Differential expression of cyclooxygenase 1 and cyclooxygenase 2 in the bovine oviduct

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Abstract

The aim of the present study was to investigate the enzymes for the local prostaglandin (PG) biosynthesis present in the bovine oviduct during the estrous cycle to influence early reproductive events. Bovine oviducts were classified into four phases: pre-ovulatory, post-ovulatory, early-to-mid luteal, and late luteal phase, subdivided further into ipsi- or contralateral site and separated into ampulla or isthmus. Oviductal cells were gained by flushing the oviductal regions. Quantitative real-time reverse transcriptase-PCR was performed for the secretory and cytosolic phospholipases A₂ (sPLA₂IB, cPLA₂α, and cPLA₂β) and cyclooxygenases (COX-1 and COX-2) as the first step enzymes of PG synthesis. COX-1 and cPLA₂β showed significant highest mRNA expression around and before ovulation compared with the luteal phase respectively. sPLA₂IB and cPLA₂α mRNA expression was unregulated during the estrous cycle. Regional differences in mRNA content were found for sPLA₂IB with higher mRNA expression in the ampulla than

in the isthmus. Western blot analysis revealed the highest COX-1 protein content in the early-to-mid luteal phase. Immunohistochemistry demonstrated that COX-1 was localized in epithelial and smooth muscle cells, whereas COX-2 was only localized in epithelial cells. COX-2 showed a differential distribution within the epithelial cell layer suggesting a regulation on a cellular level, although the COX-2 mRNA and protein amounts did not vary throughout the estrous cycle. A COX activity assay of oviductal cells revealed that COX activity originated predominantly from COX-1 than from COX-2. Treatment of primary oviductal cells with 10 pg/ml 17β-estradiol or 10 ng/ml progesterone resulted in a higher expression of COX-2 and cPLA₂α, but not of the other enzymes. The expression pattern of these enzymes suggests that an estrous-cycle dependent and region-specific PG synthesis in the bovine oviduct may be required for a successful reproduction.

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Introduction

The oviduct plays a decisive role in reproduction as it provides a beneficial environment for gamete maturation, gamete transport, fertilization, and the early embryonic development. The latter are key processes in female reproductive events, which are regulated by hormones, e.g., steroids (Murray *et al.* 1995) and prostaglandins (PGs; Lim *et al.* 1997, Murakami *et al.* 2002). For instance, PGs are central mediators concerning ovulation, fertilization, as well as establishment and maintenance of pregnancy (Lim *et al.* 1997, Kniss 1999, Sales & Jabbour 2003). Several PGs were already detected in the human and bovine oviduct (Wijayagunawardane *et al.* 1998, Huang *et al.* 2002). The predominant PGs in the human oviduct, especially in the luminal epithelium, were PGI₂ and PGE₂ (Huang *et al.* 2002). Higher PGE₂ and PGF_{2α} concentrations were observed in the bovine oviduct around ovulation compared with the luteal phase (Wijayagunawardane *et al.* 1998). In addition, ipsilateral oviducts contained higher PGE₂ and PGF_{2α} levels than the contralateral oviduct.

PG synthesis begins with the liberation of arachidonic acid (AA), the prime precursor, from membrane phospholipids by phospholipase A₂ (PLA₂). Subsequently, cyclooxygenases (COX) catalyze the rate-limiting reactions for PG synthesis comprising the bis-cyclooxygenation of AA to form PGG₂ and the peroxidative reduction of this intermediate to PGH₂ (Kniss 1999, Garavito *et al.* 2002). Then this unstable endoperoxide intermediate is converted by cell-specific synthases and isomerases to distinct PGs (Narumiya & FitzGerald 2001). These PGs exert their autocrine/paracrine effects by interacting with their G-protein-coupled cell-surface receptors (Negishi *et al.* 1995, Sales & Jabbour 2003).

Numerous isoenzymes were described possessing PLA₂ activity (Kudo & Murakami 2002). Members of the secretory PLA₂ (sPLA₂) family are involved mainly in host defense and inflammation. Until now, ten different mammalian sPLA₂ are known, including the pancreatic sPLA₂IB (Murakami & Kudo 2002). The cytosolic PLA₂ (cPLA₂) family consists of three members: cPLA₂α, β and γ. All three enzymes were considered to be expressed ubiquitously and constitutively in

most cells and tissues. However, cPLA₂α expression can be stimulated with proinflammatory cytokines or growth factors (Kudo & Murakami 2002). Therefore, this enzyme isoform is supposed as the central mediator of stimulus-induced cellular AA release. The importance of cPLA₂α in reproductive events was demonstrated by knockout studies. cPLA₂α knockout mice less frequently become pregnant and deliver small litters (Bonventre *et al.* 1997), which suggests a defect in ovulation or fertilization (Kudo & Murakami 2002).

Two different cyclooxygenases, COX-1 (*M_r* 70 000) and COX-2 (*M_r* 72 000), are known. Both COXs are expressed in a tissue- and species-specific manner (Dubois *et al.* 1998, Kniss 1999). COX-1 is considered to be a constitutive enzyme in nearly all tissues and cells, which supplies PGH₂ for the homeostatic regulation of PGs (housekeeping gene; Dubois *et al.* 1998). In contrast, COX-2, which is undetectable in most mammalian tissues, is described as the inducible isoform (Smith *et al.* 1996). Both COX enzymes are located on the luminal surface of the endoplasmic reticulum and in the membranes of the nuclear envelop (Garavito *et al.* 2002). Several studies with knockout mice revealed that COX-2 deficiency indicated a reduction in ovulation number and severe failures in fertilization, as well as in implantation and decidualization (Lim *et al.* 1997). Experiments with COX-1-deficient mice revealed that prostanoids metabolized by COX-1 are not essential for fertilization due to unaltered conception and fetal development (Sales & Jabbour 2003).

The available data in the literature about PGs in the female reproductive tract suggest that PGs are important, even essential, for the development of new life. However, information on which components of the PG biosynthesis are involved in the bovine oviduct to influence early reproductive events like the expansion of the cumulus oocyte complex, fertilization, and embryonic development, is lacking. Therefore, the aim of the present study was to investigate the estrous cycle-dependent changes and local distributions of the first step enzymes of the PG biosynthesis (sPLA₂IB, cPLA₂α, cPLA₂β, COX-1, and COX-2) in the bovine oviduct using real-time reverse transcriptase (RT)-PCR. The presence and localization of the COX proteins was examined by immunohistochemistry and western blot detection. Moreover, the contribution of COX-1 and COX-2 activities to the total COX activity was determined in bovine oviductal cells. Additionally, a potential regulation of the mRNA expression of these enzymes was investigated in cultured primary oviductal cells after treatment with sexual steroids in a time-dependent experiment.

Materials and Methods

Collection of oviductal cells

Bovine oviducts were collected at the local slaughterhouse within 15–20 min of death, immediately placed on ice, and transported to the laboratory within approximately 2 h. The

physiological status of each reproductive tract was estimated on the basis of a thorough examination of the ovarian morphology (corpus luteum and follicle), uterus, and cervix (Ireland *et al.* 1980, Arosh *et al.* 2002). Therefore, the oviducts were classified into one of the following four groups of the estrous cycle: post-ovulatory (days 1–5), early-to-mid luteal (days 6–12), late luteal (days 13–18), and pre-ovulatory (days 19–21) phase. Moreover, the oviducts of each cow were separated into the ipsilateral (to ovulation site/corpus luteum) and the contralateral oviducts. Oviducts were trimmed to remove the surrounding tissue and further divided into ampulla and isthmus. For RNA analysis and western blot detection, oviductal cells were harvested by flushing the oviductal regions three times with 1 ml Ringer solution (Berlin-Chemie AG, Berlin, Germany). After a centrifugation step at 570 *g* for 5 min at 4 °C, the supernatant was removed and the obtained cell pellets were stored at –80 °C until further analysis.

The verification of the cell types and the viability of oviductal cells were performed as described previously (Gabler *et al.* 1997). Briefly, the viability of flushed cells was confirmed by observation under a microscope of beating cilia as well as by the exclusion of Trypan blue. Immunohistochemical analysis of the flushed cells with cytokeratin as an epithelial cell-specific marker showed a positive staining of more than 60% of the cells. Therefore, the flushed cells of this study were referred to as oviductal cells.

Cell culture and treatment of bovine oviductal cells

For cell culture, complete oviducts were cut open longitudinally on the margin of the mesosalpinx with fine scissors under a laminar flow hood. The superficial oviductal cells were harvested by scraping gently with a cell scraper over the luminal cell layer. The obtained cells from both oviducts of one cow were combined, washed twice and were suspended in Earle salt buffered M199 (containing 10% heat-treated calf serum, 20 mM HEPES solution, 0.23 mg/ml sodium pyruvate, 50 µg/ml gentamycin, 2.5 µg/ml amphotericin B, and 100 µg/ml L-glutamine (all from Sigma)) to obtain approximately 1–2 × 10⁵ cells/ml. Then, 5 ml cell suspension were seeded in six-well plates and incubated at 39 °C and 5% CO₂ in a humidified atmosphere. After 72 h, medium was replaced by new medium containing only 1% heat-treated calf serum. Twelve hours after medium change, oviductal cells reached almost confluence and were treated with 10 pg/ml 17β-estradiol or 10 ng/ml progesterone (both from Sigma) for 2, 4, or 6 h respectively. Cell dishes without any hormone treatment served as controls for each time point. The viability of cultured oviductal cells at day 4, which was >95%, was determined by Trypan blue exclusion.

Total RNA extraction and reverse transcription

Total RNA from flushed or cultured bovine oviductal cells was isolated using Invisorb Spin Cell RNA Mini Kit (Invitex,

Berlin, Germany) according to the manufacturer's instructions. Cultured oviductal cells were lysed directly in six-well plates after removing the medium. Yield of total RNA was quantified photometrically at 260 nm. Quality and quantity of RNA were verified after electrophoresis on a formaldehyde-containing 1% (w/v) agarose gel by ethidium bromide staining.

To remove genomic DNA contamination, DNA digestion was performed before reverse transcription (Huang *et al.* 1996). DNase treatment was carried out in a total volume of 20 µl containing 1 µg total RNA, 1 U DNase (Promega GmbH), and the 1× buffer of the used RT. This reaction mixture was incubated at 37 °C for 30 min, heated for 5 min at 75 °C to inactivate the DNase and then placed immediately on ice for 5 min. Forty microliters premix containing 200 U Moloney-Murine Leukemia Virus reverse transcriptase (M-MLV RT; Promega), 3·75 µM random hexamers (Amersham Biosciences), 1 mM dNTPs (each) (Amersham Biosciences), and 1× of the supplied RT buffer were added to each RNA sample. Samples without M-MLV RT were performed at the same time to monitor the absence of any genomic DNA. The reverse transcription was performed at 25 °C for 10 min and 37 °C for 50 min followed by 90 °C for 2 min. The obtained cDNAs were stored at -20 °C until further investigation.

Real-time PCR

In preliminary experiments, expression of the investigated factors (18S rRNA, sPLA₂IB, cPLA₂α, cPLA₂β, COX-1, and COX-2) was examined by standard RT-PCR to confirm the expected amplicon sizes as well as to estimate the optimal annealing temperature by gradient-PCR for each primer pair. PCRs were performed in a thermocycler (Mastercycler gradient; Eppendorf AG, Hamburg, Germany). Five microliters cDNA were amplified in a total reaction volume of 25 µl containing 1× iTaq-buffer (Bio-Rad), 1·5 mM MgCl₂, 0·2 mM dNTPs (Amersham Biosciences), 0·4 µM of each primer (forward and reverse), and 0·5 U iTaq DNA polymerase (Bio-Rad). Each reaction started with an initial denaturation step for 10 min at 94 °C followed by the amplification program: 94 °C for 30 s and 60 °C for 1 min (20 cycles for 18S rRNA and 40 cycles for all the other primer pairs). As a final step, an elongation phase was performed for 2 min at 72 °C before samples were cooled down to 4 °C. Subsequently, 10 µl PCR product were subjected to 1% agarose gel electrophoresis containing ethidium bromide. Specificity of the PCR products was verified by DNA sequencing (GATC Biotech AG, Konstanz, Germany) and showed a 100% homology compared with the known bovine sequences.

The following primers were used to amplify specific bovine transcripts: 18S rRNA (337 bp, corresponding to bases 55–391 of European Molecular Biology Laboratory (EMBL) accession no. AF176811): forward 5'-GAG AAA CGG CTA CCA CAT CCA A-3', reverse 5'-GAC ACT CAG CTA AGA GCATCG A-3'; sPLA₂IB (248 bp, corresponding to bases 197–444 of EMBL accession no. Y00120): forward 5'-GGA TGA TCT

GGA CAG GTG C-3', reverse 5'-TCT TGT CCA GGT TCT TGT GC-3'; cPLA₂α (229 bp, corresponding to bases 837–1065 of EMBL accession no. XM_603415): forward 5'-AAA TGT CAG CCA CAA CCC TC-3', reverse 5'-ATG GAG ACA GGT GAA AAG CG-3'; cPLA₂β (218 bp, corresponding to bases 163–380 of EMBL accession no. XM_595922): forward 5'-CCA GCC TGG ATG AGA TCT TC-3', reverse 5'-GAA GTT GGA GTT CTC GGC TG-3'; COX-1 (313 bp, corresponding to bases 155–467 of EMBL accession no. AF004943): forward 5'-CAG ATG CGG AGT TTC TGA GTC G-3', reverse 5'-GGG TAG TGC ATC AGC ACG G-3'; and COX-2 (359 bp, corresponding to bases 519–877 of EMBL accession no. AF031698): forward 5'-CTC TTC CTC CTG TGC CTG AT-3', reverse 5'-CTG AGT ATC TTT GAC TGT GGG AG-3'. The primers were synthesized by MWG-Biotech AG (Ebersberg, Germany).

The same primers were utilized for real-time PCR (Rotor-Gene RG-3000; Corbett Research, Mortlake, Australia) using the SYBR Green I method. One microliter cDNA was used as template for the real-time PCR containing 5 µl 2× iQ SYBR Green Supermix (Bio-Rad) and 0·4 µM of each primer (forward and reverse) in a final volume of 10 µl. The following real-time PCR protocol was applied: a denaturation step at 95 °C for 10 min, a three-step amplification, including denaturation at 95 °C for 15 s, annealing at 60 °C for 20 s, and extension at 72 °C for 30 s, a melting curve program (50–99 °C) with continuous fluorescence measurement and a final cooling step to 40 °C. Data acquisition was carried out at the end of each extension step at 72 °C. The number of cycles was for 18S rRNA, sPLA₂IB, cPLA₂α, cPLA₂β, COX-1, and COX-2: 25, 40, 40, 35, 45, and 40 respectively. For mRNA quantitation, a dilution series with known quantities of the specific PCR product was amplified simultaneously with the samples as a standard. Calculations were performed using Rotor-Gene 4.6 software and contents of specific mRNA were estimated in comparison with the standard curves. The applied PCR products as standards were generated by conventional block RT-PCR and purified by Wizard SV Gel and PCR Clean-Up System (Promega) as described by the manufacturer. Concentrations of the purified PCR products were estimated using PicoGreen dsDNA quantitation kit (Molecular Probes, Karlsruhe, Germany) according to the manufacturer's instructions by fluorescence measurement (FluostarOptima; BMG Labtech GmbH, Jena, Germany).

The obtained melting points of the amplified products served as conformation for specific amplification. As negative controls, reactions containing no template (H₂O) or non-reverse transcriptased RNA were included to verify that obtained PCR products were not derived from contaminations or genomic DNA.

Immunohistochemistry

Tissue presence of COX-1 and COX-2 proteins was assessed via immunohistochemistry using the DAKO Envision+ Kit

(DAKOcytation, Hamburg, Germany). Oviducts for immunohistochemistry were collected at the slaughterhouse, classified and divided into the regions as described previously. The freshly obtained ampullae and isthmi were cut into 4 mm pieces, fixed in formalin at 4 °C for 4 days and embedded in paraffin. Oviductal cross-sections of 3–5 µm thickness were adhered to SuperFrost Plus microscope slides and fixed overnight at 56 °C. Afterwards, sections were deparaffinized in xylene and rehydrated. Endogenous peroxidase activity was blocked by incubating slides in 3% hydrogen peroxide for 15 min. Samples were then pretreated with 0.01 M citrate buffer (pH 6.0) for 6 min at 95–98 °C. The staining conditions for the primary antibodies were optimized in preliminary experiments.

For COX-1, nonspecific protein-binding sites were blocked with 10% normal goat serum diluted in 1% TBSA (Tris-buffered saline containing 1% BSA) at 37 °C for 90 min. A COX-1 polyclonal antiserum (anti-ovine COX-1, host: rabbit, catalogue no.: 160108; Cayman Chemical, Ann Arbor, MI, USA) was used in a 1:2750 dilution (in 1% TBSA, 0.1% Tween 20, and 1% normal goat serum) and incubated in a humidified chamber overnight at 4 °C.

For COX-2, a polyclonal COX-2 antibody (anti-mouse/rat COX-2, host: rabbit, catalogue no.: 160106; Cayman Chemical) was used in a 1:200 dilution in 1% TBSA and incubated in a humidified chamber for 45 min at 38 °C.

Specifically bound antibodies were detected with horseradish peroxidase (HRP) labeled polymer conjugated secondary antibodies (Envision+ System-HRP, anti-rabbit, DAKOcytation) in a humidified chamber for 30 min at room temperature (COX-1) or for 30 min at 38 °C (COX-2). Staining was visualized using DAB+ (DAKOcytation) for 10 min at room temperature. Between each step, tissues were washed with TBS. Finally, the sections were slightly counterstained in Mayer's hematoxylin and coverslipped in glycerol gelatin media. Staining of the negative controls was performed under the same staining conditions with normal rabbit serum (1:2750 dilution) or purified rabbit IgG (2.5 µg/ml) as a substitute for the primary antibody directed against COX-1 or COX-2 respectively.

Cell lysis and western blot analysis

Flushed oviductal cells were lysed for 1 h at 4 °C in 150–250 µl modified RIPA buffer containing 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 5 mM EDTA, 1% (v/v) NP-40, 0.25% Na-deoxycholate, 1% (v/v) Triton X-100, 1 mM Na₃VO₄, 1 mM NaF, 1% SDS, and 5 mM Pefabloc (Merck). To reduce the viscosity, lysates were squeezed through a 26 gauge needle with a syringe. A protein assay (Bio-Rad DC Protein assay; Bio-Rad) was performed to estimate the protein content of the cell lysates. Twenty micrograms of each sample were separated in denaturing SDS-PAGE (10% (w/v) acrylamide/bisacrylamide (37.5:1)). The electrophoresed proteins were then transferred for 1 h at 0.8 mA/cm² to nitrocellulose membranes (Hybond C+; Amersham

Biosciences) using a semidry blotter unit (NovaBlot; Amersham Biosciences) following the protocol of Kyhse-Andersen (Kyhse-Andersen 1984). Membranes were air-dried for 10 min and stained with Ponceau S (0.5% Ponceau S (Sigma), 1% acetic acid in water) to assess the quality of the transfer as well as to verify equal protein loading. To minimize unspecific antibody binding, nitrocellulose sheets were incubated for 1 h in 2% ECL-blocking agent (Amersham Biosciences) in PBST (PBS with 0.1% (v/v) Tween 20). COX-1 antiserum (same as for immunohistochemistry) or COX-2 polyclonal antiserum (anti-human COX-2, host: rabbit, catalogue no.: AB6665; Abcam, Cambridge, UK) was used in a dilution of 1:2000 or 1:2500 in 2% ECL-blocking agent in PBST respectively. Incubation with the primary antibody was performed overnight at 4 °C. Membranes were washed six times for 10 min with PBST. A peroxidase-conjugated donkey anti-rabbit secondary antibody (Amersham Biosciences) was diluted with 2% ECL-blocking agent in PBST to 1:100 000. Membranes were incubated with the secondary antibody for 60 min at room temperature. Afterwards, membranes were washed another six times for 10 min with PBST. Immunoreactive proteins were visualized using enhanced chemiluminescence (ECL Advance; Amersham Biosciences) as described by the manufacturer. Ovine COX-1 (0.5 µg) or ovine COX-2 (0.5 µg; both from Cayman Chemical) was used as a positive control to monitor the specificity of the antibodies.

COX activity assay

COX-1 and COX-2 activities in oviductal cells were investigated by using a commercial kit (Correlate-enzyme chemiluminescent cyclooxygenase activity kit; Assay Designs, Ann Arbor, MI, USA). The assay was performed as described by the manufacturer. Briefly, oviductal cells gained from ipsilateral ampullary parts ($n=5$) by scraping the luminal site were suspended in 500 µl, 0.1 M Tris-HCl (pH 7.8) containing 1 mM EDTA. The lysates were placed immediately in liquid nitrogen. After thawing, samples were centrifuged at 570 *g* for 5 min at 4 °C and the resulting supernatant was removed from cell debris. Fifty microliters of the supernatant were added in the wells of a microtiter plate. Original samples served for the estimation of the total COX activity and in the other wells 24 µg SC-560 or 10 µg DuP-697 were applied for inhibiting COX-1 or COX-2 activity respectively. After 5 min incubation, chemiluminescence was measured with FluostarOptima for 7 s after injection of arachidonic acid. The integrated light output was defined as the COX activity and the values of the inhibited sample were calculated in reference to the original sample, which was set at 100%. The COX activity was measured in duplicate for each sample. Wells containing the supernatant and all reagents, except the arachidonic acid served as negative controls. Positive controls were performed with active ovine COX-1 or COX-2 (Cayman Chemical).

Densitometric and statistical analysis

Dried films of the western blot analysis were scanned using the Bio Image System (SYNGENE, Cambridge, UK). The scanned band intensities of the immunoreactive COX were estimated using the Gene Tools Analysis software (SYNGENE) after subtraction of background. Background subtraction was individually checked for each lane.

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 time point were calculated in reference to the sample of the same cow at time 0 h. This value at 0 h was set 100%.

All data from real-time PCR and western blot analysis, presented as the mean $\pm$ S.E.M., 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. The COX activity data were analyzed by using the non-parametric Wilcoxon test comparing the original activity with the activity of the inhibited samples. The values of $P < 0.05$ 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

Specific mRNA transcripts of PLA₂ enzymes, catalyzing AA liberation, were detected in bovine oviductal cells during all stages of the estrous cycle in all regions. The mRNA expression pattern of cPLA₂ α appeared obviously unregulated during the whole estrous cycle (Fig. 1A). No differences in the cPLA₂ α mRNA contents were noted between ipsi- and contralateral oviducts or between ampulla and isthmus (Fig. 1B). One exception is the ipsilateral oviduct before ovulation: the ampulla contained about twofold more cPLA₂ α mRNA compared with the isthmus. In contrast to cPLA₂ α , a significant sevenfold increase of cPLA₂ β mRNA expression was observed from the early-to-mid luteal phase to the highest expression in the pre-ovulatory phase (Fig. 1C). This was followed by a threefold decrease in the post-ovulatory phase to the lowest cPLA₂ β mRNA content in the early-to-mid luteal phase. No difference of cPLA₂ β mRNA expression was noted between different regions in each cycle phase (Fig. 1D). sPLA₂IB mRNA expression showed no obvious regulation without considering specific oviductal regions (Fig. 1E). However, after differentiation in the ampullary or isthmus region, the expression of sPLA₂IB mRNA was significantly higher in the ampulla compared with the isthmus during the whole estrous cycle. This

regional different expression pattern was still observed after further differentiation in ipsi- or contralateral oviducts (Fig. 1F). However, no obvious differences of the sPLA₂IB mRNA expression were noted between ipsi- and contralateral oviducts. Interestingly, the highest range of absolute mRNA expression was observed for cPLA₂ β mRNA (8–66 fg/ μ g total RNA) followed by the expression level of cPLA₂ α (15–44 fg/ μ g total RNA). The lowest expression was observed for sPLA₂IB mRNA (0.13–2.2 fg/ μ g total RNA).

Specific mRNA transcripts of the second step enzymes in the PG synthesis, COX-1 and COX-2, were also detected in all regions of the bovine oviduct during the whole estrous cycle. Interestingly, COX-1, considered to be constitutively present, was expressed twofold higher around ovulation compared with the luteal phase (Fig. 2A). Local significant differences of COX-1 mRNA expression were not observed (Fig. 2B). However, COX-2, known as an inducible enzyme, showed no significant differences in mRNA expression during the whole estrous cycle (Fig. 2C). In more detail, no significant regional difference of the COX-2 mRNA expression was observed (Fig. 2D). However, two samples from the ipsilateral ampulla collected after ovulation revealed a tenfold higher COX-2 mRNA expression than the other samples from the same region. This resulted in the locally highest mean of COX-2 mRNA expression, but was not significant. In addition, the average range of COX-1 mRNA expression (0.84–3.6 fg/ μ g total RNA) was higher compared with the COX-2 mRNA expression (0.36–1.08 fg/ μ g total RNA).

mRNA analysis in oviductal cells in vitro

To reveal a suggested influence of sexual steroids on the mRNA expression of the first step enzymes of the PG biosynthesis, primary bovine oviductal cells were cultured and treated in a time-related experiment. The applied concentrations of 17 β -estradiol or progesterone correspond to the higher physiological blood serum concentrations occurring before ovulation or during the luteal phase respectively.

mRNA transcripts of cPLA₂ α and cPLA₂ β were observed in cultured oviductal cells. Treatment with 17 β -estradiol or progesterone for 6 h caused a significant increase of cPLA₂ α mRNA expression compared with the control (Fig. 3A). However, no effect was observed after 2- or 4-h incubation with 17 β -estradiol or progesterone. In contrast, cPLA₂ β mRNA expression was obviously not influenced by estradiol or progesterone treatment (Fig. 3B). However, sPLA₂IB mRNA expression decreased from the first day of cell culture and was not detectable in attached oviductal cells on day 4 in the used culture system for these studies (data not shown). Even treatment with 17 β -estradiol or progesterone did not lead to a detectable sPLA₂IB mRNA expression.

mRNA expression of both cyclooxygenases was noted in all samples of cultured oviductal cells. Estradiol or progesterone treatment after 2 or 6 h did not affect the COX-1 mRNA expression, whereas after 4 h it led to a

Figure 1 mRNA expression pattern for (A) cPLA₂α, (C) cPLA₂β, and (E) sPLA₂IB in the bovine oviduct during the estrous cycle (n=28 for each cycle phase) as well as locally-dependent mRNA expression pattern for (B) cPLA₂α, (D) cPLA₂β, and (F) sPLA₂IB 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 PLA₂ mRNA were expressed as the mean±s.e.m. ratio relative to individual 18S rRNA values as an internal control. Days 1–5, post-ovulatory phase; days 6–12, early-to-mid luteal phase; days 13–18, late luteal phase; days 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 columns; *P<0.05.

temporary lower COX-1 expression (Fig. 3C). However, after estradiol treatment, the mRNA expression of COX-2 in cultured oviductal cells showed a significant twofold increase already after 2 h and reached a maximum threefold stimulation after 4 h compared with the untreated controls. After progesterone treatment, the COX-2 mRNA expression increased continuously over the experimental period and showed a fourfold increase after 6 h compared with the untreated control (Fig. 3D).

The range of the absolute mRNA expression of the phospholipases cPLA₂α (6–33 fg/μg total RNA) and cPLA₂β

mRNA (12–48 fg/μg total RNA) was almost similar in the cultivated cells compared with the *in vivo* results. Interestingly, the mRNA concentrations of COX-1 (42–180 fg/μg total RNA) and COX-2 (300–3000 fg/μg total RNA) in culture were considerably higher than in the freshly obtained oviductal cells.

Immunohistochemistry and western blot analysis

To investigate the protein amount and localization of the rate-limiting enzymes for PG synthesis, COX-1 and COX-2, the

Figure 2 mRNA expression pattern for (A) COX-1 and (C) COX-2 in the bovine oviduct during the estrous cycle ($n=28$ for each cycle phase) as well as locally-dependent mRNA expression pattern for (B) COX-1 and (D) COX-2 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 COX mRNA were expressed as the mean $\pm$ S.E.M. ratio relative to individual 18S rRNA values as an internal control. Days 1–5, post-ovulatory phase; days 6–12, early-to-mid luteal phase; days 13–18, late luteal phase; days 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 columns; $P<0.05$.

Figure 3 Effects of estradiol or progesterone on (A) cPLA $_{2\alpha}$, (B) cPLA $_{2\beta}$, (C) COX-1, and (D) COX-2 mRNA content in bovine oviductal cell monolayers ($n=6$). The data are expressed as the mean $\pm$ S.E.M. percentage of the 0 h control.

subsequent experiments were conducted. Immunohistochemical studies revealed that COX-1 protein was present in all bovine oviductal samples. In detail, immunoreactive COX-1 was localized exclusively in epithelial cells of the mucosa, but not in stromal cells (Fig. 4A). Among epithelial cells, mainly ciliated cells stained positively for COX-1 (Fig. 4A). Furthermore, a positive COX-1 staining was noted in smooth muscle cells in the bovine oviduct (Fig. 4B). This COX-1 immunoreactivity was observed in the cytoplasm of the whole cell as well as at the nucleus. A representative negative control performed with normal rabbit serum showed no staining (Fig. 4C).

Western blot analysis was performed with flushed oviductal cells to reveal possible changes of the COX-1 protein content. The used polyclonal antiserum raised against ovine COX-1 recognized a specific protein of ovine COX-1 at approximately M_r 70 000 as well as a signal at the same size in the bovine samples (Fig. 5A). Densitometric analysis revealed a significant increase from the lowest COX-1 content during the pre-ovulatory phase to the highest level during the early-to-mid luteal phase, followed by a decrease during the late luteal phase (Fig. 5B). No differences of the band intensities were noted between the samples from the ipsi- and contralateral oviducts or between ampulla and isthmus (Fig. 5C).

The immunohistochemical staining pattern for COX-2 was different when compared with COX-1. Specific staining was observed in all investigated bovine oviductal samples. The analysis revealed that COX-2 protein was localized exclusively in epithelial cells, mainly in ciliated cells (Fig. 4D and E). COX-2 staining was detected neither in the stromal cells nor in the smooth muscle layer. Regional differences in the COX-2 localization in epithelial cells were

observed. In the ampulla, COX-2 staining was detected only at the apical site (Fig. 4D). In contrast, in the isthmus, immunoreactive COX-2 was localized apical and basal (Fig. 4E). The nuclei appeared rather unstained. Stronger staining for COX-2 protein was observed during the post-ovulatory and early-to-mid luteal phase compared with a weaker staining in the late luteal and pre-ovulatory phase. A tendency for a more intensive staining was noted in the ipsilateral oviduct compared with the other side. A representative negative control carried out with purified rabbit IgG showed no staining (Fig. 4F).

In addition, western blot analysis was conducted to quantify relatively the COX-2 protein in flushed oviductal cells. The used COX-2 antibody recognized a specific band of ovine COX-2 at approximately M_r 72 000, but a signal of approximately M_r 60 000 was detected in bovine oviductal cells (Fig. 6A). No significant estrous cycle-dependent or regional regulation of COX-2 protein content was observed after densitometric analysis of the obtained bands (Fig. 6B and C). A relative COX protein content was estimated by comparison of the signal intensity of the samples with the 0.5 µg/lane COX-1 or COX-2 standard. Therefore, the average range of COX-1 protein content (0.5–1.0 µg/20 µg oviductal protein) in oviductal cells was about four- to fivefold higher compared with COX-2 (0.1–0.3 µg/20 µg oviductal protein).

COX activity

The results of the COX activity assay demonstrated that COX activity in the bovine oviduct originated predominantly from COX-1 rather than from COX-2 (Fig. 7). The COX activity

Figure 4 Immunohistochemical staining of COX-1 and COX-2 proteins in the bovine oviduct. Both (A) luminal epithelial cells and (B) smooth muscle cells expressed COX-1. COX-1 was more often expressed in ciliated cells (narrow arrow) than in non-ciliated cells (wide arrow). (C) A representative negative control for COX-1 staining is shown. (D) COX-2 was expressed exclusively in epithelial cells preferably in ciliated cells. In the ampulla, COX-2 is localized in the apical site of the epithelial cells. (E) In the isthmus, COX-2 is localized to apical as well as basal sites in the epithelial cells. (F) A representative negative control for COX-2 staining is shown. Magnification is $\times 1000$.

Figure 5 (A) Western blot analysis of COX-1 (one representative experiment out of four is depicted). (B) COX-1 protein expression pattern in the bovine oviduct during the estrous cycle ($n=4$ animals/estrous cycle phase). (C) COX-1 protein expression pattern in the bovine oviduct during the estrous cycle subdivided into contra- and ipsilateral as well as ampulla and isthmus ($n=4$ for each region and estrous cycle phase). The signal intensities of COX-1 protein are expressed as the mean $\pm$ S.E.M. of the relative integrated absorbance. Days 1–5, post-ovulatory phase; days 6–12, early-to-mid luteal phase; days 13–18, late luteal phase; days 19–21, pre-ovulatory phase; C-A, contralateral ampulla; C-I, contralateral isthmus; I-A, ipsilateral ampulla; I-I, ipsilateral isthmus. Different letters indicate significant differences between the columns; $P<0.05$.

Figure 6 (A) Western blot analysis of COX-2 (one out of four experiments is depicted). (B) COX-2 protein expression pattern in the bovine oviduct during the estrous cycle ($n=4$ animals/estrous cycle phase). (C) COX-2 protein expression pattern in the bovine oviduct during the estrous cycle subdivided into contra- and ipsilateral as well as ampulla and isthmus ($n=4$ for each region and estrous cycle phase). The signal intensities of COX-2 protein are expressed as the mean $\pm$ S.E.M. of the relative integrated absorbance. Days 1–5, post-ovulatory phase; days 6–12, early-to-mid luteal phase; days 13–18, late luteal phase; days 19–21, pre-ovulatory phase; C-A, contralateral ampulla; C-I, contralateral isthmus; I-A, ipsilateral ampulla; I-I, ipsilateral isthmus.

Figure 7 COX activity in bovine oviductal lysates (OVI; $n=5$) which was set at 100% and the effect of 24 $\mu\text{g}/\text{well}$ COX-1 inhibitor (SC-560) and 10 $\mu\text{g}/\text{well}$ COX-2 inhibitor (DuP-697) on the COX activity in the cell lysates (OVI) after 5 min incubation. Data of the wells with inhibitors are presented as the mean $\pm$ S.E.M. percentage of the original cell lysates (OVI). Different letters indicate significant differences between the columns; $P<0.05$.

of the samples, which were treated with the specific COX-1 inhibitor SC-560 declined significantly to about 25% of the original activity. In contrast, the samples incubated with COX-2 inhibitor DuP-697 showed a weaker decrease to 80% of the original activity. The inhibition of COX-1 and COX-2 standards with SC-560 and DuP-697 reached approximately 50%. Higher inhibitor concentrations did not lead to a stronger inhibition (data not shown).

Discussion

The results of the present study demonstrate that bovine oviductal cells are the local source of the first step enzymes for prostaglandin synthesis indicating that a functional synthesis system exists to produce PGs locally in this reproductive organ. This corresponds to earlier findings showing the presence of PGE_2 and $\text{PGF}_{2\alpha}$ in the bovine oviduct (Wijayagunawardane *et al.* 1998).

Upregulated $\text{cPLA}_2\beta$ mRNA expression before ovulation in the oviduct indicates an important role of this enzyme for the processes occurring around this phase. Steroid hormone treatment did not affect the $\text{cPLA}_2\beta$ expression. Therefore, other factors must be involved in the upregulation of the $\text{cPLA}_2\beta$ expression during the estrous cycle. However, the exact role of $\text{cPLA}_2\beta$ in the bovine oviduct is still unknown. Moreover, $\text{cPLA}_2\beta$ possesses additionally a high PLA_1 activity on phosphoglycerids, besides its low PLA_2 activity (Song *et al.* 1999). In contrast to $\text{cPLA}_2\beta$, $\text{cPLA}_2\alpha$ showed an unregulated expression during the whole estrous cycle and may act as a 'housekeeping' gene to guarantee a constant level of AA in the bovine oviduct. Normally, $\text{cPLA}_2\alpha$ is described in most tissues as an inducible enzyme (Kudo & Murakami 2002) and cell culture experiments of the present study supported this because prolonged estradiol or progesterone treatment stimulated the $\text{cPLA}_2\alpha$ expression. This is also consistent

with findings that higher PLA_2 activity in oviductal epithelium was measured in estradiol-treated rabbits compared with the control (Morishita *et al.* 1993).

PLA_2 activity was observed in bovine oviductal fluid with the highest PLA_2 activity in the isthmus after ovulation (Grippio *et al.* 1994). This suggests that the generated AA may be essential for an increased PG production during this period in the lumen. Numerous enzymes contain PLA_2 activity, but it is likely that members of the secretory PLA_2 family (e.g., sPLA_2IB) contribute to this activity in the oviductal fluid. However, our findings of a higher sPLA_2IB expression in ampullary rather than in isthmic oviductal cells of the same oviduct do not explain this phenomenon completely. sPLA_2IB is possibly secreted as an inactive form (Kudo & Murakami 2002) and is subsequently accumulated in the isthmus after its activation. Furthermore, it seems that sPLA_2IB is produced only in traces compared with the cytosolic forms. This is confirmed by the literature, which described sPLA_2IB being mainly expressed in the digestive tract (Kudo & Murakami 2002). In conclusion, cytosolic PLA_2 seems to be more important for the oviduct than sPLA_2IB because of the several times higher expression levels. This indicates that the produced AA is an essential prerequisite to generate PGs through cyclooxygenases.

Surprisingly, COX-1 expression was significantly upregulated in bovine oviductal cells after ovulation indicating that COX-1 might be involved in the creation of the optimal environment for the developing embryo. In the literature, COX-1 is mainly described as a constitutive enzyme in most tissues (Kniss 1999), but only a few reports about the inducibility of COX-1 expression were published. Upregulation of COX-1 expression was observed in cancer cell lines (Schneider *et al.* 2001, Sales *et al.* 2002, Okamoto *et al.* 2003) or during differentiation (Morita 2002). However, COX-1 mRNA expression appeared unregulated in cultured oviductal cells by estradiol or progesterone treatment. These findings indicate that other factors must be involved in the upregulation during the estrous cycle, e.g., FSH or LH. Immunohistochemical studies in human (Huang *et al.* 2002) and in mouse (Huang *et al.* 2004) revealed that COX-1 was localized in the epithelial lining as well as in the smooth muscle cells, which is similar to our findings in the bovine oviduct.

In contrast to COX-1, the *in vitro* COX-2 mRNA expression was stimulated after estradiol or progesterone treatment. This is in agreement with the literature, which described COX-2 as an inducible enzyme (Kniss 1999) and elucidates the stimulatory effect on PGE_2 and $\text{PGF}_{2\alpha}$ production after treatment of bovine oviductal cells with estradiol (Wijayagunawardane *et al.* 1999). In spite of these observations, no estrous cycle-dependent change of COX-2 mRNA or protein expression was noted in the bovine oviduct. As shown by several groups, the stimulation of COX-2 mRNA was observed for merely a few hours (Han *et al.* 1996, Parent *et al.* 2003). This short-time effect is unlikely to be detected by collecting samples over a period of several days.

COX-2 was only detected in epithelial cells, which supposes that its function is limited to this cell layer. In contrast, the human fallopian tube (Huang *et al.* 2002) and mouse oviduct (Huang *et al.* 2004) showed a positive staining for COX-2 in the smooth muscle layer as well as the epithelial cells. Apparently, COX-2 protein expression pattern varies between different species, which suggests that COX-2 could have varying functions in different species. Interestingly, the COX-2 protein in the oviduct showed a reduced size of about M_r 60 000 compared with the known size of M_r 72 000, which was reported for the bovine endometrium (Arosh *et al.* 2002). In this study, a primer pair spanning all exons was used to reveal whether a splicing variant is responsible for this observation, but only the full-length transcript was found (data not shown). Therefore, the smaller COX-2 protein is believed to correspond to a specific post-translational truncated form. The cleaving may be predicted in the N-terminal region of the protein, because the antibody used recognized the C-terminus, which contains the catalytic domain. Other studies support our findings by reporting additionally smaller M_r 62 000 or 59 000 bands for COX-2 in bovine preovulatory follicles or rat granulosa cells respectively (Sirois & Richards 1992, Sirois 1994).

Generally, the quantity of COX-1 mRNA and protein as well as its activity were considerably higher than the amount and activity of COX-2 during the estrous cycle, suggesting a more prominent role for COX-1. Furthermore, the results of this study suggest that COX-1 and COX-2 play different roles in the bovine oviduct. The localization in smooth muscle cells and in the epithelial lining supports the hypothesis that COX-1 may act on two different pathways. One important role for COX-1 is to support the muscle contraction, because only COX-1, but not COX-2, was located in the smooth muscle cells. It is likely that COX-1 is involved in muscle contraction by providing the precursor for $\text{PGF}_{2\alpha}$ and PGE_2 and thus participates in transport processes. The biological activity in epithelial cells for COX-1 is indicated by the fact that the expression pattern of COX-1 is upregulated at the time when the embryo stays in the oviduct. So it is suggested that the synthesized PGs may interact with the embryo preparing for an embryo-maternal communication.

All the results of the present study support the hypothesis that an increased synthesis of PGs in the oviduct around ovulation could be necessary for the maturation and the fertilization of the oocyte as well as for the development of the early embryo. In summary, a regulated and fine-tuned expression of the first step enzymes for the PG synthesis is present in the bovine oviduct to locally supply essential PGs that may be important and pivotal for the origin and maintenance of pregnancy already in the bovine oviduct.

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