Dehydroepiandrosterone reduces preadipocyte proliferation via androgen receptor

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Obesity is one of the most important public health problems because of its association with increased risk for diabetes, hypertension, coronary heart disease, and other serious diseases. In addition to the environmental factors, including a Western style diet, hormonal changes are postulated to up- or downregulate adiposity. Recent evidence indicates that hypogonadism is a predictor of obesity, insulin resistance, diabetes, and coronary artery disease in men (22, 24). Testosterone replacement therapy improves insulin resistance and glycemic control in hypogonadal men with type 2 diabetes (23). Male hypogonadism is a distinctly defined clinical entity associated with sexual dysfunction, inactivity, depression, decreased muscle mass, bone loss, etc. Hence, testosterone replacement also improves these abnormalities (53). On the other hand, although circulating dehydroepiandrosterone (DHEA) and testosterone concentrations are known to decline during the aging process (35, 36) in men, the relationship between low plasma DHEA level and cardiovascular disease remains controversial (48). Barrett-Connor and colleagues reported negative correlation between DHEA sulfate (DHEA-S) level and death from any case and death from cardiovascular disease in men more than 50 years of age (1). The same authors concluded that DHEA-S did not predict cardiovascular death in women (2). Analyses of the relationship of plasma DHEA or DHEA-S levels with obesity or body fat distribution have revealed inconsistent results. Plasma DHEA levels are negatively correlated with total body mass index and abdominal fat accumulation; however, contradictory results have been reported for the relationship between DHEA-S and adiposity (49). Several studies supported the association of high plasma DHEA level with favorable insulin sensitivity (6, 48). Meanwhile, administration of DHEA has been reported to reduce adiposity (3), diabetes (9), and cancer (46) in animal studies. However, discrepancies have been reported in the benefit of DHEA administration in human studies. Both positive (52) and no (7) effects of DHEA replacement in elderly people or in patients with adrenal insufficiency have been reported. A major cause of these discrepancies might be the varied study designs, including the selection of subjects, dose, drug administration, and definitions of end point in these numerous studies.

Although the specific receptor of DHEA has not been identified, one proposed mechanism is that DHEA is a precursor of androgen and estrogen that is converted to active form in peripheral tissues (30). In postmenopausal women, 100% of sex steroids are made locally in peripheral target tissue, and in adult men, ~50% of androgens are made locally. The formation of each sex steroid depends upon the activity of the specific androgen- and estrogen-synthesizing enzymes in each tissue through a mechanism called intracrinology (28). DHEA is metabolized into androstenedione with 3β-hydroxysteroid dehydrogenase (3β-HSD) and then transformed to testosterone with 17β-HSD (29). The fact that androgen receptor (AR)-deficient mice are obese (43) indicates that AR acts as a mediator to prevent fat accumulation in the whole body. Accordingly, several effects of DHEA may be mediated via AR. In this regard, it is possible that DHEA and testosterone have common mechanisms to reduce adiposity. However, few comparative studies between these hormones have been reported (15, 42, 44).

We demonstrated that administration of DHEA reduced adiposity in Otsuka Long-Evans Tokushima fatty (OLETF)
rats. Moreover, we found that DHEA directly downregulated the PPARγ mRNA level in cultured adipocytes, which may explain the antiobesity effect of DHEA (21). However, contradictory data have been reported lately (25). To understand the weight-reducing effect of DHEA comprehensively, we performed a microarray study. Analysis of ~30,000 genes in epididymal fat isolated from mice after treatment with or without DHEA demonstrated that, although a few genes involved in energy metabolism (e.g., malonyl-CoA decarboxylase) were affected, genes regulating cell growth (e.g., foxl1, nuclear factor I/X, eukaryotic translation elongation factor 1α, and aurora kinase) and genes regulating apoptosis (e.g., caspase 3, tumor necrosis factor receptor superfamily, and Bcl2) were markedly up- and downregulated, respectively (unpublished results). These results indicate that DHEA regulates cell growth or death and possibly adiposity. However, we failed to find any evidence of increased apoptosis in adipose tissue and cultured adipocytes after DHEA treatment. Next, we investigated the effect of DHEA on cell growth in adipose tissue.

Adipocytes are derived from mesenchymal stem cells via unknown stimulation. These cells transform into preadipocytes, which are difficult to distinguish from stem cells, proliferate, and then terminally differentiate into mature adipocytes (40). Because proliferative activity is considered to be eliminated in mature adipocytes (16), we noted a nonmature adipocyte fraction of adipose tissue, stromal vascular fraction (SVF), consisting of adipocyte progenitor cells, mature endothelial cells, and angiogenic progenitor cells (38), and immune cells including macrophages (47). Newborn adipocytes are provided from the SVF, and the efficacy of adipogenesis vastly influences adiposity and insulin sensitivity (54). In this study, we investigated the effects of DHEA on preadipocytes and SVF to clarify whether DHEA impacts proliferation in these cells. DHEA is suggested to delay preadipocytes growth (32, 37) in vitro; therefore, we assessed this in an in vivo study. Although it is difficult to evaluate individual cell proliferation in vivo, we have estimated the effect of DHEA administration on total cell proliferation in SVF with 5-bromo-2′-deoxyuridine (BrdU) incorporation. In addition, we assessed its effect on cellular senescence, defined as the state of permanent inhibition of cell proliferation resulting from accumulation of proliferation and DNA damage. We have measured activity of senescence-associated β-galactosidase, which is known to be a biomarker of cellular senescence, similar to altered cellular morphology, and accumulation of DNA damage foci and chromosomal instability (32) in obese rats treated with or without DHEA. Furthermore, we have compared the effects of DHEA and testosterone on preadipocyte growth in vivo and in vitro to evaluate whether the DHEA signal is mediated via AR.

**MATERIALS AND METHODS**

**Animals.** Male Long-Evans Tokushima Otsuka (LETO) rats and Otsuka Long-Evans Tokushima fatty (OLETF) rats were obtained from Otsuka Long-Evans Tokushima, Japan. Animals were fed CE2 powder ad libitum and treated with or without 0.4% DHEA for 52 wk. Individual food consumptions were determined by subtracting the remaining food from the supplied one every 2–3 days, and the averages of these values in 1 wk were expressed as weekly food consumption. They were housed in a specific pathogen-free facility with a 12:12-h light-dark cycle. To obtain more accurate values, male Wistar rats were housed in metabolic cages (Tecniplast Metabolic Cage; Tecniplast, Florence, Italy) in which daily food consumption was determined. All procedures for animal care were carried out in accordance with protocols approved by the University of Gifu’s Institutional Animal Care Committee. Animals were decapitated to collect epididymal fat, liver, and skin samples. SVF were obtained by collagenase digestion. Adipose tissue was minced in KRP buffer containing 3% BSA and collagenase type I, and incubated for 30 min at 37°C with shaking. The cell suspensions were filtered through a nylon mesh and then spun at 300 g for 5 min. The pellets containing SVF were rapidly frozen in liquid nitrogen.

To compare the effects of DHEA and testosterone on adiposity, male Wistar rats were fed with (control) or without 0.4% DHEA- or 0.4% testosterone-containing food for 4 wk. Body weight, fat weight, and serum levels of glucose, aspartate aminotransferase (AST), alanine aminotransferase (ALT), triglycerides, and free fatty acid (FFA) were measured with Quickauto-Neo GLU-HK (Shino-Test, Tokyo, Japan), L-type AST-J2 kit (Wako Pure Chemical Industries, Osaka, Japan), L-type ALT-J2 kit (Wako), L-type TG-H kit (Wako), and NEFA-SS “Eiken” (Eiken Chemical, Tokyo, Japan), respectively. Immunoreactive insulin (IRI), DHEA-S, and testosterone were measured with a rat insulin RIA kit (Linco Research, St. Charles, MO), Access DHEA-S Reagent Pack (Beckman Coulter, Brea, CA), and Elecsys Testosterone II (Roche Diagnostic, Mannheim, Germany), respectively.

**Senescence-associated β-galactosidase staining.** Senescence-associated β-galactosidase staining (SA-β-Gal) staining was performed as described previously (11). Briefly, thin sections of SVF and skin were cut, mounted on glass slides, fixed in 1% formalin in phosphate-buffered saline (PBS) for 1 min, washed in PBS, and immersed overnight in SA-β-Gal solution containing 1 mg/ml 4-chloro-3-indolyl-β-D-galactoside (X-Gal), 40 mM citric acid-sodium phosphate (pH 6.0), 5 mM potassium ferrocyanide, 150 mM NaCl, and 2 mM MgCl2. The sections were washed with PBS, counterstained with eosin, and viewed under bright-field at ×50–200 magnification.

**Quantitative analysis of in vivo cell proliferation in stromal vascular fractions.** To assess the effects of DHEA and testosterone on cell proliferation in adipose SVF, 200 mg/kg BrdU was administered to rats intraperitoneally three times every 4 h. Twelve hours later, the rats were killed, and subcutaneous, epididymal, intestinal, and perirenal fat samples were collected. These tissues were fixed, paraffin-embedded, and subjected to immunohistochemical examination using the BrdU In-Situ Detection Kit (Becton-Dickinson; Downers Grove, IL). For quantitative evaluation of BrdU incorporation, we developed dot blot analysis, described below. DNA was extracted using a DNeasy blood and tissue kit (Qiagen, Hilden, Germany) from each adipose SVF. The nitrocellulose membrane (Protran BA85; Whatman, Kent, UK) was saturated in SSC, on which 0.4% BrdU was administered. After incubation with an anti-BrdU antibody (Sigma-Aldrich Japan K.K., Tokyo, Japan), followed by incubation with HRP-conjugated secondary antibody using the Snap i.d. protein detection system (Millipore, Billerica, MA). The reaction was visualized using the enhanced chemiluminescence (ECL) system.

**Cell culture.** 3T3-L1, 3T3-F442A, and 3T3-A1 fibroblasts were cultured in Dulbecco's modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Upon confluence, 3T3-L1 preadipocytes were differentiatet as described previously (19).

**Evaluation of preadipocyte proliferation.** Cell proliferation was evaluated by BrdU incorporation into the cells. Isolated cells were seeded in a 96-well microplate at a density of 3.0 × 103/well. The cells were allowed to attach for 24 h before treatment with DHEA or testosterone, following which cell proliferation was assayed with an
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enzyme-linked immunosorbent assay kit (Roche, Tokyo, Japan). To determine the effects of flutamide, an AR inhibitor, and fulvestrant, an estrogen receptor (ER) inhibitor, on DHEA-induced reduction of cell growth, inhibitors were added 2 h before the treatment. Furthermore, gene silencing of ARs or ERs was performed using specific siRNA reagents (TransIT-TKO, Mirus). Designed siRNA for AR [AR(–1): GGAGGGUCACCAAGAGGUTT and AUCCUUGGCGUAA-ACU-CCTT], and ERx [ERx(–1): GGACAACAUAGCUACAGUTT and ACAUAGUACU-UUGUGUCC(T)T] were used in parallel with manufactured products [AR(–2), ER(–2); Santa Cruz Biotechnology]. One day after transfection, cells were trypsinized and plated in a 96-well microplate to measure proliferation with or without 10 μM DHEA. Cell viability was determined using a Fluo cell double staining kit (MoBiTec, Gottingen, Germany). 3T3-L1 preadipocytes were used in parallel with 1 mM DHEA and 1 mM testosterone for 48 h incubated with calcine-AM, which stains viable cells green, and propidium iodide, which dyes dead ones red according to the manufacturer’s instructions. To obtain dead cell control, 3T3-L1 preadipocytes were incubated with 5% H2O2 for 10 min.

Real-time PCR analysis. Five days after the initiation of differentiation, 3T3-L1 adipocytes were treated with various concentrations of DHEA or testosterone for 1 or 5 days. To determine the mRNA expression levels of PPARγ, p22, LPL, SREBP-1, fatty acid synthase (FAS), leptin, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), real-time PCR analysis was performed. Total RNA was isolated and purified as mentioned above. Reverse transcription was performed using a PrimeScript Reverse Transcriptase (TAKARA Japan) according to the manufacturer’s instructions. Optimal conditions for all investigated genes were established using SYBR Premix Ex Taq Kit (TAKARA) according to the manufacturer’s instructions. Twenty microliters of the reaction solution consisted of 2 μL of the template, 10 μL of SYBR Premix Ex Taq, 0.4 μL of 10 μM of each primer and 0.4 μL of ROX Reference Dye. PCR amplification was performed as follows: predenaturation for 1 cycle at 95°C for 30 s, and 40 cycles at 95°C for 5 s, and 60–62°C for 30 s using a Thermal Cycler Dice (TAKARA, Ohtsu, Japan). Expression levels, calculated as copy numbers in each sample, were normalized to the expression level of GAPDH. Oligonucleotide primers were designed on the basis of sequences from the GenBank database (f, forward; r, reverse). PPARγ: 5′-CCCTTGGACTTCTTATGGGA-3′ (f) and 5′-CTGGCT- GAAGGT-3′ (r); FAS: 5′-AGGATGCAACATTGGAGAAG-3′ (f) and 5′-TCTCTTG-GCTTCGACCTTGT-3′ (r); FAS: 5′-AGATCTCGTGGACGAGA- CAGCAT-3′ (f) and 5′-GAGACCTGTCACCTGAGGT-3′ (r); SREBP-1: 5′-TACCAGACATGCGGTACTCATTGC-3′ (f) and 5′-AACCTGCCTCTGACACG-3′ (r); LPL: 5′-AGATGCAACATTGGAGAAG-3′ (f) and 5′-TCTCTTG-GCTTCGACCTTGT-3′ (f); GAPDH: 5′-GAGACCTGTCACCTGAGGT-3′ (f) and 5′-GAGACCTGTCACCTGAGGT-3′ (r).

Triglyceride content in liver and muscle. Liver and gastrocnemius muscle were homogenized in KRP buffer, and triglyceride of the muscle were homogenized in KRP buffer, and triglyceride of the liver and muscle were determined by BZ-II analysis application (Keyence, Japan) per rat in each group was measured.

Statistical analyses. Results were expressed as means ± SE. Statistical comparisons were performed by ANOVA. Significance was defined as P < 0.05.

RESULTS

Effect of DHEA administration on body weight, food consumption, and laboratory data in LETO and OLETF rats. DHEA administration suppressed the body weight gain in both LETO and OLETF rats over a period of 44 wk; however, a significant decrease in food consumption was observed only in the first 2 wk. This indicated that DHEA-induced reduction of body weight could not be attributed to the change in food intake (Fig. 1). As shown in Table 1, DHEA administration significantly reduced epididymal fat weight in LETO and OLETF and plasma glucose level in OLETF rats. Serum triglyceride and FFA levels were also diminished by DHEA administration.

Effect of DHEA administration on SA-β-gal staining in SVF. SA-β-Gal-positive cells were detected in SVF sections isolated from the control OLETF but not in ones isolated from DHEA-treated OLETF rats (Fig. 2, A and B). No SA-β-Gal-positive cells were observed in LETO rats (Fig. 2, C and D). Similar results were observed in skin sections (data not shown). Because SVF consist of various cells, we cannot describe the stained cell type. However, these results showed obesity-accelerated senescence in the SVF of adipose tissue, which was prevented by DHEA administration. It is possible that elevated cell turnover might have lead to telomere shortening or oxidative stress in obese adipose tissue. Considering this, we hypothesized that DHEA suppresses cell proliferation in adipose tissue; therefore, we examined the effect of DHEA on preadipocyte growth.

Effects of DHEA and testosterone on body weight and fat weight. Treatment with both 0.4% DHEA and 0.4% testosterone resulted in attenuation of body weight increase (Fig. 3A). Neither DHEA nor testosterone administration-influenced consumption was observed only in the first 2 wk in rats treated with DHEA (Fig. 3B). Treatment with these hormones equally reduced epididymal fat, intestinal fat, and perirenal fat weights and elevated rectal body temperature (Fig. 3, C and D). Because rodents lack 17α-hydroxylase (CYP17A1) in their adrenal glands, they produce little or no DHEA. Hence, DHEA-S was not detected in the control serum, whereas elevated DHEA-S levels were observed in both DHEA-treated and testosterone-treated rats (Fig. 3, E, right). Treatment with DHEA resulted in less elevated testosterone levels than treatment with testosterone (Fig. 3, E, left). No differences were observed in serum levels of glucose, triglyceride, FFA, AST, ALT, and IRI levels among these animals (data not shown). Treatment with DHEA decreased triglyceride content in liver and gastrocnemius muscle. In addition, both DHEA and testosterone administration decreased PPARγ protein levels in isolated adipocytes and adipocyte size significantly in epididymal fat (Fig. 3, F–H).

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Immunohistochemical study and dot blot. BrdU-labeled cells were markedly abundant in tissues where cells proliferate actively such as the lymph node (Fig. 4A), whereas they were less abundant around adipocytes and vessels in adipose tissue (Fig. 4B). To quantitatively measure BrdU uptake, we extracted BrdU-labeled DNA from SVF. Equal amounts of DNA (2 μg) were spotted and immobilized with baking. After denaturing, the incorporated BrdU was depicted by anti-BrdU antibody. As shown in Fig. 4C, DHEA and testosterone administration reduced BrdU uptake by SVF to 81.0 and 75.5%, respectively.

Effect of DHEA and testosterone on cultured preadipocyte proliferation. Treatment with DHEA for 24 h significantly impaired 3T3-L1 and 3T3-F442A preadipocyte proliferation at 1/1000 M concentration (Fig. 5A). Because incubation with testosterone at 1 μM for 24 h and at 0.01 μM for 48 h suppressed 3T3-L1 preadipocyte proliferation similarly to those of DHEA (Fig. 5B), we speculated that these hormones might share an identical signaling pathway. Pretreatment with 1/1000 M flutamide, but not with fulvestrant, abolished DHEA-induced suppression of proliferation (Fig. 5C). This suggests that the DHEA signal is mediated via AR. To confirm this hypothesis, we examined the effect of gene silencing by using siRNA. Both siRNA for AR and ER and AR(-11002)1 and AR(-11002)2, markedly suppressed AR mRNA levels to 3.1 and 2.5%, respectively, as well as AR protein levels in association with ablating DHEA action (Fig. 5E). These results strongly suggest that DHEA acts in an intracrine manner; therefore, we assessed the effect of pretreatment with 10 μM trilostane, an inhibitor of 3β-HSD, for 2 h on DHEA- and testosterone-induced growth.

Table 1. Effect of DHEA Administration on epididymal fat weight and laboratory data in LETO and OLETF rats

<table>
<thead>
<tr>
<th>Specimen</th>
<th>LETO</th>
<th>OLETF</th>
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<tbody>
<tr>
<td>Epididymal fat, g</td>
<td>Control</td>
<td>DHEA</td>
</tr>
<tr>
<td>Glucose, mg/ml</td>
<td>140 ± 1</td>
<td>132 ± 2</td>
</tr>
<tr>
<td>TG, mg/ml</td>
<td>101 ± 14</td>
<td>53 ± 2*</td>
</tr>
<tr>
<td>T.Chol, mg/ml</td>
<td>121 ± 5</td>
<td>139 ± 2</td>
</tr>
<tr>
<td>FFA, mEq/l</td>
<td>645 ± 115</td>
<td>490 ± 16</td>
</tr>
<tr>
<td>AST, IU/l</td>
<td>207 ± 10</td>
<td>331 ± 35*</td>
</tr>
<tr>
<td>ALT, IU/l</td>
<td>75 ± 11</td>
<td>118 ± 9</td>
</tr>
<tr>
<td>IRI, ng/ml</td>
<td>1.8 ± 0.4</td>
<td>2.4 ± 0.3</td>
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Data are expressed as means ± SE; n = 6. LETO, Long-Evans Tokushima Otsula; OLETF, Otsuka Long-Evans Tokushima fatty; TG, triglyceride; T-Chol, total cholesterol; FFA, free fatty acid; AST, asparate aminotransferase; ALT, alanine aminotransferase; IRI, immunoreactive insulin. *p < 0.05, **p < 0.01 vs. Control.

Fig. 1. Effects of dehydroepiandrosterone (DHEA) administration on body weight and food consumption in Long-Evans Tokushima Otsula (LETO) and Otsuka Long-Evans Tokushima fatty (OLETF) rats. Both LETO and OLETF rats were treated with or without (Control) 0.4% DHEA-containing food. Effects of this treatment on body weight in LETO (A) and OLETF (B) and that on food consumption in LETO (C) and OLETF rats (D) are shown. ■, Control; ○, DHEA. *P < 0.05 vs. Control; n = 15 in each group.
suppression. Trilostane prevented the effect of DHEA but not that of testosterone (Fig. 5D). In addition, we examined the possibility that these hormones might affect the viability of 3T3-L1 preadipocytes. As shown in Fig. 5F, incubation with DHEA or testosterone for 48 h did not influence cell viability.

Effect of DHEA and testosterone on gene expression of mature 3T3-L1 adipocytes. Incubation with various concentrations (10–1,000 nM) of DHEA and testosterone reduced the expression of PPARγ and its downstream genes, such as LPL and aP2, whereas they did not influence the expression of genes involved in lipogenesis, SREBP-1 and FAS (Fig. 6, A–E). A prolonged incubation period (5 days) shown in the lower panels enhanced the effects of these hormones at the lower concentration. Next, we evaluated the effects of these hormones on lipolysis. As shown in Fig. 6F, treatment with DHEA and testosterone for 1 day did not affect glycerol release.

DISCUSSION

Since the presentation by Coleman and colleagues suggested that DHEA administration prevented diabetes in db/db mice, numerous studies have been published (9). Our previous study revealed that, although treatment with DHEA fails to decrease blood glucose in Goto-Kakizaki rats, an animal model of lean type 2 diabetes, it significantly reduces blood glucose in OLETF rats (20). Therefore, we speculated that DHEA administration might improve obesity-associated diabetes in association with preventing obesity-induced insulin resistance. We found that treatment with DHEA for 2 wk reduced fat mass and that DHEA directly suppressed mRNA levels of PPARγ in isolated adipocytes (21). Since suppression of PPARγ activity leads to reduced adiposity in PPARγ-deficient mice (27) and Pro12Ala polymorphism in humans (10), our result could explain the weight-reducing effect of DHEA. On the other hand, our microarray experiment revealed that genes regulating cell proliferation were more profoundly influenced by DHEA administration than regulating energy homeostasis genes (e.g., glucose transporter 4, phosphofructokinase-1, SREBP-1, acetyl-CoA carboxylase, insulin receptor β-subunit, insulin receptor substrate-1, and protein kinase B) in adipose tissue. Our results differ from those reported by Bolduc et al. (5), presumably due to the differences in hormone administration and the underestimation in microarrays of genes coded by mtDNA. Hence, we examined the effect of DHEA on cell proliferation in adipose tissue. In this study, we demonstrated that DHEA delayed cell growth in SVF both in vivo and in vitro.

Long-term DHEA administration decreased body weight of both LETO and OLETF rats (Fig. 1). Because obesity consists of adipocyte hypertrophy and hyperplasia, detection of SA-Gal-positive cells in SVF isolated from obese animals suggests that the increased cell proliferation in obese adipose tissue resulted in accelerated senescence. The fact that DHEA-induced reduction of adiposity is accompanied by an improvement of senescence in SVF implies that DHEA acts on SVF. It is possible that DHEA-induced delay of proliferation in preadipocytes and SVF, as shown in Figs. 4 and 5, contributes to a decrease in fat mass and prevention of senescence. Epidemiological and clinical studies suggest that a high plasma DHEA-S level is associated with longevity (8, 12, 34, 41); hence, our result supports the notion that DHEA prevents senescence-associated phenomenon.

In previous studies, treatment with DHEA reduced proliferation of 3T3-L1 preadipocytes (53) and primary cultures of SVF (36). Recently, Rice et al. (37) reported that treatment with DHEA (from 10 nM) attenuated the growth of preadipo-
pocyte cell lines, including 3T3-L1 and PAZ6, as well as human primary preadipocytes. Our data are consistent with their results; however, Rice et al. could not find an inhibitory effect in testosterone. We confirmed that DHEA suppressed cell growth in 3T3-L1 and 3T3-F442A preadipocytes, but not 3T3-A31, which cannot differentiate into adipocytes. In our study, testosterone equally decreased proliferation of 3T3-L1 preadipocytes; hence, we postulate that both hormones act through a common pathway. DHEA-S is incorporated and converted into bioactive androgens and estrogens in adipose tissue (51). Therefore, we used AR and ER inhibitors and siRNA to examine whether DHEA action is mediated by AR or ER. The AR inhibitor, flutamide and AR knockdown abolised the inhibitory effect of DHEA (Fig. 5, C and E). This strongly suggests that DHEA-induced reduction of cell proliferation in adipocytes may be mediated by AR via an intracrine mechanism. The antiproliferative effect of DHEA has been reported in both cancer (45, 46, 57) and noncancer cells (50). Our data suggest that the antiproliferative effect of DHEA in preadipocytes is mediated via AR; accordingly, we speculate that DHEA may be incorporated and converted into strong androgens with \( \text{HSD} \) in preadipocytes. Since activity and mRNA of 3\( \beta \)-HSD-1 in adipocytes and preadipocytes have been detected (4), the fact that pretreatment with trilostane abolished the antiproliferative effect of DHEA (Fig. 5D) supports this hypothesis. The concentrations of both DHEA and testosterone that significantly suppress preadipocytes growth exceed the physiological value as shown in

**Fig. 3.** Effects of DHEA and testosterone administration on body weight, fat weight, serum concentration of DHEA-sulfate (DHEA-S), and testosterone, expression of PPAR\( \gamma \), and tissue triglyceride content. A: Wistar rats were housed with or without (Control, ■) 0.4% DHEA (○), or 0.4% testosterone (○)-containing food for 4 wk. DHEA and testosterone administration produced equal and significant decreases in body weight compared with Control rats. *P < 0.05 vs. each control; n = 10 in each group. B: DHEA (○) or testosterone (○) administration to Wistar rats housed in metabolic cages did not influence food consumption compared with Control rats (■); n = 9 in each group. C: DHEA and testosterone administration led to equal and significant decreases in epididymal (filled bar), intestinal (open bar), and perirenal (gray bar) fat weight. *P < 0.05 vs. each control; n = 10. D: DHEA and testosterone administration elevated rectal body temperature. *P < 0.05 vs. each control; n = 10. E: testosterone administration raised serum DHEA-S level, and DHEA administration significantly elevated serum testosterone level. **P < 0.01 vs. control; n = 6. F: treatment with DHEA significantly decreased triglyceride content in liver (filled bar) and gastrocnemius muscle (open bar). *P < 0.05 vs. Control; n = 8. G: treatment with both DHEA and testosterone reduced expression of PPAR\( \gamma \) protein in isolated adipocytes from epididymal fat. A typical result is shown at the left. *P < 0.05 vs. Control; n = 6. H: treatment with both DHEA and testosterone decreased adipocyte size in epididymal fat. H&E stain (×200). A typical result is shown at the left. Average adipocyte diameter was determined in rats from each group and is shown at the right. ***P < 0.005 vs. Control; n = 6.
Fig. 5. Since our data (Fig. 5B) indicate the time dependence of these hormones, it is possible that a lower concentration may be effective for a longer period. However, it is expected that interaction among preadipocytes and other coexisting cells, including mature adipocytes in SVF, may affect each cell proliferation, so we should apply in vitro data to in vivo phenomena carefully. Therefore, we measured in vivo cell proliferation to evaluate the effect of DHEA on SVF.

BrdU is a halogenated thymidine analog that is incorporated into the DNA of dividing cells during the S phase. BrdU-labeled cells were detected in SVF; however, quantitative analysis was difficult with immunohistochemical methods. Hence, we developed dot blot analysis to overcome this problem. (Fig. 4C).

Dot blot analysis revealed that both testosterone and DHEA administration yielded identical results. As SVF cells are heterogeneous, this result does not simply imply that both hormones suppress the proliferation of preadipocytes in vivo. However, the experiment reported by Pirke et al. and Rice et al. (36, 37), which represented that proliferation of primary cultured SVF cells and lined preadipocytes were equally suppressed with DHEA, may support the idea that DHEA administration in vivo leads to inhibition of preadipocyte proliferation. We cannot explain the difference from the result described by Rice et al. in regard to the response to testosterone in 3T3-L1 preadipocytes. However, our results that DHEA and testosterone equally suppress the proliferation of preadipocyte and SVF will explain the similarity in weight-reducing effect between these hormones.

The next problem is whether inhibition of preadipocyte proliferation explains all the weight-reducing effects of DHEA and testosterone. As shown in Fig. 3D, increased heat production is observed in both DHEA- and testosterone-treated rats. Although we could not measure O2 consumption, our data suggest that increased energy expenditure is associated with reduced adiposity. The effect of androgen on thermogenesis is a matter of controversy. AR-null mice reveal a lower O2 consumption ratio (13), whereas testosterone is reported to inhibit the transcription of PPARγ coactivator-1α (PGC1α) in brown adipose tissue (39). Recently, our preliminary experiment suggests that treatment with DHEA and testosterone may increase the expression of PGC1α in brown adipose tissue and skeletal muscle. Further study is necessary to clarify this problem. Several studies indicated that the antiobesity effect of DHEA was associated with decreased food intake. However, as shown in Fig. 1, C and D, no difference was observed in food consumption except for that in the first 2 wk in LETO and OLETF mice. In addition, as shown in Fig. 3A, DHEA and
testosterone identically decreased the increment of body weight in Wistar rats, whereas food consumption was equal among control, DHEA-treated, and testosterone-treated groups. Taking into account the fact that food intake is unchanged between AR-null and wild-type mice (43), the weight-reducing effect of these hormones cannot be attributed to suppression of food consumption. As shown in Fig. 3H, DHEA- and testosterone-induced fat weight loss is accompanied by reduced adipocyte cell size, which means that both hormones act on mature adipocytes. It is possible that impaired storage capacity of FFA in white adipose tissue due to reduced preadipocyte proliferation might lead to ectopic fat accumulation in other tissues such as liver and muscle, which was ruled out as shown in Fig. 3F. Our in vitro study shown in Fig. 6F denied another possibility that lipolysis in adipocytes might be stimulated by DHEA and testosterone. Considering the fact that expression of hormone-sensitive lipase is decreased in white adipose tissue isolated from AR-null mice (13), and that...
testosterone treatment enhances catecholamine- and forskolin-induced lipolysis in cultured adipocytes (55), our result indicates that DHEA or testosterone alone does not increase lipolysis. However, the fact that DHEA and testosterone administration do not increase serum FFA levels, it is not likely that increased lipolysis is a primary mechanism of androgen-induced reduction of adiposity. We reported previously that treatment with DHEA reduced the expression levels of PPARγ in DHEA-treated OLETF and primary cultured adipocytes (21). In this study, we demonstrated that DHEA, as well as testosterone, reduced PPARγ and its related gene mRNA levels in 3T3-L1 adipocytes and PPARγ protein level in Wistar rats. Since mild inhibition of PPARγ activity leads to decreases in triglyceride content in white adipose tissue, skeletal muscle, and liver and antagonizes high-fat diet-induced insulin resistance (56), our result explains the effects of these hormones on mature adipocytes in vivo.

A few differences exist in the effects of DHEA and testosterone in our study: food consumption in the first 2 wk and triglyceride content in muscle and liver. However, no discrepancy was observed in their effects on preadipocytes and mature adipocytes. DHEA represents only 0.1–2% the activity of that of testosterone on genital organs in mice (17), whereas DHEA has 42% of the potency of testosterone in bone formation in newborn mice (18). Although we have no evidence now, different expression levels of 3β-HSD, 17β-HSD, and other converting enzymes among tissues may explain these divergences.

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AUTHOR CONTRIBUTIONS


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