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Expression and localization of estrogen receptor α, estrogen receptor β and progesterone receptor in the bovine oviduct in vivo and in vitro☆

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Abstract

This study examined the regulation and localization of estrogen receptors α and β (ERα, ERβ) and progesterone receptor (PR) in the bovine oviduct. Oviduct epithelial cells from cycling cows (in vivo) were investigated. In addition, the reactivity of a cell suspension culture stimulated with physiological doses of estradiol-17β (E2) or progesterone (P4) was tested (in vitro). The specific steroid receptor expression of oviductal cells was quantified for mRNA using real-time RT-PCR. Furthermore, steroid receptor proteins were analyzed by Western blotting and localized by immunohistochemistry in situ. Obvious cyclic changes of receptor expression in vivo were observed and concurrent expression patterns were detected in vitro. PR and ERα mRNA transcripts were elevated in vivo during the follicular phase. The highest PR and ERα protein expression was detected subsequently during the early-luteal phase. In vitro, E2-supplementation resulted in an upregulation of PR and ERα. Both ERβ mRNA and protein expression were highest during the luteal phase in vivo and elevated ERβ expression levels were observed in vitro after P4 treatment. Evidence is provided for a varying expression of ERα, ERβ and PR in bovine oviducts at different cycle stages in vivo, respectively under steroid supplementation in vitro. The region specific and cycle dependent expression differences point towards a functional importance of the three steroid receptors in the bovine oviduct, the site of fertilization and early embryonic development.

Keywords: Bovine oviduct; Estrogen receptor α; Estrogen receptor β; Progesterone receptor; mRNA; Western blot; Immunohistochemistry

1. Introduction

A precise and functionally related synchronization of all parts of the female reproductive system is essential for fertilization and embryonic development. Sexual steroid hormones serve as conductors leading specific tissues to time specific differentiation. The mammalian oviduct is known to be under the influence of peripheral and local steroids, and remarkable changes of oviductal progesterone and estradiol contents throughout the bovine oestrus cycle are described [1]. Even before successful fertilization, the gametes located in the oviduct need optimal environment for transport, maturation and final capacitation. The oviduct is capable of transudating substances from the circulation into the oviductal lumen as well as de novo synthesizing and releasing molecules like lipids, enzymes and growth factors next to a variety of oviduct specific proteins like oviductin [2,3]. Many of those are considered to be of nutritional importance for the conceptus, but some may also contribute to sperm binding, gamete growth and developmental regulation [4]. The secretory products are suspected to be regulated from the periphery in a cycle dependent manner, but auto/paracrine regulations may occur as well [5]. Especially estrogens should induce compositional changes of the oviductal fluid with greatest protein secretion during the follicular phase [6]. The proliferation of the luminal epithelium cell layers and the differentiation of secretory cells is regulated under the influence of estrogens [7]. It has been supposed that progesterone is acting generally antagonistic to the estrogen-mediated effects described above [8].

Both hormone actions are mediated through intracellular receptors that are members of the nuclear receptor family, namely estrogen receptors α and β (ERα, ERβ) and progesterone receptor (PR). The latter regulate the expression of a wide variety of genes on a transcriptional level. ERα, the classical estrogen receptor, has been known for several years [9]. The existence of a second estrogen receptor has only recently been shown in the rat [10], mouse [11], human [12], cattle [13] and pig [14]. Within the reproductive system

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ERα appears to be the predominant subtype [15]. ERβ plays a subordinate role in most parts of the reproductive system with exception of the ovary [16,17], the mammary gland [18] and the embryo [14], where ERβ is of supreme importance mediating estrogenic action.

However, the joint expression of PR along with ER is discussed controversially [19–21]. Especially the distribution of ERβ during the normal oestrus cycle seems to be quite conflicting between and within species and tissues [22]. Nevertheless, regional expression differences within the oviduct seem to be of functional importance. Data demonstrating steroid receptor expression and localization in the endometrium and the ovary of different species are available [12,15,21,23–25]. To our knowledge no comprehensive investigation is published on the distribution and localization of steroid receptors in the bovine oviduct.

Our aim was to investigate the expression of ERα, ERβ and PR in the bovine oviduct in vivo during the course of the oestrus cycle. A focus was laid on the question whether mRNA and protein levels followed the same expression profiles. Additional in vitro experiments of exogenously applied steroids should elucidate causes and effects of the regarded expression pattern. Finally, the cellular localization of the steroid receptors within the oviduct was approached.

2. Material and methods

2.1. Tissue preparation and cell culture

Tissue preparation and cell culture were performed as described previously [26] with minor modifications. Briefly, oviducts of cycling German Fleckvieh cows were collected from a local slaughterhouse within 20 min of death and stored in ice-cold Ringer solution until further examination in the laboratory. For in vivo investigations the oviducts were grouped depending on cycle stage by examining the ovaries and uterus [27]. Oviducts were classified into four groups: early-luteal stage (days 1–5), mid-luteal stage (days 6–12), late-luteal stage (days 13–18) and follicular stage (days 19–21). Surrounding tissue was taken off and afterwards the oviducts were opened longitudinally under steril conditions (clean bench). The epithelial cells were scraped off mechanically separating the ampulla from the isthmic region [28]. These cells (∼10 mg wet weight from each oviduct) were directly used for RNA isolation. For in vitro investigations randomly selected oviducts corresponding to different reproductive stages were taken and cells collected as described above. Epithelial cells (10^5 ml^{-1} medium) were cultured in medium 199 containing 5% FCS and a mixture of antibiotics at 39 °C in 5% CO_2 and saturated humidity. After an accommodation period of 48 h either estradiol-17β (E2 10 pg/ml) or progesterone (P4 10 ng/ml) was applied in a single dose and incubated for 1, 2, 4 and 6 h ($n = 6$). P4 was incubated additionally for 24 h. A non-treated group of culture dishes served as a negative control. After centrifugation at 4000 rpm and 4 °C the cell pellets were resuspended in PBS-buffer for RNA isolation. The supernatants were stored separately at −20 °C for further enzyme-immuno assay (EIA) investigation. The identity of the collected cells was verified by microscopic observation, since ciliated cells could be detected visually. The criteria of viability of the cells used for cell culture were the beating of the cilia as well as the exclusion of trypan blue.

2.2. Total RNA extraction and mRNA analysis

RT-PCR was performed as described previously with minor modifications [29]. Briefly, total RNA was isolated from epithelial cells using spin columns (Roche), followed by DNA digestion using DNAse 1. The RNA was spectroscopically quantified at 260 nm and then stored at −80 °C. Electrophoresis on a 1% (w/v) denaturating agarose gel determined the quality and integrity of the gained RNA. One μg of total RNA was used for generating single stranded cDNA in a 60 μl reaction mixture. To exclude DNA contamination of the extracted RNA, negative controls using water instead of the reverse transcriptase enzyme were performed. The cDNA, stored at −20 °C, served as a template for polymerase chain reactions (5× buffer (Promega), 10 mM dNTPs (MBI Fermentas), 50 μM hexamere (Pharmacia), 200 U superscript (Promega)). The PCR primers were designed referring to the bovine sequences of EMBL accession number Z86041, U6496 and Y18017. The primers were synthesized (MWG-Biotech, Ebersberg, Germany) to amplify specific fragments of bovine transcripts referring to ERα (forward, 5′-AGG GAA GCT CCT ATT TGC TCC-3′; reverse, 5′-CGG TGG ATG TGG TCC TTC TCT-3′ (234 bp)), ERβ (forward, 5′-GCT TCG TGG AGC TCA GCC TG-3′; reverse, 5′-AGG ATC ATG GCC TTG ACA CAG A-3′ (262 bp)) and PR (forward, 5′-GAG AGC TCA TCA AGG CAA TTG G-3′; reverse, 5′-CAC CAT CCC TGC CAA TAT CTT G-3′b (227 bp)). The predicted size of each RT-PCR product is assigned in parenthesis. The primer pair for PR were designed to detect both A and B isoform. The amplified fragments were commercially sequenced once to specify the gained PCR product (MWG-Biotech, Ebersberg, Germany). Thereafter the melting point of the amplified product carried out within the LightCycler® standard PCR protocol served as confirmation of the product identity. To verify the integrity and the equal relative quantity of the reverse transcribed cDNA PCRs for 18 s ribosomal RNA mRNA (forward, 5′-TCA AGA ACG AAA GTC GGA GG-3′; reverse, 5′-GGA CAT CTA AGG GCA TCA CA-3′ (493 bp)) were carried out. To exclude any contamination of foreign ribonucleic acid in probes, a negative control using water instead of cDNA was added to each PCR reaction mixture. To exclude contamination with DNA a β-actin fragment (mRNA (forward, 5′-CTT CGC GGG CGA CGA TGC-3′; reverse, 5′-CGA ACA TGG CTG GGG TGT TG-3′ (341 bp)) spanning several exons was amplified (data not shown). Online PCR reactions using the LightCycler® DNA Master SYBR

Green I protocol (Roche Diagnostics, Mannheim, Germany) were performed as described previously [29]. Annealing temperatures and fluorescence acquisition points for quantification within the fourth step of the amplification segment were 63 °C respectively 86 °C for ERα, 64 °C respectively 81 °C for ERβ and 65 °C respectively 81 °C for PR. Each of the probes contained 1 μl of 17 ng/μl cDNA and was amplified in a 10 μl reaction mixture (3 mM $MgCl_2$, 0,4 μM primer forward and reverse each, 1× LightCycler DNA Master SYBR Green I) in opposition to a standard curve based on the spectroscopical quantification of a PCR product.

2.3. Statistical analysis

All data were analysed by one-way analysis of variance (ANOVA). The normal distribution was tested by the Kolmogorow–Smirnov method. If the analysis of variables showed significant differences of groups, a Tukey test was used to test the significance at a level of $P < 0.05$ (Sigma Stat).

2.4. Enzyme-immuno assay (EIA)

Enzyme-immuno assays for progesterone [30] and estradiol-17β [31] were undertaken as described previously to screen the hormone concentration in the cell culture supernatants. Additionally, the binding ability of the applied steroid hormones was verified.

2.5. Western blot analysis

For protein extraction approximately 100 μg of oviductal tissue from at least three different cows for each cycle stage and region were homogenized in lysis buffer containing proteinase inhibitor. Protein samples (36 μg per lane) were separated on a 4–12% Bis-Tris Gel (NuPage, Invitrogen, CA, USA) in MOPS running buffer and transferred onto nitrocellulose membranes. The membranes were blocked with 1% dried milk in TBS containing 0.1% Tween-20 over night. They were incubated with the monoclonal antibody against ERα (2-185, Santa Cruz Biotechnology, CA, USA), ERβ (PA1-311 Affinity BioReagents Inc., Golden, CA) or PR (Clone 10A9, Coulter Immunotech, Marseille, France) in TBS-0.1% Tween-20 1% dried milk for 75 min at room temperature. The dilutions used were 1:200 (ERα), 1:500 (ERβ) and 1:50 (PR), respectively. After washing, the membranes were incubated with anti-mouse (ERα, PR) or anti-rabbit (ERβ) horseradish peroxidase-conjugated IgG secondary antibody (DAKO, Hamburg, Germany) at a dilution of 1:2000 in TBS-0.1% Tween-20 1% dried milk for 45 min at room temperature. After washing in TBS-0.1% Tween-20 and TBS alone the membrane was incubated with enhanced chemiluminescence reagent detection solution (Amersham, Buckinghamshire, UK) for 3 min in the dark. Finally, an X-ray film was exposed to the membrane to visualize protein expression. As a positive control a recombinant human ERα protein (Sigma, Munich, Germany) was used at concentration of 30 ng per lane. The ERα protein was also employed to test for cross-reactivity between the three antibodies used.

2.6. Immunohistochemistry

The procedure for the immunohistochemical localization for PR was performed as described previously [32]. Cycle dependant oviducts were collected as described above, surrounding tissue was removed and pieces distinguishing the ampulla and the isthmus were shock-frozen in liquid nitrogen. Serial cross-sections of 7 μm thickness were cut on a cryostat (CM 1850, Leica, Bensheim, Germany) at −27 °C, mounted on poly-L-lysine coated slides and air-dried. Sections were fixed for 5 min in 4% paraformaldehyde. Endogenous peroxidase was inhibited by treating the fixed sections with 1% hydrogen peroxidase for 30 min. Non-specific protein binding was eliminated by incubation with normal goat serum (1:10 dilution in PBS-buffer) for 30 min at room temperature. A primary antibody was applied at 4 °C over night in a humidified chamber to prevent evaporation. The monoclonal mouse anti-PR antibody (MA1-410 Affinity BioReagents Inc., Golden, CA) was used which detects both PR-A 94 kDa and PR-B 120 kDa protein isoforms by nuclear staining at a concentration of 3 μg/μl. The ERα mouse monoclonal antibody from clone 1D5 (Coulter Immunotech Diagnostics, Germany) was applied at a dilution of 1:100. A rabbit polyclonal antibody (PA1-311 Affinity BioReagents Inc., Golden, CA) raised against the N-terminal region of ERβ (amino acids 54–71 of rat ERβ sequence, EMBL accession number AJ002603: AEPQKSPWCEARSLEH) was incubated as utilized by Rosenfeld et al. [13].

For control sections either primary or secondary antibodies were omitted and replaced by buffer. Sections were incubated with DAB alone to exclude residuous endogenous peroxidase activity. A lack of staining in controls showed the antigen specificity. After the incubation of PR or ERα, horseradish peroxidase-labelled goat anti-mouse IgG (DAKO, Hamburg, Germany) diluted at 1:200 was applied at room temperature for 1 h. Incubation of ERβ was followed by undiluted HRP-labelled goat secondary antibody detection system against rabbit IgG (DAKO En-Vision™+, Hamburg, Germany) on equal terms. Peroxidase was visualized by applying 0.01% 3,3′-diaminobenzidine hydrochloride (DAB, Sigma, Germany) and 0.01% hydrogen peroxide in PBS-buffer for 10 min in darkness at room temperature. Sections were counterstained in Mayer's Haemalaun (Merck, Germany), dehydrated and mounted on slides for light microscopy (Axioscope, Zeiss, Göttingen, Germany).

3. Results

3.1. In vivo transcript quantification

In bovine oviduct epithelial cells mRNA transcripts of ERα, ERβ and PR were detected. Obviously, expressions

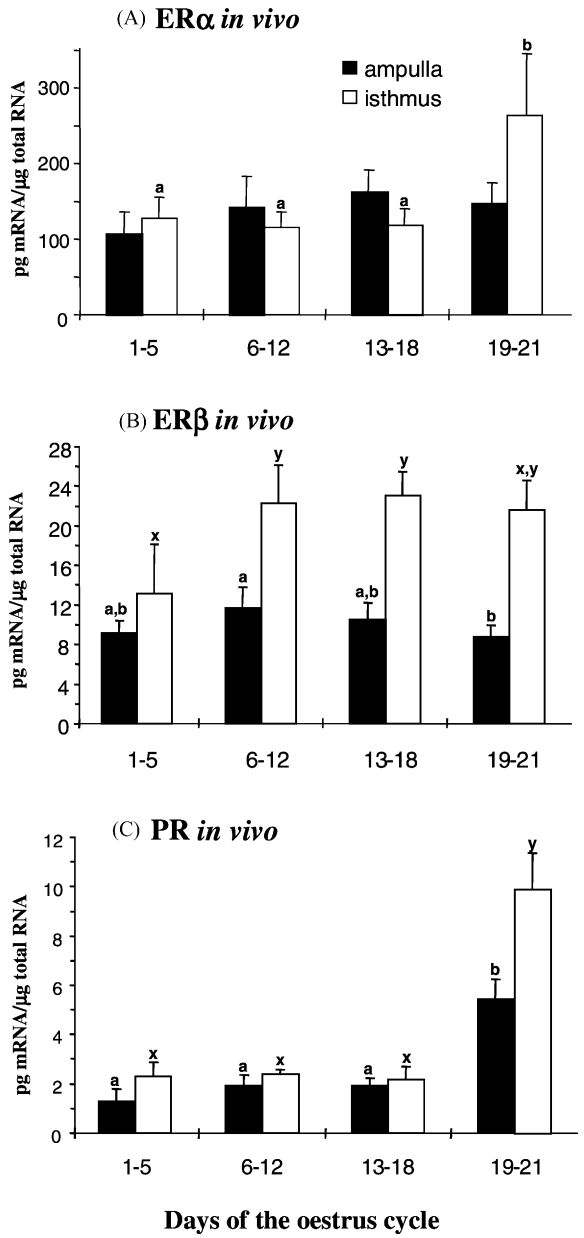

Fig. 1. ERα (A), ERβ (B) and PR (C) mRNA concentrations (LightCycler real-time RT-PCR) in vivo in bovine oviduct epithelial cells during the oestrus cycle (early-luteal phase days 1–5, mid-luteal phase days 6–12, late-luteal phase days 13–18, follicular phase days 19–21) in either ampulla (■) or isthmus (□). Results represent means of concentration of mRNA/total RNA ± S.E.M. ($n = 6$). Different superscript letters indicate significant different groups ($P < 0.05$).

changed in both the ampulla and the isthmus region throughout the oestrus cycle and after steroid treatments as initially detected by conventional block RT-PCR (data not shown).

A significant 2.5-fold increase of ERα mRNA expression (Fig. 1A) could be detected in the isthmus in vivo during the follicular phase (days 19–21) compared to the mid-luteal phase (days 6–12) (263 pg versus 116 pg mRNA/µg total RNA). In the ampulla the expression remained unchanged on a comparable level during all days of the cycle (Fig. 1A).

In bovine oviduct epithelium in vivo ERβ mRNA (Fig. 1B) was expressed highest in the isthmus during the luteal and late-luteal phase (days 6–18) and decreased during the early-luteal phase (days 1–5) (23.8 pg versus 13.1 pg mRNA/µg total RNA). Accordingly, in the ampulla the expression was highest during the luteal phase and lowest during the follicular phase (days 19–21) (11.6 pg versus 8.3 pg mRNA/µg total RNA), but on a lower absolute level (Fig. 1B). Therefore the expression in the isthmus was about twice as high as in the ampulla.

The mRNA expression of PR in vivo (Fig. 1C) was highest during the follicular phase (days 19–21) and significantly decreased to lowest levels during the early-luteal phase (days 1–5) (9.9 pg versus 2.3 pg mRNA/µg total RNA in the isthmus). The isthmus showed signals twice as high as the ampulla during the upregulated phase (Fig. 1C).

3.2. In vivo protein expression

Commercial monoclonal anti-steroid receptor antibodies were used successfully to detect ERα, ERβ and PR by Western blot analysis. The intensity of the staining allowed a semi-quantitative estimation of the protein expression during the oestrus cycle (Fig. 2A–C).

The antibody against ERα detected a 60 kDa band in the positive control (bovine endometrium) as well as in the oviduct samples. The expression varied extraordinarily between the ampulla and the isthmus. To demonstrate this difference, the blot was exposed to the X-ray film for varying times (Fig. 2A). In the ampulla, the ERα was found much higher than in the isthmus. Additionally, the signal intensity was obviously higher during the early-luteal phase compared to the other oestrus phases in both the ampulla and the isthmus.

The anti-ERβ antibody recognized two bands at approximately 62 and 58 kDa in the bovine endometrium as well as the oviduct (Fig. 2B). ERα protein was used as a negative control to specify any cross-reactivity resulting in lack of staining. This indicated the specificity of the antibody well distinguishing between ERα and ERβ. In all oviductal samples investigated these two isoforms were expressed at almost equal terms, showing lower protein expression in the ampulla than in the isthmus. In the course of the oestrus cycle the most intense staining of both bands was visible during the luteal stage.

Western blotting analysis of PR revealed three isoforms in the positive control of bovine endometrium (Fig. 2B). The bands were visible at approximately 116, 92 and 65 kDa molecular weight, corresponding to the known PR isoforms A–C, respectively [51]. Since the recognition epitope of this antibody against PR is located at the C-terminal domain of the PR molecule, all three isoforms should be detectable. Additionally, the ERα protein served as a negative control and excluded any cross-reactivity between ERα and PR. In the oviduct the PR-C isoform (65 kDa) was not detectable, whereas PR-A (92 kDa) and PR-B (116 kDa) were expressed

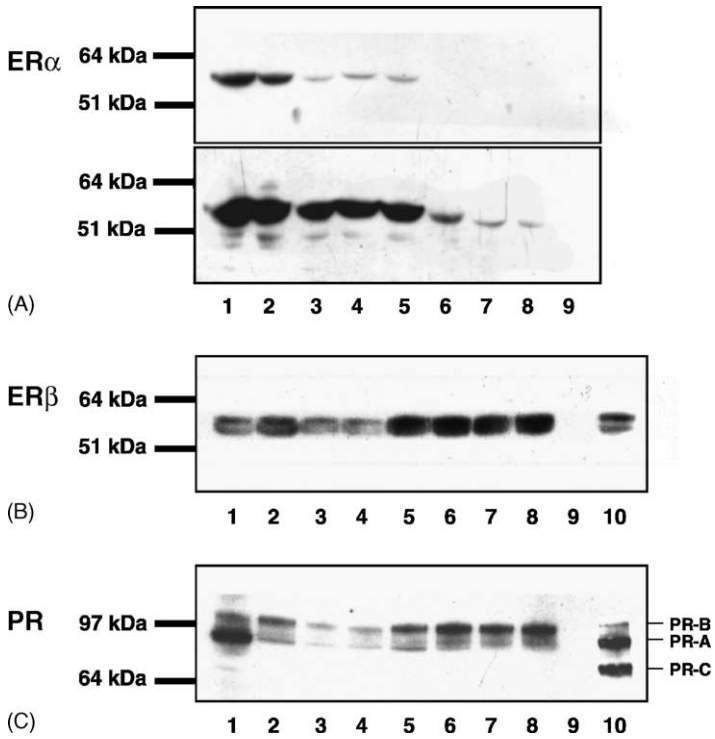

Fig. 2. Western blot analysis of ERα, ERβ and PR during the oestrus cycle. For ERα (A), the membrane was exposed to the X-ray film for varying times (upper part 1 min exposure, lower part 10 min exposure). (A) Lane 1, bovine endometrium; lane 2, ampulla early-luteal phase days 1–5; lane 3, ampulla luteal phase days 6–12; lane 4, ampulla late-luteal phase days 13–18; lane 5, ampulla follicular phase days 19–21; lane 6, isthmus early-luteal phase days 1–5; lane 7, isthmus luteal phase days 6–12; lane 8, isthmus late-luteal phase days 13–18; lane 9, isthmus follicular phase days 19–21. (B), (C) Lane 1, ampulla early-luteal phase days 1–5; lane 2, ampulla luteal phase days 6–12; lane 3, ampulla late-luteal phase days 13–18; lane 4, ampulla follicular phase days 19–21; lane 5, isthmus early-luteal phase days 1–5; lane 6, isthmus luteal phase days 6–12; lane 7, isthmus late-luteal phase days 13–18; lane 8, isthmus follicular phase days 19–21; lane 9, ERα protein; lane 10, bovine endometrium.

differentially. PR-B revealed more insense staining in the isthmus than in the ampulla. The 92 kDa band corresponding to PR-A was most intense in the ampulla during the early-luteal phase (days 1–5) and moderate during the other cycle phases. In the isthmus, the PR-A isoform stained moderate during the early-luteal phase and was absent during the luteal and oestrus phase.

3.3. *In vivo protein localization*

Immunoreactive ERα was localized to nuclei of the luminal epithelial cell layer in cross-sections of bovine oviducts during all phases of the oestrus cycle (Fig. 3A). A faint cytoplasmatic staining was visible in the muscular layer surrounding the oviduct (Fig. 3A).

The protein staining for ERβ revealed mainly nuclear signals in luminal epithelial cells of oviductal sections during all phases of the oestrus cycle (Fig. 3B). A moderate cytoplasmatic staining could be revealed in the epithelial cell layer and in the muscle layer, but not in stromal tissue.

PR was always localized to the cell nuclei in the bovine oviduct. Positive immunostaining was found in the luminal epithelial cell layer of both the ampulla and the isthmus. The most intense staining was detected in early-luteal phase oviducts (days 1–5) (Fig. 3C). Cell nuclei of longitudinal and circular muscle layers were stained intensively positive for PR in both the ampulla and the isthmus only during the early-luteal phase of the oestrus cycle (days 1–5). No cytoplasmatic staining was visible for PR. The omission of either the primary or the secondary antibodies revealed no staining as done for all three experiments (Fig. 3D).

3.4. *Cell culture, steroid application and in vitro transcript levels*

A 2-day suspension culture of the bovine oviduct epithelium was established to retain as much physiological cell morphology as possible. During the course of the experiment cells did not attach to the culture dishes and were in vital condition as judged by light microscopy of cilia-induced spinning as well as trypan-blue staining. The beginning of a typical oviduct epithelial cell arrangement in tube-form could be observed as it has been described previously [33].

The applied E2 was fully redetected in cell culture supernatants during the 6 h incubation, showing significantly higher levels of E2 than control (Table 1). During P4 stimulation constant levels of estradiol-17β were found. Astonishingly, applied P4 decreased in the course of the experiment.

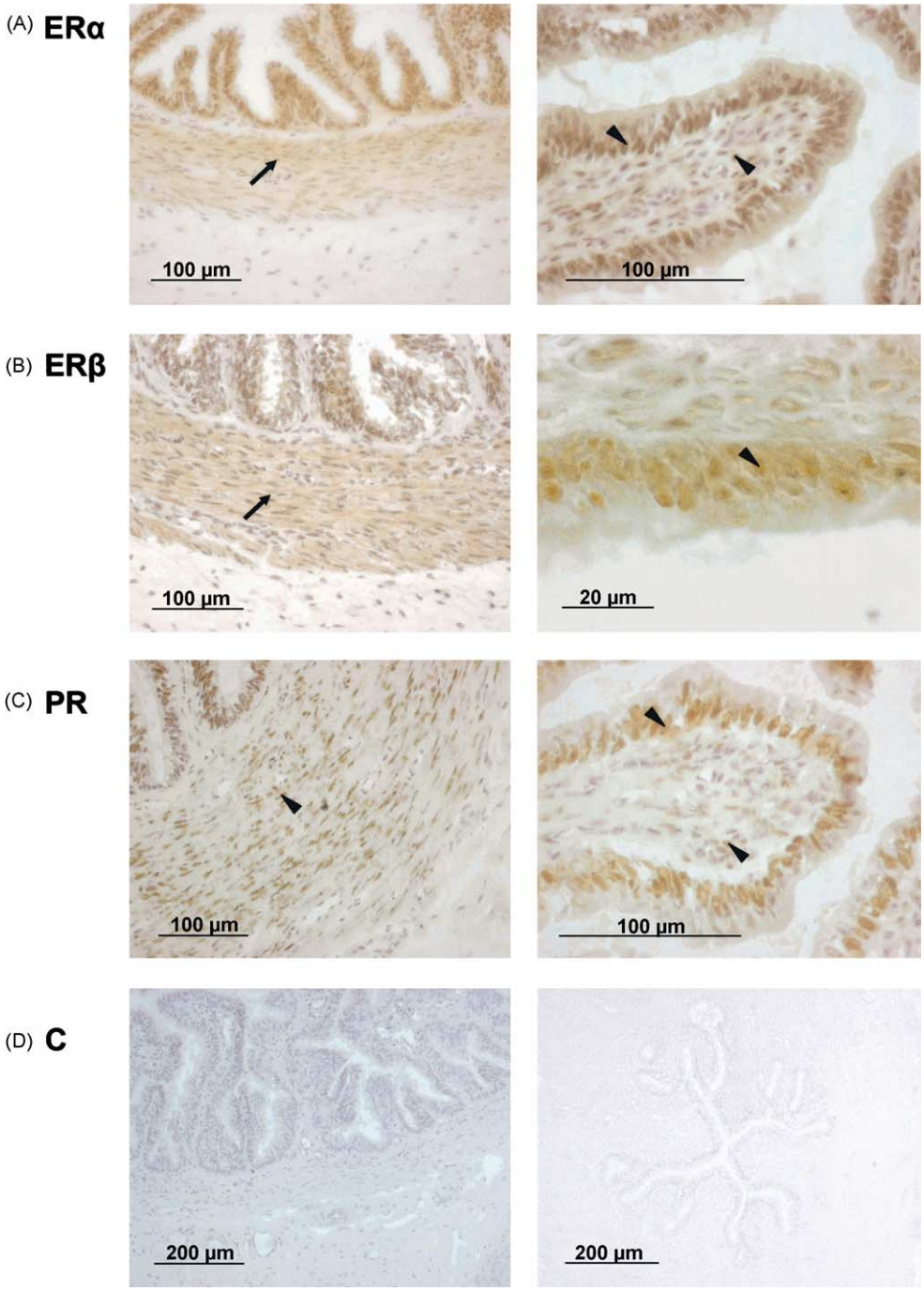

Fig. 3. Immunohistochemical localization of ERα (A), ERβ (B) and PR (C) in serial cryosections of bovine oviducts. Sections A, C and D were counterstained with haematoxilin. The black arrow point at specific nuclear staining. The spearheads indicate cytoplasmatic staining. Sections (D) exemplify negative controls with and without Haemalaun counterstaining.

After 4 h levels fell below 10 ng/ml and after 24 h levels fell below 1 ng/ml. This P4 depletion did not occur in the cell free medium (data not shown). Since the stability of P4 in culture medium was reliable over the period of the experiment, P4 was either absorbed or metabolized by epithelial cells. There was no indication of a specific enzymatic conversion (aromatase) because E2 levels remained unaffected and aromatase expression was not detectable in these cells (own unpublished data). The maintenance of the ciliated epithelial cell phenotype in our culture system points towards a retainance of characteristics associated with in situ conditions [34], reflecting predominantly physiological settings.

In vitro the expression of ERα was significantly increased by E2 for the first 2 h after stimulation (525 pg versus 220 pg

Table 1
In vitro hormone concentration in cell culture supernatants as determined by enzyme-immuno assay

	Time (h)	Progesterone (ng/ml)	Estradiol-17β (pg/ml)
Control		n.d	5.36 ± 1.96 a
E2-stimulation (10 pg/ml)	1	n.d.	10.87 ± 1.13 b
	2	n.d	11.12 ± 2.16 b
	4	n.d	10.63 ± 2.69 b
	6	n.d.	12.07 ± 2.46 b
P4-stimulation (10 ng/ml)	1	14.22 ± 1.81 a	6.96 ± 1.62 a
	2	10.61 ± 1.76 ab	5.46 ± 1.17 a
	4	9.67 ± 2.26 b	7.88 ± 4.12 a
	6	5.60 ± 0.87 c	6.66 ± 3.56 a
	24	0.93 ± 0.62 d	6.71 ± 2.74 a

Results represent mean ± S.E.M. ($n = 6$); different letters indicate significant differences between groups ($P < 0.05$); n.d., not detectable.

mRNA/μg total RNA) (Fig. 4A). Six hours later the expression level dropped below control level. P4 did not show any detectable effect on ERα transcripts regulation (Fig. 4A). Oviduct cells in vitro showed a 5.7-fold upregulation of ERβ after P4 treatment (Fig. 4B). There was only a short but not significant respondance of ERβ 1 h after E2 stimulation. In the cell culture PR was effected by E2 showing a two-fold increase after 2–4 h of incubation in comparison with untreated controls (Fig. 4C). The application of P4 had deregulating effects below the control level after 6–24 h.

4. Discussion

This study demonstrates a remarkable variability of mRNA and protein expression of PR, ERα and ERβ in the bovine oviduct during the course of the oestrus cycle. Furthermore, direct ligand effects could be demonstrated within a short time-course by the use of oviduct epithelial cells in vitro after stimulation with physiological doses of estradiol-17β and progesterone.

In peripheral blood plasma estrogen levels are found highest during the follicular phase of the bovine oestrus cycle, whereas progesterone is dominating during the mid- and late-luteal phase [31]. The oviduct is considered to be under the influence of these hormones [35] and its sensitivity towards steroid hormones should be mediated through the presence of their specific receptors. It could be shown that under the dominance of peripheral estrogens at oestrus the mRNA expression of ERα was elevated in vivo. Likewise, the in vitro stimulation with E2 was followed by significantly elevated ERα transcripts rapidly after exposure. Western blotting revealed increased ERα protein during the early-luteal phase indicating a translational delay. These results can be endorsed by binding assays performed earlier with bovine endometrium [36]. Additionally, in the human

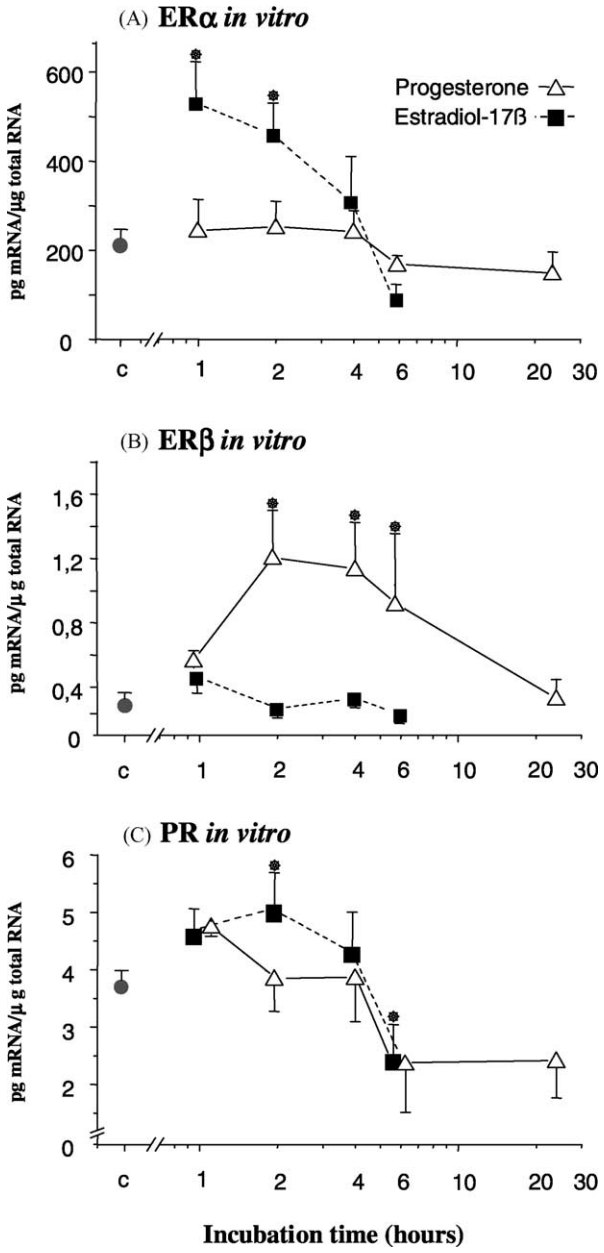

Fig. 4. ERα (A), ERβ (B) and PR (C) mRNA concentrations (LightCycler real-time RT-PCR) in vitro in bovine oviduct epithelial cells after stimulation with estradiol-17β (■) or progesterone (△) for 1, 2, 4, 6 or 24 h. Results represent means of concentration of mRNA/total RNA ± S.E.M. ($n = 6$). Significant different groups compared to control (c) are indicated with ($^*P < 0.05$).

oviduct ER protein stained positive during the follicular phase [37]. Observations of in situ hybridization detected ERα mRNA primarily in the rat oviduct epithelium of the isthmus rather than the ampulla [38], but in that study no protein data were provided. Our mRNA data revealed an enhanced oestrus dependent expression only in the isthmus, whereas Western blotting clearly demonstrated that the protein was found as well in the ampulla. The differences in half-life of specific mRNA and protein might contribute

to the contradictory finding. It must be mentioned that for protein analysis whole oviducts were taken while mRNA profiles were analyzed in epithelial cells only. Furthermore, the RT-PCR technique used is much more sensitive and therefore advantageous over the Western blotting technique concerning the quantitative prediction of expression. For this, the mRNA expression profiles seem more reliable. Anyhow, the lacking of an mRNA-rise of ERα during oestrus together with an increased number of protein at the early-luteal stage in the ampulla points towards regulatory mechanisms aside from transcriptional regulation that may affect the presence of this steroid receptor.

In the rat oviduct, Wang et al. [17] observed nuclear staining of ERα in epithelial cells as well as in stromal and muscle cells. Our results revealed immunoreactive ERα receptor protein in nuclei of the luminal epithelial layer and in addition, nuclear staining of individual stromal cells was observed. Kimmins et al. [39] suggest that stromal estrogen receptors (and progesterone receptor as well) trigger the steroid responsiveness of the epithelium as shown from mice knock-out studies [40]. This mechanism could possibly underlie our observed stromal and faint cytoplasmatic ERα staining in the muscle layer of the bovine oviduct. Overall, the upregulation of ERα was obviously found around the time of oestrus. This may account for specific compositional changes of the oviductal fluid occurring during the estrogen-dominated follicular phase.

The immunoblot studies demonstrated the presence of two isoforms encoding ERβ in bovine tissue revealing a double band of approximately 58 and 62 kDa, respectively. These data probably reflect the two isoforms of the ERβ gene, which already have been shown in the human [41]. During the early-luteal phase the ERβ protein was upregulated, which is in accordance to the mRNA data. In the cow the luteal phase is dominated by high peripheral blood levels of progesterone [31]. Accordingly, ERβ expression levels were elevated in vitro after P4 treatment, while ERβ mRNA remained unaffected by estradiol. Both in vivo and in vitro data therefore provide evidence for a direct dependency of ERβ on progesterone. In accordance to our mRNA results the ERβ protein staining was much more intense in the isthmus than in the ampulla. This may support a different regulation of ERα and ERβ indicating their distinct physiological functions.

In the rat oviduct ERβ was localized to nuclei of epithelial, stromal and muscle cells by immunhistochemistry [42]. Taylor and Al-Azzawi [43] found an almost ubiquitous immunohistochemical localization of ERβ in human tissue including the oviduct. These reports confirmed the cytoplasmatic staining found here in the bovine oviduct in addition to the expected nuclear staining within the epithelium. The cytoplasmatic staining of both ERβ and ERα in the muscle layer points towards a possible interaction with the oviduct motility.

The predominant presence of ERα has recently been shown for the rat and the human oviduct [15,43]. Because of a 10-fold higher mRNA expression of ERα versus ERβ we propose that in the bovine oviduct receptor-mediated actions may be mainly regulated through ERα. Nevertheless the occurrence of two subtypes during the oestrus cycle points towards selective time and region specific effects. The hypothesis that both ER subtypes each contribute to different biological functions is supported [38]. This is strengthened through the finding that both ER have different main target regions. Possible inhibitory effects of ERβ towards mediation of estrogenic actions should be mentioned at this point. Nevertheless its authentic physiological function especially in the bovine oviduct remains to be elucidated in more detail in the future.

During the estrogen-dominated follicular phase a distinct upregulation of PR transcripts was measured in bovine oviduct epithelium. Corresponding to this, in vitro data showed that E2 stimulated the expression of PR mRNA. In contrast, P4 stimulation resulted in a reduction of transcript numbers, indicating that the oviductal PR was suppressed during progesterone dominance. With decreasing peripheral progesterone levels during luteolysis, this inhibition assumingly diminished and entailed a strong upregulation of PR. In addition, subsequently rising peripheral estrogen levels probably stimulated PR mRNA expression during the follicular phase followed by a delayed protein expression at the beginning of the oestrus cycle.

The Western blotting analysis revealed distinct distributions of the different isoforms of PR. In bovine oviducts PR-C was not detectable, whereas this 65 kDa isoform has been found earlier in bovine endometrium and mammary tissue [44]. Most obvious was the presence of PR-A at about 92 kDa in early-luteal phase ampulla. Astonishingly, a continuously high PR-B level maintained throughout the luteal phase with even more denotation in the isthmus. Yet comparable observations were described in human endometrium throughout the menstrual cycle [45] and during the progesterone dominated gestational phase [46]. Recent studies using knock-out mice in which either the PR-A or the PR-B isoform was selectively eliminated showed that PR-B did not effect ovarian and uterine response to progesterone [47]. Thus PR-A appeared to be necessary and sufficient to maintain fertility. We therefore propose that the bovine PR-A isoform underlied the cyclical modulations induced by the change in transcript expression observed by RT-PCR, whereas PR-B seemed to be unaffected by peripheral steroid hormones. In addition, Vegeto et al. [48] demonstrated that the PR-A acted as the dominant repressor of transcription of progesterone sensitive genes, while PR-B was termed as an activator of transcription. Therefore the outlined presence of PR-B and the absence of PR-A during the luteal phase may enhance the progesterone-mediated actions in the bovine oviduct. For this reason a progesterone-mediated upregulation of ERβ in the luteal phase might have been potentiated.

Immunohistochemistry detecting PR revealed intense staining of the luminal epithelial cell layer. Because our antibody could not distinguish the two isoforms, the selective

detection of a diverse localization of PR-A and PR-B was not possible. Further observations in this respect could elucidate implications of their formerly suggested functionality [45].

Our PR protein data are corresponding to binding studies in bovine endometrium reported by Meyer et al. [19] with highest receptor concentrations found during days 1–8 of the bovine oestrus cycle. In addition, upregulated PR gene and protein expressions after E2 treatment have been shown in the rat uterus [21] as well as in the human oviduct, were high concentrations of immunoreactive PR were measured under the influence of estrogens [37].

Intense nuclear staining of the muscular layer surrounding the oviduct provide some evidence for the importance of PR mediating motility. In addition the different target regions of PR expression could possibly reflect functional importance. Hunter et al. [49] proposed progesterone interactions with sperm released from the caudal isthmus sperm reservoir. Since progesterone levels are not elevated directly in the oviduct around and after ovulation [1], minute levels of progesterone secreted by either pre-ovulatory Graafian follicles or the early corpus luteum could unfold an effect via a countercurrent transfer to the oviduct. An upregulation of PR-B along the isthmus epithelium could indicate functional active hormone-receptor complexes which may lead to controlled release of isthmus epithelial-bound sperm probably mediated through relaxation of surrounding oviductal muscular layers.

Coppens et al. [50] reported a predominant ampullary appearance of estrogen and progesterone receptors in the human oviduct. Our results, which are summarized in Fig. 5, demand a more specific differentiation of the steroidal regulation in cow oviducts. In summary, the ERα is prevalent in the ampulla, whereas ERβ is dominantly expressed in the isthmus. The indicated protein expression of PR is valid only regarding the PR isoform A. The PR isoform B seems to underlie a different regulatory mechanism due to a dominant expression in the isthmus indifferent of the cycle stage.

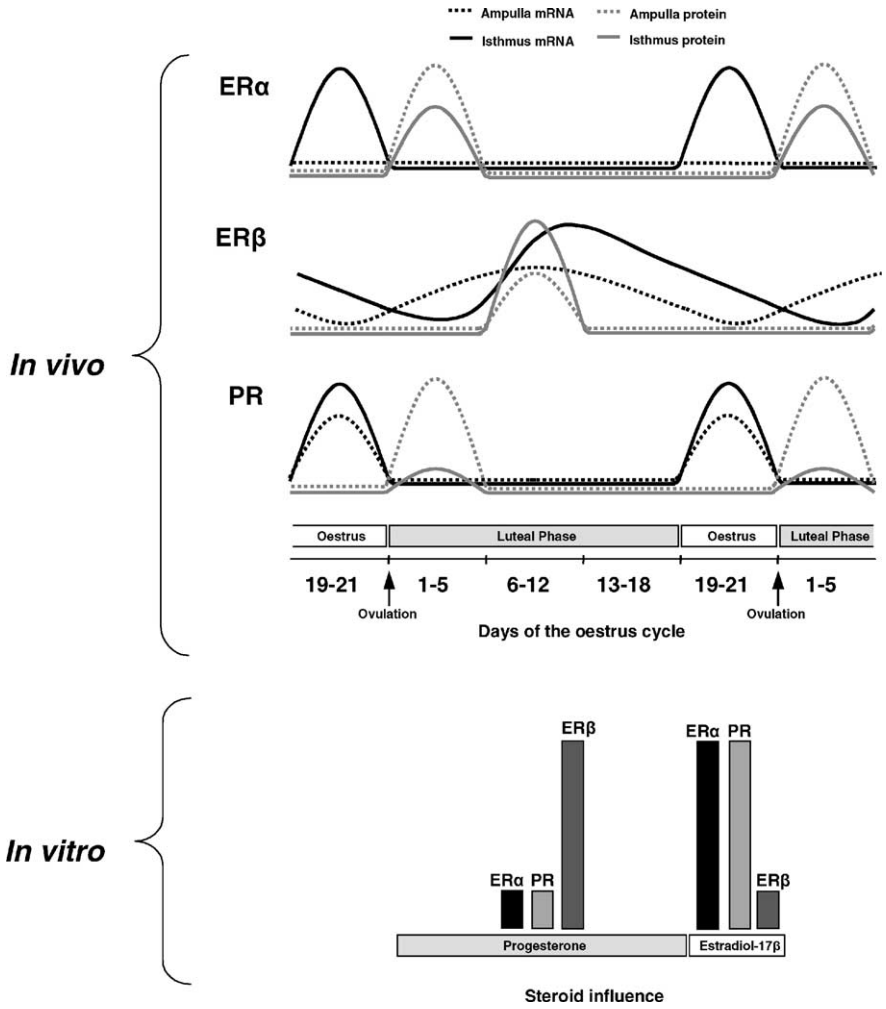

Fig. 5. Summarized results of the three steroid hormone receptors mRNA (black lines) as well as protein expression (grey lines) in the bovine oviduct during the oestrus cycle (in vivo, upper part). Dotted lines represent expression in the ampulla and bold lines expression in the isthmus of the oviduct. The upregulation of the investigated steroid hormone receptors after the exogenous application of either estradiol-17β or progesterone is presented as elevated bars (in vitro, lower part).

Enclosing possible cross-talks between each ligand-receptor system, our findings stress the importance of this fine tuned differences of the steroid receptors expression. Furthermore, it can be assumed that steroids play an important role in the regulation of their own receptors as demonstrated here with exogenous steroid applications in vitro. The in vitro experiments gave evidence for the elucidation of regulatory mechanisms in vivo on the basis of circulating steroids. Therefore, the bovine oviduct epithelial cell suspension culture could serve as a favourable and potent model to further study hormone regulations within this part of the female reproductive system.

Finally, this is the first comprehensive report, which quantified and localized ERα, ERβ and PR within the bovine oviduct. The analysis of the receptors mRNA and protein expression clearly demonstrated the cyclical and steroidal dependency and therefore gave evidence for the bovine oviduct as an important target tissue for sexual steroids. These findings may further unravel its important contribution to reproductional success, since the oviduct is the site of final gamete maturation, fertilization and early embryonic development.

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