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Successful ovulation in plasminogen-deficient mice treated with the broad-spectrum matrix metalloproteinase inhibitor galardin

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Abstract

Many studies have suggested the hypothesis that the plasminogen activator (PA) system and the matrix metalloproteinase (MMP) system, either separately or in combination, may provide the proteolytic activity that is required for rupture of the follicular wall at the time of ovulation. Our recent studies on ovulation in plasminogen (plg)-deficient mice have, however, shown that plasmin is not required for normal ovulation, leading us to the hypothesis that MMPs may be a more important source of proteolysis for this process. To investigate the role of MMPs and also the possibility of a functional overlap or synergy between the MMP and PA systems during ovulation, we have studied ovulation efficiency in wild-type and plg-deficient mice treated with the broad-spectrum MMP inhibitor galardin. We found that in both wild-type mice and heterozygous plg-deficient ($plg^{+/-}$) mice that had been treated with galardin prior to ovulation, there was a mild (18–20%) reduction in ovulation efficiency. Surprisingly, galardin treatment of plg-deficient ($plg^{-/-}$) mice only caused an additional 14% reduction in ovulation efficiency as compared to vehicle-treated $plg^{-/-}$ mice. Our data therefore suggest that although MMPs may play a role in degradation of the follicular wall, they may not be obligatory for ovulation. In contrast to previous studies on tissue remodeling during wound healing and placental development, we have demonstrated that there is no obvious functional overlap or synergy between the PA and MMP systems, which has previously been thought to be essential for the ovulatory process.

Keywords: Ovulation; Plasminogen activators; Matrix metalloproteinases; Mice

Introduction

Ovulation, the liberation of mature ova, is a fundamental process in mammals. It is required for the propagation of all mammalian species (Espey and Lipner, 1994). The ovulatory process involves follicular rupture, which is triggered by a surge of luteinizing hormone (LH) released from the pituitary, and it recurs in every reproductive cycle in female mammals (Erickson, 1986; Espey and Lipner, 1994). For breakdown of the follicular wall to take place, an extensive proteolytic degradation of basement membranes and connective tissues making up the follicular wall is required. Over the past 3 decades, many studies in both rodents and primates have suggested that two protease families, the plasminogen activator (PA) system and the matrix metalloproteinase (MMP) system, may play an important role in the degradation of the ovarian follicular wall (for references and reviews, see Beers et al., 1975; Strickland and Beers, 1976; Tsafriri and Reich, 1999; Liu et al., 1998; Curry and Osteen, 2001; Ny et al., 2002).

Although a substantial body of indirect evidence obtained from several species has suggested that plasmin has a role in ovulation, our studies in plasminogen (plg)-deficient ($plg^{-/-}$) mice have provided genetic evidence that the absence of plasmin has only a mild effect on ovulation, manifested as an insignificant (13%) reduction in ovulation efficiency (Ny et al., 1999). This marginal reduction in ovulation efficiency may also be caused by delayed maturation of the $plg^{-/-}$ mice, seen as reduced body and ovarian weight (Ny et al., 1999). Based on this

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genetic evidence, we concluded that although the PA system may participate in the ovulatory process in mice, the role it plays is not indispensable. MMPs are also widely expressed in the ovary during the periovulatory period and many reports suggest that they may play an important role in rupture of the follicular wall during ovulation (for reviews, see Woessner, 1991; Tsafriri and Reich, 1999; Curry and Osteen, 2001; Ny et al., 2002). In other biological or pathological processes requiring proteolysis, including angiogenesis, tumor metastasis, mammary gland involution, adipose tissue development, wound healing, and placental development, a coordinated function between the PA and the MMP systems has been shown to be required (Saksela and Rifkin, 1988; Mignatti and Rifkin, 1993; Khokha et al., 1995; Lund et al., 1999; Lijnen et al., 2002; Ny et al., 2002; Solberg et al., 2003). For example, when MMP activities were effectively suppressed in $plg^{-/-}$ mice by treatment with the synthetic MMP inhibitor galardin (GM6001), the processes of wound healing (Lund et al., 1999) and placental development (Solberg et al., 2003) were completely blocked. Plasminogen deficiency alone, or galardin treatment alone, had much less effect. The pronounced synergism indicates that one or more proteolytic events are essential for these processes, and that these critical proteolytic events can be the result of either plasmin activity or MMP activity (Lund et al., 1999; Solberg et al., 2003). Inspired by these studies, we wanted to test whether there is a functional overlap or synergy between plasmin and MMPs in follicular rupture during ovulation. We thus studied the effect of the broad-spectrum MMP inhibitor galardin on ovulation in wild-type and $plg^{-/-}$ mice.

Materials and methods

Materials

Pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) were purchased from Sigma (St. Louis, MO). McCoy's 5A medium (modified, without serum) was purchased from Invitrogen (Gaithersburg, MD). The gelatinolytic activity assay and purified human MMP-2 were purchased from Roche Molecular Biochemicals (Mannheim, Germany). S-2251, a chromogenic substrate for plasmin activity, was purchased from Chromogenix (Mölndal, Sweden). Ninety-six-well plates (Nunclon) were obtained from Nunc A/S (Roskilde, Denmark), and the microtiter plate reader Multiscan RC was from Labsystems (Stockholm, Sweden).

Animals

C57BL/6 wild-type mice were obtained from Bomholtgård Breeding and Research Center (Ry, Denmark). The plg-deficient mice used were as previously described (Ploplis et al., 1995). The $plg^{-/-}$ mice, and also their plasminogen heterozygous ($plg^{+/-}$) and plasminogen wild-type ($plg^{+/+}$) F2 littermates, were obtained from $plg^{+/-} \times plg^{+/-}$ breeding (backcrossed 10 generations to C57BL/6). The mice were genotyped as previously described (Ny et al., 1999). The mice were kept on a 12-h light/12-h dark cycle with the light cycle initiated at 06.00 h, and were fed chow and water ad libitum. Experimental protocols were approved by the regional ethical committee of Umeå University.

Gonadotropin-induced ovulation in mice

To induce synchronized follicular growth and ovulation, immature 25-day-old female mice were injected i.p. with 1.5 IU of PMSG to stimulate follicular development, and with 5 IU hCG 48 h later to induce ovulation. Ovulation normally takes place 10–12 h after hCG treatment (Rugh, 1990; Ny et al., 1999). Animals were sacrificed by cervical dislocation at selected time points after administration of hCG. To record the number of ova, the mice were sacrificed 20 h after hCG treatment and ova in the oviducts were counted as previously described (Leonardsson et al., 1995; Ny et al., 1999). Body weights and ovarian weights were also recorded.

Treatment with MMP inhibitor

The peptide hydroxamate MMP inhibitor galardin (Grobelny et al., 1992) was dissolved in 4% (w/v) carboxymethyl cellulose in 0.9% saline, which gave a final concentration of 20 mg/ml. At the time of hCG injection, galardin (100 mg/kg body weight) was administrated i.p. This dosage of galardin has been proven to effectively inhibit MMP activities and to disrupt wound healing and placental development in plg-deficient mice (Lund et al., 1999; Solberg et al., 2003). The same volumes of vehicle (carboxymethyl cellulose) were injected as controls. In a separate experiment, 200 mg/kg body weight of galardin was also administered during follicular development at 3 time points: at the time of PMSG administration, 24 h after PMSG administration, and at the time of hCG administration. The same volume of vehicle (carboxymethyl cellulose) was used as control.

Preparation of ovarian extract

Ovaries were dissected free of adhering tissue, washed several times in cold McCoy's 5A medium, and kept at −80°C for further analysis. To ensure that the mice responded properly to hormone treatment, only mice with a body weight of 10 g or more and with ovaries with a total weight of 4 mg or more were used in the experiments. Ovaries (n = 8–16) were transferred to a pre-cooled homogenizer and extracts were prepared on ice, followed by centrifugation at 14,000 rpm for 10 min at 4°C. The supernatants were then collected and protein concentrations were measured using the bicinchoninic acid (BCA) protein assay.

Gelatin zymography

Ovarian extracts (15 μg of total protein) were analyzed by gelatin zymography in 7.5% SDS-polyacrylamide (SDS-PAGE) gels containing 1.8 mg/ml gelatin, as previously described (Masure et al., 1990). After electrophoresis, the gels were incubated in 2.5% (v/v) Triton X-100 for 2 × 20 min to remove SDS, and then incubated in a buffer containing 50 mM Tris, pH 7.5, 5 mM $CaCl_2$, 1% Triton X-100 and 0.02% NaN_3 for 50 h.

Gelatin substrate assay

Aliquots of 15 μg of ovarian extract were tested for gelatinolytic activity, using an MMP assay detecting degradation of biotinylated gelatin. The assay was performed according to the manufacturer's instructions (Roche). Briefly, MMPs in ovarian extracts were activated immediately prior to use by 2.5 mM aminophenylmercuric acetate (APMA) at pH 7.0–7.5. Ovarian extracts containing activated MMPs were incubated at 37°C for 4 h in 50 mM Tris, 1 mM $CaCl_2$, 0.05% (v/v) Triton X-100, pH 7.5, with biotin-labeled gelatin. The sample was then transferred to a streptavidin-coated microtiter plate and incubated at room temperature for 30 min. After washing, the plate was incubated with a streptavidin–peroxidase conjugate, which would bind to remaining free biotin residues on the biotin-labeled gelatin. Finally, the remaining biotin residues could be detected after incubation with a color substrate (ABTS®). Absorbance at 405 nm was measured for 30 min at 5 min intervals with a microtiter plate reader. In this assay, high gelatinase activity yields low absorbance, while low activity results in high absorbance.

In situ gelatinase zymography

In situ gelatinase zymography was performed on 10-μm cryosections as described by Leco et al. (2001). Briefly, the sections were covered with a layer of 0.5% low melting point agarose gel containing substrate buffer and 100 μg/ml fluorescein-conjugated DQ gelatine from Molecular Probes Inc. (Eugene, OR), overlayed with coverslips, and incubated in a humidity chamber at 37°C for 16 h. As a negative control, 10 mM EDTA, which chelates Zn^{2+} and Ca^{2+}, was