Activin A specifically suppresses the expression of annexin A5 mRNA and augments gonadotropin-releasing hormone stimulation of A1 expression in LβT2 gonadotrope cells

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Abstract. Recently, we reported that gonadotropin-releasing hormone (GnRH) stimulates annexin A1 (Anxa1) and A5 (Anxa5) mRNA expression through the GnRH-receptor-mitogen-activated protein kinase cascade in LβT2 cells. As LβT2 cells respond to activin, we examined the effect of activin A on Anxa1 and a5 expression in LβT2 cells. Activin A (0.4 and 4 ng/mL) treatment decreased Anxa5 mRNA levels in a dose-dependent manner, but did not affect Anxa1 mRNA levels at concentrations up to 40 ng/mL. After activin A treatment (4 ng/mL), Anxa5 mRNA levels significantly decreased at 6 h, gradually declined until 24 h, and remained low until 48 h, whereas Anxa1 mRNA levels did not significantly change following treatment. Pretreatment with activin A for 24 h increased GnRH agonist (GnRHa)-induced Anxa1 increase by approximately 7-fold compared with GnRHa stimulation alone, but Anxa5 was not affected. As previously reported, these activin A treatments increased gonadotropin β subunit and GnRH receptor mRNA levels and slightly decreased common α-glycoprotein subunit mRNA levels. Furthermore, we examined the effect of activin A on Nr4a3, which is repressed by ANXA5 and which reduces Fshb expression, and found that activin A augmented Nr4a3 expression and slightly decreased the GnRHa-induced increase in Nr4a3. These results suggest that in gonadotrope cells, the mechanism regulating Anxa1 and a5 expression is differentially coupled with activin A signal transduction. Activin A suppresses Anxa5 expression under increased Nr4a3 expression, whereas activin A and GnRH synergistically stimulate Anxa1 expression. These GnRH-inducible annexins may have different specific functions in gonadotropes.

Key words: Activin A, Annexin A1, Annexin A5, Gonadotropin-releasing hormone (GnRH), Gonadotrope

ANNEXINS (ANXs) constitute a family of structurally related proteins that have calcium-dependent phospholipid binding abilities [1, 2]. In vertebrates, there are 12 annexins, namely ANXA1–ANXA11 and ANXA13 [3]. ANXAs consist of a conserved C-terminal core domain, four (eight in ANXA6) ~60 amino acid sequence repeats, and a variable N-terminus [1]. Annexins have various functions, including membrane repair, signaling, hormone secretion, inhibition of blood coagulation, and regulation of inflammation, and they serve as biomarkers of various pathophysiological changes [1, 2, 4]. We demonstrated that both ANXA1 and A5 are expressed in the pituitary glands of female rats and are increased by ovariectomy [5-7]. Anxa1 and a5 are also expressed and stimulated by a GnRH agonist (GnRHa) through the GnRH receptor (GnRH-R)-mitogen-activated protein kinase (MAPK) cascade in a mouse gonadotrope-derived cell line (LβT2) [8, 9]. Moreover, ANXA5 increases basal and GnRHa-induced luteinizing hormone (LH) and follicle-stimulating hormone (FSH) secretion. Furthermore, treatment with an antisense oligodeoxynucleotide targeting Anxa5 decreases GnRHa-induced LH secretion in rat anterior pituitary cell primary cultures [5], whereas ANXA1 increases basal LH secretion in LβT2 cells [7]. This suggests that these ANXAs must be directly involved in GnRH acceleration of gonadotropin secretion, however their roles are yet to be elucidated.

Activins were identified based on their ability to regulate FSH secretion from the pituitary gland [10, 11]. Activins exert diverse effects on several tissues, such as promoting cell growth, differentiation, and death [12-17]. Activins are the homo- and hetero-dimers of β-subunits which have two different forms, βA and βB. The three isoforms of activin are activin A (composed of
βAβA), activin AB (βAβB) and activin B (βBβB) [18]. Activins bind to the activin type II receptor, which forms a complex with the type I receptor and phosphorylates it, triggering the phosphorylation of SMAD2 and SMAD3 [19, 20].

Previously, we demonstrated that Anxa1 and a5 are expressed and stimulated by GnRHa through the GnRH receptor (GnRH-R)-MAPK cascade in LβT2 cells, which expresses gonadotropin subunits, LHβ, FSHβ, and common α-glycoprotein subunit (αGSU), and GnRH-R [5, 8, 9, 21, 22]. LβT2 cells also express the activin βB subunit and activin receptors [23, 24], and respond to activin A. In this cell type, activin A stimulates the secretion of FSH [25] and the expression of Lhb [26, 27], Fshb [28, 29], and Gnrh-r [26]. In addition, we demonstrated that the expression of Fshb mRNA decreased without changes in Lhb expression but with an increase in the nuclear receptor 4A3 (Nr4a3) mRNA level in the pituitary glands of Anxa5-deficient mice [30]. Moreover, Nr4a3 knockdown using siRNA resulted in an increase in Fshb mRNA levels in LβT2 cells [30]. Nr4a3 is an orphan nuclear receptor [31], and the gene encoding it is an immediate early gene that is induced by GnRH receptor [30]. Thus, it is likely that the expression of Nr4a3 is affected by ANXA5 and that Nr4a3 is involved in the regulation of FSHβ expression in gonadotrope cells.

In this study, we examined the effect of activin A on the expression levels of Anxa1 and a5, as well as Nr4a3, in LβT2 cells. Here, we demonstrated the differential regulation of Anxa1 and a5 by activin A, the drastic stimulatory effect of activin A on GnRH-induced Anxa1 expression, and the inhibitory effect of activin A on Anxa5 expression during increased Nr4a3 expression.

Materials and Methods

Materials
Activin A was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). GnRHa (Des-Gly10 [Pro9]-GnRH ethylamide; Conceral) was obtained from Nagase Pharmaceuticals Co., Ltd. (Osaka, Japan).

Cell culture
LβT2 cell line was provided by Professor P. Mellon (University of California, San Diego, CA, USA). LβT2 cells express all gonadotropin subunit genes and GnRH-R [32]. The cells were grown in 75-cm² flasks containing Dulbecco’s modified Eagle’s medium with a high concentration of glucose (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Biowest, Nuaillé, France) and an antibiotic-antimycotic mixture (penicillin 100 U/mL, streptomycin 100 μg/mL, and amphotericin B 250 ng/mL; Invitrogen). The culture was maintained in an atmosphere of 95% air, 5% CO₂, and 100% humidity. The cells were subcultured until they reached confluence. A total of 10⁶ cells in 2 mL of medium were plated in 35-mm plastic dishes, and the corresponding experiments were performed after 2 days.

Gene expression analyses
To examine the effect of different concentrations of activin A on Anxa1 and a5 mRNA levels, the cells were treated with 0.4, 4, and 40 ng/mL activin A for 24 h. To examine the time course of the changes in Anxa1 and a5 mRNA levels after activin A treatment, the cells were harvested at 1, 3, 6, 12, 24, and 48 h after changing to a medium containing 4 ng/mL activin A. Furthermore, to examine the effect of activin A on GnRHa-induced Anxa1, Anxa5, and Nr4a3 mRNA expression, the cells were treated with 4 ng/mL activin A for 24 h. The medium was replaced with fresh medium containing 10 nM GnRHα, and the cells were incubated for 6 h to measure Anxa1 and a5 expression levels and for 1 h to measure Nr4a3 expression levels.

RNA extraction and complementary DNA synthesis
The total RNA was extracted using the acid guanidium thiocyanate-phenol-chloroform extraction method with TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. Briefly, the cell growth medium was removed, and 500 μL of TRIzol reagent was added. The cell suspension was transferred to a plastic 1.5-mL centrifuge tube, and 100 μL of chloroform was added. The mixture was centrifuged at 12,000 × g for 15 min. The aqueous phase was transferred to a new tube, and the RNA was precipitated using isopropanol. The total RNA (1 μg) was treated with RNase-free DNase I (Invitrogen) to exclude genomic DNA and was reverse-transcribed using 200 U ReverTra Ace (TOYOBO, Osaka, Japan) and 10 pmol random primers (Invitrogen).

Real-time polymerase chain reaction (PCR)
Real-time PCR was performed using SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) and QuantStudio (Applied Biosystems). Previously described primers for Anxa1, Anxa5, and Rpl19 [6, 9] and for common α-glycoprotein subunit (Cga), Lhβ, Fshβ, and Nr4a3 [30] were used for each PCR assay. Primer sequences used for PCR are listed in Table 1. Each mRNA level was standardized by dividing it with the expression level of Rpl19 mRNA in the same sample.

Statistical analysis
Data were calculated by dividing the value of each sample by the mean value of the corresponding control

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group to obtain the relative amounts. Statistical analyses were performed using KaleidaGraph version 4.5 software (Synergy Software, Reading, PA, USA). Data are expressed as the mean ± standard error of the mean (SEM). They were statistically evaluated using Tukey’s multiple comparison test. Statistical significance was set at $p < 0.05$.

Results

**Effect of activin A on Anxa1, Anxa5, and Nr4a3 mRNA expression**

Treatment with 0.4 and 4 ng/mL activin A decreased the mRNA level of Anxa5 in a dose-dependent manner (Fig. 1B), whereas the Anxa1 and Nr4a3 mRNA levels were not affected by treatment with activin A at concentrations up to 40 ng/mL (Fig. 1A, C).

We then examined the time course of changes in mRNA levels after treatment with 4 ng/mL activin A. The mRNA level of Anxa1 did not significantly change (Fig. 2A). The Anxa5 mRNA level significantly decreased from 6 h, gradually declined until 24 h, and remained low until 48 h after treatment (Fig. 2B). Nr4a3 expression significantly increased at 1 h, declined until 6 h, and then began to increase again and was significantly elevated at 24 and 48 h (Fig. 2C).

**Effect of activin A on Cga, Lhb, Fshb, and Gnrh-r mRNA expression**

Treatment with 4 ng/mL activin A for 24 h significantly increased the levels of the transcripts encoding Lhb, Fshb, and Gnrh-r. Treatment with 40 ng/mL activin A caused a further increase in the mRNA levels of these molecules (Fig. 3B–D). The expression of the Cga transcript significantly decreased with 40 ng/mL activin A treatment (Fig. 3A). The effect of activin A on Fshb expression was approximately 30-fold higher than that on Lhb expression. The mRNA levels of Cga significantly decreased at 1 h and from 3 to 48 h post-treatment (Fig. 4A), whereas the mRNA levels of Lhb, Fshb, and Gnrh-r significantly increased from 3, 1, and 6 h, respectively, peaked at 24 h, and slightly declined at 48 h (Fig. 4B–D).

**Effect of activin A on GnRH-induced gene expression**

Next, the effect of 4 ng/mL activin A on GnRH-induced gene expression of Anxa1, Anxa5, and Nr4a3 was examined. GnRHa increased the Anxa1 mRNA level, and pretreatment with activin A further augmented this increase by approximately 7-fold compared with GnRHa stimulation alone (Fig. 5A). Pretreatment with activin A did not affect the GnRHa-induced increase in Anxa5 mRNA level, although the Anxa5 mRNA level decreased in the absence of GnRHa (Fig. 5B). Pretreatment with activin A slightly, but significantly, decreased the GnRHa-induced increase in Nr4a3 mRNA level (Fig. 5C).

Discussion

In the present study, we demonstrated that treatment with activin A decreased the Anxa5 mRNA level within 6 h but did not affect Anxa1 mRNA levels until 48 h in LβT2 cells. Furthermore, activin A treatment considerably enhanced the GnRHa-mediated increase in Anxa1 mRNA levels compared with those achieved with GnRHa stimulation alone. To the best of our knowledge, this is the first study to investigate the effect of activin A on the expression levels of Anxa1 and a5 mRNA in gonadotrope cells and to demonstrate that activin A differentially regulates them. A previous study reported that

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**Table 1** List of primers

| Primers | Forward (5’-3’) | Reverse (5’-3’) |
|---------|----------------|----------------|
| Anxa1   | ATGGTGCTGCTTGCATGACAAA | CCAAGGCGCTTCCATGCTC |
| Anxa5   | CTCTGGTTGGGCAGGGACCTT | GCCATGCTAGGCTCTGAG |
| Cga     | ATCCACCTGCCAGAGACAT | ACATGGACAGCAGGACAGA |
| Lhb     | GTCTGCATCCACCTTCACACC | GTAGGTGACATGGCTGAG |
| Fshb    | CTGCCATGACTGTCCGAGAAT | GAGCGTGGTCCTTATACAGCA |
| Gnrh-r  | CTCAAGACTTGGTTGGCAAGACC | ATGCCAACCAGCTGAGAGCTG |
| Nr4a3   | CCGACCTTAAACAGATGCAA | AGGTTCTGAGACAGCTCAAT |
| Rpl19   | CTGATCAAGGAGGCTGATGAT | TTCAGCCTGTGGAGTGTC |

Anxa1—annexin A1; Anxa5—annexin A5; Cga—common α-glycoprotein subunit; Lhb—LHβ subunit; Fshb—FSHβ subunit; Gnrh-r—GnRH-receptor; Nr4a3—nuclear receptor 4A3; Rpl19—ribosomal protein L19
GnRH significantly increased the expression of both Anxa1 and a5 in LβT2 cells [6]. Additionally, ANXA5 has been reported to increase LH secretion in rat anterior pituitary cells [5], and a preliminary study showed that ANXA1 promotes LH secretion in LβT2 cells [7]. Thus, ANXA1 and A5 have shown similar responses to GnRH and similar effects on LH secretion in pituitary cells. However, the responses of Anxa1 and a5 expression to activin A were notably different. Activin A inhibited Anxa5 expression, but enhanced GnRH-stimulated Anxa1 expression. Although the reason underlying this differential behavior has not yet been investigated, these results suggest that in gonadotrope cells the mechanism regulating Anxa1 and a5 expression is differentially coupled with activin A signal transduction, and that these

GnRH-inducible annexins—ANXA1 and A5—might have different specific functions in gonadotropes. The present study also demonstrated that activin A potently augmented the GnRH-mediated increase in Anxa1 expression in LβT2 cells. Activin A can augment GnRH-mediated gene expression of Fshb in LβT2 cells [26, 28, 33] and rat pituitary cells [34], Lhb in LβT2 cells [26], and Garh-r in LβT2 cells [26] and other gonadotrope cell lines, such as αT3-1 cells [35]. GnRH-mediated Fshb transcription is regulated by p38 and ERK1/2 [27, 28]. Inhibition of p38 activity completely abolished the synergistic effects of activin A and GnRH [33], whereas the inhibition of MEK activity partially reduced the synergistic effects of activin A and GnRH [33, 34]. Cotreatment with activin A and GnRH

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**Fig. 1** Effects of activin A on Anxa1 (A), Anxa5 (B), and Nr4a3 (C) mRNA levels in LβT2 cells

The cells were treated with activin A (0.4, 4, or 40 ng/mL) and harvested after 24 h of incubation. Values represent the mean ± SEM (n = 5) and are presented as a ratio to the control value. Data labeled with different letters are significantly different from each other (p < 0.05, Tukey’s multiple comparison test).

**Fig. 2** Changes in Anxa1 (A), Anxa5 (B), and Nr4a3 (C) mRNA levels in LβT2 cells after activin A stimulation

The cells were treated with activin A (4 ng/mL) and harvested after 0, 1, 3, 6, 12, 24, and 48 h of incubation. Values represent the mean ± SEM (n = 5) and are presented as a ratio to the value at 0 h. The asterisks (*) indicate a significant difference from value at 0 h of each group (p < 0.05, Tukey’s multiple comparison test).
Fig. 3  Effects of activin A on Cga (A), Lhb (B), Fshb (C), and Gnrh-r (D) mRNA levels in LβT2 cells
The cells were treated with activin A (0.4, 4, or 40 ng/mL) and harvested after 24 h of incubation. Values represent the mean ± SEM (n = 5) and are presented as a ratio to the control value. Data labeled with different letters are significantly different from each other (p < 0.05, Tukey’s multiple comparison test). Cga, common α-glycoprotein subunit; Gnrh-r, GnRH receptor.

Fig. 4  Changes in Cga (A), Lhb (B), Fshb (C), and Gnrh-r (D) mRNA levels in LβT2 cells after activin A stimulation
The cells were treated with activin A (4 ng/mL) and harvested after 0, 1, 3, 6, 12, 24, and 48 h of incubation. Values represent the mean ± SEM (n = 5) and are presented as a ratio to the value at 0 h. The asterisks (*) indicate a significant difference from the value at 0 h for each group (p < 0.05, Tukey’s multiple comparison test). Cga, common α-glycoprotein subunit; Gnrh-r, GnRH receptor.
increased SMAD3 phosphorylation compared with activin A treatment alone, and this increase was reduced by the inhibition of p38 activity [33]. Thus, MAPKs, including p38 and ERK1/2, appear to be involved in the synergistic effect of activin A and GnRH on Fshb expression. These mechanisms might be involved in the induction of Anxa1 expression by activin A and GnRH. However, in this study, activin A alone did not affect the expression of Anxa1, whereas it stimulated the expression of Fshb. Thus, activin A augments GnRH-mediated Anxa1 expression, but does not increase Anxa1 expression independently; whereas, both activin A and GnRH increase Fshb expression, and activin A synergistically (or additively) increases GnRH-mediated Fshb expression. Therefore, the mechanism regulating the effects of activin A in GnRH-induced Anxa1 expression is likely to be different from that in GnRH-induced Fshb expression.

Another possible explanation for the enhancement of GnRH-mediated Anxa1 expression by activin A may rely on the activation of GnRH-R. Activin A has been reported to stimulate Gnrh-r expression in αT3-1 cells [35, 36] and LβT2 cells [26]. This study confirmed the stimulatory effect of activin on Gnrh-r expression and that this effect persisted until 48 h after activin A treatment. Furthermore, our previous study showed that treatment with 10 nM 12-O-tetradecanoylphorbol 13-acetate (TPA) stimulated Anxa1 transcription and that 1 μM TPA further increased it in LβT2 cells, whereas the mRNA level of Anxa5 was similar after stimulation with 10 nM and 1 μM TPA [9]. Thus, the activity of PKC increased, accompanied by an increase in the expression of Anxa1, whereas Anxa5 expression remained constant. The increase in Gnrh-r expression by activin A treatment might augment the function of GnRH, resulting in an increase in PKC activity. This may partially explain how activin enhanced GnRH-mediated Anxa1 expression without affecting GnRH-mediated Anxa5 expression.

As we recently found that Nr4a3 expression is suppressed by ANXA5 and that Nr4a3 inhibits Fshb expression in gonadotropes [30], we also examined the effect of activin A on Nr4a3 expression in this study. Treatment with activin A significantly increased Nr4a3 expression, peaking at two time points. The first peak occurred at 1 h and the second started at 12 h and became significant 24–48 h after treatment. Treatment with activin A decreased the Anxa5 mRNA levels from 6 h, reaching the lowest levels at 24 and 48 h. As Nr4a3 mRNA levels increased in ANXA5-deficient mice [30], the second peak observed at 24 and 48 h could be due to the decline in Anxa5 expression. In this case, as the first peak occurred in the early phase (1 h), the first peak might be a direct effect of activin A. As the expression of Fshb is augmented by the same condition of activin A administration, it is apparent that the relationship between Nr4a3 and FSHβ is not simple. In LβT2 cells, GnRHa stimulated Nr4a3 expression with a peak at 1 h, this GnRHa-induced increase was reduced by ANXA5 treatment, and the Nr4a3 suppression with siRNA resulted in an increase in Fshb expression levels [30]. The present study showed that the GnRHa-induced increase in Nr4a3 expression was decreased by activin A treatment. Although this suppressive effect of activin A was not large, this change in Nr4a3, a GnRH-inducible immediate early gene, might be involved in GnRH-induced changes, such as Anxa1 and Fshb expression.

![Fig. 5](image-url) Effects of activin A on GnRHa stimulation of Anxa1 (A), Anxa5 (B), and Nr4a3 (C) mRNA levels in LβT2 cells

The cells were untreated or treated with activin A (4 ng/mL) for 24 h. The medium was then replaced with medium containing 10 nM GnRHa, and the incubation was continued for 6 h (A, B) and 1 h (C). Values represent the mean ± SEM (n = 5) and are presented as a ratio to the control value. Data labeled with different letters are significantly different from each other (p < 0.05, Tukey’s multiple comparison test).
Furthermore, we found a substantial increase in the \textit{Nr4a3} mRNA level in the afternoon of proestrus, just before the increase in the \textit{Fshb} mRNA level in rats. We speculated that the sequence of events in the expression of \textit{Nr4a3} and \textit{Anxa5} would be important for the significant augmentation of \textit{Fshb} expression on proestrus [37]. Although \textit{Nr4a3} expression was definitely regulated by activin A, a functional relationship of this gene with \textit{Anxas} and \textit{Fshb} needs further investigation.

Most previous studies focusing on \textit{Fshb} regulation in \textit{LβT2} cells have performed luciferase assays using reporter constructs with the \textit{33, 38}. \textit{LβT2} cells express \textit{Fshb} mRNA, and its expression is stimulated by activin. Using an RNase protection assay, Graham et al. [25] reported that treatment with activin in \textit{LβT2} cells induced \textit{Fshb} mRNA expression within 48 h. Similarly, using RT-PCR, Yamada et al. [26] reported that treatment with activin in \textit{LβT2} cells induced \textit{Fshb} mRNA expression within 6 h and that activin also induced \textit{Lhb} mRNA expression. In the present study, we confirmed the action of activin on \textit{Fshb} and \textit{Lhb} mRNA expression levels. Furthermore, we demonstrated that \textit{Fshb} and \textit{Lhb} mRNA levels significantly increased 1 and 3 h after activin A treatment, respectively, and that the increases in the mRNA levels of both were maintained until 48 h after treatment. Additionally, Yamada et al. reported that activin A did not affect the \textit{Cga} mRNA level [26]. In line with this result, the changes in the \textit{Cga} mRNA level observed in the present study were small, supporting that \textit{Cga} regulation by activin may be insignificant.

In summary, activin A decreased the \textit{Anxa5} mRNA levels in \textit{LβT2} cells until 48 h post-treatment, with an increase in \textit{Nr4a3} expression and no effect on \textit{Anxa1} mRNA levels. In contrast, activin A augmented the \textit{GnRH}-mediated increase in \textit{Anxa1} mRNA levels but did not affect \textit{Anxa5} mRNA levels. To the best of our knowledge, this is the first study to report that activin A differentially regulates the expression of \textit{Anxa1} and \textit{a5} in gonadotrope cells. Although this study does not completely elucidate the physiological significance of ANXAs, it indicates activin as one of the factors that induce ANXAs. These finding are likely to be important in understanding the physiological significance of ANXAs by revealing the relationship between annexin and activin, in addition to GnRH, in the future.

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Disclosure

The authors declare no conflicts of interest associated with this research.

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