Gene Expression of Lymphocyte Prolactin Receptor Was Suppressed in Lactating Mothers

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(Received December 16, 2009; Accepted April 15, 2010)

Prolactin (PRL) receptor (PRL-R) was proven to be ubiquitously expressed by cells in the immune system, while the physiological role of PRL was established in milk production in mammary glands. We analyzed the mRNA content of PRL-R in human lymphocytes in normo- and hyperprolactinemic conditions to document the presence of functioning PRL-R of human lymphocytes.

Blood samples were obtained prior to treatment, and with written informed consent, from outpatients with ovarian dysfunction and hyperprolactinemia (n = 8; 19 ~ 41 y/o), from breast-feeding mothers after normal delivery (n = 12; 27 ~ 36 y/o), and from healthy volunteers: men (n = 9; 33 ~ 40 y/o) and women (n = 9; 26 ~ 36 y/o). Subsequently, total RNA was prepared from the lymphocytes separated. The quantity of PRL-R mRNA was examined by reverse transcription and polymerase chain reaction and normalized with a simultaneously measured amount of β actin. The resultant mRNA level of PRL-R was analyzed for its correlation with serum concentration of PRL measured by immunoassay.

PRL-R mRNA levels of lymphocytes were significantly suppressed in lactating mothers, while there was a statistically significant negative correlation between PRL-R mRNA and serum PRL levels. However, there was no significant difference of PRL-R mRNA in the pathological condition of outpatients with ovarian dysfunction and/or hyperprolactinemia.

While a few investigators reported the extra-mammary regulation on PRL-R by PRL, our data suggest that the PRL-R levels of circulating lymphocytes could be down-regulated by the elevated serum levels of PRL and that pituitary PRL may participate in regulating the expression of PRL-R genes on cells of the human immune system, especially in physiological circumstances such as in the postpartum period.

Key words: prolactin, prolactin receptor, lactation, hyperprolactinemia, human lymphocyte

INTRODUCTION

The physiological function of prolactin (PRL) in humans has only been established in the mammary gland as a classical concept of endocrine action [1]. While there are other reported autocrine/paracrine actions, most of which are associated with the process of reproduction [2–5]. Immunoregulatory properties had received attention mainly in rodents where the PRL receptor (PRL-R) is ubiquitously expressed by cells in the immune system [6–9]. We investigated the PRL-R gene expression of human lymphocyte in normo- and hyperprolactinemic conditions and analyzed the correlation between PRL-R mRNA and serum PRL levels. We also investigated its physiological significance.

SUBJECTS

Subjects and Materials

Prior to any treatment being given, and with written informed consent, blood samples were obtained from outpatients with ovarian dysfunction and hyperprolactinemia (n = 8; 19 ~ 41 y/o), from breast-feeding mothers after normal deliveries (n = 12; 27 ~ 36 y/o), and from healthy volunteers: men (n = 9; 33 ~ 40 y/o) and women (n = 9; 26 ~ 36 y/o). Venopuncture was done in the morning of their early follicular phase of menstruating women, or on the 5th postpartum day. The breast-feeding mothers consisted of 8 primiparae and 2 multiparae without any specific medications or complications. Hyperprolactinemic patients were defined as those whose serum PRL levels were more than 15 ng/ml. And the sera were prepared immediately, frozen, and stored at -80°C until assayed. Lymphocyte fraction was separated by density gradient with Ficoll-Paque PLUS^{R} (Amersham Pharmacia Biotech AB, Uppsala, Sweden), and the total RNA was extracted using Isogen (Wako, Osaka), according to the manufacture's instructions, dissolved in 100 μl distilled water and stored at -80°C until subjected to reverse transcription and polymerase chain reaction (RT-PCR) analysis.

Hormone measurements

Serum levels of hormones were determined by commercial radioimmunoassay kits (PRL, Spac-S PRL kit; LH, luteinizing hormone, Spac-S LH kit; FSH, follicle-stimulating hormone, Spac-S FSH kit, Daiichi RI,

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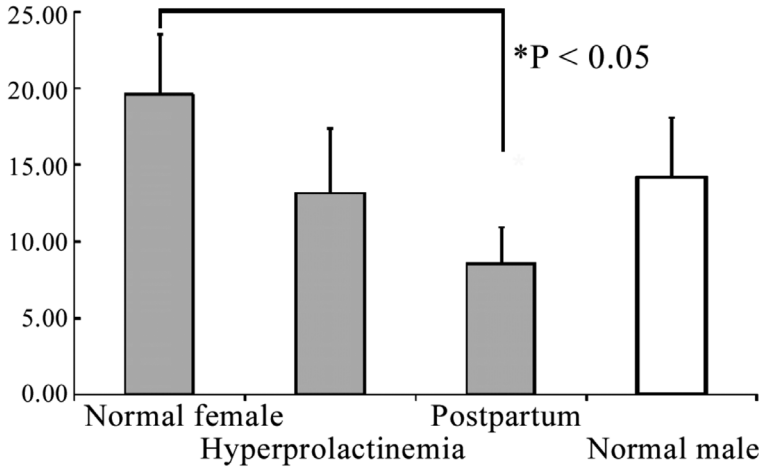

Fig. 1 Gene expression of lymphocyte PRL-R in the four groups.
Gene expression of lymphocyte PRL-R was analyzed by RT-PCR in the four groups: healthy women (n = 9; healthy women volunteers in the early follicular phase), hyperprolactinemia (n = 8; patients of ovarian dysfunction with hyperprolactinemia before treatment), postpartum (n = 12; breast-feeding mothers on the 5th day after normal delivery) and healthy men (n = 9; healthy men volunteers). All values are expressed as mean ± SEM.
*There is a statistical difference between the postpartum and the healthy women's groups ($P < 0.05$).

Tokyo) as well as estradiol (E2, Delphia, Amersham Pharmacia Biotech, Buckinghamshire, UK) and testosterone (T, Immulize, DPC, Tokyo) by enzyme-linked immunosorbent assay (ELISA) kits. Each assay was performed according to the manufacturer's instructions and duplicated in a single standard curve.

RT-PCR

RNA samples were reverse transcribed as follows. Each sample contained 10 µg of total cellular RNA, 50 mM Tris-HCl pH 8.3, 75 mM KCl, 0.5 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM of each dNTP (dATP, dTTP, dGTP, dCTP), 20 U of RNase inhibitor, 100 pmol random hexamer (TaKaRa, Kyoto, Japan), and 200 U Moloney murine leukemia virus reverse transcriptase (Gibco-BRL, Life Technologies, Inc., Gaithersburg, MD, USA) in a final volume of 20 µl. After incubation at 37°C for 60 min, the samples were heated to 94°C for 5 min to terminate the reactions and stored at -20°C until use.

The primers were synthesized on a model 391A DNA synthesizer (Applied Biosystems, Inc., Foster City, CA, USA) (using β-cyano-methylphosphoramidate derivatives), quantified by absorbance measurements at 260 nm, and stored at -20°C. Oligonucleotide primers: ACCCAGCAGAGGGAGGTAGAA (nucleotide number [nn] 1704-1724) and ATGTTGTTATCCATGACCCCG (nn 1954-1934) were constructed from the published cDNA sequences of the human PRL-R [10]. β actin served as control for the efficacy of RNA isolation and cDNA synthesis. The sets of primers used are described in our previous paper [11].

Each reverse transcription mixture was diluted 1:5 in RNase-free water, and 2 ∝ λ were then transferred to fresh tubes for amplification. Each sample contained the upstream and downstream primers (0.2 mM of each primer) spanning the given sequence for amplification, 0.2 µM of each dNTP (dATP, dCTP, dGTP, dTTP), 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 10 mM $MgCl_2$, 0.01% (w/v) gelatin, 0.5 U of AmpliTaq Gold DNA polymerase (Perkin Elmer Cetus Corporation, Norwalk, CT, USA) in a final volume of 25 µl. The reaction mixture was then overlaid with 1 drop (~ 50 µl) of mineral oil and subjected to 35 cycles of amplification for PRL-R and 30 cycles for β actin, respectively, in a Perkin Elmer Cetus thermal cycler (Norwalk). The amplification profiles consisted of denaturation at 94 °C for 30 s, primer annealing at 55°C for 30 s, and extension at 72°C for 3 min. Negative control reactions without templates were routinely included in PCR amplifications with all primer sets. The PCR products (5 µl) were separated through a 1.5% agarose gel. The gel was stained with ethidium bromide (0.3 µg/ml) and photographed on Polaroid 665 positive/negative film. The quantity of PRL-R mRNA was normalized with a simultaneously measured amount of β actin.

Statistical analysis

All values are expressed as mean ± SEM. Statistical differences were analyzed by one-way analysis of variance followed by the Bonferroni/Dunn test as a post hoc examination. PRL-R mRNA levels were analyzed for correlations with serum concentrations of PRL as well as other hormones. Values of $P < 0.05$ were considered to indicate statistical significance.

RESULTS AND DISCUSSION

Serum PRL levels of the 4 analyzed groups were 5.69 ± 0.53, 3.48 ± 0.54, 118.89 ± 58.13, and 154.70 ± 31.20 ng/ml (mean ± SEM) in healthy women controls, healthy men controls, outpatients with hyperprolactinemia, and breast-feeding mothers, respectively. Serum PRL of healthy men showed lower levels than did those of healthy women, but without statistical significance. There was no correlation among the analyzed hormones: LH, FSH, E2, and T. Expression of the PRL-R gene of lymphocytes was significantly ($P = 0.016$) suppressed in breast-feeding mothers compared with the healthy women controls (Fig. 1). In patients with hyperprolactinemia, the mRNA levels of the PRL-R showed a tendency to be suppressed but without statistical significance ($P = 0.053$). Because the serum PRL levels of the postpartum group were all above 15 ng/ml, we assumed lactating mothers all presented with physiological hyperprolactinemia. However, different from this physiological hyperprolactinemia (i.e., the postpartum group), we could not detect a clear suppression of mRNA of PRL-R in pathological hyperprolactinemia (i.e., the hyperprolactinemia group). Consistent with our data in hyperprolactinemia patients, Leite-de-Moraes *et al.* previously reported that they found no correlation between PRL-R expres-

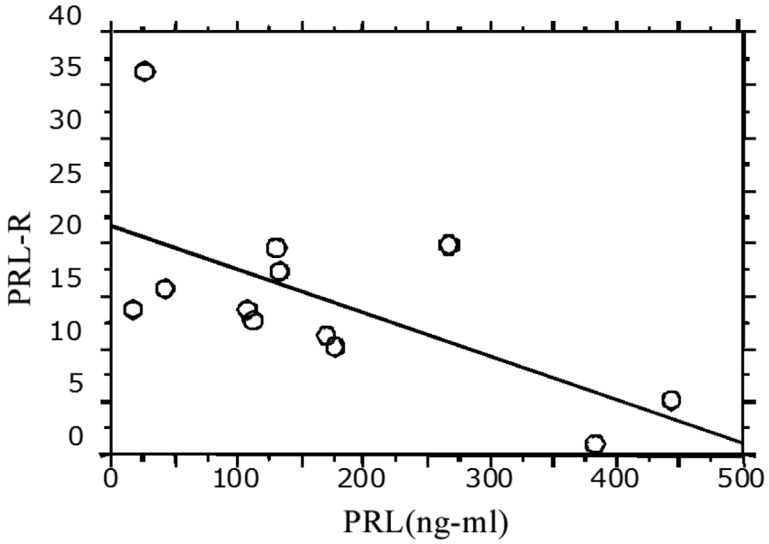

Fig. 2 Correlation between serum PRL and gene expression of lymphocyte PRL-R in lactating mothers.
There is a negative correlation (P = 0.045) between the serum levels of PRL and the gene expression of lymphocyte PRL-R in the physiologically hyperprolactinemic condition of the breast-feeding mothers on the 5th day after normal delivery. The linear regression equation is PRL-R = 21.73 − 0.041 × PRL; R^2 = 0.404. This result suggests that the PRL-R level of the circulating lymphocytes could be down-regulated by elevated serum PRL.

sion and circulating PRL levels in a flow cytometric analysis of T and B lymphocytes and monocytes from peripheral blood mononucleated cells of patients with hyperprolactinemia or acromegaly [12]. They speculated that factors other than pituitary PRL play the major role in regulating the PRL-R expression on cells of the immune system. Our results showed that lymphocyte mRNA of PRL-R had no significant correlation with serum PRL in the outpatients with ovarian dysfunction and hyperprolactinemia before treatment, therefore, we agree with Leite-de-Moraes *et al.*'s assumption.

However, in our study, the expression of the lymphocyte PRL-R gene was significantly suppressed in breast-feeding mothers. Moreover, there was a negative correlation between serum PRL and its PRL-R levels in the group (Fig. 2, P = 0.045). This result is a new finding. The linear regression equation was PRL-R = 21.73 − 0.041 × PRL (R^2 = 0.404). This new finding suggests that the PRL-R level of the circulating lymphocytes could be down-regulated by the elevated serum level of PRL and that the pituitary PRL may regulate the PRL-R expression of immune cells especially in the postpartum period. These data may support the evidence that there is some role of pituitary PRL in the human immune system in physiological circumstances.

Our data first indicate that the lymphocyte mRNA of PRL-R might be desensitized by the elevated level of circulating PRL, especially in the postpartum period. The factor of a time-scale for desensitization might be an additional reason why Leite-de-Moraes *et al.* [12] and we could not detect significant changes of lymphocyte PRL-R in pathological hyperprolactinemia. The length of time in which lymphocytes were exposed to the elevated level of circulating PRL was specifically case dependent in the outpatients with hyperprolactinemia. In our postpartum group, blood sampling was done on the 5th day after delivery. We sampled the serum from the mothers more than 6 hours after the most recent breast feeding of their babies in the morning, as suckling stimulation transiently increases serum PRL in the postpartum period [13]. Our subjects were examined postpartum with the comparably same time-scale. Because "dose" and "duration" could be important factors for the desensitization phenomenon, we could successfully fix the "duration" and analyze the effect of the "dose." In this situation, we successfully discovered that the lymphocyte mRNA of PRL-R was desensitized by the elevated level of circulating PRL during the postpartum period, i.e., evidence of the physiological function of PRL on PRL-R.

There are relatively few data available to discuss the sensitization/desensitization phenomenon of immune cells in the hyperprolactinemic condition besides that of other target cells *in vivo* [14, 15], or *in vitro* [16]. In these studies, receptor expressions were analyzed separately in either mRNA or protein levels, whereas our study evaluated only the mRNA levels. Moreover, some data revealed that a transcriptional activity was independent of its translational activity and that the translated proteins have different isoforms encoded by a single gene, such as the long and short types, with speculated different functions in certain species [17, 18]. In these studies, there are some membrane-bound isoforms generated by alternate splicing as well as a soluble form by proteolytic cleavage of the extracellular domain of the receptor. While the prototypic member of the family, the human long form is 590 amino acids long and represents the entire spectrum of signaling capabilities attributed to PRL-R, some short forms may act in a dominant negative fashion to attenuate the function of the long form. The function and transcriptional regulation should be further clarified in each tissue-specific context. Moreover, PRL-Rs of cancer cells were influenced by not only PRL levels but also by estrogen and progesterone in a complicated fashion [18]. Even if we could successfully fix a time factor, as mentioned above, such hormonal levels might differ in each individual case. Further analyses should be done while paying careful attention to both transcriptional and translational activities and to hormonal conditions with a time-course study. Further analyses should be done while paying careful attention to both transcriptional and translational activities and to hormonal conditions with a time-course study.

In conclusion, pituitary PRL could participate in regulating the expression of the PRL-R gene on cells of the human immune system, however, further

analyses of the desensitization process are warranted to clarify this point.

REFERENCES

1) **Watson CJ, Burdon TG. (1996) Prolactin signal transduction** mechanisms in the mammary gland: the role of the Jak/Stat pathway. Rev Reprod 1: 1–5.
2) **Ben-Jonathan N, Mershon JL, Allen DL, Steinmetz RW. (1996)** Extrapituitary prolactin: distribution, regulation, functions, and clinical aspects. Endocr Rev 17: 639–69.
3) **Bole-Feysot C, Goffin V, Edery M,** *et al.* (1998) Prolactin (PRL) and its receptor: actions, signal transduction pathways and phenotypes observed in PRL receptor knockout mice. Endocr Rev 19: 225–68.
4) **Tamura H, Nakamura Y, Takiguchi S,** *et al.* (1998) Pinealectomy of melatonin implantation does not affect prolactin surge or luteal function in pseudopregnant rats. Endocr J 45: 377–83.
5) **Fujikawa K, Fukuoka H, Alam KS,** *et al.* (2000) Subcutaneously administered prolactin and 20K hGH, but not rGH or 22K hGH, prevent restraint stress-induced gastric ulcers in rats. Endocr J 47: S49–52.
6) **Yu-Lee LY. (1997) Molecular actions of prolactin in the immune** system. Proc Soc Exp Biol Med 215: 35–52.
7) **Dorshkind K, Horseman ND. (2000) The roles of prolactin,** growth hormone, insulin-like growth factor-I, and thyroid hormones in lymphocyte development and function: insights from genetic models of hormone and hormone receptor deficiency. Endocr Rev 21: 292–312.
8) **De Bellis A, Bizzarro A, Pivonello R, Lombardi G, Bellastella A.** (2005) Prolactin and autoimmunity. Pituitary 8: 25–30.
9) **Orbach H, Shoenfeld Y. (2007) Hyperprolactinemia and autoim-**mune diseases. Autoimmun Rev 6: 537–42.
10) **Boutin JM, Edery M, Shirota M,** *et al.* (1989) Identification of a cDNA encoding a long form of prolactin receptor in human hepatoma and breast cancer cells. Mol Endocrinol 3: 1455–61.
11) **Chung HO, Kato T, Kato Y. (1996) Molecular cloning of c-jun** and c-fos cDNAs from porcine anterior pituitary and their involvement in gonadotropin-releasing hormone stimulation. Mol Cell Endocrinol 119: 75–82 (Erratum in: 122: 113–4).
12) **Leite-de-Moraes MC, Touraine P, Kelly PA,** *et al.* (1995) Prolactin receptor expression in lymphocytes from patients with hyperprolactinemia or acromegaly. J Endocrinol 147: 353–9.
13) **Tyson JE, Carter JN, Andreassen B, Huth J, Smith B. (1978)** Nursing-mediated prolactin and luteinizing hormone secretion during puerperal lactation. Fertil Steril 30: 154–62.
14) **Blankenstein MA, Bolt-de Vries J, Coert A,** *et al.* (1985) Effect of long-term hyperprolactinemia on the prolactin receptor content of the rat ventral prostate. Prostate 6: 277–83.
15) **Trott JF, Horigan KC, Gloviczki JM,** *et al.* (2009) Tissue-specific regulation of porcine prolactin receptor expression by estrogen, progesterone, and prolactin. J Endocrinol 202: 153–66.
16) **Brandebourg TD, Bown JL, Ben-Jonathan N. (2007) Prolactin** upregulates its receptors and inhibits lipolysis and leptin release in male rat adipose tissue. Biochem Biophys Res Commun 357: 408–13.
17) **Swaminathan G, Varghese B, Fuchs SY. (2008) Regulation** of prolactin receptor levels and activity in breast cancer. J Mammary Gland Biol Neoplasia. 13: 81–91.
18) **Hovey RC, Trott JF, Ginsburg E,** *et al.* (2001) Transcriptional and spatiotemporal regulation of prolactin receptor mRNA and cooperativity with progesterone receptor function during ductal branch growth in the mammary gland. Dev Dyn 222: 192–205.