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Research Article

Dehydroepiandrosterone Supplementation Results in Varying Tissue-specific Levels of Dihydrotestosterone in Male Mice

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Abstract

Dehydroepiandrosterone (DHEA), an adrenal androgen precursor, can be metabolized in target tissues into active sex steroids. It has been proposed that DHEA supplementation might result in restoration of physiological local sex steroid levels, but knowledge on the effect of DHEA treatment on local sex steroid levels in multiple tissues is lacking. To determine the effects of DHEA on tissue-specific levels of sex steroids, we treated orchiectomized (ORX) male mice with DHEA for 3 weeks and compared them with vehicle-treated ORX mice and gonadal intact mice. Intra-tissue levels of sex steroids were analyzed in reproductive organs (seminal vesicles, prostate, m. levator ani), major body compartments (white adipose tissue, skeletal muscle, and brain), adrenals, liver, and serum using a sensitive and validated gas chromatography–mass spectrometry method. DHEA treatment restored levels of both testosterone (T) and dihydrotestosterone (DHT) to approximately physiological levels in male reproductive organs. In contrast, this treatment did not increase DHT levels in skeletal muscle or brain. In the liver, DHEA treatment substantially increased levels of T (at least 4-fold) and DHT (+536%, $P < 0.01$) compared with vehicle-treated ORX mice. In conclusion, we provide a comprehensive map of the effect of DHEA treatment on intra-tissue sex steroid levels in ORX mice with a restoration of physiological levels of androgens in male reproductive organs while DHT levels were not restored in the skeletal muscle or brain. This, and the unexpected supraphysiological androgen levels in the liver, may be a cause for concern considering the uncontrolled use of DHEA.

Key Words: dehydroepiandrosterone, androgens, intracrinology, dihydrotestosterone, mice, reproductive organs

Abbreviations: DHEA, dehydroepiandrosterone; DHT, dihydrotestosterone; E1, estrone; E2, 17β-estradiol; LLOQ, lower limit of quantification; ORX, orchiectomized; SHBG, sex hormone–binding globulin; Srd5A1/2, steroid 5-alpha-reductase 1/2; T, testosterone.

Dehydroepiandrosterone (DHEA) is an abundant sex steroid precursor that is produced in the zona reticularis of the adrenal cortex (1). In peripheral tissues, DHEA can be metabolized by 3-ß-hydroxysteroid-dehydrogenase (HSD3B) into androstenedione, which mainly acts as a precursor for the androgen testosterone (T) and the weak estrogen estrone (E1). Testosterone can be further dehydroxylated by 5-alpha reductases (SRD5A1 and SRD5A2), yielding dihydrotestosterone (DHT) which is the most potent androgen with higher binding affinity to the androgen receptor than T (2). Alternatively, T can be aromatized yielding 17β-estradiol (E2), the most potent estrogen. Androstenedione can also be produced via a metabolic route utilizing progesterone (Supplementary Fig. S1 (3)). The enzymes needed for these conversions are expressed in several different tissues (4). Of the two 5-alpha-reductases, SRD5A1 is most abundantly expressed in the liver while SRD5A2 is most abundantly expressed in male reproductive organs (5–7).

Circulating DHEA levels decrease during adulthood and aging in men with levels in the eighth decade of life only one-

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tenth of the maximum levels observed at the age of 20 to 30 years. Hence, DHEA has been suggested as a potential "youth hormone" (4) and DHEA supplementation has by some been considered an efficient approach to restore physiological levels of androgens and estrogens in target tissues (8). The suggested target organs for the beneficial effects of DHEA supplementation in men are muscle and the central nervous system, as it is marketed for improvement of physical performance and strength, energy, and libido. DHEA is sold over the counter (in the USA where DHEA is classified as a nutritional supplement) and is widely available via online outlets (9, 10). At the same time, it is a restricted substance in sports due to its androgenic properties (11, 12). Circulating T levels indeed increase in response to DHEA administration in men (1, 13). Yet, large-scale supplementation studies in men have failed to show any major beneficial effects of DHEA treatment on metabolic parameters, sexual function, or quality of life (1, 14). Nevertheless, in certain male patient populations, DHEA treatment has shown effects on specific sex hormone-dependent phenotypes (15–17). Considering the lack of adequate clinical data, increased understanding of the effect of DHEA treatment on local sex steroid levels in different organs could be a way to predict potential benefits and risks of DHEA supplementation in men.

Mouse sex steroid biology resembles that of humans, with 2 important exceptions. First, the mouse adrenal cortex does not produce large amounts of DHEA (18) but produces other androgen precursors such as androstenedione (19). Second, mice lack sex hormone–binding globulin (SHBG) in their circulation (20), resulting in very low circulating sex steroid levels. Interestingly, transgenic male mice expressing human SHBG display increased levels of sex steroids including measurable DHEA and E2 (21). Accurate determination of the low sex steroid levels in mice has been a challenge until the recent introduction of sensitive mass spectrometry–based methods (21–24). Supplemented DHEA has been shown to exert both androgenic and estrogenic effects in male mice through downstream metabolites (25–27). However, to the best of our knowledge, no previous studies have evaluated the effect of DHEA treatment on the local sex steroid levels measured in multiple tissues.

To understand the effect of DHEA treatment on local sex steroid levels in multiple tissues, we have in this study for the first time used a sensitive and highly specific gas chromatography–mass spectrometry method to measure the local levels of sex steroids in male reproductive organs (seminal vesicles, prostate, m. levator ani), major body compartments (white adipose tissue, skeletal muscle, and brain cortex), adrenals, liver, and serum after administration of DHEA to orchiectomized (ORX) mice.

Methods

Animals and Surgeries

Male C57BL/6J mice 7 to 9 weeks of age were purchased (Taconic, Ry, Denmark) and allowed to acclimatize for 1 week before the experiment. Mice were kept in individually ventilated cages with controlled temperature (23 °C), a 12-hour light/dark cycle, and ad libitum access to chow pellets and tap water. At 8 to 10 weeks of age they were subjected to ORX or sham surgery (Sham). Surgery was performed under anesthesia with isoflurane (Baxter Medical AB, Kista, Sweden). Postoperative analgesia was Rimadyl (Orion Pharma AB, Animal Health, Sollentuna, Sweden). Starting 1 week after surgery, DHEA 1 mg/mouse per day dissolved in 100 µL vehicle (Miglyol 812, Omya Peralta GmbH, Germany) or vehicle only was given subcutaneously 5 days per week. Half of the ORX mice were treated with DHEA (ORX + DHEA group) and the other half with vehicle only (ORX group). Sham mice were treated with vehicle. The dose was chosen based on previous experience with DHEA administration (25). After 3 weeks of hormone treatment (last injections were given the day before sacrifice) the mice were anesthetized with Ketanest and Dormitor (Pfizer/Orion Pharma), bled, and euthanized by cervical dislocation. Blood was allowed to coagulate at room temperature for 30 minutes and then centrifuged to collect the serum. Liver, white adipose tissue (WAT, represented by gonadal fat), brain cortex, adrenals, m. quadriceps, m. levator ani, testicles, seminal vesicles, and prostate were collected and snap frozen. Wet weights were recorded for the reproductive organs. For technical reasons, prostate samples were only available from 3 out of 10 animals in the ORX and the ORX + DHEA groups. The results for the control mice of this study (Sham and ORX groups) have been published previously (28) and are here presented only as references for the treatment group. The experiment was conducted in accordance with all relevant legislation and was approved by the ethics committee of the University of Gothenburg.

Sample Preparation

Frozen tissues were thawed on ice. The tissue was weighed and placed in a 2-mL screw-top Eppendorf tube with 450 µL phosphate buffered saline. Tissue samples were homogenized by shaking with a 5-mm steel bead in a Tissuelyzer II for 5 minutes. Serum samples were measured volumetrically by pipetting and adjusted to a volume of 450 µL with deionized water.

Sample Extraction and Gas Chromatography–Tandem Mass Spectrometry Analysis

Sex steroids were extracted, derivatized, and measured as described previously (6, 28, 29). Briefly, after addition of isotope-labeled standards, sex steroids were first extracted by liquid-liquid extraction with 1-chlorobutane, followed by solid phase extraction (SPE) using Silica SPE columns (SupraClean, Perkin Elmer, Waltham, MA, USA) that were washed 2 times with ethylacetate-pentane-heptane [10:45:45 (vol:vol)]. For liver and adipose tissue samples 3 washing steps instead of the regular 2 were used to reach an optimal performance in the gas chromatography–tandem mass spectrometry (GC-MS/MS) assay. Then, derivatization was performed in 2 steps: oximation with pentafluorobenzylhydroxylamine hydrochloride and esterification with pentafluorobenzoyl chloride. Finally, DHEA, E2, E1, progesterone, DHT, T, and androstenedione were separated by gas chromatography (Agilent 7890, Agilent, Santa Clara, CA, USA) and detected simultaneously with negative chemical ionization by an Agilent 7000 triple quadrupole mass spectrometer (Agilent) operating in multiple reaction monitoring mode with ammonia as reagent gas. All peaks were integrated using the MassHunter quantitative analysis workstation software from Agilent. Detailed assay validation and lower limits of quantification (LLOQs) in the different types of matrices have been published previously. LLOQs were as follows: E2

and E1, 0.5 pg/mL in serum, 2.0 to 4.0 pg/g in tissues; T, 8 pg/mL in serum, 20 to 40 pg/g in tissues; DHT, 1.4 pg/mL in serum, 4.0 to 8.0 pg/g in tissues; androstenedione, 12 pg/mL in serum, 7.5 pg/g in tissues; and progesterone 56 pg/mL in serum, 75 pg/g in tissues (28, 29). For DHEA, the analysis method has only been validated in serum (LLOQ 400 pg/mL) but based on the assay performance the LLOQ in tissues was set to 2000 pg/g. Details on the validation of this method, including representative chromatograms, are presented in supplemental materials (3).

Calculations/Statistics

Sex steroid levels below the LLOQ were set to the LLOQ, and levels are only reported for groups in which at least half of the measurements were > LLOQ; otherwise, they are denoted not detected (ND). Concentrations are presented as median and interquartile range (IQR) unless stated otherwise. Differences between the 3 groups were analyzed for each hormone and tissue with Kruskal-Wallis test followed by Dunn's post hoc test. For comparison of 2 groups, Mann-Whitney U-test was used. For normally distributed data, such as tissue and body weights, mean and SEM are used and ANOVA with post hoc testing was used for comparison of 3 groups. GraphPad Prism 9 (GraphPad, San Diego, CA, USA) was used for statistical analyses. *P* values below 0.05 were considered statistically significant.

Results

DHEA was not measurable in any tissues of the gonadal intact or the vehicle-treated ORX mice. However, DHEA treatment resulted in measurable DHEA levels in all evaluated tissues and in serum (Fig. 1). In general, DHEA levels were higher in tissues than in serum, and the DHEA tissue/serum ratios were variable, with comparatively high ratios in male reproductive organs, white adipose tissue, and skeletal muscle (Fig. 1).

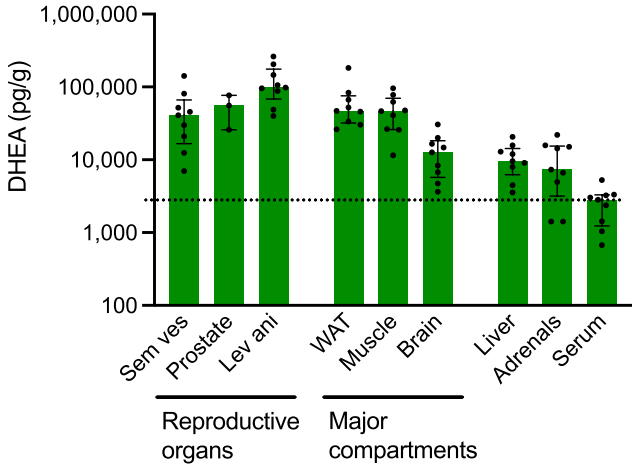

Figure 1. DHEA levels in tissues and serum as assessed by gas chromatography–tandem mass spectrometry. Young adult orchiectomized male C57BL/6 mice were treated with 1 mg DHEA/day, 5 days a week for 3 weeks, starting 1 week after orchiectomy. Bars and error bars represent median and interquartile range (IQR), the horizontal dotted line represents the median serum level. n = 9-10, for prostate n = 3. Abbreviations: DHEA, dehydroepiandrosterone; lev ani, m. levator ani; sem ves, seminal vesicles; WAT, white adipose tissue.

We next evaluated the levels of the downstream sex steroid metabolites of DHEA. We found that serum DHT levels were substantially reduced by ORX but that these levels were almost restored to physiological levels by DHEA treatment (Fig. 2A). The capacity of DHEA treatment to restore DHT levels to the levels observed in gonadal intact mice differed substantially between tissues (Fig. 2A and 2B). DHEA treatment restored levels of DHT in male reproductive organs to approximately physiological levels observed in gonadal intact mice. In contrast, the levels of DHT in brain and skeletal muscle were not significantly increased by DHEA treatment in ORX mice (Fig. 2A and 2B). These tissue-specific effects of DHEA treatment on tissue DHT levels were also reflected by high DHT tissue/serum ratios in male reproductive organs while this ratio was low in muscle and brain (Fig. 2C). In addition, the DHT/T ratio, an indicator of 5-alpha reductase activity, was lower in muscle and brain in DHEA-treated ORX mice compared with gonadal intact controls (Fig. 2D).

Similar to the observed effects on DHT, serum levels of T were substantially reduced by ORX and largely restored to physiological levels by DHEA treatment (Fig. 3). In male reproductive tissues, DHEA treatment of ORX mice restored T levels approximately to the physiological levels observed in gonadal intact mice. In skeletal muscle and brain, the results for T were less clear: DHEA increased T significantly compared with ORX in muscle but not in brain; the T levels for both these tissues in DHEA-treated mice were approximately in the middle of the T levels observed in Sham and ORX mice (Fig. 3). DHEA treatment resulted in a modest increase of the weights of the androgen-responsive organs seminal vesicles and m. levator ani compared with ORX mice (Supplementary Fig. S2A (3)). These findings support a modest DHEA-induced androgenic effect in these reproductive tissues after short term treatment, although the weights of these tissues were clearly not restored to the sham levels (Supplementary Fig. S2A and S2B (3)). A similar trend was observed for the weights of the prostate but as only 3 prostate samples were available for the ORX and ORX + DHEA groups, statistical analyses were underpowered for this comparison (Supplementary Fig. S2A and S2B (3)).

The levels of the androgen precursor androstenedione, one of the primary DHEA metabolites, were increased by DHEA treatment in most of the evaluated tissues in ORX mice (Fig. 4). As expected, progesterone levels were not increased by DHEA treatment, since progesterone is not a downstream metabolite of DHEA (Supplementary Figs. S3 and S1 (3)). E2 and E1 were not detected in any tissue from any of the groups.

In the liver, the main sex steroid metabolizing/excreting organ, DHEA treatment substantially increased levels of T (from undetectable to 4× LLOQ, $P < 0.01$), DHT (+536%, $P < 0.01$), and androstenedione (from undetectable to 16× LLOQ, $P < 0.01$) compared with vehicle-treated ORX mice, reaching clearly supraphysiological levels (Figs. 2A, 3, and 4). This was associated with a substantially increased DHT liver/serum ratio (Fig. 2C) and DHT/T ratio in the liver (Fig. 2D) of DHEA-treated ORX mice compared with gonadal intact mice.

Discussion

Notwithstanding a lack of documented clinical efficacy, DHEA is used as a supplement in men, with the intention to stimulate muscle mass, mood, libido, and energy, corresponding

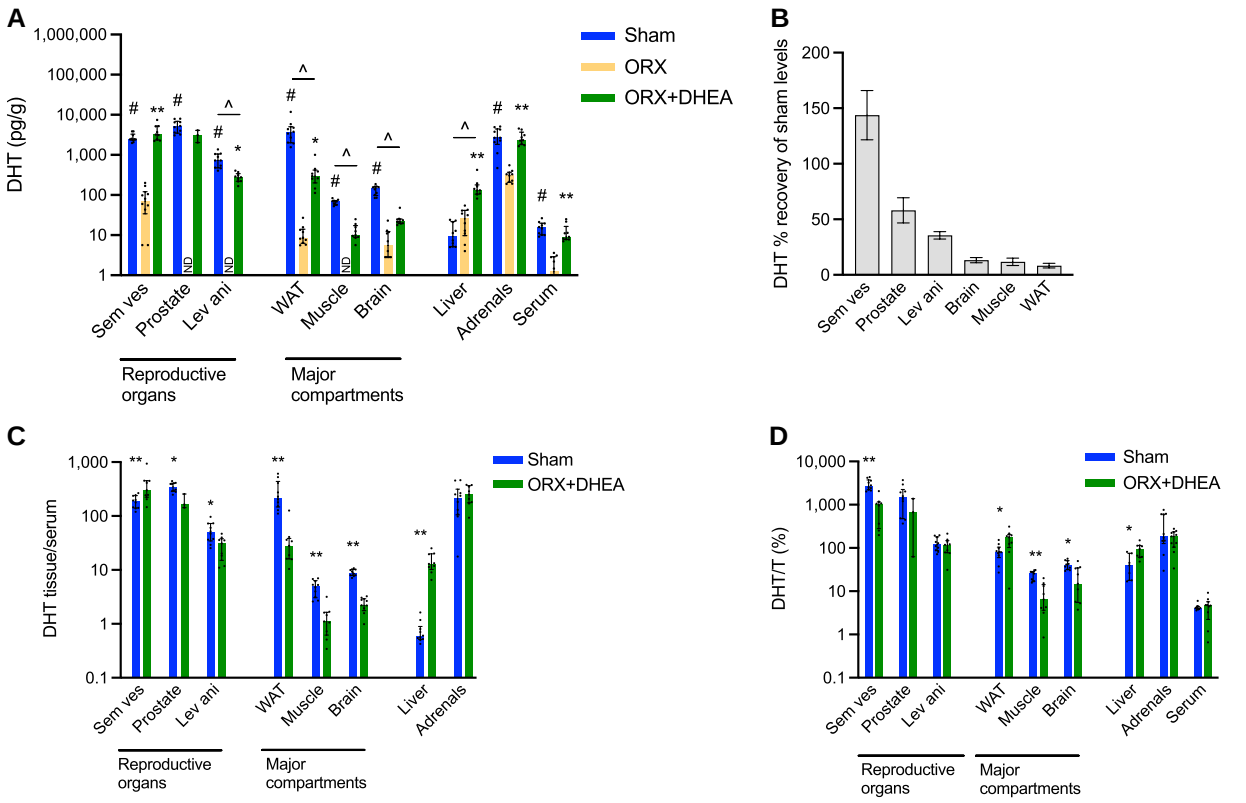

Figure 2. DHT in tissues and serum as assessed by gas chromatography–tandem mass spectrometry. Young adult orchiectomized (ORX) male C57BL/6 mice were treated with 1 mg DHEA/day, 5 days a week for 3 weeks (ORX + DHEA) and compared with sham-operated (Sham) and ORX male mice with vehicle treatment. A, DHT levels in tissues and serum. Bars and error bars represent median and interquartile range (IQR), significant differences according to Kruskal Wallis with Dunn's test are shown as: * = $P < 0.05$, ** = $P < 0.01$ ORX + DHEA vs ORX; # = $P < 0.05$ ORX vs Sham; ^ = $P < 0.05$ ORX + DHEA vs Sham. B, Recovery of precastration DHT levels in different tissues after treatment with DHEA in ORX male mice. The increase in local DHT levels with DHEA treatment is expressed as a percentage of the average decrease in local DHT levels with ORX. Calculated as (measured ORX + DHEA DHT level – mean ORX DHT level)/(mean Sham DHT level – mean ORX DHT level). Bars and error bars show mean and SEM. C, Ratio of DHT levels in tissue/serum. Significant differences according to Mann-Whitney test are shown as * = $P < 0.05$, ** $P < 0.01$. D, Ratio between DHT and T concentrations, an indicator of 5-alpha-reductase activity, in tissues and serum. Significant differences according to Mann-Whitney test are shown as * = $P < 0.05$, ** $P < 0.01$. n = 9-10, for prostate n = 3. Data for Sham and ORX control groups were previously published (28). Abbreviations: DHEA, dehydroepiandrosterone; DHT, dihydrotestosterone; lev ani, m. levator ani; sem ves, seminal vesicles; WAT, white adipose tissue; ND, not detected.

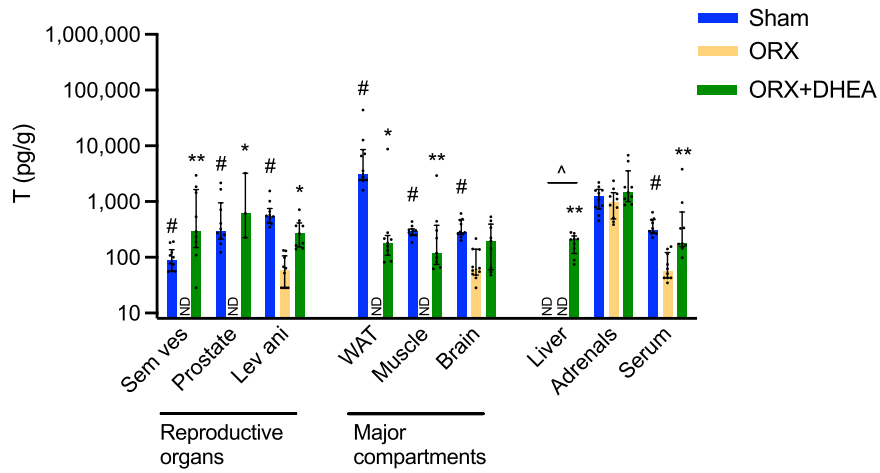

Figure 3. Testosterone levels in tissues and serum as assessed by gas chromatography–tandem mass spectrometry. Young adult orchiectomized (ORX) male C57BL/6 mice were treated with 1 mg DHEA/day, 5 days a week for 3 weeks (ORX + DHEA) and compared with sham-operated (Sham) and ORX male mice with vehicle treatment. Bars and error bars represent median and interquartile range (IQR), n = 9-10, for prostate n = 3. Significant differences according to Kruskal Wallis with Dunn's test are shown as: * = $P < 0.05$, ** = $P < 0.01$ ORX + DHEA vs ORX; # = $P < 0.05$ ORX vs Sham; ^ = $P < 0.05$ ORX + DHEA vs Sham. Data for Sham and ORX control groups were previously published (28). Abbreviations: DHEA, dehydroepiandrosterone; sem ves, seminal vesicles; lev ani, m. levator ani; T, testosterone; WAT, white adipose tissue; ND, not detected.

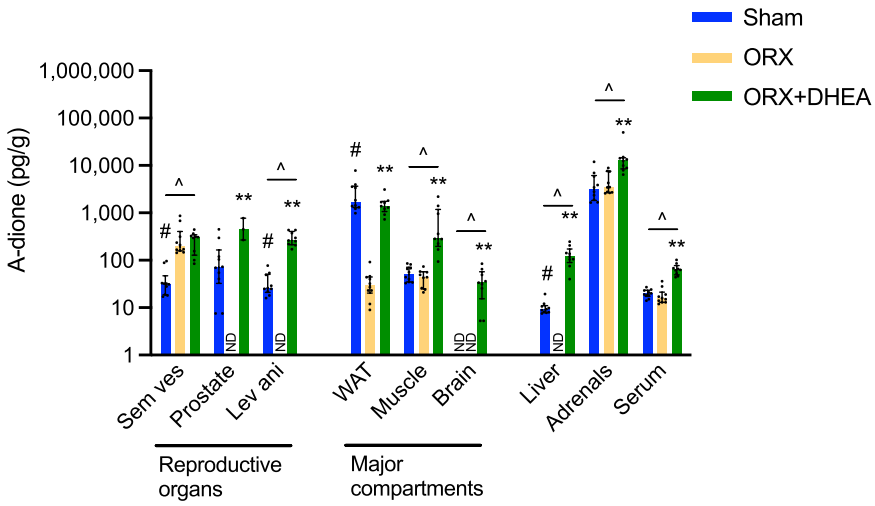

Figure 4. Androstenedione levels in tissues and serum as assessed by gas chromatography–tandem mass spectrometry. Young adult orchiectomized (ORX) male C57BL/6 mice were treated with 1 mg DHEA/day, 5 days a week for 3 weeks (ORX + DHEA) and compared with sham-operated (Sham) and ORX male mice with vehicle treatment. Bars and error bars represent median and interquartile range (IQR), n = 9-10, for prostate n = 3. Significant differences according to Kruskal Wallis with Dunn's test are shown as: * = $P < 0.05$, ** = $P < 0.01$ ORX + DHEA vs ORX; # = $P < 0.05$ ORX vs Sham; ^ = $P < 0.05$ ORX + DHEA vs Sham. Data for Sham and ORX control groups were previously published (28). Abbreviations: A-dione, androstenedione; DHEA, dehydroepiandrosterone; lev ani, m. levator ani; sem ves, seminal vesicles; WAT, white adipose tissue; ND, not detected.

to androgenic effects in skeletal muscle and brain. The effects of DHEA treatment on local sex steroid levels in potential target tissues are unknown. Using a sensitive gas chromatography–tandem mass spectrometry method for measurement of a broad panel of sex steroids, we herein observed that DHEA treatment of ORX mice increased androgen levels in reproductive organs to physiological levels, while in the liver, supraphysiological levels were reached, and no effect was observed on local DHT levels in brain or muscle.

As expected, administered DHEA was distributed to all evaluated tissues, with the highest concentrations found in male reproductive organs, white adipose tissue, and skeletal muscle. Tissue-specific sex steroid levels in ORX mice treated with DHEA and those in gonadal intact mice displayed similarities but also important differences. In male reproductive organs, DHEA treatment restored tissue levels of T and DHT in ORX mice approximately to the levels observed in gonadal intact mice. Both gonadal intact mice and DHEA-treated ORX mice had very high tissue/serum ratios of DHT and high tissue DHT/T ratios in male reproductive tissues, suggesting efficient 5-alpha-reductase enzyme activity. This was expected as male reproductive tissues display high expression of Srd5a2, encoding 5alpha-reductase (6, 7). In contrast, DHEA treatment did not restore physiological levels of DHT in skeletal muscle or brain and it only partially restored T levels in these 2 tissues. In skeletal muscle, the DHT/T ratio was substantially lower in DHEA-treated ORX mice than in gonadal intact mice. It is well known that skeletal muscle has relatively low Srd5a1 and Srd5a2 expression (5) and we interpret our finding as that the moderate DHT levels in the skeletal muscle of gonadal intact mice are testicular-derived since DHEA treatment of ORX mice cannot restore DHT levels in this tissue lacking 5-alpha reductase activity. A similar pattern for DHT was also observed in the brain. Based on our present finding, we propose that DHT in muscle and brain is mainly testicular-derived in gonadal intact males and DHT levels in these tissues cannot be restored by DHEA treatment in ORX mice.

When DHEA is used as a nutritional supplement by men, the intention is to achieve beneficial effects on energy, mood, libido, cognition (central nervous system effects of androgens), and muscle (local effects of androgens in muscle) without increased cancer risk in male reproductive organs. Epidemiological data on the role of androgens for prostate cancer risk is somewhat conflicting (30) but a recent Mendelian randomization study showed that higher lifelong exposure to circulating T levels is causally associated with increased risk of prostate cancer in men (31). Presumably, local androgen levels are more important for the development and progression of prostate cancer than circulating levels, possibly contributing to the variability in epidemiological studies examining this association (32). Based on the findings of our present study, there may be a risk that DHEA supplementation raises intra-prostatic DHT levels more than serum T levels. This could lead to an underestimation of the increase in local androgen exposure in the prostate if serum T levels are measured as a proxy for androgen exposure in studies of DHEA treatment in men.

Previous studies have in general failed to identify "pure" anabolic steroids without androgenic effects (33). Our present findings of preferentially elevated local DHT levels in androgen-responsive tissues after DHEA treatment, as well as an effect, although modest, of DHEA treatment on the weights of m. levator ani and seminal vesicles, indicate that DHEA cannot be regarded as a strong candidate for a more "physiological" or "tissue-selective" anabolic (non-androgenic) steroid treatment.

In the liver, DHEA treatment substantially increased levels of T and DHT compared with vehicle-treated ORX mice, reaching clearly supraphysiological levels. The potential health consequences of this unexpected finding are unknown. Androgens and male gender have been linked to a higher incidence of liver disease (34). The exact mechanisms for these associations are not fully known but the androgen receptor may be involved in the pathogenesis of hepatocellular carcinoma (35) and T contributes to liver fibrosis in

preclinical studies (36). In the present study we demonstrate that DHEA administered in a dose that produced physiological circulating androgen levels also caused supraphysiological local androgen levels in the liver and it may be speculated that this could result in increased risk of liver cancer and/or liver injury. In humans, DHEA effects on liver enzymes (AST, ALT) have, according to a recent systematic review, only been studied in 3 small studies with limited numbers of participants and heterogenous results (37). Thus, possible liver side-effects due to increased local androgen levels during long-term DHEA administration need to be further investigated.

A major strength of the present study is the use of a sensitive mass spectrometry–based sex steroid assay that can measure 7 sex steroids simultaneously and that has been validated across several tissue types (28). To the best of our knowledge, our study is the first to assess tissue distribution of DHEA and multiple downstream metabolites after administration of DHEA to ORX males. Another strength is the use of 2 control groups so that we can compare levels after DHEA exposure, not only with ORX mice but also with the physiological levels in gonadal intact mice. This enabled us to evaluate if DHEA treatment restored physiological tissue levels of sex steroids in ORX mice. Our study has certain limitations. Due to technical issues, we only had prostate samples from 3 individuals in the ORX and ORX + DHEA groups, making it difficult to do statistical comparisons between these groups. Also, despite using a highly sensitive method, we could not detect E2 or E1 in any group, demonstrating that improved assay sensitivity is required for estrogen analyses in male mice, since it is clear that estrogens exert crucial effects in males (25, 38–40). In agreement with this study, serum E2 was not measurable in wild-type male mice but was measurable in SHBG transgenic male mice which most likely have increased sex steroid levels including E2 (21). A final limitation of this study is the lack of examination of local expression of sex steroid metabolizing enzymes, which should be investigated in future studies.

In conclusion, we provide a comprehensive map of the effect of DHEA treatment on intra-tissue sex steroid levels in ORX mice. DHEA treatment restored physiological levels of androgens in male reproductive organs while DHT levels were not restored in skeletal muscle or brain. There was also an unexpected large increase of androgen levels in the liver by DHEA treatment. This preferential DHEA-induced increase of potent androgens in male reproductive tissues and liver but not in muscle or brain could raise concerns regarding the unsupervised use of DHEA by men.

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Author Contributions

Designed research: M.N., M.P., H.R., M.L., L.V., C.O.; Performed research: M.N., H.C., A.K.N., A.L., S.H.W., M.L., J.W., K.H., L.V.; Analyzed data: H.C., A.K.N., A.L., H.R., M.P., L.V., C.O.; Wrote the manuscript: H.C., L.V., C.O.; Revised the manuscript: H.C., M.N., A.K.N., A.L., S.H.W., J.W., M.L., K.H., M.P., H.R., M.L., L.V., C.O.; All authors approved the final version of the manuscript. H.C. and C.O. take responsibility for the integrity of the data analysis.

Disclosures

The authors state that they have no conflicting interests.

Data Availability

Some or all datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

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