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DHEA administration activates local bioactive androgen metabolism in cancellous site of tibia of ovariectomized rats

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Running title: DHEA and local androgen metabolism in bone tissue

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Abstract It is not known whether local androgen metabolism is involved in the mechanisms underlying the dehydroepiandrosterone (DHEA) administration-induced improvement of bone mineral density (BMD) in an estrogen deficiency state. The aim of the present study was to clarify whether DHEA administration would improve local androgen metabolism and BMD in cancellous site of tibia of ovariectomized (OVX) rats. Twenty-two female rats, 6 weeks old, were randomized into three groups: sham-operated rats, OVX control rats, and OVX rats that received DHEA treatment. DHEA was administered intraperitoneally at 20 mg DHEA/kg body weight for 8 weeks. The concentrations of free testosterone and dihydrotestosterone (DHT) in cancellous site of tibia did not change as a result of ovariectomy, while the DHT concentration increased following DHEA administration. We revealed that DHEA administration improved the reduction of 17β- and 3β-hydroxysteroid dehydrogenases and clearly reversed the reduction of 5α-reductase type 1 and 2 and androgen receptor in the cancellous site of tibia of OVX rats. DHEA administration suppressed estrogen deficiency relative to the decrease in the cancellous BMD, which was positively associated with local DHT concentration. These findings indicate that DHEA administration enhances local bioactive androgen metabolism in the cancellous tibia of young OVX rat, suggesting that local DHT may play a part in the DHEA administration-induced improvement of cancellous BMD.

Key words: estrogen deficiency, BMD, DHEA, androgen metabolism

Introduction

Before natural menopause, primary ovarian insufficiency defined as the premature cessation of ovarian function or menstrual dysfunction due to disordered eating or the female athlete triad syndrome is associated with severe risks for bone health [1, 2]. The ovariectomized (OVX) rat is a useful animal model for examining actions of sex steroids on bone. In young aged rats, ovariectomy induces the increases in both longitudinal and radial growth, as well as significant loss of trabecular bone [3, 4]. Although it is well known that the trabecular bone loss caused by ovariectomy is attributable to estrogen deficiency, it is not clear what actions other gonadal hormones have for bone metabolism in the estrogen deficiency state.

Dehydroepiandrosterone (DHEA), secreted mainly from the adrenal gland and ovary, plays a critical physiological role for maintaining steroidogenesis by being used as the available precursors converted to testosterone and estrogens in the various peripheral tissues such as the bones, liver, brain, skeletal muscles, and so on [5]. DHEA concentration in the blood decreases following ovariectomy in animals [6, 7]. On the other hand, DHEA replacement improves bone mineral density (BMD) in OVX animals [6, 7]. However, the mechanisms by which DHEA induces the BMD increase are largely unknown.

In osteoblast cells (OBs), DHEA can be converted to testosterone and further metabolized to dihydrotestosterone (DHT), which has the physiological strong function [8-10] and is known

to be the most powerful androgen because its higher affinity for androgen receptor (AR) than that of testosterone [11]. DHEA also increases the transcription of AR in OBs [7]. In particular, DHT prevents cancellous bone loss in OVX rats [12], and AR knockout female mice have a decreased amount of cancellous bone as well [13]. Thus, a decrease in circulating DHEA level caused by ovariectomy could deactivate the transcription of local androgen-related steroid enzymes in bone tissue, probably leading to decreased cancellous bone mass. However, it is not clear whether DHEA administration would activate the local androgen-related steroid enzymes or hormones in vivo during a period of rapid bone loss caused by estrogen deficiency.

We previously showed that estrogen deficiency reduced the cancellous BMD of tibia but not the cortical BMD in OVX rats [14]. Moreover, DHEA administration apparently improved the cancellous bone osteopenia in OVX rats [6]. Accordingly, we hypothesized that DHEA administration would activate the local androgen-related steroidogenesis with lessening the reduction of cancellous BMD of tibia caused by estrogen deficiency. In the present study, we examined androgen levels of free testosterone and DHT, steroidogenesis-related enzymes of 17β-hydroxysteroid dehydrogenase (17β-HSD), 3β-HSD, 5α-reductase type 1 and 2, and AR, and BMD in a cancellous site of the tibia in OVX rats after 8 wks of DHEA administration.

Materials and methods

Experimental animals and feeding protocol. Twenty-two female Sprague-Dawley rats, 6 weeks old, were randomized into three groups: sham-operated rats (Sham Control; SC, n=5), OVX control rats (OC, n=8), and OVX rats with DHEA treatment (OD, n=9). The OVX groups were ovariectomized via the dorsal route, and the sham-operated rats were operated on without the ovaries being removed. During the period of the experiment, all rats were fed a diet with 1.05% calcium and 1.01% phosphate purchased from CLEA Japan (CE-2, CLEA Japan, Inc., Japan). DHEA dissolved in sesame oil was administered to the OD group intraperitoneally at 20 mg DHEA/kg body weight for 8 weeks beginning one week after ovariectomy while the SC and OC groups were treated with vehicle only (sesame oil, 0.5 ml) [15]. Rats were not treated with DHEA or vehicle every fourth day (i.e., they were treated for 3 consecutive days). The rats were kept in individual cages (15×25×19.5cm) and allowed access to food and distilled water *ad libitum*. Food consumption and body weight gain were measured every second day. The room temperature was kept at 24±1℃, the humidity at 50±5%. Fluorescent lights were on from 8:00 a.m. to 8:00 p.m.. Animal care and experimental procedures were approved by the Animal Experimental Committee of the University of Tsukuba.

Measurement of bone mineral density. The left tibia of each rat was isolated by dissection and freed from any muscle and connective tissue. Thereafter, bone mineral content (BMC) and bone

mineral density (BMD) values for the whole tibia were measured by Dual-energy X-ray absorptiometry (DXA; Aloka DCS-600R instrument). We studied the proximal one-fifth of the tibia, including the epimetaphyseal region representing the cancellous sites, and middle one-fifth of the tibia representing the cortical diaphyseal region [16].

Immunoblot analysis. Western blot analysis of the cancellous site (proximal one-fifth of the tibia) of the right tibia of each rat was performed according to the procedure described in our previous paper with minor modifications [17]. After completely removing marrow and blood, the tibia fragment was homogenized by grinding the tissue in a mortar on dry ice with 10 volumes of 20 mM Tris-HCL (pH 7.8), 2 mM EDTA, 300 mM NaCl, 2 nM DTT, 2% NP 40, 0.2% SDS, 0.2% sodium deoxycholate, 0.5 mM PMSF, 60 μg/ml aprotinin, and 1 μg/ml leupeptin. The homogenate was gently rotated for 30 min at 4 ℃ and then centrifuged at 12,000 g for 15 min at 4 ℃. The protein concentration of the resulting supernatant was determined by protein assay rapid kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Samples (50 μg protein) were subjected to heat denaturation at 96 ℃ for 7 min with Laemmli buffer. Western blot analysis for the detection of 17β- and 3β-HSD, 5α-reductase type 1 and 2, and AR was performed according to the procedure described in our previous report [17, 18]. Briefly, each sample was separated on an SDS-polyacrylamide gel (10 %) and then transferred to a

polyvinylidene difluoride (PVDF, Millipore, Billerica, MA, USA) membrane. The membrane was then incubated in blocking buffer of 3% skimmed milk in phosphate-buffered saline containing 0.1% Tween-20 (PBS-T) for 1 h at room temperature followed by incubation with primary antibodies, a polyclonal anti-17β-HSD and anti-3β-HSD (1:1000 dilution, Santa Cruz Biotechnology), a polyclonal anti-5α-reductase type 1 and 2 (1:1000 dilution, Abnova Corporation, Taipei, Taiwan), a polyclonal anti-AR (1:1000 dilution, Santa Cruz Biotechnology), and a monoclonal anti-β-actin (1:1000 dilution, Santa Cruz Biotechnology), for 12 h at 4℃. Subsequently, the membrane was washed with PBS-T three times, and incubated for 1 h at room temperature with a horseradish peroxidase (HRP)-conjugated secondary antibody, either an anti-rabbit IgG (1:2000 dilution, Amersham Biosciences, Piscataway, NJ, USA) or an anti-mouse IgG (1:2000 dilution, Amersham Biosciences).. After this reaction, the membrane was washed with PBS-T five times. Finally, binding was detected by chemiluminescence with the ECL Plus system (Amersham Life Sciences), and the membrane was exposed to Hyper film (Amersham Biosciences).

Sandwich-enzyme immunoassay (EIA). Concentrations of E_2 and DHEA in serum and free testosterone and DHT in bone tissue were determined using a sandwich EIA kit (free testosterone: IBL International GMBH, Flughafenstrasse, Hamburg, Germany; E_2: Cayman

Chemical, Ann Arbor, MI, U.S.A.; DHEA: Assay Designs, Inc., Ann Arbor, MI, U.S.A.; DHT: IBL International GMBH, Flughafenstrasse, Hamburg, Germany). The protein samples of bone tissue for the EIA analysis were the same as those for the western blot analysis. All techniques and materials used in this analysis were in accordance with the manufacturers' protocols. The sensitivities of intra- and inter-assay coefficients of variation for the hormone assays were as follows: at dose of free testosterone (62 pg/ml for intra-assay precision; 76 pg/ml for inter-assay precision), 4.7% and 9.3%, respectively; at dose of E_2 (102 pg/ml for intra- and inter-assay precision), 13.0% and 8.2%, respectively; at dose of DHEA (1039 pg/ml for intra-assay precision; 1093 pg/ml for inter-assay precision), 5.8% and 6.5%, respectively; at dose of DHT (237 pg/ml for intra-assay precision; 281 pg/ml for inter-assay precision), 11.4% and 12.1%, respectively.

Statistics. All the data are expressed as mean ± SE. Statistical analysis was carried out by analysis of variance (ANOVA) followed by Fisher`s F-test for multiple comparisons. A significance level of $p < 0.05$ was used for all comparisons. Association between BMD and serum E_2 or local DHT levels was determined by the Pearson correlation test. All statistical treatments were done using the Stat View 5.01 software (SAS Institute, Inc., Cary, NC, USA, 2000-2001).

<The position of Table 1>

Results

Initial body weight did not differ among the three groups. The final body weight, body weight gain, and food intake of the OVX groups were significantly higher than those of the SC group (Table 1). The final body weight and body weight gain of the OD group were significantly lower than those of the OC group, but there were no differences in food intake. The serum concentration of DHEA and E_2 in the OC group was found to be significantly lower compared to that of the SC group and significantly higher in the OD group than in the OC group (Table 1). There were no differences in serum free testosterone concentration between the SC and OC groups, but the concentration was significantly higher in the OD group than in the OC group.

<The position of Figure 1>

The BMD of the tibial proximal metaphysis (cancellous site) in the OC group was found to be significantly lower compared to that in the SC group, but this decrease was significantly attenuated in the OD group. On the other hand, there were no differences among the 3 groups in the BMD of the tibial diaphysis (cortical-abundant region) (Fig. 1).

<The position of Figure 2>

In the tibial proximal metaphysis, free testosterone concentration did not differ among the 3 groups (Fig. 2). On the other hand, local DHT concentration was significantly higher in the

OD group than in the OC group, whereas there was no difference between the SC and OC groups (Fig. 2).

The local concentration of DHT was also significantly correlated with the BMD of the tibial proximal metaphysis in the OVX groups ($r = 0.706$, $p < 0.05$). The local DHT concentration was significantly associated with the serum concentration of DHEA ($r = 0.936$, $p < 0.001$), free testosterone ($r = 0.933$, $p < 0.001$), or E_2 ($r = 0.817$, $p < 0.01$) in the OVX groups. Also, there was a significant correlation between the BMD of tibial proximal metaphysis and the serum concentration of DHEA ($r = 0.741$, $p < 0.01$), free testosterone ($r = 0.754$, $p < 0.01$), or E_2 ($r = 0.685$, $p < 0.05$) in the OVX groups. In the correlation analysis, we used only 11 samples (n=6 for the OC group and n=5 for the OD group) because of hemolysis in blood samples.

The protein expressions of 17β- and 3β-HSD, 5α-reductase type 1 and 2, and AR were significantly lower in the OC group than in the SC group and significantly higher in the OD group than in the OC group (Fig. 3).

<The position of Figure 3>

Discussion

The present study results confirmed that DHEA administration improved the decrease in the cancellous BMD of tibia caused by estrogen deficiency. The present study demonstrated that DHEA administration increased the local DHT concentration in the cancellous site of tibia of

OVX rats. We also revealed that DHEA administration improved the reduction of 17β- and 3β-hydroxysteroid dehydrogenases and clearly reversed the reduction of 5α-reductase type 1 and 2 and androgen receptor in the cancellous site of tibia of OVX rats. Moreover, the cancellous BMD of tibia was positively correlated with the local DHT concentration in OVX rats. These results suggest that local bioactive androgen metabolism plays a role, at least in part, in the mechanism underlying DHEA administration-induced improvement of cancellous bone loss caused by estrogen deficiency.

The present study demonstrated that estrogen deficiency clearly reduced the activation of the androgen-related steroidogenic enzymes. An increase in DHEA enhances the transcriptions of the androgen-related steroidogenic enzymes in OBs [7-9]. We previously also demonstrated that androgen-related hormones could be locally synthesized by improving the transcriptions of the androgen-related steroidogenic enzymes from DHEA in muscle tissues as well [17]. Thus, we assumed in the present study that the decrease in circulating DHEA level by OVX might have partly deactivated the transcriptions of the androgen-related steroidogenic enzymes in the bone tissue. However, it is unclear whether estrogen deficiency reduced the activation of the androgen-related steroidogenic enzymes through DHEA, directly or indirectly or both.

In vitro studies using OBs demonstrated that DHEA can be metabolized to testosterone by 17β- and 3β-HSD and further metabolized to DHT by the 5α-reducatase type 1 and 2 enzymes,

binding to the AR [7-9]. These results mostly corresponded to the results of the present study in a cancellous site of the tibia in vivo that DHEA administration increased the DHT concentration and the steroidogenesis-related enzymes in 17β- and 3β-HSD, the 5α-reducatase type 1 and 2, and AR. However, in the present study, DHEA administration did not induce any increase in the local free testosterone concentration. Free testosterone is metabolized to DHT only through the 5α-reductase enzymes [11, 19]. Actually, in the present study, the expression of 5α-reductase type 1 and 2 was greatly increased to the level of sham-operated rats. Moreover, testosterone can be metabolized not only to DHT but E_2 by p450arom enzyme activity [7]. Thus, the transient increase in testosterone might have occurred, but the testosterone was soon metabolized to synthesize DHT and/or E_2.

The present study results showed that DHEA administration clearly reversed the reduction of AR induced by estrogen deficiency as well. Wang et al. [7] reported that DHEA increases the transcription of AR in OBs. AR knockout mice have a decreased amount of cancellous bone [13]. DHT also has the capacity to prevent ovariectomy-induced cancellous bone loss [12]. On the other hand, the inhibitor of 5α-reductase does not appear to have a bone-sparing effect [20]. This implies that the increase in local DHT concentration with the activation of AR by DHEA administration might play a critical role in maintaining bone-sparing in an estrogen deficiency state.

The present study showed the significant correlation between the BMD and local DHT concentration in the site of cancellous bone loss caused by estrogen deficiency. Especially, the marked increase in serum free testosterone concentration by DHEA administration was found and the serum free testosterone concentration was highly correlated with the local DHT concentration. Thus, we cautiously considered that the increase in the serum free testosterone concentration by DHEA administration may, at least in part, induce the increase in local DHT level, resulting in the increase in the cancellous BMD. On the other hand, the present study also demonstrated that DHEA administration resulted in a substantial rise in serum E_2 concentration which was also positively correlated with the cancellous BMD of tibia. The increase in serum E_2 concentration prevents severe bone loss caused by estrogen deficiency [7]. Thus, it could not be excluded that the increase in serum E_2 concentration by DHEA administration would have a sufficient bone sparing effect irrespective of the local DHT level. Therefore, to evaluate if parts of the DHEA effect was dependent on local DHT level, a further study is strongly needed to demonstrate if the DHEA effect would be reduced by co-treatment with androgen receptor antagonist or if the DHEA effect would be sustained by co-treatment with estrogen receptors or aromatase antagonist.

Although the present study results confirmed that DHEA prevented bone loss caused by estrogen deficiency and improved local bioactive androgen metabolism, DHEA concentration in

serum was approximately 13 times higher in OVX rats treated with DHEA than in sham-operated control rats. Future study should focus on the dose-dependent effect of DHEA administration on the relation between BMD and local bioactive androgen metabolism. Furthermore, the present study results confirmed that the estrogen deficiency reduced the cancellous BMD but not the cortical BMD as reported previously [14]. Moreover, DHEA administration prevented the cancellous BMD loss but did not affect the cortical BMD. However, the BMD measurement by DXA in the present study did not allow the details of bone parameters such as cortical width and periosteal or endosteal circumferences. Thus, the parameters should be examined in detail using by the methods of pQCT, μCT, or bone morphometry in further studies.

In conclusion, the present study results demonstrated for the first time that in a cancellous site of the tibia, DHEA administration increased the DHT concentration and improved the reduction of the androgen steroidogenesis-related enzymes caused by estrogen deficiency. Furthermore, DHEA administration suppressed the decrease in the cancellous BMD of tibia induced by estrogen deficiency, which was significantly associated with the local DHT concentration. These results suggested that improvement of local bioactive androgen metabolism by DHEA administration could contribute to preventing the loss of cancellous BMD in estrogen deficiency state.

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Figure legends

Figure 1. Bone mineral density of tibial proximal metaphysis (A) and diaphysis (B). The proximal one-fifth of the tibia, including the epimetaphyseal region representing the cancellous sites, and middle one-fifth of the tibia, representing the cortical diaphyseal region. SC, Sham-operated control group; OC, Ovariectomy-operated control group; OD, Ovariectomy group with DHEA treatment. *** $p < 0.001$ vs. SC; $^{\#\#}$ $p < 0.01$ vs. OC. Values are expressed as the means ± SE.

Figure 2. Concentrations of free testosterone (A) and DHT (B) in the proximal metaphysis of the tibia (trabecular bone). The tissue concentrations were normalized for total protein. SC, Sham-operated control group; OC, Ovariectomy-operated control group; OD, Ovariectomy group with DHEA treatment. $^{\#\#\#}$ $p < 0.001$ vs. OC. Values are expressed as the means ± SE.

Figure 3. Concentrations of 17ß-HSD protein (A), 3ß-HSD protein (B), 5α-reductase type 1 protein (C), 5α-reductase type 2 protein (D), and androgen receptor protein (E) in the proximal metaphysis of the tibia (trabecular bone). The tissue concentrations were

normalized for total protein. SC, Sham-operated control group; OC, Ovariectomy-operated control group; OD, Ovariectomy group with DHEA treatment. *** $p < 0.001$ vs. SC; $^{###}$ $p < 0.001$ vs. OC. Values are expressed as the means ± SE.

Figure 1.

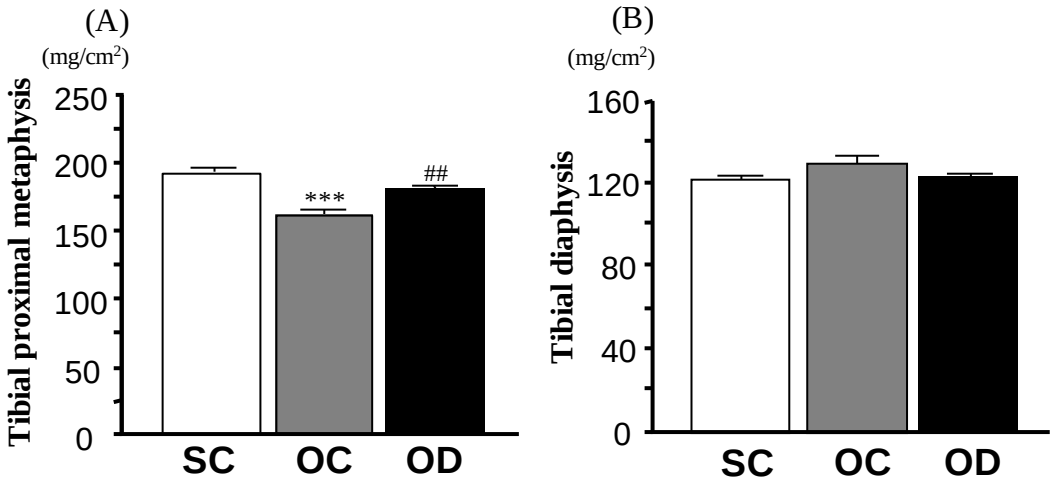
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Figure 2.

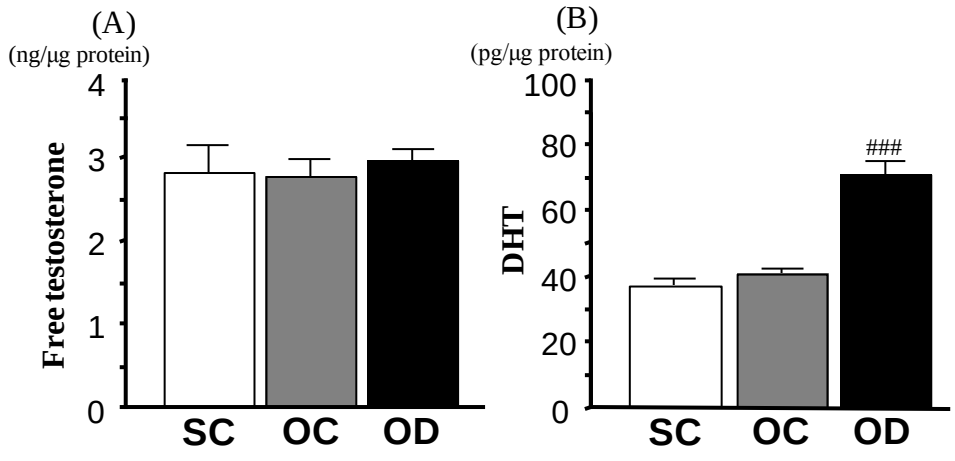
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Figure 3.

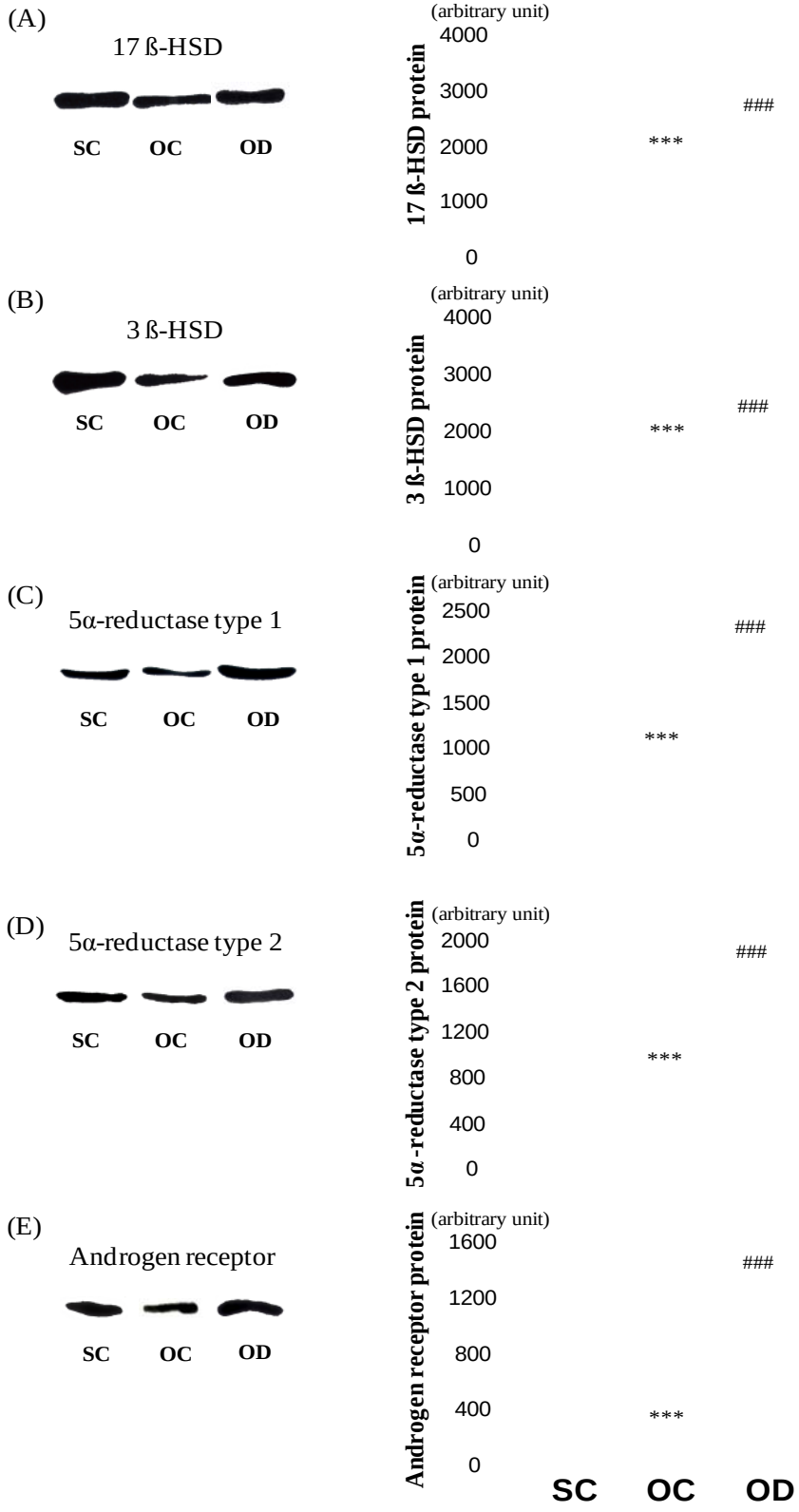
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Table 1. Body weight, food intake, and steroid hormones in serum

	Final body weight (g)	Body weight gain (g/day)	Food intake (g/day)	DHEA (ng/ml)	E_2 (pg/ml)	Free testosterone (pg/ml)
SC	274.8 ± 5.8	2.0 ± 0.1	15.4 ± 0.4	2.7 ± 0.2	35.8 ± 5.9	2.9 ± 0.2
OC	340.5 ± 6.3***	3.2 ± 0.1***	17.7 ± 0.2***	1.1 ± 0.2**	13.4 ± 1.3*	1.2 ± 0.1
OD	309.1 ± 6.8##	2.6 ± 0.1#	17.4 ± 0.5	35.0 ± 0.6###	53.1 ± 10.0###	71.7 ± 3.1###

Data show mean ± SE. SC, Sham-operated control group; OC, Ovariectomy-operated control group; OD, Ovariectomy-operated group with DHEA treatment. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. SC; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. OC.