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Effects of 3,5,3'-Triiodothyronine (T_3) and Follicle Stimulating Hormone on Apoptosis and Proliferation of Rat Ovarian Granulosa Cells

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Abstract

Thyroid hormone (TH) is important for normal reproductive functions and dysregulation of TH support is associated with reproductive disorders. We have previously reported that 3,5,3'-triiodothyronine (T_3) increases follicle stimulating hormone (FSH)-induced preantral follicle growth *in vitro*. Interaction of hormones with apoptosis and proliferation of granulosa cells is poorly understood. The present study investigated the role and the mechanism of T_3 and/or FSH on granulosa cell apoptosis and proliferation. Granulosa cells harvested from DES-primed immature rats were exposed to T_3 (1 nM) and/or FSH (100 ng/ml) for 24-48 h. We demonstrated by TUNEL assays that the hormones prevented cells from C8-ceramide-induced apoptosis. The Src/PI3K/Akt pathway was involved in the regulation of granulosa cell survival. While ineffective alone, T_3 significantly enhanced the proliferating cell nuclear antigen (PCNA) content of FSH-induced granulosa cells, consistent with the cell number pattern after treatment. Moreover, the action of the hormones on cell proliferation was also shown to be mediated by the Src/PI3K/Akt pathway. Taken together, these results suggest that T_3 potentiates the cell survival action of FSH through inhibiting cell apoptosis and promoting cell proliferation. Moreover, the protective and survival effects of hormones are mediated by the activation of Src/PI3K/Akt pathway.

Key Words: 3,5,3'-triiodothyronine, apoptosis, FSH, granulosa cell, proliferation

Introduction

In mammals, the destiny of 99% ovarian follicles is atresia involving granulosa cell apoptosis. The transition stage from preantral to antral follicle is critical for follicular development when the follicles either continue growing toward ovulation, or die by atresia. Follicles selected for further growth are thought to receive survival signals from gonadotropins and produced growth factors for development, whereas follicular atresia is a consequence of inadequate survival factors and/or stimulation by cytotoxic factors (9). The destiny of the ovarian follicle (growth/ovulation and atresia) is dependent on a delicate balance in the expression and actions of factors promoting follicular cell proliferation, differentiation and apoptosis.

Thyroid hormone (TH) is important for normal reproductive functions and dysregulation of TH support is associated with reproductive disorders, including impaired follicular development. Although hypo- and hyper-thyroidism are associated with

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dysregulation of the hypothalamic-pituitary axis and suppress ovarian follicular growth and functions (13), it is unclear whether TH exerts a direct action on ovarian cells to elicit these responses. Previous report have shown that follicle development is markedly hampered in hypothyroidism; however, this ovarian condition can be markedly improved by TH, especially in the presence of gonadotropin (12, 13), although gonadotropin alone is effective. In the presence of gonadotropin, TH decreases atresia and significantly increases the numbers of healthy large antral follicles and ovulated oocytes (13, 26). In addition, recent studies have shown that TH stimulates the growth of preantral follicles *in vitro*, suggesting that TH has a significant role in directly promoting cell survival and follicular growth during this early stage of development (2, 6). Follicle stimulating hormone (FSH) increases antral follicle development and suppresses atresia by increasing mitotic activities and inhibiting apoptosis of granulosa cells (11, 29). Whether the action of TH involves suppression of follicular cell apoptosis and increasing cell proliferation is unknown.

The activation of the PI3K/Akt pathway by T_3 is believed to be important for cell proliferation and suppression of apoptosis in a variety of cell systems (2, 3, 6). In addition, phosphorylated-Src by TH is required for phosphoinositide 3-kinase/Akt-mediated anti-apoptotic effects of T_3 in neuronal survival (3). It is not known whether these pathways are indeed regulated in granulosa cells by the actions and interactions of FSH and T_3, and if these pathways are important in promoting preantral follicle growth.

The objective of the present study was to evaluate the effects of T_3 and FSH on follicular development. We hypothesize that T_3 plays an important role in the regulation of granulosa cell apoptosis and proliferation during the development of preantral and early antral follicles. Moreover, the Src/PI3K/Akt pathway may also be involved in the regulation of hormones on follicular development.

Materials and Methods

Materials

Culture media were purchased from Gibco Bethesda Research Laboratories (Grand Island, NY, USA). 3,3',5-Triiodo-L-thyronine and eCG (equine chorionic gonadotropin) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Recombinant human FSH was obtained from the National Hormone and Peptide Program, Harbor-UCLA Medical Center (Torrance, CA, USA). The enhanced chemiluminescence (ECL) detection kit Hoechst 33258 was purchased from Amersham Life Science (Oakville, ON, Canada). Horseradish peroxidase (HRP)-conjugated anti-rabbit and anti-mouse immunoglobulin (Ig) G were products of Bio-Rad (Richmond, VA, USA). Rhodamine-conjugated goat anti-rabbit and anti-mouse IgGs were products of Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) enzyme and TUNEL label mix were from Roche Diagnostics (Indianapolis, IN, USA). C8-ceramide (N-octanoylsphingosine, D-erythro) was from Biomol International LP (Plymouth Meeting, PA, USA). Selective inhibitor of Src family tyrosine kinases (PP1) and PI3K (LY294002) was from Enzo Life Sciences, Inc. (Plymouth Meeting, PA, USA) and Sigma, respectively. The Akt inhibitor (API-2) was purchased from Calbiochem (EMD Biosciences, Inc., La Jolla, CA, USA). Rabbit polyclonal anti-mouse P-Akt (Ser473), anti-mouse total Akt, anti-human P-Src (Tyr416) antibodies and mouse monoclonal anti-human N-Src antibody were from Cell Signaling Technology (Oakville, ON, Canada). Rabbit monoclonal anti-rat PCNA (sc-6273) neutralization peptides (PCNA, sc-25280) were from Santa Cruz.

Animal Treatments

Immature female Sprague-Dawley rats (21-22 days old) were purchased from the Beijing Vital Laboratory Animal Technology Co. (Beijing, China). The study was performed in accordance with the Guidelines for the Care and Use of Laboratory Animals and China Council on Animal Care, and was approved by the Institutional Animal Care and Use Committee of Capital Normal University. All animals were caged in a controlled environment with a 12/12-h light/dark cycle and received pathogen-free water and food for maintenance. Rats were injected with DES (1 mg/day; 3 days), and ovaries were collected at 72 h.

Isolation and Culture of Rat Granulosa Cells

Granulosa cells from mainly preantral and early antral follicles were harvested by follicle puncture. Oocytes were removed from the cell preparations by filtering the cell suspensions through a nylon cell strainer (40 μm; Becton Dickinson and Co., no. 352340). On day 0, 9×10^5 viable granulosa cells (determined by Trypan blue dye-exclusion test) were cultured for 5 h in a 6-well plate with 1.2 ml M199 medium [supplemented with HEPES (10 mM), streptomycin (100 μg/ml), penicillin (100 U/ml) and fungizone (0.625 μg/ml)] and containing 10% fetal bovine serum. The media were then replaced with serum-free M199 supplemented as above for 12 h thereafter and cells were treated with FSH (100 ng/

ml) with or without T_3 (1.0 nM). Four hours thereafter, C8-ceramide (30 μm; a cell-permeable, short-chain ceramide analog) or dimethylsulfoxide (vehicle control) (28) was added. In the last experiment, cells were pretreated with the Src inhibitor PP1 (10 μM) (3), PI3K inhibitor LY294002 (10 μM) (45) or the Akt inhibitor API-2 (10 μM) (46) 1 h before FSH with or without T_3 treatment. Floating cells and cells attached to the growth surface (collected by trypsin treatment) (28) were pooled for different durations thereafter for assessment of protein/mRNA content or apoptosis.

Protein Extraction and Western Blot Analysis

Western blot analysis was performed as described previously (41, 42, 44). Protein extraction was carried out on ice, and protease inhibitors PMSF (10 μm), aprotinin (50 μg/ml) and sodium orthovanadate (1 mM) were added to the buffers, where indicated, immediately before use. Granulosa cell pellets were resuspended in lysis buffer (single-strength PBS, pH 7.4, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) containing protease inhibitors and were homogenized by sonication (5 sec/cycle, 3 cycles). Homogenates were centrifuged (14,000 ×*g*, 4°C, 30 min), and the protein content of the cell lysate was determined with the Bio-Rad DC protein assay kit. Aliquots of proteins (15 μg) were resolved by SDS-PAGE (10%) and electrotransferred to nitrocellulose membranes. The membranes were then blocked (room temperature, 1 h) with blotto [Tris-buffered saline, pH 8.0, with 0.05% Tween 20 (TBS-T), 5% dehydrated nonfat milk powder]. The membranes were then incubated at 4°C, overnight with blotto containing 0.1 g/ml PCNA (1:1000) and β-actin (1:10,000) antibody and then washed three times in TBS-T for 5 min each, followed by incubation in a HRP-conjugated secondary antibody (1:1000, 1:3000, respectively) in blotto, and washed again in TBS-T. Peroxidase activity was visualized with the ECL kit according to the manufacturer's instructions, and protein content was determined by densitometrically scanning the exposed x-ray film.

TUNEL

Apoptotic cells were identified by TUNEL (TdT-mediated dUTP nick end-labeling) (8, 22, 24). Briefly, cells were harvested by centrifugation (3000 ×*g*, 5 min, 4°C). The supernatant was removed and the cell pellet was fixed overnight with 300 μl 10% formalin. The cells were recovered by centrifugation (3000 ×*g*, 5 min, 4°C) and re-suspended on a charged glass slide in 15 μl PBS. The cells were allowed to be air-dried for 30 min and endogenous peroxidase activity was removed by treatment with 0.3% H_2O_2 (room temperature, 20 min). The cells were washed with PBS (3 × 5 min; room temperature). The cells were then incubated in 20 μl TUNEL mixture (18 μl fluorescein isothiocyanate-conjugated dUTP and 2 μl TUNEL enzyme) in a humidified chamber (60 min, 37°C), washed in PBS (3 × 10 min), and stained with Hoechst 33258 compound (1:1000; 1 min). After washing with PBS (2 × 10 min), the cells were photographed under a microscope (Leica Lasertechnik GmBH, Heidelberg, Germany) equipped with epifluorescent optics, and the images were recorded using QCapture Suite version 2.56 software (Burnaby, British Columbia, Canada). The cells incubated with 20 μl TUNEL-label solution without the TUNEL enzyme to serve as a negative control. Apoptotic cells were identified at 24 h and expressed as a percentage of the total cells.

Trypan Blue Cell Counting

Dead and viable cells in culture were distinguished by staining with trypan blue as previously described (16) and were computed using the formula:

$$\text{Cells/ml} = (\text{average count / square}) \times (\text{dilution factor}) \times 10000\ (\text{chamber conversion factor})$$

Statistical Analysis

Results were expressed as the means ± SEM of at least three independent experiments. All data were subjected to two- or three-way (repeated-measure) ANOVA Prism 5.0 statistical software (GraphPad Software, Inc., San Diego, CA, USA, or SIGMAPLOT 11 software Inc., Richmond, CA, USA). Differences between experimental groups were determined by the Bonferroni post test.

Results

Effects of T_3 and FSH on Apoptosis of Granulosa Cells

Many studies have shown that FSH or T_3 exerts anti-apoptotic effects in different cell systems (2, 38). To determine whether FSH and T_3 are survival factors that could protect granulosa cells from apoptosis, the TUNEL assay was used. In the control groups, the number of TUNEL-positive cells was 8.56 ± 1.02% (24 h) and 11.07 ± 0.09% (48 h) assayed at different time points (Fig. 1). The results showed that granulosa cell apoptosis was low irrespective of the presence of FSH [4.42 ± 0.21% (24 h), 6.53 ± 1.15% (48 h), $P > 0.05$] or T_3 [4.54 ± 0.32% (24 h), 6.83 ± 0.96% (48 h), $P > 0.05$, compared with the control group for the same duration] (Fig. 1). The combined use of the

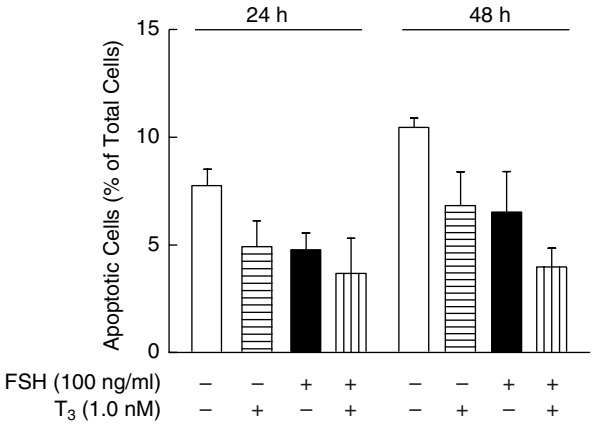

Fig. 1. **Effects of FSH and T_3 on granulosa cell apoptosis *in vitro*.** Granulosa cells were cultured for different durations in the presence of FSH (100 ng/ml) and/or T_3 (1.0 nM). Cell apoptosis were assessed by TUNEL analysis.

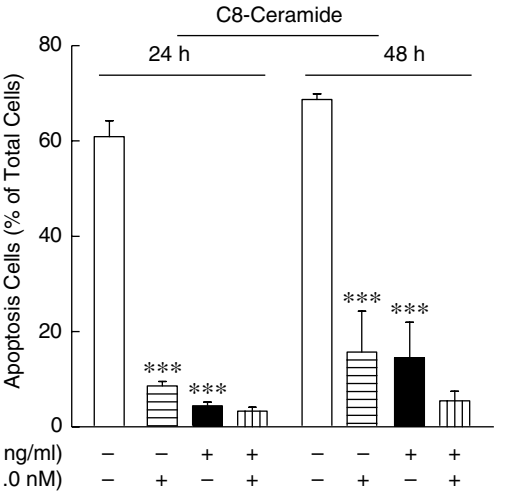

Fig. 2. **FSH and T_3 attenuate ceramide-induced granulosa cell apoptosis *in vitro*.** Granulosa cells from preantral and small antral follicles were cultured with FSH and or T_3, and ceramide (CER: 30 μM; added 4 h after addition of the hormones). The effects of hormones on ceramide-induced apoptosis were detected by TUNEL analysis and the positive cells were calculated. ***$P <$ 0.001 *vs*. the respective control (without FSH and T_3) at the same duration.

hormones also had no significant down-regulatory effects on cellular apoptosis [3.68 ± 0.85% (24 h), 3.99 ± 0.29% (48 h), $P > 0.05$] compared with the FSH or T_3 group.

T_3 and FSH Protected Granulosa Cells from Ceramide-Induced Apoptosis

Since the cell number of apoptosis in the control group in the previous experiment was very low, C8-ceramide, an inducer of cell cycle arrest and apoptosis (28), was used to induce cell death to determine whether the hormones have protective effects. Granulosa cells were cultured with FSH and/or T_3 and ceramide at 30 μM concentration, which was added 4 h after hormonal treatment. The results showed that ceramide increased the number of TUNEL-positive cells from 9.90 ± 1.84% (basal level) to 60.99 ± 3.28%. However, the TUNEL-positive cell number was reduced by T_3, FSH and FSH+T_3 to 9.29 ± 0.23%, 4.08 ± 0.17%, 3.39 ± 0.09%, respectively (Fig. 2). The same reduction trend was shown in the 48 h duration of treatment. T_3, FSH and FSH+T_3 reduced the apoptotic cell number from 67.43 ± 2.46% (control group) to 15.76 ± 4.32%, 14.55 ± 3.84%, and 5.52 ± 0.62%, respectively (Fig. 2).

Effects of FSH and T_3 on Granulosa Cell Proliferation In Vitro

PCNA (proliferating cell nuclear antigen) is an auxiliary protein of DNA polymerase delta and is required for both DNA replication and DNA repair (20, 25). PCNA labeling indices are considered to reflect cell proliferation (7, 19). A previous report showed that FSH and T_3 had important effects on proliferation in a variety of cell systems, including granulosa cells (14). To test whether T_3 induces granulosa cell proliferation, and if T_3 increases FSH-induced granulosa cells proliferation, granulosa cells were cultured with FSH and/or T_3 for 24-48 h and the cell number was counted after treatment. In addition, the cellular content of PCNA was determined by Western blot analysis.

The results showed that FSH significantly increased the number of granulosa cells at 24 h and 48 h [1.73 ± 0.38 (24 h); 1.55 ± 0.54 (48 h), $P < 0.001$], and T_3 also significantly enhanced this effect at 48 h [2.21 ± 0.34, $P < 0.05$]. Although T_3 alone had no significant influence on granulosa cell PCNA content, co-treatment with FSH and T_3 significantly decreased PCNA protein expression at 48 h [3.52 ± 0.37 (FSH+T_3) *vs*. 2.31 ± 0.78 (FSH), $P < 0.05$; Fig. 3].

Src and PI3K/Akt Pathway Are Involved in the Regulation of Cellular Apoptosis and Proliferation by FSH and T_3

Akt is a downstream effecter of PI3K, which mediates granulosa cell survival (17). The Src family of non-receptor tyrosine kinases attenuate the inhibitory activities of the regulatory p85 subunit of PI3K by inducing tyrosine phosphorylation of p85, and interaction between phospho-tyrosine and the SH2 domain of p85 (3). In our previous study, we found that T_3 enhanced FSH-induced activation of Src/PI3K/Akt pathway in granulosa cells (47). To test the hy-

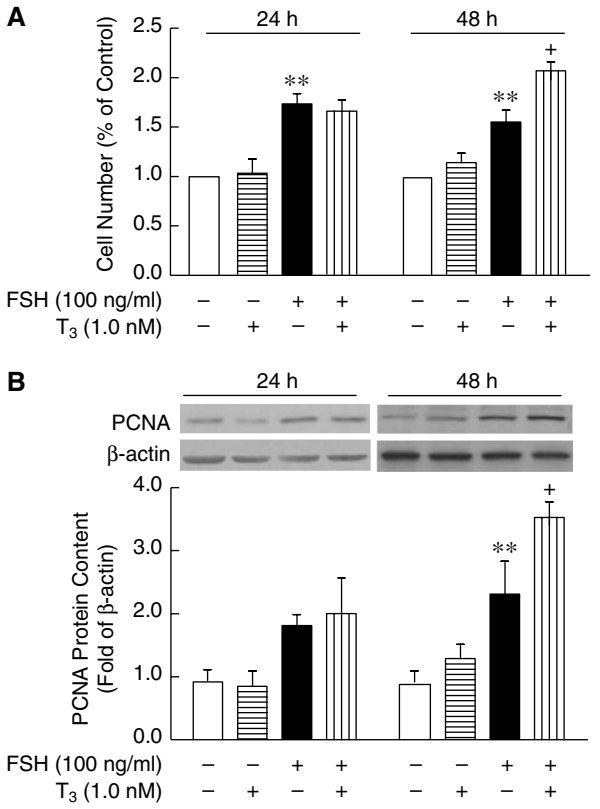

Fig. 3. **Effects of FSH and T_3 on granulosa cell proliferation *in vitro*.** Granulosa cells were cultured with FSH and/or T_3 for different durations. Cell number was counted (A) and Western blot analysis was used to detect PCNA protein content (B). $^{**}P < 0.01$ *vs.* the respective control (without FSH and T_3); $^{+}P < 0.05$ *vs.* FSH alone in the same group.

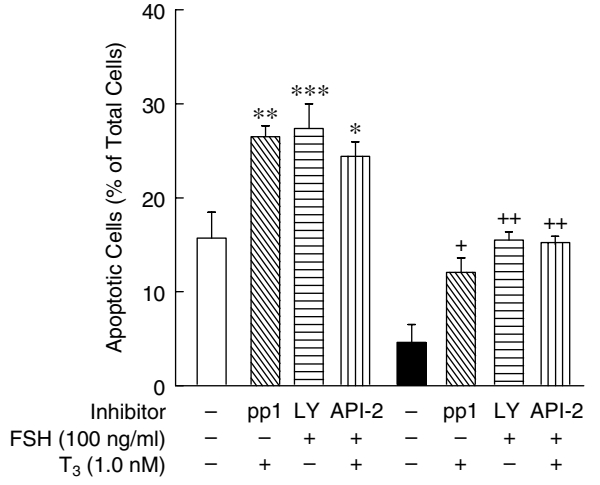

Fig. 4. **Regulation of apoptosis by FSH and T_3 is mediated by Src and the PI3K/Akt signaling.** After pretreatment with inhibitor of Src (PP1, 10 μM), PI3K (LY294002, 10 μM) or Akt (API-2, 10 μM) for 1 h, granulosa cells were cultured for 48 h with FSH and/or T_3, and cell apoptosis was analyzed by TUNEL. $^{**}P < 0.01$; $^{***}P < 0.001$ (*vs.* FSH.alone); $^{++}P < 0.01$; $^{+++}P < 0.001$ (*vs.* FSH+T_3).

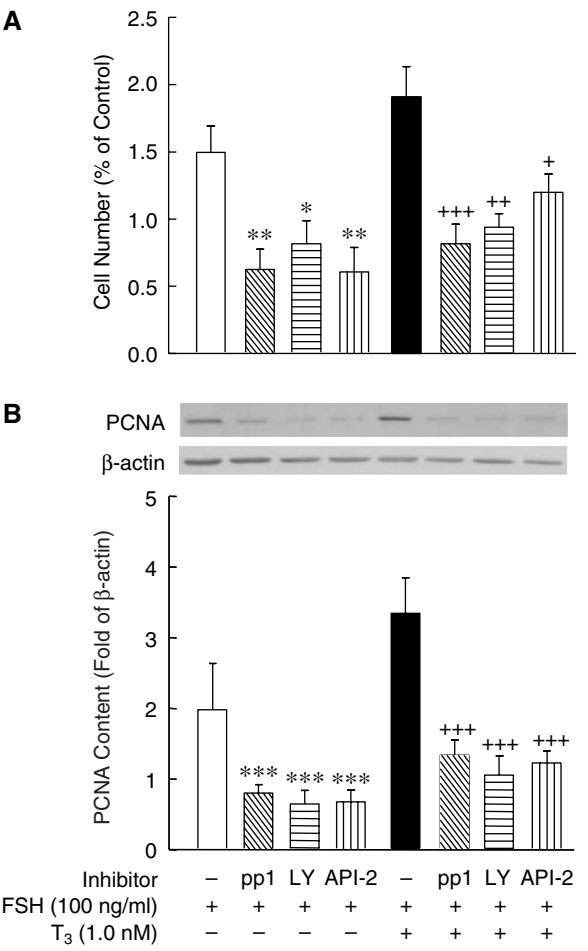

Fig. 5. **Src/PI3K/Akt pathway is involved in the regulation of granulosa cell proliferation by FSH and T_3.** Granulosa cells were pretreated with inhibitor of Src (PP1, 10 μM), PI3K (LY294002, 10 μM) or Akt (API-2, 10 μM) for 1 h before treatment with FSH and/or T_3 for 48 h. Survival cell number was determined (A) and Western blot analysis was used to detect PCNA protein content (B). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ (*vs.* FSH alone); $^{+}P < 0.05$; $^{++}P < 0.01$; $^{+++}P < 0.001$ (*vs.* FSH+T_3 group).

pothesis that Src/PI3K/Akt pathway is involved in the regulation of granulosa cell apoptosis and proliferation, and consequently promotes cell survival, granulosa cells were pretreated for 1 h with specific inhibitor of Src (PP1), PI3K (LY294002) or Akt (API-2) (47), and the cells were then treated with FSH and/or T_3 for 48 h (Figs. 4 and 5). Results showed that pretreatment with inhibitors abrogated the anti-apoptotic effects of FSH+T_3 [4.65 ± 0.71 (FSH+T_3) *vs.* 12.07 ± 0.62 (FSH+T_3+PP1), 15.51 ± 0.54 (FSH+T_3+LY), 15.24 ± 0.49 (FSH+T_3+API), $P < 0.01$; Fig. 4]. Coincidentally, the cell number and PCNA protein content were up-regulated by the presence of FSH and T_3, and these responses were also significantly attenuated by pretreatment with all three inhibitors [cell

number counts: 1.91 ± 0.18 (FSH+T_3), 0.82 ± 0.09 (FSH+T_3+PP1), 0.94 ± 0.05 (FSH+T_3+LY), 1.19 ± 0.07 (FSH+T_3+API); PCNA: 3.34 ± 0.21 (FSH+T_3), 1.34 ± 0.05 (FSH+T_3+PP1), 1.06 ± 0.08 (FSH+T_3+LY) 1.23 ± 0.03 (FSH+T_3+API); Fig. 5]. These results suggest that FSH synergizes with T_3 to promote granulosa cell survival by regulating proliferation and apoptosis *via* a Src-activated and PI3K/Akt-mediated mechanism.

Discussion

Previous reports showed that while hypothyroidism inhibited follicle development (decreased number of healthy preantral and small antral follicles, and increased number of atretic small and medium antral follicles), the response was attenuated by TH treatment (13). A previous study has also indicated that FSH increases preantral follicle growth in vitro, a response markedly enhanced by T_3 (15). However, the nature of this TH-FSH interaction is poorly understood. Furthermore, although TH nuclear receptors are expressed in the follicle and mediates the growth promoting action of TH (5, 40), the mechanism of action of TH in regulating follicle development and atresia is not known. During follicular development, the selection system ensures the release of only healthy and viable oocytes (34). The fate of the follicle depends on the delicate balance in the expression and actions of factors, which promote ovarian cell proliferation, differentiation and apoptosis (1, 4). If survival factors are not enough to maintain cellular viability, the follicle may undergo atresia. Gonadotropins stimulate ovarian follicle development. FSH inhibits apoptosis of ovarian cancer cells by regulating survival and apoptotic factors (10). Moreover, many reports have shown the effects of TH on regulating cell growth (31). T_3 protects pancreatic beta-cells from apoptosis (38). On the other hand, T_3 also down-regulates the expression of Fas and Fas ligand and suppresses caspase-3 and apoptosis in early placental extravillous trophoblasts (3, 18).

In the present study, we found that the number of apoptotic cells in the control group was low. Although hormonal treatment decreased the number of TUNEL positive cells, there were no significant differences among the treatment and control groups probably due to the low base levels. It is interesting that T_3 alone also inhibited granulosa cell apoptosis, although the effect was not significant, which indicates that T_3 exerts its anti-apoptotic effects by an unknown pathway. To further determine the effects of hormones on cellular apoptosis, cells were co-cultured with C8-ceramide and hormones. The results showed that C8-ceramide significantly increased the apoptotic cells population. However, this increased response was attenuated not only by FSH but also by T_3. The combined use of hormones had no significant enhancing effects compared with FSH or T_3 alone.

To examine the signaling pathway of the hormones regulating granulosa cells apoptosis, cells were pretreated with the Src, PI3K and Akt inhibitors PP1, LY294002 and API-2, respectively. The results showed that all inhibitors abrogated the anti-apoptotic actions of FSH+/-T_3. These results indicate that FSH and T_3 protected granulosa cells of preantral and small antral follicles from ceramide-induced apoptosis *via* the activation of the Src/PI3K/Akt signaling pathway. Furthermore, it was reported that gonadotropin induced proliferation of ovarian cancer cells (27). FSH stimulates proliferation of human epithelial ovarian cancer cells, cultured chicken ovarian germ cells (23, 33), and chick embryo testis cells (30).

Previous studies have reported the effects of TH on cellular proliferation. TH has similar action of E_2 in promoting MCF-7 cell proliferation (36). T_3 treatment results in increased cell size of human pancreatic insulinoma cells, and up-regulates cell proliferation and survival *via* the PI3K/Akt pathway (39). Lin *et al*. (21) also reported that T_3 enhanced cell proliferation in human papillary and follicular thyroid cancer cell lines by measuring quantitative changes in PCNA, In addition, thyroid hormone induces artery smooth muscle cell proliferation through up-regulating NOX1 (43). However, T_3 inhibits cell proliferation of HK2, but stimulates cell proliferation in Caki lines (32). Hence, the effects of TH on regulating cell proliferation acting as a stimulator or an inhibitor appear to be cell type-specific. However, the proliferation effects of TH on ovarian cell are still unknown.

In primary to large antral follicles, PCNA-positive staining in ovarian cells, including oocyte, granulosa cells and theca cells, is detected. Moreover, the level of PCNA protein expression is stage-dependent (31, 35, 37). In the present study, T_3 synergized with FSH to increase PCNA protein content after 48 h. Increases of this protein were correlated with the increased number of cells after co-treatment. These results suggest that T_3 is a biological enhancer of FSH action on granulosa cell proliferation. Moreover, the action of hormones on cellular proliferation may also be mediated by the Src/PI3K/Akt signaling pathway.

Overall, we found that FSH and T_3 inhibited the initiation of apoptosis and induced granulosa cell proliferation mediated by the PI3K/Akt pathway and the involvement of Src activation. Although T_3 alone was ineffective, T_3 enhanced FSH actions by inhibiting granulosa cell apoptosis and regulating cellular proliferation. T_3 may exert its effect as a bio-amplifier. However, further studies should focus on the mech-

anism of hormonal effects on granulosa cell apoptosis and proliferation.

Acknowledgments

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