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Thyroid stimulating hormone inhibits rat granulosa cell steroidogenesis in primary culture

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It is not known why hypothyroidism predisposes the ovary to develop cystic follicles in hCG-treated rats. This study examined the effect of TSH on steroidogenesis in cultured granulosa cells. Granulosa cells were isolated from the ovaries of hCG-treated Long-Evans rats. Cells were cultured in medium with either 0 or 100 nM testosterone or 25(OH)cholesterol and treated with 0, 2.5, 10 or 20 ng/ml of TSH. TSH reduced 3β-HSDI activity in these cells to 12% of control. Progesterone secretion decreased by 72% and estradiol secretion was non-detectable during the second day of treatment with 20 ng/ml of TSH. We conclude that TSH is capable of regulating the secretion of progesterone and estradiol by cultured granulosa cells.

Keywords: TSH; granulosa cells; progesterone; estradiol; 3β-HSDI

Introduction

The disruption of thyroid function by the antithyroid drug propylthiouracil (PTU) creates an internal milieu that promotes the development of cystic rather than normal follicles in rats when exposed to hCG (Adams & Leathem, 1965; Bruot, 1986; Lee *et al.*, 1986). The mechanism whereby reduced serum concentrations of T_3 and T_4 lead to the formation of cystic follicles is not known.

Thyroid hormones are known to have a direct effect on granulosa cells and increase progesterone and estrogen secretion in the presence of FSH (Maruo *et al.*, 1987). Thus, it is possible that a decrease in serum concentrations of T_3 and T_4 may alter the course of normal follicular development. However, it is also possible that the PTU-induced decrease in thyroid hormones may act indirectly by reducing the negative feedback on the thyrotropes resulting in an elevation in serum TSH.

Several studies have correlated elevated TSH with follicular failure. In 1952 Nalbandov reported increased pituitary thyrotropic hormone content in pregnant and non-pregnant female swine with ovarian cysts as compared to normal swine (Nalbandov, 1952). Leathem (1958) found that an increase in ovarian weight and cyst formation in hypothyroid, hCG-treated rat was curtailed by hypophysectomy. Since there was no cyst formation in hypophysectomized, thiouracil treated rats given hCG and FSH replacement therapy (Scommegna, 1965), it was suggested that a pituitary hormone other than the gonadotropins may have been critical for cyst formation. Moreover, Grasso and Crisp (1985a) reported that the α-subunits of the glycoprotein hormones promote progesterone secretion by luteinized granulosa cells. Finally, the occurrence of subclinical hypothyroidism in 20% of infertile women (Gerhard *et al.*, 1990), the presence of low basal TSH levels in fertile as compared to infertile women (Gerhard *et al.*, 1991) and development of multicystic ovaries in girls with long-standing hypothyroidism (Lindsay *et al.*, 1980) suggests that TSH may play a role in ovarian dysfunction.

Collectively, these studies suggest to us that elevated serum concentrations of TSH may affect granulosa cell steroidogenesis and development. Initial experiments determined the serum concentration of TSH in euthyroid and hypothyroid rats, since these were unknown to date. Further experiments were then designed to examine the effect of hypothyroid levels of TSH on steroidogenesis by luteinizing granulosa cells in an *in vitro* culture system.

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Results

Serum concentrations of T_3, T_4 and TSH in euthyroid and hypothyroid rats

Serum concentrations of T_3 and T_4 were significantly reduced from 66.7 ± 8.2 ng/dl and 3.6 ± 0.2 μg/dl to 27.8 ± 1.6 ng/dl and 0.3 ± 0.1 μg/dl respectively, after 10 days of propylthiouracil treatment. TSH concentrations were significantly increased in the hypothyroid animals. The concentration of TSH in animals receiving propylthiouracil was 12.0 ± 0.5 ng/ml compared to 2.5 ± 0.2 ng/ml in euthyroid animals.

Effect of substrate and TSH treatment on total DNA at the end of culture

The addition of testosterone or TSH had no effect on the total DNA content per well after 72 h of culture. Wells containing cells cultured without testosterone or TSH contained 776 ± 141 ng of DNA compared to 859 ± 230 ng, 764 ± 123 ng and 737 ± 175 ng for those cultured with testosterone, TSH, and testosterone and TSH respectively.

Total 3β-HSDI staining and activity

Human chorionic gonadotropin significantly increased the number of granulosa cells staining positive for 3β-HSDI. After 2 days of hCG treatment, 66 ± 2% of

the granulosa cells stained positive as compared to 23 ± 5% from non-hCG-treated rats. Total 3β-HSDI activity was also measured in granulosa cells from hCG-treated rats cultured for 2 days with TSH. TSH at a concentration of 10 ng/ml reduced 3β-HSDI activity to 12% of control at the end of 48 h of culture (Figure 1).

The effect of TSH on steroidogenesis

Progesterone secretion by granulosa cells gradually declined over time, although the addition of 25(OH)cholesterol or testosterone slowed the rate of decline (Figure 2). TSH reduced the secretion of progesterone during the second and third days of culture to 63% and 22% of basal secretion, respectively. This effect was also observed when cells were cultured in the presence of 100 nM testosterone or 100 nM 25(OH)cholesterol. Progesterone secretion by granulosa cells cultured with testosterone and TSH declined to 73% and 47% of that produced by cells cultured with only testosterone on days 2 and 3, respectively. Similarly, the addition of TSH to the culture medium supplemented with 25(OH)cholesterol resulted in a decline in progesterone secretion to 58% and 14% of those cultured with only 25(OH)cholesterol on days 2 and 3, respectively.

The addition of increasing concentrations of TSH to granulosa cells resulted in a dose dependent decrease in progesterone (Figure 3). Granulosa cells cultured with 0, 2.5 10.0 and 20.0 ng/ml TSH secreted 2.7 ± 0.2, 2.8 ± 0.1, 2.4 ± 0.1 and 1.9 ± 0.1 ng progesterone/10^5 cells respectively, during the first 24 h. TSH was more effective in reducing progesterone secretion during the second and third days in culture. TSH at a concentration of 20 ng/ml reduced progesterone secretion by 72% and 88% during the second and third days in culture, respectively.

Estradiol secretion by cells cultured in medium without testosterone was not detectable. As expected, the addition of 100 nM testosterone to the culture medium resulted in detectable levels of estradiol (Figure 4). Estradiol concentrations declined from 397 ± 90 pg/10^5 cells during the first day to 38 ± 15 pg/10^5 during the third day. The addition of TSH to the testosterone supplemented medium further reduced estradiol secretion. During the second day, cells cultured with TSH and testosterone secreted 64% less estradiol than those cultured with testosterone alone. TSH reduced estradiol concentrations during the third day to non-detectable levels.

Increasing concentrations of TSH also induced a dose dependent decrease in estradiol secretion (Figure 5). The addition of 0, 2.5, 10 and 20 ng/ml TSH to the media of cells cultured for 24 h reduced estradiol secretion to 299 ± 136, 300 ± 113, 228 ± 104 and 221 ± 86 pg/10^5 cells respectively. Similar to its effect on progesterone, TSH was more effective in reducing estradiol secretion during the second and third days in culture. During the second day in culture, 10 ng/ml TSH reduced estradiol secretion by 67% while 20 ng/ml TSH reduced estradiol to non-detectable levels. After the third days in culture, estradiol secretion by cells cultured with 10 and 20 ng/ml was non-detectable.

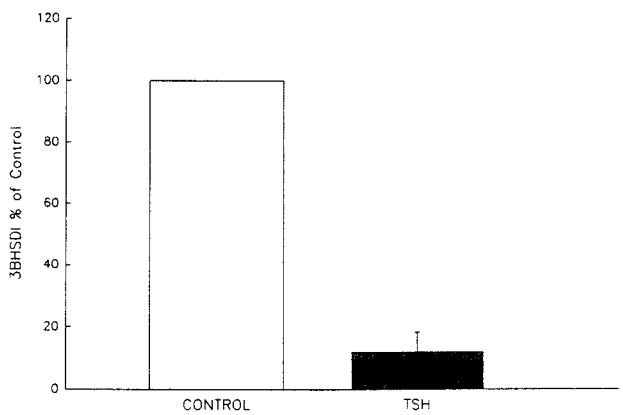

Figure 1 Percent 3β-HSDI activity in granulosa cells cultured with 0 and 10 ng/ml TSH (Solid bar, mean ± SEM). TSH significantly reduced 3β-HSDI activity after 48 h of culture

Discussion

It is well known that propylthiouracil disrupts normal thyroid function by inhibiting both the iodination of thyroglobulin and the formation of iodothyronine from iodotyrosines (Haynes, 1985). This results in a reduction in serum concentrations of thyroid hormones and the loss of the negative feedback of these hormones on

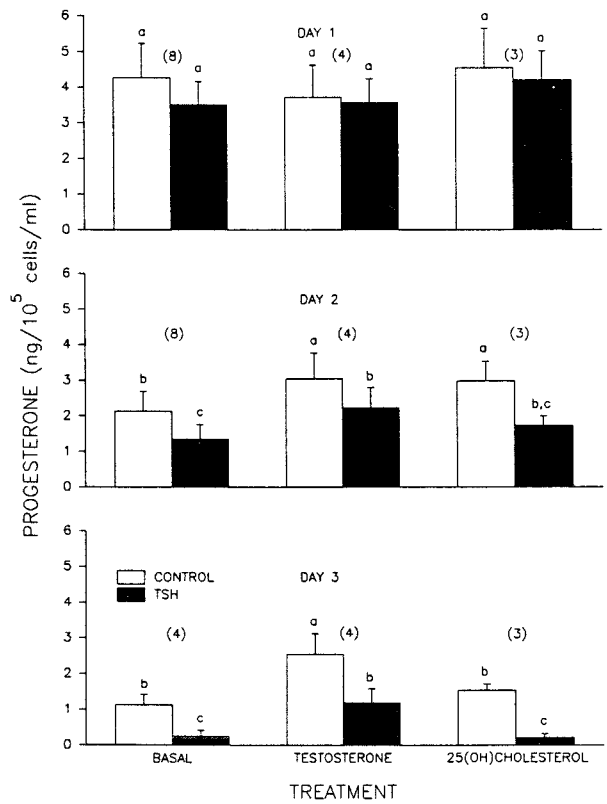

Figure 2 Progesterone secretion by granulosa cells during successive 24 h periods of culture. Some granulosa cells were cultured in medium supplemented with either 100 nM testosterone or 25(OH)cholesterol. The number of experiments in each group is shown in brackets. Solid bars represent treatment with 10 ng/ml TSH. Different letters denote significant differences between treatments (mean ± SEM). Number of experiments are shown in parenthesis

the pituitary thyrotropes and an increase in the serum concentration of TSH. This study confirmed that the serum concentration of TSH in the hypothyroid rat (10 ng/ml) was elevated compared to the euthyroid rat (≈2.5 ng/ml). Others have reported that TSH may be present in follicular fluid in concentrations at least as high as those present in serum (De Silva *et al.*, 1991). Therefore, we chose to use this physiological concentration of TSH in many of the *in vitro* studies with granulosa cells.

It has been known for many years that serum levels of TSH vary during the estrus cycle (Brown-Grant *et al.*, 1977). However, surprisingly little is known about the effect of TSH on the rat ovary. In 1981, Néauport reported an effect of TSH on the second messenger system in the rat ovary (Néauport and Emmerich, 1981). He found an increase in potassium influx and cAMP production in the ovaries of pre-pubertal rats treated with TSH. Subsequently, Grasso and Crisp (1985a,b) reported that the intact human TSH molecule and its α subunit stimulate progesterone production by luteinized granulosa cells.

The granulosa cells from euthyroid, hCG-treated rat ovaries used in these experiments appeared to be in the process of luteinization. Approximately three times more granulosa cells from euthyroid hCG-treated rat ovaries stained positive for 3β-HSDI than those from euthyroid saline treated rats. We found that TSH significantly reduced granulosa cell 3β-HSDI activity after 48 hours in culture. This is, to the best of our knowledge, the first report of an effect of TSH on 3β-HSDI activity. The time required to reduce 3β-HSDI is similar to that shown to be required to inhibit 3β-HSDI activity by GnRH in cultured granulosa cells (Fanjul *et al.*, 1992).

Progesterone secretion by granulosa cells was not limited by the availability of substrate during the first 24 h of culture. Although the secretion of progesterone declined with time, the addition of 25(OH)cholesterol predictably increased progesterone secretion especially on the 2nd day of culture. The addition of testosterone also enhanced progesterone secretion in concordance with other studies in the rat which have shown that

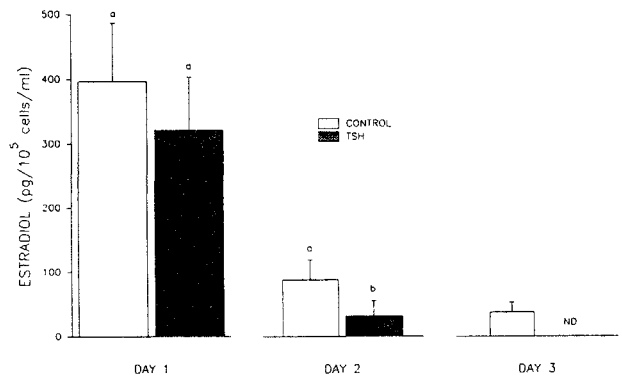

Figure 4 Estradiol secretion by granulosa cells during successive 24 h periods of culture in medium supplemented with 100 nM testosterone. Solid bars represent treatment with 10 ng/ml TSH. Data presented is the mean ± SEM of four experiments. Different letters denote significant differences between treatments

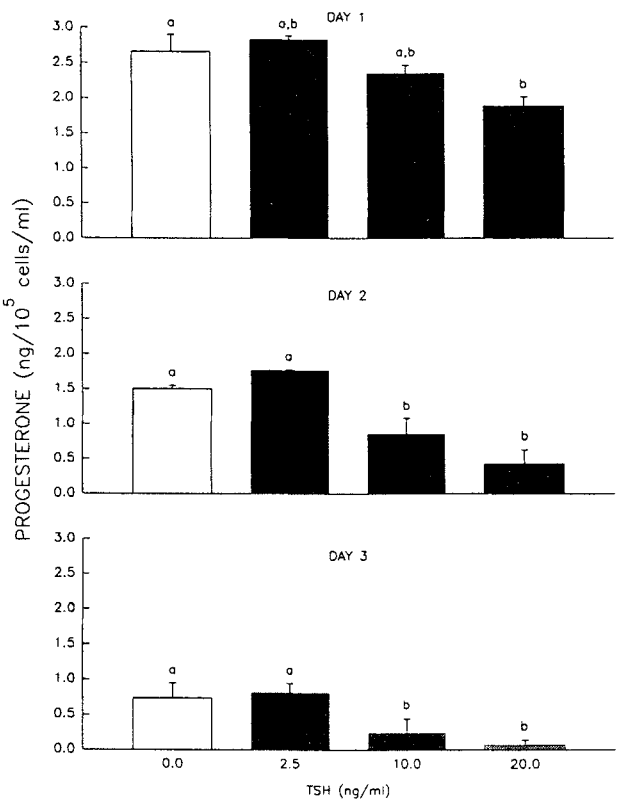

Figure 3 Progesterone secretion by granulosa cells during sucessive 24 h periods of culture in medium with 0, 2.5, 10 or 20 ng/ml TSH. Different letters denote significant differences between treatments (mean ± SEM of two experiments)

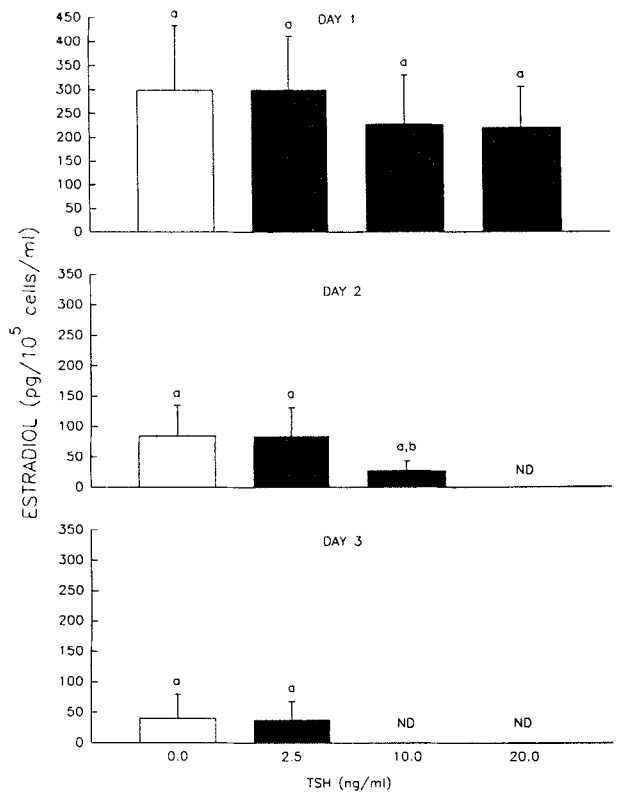

Figure 5 Estradiol secretion by granulosa cells during successive 24 h periods of culture in medium supplemented with 10 nM testosterone and treated with 0, 2.5, 10 or 20 ng/ml TSH. Different letters denote significant differences between treatments (mean ± SEM of two experiments)

testosterone stimulates progesterone production by rat granulosa cells (Armstrong and Dorrington, 1976; Duleba *et al.*, 1983; Moon *et al.*, 1984; Quirk *et al.*, 1986; Hudson *et al.*, 1987; Young and Melner, 1989).

Surprisingly, TSH significantly decreased progesterone secretion on the second and third days of culture in the presence or absence of 25(OH) cholesterol. This suggests that TSH probably does not act by limiting the availability of substrate for side chain cleavage. TSH also reduced progesterone secretion in the presence of testosterone in the medium. Since testosterone is reported to increase progesterone accumulation due to a reduction of 20α-hydroxysteroid dehydrogenase activity (Duleba *et al.*, 1983), we conclude that the effect of TSH is probably not due to increased catabolism of progesterone but a decrease in its synthesis. This hypothesis is supported by our finding that the 3β-HSDI activity in these cells is also reduced by exposure to TSH. The inhibitory effect of TSH does not appear to be due to a toxic effect on granulosa cells as the total DNA of attached (viable) cells per well at the end of culture did not change with TSH treatment.

Although these results appear to be inconsistent with those of Grasso and Crisp (1985a), a careful review reveals several significant differences in the experimental protocols. They used PMSG-primed animals as a source of granulosa cells which were cultured in plasma clot cultures. In contrast, granulosa cells from hCG-primed animals were cultured in a chemically defined medium. Furthermore, Grasso and Crisp used human TSH containing significant FSH and LH bioactivity. They concluded that the gonadotropic action of their TSH preparation was not the result of LH and FSH contamination because the addition of comparable amounts of LH and FSH failed to increase prolactin mediated progesterone production. However, they did not examine the effects of LH and FSH on basal progesterone production. Finally, they used human TSH which may have a different effect on rat granulosa cells than the rat TSH used in this experiment. Collectively, the differences in experimental protocols may explain the different effects of TSH on progesterone secretion by granulosa cells.

TSH also had no effect on estradiol secretion during the initial 24 h of culture. However, it appeared to have a significant effect on the second and third days. As little as 10 ng/ml TSH reduced estradiol secretion to non-detectable levels during the third day of culture.

Previous experiments from this lab have shown that serum levels of progesterone and estradiol were higher in hypothyroid hCG-treated animals than those in euthyroid hCG-treated animals (Lee *et al.*, 1986). Furthermore, ovarian sections from hypothyroid hCG-treated animals secreted greater amounts of estradiol during a short term incubation than those from euthyroid hCG-treated animals (Bruot, 1986). These results combined with the observation that TSH reduced granulosa cell estradiol and progesterone secretion suggests that granulosa cells may not contribute to the elevated levels of progesterone and estradiol seen during cyst development. Studies are in progress to examine the effect of TSH on other ovarian cell types.

In the testis, TSH has been reported to effect Leydig and Sertoli cells. Synthetic α-subunit peptides of the glycoprotein hormones (LH, hCG, FSH, TSH) have been shown to stimulate testosterone production by rat Leydig cells (Erickson *et al.*, 1990). Hutson and colleagues have reported an increased branching and filamentation of Sertoli cells cultured with TSH (Hutson, 1978) and increased production of androgen binding protein (Hutson and Stocco, 1978). TSH also increased plasminogen activator secretion by Sertoli cells (Hutson and Stocco, 1981). Mita *et al.* (1982) have reported a stimulatory effect of TSH on the synthesis of lactate by Sertoli cells from the rat testis. Cooke and Meisami (1991) have shown that the induction of hypothyroidism in rats from birth to 25 days of life by PTU treatment causes an increase in testicular size and DNA content in adults with a peak at 160 days of age. These studies show that TSH plays an active part in steroidogenesis in the testis and suggests to us that it may have a role in the ovary. It is noteworthy that TSH seems to increase androgen production in the testis. A similar hyperandrogenic effect in the ovary with a concomitant decrease in estradiol production that we have demonstrated could predispose a follicle to developmental failure. Studies are currently underway to investigate the effect of TSH on testosterone secretion by theca cells from these animals.

Although TSH binding has not been reported in the ovary, it has been reported in guinea pig Leydig cell membranes (Davies *et al.*, 1978) and in human Leydig cell membranes and adrenal homogenate (Trokoudes *et al.*, 1979). However, there is evidence of the presence of small amounts of TSH receptor mRNA in ovarian tissue (Akamizu *et al.*, 1990).

In conclusion, these experiments demonstrate that TSH is capable of reducing the secretion of progesterone and estradiol by granulosa cells from euthyroid hCG-treated rats. This suggests that TSH may contribute to the failure of some follicles to develop normally. As serum levels of progesterone and estradiol are elevated in the hypothyroid hCG-treated rat, it is possible that TSH may also effect theca/interstitial and luteal cell steroidogenesis. The studies of Grasso and Crisp (1985a,b) suggest that TSH may be important in the process of luteinization. Clearly, additional studies are needed to determine if TSH has a similar role in hypothyroid hCG-treated rats.

Materials and methods

Animals

Female Long-Evans rats 30 ± 2 days old were given tap water or 0.04% propylthiouracil (PTU) drinking water. After 10 days, the animals were sacrificed by decapitation, trunk blood was collected, serum separated by centrifugation and stored frozen until analysed for TSH, T_3 and T_4. In other experiments animals 30 ± 2 days old were injected with saline or 10 IU hCG (Ayerst Laboratories Inc., New York, NY) subcutaneously at 24 h intervals for 2 days. On the morning of the third day the animals were sacrificed by decapitation after Metofane anesthesia and ovaries were collected in ice-cold M199 (Sigma Chemical Company, St. Louis, MO) for harvesting granulosa cells.

Granulosa cells

Follicles ranging from 0.9 to 1 mm in diameter were microdissected from the ovaries and punctured in fresh medium to

release granulosa cells. The cells were washed and plated at a final density of ≈2 × 10^5 cells in 12 well culture plates (Corning Glass Works, Corning, NY). Cells were cultured in M199 supplemented with insulin (1 μg/ml), 0.2% BSA, and antibiotic/antimycotic (penicillin G 100 U/ml, streptomycin 100 μg/ml and amphotericin B 250 ng/ml; Sigma). Some cells were cultured in M199 that was also supplemented with 100 nM testosterone (Sigma) or 100 nM 25(OH)cholesterol (Sigma) and/or treated with 0, 2.5, 10 or 20 ng/ml TSH (NIDDK rTSH-RP-2). Media were changed each day for 3 days and spent media were collected and frozen at −20°C until analysed for progesterone and estradiol. Media from some cultures were also analysed for 20α-dihydroprogesterone. The results of this analysis were not included because the effect of TSH on 20α-dihydroprogesterone secretion was similar to its effect on progesterone secretion. At the end of the culture period total DNA content of adherent cells in each well was measured using a modification of a fluorimetric assay described by Labarca and Paigen (1980). Cells were scraped from wells and collected in assay buffer (0.05 M $NaPO_4$, 2.0 M NaCl) and stored frozen until assayed. DNA was assayed by incubating increasing volumes of 2 μg/ml of DNA (Calf thymus, Sigma) standard or 100 μl of cell suspension and 100 μl of 4 μg/ml of Hoe 33258 (Hoechst) in a final volume of 2 ml of assay buffer. At the end of one hour of incubation, fluorescence was measured at 345/450 nm excitation/emission at 10 nm slit width on a Perkin-Elmer fluorescence spectrofluorometer.

Hormone analysis

All steroids were measured by RIA without extraction. Progesterone was analysed as described by Latman *et al.* (1986) and modified by Lee *et al.* (1986). Estradiol was assayed as described by Wright *et al.* (1978) and modified by Lee *et al.* (1986).

TSH levels were measured by RIA using an rTSH RIA kit supplied by NIDDK, the standard being NIDDK-rTSH-RP-2. T_3 and T_4 levels were measured by using RIA kits from Diagnostic Products Corporation, Los Angeles CA 90045.

3βHSDI biochemical assay and histochemistry

In two experiments, cells were terminated after 48 h of culture and 3β-hydroxysteroid dehydrogenase isomerase (3β-HSDI) activity was measured using a biochemical assay as described previously by Stalvey and Clavey (1992). Briefly, cells were washed with Dulbecco's phosphate buffered saline (DPBS) and air-dried for 30 min. They were then incubated for 90 min at 37°C in 1 ml DPBS with [^{3}H]pregnenolone in dimethylsulfoxide and NAD^+. The reaction was terminated by adding methanol and progesterone. [^{14}C]progesterone was used to monitor recovery. Steroids were extracted in toluene and separated by thin layer chromatography in chloroform: ether (7:1). Radioactivity was measured by liquid scintillation counting.

Granulosa cells from saline and hCG treated rats were also stained for 3β-HSDI as described by (Stalvey and Payne, 1984). Briefly, an aliquot of cell suspension was allowed to dry on a slide and covered with PBS containing 0.1% BSA, 1.5 mM NAD^+, 0.25 mM nitroblue tetrazolium and 0.2 mM etiocholanolone. After 90 min at room temperature, the slide was gently washed and the percentage of stained cells was determined.

Statistical analysis

Data were statistically analysed by two-way analysis of variance and repeated measures analysis of variance as appropriate. When significant effects were detected comparison of means testing was done using Duncan's New Multiple Range test. All analyses were done using SAS (version 6.07) statistical software. TSH and T_3/T_4 serum concentrations are expressed as mean ± S.E. Progesterone and estradiol data are reported as mean ± S..E. of steroid produced per 24 h period. The activity of 3β-HSDI is expressed as mean ± S.E. percentage of control. Differences were considered significant for P values less than 0.05.

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