Ontogenesis of the Expression of Prolactin Receptor Messenger Ribonucleic Acid During Late Embryogenesis in Turkeys and Chickens

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ABSTRACT Changes in circulating levels of prolactin (PRL) and tissue content of PRL receptor (PRLR) messenger RNA (mRNA) in the liver, pancreas, kidney, and gonads (testis/ovary) were measured in turkey and chicken embryos from embryonic day (ED) 21 or ED15, respectively, to 1 d after hatch by real-time PCR. There were no differences between the sexes in chickens or turkeys. Both species had very similar patterns of PRL release and expression of PRLR mRNA, and no major differences were observed between turkey or chicken embryos. Plasma levels of PRL increased from low levels during the last week of embryonic development and were at significantly higher levels (about 4-fold) by 1 d after hatch. Similarly, in all tissues the content of PRLR mRNA was minimal at the outset and increased to reach maxima about the time of hatch. In both species, the highest levels of transcript were observed in the kidney followed by the gonad, liver, and pancreas. The tissue content of PRLR was correlated (0.6 to 0.8 dependent on the tissue) to circulating levels of PRL, which suggested that PRL may be associated with an increase in its receptor number around the time of hatch. Because levels of PRL and tissue content of PRLR mRNA increased around the time of hatch, this suggests that these tissues may be targets for PRL and may be involved in the physiologic changes occurring in embryos around the time of hatching.

Key words: prolactin receptor, liver, pancreas, kidney, gonad

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INTRODUCTION

Prolactin (**PRL**) is a polypeptide hormone that is produced and secreted mainly by the lactotroph cells of the adenohypophysis. In vertebrates, PRL is thought to be involved in modulating an extraordinary diversity of biological processes, and greater than 300 separate actions have been assigned to it (Bole-Feysot et al., 1998). These roles have been broadly categorized as those that affect 1) water and electrolyte metabolism, 2) growth and development, 3) endocrine systems and metabolism, 4) brain and behavior, 5) reproduction, and 6) immunoregulation and protection. The most studied role in avian species has involved the actions of PRL during the egg incubation phase of broody behavior. However, the physiological roles of PRL during avian embryogenesis have not been extensively studied.

Lactotroph cells differentiate late in embryonic development, and immunoreactive PRL is detectable by embryonic day (**ED**) 15 to 17 in chicks (Kansaku et al., 1994; Sasaki et al., 2003; Fu et al., 2004; Zheng et al., 2005) and ED23 in turkeys (Bédécarrats et al., 1999) although PRL transcripts are detectable at earlier stages (Kansaku et al., 1994; Bédécarrats et al., 1999). Subsequent to lactotroph differentiation, PRL is released into the circulation, and during the last week of embryogenesis, levels of PRL progressively increase and reach maxima at about the time of hatch (Ishida et al., 1991; Bédécarrats et al., 1999). Moreover, the time of hatch is associated with a significant shift in the circulating form of PRL isoform (Bédécarrats et al., 1999). The temporal relationship between increased levels of PRL and the hatch of the embryo suggests that PRL may participate in various physiological systems to adapt the embryo to ex ovo life.

The biological actions of PRL are mediated by binding to its receptor (**PRLR**), and receptors have been detected in the digestive, osmoregulatory, immune, reproductive, and neural system in adult chickens and turkeys (Tanaka et al., 1992; Zhou et al., 1996; Ohkubo et al., 1998b; Mao et al., 1999). The broad distribution of the receptor is consistent with the multiplicity of the physiological processes that PRL is known to be involved in. Moreover, levels of PRLR have been observed to vary according to the physiological status of the hen at least in some tissues such as the adenohypophysis and hypothalamus (Zhou et al., 1996; Ohkubo et al., 1998a). The single study measuring PRLR mRNA during embryogenesis compared levels of receptor in intestinal and kidney tissue between

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ED17 and ED19, and d 2 and 28 posthatch (Yamamoto et al., 2003). Yamamoto et al. reported that there was no change in levels of PRLR mRNA during that interval. However, because the levels of PRLR are expected to be quite low, the ribonuclease protection assay might have had insufficient sensitivity to detect changes in tissue content. Accordingly, in this study a real-time PCR (**Q-PCR**) assay was designed and validated to assess levels of PRLR mRNA in individual tissues from turkeys and chickens during the last week of embryogenesis.

MATERIALS AND METHODS

Tissue Sampling

Eggs from commercial strains of white Medium Hybrid turkeys (Hybrid Turkeys Inc., Kitchener, Ontario, Canada) and white Hyline W98 Leghorn chickens (HyLine International, West Des Moines, IA) were incubated under standard commercial conditions at 37.5°C, 85 to 86% humidity for 25 and 18 d, respectively. They were then transferred to a hatcher at 36.9°C, 90 to 92% humidity. In addition to the day of hatch and 1-d posthatch, turkey and chicken embryos (n = 10) were collected at d 21, 23, 25 and 27 or d 15, 17, and 19 of incubation, respectively, and killed by cervical dislocation. Tissue samples from the liver, kidney, pancreas, and left gonad were collected immediately after execution, snap-frozen in liquid N, and stored at –80°C until assayed.

Extraction of Total RNA

Total RNA from tissues was extracted using TRIzol reagent (Gibco BRL Products, Life Technologies, Burlington, Ontario, Canada), according to the manufacturer's protocol. The recovered RNA was dissolved in diethyl pyrocarbonate-treated water to a final concentration of 1 μg/μL. Integrity of the RNA was electrophoretically verified in 1.2% formaldehyde agarose gels stained with ethidium bromide.

Reverse Transcription and Real-Time Quantification of tPRLR mRNA

The reverse transcription, modified from Bédécarrats et al. (1999), was carried out in a final volume of 10 μL containing 2.0 μg of total RNA and 40 pmol of antisense turkey (t)PRLR-specific primer (Invitrogen Canada Inc., Burlington, Canada).

An external standard of 332 bp (Zhou et al., 1996) was used in Q-PCR quantification to semiquantify levels of PRLR cDNA. This sequence contained a 39-bp deletion of the amplicon of 371 bp amplified from tPRLR mRNA to allow for determination of coamplification efficiency by melting point analysis or gel electrophoresis or both. The external standard was amplified by PCR at 93°C for 45 s, 56°C for 2 min, and 72°C for 90 s for 35 cycles (Zhou et al., 1996), purified from 1% agarose gel before use, and stepwise diluted 10-fold to cover the range of turkey and chicken PRLR mRNA levels in tissues amplified by Q-PCR.

Conditions for Q-PCR using the LightCycler (Roche Applied Science, Laval, Quebec, Canada) were optimized for 1) $MgCl^{2+}$ concentration, 2) primer concentration, 3) annealing temperature, and 4) concentration of the cDNA. The optimized protocol for Q-PCR was as follows. An aliquot of 2 μL of a single-strand cDNA mixture was amplified by LightCycler PCR in a final volume of 20 μL containing 1× LightCycler-FastStart DNA Master SYBR Green I (Roche Applied Science), 4 m*M* $MgCl_2$, and 10 pmol of forward and reverse primers (Invitrogen Canada Inc.). For the Q-PCR, a sense and an antisense tPRLR-specific primer were as designed by Zhou et al. (1996: 5′AGGAAACATTTACCTGTTGGT and 5′AAGCCATCCAGATCTGACATC). The cDNA was diluted according to the stages of the embryo development. Samples for Q-PCR were denatured at 95°C for 10 min followed by an amplification program (95°C for 10 s, 55°C for 5 s, 72°C for 16 s) for 40 cycles. Specificity of the amplifications was determined by melting curve analysis (63°C for 15 s to 95°C in 0.1°C/sec increments) and by gel electrophoresis.

To estimate the concentration (**Co**) of PRLR mRNA in the turkey and chicken cDNA samples, the following formula was used: CP = S Log (Co) + I, where CP is the crossing point value from the turkey and chicken samples (i.e., the point at which the fluorescence rises appreciably above the background fluorescence). The standard slope (**S**) represents the overall reaction efficiency and the intercept (**I**) of the standard represents the crossing point value on the y-axis for which the concentration equals zero. The linear regression line was obtained by plotting the crossing cycle number versus the logarithm of the concentration for each unknown sample.

PRL Radioimmunoassay

Blood samples were collected into heparinized tubes from the chorio-allantoic blood in turkey and chicken embryos from d 21 until 25 and from d 15 until 19 of incubation, respectively, and thereafter by intracardiac puncture. After centrifugation, plasma samples were stored at –80°C until assayed. Levels of PRL were measured in triplicate in aliquots of plasma (100 μL: turkey or 200 μL: chicken) by radioimmunoassay as described by Guémené et al. (1994). Concentration of PRL in the samples was estimated using GraphPad Prism (version 4.03, GraphPad Software, San Diego, CA). The intra- and interassay coefficients of variation were 5.8 and 0.5%, respectively.

Statistical Analysis

Transcript levels of PRLR mRNA in tissues (**AU** = arbitrary units) and circulating levels of PRL (ng/mL) in chickens or turkeys were analyzed following log transformation using ANOVA ($P < 0.05$) followed by the Duncan multiple range test if appropriate. Correlation coefficients

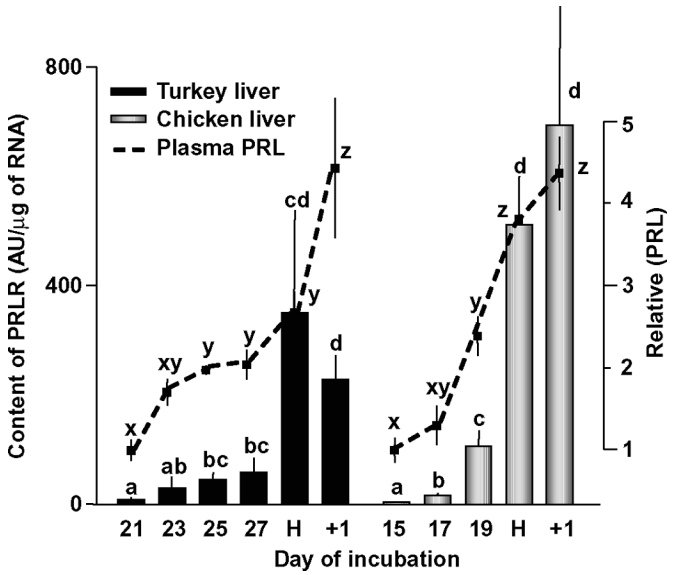

Figure 1. Changes in the expression of prolactin receptor (PRLR) mRNA [arbitrary units (AU): average ± SE] in the liver and relative levels of prolactin (PRL; dashed line) in the circulation during the last week of embryogenesis in turkeys and chickens. Developmental stages in each species with common letters (PRLR: abcd or PRL: xyz) do not significantly differ. Relative levels of PRL are based on observed values on ED21 or ED15 in chickens and turkeys, respectively. H = day of hatch.

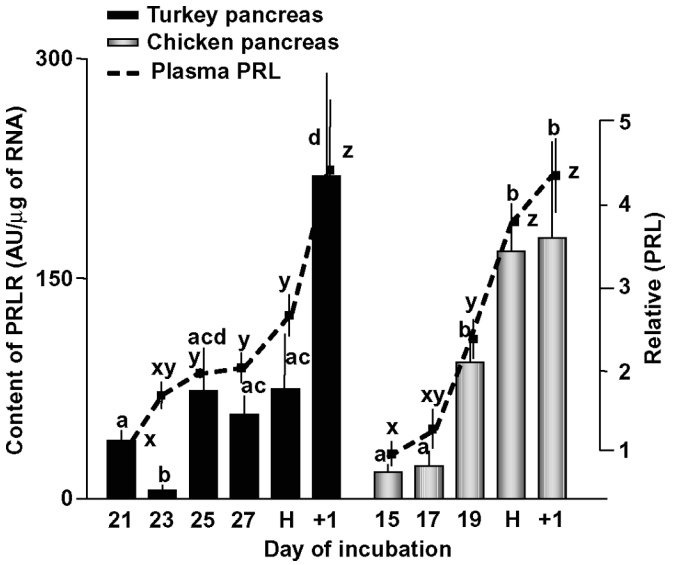

Figure 2. Changes in the expression of prolactin receptor (PRLR) mRNA [arbitrary units (AU): average ± SE] in the pancreas and relative levels of prolactin (PRL; dashed line) in the circulation during the last week of embryogenesis in turkeys and chickens. Developmental stages in each species with common letters (PRLR: abcd or PRL: xyz) do not significantly differ. Relative levels of PRL are based on observed values on ED21 or ED15 in chickens and turkeys, respectively. H = day of hatch.

were determined using linear regression analysis. Each data point represents the average ± SEM.

RESULTS

Levels of PRLR mRNA and Plasma PRL in Turkeys

Circulating levels of PRL gradually increased from ED21 (16 + 2 ng/mL) to reach a maxima of about 4-fold higher by 1 d after hatch (72 ± 14 ng/mL) in turkey embryos (Figures 1 to 4). Similarly, steady state levels of PRLR mRNA transcript in all tissues gradually increased from low levels before hatch, then subsequently increased significantly. By 1 d after hatch, tissue content of PRLR mRNA (AU/μg of total RNA) was as follows: kidney > gonad > liver > pancreas. The correlation between the plasma concentration of PRL and PRLR mRNA content in liver, kidney, pancreas, or gonad was 0.56, 0.74, 0.66, and 0.70, respectively.

Levels of PRLR mRNA and Plasma PRL in Chickens

Similar to the turkey, plasma levels of PRL were at low levels before hatch (ED15: 5 ± 1 ng/mL) then gradually increased during the late stages of embryogenesis and by 1 d after hatch were 4-fold higher (20 ± 8 ng/mL). The content of PRLR mRNA in the liver, pancreas, kidney, and gonad also gradually increased during late embryogenesis (Figures 1 to 4). Subsequently, it increased significantly and by 1 d after hatch, the tissue content of PRLR mRNA (AU/μg of total RNA) was as follows: kidney > gonad > liver > pancreas. The correlation between the plasma content of PRL and the tissue content of PRLR mRNA in the liver, kidney, pancreas, and gonad was 0.73, 0.75, 0.56 and 0.78, respectively. In all tissues, the levels of PRLR mRNA were greater in the chicken than in the turkey on the day of hatch, but this difference was not significant.

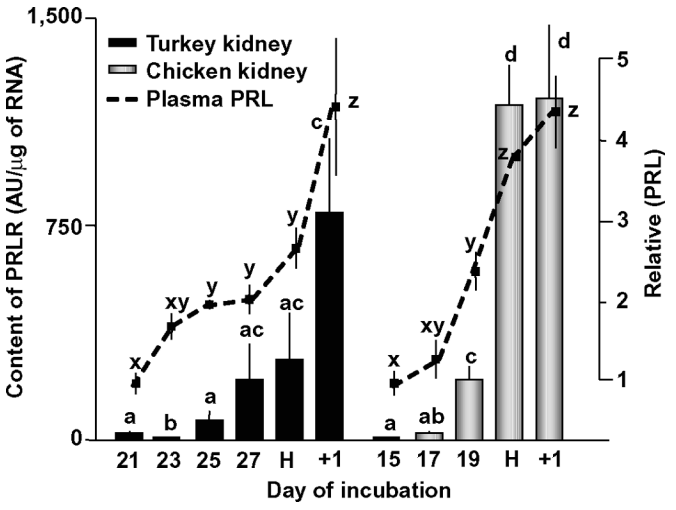

Figure 3. Changes in the expression of prolactin receptor (PRLR) mRNA [arbitrary units (AU): average ± SE] in the kidney and relative levels of prolactin (PRL; dashed line) in the circulation during the last week of embryogenesis in turkeys and chickens. Developmental stages in each species with common letters (PRLR: abcd or PRL: xyz) do not significantly differ. Relative levels of PRL are based on observed values on ED21 or ED15 in chickens and turkeys, respectively. H = day of hatch.

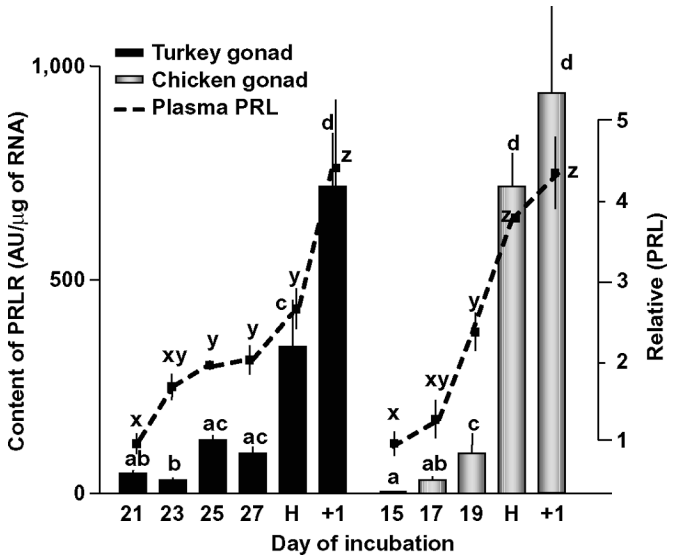

Figure 4. Changes in the expression of prolactin receptor (PRLR) mRNA [arbitrary units (AU): average ± SE] in the gonad and relative levels of prolactin (PRL; dashed line) in the circulation during the last week of embryogenesis in turkeys and chickens. Developmental stages in each species with common letters (PRLR: abcd or PRL: xyz) do not significantly differ. Relative levels of PRL are based on observed values on ED21 or ED15 in chickens and turkeys, respectively. H = day of hatch.

DISCUSSION

Real-time PCR is a sensitive method used by many laboratories to quantify levels of specific transcripts in tissues. In particular, Q-PCR is advantageous over Northern blotting, ribonuclease protection assay or semiquantitative PCR for detecting and quantifying low abundance transcripts such as PRLR or mRNA from which only limited amounts of tissue are available. Using Q-PCR, levels of PRLR mRNA were shown to increase during the last week of embryogenesis in chickens and turkeys and reach maxima about the time of hatch (Figures 1 to 4). In both species, the highest levels of PRLR were observed in the kidney, followed by the gonad, liver, and pancreas in descending order. Neither the pattern of change of tissue content of PRLR nor the absolute level of PRLR mRNA as indicated by amplification of the extracellular domain was different between the chicken and turkey. However, the latter does not preclude the possibility of differences in the levels of transcript variants as has been observed in the testes of chickens (Mao et al., 1999).

The increases in circulating level of PRL about the time of hatch are consistent with the differentiation of lactotrophs in the pituitary. In the chicken, immunoreactive PRL can be detected in the cephalic lobe of the adenohypophysis by about ED15 to ED17 (Kansaku et al., 1994; Sasaki et al., 2003; Zheng et al., 2005) and ED23 in turkeys (Bédécarrats et al., 1999), although PRL mRNA can be detected earlier (Kansaku et al. 1994; Bédécarrats et al., 1999). Consistent with lactotroph differentiation, circulating levels of PRL increased by about 4-fold from ED21 or ED15 to 1 d posthatch in turkeys and chickens, respectively, consistent with previous studies (Ishida et al., 1991; Bédécarrats et al., 1999). During the same interval, tissue content of PRLR mRNA increased in the liver, pancreas, kidney, and gonad and was correlated (0.6 to 0.8 dependent on the tissue) to the plasma concentration of PRL. This suggested that increases in PRL during embryonic development may be involved in increasing levels of its receptor in target tissues as has been observed in certain reproductive states in the hypothalamus and adenohypophysis of adult chickens and turkeys (Zhou et al., 1996; Ohkubo et al., 1998a). In support of this, in vitro stimulation of turkey pituitary glands from ED24 embryos with VIP for 4 h resulted in about a 4-fold increase in PRL and a 3-fold increase in PRLR mRNA (Leclerc et al., 2007). Conversely, Yamamoto et al. (2003) noted no change in chicken kidney or intestinal content of PRLR on ED17 and ED19 or 2 and 28 d posthatch, and they suggested that PRLR mRNA was regulated independently of the circulating levels of PRL. However, Yamamoto et al. (2003) were not able to detect PRLR mRNA in ED19 liver (detectable by ED15 using Q-PCR), and thus it is possible that their assay system (ribonuclease protection assay) might have not had sufficient sensitivity. In addition, levels of PRLR in many tissues in adult hens have no obvious relationship to circulating levels of PRL (Zhou et al., 1996; Ohkubo et al., 1998a). Furthermore, at the time of hatch, levels of many hormonal and nonhormonal factors vary in concert with PRL; hence, the observed correlation between PRL and PRLR may be spurious. Definitive proof regarding the relationship of PRL to the regulation of its receptor during embryogenesis requires further studies.

Prolactin interacts with its receptors in a wide variety of target tissues to affect physiological processes, which have been broadly grouped into actions that affect reproduction, growth and development, osmoregulation, behavior, and immunoregulation (Bole-Feysot et al., 1998). The large increases in levels of receptors in the kidney, gonad, liver, and pancreas about the time of hatch suggest that PRL may be acting in these target tissues. However, very few studies have examined the effects of PRL on avian embryonic development. For example, the increase in PRLR mRNA at hatch in pancreatic tissue may indicate that PRL is acting to stimulate the endocrine pancreas because PRL has been shown to be a mitogen in chick islet cells (Maiti et al., 1982). The latter is consistent with the observations that PRL stimulates β-cell hyperplasia and insulin secretion and downregulates proapoptotic genes in mammals (Carlsson et al., 1997; Bordin et al., 2004), whereas in PRLR knockout mice, β-cell hypoplasia occurs (Freemark et al., 2002). Similarly, the concurrent increase in circulating PRL and hepatic PRLR mRNA suggests that PRL may be active in stimulating this organ. In part, this effect may be moderated through thyroid hormones because injection of recombinant chicken PRL into ED19 chickens modulates hepatic metabolism of T3 and T4 (Kühn et al., 1996). The osmoregulatory ability of the embryonic kidney also changes around the time of hatch, and glomerular filtration rate significantly increases and resorption of allantoic water reserves occurs

in preparation for ex ovo life (Davis et al., 1988; Murphy et al., 1991). The injection of PRL into the chorio-allantoic membrane of chicken embryos has been shown to increase the Na^+-K^+-ATPase activity and reduce the concentration of Na^+ and Cl^- in allantoic fluid (Doneen and Smith, 1982) without altering the allantoic fluid volume (Murphy et al., 1986). Thus, the increase in circulating levels of PRL and kidney content of PRLR mRNA may be associated with the stimulation of NaCl resorption during the perihatch period. Evidence also suggests that PRL may affect steroid production by the gonads. During late embryogenesis, plasma levels of the sex steroids increase, which coincides with the observed increases in circulating PRL and PRLR mRNA in the gonad. In the chick embryo, gonadotrophin immunoreactive cells are the first to appear in the adenohypophysis at about ED7 (Sasaki et al., 2003), and steroid biosynthesis occurs at about the same time. At about the time of hatch, however, there is increased release of LH and FSH, and the gonads significantly increase the secretion of steroids (Gonzalez et al., 1987; Mendez-Herrera et al., 1998; Peralta et al., 2004). In many species, PRL is involved in the upregulation of gonadotrophin receptors (Bjurulf et al., 1994; Porter et al., 2000; You et al., 2000); hence, increased levels of circulating PRL and gonadal PRLR around the time of hatch suggest that PRL is involved in maintaining steroid production. The increased gonadal steroids are in turn involved in many aspects of pre- and posthatch physiology.

Many direct and indirect effects of PRL on target tissues are likely to occur during late embryogenesis in chickens and turkeys. The identification of large increases in PRLR mRNA in the kidney, gonad, liver, and pancreas at the time of hatch suggests that these tissues may be targets for PRL. To date, few physiological studies have investigated the direct effects of PRL on these tissues during embryogenesis. However, the availability of recombinant chicken (Hanks et al., 1989; Ohkubo et al., 1993) and turkey (Karatzas et al., 1993) PRL may allow for assessment of the response of these tissues to stimulation.

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