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Research Article

RFRP-3, the mammalian ortholog of GnIH, induces cell cycle arrest at G2/M in porcine ovarian granulosa cells

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ABSTRACT

RFamide-related peptide-3 (RFRP-3), the mammalian ortholog of gonadotropin-inhibitory hormone (GnIH), has been proposed as a key inhibitory regulator of mammal reproduction. Our previous studies have demonstrated that RFRP-3 inhibited the expression of proliferation-related proteins in porcine granulose cells (GCs), but the inhibitory mechanism causing this has not been discovered. Here, we aim to elucidate the underlying mechanism and determine the cell cycle regulatory sites of action of RFRP-3 on porcine GC proliferation. To this end, the viability of porcine GCs was initially estimated by cell counting kit-8 (CCK-8). We confirmed that different doses of RFRP-3 decreased the cellular viability, suggesting that RFRP-3 could inhibit the proliferation of GCs. Subsequently, we evaluated the direct effects of RFRP-3 on the expression of cell cycle regulators. Compared to the control treated cells, 10^{-6} and 10^{-8} M of RFRP-3 effectively reduced the transcription of Cyclin B1 and CDK1 mRNAs. However, treatment with RFRP-3 did not alter Cyclin A2, Cyclin D1, CDK2, or CDK4 mRNA levels. These results suggest that RFRP-3 might be inducing G2/M-phase arrest in porcine GCs. Finally, to further determine the molecular mechanism underlying RFRP-3-mediated G2/M cell cycle arrest, we observed the levels of G2/M cell cycle regulatory factors in RFRP-3-treated porcine GCs. The results showed that RFRP-3 treatment significantly increased the expression of Myt1, p-Wee1 and p-Cdc2, whereas the level of Cyclin B1 significantly decreased in porcine GCs treated with 10^{-6} M of RFRP-3. Taken together, our data suggest that RFRP-3 regulates the phosphorylation or expression of G2/M cell cycle regulatory factors to induce G2/M-phase arrest *via* inhibition Cyclin B-CDK1 complex activation in porcine GCs, which might provide an unfavorable condition for porcine GC proliferation.

1. Introduction

Reproductive physiology is very complex and is regulated by multiple factors, including a number of hypothalamic neuropeptides. In the last few decades, various neuropeptides have been discovered to be involved in stimulation or inhibition of reproduction. Among vertebrates, recent findings have indicated that gonadotropin-releasing hormone (GnRH) was not the primary factor responsible for the hypothalamic control of gonadotropin secretion. In 2000, Tsutsui discovered Gonadotropin-inhibitory hormone (GnIH) in quail brain, inhibiting gonadotrophin secretion from cultured pituitary cells, and its discovery opened a new window in reproductive neuroendocrinology [1]. GnIH orthologs, such as RFRP-3, are similar to GnIH in structure and function and were later discovered in other vertebrates from fish [2,3] to mammals [4–6] including pigs [7].

Extensive study of GnIH/RFRP-3 has confirmed that GnIH/RFRP-3 not only inhibits gonadotropin release in the hypothalamo-hypophysial system, mediated by its receptor GPR147 but also acts directly on the gonads of vertebrates [8–10]. There are accumulating data to indicate that GnIH/RFRP-3 and GPR147 are expressed in gonadal tissue. The binding sites for GnIH were localized in the interstitial layer and seminiferous tubules of the testis in males [11,12], as well as in ovarian theca and granulosa cells in females [13,14]. *In vivo* and *in vitro* studies have shown that GnIH/RFRP-3 is able to affect gonadal steroid secretion, development, and maintenance. In birds, the administration of GnIH induced testicular apoptosis in mature birds and suppressed normal testicular growth in immature birds [15]. A similar study in mice showed that GnIH treatment inhibited ovarian activity and follicular development [16]. Furthermore, our previous studies have demonstrated that RFRP-3 and its receptor are distributed in the granulosa cells of antral follicles in the pig ovary [7]. We also demonstrated that treatment with RFRP-3 in cultured porcine granulosa cells decreased the accumulation of the proliferation-related proteins ERK1/2, PCNA, and Cyclin B1 [17].

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Collectively, the studies described above suggest that RFRP-3 plays an important role in cell proliferation. However, the information provided above is mostly based on *in vivo* studies, and studies that investigated the underlying mechanism of how RFRP-3 regulates cell proliferation are limited. Thus, in this study, we investigated the effects of RFRP-3 on the cell cycle in porcine GCs and the roles of RFRP-3 in the regulation of cell cycle progression. The primary aim of this study was to elucidate the underlying mechanism and determine the cell cycle regulatory sites of action of RFRP-3 on the proliferation of porcine GCs.

2. Materials and methods

2.1. Drugs

Human RFRP-3 (RFRP-3 (Human), catalog No. 048-46, Phoenix Pharmaceuticals, USA) was used in the present study, as well as its corresponding amino acid sequences (Val - Pro - Asn - Leu - Pro - Gln - Arg - Phe - NH_2) coincidence with pig RFRP-3 sequences. Human RFRP-3 and porcine RFRP-3 are completely homologous.

2.2. Porcine granulose cells isolation, in vitro culture, and treatment

Porcine GCs were isolated and cultured as described previously by Hatey et al. [18]. Briefly, after a series of washes with D-Hanks' balanced salt solution and ethanol (70%), the granulosa cells were isolated from medium sized (3–5 mm) follicles from the ovaries of forty pubertal pigs. Approximately 1.0×10^6 live granulosa cells/well were cultured in 6-well plates in DMEM/F12 and 1% penicillin/streptomycin and incubated for 72 h at 37 °C in culture medium. Then, some cells were fixed for immunohistochemistry of FSHR to rate the purity of the cell culture, while the medium was replaced with DMEM/F12 containing different doses of RFRP-3 (10^{-6}, 10^{-8}, 10^{-10}, or 10^{-12} M). After incubation for 24 h, the medium was collected and assayed for cell viability by CCK-8. The cells were harvested and kept frozen at −70 °C for Western blot and semi-quantitative RT-PCR.

2.3. CCK-8 assay

Porcine GCs were isolated as described in Section 2.2. Then, 2×10^4 cells/well were cultured in 96-well plates. After culturing for 3 days, the medium was replaced with DMEM/F12 containing different doses of RFRP-3 (10^{-6}, 10^{-8}, 10^{-10}, or 10^{-12} M), followed by incubation for an additional 24 h. The supernatant was removed, and each well was washed with PBS, followed by the addition 10 μL of Cell Counting Kit-8 (CCK-8, Beyotime Institute of Biotechnology, China) solution. After the cells were incubated for 4 h, the absorbance was measured at 450 nm using a spectrophotometer (Thermo Varioskan, Finland). Four samples were tested in each group for each incubation time.

2.4. Semi-quantitative RT-PCR

Total RNA extraction and reverse transcription were performed as described by our previous study [7]. Amplification reactions were conducted in triplicate using gene-specific primers designed from the clone sequences shown in Table 1. The reaction protocols were performed using the following thermal cycling conditions: 5 min at 94 °C for enzyme activation, followed by different cycles of 5 s at 95 °C, 30 s at the appropriate annealing temperature, 15 s at 72 °C (Table 1), and a final extension step of 72 °C for 10 min. Products were electrophoresed on 1.2% agarose gel and analyzed.

2.5. Western blotting

After porcine GC treatment as described in Section 2.2, cells were washed with cold PBS and lysed in a cell lysis buffer (Beyotime) containing 1 mM of phenylmethylsulfonyl fluoride. An equal amount of protein (15–50 μg) was fractionated by SDS-PAGE and then blotted onto nitrocellulose membranes (Pall). Blotted membranes were blocked with 5% bovine serum albumin and then incubated with primary antibody at the appropriate dilution (Myt1 and Phospho-Wee1 (dilution 1:300, Cell Signaling Technology); Cyclin B1 (dilution 1:500, Cell Signaling Technology); Phospho-CDK1 (dilution 1:200, Cell Signaling Technology); and GAPDH (dilution 1:2000, Cell Signaling Technology)). After incubation with horseradish peroxidase-labeled goat anti-rabbit or anti-mouse IgG secondary antibody (dilution 1:2000, Cell Signaling Technology), the immunoreactive proteins were detected using ECL chemiluminescent chromogenic kit (Biouniquer) and exposure to X-ray film (Fuji Photo Film Co., Ltd., Tokyo, Japan). Densitometric quantification was performed using ImageJ (National Institutes of Health, Bethesda, MD) with corresponding GAPDH as the internal control for normalization. These experiments were performed in triplicate (n = 3), and a representative blot of three independent experiments has been depicted.

2.6. Statistical analysis

All data are shown as the means ± S.E.M. for at least three separate experiments. The differences were considered to be significant when $P < 0.05$. The statistical analysis was performed using one-way ANOVA and Tukey method with SPSS Statistics 17.0.

3. Results

3.1. RFRP-3 inhibits the proliferation of porcine granulosa cells

To investigate the influence of RFRP-3 on the proliferation of porcine GCs, porcine GCs treated with different doses of RFRP-3 were harvested at different times and were measured by CCK-8 assay. As shown in Fig. 1A, RFRP-3 inhibited the proliferation of porcine GCs in a dose-dependent manner after 12, 24 and 48 h treatment. Because the highest absorbance was measured after 24 h treatment, statistical analysis was performed on this group data. As shown in Fig. 1B, 10^{-6}, 10^{-8} and 10^{-10} M RFRP-3 treatment significantly ($P < 0.001$) reduced the porcine GC proliferation ability compared with the control, and 10^{-12} M RFRP-3 also showed a sharp inhibition ($P < 0.01$). These results indicate that RFRP-3 is closely related to porcine GC proliferation.

3.2. RFRP-3 affects the expression of cyclins and CDKs in porcine granulosa cells

Cyclin-dependent kinases (CDKs) and Cyclins play important roles in cell cycle progression. To further determine if RFRP-3 treatment induces cell cycle arrest, and if so, in which phase, we examined the relative levels of the CDKs and Cyclins mRNAs after treatment with different doses of RFRP-3. As shown in Fig. 2, 10^{-6} and 10^{-8} M RFRP-3 treatment showed a sharp ($P < 0.01$) decrease in Cyclin B1 mRNA expression, but inhibition of lower doses (10^{-10} and 10^{-12} M) of RFRP-3 showed no significant difference when compared to the control. A similar tendency was detected in CDK1 mRNA expression (Fig. 3). Additionally, 10^{-6} and 10^{-8} M RFRP-3 treatment showed a marked inhibition ($P < 0.05$). However, the expression levels of Cyclin A2, Cyclin D1, CDK2 and CDK4 did not show significant differences between the control and RFRP-3 treatment groups. The results showed that CDK1 and Cyclin B1 decrease significantly after RFRP-3 treatment, and Cyclin B1-Cdk1 complexes acting as a main coordinator advance the cell cycle into the M phase, suggesting that the cell cycle might be arrested at the G2/M phase in RFRP-3-treated porcine GCs.