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Pituitary progesterone receptor expression and plasma gonadotrophin concentrations in the reproductively dysfunctional mutant restricted ovulator chicken

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Abstract

Female mutant restricted ovulator (RO) chickens of the White Leghorn strain carry a naturally occurring single nucleotide mutation in the very low density lipoprotein receptor (VLDLR) gene. Due to this mutation, RO hens fail to express a functional VLDLR protein on the oocyte membrane, which results in an impaired uptake of circulating yolk precursor macromolecules. Mutant RO hens subsequently develop hyperlipidemia and generally fail to lay eggs due to follicular atresia. Since RO hens also reportedly have three-fold higher basal plasma estrogen concentrations, combined with four-fold lower levels of circulating progesterone as compared to wild-type (WT) hens, we hypothesized that RO hens would have an increased abundance of pituitary progesterone receptor (PR) mRNA and PR isoforms A and B as well as alterations in circulating gonadotrophin levels. Quantitative PCR assays revealed significantly greater ($P \leq 0.05$) pituitary PR mRNA abundance in RO hens as compared to WT hens. Similarly, pituitary PR isoforms A and B quantities were significantly greater ($P \leq 0.05$) in the RO hens compared to WT hens. In addition, mutant RO hens had significantly greater plasma concentrations of luteinizing hormone, follicle stimulating hormone, estrone, and estradiol,

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but lower circulating progesterone levels. Collectively, elevated circulating estrogen and/or decreased progesterone levels may have contributed to the upregulation of PR mRNA and PR isoforms A and B in the RO hen pituitary gland. Lastly, in order to gain a more complete understanding of why RO hens are reproductively dysfunctional, a model is proposed that links humoral and ovarian factors to observed and putative changes in the hypothalamic-pituitary axis.

Keywords: Chicken; Gonadotrophin; Progesterone receptor; Restricted ovulator

1. Introduction

Mutant females of the restricted ovulator (RO) strain of White Leghorn chickens have a naturally occurring point mutation of the very low density lipoprotein receptor (VLDLR) gene [1], transmitted by heterozygous roosters to one-half of their daughters [2]. A single nucleotide substitution (G → C) in the VLDLR gene on the Z chromosome converts a cysteine residue into serine, resulting in an unpaired cysteine residue and the lack of expression of a functional VLDLR protein on the oocyte surface [1]. Due to this mutation, uptake of the major yolk precursor macromolecules, namely very low density lipoprotein (VLDL) and vitellogenin (VTG) is impaired. Consequently, VLDL and VTG accumulate in the blood, resulting in endogenous hypertriglyceridemia, hypercholesterolemia, and premature atherosclerosis [3–7]. Initial studies with mutant RO hens centered on their use as a model for studying atherosclerosis [5–7] while subsequent work led to advances in our understanding of receptor-mediated growth of chicken oocytes and to the elucidation of regulatory mechanisms in avian lipid and lipoprotein metabolism [1,8–13]. Since mutant RO hens lack the cyclic calcium metabolism associated with egg formation [14] and are hyperestrogenemic and hypoprogesteronemic [15] they may also serve as valuable models for investigations of bone metabolism and reproductive dysfunction, respectively.

The RO hen ovary contains numerous large white follicles, small yellow follicles, and vitellogenic follicles, but most never reach full maturity (e.g., move from F_4 to F_1 in the follicular hierarchy [16]). Some of the follicles are atretic or become abnormally shaped or colored [3,4,11] and may contain blood or blood decomposition products. As a result, the RO hen ovary lacks the typical follicular hierarchy seen in wild-type (WT) chickens [16].

In the chicken, progesterone is primarily secreted by the largest (F_1) preovulatory follicle [17] that is absent in RO hens. In WT hens, between 24 and 12 h before ovulation, the largest preovulatory follicle secretes massive amounts of progesterone [16], stimulating the release of luteinizing hormone (LH) from the anterior pituitary [18] and inducing ovulation [19]. Due to the presence of numerous large white, small yellow, and small vitellogenic (e.g., F_7–F_5) follicles and lack of larger (e.g., F_4–F_1) preovulatory follicles, mutant RO hens have greatly increased plasma estradiol and estrone concentrations and markedly decreased plasma progesterone concentrations [15].

Progesterone exerts its genomic effects by binding to the progesterone receptor (PR) located in the nuclei of several target organs. PR isoforms A and B are the two major PR variants expressed in the chicken [20]. Progesterone receptor isoforms A (78–80 kDa) and B (97 kDa) arise from a single gene [21,22] by alternate initiation of translation from a single

mRNA transcript [21] or from multiple mRNA transcripts [23]. The presence of PR has been detected in the chicken pituitary gland and brain [24]. Moreover, specific progesterone-binding sites have been identified in the cell nuclei of the hen pituitary, suggesting that progesterone may be acting on the pituitary cells [25]. In addition, PR-immunoreactive nuclei were distributed uniformly throughout the cephalic and caudal lobes of the anterior pituitary gland in the ring dove [26]. However, PR mRNA abundance and existence of PR isoforms in the chicken pituitary gland have not been reported, while a functional role of PR in the chicken anterior pituitary gland remains to be determined. Studies on PR-null mutant mice indicate that PR is essential for generating gonadotrophin surges [27]. Therefore, we hypothesized that the abnormal follicular hierarchy, increased plasma estrogen concentrations, and decreased circulating plasma progesterone levels noted in mutant non-laying RO hens may lead to alterations in anterior pituitary gland PR gene and protein isoform expression and in plasma gonadotrophin concentrations.

2. Materials and methods

2.1. Reagents

Radioimmunoassay kits to quantify plasma estrone, estradiol, and progesterone concentrations were purchased from Diagnostic Systems Laboratories Inc. (Webster, TX). DNAse-I™ and the DNEasy™ and RNEasy™ kits used to isolate genomic DNA and RNA, respectively, were obtained from Qiagen Inc. (Valencia, CA). RNAlater™ reagent used for preservation of pituitary tissue for RNA extraction was purchased from Ambion (Austin, TX). Taq polymerase used for the polymerase chain-reaction (PCR) was purchased from New England Biolabs (Beverly, MA). Additional PCR and reverse transcription reagents (Platinum SYBR® Green qPCR Super Mix-UDG, RNAseOut) were obtained from Invitrogen Corporation (Carlsbad, CA). Powerscript reverse transcriptase and dNTP mix were purchased from Clontech (Palo Alto, CA). RIPA lysis buffer kit including protease inhibitor cocktail for protein extraction was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Reagents used for electrophoresis and Western blot analysis (Tris, Glycine, SDS, Protein Assay reagent, Laemmli sample buffer, polyacrylamide gels, polyvinyldifluoride membrane (PVDF) membrane, pre-stained and biotinylated protein markers) were obtained from Bio-Rad (Hercules, CA). Monoclonal antibodies against chicken progesterone receptor isoforms A and B (PR13 and PR22, respectively) were kindly donated by Dr. D. Toft (Department of Biochemistry and Molecular Biology, Mayo Graduate School, Rochester, MN). Monoclonal anti-chicken β-actin was purchased from Sigma Chemical Co (St. Louis, MO; Cat# A-1978). Blocking solution (10× casein) and horse anti-mouse antibody conjugated to horseradish peroxidase were obtained from Vector Laboratories (Burlingame, CA). ECL™ Plus Detection Reagent was procured from Amersham Life Sciences (Piscataway, NJ).

2.2. Animals

Mutant RO and WT Leghorn hens, arising from the same hatch, were maintained at the Poultry Education and Research Center at the Pennsylvania State University (University

Park, PA). The chickens were maintained under a 16 h light:8 h dark photoperiod (lights on between 06:00 and 22:00) and were provided with water and feed for ad libitum consumption. Hens (45–60 weeks old) were euthanized by decapitation prior to the removal of pituitary glands (see below). Following euthanasia, the presence of a normal or abnormal ovarian follicular hierarchy also was determined in order to substantiate the PCR genotype data (see below). The protocol for this study was approved by the Pennsylvania State University Institutional Animal Care and Use Committee.

2.3. RIA for estrogens, progesterone, LH and follicle stimulating hormone (FSH)

Blood samples were collected from the brachial vein 8–9 h after the onset of photophase, a time in the WT ovulatory cycle when LH or FSH surges are not anticipated. Blood samples were centrifuged at 6000 × *g* for 10 min at 4 °C. Plasma and erythrocytes were isolated and stored at −80 °C until further analysis. Plasma from both RO and WT hens were delipidated by ultracentrifugation (100,000 × *g* at 4 °C) for 15 h. Plasma estradiol, estrone and progesterone concentrations were determined in the delipidated plasma using commercial RIA kits (Cat# DSL8700, DSL4400, and DSL 3400; Diagnostic Systems Laboratories) following manufacturer's protocol. Plasma FSH and LH concentrations were estimated in the delipidated plasma by homologous RIA [28,29].

2.4. Genotyping of chickens

A DNEasy™ kit was used to extract genomic DNA from chicken erythrocytes following the manufacturer's protocol. DNA quantity and quality was ascertained by measuring absorbance at 260 and 280 nm using a spectrophotometer. A PCR-based method was utilized to genotype hens as previously described [3] and the genotyping results were substantiated by gross ovarian morphology records and egg production data.

2.5. PR mRNA quantification by real-time quantitative PCR

The anterior pituitary gland was quickly removed and minced following decapitation of WT and RO hens and stored in RNAlater™ at −80 °C until further processing. Total RNA was extracted using RNEasy™ kit following manufacturer's protocol and the quality and quantity of total RNA extracted was ascertained by measuring absorbance at 260 and 280 nm using a spectrophotometer. Following DNAse-I treatment, first strand cDNA was synthesized by reverse transcribing 1 μg of total RNA using d(T)30A/G/CA/G/C/T primer, 2 U of Powerscript reverse transcriptase in 20 μl reactions. Both PR mRNA and β-actin mRNA were quantified using 2 μl of the reverse transcription reaction (equivalent of 100 ng single stranded cDNA) as a template in the real-time quantitative PCR procedure. An 83 bp PR cDNA product (GenBank Accession No. Y00092; nt1347–1429 of PR cDNA common to PR A and B isoforms [23]) was amplified using the following primers: Forward – GGAAGGGCAGCACAACTATT; Reverse – GACACGCTGGACAGTTCTTC. Similarly a 123 bp product of chicken β-actin corresponding to nucleotides 1026-1148 (GenBank Accession No. L08165) was amplified using the following primer set: Forward – CTGGCACCTAGCACAATGAA; Reverse – CTGCTTGCTGATCCACATCT. The real

time-quantitative PCR mixture consisted of 1X Platinum SYBR® Green qPCR Super Mix-UDG and 300 nM of forward and reverse primers. The reactions were carried out in the DNA engine Opticon II (MJ Research, Reno, NV) with the following thermocycle settings: 50 °C for 2 min, 95 °C for 2 min, followed by 35 cycles of 95 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s. At the end of amplification, a melting curve analysis was done by heating the PCR products 65–95 °C while holding for 15 s at 0.2 °C increments. Fluorescence was monitored at each temperature increments to confirm the presence of a single amplification product. Each sample from WT and RO hens was run in duplicate and average log-linear threshold (C_T) values for PR mRNA or β-actin mRNA in RO or WT hens was calculated ($n = 9$). For negative controls, reverse transcription reactions using 1 μg total RNA with no reverse transcriptase (No-RT control) were used as template in place of single stranded cDNA in the real-time quantitative PCR. The CT values during the exponential phase of the PCR for PR mRNA were subtracted from that of β-actin mRNA. Progesterone receptor mRNA quantity was expressed as a proportion of β-actin mRNA quantity following $2^{-\Delta\Delta C_T}$ method for converting log-linear C_T values to linear term [30].

2.6. Pituitary protein extraction

Pituitary glands from RO and WT hens were quickly removed from the cranium following decapitation, snap-frozen in liquid nitrogen, and stored at −80 °C until further processing. Each pituitary gland was homogenized in a glass tissue grinder (Kontes Glass Co., Vineland, NJ) with 150 μl RIPA Lysis buffer (10 mM Tris–HCl, 150 mM NaCl, pH 8.0, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitor cocktail (Santa Cruz Biotechnology), 1 mM phenylmethylsulfonyl fluoride and 1 mM sodium orthovanadate. The homogenate was then passed through a 22-gauge needle and shaken in a thermomixer (Eppendorf, Westbury, NY) at 250 rpm for 15 min at 4 °C. The lysate was centrifuged at 14,000 × g for 15 min at 4 °C and the supernatant was collected. Supernatant total protein concentration was estimated by a protein dye-binding assay [31] using a commercial kit (Cat#500-0201;Bio-Rad) and chicken ovalbumin as the standard. Aliquots of pituitary protein extract were stored at −80 °C until analyzed.

2.7. SDS-polyacrylamide gel electrophoresis

One-dimensional SDS-gel electrophoresis was performed on a minigel system from Bio-Rad (Mini PROTEAN 3 Cell™) according to Laemmli [32]. Protein samples (25 μg) were combined with Laemmli Sample Buffer containing β-mercaptoethanol (2:1) and boiled for 4 min prior to electrophoresis. Samples were run on 7.5% polyacrylamide gel (Bio-Rad) under denaturing conditions at 200 V for 30–40 min. After electrophoresis, proteins were electrotransferred to Immun-Blot™ PVDF membranes (0.20 μm; Bio-Rad) at 100 V for 75 min with the Mini Trans-Blot™ system (Bio-Rad). The membrane was incubated overnight in blocking buffer (1× casein solution; Vector Laboratories) at 4 °C. Following blocking, the membrane was incubated in mouse anti-chicken PRR antibodies (PR13 and PR22, 1:1000 in blocking solution) for 1 h at room temperature with gentle agitation. Mouse monoclonal antibodies PR13 and PR22 have been previously validated to

detect PR isoforms A and B, respectively [33]. The membrane was washed once for 15 min in TBS-T (10 mM Tris, 150 mM NaCl, 0.1% Tween-20) followed by three washes for 5 min each in TBS-T. Immunoreactive proteins were detected by incubation in horseradish peroxidase conjugated to horse anti-mouse IgG (1:250 in blocking solution) for 1 h at room temperature. The membrane was washed as previously described, and treated with ECL™ Plus Chemiluminescence Detection Reagent (Amersham Biosciences). Chemiluminescent signals were detected using the Storm 860 optical scanner (Amersham Biosciences) and the signals were analyzed using Image Quant™ software (Amersham Biosciences). A linear intensity range for PR isoforms signals was first established by using different quantities of pituitary protein. The chemiluminescence signal intensity of each band (PR isoform A, B, or β-actin) was calculated using a local average background correction. Subsequently, the immunoblot was re-probed using mouse anti-chicken β-actin antibody and the chemiluminescence signal determined to normalize the amounts of protein loaded on the gel.

2.8. Statistical analyses

Genotype differences in plasma estrone, estradiol, progesterone, LH, and FSH concentrations, as well as the relative proportion of pituitary PR protein quantity to β-actin quantity, were compared by one-way ANOVA using the general linear model (GLM) of the Statistical Analysis System (SAS Institute Inc., Cary, NC). Relative PR mRNA quantity to β-actin mRNA quantity was first converted from log-linear to linear and subjected to one-way ANOVA using the GLM of the SAS. Least square mean values between genotypes were separated using a least square means test and a probability level of $P \leq 0.05$ was considered statistically significant.

3. Results

3.1. Detection of VLDLR gene mutation and ovarian morphology

PCR-amplified genomic DNA from WT hens resulted in the presence of a 413-bp product with the primer pair specific for the WT gene whereas no band was observed in the samples from mutant RO hens (data not shown) because in all Aves, females are the heterogametic sex. Conversely, PCR-amplified genomic DNA from RO hens resulted in the presence of a 413-bp product with the primer pair specific for the mutant gene, whereas no band was observed in the WT samples (data not shown). These findings also were consistent with the egg production records maintained over several months (e.g., no mutant RO hen utilized in this study laid an egg). At the time of necropsy, WT hens were all found to have a normal ovarian follicular hierarchy with a complement of 4–7 large vitellogenic follicles, while the RO ovaries lacked the largest preovulatory follicles (e.g., F_4–F_1), contained numerous smaller vitellogenic follicles (F_5 and smaller), small yellow follicles, and large white follicles, and had a much larger mass of ovarian stroma as compared to WT hens. In addition, consistent with earlier reports [3,4,11], some of the hierarchial follicles in RO hen ovaries were atretic, discolored, and/or misshapen.

3.2. Plasma LH, FSH, estrogen and progesterone concentrations

Plasma LH (Fig. 1A), FSH (Fig. 1B), estradiol (Fig. 1C), and estrone (Fig. 1D) concentrations were markedly higher ($P \leq 0.05$) in RO hens as compared to WT hens, while the opposite was true for plasma progesterone levels (Fig. 1E). In fact, plasma progesterone

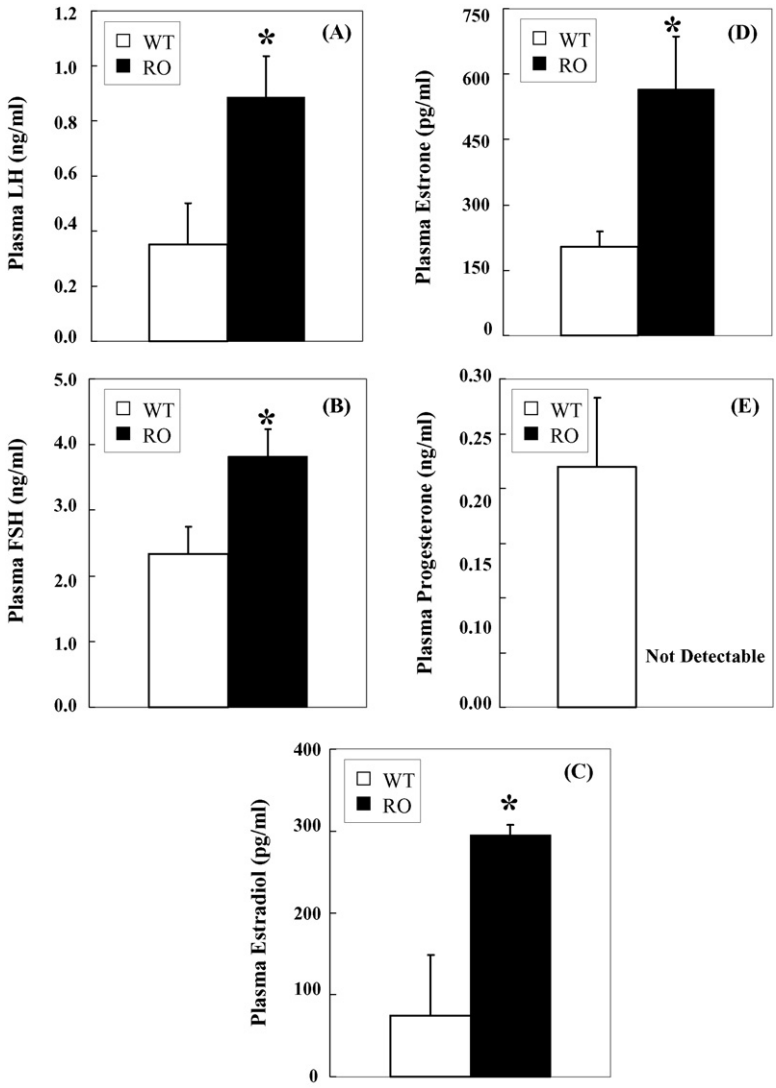

Fig. 1. Plasma concentrations of LH (A), FSH (B), estradiol (C), estrone (D) and progesterone (E) in wild-type (WT) and mutant restricted ovulator (RO) hens. Values represent mean ± standard error of the mean ($n = 4$–6). Significant differences at $P \leq 0.05$ are denoted by an asterisk.

concentrations in the RO hens were below the minimum detectable quantity (0.10 ng/ml). The intra assay coefficient of variation for the RIA were 10.03% (estradiol), 9.92% (estrone), 9.03% (progesterone), 1.6% (FSH), and 28.7% (LH). The changes in RO hen plasma estrogen and progesterone concentrations observed in the present study were consistent with the earlier report of Leszczynski et al. [15].

3.3. Pituitary PR mRNA quantification

Pituitary PR mRNA abundance was significantly greater in RO hens compared to WT hens ($P \leq 0.05$; Fig. 2). Melting curve analyses showed the presence of a single PCR prod-

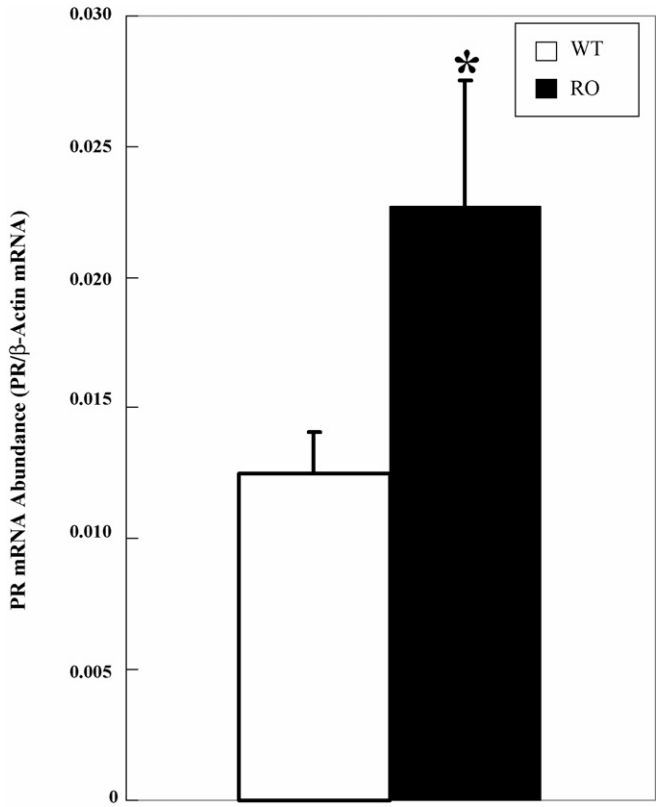

Fig. 2. Progesterone receptor (PR) mRNA abundance in the anterior pituitaries of wild-type (WT) and mutant restricted ovulator (RO) hens**.** Total RNA was extracted from the anterior pituitary gland and treated with DNAse-I. Following reverse transcription, approximately 100 ng of cDNA was used in real-time quantitative PCR using SYBR® green as the dye to quantitate progesterone receptor mRNA or β-actin mRNA in separate reactions from WT and RO hens. Each reaction was run in duplicate and the threshold (C_T) values for PR mRNA were subtracted from that of β-actin mRNA, averaged ($n = 9$) and converted from log-linear to linear term. Values represent mean ± standard error. Significant difference at $P \leq 0.05$ is denoted by an asterisk.

uct for PR or β-actin product confirming the specificity of the reaction (data not shown). Total RNA from the pituitary gland reverse transcribed in the absence of reverse transcriptase resulted in C_T values not different from blanks (>30 cycles) indicating that genomic DNA was not contributing to the PR or β-actin mRNA quantitation (data not shown).

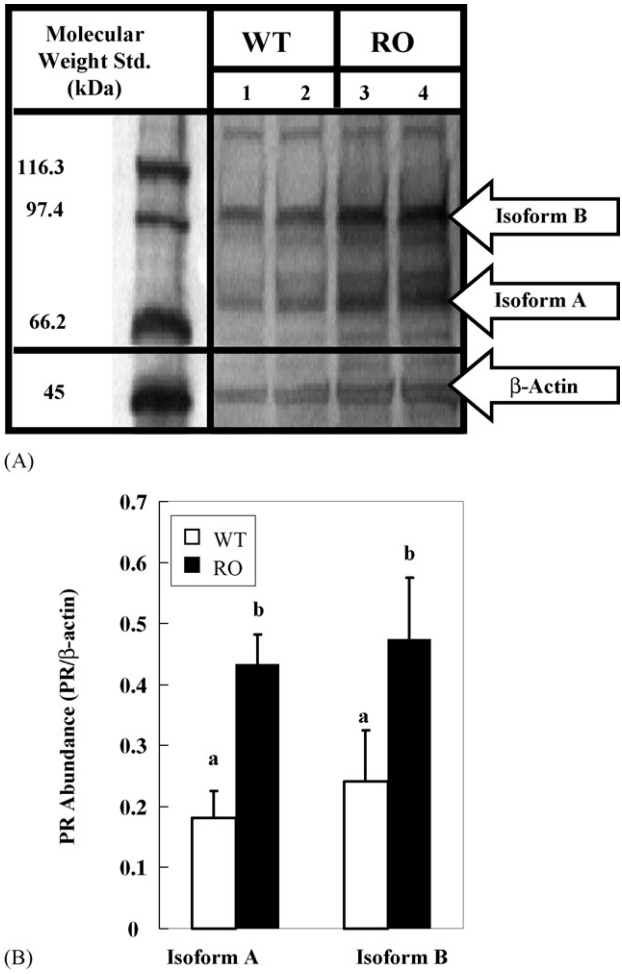

Fig. 3. Western blot analysis of pituitary progesterone receptor (PR). (A) Representative immunoblot showing PR isoforms A and B and β-actin immunoreactive bands from two wild-type (WT) hens (lanes 1 and 2) and two mutant restricted ovulator (RO) hens (lanes 3 and 4). A 25 μg of pituitary protein extract from each animal was electrophoresed and blotted on to PVDF membrane. PR isoforms and β-actin were detected by sequential immunostaining using anti-chicken PR antibodies (specific to isoform A or B), and anti-chicken β-actin antibody, respectively. (B) Progesterone receptor (PR) isoforms A and B abundance in the pituitary gland of wild-type (WT) and mutant restricted ovulator (RO) hens. Chemiluminscent immunostaining signal intensity of PR isoforms A or B was expressed as a proportion to β-actin signal intensity. Values represent means ± standard errors ($n = 6$–7). Within an isoform, bars with different superscripts are significantly different ($P \leq 0.05$).

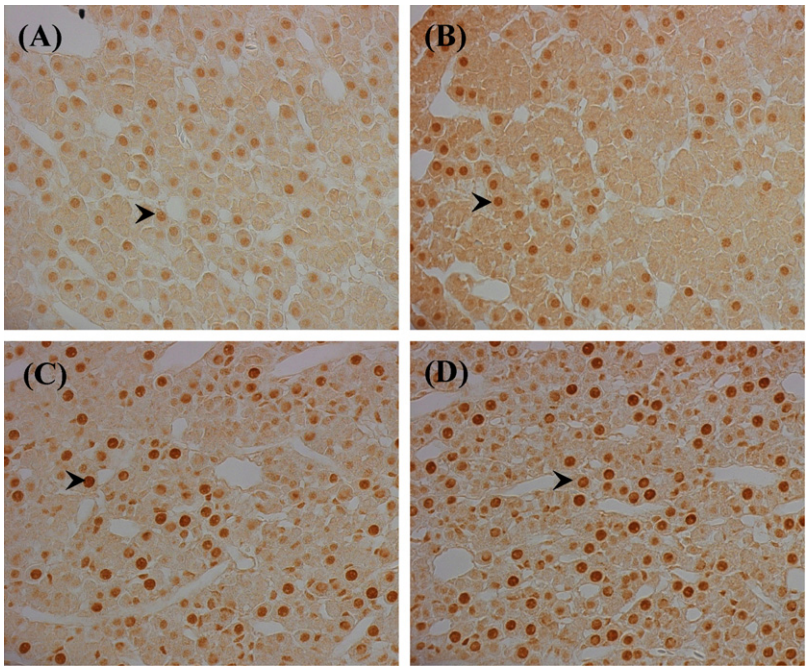

Fig. 4. Photomicrographs of the chicken anterior pituitary gland tissue sections showing nuclear immunostaining (arrows) of PR isoform A [panel A, wild-type (WT); panel B, mutant restricted ovulator (RO)] or isoform B (panel C, WT; panel D, RO). Paraffin embedded, 4 μm thin pituitary gland tissue sections were immunostained using isoform specific monoclonal anti-chicken PR antibodies.

3.4. Pituitary PR isoforms A and B quantification

Two immunoreactive PR isoforms A and B bands with approximate molecular weights of 76 and 97 kDa, respectively, were detected in the immunoblot of anterior pituitary protein extracts from RO and WT hens (Fig. 3A), and both PR isoforms were found to be more abundant ($P \leq 0.05$) in RO hens as compared to WT hens (Fig. 3B). Bands of unknown identity with MW of ∼60 and 120 kDa were also noted using anti-chicken PR antibodies specific to isoforms A and B. In this regard, multiple isoforms of PR including a 120 kDa isoform have been detected in the pituitary gland of other species [34]. Omission of primary antibody during immunostaining abolished all of the bands in the immunoblot (data not shown). Progesterone receptor antibody specificity was further verified by immunohistological staining of chicken pituitary gland sections [35]. Specific nuclear staining was noted throughout the cephalic and caudal lobes of the pituitary gland (Fig. 4A–D).

4. Discussion

This work is the first to report the abundance of PR mRNA and PR isoforms A and B in the chicken pituitary gland**.** The PR is an important molecule required for the secretion of

LH, and we hypothesized that mutant RO hen pituitaries would have an increased abundance of PR mRNA and PR isoforms A and B, as well as alterations in circulating gonadotrophin levels. Quantitative PCR assays revealed significantly greater ($P \leq 0.05$) pituitary PR mRNA abundance in RO hens as compared to WT hens. Similarly, PR isoforms A and B quantities were found to be significantly greater ($P \leq 0.05$) in the pituitary protein extracts of RO hens compared to WT hens. Plasma LH concentrations were also markedly higher in mutant RO hens as compared to WT birds.

The increase in pituitary PR isoforms A and B expression in the RO hens was possibly due to higher plasma estrogen concentrations and/or lower progesterone concentrations. Administration of estradiol to sexually immature male and female chickens induced PR expression in pituitary gland cells with a concomitant decrease in LH immunoreactivity in the same cells [24]. Progesterone receptor expression is under dual regulation of estrogen and progesterone based on the observation that PR concentrations are increased by estrogen in most species [36,37], while progesterone treatment decreases PR expression in hamster [38] and rat [39] uteri and in the chicken oviduct [37].

In the present work, the increase in pituitary PR isoforms A and B expression was accompanied by an increase in PR mRNA in the hyperestrogenemic RO hen pituitary gland. In support of our observation, estrogen treatment in rats was found to upregulate PR isoforms A and B mRNA expression in the pituitary gland [40]. Post-transcriptional modifications such as increased PR mRNA stability, decreased degradation of PR mRNA and/or protein may also have contributed to a higher PR content in the RO hen pituitary gland. The stability of the PR has been shown to be profoundly decreased by the presence of progesterone and was independent of changes in PR mRNA [41]. In addition, progesterone treatment leads to a rapid decrease in nuclear PR in rat gonadotropes primarily by increasing protein degradation without affecting PR mRNA [42]. Decreased plasma progesterone concentrations observed in the RO hens may have led to increased stability and abundance of the pituitary PR.

We also found that PR isoforms A and B were expressed in the same proportion in both WT and RO hens. In contrast, pituitary glands of rhesus macaques contained 1.6-fold more PR isoform A than PR isoform B [34]. Estradiol treatment of female rats was found to preferentially increase PR isoform B in the pituitary gland [43]. In the sexually immature chicken, administration of high doses of estradiol have been reported to upregulate expression of PR isoform A in the oviduct [44].

This work is also the first to report plasma gonadotrophin concentrations in mutant RO hens. In this regard, we observed large ($P \leq 0.05$) elevations in the circulating levels of FSH and LH. Moreover, as compared to WT hens, mutant RO hens had significantly greater plasma concentrations of estrone and estradiol, but lower circulating progesterone levels.

An increase in circulating FSH concentration could result from the lack of negative feedback inhibition from the ovary in RO hens. Large vitellogenic follicles are the major source of inhibin in the chicken [45] and removal of preovulatory follicles leads to a rise in plasma FSH levels [46]. Since we generally did not observe any large vitellogenic (e.g., F_1, F_2, or F_3) follicles in RO hen ovarian hierarchies, it was therefore logical to expect that lower inhibin secretion from the ovary may have accounted for the observed increases in circulating FSH concentrations. The increased plasma LH levels in the presence of a high plasma estrogen concentration was also intriguing, since estradiol has been reported to act as a negative regulator of LH secretion in the chicken [47]. Lower plasma progesterone

levels observed in the RO hens may be stimulating hypothalamic factor(s) responsible for increasing LH secretion from the pituitary gland. Nevertheless, altered metabolism and biotransformation leading to longer biological life of gonadotrophins may have been responsible for the higher FSH and LH concentrations observed in the RO hens.

A hereditary hyperlipidemic mammalian model with an abnormal hypothalamic-pituitary-ovarian axis, namely the Watanabe heritable hyperlipidemic (WHHL) rabbit, also exhibits increased plasma LH and FSH concentrations that are comparable to those of mutant RO hens [48]. The WHHL rabbit, which has a defective low density lipoprotein receptor and is a model of human familial hypercholesterolemia, also exhibits poor fecundity which may be due to disorders of ovulation, implantation, and gestation [48]. However, in contrast to mutant RO hens and compared to control New Zealand White rabbits, WHHL rabbits have similar baseline progesterone concentrations and 30% lower

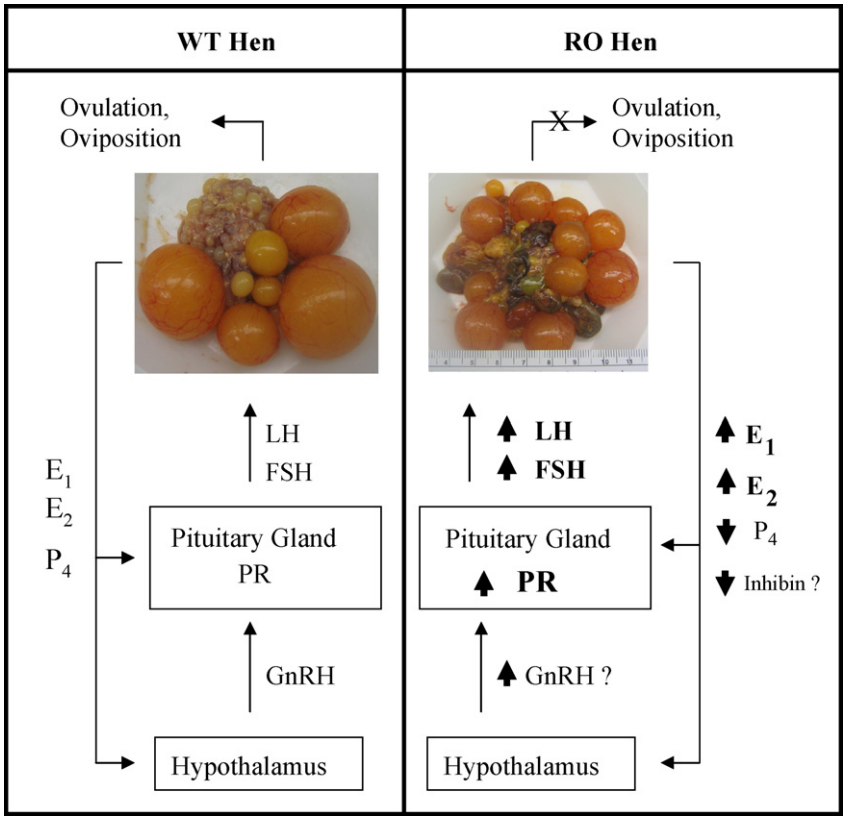

Fig. 5. Proposed model relating humoral, ovarian, pituitary, and hypothalamic factors to reproductive dysfunction in the mutant restricted ovulator (RO) hen. Lack of F1 preovulatory follicles in the RO hen ovary possibly leads to lower circulating progesterone and inhibin levels causing elevated plasma FSH, estrone, and estradiol levels as well as greater abundance of pituitary PR isoforms A and B. WT: wild-type. RO: restricted ovulator; E1: estrone; E2: estradiol; P4: progesterone; LH: luteinizing hormone; FSH: follicle stimulating hormone; GnRH: gonadotrophin-releasing hormone.

circulating estradiol levels [48]. Moreover, while it has been hypothesized that the elevated levels of gonadotrophins in WHHL rabbits may be a reflection of attenuated cholesterol transport into steroid-producing cells and thus impaired estradiol synthesis [48], different mechanisms must come into play in mutant RO hens, because estrogen levels are greatly elevated as compared to WT hens (Fig. 1 and [15]).

Thus, in order to summarize our current understanding of key factors that might be responsible for dysfunctional reproduction in mutant RO hens, a model linking humoral and ovarian factors to observed and putative changes in the hypothalamic-pituitary axis is proposed (Fig. 5). Ovarian morphology in mutant RO hens is grossly abnormal and typically characterized by a plethora of small vitellogenic (i.e., F_5–F_7) follicles, a lack of larger (i.e., F_1–F_3) preovulatory follicles, and the presence of numerous atretic and misshapen follicles (Fig. 5). The apparent inability of follicles to fully mature most likely leads to markedly lower circulating progesterone and inhibin concentrations, and results in elevated plasma FSH, estrone, and estradiol levels. Some of the above hormonal changes may be responsible for the upregulation of PR mRNA and increased abundance of PR isoforms A and B in the anterior pituitary.

The hallmark of mutant RO hens, namely hyperestrogenemia, hypoprogersteronemia, and a failure to ovulate, suggests a disruption of the follicular hierarchy despite the presence of elevated estrogen and plasma gonadotrophin concentrations. In addition, the significance of the increased abundance of PR mRNA and PR isoforms A and B in the pituitary glands of mutant RO hens remains to be determined, while the seemingly paradoxical observation of elevated circulating LH levels concomitant with hypoprogesteronemia, demands further study.

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