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NOTE

Physiology

Changes in the expression of annexin A1 in the anterior pituitary gland after ovariectomy in rats

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ABSTRACT. The expression of annexin A1 (ANXA1) is augmented by gonadotrophin releasing hormone (GnRH) in LβT2 gonadotroph. We examined the distribution of ANXA1 in the pituitary tissues and the effect of ovariectomy. ANXA1 was mainly stained on folliculostellate cell-like irregular shaped cells with extended process of adult female rats. Large gonadotroph, so called castration cells, appeared two weeks after the ovariectomy. ANXA1 in castration cells exists around cells although another GnRH responsive annexin, ANXA5, was apparent also in the cytoplasm. The pituitary expression of ANXA1 after ovariectomy was significantly higher than intact rats. These difference in tissue distribution of two annexins suggest ANXA1 and ANXA5 bear different physiological function in the gonadotroph under GnRH regulation.

KEYWORDS: annexin A1 (ANXA1), annexin A5 (ANXA5), gonadotrophin releasing hormone (GnRH), gonadotroph

Annexin A5 (ANXA5) is expressed in the rat pituitary gonadotroph and gonadotrophin releasing hormone (GnRH) stimulates the expression of ANXA5 [8, 9]. ANXA5 was shown to augment gonadotrophin secretion and enhance GnRH action on gonadotrophin secretion [7, 10]. Annexin is a family of structurally and functionally related 12 proteins [6, 11]. So, it was interesting to know whether GnRH stimulates other annexins or not. We examined this and have published recently that the expression of ANXA1 and ANXA5 mRNA but not other annexins is augmented by GnRH in the gonadotroph [5]. The response of expression rate to GnRH stimulation was much higher in ANXA1 than ANXA5 [5, 13]. The expression of ANXA1 is very low in LβT2 gonadotroph and it was strongly stimulated by GnRH agonist. LβT2 is a clonal cell line established from pituitary tumor that was induced by gene construction of promoter region of luteinizing hormone (LH) β subunit and SV40 large T antigen [18]. We showed that ANXA1 also stimulates LH release [4]. ANXA1 was found as a mediator protein of cortisol to suppress the activity of phospholipase A_2 [2]. It plays a significant role of anti-inflammatory effect of cortisol [3]. Later it was shown that ANXA1 would mediate negative feedback action of cortisol on adrenocorticotrophic hormone (ACTH) release [1].

ANXA1 would be also involved in gonadotrophin secretion. However we recently observed that activin significantly suppressed only ANXA5 expression [12]. So, it is very interesting whether ANXA1 and ANXA5 share the same function or play a different role in the gonadotroph. In the present study, we examined the effect of physiologically enhanced GnRH milieu, namely ovariectomy, on ANXA1 expression and distribution in the gonadotroph in rats.

Adult female Wistar-Imamichi rats bred in our laboratory were used. They were maintained in temperature and light conditioned (14L:10D, lights on 5:00 hr) rooms. Ovariectomy or sham-operation was performed under light anesthesia with isoflurane. All animal experiments in this study were conducted in accordance with the guideline for animal experiments and the animal management manual of the School of Veterinary Medicine, Kitasato University.

Two weeks after the operation, pituitary glands were collected after decapitation and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE, 12% gel) and Western blotting. Proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Amersham Hybond, SigmaAldrich, Tokyo, Japan). The membrane was blocked with 5% non-fat milk containing 1% Tween 20 in phosphate buffered saline (PBS) for 1 hr at room temperature with gentle shaking. ANXA1 was immunodetected

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with a 1:10,000 dilution of a polyclonal anti-ANXA1 rabbit polyclonal antibody (AB_2533983, catalog # 71-3400, Thermo Fisher Scientific, Yokohama, Japan) in blocking solution at 4°C overnight. Anti-rabbit IgG-conjugated horseradish peroxidase goat serum (AB_228341, Thermo Fisher Scientific) at a 1:50,000 dilution was used as the secondary antibody to detect ANXA1 immunoreactivity with ECL Western Blotting Detection Reagents (Cytiva, Tokyo, Japan). Chemiluminescence was detected using the ImageQuant LAS 4000 series. The membrane was directly re-probed with a 1:1,000 dilution of a monoclonal anti-β–actin antibody (sc-47778, Santa Cruz, CA, USA) as an internal control. The second antibody for β–actin immunoreactivity was anti-mouse IgG-conjugated with horseradish peroxidase goat serum (AB_2533947, Thermo Fisher Scientific) at a 1:20,000 dilution. The intensity of each protein was measured by densitometry with LAS 4000 respectively. ANXA1 content was standardized to each internal standard β-actin.

For immunohistochemistry, adult female rats were deeply anesthetized with isoflurane, and then the right atrial auricle was cut off. PBS and 4% paraformaldehyde in PBS were perfused from the left ventricle. Pituitary gland was dissected, immersed in 4% PFA solution and kept at 4°C overnight. It was then washed in PBS at 4°C overnight. Paraffin embedding was performed per standard procedure and sections (4 µm thickness) were subjected to immunohistochemistry. Primary antibodies were homemade anti ANXA5 (AB_2827744, Anti-ANXA5 rabbit serum) and a polyclonal rabbit anti-ANXA1 antibody (AB_2533983, Thermo Fisher Scientific). Anti-ANXA5 rabbit serum was raised by in our laboratory and its application for immunohistochemistry and Western blotting was already reported [15, 17]. Dried sections were processed through a series of xylene and ethanol rinses to replace the paraffin with water per standard procedure [8]

Specimens were treated with 1% H_2O_2-methanol for 20 min to suppress intrinsic peroxidase. Following a blocking treatment of 5% horse serum for 1 hr at room temperature, incubation with a primary antibody (1:5,000 for anti-ANXA5, 1:1,000 for anti-ANXA1) was performed at 4°C in a humidified atmosphere overnight. Normal rabbit serum was used instead of a primary antibody for the negative control, which showed no signal under the same conditions. The secondary antibody was from the Immpress reagent anti-rabbit IgG

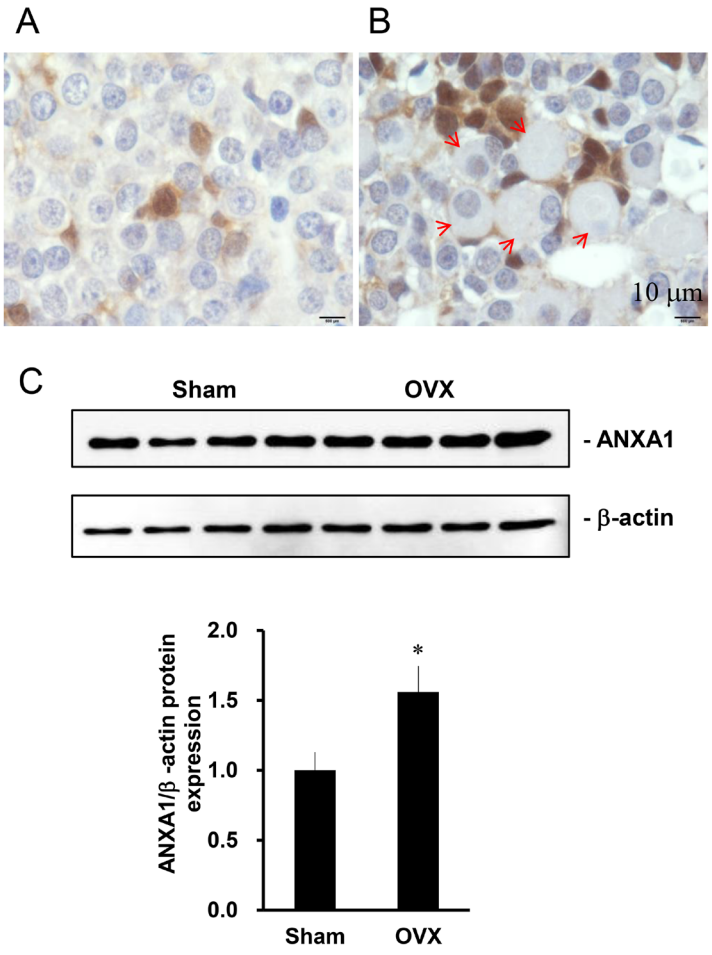

Fig. 1. Changes in annexin A1 (ANXA1) in the pituitary tissues 2 weeks after ovariectomy in rats. (**A**), (**B**) Pituitary tissues of sham operated and ovariectomized rats 2 weeks before were subjected to immunohistochemistry for ANXA1 and ANXA5. Scale bar is 10 µm. Large castration cells (red arrows) appeared 2 weeks after ovariectomy. (**C**) Whole pituitary gland of sham operated or ovariectomized rats (OVX, n=4 respectively) were homogenized and sonicated in sample buffer. Protein sample was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. β-actin was utilized as internal control. The expression of ANXA1/β-actin was shown as mean ± SEM. **P*<0.05.

POD kit (Vector Laboratories, Burlingame, CA, USA). Sections were counterstained with hematoxylin.

Some of specimens were subjected to immunofluorescence analysis. Fluorescent dye labeled second antibody was Alexa488 labeled anti-rabbit IgG (Thermo Fisher Scientific). Specimens mounted with VECTASHIELD Mounting Medium with/without 4',6-diamidino-2-phenylindol (DAPI, Vector Laboratories, Newark, CA, USA) and observed with a confocal laser microscope (Zeiss LSM7700, Tokyo, Japan).

Means of two groups were tested with Student's *t* test and when $P<0.05$, the difference of means was considered significant.

ANXA1 was distributed mainly in small cells with elongated process. They morphologically resemble folliculostellate cells (Fig. 1A). The distribution of ANXA1 to folliculostellate cells was already reported [14]. Beside folliculostellate cells, ANXA1 positive cells with lower intensity of immunostaining were also observed (Fig. 1A). Gonadotrophs are thought to be included in these positive cells, as ANXA1 was observed after ovariectomy on large round cells (Figs. 1B, 2B, 2C). They are hyperactive gonadotrophs those appeared by extinction of negative feedback by removal of the ovary and called castration cells. Present study clearly demonstrates that ANXA1 expression is increased in the pituitary gland after ovariectomy (Fig. 1C). Further we have already demonstrated that clonal cell line of gonadotroph, LβT2, expresses ANXA1 [5]. So, ANXA1 is thought to be synthesized in the gonadotroph beside folliculostellate cells as well as ANXA5. However, cytoplasm ANXA1 was rather low and ANXA1 was intensely distributed to cell periphery in the castration cell. It is different from the distribution of ANXA5 that was demonstrated intra-cellularly (Fig. 2E, 2F). Because ANXA1 was shown to be moved to plasma membrane and secreted by cortisol stimulation [16], it could be hypothesized that ANXA1 is externalized also under OVX condition.

ANXA1 and A5 are both a member of annexin family protein and have similar molecular weights (37k and 36k) showing common characteristic of calcium-phospholipid binding [6]. Both ANXA1 and ANXA5 consist of four repeats of about 60 amino acid sequence [11]. ANXA1 was first found as an inhibitor of phospholipase A_2 that mediates anti-inflammatory effect of cortisol [2]. ANXA1was shown to suppress ACTH secretion as an effector of cortisol [1]. It was proposed that ANXA1 was supplied by folliculostellate cells to the corticotroph [14]. Although an involvement of ANXA1 in ACTH secretion was already demonstrated [1], its relation to gonadotrophin secretion is a relatively new finding [5]. As ANXA1 suppresses ACTH secretion and stimulates gonadotrophin secretion, the control mechanisms in both corticotroph and gonadotroph are interesting.

Although both ANXA1 and ANXA5 are related to gonadotrophin secretion, present data reveal different response between these two annexins in the tissue distribution after ovariectomy. It is suggested distinct physiological function would be performed by ANXA1 and A5 under GnRH regulation.

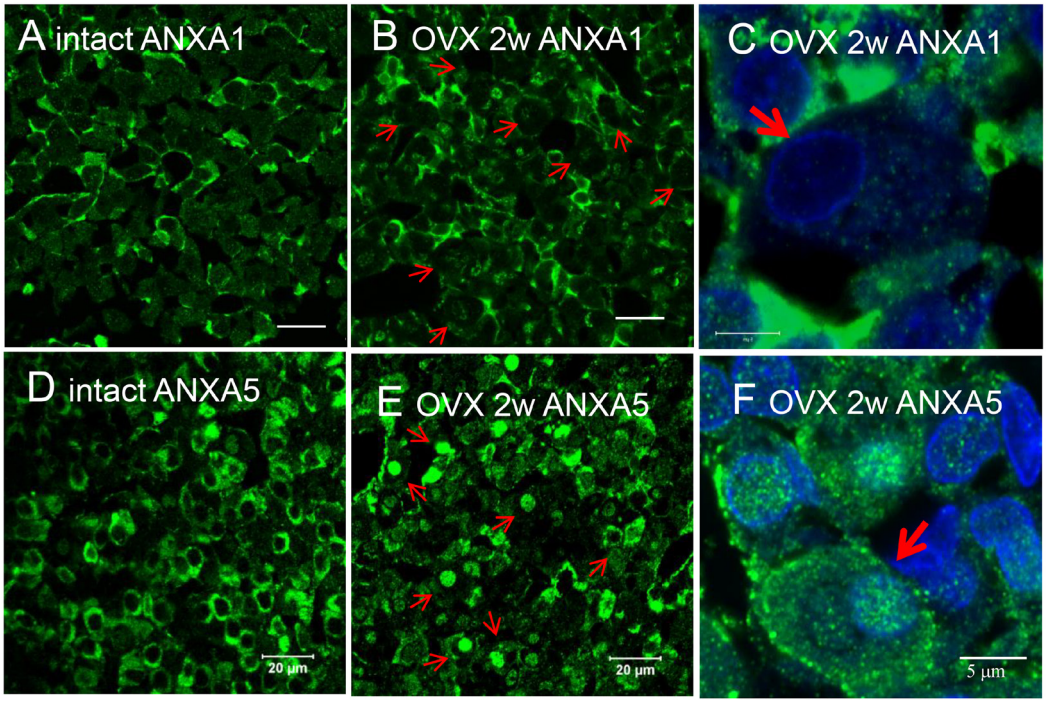

Fig. 2. Immunohistochemical analysis of ANXA1 in the pituitary tissues of 2 week after ovariectomy. Pituitary tissues of intact and ovariectomized (OVX, 2 weeks before) rats were subjected to immunohistochemistry for ANXA1 (**A**, **B**, **C**) and ANXA5 (**D**, **E**, **F**). Large castration cells (red arrows) are found in the pituitary of OVX rat. Green signal reveals ANXA1 and ANXA5. Blue in C and F is 4',6-diamidino-2-phenylindol (DAPI). Cytoplasm of castration cell shows low immune-reaction of ANXA1, and extracellular space and other cells are more intensive. ANXA5 intensely distributes to cytoplasm. Intranuclear positive staining was seen. ANXA5 is green color and blue is DAPI.

CONFLICT OF INTEREST. There is no conflict of interest in this study.

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