Effects of dihydrotestosterone administration on the expression of reproductive and body weight regulatory factors in ovariectomized and estradiol-treated female rats

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Running title
Effects of DHT injection

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Abstract

To clarify the direct effects of androgens, the changes in the hypothalamic levels of reproductive and appetite regulatory factors induced by chronic dihydrotestosterone (DHT) administration were evaluated in female rats. DHT treatment increased the BW and food intake of the ovariectomized rats, but not the estradiol (E2)-treated rats. DHT administration suppressed the expression of a hypothalamic anorexigenic factor. Although the kisspeptin (Kiss1) mRNA levels of the anterior hypothalamic block (the anteroventral periventricular nucleus, AVPV) were increased in the E2-treated rats, DHT administration did not affect the Kiss1 mRNA levels of the AVPV in the ovariectomized or E2-treated rats. Conversely, DHT administration reduced the Kiss1 mRNA levels of the posterior hypothalamic block (the arcuate nucleus, ARC) in the ovariectomized rats. Although the Kiss1 mRNA levels of the posterior hypothalamic block (ARC) were decreased in the E2-treated rats, DHT administration did not affect the Kiss1 mRNA levels of the ARC in these rats. Serum luteinizing hormone levels of these groups exhibited similar patterns to the Kiss1 mRNA levels of the ARC. These results showed that DHT affects the production of hypothalamic reproductive and appetite regulatory factors, and that these effects of DHT differ according to the estrogen milieu.
Introduction

Sex hormones influence reproductive function and body weight (BW) regulation in mammals and humans [1]. It has been well established that estrogen play pivotal roles in the regulation of gonadotropin-releasing hormone (GnRH)/luteinizing hormone (LH) secretion via positive and negative feedback effects on hypothalamic kisspeptin and its receptor Kiss1r, which is a positive regulator of GnRH [2]. Estrogen also affects the activity of hypothalamic RFamide-related peptides/gonadotropin inhibitory hormone (RFRP/GnIH) and its receptor, G protein-coupled receptor (GPR)147, which is a negative regulator of GnRH and LH [3,4]. It has been also well established that estrogen play pivotal roles in the regulation of appetite and BW through the modulation of hypothalamic orexigenic and anorexigenic factors [5-9].

In contrast to estrogen, the effects of androgens on reproductive function and BW regulation in females have not been fully established, although it has been reported that hyperandrogenemia is usually accompanied by visceral obesity and ovulatory disorders in humans [10]. Almost all of the basic studies that have investigated the effects of androgens on female physiological functions involved animal models of polycystic ovary syndrome (PCOS), which is a representative hyperandrogenism-related disorder [11]. Although the effects of androgens on reproductive and BW regulatory functions and the mechanisms underlying these effects have been partially clarified by the aforementioned studies [11], some other effects, particularly the effects of androgens on hypothalamic functions, have not been fully evaluated. Recently, we and other groups have investigated hypothalamic kisspeptin levels, as well as serum gonadotropin levels, in PCOS model
rodents; however, there were marked discrepancies between the results of these studies [12-14]. The protocols used to produce the PCOS model differed among the studies, and ovarian intact rodents were used; i.e., the rats were not only administered androgen, and androgen-induced changes in ovarian functions might affect hypothalamic functions, which could explain the discrepancies among the studies’ results. In addition, only a few studies have evaluated the effects of androgens on hypothalamic appetite regulatory factors in females [15], and there are no data about the effects of androgens on hypothalamic RFRP/GnIH in females.

To clarify the direct effects of androgens on reproductive and BW regulatory functions, we evaluated the effects of chronic dihydrotestosterone (DHT), which is a non-aromatizing androgen, on hypothalamic factors under ovariectomized (OVX) and stable estradiol (E2) milieu conditions.

**Materials and Methods**

**Animals**

Wistar female rats were purchased from Charles River Laboratories Japan, Inc., (Kanagawa, Japan) and housed in a room under controlled light (12 h light, 12 h darkness; lights turned on at 0800 and turned off at 2000) and temperature (24°C) conditions. In total, 29 rats were used in this study. All animal experiments were conducted in accordance with the ethical standards of the institutional animal care and use committee of the University of Tokushima. All surgical procedures were carried out under sodium pentobarbital- (60-80 mg/kg, intraperitoneal, i.p.) or sevoflurane-induced anesthesia.
Effects of chronic DHT administration in ovariectomized (OVX) rats and OVX and estradiol-administered rats

At 15 weeks of age, the rats were randomly divided into the no treatment (control), DHT-administered (DHT), E2-administered (E2), and E2 and DHT-administered (E2+DHT) groups. All rats were OVX bilaterally and implanted with a silastic tube filled with crystalline steroid hormones under sodium pentobarbital-induced anesthesia. The parts of the tubes filled with DHT and E2 measured 30 mm and 3.0 mm in length, respectively [16,17]. In the control group, the rats were implanted with empty tubes. All of the tubes were removed and replaced with new ones 4 weeks later under sevoflurane-induced anesthesia. The rats were individually housed after surgery, and their BW and food intake (FI) were checked every 7 days. At 8 weeks after the OVX and implantation procedures, the rats were sacrificed by decapitation under sevoflurane-induced anesthesia. The weights of visceral fat (parametrial, perirenal, and mesenteric deposits) and subcutaneous fat (inguinal deposits) were measured, and the brain and blood were collected. Serum was separated by centrifugation and stored at -20°C, and tissues were stored at -80°C.

Hormone assays

Serum E2 levels were measured by a commercial laboratory (SRL, Tokyo, Japan) using an electrochemiluminescence immunoassay (ECLIA; Roche Diagnostics GmbH, Mannheim, Germany). Serum LH levels were measured using a radioimmunoassay (RIA) (rat LH [I-125] RIA kit, Institute of Isotopes Co., Ltd., Tokyo, Japan).

Quantitative real-time polymerase chain reaction
Whole hypothalamic explants were dissected from the frozen brains, as described previously [15]. Briefly, the target region of the brain was dissected out via an anterior coronal cut at the posterior border of the mammillary bodies, parasagittal cuts along the hypothalamic fissures, and a dorsal cut 2.5 mm from the ventral surface. Subsequently, the obtained brain tissue was divided into two blocks using coronal cuts at the posterior border of the optic chiasm. The anterior hypothalamic block contained the anteroventral periventricular nucleus (AVPV), and the posterior hypothalamic block contained the arcuate nucleus (ARC) [18]. Kisspeptin in the AVPV and ARC receives positive and negative feedback signals from estrogen, respectively [2]. Total RNA was isolated from the hypothalamic explants and visceral fat using a TRIzol® reagent kit (Invitrogen Co., Carlsbad, CA, USA) and an RNeasy® mini kit (Qiagen Gmbh, Hilden, Germany). cDNA was synthesized with oligo (deoxythymidine) primers at 50°C using the SuperScript III first-strand synthesis system for the real-time polymerase chain reaction (PCR; Invitrogen Co.). The PCR analysis was performed using the StepOnePlus™ real-time PCR system (PE Applied Biosystems, Foster City, CA, USA) and FAST SYBR® green. The mRNA levels of neuropeptide Y (NPY), proopiomelanocortin (POMC), prepro-orexin (pporexin), Kiss1 (the gene encoding kisspeptin), Kiss1r, RFRP/GnIH (the gene encoding the kisspeptin receptor), and GPR147 in the posterior hypothalamus, and the Kiss1 mRNA levels of the anterior hypothalamus were measured. The mRNA expression level of each molecule was normalized to that of GAPDH. Dissociation curve analysis was also performed for each gene at the end of the PCR. Each amplicon generated a single peak. The primer sequences, product sizes, and annealing temperatures are shown in Table 1.
The PCR conditions were as follows: initial denaturation and enzyme activation at 95°C for 20 s, followed by 45 cycles of denaturation at 95°C for 3 s, and annealing and extension for 30 s.

**Statistical analyses**

All data are presented as the mean ± standard deviation (SD). The statistical analyses were conducted using one-way or two-way analysis of variance (ANOVA) together with the Tukey-Kramer post-hoc test for comparisons among the groups.

**Results**

The E2-treated groups (E2, E2+DHT) exhibited significantly smaller BW changes than the non-E2-treated groups (control, DHT). The DHT group displayed greater BW changes than the control group, whereas the BW changes in the E2 group did not differ from those seen in the E2+DHT group (Figs. 1A and B). Similarly, the DHT group demonstrated greater cumulative FI compared with the control group, whereas the cumulative FI of the E2 group did not differ from that of the E2+DHT group (Figs. 1A and B). The weights of visceral fat, subcutaneous fat, total fat, and lean body mass recorded in the E2-treated groups (E2, E2+DHT) were significantly lighter than those observed in the non-E2-treated groups (control, DHT) (Figs. 2A-D). There were no significant differences in these parameters between the control and DHT groups or between the E2 and E2+DHT groups. The serum LH levels of the DHT, E2, and E2+DHT groups were significantly lower than that of the control group (Fig. 2E). The serum E2 levels of the E2-treated groups were significantly higher than those of the non-E2-treated
There were no significant differences in the hypothalamic *NPY* or *pporexin* mRNA levels among the examined groups (Figs. 3A and B), whereas the hypothalamic *POMC* mRNA level of the E2+DHT group was significantly lower than that of the control group (Fig. 3C). In the anterior hypothalamus, the *Kiss1* mRNA levels of the E2-treated groups (E2, E2+DHT) were significantly higher than those of the non-E2-treated groups (control, DHT) (Fig. 3D). However, there were no significant differences in the anterior hypothalamic *Kiss1* mRNA level between the control and DHT groups or between the E2 and E2+DHT groups. On the other hand, the posterior hypothalamic *Kiss1* mRNA levels of the DHT, E2, and E2+DHT groups were significantly lower than that of the control group (Fig. 3E). The posterior hypothalamic *Kiss1r* mRNA level of the E2+DHT group was significantly lower than those of the other groups (Fig. 3F). The posterior hypothalamic *RFRP* and *GPR147* mRNA levels of the E2+DHT group were lower than those of the control and DHT groups (Fig. 3H).

**Discussion**

Sex hormones play roles in reproductive function and BW regulation in mammals and humans [1]. It has been well established that the effects of estrogen on GnRH/LH are mainly mediated by hypothalamic kisspeptin and RFRP/GnIH [2-4] and that its effects on appetite are mediated by hypothalamic orexigenic and anorexigenic factors [1,5-9]. In contrast to estrogen, the effects of androgens on reproductive function and BW regulation, especially on hypothalamic functions, in females have not been fully established.
Although some studies have examined the effects of androgens on hypothalamic reproductive functions, the results were disputed since ovarian intact PCOS animal models were used [12-14]. Thus, to clarify the direct effects of androgens on reproductive functions, we evaluated the changes in the hypothalamic levels of reproductive factors induced by chronic DHT administration under OVX and stable E2 milieu conditions.

Kisspeptin has been detected in both the AVPV and ARC, and it was reported that lower levels of estrogen reduce kisspeptin activity in the ARC and suppress GnRH/LH secretion, whereas higher levels of estrogen increase kisspeptin activity in the AVPV and induce surges in GnRH/LH production [2]. Thus, we separated the hypothalamus into anterior (AVPV) and posterior (ARC) blocks and evaluated the Kiss1 mRNA levels of these blocks. As a result, we found that although the Kiss1 mRNA levels of the anterior hypothalamic block (AVPV) were significantly higher in the E2-treated groups (E2 and E2+DHT groups) than in the non-E2-treated groups (control and DHT groups), they did not differ between the groups that were and were not administered DHT in the OVX or E2-treated rats. These results indicate that in the AVPV the Kiss1 gene is sensitive to E2, but not DHT. On the other hand, the Kiss1 mRNA levels in the posterior hypothalamic block (ARC) were significantly lower in the E2-treated groups than in the non-E2-treated groups. In addition, the Kiss1 mRNA levels of the posterior hypothalamic block (ARC) were significantly lower in the group treated with DHT than in the group that was not administered DHT among the OVX rats, but not the E2-treated rats. These results indicate that in the ARC the Kiss1 gene is sensitive to both E2 and DHT. As the serum LH levels of these groups exhibited similar patterns to their Kiss1 mRNA levels in the ARC; i.e.,
the levels of LH were decreased by both E2 and DHT, these steroids might have stronger effects on gonadotropin levels in the ARC than in the AVPV. Interestingly, although neither DHT nor E2 affected the hypothalamic mRNA levels of Kiss1r or RFRP/GnIH when administered alone, the co-administration of DHT and E2 suppressed the hypothalamic concentrations of these molecules. The administration of DHT reduced the hypothalamic mRNA levels of GPR147 in both the OVX and E2-treated rats. We could not evaluate the physiological roles of these alterations in this study; however, we speculate that these changes might represent a counter-regulatory mechanism that attenuates the effects of marked changes in kisspeptin activity.

In the present study, the BW gains observed in the OVX groups were significantly larger than those seen in the E2-treated groups. The BW gains and FI recorded in the group treated with DHT were significantly larger than those observed in the group that was not administered DHT in the OVX rats, whereas they did not differ in the E2-treated rats. On the other hand, the amounts of visceral and subcutaneous fat were not affected by DHT administration in either the OVX or E2-treated rats. These findings indicate that DHT itself does not affect the amount of body fat under OVX or stable E2 milieu conditions. It has been reported that both BW and fat weight were increased in DHT-treated PCOS models [11,19,20] and that they were also increased in testosterone-treated female rats [15]. As mentioned above, because androgens were administered to ovarian intact female animals and estrous cyclicity was disturbed in almost all of these animals in the aforementioned studies, some of the observed effects of androgens on BW and body fat might have been induced by disturbances in ovarian function; i.e., estrogen deficiency
or irregularities in estrogen production. In this study, the hypothalamic mRNA levels of orexigenic factors; i.e., NPY and pporexin, were not changed by the administration of DHT in the OVX or E2-treated rats. On the other hand, the hypothalamic mRNA level of POMC, an anorexigenic factor, was reduced by the co-administration of DHT and E2. As it has been reported that kisspeptin acts as a positive regulator of POMC expression in the hypothalamus, reductions in Kiss1 and Kiss1r expression might be related to the alterations in the POMC level seen in the DHT+E2 group [21,22]. However, such changes might not be directly involved in the regulation of BW or FI. Thus, we could not detect any hypothalamic mechanisms by which DHT increases FI under OVX conditions. Further studies are needed to clarify the mechanisms by which androgens affect BW and FI regulation.

In summary, this study showed that DHT affects BW, FI, and the expression of hypothalamic reproductive and appetite regulatory factors and that these effects of DHT differ according to the estrogen milieu. In addition, the effects of DHT on Kiss1 mRNA levels in the anterior (AVPV) and posterior (ARC) hypothalamus were markedly different. DHT might have stronger effects on gonadotropin levels in the ARC than in the AVPV.

Conflict of Interest

The authors declare that they have no conflict of interest.

References


Pasquali R. Effect of flutamide and metformin administered alone or in combination in dieting obese women with polycystic ovary syndrome. Clin Endocrinol (Oxf) 2004;60:241-249


**Figure legends**

**Fig. 1**
Weekly body weight (BW) changes (% of initial BW) (A, B) and cumulative food intake (FI; g/initial BW) (C, D) observed in the control, dihydrotestosterone (DHT)-treated, estradiol (E2)-treated, and DHT+E2-treated rats

Data are expressed as mean ± SEM values. Different letters (a-c) indicate significant differences ($P <0.05$).

**Fig. 2**
Visceral, subcutaneous, and total fat weight (A-C); lean body mass (D); and serum LH and serum estradiol levels (E, F) in the control, dihydrotestosterone (DHT)-treated, estradiol (E2)-treated, and DHT+E2-treated rats

Data are expressed as mean ± SEM values. Different letters (a-c) indicate significant differences ($P <0.05$).

**Fig. 3**
The mRNA levels of hypothalamic orexigenic and anorexigenic factors (A-C) and hypothalamic reproductive factors (D-H)

The mRNA expression levels of the control rats are expressed as 1.0. Data are expressed as mean ± SEM values. Different letters (a-c) indicate significant differences ($P <0.05$).
Fig. 1

A

BW change (% of initial BW)

- control
- DHT
- E2
- E2+DHT

B

BW change (% of initial BW)

- control
- DHT
- E2
- E2+DHT

C

Cumulative FI (g / initial BW)

- control
- DHT
- E2
- E2+DHT

D

Cumulative FI (g / initial BW)

- control
- DHT
- E2
- E2+DHT
Fig. 2

A. Visceral fat (g)
B. Subcutaneous fat (g)
C. Total fat (g)
D. Lean body mass (g)
E. LH (ng/mL)
F. Estradiol (pg/mL)

Legend:
- control
- DHT
- E2
- E2+DHT

Significance:
a
b
Fig. 3

A. Post hypothalamic NPY mRNA
B. Post hypothalamic proopiomelanocortin (POMC) mRNA
C. Post hypothalamic Kiss1 mRNA
D. Post hypothalamic RFRP/GnIH mRNA
E. Post hypothalamic Kiss1r mRNA
F. Post hypothalamic Kiss1r mRNA
G. Post hypothalamic RFRP/GnIH mRNA
H. Post hypothalamic GPR147 mRNA

Legend:
- control
- DHT
- E2
- E2+DHT

Bars with different letters indicate significant differences.
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