Sequential preovulatory expression of a gonadotropin-releasing hormone-inducible gene, Nr4a3, and its suppressor Anxa5 in the pituitary gland of female rats

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Abstract. Functional relationship between nuclear receptor subfamily 4 group A member 3 (Nr4a3) and annexin A5 (Anxa5), which are two gonadotropin-releasing hormone (GnRH)-inducible genes, has been established while evaluating pituitary gonadotropes in relation to follicle-stimulating hormone beta (Fshb) expression. However, the physiological variations that arise due to the differential expression of these genes in the pituitary gland during rat estrous cycle remain unknown. This study aimed to evaluate the Nr4a3 and Anxa5 mRNA expression during the estrous cycle in rats in comparison with the expression of the gonadotropin subunit genes, luteinizing hormone beta (Lhb) and Fshb. Nr4a3 mRNA expression showed a single peak at 1400 h of proestrus during the 4-d estrous cycle. Anxa5 mRNA level was elevated along with increased Fshb mRNA expression after the decline of Nr4a3 mRNA until 2300 h. Lhb mRNA expression levels were not significantly changed during the estrous cycle. Notably, addition of a GnRH antagonist at 1100 h completely eradicated luteinizing hormone secretion at 1400 h and 1700 h of proestrus, and significantly reduced the Nr4a3 mRNA expression level at both the time points. These results suggest that GnRH is, at least partly, responsible for the increase in pituitary Nr4a3, and that the interaction between NR4A3 and ANXA5 is required to regulate Fshb expression during the preovulatory gonadotropin surge.

Key words: Annexin A5, Anterior pituitary gland, Follicle-stimulating hormone beta (Fshb), Gonadotropin-releasing hormone (GnRH), Nuclear receptor 4 group A member 3 (NR4A3)

During rat estrous cycle, the two gonadotropins, namely luteinizing hormone (LH) and follicle stimulating hormone (FSH), undergo increased secretion during the afternoon of proestrus, which is known as the gonadotropin surge [1]. This surge is closely regulated by the hypothalamic neuropeptide gonadotropin-releasing hormone (GnRH) [2]. However, the secretion patterns of LH and FSH are not identical. On the contrary, the FSH surge consists of two distinct increases [1, 3], whereas that of LH is a single peak. It is assumed that different mechanisms regulate the GnRH-mediated FSH surge; for example, changes in the plasma concentration of inhibin (an FSH suppressor), changes in the secretion patterns of GnRH, and possible interactions with intracellular signaling pathways [4].

Annexin A5 (ANXA5) is a calcium-dependent phosphatidylserine-binding protein [5] that is linked to several functional pathways, including the pathogenesis of several cancers, membrane repair, suppression of blood coagulation, and inhibition of protein kinase C [6-9]. We previously reported that GnRH stimulates ANXA5 expression in the anterior pituitary gonadotrope, and that ANXA5 promotes the secretion of LH and FSH [10-12]. Recently, we demonstrated low expression levels of the FSH β subunit gene (Fshb) but not the LH β subunit gene (Lhb) and other hormone-encoding genes in the pituitary gland of ANXA5-deficient female mice, suggesting that ANXA5 upregulates FSH expression [13]. Furthermore, ANXA5 suppresses the expression of nuclear receptor 4 group A member 3 (NR4A3).

NR4A3 is a member of the orphan nuclear receptor family of Nr4a immediate early genes, namely Nr4a1, Nr4a2, and Nr4a3. NR4A3 is involved in various physiological processes, such as inflammatory, metabolic, cardiovascular, neurological, and immune functions [14]. The expression of NR4A3 is affected by various stimuli, including hormones, growth factors, inflammation, and cellular stimulation [15]. We recently demonstrated that GnRH stimulates NR4A3 expression in the clonal gonadotrope LβT2, and that NR4A3 specifically represses Fshb expression [13]. These results suggest that there is some functional interaction between ANXA5 and NR4A3 and that this interaction is involved in GnRH-dependent augmentation of FSH synthesis. Here, we investigated the transcription of pituitary Nr4a3, Anxa5, and Fshb during the estrous cycle of rats, and the potential suppression of blood coagulation, and inhibition of protein kinase C [6-9]. We previously reported that GnRH stimulates ANXA5 expression in the anterior pituitary gonadotrope, and that ANXA5 promotes the secretion of LH and FSH [10-12]. Recently, we demonstrated low expression levels of the FSH β subunit gene (Fshb) but not the LH β subunit gene (Lhb) and other hormone-encoding genes in the pituitary gland of ANXA5-deficient female mice, suggesting that ANXA5 upregulates FSH expression [13]. Furthermore, ANXA5 suppresses the expression of nuclear receptor 4 group A member 3 (NR4A3).
effect of GnRH on the regulation of Nr4a3 mRNA expression.

Materials and Methods

Animals

Wistar–Imamichi female rats (200–300 g) were purchased from SLC Japan (Shizuoka, Japan). The rats were maintained under controlled temperature and light conditions (23 ± 3°C and 14-h/10-h light–dark cycle, respectively, with lights on at 0500 h), and free access to laboratory chop and tap water. Vaginal smears were examined daily for at least two weeks before the start of the experiments. Female rats with regular 4-d estrous cycles were used in our analysis. The animals were sacrificed by decapitation, and trunk blood samples were collected immediately. Pituitary tissues were immediately collected and frozen in liquid nitrogen. All animal protocols were approved by the Animal Care and Use Committee of Kitasato University (Approval no.: 19–173).

Reverse transcription–quantitative PCR (RT–qPCR)

Total RNA was extracted from the pituitary tissues using ISOGEN (Nippon Gene, Tokyo, Japan) and reverse transcribed to cDNA using a High Capacity cDNA Synthesis Kit (Applied Biosystems, Foster City, CA, USA). The primer and probe sequences used for all RT–qPCR assays are listed in Table 1. qPCR was performed using THUNDERBIRD SYBR qPCR Mix (TOYOBO, Osaka, Japan) and TaqMan Universal PCR Master Mix (Applied Biosystems) according to the manufacturer’s protocols. Relative gene expression levels were calculated using the \( \Delta\Delta CT \) method with ribosomal protein L19 (Rpl19) as the internal control for normalization.

Determination of LH levels

Plasma LH concentrations were measured using a time-resolved fluorometric immunoassay (DELFIA System; PerkinElmer, Branchburg, NJ, USA). Briefly, purified rat LH (LH-I-9, NIDDK) was labeled with europium (Eu) using a DELFIA Eu-Labeling Kit (PerkinElmer). Anti-rabbit gamma-globulin goat serum was prepared in our laboratory and purified by ammonium sulfate precipitation. A 96-well immunoplate (Nunc, Tokyo, Japan) was coated with anti-rabbit gamma globulin and then overlaid with NIDDK anti-rat LH-S-11 (Rpl19) as the internal control for normalization.

Statistical analysis

Results are presented as mean ± standard error of the mean (SEM). Differences were statistically analyzed using Student’s \( t \)-test or one-way analysis of variance (ANOVA) followed by a Tukey–Kramer test. Simple linear regression analysis followed by Pearson’s correlation analysis was conducted to examine the correlation between the expression levels of Nr4a3, Anxa5, and Fshb. All statistical analyses were performed using IBM SPSS Statistics Subscription (Build 1.0.0.1447; IBM Japan, Tokyo, Japan). Statistical significance was set at \( P < 0.05 \).

Results and Discussion

Nr4a3 mRNA level in the anterior pituitary gland showed a specific increase at 1400 h of proestrus during the estrous cycle (Fig. 1A; proestrus 1400 and 1700 h versus proestrus 1100 h, \( P < 0.01 \)), indicating a time-specific response in Nr4a3 expression. However, Anxa5 mRNA level in the anterior pituitary gland remained low between 1100 and 1700 h, and then increased between 2000 h and 2300 h (Fig. 1B; proestrus 2000, 2300, and estrus 0200 h versus proestrus 1100 h, \( P < 0.01 \)). This increase was inversely correlated with a decline in Nr4a3 expression. Previously, we showed that GnRH stimulates Nr4a3 expression as an immediate early response.

Table 1. Sequences of the primers and probes used in this study

| Gene | Orientation | Sequence (5′–3′) |
|------|-------------|-----------------|
| Nr4a3 | Forward     | AAGACGGAACCTCCACAGAA |
|       | Reverse     | GTCGAGATAGGCCAAGCA  |
| Anxa5 | Forward     | AACGTTCCTTCCAAGGCCATG |
|       | Reverse     | CTCAGCATACTGCTGGCG |
|       | Probe       | FAM-CGACGAGGACAGCATCCTGAACCTGTT-TAMRA |
| Fshb  | Forward     | TGTTGTTAGGGCCCTAGCTGTA |
|       | Reverse     | CTCAGTACACAGCTCTCTGG |
| Lhb   | Forward     | GTCTGCATCACCTTCAAC |
|       | Reverse     | GTAGGTTGACACTGCTGAG |
| Rpl19 | Forward     | GGAGGCTCTGACTGTCAT |
|       | Reverse     | ATCCTTCGCAATCTGTCAC |
PITUITARY Nr4a3 DURING PROESTRUS

Anxa5 expression was augmented in the culture of the gonadotrope cell line LβT2 [13]. We also demonstrated that ANXA5 suppresses GnRH-induced Nr4a3 expression [13], suggesting that pituitary ANXA5 expression may inhibit Nr4a3 expression during the proestrus period.

Fshb mRNA level was significantly increased along with increasing Anxa5 level during proestrus between 1100 h and 2300 h (Fig. 1C; proestrus 2000 h, 2300 h, and estrus 0200 h versus proestrus 1100 h, P < 0.01). In contrast, Lhb expression did not change significantly over the same period (Fig. 1D). Correlation between the expression levels of Nr4a3, Anxa5, and Fshb from proestrus 1400 to 2300 h showed that the expression level of Nr4a3 was negatively correlated with those of Anxa5 (Fig. 2A; r = −0.806, r² = 0.6489, P < 0.001) and Fshb expression (Fig. 2B; r = −0.774, r² = 0.5986, P < 0.001), while the expression levels of Anxa5 and Fshb were positively correlated (Fig. 2C; r = 0.788, r² = 0.6207, P < 0.001). Several studies have reported a dramatic increase in the Fshb mRNA level, which occurs at several hours after the initial gonadotropin surge [16, 17]. The Fshb mRNA expression level increased once the Nr4a3 expression level decreased, which paralleled the Anxa5 mRNA profile, suggesting that the decline in NR4A3 and increase in ANXA5 correlated with changes in Fshb expression during the early gonadotropin surge.

Marked increases in Nr4a3 mRNA expression levels appeared to coincide with the onset of GnRH surge. Therefore, we further investigated the potential involvement of GnRH in the regulation of Nr4a3 expression during proestrus using a GnRH antagonist. Plasma LH concentrations were 4.05 ng/ml at 1400 h and 20.0 ng/ml at 1700 h in saline-treated rats, indicating that these animals experienced a normal gonadotropin surge, whereas animals treated with the GnRH antagonist presented with a dramatically reduced LH level, reaching less than 0.65 ng/ml and 0.41 ng/ml, respectively (Fig. 3A). Treatment with the GnRH antagonist also significantly reduced the Nr4a3 expression levels by 25% and 29% at 1400 h and 1700 h, respectively (Fig. 3B). Furthermore, a significant decrease was observed in Anxa5 expression at 1700 h (Fig. 3C) and Fshb expression at 1400 h and 1700 h (Fig. 3D) in the GnRH antagonist-treated group. It has been suggested that the gonadotrope Nr4a3 expression is stimulated by GnRH. Although treatment with the antagonist completely abolished LH expression, it only reduced the Nr4a3 expression level by 25–30%, suggesting that regulation of this factor is not solely mediated by GnRH. However, the decrease in the expression of another GnRH responsive gene, Anxa5, was also not substantial, suggesting a limited contribution of GnRH and/or additional player of enhancement. This is plausible since there are a variety of factors known to induce Nr4a3 as an immediate early response gene in different cells and tissues [18]. In addition, various pituitary cells, along with gonadotrope cells, also express Nr4a3.

Fshb mRNA expression levels increase between bimodal plasma FSH surges [16]. Currently, two potential mechanisms have been proposed for Fshb augmentation. One suggests that the decline in the plasma concentration of inhibin, which is inversely correlated with increasing plasma FSH concentration, facilitates the transcriptional activation of Fshb [3, 19]. Although we have previously tested the effect of inhibin antiserum in proestrus rats, Nr4a3 expression did not change despite the significantly increased expression of Fshb mRNA (data not shown). Therefore, it is suggested that inhibin is not involved.
in \textit{Nr4a3} expression during proestrus. Another theory suggests that activin/follistatin is synthesized in the pituitary gonadotrope, thereby increasing \textit{Fshb} expression [20–22]. Our data suggest an additional intracellular mechanism for the regulation of \textit{Fshb} expression. The present animal model showed a simultaneous increase in the \textit{Anxa5} and \textit{Fshb} expression levels, whereas \textit{Anxa5}-knockout mice had low \textit{Fshb} mRNA levels [13, 23], strongly suggesting that ANXA5 positively affects \textit{Fshb} mRNA expression. Taken together, our results suggest that the elevation of \textit{Nr4a3} expression is inhibited by the augmentation of \textit{Anxa5}, leading to increased \textit{Fshb} levels, and that this mechanism regulates the bimodal FSH surge.

In summary, this study shows that \textit{Nr4a3} mRNA expression levels are increased during the afternoon phases of proestrus and are regulated, at least partially, by GnRH. In addition, our data demonstrated a negative correlation between \textit{Nr4a3} and \textit{Anxa5} and \textit{Fshb}, wherein both were upregulated at midnight. This suggests that ANXA5 suppresses \textit{Nr4a3} mRNA expression and stimulates \textit{Fshb} transcription.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig2}
\caption{Scatter plot representation of correlation between mRNA expression levels of \textit{Nr4a3}, \textit{Anxa5}, and \textit{Fshb}. The plot presents simple regression line of \textit{Nr4a3} versus \textit{Anxa5} (A; \(n = 20\)), \textit{Nr4a3} versus \textit{Fshb} (B; \(n = 20\)), and \textit{Anxa5} versus \textit{Fshb} (C; \(n = 20\)) expression levels in rat anterior pituitary gland from proestus 1400 to 2300 h. Statistical analysis performed using Pearson’s correlation method.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig3}
\caption{Effect of GnRH antagonist on plasma LH and pituitary \textit{Nr4a3} expression level in proestrus rats. Proestrus rats were intraperitoneally injected with the GnRH antagonist (Cetrorelix, 100 μg/200 μl) or 200 μl saline at 1100 h, and the anterior pituitary tissues and blood samples were collected at 1400 h and 1700 h. Plasma LH concentration (A), \textit{Nr4a3} (B), \textit{Anxa5} (C), and \textit{Fshb} mRNA expression (D) were measured. Relative levels of mRNA are described in comparison to the saline group at 1400 h (set to 1.0). Data are presented as mean ± SEM (\(n = 8\)). Statistical analysis performed using Student’s \(t\)-test; * \(P < 0.05\), ** \(P < 0.01\).}
\end{figure}
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