunit have greatly relieved AMPKα1 subunit autoinhibition (36). From our present speculative docking model and mutagenesis experiments, we found the region assumed to interact with PT1 is very sensitive to maintain the autoinhibition of α1 subunit because of the increased basal activity of α1394 from most mutants. Among them, Glu-96 and Lys-156 are the most significant as stated before. Our results suggested that PT1 might interact with the region and especially residues Glu-96 and Lys-156 to relieve the autoinhibitory conformation of human AMPKα subunit. It is deliberately inferred that activator PT1 with carboxyl group has strong polar interaction such as H-bond with similar polar residues such as Glutamine acid or Lysine considering the importance of carboxy acid of PT1 from both experimental and modeling data (Fig. 3). Binding at the cleft formed between AID domain and N-lobe of AMPKα1, unfolding the CD domain and then further elongate the linker, and ultimately close the catalytic site to demonstrate the activation efficacy, is only one of devised molecule mechanisms from our viewpoint, and the details of the interaction between PT1 and α subunit as well as activation mechanism are still under further investigation in our group.

As we expected, native AMPK and the downstream pathway in L6 myotubes were phosphorylated and activated in a dose-dependent manner and in a time-dependent manner by PT1 without changing cellular AMP:ATP ratio. Moreover, PT1 stimulates cellular AMPK activity
independent of LKB1, which is predominant upstream kinase of AMPK (27), because the phosphorylations of AMPK and ACC were also induced by PT1 treatment in Hela cells deficient in LKB1. It demonstrates that PT1-induced increase in cellular AMPK activity is not by PT1-stimulated LKB1 or CaMKKβ, but by directly PT1-activated AMPK. In fat metabolism, we assessed the role of AMPK activation by PT1 in the regulation of hepatocellular lipids. We found that PT1 not only dose-dependently increased phosphorylation of AMPK and ACC, but also reduced intracellular triglyceride content in a dose-dependent manner in HepG2 cells (Fig. 7). Moreover, AMPK inhibitor compound C partly blocked both the increase in ACC phosphorylation and the decrease in triglyceride content of HepG2 cells caused by PT1 or metformin (Fig. 8). These studies suggest that the effects of PT1 on the lipid content of HepG2 cells are mediated predominantly by activation of AMPK. Previous studies have shown that AMPK activation by either AICAR or metformin stimulates fatty acid oxidation in rat hepatocytes (58, 59). This suggests that the reduction in triglyceride levels by PT1 observed in HepG2 cells is explained by increased fatty acid oxidation and/or decreased fatty acid synthesis through activation of AMPK and stimulation of ACC phosphorylation.

Recently, attention has been drawn to AMPK because of its important roles in the regulation of carbohydrate and lipid metabolism, glucose transportation and glycolysis, tumor cell growth, gene transcription and protein synthesis (60). Because abnormal AMPK activity is associated with several diseases, it is considered an important therapeutic target for the treatment of diabetes, obesity and cancer. Small-molecule activators of AMPK will be invaluable for elucidating the functions of AMPK and validating the pharmaceutical importance of AMPK as a drug target. PT1 is a novel small-molecule activator to be discovered that directly activates AMPK through regulation of a catalytic subunit autoinhibitory conformation, and will be very useful for evaluating the effects of AMPK under physiological and pathological conditions and studying its downstream signaling pathway. Further investigation of the effects of PT1 on AMPK activation and of its antidiabetic and anticancer effects in vivo could lay the foundation for a new therapeutic agent. Complexes between PT1 and α1394 could be used to determine their three-dimensional structures and to elucidate the activation mechanism, which would facilitate the discovery and design of more potent AMPK small-molecule activators. Also, this compound highlights the effort to discover novel AMPK activators and offers an alternative means by activating AMPK directly for treatment of metabolic disorders.

REFERENCES


44. The ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics (SIB) http://ca.expasy.org

**ACKNOWLEDGEMENTS**

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**FIGURE LEGENDS**

**Figure 1. Activation of human AMPK α subunits by PT1.** A. Structure of PT1, a novel small-molecule activator of the AMPK α subunit. B. The effects of PT1 on the activities of truncations at amino acid 312 or 394 (α1312, α1394). Human AMPK α1394 was activated by PT1 in a dose-dependent manner. C and D. The activation effect of PT1 on purified recombinant human AMPK α2398 and the human AMPK (α1β1γ1) heterotrimer. AMPK α2398 and AMPK (α1β1γ1) were still activated by PT1 in a dose-dependent manner. Mean ± S.D. (n = 3 replicated assays).

**Figure 2. Effects of PT1 on human AMPK α1 subunit truncations.** PT1 dose-dependently stimulates activities of α1315, α1341, α1351, α1367, α1377. Mean ± S.D. (n = 3 replicated assays).

**Figure 3.** Stereo image of the homology model of human AMPK α1 and speculative binding mode of docked PT1. KD domain (kinase domain) is colored in cyan, CD domain (common docking domain) is colored in limon, and AID (autoinhibitory domain) is colored in marine. An enlarged representation of residues 5 Å surrounding to docked PT1 (yellow, ball and stick type) is shown, where the potential hydrogen bond in a distance of 3.22 Å between NZ atom of Lys156 and carboxyl acid of PT1 is shown in black dashed line.

**Figure 4.** The effects of PT1 on mutations of predicted interacted residues in human AMPK α1 subunit. A. The effects of 0 μM and 20 μM PT1 on all mutations of the human AMPK α1 subunit. B. PT1 can’t activate the mutants of E96A-α1394, K156A-α1394. C. PT1 still activate the mutants of E96D-α1394,
Figure 5. PT1 stimulates AMPK and ACC phosphorylation in L6 myotubes time-dependently and dose-dependently without increase in cellular AMP:ATP ratio. A. 80 μM of PT1 stimulates AMPK and ACC phosphorylation in L6 myotubes time-dependently. B. PT1 stimulates AMPK and ACC phosphorylation in L6 myotubes dose-dependently after incubation for 1 h. C. PT1 didn’t change the AMP:ATP ratio after incubation for 2 h in L6 myotubes, using inhibitor PD98059 as a positive control. **, \( p < 0.01 \) compared with untreated control.

Figure 6. PT1 stimulates AMPK and ACC phosphorylation in Hela cells which lack of LKB1 without increase in cellular AMP:ATP ratio. A. PT1 stimulates AMPK and ACC phosphorylation in Hela cells in which only CaMKKβ acts as AMPK upstream kinase after incubation for 6 h, and this effects were blocked by 40 μM inhibitor Compound C, or 10 μg/mL inhibitor STO-609 pretreatment. B. PT1 didn’t change the AMP:ATP ratio after incubation for 6 h in Hela cells, using inhibitor PD98059 as a positive control. *, \( p < 0.05 \) compared with untreated control.

Figure 7. PT1 decreases intracellular lipid content in a dose-dependent manner through AMPK activation in cultured human hepatoma HepG2 cells. HepG2 cells were starved in serum-free medium overnight and treated with increasing doses of activator PT1 (5-80 μM) or 2 mM metformin for 24 h. A. Representative immunoblots of AMPK and ACC phosphorylation by PT1 or metformin. PT1 stimulates AMPK and ACC phosphorylation in a dose-dependent manner in HepG2 cells. B. Dose-response effect of activator PT1 on hepatocellular lipid concent. Levels of intracellular triglyceride and cholesterol in cells treated with PT1 for 24 h were measured by using spectrophotometric assays and expressed as μg of lipid per mg protein as described under “Materials and Methods”. The data were represented as the mean ± S.D. (n=3). *, \( p < 0.05 \), **, \( p < 0.01 \), compared with the untreated control.

Figure 8. The triglyceride-lowering effect of PT1 was diminished by pretreatment of AMPK inhibitor compound C in HepG2 cells. After starvation of HepG2 cells in serum-free medium overnight, 40 μM of compound C was added and incubated for further 60 min, followed by incubation with or without 40 μM activator PT1 or 2 mM metformin in medium for an additional 24 h. A. The ability of PT1 or metformin to phosphorylate ACC was diminished by AMPK inhibitor compound C in HepG2 cells. A representative immunoblots from three independent experiments is shown. B. Inhibition of cellular triglyceride levels by PT1 or metformin was abrogated by AMPK inhibitor compound C. Each bar represents the mean ± S.D. (n=3). \( p < 0.05 \) and \( p < 0.01 \) compared two groups as indicated.
FIGURES

Figure 1

A.

B.

C.

D.
Relative Activity (% of control)

LOG(PT1, μM)

AMPKα1β1γ1)
Figure 2

Activity (pmol/min/μM) vs. LOG (PT1, μM)

- ▲ α1(1-335)
- ▼ α1(1-341)
- ● α1(1-351)
- ○ α1(1-367)
- □ α1(1-377)
Figure 3
Figure 4
A.

![Bar graph showing CPM values for different mutations in PT1 at 0 μM and 20 μM.](image)

B.

![Graph showing Activity (pmol/min/μM) vs. LOG (PT1, μM).](image)

C.

![Graph showing CPM values for different mutations in PT1.](image)
Figure 5

A.

B.

C.
Figure 6

A.

<table>
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<th>PT1 (μM)</th>
<th>-</th>
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<th>40</th>
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<td>+</td>
<td>-</td>
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<tr>
<td>Compound C (40 μM)</td>
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<td>-</td>
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B.

![Graph showing AMP:ATP ratio with p-values](image)

- p = 0.0179
- p = 0.627
- *
Figure 7

A.

B.

PT1 (μM)  5  10  20  40  80  
2 mM Metformin  -  -  -  -  -  +  

pAMPK  
AMPK  
pACC  
ACC  
β-actin  

Lipid Content (μg/mg protein)  

DMSO  Metformin  5  10  20  40  80  

Triglyceride  
Cholesterol  

*  **  *  *  *  *  *
Figure 8
A.

<table>
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<th>Condition</th>
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<th>β-actin</th>
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<td>2 mM Metformin</td>
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<td>-</td>
</tr>
<tr>
<td>40 μM Compound C</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</tbody>
</table>

B.

![Bar chart showing TG content (μg/mg protein) for DMSO, 40 μM PT1, and 2 mM Metformin with Compound C.](chart.png)

- DMSO: P<0.01
- 40 μM PT1: P<0.05
Small molecule antagonizes autoinhibition and activates AMP-activated protein kinase in cells
Tao Pang, Zhen-Shan Zhang, Min Gu, Bei-Ying Qiu, Li-Fang Yu, Peng-Rong Cao, Wei Shao, Ming-Bo Su, Jing-Ya Li, Fa-Jun Nan and Jia Li

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