Triiodothyronine Inhibits Proliferation and Stimulates Differentiation of Cultured Neonatal Sertoli Cells: Possible Mechanism for Increased Adult Testis Weight and Sperm Production Induced by Neonatal Goitrogen Treatment

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ABSTRACT

Transient neonatal hypothyroidism in the rat causes prolonged Sertoli cell proliferation, delayed Sertoli cell maturation, and increased adult Sertoli cell number, testis weight, and sperm production. Conversely, neonatal hyperthyroidism decreases Sertoli cell proliferation and ultimate testis size. This suggests that thyroid hormones might normally directly inhibit Sertoli cell proliferation while promoting maturation. However, these Sertoli cell effects could be due to secondary hormonal or metabolic effects of hypo- or hyperthyroidism. In this study, we directly tested thyroid hormone effects on Sertoli cell proliferation and differentiation in vitro. Sertoli cells from 5-day-old rat testes were grown in serum-free medium alone (controls) or with additional triiodothyronine (T3; 1-200 nM) and/or FSH (1μg/ml). After 4 days, cultures were used to obtain RNA for Northern hybridization or for thymidine autoradiography. Labeling index (LI) for control cultures and cultures receiving 100 nM T3 alone was 5.2 ± 0.5% and 5.0 ± 0.4%, respectively. The LI of FSH-treated cultures increased to 8.4 ± 0.8% (p < 0.01 vs. control). Cultures treated with FSH and 1, 10, 100, or 200 nM T3 had LIs of 8.0 ± 0.9%, 6.1 ± 0.4%, 5.3 ± 0.6%, and 4.8 ± 0.6%, respectively; the last three values were less than for cells receiving FSH alone (p < 0.01) or FSH + 1 nM T3 (p < 0.05). Northern hybridization indicated that mRNA levels for clusterin and inhibin-βa, Sertoli cell secretory proteins whose production normally increases during postnatal differentiation in vivo, were significantly increased by T3 or FSH alone. Furthermore, mRNA levels for inhibin-βa in Sertoli cells treated with both T3 and FSH were greater than with either alone, indicating that these hormones can act synergistically to promote maturation. These results indicate that T3 directly decreases mitogenesis in FSH-stimulated Sertoli cells and stimulates production of mRNA for secretory proteins characteristic of the more mature cell. Thus, T3 may normally directly promote differentiation of Sertoli cells from the mitotic to nonmitotic state and the concomitant onset of secretory function. These results also suggest that the changes in Sertoli cell mitogenesis and differentiation, and eventual changes in adult testis size and sperm production, following neonatal hypo- or hyperthyroidism may result from direct effects of the altered levels of thyroid hormone on Sertoli cells during neonatal development.

INTRODUCTION

Transient neonatal hypothyroidism in rat pups, induced by adding the reversible goitrogen 6-propyl-2-thiouracil (PTU) to the mother’s water from birth until Day 25, results in increases in adult testis size and sperm production of 80% and 140%, respectively [1, 2]. PTU treatment is effective only when begun during early neonatal life, suggesting that these changes observed in Sertoli cells in hypothyroid rats, such as delayed histological maturation and fluid secretion [9,16], suggest that thyroid hormones may normally directly act on Sertoli cells to inhibit mitogenesis but stimulate differentiation. This suggestion is consistent with the observed effects of neonatal hyperthyroidism, in which Sertoli cell proliferation is reduced but maturation is stimulated [12]. However, neonatal hypo- or hyperthyroidism also causes other metabolic and hormonal effects, including changes in concentrations of gonadotropins, thyroid-stimulating hormone (TSH), insulin-like growth factor-I (IGF-I), inhibin, growth hormone, and prolactin [9, 12, 17]. Therefore, it was unclear whether the Sertoli cell changes in rats that had been made hypo- or hyperthyroid neonatally resulted from direct effects of altered thyroid hormone levels or indirect effects caused by other changes in the hormonal or metabolic environment.

The aim of the present study was to directly evaluate the effects of thyroid hormones on Sertoli cell mitogenesis and...
differentiation in vitro. During early postnatal life, Sertoli cells in vivo begin to produce increasing amounts of secretory proteins characteristic of the juvenile and adult cell [15, 17, 18]. The levels of production of some of these proteins clearly reflects maturational state, allowing them to be used as markers of differentiation [15]. Two such proteins, clusterin and inhibin-Bβ, were used in this study to assess the effects of T3 on differentiation of cultured Sertoli cells.

Inhibin is a dimeric protein consisting of an α-subunit and a variable β-subunit, either βα or ββ [19]. This Sertoli cell secretory product feeds back on the pituitary to inhibit FSH production. Clusterin (also called SGP-2) is a Sertoli cell secretory product produced at high levels beginning during juvenile life [20]. Clusterin is produced not only by Sertoli cells but also by testicular peritubular cells, prostate, and other organs. The mRNAs for both inhibin-βα and clusterin are produced in detectable but relatively low levels at birth; they then rise dramatically during the early postnatal period [15]. The production of the mRNA for both of these proteins and the circulating levels of immunoreactive inhibin-α are decreased by neonatal hypothyroidism [15, 17]. Therefore, these proteins are excellent markers of differentiation in vivo and also are clearly affected by the thyroid status of the animal.

Our results indicate that thyroid hormones directly inhibit FSH-stimulated Sertoli cell mitogenesis in vitro while stimulating production of mRNA for secretory proteins characteristic of the more differentiated cell. Thus, thyroid hormone may be an important direct regulator of Sertoli cell mitogenesis and the onset of secretory function in vivo. These results also suggest that the increased Sertoli cell mitogenesis and retarded differentiation that occur following neonatal hypothyroidism, as well as the converse effects produced by neonatal hyperthyroidism, may result from direct effects on the Sertoli cells themselves.

MATERIALS AND METHODS

Animal purchase, care, and breeding were as described previously [1]. At all times, rats were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Sertoli Cell Culture

Sertoli cells from 5-day-old Sprague-Dawley rats (day of birth = Day 0) were isolated using a sequential enzymatic procedure [21, 22]. For each culture, pools of Sertoli cells were obtained from 8–10 males from two liters, and the culture procedure was repeated a total of 7 times. Trypan blue exclusion was used to determine the percentage of resultant Sertoli cells that were viable after dissociation. Sertoli cells were grown in 4-chamber tissue culture slides (Lab-Tek; Nunc, Naperville, IL) coated with matrigel (Collaborative Research, Waltham, MA) diluted 1:5 with Hanks’ Balanced Salt Solution. Cells were plated at a density of 7.5 × 10⁵ cells per chamber. The nutritive medium was Dulbecco’s Minimum Essential Medium (DMEM) supplemented with Na pyruvate (1 mM) and nonessential amino acids (0.1 mM). A 1% anti-microbial solution was also added; final concentrations of fungizone, penicillin, and streptomycin were 2.5 μg/ml, 100 U/ml, and 100 μg/ml, respectively [21, 22]. Cells were grown for 4 days in a humidified atmosphere of 95% air: 5% CO₂ at 34°C. Medium was changed every 24 h.

Some cultures were grown without hormonal supplementation, while others received FSH (ovine FSH-18, 1 μg/ml) and/or T₃ (3,3',5-triiodothyronine; Sigma, St. Louis, MO) at concentrations ranging from 1 to 200 nM. In all cases, T₃ was added at the initiation of the culture while FSH was added at the beginning of the third day and fresh hormone was added when medium was changed. To assess contamination by other cell types, some cultures were stained after 24 h for β-Hydroxysteroid dehydrogenase (β-HSD) to detect Leydig cells, while others were stained for alkaline phosphatase to detect germ (gonocytes and spermatogonia) and peritubular cell contamination [22, 23]. After 4 days in vitro, some cultures were used for autoradiography, while others were used for RNA extraction.

Tritiated Thymidine Autoradiography

For autoradiography, cells were incubated with 1 μCi/ml of ³H-thymidine (specific activity = 80 Ci/mmol; American, Arlington Hts., IL) for 2 h and then washed and fixed in 10% neutral buffered formalin. Slides were dipped in Kodak (Rochester, NY) NTB-2 emulsion and stored at 4°C for 3 days or until sufficient labeling could be detected. Autoradiograms were developed by standard techniques and then stained with hematoxylin and eosin.

Sertoli cell labeling index (LI; cells incorporating tritiated thymidine/total cells counted) in the various treatment groups was determined and analyzed as described previously [24]; 1000 cells per chamber were counted. Proliferation was greater in a thin band of cells at the extreme periphery of the chamber than centrally. LI was therefore always determined in the central region of the chamber to standardize the enumeration procedure. Sertoli cells were considered labeled when they contained ≥ 5 silver grains. At least 20 chambers (20 000 total cells) were counted for all treatment groups except for the group receiving T₃ (100 nM) alone, where n = 13. Data were expressed as mean ± SEM. The LIs in the various treatment groups were compared using a one-way ANOVA test. Differences were considered significant when p < 0.05.

Northern Blot Analysis

For each experiment, a pool of Sertoli cells was prepared from 8–10 males (derived from two liters) and grown for 4 days under the conditions described above. The entire experiment was performed on three separate occa-
FIG. 1. LI of 5-day Sertoli cells cultured 4 days in serum-free medium without hormones (controls) or with FSH and/or T3. Data are expressed as mean ± SEM of 1000 cells per chamber. At least 20 chambers were counted for all groups except those receiving T3 alone, where the n = 13, and the data presented here were derived from a total of seven separate culture experiments. LI in cells exposed to FSH and 10, 100, or 200 nM T3 was lower than in cultures receiving only FSH (p < 0.01) and was not different from that for control cultures (p > 0.05). A one-sided Dunnett’s test was used to compare the LI in the treated groups with the control values, and the least significant differences test was used to compare means among treated groups.

RESULTS

Viability in the Sertoli cell preparations was > 95% as determined by trypan blue exclusion. Visual assessment of cells with the hemocytometer revealed that less than 5% were large gonocytes [22]. Immunocytochemical staining for 3β-HSD indicated that Leydig cell contamination was minimal (1% or less). Additionally, alkaline phosphatase staining indicated that germ and peritubular cell contamination was also small (5–7%).

The morphology of the vast majority of cultured cells in the autoradiograms was typical of Sertoli cells in vivo in that the cells had a pale, frequently indented nucleus and a highly visible, single nucleolus. Some large cells (gonocytes) and a more fibroblastic population (peritubular cells) were also present, as described above. However, Sertoli cells could usually be clearly identified by their size, shape, and nuclear appearance, and only Sertoli cells were counted for the autoradiographic data.

Compared to control treatment, FSH stimulated Sertoli cell labeling, while T3 alone did not (Fig. 1). The LI of cultured Sertoli cells exposed to FSH + 1 nM T3 was not different from the value in the cells FSH alone. However, exposure of cultured cells to FSH and 10, 100, or 200 nM T3
resulted in LIs that were significantly lower than those of cultures receiving only FSH ($p < 0.01$) and not different from those of control cultures ($p > 0.05$).

A Northern blot analysis comparing steady-state levels of inhibin-$\beta_B$ mRNA in Sertoli cells cultured without hormonal supplementation, with FSH or $T_3$ alone, or with FSH + $T_3$ is shown in Figure 2. Inhibin-$\beta_B$ mRNA expression was minimal in the untreated cultures (lanes 1 and 2). Both FSH alone (lanes 3 and 4) and $T_3$ alone (lanes 5 and 6) increased inhibin-$\beta_B$ mRNA expression, and the combination of these hormones produced a still larger increase (lanes 7 and 8).

Densitometric analysis of the hybridization results (Fig. 3) indicated that steady-state levels of inhibin-$\beta_B$ mRNA expression in cells treated with FSH or $T_3$ were 3.6-fold and 4.6-fold the level in the untreated controls. Cells treated with both of these hormones showed an 8-fold increase in inhibin-$\beta_B$ mRNA expression compared to control cultures, indicating that FSH and $T_3$ acted synergistically to increase inhibin-$\beta_B$ mRNA expression to a level greater than with either hormone alone.

The effects of FSH or $T_3$ alone on the steady-state level of clusterin mRNA in cultured Sertoli cells were similar to the results seen with inhibin-$\beta_B$ mRNA expression (Fig. 4). Densitometric analysis of the normalized data indicated that clusterin mRNA expression levels were low in controls and increased with either FSH or $T_3$ treatment ($2.6 \pm 0.6$ and $2.1 \pm 0.3$ times control values, respectively). However, clusterin mRNA expression levels in cultures receiving both FSH and $T_3$ ($2.4 \pm 0.4$ times control values) were not significantly greater than in those receiving only FSH or $T_3$, so the synergistic effect of FSH and $T_3$ observed with inhibin-$\beta_B$ was not seen with clusterin.

**DISCUSSION**

Recent evidence has indicated that early neonatal hypothyroidism or hyperthyroidism can affect Sertoli cell mitogenesis and maturation. The presence of thyroid hormone receptors in Sertoli cells early in development [13–15] indicates that thyroid hormones could directly produce these Sertoli cell effects. However, alterations in neonatal thyroid hormone status produce a myriad of metabolic and endocrine changes that could also possibly affect Sertoli cell development [9,12,17]. Therefore, a critical goal for understanding how thyroid hormone affects testicular development was to determine whether these hormones acted directly on Sertoli cells or whether their effects were indirect.
FIG. 5. Proposed model for the direct effects of thyroid hormone on neonatal rat Sertoli cell proliferation and differentiation.

The results of the present study indicate that T3 directly inhibits mitogenesis of cultured neonatal rat Sertoli cells. Although T3 alone did not affect the baseline mitogenic activity observed in control cultures, T3 inhibited the increased mitogenic activity in cultured Sertoli cells given exogenous FSH. The inhibitory effect of T3 was not significant at 1 nM, but became maximal at T3 concentrations of 10 nM and above (100 and 200 nM). The inhibitory effect of T3 was pronounced; indeed, the LI of cultures exposed to T3 concentrations of 10 nM T3 or greater during the 4-day culture period and subsequently given FSH for the last 2 days was not significantly greater than for those that did not receive FSH.

Our data indicate that T3 alone directly stimulated clusterin and inhibin-βb mRNA production even in the absence of FSH. The magnitude of this stimulation was substantial. Indeed, the fold increases in inhibin-βb or clusterin mRNA after T3 or FSH stimulation were almost identical, although it must be reiterated that T3 was present for the entire 4-day culture period while FSH was added only for the last 2 days.

T3 also potentiates the actions of FSH on inhibin-βb mRNA production. The increase in inhibin-βb mRNA after treatment with both FSH and T3 was approximately equal to the sum of the increases produced with either of these hormones alone. The fact that T3 can increase inhibin-βb mRNA in Sertoli cells treated with maximally stimulatory doses of FSH indicates that these hormones must be promoting this increase by independent pathways. Synergistic effects of FSH and T3 on clusterin mRNA levels were not observed, although each of these hormones alone increased clusterin mRNA levels. Thus, regulation of clusterin and inhibin-βb appears to differ in this respect.

The stimulatory effect of FSH on inhibin-βb production by 5-day Sertoli cells in this study contrasts with previous results showing that FSH did not stimulate inhibin mRNA levels in juvenile Sertoli cells [26]. Thus, there may be an age-dependent change in the regulation of inhibin-βb mRNA by FSH. This type of age-related alteration in FSH responsiveness has been described previously for other Sertoli cell proteins. For example, FSH stimulates aromatization of androgens by neonatal Sertoli cells, but Sertoli cells from older animals do not respond to FSH in this manner, reflecting a developmental change in responsiveness to FSH for aromatase activity [27].

The present study provides evidence at the molecular level that thyroid hormones can directly stimulate secretory protein production in vitro by Sertoli cells from neonatal rats. These data indicate that it may be a direct effect of thyroid hormone deficiency on the Sertoli cell that results in the delayed maturation of these cells [9, 16] and the inhibition of the postnatal increase in mRNA expression for Sertoli cell secretory proteins (such as inhibin-βb and clusterin [15]) that characterize the hypothyroid animal. Similarly, previous in vitro studies with Sertoli cells from juvenile rats have indicated that T3 produces changes in IGF-I, androgen metabolism, and overall protein synthesis [18, 28, 29] consistent with a stimulatory effect on maturation. However, other studies indicated that T3 did not affect or that it decreased production of androgen-binding protein (ABP) and its mRNA [14, 18, 28, 30] in cultured Sertoli cells from young rats; these findings are apparently inconsistent with the data in the present study showing a stimulatory effect of T3 on Sertoli cell secretory proteins.

FSH has been demonstrated to be the major endocrine factor regulating mitogenesis and differentiation of Sertoli cells and the onset of secretory activity [31]. The present results indicate that T3 may also directly promote differentiation of the neonatal Sertoli cell and concomitant changes in proliferation and secretory activity and that it must be considered alongside FSH as a major endocrine regulator of normal Sertoli cell development. Furthermore, the majority of effects on Sertoli cells caused by hypothyroidism as well as hyperthyroidism may represent direct effects on the Sertoli cells themselves rather than secondary changes in Sertoli cells caused by changes in other hormones, growth factors, metabolism, etc. We propose the model shown in Figure 5 to integrate data on direct thyroid hormone actions in normal Sertoli cell development as well as its role in the changes seen in hypo- or hyperthyroidism.

This model may offer mechanistic insights into how early neonatal hypothyroidism induced by PTU [1–4, 9–11, 15] or other goitrogens such as methimazole [16, 32] leads to unprecedented increases in Sertoli cell number, adult testis size, and sperm production. On the basis of our demonstration of T3 effects in vitro, it appears that both increased mitogenesis and retarded development in Sertoli cells of hypothyroid rats directly result from reduced thyroid hormone exposure. Thyroid hormone directly promotes the differentiation of Sertoli cells with its attendant cessation of
mitotic activity and concomitant increases in production of certain secretory proteins. Decreased thyroid hormone in rats treated with PTU or methimazole reduces an inhibitory influence on Sertoli cell proliferation and leads to extended mitogenesis [9, 10] during which secretory activity is delayed [9, 16], resulting in an increased final number of Sertoli cells [9, 11]. The increased pool of Sertoli cells is then believed to be responsible for secondary changes such as the increased germ cell number, testis weight, and sperm production that characterize adult rats treated neonatally with goitrogens. Although the major cause of hypothyroidism-induced increases in Sertoli cell mitogenesis appears to involve direct effects of decreased T₃, one cannot exclude the possibility that other factors that are increased during hypothyroidism, such as TSH, could also be contributory to the increased Sertoli cell mitogenesis [1].

Neonatal hyperthyroidism results in premature cessation of Sertoli cell proliferation and approximately a 50% decrease in adult testis weight and Sertoli cell number, along with precocious Sertoli cell maturation [12]. These effects on Sertoli cell proliferation and maturation, the exact opposite of those seen in hypothyroidism, were postulated to be due to direct thyroid hormone actions on Sertoli cells [12]. However, this could not be definitively established in vivo because hyperthyroidism, like hypothyroidism, produces endocrine and metabolic changes that could affect Sertoli cell proliferation and/or differentiation. Our present results strongly suggest that the early cessation of Sertoli cell proliferation, the decreased adult number of these cells, and the premature onset of Sertoli cell secretory activity in hyperthyroid young rats are all due to direct actions of increased thyroid hormone on the Sertoli cells.

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