学 位 論 文 の 要 旨

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学位論文名　Hyperandrogenism Induces Proportional Changes in the Expression of Kiss-1, Tac2, and DynA in Hypothalamic KNDy Neurons
発表雑誌名　Reproductive Biology and Endocrinology
（巻，初頁～終頁，年）　(20(1), 91, 2022)
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論 文 内 容 の 要 旨

INTRODUCTION

Kisspeptin released from Kiss-1 neurons in the hypothalamus plays an essential role in the control of the hypothalamic–pituitary–gonadal (HPG) axis by regulating the release of gonadotropin-releasing hormone (GnRH) from GnRH neurons in the hypothalamus. In rodents, Kiss-1 neurons in the anteroventral periventricular nucleus (AVPV) are involved in the surge secretion of GnRH/luteinizing hormone (LH), while those in the arcuate nucleus (ARC) maintain the pulsatile secretion of GnRH. Kiss-1 neurons in the ARC co-express two other neuropeptides, neurokinin B (NKB) and dynorphin A (DynA), and this population is referred to as KNDy neurons. NKB (encoded by Tca3) has a stimulatory effect on the HPG axis, while DynA is an endogenous opioid peptide that reduces LH pulse frequency. It is plausible that KNDy neurons are affected by androgens because GnRH neurons do not express estrogen receptor α or androgen receptor, but KNDy neurons express both of them. Hyperandrogenism represents one of the principale traits of Polycystic ovary syndrome (PCOS). Although the importance of androgens and their receptors in the pathogenesis of PCOS neurons has been highlighted, it still unknown how androgens affect KNDy neurons in the hypothalamus. Kiss-1-expressing mHypoA-55 cells are a model of KNDy neurons that were isolated from the ARC of an adult female mouse. Using these cells, we investigated how androgens directly affect KNDy neurons in the hypothalamus. Furthermore, we examined how androgen supplementation affects the characteristics of KNDy neurons in ovary-intact female rats.

MATERIALS AND METHODS

Cell culture

mHypoA-55 cells were plated in 35-mm tissue culture dishes and incubated with high-glucose DMEM containing 10% heat-inactivated FBS and 1% penicillin–streptomycin at 37 °C under a humidified atmosphere of 5% CO2 in air. After 24 h, cells were incubated without (control) or with various

concentrations of test reagents (testosterone, full name (DHT), NKB, Dyn A, and prolactin) for 24 h in DMEM containing 1% heat-inactivated FBS and 1% penicillin–streptomycin.

RNA preparation, reverse transcription, and real-time quantitative RT-PCR

Total RNA was extracted from the cells using TRIzol-LS and 1.0 μg total RNA was reverse-transcribed using an oligo-dT primer and prepared using a First-Strand cDNA Synthesis Kit in reverse transcription buffer. Using specific primers for Kiss-1, NKB, and DynA, the simultaneous measurement of Kiss-1, NKB, and DynA mRNA was performed. GAPDH mRNA was used to normalize the amount of cDNA added per sample. Quantification of Kiss-1, NKB, and DynA mRNA was obtained through real-time quantitative PCR using specific primer for mouse.

Western blot analysis

The cell extracts were subjected to SDS-PAGE in 10% acrylamide gel and the protein was transferred onto polyvinylidene difluoride membranes. Membranes were incubated with anti-kisspeptin antibody , anti-NKB antibody, or anti-DynA antibody. When the expression levels were compared after stimulation, the films were analyzed by densitometry, and the intensity of the target protein was normalized to that of β-actin to correct for protein loading.

Animal experiments

Six-week-old female wistar rats received a daily subcutaneous injection of DHT (5 mg/kg/day) to produce a supraphysiological androgen level in vivo, based on a previous study or a placebo control in 160 μL sesame oil for 7 days. Then, the rats were euthanized while under isoflurane anesthesia and the whole brain was removed. Hypothalamic tissues containing the ARC were extracted and used for quantitative RT-PCR analysis. This protocol was approved by the ethics committee of the Experimental Animal Center for Integrated Research at Shimane University (IZ31-51).

Statistical analysis

Data are expressed as mean ± SEM values. Statistical analysis was performed using one-way analysis of variance with Bonferroni's post hoc test or Student's t-test. $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

To examine the direct effect of testosterone, mHypoA-55 cells were stimulated with testosterone and examined how expression levels of Kiss-1, Tac3 and DynA genes and their proteins were affected by testosterone stimulating. When cells were cultured with 100 nM testosterone, Kiss-1 gene expression was significantly increased by 3.20 ± 0.44-fold compared to non-stimulated cells. Kisspeptin protein expression was also significantly increased by 10 nM testosterone stimulation to 1.5 ± 0.03-fold. Tac3 expression was significantly increased by 2.84 ± 0.98-fold and 2.69 ± 0.64-fold by 10 nM and 100 nM testosterone, respectively. NKB protein expression was also increased by 100 nM testosterone to 1.62 ± 0.64-fold. We could not detect significant changes in DynA mRNA expression by testosterone, however, western blot analysis revealed that DynA protein expression was significantly decreased by testosterone stimulation by

70%–80%.

Kisspeptin and NKB expression was significantly increased by DHT (a potent endogenous androgen synthesized from testosterone by 5α-reductase) stimulation. Conversely, DynA expression was significantly reduced by DHT. These effects of DHT on mHypoA-55 cells were the same as those observed by testosterone stimulation. Our observations using hypothalamic mHypoA-55 cells suggested that androgen have ability to increase kisspeptin and NKB levels, and probably decrease DynA expression in KNDy neurons in the ARC region of the hypothalamus.

Next, to examine the effect of supraphysiological androgens in ovary-intact female rats, subcutaneous injection of DHT (5 mg/kg/day) was administered to rats for 7 days. Kiss-1 gene expression in the ARC region of rat brain tissue was significantly increased by DHT (1.70 ± 0.25-fold). Similarly, Tac3 gene expression in this area was also upregulated by DHT treatment (2.12 ± 0.89-fold). In contrast, DynA gene expression was unchanged following DHT administration. Therefore, we speculated that androgens could increase kisspeptin and NKB levels, while decreasing or unchanging DynA expression in KNDy neurons. Neither NKB nor DynA significantly altered Kiss-1 gene expression in mHypoA-55 ARC cells.

Finally, we examined how the expression of kisspeptin, NKB, and DynA in KNDy neurons was changed by prolactin stimulation because serum level of prolactin tend to be elevated in patients with PCOS. Western blotting analysis showed that neither concentration of prolactin modulated the protein expression levels of kisspeptin, NKB, and DynA.

Our study using mHypoA-55 ARC cell models and ovary-intact female rats indicated that androgens have ability to increase kisspeptin and NKB levels, and probably decrease DynA expression in KNDy neurons in the ARC region of hypothalamus. We showed the possibility that hyperandrogenemia in PCOS could secondarily induce changes in KNDy neurons, but it is still unknown whether these changes underlie the pathogenesis of PCOS. Kisspeptin and NKB both stimulate GnRH pulse output, while DynA inhibits KNDy neuron firing during pulsation. Thus, we speculated that changes in the proportion of NKB and DynA might have an effect on kisspeptin production and release from KNDy neurons. Although NKB and DynA are considered to act in an autocrine/paracrine manner on KNDy neurons, exogenous stimulation by NKB and DynA did not alter Kiss-1 gene expression in mHypoA-55 cells. Considering this observation, the androgen-induced proportional changes in NKB, DynA, and kisspeptin expression within KNDy neurons may alter the secretory pattern of kisspeptin and eventually alter the pattern of pulsatile GnRH release, resulting in hyperactive LH secretion.

CONCLUSION

Our observations imply that hyperandrogenemia affects KNDy neurons and changes their neuronal characteristics by increasing kisspeptin and NKB levels and decreasing DynA levels. These changes might cause dysfunction of the hypothalamic–pituitary–gonadal axis.