EXPERIMENTAL STUDY

Downregulation of long-form prolactin receptor mRNA during prolactin-induced luteal regression

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Abstract

Objective: Prolactin is capable of both trophic and lytic actions in rat corpora lutea. In corpora lutea responding to a trophic prolactin signal, the long form of the prolactin receptor is the dominant form and is upregulated by prolactin. We investigated whether mRNA for the short form of the prolactin receptor was dominant in corpora lutea responding to a lytic prolactin signal, and whether the relative concentrations of the mRNAs for both forms of the prolactin receptor were changed during this response.

Design and methods: Immature rats were ovulated by injection of 5 IU equine chorionic gonadotrophin and 5 IU human chorionic gonadotrophin, and were hypophysectomized shortly after ovulation. Nine days after hypophysectomy, rats were injected with prolactin (500 µg/day) or vehicle for 24 ($n = 6$, $n = 6$) or 72 h ($n = 13$, $n = 5$). Total RNA was isolated from corpora lutea and mRNA for both types of prolactin receptor were analyzed by semiquantitative RT-PCR using the ribosomal protein S16 as the internal control.

Results: The intensities of the long- and short-form prolactin receptor signals were normalized to the S16 internal control and expressed as relative densitometric units. The normalized values at 24 h for prolactin-treated vs vehicle-treated rats were 0.23 ± 0.05 vs 0.49 ± 0.15 ($P > 0.05$) for the short form and 4.04 ± 0.8 vs 4.23 ± 0.6 ($P > 0.05$) for the long form. The values for 72 h were 0.30 ± 0.05 vs 0.24 ± 0.05 ($P > 0.05$) for the short form and 2.76 ± 0.4 vs 5.53 ± 0.3 ($P < 0.01$) for the long form respectively.

Conclusion: The long form of the prolactin receptor is the dominant form at both time-points; however, the concentration of mRNA for this receptor isoform was specifically downregulated by prolactin treatment. Our results suggest that the short form of the prolactin receptor alone is unlikely to mediate the luteolytic action of prolactin, but that luteolytic events may be influenced via a change in the ratio of the two receptor isoforms.

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Introduction

Administration of prolactin to hypophysectomized rats, after at least 3 days of hypophysectomy, results in regression of the corpora lutea; otherwise, in the absence of prolactin, these corpora lutea remain intact and steroidogenically active for an indefinite period (1–4). The luteolytic effects of prolactin include decreases in plasma 20α -dihydroprogesterone, the predominant steroid secreted by corpora lutea of the hypophysectomized rat (3, 4), and luteal weight (5). These effects of prolactin following hypophysectomy contrast sharply with the luteotrophic effect of the

hormone during pregnancy and pseudopregnancy. In the latter states, prolactin acts to maintain high progesterone secretion by the corpora lutea, by inhibiting conversion of progesterone to 20α -dihydroprogesterone (6), and by maintaining luteal receptors for luteinizing hormone (7). Prolactin also acts in concert with oestradiol to increase protein synthesis in the corpus luteum (8, 9).

Both the luteolytic and the luteotrophic effects of prolactin are active events, presumably mediated through the prolactin receptor. In the rat, there are two forms of the prolactin receptor, derived from the same gene and with identical extracellular and trans-membrane domains. The two forms have a common

intracellular sequence of 27 amino acids, followed by a unique sequence of 331 amino acids for the 'long form' of the prolactin receptor, and a unique sequence of 31 amino acids for the 'short form' (10).

The two forms of the prolactin receptor may transmit different signals. The long form of the prolactin receptor is known to activate the transcription of milk protein genes (11–13) via the transcription factor STAT 5 (14–16). In contrast, the short form of the prolactin receptor is unable to activate STAT 5 (14). The long form of the prolactin receptor is also known to activate the tyrosine kinase JAK2 (17–20), while the ability of the short form to signal through JAK2 is debated. While one report indicates that both forms of the prolactin receptor associate and activate JAK2, as well as activating the transcription factor STAT1 (20), another states that the short form is unable to activate JAK2 (21). The authors of this second report suggest that the primary role of the short form of the prolactin receptor is to act as a dominant negative by heterodimerizing with the long form of the receptor (21). However, the short form of the prolactin receptor has been shown to transmit a mitogenic signal in at least one cell type (13), indicating that it is a functional receptor. In addition, a tyrosine phosphorylated protein that associates specifically with the short form of the prolactin receptor has been identified in the corpus luteum (22, 23), suggesting that the short form of the prolactin receptor may have a specific signalling function in this tissue.

Telleria *et al.* (24) examined the expression of luteal prolactin receptor isoforms during pregnancy, when prolactin acts as a luteotrophin. They reported that mRNA for the long form of the prolactin receptor was predominant over mRNA for the short form. In addition, mRNA for the long form of the receptor was specifically increased by prolactin, while expression of mRNA for the short form was not altered (24). The luteotrophic action of prolactin in the rat is associated with increased DNA binding of several STAT proteins, specifically STATs 3, 5a and 5b (25, 26). As the short form of the prolactin receptor is unable to activate STAT 5 (14), this suggests that the luteotrophic action of prolactin is mediated through the long form of the prolactin receptor. We have proposed that the ability of prolactin to carry out both luteolytic and luteotrophic actions may result from variations in the type of receptor present on the corpus luteum. Specifically, we have suggested that the short form of the prolactin receptor would be the dominant form present in corpora lutea responding luteolytically to prolactin, and that the short form of the prolactin receptor would be upregulated during this action of prolactin.

Materials and methods

Animals

Immature female Sprague–Dawley rats were obtained from Charles River (Portage, MI, USA). Rats received an

s.c. injection of 5 IU equine chorionic gonadotrophin at 29 days of age to induce follicular development, followed by an s.c. injection of 5 IU human chorionic gonadotrophin 56 h later to induce ovulation and the development of corpora lutea. Following ovulation, the rats were hypophysectomized at 32 days of age by the vendor. All rats were provided with rat chow and 5% glucose in water *ad libitum*, and were supplemented with sliced oranges. Animal procedures were approved by the University Committee on the Use and Care of Animals at the University of Michigan.

Experimental design

Rats ($n = 30$) were divided into four groups. Treated rats ($n = 19$) received injections of ovine prolactin (250 μg in 0.2 ml vehicle, s.c.) at 12-h intervals (1000 and 2200 h) on days 9–11 post-hypophysectomy, and were killed by decapitation 24 h ($n = 6$) or 72 h ($n = 13$) after the onset of prolactin treatment. Control rats ($n = 11$) were given injections of 0.2 ml vehicle (0.1% bovine serum albumin (BSA) and 0.03 mol/l NaHCO_3 in 0.15 mol/l NaCl, s.c.) and were also killed at 24 ($n = 6$) or 72 ($n = 5$) h after the beginning of treatment. Trunk blood was collected from all rats, and plasma was obtained for later radioimmunoassay of 20α -dihydroprogesterone. The ovaries were removed immediately and the corpora lutea dissected out and weighed as a group. The corpora lutea from each rat (eight to fifteen) were frozen in liquid nitrogen and stored at -70°C until later extraction of total RNA. The sella turcica was visually inspected in all rats for the presence of pituitary fragments and rats with incomplete hypophysectomy were removed from the experiment (reflected in the numbers given above).

Hormones

Ovine prolactin (NIDDK-oPRL, lot no. AFP10677C) was obtained from the National Institute of Diabetes and Digestive and Kidney Diseases of the NIH (Bethesda, MD, USA). The prolactin was diluted in 0.15 M NaCl, 0.03 M NaHCO_3 and 0.1% BSA to a final concentration of 1.25 mg/ml and pH of 8.2–8.6. Diluted prolactin was stored at 4°C and used within 1 week.

Radioimmunoassay

Plasma concentration of 20α -dihydroprogesterone was determined by radioimmunoassay. After decapitation of the rats, trunk blood was collected into heparinized tubes and then centrifuged at 1740 *g* for 20 min. The plasma was frozen in liquid nitrogen, and stored at -20°C until extracted and assayed for 20α -dihydroprogesterone according to the method previously described by Bender *et al.* (27).

RNA isolation and RT-PCR analysis

Total RNA was extracted from homogenates of isolated corpora lutea using TRIZOL Reagent (phenol and guanidine isothiocyanate solution; LifeTechnologies Inc., Grand Island, NY, USA) and the protocol provided by the company. Each sample contained luteal tissue from one rat with the exception of the prolactin-treated group at 72 h, for which each sample contained luteal tissue from two to three rats. Due to one poor round of RNA extraction, the total number of samples was decreased by one in each group, with the exception of the 72-h prolactin-treated animals. Detection of long- and short-form prolactin receptor mRNAs was carried out by RT-PCR using three oligonucleotide primers described previously (24). Briefly, a sense strand oligonucleotide from the common extracellular domain coding region was combined with either of two primers corresponding to the first 23 nucleotides of the unique cytoplasmic sequence for the long form or short form of the receptor respectively. The predicted size of the PCR-amplified product was 279 bp for both forms of the prolactin receptor. An additional pair of primers specific to the rat ribosomal protein S16 (5'-TCCAAGGGTCCGCTGCAGTC-3' and 5'-CGTTCACCTTGATGAGCTCATT-3') were used in each reaction as an internal control. The predicted size of the PCR-amplified product for S16 was 100 bp. Three micrograms of total RNA were reverse transcribed at 42 °C using random hexamer primers (Pharmacia, Piscataway, NJ, USA) and Moloney murine leukaemia virus reverse transcriptase (LifeTechnologies Inc.) in a 20 µl reaction mixture. Reverse transcription was carried out in duplicate for each sample. The reaction mixtures for each sample were added to either a tube containing 50 pmol each of the oligonucleotide primers for amplification of the short form of the prolactin receptor or a tube containing 50 pmol each of the oligonucleotide primers for amplification of the long form of the prolactin receptor. Each tube also contained 16.5 pmol each of the oligonucleotide primers for amplification of S16. PCR was carried out using [α -³²P]deoxy-CTP as previously described (24) for 25 cycles, with an annealing temperature of 65 °C. Under these conditions, amplification of prolactin receptor and S16 products from corpus luteum mRNA is in the exponential phase and the assay is linear with respect to the amount of input RNA. The kinetics of amplification are similar for cDNAs of the two forms of the prolactin receptor due to the similarity of the primer sets used and the identical size of the reaction products.

PCR reaction products were electrophoresed on 6% polyacrylamide non-denaturing gels. There was a total of four gels containing reaction products for both prolactin-treated and control groups as follows: long-form prolactin receptor at 24 h, long-form prolactin receptor at 72 h, short-form prolactin receptor at 24 h, and short-form prolactin receptor at 72 h. Total RNA

from the GGCL cell line, which does not contain the prolactin receptor (28), was reverse transcribed and submitted to PCR in parallel with the samples to be tested, as a negative control, and was run on each gel. Autoradiographic data were analyzed using a Molecular Dynamics PhosphorImager and ImageQuant version 3 software (Molecular Dynamics, Sunnyvale, CA, USA). The intensities of the long- and short-form prolactin receptor signals were normalized to the S16 internal control, and are expressed as relative densitometric units.

Statistics

Luteal weight and plasma 20 α -dihydroprogesterone values were compared between groups using the Bonferroni multiple comparisons test. Plasma 20 α -dihydroprogesterone values were log transformed prior to statistical analysis to normalize variance. Comparisons of densitometric values within a gel were made using Student's *t*-test.

Results

Luteal weight

Weight is expressed as mg/corpus luteum (mean $\pm$ S.E.M.) and was 0.77 ± 0.03 mg for control rats at 24 h ($n = 6$), 0.57 ± 0.04 for prolactin-treated rats at 24 h ($n = 6$), 0.74 ± 0.03 for control rats at 72 h ($n = 5$), and 0.38 ± 0.03 for prolactin-treated rats at 72 h ($n = 13$; Fig. 1). There was no difference in weight between the control groups at 24 and 72 h ($P > 0.05$). Luteal weight was significantly lower in the prolactin-treated groups at both time-points, when compared with the control groups ($P < 0.05$). In addition, luteal weight declined from 24 to 72 h after the onset of prolactin treatment ($P < 0.05$).

Figure 1 Luteal weight in mg/corpus luteum (mean $\pm$ S.E.M.) for corpora lutea of rats injected with either vehicle (VEH; solid bars) or prolactin (PRL; open bars) and killed at 24 or 72 h after the onset of treatment. Different letters indicate significant differences at $P < 0.05$.

Figure 2 Plasma 20 α -dihydroprogesterone concentration in ng/ml plasma (mean $\pm$ S.E.M.) for rats injected with either vehicle (VEH; solid bars) or prolactin (PRL; open bars) and killed at 24 or 72 h after the onset of treatment. Different letters indicate significant differences at $P < 0.05$.

20 α -Dihydroprogesterone

Plasma concentration of 20 α -dihydroprogesterone is expressed as ng/ml (mean $\pm$ S.E.M.) and was 18.2 ± 1.7 for control rats at 24 h ($n = 6$), 8.3 ± 1.0 for prolactin-treated rats at 24 h ($n = 6$), 22.0 ± 3.4 for control rats at 72 h ($n = 5$), and 7.5 ± 0.7 for prolactin-treated rats at 72 h ($n = 13$; Fig. 2). There were no significant differences between the two control groups or the two prolactin-treated groups ($P > 0.05$). However, plasma 20 α -dihydroprogesterone was significantly decreased in prolactin-treated groups when compared with control groups ($P < 0.05$).

Prolactin receptor mRNA expression

The abundance of mRNA for the long form of the prolactin receptor appeared to be greater than for the short form of the prolactin receptor following both 24 h (Fig. 3) and 72 h (Fig. 4) of prolactin treatment. Using densitometry, the amount of RT-PCR product specific for the long and short forms of the receptor was quantified. The intensity of the signal for each sample was normalized to the intensity of the S16 signal for that sample, and the resulting value is expressed in relative densitometric units. At 24 h following the onset of prolactin treatment, the short form of the prolactin receptor was at 0.23 ± 0.05 units (mean $\pm$ S.E.M.) for the prolactin-treated group ($n = 5$) and at 0.49 ± 0.15 units for the control group ($n = 5$; Fig. 3). These values were not significantly different ($P > 0.05$). At 72 h following prolactin, the short form of the prolactin receptor was at 0.30 ± 0.05 units for the prolactin-treated group ($n = 6$) and at 0.24 ± 0.05 units for the control group ($n = 4$; Fig. 4). Again, these values were not different ($P > 0.05$). Although there were no significant differences in the amount of cDNA for the short form of the receptor between treatment groups, this may be due to the great variation in abundance of

this mRNA among the samples. The mean value was lower in corpora lutea of prolactin-treated than of vehicle-treated rats at 24 h, and greater in prolactin-treated than in vehicle-treated at 72 h, which does suggest an increase in the amount of mRNA for the short form of the prolactin receptor during this period of luteal regression.

For the long form of the prolactin receptor, values at 24 h following the onset of prolactin treatment were 4.04 ± 0.8 units for the prolactin-treated group ($n = 5$) and 4.23 ± 0.6 units for the control group ($n = 5$) and were not different ($P > 0.05$; Fig. 3). However, at 72 h following the onset of prolactin treatment, values for the long form of the prolactin receptor were 2.76 ± 0.4 units for the prolactin-treated group ($n = 6$) and 5.53 ± 0.3 units for the control group ($n = 4$) and these values were significantly different ($P < 0.01$; Fig. 4). The data suggest that the abundance of mRNA for the long form of the prolactin receptor was essentially identical for corpora lutea of prolactin-treated and vehicle-treated rats at 24 h, but by 72 h after the onset of treatment the mRNA for the long form of the prolactin receptor was significantly decreased in corpora lutea of prolactin-treated rats. This indicates downregulation of mRNA for this form of the prolactin receptor during prolactin-induced luteal regression.

Discussion

The decreases in weight per corpus luteum and in steroid production, as indicated by a drop in plasma 20 α -dihydroprogesterone, indicate that prolactin treatment resulted in the expected regressive changes in the corpora lutea. These changes occurred as early as 24 h after the onset of prolactin treatment, following two injections of prolactin, and luteal weight continued to decline over the next 48 h of treatment. Analysis of mRNA expression of the long and short forms of the prolactin receptor revealed that the long form of the prolactin receptor was clearly the dominant form present in these corpora lutea at both these time-points. However, expression of mRNA for the long form of the prolactin receptor appeared to be downregulated by 72 h after the onset of prolactin treatment. The data also indicated a trend towards increased abundance of mRNA for the short form of the prolactin receptor at this 72-h time-point; however, this was not significant, probably due to the considerable variation in expression of mRNA for this form of the prolactin receptor. RT-PCR analysis was used to determine changes in prolactin receptor isoforms in this study due to low abundance of prolactin receptor in the rat ovary (29), and thus difficulties in quantifying this mRNA using Northern analysis.

Telleria *et al.* (24) have shown that the long form of the prolactin receptor is the most abundant form in the corpora lutea at all stages of pregnancy. Corpora lutea of pregnancy respond to prolactin with enhanced progesterone production and luteal development (6, 8), and at

Figure 3 Expression of mRNA for prolactin receptor (PRL-R) long form (top) and short form (bottom) for corpora lutea of prolactin-treated (PRL; lanes 2–6) and vehicle-treated (VEH; lanes 8–12) rats at 24 h after the onset of treatment. The PCR reaction product for both forms of the PRL-R runs at 279 bp, while the internal control, S16, runs at 100 bp. Each lane contains cDNA derived from corpora lutea of one rat. Lane 7 contains cDNA from the GGCL cell line, a negative control for PRL-R. The times of exposure are identical for the two autoradiograms. Results from each autoradiogram are shown graphically on the right-hand side, as PRL-R/S16 expressed as relative densitometric units.

least part of this luteotrophic action of prolactin appears to be transmitted through the long form of the prolactin receptor (25, 26). The short form of the prolactin receptor may signal through a different pathway than the long form in the rat corpus luteum; certainly it appears to have some different protein associations in this tissue (22). An intriguing hypothesis, therefore, was that the short form of the prolactin receptor, acting through an independent signalling pathway, was responsible for the luteolytic actions of prolactin. We proposed that, if this were the case, the short form of the prolactin receptor would be dominant in corpora lutea of the hypophysectomized rat, which are able to respond to prolactin with luteal regression, and that prolactin would act to upregulate this form of the prolactin

receptor. Our results do not support this proposal, as the long form of the prolactin receptor is still dominant in these corpora lutea, as it is during pregnancy, and there is no clear upregulation of the short form of the prolactin receptor. However, we do observe some interesting changes in the expression of mRNA for the two forms of the prolactin receptor, once prolactin-induced luteal regression has been initiated.

Prolactin treatment causes an increase in abundance of mRNA for the long form of the prolactin receptor when it is acting as a luteotrophin, during pregnancy (24). The short form does not appear to be affected, resulting in an alteration in the ratio of the short to the long form of the prolactin receptor. During prolactin-induced luteolysis, as examined in the current study,

Figure 4 Expression of mRNA for prolactin receptor (PRL-R) long form (top) and short form (bottom) for corpora lutea of prolactin-treated (PRL; lanes 2–7) and vehicle-treated (VEH; lanes 9–12) rats at 72 h after the onset of treatment. The PCR reaction product for both forms of the PRL-R runs at 279 bp, while the internal control, S16, runs at 100 bp. Each PRL lane contains cDNA from corpora lutea of two to three rats, while each VEH lane contains cDNA from corpora lutea of one rat. Lane 8 contains cDNA from the GGCL cell line, a negative control for PRL-R. The times of exposure are identical for the two autoradiograms. Results from each autoradiogram are shown graphically on the right-hand side, as PRL-R/S16 expressed as relative densitometric units. The asterisk indicates a significant difference at $P < 0.01$.

there also appears to be an alteration in this ratio. However, the alteration in this case is in the inverse direction of the alteration during the luteotrophic action of prolactin. The ratio between the long and short forms of the prolactin receptor has been previously shown to be an important parameter in determining response to prolactin in different tissue types (30), and changes in this ratio may help to explain how prolactin is able to carry out both luteotrophic and luteolytic actions in the corpus luteum. Alteration in the ratio of the receptor isoforms is, however, insufficient to explain the ability of prolactin to induce luteal regression in the hypophysectomized rat, since this change in ratio appears to occur only after prolactin-induced luteal regression has been initiated.

The conversion of the corpus luteum to a tissue which

is able to respond luteolytically to prolactin begins with the removal of prolactin. This conversion apparently takes 3 days to complete, as prolactin is able to act as a luteolysin only 3 or more days after hypophysectomy (1, 2). The exact changes in the corpus luteum are unknown; however, they may include alterations in the signalling pathways coupled to the prolactin receptor. These alterations may consist of changes in specific transcription factors or other key proteins in these pathways. The overall result appears to be a restructuring of the signalling pathways linked to the prolactin receptors such that restored prolactin acts as a luteolysin. The apparent change in ratio of short form to long form prolactin receptor observed in this study appears to be initiated as part of the luteolytic action of

prolactin, and may play a part in maintaining the luteolytic response of the tissue, once it has been initiated. The means by which prolactin can execute both luteotrophic and luteolytic responses in the rat corpus luteum remains an intriguing subject for further investigation. In particular, elaboration of the signalling pathways linked to the prolactin receptor isoforms in these two systems is required.

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