Regular Article

Atractylenolide III Enhances Energy Metabolism by Increasing the SIRT-1 and PGC1 α Expression with AMPK Phosphorylation in C2C12 Mouse Skeletal Muscle Cells

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Targeting energy expenditure provides a potential alternative strategy for achieving energy balance to combat obesity and the development of type 2 diabetes mellitus (T2DM). In the present study, we investigated whether atractylenolide III (AIII) regulates energy metabolism in skeletal muscle cells. Differentiated C2C12 myotubes were treated with AIII (10, 20, or 50 μ M) or metformin (2.5 mM) for indicated times. The levels of glucose uptake, the expressions of key mitochondrial biogenesis-related factors and their target genes were measured in C2C12 myotubes. AIII significantly increased the glucose uptake levels, and significantly increased the expressions of peroxisome proliferator-activated receptor coactivator-1 α (PGC1 α) and mitochondrial biogenesis-related markers, such as, nuclear respiratory factor-1 (NRF-1), and mitochondrial transcription factor A (TFAM) and mitochondrial mass and total ATP contents. In addition, AIII significantly increased the phosphorylation of AMP-activated protein kinase (AMPK) and the expression of sirtuin1 (SIRT1). These results suggest that AIII may have beneficial effects on obesity and T2DM by improving energy metabolism in skeletal muscle.

Key words atractylenolide III; mitochondrial biogenesis; AMP-activated protein kinase; peroxisome proliferator-activated receptor coactivator-1 α ; sirtuin1; C2C12 cell

The prevalence of obesity, metabolic syndrome, and type 2 diabetes mellitus (T2DM) has increased over past decades,¹⁾ and it is estimated over 1.9 billion adults worldwide are overweight, and that more than 600 million are clinically obese.²⁾ Obesity, T2DM, and metabolic syndrome are among the most frequent pathological consequences of chronic energy metabolism imbalance.³⁾ The maintenance of energy balance depends on the regulations of energy intake and expenditure. However, the long-term efficacies of reduced energy intake, such as, by caloric restriction, are problematic, and thus, the targeting of energy expenditure could provide a more effective means of promoting energy balance and combating obesity and T2DM.⁴⁾

Skeletal muscle is regarded a target organ in the context of cellular bioenergetics, and is known to play an important role in the maintenance of glucose homeostasis and insulin sensitivity. Insulin resistance is associated with myocellular lipid accumulation, and it has been shown that insulin resistance is caused or accelerated by impaired oxidative capacity of skeletal muscle.^{5,6)} One study conducted under euglycemic conditions showed that skeletal muscle is responsible for ca. 80% of total body glucose uptake.⁷⁾

Peroxisome proliferator-activated receptor coactivator 1 α (PGC1 α), AMP-activated protein kinase (AMPK) and sirtuin1 (SIRT1) compose an energy sensing network that controls energy expenditure in skeletal muscle.⁸⁾ PGC1 α plays a central role in the regulation of cellular energy metabolism, which results from the up-regulation of oxidative metabolism and the stimulation of mitochondrial biogenesis.⁹⁾ Furthermore, it is known metabolic sensors, such as AMPK, SIRT1 and PGC1 α ,

constitute vital links in the network responsible for the regulation of cellular energy metabolism.⁸⁾

In recent years, a number of natural compounds have been shown to increase energy expenditure in skeletal muscle by increasing mitochondrial biogenesis and oxidative phosphorylation via the activations of PGC1 α , AMPK, and SIRT1.^{10,11)} And, in a previous study, we found the root extract of *Atractylodes macrocephala* KOIDZUMI (Atractylodis Rhizoma Alba; ARA) increases glucose and lipid metabolism in skeletal muscle by enhancing the activities of PGC1 α , AMPK and SIRT1.¹²⁾ Atractylenolide III (AIII) is the primary bioactive compound of this extract.¹³⁾ AIII has been previously reported to have anti-inflammatory^{14,15)} and gastroprotective effects,¹⁶⁾ however its effects on skeletal muscle energy metabolism have not been investigated. Therefore, in the present study, we investigated the effects of AIII on energy metabolism via PGC1 α /SIRT1/AMPK pathway activation in mouse skeletal muscle cells.

MATERIALS AND METHODS

Cell Culture and Treatments Mouse C2C12 myoblasts (CRL-1772; Manassas, VA, U.S.A.) maintained in Dulbecco's Modified Eagle's Medium (DMEM, GibcoBRL, U.S.A.) supplemented with 10% fetal bovine serum (FBS) and a penicillin/streptomycin mix (Invitrogen, Grand Island, NY, U.S.A.) were used in the study. When cells were confluent, they were induced to differentiate into myotubes by placing them in differentiation medium (DMEM supplemented with 2% horse serum; Invitrogen) for 4 d. C2C12 myotubes were then treated with or without different concentrations of AIII (A2987,

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Sigma-Aldrich, St. Louis, MO, U.S.A.) at 10, 20 and 50 μM or metformin (the reference drug) at 2.5 mM.

Glucose Uptake Assay The amount of glucose uptake in C2C12 myotubes were measured using Glucose uptake cell-based assay kit (Cayman Chemical Co., Ann Arbor, MI, U.S.A.). Briefly, C2C12 myotubes were placed in 1 mL of glucose-free medium containing 150 $\mu\text{g}/\text{mL}$ of 2-[N-(7-nitro-benz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (NBDG) the wells of a 4-well plate for 4 h. Supernatants were then removed and 200 μL of cell-based assay buffer was added to each well. Amounts of 2-NBDG taken up by cells were determined by fluorescence microscopy (Leica Biosystems, Wetzlar, Germany).

Western Blot Cells were lysed in ice-cold lysis buffer (0.1 mL of 50 mM Tris-HCl (pH 7.2) containing 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.15 M NaCl, and 1% NP-40), and lysates were centrifuged at 12000 $\times g$ for 20 min at 4°C. After measuring protein content using a bicinchoninic acid (BCA) assay, equal amounts of protein (20 $\mu\text{g}/\text{mL}$) were electrophoresed on 10% SDS-acrylamide gels, and then transferred to nitrocellulose membranes using an electrical transfer system. Non-specific binding was blocked by treating membranes with 3% skim milk in TBST buffer (5 mM Tris-HCl, pH 7.6, 136 mM NaCl, and 0.1% Tween-20) for 1 h. Blots were incubated for 1 h at room temperature (RT) with primary antibodies against anti-PGC1 α , anti-mitochondrial transcription factor A (TFAM), anti-nuclear respiratory factor-1 (NRF-1), anti-phospho-AMPK α (Thr 172), anti-AMPK α , anti-SIRT1 (Cell Signaling Technology, Danvers, MA, U.S.A.), and anti- β -Actin (Sigma-Aldrich), incubated for 1 h at RT with horseradish peroxidase (HRP)-labeled anti-mouse immunoglobulin G (IgG) (1:1000, Santa Cruz Biotechnology, MN, U.S.A.), washed three times with 1 $\times$ TBST, and developed using ECL Western detection reagents (GE Healthcare Bio-Sciences, PA, U.S.A.). Protein bands were quantified by densitometry using Image J software.

RT-PCR Total RNA was isolated from cells using TRIzol reagent, according to the manufacturer's instructions (Gibco-BRL Life Technologies Inc., Grand Island, NY, U.S.A.), and quantified using a NanoDrop ND-1000 spectrophotometer (Nadrop Technologies, Inc., Wilmington, DE, U.S.A.). cDNA was generated from 2 μg of total RNA using a Reverse Transcription System kit (Promega, Fitchburg, WI, U.S.A.).

PCR was conducted using a Blend Taq PCR kit (Toyobo, Osaka, Japan) using the following conditions; 2 min at 94°C (pre-denaturation), 30 s at 94°C (denaturation), 30 s at 60°C (annealing), and 1 min at 72°C (extension) for 30 cycles. The following primers were used. PGC1 α : Forward; 5'-CAC CAAACCCACAGAAAAACAG-3', Reverse; 5'-GGGTACAGAG GAAAGAGATAAAGTTG, NRF-1 : Forward; 5'-AATGTC CGCAGTGATGTCC-3', Reverse; 5'-GCCTGAGTTTGTGTT TGCTG-3', TFAM : Forward; 5'-CACCCAGATGCAAAA CTTTACAG-3', Reverse; 5'-CTGCTCTTTATACTTGCTCAC AG-3', SIRT1 : Forward; 5'-GATCCTTCAGTGTCATGG TT-3', Reverse; 5'-GAAGACAATCTCTGGCTTCA-3', and glyceraldehyde-3-phosphate dehydrogenase (GAPDH): Forward 5'-GACATCATACCTTGGCAGG-3', Reverse; 5'-CTC GTGAGTCTACTGGT-3'/ACTGGT-3' used as the internal control. The bands were detected by UV and quantified by densitometry using Image J software (v. 1.48, NIH, U.S.A.).

Mitochondrial Mass Assay Mitochondrial mass was evaluated using 10-N-Nonyl acridine orange (NAO; a fluorescent probe) (ENZO Life Sciences, NY, U.S.A.). Briefly, C2C12 cells were incubated in DMEM containing 10 nM of NAO for 1 h at 37°C in the dark, trypsinized, and resuspended in DMEM without NAO. Fluorescence intensities were measured using a luminometer (Promega) using excitation and emission wavelengths of 495 and 519 nm, respectively.

ATP Content Assay Total ATP contents were determined using an ATP calorimetric assay kit (BioVision, Inc., Headquarters, CA, U.S.A.). Absorbance was measured at 570 nm.

Data Analysis All experiments were performed in triplicate. Results are expressed as mean $\pm$ standard errors of mean (S.E.M.). Statistical significance was determined by ANOVA followed by Tukey's test in GraphPad Prism (GraphPad Software, Inc., San Diego, CA, U.S.A.). Statistical significance was accepted for p values <0.05 .

RESULTS

Effect of AIII on Glucose Uptake in C2C12 Myotubes

The glucose uptake assay showed that AIII at 20 or 50 μM significantly increased myotube glucose uptake as compared with untreated control or cells treated with apigenin (100 μM ; a glucose transporter inhibitor) (Fig. 1). Using an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell

Fig. 1. Effect of AIII on Glucose Uptake in C2C12 Myotubes

Cells were treated with AIII, metformin (2.5 mM), or apigenin (100 μM) for 4 h. Glucose uptakes were observed by fluorescence microscopy after staining with 2-NBDG ($\times 100$ original magnification).

Fig. 2. Effect of AIII on PGC1 α Expression in C2C12 Myotubes

Cells were treated with AIII (10, 20, or 50 μ M) or (2.5 mM) for 24 h (for mRNA) or 45 min (for protein). (A) PGC1 α mRNA levels were assessed by RT-PCR. (B) PGC1 α protein levels were assessed by Western blotting. Results are presented as mean $\pm$ S.E.M. of three independent experiments. * p < 0.05 vs. untreated cells.

Fig. 3. Effect of AIII on Mitochondrial Biogenesis in C2C12 Myotubes

Cells were treated with AIII (10, 20, or 50 μ M) or (2.5 mM) for 24 h (for mRNA) or 45 min (for protein). (A) The mRNA expressions of NRF and TFAM were assessed by RT-PCR. (B and C) The protein expressions of NRF and TFAM were analyzed by Western blotting. (D) Mitochondrial mass and (E) total ATP content were measured by fluorescence staining with NAO or using an ATP assay, respectively. Results are presented as mean $\pm$ S.E.M. of three independent experiments. * p < 0.05 vs. untreated cells.

viability assay, AIII at 20 and 50 μM have no toxicity in the cells (data not shown). Meanwhile, treatment with metformin also increased glucose uptake levels.

Effect of AIII on PGC1 α Expression in C2C12 Myotubes PGC1 α is a critical regulator of mitochondrial biogenesis and glucose metabolism in skeletal muscle and promotes response to exercise to maintain the balance between energy requirements and supply,⁹⁾ and thus, we investigated the effects of AIII on PGC1 α expression in C2C12 myotubes by RT-PCR and Western blotting. The expression of PGC1 α mRNA (Fig. 2A) and protein (Fig. 2B) was increased in C2C12 myotubes treated with AIII, indicating AIII enhances PGC1 α activity in skeletal muscle cells.

Effect of AIII on Mitochondrial Biogenesis in C2C12 Myotubes NRF-1 and TFAM are transcription factors targeted by PGC-1 α for the induction of mitochondrial biogenesis.¹⁷⁾ Thus, to determine whether AIII increases mitochondrial biogenesis in skeletal muscle, we examined the expressions of NRF-1 and TFAM in C2C12 myotubes by PCR and Western blotting, respectively. Treating C2C12 myotubes with AIII dose-dependently increased the expressions of NRF-1 and TFAM at the mRNA (Fig. 3A) and protein levels (Figs. 3B, C), and similar increases were observed in metformin-treated cells.

Mitochondria are responsible for producing most of the ATP needed for energy-requiring reactions in eukaryotic cells, and ATP synthesis occurs in concert with expressional increases of key regulators of mitochondrial biogenesis.¹⁸⁾ Therefore, we measured the effect of AIII on ATP levels and mitochondrial mass using an ATP assay and by staining with NAO, which binds to cardiolipin (a phospholipid specifically present on the mitochondrial membranes),¹⁹⁾ respectively. AIII dose-dependently increased myotube NAO fluorescence intensities (Fig. 3D) and total ATP contents (Fig. 3E), and similar results were observed in metformin-treated cells. These results indicate AIII increases mitochondrial biogenesis in skeletal muscle cells.

Effect of AIII on the Phosphorylation of AMPK in C2C12 Myotubes AMPK is a master sensor and regulator of

energy homeostasis at the cellular level, and plays fundamental roles in the regulations of glucose and lipid metabolism in skeletal muscle.²⁰⁾ In addition, AMPK is also required for mitochondrial biogenesis and for the expressional up-regulation of PGC1 α in muscle.²¹⁾ Therefore, we investigated whether AIII regulates AMPK activity in C2C12 myotubes by Western blot. AIII at 20 and 50 μM was found to increase AMPK phosphorylation in C2C12 myotubes *versus* untreated controls (Fig. 4). These results indicate that AIII increases glucose metabolism by activating the AMPK pathway in skeletal muscle cells.

Effect of AIII on SIRT1 Expression in C2C12 Myotubes AMPK enhances SIRT1 activity by increasing cellular oxidized form of nicotinamide adenine dinucleotide (NAD⁺ levels, which results in the deacetylations and modulations of the activities of downstream SIRT1 targets. Furthermore, this ex-

Fig. 4. Effect of AIII on the Phosphorylation of AMPK in C2C12 Myotubes

Cells were treated with AIII (10, 20, and 50 μM) or metformin (2.5 mM) for 45 min. The phosphorylation of AMPK was determined by Western blot, and histogram was presented as phospho- to total AMPK ratios. Results are presented as mean $\pm$ S.E.M. of three independent experiments. * p <0.05 and ** p <0.01 vs. untreated cells.

Fig. 5. Effect of AIII on the Expression of SIRT1 in C2C12 Myotubes

C2C12 myotubes were treated with AIII (10, 20, or 50 μM) or metformin (2.5 mM) for 24 h. The expressions of SIRT1 at the mRNA (A) and protein (B) levels were analyzed by RT-PCR and Western blotting, respectively. Data was presented as mean $\pm$ S.E.M. of three independent experiments. * p <0.05 and ** p <0.01 vs. untreated cells.

plains many of the convergent biological effects of AMPK and SIRT1 in the context of energy metabolism.²²⁾ To investigate the effects of AIII on SIRT1 activity, we used RT-PCR and Western blotting to assess SIRT1 mRNA and protein levels. Treatment of C2C12 myotubes with AIII was found to dose-dependently increase SIRT1 mRNA (Fig. 5A) and protein (Fig. 5B) levels. These results indicate that AIII upregulates AMPK activation-mediated SIRT1 activity in skeletal muscle cells.

DISCUSSION

We previously reported that ARA extract increases glucose and lipid metabolism in C2C12 myotubes by enhancing the activities of PGC1 α , SIRT1, and AMPK.¹²⁾ This study was undertaken to determine whether AIII affects skeletal muscle cell energy metabolism. ARA actually contains three types of atractylenolides (I, II, and III),²³⁾ although AIII is the most abundant.¹³⁾ AIII has been reported to possess various biological activities, which include the inhibition of lipopolysaccharide-induced inflammatory responses in macrophages,¹⁴⁾ the suppression of histamine release from mast cells *via* the regulation of interleukin (IL)-6 at the cellular level,²⁴⁾ and the induction of lung carcinoma cell apoptosis *via* cell growth inhibition and increasing lactate dehydrogenase release.¹⁵⁾ Here, we describe for the first time the regulatory effects of AIII on glucose homeostasis and energy metabolism in skeletal muscle cells.

The PGC1 α /SIRT1/AMPK pathway plays important roles in mitochondrial biogenesis and glucose metabolism in skeletal muscle.^{8,22,25)} In the present study, we found that AIII enhanced mitochondrial energy levels in C2C12 myotubes and their glucose uptakes by activating this pathway. It has been shown PGC1 α increases energy expenditure by increasing mitochondrial biogenesis and respiration rates in skeletal muscle.⁹⁾ PGC1 α also interacts with NRF-1, a transcription factor of many mitochondrial genes, including TFAM a direct regulator of mitochondrial DNA replication and transcription.²⁶⁾ In the present study, the mRNA and protein levels of PGC1 α , NRF-1 and TFAM were dose-dependently increased by AIII treatment, and AIII significantly increased mitochondrial mass and total ATP content in C2C12 myotubes. These results indicate that AIII can increase mitochondrial biogenesis by activating PGC1 α , NRF-1, and TFAM, in skeletal muscle cells.

AMPK and SIRT1 play major roles in the metabolic regulation of skeletal muscle, and have been reported to impact PGC1 α expression, and thus, to transcriptionally regulate energy expenditure.⁸⁾ AMPK switches on catabolic processes that provide alternative routes for ATP generation, glucose uptake, glycolysis, fatty acid oxidation, and mitochondrial biogenesis, but switches off anabolic processes that consume ATP for the syntheses of fatty acids, triglyceride, cholesterol, and glucose.²⁰⁾ Therefore, AMPK activation in skeletal muscle is associated with lower plasma glucose levels because it increases glucose uptake for fatty acid oxidation by inhibiting fatty acid synthesis by directly phosphorylating acetyl-CoA carboxylase (ACC) and PGC1 α .²⁰⁾ In the present study, AIII was found to increase glucose metabolism by directly activating PGC1 α , and thus, activating the AMPK pathway.

Under conditions of energy deficiency, such as, caloric restriction, which increases intracellular NAD⁺ levels and ac-

tivates SIRT1 *via* PGC1 α deacetylation.²⁷⁾ AMPK and SIRT1 regulate each other and target molecules, such as, PGC1 α , forkhead box-containing protein, and endothelial NO synthase.²⁵⁾ Previous studies have shown that AMPK activation and PGC1 α phosphorylation precede SIRT1 activation and PGC1 α deacetylation, and in the present study, SIRT1 expression was found to be significantly increased by AIII in C2C12 myotubes.

Summarizing, our data showed that AIII improves mitochondrial biogenesis and glucose metabolism by activating the PGC1 α /SIRT1/AMPK pathway in skeletal muscle cells. Thus, this finding suggests that AII has therapeutic potential for the treatment of obesity and T2DM by improving skeletal muscle energy metabolism.

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Conflict of Interest The authors declare no conflict of interest.

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