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Effects of Hyperprolactinemia on Testosterone Production in Rat Leydig Cells

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Abstract The pathogenesis of hyperprolactinemia (hyperPRL) induced hypogonadism has been suggested to be related with a dysfunction of hypothalamus–pituitary–testis axis. While the direct inhibitory effects of prolactin (PRL) on testosterone (T) release have been demonstrated, the mechanism is still unclear. Our previous study demonstrated a diminished T release in the testicular interstitial cells (TICs) from the anterior pituitary (AP)-grafted rats as compared with the control, and the pattern was in agreement with the in vivo model. However, TICs incubation cannot totally represent the response of the Leydig cells. Therefore, a Percoll gradient purified Leydig cell model was adopted to explore the response of T release under similar challenges in this study to investigate the effects of hyperPRL on the Leydig cells per se. HyperPRL in male rats was induced by grafting rat AP under the renal capsule. The control animals were grafted with rat brain cortex tissue (CX). Six weeks after grafting, the rats were sacrificed. Either TICs or Leydig cells were isolated, respectively, for in vitro incubation and challenge. Challenge drugs included human chorionic gonadotropin (hCG, 0.05 IU/ml), steroidogenic precursors (25-OH-cholesterol, 10^{-6} M; pregnenolone, 10^{-6} M), forskolin (an anenylyl cyclase activator, 10^{-4} M) and 8-bromo-3′:5′ cyclic adenosine monophosphate (cAMP) (8-Br-cAMP 10^{-4} M). T released by TICs or Leydig cells was determined by radioimmunoassay. The TICs from the AP-grafted rats showed lower levels of T release than the control group while the purified Leydig cells demonstrated a reverse pattern in response to challenges of hCG, steroidogenic precursors, forskolin and 8-Br-cAMP. In hyperPRL rats, a paradoxical pattern of T release between TICs and purified Leydig cells is observed. The purified Leydig cells from AP-grafted rats demonstrated a higher level amount of T release than the control after stimulation. The phenomenon can be attributed to the change of Leydig cell sensitivity to the stimulation after the effects of chronic hyperPRL. Moreover, another possibility is the role played by other interstitial cells to modulate steroidogenesis in Leydig cells. J. Cell. Biochem. 80:313–320, 2001. © 2001 Wiley-Liss, Inc.

Key words: prolactin; hyperprolactinemia; leydig cells; testosterone; testicular interstitial cells; male rats

Prolactin (PRL) acts as a trophic hormone in various aspects of reproductive functions such as helping gonadal maturation in the adolescent animals and regulating Leydig cell's T production [Dombrowicz et al., 1992]. However, high levels of circulating PRL result in testicular hypofunction and contribute to the appearance of sexual impotence and infertility in the mammals [Abbassy and Saikali, 1982; Bartke et al., 1986; Carter et al., 1978; Svare et al., 1979]. A precisely controlled homeostasis of plasma PRL is crucial to the competence of reproductive functions.

The mechanism of hyperPRL-induced hypogonadism has been suggested by a pre-appearance of gonadotropin-releasing hormone (GnRH) neuron dysfunction, presenting as reduced GnRH secretion [deGreef et al., 1995]. As a result, the gonads are exposed to a lesser extent of stimulation from the gonadotropins, in both amount and frequency. Hence, the picture of hypogonadism follows.

Besides the impact on the hypothalamus–pituitary–testis axis, a direct effect of PRL on

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the gonad had been proposed for decades. PRL receptors have been noted to be widely distributed in the testicular cells [Aragona et al., 1977; Barkey et al., 1977; Costlow and McGuire, 1977; Jabbour and Lincoln, 1999]. The role of PRL in regulating Leydig cells' steroidogenesis is still controversial. Direct biphasic modulation of PRL has also been found in gonadotropin-stimulated testicular androgen biosynthesis in vitro [Welsh et al., 1986], where the whole testicular cells responded with stimulatory effects of T release to a low concentration of PRL and inhibitory effects at higher concentration of PRL. Direct stimulatory effects of high concentration of PRL (up to 1 μg/ml) on aromatase activity in vitro in Leydig cells have also been documented [Papadopoulos et al., 1986]. Similarly, high doses of PRL (0.1–1 μg/ml) are noted to be stimulatory to luteinizing hormone (LH)-induced T release in vitro in a rat interstitial cells model [Odell and Larsen, 1984].

In studies with induced hyperPRL animals, increase of LH receptors and binding of LH with LH receptors on the Leydig cells have been demonstrated [Sharpe and McNeilly, 1980; Waeber et al., 1983]. The increase of LH receptors in the Leydig cells of hyperPRL rats is contributory to the increase of responsiveness or sensitivity in T release to the gonadotropin stimulation [Sharpe and McNeilly, 1980]. Ironically, chronic hyperPRL has made a pronounced impairment of steroidogenesis so that the overall amount of in vitro T production (efficacy) in these "sensitized" dispersed Leydig cells (interstitial cells in nature) is significantly lower than that in the control group [Sharpe and McNeilly, 1980]. Unfortunately, the fact is that chronic hyperPRL increases the sensitivity of Leydig cells to LH/hCG challenge, but the overall decreased T production in the interstitial cells isolated from the hyperPRL animals draws little attention. The conflicting faces between "sensitivity" and "efficacy" of PRL-modulated Leydig cell steroidogenesis and the overall effects of T production might be the clues to answer the mechanism of hyperPRL induced hypogonadism.

Interestingly, the effect of hyperPRL on the steroidogenesis of the Leydig cells had been studies in different in vitro models, such as the whole testis culture [Klemcke and Bartke, 1981; Sharpe and McNeilly, 1980] and interstitial cells [Sharpe and McNeilly, 1980; Huang et al., 1999]. All these reports indicated that a diminished amount of T was released from the group of hyperPRL in response to LH/hCG challenge. However, we can hardly find an in vitro study of LH/hCG-stimulated T release from Percoll gradient purified Leydig cells isolated from chronic hyperPRL animals. The purpose of this study is to investigate the effect of chronic hyperPRL on the Percoll gradient purified Leydig cells. Meanwhile, the responses of interstitial cells from chronic hyperPRL rats were also presented for comparison.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats weighing 250–300 g were housed in a temperature-controlled room ($22 \pm 1^{\circ}C$) with 14 h (0600–2000) artificial illumination daily. Food and water were provided ad libitum. All animal experimentation has been conducted humanely and in conformance with the policy statement of the Committee of National Yang-Ming University.

Induction of Hyperprolactinemia

Under light ether anesthesia, an incision was made in the left flank to expose the kidney. A small slit in the renal capsule was made to allow implantation of two anterior pituitary (AP) glands in the space beneath [Everett, 1954; Huang, 1999]. Rats of the control group were implanted with a similar amount of brain cortex (CX) in a like manner. Usually, seven or eight rats were grafted with AP or CX in each experiment. The in vitro studies were performed following the seventh week of survival. The rats were decapitated and the testes were collected for further preparation. Meanwhile, the kidney with the grafts was also examined. The criteria of vascularization and size of the viable grafted pituitary tissue were confirmed grossly and later microscopically by the method reported earlier [Merchenthaler et al., 1995]. Only those animals whose grafts showed the signs of survival and exhibited elevated plasma PRL levels over the mean of control level by at least 50% were employed. Overall, a successful rate of grafting in this experiment was 95%.

Preparation of Rat Testicular Interstitial Cells (TICs)

The method used for the collagenase dispersion of TICs has been described elsewhere [Tsai

et al., 1997]. Briefly, the medium (1% bovine serum albumin in Hank's balanced salt solution, with HEPES 25 mM, sodium bicarbonate 0.35 g/l, penicillin-G 100 IU/ml, streptomycin sulfate 50 mg/ml, heparin 2550 USP K units/l, pH 7.3) was aerated and saturated with 95% O_2 and 5% CO_2 before use. Collagenase (type IA, Sigma, St. Louis, MO) at a concentration of 700 μg/5 ml was used to disperse the TICs from rat testis blocks. After filtration of rat testicular tissues, the filtrate containing TICs was centrifuged, washed, then resuspended in the medium. TICs were plated into test tubes at a density of 1×10^6 cells/ml/tube. All the tubes were arrayed in a 34°C water bath, with gentle shaking at a speed of 50 cycles/min. The water bath chamber was kept saturated with 95% O_2 and 5% CO_2. Each preparation was found to contain approximately 20% Leydig cells [Lin et al., 1998].

Preparation of Leydig Cells

Following the procedure described above, the TICs were collected by centrifugation at 4°C, $200 \times g$ for 10 min. The cell pellet volume was recovered by the incubation medium to 5 ml and then added gently to the upper layer of a continuous gradient Percoll. The continuous gradient Percoll (25 ml/dispersion) was composed of nine parts of Percoll plus 11 parts of Medium 199 and 0.1% BSA before centrifugation at 4°C, $20{,}000 \times g$ for 60 min. The mixture of TICs with continuous gradient Percoll was centrifuged at 4°C, $800 \times g$ for 20 min. The Leydig cells were located at 3–7 ml layers from the bottom. The Leydig cells layer was diluted to 5 ml and then centrifuged $80 \times g$ at room temperature for 8 min. After repeating the wash step, the cell pellet was resuspended to 10 ml by the incubation medium. Cell concentration (1×10^5 cells/ml), and viability (over 95%), were determined using a hemocytometer and the trypan blue method. For the experiments of 1 h challenge, the Leydig cells were plated into test tubes at a density of 1×10^5 cells/ml. The following procedures were similar to those of TICs incubation. For the experiments of 24 h challenge, the Leydig cells were plated into 24-well culture dishes (Falcon, Franklin Lakes, NJ) at a density of 1×10^5 cells/ml. The culture dishes were incubated in a incubating chamber kept aerated with 95% air and 5% CO_2 at 37°C. To measure the abundance of Leydig cells in our preparation, the 3β-hydroxysteroid dehydrogenase (3β-HSD) staining method was used [Dirami et al., 1991; Krummen et al., 1994]. The cells (1×10^5 cells/ml), were incubated with a solution containing 0.2 mg/ml nitro blue tetrozolium (Sigma), 0.12 mg/ml 5-androstane-3β-ol-one (Sigma), and 1 mg/ml NAD^+ (Sigma) in 0.05 M PBS, pH 7.4 at 34°C for 90 min. Upon development of the blue formazan deposit sites of 3β-HSD activity, the abundance of Leydig cells was determined by using a hemocytometer. Our preparation was found to contain approximately 87% Leydig cells.

Treatment of TICs and Leydig Cells with hCG

The TICs (n = 8) and Leydig cells (n = 6) prepared from both CX- and AP-grafted rats were challenged with 200 μl hCG (0.05 IU/ml). A 90-min preincubation was followed by a 60-min drug-challenged incubation which was stopped by adding 2 ml of ice-cold gelatin phosphate buffer saline (PBSG, 0.1% gelatin in 0.01 M phosphate buffer, 0.15 M sodium chloride, pH 7.5). After centrifugation at $100 \times g$ for 10 min, the supernatant was collected and freeze-stored for T radioimmunoassay (RIA).

Treatment of TICs and Leydig Cells with 25-OH-Cholesterol and Pregnenolone

Pregnenolone and 25-OH-cholesterol are the precursors for steroidogenesis. The TICs (n = 8) and Leydig cells (n = 6) prepared from both CX- and AP-grafted rats were challenged with 200 μl of 25-OH-cholesterol (10^{-6} M) and pregnenolone (10^{-6} M),respectively. A 90-min preincubation was followed by a 60-min drug-challenged incubation. Addition of 2 ml of ice-cold PBSG stopped the reaction. After centrifugation at $100 \times g$ for 10 min, the supernatant was freeze-stored for T RIA.

Treatment of TICs and Leydig Cells with Forskolin and 8-Br-3′:5′ Cyclic Adenosine Monophosphate (cAMP)

Forskolin is an activator of adenylyl cyclase, by which cAMP is synthesized and acts as a secondary messenger for subsequent steroidogenesis in the Leydig cell, while 8-Br-cAMP is a soluble cAMP molecule that is permeable to the cell membrane, and subsequently activates the steroidogenesis process. The TICs (n = 8) and Leydig cells (n = 6) prepared from both CX- and AP-grafted rats were challenged with 200 μl of forskolin (10^{-4} M) and 8-Br-cAMP (10^{-4} M),

respectively. A 90-min preincubation was followed by a 60-min drug-challenged incubation. Addition of 2 ml of ice-cold PBSG stopped the reaction. After centrifugation at $100 \times g$ for 10 min, the supernatant was freeze-stored for T RIA.

RIA of T and PRL

The concentration of medium T was determined by RIA as described elsewhere [Wang et al., 1994]. The sensitivity of anti-T serum (W8) was 2 pg per assay tube. The intra- and interassay coefficient of variation (CV) were 4.1% (n = 6) and 4.7% (n = 10), respectively.

The concentration of PRL in plasma was determined by RIA as described elsewhere [Tang et al., 1986]. The rat PRL RIA kit was provided by the National Institute of Diabetes and Digestive and Kidney Disease (NIDDK), the National Institute of Child Health and Human Development, and the U.S. Department of Agriculture, USA. Rat PRL-I-5 was used for ionization. Rat PRL-RP-3 served as standards. The intra- and interassay CV were 3.8 and 3.2%, respectively. The sensitivity was 30 pg/tube.

Statistics

All values were expressed as mean ± standard error of the mean (SEM). For multiple comparison, the means from different treatments were tested for homogeneity by a two-way analysis of variance (ANOVA), and the difference between specific means was tested for significance by Duncan's multiple-range test. The difference between means from two different groups was analyzed by Student's *t*-test [Steel and Torie, 1960]. A difference between two means was considered statistically significant when $P < 0.05$.

RESULTS

Plasma PRL Levels in AP- and CX- Grafted Rats

The plasma sample was collected at the time of decapitation. Plasma PRL levels in the AP-grafted rats were significantly higher than that in the CX-grafted ones ($P < 0.01$, Fig. 1).

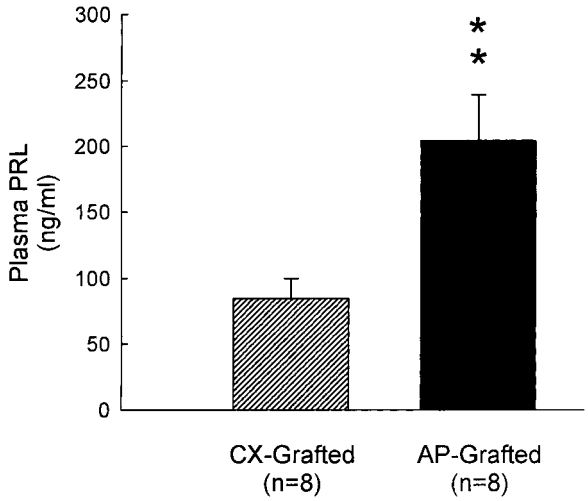

Fig. 1. Plasma PRL levels in AP- and CX- grafted rats. The blood sample was obtained at sixth week of grafting operation. $^{**}P < 0.01$ as compared with the CX- grafted rats.

Effects of HyperPRL on T Release by TICs and Leydig Cells in Response to hCG

The T release after hCG (0.05 IU/ml/h) challenge was evident in both TICs and Leydig cells after incubation. In TICs setting, AP-grafted groups showed a diminished amount of T release following hCG stimulation ($P < 0.05$). While in the Leydig cell preparations, AP-grafted groups were greater in T release compared with CX-grafted groups after hCG challenge ($P < 0.01$) (Fig. 2).

Effects of HyperPRL on T Release by TICs and Leydig Cells in Response to Steroidogenesis Precursors in AP- and CX-Grafted Rats

The T release after 25-OH-cholesterol (10^{-6} M) and pregnenolone (10^{-6} M) challenge was evident in both TICs and Leydig cells after incubation. In TICs, AP-grafted groups showed a diminished amount of T release to co-incubation of either precursors compared with CX-grafted groups (vs. 25-OH-cholesterol, $P < 0.05$; vs. pregnenolone, $P < 0.01$), while in the Leydig cell preparations, AP-grafted groups were higher in amount of T release after challenges of either precursors ($P < 0.01$) (Fig. 3).

Effects of HyperPRL on T Release by TICs and Leydig Cells in Response to Forskolin and 8-Br-cAMP in AP- and CX-Grafted Rats

The T release after forskolin (10^{-4} M) and 8-Br-cAMP (10^{-4} M) challenge was evident in both TICs and Leydig cells after incubation. In TICs, AP-grafted groups showed a diminished amount of T release to both stimulants compared with CX-grafted groups (vs. forskolin,

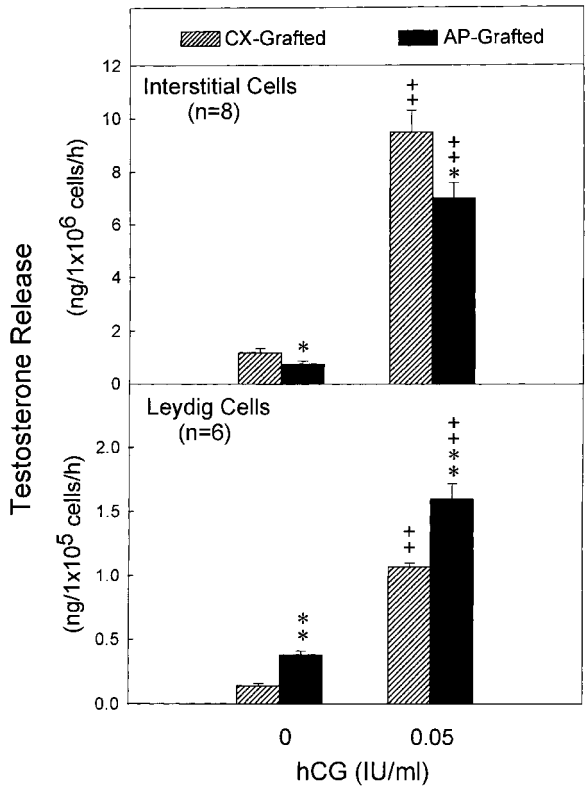

Fig. 2. T release by rat testicular interstitial cells (TICs, 1×10^6 cells/h), upper panel, or Leydig cells (1×10^5 cells/h), lower panel, in response to hCG (0.05 IU/ml). $^*P < 0.05$, and $^{**}P < 0.01$, vs. the CX-grafted group, respectively. $^+P < 0.05$, and $^{++}P < 0.01$ vs. the corresponding basal level, respectively.

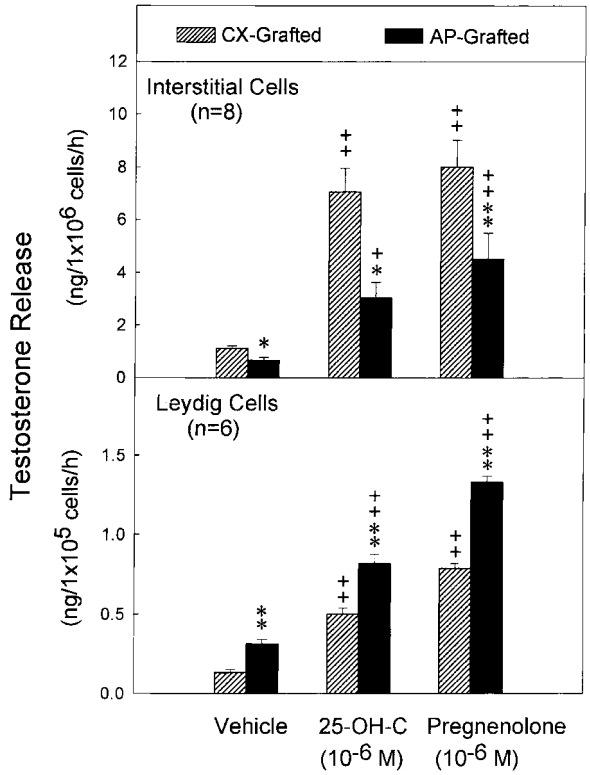

Fig. 3. T release by rat testicular interstitial cells (TICs, 1×10^6 cells/h), upper panel, or Leydig cells (1×10^5 cells/h), lower panel, in response to steroidogenic precursors, 25-OH-cholesterol (25-OH-C, 10^{-6} M) and pregnenolone (10^{-6} M). $^*P < 0.05$, and $^{**}P < 0.01$, vs. the CX-grafted group, respectively. $^+P < 0.05$, and $^{++}P < 0.01$ vs. the corresponding basal level, respectively.

$P < 0.05$; vs. 8-Br-cAMP, $P < 0.01$), while in the Leydig cell preparations, AP-grafted groups were greater in amount of T release compared with CX-grafted groups after either challenges ($P < 0.01$) (Fig. 4).

DISCUSSION

PRL is a pituitary-secreted protein hormone whose secretion is delicately controlled by the hypothalamic dopamine tonus. PRL is not only necessary in the differentiation or multiplication of the Leydig cells during gonadal maturation, but it is also important in modifying T release by Leydig cells. A homeostasis of plasma PRL is crucial for a normally functioning reproductive system. However, abnormally elevated plasma PRL levels for a long period of time are harmful for the gonadal functions [Katovich et al., 1985; Sharpe and McNeilly, 1980; Waeber et al., 1983].

The direct effects of PRL on the gonad steroidogenesis have been studied in many different ways, but the role of PRL is still inconclusive in its either stimulatory or inhibitory effects. One of the two major in vitro approaches of testis response to stimulation under PRL manipulation is to use the dispersed testicular cells, or whole testis, as study materials, where PRL is added in vitro. In the study of whole testicular cells, administration of higher dose of PRL inhibits the T release after LH/hCG challenge, while a smaller amount of PRL is stimulatory [Welsh et al., 1986]. In a TIC setting, as the given amount of PRL increased, the maximal LH response of T release decreased. Moreover, the dose–response curve for LH-stimulated T release shifted to the right, that was to say that the responsiveness of Leydig cells to LH stimulation decreased as more PRL was given [Odell and Larsen, 1984]. Paradoxically, high doses of PRL have also been found to be stimulatory in

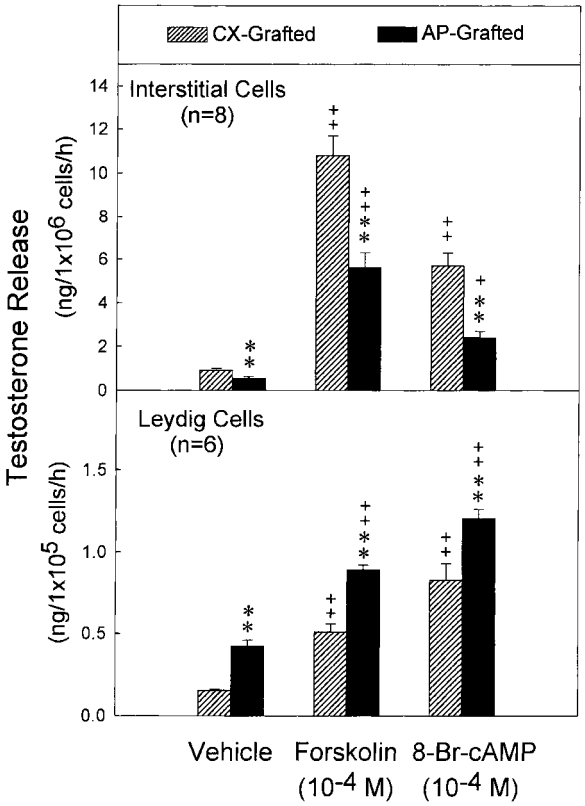

Fig. 4. T release by rat testicular interstitial cells (TICs, 1×10^6 cells/h), upper panel, or Leydig cells (1×10^5 cells/h), lower panel, in response to forskolin (an adenylyl cyclase activator, 10^{-4} M) and 8-Br-cAMP (a membrane permeable analog of cAMP, 10^{-4} M). $^{*}P < 0.05$, and $^{**}P < 0.01$, vs. the CX-grafted group, respectively. $^{+}P < 0.05$, and $^{++}P < 0.01$ vs. the corresponding basal level, respectively.

T release in the same study [Odell and Larsen, 1984]. In a Percoll-purified rat Leydig cell setting, PRL alone at high concentration (1 μg/ml) increases the aramatase activity; however, co-incubation of PRL with LH diminished the usual activity of LH [Papadopoulus et al., 1985].

The other in vitro approach of the study of testicular response to stimulation under PRL manipulation is to establish hyperPRL animals beforehand and to use the isolated testicular cells as study materials. In these studies with either whole testicular cells [Klemcke and Bartke, 1981] or dispersed Leydig cells [Huang et al., 1999; Sharpe and McNeilly, 1980; Waeber et al., 1983], a reduced amount of in vitro T release was noted in the hyperPRL groups in both basal and LH/hCG challenged conditions.

In our previous experiments using AP-grafted hyperPRL rats, the results obtained from isolated TIC models disclosed the reduction of overall T release in the hyperPRL group [Huang et al., 1999]. These findings are in agreement with the in vivo states, where reduced T release was found in the AP-grafted hyperPRL rats after the hCG stimulation. However, in the present study the results from the Percoll gradient purified Leydig cell incubation showed that, under similar challenges as in the TIC model, more T was released in vitro in the Leydig cells of the hyperPRL group than in the control. The paradoxical patterns of T release between TICs and Percoll gradient purified Leydig cells in vitro also happened in studies challenged with androgen biosynthesis precursors, adenylyl cyclase activator forskolin and membrane-permeable 8-Br-cAMP. These findings suggested that the discrepancy between TIC and purified Leydig cell models could be due to an overall impairment of steroidogenesis in the Leydig cell situated in the TICs environment in the hyperPRL rats. This is a novel finding and has not been reported elsewhere.

The possible reasons to explain the paradoxical patterns of T release between TIC and Percoll-purified Leydig cell setting deserve a more detailed discussion. First of all, hyperPRL has been suggested to increase testicular sensitivity to LH challenge [Sharpe and McNeilly, 1980]. In a rat model, Bartke and Dalterio [1976] have demonstrated that the AP-graft-induced hyperPRL could enhance testicular responsiveness to the action of gonadotropins, particularly in the period before sexual maturation. PRL has also been noted to be stimulatory in the in vitro T biosynthesis at a lower concentration [Welsh et al., 1986] or at higher concentrations [Odell and Larson, 1984; Papadopoulos et al., 1986]. Therefore, hyperPRL itself is a beneficial factor for the responsiveness of Leydig cells to gonadotropin action.

Secondly, the cell compositions are different between TIC and Percoll-purified Leydig cell models. The major difference is in the proportion of Leydig cells and the existence of the other interstitial cells in the TIC preparation. It has been measured that in the testicular interstitium the testicular macrophage is the major cellular population second to the Leydig cells. According to several reports, the percentage of macrophage in the interstitial cells varies from 20 to 30% [Miller et al., 1983; Niemi et al., 1986]. In the TIC preparation around

60% of the cells were macrophages (our unpublished data). Macrophage has also been acknowledged as a major source of cytokines, particularly tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) [Le and Vilcek, 1987]. Through the cytokines the macrophages may cross-talk with the other cells in the testis. Besides, the macrophage is in close contact with the Leydig cell as shown in the electron microscopic pictures, which indicates the potential interactions between them [Gaytan et al., 1994; Miller et al., 1983]. There is increasing evidence indicating that macrophages play an important role in regulating steroidogenesis of the Leydig cells [Gaytan et al., 1994, 1995]. TNF-α has also been found to be present in the TICs medium [unpublished data]. The role of testicular macrophage and its effects on the Leydig cells under the action of PRL or under the circumstance of preexisting chronic hyperPRL deserve careful verification.

Thirdly, the minor cell populations in the testicular interstitium or cells from the tubular compartments might also have some effects on the steroidogenesis in Leydig cells.

It is evident that AP-grafting-induced chronic hyperPRL has made a fundamental change in Leydig cells. It is possible that chronic hyperPRL has also made a change in the cross-talk among the cells in the testis.

In summary, in a chronic hyperPRL rat model, a discrepancy of in vitro T release between the TIC setting and the Percoll-purified Leydig cell preparation is present. The exploration of the mechanism contributing to this paradoxical phenomenon might be the key to answer the pathogenesis of hyperPRL-induced hypogonadism. We are currently doing the experiments for characterizing the role of the testicular macrophage in the Leydig cells' steroidogenesis in the scenario of hyperPRL.

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