NOTE

Gonadotropin-releasing hormone is prerequisite for the constitutive expression of pituitary annexin A5

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Abstract. Annexin A5 (ANXA5), a member of the structurally related family of annexin proteins, is expressed in pituitary gonadotropes. We previously reported that ANXA5 expression is stimulated by gonadotropin-releasing hormone (GnRH). In the present study, we investigated ANXA5 expression in the anterior pituitary gland of GnRH-deficient mutant hypogonadal (*hpg*) mice. RT-PCR demonstrated that luteinizing hormone β subunit (LH β) and ANXA5 mRNA levels were both lower in the pituitary gland of *hpg* mice than in wild-type mice. Immunohistochemistry showed that ANXA5 expression throughout the pituitary gland was very low in *hpg* mice, suggesting that ANXA5 is diminished in gonadotropes and also in other cell types. Subcutaneous administration of a GnRH analogue, des-gly10 (Pro9)-GnRH ethylamide (1 μ g/day for 7 days), augmented the expression of LH β and ANXA5 in the pituitary gland in *hpg* mice. However, LH β - and ANXA5-positive cells did not show exactly matched spatial distributions. These findings suggest that GnRH is necessary for constitutive ANXA5 expression in the pituitary gland, not only in gonadotropes but also in other pituitary gland cell types. A close relationship between ANXA5 and LH β expression was confirmed. It is suggested that a significant role of ANXA5 in the physiologic secretion of LH.

Key words: Annexin A5 (ANXA5), Gonadotropin-releasing hormone (GnRH), Hypogonadal (*hpg*) mouse, Gene expression

ANNEXIN A5 (ANXA5) is a member of the annexin family of structurally related proteins, which are characterized by calcium-dependent phospholipid binding [1, 2]. Although found in a variety of tissues, the distribution of ANXA5 is specific to cell type [3, 4]. We previously reported that ANXA5 is expressed in gonadotropes of the anterior pituitary gland and that its expression is enhanced after ovariectomy in rats [4, 5]. We also demonstrated that gonadotropin-releasing hormone (GnRH) stimulates ANXA5 synthesis *in vivo* and *in vitro* [6, 7], indicating the expression of ANXA5 is directly regulated by the GnRH receptor. Finally, we showed that the suppression of ANXA5 synthesis with antisense oligonucleotides retards GnRH-stimulated luteinizing hormone (LH) release [8].

Hypogonadal (*hpg*) mutant mice have a truncation in the gene encoding GnRH-associated peptide, leading to the absence of GnRH translation [9]. GnRH deficiency results in very low levels of gonadotropins and infertility [10, 11]. The incompletely developed genital tract of *hpg* mice is restored by the administration of exogenous GnRH [12], indicating an intact GnRH receptor system in *hpg* mice. Thus, the *hpg* mouse is a useful animal model for studying exogenous GnRH action with no background of endogenous GnRH.

In this study, we investigated the expression of ANXA5 in the anterior pituitary gland of *hpg* mice and observed changes in its expression after GnRH analogue (GnRHa) administration.

Materials and Methods

All experiments were approved by the Animal Experiments Ethics Committees of Kitasato University. Mutant *hpg* mice were obtained from Jackson Laboratory. Mice were maintained in a room with a

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14/10-h light/dark cycle and fed standard rodent chow. Heterozygous mice were mated to generate homozygous *hpg* mice. Genotypes were determined using a standard PCR procedure with the following primers: *hpg* GnRH gene, 5'-AGT CTC TTC CCA GGT GAT T-3' (forward) and 5'-TTC CAG GTT TCA GTG CAT C-3' (reverse) (product size: 324 bp); wild-type (WT) GnRH gene, 5'-AAT CAATCC AAC CTC GCT G-3' (forward) and 5'-GAA TGA GCT GGA GTT TAG G-3' (reverse) (product size: 693 bp). Adult homozygous *hpg* mice and their WT littermates were used for all experiments.

Pituitary glands of *hpg* and WT mice ($n = 3$ per group) were collected immediately after decapitation for reverse transcription (RT)-PCR analysis of LH β and ANXA5 expression. Pituitary glands were submerged in extraction solution (TRIzol, Invitrogen, Carlsbad, CA), and total RNA was extracted from the tissues according to the manufacturer's instructions. The concentration of total RNA was measured by ultraviolet spectrophotometry. Total RNA (500 ng) was reverse-transcribed for each sample using a standard reverse transcription kit (Multiscribe High Capacity cDNA Reverse Transcription Kits, Applied Biosystems, Foster City, CA) with random primers according to the manufacturer's instructions. RT-PCR was performed using a Veriti Thermal Cycler (Applied Biosystems). The primers were designed as follows: LH β , 5'-TGG AGA GGC TCC AGG GGC TG-3' (forward) and 5'-GGG GAG GTG GGG GAG GTC AC-3' (reverse) (product size: 407 bp); ANXA5, 5'-AGA TGA TGT GGT GGG GGA TA-3' (forward) and 5'-TCT CTG CAA GGT AGG CAG GT-3' (reverse) (product size: 342 bp); ribosomal RNA RPL19 as the internal control, 5'-CTG ATC AAG GAT GGG CTG AT-3' (forward) and 5'-TTC AGC TTG TGG ATG TGC TC-3' (reverse) (product size: 308 bp). Each RT-PCR reaction used 0.4 μ L forward primer (final concentration: 2 μ M), 0.4 μ L reverse primer, 10 μ L Premix Taq reagent (Takara Shuzo, Shiga, Japan), and 7.2 μ L nuclease-free water. The thermal cycling conditions were 95°C for 5 min for preincubation; 24-28 cycles at 94°C for 30 s, 58-64°C for 30 s, and 72°C for 1 min; and 72°C for 5 min for postincubation. After electroplating, the intensities of each band were measured using densitometry analysis (ImageJ 1.42q, National Institutes of Health, Bethesda, MD). The mRNA levels of LH β and ANXA5 were normalized to that of RPL19.

For fluorescent immunohistochemistry, adult *hpg* and WT mice ($n = 3$ -6 per group) were killed by anesthetic

overdose of diethyl ether and intracardiac perfusion of 4% buffered paraformaldehyde (30 mL). Pituitaries were removed and postfixed overnight. Paraffin sections (4 μ m thick) were processed for immunohistochemistry. Primary antibodies were monoclonal anti-human ANXA5 (Anti-ANXA5, dilution: 1:5,000; Kowa, Aichi, Japan) and rabbit anti-rat LH β (Anti-LH β , dilution 1:10,000; NIDDK (Bethesda, MD, USA)). Dried sections were processed through a series of xylene and ethanol rinses to replace the paraffin with water. Following a blocking treatment of 1% normal goat serum for 1 h at room temperature, incubation with a primary antibody was performed at 4°C in a humidified atmosphere overnight. Secondary antibodies were Alexa 488 goat anti-mouse IgG (1:10,000; Invitrogen, Carlsbad, CA) or Alexa 568 goat anti-rabbit IgG (1:10,000; Invitrogen). Sections were observed by confocal microscopy (LSM710, Carl Zeiss, Freistaat Thüringen, Germany). Normal rabbit or mouse serum was used instead of a primary antibody for the negative control, which showed no signal under the same conditions.

For bright-field immunohistochemistry, sections were incubated with 2.5% normal horse serum for 30 min to reduce non-specific antibody binding. The primary antibody was anti-ANXA5 rabbit serum raised in our laboratory [13]. The antiserum was diluted to 1:10,000, and sections were incubated with the serum overnight at 4°C. The secondary antibody was from the ImmPRESS reagent anti-rabbit IgG POD kit (Vector Laboratories, Burlingame, CA). Sections were counterstained with hematoxylin.

In one experiment, GnRH agonist (GnRHa) or saline was subcutaneously administered to adult *hpg* mice ($n = 4$ per group). The GnRHa (des-gly10 (Pro9)-GnRH ethylamide; 1.0 μ g/0.2 mL) or saline (0.2 mL) was injected once a day for 7 days. Pituitaries were collected 3 h after the last injection.

RT-PCR results were analyzed using Student's *t*-tests. A *p*-value < 0.05 was considered statistically significant.

Results and Discussion

We found that the expression of LH β and ANXA5 mRNA in the pituitary gland of *hpg* mice compared with that of WT mice (Fig. 1A-B), which was expected based on previous findings [9]. In earlier studies, we demonstrated that GnRH directly stimulates the expression of ANXA5 in primary cultures of pituitary cells and the gonadotrope cell line L β T2 [6, 7]. The

Fig. 1 RT-PCR analysis of LH β and ANXA5 mRNA levels in WT and *hpg* mice. (A) DNA products in an agarose gel visualized with ethidium bromide. RPL19 is a ribosomal RNA used as an internal control. (B) Each band was scanned, and its density was measured using ImageJ. Intensity was normalized to that of RPL19. Data are shown as mean $\pm$ standard error of the mean ($n = 3$ per group). * $p < 0.05$.

present results, therefore, indicate that basal ANXA5 synthesis in the pituitary gland is maintained by constitutive GnRH stimulation. Immunohistochemistry revealed a low expression of ANXA5 throughout the anterior pituitary gland of *hpg* mice (Fig. 2A-B). As gonadotropes comprise approximately 10% of pituitary gland cells [14], other hormone-secreting cells apart from gonadotropes may contribute to this reduction of ANXA5. Although further studies are needed, it is possible that gonadotropes are the primary cell type responsible for supplying ANXA5 in the pituitary gland. Alternatively, as the GnRH receptor is found not only in gonadotropes but also lactotropes [15], GnRH could also act through this cell type. If this is the case, GnRH could affect the function of lactotropes through ANXA5 synthesis, as GnRH has been shown to modify prolactin secretion [16].

The annexin family is made up of 12 structurally related proteins (ANXA1 to 13, ANXA12 is not assigned) in mammals. The annexin molecule consists of four repeats of an approximately 60 amino acid conserved sequence (eight repeats in ANXA6) and a variable N-terminal sequence [17]. The N-terminal sequence is thought to serve a specific function for each annexin, although the N-terminus of ANXA5 is missing [17]. Despite these interesting biochemical

Fig. 2 Immunohistochemistry analysis of LH β and ANXA5 expression in WT and *hpg* mice. (A) Bright-field images of pituitary tissue. Brown-black staining indicates ANXA5 immunoreactivity. Sections were counterstained with hematoxylin. (B) Fluorescent images of anterior pituitary tissue. Red and green signals indicate LH β and ANXA5 immunoreactivity, respectively. Scale bars: 200 μ m.

Fig. 3 RT-PCR analysis of the effect of GnRHa administration on LHβ and ANXA5 mRNA levels in *hpg* mice. (A) DNA products in an agarose gel visualized with ethidium bromide. Saline or GnRHa was subcutaneously administered daily for 7 days. (B) Each band was scanned, and its density was measured using ImageJ. Intensity was normalized to that of RPL19. Data are shown as mean $\pm$ standard error of the mean ($n = 3$ per group). * $p < 0.05$.

Fig. 4 Immunohistochemistry analysis of LHβ and ANXA5 expression in saline- or GnRHa-treated *hpg* mice. Fluorescent images of anterior pituitary tissue. Green signal indicates ANXA5 immunoreactivity. Scale bars: 100 μ m.

characteristics, the physiological function of annexins is not well elucidated. Previous studies indicate that ANXA1 mediates the anti-inflammatory effects of glucocorticoids by inhibiting phospholipase A₂ [18, 19], and ANXA 7 is related to the release of secretory granules in adrenal medulla cells [20]. Among all annexins, however, ANXA5 has been the best studied. Since its discovery, ANXA5 has been postulated to suppress blood coagulation in pregnant woman [21]. We recently showed that ANXA5 deficiency increases placental thrombosis and pregnancy loss in ANXA5-null mice [22]. Although we do not know whether placental ANXA5 expression is induced by local GnRH, we previously demonstrated an intimate relationship between GnRH and ANXA5 in tissues other than the anterior pituitary gland. Specifically, we found that ovarian GnRH increases ANXA5 expression and apoptosis in the corpus luteum [23], and testicular GnRH stimulates ANXA5 synthesis in Leydig cells [24]. These results suggest that ANXA5 is involved in basic cell functions such as cell growth rather than serving a gonadotrope-specific function. However, although ANXA5 seems to have multiple functions in various tissues, future studies are expected to show a consistent function of ANXA5 in gonadotropin secretion, inhibition of blood coagulation, apoptosis, and cell cycle.

Next, we found that administration of GnRHa for 7 days significantly increased LHβ and ANXA5 mRNA levels in the pituitary gland of *hpg* mice (Fig. 3A-B), suggesting that ANXA5 expression is sustained by GnRH in wild mouse. Immunohistochemistry also showed that GnRH-stimulated LHβ and ANXA5 expression (Fig. 4). Not only the intensity of the LHβ signal but also the area of LHβ-positive cells was increased after GnRHa administration. As GnRH stimulates the proliferation of gonadotropes [25], it appears that GnRH increases the number of gonadotropes in the pituitary gland of *hpg* mice.

Differences in the spatial distribution of ANXA5 and LHβ (Fig. 4) suggest the abundance of ANXA5-positive but LHβ-negative cells in the pituitary gland of *hpg* mice after GnRHa administration. Furthermore, the distribution of ANXA5 in GnRHa-treated *hpg* mice differed from that of untreated WT mice (Fig. 2B), suggesting that normal secretion of GnRH in WT mice regulates ANXA5 expression differently than repetitive administration of GnRHa in *hpg* mice. GnRHa administration has been suggested to facilitate gonadotropin secretion, and thus ANXA5 synthesis may pre-

cede LH β synthesis in premature gonadotropes. Also, ANXA5 has been postulated to be involved in cell cycle-related phenomena [26] and apoptosis [23].

The results of the present study demonstrate that ANXA5 expression is supported by secretion of hypothalamic GnRH and suggest a close relationship between gonadotropin synthesis and ANXA5 expression. Thus, ANXA5 could serve to regulate the physiologic secretion of LH.

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