Expression of Prolactin Receptor on the Surface of Quail Spermatozoa

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Prolactin receptor (PRLR) is expressed in a wide variety of tissues and mediates diverse biological actions of prolactin (PRL). In mammals, PRL signaling is thought to be involved not only in the process of spermatogenesis and steroidogenesis in the testis, but also in the survival of ejaculated sperm. In avian species, although the expression of PRLR with several variants in the testis was reported, the role of PRL in testicular function is still unclear. The aim of this study was to examine the expression of PRLR in the testis and mature sperm in quail. It is revealed that PRLR was mainly localized in the round- and elongated-spermatid by immunohistochemical analysis on the testis suggesting that PRL signaling may participate in the spermatogenesis. Western blot analysis confirmed the presence of PRLR in the plasma membrane of the ejaculated sperm (SPML), whereas the size of PRLR in the sperm was smaller than that in the hypothalamus. Moreover, PRLR was detected on the surface of the midpiece and flagellum of sperm by immunostaining. To evaluate the functionality of the sperm PRLR, the dot blot assay was performed to test the binding of pituitary PRL to PRLR in the SPML, and resulted in the detection of specific binding of PRL to the component of SPML, most likely to sperm PRLR. Furthermore, when the ejaculates were incubated with pituitary PRL to investigate the role of PRL on the sperm, the occurrence of spontaneous acrosome reaction was significantly decreased. In addition, the expression of PRL on the surface of utero-vaginal junction of oviduct was detected by immunohistochemistry. These results may suggest a novel system that the interaction between oviductal PRL and sperm PRLR is involved in the maintenance of the fertilizability of the spermatozoa through the prevention of the spontaneous acrosome reaction in Japanese quail.

Key words: acrosome reaction, prolactin receptor, quail, sperm, testis, utero-vaginal junction

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Introduction

Prolactin (PRL) is mainly produced by lactotroph cells in anterior pituitary gland, and exerts diverse biological actions. PRL exerts its function through binding to its membrane-bound receptor (PRLR), which is expressed in a wide variety of target tissues (Bole-Feyt et al., 1998; Marano and Ben-Jonathan, 2014). The PRLR gene in mammalian species is comprised of 11 exons and produces the receptor with extracellular, transmembrane and cytoplasmic domains. There are two major isoforms referred to as long and short form with intact- or truncated-cytoplasmic domain of carboxy terminus, respectively (Boutin et al., 1988; Shirota et al., 1990). Although the functions of the isoforms are not well established, the existence of several splice variants of the receptor had been reported (Freeman et al., 2000; Trott et al., 2003). In addition, the presence of many types of PRL-like peptide (Duckworth et al., 1986; Toft and Lünzer, 1999; Dai et al., 2000; Soares, 2004) as well as extra-pituitary PRL had been reported (Ben-Jonathan et al., 1996; Bole-Feyt et al., 1998; Harvey et al., 2014), thus the function of PRL might be more complex than our classical understanding.

In avian species, multiple actions of PRL have also been reported including the modulation of immune responsiveness (Skwarlo-Sonta et al., 1986), crop-sac development (Nicoll et al., 1967), the induction of broody behavior (Riddle et al., 1935) and control of follicular development and egg production (Peters and Burke 1977, Sharp et al., 1989, Zadworny et al., 1989, Li et al., 2011). In birds, such as chicken (Tanaka et al., 1992), turkey (Zhou et al., 1996) and pigeon (Chen and Horseman, 1994), PRLR had been identified by cDNA cloning, and the tissue distribution of the receptor gene is as ubiquitous as in mammalian species (Zhou et al., 1996).
Although a wide spread expression of the receptor may indicate the diverse physiological functions of PRL signaling via PRLR, an elucidation of authentic functions of avian PRL based on experimental evidence is not well investigated.

In mammalian species including human, mice and rats, it is reported that both PRL and its receptor are expressed in the testis (Ouhtit et al., 1993; Hondo et al., 1995; Untergasser et al., 1997; Hair et al., 2002; Parthasarathy and Balasubramanian, 2008; Ishida et al., 2010). Because the receptor had been expressed in either spermatogenic or somatic cells (i.e. Leydig and Sertoli cells), PRL is thought to be involved in the process of both spermatogenesis and steroidogenesis in the testis. In contrast, Binart et al. (2003) demonstrated that male mice with a targeted disruption of the PRLR appeared normal in their reproductive parameters (e.g. circulating gonadotropins level, prostate weight, testis morphology and total number of sperm) and were fertile with natural mating.

In birds, the expression of several kinds of receptor variant in the testis was reported (Mao et al., 1999; Tanaka et al., 2000), however, the role of PRL in testicular function such as sperm production as well as fertilization is poorly understood. In this study, we aimed to study the expression of PRLR in the testis and mature sperm to advance our understanding of the role of PRL signaling in fertilization in birds. In addition, we reported here that the PRLR expressed on the surface of quail spermatozoa might contribute to the preservation of the sperm fertilizability by inhibiting spontaneous acrosome reaction.

Materials and Methods

Animals and Tissue Preparation
Male and female Japanese quail, Coturnix japonica, 8–20 weeks of age (Motoki Corporation, Tokorozawa, Japan), were maintained individually under a photoperiod of 14L:10D (with the light on at 05:00) and provided with water and food ad libitum (Molecular Probes, Eugene, OR, USA). Sperm were incubated in the presence or absence of chicken PRL (generous gift from NIDDK) at 39°C for 24h, and the protein was purified from the cell lysate using nickel resin (Novagen, Madison, WI, USA) according to the manufacturer’s instructions. The purity of the recombinant protein was verified by SDS-PAGE followed by CBB staining.

Female New Zealand White rabbits (SLC, Hamamatsu, Japan) were immunized with the recombinant PRLR (300 μg of protein) as described previously (Kuroki and Mori, 1995) and resulting antisera raised against extracellular or intracellular domain of the receptor were mixed at 1:1 ratio. To verify the specificity of anti-PRLR antiserum, we preincubated the antiserum with the antigen protein (1 mg/ml) at 4°C overnight before use.

The antiserum against chicken PRL (anti-PRL) used in the present study was raised in rabbit, and was characterized as described previously (Hiyama et al., 2009). The amino acid sequence similarity of chicken and quail PRL shows more than 90%.

Semen Collection and Preparation
Ejaculated semen was obtained from male quail during mating prior to ejaculation according to the procedure of Kuroki and Mori (1997). Semen obtained from two to three males was suspended in Hanks balanced salt solution containing 1.25 mM CaCl2 and 1.8 mM MgSO4. The concentrations of sperm were measured with a hemocytometer and sperm viability was assessed using a LIVE/DEAD sperm viability kit according to the manufacturer’s instructions (Molecular Probes, Eugene, OR, USA). Sperm were incubated in the presence or absence of chicken PRL (generous gift from NIDDK) at 39°C. The acrosome status of the spermatozoa was observed based on the presence (acrosome-intact sperm) or absence (acrosome-reacted sperm) of the acrosome, which is observed as a propidium iodide-negative structure located on the tip of the sperm nucleus as previously described (Sasanami et al., 2007).

To obtain the sperm membrane lysates (SPML), the ejaculates were washed three times with ice-cold PBS with repeated centrifugation at 800 × g for 3 min at 4°C, and the final pellet was suspended in cavitation buffer containing 150 mM NaCl, 20 mM HEPES (pH 7.4). The suspension was cavitated with a cell disruptor (Parr Instrument Company, Moline, IL, USA) at 400 pound per square inch gauge (psig), and the cellular debris was removed by centrifugation at 10,000 × g for 10 min. The supernatants were further centrifuged at 158,000 × g for 30 min, and the precipitates were suspended in cavitation buffer containing 0.1% Triton X-100, 0.1 mM PMSF, 0.5 μg/ml leupeptin and 10 μg/ml soybean trypsin inhibitor. The SPML samples were stored at −80°C until use.
**Western Blot Analysis**

Testis, hypothalamus or pituitary was homogenized in PBS containing 0.1 mM PMSF, 0.5 μg/ml leupeptin and 10 μg/ml soybean trypsin inhibitor and the supernatant was obtained by centrifugation at 20,000×g for 10 min. Protein concentration of each lysate was determined using a BCA protein assay kit (Pierce, Rockford, IL). Aliquots (10 μg) of the solubilized materials were subjected to one-dimensional SDS-PAGE with 12% separating gel as described previously (Laemmli, 1970). The protein was transferred onto polyvinylidene difluoride (PVDF) membrane (Immobilon, Millipore, Bedford, MA). PVDF membrane was blocked with 5% non-fat skim milk in saline buffered at pH 7.4 with 10 mM Tris-HCl containing 0.1% Tween 20 (TBS-T) and incubated for 1 h with anti-PRLR (1:2,000) or anti-PRL (1:5,000). After reacting, bands were visualized by a chemiluminescent technique (LAS500, GE Healthcare) using horseradish peroxidase-conjugated anti-rabbit IgG (Cappel, Durham, NC) as secondary antibody.

**Dot Blot Analysis**

The dot blot assay was performed according to Kubo et al. (2010). A PVDF sheet (Immobilon-P; Millipore) wetted with methanol and then with PBS was set in a dot blotter (Bio-Rad Laboratories), and the sperm plasma membrane extracts was added to each well. Twenty minutes later, the solution was removed by suction, and the wells were washed three times with repeated centrifugation at 800×g for 3 min. After washing, sperm were fixed in formaldehyde (final concentration, 3.7% v/v). The fixed sperm were then smeared on a microscope glass slide and the PRLR on the surface of the spermatozoa was detected immunologically as described previously (Sasanami et al., 2011).

**Immunohistochemistry**

To observe the localization of the PRLR in the testis, the isolated testis were fixed in Bouin’s fixative solution and embedded in Paraplast (Oxford Labware, St. Louis, MO). The isolated UVJ mucosa was also processed for immunohistochemistry to observe the expression of PRL protein. Immunohistochemical techniques were as described previously (Sasanami et al., 2002) using anti-PRLR (1:200) or anti-PRL (1:200) for the sections of testis or UVJ, respectively. The immunolabeled sections were examined under a interference–contrast photomicroscope (BX 50, Olympus Optics, Tokyo, Japan).

Ejaculated sperm were suspended in sperm extender and washed three times with repeated centrifugation at 800×g for 3 min. After washing, sperm were fixed in formaldehyde (final concentration, 3.7% v/v). The fixed sperm were then smeared on a microscope glass slide, and the PRLR on the surface of the spermatozoa was detected immunologically as described previously (Sasanami et al., 2011).

**Data Analysis**

Data were expressed as means±SE. An arcsine square-root transformation of the percentage data was performed and the transformed data was compared by Student’s t-test. Differences were considered statistically significant when P<0.05.

**Results and Discussion**

To investigate the expression of PRLR, we produced antiserum against bacterially expressed PRLR. Western blot analysis demonstrated that the SPML contain immunoreactive band migrating around 70kDa (Fig 1, left panel, lane 1). This is not due to the contamination of seminal plasma protein since we failed to detect 70kDa band in the sample of seminal plasma (Fig 1, left panel, lane 2). Importantly, the size of this molecule is much smaller than that of the immunoreactive band in the lysate of hypothalamus (approximately 97kDa, Fig 1, lane 3), indicating the presence of
structural differences in the receptor on the sperm with that of hypothalamus. Although the nature of this difference in size is not known, Mao et al. (1999) reported that several kind of mRNAs derived from alternative splicing of PRLR gene are present in the testis of chicken. In addition, differential N oligosaccharides modifications of the protein might contribute to the structural difference between them since there are totally 13 sites for a potential N glycosylation in the amino acid sequence of quail PRLR (GenBank accession number: LC074729). Further studies are required to uncover the structural properties of sperm PRLR. Because the bands detected around 33 kDa that reacted with anti-PRLR did not fade away by antigen absorption (right panel, lanes 1–3), this protein appears to react non-specifically with the antibody.

The immunohistochemical analysis on the sections of the testis shown in Fig. 2A revealed that immunoreactive PRLR proteins were mainly localized in the round spermatids and elongated spermatids. Although a weak signal nearly equivalent to background level was observed, such strong signals were not seen in the spermatocytes, spermatogonia, Sertoli cells as well as Leydig cells. Importantly, no such intense signals were observed when the specimens were incubated with anti-PRLR pre-incubated with antigen proteins (Fig. 2B), indicating that the 33 kDa proteins on the blot are not reactive to our antibody on the paraffin sections. In mammalian species, it is reported that PRLR protein are detected in Leydig cells in addition to spermatogenic cells, indicating the involvement of PRL signaling in the process of steroidogenesis (Hair et al., 2002). Although we can not explain this discrepancy, mRNA of PRLR was detected in the isolated Leydig cells by conventional RT-PCR (data not shown).

Additional experiments elucidating the role of PRL signaling on the steroidogenesis in the testis of quail will remain in the future studies.

To localize the PRLR proteins in the spermatozoa, we next performed immunostaining of the ejaculated sperm with anti-PRLR. As a result, immunoreactive signals were clearly observed on the surface of the midpiece and flagellum of the sperm (Fig. 3, panels A and C). On the other hand, sperm head was not stained by the antibody (arrows in panels A and C), indicating PRLR proteins mainly localized on the surface of the midpiece and flagellum of the sperm. These signals were considered specific as no such signal was observed when the same preparations were incubated with anti-PRLR antiserum pre-incubated with antigen proteins (panels B and D). This staining pattern is coincident with that of human sperm in that immunoreactive PRLR are mainly localized in the midpiece and flagellum (Pujianto et al., 2010). To evaluate the binding of the sperm PRLR to PRL, we tested whether the PRLR in SPML interacts with pituitary PRL based on the dot blot assay (Kubo et al., 2010). Our anti-PRL is confirmed to recognize pituitary PRL (Fig. 4A) in accordance with the results of our previous study (Hiyama et al., 2009).

As shown in Figure 4B, anti-PRL recognized immunoreactive materials that interact with the SPML component immobilized on a PVDF membrane. When the pituitary extracts and/or SPML were omitted from the assay, the immunoreactive signal was diminished to a background level or lower, indicating the specificity and the reliability of the assay performed here. These results indicated that pituitary PRL interact with SPML component, most probably via sperm PRLR. Taken together with the results of Western blot analysis, the 70 kDa PRLR on the surface of the ejaculated spermatozoa is functional receptor and might play a role for sperm function.

These results prompt us to investigate the role of PRL on the sperm functions. When we incubated the ejaculates with purified chicken PRL, the occurrence of spontaneous acrosome reaction was decreased in a dose related manner (Fig. 5A). The percentage of acrosome reacted sperm during incubation without PRL increased markedly over time, however, the change was minimal when PRL was added in the incubation mixture (Fig. 5B). This PRL effects is not thought to be a nonspecific action due to the presence of a protein because our previous results indicated the purified ZP3 protein does not affect the occurrence of the spontaneous acrosome reaction (Sasanami et al., 2007). In contrast, neither sperm motility nor intracellular ATP level was affected in the presence of the PRL during incubation (data not shown). These results indicated that PRL might inhibit spontaneous acrosome reaction that will impede the fertilizability of the sperm, thus this hormone might maintain the sperm fertilizability until the time of fertilization in birds. The acrosomal contents were required to penetrate the perivitelline membrane, an investment of avian ovum homologous to mammalian zona pellucida at the time of fertilization. Thus, acrosomal components such as sperm acrosin (Sasanami et al., 2011) or proteasome (Sasanami et al., 2012) that are required for fertilization are not prematurely released until the time of encounter of the sperm and eggs. Recent findings in human indicated that PRLR is expressed on the surface of the spermatozoa and PRL may act as a prosurvival factor for sperm that is responsible for preventing caspase activation thereby inhibiting capacitation (Pujianto et al., 2010). They concluded that PRL in the seminal plasma that is transported to the female genital tract along with sperm may exert pro-survival effects for successful fertilization. In contrast, when we examined the quail seminal plasma by Western blot using anti-PRL, a positive signal was not detected (data not shown), instead, the immunohistochemical detection clearly demonstrated that PRL is expressed in the utero-vaginal junction of the oviduct (Fig. 6). It is now accepted that the female reproductive tract actively eliminates the spermatozoa as foreign body. Indeed, Kawano et al. (2007, 2014) discovered a sperm protection system in mice in that seminal vesicle secretion 2 (SVS2) protein coats sperm surface and protects the sperm from the cytotoxic factor derived from the uterus. In birds, the oviductal immune system may also affect the survivability of the sperm in the oviduct (Das et al., 2008). Very recently we also found that cloacal gland secretions, the major seminal plasma in quail, contains prostaglandin F2α, which act to open the entrance of sperm storage tubules that serve as “sperm shelter” for sperm in the oviduct (Sasanami
Fig. 2  Immunohistochemical detection of prolactin receptor in testis. Sections of testis of mature male were processed for immunohistochemical observation using anti-PRLR (A) or anti-PRLR preincubated with antigen protein (B). The sections were lightly stained by hematoxylin. Arrows shown in panel A represent Sertoli cell. Shown are representative of repeated experiments. Bar=100 μm.

Fig. 3  Immunohistochemical localization of prolactin receptor in ejaculated sperm. The smeared specimens of the ejaculated sperm were detected with anti-PRLR. The immunoreactive materials with anti-PRLR (A) can be seen in the midpiece and flagellum but not detected in the head of the sperm. (B) These signals were not observed when the specimen was detected by normal rabbit serum. The sperm nuclei in (A) were stained with DAPI (blue) and the image was merged (C). The control specimen was also stained with DAPI and merged with immunostaining (D). Arrows shown in panels (A) and (C) represent sperm head. Shown are representative of repeated experiments. Bar=100 μm.
The results obtained in this study suggest a novel system that a female factor, oviductal PRL prevents spontaneous acrosome reaction that might directly link to the maintenance of the fertilizability of the spermatozoa in Japanese quail. The question of whether this unique mechanism works in the quail reproductive system is also functional in other birds remains to be solved in the future studies.

Fig. 4. Detection of prolactin binding to the sperm plasma membrane. (A) The extracts of pituitary (1 μg proteins) was separated on SDS-PAGE and was detected by Western blotting using anti-prolactin antiserum (lane 1) or anti-PRL antiserum preincubated with antigen protein (lane 2). SDS-solubilized sperm plasma membrane (0, 0.5 or 1 μg proteins) was dot blotted onto a PVDF and then was incubated with the extracts of pituitary (1 μg/ml). The dots were incubated with anti-PRL antiserum and the bound prolactin was immunologically detected. Shown are results representative of three repeated experiments.

Fig. 5. Effects of chicken prolactin on the acrosome reaction of quail sperm. (A) The ejaculated sperm were suspended in the medium containing 0, 2.5 or 5 μg/ml chicken PRL and were incubated at 39°C for 30 min. Data shown are the mean ± SE of 3 experiments. Value with * is significantly different from 0 μg/ml control (P < 0.05). (B) The ejaculated sperm were incubated in the presence (+PRL) or absence (-PRL) of chicken PRL (5 μg/ml) at 39°C for 0, 10, 30 or 60 min. The acrosome status of the sperm after the incubation was observed under fluorescent microscope. Data shown are the mean ± SE of 3 experiments. Value with * or ** is significantly different from “-PRL” at respective incubation time (* or ** denotes P < 0.05 or P < 0.01, respectively).
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