

Role of Androgen and Estrogen Receptors for the Action of Dehydroepiandrosterone (DHEA)

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Dehydroepiandrosterone (DHEA) is an abundant steroid hormone, and its mechanism of action is yet to be determined. The aim of this study was to elucidate the importance of androgen receptors (ARs) and estrogen receptors (ERs) for DHEA function. Orchidectomized C57BL/6 mice were treated with DHEA, DHT, 17 β -estradiol-3-benzoate (E2), or vehicle. Orchidectomized AR-deficient (ARKO) mice and wild-type (WT) littermates were treated with DHEA or vehicle for 2.5 weeks. At termination, bone mineral density (BMD) was evaluated, thymus and seminal vesicles were weighted, and submandibular glands (SMGs) were histologically examined. To evaluate the *in vivo* ER activation of the classical estrogen signaling pathway, estrogen response element reporter mice were treated with DHEA, DHT, E2, or vehicle, and a reporter gene was investigated in different sex steroid-sensitive organs after 24 hours. DHEA treatment increased trabecular BMD and thymic atrophy in both WT and ARKO mice. In WT mice, DHEA induced enlargement of glands in the SMGs, whereas this effect was absent in ARKO mice. Furthermore, DHEA was able to induce activation of classical estrogen signaling in bone, thymus, and seminal vesicles but not in the SMGs. In summary, the DHEA effects on trabecular BMD and thymus do not require signaling via AR and DHEA can activate the classical estrogen signaling in these organs. In contrast, DHEA induction of gland size in the SMGs is dependent on AR and does not involve classical estrogen signaling. Thus, both ERs and ARs are involved in mediating the effects of DHEA in an organ-dependent manner. (*Endocrinology* 155: 889–896, 2014)

Dehydroepiandrosterone (DHEA) and its sulfated derivative (DHEA-S) are the most abundant steroid hormones in humans and are produced mainly in the adrenal cortex as well as to a limited extent by the testes or ovaries and may be converted to androgens and estrogens in peripheral tissues. In contrast to humans, rodents have low serum levels of DHEA (1, 2). Several beneficial effects of DHEA therapy have been found in human studies of various conditions, including atherosclerosis, cancer, diabetes, obesity, depression, sexual dysfunction, and osteoporosis (3). DHEA replacement after estrogen deprivation caused by ovariectomy in animal models improves bone

mineral density (BMD), but the mechanism behind these bone-protective effects is largely unknown (2, 4). Clinical trials with DHEA have also been assessed in inflammatory rheumatic diseases (5, 6). Primary Sjögren's syndrome is a common rheumatic disease characterized by dry eyes and dry mouth due to lacrimal and salivary gland involvement (7, 8). It has been found in rodents that sex steroids, mainly androgens, are important in the regulation of salivary secretion and submandibular glands (SMGs), resulting in enhanced glands in males in contrast to females (9, 10). A reduced serum concentration of DHEA-S has been described in primary Sjögren's syndrome (11–13) compared

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Abbreviations: AR, androgen receptor; ARKO, AR knockout; BMD, bone mineral density; CT, computed tomography; DHEA, dehydroepiandrosterone; ER, estrogen receptor; ERE, estrogen response element; orx, orchidectomized; SMG, submandibular gland; WT, wild-type.

with healthy controls, and there is evidence of hypofunctioning of the hypothalamic-pituitary-adrenal axis (11). Furthermore, we have previously shown that low serum levels of sex steroids are associated with several disease characteristics in primary Sjögren's syndrome, including sicca symptoms, and supplementation with DHEA restores the hormonal levels and decreases sicca symptoms in the mouth (5). However, most human studies on DHEA have been performed with a limited number of patients and are short-time trials, and not much is known about the mechanism behind the positive effects of DHEA. Thus, experimental studies are needed to improve our knowledge regarding the mechanism behind the effect of DHEA in sex steroid-sensitive organs.

DHEA exerts many of its effects via the sex steroid receptors androgen receptor (AR) and/or estrogen receptors (ER) after peripheral enzymatic conversion to androgens or estrogens within target cells (14, 15). There are also reports suggesting that DHEA might be able to bind sex steroid receptors directly without previous conversion (16–18); however, a nuclear receptor specific for DHEA is yet to be identified. There are 2 ER subtypes, ER α and ER β , which are differentially expressed in different tissues, whereas ARs are ubiquitously expressed in all tissues. The AR and ERs function mainly as nuclear receptors and are able to affect gene transcription in target cells. Estrogen can affect gene transcription either via the classical estrogen signaling pathway, involving direct interaction of ERs with estrogen response elements (EREs) located in promoter regions of target genes, which initiates gene transcription (19), or the nonclassical pathway, involving binding of ERs to other transcription factors (20, 21). Androgens affect gene transcription via ARs in similar ways (22). Several studies have also shown that both estrogens and androgens can exert rapid activating or repressing effects in cells without interaction with the DNA (23, 24).

DHEA has several beneficial effects in humans, and it is therefore of interest to determine the mechanisms behind these diverse effects. Hence, the aim of this study was to determine the effect of DHEA treatment on the sex steroid-sensitive organs bone, thymus, seminal vesicles, and SMGs. We have used ERE-reporter mice (25) to elucidate the ability of DHEA to activate the ER-dependent classical estrogen signaling pathway, and AR knockout (ARKO) mice (26) to elucidate the importance of ARs for the DHEA effects.

Materials and Methods

Animals and experimental procedures

The ethical committee for animal experiments at the University of Gothenburg approved this study. Male C57BL/6 mice

(Scanbur, NOVA-SCB AB), ARKO mice with corresponding wild-type (WT) littermates and 3xERE-TAT-Luc (ERE-reporter) mice were kept, 5 to 10 animals per cage, under standard environmental conditions and were fed with standard laboratory chow and tap water ad libitum. All mice used in this study were orchidectomized (orx) at 2 to 3 months of age, after pubertal maturation. The skin incision after orx was closed with a metallic clip. Surgery was performed after anesthesia with isoflurane (Baxter Medical AB) or ketamine (Pfizer) and medetomidine (OrionPharma). Carprofen (OrionPharma) was used for post-operative pain relief. At termination, the mice were anesthetized for blood collection and then killed by cervical dislocation.

Breeding of male ARKO mice and WT littermates was performed as previously described (25). In brief, male mice expressing *Cre* recombinase driven by the ubiquitous promoter *PGK* were mated with female mice heterozygous for a floxed allele of the *AR* gene. We assessed *AR* genotype by PCR amplification of genomic DNA using primers 5'-AAT GCA TCA CAT TAA GTT GAT ACC-3' and 5'-AGC CTG TAT ACT CAG TTG GGG-3', with additional genetic determination of sex, using primers specific for the *Zfy* gene on the Y chromosome.

The ERE-reporter mice used in the study have a luciferase reporter gene under the control of 3 consensus EREs coupled to a minimal TATA box (26). ER binding to the EREs results in transcription of the enzyme luciferase, and the ERE-dependent transcription (corresponding to classical estrogen signaling) can be quantified using an enzymatic reaction.

Treatment

The C57BL/6 mice were given sc injections, 5 d/wk, of DHEA (1.0 mg/mouse/d; Sigma) (27), DHT (172 μ g/mouse/d; Sigma), E2 (1.0 μ g/mouse/d; Sigma) dissolved in 100 μ L Miglyol812 (OmyaPeralta GmbH) (vehicle), or vehicle. The ARKO and WT littermates were administered sc injections, 5 d/wk, with vehicle or DHEA (1.0 mg/mouse/d) dissolved in 100 μ L vehicle. Treatment of the C57BL/6, ARKO, and WT littermates started 10 days after orx and lasted until termination 2.5 weeks later.

The ERE-reporter mice were given a single sc injection of treatment 10 days after orx and were terminated 24 hours after the injection. In one experiment, the mice were injected with DHEA (1.0 mg/mouse) or E2 (1.0 μ g/mouse) dissolved in 100 μ L vehicle, or vehicle alone. In a second experiment, the mice were injected with DHT (172 μ g/mouse) or E2 (1.0 μ g/mouse) dissolved in 100 μ L vehicle, or vehicle alone.

The E2 dose chosen results in serum levels of estradiol within the physiological range in female mice, corresponding to levels seen in the estrous phase (28, 29). The DHT dose was chosen to ensure a strong androgenic response and is within the supra-physiological range, since it is 3 to 4 times the dose needed to preserve the size of the seminal vesicles after orx (30). The DHEA dose was chosen after a pilot study demonstrating that 1.0 mg/mouse of DHEA can activate ERE-dependent transcription after 24 hours.

Tissue weight and cellular parameters

Thymus and seminal vesicles from the C57BL/6 and ARKO mice were removed and weighed. The SMGs were removed, weighed, collected in 4% formaldehyde, fixated, dehydrated, and embedded in paraffin, and 4- μ m-thick tissue sections were prepared. Histological examination of SMGs was performed in

a light microscope ($\times 100$). Sections were stained with hematoxylin and eosin, and the androgenic phenotype was scored 0 to 3 according to the size of the granular convoluted tubules by a blinded examiner (C.E.) (31).

Assessment of BMD

The femur was analyzed by peripheral quantitative computed tomography (CT) with a Stratec pQCT XCT Research M, software version 5.4B (Norland) at a resolution of $70 \mu\text{m}$, as previously described (32). Trabecular BMD was determined with a metaphyseal scan at a distance from the growth plate corresponding to 3% of the length of the femur. The inner 45% of the area was defined as the trabecular bone compartment. Cortical parameters were determined with a mid-diaphyseal scan.

Tissue collection, protein preparation, and luciferase analysis

Organs (bone, thymus, seminal vesicles, and SMGs) from the ERE-reporter mice were collected and individually frozen in liquid nitrogen. The bone, a femur, consisting of cortical and trabecular bone as well as bone marrow was cut into small pieces using a scalpel before homogenization. The frozen tissues were individually homogenized, using a Mixer Mill (RETSCH GMBR MM301), in lysis buffer (25mM Tris [pH 7.8], 1.5mM EDTA, 10% glycerol, 1% Triton X-100, 2mM dithiothreitol, and com-

plete protease inhibitors; Roche Diagnostic) and separated by centrifugation at $10\ 650g$ for 30 minutes. The supernatant was stored at -20°C until further analysis. The luciferase activity was analyzed using a standard luciferase assay (Promega) according to the manufacturer's instructions and measured on a GloMax 20/20 Luminometer (Promega) and related to the protein content measured using BioRad DC protein assay (Promega), according to the manufacturer's instructions.

Statistical analysis

For statistical evaluation, the Mann-Whitney U test was used when comparing 2 groups and Kruskal-Wallis test followed by a χ^2 post hoc test was used when comparing more than 2 groups. All statistical calculations were performed in GraphPad 5.0d Macintosh version (Software MacKiev). $P < .05$ was considered statistically significant. Values are given as mean \pm SD.

Results

Effects of DHEA, DHT, and E2 on bone

Treatment of orx C57BL/6 mice with DHT ($172 \mu\text{g}/\text{mouse}/\text{d}$) and E2 ($1.0 \mu\text{g}/\text{mouse}/\text{d}$) resulted in increased trabecular BMD compared with vehicle treatment (Figure 1, A and B) ($+27\%$, $P < .01$, and $+38\%$, $P < .01$, respectively).

The same pattern was seen after DHEA ($1.0 \text{ mg}/\text{mouse}/\text{d}$) treatment with a significant increase in trabecular BMD ($+19\%$, $P < .05$). E2, but neither DHT nor DHEA, significantly affected the cortical bone content ($+15\%$, $P < .01$) and cortical thickness ($+12\%$, $P < .01$).

In the orx ERE-reporter mice, E2 and DHEA treatment significantly increased luciferase activity in bone compared with vehicle (Table 1) ($+42\ 445\%$, $P < .05$, and $+30\ 380\%$, $P < .05$, respectively). In contrast, DHT treatment did not cause any induction of luciferase activity compared with vehicle treatment. These data demonstrate that DHEA can activate the classical estrogen signaling pathway in bone.

To determine whether the DHEA effect on trabecular BMD was dependent on signaling via the AR, orx ARKO mice and WT littermates were given sc injections of vehicle or DHEA for 2.5 weeks. As expected, DHEA increased trabecular BMD in the WT littermates (Figure 1, C and

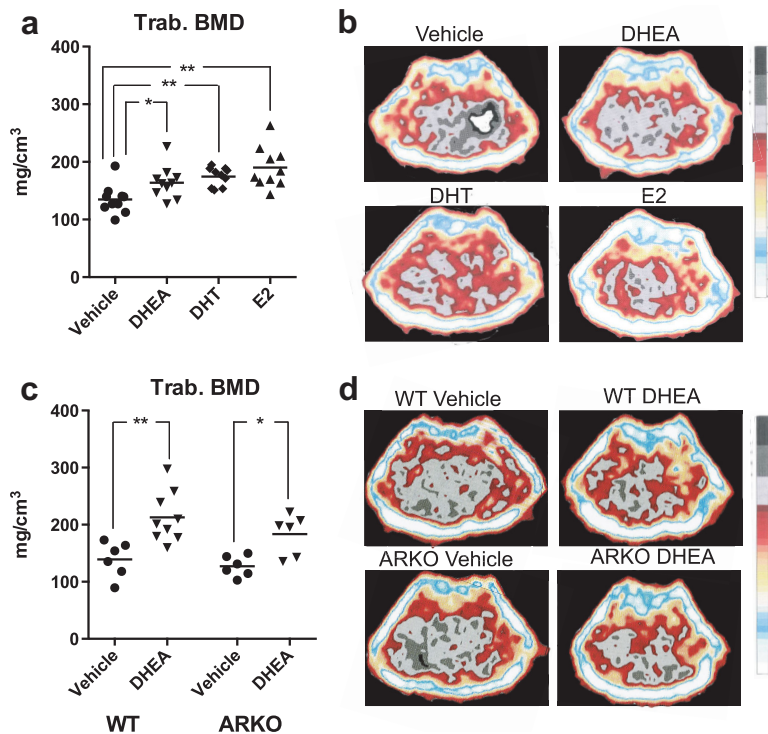


Figure 1. Treatment with DHEA, DHT, or E2 increases BMD. C57BL/6 mice were orx and treated with vehicle, DHEA, DHT, or E2. A, Scatter plots of individual data (bars indicate the mean per group) showing the trabecular BMD. *, $P < .05$; **, $P < .01$ vs vehicle, ($n = 10$). B, Representative peripheral quantitative CT images of cross-sections of the femur showing trabecular BMD. The scale indicates the density of the bone, from 0 (black) to $750 \text{ mg}/\text{cm}^3$ (white). ARKO and WT littermates were orx and treated with vehicle or DHEA. C, Scatter plots of individual data (bars indicate the mean per group) showing the trabecular BMD. *, $P < .05$; **, $P < .01$ vs vehicle ($n = 6-9$). D, Representative peripheral quantitative CT images of cross-sections of the femur showing trabecular BMD.

Table 1. Activation of the Classical Estrogen-Signaling Pathway in Bone, Thymus, Seminal Vesicles, and SMGs After Administration of DHEA, DHT, or E2^a

	Bone	Thymus	Seminal vesicle	SMG
Vehicle	38 ± 12	282 ± 187	134 ± 113	20 ± 6
DHEA	11621 ± 5816 ^b	807 ± 262 ^b	406 ± 210 ^b	12 ± 3
E2	16221 ± 16432 ^b	1280 ± 833 ^b	1477 ± 1216 ^b	52 ± 30
Vehicle	128 ± 74	118 ± 46	19 ± 7	13 ± 3
DHT	123 ± 23	164 ± 96	46 ± 40	14 ± 3
E2	1670 ± 1447 ^b	614 ± 461 ^b	217 ± 186 ^b	13 ± 2

^a Results are presented as mean ± SD.

^b $P < .05$ vs vehicle ($n = 5-6$).

D) (+35%, $P < .01$). An increase in trabecular BMD was also seen in the ARKO mice (Figure 1, C and D) (+44%, $P < .05$), indicating that the effect of DHEA on trabecular bone does not require signaling via ARs. DHEA did not influence the cortical BMD in ARKO or WT littermates.

Effects of DHEA, DHT, and E2 on the thymus

DHEA, DHT, and E2 induced involution of the thymus, as demonstrated by reduction of thymus weight compared with vehicle treatment (Figure 2A) (−56%, $P < .05$; −87%, $P < .001$; and −71%, $P < .001$ respectively).

Luciferase activity in the thymus of ERE-reporter mice was induced by E2 and DHEA compared with vehicle-treated mice (Table 1) (+355%, $P < .05$, and +187%, $P < .05$, respectively), whereas no induction was detectable after administration with DHT. These data demonstrate that DHEA can activate the classical estrogen signaling pathway in the thymus.

To determine whether the effect of DHEA on thymic atrophy was dependent on signaling via ARs, sex steroid-deficient ARKO and WT littermates were treated with DHEA or vehicle. DHEA induced thymus involution in both ARKO mice and WT littermates (−29%, $P < .05$, and −27%, $P < .01$, respectively), demonstrating that the DHEA effect on thymic atrophy does not require signaling via AR (Figure 2B).

Effects of DHEA, DHT, and E2 on seminal vesicles

Administration of DHEA and DHT induced growth of the seminal vesicles, as demonstrated by increased weight compared with vehicle treatment (Figure 3A) (216%, $P < .001$, and 960%, $P < .001$, respectively), whereas E2 had no effect on seminal vesicle weight.

The luciferase activity in ERE-reporter mice was induced by E2 and DHEA compared with vehicle treatment (Table 1) (+1004%, $P < .05$, and +204%, $P < .05$, respectively), whereas DHT treatment had no effect on luciferase activity in seminal vesicle.

To determine whether the effect of DHEA on seminal vesicles was AR-dependent was not possible because the

ARKO mice do not develop seminal vesicles due to lack of androgen signaling (Figure 3B).

Effects of DHEA, DHT, and E2 on SMGs

DHT treatment induced an enlargement of the granular convoluted tubules, as demonstrated by an increase in SMG score compared with vehicle treatment (Figure

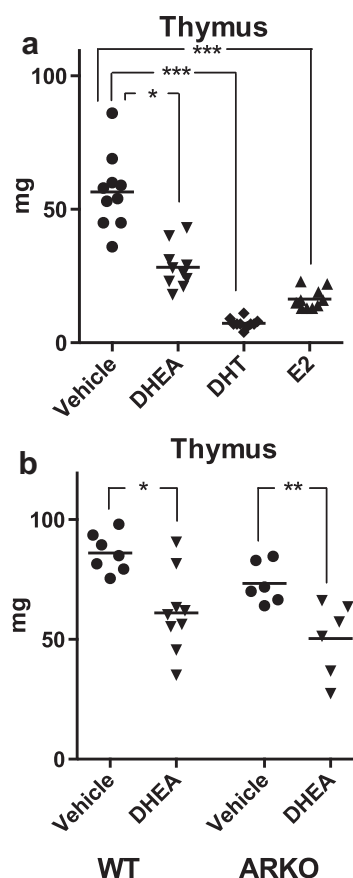


Figure 2. Treatment with DHEA, DHT, and E2 decreases thymus weight. C57BL/6 mice were orx and treated with vehicle, DHEA, DHT, or E2. A, Scatter plots of individual data (bars indicate the mean per group) showing the thymus weight. *, $P < .05$; ***, $P < .001$ vs vehicle ($n = 10$). ARKO and WT littermates were orx and treated with vehicle or DHEA. B, Scatter plots of individual data (bars indicate the mean per group) showing the thymus weight. *, $P < .05$; **, $P < .01$ vs vehicle ($n = 6-9$).

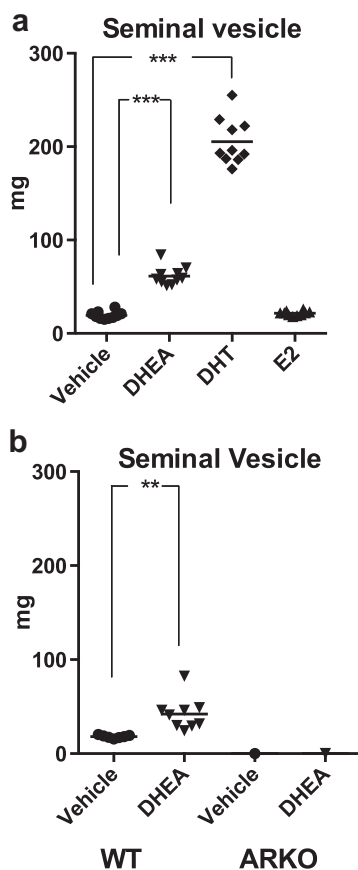


Figure 3. Treatment with DHEA or DHT increases seminal vesicle weight. C57BL/6 mice were orx and treated with vehicle, DHEA, DHT, or E2. A, Scatter plots of individual data (bars indicate the mean per group) showing the seminal vesicle weight. ***, $P < .001$ vs vehicle ($n = 10$). ARKO and WT littermates were orx and treated with vehicle or DHEA. B, Scatter plots of individual data (bars indicate the mean per group) showing the seminal vesicle weight. **, $P < .01$ vs vehicle ($n = 6-9$).

4, A and B) (+234%, $P < .001$). DHEA treatment also increased the SMG score (+122%, $P < .01$), whereas E2 achieved no change in the score (Figure 4, A and B).

There was no induction in luciferase activity in the SMGs in the ERE-reporter mice after any treatment, demonstrating that the classical estrogen pathway is not activated in the SMGs by E2, DHEA, or DHT.

To determine whether the effect of DHEA on SMGs was dependent on signaling via AR, sex steroid-deficient ARKO and WT littermates were treated with DHEA or vehicle. WT littermates displayed an increase in the SMG score after DHEA treatment (Figure 4, C and D) (+309%, $P < .01$), but this induction was not visible in the ARKO mice, demonstrating that the effect of DHEA on SMGs is AR-dependent.

Discussion

Steroids, including estrogens, androgens, DHEA, and DHEA-S, are important in the regulation of various body

functions. DHEA is an important precursor for androgens and estrogens and members of the cytochrome P450, 3 β -hydroxysteroid dehydrogenase, and 17 β -hydroxysteroid dehydrogenase enzyme families catalyze the various steps of sex steroid conversion (33, 34). Enzymes required for converting DHEA into androgens and/or estrogens are expressed in a cell-specific manner in peripheral tissues (14, 35), regulating the internal levels of androgens and estrogens in different cell populations and tissues. There is some evidence of direct effects of DHEA in cell-based assays, suggesting agonistic effects on ERs and antagonistic effects on the AR (17). A direct androgenic activity of DHEA on the AR, using transient cotransfection of the AR with an androgenic response element-luciferase reporter, has also been proposed (36). In addition to the classical sex steroid receptors, there are suggestions that DHEA may promote production of endothelial nitric oxide synthase directly via a nonnuclear membrane bound DHEA-specific receptor (37). However, many effects of DHEA are shown to be mediated via conversion into estrogen and androgen, and the role of ER and AR for the effect of DHEA on various organs is not completely elucidated. We have therefore used sex steroid-deficient orx ARKO mice to determine the importance of AR for the physiological effects of DHEA and orx ERE-luciferase mice to determine whether DHEA can activate the classical estrogen signaling in various sex steroid-sensitive organs. These 2 different mice strains are widely used and well described (25, 26).

Bone is regulated by both ERs and the AR (38). Accordingly, both E2 and DHT increased trabecular BMD in orx male mice in our study. Previously it has been shown that DHEA treatment increased BMD in female mice (2, 39), while positive effects of DHEA treatment on trabecular BMD in orx male mice has not previously been reported. It is known that DHEA can be converted into testosterone in osteoblasts (40) and also increase transcription of AR in osteoblasts (2). Interestingly, the effect of DHEA on trabecular BMD did not require activation of the AR, because similar effects of DHEA were seen in sex steroid-deficient, orx WT and ARKO mice. We also detected a significant elevation in luciferase activation in intact bone after DHEA treatment in ERE-reporter mice, which demonstrates that administration of DHEA can activate the classical estrogen signaling pathway in bone. It is well known that E2 activates the classical estrogen signaling pathway in bone and that this activation is involved in the E2 bone-protecting effect (41–43). Thus, our finding that DHEA can activate the classical estrogen signaling pathway in bone suggests that the effect of DHEA on trabecular BMD, at least partly, may be mediated via this pathway. In contrast to E2 treatment, treatment with

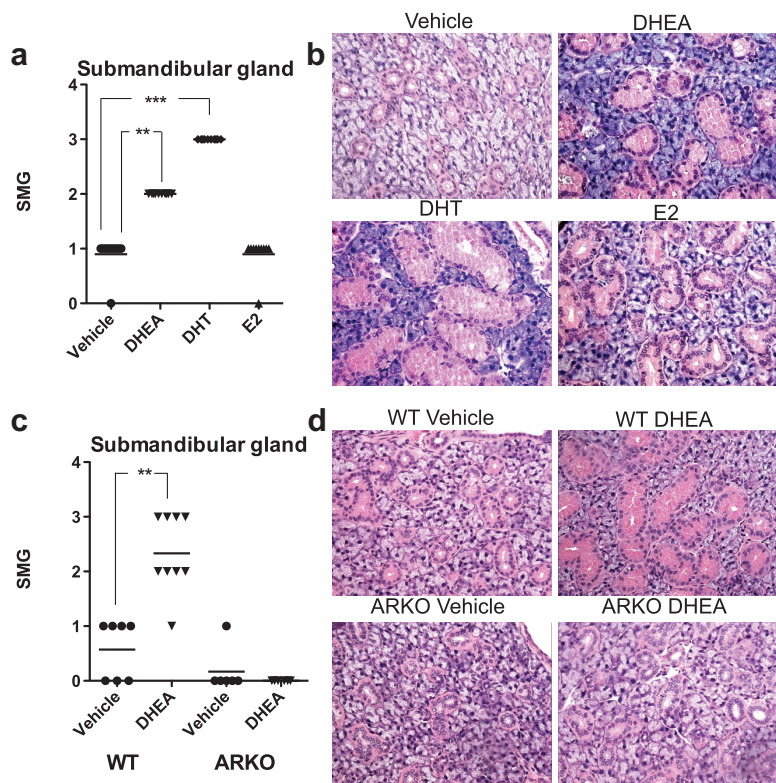


Figure 4. Treatment with DHEA or DHT increases SMG score. C57BL/6 mice were orx and treated with vehicle, DHEA, E2, or DHT. A, Scatter plots of individual data (bars indicate the mean per group) showing the SMG score. **, $P < .01$; ***, $P < .001$ vs vehicle ($n = 10$). B, Representative photos of SMGs from all groups. The degree of the androgen phenotype in the SMG sections were scored from 0 (no androgen phenotype) to 3 (maximal androgen phenotype). ARKO and WT littermates were orx and treated with vehicle or DHEA. C, Scatter plots of individual data (bars indicate the mean per group) showing the SMG score. **, $P < .01$ vs vehicle ($n = 6-9$). D, Representative photos of the SMG from ARKO mice and littermates.

DHEA and DHT did not affect cortical bone parameters in sex steroid-deficient orx male mice. AR activation is known to increase periosteal bone formation (44), leading to increased cortical thickness, and the discrepancy between our study and previous findings may be due to the short duration of treatment in the present study. Furthermore, the serum levels of DHEA or the converted sex steroids were not measured, and it is possible that the finding that DHEA did not significantly affect cortical bone may be related to the dose of DHEA.

Treatment with E2, DHT, or DHEA induced thymic atrophy in sex steroid-deficient orx male mice. E2 and DHEA treatment also resulted in increased luciferase activation in the thymus of ERE-reporter mice, indicating that the thymic atrophy mediated by DHEA may involve the classical estrogen signaling pathway. However, we have previously suggested that E2-induced thymic atrophy may involve nonclassical signaling or a nongenomic membrane-associated pathway (43). Thus, the exact mechanism behind the effect of sex steroids on thymic atrophy is not completely understood and needs further study. Moreover, DHEA treatment induced thymus atro-

phy in the sex steroid-deficient orx male ARKO mice, demonstrating that the effect of DHEA on thymic atrophy does not require signaling via the AR.

Both DHEA and DHT treatment increased the weight of the seminal vesicles, but because the seminal vesicles are undetectable in ARKO mice, we were not able to determine the importance of the AR for the effect. Perinatal exposure of estrogen has been shown to affect seminal vesicles via $ER\alpha$ (45); however, we found no effect of E2 on the weight of the seminal vesicles. Because the classical estrogen signaling pathway was induced after both E2 and DHEA treatment in the seminal vesicles, we suggest that the effect of DHEA on the seminal vesicle weight is most likely independent of ER signaling via the classical estrogen pathway.

We also studied effects of sex steroids on SMGs in different animal model of sex steroid-deficient orx male mice. SMGs are sexually dimorphic in rodents, resulting in enlargement of the granular convoluted tubules in males compared with females (10, 31). We found that DHEA influenced the SMG scores to a similar extent as DHT, whereas E2 had no effect on SMG histology. In the ERE-reporter mice, there was no induction of the classical estrogen signaling pathway in the SMGs, indicating that this pathway is insignificant for the effects of DHEA on these glands. In ARKO mice, there was no effect of DHEA, indicating that DHEA exerts its effect via activation of AR on the SMG score. The finding that DHEA is dependent on AR for the effect on the SMGs suggests diverse pathways for the effect of DHEA in different organs.

One potential confounding factor in this study is that treatment with DHEA, E2, and DHT may influence the hypothalamic-pituitary-gonadal axis differentially, which could lead to different secondary effects on the sex steroid-sensitive organs by GnRH and gonadotropins (LH and FSH). Furthermore, this study was conducted in orx male mice, and these results cannot necessarily be extrapolated to female mice.

We demonstrate that the effect of DHEA on trabecular BMD and thymic atrophy does not require signaling via

the AR and that DHEA can activate the classical estrogen signaling pathway in bone and thymus in sex steroid-deficient orx male mice. These findings indicate that ERs, and not the AR, are important for the action on bone and thymus by DHEA under the present experimental conditions. In contrast, the induction of an androgenic phenotype in the SMGs by DHEA is dependent on ARs. Thus, both ERs and AR are important for mediating the effects of DHEA in an organ-dependent manner. However, we cannot exclude the possibility that DHEA may act on an as yet unknown steroid receptor or via the suggested membrane-bound nonnuclear DHEA receptor (37).

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