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Original article

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EXPLORING CUMULUS-OOCYTE-COMPLEX-OVIDUCTAL CELL INTERACTIONS: GENE PROFILING IN THE BOVINE OVIDUCT

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Prostaglandin E_2 (PGE_2) is present in the bovine oviduct and may play an important role in muscle contraction or as survival factor providing the optimal environment for fertilization and the early embryo. The aim of the present study was to investigate the estrous cycle-dependent changes and local distributions of PGE_2 receptors (EP) and members of the trefoil factor (TFF)-family in the bovine oviduct using real-time RT-PCR. A co-cultivation system of cumulus-oocyte-complexes (COC) with primary oviductal cells was screened for the mRNA expression pattern of selected factors. An oviductal primary cell culture was used for investigating effects of estradiol on signal transduction pathways. Quantitative RT-PCR revealed significant higher expression of EP2 and EP4 in the pre-ovulatory phase compared with the luteal phase. TFF3 mRNA was expressed during the estrous cycle with highest level in the post-ovulatory phase showing higher expression levels in the isthmus compared with the ampulla. A different mRNA expression pattern was observed for factors involved in extracellular matrix formation in co-cultured oviductal cells compared to untreated controls. *In vitro*, NF-κB was found activated after estradiol treatment. These results suggest that a fine-tuned PGE_2 signal transduction pathway may support fertilization, early embryonic development and gamete transport in the bovine oviduct.

Key words: *bovine, estrous cycle, oviduct, prostaglandin, trefoil factor*

INTRODUCTION

Mammalian fertilization occurs in the oviduct of the female genital tract. The oviduct and the fluid contained within provide a beneficial environment for gamete maturation, gamete transport, fertilization, and early embryonic development. These events are key processes in mammalian reproduction which

are under control of hormones, *e.g.* steroids (1) and prostaglandins (PG) (2,3). Higher PGE_2 and $PGF_{2\alpha}$ concentrations were observed around ovulation in the bovine oviduct compared with the luteal phase (4). In addition, ipsilateral oviducts contained higher PGE_2 and $PGF_{2\alpha}$ levels than the contralateral oviduct. *In vitro* experiments revealed that PGE_2 synthesis is up-regulated in bovine oviductal cells by treatment with estradiol (5). Estradiol acts via its receptors and results in activation of NF-κB (6). This signal transduction pathway led in human bronchial epithelial cells to higher PGE_2 secretion (7).

Cyclooxygenases (COX) synthesize the intermediate PGH_2, the precursor for different prostaglandins, including PGE_2 (8, 9). COX-1 showed significantly increased mRNA expression in the bovine oviduct around ovulation compared with the luteal phase resulting in the highest COX-1 protein content in the early-to-mid luteal phase (10). In contrast, COX-2 mRNA and protein amounts did not vary throughout the estrous cycle in the bovine oviduct. COX activity in oviductal cells originated predominantly from COX-1 (10). The unstable PGH_2 intermediate is transformed into PGE_2 by one of the three specific PGE synthases (PGES) (11). The autocrine/paracrine effects of PGE_2 were mediated by interacting with their G-protein-coupled cell-surface receptors (12). PGE_2 has four receptor subtypes: EP1, EP2, EP3, and EP4. These receptor subtypes are encoded by different genes and differ in their signal transduction pathways (13). Activation of EP1 and EP3 generally results in contraction of smooth muscle, while activation of EP2 and EP4 results in relaxation.

The integrity of the mucus lining in the female reproductive tract is important for its physiological function and its defense. Prostaglandins are described as important cytoprotective molecules (14) and mediate epithelial cell migration in the gastrointestinal tract (15). It has been demonstrated that trefoil factor 3 (TFF3) stimulated PGE_2 production (16). Three TFF-peptides have been characterized as constituents of mucous gels: TFF1, TFF2, and TFF3 (17). Expression of members of the TFF family has been detected in the human (18) and bovine uterus (19). Prostaglandins induce the cumulus expansion in the bovine cumulus-oocyte-complex (COC) (20). Furthermore, it was suggested that a cross-talk takes place between the COC and oviductal cells, probably mediated by growth factors and extracellular matrix (ECM) components (21). A finely tuned extracellular proteolysis of bovine COC possibly facilitates cumulus expansion (22), which is important for a successful fertilization. An important proteolytic system is the matrix metalloproteinase (MMP)/tissue inhibitor of metalloproteinase (TIMP) system (23). MMP-1 and MMP-2, also known as collagenase-1 and gelatinase A, are enzymes which degrade components of the ECM. Members of the TIMP family can inhibit activity of MMP resulting in a fine-tuned proteolytic cascade. In the interaction between proteases and their inhibitors are involved several growth factors, *e.g.* fibroblast growth factor (FGF) and transforming growth factor beta (TGFß) families (24). MMP are responsible for liberating active growth factors and cytokines by degrading for example TGF-β binding proteins

to release and activate latent TGF-β. On the other hand, TGF-β has a positive effect on extracellular matrix production (25).

The available data in the literature about PG in the female reproductive tract suggest that PG are important, even essential, for the development of new life. But information is lacking which components of the PGE_2 signal transduction system are present in the bovine oviduct to possibly influence embryo-maternal interactions. Therefore, the aim of the present study was to investigate the estrous cycle-dependent changes and local distributions of PGE_2 receptors and members of the TFF-family in the bovine oviduct using real-time RT-PCR. In addition, a co-cultivation system of COC with primary oviductal cells was screened for the mRNA expression pattern of selected factors and enzymes of the PGE_2 synthesis as well as ECM components. An oviductal primary cell culture was used for investigating effects of estradiol on signal transduction pathways including NF-κB activation.

MATERIALS AND METHODS

Collection of oviductal cells

This study was approved by Institutional Ethic Committee of University of Berlin, Germany.

Oviducts from adult cows were collected at the local slaughterhouse within 15-20 minutes of death and transported on ice to the laboratory. The phase of the estrous cycle was defined by careful examination of the ovaries (corpus luteum, follicle), uterus, and cervix (26, 27). These criteria were used to classify the oviducts into one of the four groups: post-ovulatory [d 1-5], early-to-mid luteal [d 6-12], late luteal [d 13-18], and pre-ovulatory [d 19-21] phase. In addition, oviducts of each cow were separated into the ipsilateral (to ovulation site/corpus luteum) and the contralateral oviduct. The surrounding tissue was removed and the oviducts were further divided into ampulla and isthmus. Oviductal cells were harvested from each distinct region as previously described and were stored at -80°C until further analysis (10).

Co-culturing of bovine primary oviductal cells with cumulus-oocyte-complexes

The isolation of oviductal cells and culture conditions for a primary cell culture were slightly modified as previously published (10). Briefly, 2 ml suspended mucosal oviductal cells (approximately 1-2 x 10^5 cells/ml) in medium (M199 with supplements) were plated in 24-well plates and incubated at 39°C and 5% CO_2 in a humidified atmosphere. At day 4, medium of cultured oviductal cells was replaced. The cells were attached to the plastic surface and the cell culture was nearly confluent at this day. At the same day, ovaries of healthy and non-pregnant cows were collected at the slaughterhouse and transported to the laboratory in 37°C PBS containing penicillin (100 U/ml) and streptomycin (100 µg/ml). Cumulus-oocyte-complexes (COC) were aspirated and picked under stereomicroscopic control. COC were washed three times and subsequently transferred into maturation medium (M199 containing 1 µg/ml 17β-estradiol, 1 µg/ml follicle stimulating hormone (FSH), 0.3 IE/ml luteinizing hormone (LH), 0.25 mM sodium pyruvate, 50 µg/ml gentamicin and 5% estrous cow serum (all from Sigma, Deisenhofen, Germany) for 24h. At day 5, oviductal cell culture was almost confluent and viability of the cultured oviductal cells (>95%) was assessed by trypan blue exclusion. The 24h matured COC were washed two times. Three approaches were performed: 1) matured COC (10/well) were added to the oviductal cells and co-incubated for 5 hours (5h co-incubation); 2) matured COC (10/well) were subjected together

with oviductal cells at the timepoint 0h to lysis (0h control group); 3) matured COC (10/well) and oviductal cells (one well of 24-well plate) were cultured separately for 5 hours in oviductal cell culture medium, then combined and lysed together (5h control group).

RNA isolation and reverse transcription

Total RNA was extracted from oviductal cells or from cells of the co-culturing experiments using Invisorb Spin Cell RNA Mini Kit (Invitek, Berlin, Germany) following the manufacturer's instructions. Total RNA concentration was measured photometrically at the wavelength of 260 nm. Integrity of RNA was confirmed after electrophoresis on a formaldehyde-containing 1% (w/v) agarose gel by ethidium bromide staining. To generate single stranded cDNA, 500 ng total RNA were subjected using 3.75 µM random hexamers (Amersham Biosciences, Freiburg, Germany) and 200 U Moloney Murine Leukemia Virus reverse transcriptase (Fermentas, St. Leon-Rot, Germany) in a 60 µl reaction mixture (10). The generated cDNA served as template for polymerase chain reactions and was stored at -20°C until use.

Real-Time PCR

Quantitative real-time PCR was performed on a Rotor-Gene 3000 (Corbett Research, Mortlake, Australia) using SensiMix dT (Quantace, Berlin, Germany) (10) and specific sets of primers (synthesized by MWG Biotech, Ebersberg, Germany) for target gene amplicons as indicated in *Table 1.* The cycling conditions comprised an initial denaturation step at 95°C for 10 min, a three-step amplification including denaturation at 95°C for 15 sec, annealing at the temperature as indicated in *Table 1* for 20 sec and extension at 72°C for 30 sec, a melting curve program (50-99°C) to confirm specific amplification, and a final cooling step to 40°C. Quantities of the genes of interest were calculated in comparison with a simultaneously amplified standard curve of known quantities of the specific amplicon. As negative controls, reactions containing no template (H_2O) or non-reverse transcriptased RNA were included to verify that obtained PCR products were not derived from contaminations or genomic DNA.

Analysis of signal transduction pathways

The above described oviductal cell culture system was used for the analysis of signal transduction pathways. Oviductal cells were transiently transfected with reporter vectors of the Mercury Pathway

Table 1. Gene transcript, primer sequences and annealing temperature used for real-time PCR with subsequently resulting amplicon length.

Gene	Primer sequence	EMBL accession no.	Nucleotide no.	Amplicon length (bp)	Annealing temperature
EP2	forward 5'-GGA GCG CTA CCT AGC CAT C-3' reverse 5'-GAT GAG CAA CAG CAG CAG AG-3'	AF539402	401-629	229 bp	56°C
EP3	forward 5'-TAT ACT ATC CAG TGG CCC GG-3' reverse 5'-TCA ACA TCA TTA TCA GCA ACG G-3'	NM_181032	592-907	316 bp	63°C
EP4	forward 5'-CGG TGA TGT TCA TCT TCG G-3' reverse 5'-GTA GGC GTG GTT GAT GGC-3'	AF 539403	79-380	302 bp	60°C
MMP-1	forward 5'-GAT GAT GAT GAA TGG TGG ACC-3' reverse 5'-TCC ACT TCT GGG TAC AAG GGA-3'	(22)		347 bp	60°C
MMP-2	forward 5'-CCC AGA CAG TGG ATG ATG C-3' reverse 5'-ACA CGG ACC ACT TGT CCT TC-3'	(42)		259 bp	60°C
$TGF\beta_3$	forward 5'-AGA ACT GCT GTG TGC GTC C-3' reverse 5'-GCA GGA CTT CAC CAC CAT G-3'	BC149207	1165-1454	290 bp	61°C
18S rRNA	forward 5'-GAG AAA CGG CTA CCA CAT CCA A-3' reverse 5'-GAC ACT CAG CTA AGA GCA TCG A-3'	(10)		337 bp	61°C

Profiling System (Clontech, Mountain View, CA, USA) containing response elements fused to the alkaline phosphatase reporter gene. Oviductal cells were stimulated with 80 pg/ml 17β-estradiol for 4h and subsequently secreted alkaline phosphatase (SEAP) activity was measured using a SEAP-Reporter Gene Assay (Roche, Mannheim, Germany) by chemiluminescence. In addition, transiently transfected oviductal cells with the vector carrying the NF-κB response element were treated with different concentrations of 17β-estradiol (10 pg/ml, 50 pg/ml, 100 pg/ml, 10 ng/ml or 250 ng/ml, respectively) and the resulting chemiluminescence was estimated.

Statistical analysis

The content for each specific mRNA was normalized with the 18S rRNA data. For the analysis of the *in vitro* experiments, the data of the treatments or controls at each timepoint were calculated in reference to the sample of the same cow at time 0h. This value at 0h was set 100%.

All data from real-time PCR, presented as the mean ± SEM, were analyzed by the univariate variance analysis. If this statistical test revealed significant differences, the post-hoc tests Tukey or Dunnett-T3 were performed after considering the results of the Levene test for the homogeneity of variances. The paired t-test was used for the results of the cell culture experiments by comparing the controls with the treated samples at the same time. P-values < 0.05 were considered to be significant. All the statistical evaluations were performed by using the SPSS for windows version 12.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

mRNA analysis in oviductal cells in vivo

Quantitative real-time RT-PCR revealed mRNA expression of the PGE_2-receptors EP2, EP3 and EP4 in bovine oviductal cells during all stages of the estrous cycle in all regions (*Fig. 1*). However, the expression pattern for each receptor subtype was different. In detail, a significant higher expression of EP2 mRNA was observed in the pre-ovulatory phase compared with the early-to-mid luteal phase (*Fig. 1A*). In addition, significant increased expression of EP4 mRNA was observed in the phase before ovulation compared with the post-ovulatory and late luteal phase (*Fig. 1E*). Interestingly, the expression of EP2 and EP4 was twofold higher in the ipsilateral oviduct before ovulation compared with the same part after ovulation (*Fig. 1B* and *1F*). No difference between the ampulla and the isthmus was noted for these PGE_2 receptor subtypes (data not shown). The expression of EP3 showed no obvious estrous cycle-dependent or region-specific pattern (*Fig. 1C* and *1D*). EP1 mRNA expression was not detectable in the bovine oviduct, but in bovine endometrial cells.

Only one member out of the three trefoil factors (TFF), TFF3, was detected in bovine oviductal cells during all stages of the estrous cycle in all regions. However, the analysis of oviductal cells *in vivo* revealed a significant change of TFF3 mRNA contents during the estrous cycle with the highest expression in the post-ovulatory phase (*Fig. 2A*). This expression pattern was contributed to the expression level in the isthmus, whereas in the ampulla TFF3 mRNA was expressed without variation on a low level. In detail, TFF3 mRNA expression was

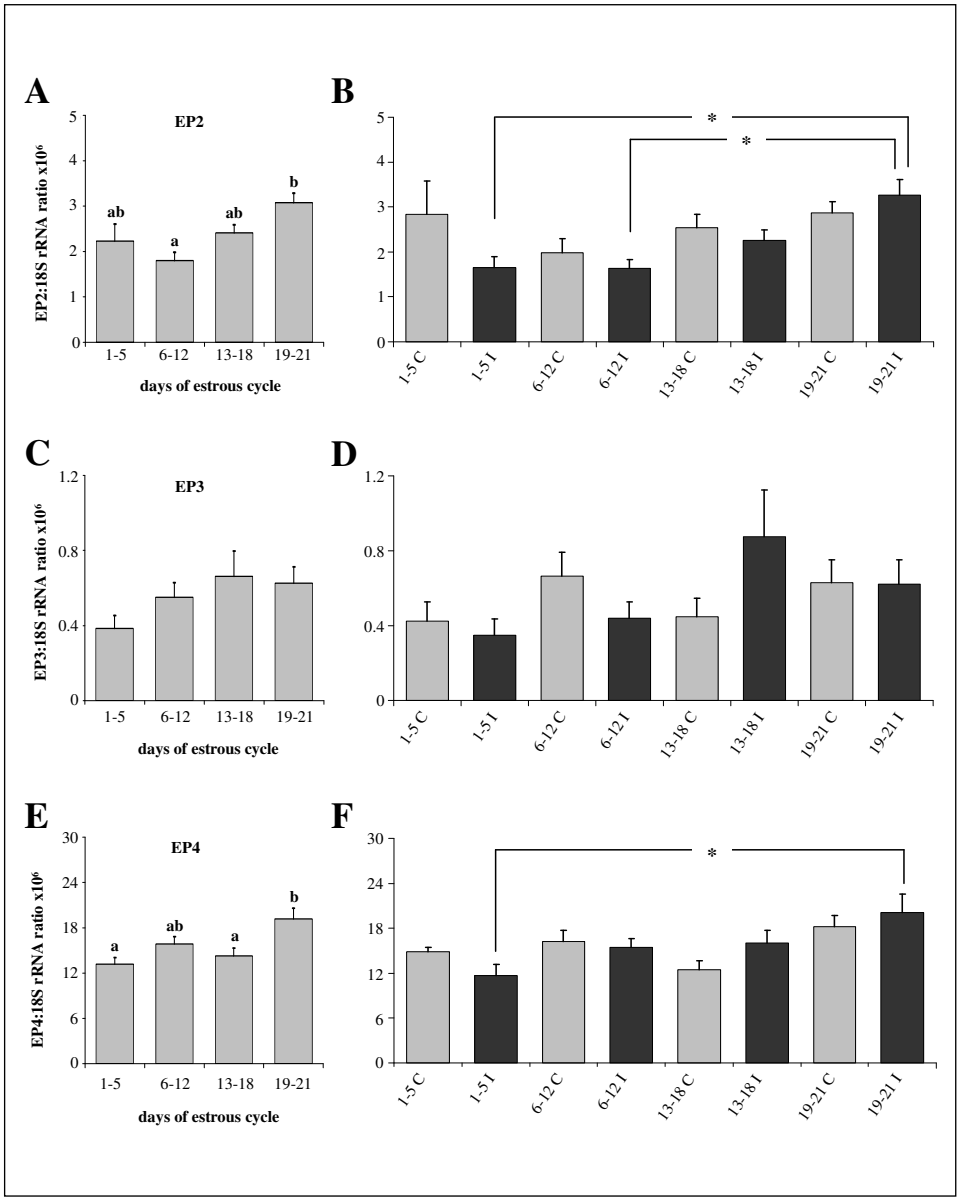

Fig. 1. mRNA expression pattern for EP2 (**A**), EP3 (**C**) and EP4 (**E**) in the bovine oviduct during the estrous cycle (n=28 for each cycle phase) as well as local-dependant mRNA expression pattern for EP2 (**B**), EP3 (**D**) and EP4 (**F**) in the bovine oviduct during the estrous cycle subdivided into contra- and ipsilateral (n=14 for each region and estrous cycle phase). The contents of the EP mRNA were expressed as the mean ± SEM ratio relative to individually 18S rRNA values as an internal control. 1-5: post-ovulatory phase; 6-12: early-to-mid luteal phase; 13-18: late luteal phase; 19-21: pre-ovulatory phase; C: contralateral oviduct; I: ipsilateral oviduct. Different letters above the columns indicate significant differences between the groups; $P<0.05$.

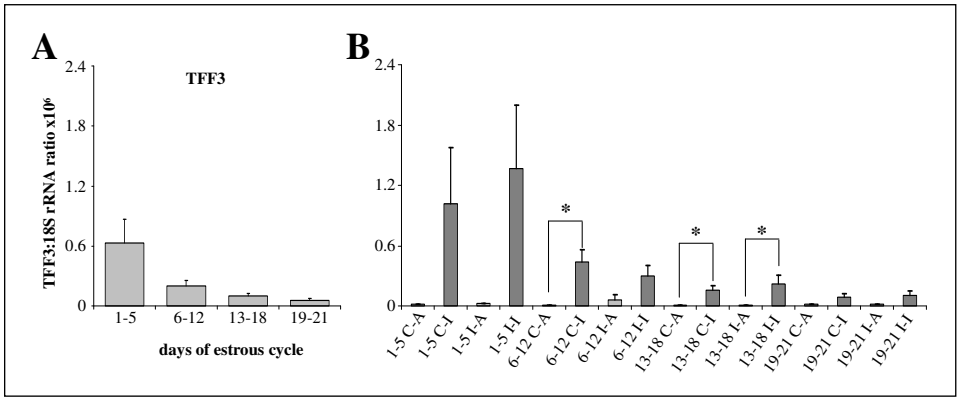

Fig. 2. mRNA expression pattern for TFF3 (**A**) in the bovine oviduct during the estrous cycle (n=28 for each cycle phase) as well as local-dependant mRNA expression pattern for TFF3 (**B**) in the bovine oviduct during the estrous cycle subdivided into contra- and ipsilateral as well as ampulla and isthmus (n=7 for each region and estrous cycle phase). The contents of the TFF3 mRNA were expressed as the mean ± SEM ratio relative to individually 18S rRNA values as an internal control. 1-5: post-ovulatory phase; 6-12: early-to-mid luteal phase; 13-18: late luteal phase; 19-21: pre-ovulatory phase; C-A: contralateral ampulla; C-I: contralateral isthmus; I-A: ipsilateral ampulla; I-I: ipsilateral isthmus. Different letters above the columns indicate significant differences between the groups; $P<0.05$.

significantly higher expressed in the isthmus compared with the ampulla in all phases (*Fig. 2B*). This was only significant in the early-to-mid and late luteal phase in the ipsi- as well as contralateral oviduct. In the post-ovulatory phase, the expression in all samples was higher in the isthmus than in the ampulla, but the biological variation was very high in the isthmus. Therefore, statistical analysis did not result in a significant difference. No differences were detected between ipsi- and contralateral oviducts.

mRNA analysis in oviductal cells in vitro

To reveal a suggested influence of COC on the mRNA expression in bovine oviductal cells, primary bovine oviductal cells were cultured and treated in a co-culture experiment.

Different mRNA expression pattern were observed in co-cultured oviductal cells compared to the untreated control predominantly for factors involved in extracellular matrix formation such as: MMP-1, MMP-2, and TGF-β_3. MMP-1 (*Fig. 3A*) and MMP-2 (*Fig. 3B*) mRNA expression was significantly increased after COC-co-cultivation. The expression of the inhibitor of MMP, TIMP-1 was unaffected by this treatment (data not shown). mRNA expression of TGF-β_3 increased twofold in contrast to the untreated control (*Fig. 3C*). TGF-β_1 and TGF-β_2 mRNA expression was not influenced by COC-co-culturing (data not shown).

The influence of COC on the expression in oviductal cells on enzymes of the PGE_2 synthesis was also investigated. The expression pattern of phospholipases,

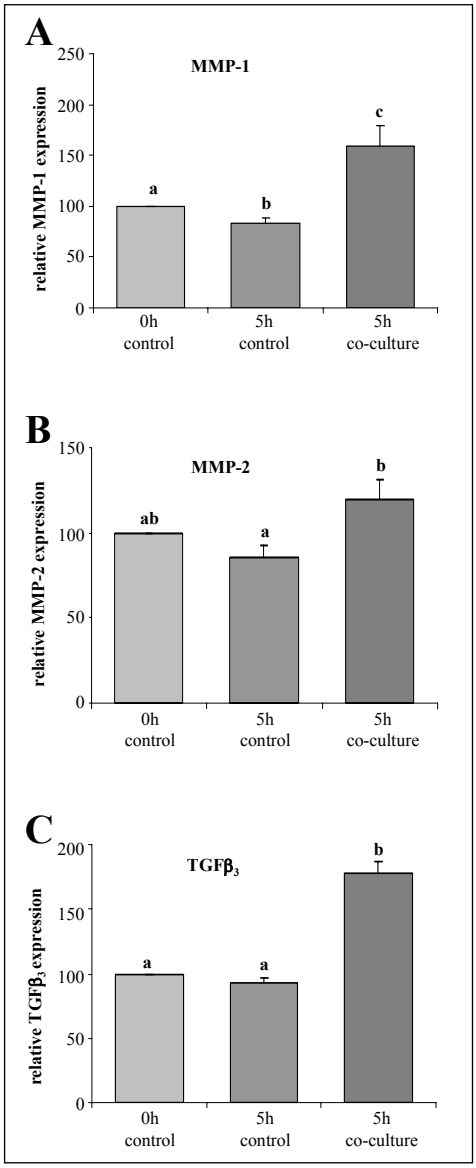

Fig. 3. Effects of co-culturing of bovine oviductal cells with COC on MMP-1 (**A**), MMP-2 (**B**), and TGF-β_3 (**C**) mRNA content (n=8 cows). The data are expressed as the mean ± SEM percentage of the 0h control. Different letters above the columns indicate significant differences between the groups; $P<0.05$.

COX-1, COX-2 as well as PGES was not affected by the presence of COC compared with the untreated control (data not shown).

Analysis of signal transduction pathways

Increased SEAP activity resulted only in estradiol treated samples transfected with the vector including the NF-κB response element (*Fig. 4A*). The other

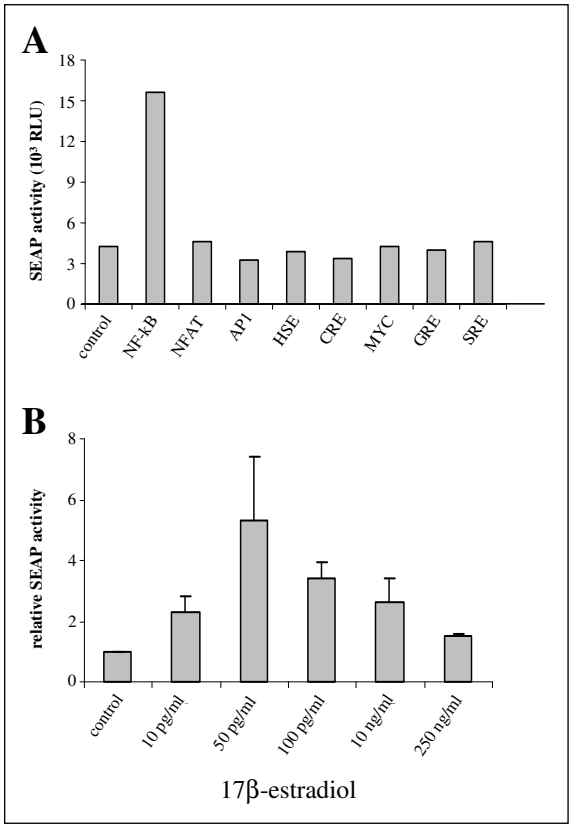

Fig. 4. (**A**) Activation of NF-κB in primary bovine oviductal cells stimulated with 80 pg/ml 17β-estradiol for 4h. Cells were transiently transfected with vectors containing different response elements fused to the alkaline phosphatase reporter gene. The secreted alkaline phosphatase (SEAP) activity of the non-stimulated controls is in the range of 4000 RLU (relative light units). (**B**) Activation of NF-κB in response to different concentrations of 17β-estradiol in primary bovine oviductal cells. The relative SEAP activity is presented as the mean ± SEM related to the control (relative value of one).

investigated response elements containing AP1, CRE, GRE, HSE, MYC, NFAT or SRE were not affected by estradiol treatment, respectively. Furthermore, different concentrations of 17β-estradiol resulted in the highest activation of NF-κB for 50 pg/ml and 100 pg/ml (*Fig. 4B*).

DISCUSSION

The oviduct is considered to be under the influence of hormones including estradiol. Its sensitivity towards these molecules should be mediated through the presence of their specific receptors. Protein and mRNA for both receptors for estradiol, estrogen receptor alpha (ERα) and beta (ERβ), were detected in the bovine oviduct (28). Immunohistochemical stainings revealed the presence of both receptors in luminal epithelial cells, which are supposed to be responsible to take part in the cross-talk between the oviduct and the gametes or the embryo. In peripheral blood plasma, estrogen levels are found high around ovulation. Interestingly, highest estradiol concentrations were observed in the ipsilateral

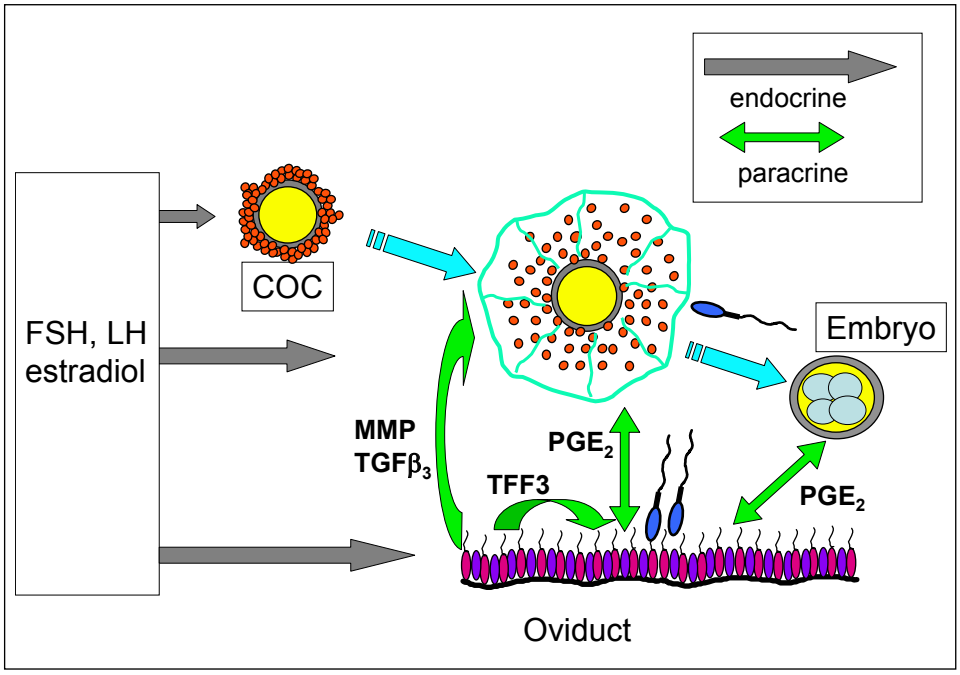

Fig. 5. Proposed local interactions of the PGE_2 and ECM system in the bovine oviduct.

oviduct during the follicular phase (4). These findings support the hypothesis that estrogen will bind to one of the receptors exerting its biological activity when gametes or the embryo are present. The present study revealed that the estradiol signal transduction will lead to a NF-κB activation.

One of the estradiol stimulating effects in the bovine oviduct is the increased PGE_2 synthesis (5). It was reported that in contrast to COX-1, the COX-2 mRNA expression was stimulated *in vitro* after estradiol treatment (10). This is in agreement with the literature, which described COX-2 as an inducible enzyme (8). Therefore, all these results indicate that a complete signal transduction pathway is present in the bovine oviduct: starting with increasing estradiol concentrations in the oviduct, binding to its receptor resulting in the signalling cascade by activating NF-κB, leading to an increased conversion of arachidonic acid finally resulting in PGE_2. This pathway was shown to be responsible for increased cilia beating (29) and muscle contraction regulation (30, 31) to guarantee highest oviductal motility.

The data of the present study showed that several types of receptors for PGE_2 were synthesized in the bovine oviduct. Especially the increased expression of EP2 and EP4 before ovulation indicates that the higher amount of PGE_2 around ovulation could exert its biological activity through its specific receptors. Activation of EP2 and EP4 results in increased cAMP content (12). This pathway

was suggested to increase the expression of vascular endothelial growth factor (VEGF) and fibroblast growth factor 2 (FGF-2) (32, 33). VEGF is known to support COC maturation (34) and was elevated in the bovine oviduct before ovulation (35). Furthermore, several growth factors are known to play an important role in preimplantion development of human embryos (36).

These results explain the finding that PGE_2 supports the final maturation of COC (20). An increased amount of PGE_2 was delivered steroid-driven in the bovine oviduct (5). So it is not surprising that co-culturing of oviductal cells with COC showed no effect on the expression of enzymes of the prostaglandin synthesis, because the regulation is hormone-dependent and needs no cell-to-cell-contact. Sperm are stored in the bovine oviduct for a short time (37) and they are able to increase locally the PGE_2 production *via* COX-2 in the bovine oviduct (38).

It has been shown that synthesis of PGE_2 is influenced by TFF3 (16). The TFF3 expression level was higher in the post-ovulatory phase which indicates that it may be involved in the embryo-maternal communication. In modulating mucosal immune responses and tissue repair functions (17), TFF have a major impact on maintenance of healthy mucosal surfaces. So it is likely that TFF3 is responsible for the intact epithelium that is optimal prepared for embryo-maternal interaction and that the epithelium can provide the early embryo with all nutrients and factors for survival. PGE_2 was suggested to be involved in the maternal-embryo interaction to prevent the rejection of the embryo in the uterus (39). The same could be responsible for the embryo survival in the first days in the oviduct, because immune cells are also present in the oviduct (40). In addition, PGE_2 stimulates the FGF-2 synthesis (32), which is a pivotal factor for embryo survival (41).

Another aim of this study was to investigate the modulation of oviductal expression of components of the extracellular matrix in oviductal cells in the presence of COC. As reported earlier, the ECM was influenced by the final maturation of COC in the presence of FSH (22). MMP-1 and MMP-2 as well TIMP-1 were up-regulated in a time-dependent manner. In addition, the highest mRNA expression as well as activity of MMP-2 was detected in oviductal cells before ovulation and also for TIMP-1 (42). A proteolytic balance has been reported to be enhanced in presence of FGF-2, while TGF-β acts antiproteolytic (43). All these data indicate that a fine-tuned system of proteases and inhibitors is present in the bovine oviduct and after the suitable stimulus the system can be activated.

The present study supports the hypothesis that an increased synthesis of PGE_2 in the oviduct around ovulation could be necessary for maturation and fertilization of the oocyte as well as for the development of the early embryo (*Fig. 5*). In summary, a regulated and fine-tuned network with PGE_2 as major player in the bovine oviduct may be important and pivotal for the origin and maintenance of pregnancy already in the bovine oviduct.

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Conflicts of interest statement: None declared.

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