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# Dehydroepiandrosterone-sulfate (DHEAS) promotes MIN6 cells insulin secretion via inhibition of AMP-activated protein kinase

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ABSTRACT

Derived from adrenal cortical, dehydroepiandrosterone-sulfate (DHEAS) is a precursor to androgens and estrogens, with various bioactivities. Although it has the property of anti-diabetes, the long-term effect of DHEAS on insulin secretion in beta-cells is still unclear. In this study, the effect of DHEAS on the insulin secretion activity in MIN6 cell lines in vitro was assessed. Insulin biosynthesis and secretion were stimulated by DHEAS for 24 h. DHEAS inhibited the AMPK activation and upregulated the expression of ACC-1. These findings indicate that DHEAS may exert prominent stimulatory effects on insulin secretion partly via AMPK inhibition and ACC-1 upregulation.



## 1. Introduction

Type 2 diabetes (T2DM) is characterized by hyperglycemia that results from insufficient insulin to metabolize the glucose, which in turns destroys the balance between the insulin secretion and metabolic demand. Although there are debates on whether beta-cell abnormality is the cause or the result of the type 2 diabetes [1], in fact, impaired insulin secretion has been consistently demonstrated to occur during the aging process [2].

Dehydroepiandrosterone (DHEA), an androgenic 17-ketosteroid derived from pregnenolone, is an intermediate in the biosynthesis of testosterone and estrogens. DHEA and its sulfated metabolite (DHEAS) are the most abundant circulating adrenal steroids in humans. DHEAS, whose level is higher in men than women, peaks at 100 μmol/L sometime in the third decade, and falls gradually thereafter to levels of 20% of peak by the eighth decade [3]. In other words, the production of DHEAS by adrenal glands is significantly decreased during aging [3]. Therefore, we may infer that the decline of DHEAS and islet cell function is synchronized during aging. Although DHEAS has widespread beneficial biological activities, including effects on cell proliferation, immunomodulation, atherogenesis, diabetes, obesity and satiety [4,5], unlike other steroid hormones, an intracellular receptor for DHEAS has not been isolated [6], so its molecular mechanism is still unclear.

Adenosine monophosphate-activated protein kinase (AMPK), which serves as a metabolic master switch in response to alterations in the cellular energy charge, has been reported to be involved in the regulation of glucose and lipid homeostasis and insulin sensitivity [7–9]. Once activated, AMPK phosphorylates and inhibits the rate-limiting enzymes in cholesterol and fatty acid synthesis. AMPK-activation promotes fatty acid oxidation and glucose uptake in myocytes, reduces gluconeogenesis in hepatocytes, and inhibits lipogenesis in adipocytes [10]. It has also been shown that activation of AMPK by AICAR inhibits insulin release in beta-cells [11–13]. So, it is a plausible assumption that pancreatic beta-cells appear to be functionally affected by AMPK activation.

Acetyl CoA carboxylase-1 (ACC-1) was mediated by AMPK. Prentki et al. have shown that lipid metabolites, specifically cytoplasmic long-chain fatty acyl coenzyme A (LC-CoA) or its products, play a major signaling role in insulin secretion [14,15]. While a role for AMPK in the beta-cells continues to unfold, evidence for a role of DHEAS in islet function is lacking. In this report, we investigated the possible role of DHEAS in beta-cell function using MIN6 cell lines.

## 2. Materials and methods

### 2.1. Materials

DHEAS was purchased from International Laboratory (IL, USA). Dulbecco's modified Eagle's medium (DMEM) and other culture reagents were obtained from Gibco Life Technologies (Grand Island, NY, USA). AICA-riboside (AICAR) was sourced from Toronto Research Chemicals (North York, ON, Canada). Antibodies against phosphospecific and non-phosphospecific-AMPK, acetyl CoA carboxylase (ACC), acetyl CoA carboxylase-1 (ACC-1) were

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purchased from cell signaling technology (Beverly, MA, USA). Anti-beta-actin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mice insulin detection ELISA kit was from Millipore Corporation (Billerica, MA, USA). All other reagents were of the highest analytical grade commercially available.

### 2.2. Cell culture and treatment

MIN6 cell lines (passage 23–30), which kept extremely sensitive to glucose, were grown in DMEM with 25 mmol/L glucose, supplemented with 15% (v/v) heat-inactivated fetal calf serum, 2 mmol/L glutamine, β-mercaptoethanol (1 μl/L), 100 U/ml penicillin G sodium and 100 μg/ml streptomycin sulfate in a humidified atmosphere at 37 °C with 5% $CO_2$. MIN6 cells were seeded in a 24-well dish ($1 \times 10^5$ cells per well) for secretary experiment, which were also seeded in a 6-well dish ($2 \times 10^6$ cells per well) for Real Time-PCR and Western blotting. Medium containing 0.1% DMSO was enrolled as vehicle. When the MIN6 cells were 80–90% confluent, the medium was replaced with fresh medium and treatment.

### 2.3. The effect of DHEAS and AICAR on the viability of MIN6 cell lines

MIN6 cells were plated at 5000 per well in DMEM containing 15% FBS in 96-well plates and incubated at 37 °C in a humidified circumstance containing 5% $CO_2$ for 24 h. Then the cells were treated with different concentrations of DEHAS (100 nmol/L–200 μmol/L) or AICAR (200–1000 μmol/L) for 24 h (As group), which was not added DHEAS or AICAR as a control (Ac group). After 24 h, reagent of CCK-8 was added in each well of 96-well plate, and then cultured in the incubator for another 2 h, which contains no CCK-8 as a blank (Ab group). Cell viability was determined using a CCK-8 assay kit according to the manufacturer's instructions. The survival rate of the cells in each well was calculated by the following formula: $[(As-Ab)/(Ac-Ab)] \times 100\%$.

### 2.4. Assay for insulin secretion

For insulin secretion experiments, MIN6 cells were seeded in 24-well plates. After 24 h incubation with DHEAS and/or AICAR, the cells were preincubated at 37 °C for 0.5 h in Kreb-Ringer bicarbonate Hepes buffer (129 mmol/L NaCl, 4.8 mmol/L KCl, 2.5 mmol/L $CaCl_2$, 1.2 mmol/L $MgSO_4$, 1.2 mmol/L $KH_2PO_4$, 5 mmol/L $NaHCO_3$, 10 mmol/L Hepes, and 0.1% BSA) containing 2.8 mmol/L glucose. Cells were then treated in Kreb-Ringer bicarbonate Hepes buffer containing 2.8 or 16.7 mmol/L glucose and DHEAS and/or AICAR for 1 h. Cell supernatants were collected and centrifuged at 1000 rpm/min, 4 °C for 5 min. Insulin was measured by ELISA kit. Treated cells were digested by 0.25% trypsogen–EDTA for counting the cells number in order to correct the concentrations of insulin in the supernatants. Treated cells were also homogenized in ice-cold lysis buffer, which were determined by BCA Protein Assay Kit to normalizing its insulin level (data not shown).

### 2.5. Measurement of insulin content

Cells were seeded and then treated with DHEAS for 24 h. The cells were washed twice with ice-cold PBS and extracted with acid ethanol (15 mmol/L HCl, 75% ethanol) for 18 h at 4 °C. The cells were scraped off with a rubber policeman. After centrifugation at 3500 rpm/min for 25 min at 4 °C, the extract was stored at −20 °C until assayed by ELISA kit.

### 2.6. RNA extraction and Real Time-PCR

Total cellular RNA from the treated MIN6 cells with DHEAS were obtained by using TRIzol reagent. Total RNA (1 μg) in each RNAase-free tube was then reverse transcribed into cDNA with a high-capacity reverse transcription kit according to the manufacturer's instructions. Real Time-PCR was performed in a total volume of 10 μl containing 1.0 μl of reverse-transcribed total RNA and 0.2 μl of forward and reverse primers, respectively. The reactions were performed on the ABI-7900HT Prism Real Time-PCR instrument using SYBR green master mix with initial denaturation at 95 °C for 30 s followed by 40 cycles consisting of 95 °C for 5 s, 60 °C for 30 s and final solubility curve at 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s. All quantifications were performed with beta-actin as internal standard and presented as fold over control. The following formula was used: target amount = $2^{-\Delta\Delta^{Ct}}$, where $\Delta\Delta Ct$ = {[Ct (target gene sample) – Ct (beta-actin sample)] – [Ct (control sample) – Ct (beta-actin control)]}. Sequences of primers used in this study were as follows: insulin: forward, 5′-TGAAGTGGAGGACCCACAAGTG-3′, reverse, 5′-TACAATGCCACGCTTCTGCTG-3′; ACC-1: forward, 5′-ATGTCCGCACTGACTGTAACCA-3′, reverse, 5′-TGCTCCGCACAGATTCTTCAA-3′; beta-actin: forward, 5′-GAAATCGTGCGTGACATCAAAGAG-3′, reverse, 5′-CAATAGTGATGACCTGGCCGTC-3′.

### 2.7. Immunoblotting

Treated cells with DHEAS and/or AICAR in 6-well plates were washed twice with ice-cold PBS and lysed immediately in RIPA buffer containing protease and phosphatase inhibitors [20 mmol/L Tris–HCl, 137 mmol/L NaCl, 10% glycerol, 1% Triton X-100, 2 mmol/L EDTA, 1 mmol/L PMSF, 1× Phosphatase Inhibitor-Cocktail Set II (Calbiochem, Darmstadt, Germany), and 1× protease inhibitor (Roche Applied Science, Mannheim, Germany)]. Lysates were gently mixed for 5 min at 4 °C and placed in the ice for 30 min, which was then centrifuged at 12,000*g* for 30 min at 4 °C. Protein concentration in each tube was assayed by using the BCA protein assay kit. Protein extracts (40 μg) mixed with SDS loading buffer were boiled for 15 min at 95 °C, which then were separated by SDS–PAGE on 6% or 10% polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked and incubated in 5% BSA (w/v) in TBS/Tween. Blotted proteins were probed with antibodies against pi-AMPK (1:2000 dilution), AMPK (1:1000 dilution), pi-ACC (1:1000 dilution), ACC-1 (1:1000 dilution), ACC (1:1000 dilution) according to the suppliers' protocols. Anti-rabbit IgG at 1:2000 for 2 h at room temperature was used to detect the above primary antibodies. Signal detection was performed by using enhanced chemiluminescence (ECL) detection kit (Amersham Biosciences) and the Fluor-S MAX Multi-Imager (Bio-Rad). Phospho-blots were normalized by stripping membranes and re-probing with antibodies to beta-actin to control loadings. Signals were quantified by using the Quantity One software (Bio-Rad).

### 2.8. Statistics

All the experiments were performed at least three times. Average values were expressed as mean ± SE. Significance between groups were performed by using independent Student's *t*-test or one-way ANOVA when suitable. Differences between groups were considered significant when $P$ values of $<0.05$ or $P$ values of $<0.01$.

## 3. Results

### 3.1. DHEAS and AICAR have no impact on the viability of MIN6 cell lines

To investigate the effect of DHEAS or AICAR on cell viability, MIN6 cells were exposed to various concentration loads for 24 h containing 2.8 or 16.7 mmol/L glucose (data not showed). When the MIN6 cells were treated with different concentrations of DHEAS, either at 2.8 or 16.7 mmol/L glucose, the survival rate of MIN6 cell was similar to the controls (>95%, respectively), excluding DHEAS concentration at 200 μmol/L in the presence of 2.8 mmol/L glucose, the survival rate was 90%. The parallels happened in the AICAR groups. It showed that there was no poisonous effect of DHEAS or AICAR on MIN6 cell at the concentration we used in this study.

### 3.2. DHEAS promoted insulin secretion and biosynthesis of insulin content, while AICAR decreased insulin release

We incubated MIN6 cells with various concentrations of DHEAS for 24 h. The insulin secretion was in a dose-dependence curve. When the cells were incubated in the presence of 16.7 mmol/L glucose, the insulin secretion was significantly increased in many DHEAS-treated groups (1–100 μm) compared with the control one ($P < 0.05$) (Fig. 1A). No change existed in the insulin secretion of MIN6 cells containing 2.8 mmol/L glucose ($P > 0.05$) (Fig. 1A).

We incubated MIN6 cell lines with various concentrations of AICAR for 24 h. When the cells were incubated in the presence of 16.7 mmol/L glucose, the insulin secretion was significantly decreased in AICAR-treated groups (200, 500 μmol/L) compared with the control one ($P < 0.05$) (Fig. 1B). There was no change existing in the presence of 2.8 mmol/L glucose ($P > 0.05$) (Fig. 1B). Therefore, we chose 200 μmol/L AICAR for further study. Many results showed that activation of AMPK in beta-cells using AICAR resulted in a reduced capacity for insulin secretion [16,17]. Whereas, when MIN6 cells were treated with AICAR (200 μmol/L) and DHEAS (100 μmol/L), insulin secretion sharply decreased compared with those only cultured with DHEAS containing 16.7 mmol/L ($P < 0.05$) (Fig. 1C). No change existed in the presence of 2.8 mmol/L glucose ($P > 0.05$) (Fig. 1C).

There are species-divergent effects of DHEAS on pancreatic insulin content in rodents. In db/db mice, DHEAS treatment is associated with increased islet cell number, size, mitotic figures, and insulin content [11]. To explore a DHEAS-stimulated increase in intracellular insulin content, it was assessed in MIN6 cells after 24 h of incubation with various concentrations of DHEAS. When the cells were incubated in the presence of 16.7 mmol/L glucose, the insulin biosynthesis was markedly increased in many DHEAS-treated groups (1–100 μmol/L) compared with the control one ($P < 0.05$) (Fig. 1D). There was no change existing in the presence of 2.8 mmol/L glucose, in which the concentration of DHEAS was from 10 nmol/L to 100 μmol/L ($P > 0.05$) (Fig. 1D). These results suggested that DHEAS may increase the biosynthesis of intracellular insulin content.

### 3.3. Expression of insulin and ACC-1 mRNA in MIN6 cells

Real Time-PCR results showed that in MIN6 cells, when the cells were incubated with DHEAS (1–200 μmol/L) at 16.7 mmol/L glucose for 24 h, the insulin mRNA levels were upregulated compared with the control one ($P < 0.05$) (Fig. 2A), while the glucose concentration was 2.8 mmol/L, the insulin mRNA levels did not reach the significance ($P > 0.05$) (Fig. 2A). Our result is consistent with previous studies of pancreatic beta-cells [11,18]. As expected, when the glucose concentration was 16.7 mmol/L, the ACC-1 mRNA levels

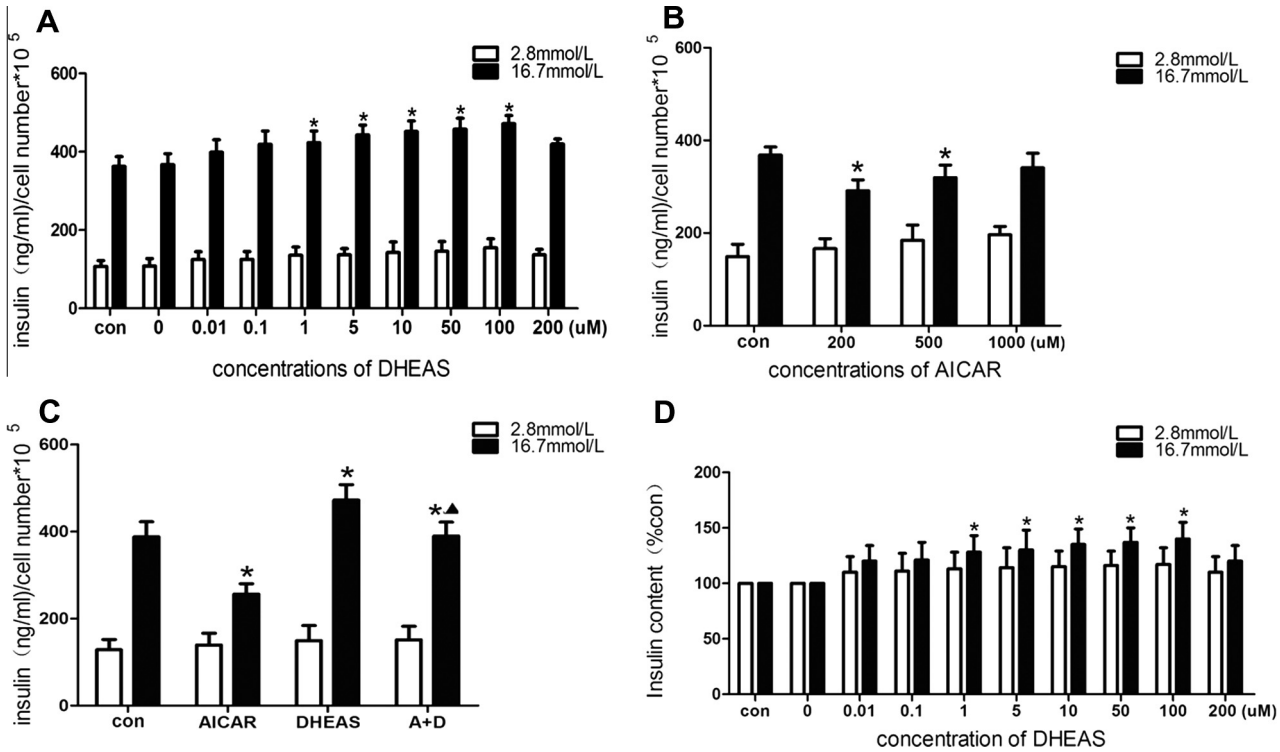


**Fig. 1.** Regulation of insulin secretion by DHEAS and/or AICAR in MIN6 cells for24 h. (A) The different concentrations of DHEAS containing 2.8 or 16.7 mmol/L glucose. (B) Different concentrations of AICAR containing 2.8 or 16.7 mmol/L glucose. (C) DHEAS (100 μmol/L) and/or AICAR (200 μmol/L). (D) The intracellular insulin content treated by DHEAS. All values were normalized to cell numbers. Results were shown as means ± SE, $^*P < 0.05$ vs. untreated controls. ▲ $P < 0.05$ for the effect of the AICAR and DHEAS vs. DHEAS-treated. A + D: AICAR plus DHEAS.

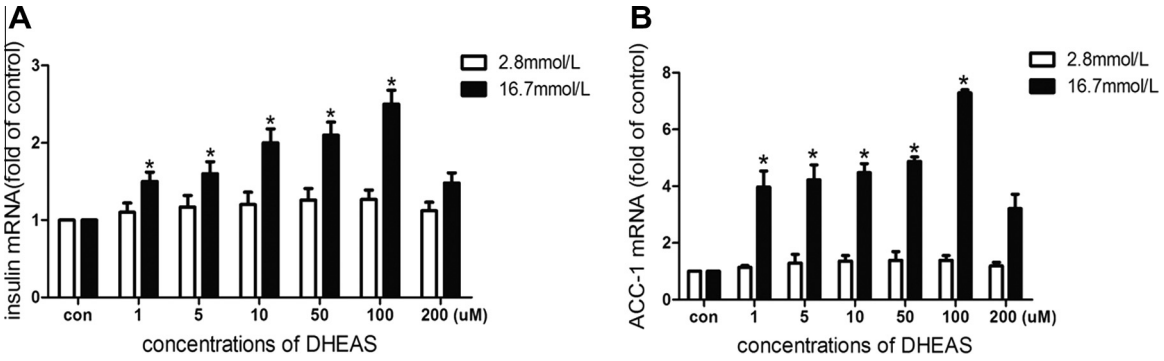


**Fig. 2.** Expression of insulin and ACC-1 mRNA in MIN6 cells treated by DHEAS for 24 h. (A) Expression of insulin mRNA in MIN6 cells. (B) Expression of ACC-1 mRNA in MIN6 cells. Results were expressed as means ± SE, $^{*}P < 0.05$ compared with control values.

treated with DHEAS (1–100 μmol/L) for the long-term were significantly higher than the control one ($P < 0.05$) (Fig. 2B). No change existed in the 2.8 mmol/L glucose ($P > 0.05$) (Fig. 2B).

### 3.4. Effect of DHEAS and/or AICAR on AMPKα phosphorylation

To assess activation or inhibition of AMPK, immunoblotting using antibodies against AMPKα, its phosphorylated form: p-AMPKαThr172 and beta-actin were performed after 24 h of incubation in serum-free DHEAS medium containing 2.8 mmol/L or 16.7 mmol/L glucose in the absence or presence of DHEAS (1–100 μmol/L), ACC phosphorylation and ACC-1 were also detected in the MIN6 cells. The amount of Thr172-phosphorylated AMPKα was significantly decreased in the cells incubated with DHEAS containing 16.7 mmol/L glucose compared to untreated group ($P < 0.01$) (Fig. 3A and B). The amounts of total AMPKα and beta-actin were unaffected by either treatment (Fig. 3A). The amount of phosphorylated ACC, a known target of AMPK was also significantly decreased in the cells treated with DHEAS containing 16.7 mmol/L glucose compared to untreated cells ($P < 0.01$) (Fig. 3A and C). Wherein, the amount of ACC-1 was significantly increased when treated with DHEAS containing 16.7 mmol/L glucose compared to untreated cells ($P < 0.01$) (Fig. 3A and D). No change existed in the phosphorylation of AMPK, ACC and the quantity of ACC-1 in MIN6 cells containing 2.8 mmol/L glucose, as DHEAS concentration was from 1 μmol/L to 100 μmol/L ($P > 0.05$) (Fig. 3E–H).

We then investigated whether DHEAS promoted insulin secretion via AMPK pathway by using 200 μmol/L AICAR (AMPK activator). AICAR stimulated AMPK phosphorylation and blocked the effect of DHEAS as expected. Meanwhile, phosphorylated-ACC was also blocked by AICAR containing 16.7 mmol/L glucose ($P < 0.01$) (Fig. 4A–C), which did not happen in MIN6 cells containing

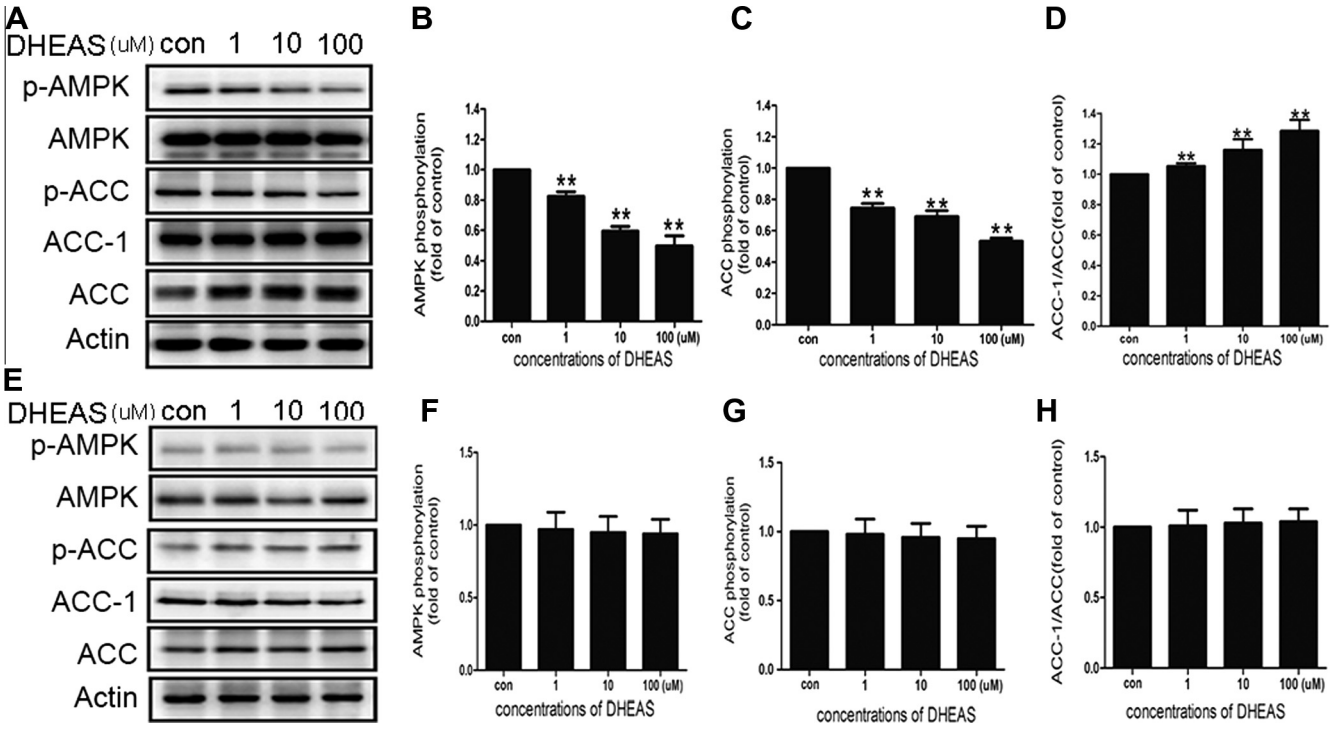


**Fig. 3.** DHEAS induced AMPK and ACC phosphorylation and ACC-1 in MIN6 cell lines for long-term. (A and E) Phosphorylated AMPK and ACC and ACC-1 were immunoblotted with specific antibodies as indicated. Representative blots for p-AMPK, total AMPK, p-ACC, total ACC and ACC-1 in MIN6 cells treated with DHEAS containing 2.8 or 16.7 mmol/L glucose for 24 h. Results were shown as means ± SE. $^{**}P < 0.01$ vs. untreated controls.

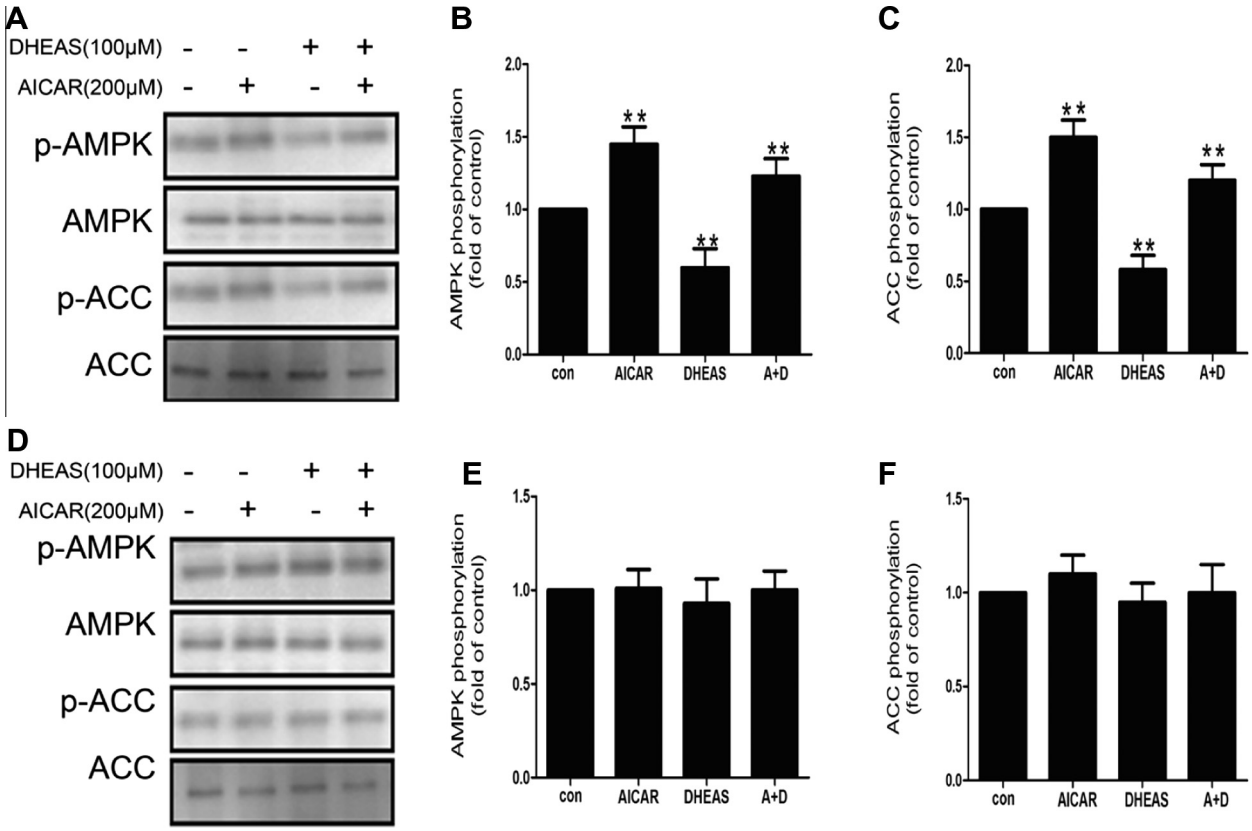


**Fig. 4.** Specificity of DHEAS in AMPK pathway. (A and D) Phosphorylated AMPK and ACC were immunoblotted with specific antibodies as indicated. Representative blots for p-AMPK, total AMPK, p-ACC, total ACC in MIN6 cells treated with AICAR (200 μmol/L) and/or DHEAS (100 μmol/L) for 24 h. A + D: AICAR plus DHEAS. Results were shown as means ± SE. $^{**}P < 0.01$ vs. basal.

2.8 mmol/L glucose ($P > 0.05$) (Fig. 4D–F). Our results suggested that DHEAS enhanced insulin secretion by inhibition of AMPK expression and activation of ACC-1 expression.

## 4. Discussion

DHEAS has been demonstrated to have a series of beneficial effects in different kinds of cells, tissues, organs and animal models. In this report, we found DHEAS could promote insulin secretion and biosynthesis from MIN6 cells for 24 h. Unlike other steroid hormones, an intracellular receptor for DHEAS has not been isolated [6]. It was unclear what the physiological functions of DHEAS and its cellular mechanisms of action really were. Here, we had demonstrated that DHEAS promoted insulin secretion from beta-cells through inhibiting the expression of AMPK and upregulating the expression of ACC-1 for 24 h. Chronic inactivation of AMPK and then activation of ACC by DHEAS may be involved in the elevation of insulin secretion from beta-cells.

The present findings that the expression of mRNA and protein levels of ACC-1 in beta-cells were significantly elevated when incubated with DHEAS for 24 h, raised the question of the possible role of AMPK and ACC-1 in insulin secretion of beta-cells. AMPK is a key regulator of cellular energy status, which is inhibited with ensuing rise of ATP:ADP ratio [19]. It is widely known that AMPK is mainly activated by phosphorylation of the catalytic subunit on Thr172. A significant target of AMPK is ACC, which is involved in the rate-determining steps of important biosynthetic pathways and is a major site of regulation for fatty acid synthesis. When the cells were cultured with fuel-free medium, cellular 5′-AMP levels rose, by which AMPK was activated. This could result in phosphorylation of ACC subunit on Ser79, which was inactivation. Conversely, the decrease in 5′-AMP levels would then lead to a decrease in the activity of the AMPK and diminish ACC on Ser79 phosphorylation, which stimulates insulin secretion [20]. AICAR and various amino acids, as insulin secretagogues, were used to show that AMPK activation decreases insulin secretion. This theory was also supported by other studies, which used thiazolidinediones to observe a decrease in insulin secretion and activation of AMPK [21,22].

To determine whether insulin secretion treated with DHEAS was related to AMPK, we employed AICAR and examined its effects. It was found that preincubated with AICAR blocked DHEAS-stimulated insulin secretion in MIN6 cells. Our results showed that DHEAS could make AMPK inactive and then ACC active, which led to the increased activity of ACC-1 and promoted the insulin secretion from MIN6 cells by a series of signal pathway. Several studies have shown that AMPK suppresses insulin secretion and synthesis in beta-cells [11,23,24], which is consistent with its role as an energy-conserving enzyme. Targonsky et al. reported that chronic treatment of islets and MIN6 cells with α-Lipoic acid (α-LA) led to dose-dependent rises in phosphorylation of the AMPKα-subunit and ACC [25]. Chronic exposure to α-LA, AICAR or metformin caused a reduction in insulin secretion. The chronic effects of α-LA include AMPK activation and reductions in insulin secretion and content, and cell growth.

The role of AMPK in insulin secretion of beta-cells has been studied extensively, but the results are still markedly controversial. It had been reported that during the activation of AMPK with AICAR in pancreatic beta-cells, the insulin secretion was decreased [11,21,23,26,27]. Gleason et al. reported that activation of AMPK by AICAR failed to suppress insulin secretion in a study involving three different beta-cells model systems including primary islets

[28]. A number of more studies found that activation of AMPK by AICAR increased insulin secretion. Recently, Fu et al. in reviewing the role of AMPK in insulin secretion of beta-cells have reported that treatment of MIN6 cells with AICAR increased both AMPK phosphorylation and insulin secretion [21]. Possible reason may be inferred that the effect of AMPK on insulin secretion may be influenced by glucose concentrations in the medium during the entire assay, short or long term activation of AMPK, cell culture conditions, etc.

There are a number of possible mechanisms by which DHEAS may enhance insulin secretion through AMPK. This enzyme has been shown to have an important role in suppressing preproinsulin promoter activity [11]. It has also been demonstrated that activation of AMPK leads to modulation of secretory vesicle dynamics [24]. Eight years ago, Richards et al. showed that mouse islets infected with a constitutively active AMPKα-subunit had reduced glucose oxidation and insulin secretion, and were associated with poorer glycaemic control when transplanted into streptozotocin diabetic mice compared with islets infected with null or dominant-negative AMPK viruses [17].

In our study, we also demonstrated that DHEAS could enhance the increased level of ACC-1, not only the level of mRNA but also its protein. In accordance with our findings, Ronnebaum et al. had studied the effects of knockdown of ACC-1, the significant step producing a malonyl-CoA and found that chronic, but not acute, suppression of this enzyme by pharmacological or siRNA methods resulted in impaired insulin secretion both in 832/13 cells and in primary rat islets [29]. MacDonald et al. had also reported that inhibitors of ACC-1 caused decreased insulin secretion in 832/13 cells and rat islets, but in their studies, these effects were seen with both acute and chronic administration of inhibitors [30]. However, our findings do not exclude other signal transduction pathways unrelated to AMPK concerning the insulin secretion. Further studies on DHEAS and its relationship with AMPK are needed to gain a better understanding of the antidiabetic properties of this molecule and the precise mechanism by which AMPK regulation occurs.

In summary, our study provided potential biochemical evidence that chronic administration of DHEAS could promote insulin secretion, which may be partly via the AMPK inhibition and ACC-1 upregulation. Our findings support the assumption that DHEAS might be a useful therapeutic for the treatment of diabetes, especially in the patients with insulin secretion defects.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.10.002.

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