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The Expression of Prolactin and its Cathepsin D-mediated Cleavage in the bovine Corpus luteum vary with the Oestrous cycle

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Running head: PRL cleavage activity in the CL

Abbreviations: PRL, prolactin; 16-K PRL, the N-terminal 16-K fragment of PRL; Cath D, cathepsin D; CL, corpus luteum; EC, endothelial cells; GLC, granulosa-like cells

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Abstract

In the corpus luteum (CL), blood vessels develop, stabilize, and regress. This depends on the ratio of pro- and anti-angiogenic factors, which change during the ovarian cycle. The present study focuses on the possible roles of 23-K prolactin (PRL) in the bovine CL and its anti-angiogenic N-terminal fragments after extracellular cleavage by cathepsin D (Cath D).

Prolactin RNA and protein were demonstrated in the CL tissue, in luteal endothelial cells and steroidogenic cells. Cath D was detected in CL tissue, cell extracts and corresponding cell supernatants. In the intact CL, 23-K PRL levels decreased gradually, whereas Cath D levels concomitantly increased between early to late luteal stages. In vitro, PRL cleavage occurred in the presence of acidified homogenates of CL tissue, cells and corresponding cell supernatants. Similar fragments were obtained with purified Cath D, and their appearance was inhibited by pepstatin A. The aspartic protease specific substrate MOCAc-GKPILF~FRLK(Dnp)-*D*-R-NH_2 was cleaved by CL cell supernatants, providing further evidence for Cath D activity. 16-K PRL inhibited proliferation of luteal endothelial cells accompanied by an increase in cleaved caspase-3.

In conclusion: (1) The bovine CL is able to produce PRL and to process it into anti-angiogenic fragments by Cath D activity. (2) PRL cleavage might mediate angioregression during luteolysis.

Key words

prolactin fragments; lysosomal proteases; acidic microenvironment, extracellular proteolysis; ovary; cow

Introduction

The peptide hormone prolactin (PRL) is involved in a broad spectrum of physiological processes (6, 10, 28). It is synthesized in the pituitary gland and, at many extra-pituitary sites, binds to PRL receptors, which are almost ubiquitously expressed (6, 10, 28). In recent years, interest has grown in the potential involvement of PRL in angiogenesis. Full-length 23-K PRL appears to be pro-angiogenic (82), whereas its N-terminal 14- and 16-K PRL cleavage products appear to be anti-angiogenic (17). These 14- and 16-K PRL cleavage products arise through partial proteolysis by the lysosomal protease cathepsin D (Cath D) (4, 66) like the angiostatic molecule angiostatin by cleaved plasminogen (55, 63).

Lysosomal Cath D is synthesized as an inactive precursor, procathepsin D, in the rough endoplasmatic reticulum. It is translocated to the Golgi apparatus and finally targeted to the lysosomes thanks to the presence of acquired mannose 6-phosphate residues (32). There, procathepsin D is converted to an active 44-47-K single-chain form, which, depending on the species, is further cleaved into an active two-chain form (27). Procathepsin D can escape transfer to lysosomes and be secreted instead into the extracellular space. Secreted procathepsin D is detected at higher levels in supernatants of tumor-cell cultures than in supernatants of normal-cell cultures (55). In malignant tumors, the presence of active Cath D forms in the extracellular space is well documented (42, 90). Furthermore, physiological release of active Cath D forms has recently been described in enriched mammary epithelial-cell preparations (42, 50) and various tissues (65). Proton extruders can trigger PRL cleavage under certain physiological conditions (65). Extracellular Cath D might thus participate in generating bioactive proteins under such conditions. It might additionally be involved in cell migration and proliferation through actions other than proteolytic cleavage (34, 48, 90).

The corpus luteum (CL) is one of the few organs in the adult body that exhibits angiogenesis and angioregression under physiological conditions. Capillary formation and maintenance occur during

formation and functioning of the cyclic CL and the CL of pregnancy (19). Along with other autocrine/paracrine angiogenic factors (e.g. prostaglandin E2, transforming growth factor β1, basic fibroblast growth factor, vascular endothelial growth factor, and angiogenin), 23-K PRL may contribute to establishing the microvascular bed. This is supported by the finding of Gaytan et al. (31) that PRL treatment can enhance the proliferative activity of CL endothelial cells (EC), and even more by the demonstration by Grosdemouge et al. (37) that angiogenesis during CL development is strongly inhibited in mice with targeted disruption of the PRL-R gene.

Blood vessel breakdown and endothelial cell loss are inherent features of CL regression (53). Prostaglandin F2α (PGF2α) of uterine origin is the key luteolytic hormone in hoofed ruminants such as cows (53, 57). It initiates a rapid decline in luteal progesterone (functional luteolysis), followed by structural CL regression (structural luteolysis). It acts in concert with other vasoconstrictors such as endothelin-1 and angiotensin II (75), causing the levels of these factors to rise in the CL (33). Long-term vasoconstriction of luteal arterioles is followed by endothelial injury, endothelial cell depletion, blood vessel occlusion, degenerative changes (54, 58), and influx of cytokine- and protease-releasing leukocytes, notably macrophages (38, 54). The observed changes resemble hypoxic damage in other tissues, in which lysosomes participate (20, 21, 38, 93). Not surprisingly, therefore, in various species (47, 67) including ruminants (23, 81), cells of the regressing CL show an increased lysosome content, increased lysosomal enzyme activity, and release of enzymes from fragile lysosomes into the cytosol of degenerating luteal cells**.** In a hypoxic microenvironment, full-length 23-K PRL is likely to be cleaved into 14- and 16-K PRL and thus to participate in the involution process.

In the present study we have examined whether PRL is synthesized by bovine CL-derived EC and granulosa-like cells (GLC), whether it undergoes posttranslational processing by Cath D, and how full-length PRL and its fragments affect CL-derived EC in vitro. Both studied cell types have been extensively characterized by our group (78-80). We show that PRL is synthesized in the bovine CL and that full-

length PRL declines during the estrous cycle concomitantly with an increase in total active Cath D. We demonstrate that the CL is equipped enzymatically to generate 16-K PRL fragments and that CL tissue extracts do generate PRL fragments similar to those produced by bovine Cath D. Our data suggest that PRL cleavage may contribute to the CL regression mechanism.

Materials and Methods

Bovine ovaries with CL were collected at the local slaughterhouse. Bovine pituitary glands were kindly prepared and provided by Dr. Christine Ellenberger, Institute of Veterinary Pathology, Faculty of Veterinary Medicine, University of Leipzig, Leipzig, Germany.

The CL were assigned to the early (days 1 to 4 after ovulation), mid (days 5 to 17), and late (days 18 to 21) luteal stages of the estrous cycle according to their macroscopic appearance (41). These assignments were confirmed by histologic examination (41, 69).

Corpora lutea pieces to be used in molecular biology experiments were immediately shock-frozen in liquid nitrogen. Corpora lutea for cell isolation were transported in ice-cold phosphate-buffered saline (PBS, pH 7.4) to the laboratory, where they were dissected and mechanically disrupted as described in detail (78-80). Briefly, CL were chopped up into small pieces, first with a scalpel and then with a multi-blade knife. The material was suspended in medium, further broken up by vigorous pipetting, and filtered through a 150-µm-pore-size metal sieve and then a 70-µm-pore-size nylon sieve. Cells having passed through these filters were pelleted and resuspended in DF medium (Dulbecco's Modified Eagle's Medium mixed 1:1 with Ham's F-12 medium, Invitrogen-Gibco Cell Culture Products, Karlsruhe, Germany, adjusted to pH 7.2 with 15 mM Hepes and 22 mM $NaHCO_3$) supplemented with 5% fetal calf serum. Cells were seeded under semiclonal conditions onto 24-well culture plates precoated with collagen I (1% Vitrogen, Nutacon, Leimuiden, The Netherlands). Non-adherent cells and erythrocytes were removed by medium changes at 24 h and every 4 days thereafter. Between weeks one and four, colonies of CL-derived EC and GLC were obtained, trypsinized, dislodged, and transferred to new culture dishes (1:4 split ratio). Pure cultures showing the marker characteristics of either endothelial or steroidogenic cells (Fig 1) were harvested at confluency. The cells were stored in liquid nitrogen until use. At this time, they were replated in either 24- or 96-well culture plates or in 75-cm^2 flasks.

The rat pituitary cell line GH4-C1 was used as a positive control cell line for PRL secretion. It was purchased from the German Resource Centre for Biological Material (DMSZ # ACC 482, Braunschweig,

Germany) and then maintained and propagated in the laboratory in Ham´s F10 medium (Invitrogen-Gibco Cell Culture Products) supplemented with 15% horse serum (Sigma-Aldrich Chemie, Munich, Germany) and 2.5% FCS (Biochrom, Berlin, Germany).

Detection of PRL transcripts

PRL Primer: Primer pairs were chosen from different exons to avoid misinterpretation of results due to possible contaminating genomic DNA. The upstream sense primer (5' - GGGCAGTCATGGTGTCCCACTA – 3') is located in exon 2 (GenBank Accession # AY337764), the downstream antisense primer (5' - AAGTGTCAATCTTGCTTGAATC – 3') in exon 5 (GenBank Accession # M34535) of the bovine PRL gene (15).

RT-PCR: Total RNA was reverse-transcribed and probed with the primer pair. Briefly, total RNA was prepared from shock-frozen CL tissue and cultured cells by means of the pegGold RNAPure ™ Kit (PEQLAB Bitotechnology, Erlangen Germany). This was followed by chloroform extraction and alcohol precipitation. Reverse transcription was carried out in a final volume of 20 µl containing 5 µg DNase-I-treated (2 units / 5 µg RNA) RNA, 500 ng oligo $(dT)_{15}$ primers (Promega, Mannheim, Germany), 4 µl first strand buffer (Invitrogen, Karlsruhe, Germany), 2 µl of 0.1 M dithiothreitol, 1 µl of dNTP mix (10 mM each) 1 µl of RNaseOut (Invitrogen) and 200 units SuperScript ™ Reverse Transcriptase (Invitrogen). Polymerase chain reactions were performed in a reaction mixture (25 µl final volume) containing 1 µl generated cDNA, 0.5 µM sense and antisense primers, 2.5 µl PCR Buffer (10x; Roche, Mannheim, Germany), 2 µl dNTP mix, and 2.5 units Taq DNA Polymerase (Roche). Initial denaturing at 94°C for 4 min was followed by 35 cycles of denaturing at 94°C for 30 sec, annealing at 60°C for 30 sec, extension at 72°C for 30 sec and a final extension at 72°C for 10 min. PCR products were run on 2% agarose gels stained with ethidium bromide, and visualized under UV light. As an internal control, the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified in a separate reaction using the same cDNA and 25 cycles. The corresponding upstream and downstream primers were based on the bovine GAPDH sequence (GenBank Accession # NM_001034034) and were 5' –

TGAAGGGTGGCGCCAAGAGG – 3' and 5' – TGCCAGCCCCAGCATCGAAG – 3', respectively. Amplified PCR products were cloned into pGEM-T (Promega). Transformants harboring inserts of adequate size were identified and analyzed by DNA sequencing and interrogation of GenBank.

Detection of PRL and Cath D proteins and fragments

Preparation of total tissue protein extracts: Shock-frozen tissue pieces were ground with a mortar and pestle and immediately suspended in lysis buffer (10 mM Tris-HCl, 1 mM EDTA, 1 mM $MgCl_2$, 0.02% Triton X-100) containing freshly added protease inhibitors (protease inhibitor cocktail and 1 mM phenylmethylsulphonylfluoride (PMSF), both from Sigma-Aldrich Chemie, Munich, Germany). The suspension was homogenized with the Ultra Turrax T8 homogenizer and centrifuged in a table centrifuge (16'000 x g, 4°C, 15 min). The protein concentration of the supernatant was determined with the Micro BCA Protein Assay Kit (Perbio Science, Bonn, Germany).

Preparation of cultured-cell protein extracts: Cells were cultured to confluency in 75-cm^2 flasks. Culture medium was removed, adherent cells were washed with PBS, scraped off, and transferred to supplemented lysis buffer. The crude cell lysate was homogenized and centrifuged; the protein concentration was determined in the supernatant.

Preparation of culture supernatant concentrates: Cells were grown to confluency in 24-well culture plates. After a 24 h incubation in serum-free culture medium, the supernatants of 4 wells (2 ml) were collected, concentrated in Centricon-YM 10 filter devices (Millipore, Schwalbach, Germany) at 8500 g, and adjusted to a final volume of 150 µl.

Antibodies: For each protein of interest several antibodies were used to investigate protein synthesis and processing. Three different monoclonal anti-bovine PRL antibodies (mAbs) recognizing epitopes at the N- (5G2 and 4C10) or C-terminal end (6F11) of PRL (73) were used to distinguish N-terminal from C-terminal fragments after digestion of the full-length protein. They were obtained from Acris Antibodies GmbH (Hiddenausen, Germany) or kindly donated by Dr. Jonathan G. Scammell (Department of Pharmacology, University of South Alabama, AL, USA). In addition, we used a polyclonal anti-bovine

PRL antiserum. This antiserum is part of the kit distributed by the National Hormone and Peptide Program (NHPP, Dr. A.F. Parlow, Harbor-UCLA Medical Center, Torrance, CA, USA) for RIA determinations of bovine PRL. The ability of the antiserum to react with N-terminal bovine PRL fragments of various size is documented in the literature (15). The monoclonal anti-bovine PRL antibodies were used at a final concentration of 0.2 µg/ml, the anti-bovine PRL antiserum at a final dilution of 1:3'000 for Western blotting.

A rabbit anti-Cath D polyclonal antibody produced in C. Isidoro's laboratory (12), a rabbit anti-human Cath D antiserum Ab-2 obtained from Merck Biosciences GmbH (Bad Soden Germany), and a rabbit anti- bovine Cath D antiserum (51) kindly donated by Dr. Hoflack (Biotechnological Centre, Dresden University of Technology, Dresden, Germany) were used to detect inactive procathepsin D and its cleaved fragments. The antisera were used at the final concentration of 0.1 µg/ml (Ab-2) or at the final dilution of 1:1000.

Protein electrophoreses and blotting: Protein samples were boiled in denaturing loading buffer at 95°C for 5 min. Either 20 µg total protein lysate or 20 µl concentrated supernatant was loaded per lane of a 15 % polyacrylamide–sodium dodecyl sulfate minigel and separated in parallel with a molecular weight marker (Rainbow™ Molecular Weight Marker, Amersham Bioscience, Freiburg, Germany). Electrophoresed proteins were semi-dry blotted onto a 0.45 µm-pore-size nitrocellulose membrane (Biometra, Göttingen, Germany). Protein loading and transfer efficiency were checked by Ponceau Red staining. Destained membranes were blocked with 5 % (w/v) non-fat dried milk powder at RT for 1 h. Subsequently, the protein of interest was labeled overnight at 4 °C with the specific primary antibody or antiserum. This was followed by incubation for 1 h at RT with the relevant peroxidase-conjugated secondary antibody (0.2 µg/ml). Binding sites were visualized on Hyperfilm ECL™ (Amersham Biosciences) by the enhanced chemiluminescent method (ECL Western blotting detection reagents and analysis system, Amersham Biosciences). The blots produced with lysates from tissue and cells were routinely stripped and reprobed for actin, using the mouse monoclonal antibody JLA20 (VWR Deutschland, Darmstadt, Germany) to check for equal loading and transfer of proteins.

Processing of PRL by purified Cath D, CL tissue extracts, and culture supernatant concentrates in vitro

Prolactin cleavage by purified Cath D: Human recombinant 23-K PRL was produced in bacteria (pT7L-PRL vector) and purified as previously reported (61). Purified rat and bovine PRL were purchased from the National Hormone and Peptide Program (NHPP, Dr. A.F. Parlow, Harbor-UCLA Medical Center, Torrance, CA, USA). Cleavage of PRL (150 ng) was performed with Cath D from bovine spleen (Sigma-Aldrich Chemie, Munich, Germany) in 100 µl of 20 mM citrate/phosphate buffer at various enzyme to substrate ratios. The pH was adjusted as needed by mixing the two buffers (0.2 M $NaHPO_4$ and 0.1 M citric acid) in various amounts. Bovine serum albumin (100 µg, BSA) and/or the inhibitor pepstatin A (1.5 µM) were added when specified. Cleavage was allowed to proceed at 37 °C for 3 h. Cleavage products were separated by SDS-PAGE and analyzed with anti-PRL antibodies by Western blotting.

Prolactin cleavage by tissue extracts: Shock-frozen CL pieces were pulverized with mortar and pestle and cultured cells were scraped from the bottom of the flask. The samples were immediately transferred into 7.5 vol (wt/vol) ice-cold 0.25 M sucrose/0.1M Tris-HCl (pH 7.4) buffer and homogenized with an Ultra Turrax T8 homogenizer (IKA Werke, Staufen, Germany). Subsequent successive 10-min centrifugations at 600 x g, 3300 x g, and 25'000 x g resulted in a high-speed pellet, which was washed twice. The pellet was weighed with an analytical balance, resuspended in reaction buffer (50 mM citrate-phosphate/75 mM NaCl) and stored at –80 °C. 2.5 µg PRL was incubated with 50 µg pellet in a final volume of 25 µl reaction buffer at various pH values. The inhibitor pepstatin A (1 µl of a 1.5 µM solution) was added to some of the incubation tubes. Proteolysis was allowed to proceed at 37 °C for 3 h. PRL fragments were electrophoresed, transferred to a nitrocellulose membrane, and analyzed by Western blotting.

Prolactin cleavage by culture supernatant concentrates: Culture supernatant concentrates were prepared as described above. 2.5 µl supernatant concentrate was mixed with 2.5 µl PRL (1 µg/µl) in 20 µl of 50 mM citrate-phosphate/75 mM NaCl buffer at various pH values. Pepstatin A (1 µl of a 1.5 µM solution) was selectively added to the reaction mix. After 3 h of incubation at 37° C, the reaction mix was analyzed for the presence of PRL fragments by Western blotting.

Preparation of lysosomal fractions

CL tissue was pulverized in liquid nitrogen, transferred to TSE buffer (10 mM Tris HCl, 0.3 M Sucrose, 1mM EDTA; pH 7.5), and homogenized on ice with the Ultra Turrax homogenizer. This was followed by three successive centrifugations at 900 x g (5 min), 3000 x g (10 min), 10'000 x g (25 min), respectively. The procedure resulted in pure lysosomal pellets, confirmed by the presence of electron-dense lysosomes observed by transmission electron microscopy in routinely fixed and embedded samples (not shown). The lysosomal pellet was considered to contain membrane "bound" lysosomal enzymes, whereas the resulting post-lysosomal supernatant was considered to contain "free" lysosomal enzymes due to lysosomal fragility (47, 83). For Western blot analysis, the lysosomal pellet was further processed in lysis buffer (PBS, 0.2 % Triton X-100, 20 mM EDTA, protease inhibitors; pH 5.0).

Detection of enzymatic activity

Total aspartic protease activity was detected with the internally quenched fluorescent substrate MOCAc-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)-*D*-Arg-NH2 (where MOCAc is [7-methoxycoumarin-4-yl]acetyl and Dnp is dinitrophenyl, Calbiochem, Merck Biosciences, Darmstadt) (94). The supernatants from 12 wells of a 96 well plate were collected and pooled. The corresponding cell monolayers were treated with 200 µl of 0,9 % Triton-X 100 added to basal medium for 2 min at room temperature in order to disrupt the cell membranes. The cell lysates were collected, pooled and cellular debris removed by centrifuging at 1200 x g for 5 min. Supernatant and the cell lysates were than concentrated with Centricon filter devices as described above. Subsequently 10 µl sample and substrate (20 µM) were incubated for 10 min at 40° C in 50 mM sodium acetate buffer, pH 4.0. The reaction was stopped by addition of 5 % trichloroacetic acid (TCA) and the fluorescence of the intramolecularly cleaved fluorogenic substrate was measured in a monochromator (Molecular Devices, Ismaning/München, Germany). The excitation wavelength was set at 328 nm and the emission monitored at wavelength 393 nm. Results expressed in relative fluorescence units (RFU) were determined by subtracting the RFU measured in the substrate mix alone from the RFU measured in the substrate mix plus supernatant. The experiment was repeated four

times. The values obtained in the individual experiments were averaged and expressed as means with standard deviations.

The release of lactate dehydrogenase (LDH) was measured as an indicator of spontaneous cell lysis. LDH release was assayed with the CytoTox-ONE homogeneous membrane integrity kit (Promega). In this assay, 100 µl culture supernatant concentrate or cell lysate were mixed with 100µl CytoTox-ONE reagent (substrate mix mixed 1:1 with assay buffer) and incubated at RT for 10 min. Stop solution was added. The excitation wavelength was set at 560 nm and the emission recorded at 590 nm to determine the generated fluorescent resorufin product.

Determination of the influence of 16-K PRL on cell number and demonstration of caspase-3 cleavage

To determine whether 16-K PRL alters proliferation of luteal EC, 10'000 cells were seeded per well of a 24-well culture plate. After 24 h and 48 h, the cultures were exposed or not to bovine 23-K PRL (500 ng/ml, NHPP) or to recombinant human 16- or 23-K PRL (500 ng/ml). Generation of these recombinant molecules has been described in detail elsewhere (86). At 24-h intervals, the cells were trypsinized. Cells excluding Trypan Blue were counted with a hemocytometer. The experiment was repeated three times. The values obtained in the individual experiments were averaged and expressed as means with standard deviation.

We assayed EC cultures for cleavage of caspase-3 into its active fragments. To this end, 375000 EC were seeded per 75-cm^2 flask and, at confluency, the cultures were exposed or not for 48 h to bovine 23-K PRL (500 ng/ml) or to recombinant human 16- or 23-K PRL (500 ng / ml), or for 6 h to 200 nM staurosporine. At the end of the exposure, proteins were extracted from the cultures and analyzed by Western blotting with rabbit monoclonal anti-cleaved caspase-3 antibody 5A1 (New England Biolabs, Frankfurt am Main, Germany), which detects endogenous levels of the large fragment (17/19-K) of activated caspase-3 resulting from cleavage adjacent to Asp175. Lysates of cytochrome c-treated or -untreated Jurkat cells (New England Biolabs) were used, respectively, as positive and negative controls.

Reliability and statistical methods

Experiments were performed at least in triplicate. Only representative results are reported.

Statistically evaluated data are depicted as mean values ± SEM. Statistical analysis were done by using Kruskal-Wallis one way analysis of variance on ranks followed by Tukey's test for pairwise comparisons. Statistical computations were performed with Sigma Stat (Systat Software, Erkrath, Germany). Differences are reported as statistically significant at $p < 0.05$.

Results

Classification of bovine CL and identity and purity of CL cell cultures

Corpora lutea of bovine ovaries could be assigned to early (days 1 to 4 after ovulation), mid (days 5 to 17) and late (days 18 to 21) luteal stage by their macroscopic and histologic appearance. A positive factor eight-related antigen labelling confirmed the cells with a cobble-stone morphology to be endothelial cells. Abundant Nil red positive droplets and high 3β-hydroxysteroid dehydrogenase (3β-HSD) activity characterized flat granulosa-like cell cultures (Fig. 1).

PRL is synthesized in the bovine CL.

Although investigators have reported the presence of PRL mRNA in the bovine CL and in CL-derived cells (25, 74), it was necessary to check for the presence of PRL protein itself.

We first confirmed the presence of PRL mRNA in CL tissue (Fig. 2) and cells (EC and GLC, not shown). Amplification of the corresponding cDNA with PRL-specific primers yielded a single band corresponding to a PCR product of the expected length (525 bp), similar to the control RT-PCR bands obtained from bovine pituitary (Fig. 2) and GH4-C1 cells (not shown), both known as sources of PRL (22). Restriction with *NcoI* and *RcaI* yielded the expected digestion products and sequencing of the PCR product cloned into pGEM-T revealed an exact match with the submitted nucleotide sequence of bos taurus PRL at GenBank (Accession # NM_173953, not shown). Although the RT-PCR analysis was not quantitative, the amplified PRL and internal control fragments appeared similar when the results of different CL estrous-cycle stages were compared.

Next, protein extracts of cultured luteal EC and GLC were tested for the presence of PRL by Western blotting. They gave rise to a band of the expected relative molecular mass, similar to that seen with extracts of CL and pituitary tissue and control cells (GH4-C1 cells, Fig. 2), when probed with specific

monoclonal antibodies or with anti-PRL antiserum from NHPP (Fig. 3). The position of the band was also in agreement with the expected molecular mass as calculated on the basis of the published bovine PRL amino-acid sequence deposited at Swiss Prot (Accession # P01239).

In the CL, the PRL and Cath D levels change in opposite directions in the course of the estrous cycle.

We next examined the variation of CL PRL and Cath D levels in the course of the bovine estrous cycle. Prolactin was detected in the CL at all estrous-cycle stages. The intensity of the 23-K PRL band declined with respect to the actin band from early to late luteal stage (Fig. 3). Bands beneath the 23-K band, likely to correspond to PRL fragments, showed only weak staining but became clearly visible when 100 µg protein extract were loaded in the lanes. Blots probed for Cath D showed two intense bands, one at 44 K and one at 30 K, corresponding respectively to the enzymatically active single-chain form and to the heavy chain of the enzymatically active two-chain form (60). In addition to these bands, we occasionally distinguished a very faint band above the 44-K band, which may correspond to the inactive Cath D precursor, procathepsin D (e.g. see Figs. 3 and 5).

In contrast to the bands in the PRL blots, the Cath D bands increased in intensity with respect to the actin bands from early to late luteal stage. Intense Cath D signals were observed in both the lysosomal fraction ("lysosome-bound" Cath D) and the post-lysosomal supernatant ("free" Cath D) obtained from late-stage CL.

Together, these Western blot data indicate that the Cath D protein content increases in the CL as the latter progresses towards the middle and end of its lifespan. Over the same period, the level of 23-K PRL protein decreases. This could mean that 23-K PRL becomes increasingly cleaved towards the end of the CL lifespan. We thus addressed the following questions: can Cath D cleave bovine PRL and can bovine CL tissue cleave it in a similar manner?

Cathepsin D processes bovine PRL into multiple fragments.

Initially, N-terminal 14- and 16-K PRL fragments were reported to result from Cath D cleavage of rat PRL (4) but subsequently, human PRL was reported to be resistant to Cath D cleavage (46). Although this was disproved by a later study (66), we found it necessary to test whether bovine PRL is cleaved by Cath D.

When bovine PRL was incubated with bovine Cath D at a 1:100 enzyme to substrate ratio for 3 h, the 23-K hormone was cleaved into 14- and 16-K fragments under acidic conditions (Fig. 4, bottom left). Incubation of rat or human 23-K PRL with bovine Cath D at the same enzyme to substrate ratio yielded only a 16-K fragment (Fig. 4, top and middle left). Cleavage was blocked when the pH was raised to 7 and when the specific Cath D inhibitor pepstatin A was added to the reaction mixture. The optimal pH for cleavage was pH 3, and the presence of bovine serum albumin in the digestion mixture facilitated cleavage at a higher pH, up to pH 5.5 (not shown). In similar experiments (Fig. 4, right), the enzyme to substrate ratio was varied. At the highest ratio tested, the major band observed corresponds to an apparent molecular mass of 14-K. Between the ratios of 1:20 and 1:100, both 16- and 14-K fragments were detected. Additional fragments of higher relative molecular mass, such as 21 K, were noticed in acidic preparations, but were considered nonspecific, as they occurred independently of the presence or absence of pepstatin A and of any source of Cath D (Fig. 4 and also Figs. 5 and 7). The 16- and 14-K fragments appeared to be N-terminal fragments, since they produced a signal when probed with monoclonal anti-PRL antibodies (mAb 5G2 and mAb 4C10) recognizing an epitope near the N-terminus (not shown). It emerges from these results that Cath D cleaves bovine PRL even more efficiently than it cleaves rat and human PRL.

Bovine CL tissue extracts cleave PRL in a Cath D-like manner.

Tissue homogenates from mammary gland (14), liver (14), and prostate (16) have been shown to process PRL into 16-K fragments in vitro. Since full-length PRL declines in the CL between the early and late

luteal stages, we wondered whether this decline reflected the processing of PRL in the CL. We therefore tested whether bovine CL tissue extracts were able to cleave PRL in vitro.

Bovine 23-K PRL was incubated at acidic pH with CL tissue extracts corresponding to different estrous-cycle stages. Western blot analysis of the reaction mixture revealed that the extracts could generate cleaved PRL forms (Fig. 5). The two major bands beneath that corresponding to 23-K PRL, and showing estimated molecular masses of 16 and 14 K, were comparable to those produced by Cath D-mediated proteolysis in vitro. Proteolysis was maximal under acidic conditions and was blocked by the Cath D inhibitor pepstatin A. No differences in cleavage activity were noted between CL extracts corresponding to different estrous-cycle stages (not shown). These results strongly suggest that PRL is cleaved in bovine CL tissue and that Cath D may effect this cleavage.

Cultured bovine CL-derived cells synthesize and release active forms of Cath D.

We next focused on cultured luteal EC and GLC to see whether these cells can produce and secrete Cath D.

Cell lysates and concentrated conditioned media (Fig. 6) revealed the presence of two major bands, of 44 K and 30 K, when probed with anti-Cath D. These molecular masses match with those of the active Cath D single-chain form and the heavy chain of the active Cath D two-chain form, respectively (60). The presence of this aspartic protease in the cell supernatant was further confirmed by testing the ability of culture supernatants to quench a fluorescent Cath D substrate. Both media from EC and GLC cultures displayed Cath D activity clearly above the basal level. They also displayed LDH activity above the basal level, but this activity was comparatively much lower than if staurosporine-induced enzyme release had occurred, due to membrane leakage in the late phase of staurosporine-induced apoptosis (1, 45).

16-K PRL is generated by culture medium conditioned by CL cells.

We next tested whether the media of luteal EC and GLC cultures have the ability to generate PRL fragments.

Exogenous bovine PRL added to acidified non-conditioned medium incubated at 37° C for 3 h was detectable as a major 23-K and a minor 21-K band. In contrast, exogenous bovine PRL incubated similarly in media conditioned by CL-derived cells gave rise to 16-K and 14-K PRL fragments (Fig. 7). The fragments migrated like the fragments generated in vitro by Cath D-mediated proteolysis. The fragments were not detected when pepstatin A was added to the conditioned medium or when the incubation was performed at pH 7.

Recombinant 16-K PRL inhibits proliferation of CL-derived EC.

To determine whether PRL cleavage has an effect on luteal EC, we exposed cultures of these cells to recombinant human 16-K PRL. Cultures exposed to 16-K PRL showed a significantly reduced growth rate compared to unexposed cultures, as early as 8 h after the start of treatment (Fig. 8, left).

Since the 16-K hPRL has been shown to exert anti-angiogenic action in human umbilical vein endothelial cells through its ability to induce apoptosis by caspase-3 activation (86), lysates of CL-derived EC were tested for the presence of caspase-3 active fragments. The treatment with 16-K hPRL indeed increased the level of cleaved caspase-3 in these cells (Fig. 8, right), consistent with ongoing apoptosis.

Discussion

In rodents, the CL is a well-established PRL target tissue (3): during CL formation and activity, PRL stabilizes progesterone production, promotes LH-receptor expression, and increases overall protein synthesis, but during CL regression, PRL triggers programmed cell death. In contrast, PRL signaling appears not to affect all these processes in ruminants (8) and primates (68), although all the PRL receptor forms described to date are expressed in the gland (64, 70, 74).

Data on PRL-receptor knockout mice (37) and from experiments with the anti-angiogenic 16-K PRL fragment point to a new role for PRL signaling in the CL: 23-K PRL signaling may be required to promote CL vascularization, whereas its bioactive fragments are likely to favor CL regression. Thus, the balance between 23-K and 16-K PRL might determine whether the CL persists and develops further to maintain pregnancy or regresses and allows the development of a new ovulatory follicle.

PRL synthesis in the CL

Here we provide evidence that luteal GLC and EC, two major cell types of the bovine CL, synthesize and secrete PRL. This confirms and extends results of very recent studies conducted by us (70) and by Shibaya et al. (74), revealing the presence of PRL and PRL-receptor mRNA in the bovine CL. Local PRL synthesis and secretion raises the question of the role the hormone might play in the CL. Since the cell types studied here express both the long and short forms of the PRL-receptor (70, 74), and since treatment with receptor-neutralizing antibodies inhibits the growth of these cells in culture (70, 74), autocrine or paracrine effects of PRL are hypothesized.

PRL and PRL fragments in the CL

We also show that full-length 23-K PRL drops in the bovine CL to almost undetectable levels toward the end of the CL lifespan. This might be caused by decreased local PRL supply, decreased local synthesis, and/or increased generation of PRL fragments. Reduced synthesis is in line with the reduced PRL mRNA

level observed at the end of the CL lifespan (74); enhanced proteolysis is suggested by two findings presented here: the fact that the Cath D rises as the 23-K PRL level falls and the ability of Cath D to cleave 23-K PRL in a manner similar to that reported for rat and human PRL (4, 66). We further show that CL tissue extracts can cleave PRL under acidic conditions. The CL is thus equipped for partial PRL proteolysis.

Increased synthesis of hydrolytic enzymes like Cath D and their release from lysosomes are known to occur in many instances of cell destruction (20).They also herald CL regression. At the onset of luteal regression, autophagic vacuoles and lysosomes increase in number and size (23) and we and others observe an increase in lysosomal bound and free Cath D. PGF2α is proposed to affect the lysosomal membrane, causing the release of bound Cath D into the cytosol (47, 67). Both autophagosomes/autolysosomes and the Cath D free in the cytosol may initiate cell death without (88) and with caspase involvement (43), respectively.

The ideal way to demonstrate PRL cleavage at the end of the CL lifespan would be to detect specific cleavage products in the regressing CL. These products appear to be short-lived and transiently appear in earlier stages. However, as suggested by the progressive degradation of bovine PRL at high enzyme to substrate ratios it is also imaginable that Cath D might act in concert with other proteases (52) or activate other proteases present in the CL (59), either directly or indirectly (76, 89), causing the fragments to be further processed.

Lysosomes / Cath D exocytosis in the CL

In addition to cell-associated Cath D, secreted or released Cath D might play a role in the CL. We have detected the presence of 44-K and 30-K Cath D forms from either endosomes or lysosomes in supernatants of cultured CL-derived cells. This contrasts with most of the established literature, which suggests that in mammalian cell cultures, only the inactive precursor procathepsin D is secreted (90). Yet, the secretion of mature Cath D forms active on native PRL has been shown to occur in cultured rat mammary epithelial cells (15). Nonetheless, we cannot exclude that in our cultures small amounts of Cath

D could arise from spontaneous cytolysis after normal cell shedding, since LDH activity also slightly increased over time in the supernatants.

Procathepsin D is reported to require an acidic pH to be proteolytically processed to the active intermediate 44-K single chain and to the more stable 30-K and 14-K two-chain forms (39). Here we demonstrate the presence of the 44- and 30-K Cath D forms in culture supernatants, in keeping with the finding that Cath D can cleave PRL to 16-K PRL outside the cell compartment (15,16). Yet only at acidic pH do our supernatants display Cath D-like enzymatic activity that can cleave native 23-K PRL to 16-K PRL. PRL cleavage activity is highest at pH 3 and disappears at pH 5.5. This is in agreement with the observation that an acidic pH is required for Cath D activity in vitro (12, 55, 91). Yet, assays in vitro have shown that Cath D is stable between pH 1 and 9 (44) and that it can cleave some substrates at pH 5.5 (44). Imaginable, furthermore, aggregate or spheroid cultures, instead of a two-dimensional culture setup, might allow a more physiological pH for PRL cleavage (5). We have noted PRL cleavage up to pH 5.5 after addition of BSA, in keeping with the finding that physiological activators may facilitate Cath D cleavage at a more basic pH (50).

Hypoxia, glycolysis, and acidity in the CL

In solid tumors, the extracellular environment where Cath D is suggested to occur (50, 76, 91) is known to be continuously acidic (36) because of hypoxia (40), changes in energy metabolism (30), and a slow rate of exchange between tissue and body fluids. Hypovascularity and anaerobic glycolysis are observed in the ovary as well, during late folliculogenesis and early CL development (11, 56). At these times the extracellular microenvironment resembles that of solid tumors (9, 56). Mathematical models (35) and intrafollicular oxygen measurements suggest that mature follicles are heavily underoxygenated. One study showed the oxygen level in follicular fluid to fall from 90 to 50-60 mm Hg (26) at this time, when glucose is preferentially converted to lactic acid.

Hypoxia and its metabolic consequences again prevail when the CL is regressing. Vasoconstriction, detachment of EC, and occlusion of blood vessels are early events of CL regression (54, 57, 58). The

breakdown of blood vessels results in a diminished oxygen supply to the luteal parenchyma. When oxygen tension is experimentally reduced in the media of CL from pseudopregnant rats, lactic acid accumulates (29). Acidification of luteal cells leads to hydrolase release and cell death due to activation of p53- and caspase-3 dependent apoptosis pathways, autophagy, and loss of function of critical pH-sensitive genes (77). Protein H^+-pumps, which actively extrude H^+ in exchange for extracellular cations, are present and active in EC (2, 71) and macrophages (84) populating the CL. Their role in pH homeostasis in the CL requires further investigation. In addition, it is interesting to note that short periods of hypoxia may stimulate the exocytosis of mature lysosomal enzymes, among which is Cath D, as it has been shown to occur in hepatocytes (13).

On the basis of the above findings, an acidic extracellular environment allowing PRL cleavage by Cath D appears likely in the regressing CL. Cath D might also perform functions independent of this protease activity. For instance, it might recruit other proteases, such as cathepsin L in the CL (59), by binding to their natural inhibitors (48), or it might act via a putative activation peptide receptor (90) to stimulate the proliferation, motility, and survival of certain CL cells, such as fibroblasts (48) and EC (7). At present we cannot exclude that part of the aspartic proteolytic capacity observed in the ovary is due to cathepsin E (Cath E) and not to Cath D. Cath E has similar enzymatic properties as Cath D (e.g. susceptibility to pepstatin A, acidic pH optimum (92)) and MOCAc-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)-*D*-Arg-NH2 is as substrate for both proteases (95). Cath E is of non lysosomal origin and not secretory (87). Cath E is retained within vesicular structures mainly associated with the endoplasmatic-endosome compartment. Its tissue and cellular distribution is more restricted than the one of Cath D (72). It is of note that particular cells of the immune system such as macrophages and lymphocytes contain Cath E. Whether PRL is a Cath E target and whether Cath E is present in the CL is presently unknown.

16-K PRL affects CL-derived EC.

We finally show that 16-K PRL, the product of PRL cleavage by Cath D, inhibits the growth of CL-derived EC and is thus likely to affect CL vascularization in vivo. This growth inhibition correlates with

caspase-3 activation. Such a correlation has been described in the literature (86) and is in line with the observation that 16-K PRL promotes apoptosis-induced vascular regression (24). It may well be that 16-K PRL exerts further effects on positive and negative regulators of cell cycle progression in the CL (85), but this is beyond the scope of the present paper.

Conclusion

In conclusion, our study demonstrates that the bovine CL is not only a PRL target organ but also a site of PRL synthesis. We further show that CL-derived cells deliver Cath D to the extracellular space, and that this protease can cleave PRL into bioactive fragments affecting CL-derived EC. Therefore, given the importance of vascular changes in the course of the estrous cycle, we propose that the balance between 23-K and 16- K PRL in the CL might influence whether it persists and develops for the maintenance of pregnancy or regresses and allows the development of a new preovulatory follicle. We are only beginning to appreciate the influence of the microenvironment on CL biology. An understanding of how the microenvironment determines the generation of bioactive fragments should prove useful not only in reproductive biology but also in other fields, such as regenerative medicine, therapeutic angiogenesis, and tissue remodeling.

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Figure Legends

Fig. 1: Characteristics of CL-derived endothelial and granulosa-like cells.

CL-derived endothelial cells: EC, CL-derived granulosa-like cells: GLC

Top, phase contrast morphology of EC and GLC in culture. The EC monolayer shows typical cobblestone-like morphology. The GLC culture has a flat appearance. Magnification: 125 x.

Middle, Immunofluorescence staining of von Willebrand factor (FVIII-related antigen, FVIIIr). The EC monolayers shows intense punctate cytoplasmic staining. The GLC culture is von Willebrand factor negative. Simultaneous 4',6-diamidino-2-phenylindole (DAPI) staining confirms the presence of cells (insert). The cells were formalin-fixed, permeabilzed with 0.2 % Triton, and probed with polyclonal antibodies against von Willebrand factor (DAKO). Immunoreactive sites were visualized with a Cy3-conjugated secondary antibody and a laser scanning microscope (LSM 510 - Meta, Zeiss, Jena, Germany). Magnification: 200 x; insert 100 x.

Bottom left, Oil Red O stain. The EC monolayer doed not stain with Oil Red O. The GLC monolayer contains Oil-Red-O stained lipid droplets. The cells were formalin-fixed, stained by the Oil red method (49), and lightly counterstained with hemalaun. Magnification: 500 x.

Bottom right, 3β-hydroxysteroid dehydrogenase (3β-HSD) enzyme histochemistry. The EC monlayer shows no 3β-HSD activity. In the GLC culture 3β-HSD activity is detectable. The cultures were air dried and probed (37 °C, 90 min) for 3β-HSD, a key enzyme of steroid hormone biosynthesis, according to the method of Payne et al. (18, 62), using 5-beta-androstane-3,17-dione (etiocholane-3,17-dione) as substrate. Magnification: 500 x.

Fig. 2: PRL is expressed in CL tissue and cells.

Early-, mid-, and late-luteal-stage-CL. CL-derived endothelial cells: EC, CL-derived granulosa-like cells: GLC, cells of the PRL-producing rat pituitary adenoma cell line GH4-C1: GH4-C1.

Left, PRL transcripts are present in CL total RNA extracts at all estrous cycle stages and also in pituitary total RNA extracts (positive control). Amplification of GAPDH mRNA reveals equal amounts of cDNA template. The corresponding molecular sizes are indicated on the left.

Right, proteins from electrophoresed CL-derived cell lysates (lanes 1 and 2) were probed on a nitrocellulose membrane with the anti-PRL monoclonal antibody 6F11. A strong chemiluminescence signal was captured on X-ray film at 23 K, the expected molecular mass for intact bovine PRL. Comparable bands resulted when lysates of GH4-C1 cells and 15 ng purified bovine PRL were loaded. Bottom: Blots stripped and then reprobed with anti-actin antibody to ensure equal protein loading in each lane. Molecular masses are indicated on the left side.

Fig. 3: The PRL level is lower and the Cath D level higher in the late-luteal-stage CL than in the CL at earlier stages.

Early-, mid-, and late-luteal-stage CL.

Top left, the PRL level drops with CL age. Twenty µg of total proteins extracted from CL at different luteal stages were run on SDS gels, transferred to a nitrocellulose membrane and analyzed with anti-PRL polyclonal antiserum from NHPP. Extracts of early-luteal-stage CL show a strong chemiluminescence signal at the position of PRL (lane 1). Extracts of mid- and late-luteal-stage CL show, respectively, a moderate (lane 2) and a weak signal (lane 3). Note that pituitary extracts and purified PRL give signals at the same position (lanes 4 and 5). Bottom: Membranes stripped and reprobed with anti-actin antibody, demonstrating equal protein loading.

Top right, extract of mid luteal stage CL demonstrates strong 16 K and 14 K PRL bands, comparable to those gained by PRL cleavage with bovine Cath D (cleaved PRL, see Fig. 4 for detailed explanation), when 100 µg protein extract is loaded on the lanes.

Bottom left, Cath D expression and processing changes in the bovine CL. The overall Cath D protein content increases in the CL as it progresses through the cycle (from lane 1 to lane 3). Concomitantly, the Cath D signal shifts from the mature single-chain form (44-K band) to the heavy chain of the two-chain

form (30-K band). CL extracts were prepared form CL at the indicated luteal stage and tested for the presence of Cath D forms by Western blotting with the polyclonal anti-human Cath D antiserum Ab-2. Actin immunoreactivity at the bottom of the blots demonstrates equal protein loading**.**

Bottom right, the ratio of "free" to "lysosome-bound" Cath D increases from early to late luteal stage. Corpora lutea at different stages of the estrous cycle were subjected to subcellular fractionation. On Western blots, enzymes released from the lysosomal fraction were probed in parallel with samples of post-lysosomal supernatant (containing "free Cath D"), with antiserum Ab-2. The protein bands at 44 K and 30 K correspond, respectively, to the mature single-chain form and the heavy chain of the two-chain form of Cath D. Note the concomitant increase of Cath D in both fractions.

Fig. 4: Cath D cleaves bovine PRL in vitro.

Left, bovine PRL appears to be more efficiently digested than rat or human PRL by Cath D from bovine spleen. PRL from all three species (150 ng) was subjected for 3 h to bovine Cath D (1.5 ng) hydrolysis at 37 °C and at acidic or neutral pH in the presence or absence of the Cath D inhibitor pepstatin A. At the end of the digestion period, undigested and digested PRL were electrophoretically separated, transferred to a nitrocellulose membrane, and stained with anti-PRL polyclonal antiserum from NHPP. Prolactin from all three species yields a band corresponding to 16-K PRL at acidic pH in the absence of inhibitor (lanes 2). Only bovine 16-K PRL (bottom) appears to be readily digested to 14-K PRL, while the 16-K fragments of the other two species appear more resistant.

Right, the rate and degree of in vitro digestion of bovine PRL by Cath D depend on the enzyme to substrate ratio. At an enzyme to substrate ratio of 1:10 to 1:100, 16- and 14-K fragments are recognized by the anti-PRL polyclonal antiserum (lanes 2 to 4). This hydrolysis reaction is sensitive to pH (lane 7) and to the presence of the Cath D inhibitor pepstatin A.

Fig. 5: Bovine PRL is cleaved by CL tissue extracts.

Prolactin (2.5 µg) was incubated with whole-tissue extract (50 µg) prepared from a CL at the mid-luteal-stage. The Western blot on the left shows the amount of PRL cleavage obtained after 3 h of incubation under acidic and neutral conditions, and in the presence and absence of pepstatin A. Both16-K and 14-K PRL fragments are seen in lane 1, corresponding to conditions favorable to Cath D-mediated cleavage (acidic pH and no specific inhibitor present). The other labeled bands in lane 1 are most likely not due to Cath D-mediated cleavage, since they also occur under conditions unfavorable to this cleavage (lane 3). The Western blot on the right shows the similarity of the fragments obtained in the presence of tissue extracts and Cath D.

Fig. 6: Cath D is present in its active single- and two-chain forms in extracts and supernatants of cultured luteal EC and GLC.

CL-derived endothelial cells: EC, CL-derived granulosa-like cells: GLC.

Left, extracts and supernatants of CL cells were tested for the presence of Cath D by Western blotting. Cells were seeded in either 24-well culture plates or 75-cm^2 flasks at a density of $5x10^4$ cells per cm^2. The medium was changed to serum-free medium 24 h later. After a further 24 h of culture, the cells were scraped off and subjected to protein extraction. Cell supernatants were concentrated with Centricon-YM 10 filter devices. Equal protein loads were run through an SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and probed for Cath D with anti-Cath D antiserum Ab-2. The corresponding lanes show two bands at approximately 44 and 30 K, representing respectively the mature single-chain form and the heavy chain of the two-chain form of Cath D. Only conditioned medium contained these bands.

Right, Cath D and LDH activity was measured in supernatant and cell lysate concentrates of EC and GLC. Prior to measurement, part of the cultures were exposed to 10 µM / ml staurosporine for 24 h. Cleavage of the fluorogenic Cath D peptide substrate MOCAc-GKPILF~FRLK(Dnp)-*D*-R-NH_2 was measured with the excitation monochromator set at 328 nm and the emission monochromator set at 393

nm. LDH presence was determined with the CytoTox-ONE assay. The bar chart depicts the activities in the supernatants as percentage of the activities in the supernatant plus the activities in the cell lysates. Data represent means ± standard deviations of quadruplicate experiments. Media from EC and GLC cultures display Cath D activity. Cath D activity is already comparatively high in supernatants of unexposed cultures, while LDH activity is comparatively low, and increases dramatically during prolonged staurosporine exposure due to membrane leakage in the late phase of staurosporine-induced apoptosis. *Significant difference (P <<0.05) compared with samples exposed to staurosporine.

Fig. 7: Bovine PRL is cleaved by CL cell supernatants.

CL-derived endothelial cells: EC, CL-derived granulosa-like cells: GLC.

Intact 23-K PRL (2.5 µg) was incubated with concentrated CL-cell supernatants in reaction buffer at 37 °C for 3 h. Western blots show PRL cleavage products of approximately 16 K and 14 K after incubation at acidic pH and in the absence of pepstatin A (lane 1 and 2). No such bands are seen after incubation at a pH unfavorable to cleavage by Cath D (lane 3 and 4) or in the presence of the Cath D inhibitor pepstatin A (lane 5 and 6). Lanes further right (lanes 7 to 9) highlight the similar cleavage profiles of supernatant-cleaved and Cath-D-cleaved 23-K PRL.

Fig. 8: Recombinant human 16 K PRL (rh 16 K PRL) inhibits EC growth via a caspase-3-dependent pathway.

CL-derived endothelial cells: EC.

Left, recombinant human 16 K PRL inhibits the growth of cultured EC**.** 10’000 EC were seeded per well in a 24-well culture plate. 24 and 48 h later, the cultures were treated with recombinant human 16 K PRL (500 ng/ml) or 23 K PRL (500 ng/ml) or left untreated. At the indicated time, cells were harvested by trypsinization and Trypan-Blue-excluding cells were counted with a hemocytometer. Recombinant human 16 K PRL significantly decreases the number of cells within the first hours of treatment, whereas the cell

count is closer to that of untreated cultures in cultures exposed to bovine or recombinant human 23-K PRL. The indicated values are means of quadruplicate determinations in three independent experiments; vertical bars represent standard deviations.

Right, caspase-3 is highly activated in EC exposed to recombinant human 16 K PRL. EC were seeded into 75-cm^2 flasks at a density of 50'000 cells per cm^2. At confluency, the cultures were treated once with recombinant human 16 K PRL (500 ng/ml) or 23 K PRL (500 ng/ml) for 48 h or left untreated. For comparison, additional cultures were exposed to staurosporine (200 nM) for 6 h. At the end of the exposure time proteins were extracted, electrophoretically separated, transferred to a nitrocellulose membrane, and probed for the presence of activated, e.g. cleaved caspase-3 with rabbit monoclonal antibody 5A1, which solely detects the large fragment (17/19 K) of caspase-3 resulting from cleavage at aspartic acid 175. Control cell extracts of Jurkat cells treated (+) or not (-) with cytochrome c in vitro were run in parallel in order to show specific detection of cleaved caspase-3 (Asp175) by the antibody. Untreated cultures and those treated with bovine or recombinant human 23 K PRL show basal levels of cleaved caspase-3. In contrast, cleaved caspase-3 levels are high in extracts of cultures treated with rh 16-K PRL or staurosporine.

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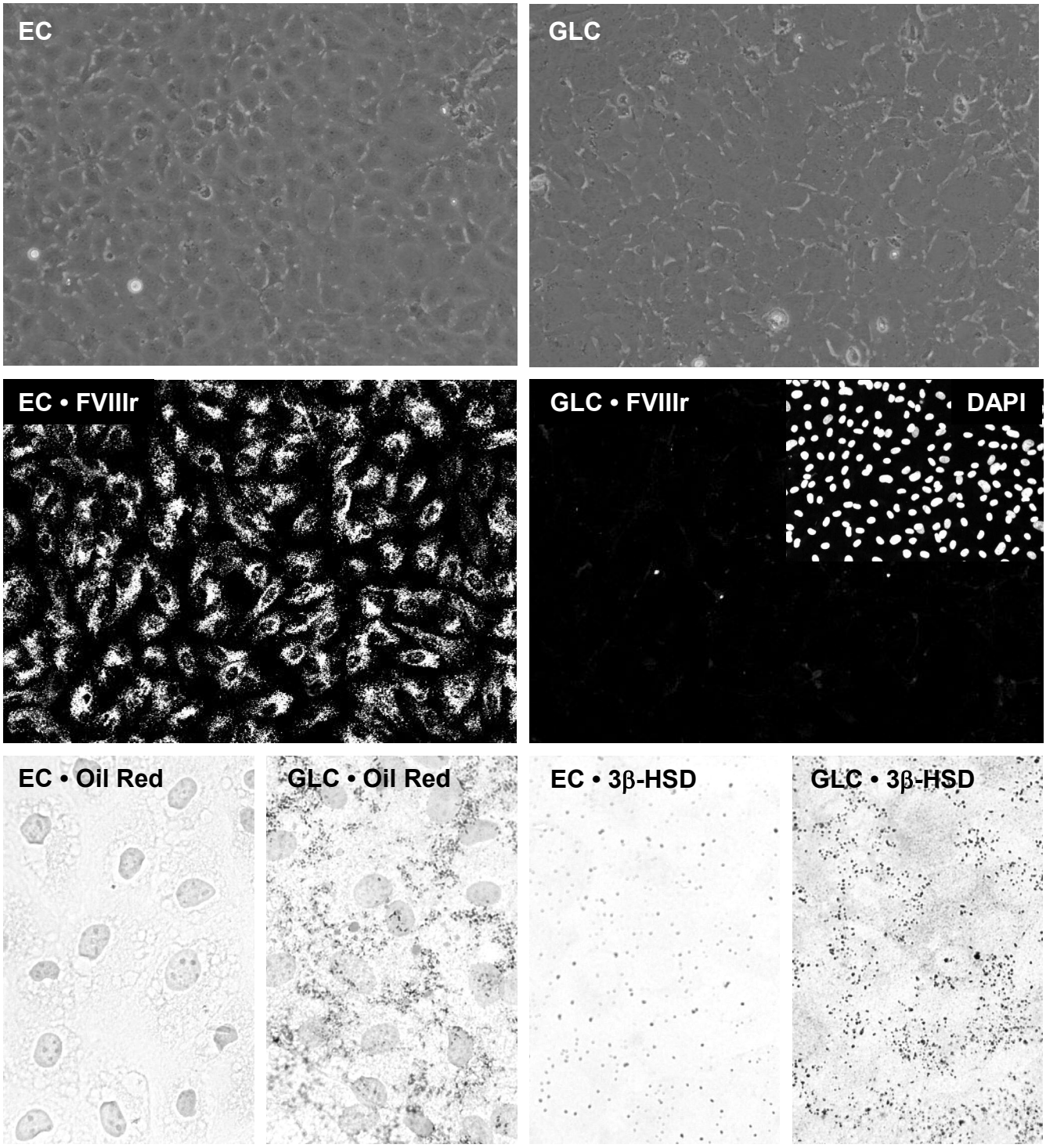
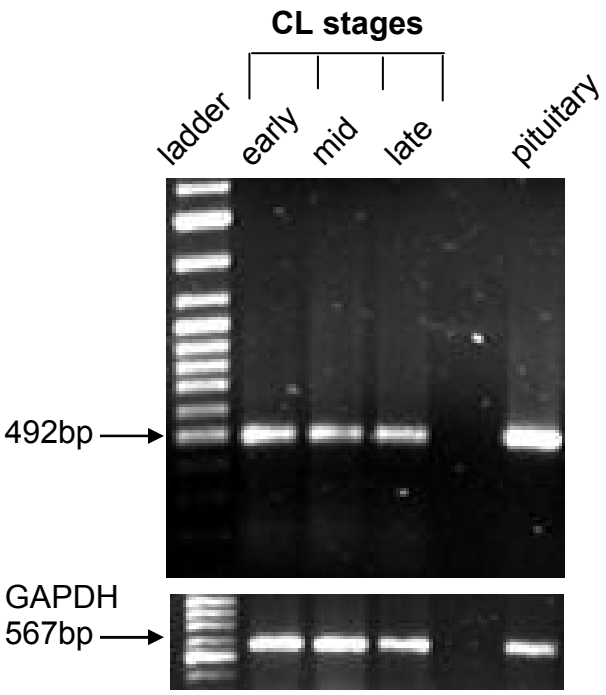

EC
GLC
EC • FVIIIr
GLC • FVIIIr
DAPI
EC • Oil Red
GLC • Oil Red
EC • 3β-HSD
GLC • 3β-HSD

Fig. 1

PRL transcripts



protein

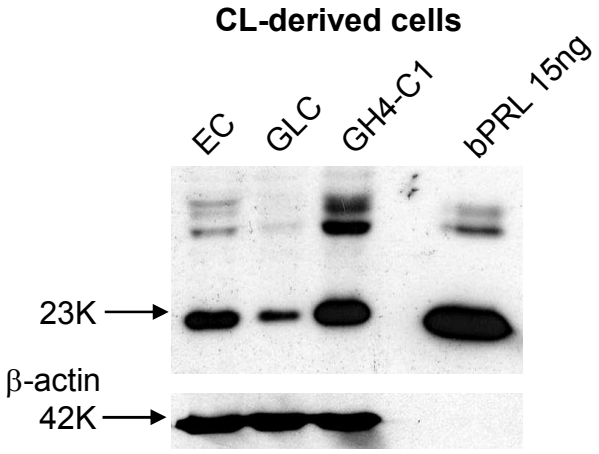


Fig. 2

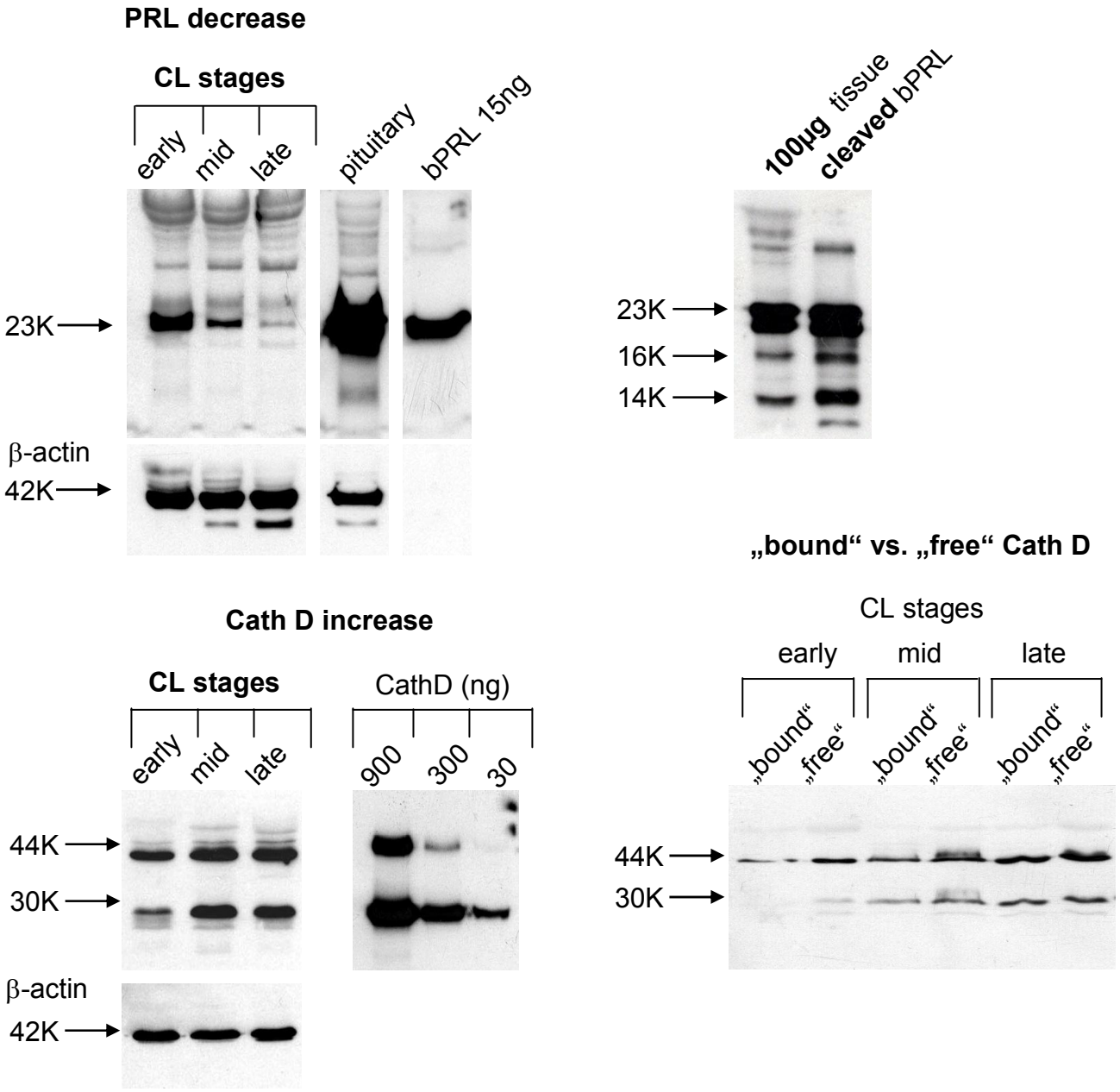

PRL decrease
CL stages
early
mid
late
pituitary
bPRL 15ng
23K
β-actin
42K
100µg tissue
cleaved bPRL
23K
16K
14K
Cath D increase
CL stages
early
mid
late
CathD (ng)
900
300
30
44K
30K
β-actin
42K
„bound“ vs. „free“ Cath D
CL stages
early
mid
late
„bound“
„free“
„bound“
„free“
„bound“
„free“
44K
30K

Fig. 3

Bovine PRL cleavage by Cath D

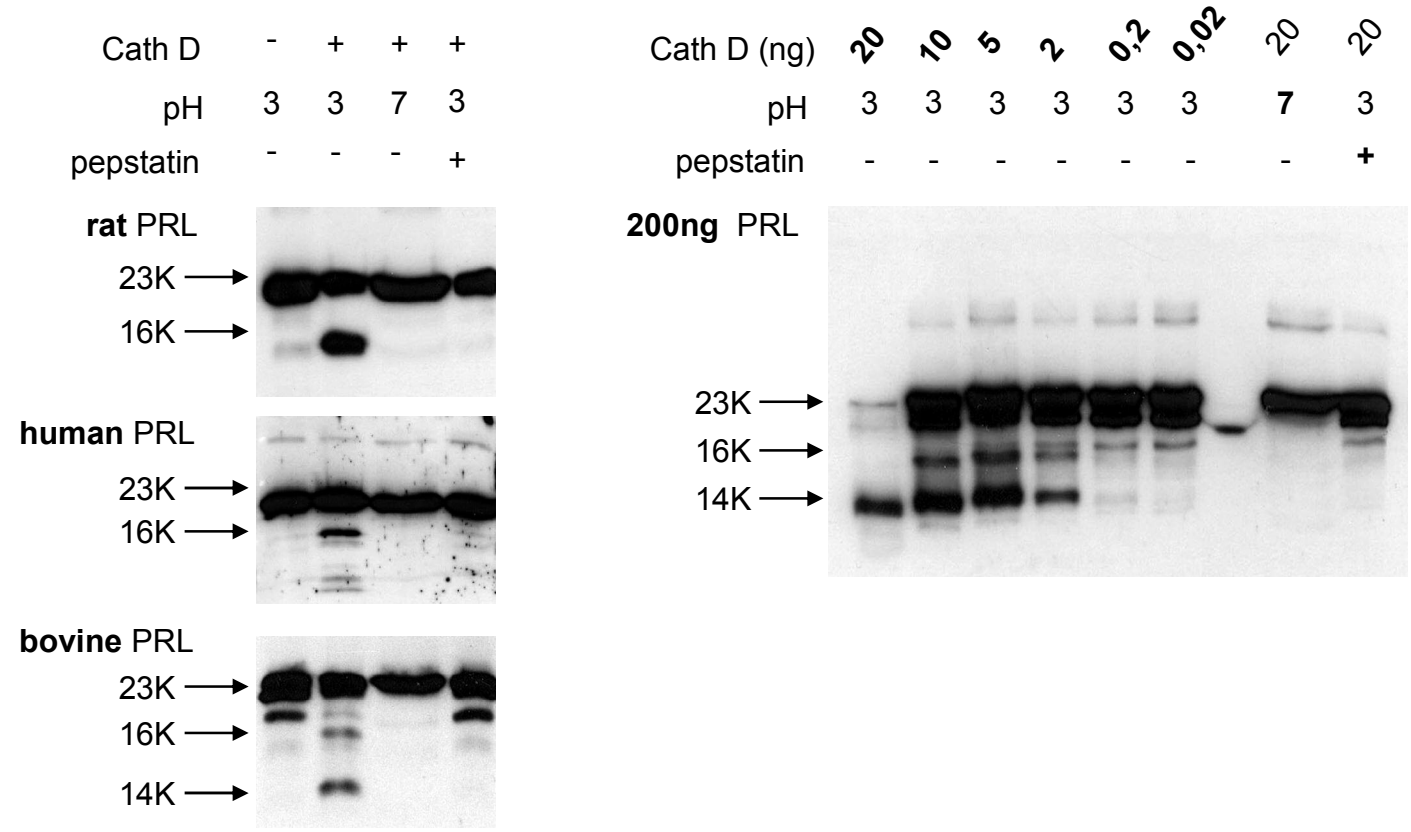


Fig. 4

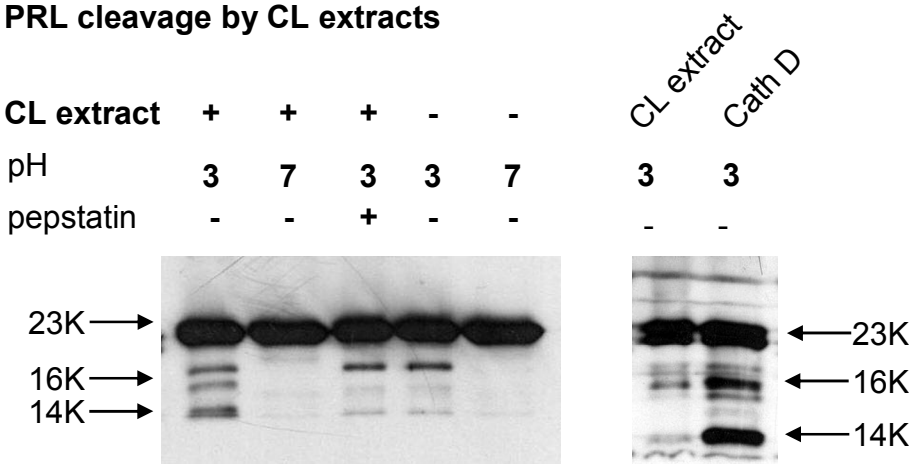

PRL cleavage by CL extracts
CL extract + + + - -
pH 3 7 3 3 7
pepstatin - - + - -
23K
16K
14K
CL extract
Cath D
3 3
- -
23K
16K
14K

Fig. 5

Cath D in CL-derived cell supernatants and cells

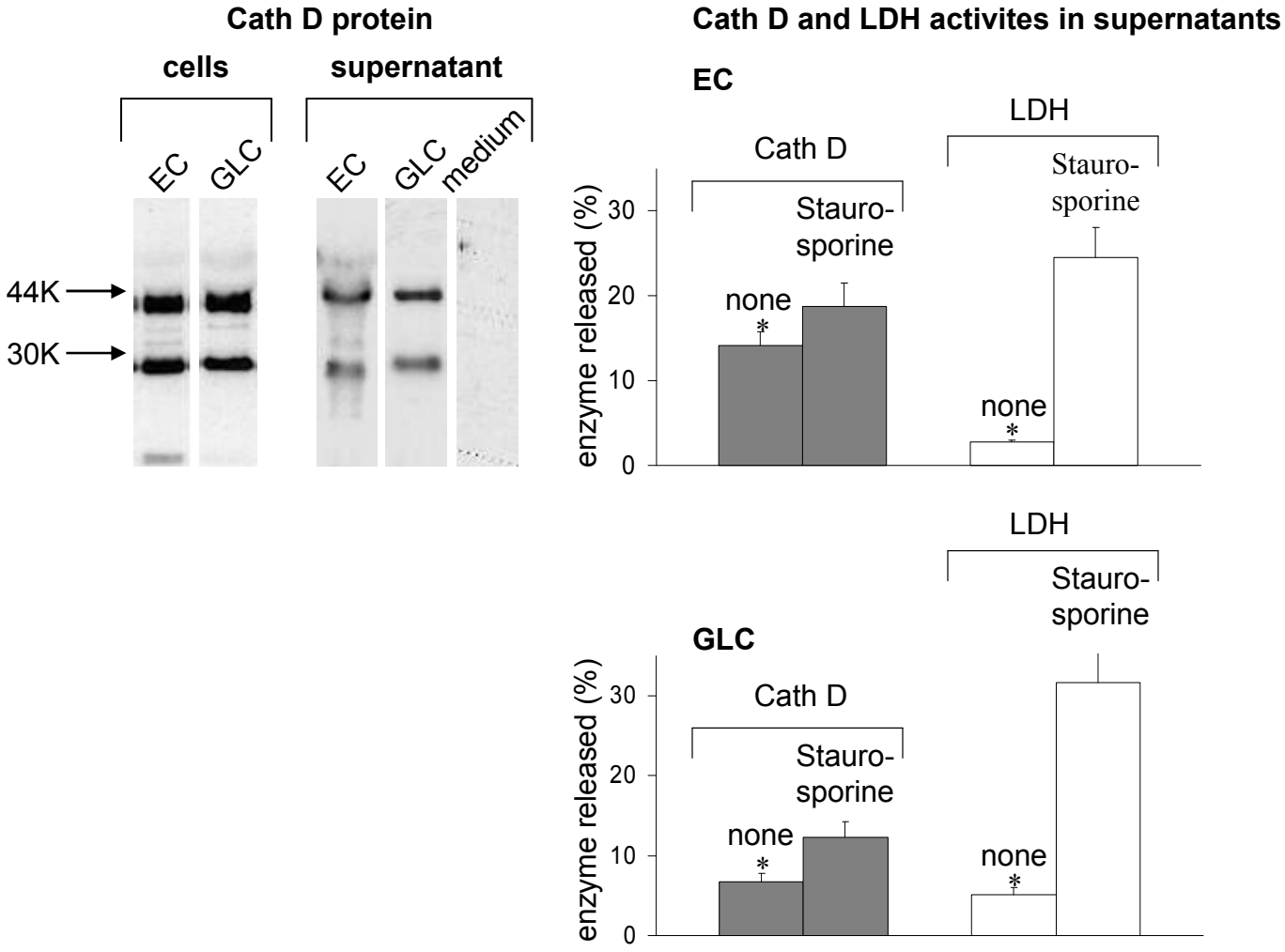


Fig. 6

PRL cleavage by CL-derived cell supernatants

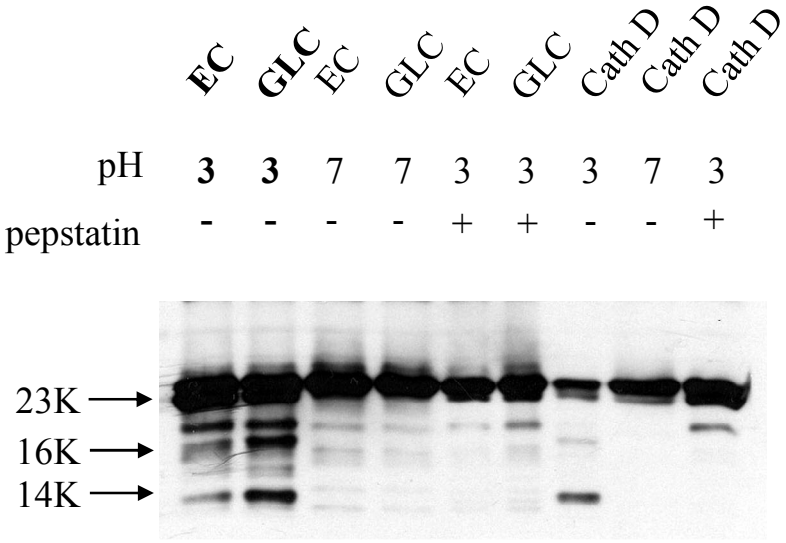


Fig. 7

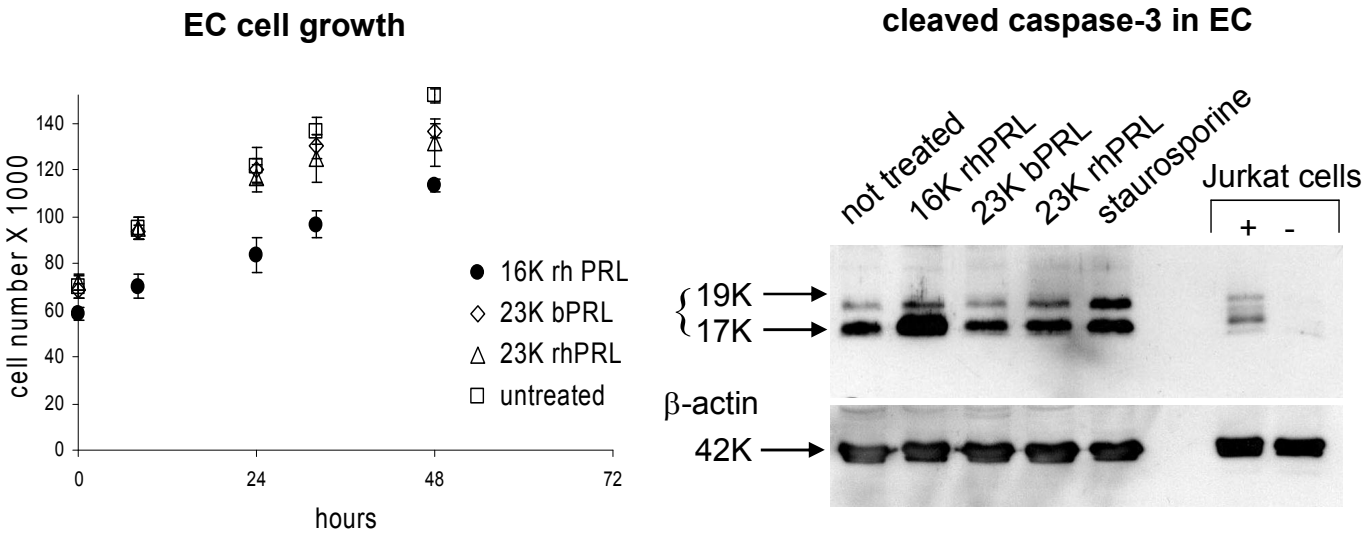

EC cell growth
cell number X 1000
140
120
100
80
60
40
20
0
0
24
48
72
hours
16K rh PRL
23K bPRL
23K rhPRL
untreated
cleaved caspase-3 in EC
not treated
16K rhPRL
23K bPRL
23K rhPRL
staurosporine
Jurkat cells
+
-
19K
17K
β-actin
42K

Fig. 8