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Ascofuranone prevents ER stress-induced insulin resistance via activation of AMP-activated protein kinase in L6 myotube cells

Seung-Lark Hwang [a,b], Hyeun-Wook Chang [b], In-Kyu Lee [c], Byung-Keun Yang [d], Junji Magae [e], Young-Chae Chang [a,*]

[a] Research Institute of Biomedical Engineering and Department of Pathology, Catholic University of Daegu School of Medicine, Daegu 705-718, Republic of Korea
[b] College of Pharmacy, Yeungnam University, Gyeongsan 712-749, Republic of Korea
[c] Department of Internal Medicine, Kyungpook National University School of Medicine, Daegu 700-721, Republic of Korea
[d] Heuksalim Soil Research Institute, Ochang-eup, Cheongwon-Gun, Chungbuk 363-885, Republic of Korea
[e] Radiation Safety Research Center, Nuclear Technology Research Laboratory, Central Research Institute of Electric Power Industry, Tokyo 201-8511, Japan

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ABSTRACT

The current study presents that ascofuranone isolated from a phytopathogenic fungus, *Ascochyta viciae*, has antitumor activity against various transplantable tumors and a considerable hypolipidemic activity. AMP-activated protein kinase (AMPK) plays a critical role in cellular glucose and lipid homeostasis. We found that ascofuranone improves ER stress-induced insulin resistance by activating AMPK through the LKB1 pathway. In L6 myotube cells, ascofuranone treatment increased the phosphorylation of the Thr-172 residue of the AMPKα subunit and the Ser-79 subunit of acetyl-CoA carboxylase (ACC) and cellular glucose uptake. Ascofuranone-induced phosphorylation of AMPK and ACC was not increased in A549 cells lacking LKB1. Interestingly, ascofuranone treatment also improved insulin signaling impaired by ER stress in L6 myotube cells. These effects were all reversed by pretreatment with Compound C, an AMPK inhibitor or with adenoviral-mediated dominant-negative AMPKα2. Taken together, these results indicated that ascofuranone-mediated enhancement of glucose uptake and reduction of impaired insulin sensitivity in L6 cells is predominantly accomplished by activating AMPK, thereby mediating beneficial effects in type 2 diabetes and insulin resistance.

1. Introduction

Ascofuranone is a prenylphenol antifungal antibiotics isolated from an incomplete fungus, *Ascochyta viciae.* Although originally reported to be an antiviral antibiotics [1–3], ascofuranone exhibits a large variety of physiological activities including hypolipidemic activity [2], suppression of hypertension [4], immune-modulation [5] and antitumor activity [6,7]. In addition, the plasma glucose concentration was found to be significantly reduced in type 1 and 2 diabetes mice treated with ascofuranone [8,9], suggesting a possible role of ascofuranone in lowering the blood glucose level. However, the molecular mechanisms underlying ascofuranone's beneficial effects are largely unknown.

AMP-activated protein kinase (AMPK) is a phylogenetically conserved intracellular energy sensor that has been implicated in the regulation of food intake, body weight, glucose uptake and lipid metabolism [10,11]. AMPK activity is stimulated through phosphorylation of Thr^{172} within the activation domain of the α subunit by upstream AMPK kinases, the tumor suppressor LKB1 and calcium/calmodulin-dependent protein kinase kinase (CaMKK) [8–12].

Once activated, AMPK phosphorylates its downstream substrates to reduce ATP-consuming pathways, including fatty acid, cholesterol, and triacylglycerol synthesis, and increases ATP-generating pathways, including fatty acid oxidation and glycolysis [13–15]. Type 2 diabetes and obesity contribute to insulin resistance in skeletal muscle [16]. Recent reports have shown that AMPK activation may account for at least some of the beneficial effects of exercise such as increased fatty acid oxidation [17,18], increased mitochondrial biogenesis [19], and possibly glucose uptake [20]. AMPK is activated indirectly by metformin [21] and thiazolidinediones [22], widely used treatments for type 2 diabetes, suggesting that AMPK activity regulates insulin sensitivity and reduces plasma glucose and lipids. The present study was performed to determine whether the effects of ascofuranone on glucose uptake is mediated by AMPK and whether AMPK regulates glucose transport in ER stress-induce insulin resistance in L6 myotube cells. We found that treating L6 myotubes with ER stressor (tunicamycin) inhibited the ability of insulin to stimulate Akt phosphorylation and glucose uptake. In contrast, ER stress significantly increased JNK and mTOR-S6K activity. ER stress-induced JNK and mTOR-S6K activation

* Corresponding author. Fax: +82 53 650 4834.
E-mail address: ycchang@cu.ac.kr (Y.-C. Chang).

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was diminished by ascofuranone. Finally, overexpression of dominant-negative AMPKα2 abolished the re-repressed insulin-sensitizing glucose uptake through treatment of ascofuranone under ER stress conditions. Taken together, these findings demonstrate that AMPK might have protective effects against ER stress-induced insulin resistance. Also, we have found that activation of AMPK by ascofuranone reverses the defects in ER stress-impaired insulin signaling and glucose uptake, and these effects were blocked by selective AMPK inhibitor, Compound C, or dominant-negative AMPKα2 in L6 myotube cells.

2. Materials and methods

2.1. Chemicals and reagents

L6 myotube, H1299 and A549 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Fetal Bovine Serum (FBS), minimum essential medium α-MEM) and Dulbecco's modified Eagle's medium (DMEM) were obtained from Invitrogen (Carlsbad, CA, USA). Tunicamycin, 2-deoxy-glucose, Compound C and 5-amino-4-imidazole carboxamide ribose (AICAR) were purchased from Calbiochem (Merk, Darmstadt, Germany). Insulin, JNK inhibitor (SP600125), BSA, and other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). 2-Deoxy-[3H] D-glucose was obtained from Perkin–Elmer Life Sciences (Boston, MA, USA). Antibodies against phospho-AMPKα (Thr^{172}), phospho-acetyl-CoA carboxylase (ACC) (Ser^{79}), phospho-mTOR (Ser^{2448}), phosphor-S6 K (Thr^{389}), and phopho-Akt (Ser^{473}) were purchased from Cell Signaling Technology (Beverly, MA, USA). β-tubulin antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Ascofuranone was purified from a broth culture of *A. visiae* as described previously [2]. All other reagents were of the highest analytical grade.

2.2. Cell culture

L6 cells (American Type Culture Collection, Manassas, VA, USA) were maintained in α-MEM (minimum essential medium α Invitrogen) supplemented with 10% (v/v) FBS (fetal bovine serum), 100 units/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C. L6 cells were allowed to differentiate into myotube cells as reported previously [23]. Lung cancer cell lines A549, H1299 cells were maintained in RPMI and supplemented with 10% (v/v) fetal bovine serum (FBS), 100 units/ml of penicillin. Cells were incubated at 37 °C in a 5.0% CO_2 atmosphere.

2.3. 2-Deoxy-[^{3}H]D-glucose uptake

The radiolabeled 2-deoxyglucose uptake assay was carried out as previously described [23].

2.4. Western blot

Western blot experiments have been described in detail previously [23].

2.5. Recombinant adenovirus

Plasmids encoding c-Myc-tagged forms of dominant-negative α1 and α2AMPK (DN-AMPK) and a constitutively active form of AMPK (CA-AMPK) recombinant adenovirus [24] were kind gifts from Dr. In-Kyu Lee (Department of Internal Medicine, Kyungpook National University School of Medicine, Daegu, Korea). We used adenovirus-mediated gene transfer to express this mutant gene in cultured L6 myotubes. Cells were infected with adenovirus diluted in AMPK (DN-AMPK) and a constitutively active form α-MEM containing 10% FBS and incubated for 4 h. After removal of viral suspension, L6 myotubes were incubated with α-MEM containing 2% FBS for 18 h and stimulated with reagents or exposed to ER stress. Adenovirus vector expressing β-galactosidase (Ad-β-gal) was used as a control. Efficiency of infection, determined by *lacZ* gene expression in cultured L6 myotubes, was consistently at >90% with this method.

2.6. Statistics

Results are expressed as the means ± the standard error of the mean (SE). Significance was analyzed using two-tailed unpaired Student's *t* tests.

3. Results

3.1. Ascofuranone stimulates AMPK activation in L6 myotubes

We found that ascofuranone (Fig. 1A) from *A. visiae* exhibited potent AMPK activation (Fig. 1B). Ascofuranone increased phosphorylation of the Thr-172 residue of the AMPKα subunit and the Ser-79 subunit of ACC in a dose-dependent manner without changing the total protein levels of AMPKα, and ACC in L6 myotubes. We used phosphorylation state-specific antibodies to probe the immunoblots prepared from L6 myotube cells treated with varying concentrations of ascofuranone for 2 h (Fig. 1B). We found that ascofuranone promotes the dose-dependent phosphorylation of AMPKα and ACC (Fig. 1B). We next examined the effect of ascofuranone on glucose uptake in L6 myotubes. For short term treatment of ascofuranone, L6 myotubes were incubated in serum-free media for 5 h, and then the indicated concentrations of ascofuranone for 2 h (Fig. 1C). Glucose uptake was induced in a dose-dependent manner, showing 1.7-fold (2.2 ± 0.1 pmol/mg/min) induction at 5 μM and a maximum 2.2-fold (2.8 ± 0.2 pmol/mg/min) ($p < 0.01$) induction at 10 μM of ascofuranone, compared with untreated control cells (1.3 ± 0.1 pmol/mg/min) (Fig. 1C). Similarly, AMPK activator, AICAR also increased glucose uptake by 2-fold (2.6 ± 0.2 pmol/mg/min) ($p < 0.01$) compared with untreated cells (Fig. 1C).

3.2. Ascofuranone causes increase glucose uptake in L6 myotubes by LKB1-AMPK pathway

To identify the specificity of ascofurnaone in AMPK activity, we inhibited the AMPK activity using Compound C, an AMPK inhibitor [25], preventing the phosphorylation of AMPK and its downstream target ACC. Pretreatment of L6 myotubes with Compound C significantly suppressed ascofuranone-induced phosphorylation of AMPK and ACC without changing the total expression levels of AMPK and, ACC (Fig. 2A). Accordingly, pretreatment of L6 myotubes with Compound C significantly attenuated the ascofuranone-induced glucose uptake (Fig. 2B). Glucose uptake increased 2.1-fold in cells with ascofuranone treatment (3.5 ± 0.2 pmol/mg/min) ($p < 0.01$) compared with untreated control cells (1.7 ± 0.1 pmol/mg/min) (Fig. 2B). Pretreatment of the cells with Compound C before ascofuranone treatment abolished glucose uptake to 43% of the levels in cells treated with ascofuranone alone (3.5 ± 0.2 pmol/mg/min in ascofuranone-treated versus 1.7 ± 0.1 pmol/mg/min in Compound C-pretreated) (Fig. 2B). The kinase LKB1 is biochemically sufficient to activate AMPK in vitro and is genetically required for AMPK activation during energy stress in a number of mammalian cell lines [26,27]. To determine if AMPK activation in response to ascofuranone is mediated by LKB1, we investigated the effects of ascofuranone in condition of mutated LKB1. We treated LKB1-mutant (A549) and wild-type (H1299) lung caner cell lines [28] with ascof-