ADRENOMEDULLIN SIGNALLING IN BOVINE CORPUS LUTEUM DURING OESTROUS CYCLE, GRAVIDITY AND INDUCED LUTEOLYSIS

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Abstract

Investigations on "nonclassic" regulators of angiogenesis could open new perspectives in understanding angiogenesis under physiological and pathological conditions. A pro-angiogenic effect has been demonstrated for adrenomedullin (AM), it´s co-factor activity-modifying protein 2 (RAMP-2) and the calcitonin receptor-like receptor (CALCLR). This signalling pathway is besides associated with lymphangiogenesis, that (in a physiologically way) is still only poorly understood in ovary and its structures. The aim of this study was therefore to evaluate mRNA expression of AM, RAMP-2 and CALCLR in bovine corpora lutea (CL) during different physiological stages. Experiment 1: CL were assigned days 1-2, 3-4, 5-7, 8-12, 13-16, >18 (after regression) of oestrous cycle and of gravidity (month <3, 3-5, 6-7 and >8). Experiment 2: Induced luteolysis. Cows on days 8-12 were injected with a PGF2α analogue and CL were collected by transvaginal ovariectomy before and 0.5, 2, 4, 12, 24, 48 and 64 h after PGF2α injection. Tissue levels of mRNA were characterized by qPCR. All 3 factors were clearly expressed. AM and CALCRL showed significant changes in both experiments, RAMP-2 during induced luteolysis. For AM highest levels could be observed in the beginning and the end of the luteal phase, CALCRL remained at around the same levels. Both factors showed their most expression at the end of pregnancy. During induced luteolysis all factors started to decline not until structural luteolysis. In conclusion, our results could lead to the assumption that factors investigated may be involved in mechanisms regulating CL formation, function and regression – and a special impact in the final month of pregnancy.

Keywords: corpus luteum function, lymphangiogenesis, bovine oestrous cycle, bovine pregnancy.

1.Introduction

Angiogenesis, the process through which new blood vessels arise from pre-existing ones, is regulated by several "classic" factors, among which the most studied are vascular endothelial growth factor (VEGF) [1], [2], [3]. In recent years, investigations showed that, in addition to the classic factors, numerous endogenous peptides play a relevant regulatory role in ovary angiogenesis [4], [5], [6]. Such regulatory peptides, each of which exerts well-known specific biological activities, are present, along with their receptors, in the blood vessels and may take part in the

control of the "angiogenic switch". An in vivo and in vitro pro-angiogenic effect has been demonstrated for adrenomedullin (AM). AM raises the expression of VEGF in endothelial cells, but VEGF blockade does not affect the pro-angiogenic action of AM [7]. AM is a ubiquitously expressed 52 amino acid peptide that is a member of the family of calcitonin gene–related peptides (CGRPs) and was initially isolated from phaechromyctoma in 1933 [23]. It was initially demonstrated to have profound effects in vascular cell biology, since AM protects endothelial cells from apoptosis, promotes angiogenesis and affects vascular tone and permeability [8]. Receptor activity–modifying proteins (RAMPs), single pass transmembrane proteins that associate with GPCRs modulate the ligand binding of the calcitonin receptor–like receptor (CALCRL) such that RAMP1-CALCRL functions primarily as a CGRP receptor and RAMP2/3-CALCRL functions primarily as an AM receptor [9]. The AM signalling pathway is besides associated with lymphangiogenesis, that (in a physiologically way) is still only poorly understood in ovary and its structures. In contrast to angiogenesis and its involved factors that have been investigated intensively for the last decade, no attention to lymphangiogenesis has been paid until recently [10] [11]. Studies by [12] and [21] demonstrated that loss of signalling by the AM results in embryonic oedema and death. Remarkably, this phenotype is attributed to defects in lymphatic vessels by one group and to defects in blood vessels by the other [22]. The objective of this study was the characterization of AM, RAMP-2 and CALCLR mRNA expression in the bovine CL. Since blood vessel growth is known to be essential for CL development, maturation and function, it is of much interest to find out if these “nonclassic” factors are involved in this context.

2. Material and Methods

Experiment 1: Collection of bovine CL during oestrous cycle and pregnancy

The bovine CL were collected at the local slaughterhouse within 10-20 min after slaughter. The stage of oestrous cycle was determined by examining macroscopically the size, colour, consistency, connective tissue and mucus of the ovaries and uteri as previously described [2]. CL were assigned to the stages days 1-2, 3-4, 5-7, 8-12, 13-16, >18 (after regression) of oestrous cycle and of pregnancy month 1-2, 3-4, 6-7, >8 (the crown-rump length of the foetus was measured to evaluate the month of pregnancy) (n=6-7/group from different animals). Luteal tissue was frozen in liquid nitrogen and stored at -80°C until RNA extraction.

Experiment 2: Collection of bovine CL during induced luteolysis

Cows (Holstein Frisians and Brown Swiss) at mid-luteal phase (days 8–12) were injected i.m. with 500 µg PGF2α analogue Cloprostenol (Estrumate®, Intervet,

Germany). The CL were collected by transvaginal ovariectomy 0.5, 2, 4, 12, 24, 48 and 64 h (*n*=5/group from 5 different animals) after PGF2α injection. Control CL (0 h) were collected from a local slaughter house (previously described by [4].

Total RNA extraction and reverse transcription

Constant amounts of 1 µg of total RNA were reverse transcribed to cDNA using the following master mix: 26 µl Rnase-free water, 12 µl 5x Buffer (Promega, Mannheim, Germany), 3 µl Random Primers (50 mM) (Invitrogen™, Carlsbad, Germany), 3 µl dNTPs (10 mM) (Fermentas, St. Leon-Rot, Germany) and 200 U of MMLV Reverse Transcriptase (Promega, Mannheim, Germany) according to the manufacturer's instructions as previously described [1]

Quality determination of total RNA

Degradation of the RNA was measured with the Agilent 2100 bioanalyzer (Agilent Technologies, Deutschland Gmbh, Waldbronn, Germany) in conjunction with the RNA 6000 Nano Assay, according to the manufacturer's instructions. The bioanalyzer enables the standardization of RNA quality control. RNA samples are electrophoretically separated on a microfabricated chip, and subsequently detected with laser-induced fluorescence induction. The Bioanalyzer electropherogram of total RNA shows two distinct ribosomal peaks corresponding to either 18S or 28S for eukaryotic RNA, and a relatively flat baseline between the 5S and 18S ribosomal peaks. The automatically calculated RNA integrity number (RIN) allows classification of total RNA based on a numbering system from 1 to 10, with 1 being the most degraded profile and 10 being the most intact [13]

Real-Time PCR quantification

A master mix of the following reaction components was prepared: 6.4 µl water, 1.2 µl MgCl2 (4 mM), 0.2 µl forward primer (0.2 mM), 0.2 µl reverse primer (0.2 mM), and 1.0 µl LightCycler® Fast Start DNA Master SYBR Green I (Roche Diagnostics, Mannheim, Germany). The master mix (9 µl) was added to the strip tubes and 1 µl PCR template containing 16.66 ng reverse transcribed total RNA was added. The following general real-time PCR protocol was employed for all investigated factors: denaturation for 10 min at 95°C, 40 cycles of a three segmented amplification and quantification program (denaturation for 10 sec at 95°C, annealing for 10 sec at the primer specific temperature, elongation for 15 sec at 72°C), a melting step by slow heating from 60 to 99°C with a rate of 0.58°C/sec and continuous fluorescence measurement, and a final cooling down to 40°C. Crossing point (cp) values were acquired by using the second derivative maximum method of the Rotor-Gene 6 software (Corbett Research, Mortlake, Australia). Real-time PCR efficiencies were determined by amplification of a standardized dilution series, and slopes were

calculated using Rotor-Gene 6 software (Corbett Research, Mortlake, Australia). The specificity of the desired products in bovine CL was documented using a high resolution gel electrophoresis and analysis of the melting temperature, which is product specific.

Primers

For primer design the HUSAR® (DKFZ) software was used. The primers used for real-time PCR were as follows: histone (232bp) forward 5'-ACT GCT ACA AAA GCC GCT C-3' and reverse 5'-ACT GCC TCC TGC AAA GCAC-3'. [14], Adrenomedullin (190bp) forward 5'- CCA GAC TCT TAT TCG GCC C -3' and reverse 5'- GAA ATG GTA GAT CTG ATG CGC -3' (NM_173888), RAMP-2 forward 5'-CCA AGT CAG AAG GGA AAA CG-3' and reverse 5'- CAA AGA TGA TCT CTT CCG CC -3' (NM_001098860) and calcrl forward 5'- TCC CAG TTC ATC CAT CTC TAC C-3' and reverse 5'- TCT GGC AAC AGC GTG AAT AC -3' (NM_001102107).

Statistical analysis

The statistical significance of differences in mRNA expressions of the examined factors was assessed by one-way ANOVA followed by the Holm-Sidak as a multiple comparison test. Differences were considered significant if $P<0.05$. All experimental data are shown as 40 minus the mean of (normalized) Δcp ± SEM (standard error of the mean), so that a *high* "40-cp" value indicated a *high* gene expression level and vice versa.

3. Results

RNA Quality Determination

RIN values of the used samples ranged between 7.1 and 9.2. All samples revealed two distinct ribosomal peaks, corresponding to 18S and 28S for eukaryotic RNA.

Confirmation of Primer Specificity and Sequence Analysis

The mRNA expression was analyzed by real-time reverse transcription PCR (RT-PCR) with the Rotor-Gene 3000. Initial RT-PCR experiments verified specific transcripts for all factors in bovine CL. For exact length verification, RT-PCR products were separated on 2% high-resolution agarose gel electrophoresis. PCR products were verified by commercial DNA sequencing (TopLab, Munich, Germany). Each PCR product showed 100% homology to the known genes after sequencing.

Housekeeping Gene Expression

To evaluate equal quantity and quality of the preceding reverse transcription reaction in each sample, the housekeeping genes, ubiquitin and histone, were examined in all samples. As both housekeeping genes were constantly expressed in all samples, we chose histone as normaliser. The results of mRNA expression of the examined factors are presented as changes (40 − ΔCP ± SEM

from 5 CL per group) in the target gene expression, normalized to *Histon.*

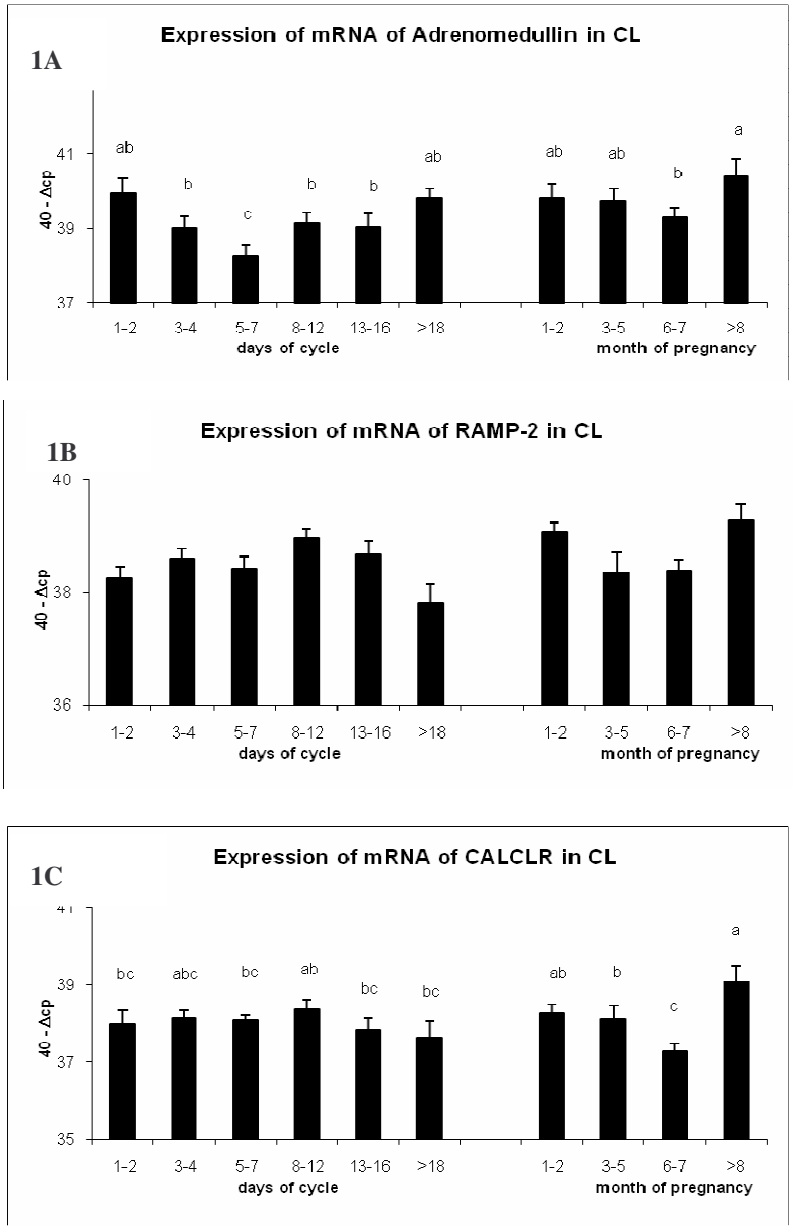

Figure 1. Expression of mRNA of **(A)** Adrenomedullin, **(B)** CALCLR and **(C)** CALCLR in the CL of oestrous cycle and gravidity. Results are presented by 40 minus mean of normalized crossing points (Δcp) ± SEM (n=6-7). Different superscripts denote statistically different values (P<0.05).

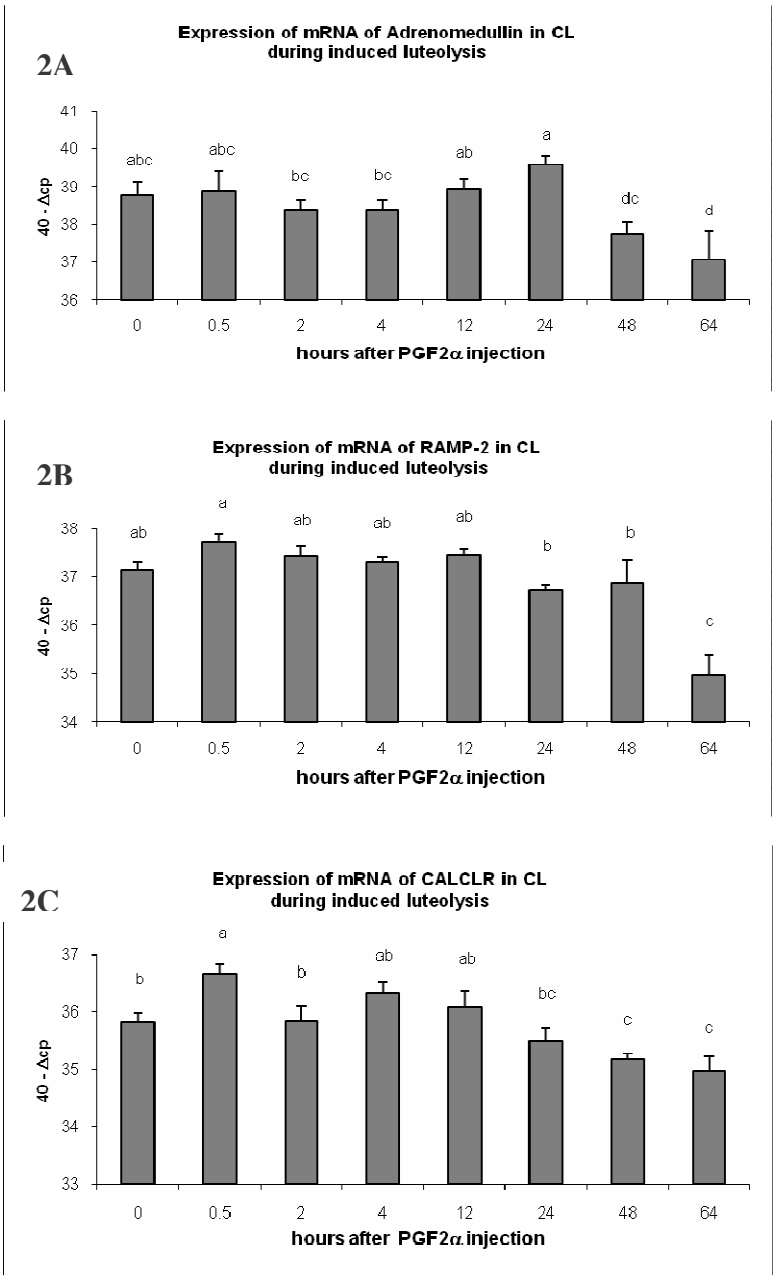

Figure 2. Expression of mRNA of **(A)** Adrenomedullin, **(B)** RAMP-2 and **(C)** CALCLR in the CL during induced luteolysis. Results are presented by 40 minus mean of normalized crossing points (Δcp) ± SEM (n=6-7). Different superscripts denote statistically different values ($P<0.05$).

Expression of AM, RAMP-2 and CALCLR in CL during oestrous cycle and gravidity

As shown in fig. 1A mRNA expression of AM starts highly in the beginning of the luteal phase and decreases significantly on days 3-4 and 5-7. Midluteal the expression rises again and reaches levels from the start during regression. For RAMP-2 no significant changes of mRNA expression could be obtained. Expression performance of the receptor CALCLR is shown in fig. 1B. Here the highest levels are midluteal on days 8-12 but those in the beginning and the end are similar again. Both factors have their peak at the end of pregnancy in month >8.

Expression of AM, RAMP-2 and CALCLR in CL during induced luteolysis

In CL during induced luteolysis mRNA expression of AM and RAMP-1 not changes significantly until 24 h (AM) and 48 h (RAMP-2) after PGF2α. AM and CALCLR levels drop down significantly after 48 h the one of RAMP-2 not until 64 h after induction.

4. Discussion

AM signalling is of particular significance in endothelial cell biology since the peptide protects cells from apoptosis, promotes angiogenesis, and affects vascular tone and permeability [15]. That angiogenesis in the reproductive tract and especially in the ovary is of great importance for its accurate function is nowadays well established [16][24], [1, 2, 17]. What is more AM is demanding in various tumours [25] and when cardiovascular problems occur [18]. Whether in pathologic circumstances or physiologically, hypoxia is a key stimulus for angiogenesis. Hypoxia-inducible factor 1 (HIF-1) is the master regulator of oxygen homeostasis [19] Low oxygen levels and therefore HIF-1α effects do not only up regulate the expression of VEGF, endothelin-1 and -2 und angiogenin but also the one of AM [8]. Its receptor CALCLR is also up regulated under hypoxic conditions in micro vascular endothelial cells (EC), whereas RAMP-2 in EC is not activated by a lack of oxygen [20]. Our results during regression of CL (>18 day of cycle) conform to these findings. When the CL undergoes degeneration unavoidable hypoxia comes into being. Thus HIF-1α is up regulated, that in turn activates AM and CALCLR expression [8]. High mRNA expression in the beginning of luteal phase is due to the enormous angiogenic rate needed during CL formation [2]. The high levels at the end of pregnancy could also be in agreement with this theory, as well as the fact that expression of all three factors starts to decline significantly very late (48 or even 64 h after PGF2α). Hypoxia activates AM and its receptor. Maybe they act in a kind of survival strategy for the cells that undergo regression. RAMP-2 follows. As recently as structural luteolysis is in full activity its mRNA levels decline.

5. Acknowledgements

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