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p-Coumaric acid modulates glucose and lipid metabolism via AMP-activated protein kinase in L6 skeletal muscle cells

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ABSTRACT

p-Coumaric acid (3-[4-hydroxyphenyl]-2-propenoic acid) is a ubiquitous plant metabolite with antioxidant, anti-inflammatory, and anticancer properties. In this study, we examined whether *p*-coumaric acid modulates glucose and lipid metabolism via AMP-activated protein kinase (AMPK) in L6 skeletal muscle cells. *p*-Coumaric acid increased the phosphorylation of AMPK in a dose-dependent manner in differentiated L6 skeletal muscle cells. It also increased the phosphorylation of acetyl-CoA carboxylase (ACC) and the expression of *CPT-1* mRNA and PPARα, suggesting that it promotes the β-oxidation of fatty acids. Also, it suppressed oleic acid-induced triglyceride accumulation, and enhanced 2-NBDG uptake in differentiated L6 muscle cells. Pretreatment with compound C inhibited AMPK activation, reduced ACC phosphorylation and 2-NBDG uptake, and increased triglyceride accumulation. However, *p*-coumaric acid counterbalanced the inhibitory effects of compound C. Taken together, these results suggest that *p*-coumaric acid modulates glucose and lipid metabolism via AMPK activation in L6 skeletal muscle cells and that it has potentially beneficial effects in improving or treating metabolic disorders.

1. Introduction

Skeletal muscle is a major site of human energy production; it is responsible for approximately 70% of glucose uptake and oxidation in the body [1]. Skeletal muscle also plays crucial roles in lipid homeostasis, especially fatty acids [2]. Although glucose and fatty acids are important sources of energy in skeletal muscle, an excess of glucose and lipids in human blood can cause insulin resistance, leading to the development of type 2 diabetes [3–5].

AMP-activated protein kinase (AMPK), which is involved in the regulation of energy homeostasis, is composed of a catalytic α-subunit and regulatory β- and γ-subunits [6]. AMPK is activated by the phosphorylation of its α-subunit at threonine-172 [7,8]. Under conditions of metabolic stress, including oxidative stress, ischemia, and excessive exercise, AMPK promotes the production of energy by mitochondria to restore energy homeostasis [6,9]. Activated AMPK has exercise-like beneficial effects on the human body, including an increase in fatty acid β-oxidation, a decrease in fatty acid and triacylglycerol synthesis, and an increase in glucose uptake [7–9]. Anti-diabetic agents such as 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside (AICAR) and metformin modulate the metabolism of glucose and lipids as AMPK activators. Therefore, AMPK is considered to be a therapeutic factor in the treatment of metabolic diseases, including lipid-related disorders and type 2 diabetes [10,11].

Recently, there has been increasing interest in natural products derived from edible plants with AMPK-activating properties and fewer side effects for the management of lifestyle-related diseases, which are caused by an imbalance in energy homeostasis [12–14]. Natural products play a remarkable role in traditional medicine due to their various health-promoting properties. There is an inverse association between dietary phenolic compound intake and mortality from various diseases [15].

p-Coumaric acid (3-[4-hydroxyphenyl]-2-propenoic acid) is a ubiquitous plant metabolite found in various edible plants [16]. Previous studies revealed that *p*-coumaric acid has antioxidant properties [17–19] and can reduce the risk of stomach cancer [20]. In addition, *p*-coumaric acid prevents lysosomal dysfunction and reduces myocardial infarct size in rats with an isoproterenol-induced myocardial infarct [21]. In addition, it inhibits melanogenesis and prevents hepatotoxicity in ethanol-induced mice [22,23]. We previously showed that a bamboo grass (*Sasa quelpaertensis* Nakai) extract, which contained a considerable amount of *p*-coumaric acid, had anti-obesity properties via AMPK in mice fed a high-fat diet and 3T3-L1 cells [24]. Thus, in this study we explored

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whether *p*-coumaric acid may modulate the metabolism of glucose and lipids in L6 skeletal muscle cells.

2. Materials and methods

2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin–streptomycin (P/S) were purchased from Gibco–BRL (Grand Island, NY, USA). A Lactate Dehydrogenase (LDH) Cytotoxicity Detection Kit was purchased from Takara Shuzo Co. (Otsu, Shiga, Japan). Antibodies against AMPKα, phospho-AMPK (p-AMPK) α^{Thr172}, acetyl-CoA carboxylase (ACC), and phospho-ACC (p-ACC)^{ser79} were acquired from Cell Signaling Technology (Beverly, MA, USA). Antibodies against peroxisome proliferator-activated receptor (PPAR)α and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Sigma Chemical Co. (St. Louis, MO, USA), respectively. 2-[N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxyglucose (2-NBDG) was acquired from Invitrogen (Carlsbad, CA, USA). Compound C was purchased from Calbiochem (Merck, Darmstadt, Germany). All other reagents were purchased from Sigma Chemical Co. unless otherwise stated.

2.2. Cell culture

L6 rat skeletal muscle cells obtained from the American Type Culture Collection (Manassas, VA, USA) were maintained in DMEM containing 10% (v/v) FBS and 1% P/S, and cultured in a humidified atmosphere of 5% CO_2 at 37 °C. For differentiation into myotubes, the medium was switched to DMEM containing 2% (v/v) FBS and replaced every 2 days prior to use in our experiments. The differentiated L6 myotubes were serum deprived for 4 or 16 h in serum-free DMEM supplemented with 0.5% bovine serum albumin (BSA).

2.3. Cell viability and cytotoxicity assays

Cell viability and cytotoxicity were examined by the MTT assay and LDH assay, respectively. Cell viability was examined using MTT solution after 48 h treatment with *p*-coumaric acid. The MTT solution in each well was completely removed, and 100 μL DMSO was added. The dissolved formazan was read using a microplate reader at 595 nm. The LDH assay was performed using an LDH Cytotoxicity Detection Kit. The cells were treated with 1% Triton X-100 to cause maximal LDH release (positive control). The absorbance was measured at 490 nm. Cell viability and cytotoxicity were calculated as the relative absorbance compared to that of the control.

2.4. Western blotting

The cell pellets were resuspended in lysis buffer containing 1× RIPA, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, 1 mM NaF, 1 μg/mL aprotinin, 1 μg/mL pepstatin, and 1 μg/mL leupeptin and incubated on ice for 1 h. After the cell debris was removed by centrifugation, the protein concentration in each lysate was measured using Bio-Rad Protein Assay reagent (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein were electrophoresed on 8% or 10% SDS–polyacrylamide gels, and then transferred to polyvinylidene difluoride membranes. The membranes were blocked through incubation for 1 h at room temperature with a 0.1% solution of Tween 20 in Tris-buffered saline containing 5% nonfat dry milk or 5% BSA. Incubation overnight at 4 °C with primary antibodies was followed by incubation with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Then the blots were visualized using the WEST-ZOL Western Blot Detection System (Intron Biotechnology, Sungnam, Korea).

2.5. RNA isolation and real-time PCR

Total RNA was isolated using total RNA extraction reagent (RNAiso Plus; Takara Shuzo Co.). The absorbance of each extracted RNA was measured using a NanoDrop 2000 spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) at 260/280 nm. cDNA synthesis was carried out using a Maxime RT PreMix Kit (Intron Biotechnology). For real-time PCR, cDNA and primers were prepared with a DyNAmo™ ColorFlash SYBR Green qPCR Kit (Thermo Fisher Scientific Inc.) according to the instruction manual. The following primer sequences were used for real-time PCR: carnitine palmitoyltransferase-1 (CPT-1), forward 5′-GCAAACTGGACCGAGAAGAG-3′, and reverse 5′-CGACCTTTGTGGTAGACAGC-3′; and β-actin, forward 5′-CCCAC ACTGTGCCCATCTAT-3′, and reverse 5′-CA CGCACGATTTCCCTCTCA-3′. The amplification conditions were as follows: 95 °C (5 min) for DNA polymerase activation, followed by 95 °C (20 s), 63 °C (20 s), and 72 °C (30 s). Once the reactions were completed, a melting curve was constructed to confirm that amplification had been properly performed. β-Actin was used to normalize CPT-1 expression. The data were analyzed using Opticon Monitor software (ver. 3.1; Bio-Rad Laboratories).

2.6. Oil Red O staining and measurement of the triglyceride content

Cells were washed and fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) for 1 h. The fixed cells were washed an additional three times with water and then stained with Oil Red O solution for 40 min [25]. Excess stain was removed by washing with water, and the stained cells were dried completely. The stained lipid droplets were dissolved with 4% Nonidet P-40 in isopropanol, and then quantified by measuring the absorbance at 520 nm.

2.7. 2-NBDG uptake assay

2-NBDG uptake was measured using fluorescent 2-NBDG reagent [26]. L6 skeletal muscle cells were incubated with low-glucose DMEM containing 0.5% BSA overnight. Serum-deprived cells were treated with fluorescent 2-NBDG and *p*-coumaric acid; subsequently, the culture medium was removed and the cells were washed with 1× PBS. The fluorescence intensity was measured at an excitation of 485 nm and emission of 520 nm using a fluorescence micro reader.

2.8. Statistical analysis

All results are presented as the mean ± standard deviation (SD) of three independent experiments. The data were analyzed using a Student's *t*-test, and $p < 0.05$ was considered to be a significant value.

3. Results and discussion

The appropriate concentrations of *p*-coumaric acid and oleic acid for cellular treatment were determined by MTT and LDH assays. *p*-Coumaric acid (up to 100 μM) and oleic acid (1 mM) did not influence the viability and cytotoxicity of L6 skeletal muscle cells (Fig. 1A and B). When differentiated L6 skeletal muscle cells were exposed to *p*-coumaric acid, the phosphorylation of AMPK was increased in a time- and dose-dependent manner (Fig. 1C and D). These results suggest that *p*-coumaric acid acts as an AMPK activator in L6 skeletal muscle cells. Thus, we investigated the key

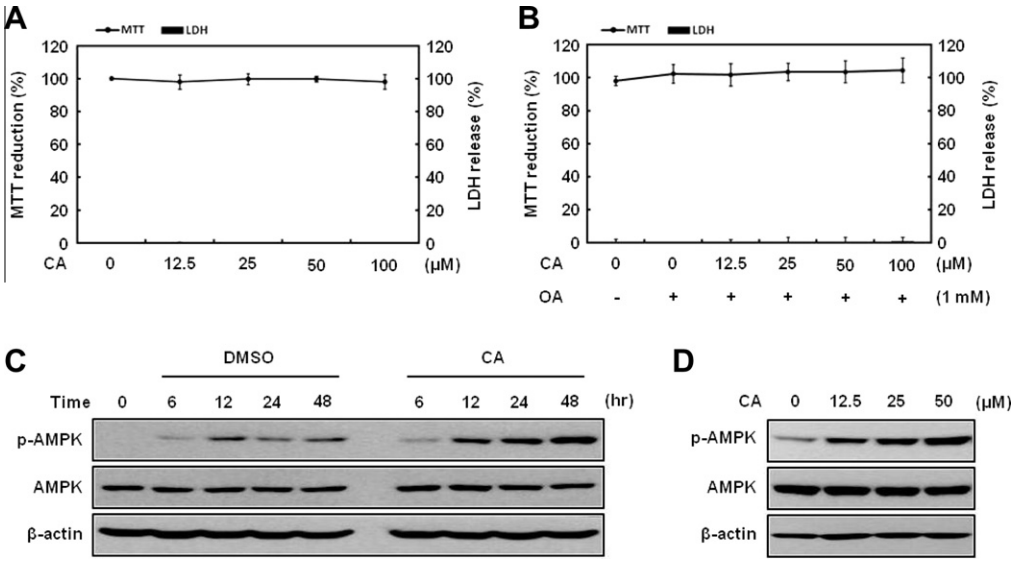

Fig. 1. Effects of *p*-coumaric acid on cell viability and the phosphorylation of AMPK. (A) Differentiated L6 skeletal muscle cells were preincubated in serum-free medium for 4 h then exposed to *p*-coumaric acid for 48 h. (B) Serum-deprived cells were exposed to oleic acid for 24 h, and then exposed to *p*-coumaric acid for 48 h. Cell viability and cytotoxicity were measured by MTT and LDH assays, respectively. The data are presented as the mean ± SD of three independent experiments. (C and D) Serum-deprived cells were exposed to *p*-coumaric acid (50 μM) for the indicated time period or to the indicated concentrations of *p*-coumaric acid for 24 h. The protein levels were examined by Western blotting. The results are representative of three independent experiments. CA, *p*-coumaric acid; OA, oleic acid.

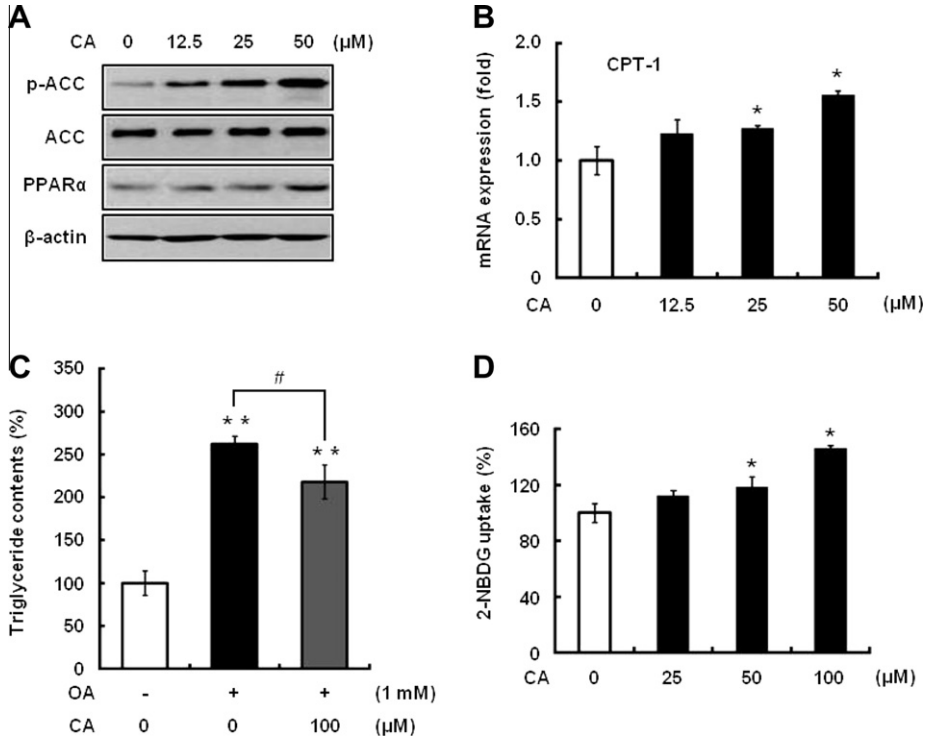

Fig. 2. Effects of *p*-coumaric acid on ACC phosphorylation, PPARα expression, *CPT-1* mRNA expression, lipid accumulation, and 2-NBDG uptake. (A) Serum-deprived cells were exposed to *p*-coumaric acid for 24 h, and the protein levels were examined by Western blotting using their corresponding antibodies. (B) Serum-deprived cells were treated with *p*-coumaric acid for 24 h, and the *CPT-1* mRNA levels were quantified by real-time PCR. The data are presented as the mean ± SD of three independent experiments. $^{*}p < 0.05$ compared to the *p*-coumaric acid-untreated group. (C) Serum-deprived cells were preincubated with oleic acid for 24 h, and then were exposed to *p*-coumaric acid (100 μM) for 12 h. The triglyceride contents were measured by Oil Red O staining. The data are presented as the mean ± SD of three independent experiments. (D) Serum-deprived cells were co-exposed to the indicated concentration of *p*-coumaric acid and 2-NBDG (50 μM) for 12 h, and the fluorescence intensity was measured using a fluorescence micro reader. The data are presented as the mean ± SD of three independent experiments. $^{*}p < 0.05$ compared to the *p*-coumaric acid-untreated group. CA, *p*-coumaric acid; OA, oleic acid.

downstream events related to fatty acid β-oxidation. *p*-Coumaric acid (up to 50 μM) increased the phosphorylation of ACC in a dose-dependent manner (Fig. 2A). It also increased the mRNA expression of *CPT-1* and its transcription factor, PPARα, at a concentration between 25 and 50 μM (Fig. 2A and B), suggesting that it promotes fatty acid β-oxidation in L6 skeletal muscle cells.

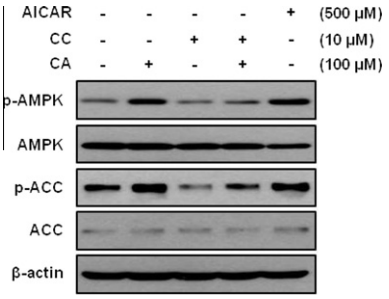

Fig. 3. Effects of *p*-coumaric acid on AMPK and ACC phosphorylation in cells preincubated with compound C. Serum-deprived L6 muscle cells were preincubated with compound C (10 μM) for 30 min, and then exposed to *p*-coumaric acid (100 μM) for 12 h. The protein levels were examined by Western blotting using their corresponding antibodies. AICAR was used as a positive control. The results are representative of three independent experiments. CA, *p*-coumaric acid; CC, compound C.

We also investigated the effect of *p*-coumaric acid on oleic acid-induced triglyceride accumulation. Oleic acid (1 mM) increased triglyceride accumulation by 2.6-fold compared to control cells. However, oleic acid-induced triglyceride accumulation was decreased significantly by *p*-coumaric acid (Fig. 2C). Furthermore, it significantly increased the rate of 2-NBDG uptake in L6 skeletal muscle cells (Fig. 2D). These results suggest that *p*-coumaric acid exerts its health-promoting effects by inhibiting lipid accumulation and promoting glucose uptake in L6 skeletal muscle cells. *p*-Coumaric acid did not affect lipolysis in L6 skeletal muscle cells (data not shown). It was previously reported that AMPK activation inhibits lipolysis [27]. Thus, it is believed that the inhibitory effect of *p*-coumaric acid on oleic acid-induced triglyceride accumulation may be caused by the inhibition of triglyceride synthesis.

Next, we investigated the effects of compound C (a selective AMPK inhibitor) on *p*-coumaric acid-mediated AMPK signaling. As shown in Fig. 3, the phosphorylation of AMPK and ACC was attenuated by pretreatment with compound C. However, *p*-coumaric acid compensated for this effect. AICAR has been shown to activate skeletal muscle AMPK and increase glucose transport activity in insulin-resistant rat models [10]. AICAR may have therapeutic potential for the treatment of insulin resistance and type 2 diabetes either by bypassing the defective insulin signaling pathway or by creating metabolic conditions that improve insulin action [28]; thus, it is interesting that *p*-coumaric acid (100 μM) showed activity that was equivalent to AICAR (500 μM), a synthetic AMPK activator.

Oleic acid-induced triglyceride accumulation was increased by pretreatment with compound C; however, the exposure of cells to *p*-coumaric acid attenuated the increasing effect of compound C on oleic acid-induced triglyceride accumulation in L6 skeletal muscle cells (Fig. 4A). In the same manner, 2-NBDG uptake was decreased by pretreatment with compound C, and exposure to *p*-coumaric acid significantly attenuated the decreasing effect of compound C in L6 skeletal muscle cells (Fig. 4B). These results indicate that the beneficial properties of *p*-coumaric acid in L6 skeletal muscle cells resulted mainly from its action as an AMPK activator.

The beneficial effects of plant-derived compounds and lack of side effects make them attractive alternatives as a cure for diabetic disorders. It has been reported that β-sitosterol, a plant-derived product, promotes glucose and lipid metabolism by enhancing glucose uptake and reducing the triglyceride and cholesterol cellular contents via AMPK activation [29]. Moreover, naringenin [30] and resveratrol [31] have been shown to increase glucose uptake through AMPK activation rather than the PI3K/Akt pathway. Thus, *p*-coumaric acid may have similar health-promoting properties to these phytochemicals.

In conclusion, in L6 skeletal muscle cells, *p*-coumaric acid promoted fatty acid β-oxidation, decreased oleic acid-induced triglyceride accumulation, and enhanced glucose uptake via its role as an AMPK activator. These results suggest that *p*-coumaric acid has the potential to prevent or improve insulin resistance and type 2 diabetes by modulating glucose and lipid metabolism.

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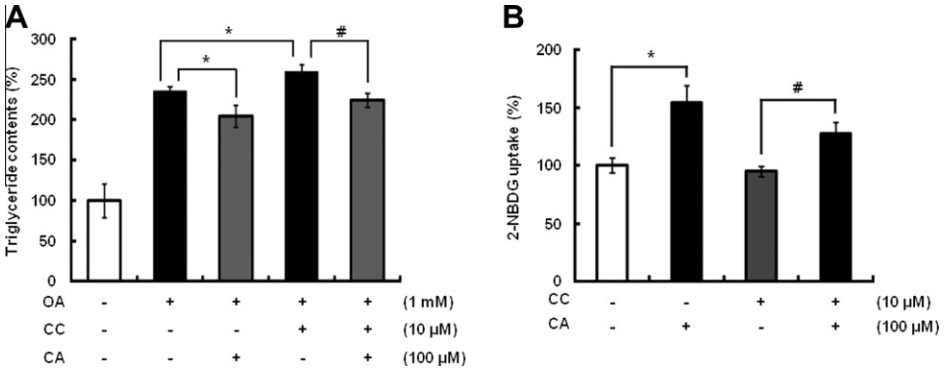

Fig. 4. Effects of *p*-coumaric acid on triglyceride accumulation and 2-NBDG uptake in cells preincubated with compound C. (A) Serum-deprived cells were preincubated with compound C (10 μM) for 30 min and then exposed to oleic acid for 24 h before *p*-coumaric acid treatment. (B) Serum-deprived cells were co-treated with the indicated concentration of *p*-coumaric acid and 2-NBDG (50 μM) for 12 h. 2-NBDG uptake was measured using a fluorescence micro reader. The data shown are presented as the mean ± SD of three independent experiments. $^{*}p < 0.05$ compared to the compound C-untreated group. $^{\#}p < 0.05$ compared to the compound C-treated group. CA, *p*-coumaric acid; OA, oleic acid; CC, compound C.

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