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Unmodified Prolactin (PRL) Promotes PRL Secretion and Acidophil Hypertrophy and Is Associated with Pituitary Hyperplasia in Female Rats

Terence E. Johnson, Mayza Vue, Sharyn Brekhus, Amy Khong, Timothy W.C. Ho, and Ameae M. Walker*

Division of Biomedical Sciences, University of California, Riverside, CA

In this study, we have tested the hypothesis that unmodified prolactin (U-PRL) and phosphorylated prolactin (P-PRL) have differential roles in the autoregulation of PRL secretion in vivo. Recombinant human U-PRL and a molecular mimic of P-PRL (S179D PRL) were administered to male rats and to female rats in different physiological states and the effect on rat PRL release was measured. Administration of U-PRL elevated rat PRL in all female animals, but was without effect in males. By contrast, S179D PRL was inactive in females, but inhibited PRL release in males. Morphometric and immunohistochemical analyses demonstrated acidophil hypertrophy and evidence of increased PRL secretion in the pituitaries of U-PRL-treated females. Analysis of the two forms of PRL during prolactinoma induction in two differentially susceptible strains of rats found a strong temporal correlation among increased ratios of U-PRL: P-PRL, increased circulating PRL, and increased cell proliferation. We conclude (1) that the autoregulatory mechanism(s) can distinguish between the two major forms of PRL and that higher proportions of U-PRL not only allow for higher circulating levels of PRL, but are also autostimulatory, (2) that the autoregulatory mechanism(s) are set differently in males and females such that females are more sensitive to autostimulation by U-PRL and less sensitive to inhibition by P-PRL, and (3) that U-PRL and P-PRL may also have differential roles in the regulation of pituitary cell proliferation.

Key Words: Unmodified prolactin; phosphorylated prolactin; hyperprolactinemia; hypertrophy; hyperplasia; prolactinoma.

Introduction

PRL secretion is regulated by factors released from the hypothalamus, from the posterior and intermediate lobes of the pituitary, and by autocrine/paracrine interactions within the anterior pituitary itself (for review see ref. *1*). Unlike most pituitary hormones, there is no target tissue feedback molecule that regulates PRL secretion. Instead, it is PRL itself that feeds back to regulate its own secretion. This has been convincingly documented for hypothalamic feedback regulation (for review see ref. *2*), and has been demonstrated via in vitro experimentation for paracrine/autocrine regulation *(3–7)*, but descriptions of posterior/intermediate lobe feedback mechanisms are not yet forthcoming. This article does not address the relative importance of each potential site for feedback regulation, but instead investigates a possible mechanism that allows for physiological hyperprolactinemia. In other words, a mechanism which allows for appropriate periods of increased PRL release.

Previous work from this laboratory has demonstrated a change in the composition of released PRL during physiological hyperprolactinemia *(8,9)* and so we have examined the relative roles of the two major forms of PRL in rodents in terms of feedback regulation. These two forms of PRL are unmodified PRL (U-PRL) and phosphorylated PRL (P-PRL). In addition, we have examined the production of these two forms of PRL during 17β-estradiol-induced hyperprolactinemia and the pathological hyperprolactinemia induced by diethylstilbestrol (DES).

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Author to whom all correspondence and reprint requests should be addressed: Division of Biomedical Sciences, University of California, Riverside, CA 92521-0121. E-mail: ameae.walker@ucr.edu

Results

Treatment of Rats with U-PRL or a Molecular Mimic of P-PRL

Recombinant versions of human U-PRL or a molecular mimic of human P-PRL (S179D PRL) were administered to males, nonpregnant females, pregnant females, and pregnant then postpartum females at a dose previously demonstrated to result in circulating levels of the administered hormone of about 50 ng/mL *(10)*. Table 1 shows the effect on endogenous rat PRL levels after 19.5–21.5 d of treatment. This time period was chosen so that we could look at almost term pregnant females and postpartum females and compare them to similarly treated males and nonpregnant females. Preliminary experiments had demonstrated that the kit used to assay rat PRL levels did not recognize either of the two recombinant human PRLs.

Table 1
Circulating Rat PRL Levels[a]

Animal	Rat PRL (ng/mL ± SE)		
	No PRL	U-PRL	S179D PRL
Female, 21 d, nonpregnant	33.7 ± 7.2**	74 ± 6.5**	35 ± 2.9
Female, 19.5 d, pregnant	40.8 ± 10.6***	97.3 ± 7.9***	45 ± 8.2
Female, 21.5 d = 1 d postpartum	91.2 ± 9.7*	141 ± 10.9*	109 ± 8.9
Male, 21 d	33.8 ± 5.2*	41 ± 3.6*	18.6 ± 3.5

[a]Animals were treated for the indicated number of days with saline (No PRL), unmodified PRL (U-PRL), or the molecular mimic of phosphorylated PRL (S179D PRL). Levels of rat PRL were measured in serum derived from trunk blood. $n = 5$ animals except for the 19.5 d pregnant animals where $n = 10$. ***, different with $p < 0.001$; **, different with $p < 0.01$; * No PRL, different from U-PRL with $p < 0.05$.

Table 2
Acidophils and Basophils in a Set Area of the Anterior Pituitaries of 7 d-Treated Rats[a]

	No PRL	U-PRL	S179D PRL
Number of acidophils	49.4 ± 3.35*	45.7 ± 3.1*	45.1 ± 3.7*
Size of acidophils	19.9 ± 0.6**	21.8 ± 0.58**	20.3 ± 0.63
Size of acidophil nucleus	12 ± 0.3**	14.26 ± 0.23**	12.6 ± 0.27
Number of basophils	110 ± 12***	77 ± 5.3***	95 ± 11.1

[a]Numbers are arbitrary units as explained under Materials and Methods. *, not significantly different ($p > 0.05$); **, No PRL, different from U-PRL with $p < 0.001$, $n = 120$; ***, different with $p < 0.02$, $n = 9$.

As can be seen from the results, administration of U-PRL raised the circulating level of endogenous rat PRL in female animals, regardless of physiologic state, although the relative degree of elevation was reduced in postpartum animals (1.5-fold vs 2-fold in the other females). Similar administration of U-PRL to male animals had no effect. By contrast, administration of S179D PRL had no effect on the circulating rat PRL levels in female animals, but reduced PRL levels in the males to approx 50% of control. Differential results with the two administered hormones between male and female animals confirms the lack of recognition of the administered hormones in the rat PRL assay. The presence of very similar endogenous rat PRL in the untreated male and female animals also makes it clear that the difference between the male and female response is not related to the relative dose of the administered hormone.

In other, shorter term experiments, nonpregnant females were given four times the dose of the recombinant PRLs for 7 d. A similar elevation of circulating rat PRL was observed in response to U-PRL (no PRL, 12 ± 3.2*; U-PRL, 28.3 ± 3.1*; S179D PRL, 13.5 ± 2.9; * different with $p < 0.05$) and even at this higher dose, S179D PRL was inactive in this time frame. In these animals, morphometric analysis of pituitary sections (Table 2) demonstrated that there was no increase in acidophil number, but that there was an increase in overall acidophil size and size of acidophil nuclei after treatment with U-PRL. These hypertrophic changes are consistent with increased secretion of PRL. In the same areas of tissue, there was no effect on the size of basophils, although the number of basophils decreased with U-PRL because of the increased area occupied by acidophils. S179D PRL also had no effect on cell number in this time frame and had no effect on acidophil or basophil size.

Immunohistochemical staining of consecutive sections with anti-PRL followed by hematoxylin and eosin (H&E) staining showed approx 60% of the acidophils to be mammotrophs. This result is reported in approximate terms because it was not always possible to identify every cell before and after H&E staining. The immunohistochemistry also provided some evidence consistent with an increased rate of PRL secretion from these pituitaries. Thus, PRL was seen to accumulate around blood vessels in the U-PRL-treated pituitaries (arrowheads in Fig. 1B), something that was not observed in the other two groups (compare Fig. 1B with 1A).

U-PRL and P-PRL During DES-Induced Prolactinoma Production

As anticipated from numerous previous reports (e.g., *11*), DES treatment of rats resulted in pituitary tumor formation. Fig. 2 illustrates changes in the DNA content of pituitaries, the pituitary ratio of U-PRL:P-PRL (panel A) and circulating levels of total PRL (panel B) with time of treatment in Fischer 344 rats. DNA is expressed as DES: placebo to correct for animal growth with time. This correction is more important for the longer trial with Sprague-Dawley rats, but is used here in order to be consistent in presentation of the data.

In the Fischer rat study (Fig. 2), animals were sacrificed at weekly intervals. There was an initial and transient drop in animal weight between the first and second weeks of DES treatment, and so values were obtained for DNA content, U-PRL:P-PRL ratio, and circulating PRL from the second week on to be sure that they reflected responses in healthy animals.

Between wk 2 and 4, DNA content tripled, the ratio of U-PRL:P-PRL increased 1.5-fold, and circulating PRL went up 8-fold. Between wk 4 and 5 there was a drop in DNA content that was not accompanied by any decrease in body weight. This drop in DNA content was also observed in all animals of each of the three trials. During this time, there was a

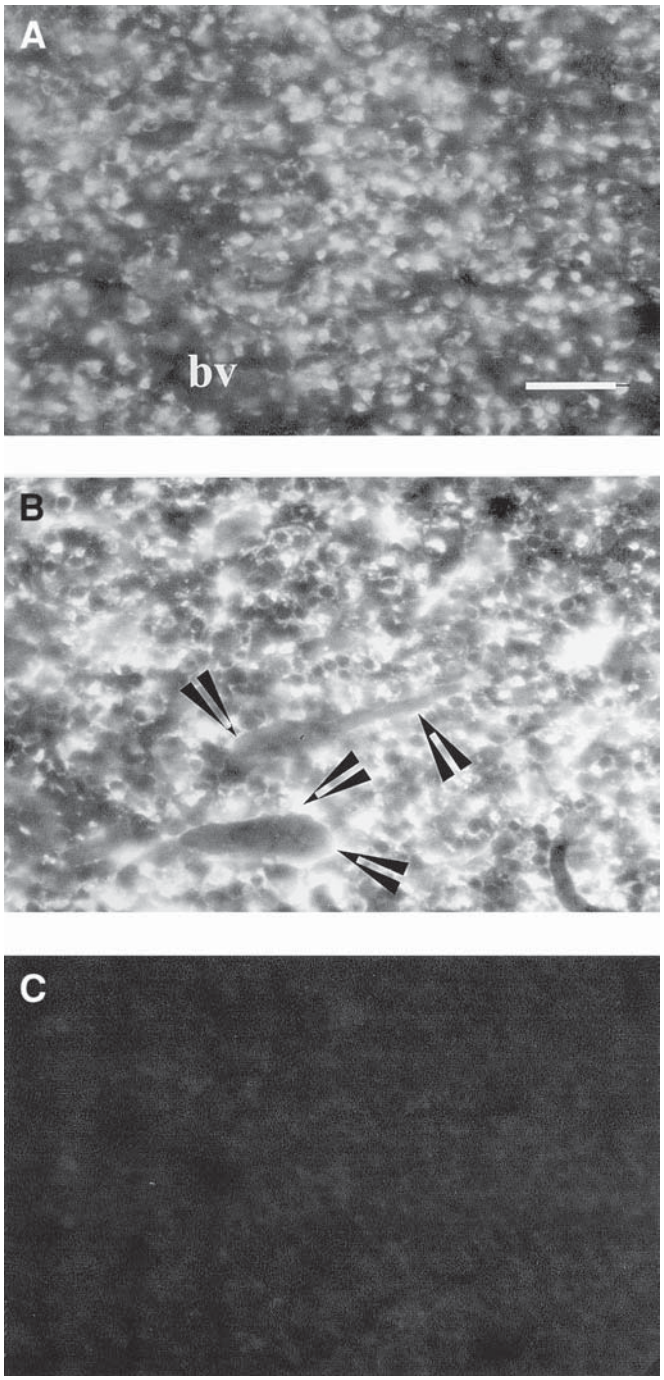

Fig. 1. Effect of administered PRL on pituitary PRL distribution. Panel A shows anti-PRL fluorescence in pituitaries of animals bearing saline pumps. Panel B shows fluorescence in pituitaries of animals bearing pumps delivering U-PRL. Panel C shows the use of a control antibody on a section from the U-PRL-treated animals. Note the accumulation of PRL in the perivascular regions in panel B (arrows). This was not seen in any sections from the saline- or S179D PRL-treated groups (latter not shown). bv, blood vessel. Bar = 70 μm.

plateau in the ratio of U-PRL:P-PRL and in circulating levels of PRL. Between wk 5 and 6, DNA content, the ratio of U-PRL:P-PRL, and circulating PRL all climbed once again. In other words, increased ratios of U-PRL:P-PRL were associated with hyperprolactinemia and pituitary cell proliferation. Plateaus in the U-PRL:P-PRL and circulating PRL levels were associated with a decreased DNA content.

Sprague-Dawley rats were slower to develop pituitary tumors in response to DES, and so these animals were monitored at 4 wk intervals for a total of 24 wk. A biphasic response in terms of pituitary DNA content was observed and in this strain of rats recovery from the first proliferative phase was complete (Fig. 3A). The first proliferative phase was associated with an increase in the ratio of U-PRL:P-

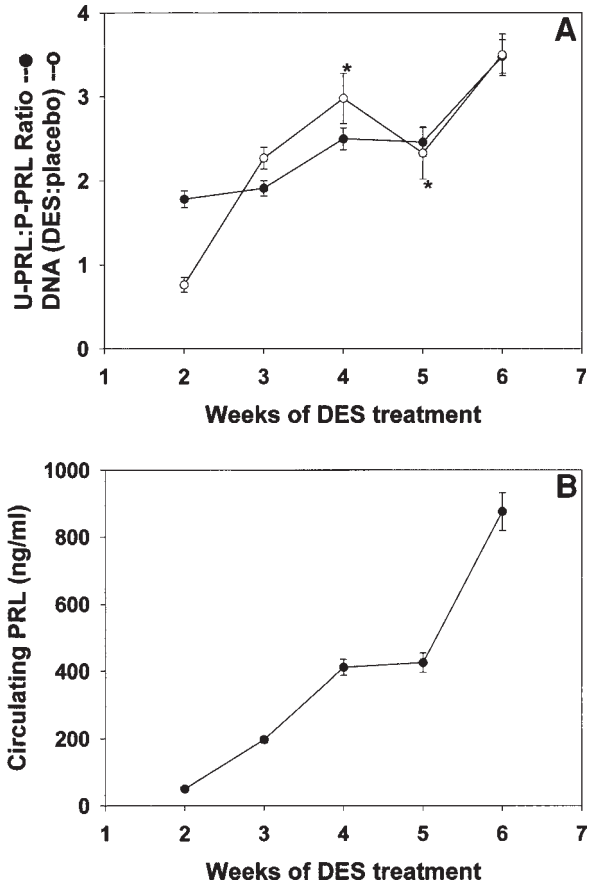

Fig. 2. Development of prolactinomas in Fischer 344 rats. Panel A shows the pituitary DNA content and the U-PRL:P-PRL ratio and panel B, the circulating PRL as a function of time of treatment with DES. Note the similar patterns in all three measured parameters. *, marginally significantly different from each other, $p < 0.06$. Data are presented as the mean ± SE and are derived from nine animals at each point.

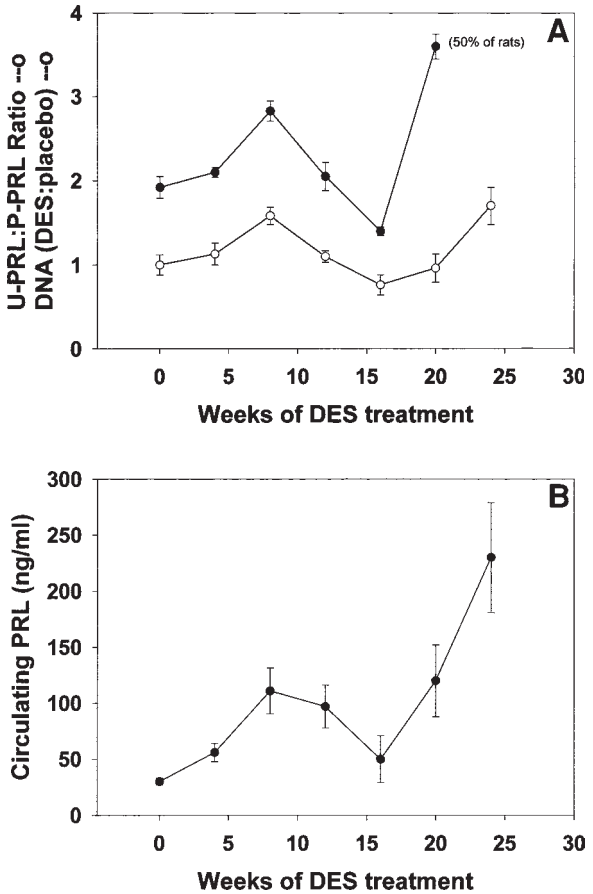

Fig. 3. Development of prolactinomas in Sprague-Dawley rats. The panels are the same as for Fig. 2. Once again, note the similar patterns in all three measured parameters. The final timepoint for the U-PRL:P-PRL ratio at 20 wk was only representative of 3 animals rather than the six for all the other time points and parameters. The other three animals showed hyperphosphorylated PRL as illustrated in Fig. 4.

PRL (panel A) and an increase in circulating PRL (panel B). During the recovery phase, the ratio of U-PRL:P-PRL decreased and the amount of circulating PRL decreased. The second proliferative phase was accompanied in 50% of the animals by a major increase in the U-PRL:P-PRL ratio to 3.6 ± 0.15. In the other 50%, abnormal hyperphosphorylated forms appeared that could not be quantified in terms of U-PRL:P-PRL and hence are not presented on the graph, but are illustrated in Fig. 4. Thus, in this second strain of rats, cell proliferation is associated with an increase in the ratio of U-PRL:P-PRL and reversal is associated with a decrease. The major differences between the two strains of rats were the time frames of each phase and the superior ability in the Sprague-Dawley rats to normalize DNA content after the first proliferative phase. In addition, pituitaries from the Sprague-Dawley rats showed a capacity to produce abnormal, hyperphosphorylated forms.

DES is considered an estrogen, but to be sure that it behaved similarly to 17β-estradiol in increasing the proportion of PRL, that is, U-PRL, Sprague-Dawley rats were treated with 17β-estradiol for 4 wk. Figure 5A shows a typical two-dimensional gel of pituitary proteins after 4 wk of treatment. When compared to the control shown in Fig. 5B, one can appreciate that treatment caused an increase in the amount of PRL present that was unmodified. In other words, 17β-estradiol and DES had similar effects, although 17β-estradiol was surprisingly more effective in an equivalent time frame (compare Fig. 5A with the 4 wk time point of Fig. 3).

Discussion

By administration of the recombinant human PRLs to the experimental animals, we expected to initiate one or more of the feedback mechanisms for PRL secretion. One feedback mechanism involves the increased production and release of dopamine from the hypothalamus *(1)*. This dopamine then interacts with D2 dopamine receptors on mammotrophs and inhibits PRL release *(1)*. Recent analysis of the D2 recep-

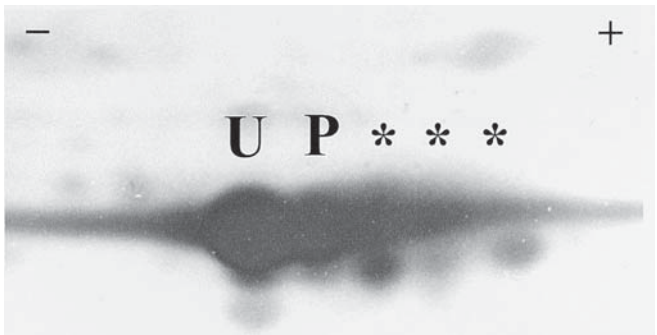

Fig. 4. A silver-stained two-dimensional gel of pituitary proteins following DES treatment of Sprague-Dawley rats for 20 wk. Note the hyperphosphorylated forms marked with asterisks. This occurred in 50% of the animals at this time. U, unmodified PRL, P, phosphorylated PRL. For comparison with a normal control, see Fig. 5B.

tor null mouse has demonstrated that these animals are hyperprolactinemic, as expected, and, moreover, that they develop prolactinomas *(12)*. In like manner, the PRL receptor null mouse is also hyperprolactinemic and also develops prolactinomas *(13)*. This was originally thought to be due only to the absence of PRL receptors on hypothalamic neurons, and, hence, to the effective production of the same result as the D2 receptor null mouse. More recently, comparisons between the D2 receptor and PRL receptor null mouse have found that the PRL receptor null mouse develops prolactinomas more rapidly *(13)*. Further analysis suggests an important role for PRL signaling at the level of the pituitary in the control of PRL release and the development of prolactinomas *(13)*. This is in addition to a very clear effect of dopamine on the inhibition of mammotroph proliferation, most recently demonstrated in the PRL null mouse *(14)*.

In previous publications, we and others have demonstrated that PRL acts in vitro as an autocrine regulator of PRL secretion and mammotroph proliferation *(3–7,15,16)*. We and others have demonstrated the presence of PRL receptors on mammotrophs in the normal rat pituitary *(17–19)* and similar observations have been made in human pituitaries *(20)*. In addition, work from this laboratory has shown that the different forms of PRL have different activities in terms of their ability to autoregulate PRL secretion and mammotroph proliferation in vitro. Thus, P-PRL inhibits further PRL release and inhibits pituitary tumor cell proliferation *(6,15)*. U-PRL, by contrast, promotes pituitary tumor cell proliferation, with its effect on PRL release still unclear *(15)*. In the PRL receptor null mouse, neither of those two forms of PRL would be able to exert its effect at either the hypothalamus or pituitary. The fact that these mice develop prolactinomas at a faster rate than the D2 receptor null mice, however, suggests that an absence of inhibitory signaling at the level of the pituitary contributes to the pathology.

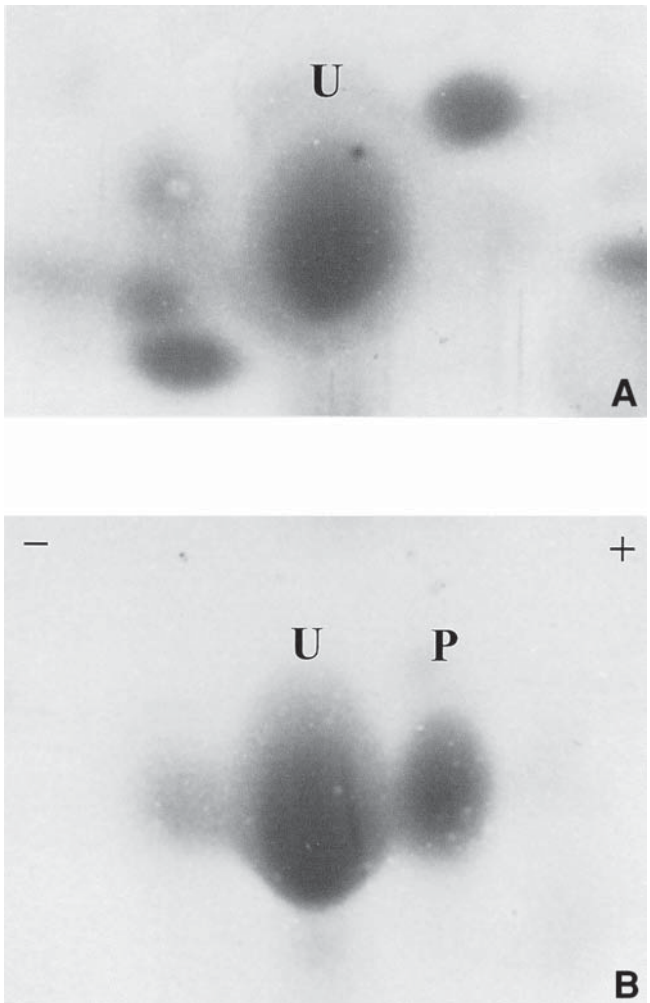

Fig. 5. Silver-stained two-dimensional gels of pituitary proteins with and without 17β-estradiol treatment of Sprague-Dawley rats for 4 wk. The majority of PRL present in those treated with 17β-estradiol is U-PRL, designated by a U above the spot (Panel A). Panel B shows a typical pattern from a nontreated female. This does vary with the estrous cycle *(8)*. These gels are representative of greater than 60 produced for this and other studies.

In the current study, the methodology does not allow us to determine the relative importance of hypothalamic and pituitary (or other) feedback mechanisms, but it does allow us to address the importance of the different forms of PRL. To conduct these experiments, it was necessary to use PRL of a different species so that it would not cross react with antibodies used to measure endogenous PRL. The production of recombinant human PRLs allowed us to produce sufficient protein for these experiments, proteins that were not recognized in the rat PRL assay. In these experiments, it was also crucial that P-PRL could not be dephosphorylated to produce U-PRL. This was accomplished by using a molecular mimic of P-PRL, which cannot be converted into U-PRL. This molecular mimic, S179D PRL, substitutes an aspartate residue (D) for the normally phosphorylated

serine (S) in PRL. In rats this is S177 *(21)* and the equivalent in humans is S179 *(22)*. Mimicry of phosphoserine with an aspartate residue has become fairly commonplace in recent years (e.g., *23–25*). The aspartate mimics a phosphoserine by approximate size of the side chain and by carrying a negative charge. In several instances, structural and functional analyses have shown faithful concurrence of 3-D structure and biological activity between the mimic and the naturally phosphorylated protein *(23–25)*. In the case of P-PRL and S179D PRL, we have shown that both act similarly in an Nb2 cell bioassay (compare refs. *26* and *27*).

Based on our previous in vitro data, we anticipated that S179D PRL, as a mimic of P-PRL, would inhibit PRL release, at least at the level of the pituitary. Our anticipated result was, however, only observed in male animals, leading to the conclusion that feedback regulatory mechanisms are complex and impacted by different factors in male and female animals. This concept is carried further by the observation that administration of U-PRL only elevated circulating rat PRL in the female animals. Hyperprolactinemia and prolactinoma formation occur more readily in females (reviewed in ref. *28*). Because this remains true for the two receptor null mice *(12,13)*, we can conclude that it is a pituitary difference. Thus, some aspect of the female autoregulatory mechanisms is different from that of males, and from our studies we know that this involves the ability to differentiate between the two forms of administered PRL. All forms of the PRL receptor found in rodents have identical extracellular domains *(29)* and so there is at present no reason to suggest that the two forms of PRL bind to different receptors. The two main forms of PRL receptor do, however, have different signaling capacities *(29)*. Binding of the different forms of PRL to the receptors does result in different intracellular signaling in other tissues *(30)*. These differences in signaling are also amplified over time of treatment with the two PRLs in the other tissues by upregulation of the expression of one or other major form of the receptor *(30)*. The necessity for slow changes in receptor expression with time could account for the effectiveness of P-PRL in reducing PRL secretion between wk 8 and 16 of prolactinoma induction in the Sprague-Dawley rats while a 7 d exposure to higher proportions (although not higher concentrations) of S179D PRL was without effect. It would now be of interest to compare receptor expression and signaling in the pituitaries of male and female PRL-treated and untreated animals and to determine whether estrogen and/or progesterone were key to the differences observed. In other words, does estrogen, for example, slow the process of receptor change? Differences in the expression of the two major PRL receptors has already been noted between the pituitaries of male and female rats *(18)* and ratios of long and short receptors do vary with the estrous cycle *(19)*.

An increase in the amount of rat PRL in the circulation in response to U-PRL could have been accomplished either by decreased clearance or by increased secretion. Thus, one could envisage that administered hormone might occupy PRL binding proteins and tissue PRL receptors thereby leaving more rat PRL in the circulation. If this had been the operative mechanism, however, we should have observed a greater increase in circulating rat PRL when U-PRL was given to male rats because they express lower amounts of receptor *(18)*. In fact, male rats did not show any response to U-PRL at the dose used. It is therefore likely that increased circulating rat PRL is a consequence of increased secretion. Consistent with this interpretation is the observed hypertrophy of acidophils, approx 60% of which were mammotrophs, and the accumulation of PRL in perivascular regions. PRL is stored in oligomeric form and normally monomerizes during the process of exocytosis *(31)*. Several reports have demonstrated pericellular oligomeric granule cores following periods of accelerated release (e.g., *32*), and many reports have documented an increase in smaller, but still oligomeric, forms in the circulation of hyperprolactinemic patients (e.g., *33*). Oligomeric PRL accumulates in the perivascular regions because in its large oligomeric form it cannot pass through the basement membrane surrounding the vessel endothelium.

The evidence therefore suggests that U-PRL promotes further secretion. In addition, we have demonstrated that estrogen and DES promote an increased ratio of U-PRL:P-PRL and therefore that circulating PRL release will increase in response to estrogen, both as a function of the autostimulatory effect of U-PRL and also via estrogen stimulation of overall PRL synthesis *(34)*. Why this does not initiate counteracting increased dopamine production is unclear at present, but suggests the possibility that the hypothalamus responds differently to U-PRL vs P-PRL. How far this U-PRL autostimulatory loop will go before it is counteracted is also unclear, but there may be clues in the tumor induction studies that show an apparent counteraction after 4 wk at over 100 ng/mL and a U-PRL:P-PRL ratio of 2–2.8 (Sprague Dawley) or after 1 wk at 400 ng/mL and a U-PRL:P-PRL ratio of 2.5 (Fischer). Many previous studies demonstrating PRL feedback regulation have utilized male rats, male rat pituitaries, short exposures, and higher concentrations of administered PRL (e.g., *35,36*) than were used in the present study.

Our previous in vitro data had demonstrated that U-PRL was also an autocrine growth factor for pituitary tumor cells and that P-PRL inhibited proliferation of pituitary tumor cells *(15)*. By examining two strains of rats with very different susceptibility to prolactinoma induction, we can correlate pituitary cell proliferation with the U-PRL:P-PRL ratio and overall output of PRL. There should be different timecourses of effects on the U-PRL:P-PRL ratio and PRL in the circulation between the two strains if these are related to cell proliferation. Very different timecourses were observed. In both strains of rats, cell proliferation was associated with an increase in the U-PRL:P-PRL ratio, as was an increase in total circulating PRL. In both strains of rats, there appears to be an attempt by the system to counteract excess prolif-

eration. In the Fischer rats this was between wk 4 and 5. In the Sprague-Dawley rats this was between wk 8 and 16. During this time, the ratio of U-PRL:P-PRL decreased, DNA content of the pituitaries decreased, and circulating PRL either plateaued (Fischer) or decreased (Sprague-Dawley). Thus some mechanism was induced which resulted in the phosphorylation of a greater proportion of the PRL. This recovery mechanism was far more effective in the Sprague-Dawley animals where pituitary DNA content returned to normal. That the recovery is related to increased activity/amount of PRL kinase is also suggested by the subsequent appearance of hyperphosphorylated forms of PRL in half of the Sprague-Dawley animals. Judging by the reinitiation of proliferation after 16 wk in this strain, however, it is clear that these hyperphosphorylated, as opposed to monophosphorylated forms, have lost their ability to inhibit secretion and cell proliferation.

Based on our previous in vitro data using pituitary tumor cell lines and the current correlations in vivo, it appears likely that exposure to sufficient U-PRL for a long enough period of time would induce a prolactinoma, but this remains to be directly demonstrated. The circulating concentrations required to test the effect of U-PRL on prolactinoma development (>100 ng/mL for >4 wk) make this experiment impossible at present with the approach used for the 21 d and 7 d experiments. Other delivery mechanisms are, however, currently being explored.

In summary, these data provide evidence in support of a differential role for the two forms of PRL in the autoregulation of PRL secretion and mammotroph proliferation. An increase in the proportion of U-PRL, which can be accomplished by estrogen exposure, results in autostimulation of PRL secretion leading to increased release and is associated in the longer term with the development of prolactinomas. Female animals are also less sensitive to the inhibitory actions of P-PRL and this may contribute to their greater susceptibility to prolactinoma formation.

Materials and Methods

Administration of Recombinant Human PRLs

Both recombinant proteins were produced in *Escherichia coli* and were extracted, folded, and concentrated in parallel as previously described *(27)*. The proteins were concentrated to 1 mg/mL saline for the females and 1.3 mg/mL for the males and were loaded into Alzet minipumps (Alza, Palo Alto, CA). Model 2004, which releases 6 µL/24 h, was used for the 19.5–21.5 d experiment and model 2001, which releases 24 µL/24 h, was used for the 7 d experiment. All pumps were inserted subcutaneously under local anesthesia. For the pregnant animals, this was done the morning of vaginal plug observation and this was considered d 0.5 of pregnancy. The animals tested 1 d postpartum had also received the PRLs throughout pregnancy and delivery for a total treatment period of 21.5 d. In these animals, the pups were removed after delivery to eliminate variation from different times of suckling. The animals were killed at the indicated times and trunk blood was collected and processed to produce serum.

Treatment of Rats with 17β-Estradiol

Female Sprague-Dawley rats (approx 250 g) (Bantin and Kingman, Fremont, CA) received a subcutaneous injection three times a week for 4 wk. On each occasion, they received 0.75 mL of 17β-estradiol (15 mg in 2:98 butanol:water). The last dose was given 24 h before sacrifice of the animals.

Induction of Prolactinomas

Female Fischer 344 and Sprague-Dawley rats at about 5 mo of age received 7.5 mg DES or placebo pellets (Preclinical Products, Boston, MA) subcutaneously using a trochar and local anesthesia with xylocaine. The pellets give constant release for a period of up to 6 mo. Constant release pellets were considered essential for interanimal and intertrial reproducibility. All animals were weighed at weekly intervals.

At weekly intervals for the Fisher rats and every 4 wk for the Sprague-Dawley rats, three animals from the DES- and three from the placebo-treated group were killed and trunk blood was collected. The pituitaries were carefully hemisected and processed for two-dimensional gel electrophoresis and assay of DNA content. Three separate trials were performed for the Fischer rats and two separate trials for the Sprague-Dawley rats. Data presented therefore have an *n* of 9 or 6 animals.

All animals were housed in light- and temperature-controlled conditions and were allowed free access to food and water. All animal procedures were approved by the University of California, Riverside, Committee on Laboratory Animal Care and were within NIH guidelines.

Radioimmunoassay for Rat PRL

Rat PRL was assayed either using a commercial kit purchased from ALPCO (American Laboratory Products Corporated, Windham, NH) or using reagents provided through the Hormone and Pituitary Program of the NIDDK, NICHD, and USDA. The intraassay variation and interassay variation were 1.5% and 5.6%, respectively, when purchasing ALPCO kits within a 3-mo period. The interassay variation rose to 7.2% for kits purchased more than 3 mo apart. For the data presented in Table 1, all of the 19.5–21.5 female animals were assayed in a similar time frame. The male animals and the 7-d-treated female animals were assayed at different times, but comparisons among treatments were all in the same assay.

Preliminary analyses showed that this assay did not recognize either recombinant human PRL at concentrations up to 250 ng/mL (highest concentration tested).

PRL levels during 17β-estradiol treatment and during prolactinoma development were assayed as described previously

using the NIDDK materials *(8)*. All samples were assayed in duplicate at more than one dilution.

Morphometric Analysis of the 7-d Treated Pituitaries

Pituitaries were fixed in periodate-lysine-paraformaldehyde *(37)*, dehydrated in graded ethanols, and then embedded in paraplast via Hemo D. Pituitaries were mounted in blocks such that sections would be made through the whole anterior pituitary. Sections were stained with hemotoxylin and eosin. Three sections from each of three animals were analyzed for each group. A random area at constant magnification was displayed and the image was digitized via PAXIT™ software (Midwest Information Systems, Franklin Park, IL). The number of cells staining blue or pink within the constant area was recorded. If a whole nucleus was present in the field of view, this was counted as a whole cell. If a half nucleus was present on the right or bottom of the screen, this was not counted. Those to the left and top were counted. With this approach a significant increase in the size of one cell type can cause a decrease in the number of another cell type in the field of view and vice versa. One hundred and twenty cells and their nuclei were also measured for each group. This was done on the computer monitor and hence the units are reported as arbitrary.

Immunohistochemical Staining

When sectioning for morphometric analyses, every third section was chosen. The intervening two were used for immunohistochemistry. Paraplast was removed from the sections with Hemo D, and they were then rehydrated through an ethanol series and incubated in Dulbecco A (0.01 *M* phosphate-buffered saline). Intrinsic fluorescence was reduced by incubation of the sections in 1% sodium borohydride in Dulbecco A. Nonspecific sticking of antibodies was reduced by blocking the sections in Dulbecco A containing 20% horse serum (HS), 0.2% bovine serum albumin (BSA), 25 m*M* NH_4Cl, 25 m*M* glycine, and 25 m*M* lysine. Sections were then incubated in anti-rat PRL or a control antibody diluted 1:100 in Dulbecco A plus 20% HS and 0.2% BSA for 2 h at 37°C in a humidified chamber. The anti-rat PRL was a gift from Dr. A. Parlow (Harbor-UCLA Medical Center, Torrance, CA), and the production and distribution of the antiserum was also partially supported through the Hormone and Pituitary Program of the NIDDK, NICHD, and USDA. The primary antibody was made in rabbits and was designated IC5 (AFP425-10-91). At the end of this incubation, the sections were washed three times in Dulbecco A plus HS and BSA and then incubated in fluorescently labeled anti-rabbit antibody (Cappel, Westchester, PA) also diluted 1:100 in Dulbecco A plus HS and BSA, for 2 h at room temperature in a humidified chamber. After three more washes in Dulbecco A plus HS and BSA, the sections were washed once in Dulbecco A with no additives and then were mounted in Vectashield (Vector Laboratories, Burlingame, CA). Fluorescent images were captured and the slide coordinates noted. The sections were then stained with hemotoxylin and eosin and, using the same coordinates, the same area was identified. In this manner, it was possible to approximate the percentage of acidophils that stained positively for PRL.

Two-Dimensional Gel Analysis

Hemipituitaries were homogenized on ice in 50 m*M* Tris buffer containing 150 m*M* NaCl, 5 m*M* EDTA, 3 m*M* NaN_3, 0.5% Nonidet P40, and 10^{-5} *M* leupeptin at 4ºC using 20 strokes of a Teflon pestle. This was followed by sonication of the homogenate for 10 s at setting 4 with a sonicator (Heat Systems-Ultrasonics, Plainview, NY). Proteins in the resultant suspension were then precipitated with 4 vol of cold (–20°C) acetone for 48 h at –20ºC. Precipitated proteins were collected by centrifugation and were dissolved in urea-lysis buffer [9 *M* urea, 5% β-mercaptoethanol, 4% ampholines, pH 4–6.5 (Sigma) and 2% nonidet P40]. Prior to electrophoresis, molecular weight and pI markers were added for accurate identification of the PRL spots by three-point coordination. The gels were run and stained as previously described *(8,9)*. Quantification was achieved using a Bio image analysis system (Ann Arbor, MI).

DNA Assay

Hemipituitaries were placed in 1 mL of 0.5 *N* perchloric acid (PCA) at 4ºC for a period of 48 h. The brittle tissue was then briefly homogenized using a Teflon pestle and then allowed to sit for a further 2 h at 4ºC before heating to 70ºC with occasional agitation for 15 min to solubilize the DNA. After cooling, protein was removed by centrifugation at 120*g* for 15 min, 4ºC. Some aliquots of the supernatant were placed immediately in the assay, while others were frozen at –20ºC to be later compared within one assay of all time points. DNA in the supernatants was assayed according to the method of Burton *(38)* using calf thymus DNA (Sigma Chemical Co., St. Louis, MO) as standard. The intraassay variation was 3% and the interassay variation was 7%. All data within one figure are from the same assay, and each sample was assayed in duplicate.

Statistical Analysis

Data in the tables are presented as the mean plus/minus standard error. All comparisons were made using analysis of variance followed by *t* tests among all groups. Because these latter compared more than one test group against a single control, Bonfferoni corrections were applied. Results yielding a confidence limit of 95% were considered statistically significant. For the time course experiments, *t* tests were applied to different time points to establish the significance of individual changes.

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