Effects of Dehydroepiandrosterone on Gluconeogenic Enzymes and Glucose Uptake in Human Hepatoma Cell Line, HepG2

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Abstract. Dehydroepiandrosterone (DHEA), the most abundant human adrenal steroid, improves insulin sensitivity and obesity in human and model animals. In a previous study, we reported that orally administered DHEA suppresses the elevated activities of hepatic gluconeogenic enzymes like glucose-6-phosphatase (G6Pase) in C57BL/KsJ-db/db mice (Aoki K, Saito T, Satoh S, Mukasa K, Kaneshiro M, Kawasaki S, Okamura A, Sekihara H (1999) Diabetes 48: 1579–1585). However, the molecular mechanisms by which DHEA ameliorates insulin resistance are not clearly understood. In the present study, we cultured the human hepatoma cell line HepG2 with DHEA and measured the enzyme activity and protein expression of G6Pase to investigate the direct effect of DHEA on glucose metabolism in hepatocytes. DHEA significantly suppressed both the activity and protein expression of G6Pase. Moreover, DHEA decreased the gene expression of G6Pase and phosphoenolpyruvate carboxykinase, both of which were maximal at 1 μM DHEA, whereas the mRNA level of glucose-6-phosphate translocase was unchanged. Furthermore, DHEA enhanced 2-deoxyglucose uptake, although its effect was much smaller than that of insulin. These results suggest that DHEA may act at multiple steps in the regulation of glucose metabolism in the liver.

Key words: DHEA, Glucose-6-phosphatase, PEPCK, Gluconeogenesis, Glucose uptake


DEHYDROEPIANDROSTERONE (DHEA) is the most abundant human adrenal steroid hormone [1]. The serum concentration of DHEA reaches a maximum between the ages of 25 and 30 years but steadily declines thereafter, and by the age of 60 the concentration of DHEA is only 5–10% of that in young adults [1]. The physiological function of DHEA remains unclear. Many protective functions against age-related processes in humans including anti-obese, anti-diabetic [2–4], immuno-potentiating [5], anti-atherosclerotic [6], anti-carcinogenic [7], and anti-aging [8] effects have been proposed, although none of these effects have been confirmed.

Coleman et al. previously reported that the dietary administration of DHEA to genetically diabetic db/db mice induces the remission of hyperglycemia and ameliorates insulin resistance [2–4]. Another study also indicated that DHEA protects against visceral obesity and muscle insulin resistance in rats fed a high-fat diet [9]. In a previous study, we evaluated the effects of orally administered DHEA on key enzymes involved in hepatic gluconeogenesis, an important process in glucose metabolism, in db/db mice to elucidate the mechanism by which DHEA improves insulin sensitivity [10]. In these mice, DHEA suppressed the
elevated activities of hepatic glucose-6-phosphatase (G6Pase) and fructose-1, 6-bisphosphatase, and ameliorated hyperglycemia. These effects of DHEA on hepatic gluconeogenic enzymes may be due to DHEA itself, since the administration of androstenedione, a metabolite derived from DHEA, barely affected the blood glucose level or the activities of these gluconeogenic enzymes [10]. We also showed in another study that the oral administration of DHEA decreased the elevated gene expression of G6Pase in db/db mice, suggesting that this enzyme is at least one target of the hypoglycemic effects of DHEA [11]. In addition, we recently reported that glucose production in primary cultured hepatocytes from db/db mice is significantly decreased by the addition of DHEA or its sulfate ester (DHEA-S) to the culture medium, indicating that this suppressive effect on liver glucose production may be directly caused by either DHEA or DHEA-S [12].

To analyze the molecular mechanisms of the DHEA-induced down-regulation of hepatic gluconeogenic enzymes more precisely, we investigated in the present study the direct effects of DHEA on the enzyme activity, mRNA level and protein expression of hepatic G6Pase in a human hepatoma cell line, HepG2. We also evaluated the gene expression of another gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK), as well as that of glucose-6-phosphate transporter (G6PT), which is involved in the gluconeogenic process via the translocation of glucose-6-phosphate (G6P) from the cytoplasm to the endoplasmic reticulum (ER). Moreover, the effect of DHEA on 2-deoxyglucose (2-DOG) uptake in HepG2 cells was also examined.

### Materials and Methods

#### Cell culture

HepG2 cells obtained from Riken (Tsukuba, Japan) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% bovine fetal serum (FBS) at 37°C in 5% CO₂. To investigate the effect of each hormone, the cells were incubated for 24 h under the conditions indicated in the figure legends.

#### Assay of G6Pase activity

HepG2 cells were sonicated in ice-cold homogenization buffer (20 mM Tris-HCl; pH 7.0, 5 mM EDTA, 250 mM sucrose, 1 mM AEBSF, 10 µM leupeptin, and 10 µM pepstatin), followed by centrifugation at 12,000 x g for 15 min at 4°C. The supernatant was subsequently centrifuged at 105,000 x g for 60 min at 4°C. Then, the pellet containing the microsomal proteins was resuspended in homogenization buffer, and used for the enzyme assay as previously described [13–15]. Briefly, the microsomal suspension was incubated with 20 mM glucose-6-phosphate and 50 mM Tris-cacodylate; pH 6.5 in a final volume of 200 µl for 10 min at 35°C. The reaction was stopped by adding 700 µl of 10% SDS. To determine the amount of inorganic phosphate formed during the reaction, 2.1 ml of 0.36% ammonium molybdate containing 1 mM H₂SO₄ and 1.4% ascorbic acid was added, and after incubation at 45°C for 20 min, the optical density at 820 nm was measured. The protein concentration of the microsomal suspension was determined using DC Protein assay kit (Bio-Rad). The enzyme activity was expressed as the molar value of glucose-6-phosphate hydrolyzed by 1 mg of microsomal protein in 1 min.

#### Immunoblotting analysis

Thirty µg of the proteins contained in the microsomal pellet was separated by electrophoresis on a 10% SDS-polyacrylamide gel, and electrophoretically transferred to polyvinylidene fluoride membranes (ATTO). The membranes were probed with antibodies against G6Pase, which were raised from rabbits immunized with a synthetic polypeptide (FGIQSTHYLQV-NYQDSC) consisting of a sequence corresponding to that of human G6Pase (amino acid 11–26). Then, the blotted proteins were visualized using ECL plus Western blotting detection system (Amersham Biosciences).

#### Northern blot analysis

HepG2 cells were incubated with the indicated concentrations of DHEA in DMEM containing 10% dextran-charcoal-treated FBS for 24 h, and the total RNA was isolated from the cells using Trizol (Invitrogen) according to the manufacturer’s protocol. The RT-PCR reaction was performed using the extracted RNA as a template to obtain the cDNA fragments for G6Pase (1.1-kb), PEPCK (1.3-kb), and G6PT (1.5-kb). The primers used for the PCR reactions were synthesized according to the sequence data in Genbank as follows:
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G6Pase, 5'-ATGGAGGAAGGAATGAAT-3' (sense) and 5'-TTACAACGACTTTGTGC-3' (antisense); PEPCk, 5'-TTATCGTCACCCAAGAGC-3' (sense) and 5'-GGAAGATCTTGGGCAGTT-3' (antisense); and G6PT, 5'-GTTCATCACCAGCGCCAGTC-3' (sense) and 5'-GAAGTCAAGGGTCATTAGTGC-3' (antisense). The cDNA fragments obtained were further amplified by the second PCR reaction. Each cDNA fragment obtained was subcloned into pGEM®-T Easy Vector (Promega) and its nucleotide sequence was confirmed by ABI PRISM® 310 Genetic Analyzer (PE Biosystems). cDNAs were labeled with 32P by the random primer method using Ready-To-Go DNA labeling beads (Amersham Biosciences), and purified in MicroSpinTM G-50 columns (Amersham Biosciences) before being used as probes. Twenty µg of total RNA was electrophoresed on a 1% formaldehyde-denatured agarose gel using 1 × MOPS running buffer. The RNA was transferred to a nylon membrane (Millipore) by capillary action and UV-cross-linked in a Funakoshi. The membrane was then hybridized with 32P-labeled probes at 68°C for 1 h in ExpressHybTM Hybridization solution (Clontech). Detection was performed as described [16]. As an internal control, the signal intensity of each enzyme mRNA was normalized using that for ribosomal protein S9 (Clontech).

2-Deoxyglucose (DOG) uptake analysis

HepG2 cells were incubated with 10 µM DHEA or 0.1 µM insulin in DMEM containing 10% dextran-charcoal-treated FBS for 24 h, and 2-DOG uptake was measured as previously described [17]. Briefly, HepG2 cells were preincubated with the indicated concentration of each hormone in 900 µl of glucose-free HEPES-Krebs-Ringer (HKR) buffer containing 5% BSA, 20 mM HEPES; pH 7.4, 140 mM NaCl, 5 mM KCl, 2.5 mM MgSO4 and 1 mM CaCl2 for 4 h at 37°C. After adding 100 µl of [3H] 2-DOG (1.25 µCi/5.2 nmol) in HKR, the cells were incubated for 2 min at 37°C. The cells were then quickly washed three times with ice-cold PBS, and mixed with 500 µl of cell lysis solution (0.2 N NaOH, 1% SDS). A portion of the cell lysate was used in a radioactivity assay performed using a liquid scintillation counter.

Statistical analysis

The results were expressed as the means ± SEM, and analyzed using Student’s t test with StatView 5 software (SAS Institute). A P value of less than 0.05 was considered statistically significant.

Results

Effects of DHEA on G6Pase enzyme activity and protein level

We previously elucidated that DHEA suppresses the G6Pase activity and the G6Pase mRNA level in vivo, although the possibility that some DHEA metabolite, but not DHEA itself, may be involved in this process could not be ruled out [11]. To test the direct effect of DHEA on gluconeogenic enzyme activity, we first examined the in vitro effect of DHEA on G6Pase activity in HepG2 cells. The cells were cultured with 1 µM dexamethasone, 0.1 µM insulin or 0.1 µM DHEA for 24 h, and the microsomal fraction containing G6Pase was isolated. Glucose-6-phosphate was added to the fraction as a substrate, and the inorganic phosphate derived from the enzyme reaction was measured. The G6Pase enzyme activity of the control was approximately 7.8 nmol/mg protein/min. As shown in Fig. 1, the G6Pase activity was significantly elevated to 128% (vs. control, p<0.01) by the addition of 1 µM dexamethasone, whereas it was suppressed to 72% by the addition of 0.1 µM insulin (vs. control, p<0.01). Treatment with 0.1 µM DHEA decreased G6Pase activity to 82% (vs. control, p<0.05), demonstrating a direct suppressive effect of DHEA on G6Pase activity. We also determined the amount of G6Pase protein in isolated microsomal fractions by immunoblotting with anti-G6Pase antibodies. The treatment of HepG2 cells with 0.1 µM DHEA lowered the protein expression of G6Pase, compared to that in the control, suggesting that DHEA also suppresses the protein expression of this gluconeogenic enzyme (Fig. 2).

Effects of DHEA on mRNA levels of enzymes involved in gluconeogenesis

Next, we investigated whether DHEA affects the gene expression of enzymes involved in gluconeogenesis. HepG2 cells were cultured for 24 h with increasing...
concentrations of DHEA (0.1–10 μM), and RNA was isolated and used in a Northern blot analysis to measure the mRNA levels of G6Pase, PEPCK, G6PT and ribosomal protein S9 (as an inner control) (Fig. 3). DHEA significantly decreased the gene expression of G6Pase in a dose-dependent manner, compared to that in the control, and this effect was maximized at 1 μM DHEA.

Fig. 1. Effects of DHEA, dexamethasone and insulin on G6Pase activity in HepG2 cells. HepG2 cells were cultured for 24 h in the presence of 1 μM dexamethasone, 0.1 μM DHEA, or 0.1 μM insulin in serum-free DMEM. The G6Pase activity in the microsomal suspensions was measured as described under “Materials and Methods”. Values represent the means and standard errors of data for three independent experiments. *p<0.05 vs. control; **p<0.01 vs. control.

Fig. 2. Effects of DHEA on G6Pase protein expression in HepG2 cells. HepG2 cells were cultured in the presence or absence of 0.1 μM DHEA for 24 h in serum-free DMEM. The microsomal suspensions isolated from HepG2 cells were subjected to immunoblotting as described under “Materials and Methods”.

Fig. 3. Effects of DHEA on G6Pase, PEPCK and G6PT mRNA level in HepG2 cells. HepG2 cells were incubated with the indicated concentrations of DHEA in DMEM containing 10% dextran-charcoal-treated FBS for 24 h. RNA was extracted and analyzed for the expression of G6Pase, PEPCK, G6PT and S9 mRNAs by Northern blot analysis. The results were normalized according to the S9 signal and expressed relative to the values in the control. Values represent the means and standard errors of data for three independent experiments. *p<0.05 vs. control.
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(43.3 ± 13%, p<0.05). DHEA also significantly reduced the mRNA level of PEPCK, which was maximally suppressed at a DHEA concentration of 1 μM (66.7 ± 9.5%, p<0.05), whereas it did not affect the gene expression of G6PT.

Effects of DHEA on 2-DOG uptake in HepG2 cells

DHEA is known to increase glucose uptake in human fibroblasts and rat adipocytes [18, 19], although its effect on hepatocytes has not been examined. To analyze the effect of this steroid on liver glucose transport, we assessed DHEA-induced glucose uptake in HepG2 cells. The cells were preincubated with 10 μM DHEA or 0.1 μM insulin for 24 h, and were incubated with HKR buffer containing [3H]-labeled 2-deoxyglucose for 2 min, and then the radioactivity of the isolated cell lysate was measured. Insulin significantly enhanced 2-DOG uptake into HepG2 cells by 33%, compared with that in the control (p<0.01). Although DHEA at concentrations of 0.1–1 μM DHEA did not affect 2-DOG uptake (data not shown), 10 μM of DHEA significantly increased 2-DOG uptake by 11% as compared with that in the control (p<0.05), indicating that DHEA may also directly promote glucose uptake in hepatocytes.

Discussion

The age-related decline in serum DHEA suggests that a relative deficiency in this steroid may be causally related to the development of chronic diseases generally associated with aging, including insulin resistance and obesity [8]. A recent clinical study reported that DHEA replacement therapy for 12 weeks significantly increased insulin sensitivity in hypoadrenal women [20]. We also showed that the dietary administration of DHEA lowered glucose levels and reduced hepatic gluconeogenic enzyme activities in diabetic db/db mice [10].

Among the hepatic gluconeogenic enzymes, G6Pase is a key enzyme involved in the homeostatic regulation of blood glucose, catalyzing the hydrolysis of G6P derived from gluconeogenesis and glycogenolysis. We previously demonstrated that G6Pase mRNA and enzyme activity levels in the livers of diabetic db/db mice are elevated, compared with those in non-diabetic db/+m mice, and that hepatic G6Pase activity and blood glucose levels are significantly correlated in db/+m mice, DHEA-treated db/db mice, and non-treated db/db mice, suggesting that G6Pase may be one of the targets of the hypoglycemic effects of DHEA [10, 11]. Consistent with these previous reports, we showed in this study that DHEA directly suppresses the enzyme activity, mRNA level and protein expression of G6Pase in HepG2 cells (Fig. 1, 2). In addition, we observed that the gene expression of PEPCK, another key enzyme involved in gluconeogenesis, is reduced by DHEA (Fig. 2), indicating that PEPCK may be another molecular target for the insulin-sensitizing effect of DHEA. However, the molecular mechanism of decreased gene expression of these gluconeogenic enzymes was unclear. In our analysis, the promoter activity of the -1184/+112 region of the G6Pase gene was not changed by DHEA treatment in HepG2 cells (data not shown), raising the possibility that these effects of DHEA may be mediated through other promoter regions of G6Pase or caused by the decreased stability of G6Pase mRNA. These hypotheses should be subjected to experimental testing in future studies.

The hydrolysis of G6P is thought to require at least two ER-associated membrane proteins: G6PT, which translocates G6P from the cytoplasm to the ER lumen, and G6Pase, which hydrolyzes G6P to glucose and phosphate in the ER [13]. According to previous reports, the mRNA level of G6PT in hepatocytes parallels that of G6Pase, which is increased by dexamethasone and reduced by insulin [21, 22]. In contrast to these experimental findings, we showed that DHEA suppressed the mRNA level of G6Pase but did not suppress that of G6PT (Fig. 3), suggesting that DHEA may affect gluconeogenesis at sites that are different from those of dexamethasone and insulin.

Insulin-stimulated glucose uptake in adipose tissue and striated muscle is known to be critical for reducing postprandial blood glucose concentrations [23]. Several studies have reported that DHEA increases glucose uptake in a manner similar to insulin-induced glucose uptake in human fibroblasts and rat adipocytes [18, 19]. In addition, a recent study demonstrated that DHEA increases glucose uptake in both human and 3T3-L1 adipocytes by stimulating the translocation of both Glut4 and Glut1 to the plasma membrane [24]. However, the effect of DHEA on glucose transport in hepatocytes has not been analyzed. In the present study, we showed that DHEA at a concentration of 10 μM increased 2-DOG uptake in HepG2 cells (Fig.)
4), although the degree of enhancement appeared to be much smaller than that in adipocytes in which 10μg/mL DHEA reportedly provoked a two-fold increase in 2-DOG uptake [24]. Therefore, these results suggest that enhanced glucose uptake in the liver may also play at least a partial role in the anti-diabetic effects of DHEA. In this study, DHEA was effective at concentrations from 0.1μg/mL to 10μg/mL, which are higher than human physiological concentrations of DHEA (0.7–50 nM). In fact, plasma DHEA level in DHEA-treated db/db mice were elevated to 10-fold concentrations as compared with non-treated mice, at which a significant anti-diabetic effect of DHEA was observed in our previous study [10], suggesting that the in vivo effects of DHEA on G6Pase down-regulation may be also caused by DHEA levels above the human physiological DHEA concentrations. Consequently, control of the serum DHEA concentration to an effective pharmacological level by DHEA supplementation could potentially lead to improve insulin resistance in diabetic patients.

In conclusion, we analyzed the direct effects of DHEA in HepG2 cells and showed that: 1) DHEA decreases the enzyme activity and protein expression of G6Pase; 2) DHEA suppresses gene expression of G6Pase and PEPCK, but not G6PT, and; 3) DHEA enhances glucose uptake. These results suggest that DHEA may act at multiple steps in the regulation of glucose metabolism in the liver.

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