Increased Plasma and Pituitary Prolactin Concentrations in Adult Male Rats with Selective Elevation of FSH Levels May Be Explained by Reduced Testosterone and Increased Estradiol Production

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The roles of testosterone and estradiol in regulating prolactin concentrations were studied in acutely castrated adult male rats receiving subcutaneous Silastic® implants of the sex steroids. Testosterone was administered in increasing doses, from subphysiologic to intact levels, both alone and in combination with a small, single dose of estradiol. The study was designed to assess whether a change in the relative rates of sex steroid production could account for an increase in PRL release in the absence of other testicular factors. At very low levels of plasma testosterone, FSH and LH levels were indistinguishable from castrate controls. As plasma testosterone concentration increased, both plasma FSH and LH levels were suppressed progressively to intact levels. When a subphysiologic dose of testosterone was coadministered with a small dose of estradiol, the combined effects produced a midcastrate level of FSH but maintained a normal level of LH similar to the selective increase in FSH concentration observed in men with germinal aplasia. Although PRL levels were indistinguishable in intact and castrate controls, testosterone replacement by capsule increased prolactin in a dose-related manner so that, at the physiologic level of testosterone, prolactin was elevated two-fold ($P < 0.01$), similar to the level achieved with estradiol replacement alone. Pituitary prolactin levels also increased with increasing doses of testosterone but values remained within the range measured in intact controls. When estradiol was coadministered with testosterone, the combination produced different effects depending on the testosterone dose. With subphysiologic testosterone replacement, when plasma FSH was selectively increased, plasma PRL was elevated four-fold ($P < 0.01$) compared with intact controls and two-fold compared with rats receiving estradiol alone ($P < 0.01$). A smaller increase in pituitary PRL content was noted similar to the level achieved with estradiol replacement alone. As the testosterone dose increased toward midphysiologic values, plasma and pituitary prolactin levels fell progressively, but remained elevated above intact levels ($P < 0.05$). It was concluded that testosterone and estradiol both modulate prolactin secretion in the absence of other testicular factors, but the greatest effect is seen when the testosterone-estradiol ratio is reduced, producing a selective increase in FSH concentration. These results are similar to the increased prolactin release observed in men with testicular dysfunction associated with selectively elevated FSH concentrations. The precise mechanism of these changes of prolactin release remain to be elucidated.

Key words: pituitary, prolactin, testosterone, estradiol, LH, FSH, rat.

J Androl 1986; 7:105-111.

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Submitted for publication June 19, 1985; revised version received August 5, 1985; accepted for publication September 4, 1985.

Recent reports show that although the mean basal plasma prolactin (PRL) level is normal in men with oligozoospermia, germinal aplasia, or Klinefelter's syndrome (Hagen et al, 1974; Cheikh et al, 1975; Spitz et al, 1980a, 1981; LeRoith et al, 1981), the plasma PRL response to thyrotropin-releasing-hormone (Burman et al, 1975; Cheikh et al, 1975; Spitz et al, 1979; 1980b; 1981; LeRoith et al, 1981) and metoclopramide, a dopaminergic antagonist (Spitz et al, 1980a; 1981; LeRoith et al, 1981), is increased significantly. The exaggerated PRL response is reversed with replacement of testosterone (T) (Burman et al, 1975) or treatment with the estrogen antagonist

clomiphene citrate (Spitz et al, 1980b; 1981). Since the plasma estradiol level may be high in primary testicular failure (LeRoith et al, 1981; Spitz et al, 1981; Wu et al, 1982) and estrogen is known to increase PRL in both men (Buckman and Peake, 1973) and women (Buckman and Peake, 1973; Yen et al, 1974), the data suggest that the exaggerated PRL response in patients with testicular dysfunction is estrogen induced.

Recently, we have demonstrated that when the castrate adult male rat is replaced with a subphysiologic dose of T coupled with an additional small dose of estradiol (E_2), a selective elevation in FSH concentration occurs similar to that observed in males with germinal aplasia (Sherins et al, 1982). Accordingly, the following study in the male rat was designed to examine whether such a change in the relative rates of sex-steroid production could also account for an increase in prolactin secretion.

Materials and Methods

Animals

Adult male Holtzman rats initially weighing between 250 and 300 g were used throughout the study. All animals received Purina rat chow (Farmer Supply Company, Walkersville, MD) and tap water *ad libitum*. Eight to ten animals were housed in each cage as one experimental group and provided with artificial lighting from 0800 to 1900 h.

Capsule Preparation

Steroid-containing Silastic capsules (Dow-Corning Corp.) were prepared as previously described (Kincl et al, 1968). The dose of steroid administered is directly proportional to capsule surface area but inversely proportional to capsule wall thickness (Kincl et al, 1968; Stratton et al, 1973). To permit differing rates of steroid diffusion, capsules of two different diameters were constructed. Testosterone was placed in a "single" capsule of one layer of polydimethylsiloxane (PDS) tubing (0.062" ID, 0.125" OD) surrounding a specified length of hormone. E_2 was placed in a "double" capsule constructed by placing an E_2-filled, sealed single capsule inside a length of PDS tubing (0.125" ID, 0.25" OD) and sealing each end.

General Experimental Protocol

T and/or E_2 were administered for 4 weeks to acutely castrated adult male rats via subcutaneous Silastic capsules. The influences of varying doses of sex steroids on plasma and pituitary PRL concentrations were assessed in relationship to plasma gonadotropin levels. Normal intact and acutely castrated littermates were used as controls throughout each experiment.

The uniformity of steroid release from the capsule was ensured by preimplanting all capsules into intact rats for 7 days prior to reimplantation into the experimental animals. Bilateral orchiectomies were done under ether anesthesia via a transscrotal approach. Steroid-filled capsules were implanted subcutaneously immediately after castration to avoid the changes in steroid feedback sensitivity noted after subacute and chronic castration (Gay and Bogdanove, 1969). They were kept in place for 4 weeks to achieve chronic steady-state levels of steroid before the rats were killed. Previous experiments demonstrated that T capsule lengths ranging from 1.5 to 6.0 cm maintain plasma T concentrations within the physiologic range (Sherins et al, 1982). Additionally, we have noted that while we use a relatively sensitive radioimmunoassay (RIA), the mean plasma E_2 level in the castrate adult male rat (9.4 ± 3.6 pg/ml) is indistinguishable from the very low level measured in intact adults (11.0 ± 3.7 pg/ml, NS). Nevertheless, when thick-wall capsules are constructed to deliver very small amounts of E_2, where plasma levels remain below detection limits (10 pg/tube), even such small quantities of E_2 produce selective dose-dependent (variable capsule length) suppression of LH release (Sherins et al, 1982). When a subphysiologic dose of T, which by itself would allow escape of both gonadotropins to castrate levels, is coadministered with a small supplemental quantity of E_2, the FSH concentration increases selectively to half castrate levels similar to that noted among men with germinal aplasia (Sherins et al, 1982).

The experimental animals received either 1) a T capsule of 0.5, 1.5, or 3.0 cm in length, 2) an E_2 capsule of 0.1 cm in length, or 3) a T capsule of 0.5, 1.5, or 3.0 cm in length together with a 0.1-cm E_2 capsule. After 4 weeks, the experiment was terminated by decapitation of the rats. Care was taken to avoid stress-induced release of PRL. Decapitation of rats was performed at the opposite end of a large animal room where the remaining cages of animals were visually blocked from the procedure. Additionally, a separate group of intact and castrate littermates was decapitated after all other animals in the study had been killed; PRL concentrations were indistinguishable regardless of the order of decapitation (data not shown). Trunk blood was collected in heparinized beakers, centrifuged and the individual plasma samples were stored at -20 C for subsequent hormone assay. The pituitary gland was removed quickly from each animal and placed in 1 ml of radioimmunoassay buffer on ice, sonicated, frozen and then stored at -20 C for subsequent assay of PRL.

Pituitary Hormone Measurements

FSH, LH, and PRL concentrations were measured by double antibody RIA using FSH-RP-1, LH-RP-1 and PRL-RP-1 as standards. Reagents were provided by the NIADDK National Hormone and Pituitary Program. Plasma and pituitary samples were assayed individually in duplicate in single assays to eliminate between-assay variation.

FSH and LH were iodinated by lactoperoxidase, desalted with Sephadex G-25 coarse, and purified with Concanavalin A-sepharose 4B chromatography prior to use in the assays. Other details of the assay have been described previously (Krueger et al, 1974). The assay detection limits were 25 ng FSH/ml and 2 ng LH/ml.

PRL also was iodinated by lactoperoxidase. The labeled ligand was separated by Sephadex G-75. The procedures

of the assay were the same as those reported by Neill and Reichert (1971) with minor modifications. The sensitivity of the assay was 0.1 ng PRL/ml and the intraassay coefficient of variation was 10%.

Testosterone Assay

Plasma T levels were measured by RIA using an antiserum generated against testosterone-3-oxime conjugated to albumin as described previously (Nieschlag and Loriaux, 1972). The steroid was separated by celite-545 column chromatography (Abraham et al, 1972) prior to assay measurement. Assay sensitivity was 180 pg/ml. The intra-assay variation was 14% at 300 pg/ml. For each treatment group, aliquots of individual plasma samples (0.2 ml) were pooled before extraction for the assay.

Estradiol Assay

Plasma E_2 was measured by RIA using an antiserum generated against E_2-6-oxime conjugated to albumin. The cross reactivity was < 0.2% for a wide range of steroids, but was 4.4% and 0.4% for estrone and estriol, respectively. Specificity was improved further by complete separation of E_2 following celite-545 column chromatography (Abraham et al, 1972). The antiserum was used at a final dilution of 1:160,000. Other assay procedures were as previously described (Loriaux et al, 1971). Dextran-coated charcoal was used to separate bound from free hormone, slope = −1.0, 50% intercept = 60 pg, sensitivity = 10 pg/tube; precision was 10% for intra-assay and 17% for interassay variation. Accuracy of the assay was confirmed by the quantitative recovery of added authentic E_2 (50–100 pg) to charcoal-stripped plasma. Because of the very low levels of E_2 in rat plasma, plasma samples from 2 or 3 rats of each experimental group were pooled as 5-ml aliquots before extraction for the assay.

Statistical Analysis

All results were presented as the mean ± SEM. Comparisons between treated animals and castrate and intact control groups were made using a two-tailed Student's *t* test. The 95% confidence limit was used to determine statistical significance.

Results

Effect of Hormone Replacement on Gonadotropin Concentrations

The plasma T and E_2 concentrations achieved by the varying lengths of steroid-filled capsules are summarized in Table 1. T capsules of 3.0 cm in length maintained plasma T at levels similar to intact controls.

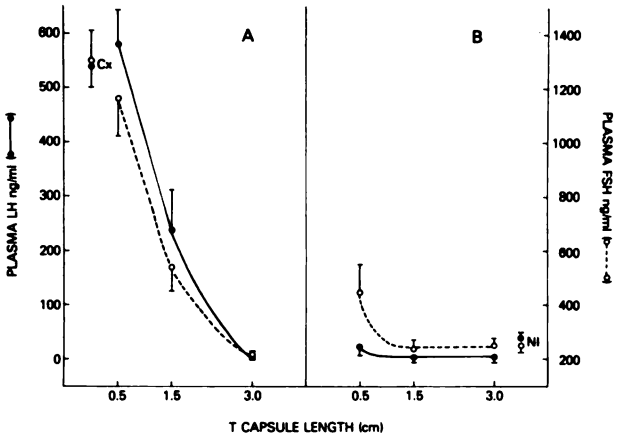

Fig. 1. Influence of sex steroids delivered by Silastic® capsule on plasma LH and FSH concentrations in castrate male rats. A. Testosterone administered alone at increasing doses. B. Testosterone administered at increasing doses with estradiol capsule (0.1 cm). Each group contained nine animals. Data plotted as mean ± SEM. N1 = normal intact controls. Cx = castrate controls.

The effects of the varying doses of T alone or T in combination with E_2 upon plasma FSH and LH levels are shown in Fig. 1. Castration increased the mean plasma FSH level six-fold and mean LH concentrations 17-fold. Capsules containing T suppressed plasma FSH and LH levels linearly toward normal in a dose-dependent manner, with similar slopes for both gonadotropins (FSH, $r = 0.973$, $P < 0.02$; LH $r = 0.969$, $P < 0.03$). For rats receiving the 3.0-cm T capsule, the mean FSH level was indistinguishable from intact littermate controls, while the mean LH level was minimally reduced ($P < 0.05$), although still within the normal range (Fig. 1A). When E_2 was administered alone, plasma FSH increased to 745 ± 118 ng/ml and LH increased to 215 ± 75 ng/ml, both higher than intact controls ($P < 0.01$).

When T and E_2 were coadministered (Fig. 1B), a dissociation of plasma FSH and LH concentrations

TABLE 1. Effect of Varying Length of T- and/or E_2-filled Silastic® Capsules on Plasma T and E_2 Concentrations

Treatment Group	Plasma T (ng/dl)*				Plasma E_2 (pg/ml)*	
	T Alone		T Plus E_2		T Alone	T Plus E_2
Castrate	< 10	(18)†	< 10	(8)	9.4 ± 3.6 (18)	12.5 ± 0.6 (8)
Castrate + T 0.5 cm	58	(9)	46	(10)	9.4 ± 1.8 (9)	13.6 ± 1.4 (10)
+ T 1.5 cm	113	(10)	110	(10)	17.0 ± 1.1 (10)	9.0 ± 1.1 (10)
+ T 3.0 cm	203	(10)	209	(9)	6.4 ± 1.8 (10)	16.0 ± 5.3 (9)

*Among intact controls plasma T = 211 ng/dl (n = 9) and plasma E_2 = 11.0 ± 3.7 pg/ml (n = 17).
†Number of rats in each experimental group.

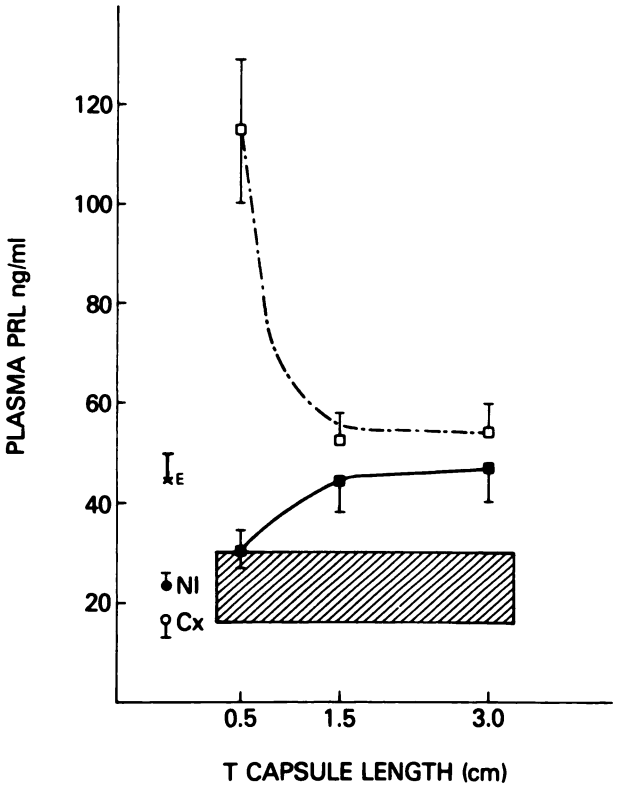

Fig. 2. Effect of sex steroids delivered by Silastic® capsule on plasma PRL levels in castrate male rats: testosterone administered at increasing doses alone (■——■), E = estradiol capsule (0.1 cm), testosterone administered at increasing doses with estradiol (□—·—□). Each group contained nine animals. Data plotted as mean ± SEM. The hatched area represents the range of PRL levels measured in normal intact rats (N1). Cx = castrate controls.

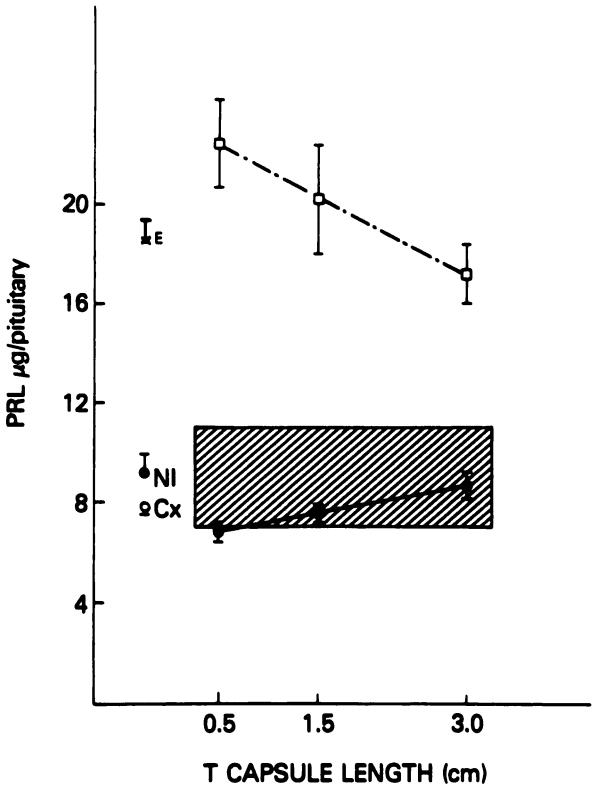

Fig. 3. Effects of sex steroids delivered by Silastic® capsule on pituitary PRL levels in castrate male rats: testosterone administered at increasing doses alone (■——■), E = estradiol administered alone, testosterone administered at increasing doses with estradiol capsule (0.1 cm) (□—·—□). Each group contained nine animals. Data plotted as mean ± SEM. The hatched area represents the range of PRL levels measured in normal intact rats (N1). Cx = castrate controls.

was observed at the lower subphysiologic dose of T (0.5-cm capsule) as previously reported (Sherins et al, 1982). The mean plasma LH concentration (25.0 ± 13.7 ng/ml) was indistinguishable from that of intact littermates (41.7 ± 6.9 ng/ml), while the mean plasma FSH level (449.2 ± 101.1 ng/ml) was increased compared with controls (250 ± 50 ng/ml, $P < 0.002$). As the amount of T administered increased toward the midphysiologic dose, the plasma gonadotropin concentrations remained indistinguishable from intact littermates.

Effects of Hormone Replacement on Prolactin Concentrations

The mean plasma PRL level in castrates (17.3 ± 4.1 ng/ml) was indistinguishable from that of intact controls (23.2 ± 2.8 ng/ml). PRL concentrations of castrates exposed to E_2 alone, however, increased two-fold (44.1 ± 8.7 ng/ml, $P < 0.05$) (Fig. 2). The effect of T or of T plus E_2 on the plasma PRL level is shown in Fig. 2. T replacement alone by capsule increased PRL concentrations in a dose-related manner so that, at physiologic T levels, the PRL values were elevated two-fold above intact controls ($P < 0.01$).

When E_2 was coadministered with T, the combination produced different effects depending on the T dose. With subphysiologic T replacement (0.5 cm T capsule), when the plasma FSH concentration is increased selectively (Fig. 1B), the mean PRL level is increased markedly (110.7 ± 19.0 ng/ml, $P < 0.01$) compared with values obtained both in intact controls and in castrate rats treated with E_2 alone. At physiologic T dosages (1.5- and 3.0-cm T capsules), plasma PRL levels are identical to those of castrates receiving similar T capsules alone.

As with plasma concentrations, the pituitary PRL content in castrates (7.8 ± 0.3 g/pituitary) was indistinguishable from levels measured in intact controls (9.1 ± 0.8 g/pituitary) and increased two-fold above normal (18.7 ± 0.8 g/pituitary, $P < 0.05$) in castrates

receiving E_2 replacement alone (Fig. 3). In response to increasing dosages of T, pituitary PRL content increased progressively within the normal range.

When E_2 was coadministered with T, the combination produced markedly elevated PRL content at all T doses ($P < 0.05$). With increasing T dosages, however, PRL content fell progressively. At midphysiologic T replacement (3.0-cm T capsule), pituitary PRL content was similar to that of rats receiving E_2 alone, but greater than that observed among castrate controls ($P < 0.05$).

Discussion

Previous studies in men show that an increase in releasable prolactin is present in association with testicular injury (Burman et al, 1975; Spitz et al, 1980a; LeRoith et al, 1981). Since the exaggerated prolactin release is reversible with administration of androgen or antiestrogens, the data suggest that the prolactin response results from changes in Leydig cell function and increased estrogen production (Burman et al, 1975; Spitz et al, 1979; 1980b; 1981). Recent studies in our laboratory indicate that when the castrate adult male rat is replaced with a subphysiologic dose of T coupled with an additional small dose of E_2, a selective elevation in FSH concentration occurs similar to that noted in men with germinal aplasia (Sherins et al, 1982). It therefore seemed appropriate to determine whether there would be exaggerated prolactin release in an experimental setting where there was a reduction of the normal T: E_2 production ratios.

In our current study, we demonstrate that when we alter the normal male sex steroid ratio by decreasing T and increasing E_2 plasma concentrations in the absence of the testis, we stimulate markedly pituitary and plasma prolactin levels. Part of the augmentation of prolactin production can be accounted for by changes induced by E_2 alone, but the most profound increase in prolactin levels occurs when a small supplemental dose of E_2 is coupled with a subphysiologic dose of T. The effects of T in modulating prolactin release are complex, however, since when given alone, T increases prolactin release while when coupled with E_2, T actually reduces elevated levels of prolactin in a dose-dependent manner. The high prolactin levels achieved with T plus E_2 most closely correlate with changes in plasma FSH levels since PRL and FSH concentrations are both highest when the lowest dose of T is coupled with supplemental E_2. A similar association between PRL and FSH concentration has also been demonstrated in men where the release of PRL to both TRH and metoclopramide was highly correlated with the plasma FSH level. PRL release was most pronounced in men with severe germ cell loss in whom FSH, but not LH, was elevated (Spitz et al, 1980a).

Estrogens are well known to modulate PRL secretion. Estrogen administration in animals and man increases PRL pituitary content (Ajika et al, 1972), PRL blood levels, and PRL release (Nicoll and Meites, 1964; Frantz et al, 1972; Carlson et al, 1973; De Lean et al, 1977; Ojeda et al, 1977; Rutlin et al, 1977). However, there are no data that demonstrate that estrogens contribute in maintaining PRL blood levels under physiologic conditions. Recently D'Agata (D'Agata, 1980) and Gooren (Gooren et al, 1984) both have shown in normal men that decreasing plasma E_2 levels by administering an aromatase inhibitor produces a corresponding decrease in PRL blood levels.

Since T serves as a prehormone for the production of E_2 in the peripheral circulation (Baird et al, 1968), the increased plasma PRL levels that we observed when T was given at physiologic dosages may be accounted for by increased aromatization of the T administered by capsule. We recognize that the animal model that we have constructed does not provide the full functional equivalent of a normal gonad because the dynamic readjustments of the hypothalamic-pituitary-gonadal axis are missing. The steroid-filled capsules maintain steady-state release of steroid with consequent elimination of pulsatile secretions and diurnal variation of both gonadotropin and T (Dykman et al, 1981). In this regard, it might be reasonable to assume that constant release of T might stimulate PRL secretion more effectively than fluctuating T levels within the same range of physiologic concentrations.

Since T also serves as the prehormone for the nonaromatizable androgen, dihydrotestosterone (DHT) (Baird et al, 1968), the effects of T on prolactin release could also be mediated by DHT. Nolin found, however, that DHT consistently failed to stimulate PRL secretion in male rats, although it inhibited LH release and stimulated ventral prostate growth (Nolin et al, 1977). Rennels and Herbert also were unable to demonstrate an effect of DHT in altering serum or pituitary levels of PRL in female rats (Rennels and Herbert, 1977), and Labrie and his colleagues found no effect of DHT on PRL secretion in rat pituitary cell cultures (Labrie et al, 1980). In our current study, we show that the markedly increased plasma prolactin level achieved when the smallest dose of T is

coadministered with E_2 is attenuated when the dose of T increases toward physiologic levels (to PRL levels achieved when E_2 is given alone). In this regard it seems that the androgenic action of T partly counteracts the stimulatory effect of E_2 on PRL secretion. This is consistent with Labrie's observation that DHT, although inactive alone, can reverse a portion of the stimulatory effect of E_2 on PRL secretion at both the pituitary and hypothalamic level in rat pituitary cell cultures (Labrie et al, 1980).

It is clear from our studies that reduced T production coupled with increased E_2 production markedly augments PRL release in the absence of other testicular factors. The precise cellular mechanism(s) by which the change in lactotroph function takes place, however, is not clear. While androgen (Lieberburg et al, 1977) and estrogen (Kato, 1975) receptors have been demonstrated in pituitary tissue, there is no evidence for a direct effect of sex steroids on the lactotroph. There is growing evidence, however, for an important association between gonadotroph and lactotroph function. Recent studies show that there is synchronous endogenous episodic release of LH and PRL in both men (Barbarino et al, 1982) and women (Cetel et al, 1982; Braund et al, 1984) as well as to exogenous GnRH administration (Barbarino et al, 1982). But the synchrony of LH and PRL pulses is abolished by metoclopramide, a dopamine antagonist, suggesting the GnRH effect on PRL is mediated via a dopaminergic pathway (Braund et al, 1984).

A recent study in normal men by Veldhuis et al (1984) shows that endogeneous LH pulses usually suppressed by androgen and estrogen are restored by opiate receptor blockade administered with the sex steroids, which suggests that the negative feedback of gonadal steroids is functionally coupled to endogenous opiate pathways at the level of the hypothalamus. Since LH and PRL pulse synchrony is not abolished by an opioid antagonist (Cetel et al, 1985), such evidence suggests that the actions of T and E_2 in modulating PRL release are not mediated directly via opioid pathways.

The effect of GnRH on PRL release appears to be indirect, involving a paracrine interaction between gonadotrophs and lactotrophs. Lactotrophs and gonadotrophs are found closely associated *in situ* (Sato, 1980). Using cell cultures of enriched fractions of gonadotrophs and lactotrophs, Denef (Denef and Andries, 1983; Denef, 1984) has shown convincingly that GnRH stimulates PRL release from lactotrophs only in the presence of gonadotrophs, and this stimulation of basal and dopamine-inhibited PRL release is proportional to the number of gonadotrophs present. Media conditioned by purified gonadotrophs also stimulate PRL release by an ultrafiltrable factor of less than 10,000 MW (Denef, 1984). The authors speculate that this paracrine factor might be vasoactive intestinal peptide and/or angiotensin II since angiotensin-like immunoreactivity has been shown in both gonadotrophs and lactotrophs; angiotensin II stimulation of PRL release from superfused pituitary glands can be blocked by an angiotensin II receptor antagonist (Schramme and Denef, 1983), and vasoactive intestinal peptide can stimulate PRL release of cells in culture directly (Denef, 1984). Additionally, it has been shown that T and E_2 can inhibit the PRL response to GnRH in cocultured cells (Denef, 1984), emphasing an important action of sex steroids directly on the gonadotroph as well as the hypothalamus.

The data from our study suggest that, in the absence of other testicular factors, both T and E_2 modulate PRL secretion, and that a decrease of the T:E_2 ratio augments PRL secretion. In our model, prolactin concentrations most closely correlate with changes in FSH levels. While the precise mechanisms(s) for this association remains unclear, recent studies suggest an important paracrine interaction may exist between gonadotrophs and lactotrophs whereby changes in gonadotroph function influence prolactin release.

Acknowledgment

The authors express their appreciation to the NIADDK National Hormone and Pituitary Program for the generous gifts of rat FSH, LH, and PRL reagents, and to Mrs. Penny Colbert, Mrs. Mary Hall, Ms. Allison Williams, and Wylbur for their expert technical assistance in the preparation of this manuscript.

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