Developmental aspects of prolactin receptor gene expression in fetal and neonatal mice

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The prolactin receptor (PRL-R), a member of the hematopoietin cytokine receptor superfamily, is widely distributed among mammalian tissues. To understand better the potential sites of action and onset of potential PRL responsiveness, the developmental distribution pattern of PRL-R mRNA expression in fetal and neonatal mice was examined. Fetal mouse tissues were collected at distinct stages from timed pregnancies. Following extraction of total RNA, onset of gene expression was evaluated via reverse transcription-polymerase chain reaction (RT-PCR) and Southern hybridization was employed for verification. Expression of PRL-R mRNA was first observed on day 14 in the liver and cranium and on day 15 in the kidney, lung and thymus gland. Pituitary and adrenal glands were positive for PRL-R at day 18 of gestation through to day 1 of postnatal life. Neither whole fetuses prior to day 14 (days 10–13) of gestation nor skin and bladder tissues from 2-day-old mice generated detectable RT-PCR signals for PRL-R. The presence of PRL-R mRNA in fetal thymus and spleen tissues suggests a possible role for PRL in the development of the immune system. Prolactin may act directly on the pituitary to influence its own secretion and/or that of other pituitary-derived factors, as evidenced by the presence of PRL-R mRNA in the pituitary glands of fetal and 1-day-old mice. These data are the first to show the presence of PRL-R gene expression in various organ systems in fetal mice and suggest that PRL is among several factors necessary to coordinate developmental activities.

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Prolactin (PRL) is a multifunctional hormone that has been shown to regulate growth and differentiation of many different cell types (1). Prolactin is best known for stimulating the development of mammary glands and lactation (2). However, this protein is also known to be involved in the regulation of other physiological functions (3), including reproduction, water and salt balance (4), metabolism, behavior (5) and, according to more recent data, immunoregulation (6).

The PRL receptor (PRL-R) belongs to the hematopoietin cytokine receptor superfamily (7), which includes the receptors for interleukins (2–7) and colony-stimulating factors in addition to growth hormone and placental lactogens. Prolactin receptors have been found in several tissues, including mammary gland, ovary, testis, prostate, liver, kidney, adrenal gland, hypothalamus, pancreatic islets and lymphoid tissue (8). In addition, PRL is expressed in cells of the immune system and PRL can affect these cells directly, both in vivo and in vitro (9, 10), although the precise nature of its modulatory action (stimulation vs inhibition) in this system remains controversial.

Very little information is available on the ontogeny of PRL-R expression in mice. To better understand the potential sites of action and onset of potential PRL responsiveness, the developmental distribution pattern of PRL-R mRNA expression in various organ systems over several fetal and early neonatal ages was examined. We were especially interested in gaining insights into the development of the neuroendocrine control of the immune system.

Materials and methods

The animals used in this study were maintained in wire cages under controlled conditions of photoperiod (12 h light/dark) and temperature (22 ± 1°C) and ad libitum access to food (Purina Lab Chow, Purina Mills, St. Louis, MO) and water. Individual female mice derived from crosses of C57BL/6 and C3H strains, acquired from an in-house colony, were housed with a male until a cervical plug was observed (designated as day 0). Males were then removed from cages. Female mice were sacrificed using institutionally reviewed and approved protocols. Fetal tissues were collected at distinct stages from timed pregnancies. Tissues were placed in denaturing solution (4 mol/l guanidinium thiocyanate, 25 mmol/l sodium citrate (pH 7.0), 0.5% Na Na-laurolysosarcosine and 0.1 mol/l 2-mercaptoethanol), rapidly frozen and stored at −70°C following collection. Total cellular RNA was extracted using a single-step acid guanidinium thiocyanate–phenol–chloroform method.
of Chomczynski and Sacchi (11). This isolation procedure produced RNA that had 260/280 absorbance ratios between 1.85 and 2.10.

The isolated RNA was used for reverse transcription-polymerase chain reaction (RT-PCR) analyses. The concentration of total RNA was assessed by spectrophotometric absorbance at 260 nm. Three micrograms of RNA from each tissue were reverse transcribed to cDNA using 15 U of avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI) in 10 mmol/l TRIS-HCl (pH 8.8 at 25°C), 50 mmol/l KCl, 0.1% Triton X-100, 5 mmol/l MgCl2, 1 mmol/l of each deoxynucleoside triphosphate (dNTP), 1 U of ribonuclease inhibitor and 1.2 ng of PR2 primer (details later in this section). First-strand cDNA synthesis of total RNA (final volume = 20 µl) was performed for 20 min at 42°C. Two negative controls were performed for the first-strand synthesis: samples that contained the RT reagents but no RNA; and samples that contained RNA but no reverse transcriptase. Samples were denatured for 5 min at 99°C prior to PCR. A 698-bp PCR product was generated following the addition of 1.25 µl of cDNA to the PCR reaction buffer: 10 mmol/l TRIS-HCl, 1.5 mmol/l MgCl2, 50 mmol/l KCl (pH 8.3). Five mmol/l of each dNTP, 150 µg/µl of PR1 and PR2 and 2.5 U of Taq DNA polymerase (Boehringer Mannheim, Indianapolis, IN) were added to the reaction for a final volume of 25 µl. The conditions for PCR of all tissues were as follows: 96°C for 1 min, 54°C for 1.5 min, 72°C for 2 min for 37 cycles and a final extension period of 5 min at 72°C. The number of cycles for amplification was chosen following optimization of PCR conditions in our laboratory and based on previous reports (Brown-Borg, unpubl. data: 12).

For identification of amplification products, 7.5 µl of each reaction mixture was electrophoresed on a 1.5% agarose gel containing ethidium bromide. A positive control (adult liver), negative control (no template) and DNA size marker were run in conjunction with mouse tissue cDNA.

Southern hybridization was performed to verify correct amplification of PRL-R mRNA. An internal oligonucleotide probe was labeled using a non-radioactive labeling system, digoxigenin-dUTP (DIG; Boehringer Mannheim). Briefly, 100 pmol of the oligonucleotide probe was added to the reaction buffer (1 mol/l potassium cacodylate, 1.25 mmol/l TRIS·HCl, 1.25 mg/ml BSA; pH 6.6), 5 mmol/l CoCl2, 0.05 mmol/l DIG·dUTP, 0.5 mmol/l dATP and 2.5 U of terminal transferase. The reaction mixture was incubated for 15 min at 37°C, placed on ice and 1 µl of glycogen + 200 mmol/l EDTA (pH 8.0) was added. The labeled oligonucleotide was precipitated in 4 mol/l LiCl and ethanol for 30 min at −70°C. The precipitate was centrifuged (13,000 g) for 15 min and washed in 70% ethanol. The precipitate was dried and resuspended in a solution containing 10 mmol/l TRIS·HCl and 1 mmol/l EDTA with 0.1% sodium dodecyl sulfate.

Gels were denatured (0.5 mol/l NaOH, 1.5 mol/l NaCl), neutralized [1.0 mol/l TRIS·HCl (pH 8.0), 1.5 mol/l NaCl] and transferred to nylon membranes in 20× SSC buffer (3 mol/l NaCl and 300 mmol/l sodium citrate, pH 7.0) overnight. Following fixation of DNA to membrane (80°C for 1 h), hybridization of the DIG-labeled oligonucleotide to target DNA was performed at 63°C for 2 h. Chemiluminescent detection was performed using Luminen PPD (Boehringer Mannheim) as substrate for the anti-DIG-alkaline phosphatase conjugate to examine specific hybridization following exposure to X-ray film.

Prolactin receptor primers (PR1 and oligonucleotide probe) used for PCR and Southern hybridization correspond to the extracellular domain of the rat ovary PRL-R gene sequence (7). Primer PR2 corresponds to the intracellular domain.

PR1-5′ GAC AAG GAA ACA TTC ACC TGC TGG TG 3′
PR2-5′ GG AAC TGG TGG AAA GAT GCA GGT CAT C 3′

The oligonucleotide sequence for Southern analysis is:

5′ TTC GCA TCC ACT TTA TGT GGA TGT G 3′

The predicted length of the RT-PCR products generated with PR1 and PR2 is 698 bp, and this is common to both forms (short and long) of the PRL-R (13).

Results

Expression of the PRL-R mRNA was observed in several fetal and neonatal tissues. The tissues evaluated in the present study included brain, liver, thymus, spleen, lung, kidney, stomach, skin, bladder, adrenal gland and pituitary gland. Three to six animals were evaluated, with similar results for all tissues at each age. The RNA isolation method employed extracts of total RNA, excluding genomic DNA, and DNA was not apparent in negative controls performed to detect contamination. The sex of each animal was recorded during gestation on days 18 and 19 and on day 1 of postnatal life. No obvious sex differences in PRL-R expression were apparent in fetal mice (data not shown). Tissues represent individual animals unless noted otherwise. Southern analysis of PRL-R mRNA agreed with the presence of detectable RT-PCR bands in every sample that was tested. Forty-five cycles of amplification were performed before a tissue was considered negative for PRL-R mRNA. In addition, having negative samples following that many cycles provides additional confidence as to specificity of the PCR and lack of cross-contamination during dissection.

Whole fetuses from day 10 of gestation were negative for PRL-R mRNA when all remnants of the placenta were removed (Fig. 1). Other samples extracted and amplified simultaneously generated positive PRL-R cDNA. Lane 4 depicts a positive signal for PRL-R and represents a fetus that was apparently contaminated with placental tissue. Day 12 and 13 whole fetuses (N = 4 each) were also negative for PRL-R mRNA (data
Development of PRL receptor expression

In embryos from days 6–9 mixed results were noted, presumably due to difficulty in removing the placenta (data not shown). Bladder and skin tissues from postnatal day 2 and the negative control (no template; lane 12) were consistently negative for PRL-R expression. However, stomach tissue from this same group generated a weak but positive signal for the PRL-R.

Liver tissues expressed the PRL-R message as early as day 14 of fetal life, while day 12 and 13 livers were negative (Fig. 2). These observations were also corroborated using Southern hybridization. Livers from fetuses at every age tested (fetal days 14, 15, 16, 17, 18 and 19, and postnatal day 1; N = 2/age shown), starting from day 14, generated positive signals for PRL-R mRNA.

On days 12 and 13 of gestation, PRL-R mRNA was not detected in fetal cranium tissues (Fig. 2). Craniums from fetal day 14 and whole brains from all ages examined, i.e. days 14, 15, 16, 17, 18 and 19 of gestation and postnatal day 1 (N = 2/age shown), revealed positive signals for the PRL-R.

Paired kidneys were evaluated for PRL-R message expression and found to be positive as early as day 15 of gestation (band represents a pool of three animals: Fig. 2). Kidney tissues from all ages falling between fetal day 15 and postnatal day 1 (days 16–19) generated positive RT-PCR signals. Similarly, total RNA extracted from fetal lung tissue produced positive PRL-R signals as early as day 15 and for all ages evaluated (fetal day 15 through to postnatal day 1: Fig. 3).

In the thymus gland, the mRNA for PRL-R was expressed as early as day 15 of fetal life (Fig. 3). Weak but positive PCR and Southern analysis signals were generated from thymic tissue. In addition to the experiment shown in Fig. 3, three additional samples from each age (fetal days 18 and 19 and postnatal day 1) were assayed and they verified the presence of PRL-R mRNA (data not shown).

Splenic tissue, which represents a secondary lymphoid organ, was not collected prior to day 18. Although the bands generated via ethidium bromide were weak, Southern analysis clearly indicated positive signals for PRL-R in spleens on fetal days 18 and 19 and postnatal day 1 (Fig. 3). Adrenal gland tissues produced intense signals for PRL-R mRNA at all ages tested (Fig. 3). Each age (fetal days 18 and 19 and postnatal day 1) evaluated consisted of a pool of tissues collected from five animals.

Messenger RNA from the pituitary glands was evaluated for receptor expression using pools of five pituitaries at each age examined. Pituitaries from day 18 and 19 fetuses and from 1-day-old pups revealed robust signals for PRL-R (Fig. 3). The intensity of the PCR bands was confirmed in the Southern analysis (Fig. 3).

Discussion

This study represents the first developmental survey of PRL-R mRNA expression in the mouse and clearly shows that this receptor mRNA is widely expressed in different organ systems prior to birth. While several reports
Fig. 2. Liver, brain and kidney tissue PRL-R expression in fetal (days 12–18) and neonatal mice (day 1). Lane numbers are noted above the gel, and animal age and specific tissues are stated below the gel. (Upper panel) Ethidium bromide-stained gel of RT-PCR products. Lane 16 represents DNA marker (m). (Lower panel) Southern hybridization of gel in upper panel.

Fig. 3. Expression of PRL-R in tissues collected from fetal (days 15–19) and neonatal mice (day 1). The band generated from day 15 thymus gland represents a pool of three animals. Bands generated from adrenal and pituitary glands represent pools of five animals per age (upper panel). Lane numbers are noted above the gel, and animal age and specific tissues are stated below the gel. (Lower panel) Southern hybridization of ethidium bromide-stained gel in upper panel.
provided evidence for wide distribution of PRL-R messages in late fetal development (during the immediate prenatal period), we have examined expression of the PRL-R gene starting at fetal day 10. This allowed the times when this gene is first expressed to be determined. The primers utilized in this study were chosen to amplify multiple forms of the receptor mRNA in an effort to identify presence or absence of PRL-R gene expression. Correspondingly, it has been shown recently that the distribution of the long and short forms of the receptor in fetal rat tissues was identical (14). The entire fetus appears to be devoid of PRL-R mRNA prior to day 14 of gestation. This developmental onset of PRL-R gene expression may coincide with, or slightly precede, the onset of PRL secretion during development. Prolactin gene expression was first detected in the pituitaries of day 15.5 fetal mice (15). In addition, differences in PRL-R mRNA expression were not observed between male and female fetal tissues. Similarly, no differences in liver GH receptor mRNA expression (using northern blot analysis) have been noted between sexes in neonatal or adult rats (16).

The onset of PRL-R expression in mice has not been addressed to this extent previously. The current study documents expression of PRL-R mRNA in several organ systems during fetal life. Other investigators (17) have used northern blotting techniques to identify receptor expression in adult mouse tissues, and our results obtained in 1-day-old mice and adults (data not shown) generally agree with their observations. However, Buck and co-workers (17) did not observe PRL-R message expression in thymus, spleen and lung tissues, which may reflect the low abundance of this mRNA and the sensitivity of RT-PCR versus northern analysis.

Because lymphoid cells originate in the fetal liver and migrate to lymphoid organs later in development (18-19), the onset of PRL-R gene expression in day 14 fetal liver tissue is consistent with the hypothesis that PRL plays a role in the developing immune system. The thymus is colonized in major waves during fetal development (20); one around day 14 of gestation and another shortly before birth. These developmental colonization timepoints correspond to the appearance of PRL-R mRNA expression in our studies: first in the fetal liver at day 14, followed by the fetal thymus at day 15. The spleen is a secondary lymphoid organ that is populated by lymphocytes towards the end of gestation and during the first week following birth (21). Presence of messenger RNA for PRL-R in splenic tissue is consistent with results showing PRL-R on the cell surface of splenocytes (22, 23). Early expression of this receptor in lymphoid tissues (thymus and spleen) has also been evidenced in 5-6-month-old (mid-gestation) fetal bovines (24).

The presence of PRL-R mRNA in mouse liver tissues throughout the latter half of gestation is concordant with recent studies in the fetal rat (14). Growth hormone induces liver PRL-R in the mouse (25) and PRL-R mRNA is up-regulated in rat liver following treatment with GH (26). The levels of plasma GH continually increase until birth in rats (27, 28), thus the actions of GH may partially explain the apparent abundance of fetal liver PRL-R message observed at the older ages in this study.

This study is the first to show the presence of PRL-R mRNA in the pituitary glands of fetal and 1-day-old mice. These findings suggests that at these stages of development PRL may influence its own secretion directly at the pituitary level and/or modulate the secretion of other pituitary-derived factors, i.e. LH, GH or PRL-releasing factor, prior to birth. Prolactin is known to feedback directly on dopaminergic neurons in the hypothalamus, to influence its own secretion and to possibly regulate GnRH and CRH neurons (29, 30). The expression of PRL-R in the pituitary glands of adult rats was detected using both RT-PCR (31) and in situ hybridization (32).

Expression of PRL-R message was noted in brain tissue of mice (strain C57B1/6 × DBA/2) at day 15 of gestation by Zhang and co-workers (12). In the present study, whole heads were positive at day 14, but contribution of tissues other than brain to this signal cannot be ruled out. Prolactin binding studies in rodents suggest the presence of the PRL-R in many areas of the brain (33). Expression of PRL-R in the hypothalamus suggests involvement of PRL in the regulation of adenohypophysial function and other brain activity (31, 34). We are currently evaluating PRL-R mRNA expression in discrete fetal brain structures.

The presence of mRNA for PRL-R in kidney tissues is not surprising because PRL is thought to play a role in water and salt balance and to effect uptake processes in the mammalian kidney (4, 35). However, the appearance of this message on fetal day 15 suggests a possible role for PRL in the development of this system. Renal PRL-R is regulated in part by thyroid status, with hypothyroidism reducing and hyperthyroidism increasing PRL-R (36). Concentrations of TSH rise during gestation and the first week of life (37, 38). In our qualitative study, the abundance of mRNA for PRL-R in older fetuses may reflect the continual rise in plasma TSH and subsequently the thyroxine levels.

Prolactin has been shown to modulate fetal adrenocortical steroidogenesis in the monkey (39), effect catecholamine release from rat medullary cells and stimulate output of corticosteroids by inhibiting formation of steroid metabolites (40). Hence, the identification of PRL-R mRNA in fetal adrenal tissues suggests that this modulation may begin as early as day 18 in mice. In addition, the early expression of PRL-R mRNA in the lung (day 15) is interesting because it has been suggested that PRL stimulates lung development via regulation of surfactant synthesis (41) and stimulation of adrenal corticosteroid production (42). Furthermore, serum PRL concentrations increase before secretion of surfactant and low levels of PRL are associated with the
occurrence of respiratory distress syndrome in premature infants (43). The presence of PRL-R mRNA in fetal lung tissues is consistent with a role for this hormone in the development of this organ.

Stomach tissue isolated from 2-day-old mice exhibited weak but positive signals for PRL-R mRNA via RT-PCR. Prolactin modulates the function of the gastrointestinal tract (44), of which the most prominent action is stimulation of ion/saccharide/amino acid transport and islet cell insulin secretion (45, 46). The PRL-R and receptor mRNA have been visualized in rabbit stomach (47) and rat digestive tissues (48).

The current study clearly shows that the PRL-R message is widely distributed in most organ systems prior to birth. The early presence of PRL-R mRNA in fetal life suggests a role for PRL in both the structural and functional organization of target organ and cells. Interference of plasma PRL concentrations during the neonatal period has been shown to alter subsequent expression of T and B lymphocyte differentiation antigens in thymus and spleen cell populations of adult mice (49). The opposite is also true; disturbance of the immune system early in development (athymic nude mice; neonatally thymectomized mice) has been shown to alter greatly the functioning of the adult neuroendocrine system (50). Therefore, the onset of PRL-R message expression in various organ systems in mice suggests that PRL is among factors necessary to coordinate developmental activities.

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