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Dehydroepiandrosterone activates AMP kinase and regulates GLUT4 and PGC-1α expression in C2C12 myotubes

Takumi Yokokawa [a], Koji Sato [b], Nobumasa Iwanaka [c], Hiroki Honda [b], Kazuhiko Higashida [d], Motoyuki Iemitsu [b], Tatsuya Hayashi [a], Takeshi Hashimoto [b, *]

[a] Laboratory of Sports and Exercise Medicine, Graduate School of Human and Environmental Studies, Kyoto University, Kyoto, Japan
[b] Graduate School of Sport & Health Science, Ritsumeikan University, Shiga, Japan
[c] The Graduate School of Science and Engineering, Ritsumeikan University, Shiga, Japan
[d] Faculty of Sport Science, Waseda University, Saitama, Japan

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ABSTRACT

Exercise and caloric restriction (CR) have been reported to have anti-ageing, anti-obesity, and health-promoting effects. Both interventions increase the level of dehydroepiandrosterone (DHEA) in muscle and blood, suggesting that DHEA might partially mediate these effects. In addition, it is thought that either 5′-adenosine monophosphate-activated protein kinase (AMPK) or peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) mediates the beneficial effects of exercise and CR. However, the effects of DHEA on AMPK activity and PGC-1α expression remain unclear. Therefore, we explored whether DHEA in myotubes acts as an activator of AMPK and increases PGC-1α.

DHEA exposure increased glucose uptake but not the phosphorylation levels of Akt and PKCζ/λ in C2C12 myotubes. In contrast, the phosphorylation levels of AMPK were elevated by DHEA exposure. Finally, we found that DHEA induced the expression of the genes PGC-1α and GLUT4.

Our current results might reveal a previously unrecognized physiological role of DHEA; the activation of AMPK and the induction of PGC-1α by DHEA might mediate its anti-obesity and health-promoting effects in living organisms.

1. Introduction

Exercise and dietary caloric restriction (CR) have anti-ageing, anti-obesity, and health-promoting effects [1–3]. While ageing, obesity, and diabetes lead to reduced serum levels of dehydroepiandrosterone (DHEA), a precursor of sex steroid hormones secreted by the adrenal gland, both exercise and CR up-regulate DHEA in muscle and blood. For example, acute and chronic exercise increase the concentrations of muscular DHEA and dihydrotestosterone (DHT), a metabolite of DHEA, and also increase the expression of steroidogenesis-related enzymes in the skeletal muscle of normal and obese rats [4–7]. In addition, CR is reported to slow the gradual decline of serum DHEA sulfate levels in rhesus monkeys with ageing [8,9]. Therefore, DHEA might play an important role in the beneficial effects induced by exercise and CR.

We have reported that exposure of primary muscular cultures to DHEA and testosterone increased the phosphorylation levels of PKCζ/λ Thr$^{410/403}$ and Akt Ser^{473} and led to an increase in glucose transporter type 4 (GLUT4) expression and translocation [10]. We also demonstrated that the chronic administration of DHEA to dietary-induced obese rats activated the PKCζ/λ and Akt pathways, and increased GLUT4 expression and translocation [7]. While these results suggest that PKCζ/λ and Akt might mediate DHEA-induced GLUT4 translocation, the possibility that DHEA affects other glucose metabolism-related signaling in skeletal muscle has not been examined.

The activation of 5′-adenosine monophosphate-activated protein kinase (AMPK), as well as Akt and PKCζ/λ, up-regulates glucose uptake through GLUT4 translocation [11,12]. AMPK signaling is also involved in mitochondrial biogenesis [13], lipid metabolism [14,15], and protein synthesis [16]. A number of studies have suggested that AMPK mediates the anti-obesity and longevity effects of exercise and CR [1,2], the latter of which is thought to be caused in part by the inhibitory effect of AMPK activation on the mammalian target

* Corresponding author. Faculty of Sport & Health Science, Ritsumeikan University, 1-1-1 Nojihigashi, Kusatsu, Shiga 525-8577, Japan.
E-mail address: thashimo@fc.ritsumei.ac.jp (T. Hashimoto).

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of rapamycin (mTOR) pathway [16]. Given the anti-obesity effect of DHEA in animal studies [17], the effect of DHEA may be partly mediated by AMPK signaling. Results from studies have indicated that DHEA induced a decrease in intracellular energy, activated AMPKα2 in rat cardiac myocytes [18] and increased the phosphorylation levels of AMPKα Thr^{172} in mouse ovarian tissue [19]. In contrast, in human osteosarcoma SaOS-2 cells, DHEA had no effect on AMPK activation [20]. Therefore, the effects of DHEA on AMPK phosphorylation and activation, especially in skeletal muscle, remain to be elucidated.

Peroxisome proliferator-activated receptor (PPAR)-γ coactivator-1 α (PGC-1α) regulates the activity of several transcriptional factors (e.g., PPARγ [21], PPARα [22], and estrogen related-receptors (ERRs) [23] and nuclear respiratory factors (NRFs) [24]). The regulation of transcriptional activity by PGC-1α modulates a number of metabolic genes involved in glucose metabolism, fatty acid oxidation, and mitochondrial biogenesis [25]. The expression of the PGC-1α gene is regulated by AMPK [13,26] in addition to calmodulin-dependent protein kinase (CaMK) II and p38 mitogen-activated protein kinase (MAPK) [27]. Several anti-obesity and anti-diabetic compounds, such as butyrate [28], berberine [29] and resveratrol [30], were reported to increase PGC-1α expression. Thus, it is plausible that AMPK might mediate the anti-diabetic and anti-obesity effects of DHEA in skeletal muscle through increasing PGC-1α and its regulation of a number of metabolic genes.

Thus, we hypothesized that DHEA activates AMPK signaling and regulates glucose uptake and the expression of metabolic genes, including GLUT4 and PGC-1α, in skeletal muscle cells. To test these hypotheses, we first examined whether DHEA exposure increases glucose uptake in C2C12 myotubes. We then investigated whether DHEA exposure affects the phosphorylation levels of Akt, PKCζ/λ, and AMPK. Finally, we examined whether DHEA increases the expression levels of GLUT4 and PGC-1α.

2. Materials and methods

2.1. Cell culture

Mouse C2C12 muscle cells (American Type Culture Collection, Manassas, VA) were grown in a 5% CO_2 atmosphere at 37 °C as described previously [31,32]. Myoblasts were incubated in Dulbecco's modified Eagle's medium (DMEM; 4.5 g glucose/L, Nacalai Tesque, Kyoto, Japan) containing 10% fetal bovine serum and 1% penicillin-streptomycin (P/S). Differentiation was induced by switching to medium containing 2% horse serum and 1% P/S when myoblasts were 90–100% confluent. Differentiated myotubes were serum-starved overnight, and then were incubated in serum-free media containing 25, 50 or 100 μM trans-dehydroepiandrosterone (Sigma–Aldrich, St Louis, MO), 1 μM insulin (Sigma–Aldrich), or 10 mM metformin hydrochloride (Enzo Life Sciences, Farmingdale, NY). Stimulated cells were harvested and lysed for western blotting or real-time PCR.

2.2. Western blotting

Myotubes were washed with PBS and directly lysed by SDS sample buffer (62.5 mM Tris–HCl pH 6.8, 2% SDS, 10% glycerol, 50 mM dithiothreitol), then were sonicated and boiled. Supernatants of the extracts were subjected to SDS-PAGE and transferred to PVDF membranes (Bio-Rad Laboratories, Inc., Hercules, California, USA). Proteins were probed with the following primary antibodies: phospho-AMPKα Thr^{172} (Cell Signaling Technology, Beverly, Massachusetts, USA, #2535, 1:1000), AMPK (Cell Signaling Technology, #2793, 1:1000), phospho-acetyl-CoA carboxylase (ACC) Ser^{79} (Cell Signaling Technology, #3661, 1:1000), ACC (Cell Signaling Technology, #3662, 1:1000), phospho-Akt Ser^{473} (Cell Signaling, #9271, 1:1000), phospho-Akt Thr^{308} (Cell Signaling, #13038, 1:1000), Akt (Cell Signaling, #9272, 1:1000), phosphor-PKCζ/λ $Thr^{410/403}$ (Cell Signaling, #9378, 1:1000), and α-Tubulin (Sigma–Aldrich, T8203, 1:5000). Then, the membranes were incubated with the appropriate horseradish-conjugated anti-IgG secondary antibodies. The immunoblotted membranes were scanned with an ImageQuant LAS-4000 (GE Healthcare, Milwaukee, WI, USA) luminescent image analyzer.

2.3. Quantitative real-time PCR

After stimulation as described above, total RNA was extracted using a Purelink RNA Mini Kit (Life technologies, Gaithersburg, MD). RNA concentration and purity were measured with a NanoDrop 2000 (Themo Fisher Scientific Inc., Yokohama, Japan). Total RNA was reverse-transcribed to single-strand cDNA by ReverTra Ace qRNA RT Master Mix with gDNA Remover (Toyobo Co., Osaka, Japan). The cDNA samples were mixed with 2 × KAPA master (Nippon Genetics Co. Itd., Tokyo, Japan) and primer pairs, then were amplified on a Piko Real PCR system (Themo Fisher Scientific Inc.). The following primer sequences were used: PGC-1α forward 5′- GCTGCATGGTTCTGAGTGCTAAG -3′, PGC-1α Reverse 5′- AGCCGTGACCACTGACAACGAG -3′; PGC-1β forward 5′- GGCAGGTTCAACCCCGA -3′, PGC-1β reverse 5′- CTTGCTAACATCACAGAGGATATCTTG -3′; GLUT4 forward 5′ CAGCTCTCAGGCATCAAT -3′, GLUT4 Reverse 5′- TCTACTAAGAGCACCGAG -3′; uncoupling protein 3 (UCP3) forward 5′- GAGTCAGGGGCCTGTGGAAA -3′, UCP3 reverse 5′- GCGTTCATGTATCGGGTCTT -3′; and glyceroldehyde 3-phosphate dehydrogenase (GAPDH) forward 5′- CCCAGCTGAATGTGTAAGCC -3′, GAPDH Reverse 5′- CAAACAAGCTTCGGTCTGGA -3′. The housekeeping gene GAPDH was used as an internal control and for normalization of cDNA concentrations.

2.4. Measurement of medium glucose concentration

Differentiated C2C12 myotubes were exposed to DHEA, insulin, or metformin in DMEM (Life Technologies) without phenol red. The culture media were collected 3 or 6 h after initiation of exposure and assayed glucose concentration using a Glucose II-test kit (Wako, Osaka, Japan).

2.5. Statistical analysis

Multiple means were compared by ANOVA, followed by a post hoc comparison with Dunnett's test when appropriate. The significance level was set at $p < 0.05$. All results are presented as the means ± SE.

3. Results

3.1. DHEA exposure to C2C12 myotubes decreased glucose concentration in culture media

The results from a previous study indicated that DHEA exposure at concentrations of 1–100 μM increased glucose uptake in 3T3-L1 adipocytes [33]. Although we reported that 300 μM DHEA exposure for 24 h significantly increased GLUT4 translocation to the membrane in skeletal muscle cells [10], it remains unclear whether DHEA increases glucose uptake in myotubes. Therefore, we measured the glucose concentration in incubated media after DHEA exposure to C2C12 myotubes. Exposure of 1 μM insulin, an activator of Akt signaling, or 10 mM metformin, an activator of

AMPK, for 3 or 6 h significantly decreased glucose concentration in media. DHEA exposure at a concentration of 100 μM for 3 or 6 h also significantly decreased the glucose concentration of the medium.

3.2. DHEA did not affect phosphorylation of Akt and PKCζ/λ in C2C12 myotubes

We reported that DHEA exposure at a concentration of 300 μM, but not 100 μM, increases phosphorylation of Akt and PKCζ/λ, suggesting that activation of these kinases mediates the DHEA-induced enhancement of glucose uptake in incubated skeletal muscle cells [10]. However, in C2C12 myotubes, 100 μM DHEA stimulation increased glucose uptake (Fig. 1). To elucidate the contribution of Akt and PKCζ/λ to DHEA-induced glucose uptake in C2C12 myotubes, we investigated whether DHEA exposure at a concentration of 100 μM affects the phosphorylation levels of Akt residues Ser^{473} and Thr^{308} and PKCζ/λ residue $Thr^{410/403}$ in C2C12 myotubes. Stimulation by 1 μM insulin for 3 or 6 h significantly increased the phosphorylation of Akt Ser^{473} and Thr^{308}, while treatment with 100 μM DHEA and 10 mM metformin had no effect on either Akt phosphorylation site (Fig. 2A and B). In addition, a cocktail of 1 μM insulin, 10 mM metformin, and 100 μM DHEA did not affect the phosphorylation of PKCζ/λ $Thr^{410/403}$ in C2C12 myotubes (Fig. 2A and C).

3.3. DHEA activates AMPK in C2C12 myotubes

Although treatment with 100 μM DHEA significantly increased glucose uptake, it had no effect on the phosphorylation of Akt and PKCζ/λ in C2C12 myotubes. These results suggest that DHEA might regulate glucose uptake by activating another signaling pathway. We therefore investigated the effect of DHEA on AMPK signaling, which regulates GLUT4 translocation [11] and expression [13], in C2C12 myotubes. DHEA significantly increased the phosphorylation of AMPK and ACC after 1 h at a concentrations exceeding 25 μM (Fig. 3A and B). In addition, exposure to 100 μM DHEA, or 10 mM Metformin significantly increased the phosphorylation of AMPKα Thr^{172} and ACC Ser^{79}, a downstream effector of AMPK, after 3 and 6 h of exposure (Fig. 3C and D). In contrast, insulin stimulation had no effect on the phosphorylation of AMPKα Thr^{172} and ACC Ser^{79} at these time points (Fig. 3C and D).

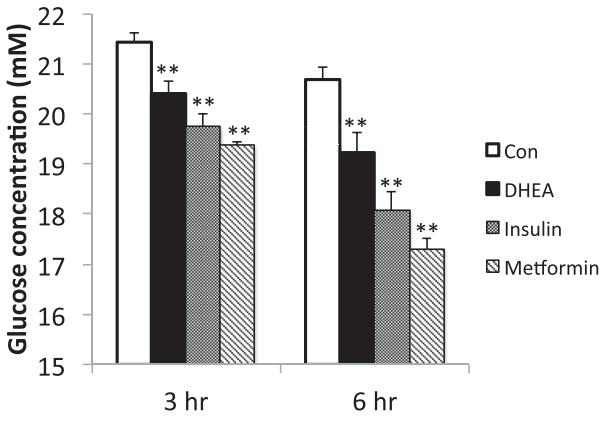

Fig. 1. DHEA, insulin and metformin increase glucose utilization in C2C12 myotubes. Serum-starved C2C12 myotubes were exposed to 100 μM DHEA, 1 μM insulin or 10 mM metformin for 3 or 6 h. The glucose concentrations of collected media were analyzed by spectrophotometry. Values are mean ± SE; $n = 6$ per group. $^{**}P < 0.01$ vs control.

3.4. DHEA increased AMPK-dependent gene expression

AMPK activation increases the expression of AMPK-dependent genes, such as PGC-1α and GLUT4, and regulates metabolism [13]. Thus, we investigated whether DHEA increased the expression of AMPK-dependent genes. DHEA exposure for 6 h at concentrations of 50 and 100 μM increases PGC-1α and GLUT4 expression (Fig. 4). In addition, DHEA exposure significantly increased the expression of UCP3, a gene down-stream of PGC-1α, in C2C12 myotubes (Fig. 4). In contrast, the expression of PGC-1β, a member of the AMPK-independent PGC-1 family, was not changed by DHEA exposure (Fig. 4).

4. Discussion

Our results provide evidence that DHEA activates AMPK signaling and induces the expression of AMPK-dependent genes in C2C12 myotubes. In this study, DHEA exposure increased glucose uptake (Fig. 1) but not the phosphorylation of Akt and PKCζ/λ (Fig. 2). Interestingly, the phosphorylation of AMPK, another regulator of glucose uptake, was increased by DHEA exposure (Fig. 3). Finally, we found that DHEA induced the expression of the AMPK-dependent genes PGC-1α and GLUT4 (Fig. 4).

DHEA has been reported to activate insulin-like signaling, which increases the phosphorylation of the insulin receptor substrates Akt and PKC in skeletal muscle cells and adipocytes. These activations are suggested to mediate the anti-diabetic effect of DHEA [7,10,33]. However, insulin signaling is a well-known adipogenic pathway [34]. Thus, another signaling pathway is thought to mediate the anti-obesity effect of DHEA. In this study, exposure to 100 μM DHEA, as well as insulin and metformin, increased glucose utilization (Fig. 1), consistent with a previous study conducted with 3T3-L1 adipocytes [33]. However, DHEA did not affect the phosphorylation levels of Akt Ser^{473} and Thr^{308} or PKCζ/λ $Thr^{410/403}$ (Fig. 2), suggesting that the DHEA-induced enhancement of glucose uptake is regulated, at least in part, by another signaling pathway. Results from our previous study indicated that exposure to DHEA at concentrations exceeding 300 μM for 24 h increased the phosphorylation of Akt and PKCζ/λ in skeletal muscle cells [10]. Therefore, DHEA-induced Akt and PKCζ/λ phosphorylation might be dependent on exposure time and concentration. However, DHEA significantly increased the phosphorylation levels of AMPK Thr^{172} and ACC Ser^{79} at concentrations of 25 μM or higher (Fig. 3). Although differences between incubated skeletal muscle cells and C2C12 cell line should be carefully considered, AMPK might be more responsive than Akt and PKCζ/λ to DHEA and could regulate glucose uptake in skeletal muscle cells.

AMPK is a potent metabolic regulator and plays an important role in various metabolic pathways. We reported that exposing incubated skeletal muscle cell cultures to DHEA increased GLUT4 protein expression [10]. However, the molecular pathway underlying this effect remains unknown. Results of a previous study indicated that 5-aminoimidazole-4-carbozamide ribonucleoside (AICAR), an AMPK activator, increased GLUT4 protein expression in L6 cells [35]. In addition, AMPK activation increased GLUT4 gene expression and promoter activity via histone deacetylase (HDAC) 5 and myocyte enhancer factor (Mef) 2 in human primary skeletal muscle cells [36]. Thus, AMPK might be involved in the DHEA-induced enhancement of GLUT4 gene expression in skeletal muscle cells. Furthermore, activated AMPK phosphorylates ACC and enhances fatty acid oxidation [14]; in fact, DHEA exposure increased ACC phosphorylation (Fig. 3), suggesting that DHEA also acts as an activator of fatty acid oxidation in muscle tissue. In addition, AICAR and metformin induction of AMPK up-regulated PGC-1α, a master regulator of mitochondrial biogenesis, and

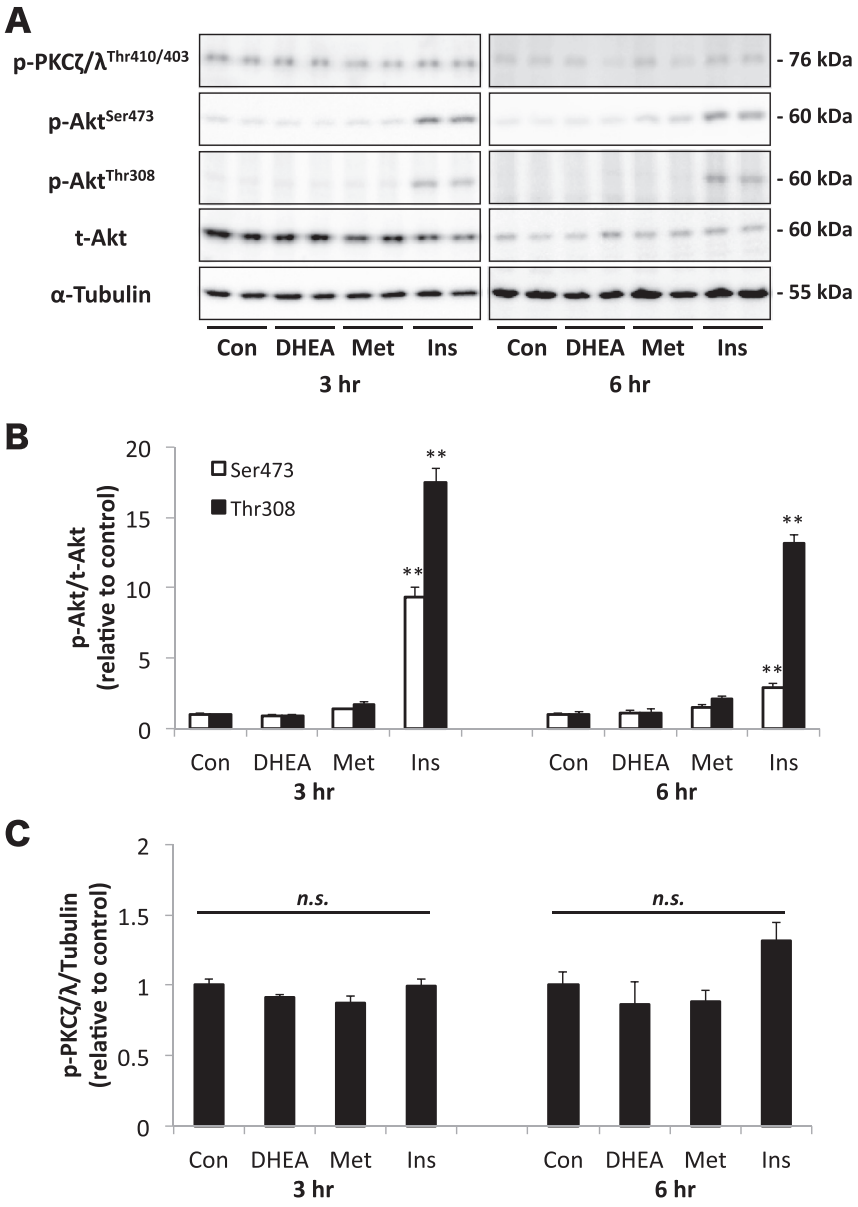

Fig. 2. Effect of DHEA on the phosphorylation of Akt and PKCζ/λ in C2C12 myotubes. Serum-starved C2C12 myotubes were exposed to 100 μM DHEA, 10 mM metformin or 1 μM insulin for 3 or 6 h. The phosphorylation levels of Akt and PKCζ/λ were analyzed by western blot. Representative immunoblots (A) and quantification (B–C) of phospho- or total- Akt and PKCζ/λ as well as α-tubulin in C2C12 myotubes. Values are mean ± SE; $n = 4$ per group. $^{**}P < 0.01$ vs control.

increased its phosphorylation and nuclear translocation, resulting in the increased expression of mitochondrial-related genes [13,26]. In this study, DHEA increased the expression of PGC-1α and UCP3, a downstream gene of PGC-1α, suggesting that DHEA-induced AMPK signaling functionally activates down-stream pathways in C2C12 myotubes. PGC-1α regulates a number of genes involved in glucose metabolism, lipid oxidation, and mitochondrial function. UCP3 is a mitochondrial gene reported to enhance fatty acid oxidation in skeletal muscle cells [37]. These results suggest that DHEA increases mitochondrial biogenesis as well as glucose and fat metabolism. In contrast, DHEA had no effect on the expression of PGC-1β in myotubes, which is independent of AMPK activation [13]. Thus, AMPK might mediate the anti-diabetic and anti-obesity effects of DHEA in skeletal muscle through increasing the level of PGC-1α.

In this study, we did not elucidate the molecular mechanism by which DHEA activates AMPK in myotubes. The results from previous study indicated that DHEA inhibited complex I of the mitochondrial respiratory chain in primary neuronal culture [38]. Thus, it is likely that the inhibition of mitochondrial respiration induces ATP depression, leading to AMPK activation in skeletal muscle cells. To validate this mechanism, further experiments focusing on DHEA-induced ATP depression must be performed. In addition, the contribution of AMPK to glucose uptake and PGC-1α gene expression remains unclear. As discussed above, DHEA has been reported to activate various pathways [10,33]. Thus, an inhibition study is necessary to uncover the contribution of each DHEA-induced pathway. Furthermore, it is unclear whether DHEA activates AMPK *in vivo* due to the lack of animal studies. Further *in vivo* investigation is required to assess the biophysiological significance of our *in vitro* evidence that DHEA acts at a cellular level.

In conclusion, the results of the present study indicate that exposing C2C12 myotubes to DHEA significantly increased glucose uptake, but not phosphorylation of Akt and PCKζ/λ. Furthermore,

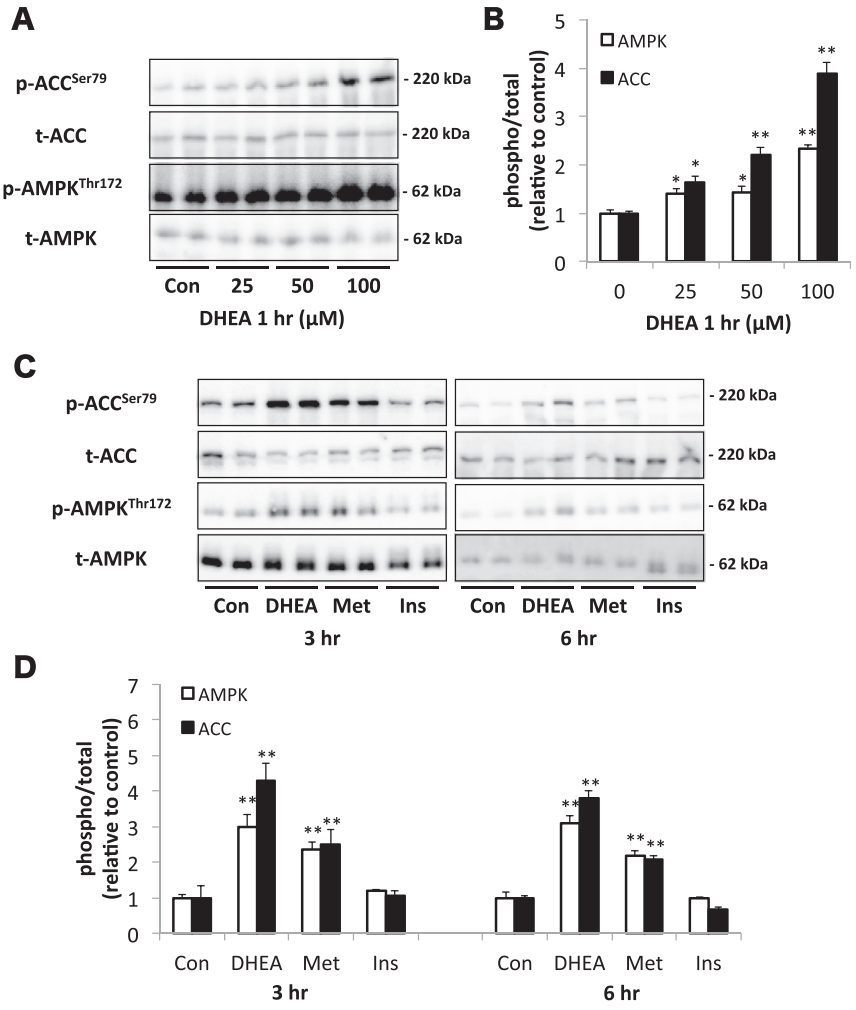

Fig. 3. Effect of DHEA on the phosphorylation of AMPK and ACC in C2C12 myotubes. Serum-starved C2C12 myotubes were exposed to 25, 50, and 100 μM DHEA for 1 h (A and B), and 100 μM DHEA, 10 mM metformin or 1 μM insulin for 3 or 6 h (C and D). The phosphorylation levels of AMPK and ACC were analyzed by western blot. Representative immunoblots (A and C) and quantification (B and D) of phospho- or total- AMPK and ACC in C2C12 myotubes. Values are mean ± SE; $n = 4$ per group. $^{*}P < 0.05$ vs control. $^{**}P < 0.01$ vs control.

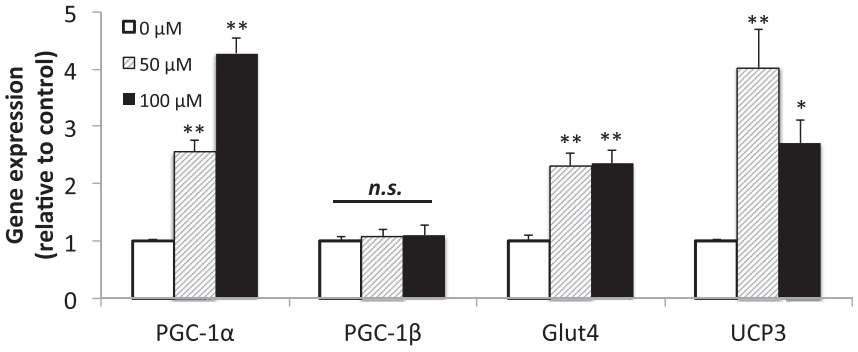

Fig. 4. Effect of DHEA on the expression of PGC-1α, PGC-1β, GLUT4 and UCP3 in C2C12 myotubes. Serum-starved C2C12 myotubes were exposed to 0, 50 or 100 μM DHEA for 6 h. The gene expression levels of PGC-1α, PGC-1β, GLUT4 and UCP3 were analyzed by real-time PCR. Values are mean ± SE; $n = 4$ per group. $^{*}P < 0.05$ vs control. $^{**}P < 0.01$ vs control. *n.s.*: not significant.

DHEA activated AMPK signaling in a dose-dependent manner and was sustained for 6 h. Finally, DHEA increased the expression of the AMPK-dependent genes GLUT4, PGC-1α and UCP3, but not the AMPK-independent gene PGC-1β. Our results might reveal a physiological role for DHEA not observed in previous *in vivo* studies [7,17]. Considering the numerous bio-activities of AMPK, its activation AMPK might mediate the anti-obesity and health-promoting effects of DHEA in living organisms.

Conflict of interest

None.

Acknowledgments

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