Vasoinhibins, N-terminal mouse prolactin fragments, participate in mammary gland involution

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Abstract

Vasoinhibins are a family of peptides that act on endothelial cells to suppress angiogenesis and promote apoptosis-mediated vascular regression. Vasoinhibins include the N-terminal fragments from prolactin (PRL), GH, and placental lactogen. One of the vasoinhibins, the N-terminal PRL fragment of 16 kDa, is generated by the lysosomal representative protease cathepsin D (Cath D). Because the normal growth and involution of the mammary gland (MG) are profoundly affected by the expansion and regression of blood vessels and also because PRL stimulates the growth and differentiation of MG, we proposed that intact PRL produced during lactation contributes to MG angiogenesis and increased blood flow, whereas during involution, the N-terminal PRL fragment would have proapoptotic effects on mammary epithelial cells (MECs). Therefore, we investigated the production of the N-terminal PRL fragment and its direct effect on the MG. Mouse PRL (mPRL) was proteolytically cleaved by Cath D between amino acids 148 and 149. N-terminal PRL fragment and Cath D expression increased during MG involution. Furthermore, incubation of MG fragments and MCF7 with recombinant 16 kDa mPRL revealed a proapoptotic effect in MECs. Ectopic mPRL in MECs was cleaved to 16 kDa PRL by Cath D in the MG lysosomal fraction. The majority of PRL derived from pituitary gland was cleaved to 16 kDa PRL in culture medium. Therefore, N-terminal PRL fragment increases during the involution period, has a proapoptotic effect on MECs, and is mainly generated by secreted Cath D in the extracellular space of MG.

Key Words

- ▶ prolactin
- ▶ ectopic expression
- ▶ mouse
- ▶ mammary gland

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Introduction

The development and functional differentiation of the mammary gland (MG) are governed by the coordinated action of estrogen, progesterone, and prolactin (PRL) (Hovey *et al.* 2002). PRL is a 23 kDa polypeptide hormone, produced mainly in the anterior lobe of the pituitary gland, which stimulates the growth and differentiation of mammary epithelial cells (MECs) via the PRL receptor

(PRLR). It has been reported that PRL can be detected in lactating MECs and in milk and that these extra-pituitary PRLs have a similar function to pituitary-derived PRL (BenJonathan *et al.* 1996, Hovey *et al.* 2002).

The action of PRL is complicated by the fact that PRL itself is angiogenic, but N-terminal PRL fragments are antiangiogenic. The family of N-terminal peptide fragments

derived from PRL, growth hormone (GH), and placental lactogen (PL) was named vasoinhibins by Clapp *et al.* (2006). Experiments with recombinant proteins revealed that vasoinhibin derived from human PRL is proteolytically cleaved by cathepsin D (Cath D), matrix metalloproteases (MMP1, MMP2, MMP3, MMP8, MMP9, and MMP13), or bone morphogenetic protein 1 (Clapp *et al.* 2006, Ge *et al.* 2007). Cleavage of rat PRL by Cath D produces a single 16 kDa vasoinhibin (amino acids (aa) 1–145), whereas from human PRL, this enzyme generates vasoinhibins 1–132 (15 kDa), 1–147 (16.8 kDa), and 1–150 (17.2 kDa). All these cleavages of full-length native PRL by proteases generated N-terminal 14–18 kDa fragments (Cruz-Soto *et al.* 2009).

Similar to most antiangiogenic factors, the N-terminal PRL fragment vasoinhibin blocks various steps of the proangiogenic process by mechanisms that involve interference with growth factor activity, inhibition of protease production, and stimulation of endothelial cell apoptosis (Clapp *et al.* 2006). Although the MG is a main target organ of PRL, there has been little reported about the function of vasoinhibin. Data available to date indicate that low levels of Cath D activity are insufficient to generate vasoinhibins and that PRL exerts proangiogenic effects in the MG during pregnancy and lactation (Clapp *et al.* 2008). On the other hand, breast cancer cell lines expressing 16 kDa PRL exhibited inhibition of angiogenesis *in vivo* but no reduction in tumor size or formation (Faupel-Badger *et al.* 2010).

Conversely, high levels of Cath D activity during involution would favor the generation of the N-terminal mouse PRL (mPRL) fragment and thus would inhibit blood vessel growth and promote vascular regression after weaning. To clarify these issues, we investigated the endogenous levels of PRL and N-terminal mPRL fragment in the MG and the relative contribution of Cath D and its direct effect on MECs.

Materials and methods

Animals

This study complied with the guidelines for animal experiments of our institution. The ICR mice were housed under controlled-temperature conditions ($22 \pm 2^\circ\text{C}$) in an artificially illuminated room (12 h light:12 h darkness cycle). Food and tap water were freely available. The day on which a vaginal plug was first observed was designated as day 0 of pregnancy, and the day of birth was designated as infant day 0. Mice were killed under anesthesia and removed organs were stored at -80°C until use.

Hormones, enzymes, antibodies, and inhibitors

mPRL was obtained from Dr A Parlow, National Hormone and Pituitary Program (Torrance, CA, USA). Biotinylated mPRL (mPRL^{biot}) was prepared using a Biotin Labeling Kit (Roche Diagnostics K.K.). mPRL^{biot} was quantified using a 1/10 000 dilution of streptavidin–HRP. The 16 kDa mPRL recombinant (1–145mPRL) was produced in *Escherichia coli* and purified via its His tag (Kitayama Labs Co., Ltd, Nagano, Japan). As the 1–145mPRL preparation contained a very low concentration of endotoxin (0.028 EU/mg protein), it was judged suitable for *in vitro* experiments.

Cath D from bovine spleen and the aspartic protease inhibitor, pepstatin A (Pep A), were from Sigma–Aldrich. Thrombin was from GE Healthcare Japan K.K. (Tokyo, Japan), and MMP8 was from R&D Systems, Inc. (Minneapolis, MN, USA). Rabbit antiserum against the oligopeptide corresponding to aa 39–56 of mPRL (N-PRL Ab) was generated in our laboratory and recognized both the full-length PRL and the N-terminal mPRL fragment. Cath D-purified goat polyclonal antibody (Ab) (Santa Cruz Biotechnology, Inc.) recognized immature (52–60 kDa), intermediate (48 kDa), and mature (33 kDa) Cath D. Two secondary antibodies, biotin-conjugated goat anti-rabbit (Vector Laboratories, Burlingame, CA, USA) and donkey anti-goat IgG–HRP Ab (R&D Systems, Inc.), were used for immunoblotting. mPRL^{biot} recognized streptavidin conjugated with peroxidase (Immuno Bio Science Corp., Mukilteo, WA, USA).

Preparation and incubation of MG fragments

MG fragments were prepared from day 14 of lactation and day 3 of weaning. Connective and adipose tissues were removed from the MG, and then the MG was cut into small fragments with a surgical knife. MG fragments (100 mg) were incubated in 200 μl Medium 199 (Life Technologies), pH 7.5, at 37°C at 95% relative humidity and 5% CO_2 . After the incubation period, cultured MG fragments were used for each assay.

Cell cultures

The human breast cancer-derived cell line MCF7 and the vascular endothelial cell line normal human umbilical vein endothelial cells (HUVECs) were obtained from Cell Bank (RIKEN BioResource Center, Ibaraki, Japan) and American Type Culture Collection (ATCC, Manassas, VA, USA) respectively. Cells were grown in DMEM with 10% fetal bovine serum and 100 units

penicillin/streptomycin as a monolayer at 37 °C at 95% relative humidity and 5% CO₂. Subsequently, cultured cells were used for the TUNEL assay.

PRL cleavage analyses

The activity of the enzymes that cleave mPRL into the N-terminal mPRL fragment was assessed by incubating 2 µg mPRL with Cath D in incubation buffer (0.1 M citrate-phosphate buffer, pH 3.5) or with thrombin (0.1 M Tris-HCl, pH 7.4) or MMP8 in incubation buffer (0.05 M Tris-HCl, 0.15 M NaCl and 0.01 M CaCl₂, pH 6.5, 7, or 7.5).

mPRL cleavage was assessed by incubating 2 µg mPRL with 2 µg of protein from each cell fraction in a final volume of 20 µl for 12 h at 37 °C. Inhibition of Cath D activity by the aspartic protease inhibitor, Pep A (final concentration, 1.4 nM), was investigated by preincubating each of the above test mixtures for 10–30 min with Pep A at room temperature. Each reaction was stopped by adding sample buffer (0.5 M Tris-HCl (pH 6.8), 20% (v/v) glycerol, 1% (w/v) SDS, 1% (v/v) 2-mercaptoethanol, and 0.01% (w/v) bromophenol blue), followed by boiling the samples for 5 min prior to their being applied to a 15% SDS-PAGE gel.

Amino acid sequencing

Amino acid sequence analysis was performed on the major protein bands that reacted with mPRL and MG lysosomal fractions (5 µg of protein) incubated with 600 ng of Cath D adjusted to a final volume of 50 µl with incubation buffer, pH 3.5. After incubation, samples were resolved under reducing conditions using 15% SDS-PAGE. The proteins were transferred to Sequi-Blot PVDF membranes (Bio-Rad K.K.), visualized with 0.1% Coomassie blue R-250, excised, and sequenced by automated Edman degradation (Hokkaido System Science Co., Ltd, Hokkaido, Japan).

Immunoblotting

Each sample was disrupted ultrasonically using tissue protein extraction reagent and a protease inhibitor cocktail kit (Thermo Fisher Scientific K.K., Yokohama, Japan) and separated by centrifugation at 15 000 *g* for 10 min. Proteins were quantitated using a 2-D Quant Kit (GE Healthcare).

An equivalent amount of sample buffer was added to the supernatants, and the mixture was heated at 95 °C for 5 min. The separated proteins were electrophoretically transferred onto Immobilon-P membranes (Nihon Millipore K.K., Tokyo, Japan) and incubated with the primary

antibodies, N-PRL Ab (1:1000) or Cath D (1:50 000). After washing with TBST (50 mM Tris, 100 mM NaCl, 0.1% v/v Tween 20, and pH 7.4), the membranes were incubated with secondary antibodies (1:5000). The signals were visualized using an ECL Plus Western Blotting Detection System (GE Healthcare) and LAS-4000 (Fuji Film K.K., Tokyo, Japan).

Histochemistry

For histochemistry studies, MG and MG fragments were fixed in 4% paraformaldehyde overnight and embedded in paraffin. Sections 6 µm thick were cut on a microtome and mounted on FRONTIER-coated slides (Matsunami Glass, Inc., Ltd, Osaka, Japan) and were used for hematoxylin-eosin (HE) staining and immunohistochemistry. MG sections were incubated with primary antibody N-PRL Ab (1:200) and secondary antibodies. Slides were examined under a BioRevo microscope (Keyence Corporation, Tokyo, Japan).

TUNEL assay and cell counting

MCF7 and HUVEC were seeded on an eight-well culture slide glass (Japan BD K.K., Tokyo, Japan), and cultured cells were fixed with 4% paraformaldehyde for 1 h. In the presence or absence of 7.65 µM 1–145mPRL, MG fragments and cell lines were incubated for 8 and 3 h respectively.

Apoptotic nuclei were detected by TUNEL using an *In Situ* Cell Death Detection Kit (Roche Diagnostics K.K.). Slides were counterstained with 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI; Vector Laboratories) to confirm the apoptotic morphology of individual nuclei. Nuclei with TUNEL-positive apoptotic morphology were counted using TUNEL/DAPI staining using the dynamic cell count function of the BIOREVO microscope. High-power fields (400×, 200 µm×200 µm, and five fields per section) were examined to calculate the frequency of TUNEL/DAPI-positive nuclei.

Microdissection and RT-PCR

The MECs, anterior pituitary gland, and posterior pituitary gland from female mice were collected from frozen MG and pituitary gland by microdissection (Leica Application Solutions Laser Micro Dissection Systems, Leica Microsystems Japan, Tokyo, Japan). Total RNA was isolated using an RNeasy Micro Kit (Qiagen K.K.). First-strand cDNA synthesis from 1 µg total RNA was performed using

a QuantiTect RT Kit (Qiagen) according to the manufacturer's protocol. The cDNAs were then subjected to PCR amplification by 35 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, with TaKaRa Taq DNA polymerase (Takara Bio K.K, Shiga, Japan) and a GeneAmp PCR system 9600 (Bio-Rad). For mouse *Prl*, the nucleotide sequences of the primers were the following: sense, 5'-CAAGCCCT-GAAAGTCCCTCCGGAAG-3' and antisense, 5'-CTCA-GAAAGAGATGGACTGAATGT-3'. The predicted size of the PCR product was 418 bp. For mouse *Prlr*, the nucleotide sequences of the primers were the following: sense, 5'-CAAGCCAGACCATGGATACTGGAG-3' and antisense, 5'-AGCAGTTCTTCAGACTTGCCCTT-3'. For *Cathd* (*Ctsd*), the nucleotide sequences of the primers were as follows: sense, 5'-CGTCTTTGATACTGGCTCCT-3' and antisense, 5'-GCCTTTGACTGGTCAGACTT-3'. The predicted size of the PCR product was 219 bp. The control RT-PCR included glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) primers: sense, 5'-CACCCAGAAGACTGTGGA-3' and antisense, 5'-TGTTGAAGTCGCAGGAGA-3'. The predicted size of the *Gapdh* PCR product was 313 bp. To test the specificity of the RT-PCR assay, negative control experiments were performed without reverse transcriptase in each reaction buffer.

Subcellular fractionation

MG (200 mg) was homogenized in 0.25 M sucrose solution using a Teflon homogenizer, centrifuged at 600 *g* for 10 min, and the precipitate containing nuclei was collected. The supernatant was centrifuged at 8000 *g* for 10 min, and the precipitate containing mitochondria was collected. The supernatant was centrifuged again at 105 000 *g* for 1 h and the precipitate containing microsome was collected. The collected fractions were dissolved in 1 ml 0.1 M Tris-HCl (pH 7.5), and proteins were quantified using 2-D Quant Kit.

In vitro cleavage of mPRL and Cath D secretion

MG fragments from day 3 of weaning were incubated with mPRL^{biot} to track the additional mPRL. MG fragments were incubated for 12 h with mPRL^{biot}, mPRL^{biot}, and Pep A. MG fragments were also incubated with phosphate buffer as controls. Then, streptavidin-HRP was used to detect the biotin of cleaved mPRL^{biot} in the MG fragments and the cultured medium separated by SDS-PAGE. Cath D Ab was used to detect the mature (33 kDa) Cath D in the MG fragments and the cultured medium by immunoblotting.

Statistical analyses

Student's *t*-test was used to test for differences between the means, and all data are expressed as mean $\pm$ S.E.M.

Results

Protease PRL cleavage

Incubation of mPRL with Cath D resulted in its partial conversion into two fragments with apparent molecular masses of 16 and 6 kDa as revealed by SDS-PAGE (Fig. 1A). The amount of mPRL fragments increased dose dependently with Cath D and the reaction was inhibited by Pep A (Fig. 1A). We also investigated the potential involvement of MMP8 and thrombin in mPRL cleavage, but observed no proteolysis products (Fig. 1B). Figure 1C shows that, under reducing conditions, the N-terminal mPRL fragment sequence, LPIC, remains the same as that of undigested mPRL, verifying that it corresponds to the N-terminal fragment. The ~6 kDa fragment had the N-terminal sequence SQLPS, corresponding to residues 149–153, indicating that this peptide is the C-terminal mPRL fragment.

Vasoinhibin and Cath D expression level in MG

The molecular sizes of the proteins that reacted with N-PRL Ab from MGs in various physiological states were ~14 kDa, 23 kDa, and several bands of roughly 23–28 kDa. We investigated the bands in the 23–28 kDa range, whether these represented non-specific or specific proteins (data not shown).

The N-terminal mPRL fragment expression level gradually increased during the involutional period, peaking on day 3 after weaning, but the N-terminal mPRL fragment molecular size was mainly 14 kDa with a small amount of 16 kDa (Fig. 2A). The expression level of mature Cath D (33 kDa) gradually increased and peaked at W3, similar to the N-terminal mPRL fragment expression pattern (Fig. 2B).

Effects of 1–145mPRL on MG fragments and MCF7

mPRL and the N-terminal mPRL fragment were detected in apoptotic MECs after weaning, and the total number of apoptotic cells peaked after 24–30 h. Four days after weaning, the parenchyma consisted mainly of ducts, vessels, and clusters of epithelial cords, some with small lumina, all surrounded by condensed connective tissue and increasing numbers of adipocytes as determined by

Figure 1

mPRL protease cleavage and N-terminal sequences. (A) Effect of Cath D concentration on mPRL cleavage. (Mr) molecular mass marker (masses given in kDa at left and right of blot) (1) mPRL 2 μ g, (2) mPRL 2 μ g + Cath D 80 ng + Pep A 8 ng, (3) mPRL 2 μ g + Cath D 80 ng, (4) mPRL 2 μ g + Cath D 120 ng, and (5) mPRL 2 μ g + Cath D 150 ng. (B) Effects of thrombin and MMP8 on mPRL cleavage. (Mr) molecular mass marker (masses given in kDa at left and right of blot) (1) mPRL 2 μ g + thrombin 0.05 units, (2) mPRL 2 μ g

+thrombin 0.5 units, (3) mPRL 2 μ g + thrombin 1 unit, (4) mPRL 2 μ g + MMP8 0.4 ng, (5) mPRL 2 μ g + MMP8 4 ng, and (6) mPRL 2 μ g + MMP8 8 ng. (C) Amino acid sequence analysis of the site of mPRL cleavage by Cath D. mPRL consisted of 197 amino acids and was cleaved between residues 148 (Trp) and 149 (Ser) by Cath D. (A) and (B) shows a representative result from three independent experiments.

HE staining and immunohistochemistry using N-PRL Ab (data not shown). These results raised the possibility that the N-terminal mPRL fragment participates in MG involution. However, N-PRL Ab also recognizes PRL and is not specific for the N-terminal mPRL fragment. We therefore investigated the effect of 1–145mPRL on MEC apoptosis, with use of MG fragments from day 14 of lactation. The number of apoptotic nuclei had significantly increased when incubated with 1–145mPRL compared with when incubated with 1–145mPRL dilution buffer (Fig. 3A). MCF7 was used for the purpose of investigating whether 1–145mPRL acts directly on MECs or via vascular endothelial cells. The number of apoptotic nuclei of MCF7 was significantly increased by the addition of 1–145mPRL compared with the addition of 1–145mPRL dilution buffer (Fig. 3B). We also investigated the effect

of 1–145mPRL on HUVEC directly tested in a similar manner. The number of apoptotic nuclei of HUVEC was significantly increased by the addition of 1–145mPRL, and we consider that cultures/chamber slide analyses would be appropriate (data not shown).

PRL, PRLR, and Cath D mRNA expression

RT-PCR was performed for mouse *Prl*, *Prlr*, and *Cath D* from MECs obtained by laser microdissection at various physiological states. *Prl* was expressed during the virgin, pregnancy and lactational periods and especially during the involutional period, although the levels of expression were very low. *Prlr* was expressed at all periods and increased during the lactation period from the beginning of pregnancy (Fig. 4). *Cath D* was ubiquitously expressed (Fig. 4).

Figure 2

Immunoblotting of PRL, N-terminal mPRL fragment, and Cath D in MG. (A) Immunoblot analysis of various physiological states of mouse MG with N-PRL Ab. The molecular sizes of the protein bands show that the reactants were full-length PRL (23 kDa) and the N-terminal mPRL fragment. (B) Immunoblots of various physiological states of mouse MG tissue reacted with Cath D Ab. The molecular sizes of the protein bands show that the reactants were mature Cath D (33 kDa) and intermediate Cath D (48 kDa).

Mr, molecular mass marker (masses given in kDa at left and right of blots); P10, day 10 of pregnancy; L10, day 10 of lactation; W0, delivery day; W1–5, day 1–5 of weaning; Pit, mouse pituitary (female L10); in (A) the positive control (PC) was mPRL incubated with Cath D, at 37 °C for 10 min; in (B) the PC was mouse liver (female L10). In (A) and (B), total protein for each column was quantified and equivalent amounts were applied. (A) and (B) each show a representative result from three independent experiments.

mPRL cleavage by intracellular organelle or extracellular space of MG

To identify whether mPRL cleavage occurred in the intracellular or extracellular compartment of involutational MG, we performed cell fractionation and fragment cultures of MG.

Incubation of mPRL with the MG lysosomal fraction at pH 3.5 resulted in its partial conversion to fragments with apparent molecular masses of 16, 10, and 8 kDa. However, the ability of Cath D to cleave mPRL was not specific to the involutational period of MG. No proteolysis

was observed when mPRL was incubated at neutral pH or in the absence of MG lysosomal fraction (Fig. 5A). Addition of Pep A, an inhibitor of Cath D, to the incubation mixture completely abolished proteolytic cleavage by the MG lysosomal fraction (Fig. 5A). Under reducing conditions, the N-terminal mPRL fragment with the sequence MLPIC and a smaller fragment with the N-terminal sequence SQLPS (starting at residue 148) are generated. These sequences match the Cath D cleavage site of mPRL (Fig. 1C).

In fragment cultures of day 3 after weaning MG with mPRL^{biot} showed the presence of 14 and 17 kDa bands other

Figure 3

Effects of 1–145mPRL on MG fragments and MCF7. Representative images of three independent experiments shown for DNA fragmentation. (A) The blue signals in (a) and (c) show DAPI staining of each right-side panel. The green signals in (b) (–1–145mPRL) and (d) (+1–145mPRL) show TUNEL-positive cells after 8 h incubation with day 14 lactation MG fragments. Bars=50 µm. –1–145mPRL, 8 h after addition of 1–145mPRL buffer; +1–145mPRL, 8 h after addition of 1–145mPRL. (B) The blue signals in (a) and (c) show DAPI staining of each right-side panel. The green signals in (b) (–1–145mPRL) and (d) (+1–145mPRL) show TUNEL-positive cells after

3 h incubation with MCF cells. Bars=100 µm. –1–145mPRL, 3 h after addition of 1–145mPRL buffer; +1–145mPRL, 3 h after addition of 1–145mPRL. Each graph column shows the percentage TUNEL-positive MG fragment cells/DAPI (–1–145mPRL 33.69% and +1–145mPRL 75.39%) or TUNEL-positive MCF7 cells/DAPI (1–145mPRL buffer 20.44% and 1–145mPRL 63.3%). Cell counts were examined in five fields per section and in triplicate. Data were analyzed by Student's *t*-test. **P*<0.01 vs respective control without 1–145mPRL.

Figure 4

Prl, *Prlr*, and *Cath D* mRNA expression. Mr, molecular mass marker; VG, virgin MG; P18, pregnancy day 18 MG; L0, delivery day; L8, lactation day 8; L18, lactation day 18; W3, weaning day 3; A pit, anterior pituitary gland; P pit, posterior pituitary gland; NC, no template control. A result representative of three independent experiments was shown.

than that due to mPRL^{biot} according to screening with streptavidin–HRP. However, these bands were present regardless of the added mPRL^{biot} (Fig. 5B, left), and we could not determine the specificity of these bands. Mature high-molecular-weight Cath D (33 kDa) was detected when mPRL^{biot} was incubated with MG fragment (Fig. 5B, right). MG fragment culture medium incubated with mPRL^{biot} was also screened using streptavidin–HRP. A small amount of the 16 kDa band was detected but disappeared upon addition of Pep A (Fig. 5C, left). Mature Cath D (33 kDa) was detected in mPRL^{biot} conditioned medium (Fig. 5C, right).

Discussion

Vasoinhibins block various steps of the proangiogenic process and induce the apoptosis of endothelial cells. We therefore investigated whether vasoinhibin derived from PRL inhibits angiogenesis of MG involution, in contrast with intact PRL, which participates in lactational MG angiogenesis. N-terminal sequencing of mPRL cleaved by Cath D, expression analysis of N-terminal mPRL fragment and Cath D, TUNEL staining, cell fractionation, and MG fragment culture all verified the function of the N-terminal mPRL fragment.

Human and rat N-terminal antiangiogenic PRL, GH, and PL peptides (vasoinhibins) are generated by proteolytic cleavage near or within the long loop connecting the third and fourth α -helices, comprising residues 138–160 in PRL and 129–153 in GH and PL. As the proteolytic enzyme responsible for generating mPRL was unknown, cleavage experiments and amino acid sequencing of the bands corresponding to these peptides were performed. mPRL is an approximately 22.5 kDa protein comprising 197 residues which form four α -helices organized in an ‘up–up–down–down’ configuration and containing three disulfide bonds. Recombinant mPRL was proteolyzed by Cath D

between aa 148 and 149, generating a 16.5 kDa (digested aa 1–148) fragment. 1–148mPRL was detected by the incubation of mPRL with the MG lysosomal fraction. Cath D cleaved rPRL at two sites (145–146 and 148–149) to generate two fragments, 16 kDa (residues 1–145) and 16.5 kDa (residues 1–148) (Baldocchi *et al.* 1993). If digestion by Cath D was taken to completion, only the 16 kDa PRL fragment is observed in the rat. On the other hand, complete digestion of mPRL by Cath D preferentially produced a fragment corresponding to residues 1–148 (16.5 kDa). These results support the findings of Cruz-Soto *et al.* (2009) who demonstrated the presence of a 16-kDa vasoinhibin in Cath D null mice and a site at aa 148–149 cleaved by Cath D. No significant mPRL cleavage by MMPs was observed.

Immunoblotting results showed that the expression pattern of the N-terminal mPRL fragment was similar to that of Cath D during the pregnancy period and involutional period of MG. In particular, the expression of N-terminal mPRL fragment and mature Cath D during the MG involutional period gradually increased and peaked on day 3 after weaning. The molecular mass of the N-terminal mPRL fragment was 14 kDa, and there has been no report in mouse, but there are several reports in rats. Torner *et al.* (1995) reported that a 14 kDa immunoreactive PRL-like protein was released into the medium by incubated rat neurohypophyseal lobes and could be detected in the circulation of the rat by immunoblotting. In addition, Macotela *et al.* (2006) reported that acidic proteases in rat chondrocytes can cleave PRL to generate predominantly 16- and 14-kDa fragments.

The process of involution has been suggested to occur in the following three stages: i) cessation of milk secretion; ii) collapse of the alveoli and apoptosis of the milk-secreting alveolar cells, followed by clearance of both residual milk and the apoptotic cells; and iii) regrowth of the stromal adipose tissue and return to a structure similar to the postpubertal virgin gland (Monks *et al.* 2008). From histological observation, we examined MG 3 days after weaning, which was the second stage of involution, because the lobulo-alveolar structure had collapsed and apoptosis of the MECs had occurred. Three days after weaning, the expression of N-terminal mPRL fragment and Cath D had peaked, so we thought that the N-terminal PRL fragment might help promote the apoptosis of MECs.

To test this possibility, MG fragments from lactating mice were incubated with 1–145mPRL for 8 h and evaluated by TUNEL. The data indicated that the N-terminal mPRL fragment has a proapoptotic action in MECs. As recently reported (Ferraris *et al.* 2011), vasoinhibin has an anti-proliferative and estradiol-dependent proapoptotic effect

Figure 5

mPRL cleavage by intracellular organelles or extracellular products of MG. (A) mPRL was cleaved by VG and W3 MG microsomal fractions dose-dependently. (Mr) molecular mass marker (masses given in kDa at left and right of blot); (1–3) VG; (4–6) W3. (1, 4) MG microsomal fraction 1 μ g + mPRL 2 μ g. (2, 5) MG microsomal fraction 5 μ g + mPRL 2 μ g. (3, 6) MG microsomal fraction 10 μ g + mPRL 2 μ g. PC, positive control Cath D 200 ng + mPRL 2 μ g. (7) MG microsomal fraction 10 μ g + mPRL 2 μ g + Pep A 30 ng. (8) MG microsomal fraction 10 μ g + mPRL 2 μ g (pH 7.4). NC, negative control Cath D 200 ng + mPRL 2 μ g. (B, left panel) Detection of biotin of cleaved mPRL from MG fragments incubated with mPRL^{biot}. Mr: molecular mass marker (masses given in kDa at left and right of blot), -mPRL^{biot}: addition of phosphate buffer (dilutions of mPRL^{biot}), +mPRL^{biot}: addition of biotinylated mPRL, Positive control (PC): mPRL^{biot} was cleaved by purified Cath D, negative control (NC): mouse pituitary gland. (B, right panel) Detection of

Cath D by immunoblotting from MG fragments incubated with mPRL^{biot}. Mr: molecular mass marker (masses given in kDa at left and right of blot), +mPRL^{biot}: addition of biotinylated mPRL, Liver: positive control for Cath D. (C, left panel) Detection of biotin of cleaved mPRL from MG fragment culture medium incubated with mPRL^{biot}. Mr: molecular mass marker (masses given in kDa at left and right of blot), -mPRL^{biot}: addition of phosphate buffer (dilutions of mPRL^{biot}), +mPRL^{biot}: addition of biotinylated mPRL, PC: mPRL^{biot} was cleaved by purified Cath D, NC: mouse pituitary gland. Pep A⁺: addition of an aspartic proteinase inhibitor. (C, right panel) Detection of Cath D by immunoblotting of MG fragment culture medium incubated with mPRL^{biot}. Mr: molecular mass marker (masses given in kDa at left and right of blot); +mPRL^{biot}: addition of biotinylated mPRL, Liver: positive control for Cath D. (A) and (B) each show a sample representative of three independent experiments.

on anterior pituitary cells. Ferraris *et al.* (2011) generated primary cultures of anterior pituitary cells from OVX rats and then incubated them with vasoinhibin for 4 h followed by TUNEL analysis. However, our experiments with MG fragments cultured with 1–145mPRL for 4 h could not detect significant differences in proapoptotic effects compared with fragments cultured without 1–145mPRL.

However, the two experiments differed in the MG fragments and primary cultures of anterior pituitary cells used. Our results demonstrate that the presence of 1–145mPRL does not trigger the apoptosis of MECs but rather has a proapoptotic effect. Because the source of

N-terminal mPRL fragment in our experiments was derived either from MECs or from vascular endothelial cells, we performed TUNEL assays using MCF7 and showed that apoptotic nuclei increased following the addition of 1–145mPRL, indicating that the N-terminal mPRL fragment acts on MECs directly.

In addition, we investigated the source of N-terminal mPRL fragment in the involutinal MG. Mouse *Pr1* mRNA was ectopically expressed in MECs and increased 3 days after weaning, similar to the results obtained by immunoblotting. However, mPRL was cleaved to vasoinhibin by Cath D in the MG lysosomal fraction regardless of the

physiological state of the MG. Cath D is secreted into the extracellular compartment (Lkhider *et al.* 2004). Rat MECs that secrete Cath D can produce 16 kDa PRL at pH 7 in the extracellular milieu (Castino *et al.* 2008). Under normal conditions, a pericellular acidic pH can be produced by the activity of the Na⁺/H⁺ exchanger and H⁺/ATPase (Piwnica *et al.* 2006). In rat anterior pituitary cells, Cath D was present in PRL secretory granules and has been proposed to be the main protease involved in 16 kDa PRL production (Cruz-Soto *et al.* 2009). Our present data show the cleavage of mPRL to N-terminal mPRL fragment in media from cultures of MG fragments, but this reaction did not occur in the absence of MG fragments, supporting the hypothesis that mature Cath D secreted from MECs produces the N-terminal mPRL fragment in the extracellular milieu.

In summary, our study demonstrated for the first time that N-terminal mPRL fragment generated by secreted Cath D contributes to MG involution.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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