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Regulation of Testosterone Secretion by Prolactin in Male Rats

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Abstract The goal of this study was to characterize the mechanism by which hyperprolactinemia alters testosterone production in rat testicular interstitial cells (TICs). Hyperprolactinemia was induced by grafting 2 anterior pituitary (AP) glands under the subcapsular space of the kidney in experimental rats. Control rats were grafted with brain cortex (CX). Six weeks post-grafting, rats were challenged with human chorionic gonadotropin (hCG) then, the changes in either plasma testosterone or luteinizing hormone was measured. Additionally, TICs were isolated and challenged in vitro with hCG or prolactin, and the testosterone release measured by radioimmunoassay. Further investigation in signal transduction as intracellular 3′:5′ cyclic adenosine monophosphate (cAMP) production was observed under a regulation of forskolin or SQ22536. After the challenge of hCG or GnRH, the AP-grafted rats showed a suppressed response in testosterone release as compared to those in the CX-grafted group. The in vitro data from the AP-grafted rats compared to the CX-grafted animals showed a diminished response in testosterone release upon hCG stimulation. Administration of forskolin or SQ22536 disclosed dysfunction of adenylate cyclase in TICs from the AP-grafted rats. When 8-Br-cAMP was incubated with TICs, the testosterone production was lower in the AP-grafted compared to the CX-grafted group. These results suggest that in addition to adenylate cyclase dysfunction, inefficiency of post-cAMP pathways are also involved in the hypogonadism elicited by hyperprolactinemia in rats. J. Cell. Biochem. 74:111–118, 1999. © 1999 Wiley-Liss, Inc.

Key words: PRL; hyperprolactinemia; testosterone; cAMP; male rats

Prolactin (PRL) is a protein hormone secreted by the anterior pituitary gland (AP) and is controlled by tonic inhibition of hypothalamic dopamine. In male animals, PRL plays an important role in the maturation of gonads. It has been reported that PRL supplementation to immature hypophysectomized rats stimulates the multiplication and differentiation of Leydig cells and germ cells in a dose-dependent manner [Dombrowicz et al., 1992]. Furthermore, PRL is necessary in regulating the interaction between gonadotropins and the Leydig cells during testosterone secretion [Bartke, 1971; Evans et al., 1982; Martikainen et al., 1983]. Some investigators have provided evidence that PRL receptors are expressed in Leydig cells, Sertoli cells and even spermatogonia [Hondo et al., 1995; Barkey et al., 1994]. A stimulatory effect of PRL on steroidogenesis has also been established [Rubin et al., 1975]. These observations indicate that PRL is a trophic hormone targeting the testes, and may exert its effect by acting directly on the testicular tissue.

However, hyperprolactinemia has been acknowledged as one of the causes of sexual impotence and infertility in humans and other mam-

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mals [Bartke, 1971; Boyar et al., 1974; Thorner et al., 1974; Saidi et al., 1977]. This phenomenon has been attributed to the decreased activity of GnRH neurons and a reduced amount and frequency of GnRH secretion. AP responds to the altered GnRH secretion by decreasing the amount of gonadotropins. As a result, the testes show hypogonadism in response to the diminished gonadotropins [Bartke, 1971; deGreef et al., 1995].

Furthermore, hyperprolactin-induced suppression of gonadotropin release may also be accompanied by unchanged testosterone levels [Boyar et al., 1974; Bartke and Dalterio, 1976; McNeilly et al., 1978]. Likewise, hyperprolactin-induced suppression of testosterone secretion is sometimes present with an unchanged serum LH concentration [Saidi et al., 1977]. Klemcke and Bartke [1981] reported chronic hyperprolactinemia in mouse models that showed plasma LH and FSH were significantly increased and testosterone remained unchanged. These features seem not to be fully satisfied by the explanation of pathogenesis from previous observations [Bartke, 1971; deGreef et al., 1995]. In hyperprolactinemic patients, it has been suspected that the hypogonadism is in part due to a PRL-induced blockade of the action of the gonadotropins on the gonads [Thorner et al., 1974; McNatty et al., 1974]. However, the direct effects of PRL and its mechanism of action on steroidogenesis in testicular cells have not been well studied and remain poorly defined [Barkey et al., 1994].

Testosterone is released by the Leydig cells through a 3′:5′cyclic adenosine monophosphate (cAMP) mediated steroidogenesis pathway. It has been shown that PRL exerts inhibitory effects on hCG-induced intracellular cAMP accumulation in a human granulosa cell culture system [Kraiem, 1984], nevertheless, no previous report has characterized hyperprolactin-related inhibition of cAMP production in a testicular interstitial cell (TIC) system. The underlying rationale for this study was to determine how PRL exerts its inhibitory influence on hCG-induced testosterone release of TICs under a hyperprolactin-induced environment, and to define the intracellular mechanism that may be involved.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats weighing 250–300 g were housed in a temperature controlled room (22 ± 1°C) with 14 h (0600–2000) artificial illumination daily. Food and water were provided *ad libitum*.

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 APs in the space beneath [Everett, 1954]. Rats of the control group were implanted with a similar amount of brain cortex (CX) in a like manner. Usually, seven to eight rats were grafted with AP or CX in each experiment. The in vivo or in vitro experiments were performed following the 7th week of survival. For the in vivo study, an indwelling polyethylene catheter was positioned in the left internal jugular vein 1 day prior to each experiment. On the following day, the challenge drug was administered and blood samples were collected via the catheter so as to minimize stress. The lost blood volume was replenished with donor blood immediately after each bleeding [Sheu et al., 1987]. For the in vitro study, the rats were decapitated and the testes were retrieved for preparation of TICs. Meanwhile, the kidney with grafts was also examined. The criteria of vascularization and size of the viable grafted pituitary tissue were confirmed grossly and later microscopically as per 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 94.4%.

Preparation of TICs

The method used for the collagenase dispersion of TICs is described elsewhere [Tsai et al., 1997]. Briefly, the medium 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 mg/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 prepa-

ration was found to contain approximately 20% Leydig cells [Tsai et al., 1997].

Experiment 1: Effects of Hyperprolactinemia on the In Vivo Release of Testosterone in Response to hCG

Eight AP-grafted and eight CX-grafted pre-catheterized rats were maintained undisturbed for at least 90 min before drug challenge. The hCG (5 IU/ml/kg) was administered as an intravenous bolus. Blood samples (0.6 ml each) were collected at 0, 30, 60, 120, and 240 min post hCG injection. The plasma was separated and freeze-stored for later testosterone and PRL RIA.

Experiment 2: Effects of PRL In Vivo on the Testosterone Release by TICs

The TICs prepared from both CX- and AP-grafted rats (n = 8) were challenged with 200 μl hCG at different concentrations (0.01, 0.05, 0.1, and 0.5 IU/ml). A 90 min preincubation was followed by a 60 min hCG challenged incubation. At the end of incubation, the reaction 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*g* for 10 min, the supernatant was collected and freeze-stored for testosterone RIA.

Experiment 3: Effects of PRL In Vitro on the Release of Testosterone in Hyperprolactinemic Rats

Different doses (0.01, 0.1, 1, 5, to 10 mg/ml) of ovine PRL (oPRL) in 200 μl aliquots were incubated with TICs prepared from CX- and AP-grafted rats. Each incubation period was 60 min, as per the method described in Experiment 2, the incubation media were collected for testosterone RIA.

Experiment 4: Effects of Hyperprolactinemia on Signal Transduction in TICs

The signal transduction in terms of intracellular cAMP production and the subsequent testosterone release in TICs from both the AP- and CX-grafted (n = 8) rats was studied. The cAMP production machinery was manipulated by adding 1 μM forskolin (Sigma), an adenylate cyclase activator, to increase intracellular cAMP, or, by adding 10 μM SQ22536 (Sigma), an adenylate cyclase inhibitor, to suppress intracellular cAMP. In this setting, cells were incubated for two consecutive 60 min periods. The medium collected after the second period of incubation was freeze-stored for testosterone RIA. The spun down cell pellet in each test tube was mixed with 1 ml of 65% ice-cold ethanol, then, was homogenized on a polytron (PT3000, Kinematica Ag., Luzern, Switzerland). The homogenate was centrifuged at 1,500*g* for 15 min. The ethanol supernatant was lyophilized in a vacuum concentrator and stored at −20°C until analyzed by cAMP RIA.

A cAMP analogue, 8-Br-cAMP was incubated with TICs from the AP- and CX-grafted rats in order to assess the mediation of cAMP on testosterone production by-passing the change of the activity of adenylate cyclase.

RIA of Testosterone, Corticosterone, PRL, FSH, and LH

The concentration of medium and plasma testosterone was determined by RIA as described elsewhere [Wang et al., 1994; Tsai et al., 1996a]. The sensitivity of anti-testosterone serum (W8) was 2 pg per assay tube. The intra- and interassay (CV) were 4.1% (n = 6) and 4.7% (n = 10), respectively.

The concentration of plasma corticosterone was measured by RIA as described elsewhere [Chen et al., 1997; Lo et al., 1998] with anti-corticosterone PSW#4-9, the sensitivity of corticosterone RIA was 5 pg per tube. The intra- and interassay CV were 3.3% (n = 5) and 9.2% (n = 4), respectively.

The concentration of PRL in plasma was determined by RIA as described elsewhere [Tang et al., 1986; Tsai et al., 1996b]. The rat prolactin 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. Rat PRL-I-5 was used for iodination. 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.

The concentration of plasma FSH was determined by RIA using anti-FSH serum S-11. The rat FSH-I-8 used for iodination and the rat FSH RP-2 which served as standard preparation was provided by the NIDDK. The sensitivity was 0.1 ng/tube for FSH RIA. The intra- and interassay CV were 3.9% (n = 6) and 6.2% (n = 3), respectively.

The concentration of plasma LH was determined by RIA as described previously using

anti-LH serum PW11-2 [Wang et al., 1994]. The rat LH-I-6 used for iodination and the rat LH-RP-3 that served as a standard preparation was provided by the NIDDK. The sensitivity was 0.1 ng/tube for LH RIA. The intra- and interassay CV were 3.8% (n = 4) and 6.6% (n = 5), respectively.

RIA of cAMP

The concentration of cAMP was measured by RIA as previously described [Tsai et al., 1996a; Lu et al., 1996]. The anti-cAMP (CV-27 pool) was provided by NIDDK. The sensitivity of cAMP assay was 2 fmol per assay tube. The intra- and interassay CV was 6.9% (n = 5) and 11.9% (n = 5), respectively.

Statistical Analysis

All values are 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 [Steel and Torrie, 1960]. A difference between two means was considered statistically significant when $P < 0.05$.

RESULTS

Plasma PRL

There was no significant difference in the levels of plasma LH, FSH and corticosterone between the CX-grafted (LH: 3.17 ± 1.35 ng/ml; FSH: 6.11 ± 0.48 ng/ml; corticosterone: 44.84 ± 8.03 ng/ml, n = 7–8) and the AP-grafted rats (LH: 2.54 ± 0.90 ng/ml; FSH: 6.21 ± 0.52 ng/ml; corticosterone: 46.49 ± 5.17 ng/ml, n = 7–8).

The concentrations of plasma PRL were higher in the hyperprolactinemic (AP-grafted) rats than in the control (CX-grafted) animals (Fig. 1).

Effects of Hyperprolactinemia on In Vivo Testosterone Release in Response to hCG

The basal levels of plasma testosterone remained unchanged in the AP-grafted rats as compared to CX-grafted animals (Fig. 2). In both the AP- and CX-grafted rats, the bolus injection of hCG (5 IU/ml/kg) induced an increase in testosterone release which reached a peak at 30 min post-challenge. However, the level or responsiveness of testosterone release in the AP-grafted rats was significantly lower than that in the control animals (Fig. 2).

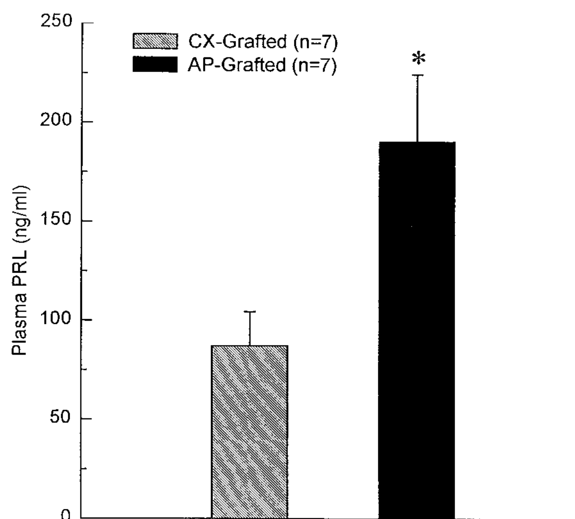

Fig. 1. Plasma PRL levels in the AP- and CX-grafted rats. $^{*}P <$ 0.05 as compared with the CX-grafted rats.

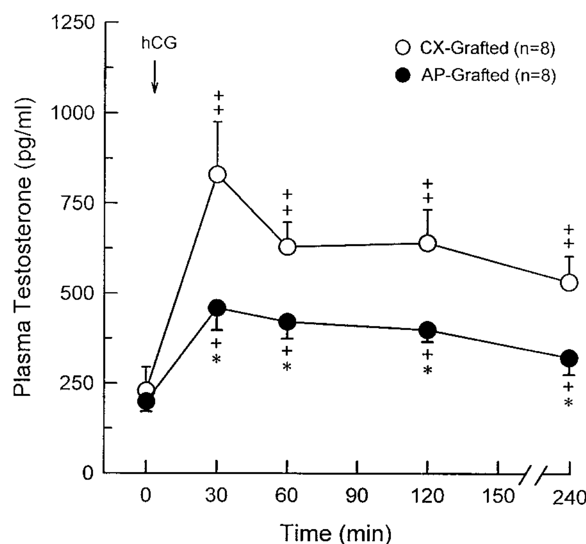

Fig. 2. Plasma testosterone levels in response to intravenous injection of hCG (5 IU/ml/kg) at time of 0 min. $^{*}P < 0.05$ vs. CX-grafted group; +,++$P < 0.05$, and $P < 0.01$ vs. basal level at 0 min.

Effects of Hyperprolactinemia on Testosterone Release In Vitro in Response to hCG and oPRL

In TICs, the stimulatory effect of hCG was evident in both the AP- and CX-grafted groups. However, in the AP-grafted group, the released amount of testosterone was significantly less than that of the CX-grafted group, and the

response failed to increase when more than 0.05 IU/ml of hCG was given (Fig. 3).

In both the AP- and CX-grafted groups, incubation of oPRL with TICs showed no effect on testosterone release at low concentrations (up to 1 μg/ml). However, when the concentration of oPRL was elevated to above 1 μg/ml, a dose-related response of testosterone secretion was observed, in spite of the fact that the AP-grafted group showed a decrease in the release of testosterone (Fig. 4).

Effects of Hyperprolactinemia on Signal Transduction in TICs

The elevation of testosterone release in response to forskolin (1 μM) was exhibited only in the CX-grafted but not in the AP-grafted group (Fig. 5). While for the effects of SQ22536 (10 μM), as an inhibitor of adenylate cyclase, both groups yielded a markedly diminished performance in testosterone secretion. For both the AP- and CX-grafted groups, administration of 8-Br-cAMP resulted in a dramatic increase of testosterone release. However, the response between both groups was not the same, the AP-grafted group had a weaker performance (Fig. 5).

After administration of forskolin, the production of cellular cAMP in TICs was lower in the AP-grafted than in the CX-grafted group (Fig. 6).

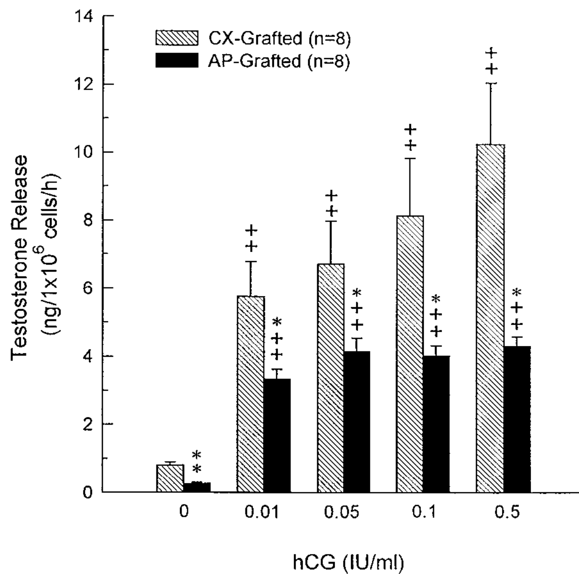

Fig. 3. The testosterone release by TICs (1 × 10^6 cells/h) in response to hCG challenge. *,**$P < 0.05$, and $P < 0.01$ vs. the CX-grafted group; ++$P < 0.01$ vs. basal testosterone release.

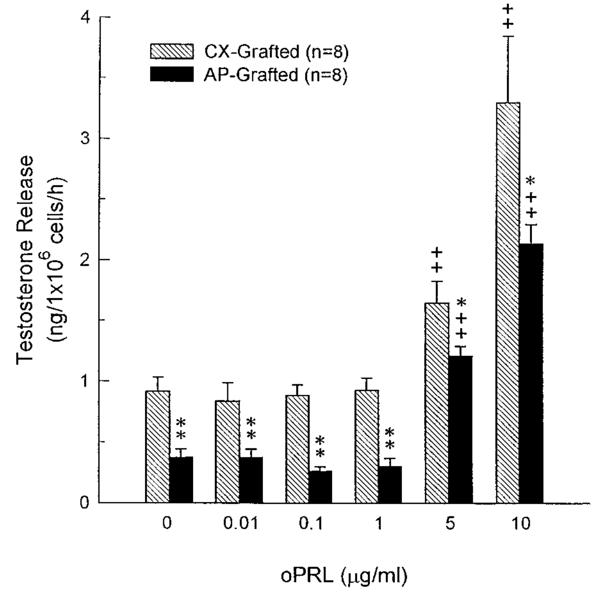

Fig. 4. The testosterone release by TICs (1 × 10^6 cells/h) in response to oPRL challenge. *,**$P < 0.05$, and $P < 0.01$ vs. the CX-grafted group; ++ $P < 0.01$ vs. basal testosterone release.

DISCUSSION

Although the basal level of plasma testosterone was not decreased in the AP-grafted group, the sensitivity or responsiveness of testosterone to hCG stimulation was markedly suppressed by hyperprolactinemia (Fig. 2). The suppressed response of testosterone release in the AP-grafted rats to the in vivo challenge of hCG corresponds to the findings of previous studies [Sharpe and McNeilly, 1980; Waeber et al., 1983]. This result suggests that an inhibitory mechanism may exist which suppresses the interstitial and Leydig cells from normal LH stimulation. This also confirms the hypothesis that the mechanism underlying hyperprolactin-induced hypogonadism is explained by dysfunction of AP-testis axis, which originated from an influence elicited by hyperprolactinemia action directly on the testes. The consensus of these aspects indicates two possibilities for the reason of suppressed responsiveness to hCG stimulation. The first, coming from within the TICs that is related to some specific functional changes in the cell; and the second, via an external route outside the TICs that certain circulatory factors may hinder it from normal gonadotropin stimulation.

Theoretically, the in vitro incubation using TICs preparation is free of circulatory inhibitory factors. This model can be utilized to ob-

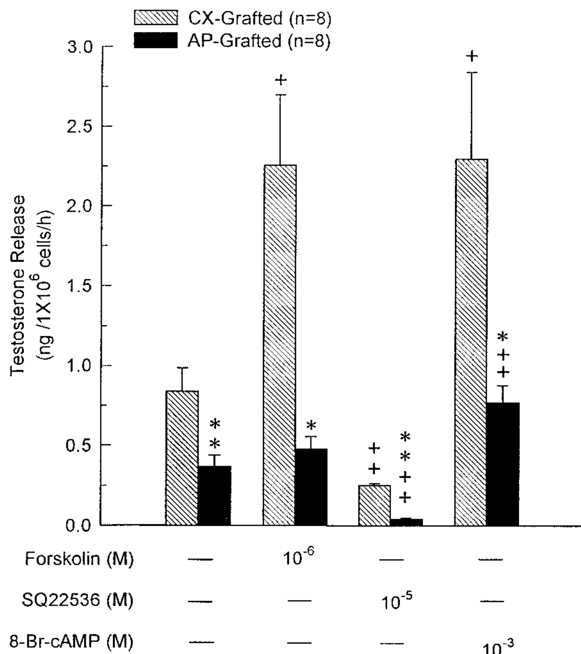

Fig. 5. The testosterone release by TICs (1 × 10^6 cells/h) in response to the action of forskolin (an adenylate cyclase activator, 10^{-6} M) and SQ22536 (an adenylate cyclase inhibitor, 10^{-5} M) as well as 8-Br-cAMP (a membrane permeable analog of cAMP, 10^{-3} M). *,**$P < 0.05$, and $P < 0.01$, vs. the CX-grafted group; +,++$P < 0.05$, and $P < 0.01$ vs. basal testosterone release.

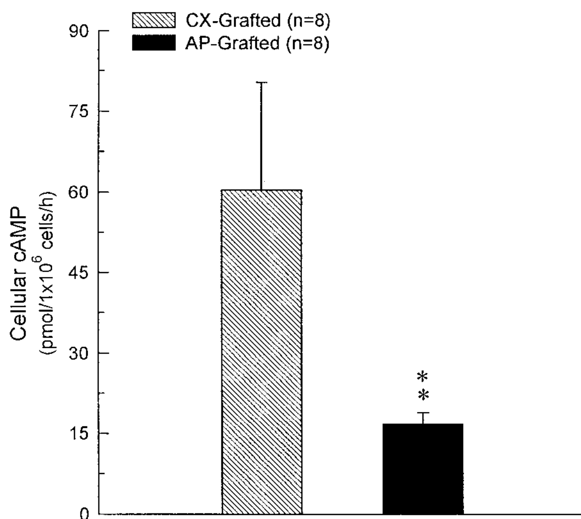

Fig. 6. The production of intracellular cAMP (pmol/10^6 cells/h) in TICs after challenge of forskolin (10^{-6} M) in the AP- and CX-grafted rats. **$P < 0.01$, vs. the CX-grafted group.

serve if there are any intracellular functional abnormalities of either the TICs or Leydig cells by hyperprolactinemia. The challenge of hCG (from 0.01 to 0.5 IU/ml) to the TICs demonstrated a dose-dependent testosterone release in both the AP- and CX-grafted groups. It was similar to the findings shown in the in vivo study that the AP-grafted group revealed a suppressed response. The basal testosterone level as well, was lowered (Fig. 3). These results support the existence of functional changes intrinsic to the TICs or Leydig cells.

The investigation on the signal transduction pathways of cAMP by adding forskolin or SQ22536 to TICs incubation provided a meaningful approach to determine the competence of adenylate cyclase. Our data confirms that the activity of adenylate cyclase is impaired by hyperprolactinemia. It is well known that 8-Br-cAMP mediates the signals directly to the machinery of steroidogenesis, which allows an observation on the response of testosterone production to hyperprolactinemia by by-passing the adenylate cyclase. Using 8-Br-cAMP to bypass the action of adenylate cyclase revealed that TICs of the AP-grafted group may also have dysfunctional steroidogenesis of the post-cAMP pathways. These results suggest that a functional change inside the TICs or Leydig cells occurred in hyperprolactinemic rats. A recent study using a murine Leydig tumor cell line also suggests a possible hyperprolactin-induced dysfunction at the LH/hCG receptor or at the signal transduction levels in Leydig cells [Weiss-Messer et al., 1996].

Whether PRL itself functions as an inhibitory factor on steroidogenesis in the Leydig cell remains controversial [Barkey et al., 1987; Papadopoulos et al., 1986; Bartke et al., 1986]. Comparing several studies on PRL-related hypogonadism, the given doses of PRL should be carefully examined. It has been reported that during short term incubation, PRL (0.001 to 1 μg/ml) has no effect on testosterone synthesis in isolated Leydig cell culture, but an inhibitory influence on testosterone synthesis is shown for a 3-day culture [Barkey et al., 1987]. In agreement with their findings, we found that administration of low doses of oPRL (from 0.01 to 1 μg/ml) to the TICs incubation did not affect testosterone synthesis. However, while at higher doses (1 to 10 μg/ml) of PRL, a dose-dependent stimulatory response was shown in both the AP- and CX-grafted groups. Although the AP-grafted group expressed lower levels in oPRL-challenged testosterone release as compared to the control, the responsiveness (the ratio of response level to the baseline level) to the challenge was higher. These data are compatible

with the idea that PRL is a trophic hormone that stimulates steroidogenesis in the Leydig cells [Papadopoulos et al., 1986]. This study shows the results of basal in vitro testosterone release, and the AP-grafted group had a significantly lower level than did the CX-grafted (Figs. 3 to 5). We also showed oPRL stimulated testosterone production in vitro, in both the AP- and CX-grafted rats (Fig. 4). In the in vivo condition, a prominently higher level of PRL was observed in AP-grafted rats. It is very likely, the increased PRL helps to maintain an unchanged basal testosterone level in the hyperprolactinemic animals (Fig. 2). Based on these findings, PRL in circulation seems not to be the answer to explain the inhibition of steroidogenesis in vivo. Therefore, we postulate that there could be some factors other than PRL in the circulation that interferes with testosterone production under hyperprolactinemia, although it has been demonstrated that the exposure of a higher concentration of PRL (1000 ng/ml) induced a reduction in hCG binding [Weiss-Messer et al., 1996].

In summary, a chronic hyperprolactinemic status induced by AP-implantation leads to an abnormal performance of the TICs in terms of a decreased responsiveness of testosterone to gonadotropin stimulation. The mechanism is in part related to a direct inhibitory effect on the TICs. An impaired function of adenylate cyclase in cAMP production and cAMP action are believed to be involved in this mechanism.

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