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Thyroid Hormone Synergizes with Follicle Stimulating Hormone to Inhibit Apoptosis in Porcine Granulosa Cells Selectively from Small Follicles

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To investigate the effect of thyroid hormone on the proliferative activity and apoptosis of granulosa cells at the varying stages of follicular growth, porcine granulosa cells obtained from small (1-2 mm), medium (3-5 mm) and large (6-11 mm) follicles were cultured under a serum-free condition in the presence or absence of follicle stimulating hormone (FSH; 20 ng/ml), with or without triiodothyronine (T_3; 10^{-8}M). Relative viability, proliferative activity, and apoptosis of cultured granulosa cells were evaluated with 3-(4.5-dimethylahiazol-2-yl)-2,5-diphenyltetrazolium bromide [MTT] assay, Ki67 expression and activated caspase-3 protein expression, respectively. MTT assay showed that T_3 had no significant effect on the relative viability of granulosa cells regardless of the follicle size. Ki67-positive rate in small follicle granulosa cells was augmented by treatment with FSH whereas it was not affected by T_3. Furthermore, FSH treatment decreased activated caspase-3 protein-positive rate of small follicle granulosa cells. Relative to the treatment with FSH alone, concomitant treatment with FSH and T_3 resulted in further decrease in caspase-3 protein-positive rate in small follicle granulosa cells. Treatment with T_3 alone did not affect the caspase-3 protein-positive rate. These results suggest that thyroid hormone synergizes with FSH to inhibit apoptosis in small follicle granulosa cells without affecting the proliferative potential of those cells.

It is clinically evident that women suffering from thyroid disorders are associated with frequent occurrence of menstrual disturbances and impaired fertility, and these abnormalities are improved by restoring the euthyroid state [3, 12, 18-20]. A possible relationship between the thyroid hormone and ovarian function has been well documented in the literatures based on in vivo studies. Fredrikson and Ryden [9] noted that thyroidectomy in rabbits arrested the maturation of the ovarian follicles. Furthermore, investigations on rats by Folley [8], on rabbits by Krohn [15], and on humans by Williams and co-workers [40] showed that treatment with thyroid-suppressive drugs caused ovarian atrophy, with arrest of follicular maturation. The exact mechanism for such reproductive aberrations is not well known; however, it is conceivable that thyroid hormones might have a direct role in ovarian physiology via receptors in granulosa cells.

We have demonstrated in a monolayer culture system of porcine granulosa cells that thyroid hormone synergizes with FSH to exert direct stimulatory effects on granulosa cell

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functions, including luteinizing hormone (LH) /chorionic gonadotropin (CG) receptor formation and induction of steroidogenic enzyme, such as 3β-hydroxysteroid dehydrogenase and aromatase [21]. In addition, physiological concentration of thyroid hormone has been shown to exert optimal effects on FSH-mediated morphological differentiation of granulosa cells [21].

During ovarian follicular development, only limited numbers of follicles are selected for ovulation, whereas the remaining majority of follicles undergo atresia. It is now evident that follicular atresia is associated with internucleosomal DNA fragmentation of granulosa cells [13, 14, 35, 36]. Homeostatic control of follicular growth is thought to be the result of the dynamic balance between cell proliferation and cell death.

Thyroid hormone plays important roles in proliferation and apoptosis of various kinds of cells, such as human lymphocytes [23], chick muscle cells [27], amphibian intestinal cells and muscle cells [7, 16]. However, little information is available concerning biological role of thyroid hormone in proliferation and apoptosis of granulosa cells. The purpose of this study is to elucidate the effect of thyroid hormone on granulosa cell proliferation and apoptosis.

MATERIALS AND METHODS

Monolayer Cultures of Porcine Granulosa Cells

Porcine ovaries were obtained at a local slaughterhouse and placed into physiological saline containing 1×10^5 U/L of penicillin and 100 mg/L of streptomycin. Follicular fluids were harvested aseptically by the needle aspiration method [6] from small (1-2 mm), medium (3-5 mm), and large (6-11 mm) follicles as previously described [21]. Granulosa cells were separated from follicular fluids by centrifugation at 500 g for 5min at 4 °C, and washed twice with Dulbecco's phosphate-buffered saline (PBS). The isolated granulosa cells were plated at 2×10^6cells/well in 2-well chamber slides, and 5×10^4cells/well in 96-well tissue-culture plates. The granulosa cells were pre-cultured for 2days at 37 °C in a humidified atmosphere of 5% CO_2-95% air in DMEM/F12 supplemented with 10% fetal bovine serum (BioWhittaker, Portland, OR). Thereafter, the cultured cells were stepped down to serum-free conditions by incubating with DMEM/F12 in the presence or absence of porcine FSH (20 ng/mL, Sigma, St. Louis, MO), with or without T_3 (10^{-8} M, Sigma, St. Louis, MO) for the subsequent 48h.

3-(4.5-dimethylahiazol-2-yl)-2,5-diphenyltetrazolium Bromide [MTT] Assay for Porcine Granulosa Cell Growth and Viability

The term "growth" is used to denote a net increase in cell numbers, reflecting both proliferation and apoptosis. In the present study, granulosa cell numbers and viability were evaluated with MTT assay using the Mosmann's method [24]. Porcine granulosa cells were incubated with serum free DMEM/F12 in the presence or absence of porcine FSH (20 ng/mL), with or without T_3 (10^{-8} M) for 48h in a 96-well tissue culture plate. 10 μL of MTT (Chemicon International Inc., Temecula, CA) solution was added to each well, and the cultured cells were incubated at 37 °C for another 4h. Subsequently, 100 μL of isopropanol/0.04 N hydrochloric acid solution was added in each well and mixed thoroughly. The absorbance was measured in an MTP-120 ELISA plate reader (Corona Electric Co., Osaka, Japan) with a test wavelength of 570 nm and a reference wavelength of 630 nm.

Immunocytochemical Staining for Ki67 Antigen and Activated Caspase-3 Antigen

Granulosa cells cultured in 2-well chamber slides were washed for three times with PBS, fixed in 100% ethanol at 4 °C for 30 min, and washed with PBS for three times. The fixed cells were subjected to immunostaining, by the avidin/biotin immunoperoxidase method, using a polyvalent immunoperoxidase kit (Vectastain ABC kit, Vector Laboratories,

Burlingame, CA and Histofine Simplestain MAX-PO (Multi) kit, Nichirei, Tokyo, Japan) according to the manufacturer's instruction. A mouse monoclonal antibody against Ki67 protein at a dilution of 1:80 (Novocastra Laboratories, New Castle Upon Tyme, UK) or a rabbit polyclonal antibody against activated caspase-3 protein at a dilution of 1:250 (Promega, Madison, WI) was used as the primary antibody. To assure the specificity of the immunoperoxidase method, the primary antibody was replaced by non-immune murine IgG (Miles, Erkhardt, IN) or non-immune rabbit serum (Sigma, St. Louis, MO) at the same dilution as the specific antibody, and the replacement of the specific primary antibody with non-immune IgG or serum resulted in a lack of positive immunostaining. Immunostained slides were analyzed, in a blinded fashion, without knowledge of the experimental group. The Ki67 and activated caspase-3 labeling indices were determined by observing more than 1000 nuclei for each experimental sample and were used for evaluating the proliferating activity and apoptosis of the cells.

Data Analysis

The data were expressed as the mean±SEM. Statistical analysis was performed using one-way ANOVA with the StatView 4.1 software for Macintosh (SAS Institute, Inc., Cary, NC), followed by post hoc testing using Fisher's protected least-significant-difference test. Difference with a $P<0.05$ was considered statistically significant.

RESULTS

Effect of Thyroid Hormone on Porcine Granulosa Cell Viability

In MTT assay, treatment with T_3 had no significant effect on relative viability of small, medium and large follicle granulosa cells compared with that of control cultures (Fig. 1). On the other hand, relative viability of granulosa cells was augmented by FSH treatment,

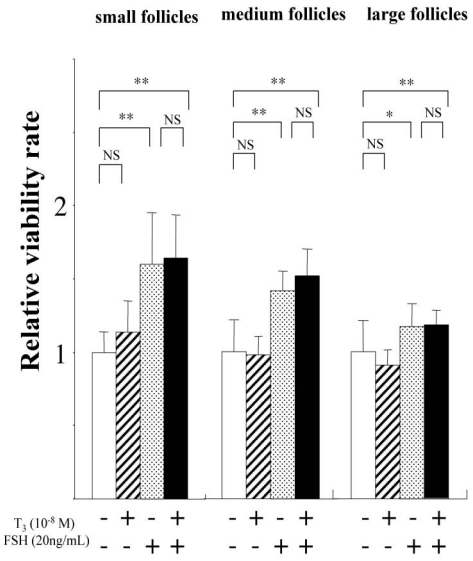

Fig. 1 Effects of FSH and T_3 on the cell viability of porcine granulosa cells from varying sizes of follicles. Granulosa cells were cultured for 48 h under serum-free conditions and cell viability was assessed by MTT assay. Relative viability rate is the ratio of the absorbance against each control culture. Results represent the mean ± SEM of three determinations.
* : $p<0.05$, * * : $p<0.01$, NS: not significant.

1.59±0.35 times from small, 1.41±0.13 times from medium and 1.17±0.14 times from large follicles, compared with that of control cultures. However, concomitant treatment with T_3 and FSH had no significant effect on the relative viability of granulosa cells from small, medium and large follicle granulosa cells compared with that of treatment with FSH alone. The augmentation of granulosa cell viabilities by FSH was observed particularly after 48 h incubation and similar results were obtained up to 60 h (data not shown). Therefore, granulosa cells were incubated for 48 h under serum free condition in the following experiments.

Effect of Thyroid Hormone on Ki67 Expression in Porcine Granulosa Cells

Ki67 expression in porcine granulosa cells was studied in order to assess the cell proliferation since Ki67 is the nuclear antigen expressed exclusively in the nuclei of all proliferating cells during late G1, S, M1 and G2 phases of the cell cycle, and its expression has been advocated as an effective marker of cell proliferation [10, 11, 32].

Ki67 was immunolocalized exclusively in the nuclei of granulosa cells (Fig. 2-A). In small follicle granulosa cells, Ki67 positive nuclei were more abundant when cultured in the presence of FSH (Fig. 2-B, Fig. 3). On the other hand, treatment with T_3 showed no significant effect on Ki67-positive rate of granulosa cells from small follicles compared with the control culture (Fig. 2-C, Fig. 3). Concomitant treatment with FSH and T_3 showed no significant difference in Ki67 expression in small follicle granulosa cells compared to treated with FSH alone (Fig. 2-D, Fig. 3). Medium and large follicle granulosa cells responded to neither FSH nor T_3 in terms of Ki67 expression (Fig. 3).

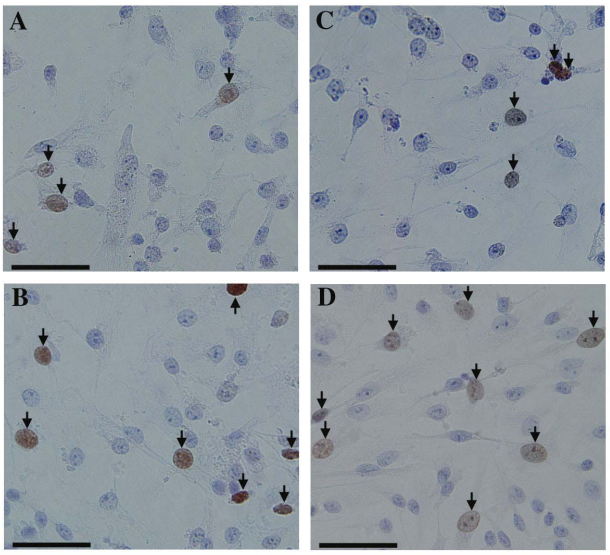

Fig. 2 Immunocytochemical staining for Ki67 in porcine granulosa cells from small follicles cultured for 48 h under serum-free conditions. (A) Without any hormonal treatment; (B) in the presence of FSH (20 ng/mL); (C) in the presence of T_3 (10^{-8} M); (D) in the presence of both FSH (20 ng/mL) and T_3 (10^{-8} M).
Bars represent 50 μm.

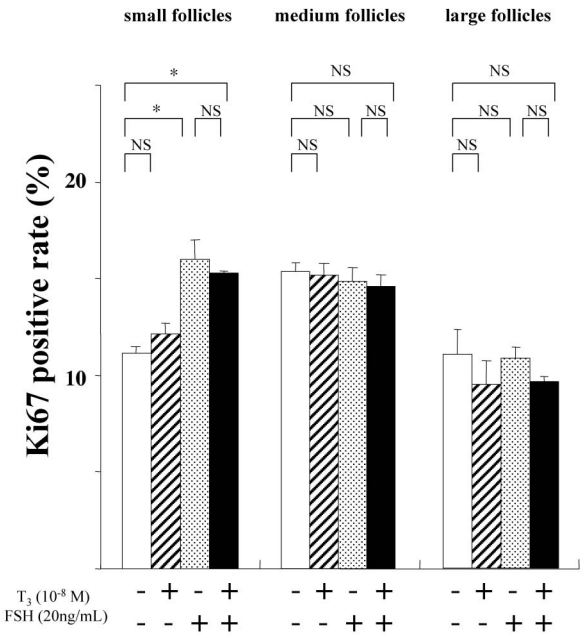

Fig. 3 Ki67 positive rate of porcine granulosa cells from varying sizes of follicles cultured for 48 h under serum-free conditions. Results represent the mean ± SEM of three determinations.
* : $p<0.01$ NS: not significant.

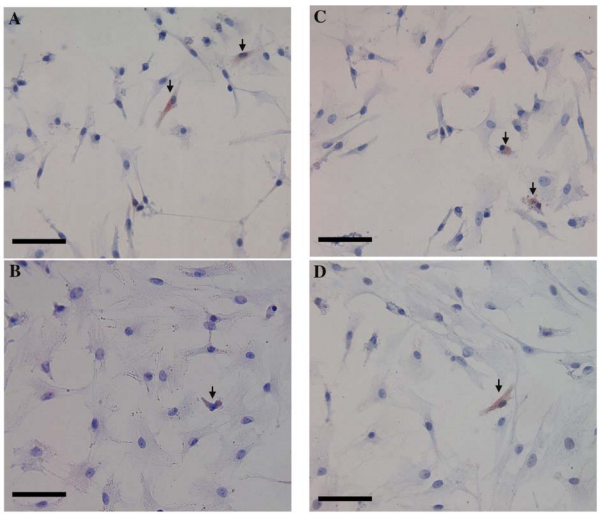

Fig. 4 Immunocytochemical staining for activated caspase-3 protein in porcine granulosa cells from small follicles cultured for 48 h under serum-free conditions. (A) Without any hormonal treatment; (B) in the presence of FSH (20 ng/mL); (C) in the presence of T_3 (10^{-8} M); (D) in the presence of both FSH (20 ng/mL) and T_3 (10^{-8} M). Activated caspase-3 protein was immunolocalized exclusively in the cytoplasm of granulosa cells.
Bars represent 50μm.

Expression of Activated Caspase-3 Protein in Porcine Granulosa Cells

Immunocytochemical analysis of activated caspase-3 protein expression demonstrated that activated caspase-3 protein was immunolocalized exclusively in the cytoplasm of small follicle granulosa cells (Fig. 4-A). In small follicle granulosa cells cultured in the presence of FSH, activated caspase-3 protein-positive cells were less abundant than those in untreated cultures (Fig. 4-B, Fig. 5). Concomitant treatment with FSH and T_3 resulted in further decrease in the activated caspase-3 protein-positive rate in small follicle granulosa cells compared with that in the cells treated with FSH alone (Fig. 4-D, Fig. 5). Treatment with T_3 alone did not affect the activated caspase-3 protein-positive rate of granulosa cells from small follicles compared with that in untreated cultures (Fig. 4-C, Fig. 5). Medium and large follicle granulosa cells responded to neither FSH nor T_3 in terms of activated caspase-3 protein expression (Fig. 5).

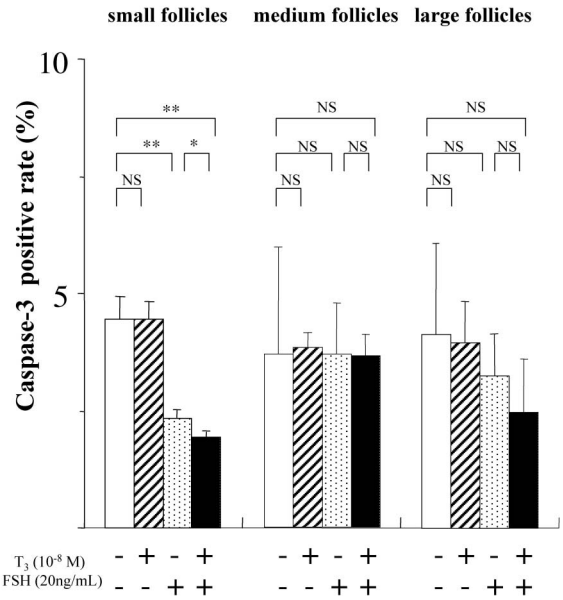

Fig. 5 Effect of FSH and T_3 on the activated caspase-3 positive rate in cultured porcine granulosa cells. Results represent the mean ± SEM of three determinations. * : $p<0.05$, * * : $p<0.01$, NS: not significant.

DISCUSSION

Thyroid hormone contributes to vertebrate development and homeostasis by serving as biological signals to control cellular functions, including cell growth, differentiation and cell death [2]. T_3 prevents apoptosis of early differentiating cerebellar granule neurons [25]. On the other hand, T_3 induces apoptosis of cultured human T lymphocyte cell line as well as peripheral blood lymphocytes in human [23]. T_3 inhibits cell proliferation and stimulates protein synthesis and apoptosis in chick muscle cells [27]. It was shown that thyroid hormone-induced cell death is required for the adsorption of tadpole tail during metamorphosis of Xenopus laevis [7]. In larval epithelium during Xenopus metamorphosis, T_3

is the universal trigger for both the cell death and the subsequent cell proliferation.

A lack or excess of thyroid hormone has a marked influence on the structural, biochemical and functional development, especially of the central nervous system in early life [29, 33]. Thyroid disorders are also associated with a broad spectrum of reproductive disturbances, ranging from abnormal sexual development to menstrual disturbance and impaired fertility [19, 20]. Hyperthyroidism is associated with the elevated plasma estrogen level in the adult human female [28, 30]. Several reports have shown the ability of thyroid hormones to modulate gonadotropin-induced steroidogenic activity in cultured granulosa cells [4, 5, 21]. However, little information is available concerning the effect of thyroid hormone on the cell proliferation and apoptosis of granulosa cells.

In the present study, much attention was paid to elucidate the effects of T_3 on apoptosis in granulosa cell from varying stages of follicular development. Thus, apoptosis in cultured granulosa cells was assessed not only by activated caspase-3 protein expression but also by terminal deoxynucleotide transferase (TdT) mediated dUTP-biotin nick-end-labeling (TUNEL) analysis using the in situ apoptosis detection kit (Oncor, Gaithersburg. MD) . Treatment with T_3 alone did not affect the cell viability, Ki67-positive rate, activated caspase-3 protein positive rate and apoptotic indices assessed by TUNEL (data not shown) of the granulosa cells regardless of the derived follicle size. In contrast, FSH significantly increased the relative viability rate and the Ki67-positive rate, and decreased the activated caspase-3 protein-positive rate and apoptotic indices assessed by TUNEL (data not shown) of porcine granulosa cells retrieved from small follicles. In comparison with the treatment with FSH alone, concomitant treatment with FSH and T_3 did not alter the relative viability rate and the Ki67-positive rate of cultured granulosa cells, but further decreased the activated caspase-3 protein-positive rate and apoptotic indices assessed by TUNEL (data not shown) of granulosa cells from small follicles. The data obtained with TUNEL analysis were similar to those attained on the basis of activated caspase-3 protein expression. This is consistent with our previous findings that thyroid hormones synergize with FSH to exert direct stimulatory effects on granulosa cells selectively from small follicles, including morphological differentiation, LH/hCG receptor formation and steroidogenic enzyme induction [21, 22]. The inhibitory effect of T_3 on apoptosis in small follicle granulosa cells was observed only in the simultaneous presence of FSH. These results suggest that T_3 interacts synergistically with FSH to inhibit apoptosis of small follicle granulosa cells without affecting the cell proliferation.

Thyroid hormones are present in the follicular fluid of human's [37], and a large majority of the thyroid hormone values from follicular fluid fell within the normal range [37] or slightly below in the serum [42]. Thyroid hormone action is mediated through nuclear thyroid hormone receptors and expression of thyroid hormone receptors in granulosa cells from human [37]; rat [1] and porcine [38] were also reported. Porcine granulosa cells from small follicles bound significantly higher amount of ^{125}I-labeled-T_3 compared with those from large follicles [22, 38].

There are two isoforms of thyroid hormone receptors, α and β [31, 39] and each isoforms has subtypes derived through alternative splicing or use of different promoters [17]. The level of thyroid hormone receptor β mRNA rises during amphibian metamorphosis, especially in the developing limbs and regressing tail, and drops after metamorphosis [41]. High expression of thyroid hormone receptor β1 mRNA is also observed in the developing limbs and face in the fetal mouse [26]. In contrast, Wakim and colleagues demonstrated that human granulosa cells preponderantly expressed thyroid hormone receptor α1 than β1 [37]. Thyroid hormone receptor α has been shown to elicit a greater activation of transcription of thyroid hormone responsive promoter regions than thyroid hormone receptor β [34]. However, whether this

hypothesis is true in the porcine granulosa cells is yet to be established.

In conclusion, this is the first study to demonstrate that thyroid hormone T_3, does not affect the proliferation of porcine granulosa cells, but interact synergistically with FSH to inhibit apoptosis of granulosa cells selectively from small follicles.

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