[Frontiers in Bioscience E3, 1401-1413, June 1, 2011]

Interactions of thyroid hormone and FSH in the regulation of rat granulosa cell apoptosis

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1. ABSTRACT

Thyroid hormone (TH) is important for normal reproductive function. Our previous studies indicate that FSH increases preantral follicle growth in vitro, a response markedly enhanced by triiodothyronine (T_3). However, the nature of this hormonal interaction is poorly understood. The objective of this study was to determine if and how T_3 modulate FSH-induced expression and actions of granulosa cell intracellular survival and death intermediates. We investigated the possible involvement of Src and PI3K/Akt pathway in the regulation of granulosa cell survival. We demonstrated that, while ineffective alone (0.1-100 nM), T_3 markedly enhanced FSH (100 ng/ml)-induced granulosa cell phospho-Src and phospho-Akt contents and Xiap expression in vitro. The effects of T_3 were concentration-dependent, with maximal responses at 1.0 nM. FSH alone decreased Fas Ligand (FasL) content irrespective of the presence of T_3. Co-treatment of cell with T_3 and FSH decreased Fas content, although neither hormone alone elicited a significant response. Taken together, the present study demonstrates that T_3 potentiates the cell survival action of FSH through Src- and PI3K-mediated Xiap up-regulation and decreased Fas and FasL expression.

2. INTRODUCTION

Mammalian ovarian follicular development is a highly selective process. While the dominant follicle(s) eventually ovulates, the majority of the follicles undergo atresia, a process involving granulosa cell apoptosis (1-6). The preantral-early antral follicle transition is the penultimate stage of follicular development in terms of gonadotropin dependence and follicle destiny (growth versus atresia). The survival of granulosa cells in follicles that escape atresia and selected to ovulate in each reproductive cycle may occur as a consequence of up-regulation of the survival factors and/or removal of the cell death inducers. Apoptosis could be triggered by the removal of survival factors and/or stimulation by cytotoxic factors (4).

FSH increases follicle development and suppresses atresia by increasing granulosa cell mitotic activity and inhibiting apoptosis (5, 7). Thyroid hormone is important for normal reproductive function and dysregulation of TH support is associated with reproductive disorders, including impaired follicular development. Although hypothyroidism markedly hampered the follicle development, TH

significantly improve ovarian condition, especially in the presence of gonadotropin (8, 9). In our previous study, FSH increases preantral follicle growth in vitro, a response markedly enhanced by T_3 (10). However, the mechanisms involved are not fully understood.

Xiap, a member of inhibitor of apoptosis protein family, inhibits caspases at both the initiation phase (caspase-9) and the execution phase (caspase-3 and 7) of apoptosis (11, 12). Xiap is highly expressed in healthy but not atretic follicles (13). Rat granulosa cell Xiap expression is upregulated by gonadotropin and suppressed during induction of apoptosis (14, 15). In contrast, Fas antigen (APO-1, CD95), a type I transmembranous glycoprotein belonging to the TNF/nerve growth factor receptor family (1), is expressed abundantly and mediates apoptosis in a variety of cells (2, 16, 17). Fas induces apoptosis when activated by Fas ligand (FasL) (18), a type II membrane protein processed to a soluble form by a metalloproteinase (19). Fas and FasL are detected in the granulosa cells and are associated with follicular atresia (20-28). Induction of apoptosis through the Fas/FasL system involves activation of a caspase 8-mediated cascade.

It has been demonstrated that gonadotropic stimulation down-regulates both Fas and FasL expression and increases Xiap content in rat granulosa cells (21). The latter response is mediated through PI3K/Akt pathway (29), which is critical for the maintenance of normal follicular growth and development (30). Moreover, T_3 decreases Fas and FasL expression and caspase-3 activation, suppressed apoptosis in early plancental extravillous trophoblasts (EVTs) (31).

Although TH are well known for their classic genomic effects which are mediated by their receptors (TR), recent studies has led to increased interest on their nongenomic actions. Evidence indicates that TH exerts important nongenomic effects (32) resulting from their binding to plasma membrane, cytoplasm or mitochondrial receptors that subsequent lead to rapid cellular regulation, such as modulation of plasma membrane ion channels (33) and αvβ3 integrin-mediated extracellular signal-regulated/mitogen activated kinases (ERK/MAPK) pathways (32) and activation of protein kinase Cα (32) and protein kinase Cδ (34), PI3K and Akt (32, 35).

Although activation of the PI3K/Akt pathway by T_3 is believed to be important for cell proliferation, differentiation and suppression of apoptosis in a variety of cell systems (36-38), whether these pathways are indeed regulated in granulosa cells by the actions and interactions of FSH and T_3 and their importance in promoting preantral follicle growth, is not known. Src is a member of non-receptor tyrosine kinases family, which has been shown to be required for PI3K activation by estrogen and androgen receptors (39, 40). p85α (subunit of PI3K) is a substrate of Src family kinases (41). It has also been reported that activation of Src by TH is required for PI3K /Akt - mediated in neuronal survival (36).

In the present study, we have examined the hypothesis that TH interacts with FSH in promoting ovarian preantral follicle growth by modulation of Fas, FasL and Xiap content and preventing granulosa cell apoptosis, a process involving Src activation and mediated by the PI3K/Akt pathway. We have demonstrated for the first time that FSH and T_3 inhibit granulosa cell apoptosis by down-regulating the Fas/FasL pathway and increasing cellular Xiap content. These responses appeared to be mediated by Src activation of the PI3K/Akt pathway.

3. MATERIALS AND METHODS

3.1. Materials

Culture media were purchased from Gibco Bethesda Research Laboratories (Burlington, ON, Canada). 3,3',5-Triiodo-L-thyronine, HEPES, BSA, bovine insulin and Tween 20 were obtained from Sigma Chemical Co. (St. Louis, MO). Recombinant human FSH was obtained from the National Hormone and Peptide Program, Harbor-UCLA Medical Center (Torrance, CA). The 6-well plate was product of Becton Dickinson (Becton Dickinson Labware, USA). The enhanced chemiluminescence (ECL) detection kit and Hoechst 33258 were obtained from Amersham Life Science (Oakville, ON, Canada). Acrylamide (electrophoresis grade), N,N′-methylene-bis-acrylamide, ammonium persulfate, glycine, SDS-PAGE prestained molecular weight standards (low range), nitrocellulose membranes, horse radish peroxidase (HRP)-conjugated anti-rabbit and anti-mouse immunoglobulin (IgG) were products of Bio-Rad (Richmond, CA). Rhodamine-conjugated goat anti-rabbit IgG, and mouse IgG were product of Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) enzyme and TUNEL label mix were from Roche Diagnostics (Indianapolis,IN). C8-ceramide (N-octanoylsphingosine, D-erythro) was from Biomol International LP (Plymouth Meeting, PA). Selective inhibitor of Src family tyrosine kinases (PP1) and PI3K (LY294002) was from Enzo Life Sciences, Inc. (Plymouth Meeting, PA) and Sigma Chemical Co. (St. Louis, MO), respectively. The Akt inhibitor (API-2) was purchased from Calbiochem (EMD Biosciences, Inc. La Jolla, CA). Rabbit polyclonal anti-mouse Fas (sc-716), rabbit polyclonal anti-rat FasL (sc-834) and rabbit monoclonal anti-rat FasL (sc-6273) neutralization peptides (Fas, sc-716P; FasL, sc-834P) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit polyclonal anti-mouse Xiap (2323-PC-050) antibody was purchased from Trevigen Inc. (Trevigen Inc. 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, Ontario, Canada). Random decamer primers were from Ambion, Inc. (Austin, TX). Ribonuclease (RNase) inhibitor, RevertAid H Minus M-MULV RT Enzyme, 5 X reaction buffer, and dNTP were from Fermentas International Inc. (Burlington, Ontario, Canada). RNeasy Micro kit, deoxyribonuclease I in RNase-free deoxyribonuclease set and QuantiTect SYBR Green PCR kit were purchased from QIAGEN Inc. (Mississauga,

Ontario, Canada). PCR primers for Xiap, Fas, FasL and 18S rRNA were from Invitrogen Canada.

3.2. Rat granulosa cell isolation and culture

All animal work reported herein were carried in accordance with the Guidelines for the Care and Use of Laboratory Animals and Canadian Council on Animal Care. Immature female Sprague-Dawley rats (21–22 days old) from Charles River Canada (Montreal, PQ, Canada) were injected with DES (1mg/day; 3 days), and ovaries were collected at 72 h. 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). At 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 of M199 medium [supplemented with HEPES (10 mM), streptomycin (100 µg/ml), penicillin (100 U/ml), and fungizone (0.625 µg/ml)] containing fetal bovine serum (10%, wt/vol). 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) ± T_3 (0.10-100 nM). Four hours thereafter, C8-ceramide (30µm; a cell-permeable, short-chain ceramide analog) or dimethylsulfoxide (vehicle control) (42) were added. In some experiments, cells were pretreated with the Src inhibitor PP1 (10µM) (36), PI3K inhibitor LY294002 (10µM) (29) and Akt inhibitor API-2 (10µM) (43) 1h before FSH ± T_3 treatment. Floating cells and cells attached to the growth surface (collected by trypsin treatment) (42) were pooled for different duration thereafter for assessment of protein/mRNA content or apoptosis.

3.3. Protein extraction and western blot analysis

Western blot analysis was performed as described previously (44). All protein extraction was carried out on ice, and protease inhibitors PMSF (10 µm), aprotinin (50 ug/ml) and sodium orthovanadate (1 mM) were added to 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 homogenized by sonication (5 sec/cycle, 3 cycles). Homogenates were centrifuged (14,000 x g, 4C, 30 min), and the protein content of the supernatant (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 (RT, 1 h) with blotto [Tris-buffered saline (pH 8.0) with 0.05% Tween 20 (TBS-T), 5% dehydrated nonfat milk powder]; they were then incubated 4 ºC, overnight with blotto containing 0.1 g/ml p-Akt (1:1000), t-Akt (1:1000), Xiap (1:1000), Fas (1:1000), FasL (1:1000), p-Src (1:1000) , N-Src (1:3000), β-actin (1:10,000) antibody and then washed in TBS-T (3 x 5 min), incubated in HRP-conjugated secondary antibody (1:3000, 1:3000, 1:1000, 1:1000, 1:1000, 1:3000, 1:1000, 1:1000, 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. Antibody specificity for Fas and FasL was confirmed by antibody preabsorption test, using 0.1 µg/ml of control peptides for Fas (sc-716P) and FasL (sc-834P), respectively.

3.4. RNA extraction, cDNA synthesis, and real-time PCR analysis

Total RNAs were extracted from granulosa cells that were collected from the above indicated culture using RNeasy Micro kit. Briefly, 0.2 µg total RNAs were reverse transcribed in a final volume of 20 µl solution containing 4µl 5×reaction buffer, 2µl 10 mM dNTP, 20 U of RNase inhibitor, 200 U RevertAid H Minus M-MULV RT enzyme, random decamer primers, and RNase free H_2O. Quantitative PCR analysis for Xiap, Fas, FasL and 18S rRNA were performed, using a LightCycler 2.0 System (Roche Diagnostics). The Xiap primers used for amplification were a 5' forward primer (5'-CAGTGTGCGGAAGCAGTTGACAAA-3') and a 3' reverse primer (5'-TAACATGCCTACTGTGGTGCTGGA-3'). The Fas primer sequences were 5'-ACCACTGTTATCACTGCACCTCGT-3' (5' forward primer) and 5'- AGGATCAGCAGCCAAAGGAGCTTA-3' (3' reverse primer). The FasL primers used for amplification were a 5' forward primer (5' -AAGACCACAAGGTCCAACAGGTCA-3') and a 3' reverse primer (5'- TTCTCTTTGCCTCTGCATTGCCAC-3'). The 18S rRNA primer sequences used were a 5' forward primer (5'-CGCGGTTCTATTTTGTTGGT-3') and a 3' reverse primer (5'-AGTCGGCATCGTTTATGGTC-3'). Amplification reaction was then performed using the QuantiTect SYBR Green PCR kit. The thermal cycling conditions were comprised of an initial denaturation step (95 ºC, 15 min) followed by 45 cycles amplification for Xiap, Fas, FasL and 40 cycles amplification for 18S rRNA (95 ºC for 15 sec, 58 ºC for 20 sec, and 72 ºC for 30 sec, respectively). PCR products were subsequently melted at 60 ºC for 30 sec. The melting curve analysis shows a single peak with no primer-dimers at the described PCR working conditions for Xiap, Fas, FasL and 18S primer sets, respectively. A standard curve was included for each gene. All the real time-PCR assay for Xiap, Fas and FasL mRNA abundance were quantified within the linear range, normalized against their respective 18S rRNA mRNA and expressed as fold of 0 h respectively before statistical analysis (45). PCR without reverse-transcribed cDNA were used as negative controls.

3.5. TUNEL

Apoptotic cells were identified by TUNEL (TdT-mediated dUTP nick end-labeling). Briefly, cells were harvested by centrifugation (3000 x g, 5 min, 4 ºC). The supernatant was removed and the cell pellet was fixed overnight with 300 µl 10% formalin. The cells were recovered by centrifugation (3000 x g, 5 `min, 4 ºC) and re-suspended on a charged glass slide in 15µl PBS. The cells were allowed to be air-dried (30 min) and endogenous peroxidase activity was removed by treatment with 0.3% H_2O_2 (RT, 20 min). The cells were washed with PBS (3 x 5 min; RT). The cells were then incubated in 20 µl of the

TUNEL mixture (18µl fluorescein isothiocyanate-conjugated dUTP and 2µl TUNEL enzyme) in a humidified chamber (60 min, 37 ºC), washed in PBS (3 x 10 min), and stained with Hoechst 33258 compound (1:1000; 1 min). After washed with PBS (2 x 10 min), the cells were photographed with a Leica 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 20µl TUNEL-label solution without TUNEL enzyme served as a negative control. Apoptotic cells were identified at 24h and expressed as a percentage of the total cells.

3.6. Statistical analysis

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

4. RESULTS

4.1. The effect of FSH and T_3 on Fas and FasL protein and mRNA content

It has been demonstrated that the Fas-FasL system is involved in the induction of apoptosis in ovarian cells of the human (28), mouse (23), pig (46) and rat (21). To examine if TH and FSH regulate Fas and FasL protein expression, granulosa cells were cultured with FSH and/or T_3 for 24 h and cellular content of Fas and FasL were determined by Western blot. Although FSH or T_3 alone had no significant influence on granulosa cell Fas content, co-treatment with FSH and T_3 (1.0 nM) significantly decreased Fas protein content [0.19±0.04 (FSH+T_3) vs. 1.32±0.1 (FSH), $p<0.001$; Figure 1A]. FasL content was significantly decreased in the presence of FSH [0.39±0.05 (FSH) vs. 0.925±0.09 (CTL without T_3), $p<0.05$; Figure 1A] and appeared to be decreased further though not significantly, with co-treatment with T_3. To better understand if the Fas/FasL protein content are regulated at the mRNA level by these hormones, Fas/FasL mRNA abundance were determined by qPCR. Granulosa cells cultured for 6 h to 12 h with FSH alone had lower Fas mRNA level when compared to the respective control [FSH vs. CTL: 1.08±0.09 vs. 1.99±0.21(6h); 1.17±0.15 vs. 1.74±0.26 (12h), Figure 1B] , although this response was not altered by the presence of T_3 [FSH+T_3, 1.22±0.23 (6h); 1.02±0.14 (12h)]. Expression of FasL mRNA was not affected by FSH irrespective of the presence of T_3 (Figure 1B). Moreover, T_3 alone had no effect on Fas/FasL mRNA content, suggesting that the regulation of granulosa cell FasL expression by FSH and T_3 is at the translational level or via post-translational processing.

4.2. T_3 significantly increased FSH-induced Xiap content *in vitro*

It is well established that the pro-survival factor, Xiap, directly inhibits caspase-3,-7 and -9 (11). Our previous studies have shown that extensive granulosa cell apoptosis in preantral and early antral follicles is associated with reduced Xiap levels. Withdrawal of gonadotropin negatively regulate Xiap expression and induced apoptosis in vivo (13). FSH increased Xiap mRNA and protein content in granulosa cells from large antral follicles in vitro, a process mediated by activated NFκB (29). To determine whether T_3 has a direct effect on Xiap content and can elicit a mitogenic response, granulosa cells were cultured in the FSH and/or T_3 for 24 h. FSH significantly increased granulosa cell Xiap content [2.06±0.07 (FSH) vs. 1.18±0.14 (CTL without T_3), $P<0.05$; Figure 2A]. Although T_3 alone was ineffective, a further and significant increase in Xiap content was detected in the presence of both FSH and T_3 (3.29±0.11, $p<0.01$) at 1.0 nM but not 0.1 (2.25±0.32), 10 (2.09±0.25) or 100 (2.1±0.34) nM T_3 (Figure 2A), when compared with those of FSH alone. Xiap mRNA abundance at 18h is significantly higher in FSH + T_3 group (1.95±0.14) than in FSH alone group (1.33±0.05) ($P<0.05$; Figure 2B), although the differences between the FSH alone and control group (1.06±0.05) were not statistically significant. Xiap protein content increased along with the up-regulation of Xiap mRNA abundance, suggesting that increased gene transcription may at least partially account for the increased protein content induced by hormonal co-treatment.

4.3. T_3 and FSH protected granulosa cells from ceramide-induced apoptosis

Granulosa cell apoptosis, as assessed by TUNEL, was low irrespective of the presence of FSH or T_3. To determine whether FSH and T_3 are survival factors and could protect granulosa cells from C8-ceramide-induced cell death, we cultured granulosa cells with FSH and/or T_3 and ceramide [an inducer of cell cycle arrest and apoptosis; 30 µM, added 4 h after hormonal treatment (42)]. Ceramide increased the number of TUNEL-positive cells from about 9.90±1.84% (basal level) to 60.99±3.28%, which was reduced by T_3, FSH and FSH+T_3 to 9.29±0.41%, 4.08±0.47%, 3.39±0.77 %, respectively (Figure 2C).

4.4. T_3 enhanced FSH-induced up-regulation of granulosa cell P-Akt content

Akt is a downstream effector of PI3K, which mediates granulosa cell survival (47). To determine if the combined treatment with FSH and T_3 activate granulosa cells PI3K pathway in vitro, granulosa cells were incubated with FSH and/or T_3 for 30 minutes and phospho-Akt content was determined. Phospho-Ser 473 Akt content was significantly increased in granulosa cells incubated with FSH [36.11±6.89 (FSH) vs. 0.89±0.06 (CTL without T3), $p<0.01$], a response significantly enhanced by T3 (1.0 nM, 57.81±10.82; $p<0.05$), although T3 alone (0.10-100 nM) was ineffective (Figure 3A). Remarkably, T3 enhanced FSH-induced Akt activation was PI3K-dependent, as pre-treatment of granulosa cells with the PI3K inhibitor LY294002 was capable of preventing FSH and T_3-induced Akt phosphorylation (Figure 3D). Additionally, granulosa cells treated with Akt inhibitor API-2 (48) suppressed phospho-Akt content induced by FSH and T_3.

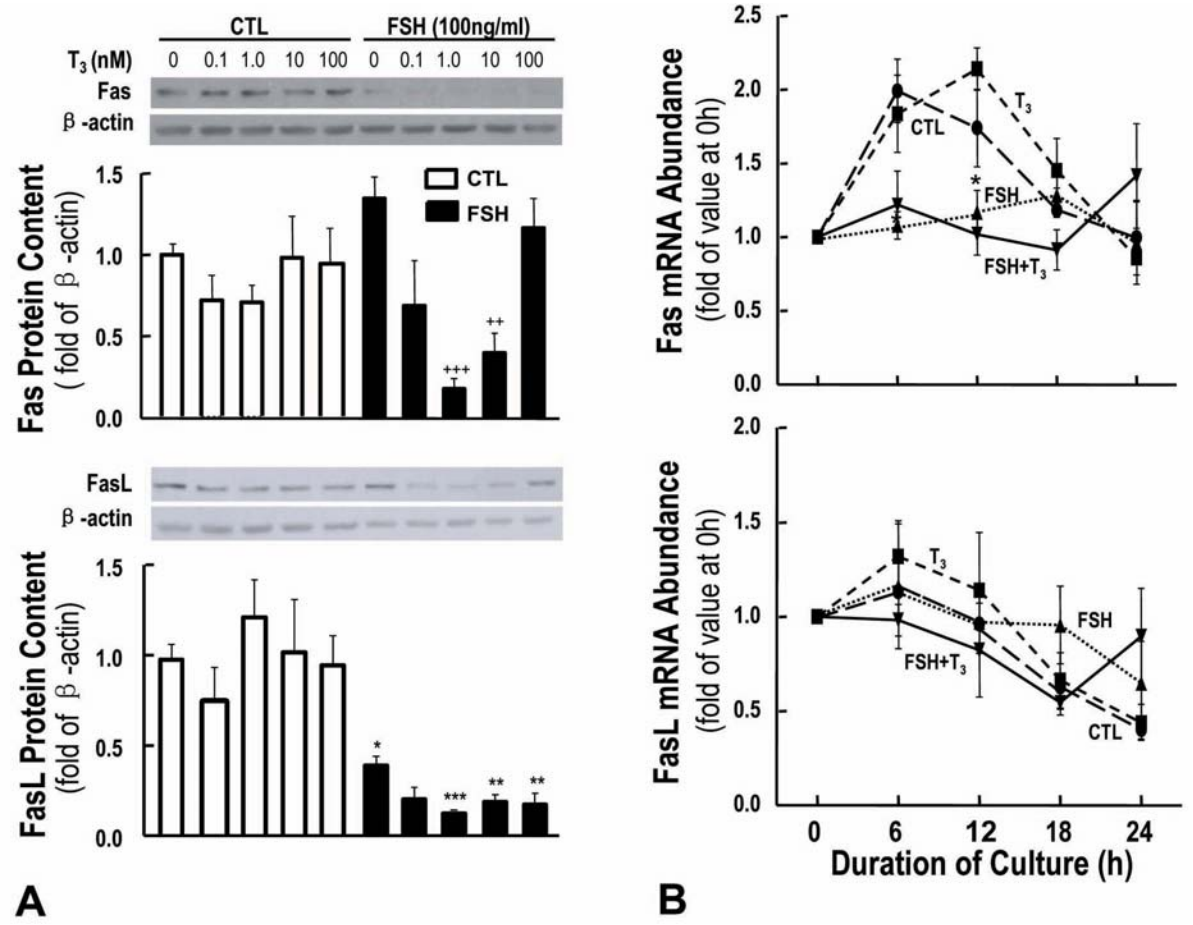

Figure 1. Effect of FSH and/or T_3 on granulosa cell Fas/FasL protein and mRNA content *in vitro.* Immature rats were treated with DES (1mg/day, 3 consecutive days) for granulosa cell isolation from preantral and early antral follicles. Granulosa cells were cultured up to 24h in the absence or presence of FSH (100 ng/ml) and/or various concentrations of T_3 (0-100 nM). The cells were harvested for Fas/FasL protein (Western Blot) and mRNA (real-time PCR) analysis. The Fas and FasL protein contents and mRNA abundance were normalized by β-actin and 18S rRNA respectively, and results are expressed as the fold of 0 time value for each group (B, real-time PCR). (A) Although FSH had no significant influence on Fas content, this response was significantly down-regulated by the presence of both FSH and T_3 (1.0 nM; $P<0.001$). FSH significantly down-regulated FasL expression ($P<0.05$), which was not affected by T_3. (B) FSH alone dramatically decreased Fas mRNA level during 6-12 h compared with the respective control ($P<0.05$). The combination of two hormones failed to enhance the down-regulated effect. T_3 alone was also ineffective. Neither of the hormone alone nor together elicited a significant response in the FasL mRNA regulation at all time points examined. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ (vs respective CTL); ++, $P<0.01$; +++, $P<0.001$ (vs FSH alone).

4.5. T_3-FSH interaction increased granulosa cell P-Src content

The Src family of non-receptor tyrosine kinases attenuate the inhibitory activity of regulatory subunit (p85) of PI3K by inducing tyrosine phosphorylation of p85, and interaction between phospho-tyrosine and the SH2 domain of p85 (36). To determine whether activated Src is the upstream of PI3K/Akt pathway, granulosa cells were incubated for 10 min with FSH and/or T_3. As shown in Figure 3B, FSH dramatically increased the phospho-Src content [0.92±0.06 (FSH) vs. 0.49±0.03 (CTL), $P<0.001$]. While T_3 alone had no effective on Src phosphorylation, co-treatment with the two hormones significantly enhanced Src activation when compared with FSH alone [1.33±0.06 (FSH+T_3) vs.0.92±0.06 (FSH), $p<0.01$; Figure 3B]. Pre-treatment of granulosa cells with Src kinase inhibitor PP1 for 1 hour, resulted in the inhibition of the FSH- and/or T_3 -induced Src phosphorylation. To address the possible role of activated Src in the regulation of the PI3K/Akt pathway, granulosa cells were also pre-treated with PP1 and phospho-Akt content was determined. PP1 attenuated the FSH- and or T_3- induced phospho-Akt content, but had no effect on total Akt content (Figure 3D).

4.6. Src & PI-3K/Akt pathway is involved the regulation of Fas, FasL and Xiap by FSH and T_3

Gonadotropic stimulation down-regulates both Fas and FasL expression in rat granulosa cells (21). FSH increased follicular Xiap expression via PI3K/Akt pathway in vitro (15). To test the hypothesis that Src/PI3K/Akt pathway is involved the regulation of granulosa cell Fas,

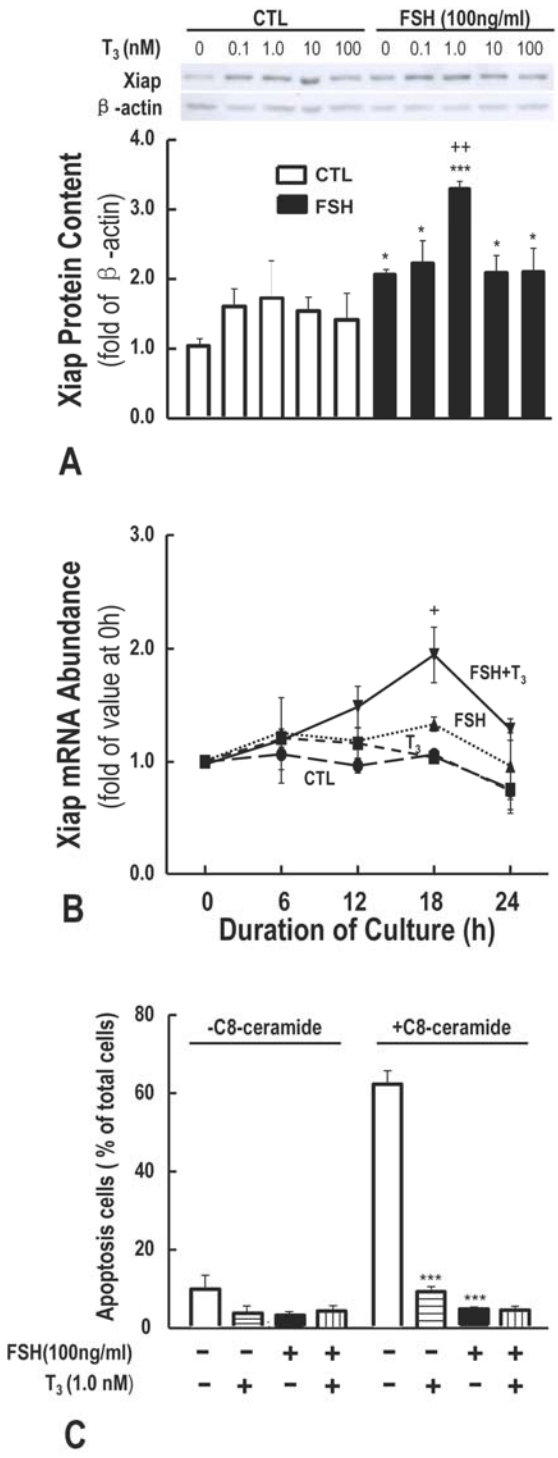

Figure 2. Effect of FSH and T_3 on granulosa cell Xiap content and apoptosis *in vitro*.Granulosa cells were cultured for up to 24h with FSH and/or T_3. As described in the Material and Methods, cells were collected for Western, real-time PCR analysis and TUNEL measurement. Xiap protein content and mRNA abundance was normalized by β-actin or 18S rRNA respectively, and expressed as the fold of 0h value for each group (B, real-time PCR). (A) T_3, although ineffective alone, significantly increased FSH-induced Xiap expression in the presence of T_3 (10^{-9} M; $P<0.01$). (B) T_3 (1.0 nM) plus FSH (100 ng/ml) significantly increased Xiap mRNA at 18 h ($P<0.05$). FSH or T_3 alone was ineffective at all time points examined. (C) Ceramide (30μm)-induced granulosa cell apoptosis was attenuated by the presence of FSH and/or T_3. *, $P<0.05$; ***,$P<0.001$ (vs respective CTL). +, $P<0.05$; ++, $P<0.01$ (vs FSH alone in the groups of same culture duration).

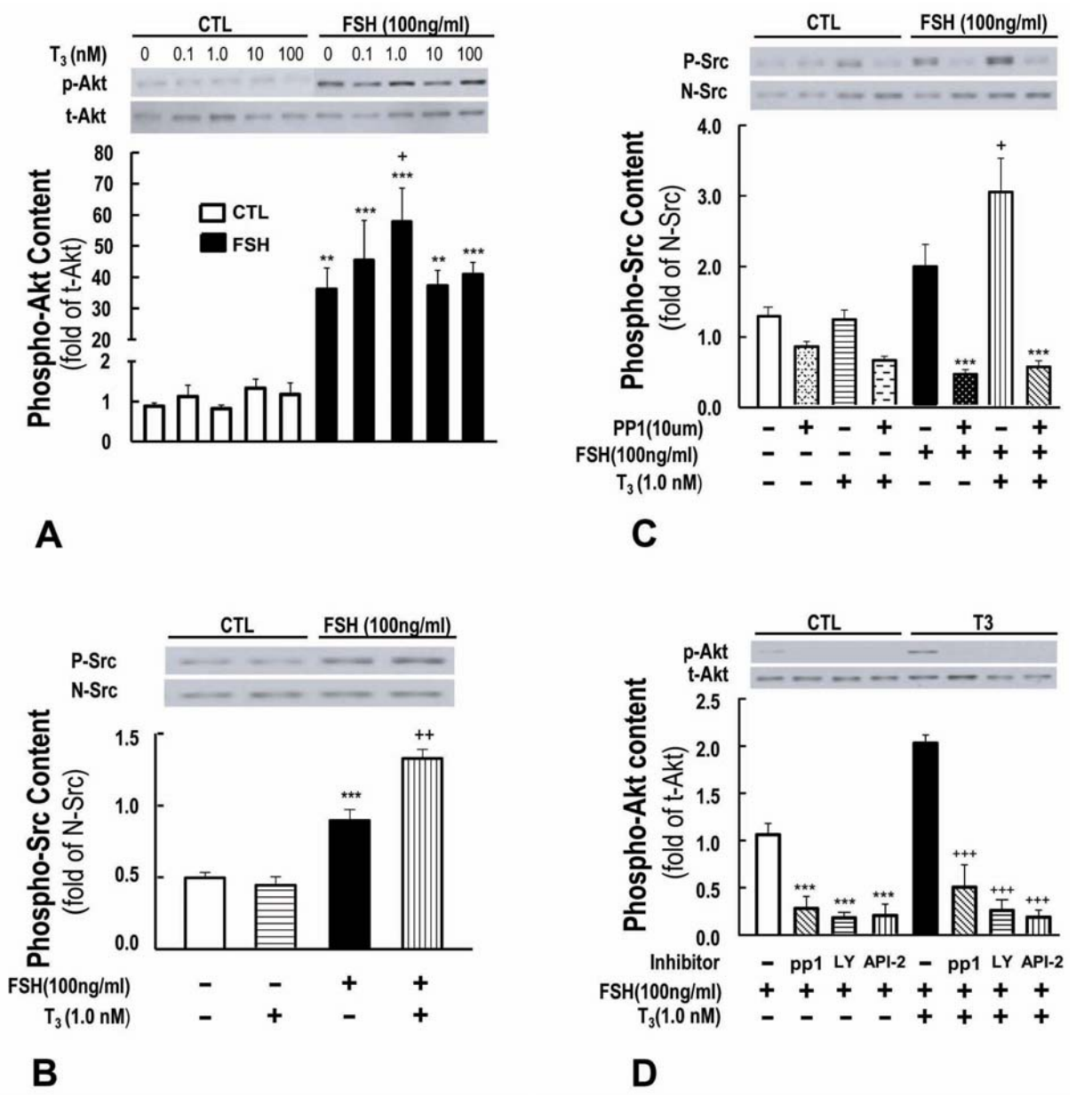

Figure 3. Effect of T_3 and FSH in vitro on P-Akt and P-Src content in granulosa cells .Granulosa cells were cultured for different duration in the presence of FSH (100 ng/ml) and/or T_3. Proteins from whole-cell lysate were assessed by Western blot analysis. Phospho-Akt and phospho-Src contents were normalized by t-Akt and N-src, respectively. (A) FSH significantly increase phospho-Akt content after 30 min treatment ($P<0.01$), a response enhanced by T_3 (1.0 nM; $P<0.05$). T_3 alone was ineffective. (B) FSH (100 ng/ml) significantly increased the phospho-Src content at 10 min ($P<0.001$). After co-treatment with FSH (100 ng/ml) and T_3 (1.0 nM) for 10 min, the level of phosphor-Src was further enhanced ($P<0.01$; compared with FSH alone). However, T_3 alone was without effect on phospho-Src content. (C) T_3 (1.0 nM) significantly synergized with FSH (100 ng/ml) in the upregulation of phospho-Src content in a 10 min incubation. The increased phospho-Src in the presence of FSH plusT_3 was blocked by pretreatment with the Src inhibitor PP1 (10 μM). (D) Inhibitor of Src, PI3K and Akt decreased basal and FSH plus T_3-induced phospho-Akt content. A,B,C, **, $P<0.01$; ***, $P<0.001$ (vs respective CTL); +, $P<0.05$; ++, $P<0.01$ (vs FSH alone); D, ***, $P<0.001$ (vs FSH alone); +++, $P<0.001$ (vs FSH+T_3).

FasL and Xiap expression, and consequently promoting cell survival, granulosa cells were pretreated for 1 hour with specific inhibitor of Src (PP1), PI3K (LY294002) or Akt (API-2) and then treated with FSH and/or T_3 for 24 hours (Figure 4A, B & C). While Fas and FasL protein contents were down-regulated by the presence of FSH and T_3, these responses were significantly attenuated by pretreatment with all three inhibitors [Fas:

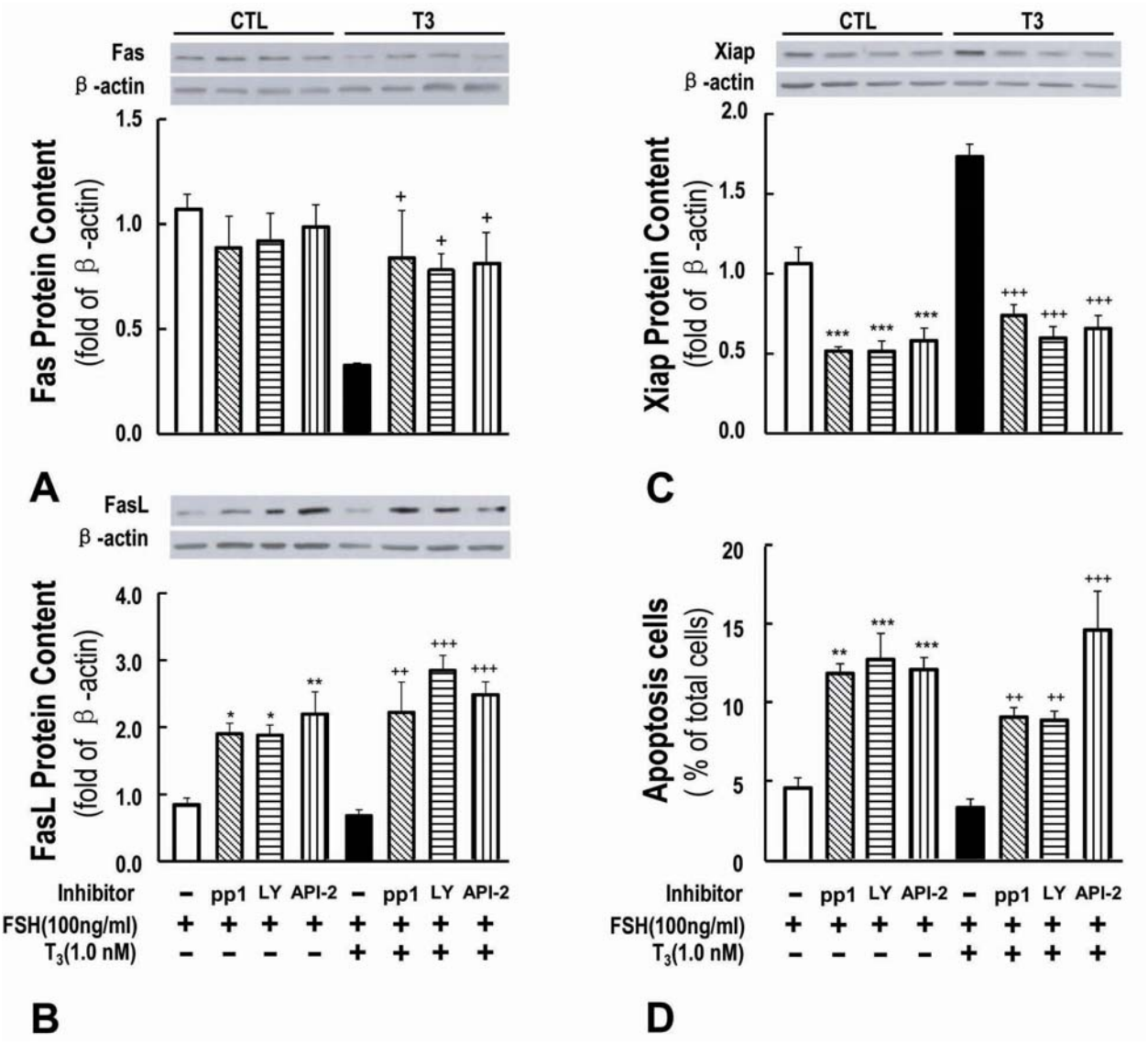

Figure 4. Regulation of Fas/FasL and Xiap by FSH and T_3 is mediated by Src and PI3K/Akt signaling. After pretreatment of with inhibitor of src (PP1, 10μm), PI3K (LY294002, 10μm) or Akt (API-2, 10μm) for 1 h, granulosa cells were cultured for 24 h with FSH and/or T_3, and Fas, FasL and Xiap contents and apoptosis were analyzed by Western Blot and TUNEL measurement respectively. (A) Although the inhibitors alone failed to affect Fas expression, they attenuated the inhibitory effect of FSH and T_3 on Fas content. **(**B) The **i**nhibitors attenuated the inhibitory effect of FSH and T_3 on FasL content. **(**C) FSH plus T_3 -induced Xiap content was decreased by all three inhibitors. (D) Pretreatment with the inhibitors abrogated the anti-apoptotic effect of FSH plus T_3. *, 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).

0.32±0.12 (FSH+T_3), 0.83±0.23 (FSH+T_3+PP1), 0.78±0.08 (FSH+T_3+LY), 0.81±0.15 (FSH+T_3+API); FasL: 0.74±0.06 (FSH+T_3), 2.20±0.45 (FSH+T_3+PP1), 2.84±0.23 (FSH+T_3+LY), 2.46±0.19 (FSH+T_3+API)] Coincidentally, the inhibitors also abrogated the up-regulation of Xiap induced by FSH+T_3 [1.73±0.08 (FSH+T_3) vs 0.74±0.07 (FSH+T_3+PP1), 0.59±0.07 (FSH+T_3+LY), 0.65±0.08 (FSH+T_3+API), p<0.001; Figure 4C]. Pretreatment with inhibitors also abrogated the anti-apoptotic effect of FSH+T_3 [3.36±0.51 (FSH+T_3) vs 9.07±0.58 (FSH+T_3+PP1), 8.84±0.58 (FSH+T_3+LY), 14.57±2.48 (FSH+T_3+API), P<0.01; Figure 4D]. These results suggest that FSH synergizes with T_3 to promote granulosa cell survival by regulating Fas, FasL and Xiap content via a Src-activated and PI3K/Akt-mediated mechanism.

5. DISCUSSION

We have previously demonstrated that gonadotropin up-regulates antral follicular Xiap expression and down-regulates the Fas/FasL pathway, and suppressed follicular apoptosis (15, 21). In addition, TH synergizes with FSH in promoting preantral follicular development (10). However, precisely how TH interacts with FSH in the regulation of

preantral follicular growth is not known. Although TH regulates genes expression by binding to its specific nuclear thyroid receptors (TRs), several rapid actions of TH appears mediated by other non-genomic pathways (32). Our results are also consistent with the non-genomic concept.

In the present study, we have demonstrated for the first time that FSH and T_3 increases granulosa cell Xiap content, down-regulates Fas/FasL pathway, and inhibits apoptosis. This regulation is mediated through the activation of Src-dependent PI3K/Akt pathway. The effect of T_3 was biphasic and within the physiological range of circulating levels of T_3 (38), and maximum response was evident at 1.0 nM. These findings are consistent with earlier studies showing an induction by T_3 of a biphasic increase in preantral follicular FSH receptor mRNA abundance with a maximal stimulatory concentration of the hormone also at 1.0 nM (10). Additionally, it is well established that both hypo- and hyper-thyroidism result in suppressed ovarian follicular growth (49, 50). Whether our current observations are related to these pathologic conditions remains to be determined.

It is well established that Xiap suppresses apoptosis by inhibiting caspase-9,-3 and -7 (11, 51-53). Xiap also ubiquitinates caspases and smac, leading to decreased contents of these proteins by proteasomal degradation (54). We have previously reported that FSH increased follicular Xiap expression, suppressed apoptosis and stimulated antral follicle development (15) and that TH and FSH synergize to promote growth of preantral/early antral follicles in vitro (10). The present studies extent these findings and demonstrate that co-treatment of granulosa cells from preantral/early antral follicles with FSH and T_3 increases Xiap expression, as evident by an increased Xiap protein and mRNA content following the hormonal treatment. Whether the increase in Xiap mRNA abundance is due to increased gene transcription and stabilization of the message in response to FSH and T_3, remains to be determined.

In the present study, granulosa cell Fas protein and mRNA levels were down-regulated by co-treatment of FSH and T_3, although neither FSH nor T_3 alone had any significant effect. Whereas FasL protein level was dramatically decreased by FSH, this effect was not significantly enhanced by T_3. Although FasL mRNA abundance was not significantly changed by the hormonal treatment, the level of FasL was significantly decreased, suggesting that the regulation of FasL protein may be at the translation level or via post-translational processing. Whether the latter possibility includes T_3-induced FasL degradation, maturation, stability, secretion, surface appearance and storage (55, 56), remains to be determined. Moreover, since FSH decreased the FasL but not Fas protein level and stimulated follicular development (10), it is possible that the control of FasL content might be more important in the regulation of granulosa cell apoptosis. It is of note that FasL expression and apoptosis were higher in the control groups than hormone-treated groups, the latter was not significantly different (Figure 1A and 2C). This is not surprising, considering the low level of apoptosis in the control group. These findings are consistent with our previous data that follicles showing intense immuno-activities for Fas and FasL also exhibit minimal or no TUNEL signals (17). In this context, while treatment of granulosa cells with C8-ceramide dramatically increased the number of TUNEL-positive cells, this was significantly decreased by treatment with FSH and/or T_3. These findings demonstrate that FSH and T_3 are anti-apoptotic and exert their action through inhibition of the Fas/FasL death pathway and the up-regulation the anti-apoptotic factor Xiap.

Activation of the PI3K/Akt pathway inhibits apoptosis via both the intrinsic and extrinsic death pathway (48, 57). Although FSH or TH rapidly activates the PI3K/Akt pathway in different cell type (29, 36), whether the activation of PI3K/Akt pathway by FSH and T_3 would indeed suppress granulosa cell apoptosis is not known. In the present study, we have demonstrated for the first time that T_3 rapidly enhanced FSH-induced Akt activation in granulosa cells, a response markedly attenuated by pretreatment with inhibitor of PI3K (LY294002) and Akt (API-2). Both inhibitors also abrogated the changes on Xiap, Fas, FasL and apoptosis induced by FSH and T_3, suggesting that the activation of PI3K/Akt pathway is required for the regulation of Xiap, Fas, FasL and apoptosis.

The non-receptor tyrosine kinase Src is localized to cellular membrane and is involved in the regulation of cell proliferation, apoptosis and motility (58). Activation of G-protein-coupled receptors and steroid hormone receptors, including thyroid hormone receptor (TR) leads to Src activation and autophosphorylation and tyrosine phosphorylation of p85, the regulatory subunit of the PI3K (36). It has been demonstrated that TH activates Akt via TR/Src/PI3K complex (36). However, whether Src is involved in the FSH- and T_3-induced Akt activation in granulosa cells is not known. In the present study, inhibition of Src by PP1 suppressed FSH- and T_3-induced Src activation and Akt phosphorylation as well as changes in Xiap, Fas and FasL contents, suggesting that Src is essential for Akt activation involved in anti-apoptotic signaling in granulosa cells in response to FSH and T_3.

Although our present studies provide strong evidence supporting the hypothesis that FSH and T_3 modulate the granulosa cell Xiap, Fas/Fasl content and apoptosis via Src activation of the PI3K/Akt pathway, the precise mechanism involved needs further investigated. Thyroid hormone receptor are present in rat granulosa cells (59) and thyroid receptor β1 (TRβ1) is able to interact with the regulatory subunit p85α of the PI3K (60). Although T_3 induces the activation of PI3K/Akt, a process involving the TRα1/p85/Src complex, the involvement of this complex in the PI3K/Akt pathway in the action of FSH and T_3 in granulosa cell has not been demonstrated. Our results suggest that TH interacted with FSH in increasing phospho-Src and phospho-Akt content and modulate Xiap, Fas and FasL expression. In this regard, we speculated that TR/Src/PI3K form complex and then increase Akt activity

and induce the downstream regulation. Alternatively, since TH increases intracellular Ca^{2+} concentration, which contributes to increase phospho-Src level (61), it is thus possible that increased intracellular Ca^{2+} level activates Src, which then activates the PI3K/Akt pathway without the TR/Src/PI3K complex. The another possibility is that Src is activated by NO/cGMP/PKG-I alpha signaling pathway (62), and subsequently the activation of the PI3K/Akt pathway and the up-regulation of Xiap. Whether any of these mechanisms is operational in the granulosa cells in response to FSH/T_3 remains to be determined.

6. ACKNOWLEDGMENTS

This work was supported by grants from the Canadian Institutes of Health Research (MOP-10369 to BKT), the Canada-China Joint Health Research Initiative of the Canadian Institutes of Health Research and the National Natural Science Foundation of China (CCI-109620; to BKY and GLX) and the World Class University (WCU) program (R31-10056 to BKT) through the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology. Cheng Zhang is a recipient of scholarships from the China Scholarship Council, Ministry of Education, Peoples' Republic of China and CIHR Institute of Human Development, Child and Youth Health REDIH Scholarship.

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Abbreviations: CTL, control; DES, Diethylstilbestrol; ECL, chemiluminescence; EVTs, extravillous trophoblasts; FasL, Fas ligand; FBS, fetal bovine serum; FSH, follicle-stimulating hormone; HRP, horseradish peroxidase; LY, LY294002; N-Src, Non-phospho-Src; p-Akt, phospho-Akt; p-Src, phospho-Src; PI3K, phosphatidylinositol 3-kinase; RT, room temperature; SH2, Src homology 2; t-Akt, total Akt; T3, triiodothyronine; TBS-T, Tris-buffered saline with tween; TH, thyroid hormone; TR, thyroid hormone receptor; TNF, tumor necrosis factor; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling.

Key Words: thyroid hormone, FSH, granulosa cell, apoptosis, Xiap, Fas, Fas ligand, Src.

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