Effects of metoclopramide-induced hyperprolactinemia on the prolactin receptor of murine endometrium

FERTILITY AND STERILITY, NEW YORK, v.93, n.5, p.1643-1649, 2010
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The uterus is one of the first extrahypophyseal sites described as a possible source of PRL synthesis and secretion (9). In nonpregnant uteri, PRL synthesis is detected at the peak of the secreting and menstrual phases, coinciding with the first histologic signs of decidualization. If pregnancy occurs, the number of differentiated decidual cells, as well as the synthesis of decidual PRL, increases after implantation, peaking between 20 and 25 weeks, and declining close to term (10, 11).

Received August 7, 2008; revised January 16, 2009; accepted February 9, 2009; published online March 21, 2009.

A.G.Z.R. has nothing to declare. R.C.T.G. has nothing to declare. M.d.J.S. has nothing to declare. R.d.S.S. has nothing to declare. P.B.O. has nothing to declare.

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Jones et al. (12) and Jabbour et al. (13) confirmed the PRL receptor (PRL-R) expression in the endometrium and the decidua. In situ immunohistochemical and hybridization methods revealed that PRL-R is strongly expressed by the glandular epithelium and stromal cells in the decidualized and pseudodecidualized endometrium but minimally so or absent in the proliferative and initial secretory phases. Prolactin-receptor temporal expression is identical to PRL-R endometrial expression. In addition, PRL acutely stimulated anion secretion across the porcine endometrial epithelial cells, possibly through the short isoform of the PRL-R present in both apical and basolateral membranes. This pathway may be regulated by 17β-estradiol (14).

When Stewart et al. (15) studied PRL-R in ovine endometrium during the estrous cycle, they reached the conclusion that endometrial PRL stimulated the development and the function of endometrial glands during pregnancy to aid implantation and placentation. Arie et al. (16), in a detailed study of endometrial PRL behavior, concluded that the concentration of serum PRL was not a correlate of endometrial PRL, and that PRL synthesis in the endometrium was directly related to stromal cell differentiation, progesterone induced in the decidualized endometrium. In our laboratory, the PRL-R was only barely identified through immunohistochemical analyses in rat endometrium after castration (data not published). Tanaka et al. (17) did not identify at all the mRNA of the PRL-R in the liver of castrated animals after estrogen replacement. Because the effect of hyperprolactinemia on the endometrial mRNA PRL-R is not known, there is obviously a need for more knowledge on tissue PRL and its receptors in the endometrium and its interaction with estrogen and PRL.

MATERIALS AND METHODS

Seventy-two virgin, female albino Swiss mice (Mus musculus), approximately 100 days old and weighing about 30 g each, were used. They all came from CEDEME—Centro de Desenvolvimento de Modelos Experimentais da Universidade Federal de São Paulo [Center for the Development of Experimental Models of the São Paulo Federal University]—Escola Paulista de Medicina [Paulista School of Medicine] (UNIFESP-EPM). The project was approved by the UNIFESP-EPM institutional review board (IRB No. 1205/03).

All the 72 animals were previously screened for 7 days with a colpocytologic exam to check for the presence of normal estrous cycles. Of this total, 48 were submitted to bilateral oophorectomy and then housed in cages for a 20-day intervening period of adjustment until the beginning of the experiment. The 24 noncastrated mice were placed together with the others. They were all kept on food and water ad libitum, at 22°C room temperature, with artificial lighting provided by 40-watt daylight fluorescent lamps (Philips, Andover, MA), in a photoperiodic cycle LD 12:12, with light period 7 a.m. to 7 p.m. throughout the experiment.

To perform the oophorectomy, the mice were first anesthetized intraperitoneally with ketamina (ketamine [30 mg/kg]) and xilazina (xilazine [5 mg/kg]) and then submitted to dorsal trichotomy, a longitudinal incision and penetration of the peritoneal cavity. With the aid of tweezers, the ovaries and the distal portions of the uterine horns were isolated from the periovarian fat deposits. After ligation of the ovarian pedicles with cotton thread, the ovaries were removed, and the surgical sites, closed.

Afterward, the mice were randomly divided into six 12-animal groups: (GI) nonoophorectomized mice given vehicle (saline solution 0.9%); (GII) nonoophorectomized mice treated with metoclopramide; (GIII) oophorectomized mice medicated with metoclopramide; (GIV) oophorectomized mice administered metoclopramide along with 17β-estradiol; (GV) oophorectomized mice injected with metoclopramide together with micronized progesterone; (GVI) oophorectomized mice treated with metoclopramide as well as 17β-estradiol and micronized progesterone.

The medications and the vehicle were administered once a day at 10 a.m. for 50 consecutive days via the subcutaneous route. The daily dose of metoclopramide (Sigma, St. Louis, MO) was 200 μg, and the vehicle for it was a 0.9% saline solution. 17β-Estradiol (Sigma) and micronized progesterone (Sigma) were both administered in oil solution at a daily dose of 1 μg and 1 mg, respectively. The dosages of the steroid hormones were established based on a study by Baranão et al. (18).

The administration of the drug or physiologic solution was followed every day by the collection of material from the noncastrated groups for colpocytology. The interpretation of the slides came next after the following preparations: the vaginal content was obtained using a very small spatula with cotton wound around one of the ends; the implement was introduced in the vaginal orifice and rotated to collect material for cytologic analysis. This material was smeared on glass slides, fixed in a 50:50 alcohol:ether solution, and then stained by the Harris-Schorr technique. Thus, the characteristics of the estrous cycle were determined throughout the 50 days of the experiment. On the last day, 1 hour after the administration of the solutions, the animals were decapitated, except for those not in the proestrus phase, which kept on receiving the injections and were killed as soon as they reached the estrous phase.

The mice were killed by guillotine decapitation, and their blood was promptly collected for the determination of PRL and sex steroids. Immediately afterward, the middle portions of the uterine horns were swiftly excised, sectioned, and frozen in liquid nitrogen for the extraction of RNA. The blood samples were centrifuged for 10 minutes at 3,000 rpm, and the plasma was transferred to Eppendorf tubes and frozen at 20°C for later measurement of PRL, estrogen, and serum progesterone by radioimmune assay.

The levels of 17β-estradiol, progesterone, and PRL in the frozen serum were determined by radioimmune assay, using

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double antibodies (ICN Biomedicals Inc, Costa Mera, CA). These measurements were made at the Central Laboratory of the São Paulo Hospital, UNIFESP. Following extraction of the serum, the material was incubated with an antibody labeled with radioactive iodine at 4°C for 16 to 18 hours. Measurements were duplicated. The maximum percentage of the crossreactivity of this kit was lower than 0.01% (specificity).

RNA Extraction
To isolate the total amount of uterine RNA, the samples were homogenized in Trizol reagent (Invitrogen Molecular Probe, Carlsbad, CA) according to the manufacturer’s protocol. The precipitated RNA was washed with 70% ethanol to eliminate salt and phenol residues and then solubilized in diethyl pirocarbonate (DEPC)-treated water. The concentration of the total RNA samples was determined by spectrophotometry using Spectronic Genesys 5 equipment (made in the United States) at a wavelength of 260 nm.

Reverse Transcriptase Reaction
To synthesize cDNA (complementary DNA), RNA pools were made from each group so as to obtain a final concentration of 1 μg/μL from the total RNA extracted. Subsequently, the samples were incubated with oligo dT at 65°C for 5 minutes and again incubated at 50°C for 60 minutes in the presence of reverse transcriptase Superscript III, magnesium chloride, dNTPs, and buffer solution in a final volume of 20 μL of DEPC water. The cDNA thus obtained was stored at −20°C for later analyses.

Semiquantitative Analysis of Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)
The cDNA mentioned above was submitted to a PCR using specific primers for PRL-R isoforms and β-actin genes (Table 1).

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Primer sequences for Semiquantitative Reverse Transcriptase-Polymerase Chain Reaction Analysis (RT-PCR).</th>
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<tbody>
<tr>
<td>Gene</td>
<td>Primer sequences</td>
</tr>
<tr>
<td></td>
<td>(5’ to 3’)</td>
</tr>
<tr>
<td></td>
<td>bp</td>
</tr>
<tr>
<td>Same</td>
<td>Sense AAG CCA AGA CCA</td>
</tr>
<tr>
<td></td>
<td>TGG ATA CTG GAG</td>
</tr>
<tr>
<td>Prl-R-L</td>
<td>Anti-sense AGC AGT TCT</td>
</tr>
<tr>
<td></td>
<td>TCA GAC TTG CCC TT</td>
</tr>
<tr>
<td>Prl-R-S1</td>
<td>Anti-sense AAC TGG AGA</td>
</tr>
<tr>
<td></td>
<td>ATA GAA CAC CAG AG</td>
</tr>
<tr>
<td>Prl-R-S2</td>
<td>Anti-sense TCA AGT TGC</td>
</tr>
<tr>
<td></td>
<td>TCT TTG TTG TGA AC</td>
</tr>
<tr>
<td>Prl-R-S3</td>
<td>Anti-sense TTG TAT TTG</td>
</tr>
<tr>
<td></td>
<td>CTT GGA GAG CCA GT</td>
</tr>
<tr>
<td>β-actin</td>
<td>Sense AAT TGT GGC TGA</td>
</tr>
<tr>
<td></td>
<td>GGA CTT TG 3’</td>
</tr>
<tr>
<td></td>
<td>Anti-sense CAC AGA AGC</td>
</tr>
<tr>
<td></td>
<td>AAT GCT GTC AC</td>
</tr>
</tbody>
</table>

Note: Long (Prl-R-L), Short 1 (Prl-R-S1), Short 2 (Prl-R-S2) and Short 3 (Prl-R-S3); prolactin receptor isoforms (GenBank accession number D10214) and β-actin (GenBank accession number X03672).

Semiquantitative Analysis of Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)
The cDNA mentioned above was submitted to a PCR using specific primers for PRL-R isoforms and β-actin genes (Table 1).

The PCR reaction itself took place in a Perkin-Elmer (a6859; Norwalk CT) thermocycler (Gene Amp PCR System 9700). The amplified products were then submitted to electrophoretic analysis in agarose gel at 2%, which standardized the amounts of cDNA for the subsequent performance of semiquantitative PCR using specific primers for the PRL isoform receptor gene. The expression of the four PRL isoforms

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Determination of serum estradiol, progesterone, and prolactin concentrations in the six groups.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estradiol (pg/mL)</td>
<td>Progesterone (ng/mL)</td>
</tr>
<tr>
<td>GI</td>
<td>119.13 ± 12.33</td>
</tr>
<tr>
<td>GII</td>
<td>122.69 ± 8.45</td>
</tr>
<tr>
<td>GIII</td>
<td>25.82 ± 3.47a</td>
</tr>
<tr>
<td>GIV</td>
<td>125.29 ± 10.01</td>
</tr>
<tr>
<td>GV</td>
<td>28.23 ± 1.85a</td>
</tr>
<tr>
<td>GVI</td>
<td>127.69 ± 7.97</td>
</tr>
</tbody>
</table>

Note: Mean ± SEM: each value is the average of 12 animals per group. (GI) nonoophorectomized mice given vehicle; (GII) oophorectomized mice treated with metoclopramide; (GIII) oophorectomized mice treated with metoclopramide and 17β-estradiol; (GIV) oophorectomized mice treated with metoclopramide and micronized progesterone; (GV) oophorectomized mice treated with metoclopramide and a solution of 17β-estradiol and micronized progesterone.

a P<.01 compared with other groups.
b P<.01 compared with other groups.
c P<.01 compared with other groups.

that is, of the four subtypes L, S1, S2, and S3, was analyzed using a PCR primer corresponding to the first 23 nucleotides of the receptor isoforms. The primer sequences were those used by Ling et al. as shown in Table 1. Thus, 254-bp products were generated for the PRL-R-PCR isoforms.

Protocol Used for Agarose Gel Electrophoresis

The SYBR Gold stain stock (Invitrogen Molecular Probes) was diluted 10,000-fold into TBE buffer (89 mM Tris base, 89 mM boric acid, 1 mM EDTA, pH 8.0) to make a 1 x staining solution. The samples were then incubated in the staining solution for 10 to 40 minutes with the gel in the container. Enough staining solution (50 mL) was made to cover the agarose gel. (To stain large amounts of agarose gel, scale up the volume of staining solution in proportion to the increased gel volume and ensure that all the gel is fully immersed during staining. Protect the staining solution from light by covering it with aluminum foil and agitate the gel gently at room temperature for 10–40 minutes; the optimal staining time is typically 10–40 minutes, depending on the thickness of the gel and the percentage of agarose).

Viewing and Analyzing the Band Intensity

After electrophoresis, the intensity of RT-PCR products was visualized through an ultraviolet Tran illuminator (300-nm transillumination) and the band intensity was analyzed by using an image analysis computer-assisted program (ImageLab Softium, São Paulo, Brazil).

Statistical Analysis

Analyses were performed with one-way analysis of variance and the multiple comparisons of Turkey-Kramer, and their results expressed as means and standard error of the mean. After the quantification of RT-PCR band intensity, the unpaired t test was performed for analyzing the ratios of long isoform (Prl-R-L)/β-actin and short 1 isoform (Prl-R-S1)/β-actin. A significance level of 5% (α) was adopted for all the tests. The descriptive level (P) was calculated and presented for all the statistics.

RESULTS

Hormone Determination

The estrogen and progesterone determination data are annotated in Table 2. The estradiol levels were lower in groups III (oophorectomized mice given metoclopramide) and V (oophorectomized mice treated with metoclopramide and micronized progesterone) in comparison with those of the other groups (P < 0.01). Serum progesterone values were significantly (P < 0.01) smaller in groups III and IV (oophorectomized mice injected with metoclopramide and 17β-estradiol). The PRL levels of group I were significantly lower than those of other groups (P < 0.01). No significant differences were observed among groups II, III, IV, V, and VI.

Only the L and S1 PRL-R isoforms were expressed, and their expressions were limited to groups I (control) and II (mice treated with metoclopramide), those consisting of animals with intact ovaries (Fig. 1). To confirm the extraction and reaction of RT, an electrophoretic analysis of the β-actin gene was performed (Fig. 1). The PRL expression was more intense in group II as was that of its isoform L. There was no expression of the other isoforms of the PRL-R in any of the groups. Figure 2 shows the graphic representation of the amount of DNAc of PRF-R and β-actin as well as the ratios of long isoform (Prl-R-L)/β-actin and short 1 isoform (Prl-R-S1)/β-actin. The amount of long and short 1 isoforms of

FIGURE 1

Result of agarose gel electrophoresis of RT-PCR products stained with SYBR Gold. (A) Detection of long (Prl-R-L) mRNA expression in the uteri from all of the groups; (B) detection of short 1 (Prl-R-S1) mRNA expression in the uteri from all of the groups; (C) detection of β-actin (internal control) mRNA expression in the uteri from all of the groups. GI—nonoophorectomized mice given vehicle; GII—nonoophorectomized mice treated with metoclopramide; GIII—oophorectomized mice treated with metoclopramide; GIV—oophorectomized mice treated with metoclopramide and 17β-estradiol; GV—oophorectomized mice treated with metoclopramide and micronized progesterone; GVI—oophorectomized mice treated with metoclopramide and a solution of 17β-estradiol and micronized progesterone. The gels were subjected to 300 nm transillumination.
GII was significantly higher than GI \((P < .01\)). There is not a difference of \(\beta\)-actin values among the groups.

**DISCUSSION**

Hyperprolactinemia induction in the female mice was performed according to the protocol recommended by Singtrippop et al. (20), who chose metoclopramide from among diverse dopamine antagonists. The investigators administered a daily dose of 200 \(\mu\)g of the drug for 50 consecutive days and detected a longer estrous cycle and higher serum PRL levels.

In our study, the amount of serum PRL was measured at the end of the experiment, and we were able to ascertain that the administered dose (200 \(\mu\)g/animal/day) of metoclopramide was able to produce a hyperprolactinemic state after 50 days. Serum measurements of the sex steroids (estradiol and progesterone) were also performed in all the groups to assess the serum levels of these hormones in the castrated and noncastrated animals. We observed that the serum values of the castrated mice given hormone (estrogen and/or progesterone) therapy were similar to those of the noncastrated mice not subjected to hyperprolactinemia induction.

Prolactin acts in multiple ways depending on the concentration of the ovarian steroids (21–23). Negami and Tomimnaga (24) showed that the effects of PRL on the cohesion and growth of the in vitro epithelial and stromal cells are dose dependent, that is, low concentrations of PRL have a stimulating effect, and high concentrations, an inhibiting effect. It must be emphasized that these studies were conducted in the absence of physiologic concentrations of ovarian steroids.

Another important aspect to understand is the behavior of the PRL-R in animals submitted to a hyperprolactinemia state. In our study, we did not find expression of the PRL-R or any of its isoforms in any of the castrated mice that received metoclopramide. However, those with intact ovaries expressed the PRL-R and its isoform L, and further, that expression was greater in the group given metoclopramide (hyperprolactinemia).
There are studies in the literature focusing on the synthesis and action of PRL in the endometrium; however, few analyze the effect of hypophyseal PRL on the uterine mucous membrane. Nor are there many data comparing and associating serum PRL levels with those of the endometrium, or the latter's behavior in the hyperprolactinemia state. In our study, we found that there was a decrease in the PRL-R expression following treatment with metoclopramide and an absence of expression in the oophorectomized animals. This fact had already been identified in the liver of castrated female mice (17): after removal of the ovaries, not even with estrogen and progesterone replacement at physiologic concentrations did the PRL-R expression occur.

In the study by Tanaka et al. (17), a new exon was identified, namely E15, complementing the previously discovered exons: E11, E12, E13, and E14. It was found in the PRL-R gene of rats by cDNA and genomic clones of DNA in the most proximal position of exon 2, and it was preferentially expressed in the liver, brain, and kidneys. In mice, exons E11, E12, E13, and E15 are located in region 5’ of exon 2 of the PRL-R gene contained in a bacterial artificial chromosome clone derived from the animals’ chromosome 15. Although exons E11 and E13 have been shown to express in cell lineages of Leydig tumors (25), the expression profile of each exon in mouse tissues is still unknown.

What is known is that the PRL-R is present in large concentrations in mouse liver and that sex steroids are strongly influential in its expression levels (26, 27). Its expression increases in female rats and decreases in male rats during sexual maturation (28).

Tanaka et al. (17) observed that exons E12, E13, and E15 contained in the mRNA of rat PRL-R were regulated differently by the sex steroids in the liver. In female rats, the E12 levels of mRNA of the PRL-R rose during the sexual maturity phase, whereas in male rats, the mRNA levels remained very low, regardless of their sexual maturity. The E15 PRL-R-mRNA levels fell with sexual maturity in both sexes, whereas those of E13 PRL-R-mRNA did not change with either sex or age. In the female rat liver, estrogen therapy following castration resulted in the increase in the expression of E12 mRNA-PRL-R and the reduction in the expression of E15. This decrease might interfere in the total expression of the PRL messenger RNA, which would explain why the expression was not detected in our study. Regardless of the estrogen and progesterone levels, it is important to emphasize which substances may influence the expression of the PRL-R, such as androgen, which increases the expression of exon E15, suggesting that androgens are important for PRL-R expression.

Reese et al. (29) observed that defects in the PRL-R might change implantation and endometrial decidualization in female rats. They determined that the occurrence of these phenomena was dependent on the presence of progesterone; in oophorectomized animals, however, despite replacement of this hormone, there were many more cases of deficient decidualization and miscarriages than in those with intact ovaries. They concluded that, as PRL-Rs mediated progesterone production at the ovarian level, they were crucial for implantation and decidualization.

Gu et al. (30) analyzed the PRL-Rs and their regulation by decidua-derived factors in female rats. They observed that both the long and the short forms of receptors are expressed in the decidualized endometrium. In addition, they demonstrated that activin A might cause decidual cells to lose PRL-R expression, but that the presence of a binding α2-macroglobulin (α2MG) protein would recover that expression (30, 31). Activin A is a protein that occurs and expresses itself in the decidua, especially in the preimplantation phase, and is responsible for decidual apoptosis: its presence decreases the expression of the PRL-Rs in the decidua (32). In rats, the sites of greatest α2MG expression are the liver, the ovaries (corpus luteum), the mammary glands, and the decidua (33–35). Prolactin regulates most α2MG expression of ovarian and decidual origin (31, 34, 36). In short, there seems to be a feedback mechanism between α2MG and the PRL-R: the latter would stimulate the former at the ovarian and decidual level, and α2MG would bind to activin A, enabling expression of the PRL-Rs. Hence, it seems that the larger the quantity of α2MG, the greater the expression of the PRL-Rs; the presence of the ovaries appears to be key in this process. This mechanism would partly explain our finding that, in the oophorectomized mice, there was no expression of the PRL-Rs, but in the animals with ovaries submitted to high PRL levels, there was a greater expression of these receptors.

Our results suggest that estrogen and progesterin replacement may not increase the mRNA of endometrial PRL-R in the metoclopramide-induced hyperprolactinemia rats after castration. Thus, future studies along this research line should be conducted to clarify further the molecular mechanisms related to PRL-Rs and their interaction with ovarian steroids in the endometrium.

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