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Importance of

steroid hormone receptors, nitric oxide synthesis and hyaluronan turnover
for the control of oviduct function

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CONTENT

ABBREVIATIONS

ANOVA	analysis of variance	iNOS	inducible nitric oxide synthase
App.	appendix		
BOEC	bovine oviduct epithelial cells	luteal p.	luteal phase
bp	base pair	mRNA	messengerRNA
Ca^{2+}	calcium	n	numbers
CD44	cluster of differentiation 44	NADPH	nicotinamide adenin dinucleotide phosphate
cDNA	complementary DNA		
cGMP	cyclic guanosine monophosphate	nNOS	neuronal nitric oxide synthase
CP	crossing point	NO	nitric oxide
DNA	desoxyribonucleic acid	NOS	nitric oxide synthase
DNAse	desoxyribonuclease	O_2	oxygen
ECM	extra cellular matrix	PCR	polymerase chain reaction
EIA	enzyme immuno assay	p.	page
EMBL	European Molecular Biology Laboratory	PR	progestin receptor
		PR-A (-B, -C)	progestin receptor isoform A (B,C)
eNOS	endothelial nitric oxide synthase	RHAMM	receptor for HA-mediated motility
ERα	estrogen receptor alpha		
ERβ	estrogen receptor beta	RNA	ribonucleic acid
FAD	flavin adenin dinucleotide	rRNA	ribosomal RNA
Fe	iron ion	RT-PCR	reverse transcription-polymerase chain reaction
Fig.	figure		
follicular p.	follicular phase	SEM	standard error of mean
FMN	flavin mononucleotide		
GAG	glucosaminoglycans		
GTP	guanosine triphosphate		
HA	hyaluronan		
HABP	hyaluronan binding protein		
HARE	hyaluronan receptor for endocytosis		
HAS	hyaluronan synthase		
HAS2 (3)	hyaluronan synthase isoform 2 (3)		

ABSTRACT

The bovine oviduct is responsible for the accomodation of the gametes and the early embryo by providing an optimal environment for successful fertilization. In addition, it accounts for the transport of the gametes and the embryo into the uterus. Steroid hormones serve as conductors leading specific tissues to time specific differentiation. This guarantees that cyclic events occur orchestrated between the different organs and tissues involved in the reproductive tract. Although the oviduct does not synthesize steroid hormones itself, it is a target tissue for the action of peripheral hormones. The sensitivity of tissues towards the actions of steroid hormones is determined by the presence of steroid hormone receptors. Therefore, in a first approach the steroid hormone receptor mRNA expression and protein localization in the oviduct during the estrous cycle and *in vitro* in a bovine epithelial cell suspension culture were investigated. Obvious cyclic changes of steroid hormone receptor expression *in vivo* in the bovine oviduct were observed and concurrent expression patterns were detected *in vitro*. Estrogen receptor alpha (ERα) was stimulated by estradiol-17β, whereas estrogen receptor beta (ERβ) depended on progesterone. In the bovine oviduct receptor-mediated actions of estrogens are mainly regulated through ERα rather than ERβ. Progestin receptor (PR) was upregulated by estradiol-17β, but progesterone had a hampered effect. The regulation of steroid receptors enables the determination of a distinct responsiveness of the oviduct towards steroids.

Nitric oxide (NO) has emerged as major paracrine mediator in a variety of different physiological processes. To approach this signalling molecule with a specific regulatory role of the oviduct function the expression of the two isoenzymes endothelial (eNOS) and inducible (iNOS) NO synthase (NOS) were studied. The present results provide evidence for the presence of NOS derived NO in the bovine oviduct. Again, pronounced changes during the estrous cycle occurred for both isoenzymes, predominantly a conspicious downregulation of iNOS at estrus in the isthmus. The hypothesis is that the downregulation of iNOS at estrus in the isthmus leads to an increase of oviduct motility by circular smooth muscles and/or ciliary activity.

During the passage local interactions between the oviduct and the gametes are of significant importance for fertilization and subsequent embryogenesis. Concerning cell-to-cell contacts, components of the extracellular matrix (ECM) may play important roles. Hyaluronan (HA) is a major component of the ECM and could be demonstrated in oviduct epithelial cells in addition to a functional HA-system. The characterization of selected components of this functional HA-system was aimed to outline their potential contribution to the early reproductive events. Most interestingly, the principal HA receptor CD44 was conspicuously regulated between the ampulla and the isthmus part of the oviduct, which was conversily for

its ligand HA. Because HA-synthases (HAS) remained unchanged these results suggest that in the bovine oviduct the cell surface receptor CD44 in particular might inversely regulate HA through internalization. This local turnover may influence the HA concentration in the oviduct beneficial for the development of the passing gametes.

Furthermore, using a potent cell culture model, HA was found to stimulate iNOS mRNA *in vitro*. Possible functional implications can be linked to local contractions or relaxation, cilliary beating of epithelial cells or a participation in the regulation of inflammatory immune response of the oviduct epithelium towards gametes. Taking together these findings underline a physiological importance of the oviduct environment towards supporting successful reproduction.

ZUSAMMENFASSUNG

Die Bedeutung von Steroidhormonrezeptoren, der Stickstoffmonoxidsynthese und des Umsatzes von Hyaluronsäure für die Steuerung der Eileiterfunktion

Der Rindereileiter ist für die Unterbringung der Gameten und des frühen Embryos durch die Bereitstellung eines optimalen Milieus für eine erfolgreiche Befruchtung verantwortlich. Außerdem ist er für den Transport der Gameten und des Embryos in den Uterus zuständig. Steroidhormone dienen als Vermittler, um spezifische Gewebe zu zeitgerechter Differenzierung zu lenken. Dies garantiert, dass zyklische Ereignisse orchestriert zwischen den verschiedenen Organen und Geweben, die sich im Reproduktionstrakt befinden, stattfinden. Obwohl der Eileiter selbst keine Steroidhormone produziert, ist er ein Zielorgan für die Wirkung peripherer Hormone. Die Sensitivität eines Gewebes gegenüber der Wirkung von Steroidhormonen wird durch die Anwesenheit von Steroidhormonrezeptoren bestimmt. Daher wurde in einem ersten Versuch die Expression der mRNA der Steroidhormonrezeptoren und deren Proteinlokalisation im Eileiter während des östrischen Zyklus und *in vitro* in einer Epithelzellsuspensionskultur des Rindes untersucht. *In vivo* wurden deutliche zyklische Änderungen der Expression der Steroidhormonrezeptoren im Rindereileiter festgestellt und *in vivo* übereinstimmende Expressionsmuster gefunden. Der Östradiolrezeptor alpha (ERα) wurde durch Östradiol-17β stimuliert, Östradiolrezeptor beta (ERβ) hingegen war von Progesteron abhängig. Rezeptor-vermittelte Wirkungen von Östrogenen werden im Rindereileiter mehr durch ERα als durch ERβ reguliert. Der Progestinrezeptor (PR) wurde durch Östradiol-17β aufreguliert, aber Progesteron hatte einen dämpfenden Effekt. Die Regulation der Steroidhormonrezeptoren ermöglicht die Feststellung der konkreten Ansprechbarkeit des Eileiters gegenüber Steroidhormonen.

Stickstoffmonoxid (NO) hat als wichtiger parakriner Vermittler einer Vielzahl verschiedener physiologischer Prozesse Bedeutung erlangt. Zur näheren Untersuchung dieses Signalmoleküls mit spezifischer Rolle bei der Regulation der Eileiterfunktion wurde die Expression der zwei Isoenzyme endotheliale (eNOS) und induzierbare (iNOS) NO-Synthase (NOS) untersucht. Die gegenwärtig vorliegenden Ergebnisse liefern Beweise für das Vorliegen von aus NOS stammendem NO im Rindereileiter. Wieder ließen sich für beide Isoenzyme ausgeprägte Änderungen während des östrischen Zyklus nachweisen, hauptsächlich eine bemerkenswerte Herabregulierung von iNOS während des Östrus im Isthmus. Die Hypothese ist, dass die Herabregulierung von iNOS während des Östrus im Isthmus zu einer Zunahme an Bewegung des Eileiters durch die zirkuläre glatte Muskulatur und/oder durch Aktivität der Zilien führt.

Während der Passage sind lokale Interaktionen zwischen dem Eileiter und den Gameten für die Fertilisierung und die darauf folgende Embryogenese von signifikanter Bedeutung. Komponenten der Extrazellulären Matrix (ECM) könnten bezüglich Zell-zu-Zell Kontakten eine wichtige Rolle spielen. Hyaluronsäure (HA) ist ein bedeutender Bestandteil der ECM und konnte in Eileiterepithelzellen zusätzlich zu einem funktionierenden HA-System nachgewiesen werden. Die Charakterisierung von ausgewählten Komponenten dieses HA-Systems könnte einen möglichen Beitrag zu frühen Reproduktionsereignissen darstellen. Am interessantesten war die auffallende Regulierung des hauptsächlichen HA-Rezeptors CD44, was in umgekehrter Weise für den Liganden HA der Fall war, zwischen dem Ampulle- und dem Isthmusbereich des Eileiters. Da die HA-Synthasen (HAS) unverändert blieben deuten diese Ergebnisse an, dass im Rindereileiter insbesondere der Zelloberflächenrezeptor CD44 HA durch Internalisierung gegenläufig regulieren könnte. Dieser lokale Umsatz könnte, günstig für die Entwicklung der durchwandernden Gameten, die HA-Konzentration im Eileiter beeinflussen.

Mit Hilfe eines etablierten Zellsuspensionskulturmodells wurde weiterhin gefunden, dass HA *in vitro* iNOS mRNA stimulierte. Mögliche funktionelle Implikationen können mit lokalen Kontraktionen oder Relaxationen sowie mit dem Zilienschlag der Epithelzellen in Verbindung gebracht werden oder mit einer Beteiligung des Eileiterepithels an der Regulierung der inflammatorischen Immunantwort gegenüber den Gameten. Zusammengenommen unterstreichen die Befunde eine physiologische Bedeutung des Eileitermilieus für die Unterstützung einer erfolgreichen Reproduktion.

INTRODUCTION

The bovine female reproductive tract is mainly influenced by ovarian steroids. While the luteal phase is characterized by rising levels of progesterone from a functional corpus luteum, the follicular phase is dominated by high levels of circulating estrogens (Fig.1). The latter originate from developing follicles, which successively grow and emerge in the rupture of one follicle at ovulation. Located between the ovary and the uterus, the bovine oviduct resembles an organ that is transiently used for the capturing of the oocyte from the follicle and its transport into the uterus (Fig.1).
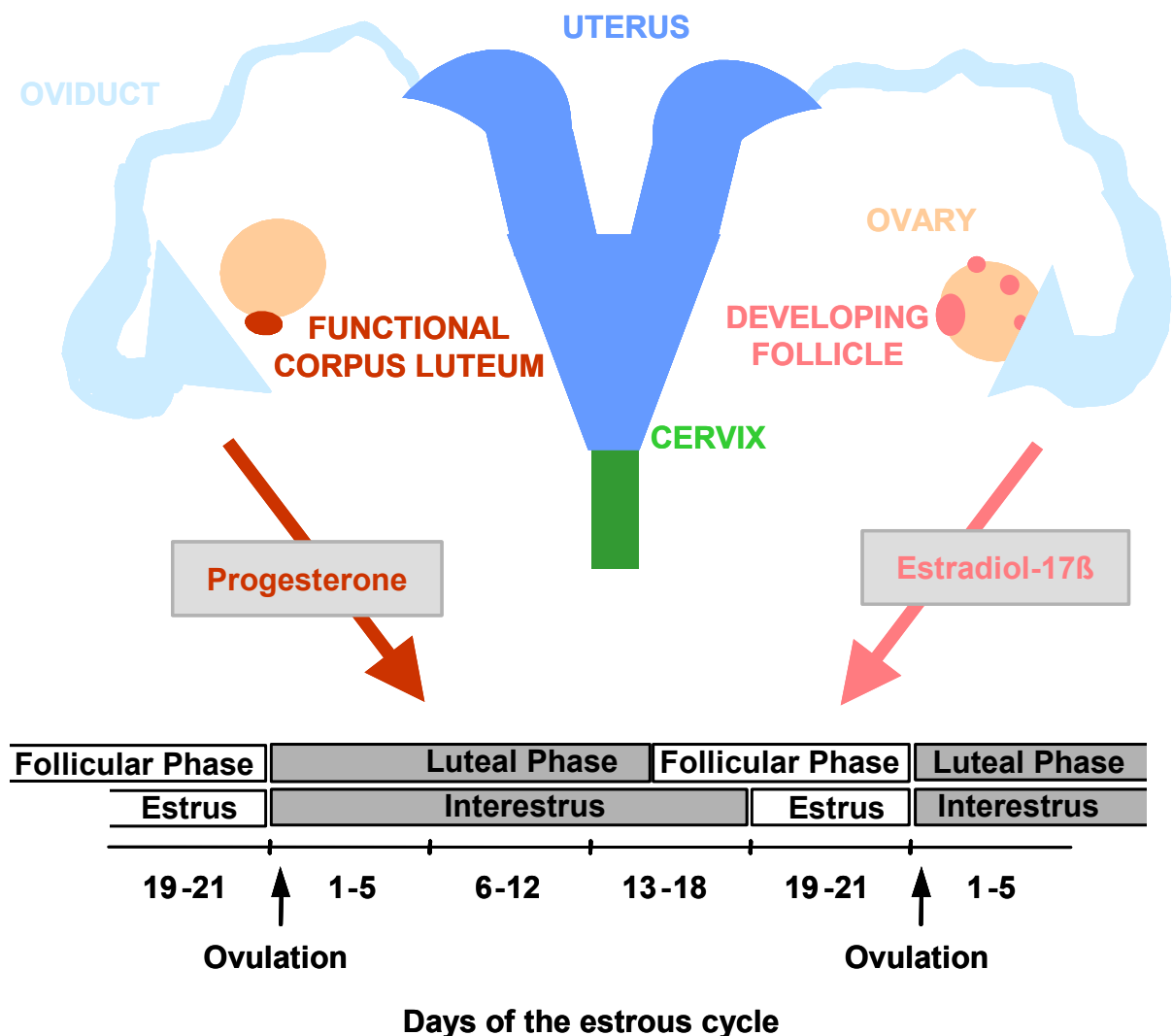

Fig.1: Scheme of the female reproductive tract with the functional corpus luteum producing progesterone during the luteal phase of the estrous cycle and follicles producing estrogens during the follicular phase.

The advancing sperm capacitate in the oviduct and after the final maturation of the oocyte fertilization occurs here (Fig.2). But the oviduct is more than a simplified tube for the passage of gametes and the embryo. Its necessity became obvious by cultivating embryos *in vitro*. Namely in case of an absent oviduct environment, the embryo underwent a developmental blockade. Until today, the supporting role of the oviduct further remains to be enlightened.

The oviduct is lined with a mucosal epithelium and surrounded by a lamina propria and a lamina muscularis. Due to morphological particularities, already supposing functional disparities, the oviduct is divided into the fimbria, which allows the pick-up of the ovulated cumulus-oocyte-complex, the ampulla and the isthmus (Fig.2). The inner surface of the oviduct is enlarged because of numerous foldings, predominantly in the ampulla. The epithelium consists of secretory and ciliated cells, the latter being also more numerously in the ampulla. The beating of the ciliated cells are most pronounced at estrus, indicating a dependency on estrogens (Hunter 1988) as well as a possible participation in the transport of the gametes and the embryo. The transport can also be influenced by the lamina muscularis through its contractibility, which is most pronounced in the isthmus.
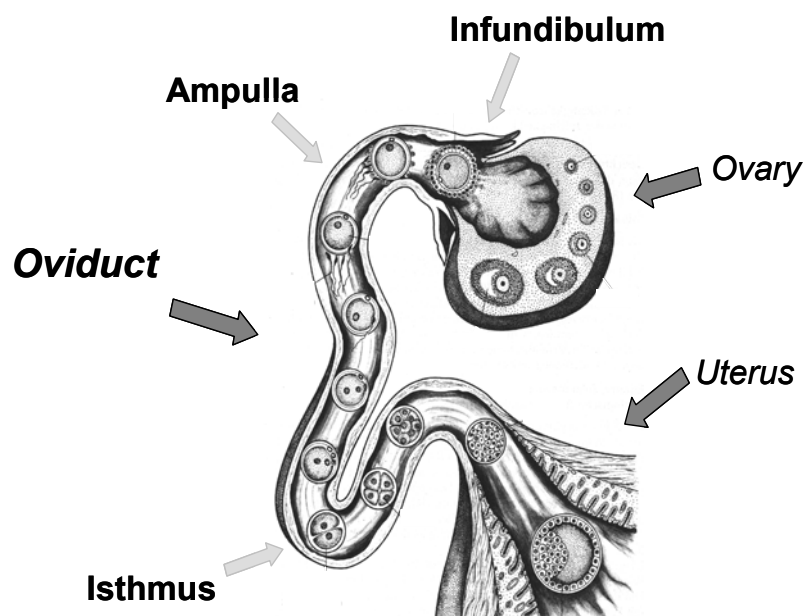

Fig.2: Scheme of the oviduct as the site of fertilization and early embryonic development (following Löffler 1991).

The oviduct is capable of transferring substances from the circulation into the oviduct lumen as well as *de novo* synthesizing and releasing molecules. These lipids, enzymes and growth factors next to a variety of oviduct specific proteins like oviductin altogether form the oviduct fluid (Henault & Killian 1993; Boatman 1997). Many of these proteins are considered to be of nutritional importance for the conceptus, but some may also contribute to sperm binding,

gamete growth and developmental regulation (Gandolfi 1995). The secretory products are suspected to be regulated from the periphery in a cycle dependent manner, but auto-/paracrine regulations may occur as well (Einspanier *et al.* 1997).

A precise and functionally related synchronization of all parts of the female reproductive system is essential for fertilization and embryonic development. The oviduct is known to be under the influence of peripheral steroids (Fig.1), and remarkable changes of progesterone and estradiol contents in the oviduct throughout the estrous cycle are described (Wijayagunawardane *et al.* 1998). Especially estrogens should induce compositional changes of the oviduct fluid with greatest protein secrection during the follicular phase (Buhi *et al.* 2000). The proliferation of the luminal epithelium cell layers and the differentiation of secretory cells is regulated under the influence of estrogens (Abe & Oikawa 1993). It has been supposed that progesterone is acting generally antagonistic to the estrogen-mediated effects described above (Clark *et al.* 1992).

Both steroid hormone actions are mediated through intracellular receptors representing members of the nuclear receptor family, namely estrogen receptor alpha (ERα), beta (ERβ) and progestin receptor (PR). The latter regulate the expression of a wide variety of genes on a transcriptional level. The existence of a second estrogen receptor next to ERα has only recently been shown in the rat, mouse, human, cattle and pig (Kuiper *et al.* 1996; Tremblay *et al.* 1997; Mosselman *et al.* 1996; Rosenfeld *et al.* 1999; Kowalski *et al.* 2002). However, the joint expression of PR along with estrogen receptors is discussed controversially (Meyer *et al.* 1988; Meikle *et al.* 2001; Kraus & Katzenellenbogen 1993). Especially the distribution of ERβ during the normal estrous cycle seems to be quite conflicting between and within species and tissues (Walther *et al.* 1999). Nevertheless, regional expression differences within the oviduct seem to be of functional importance. Although data demonstrating steroid hormone receptor expression and localization in the endometrium and the ovary of different species are available (Mosselman *et al.* 1996; Pelletier *et al.* 2000; Kraus & Katzenellenbogen 1993; Byers *et al.* 1997; Slomczynska & Wozniak 2001; Berisha *et al.* 2002), a thourough investigation focusing on the bovine oviduct is missing.

Next to endocrine mediators of steroid action other mechanisms might regulate oviduct function as well. The mediating effects of nitric oxide (NO) exemplary for a paracrine mediator were therefore evaluated.

NO is an important intercellular regulatory molecule and a major paracrine mediator, functioning as a vascular, immune and neuronal signalling molecule (Ignarro *et al.* 2001). NO is able to diffuse freely through membranes (Fig.3) to react with several targets by exerting an effect as agent for unspecific immune response (Guzik *et al.* 2003) or as modulator of gene expression (Nathan & Xie 1994). The effect of NO on vasodilatation has been shown

thouroughly (Furchgott & Vanhoutte 1989). NO is supposed to be involved in a variety of different functions as well as reproductive processes such as oocyte maturation, ovulation, implantation, pregnancy maintenance, labor and delivery (Jablonka-Shariff *et al.* 1999; Shukovski & Tsafriri 1994; Sengoku *et al.* 2001; Maul *et al.* 2003). NO is produced by the conversion of L-arginine to L-citrullin by the enzyme NO-synthase (NOS) in a number of different tissues and cell types (Fig.3). Up to date, three isoforms of NOS, products of separate genes with different molecular weight but apparently similar molecular structure, have been described: neuronal NOS (nNOS) in brain and peripheral nervous system, endothelial NOS (eNOS) as a constitutive NOS primarily in the endothelium and inducible NOS (iNOS) synthesized by activated macrophages, hepatocytes and neutrophiles in several tissue types and organes and upon inflammatory stimulation (Moncada *et al.* 1997). NOS have been identified in the human, bovine and rat oviduct (Ekerhovd *et al.* 1997; Bryant *et al.* 1995; Rosselli *et al.* 1996). A specific regulatory role of the oviduct function has been subscribed to NOS.
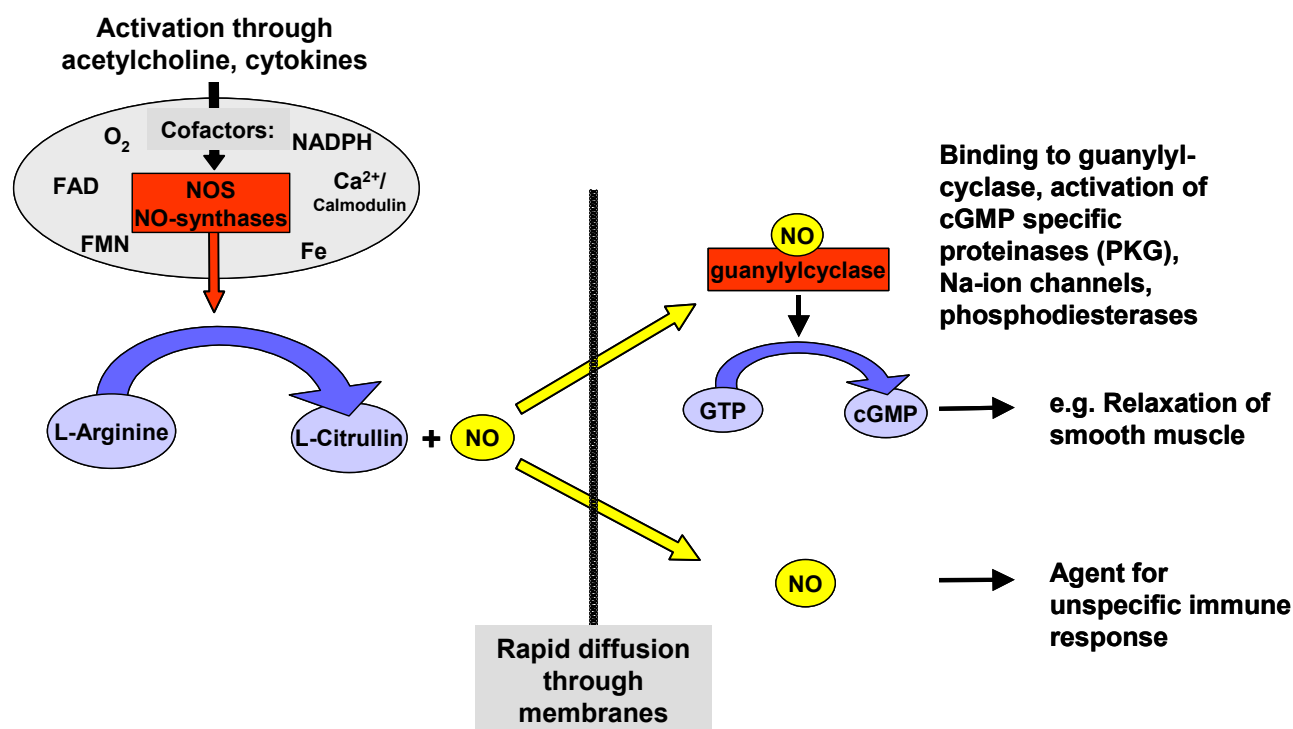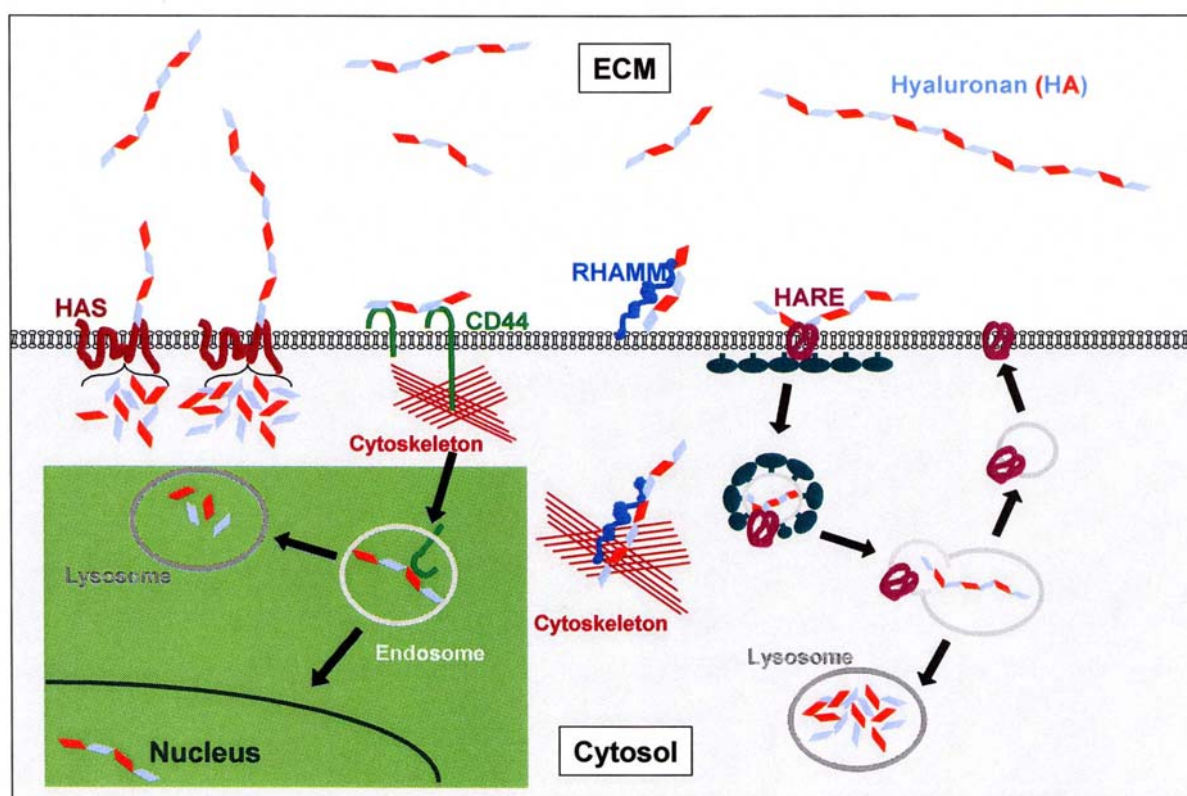

Fig.3: Scheme of NO production by NO-synthases. NO is generated by the conversion of L-arginine to L-citrullin by the enzyme NO-synthase (NOS) involving several co-factors. It exerts its numerous effects either directly or indirectly via cGMP.

Recently, the supportive role of the oviduct towards sperm competence has been shown (Gualtieri & Talevi 2003). In addition, the maturing cumulus-oocyte-complex is considered to

interact with the oviduct. Concerning cell-to-cell contacts, components of the extracellular matrix (ECM) may play important roles. Hyaluronan (HA) is a major component of the ECM, and therefore selected components of the prominent HA-system were outlined (Fig.4) concerning their potential contribution towards early reproductive events.

HA has been demonstrated in the oviduct fluid (Tienthai *et al.* 2000). It forms linear polymers of nonsulphated glucosaminoglycans (GAG) of high-molecular weight (10^4-10^7 Da) and is able to expand dramatically by hydratization. Its biological functions have primarily been associated with matrix structure and plasma protein distribution. More recently, HA has been linked to local cell proliferation and angiogenesis, inflammation, cell recognition and migration beyond simply being a structural component (Laurent & Fraser 1992).

Exclusively synthesized by HA synthases (HAS) which are located at the plasma membrane (Fig.4) (Prehm 1984), the expanding GAG is extruded through the membrane into the ECM (Weigel *et al.* 1997). Several growth factors and cytokines have been demonstrated to trigger either one or more HAS isoforms in a tissue and cell specific manner (Kuroda *et al.* 2001).

following Weigel 1997

Fig.4: Scheme of the hyaluronan metabolism. HA is produced by HAS located at the cell membrane. Next to its direct action in the ECM HA acts via the receptors CD44, RHAMM and HARE, which additionally participate in the regulation of HA turnover.

Through specific cell surface receptors HA is known to contribute to cellular signaling (Salamonsen *et al.* 2001). The cluster of differentiation CD44 is suggested to represent the principal cell surface receptor for HA (Fig.4) (Aruffo *et al.* 1990). It belongs to a larger group of hyaluronan-binding proteins (HABPs), which are of importance for cell-cell and cell-ECM interactions as well as cell proliferation, adhesion and migration (Lesley *et al.* 1993). Additionally, CD44 has been implicated in the binding and the presentation of growth factors (Behzad *et al.* 1994). The binding of HA to the cell surface is a multivalent interaction, since several CD44 molecules are able to bind to the same HA molecule (Underhill 1992). CD44 is therefore capable of assembling pericellular matrices as well as internalizing HA (Jiang *et al.* 2002).

A second receptor for HA-mediated motility (RHAMM) has been described stimulating cell migration and locomotion via an activation of a signal transduction cascade upon HA binding (Fig.4) (Assmann *et al.* 1999). Concerning reproductive tissues, several reports describe the RHAMM-mediated promotion of cell growth and movement, sperm motility, angiogenesis and embryonic development (Kornovski *et al.* 1994; Savani *et al.* 2001; Stojkovic *et al.* 2003).

Recently, a third HA receptor has been purified from rat liver, the hyaluronan receptor for endocytosis (HARE) (Zhou *et al.* 2000). The ability of HARE to internalize HA by endocytosis (Fig.4) via the clathrin-coated pit pathway was demonstrated in liver sinusoids. Therefore a responsibility for the clearance of systemic, circulating HA was suggested. Since HARE was found not to be restricted to the liver, a general survey of the bovine tissue distribution of putative HARE was undertaken in the present study by RT-PCR.

Using an *in vitro* cell culture approach simplifies the complexity of any tissue and organ association of a resembled organism. Single peripheral parameters can be modulated in order to investigate monocausal responses. Nevertheless, the disadvantage is that *in vitro* approaches disregard the number of possible compensatory reactions. In addition, cells in culture might not behave equally as *in vivo* due to the artificial surrounding of the experimental construct. Therefore, there is a definite need to check the validity of the cells in culture. Next to a morphological examination compared to the appearance *in vivo* the synthesis of cell specific proteins may support the sheer viability aspect of resembling culture conditions. Specifically important for steroid sensitive cells is the response of the cultured cells towards a sexual steroid application. Therefore, a bovine oviduct epithelial cell (BOEC) suspension culture was established and validated. It was then used to observe the regulation of the candidate genes of interest under the influence of steroids as well as HA.

AIM OF THE STUDY

The bovine oviduct undergoes specific cyclic changes to contribute to an optimal environment for the passing gametes and the embryo. The present work was undertaken to enlighten the cyclic modulations of steroid hormone receptors, synthase enzymes of nitric oxide and members of the hyaluronan system of the extracellular matrix through real-time RT-PCR, Western blot and immunohistochemistry. To specify the cause of the modulations a bovine epithelial cell supension culture was established and stimulated with physiological doses of steroid hormones and hyaluronan, respectively. Proceeding from the *in vitro* together with the *in vivo* observations possible regulatory roles in the questioning context were deduced. A focus was layed on the possible involvement of the investigated candidates in the regulation of oviduct secretion capacity, motility and ciliary beating.

MATERIAL AND METHODS

Collection of Tissue Samples

Bovine oviducts from healthy cows were collected from the local slaughterhouse. They were grouped depending on the cycle stage directly distinguishing the ampulla from the isthmus (Ireland *et al.* 1980). For analyzation of epithelial cells the oviducts were opened longitudinally under steril conditions and epithelial cells were scraped off mechanically seperating the ampulla from the isthmic region. For *in vitro* as well as histological investigations, pieces of whole oviducts were taken. All *in vitro* investigations were performed with randomly selected oviducts corresponding to different reproductive stages. For the expression analysis of eNOS and iNOS oviduct epithelial cells from synchronized animals were obtained as stated previously (Bauersachs *et al.* 2003; Rottmayer *et al.* 2005).

	PCR-Product [bp]	Annealing temperature [°C]	Fluorescence acquisition temperature [°C]	Primer sequence	EMBL Accession number
18S	488	60	80	For 5´ AAG TCT TTG GGT TCC GGG 3´ Rev 5´ GGA CAT CTA AGG GCA TCA CA 3´	AF176811
ERα	234	63	86	For 5´ AGG GAA GCT CCT ATT TGC TCC 3´ Rev 5´ CGG TGG ATG TGG TCC TTC TCT 3´	Z86041
ERβ	262	64	81	For 5´ GCT TCG TGG AGC TCA GCC TG 3´ Rev 5´ AGG ATC ATG GCC TTG ACA CAG A 3´	U6496
PR	227	65	81	For 5´ GAG AGC TCA TCA AGG CAA TTG G 3´ Rev 5´ CAC CAT CCC TGC CAA TAT CTT G 3´	Y18017
HAS 2	144	68	80	For 5´ GGM TGT GTC CAG TGC ATT AGC GGA 3´ Rev 5´ CAG CAC TCG GTT CGT TAG RTG CCT 3´	U54804
HAS 3	166	60	84	For 5´ ACA GGT TTC TTC CCC TTC TTC C3´ Rev 5´ GCG ACA TGA AGA TCA TCT CTG C 3´	AJ293889
CD44	221	64	83	For 5´ TAT AAC CTG CCG ATA TGC AGG 3´ Rev 5´ CAG CAC AGA TGG AAT TGG G 3´	X62881
RHAMM	249	61	77	For 5´ TGT TGA ATG AAC ATG GTG CAG CTC 3´ Rev 5´ CCT TAG AAG GGT CAA AGT GTT TGA 3´	AF310973
putative HARE	245	62	84	For 5´ATC ACT GAC TCC ATC CAC ACC C 3´ Rev 5´GGT GTG GAA CTG GCA GTG ACA T 3´	AJ550060
iNOS	216	61	86	For 5´ ACC TAC CAG CTG ACG GGA GAT 3´ Rev 5´ TGG CAG GGT CCC CTG TGA TG 3´	AJ699400
eNOS	126	66	87	For 5´ AGG AGT GGA AGT GGT TCC G 3´ Rev 5´ GCC CCG GTA CTA CTC TGT CA 3´	NM181037

Table 1: Primer sequences and parameters used for quantitative real-time PCR.

Total RNA extraction and mRNA analysis

Total RNA was extracted as described by Chomczynski & Sacchi (1987). The RNA was reverse transcribed as described previously (Ulbrich *et al.* 2003). PCR primers were generated commercially and introduced as depicted in table 1. The primer pair for PR were designed to detect both A and B isoform. Quantitative real-time PCR reactions were performed as described recently (Ulbrich *et al.* 2004). Annealing temperatures and fluorescence acquisition points for quantification are outlined in table 1. Full-length cDNA sequencing of putative HARE was performed introducing total RNA from bovine liver (Ulbrich *et al.* 2004). Two cDNA libraries were subsequently used for the amplification of specific PCR products in a touch-down PCR. The alignment of the partial putative HARE sequence to other known sequences was done using the software package DNAsis (Version 3.5 Pro).

Cell culture

Epithelial cells (10^5/mL medium) were cultured as described recently (Ulbrich *et al.* 2003). After an accomodation period of 48h either estradiol-17β (10 pg/mL), progesterone (10 ng/mL) or hyaluronan (1mg/mL) was applied in a single dose and incubated for 1, 2, 4, 6 and 24 hours, respectively. A nontreated group of culture dishes served as a negative control. After centrifugation of the cell pellets the RNA was isolated subsequently (Ulbrich *et al.* 2004). Cell culture supernatants were stored seperately at –20°C for further EIA investigation. The identity of the collected cells was verified by microscopic observation, since ciliated cells could be detected visually. Cytokeratine-specific staining revealed more than 90% epithelial cells (Rottmayer *et al.* 2005). 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.

Enzyme-immuno-assay

Enzyme-immuno-assays for progesterone (Prakash *et al.* 1987) and estradiol-17β (Meyer *et al.* 1990) were undertaken as described previously to screen the hormone concentration in the cell culture supernatants.

Data analysis of Real-time RT-PCR

The cycle number (CP) required to achieve a definite SYBR Green fluorescence signal was calculated by the second derivative maximum method (LightCycler software version 3.5.28) (Ulbrich *et al.* 2005). The CP is correlated inversely with the logarithm of the initial template concentration. To verify the equal relative quantity of the reverse transcribed cDNA PCR for 18s ribosomal RNA mRNA were carried out. The CP determined for the target genes were normalized against the housekeeping gene 18S if stated. Otherwise the housekeeping genes revealed balanced expression and were therefore not taken into consideration. Differences

between groups were analyzed using a one-way ANOVA. The normal distribution was tested by the Kolmogorow-Smirnov method, followed by a student's t-test to find significant differences (Sigma-Stat, version 2.03) at $p<0.05$.

Western blot analysis

For protein extraction oviduct tissue from at least three different cows for each cycle stage and region were homogenized as reported earlier (Ulbrich *et al.* 2003). Protein samples were separated on a polyacrylamide gels, blotted onto membranes and incubated with monoclonal antibodies against ERα, ERβ or PR. After washing, the membranes were incubated with anti-mouse (ERα, PR) or anti-rabbit (ERβ) horse radish peroxidase-conjugated IgG secondary antibody. As a positive control a recombinant human ERα protein was used. The ERα protein was also employed to test for cross-reactivity between the three antibodies used.

Immunohistochemistry

Detailed immunohistochemical procedures have been described in detail (Ulbrich *et al.* 2003). Serial cryo-cross-sections of 7µm thickness were cut. The ERα mouse monoclonal antibody and a rabbit polyclonal antibody raised against the N-terminal region of ERβ (Rosenfeld *et al.* 1999) were used as well as the monoclonal mouse anti-PR antibody which detects both PR-A 94 kDa and PR-B 120 kDa protein isoforms by nuclear staining (Ulbrich *et al.* 2003). For eNOS and iNOS rabbit anti-mouse primary antibodies (Ulbrich *et al.* 2005) and additionally a monoclonal rat-anti-porcine immunoglobulin IgG1 fraction raised against CD44 were used (Ulbrich *et al.* 2004). For the HA detection a biotinylated hyaluronan acid binding protein (HABP) from bovine nasal cartilage was used binding specifically to HA (Ulbrich *et al.* 2004). Tissue slices of eight oviducts referring to each cycle stage and region were mounted together on a single slide. Two sets of embedded oviducts from 16 different cycling cows were taken under investigation. The antibody staining procedure was reproduced at least twice in each case.

RESULTS AND DISCUSSION

The present results demonstrate the cyclic expression patterns of steroid receptors (Fig.5, Ulbrich *et al.* 2003), nitric oxide synthases (Fig.6, Ulbrich *et al.* 2005) and members of the hyaluronan turnover (Fig.7, Ulbrich *et al.* 2004) in the bovine oviduct as summarized in the corresponding figures. Next to the *in vivo* investigation a two-day suspension culture of BOEC was established to retain as much physiological cell morphology as possible. During the course of the short-time 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 (Ulbrich *et al.* 2003). The beginning of a typical oviduct epithelial cell arrangement in tube-form could be observed as it has been described previously (Joshi 1995). The maintainance of the ciliated epithelial cell phenotype in our culture system (Rottmayer *et al.* 2005) points towards a retention of characteristics associated with *in situ* conditions, reflecting predominantly physiological settings (Comer *et al.* 1998).

In peripheral blood plasma estrogen levels are found highest during the follicular phase of the bovine estrous cycle (Meyer *et al.* 1990). Under the dominance of peripheral estrogens at estrus the mRNA expression of ERα was elevated *in vivo* predominantly in the ampulla (Fig.5A, App. p.43). Likewise, the *in vitro* stimulation with estradiol-17β was followed by significantly elevated ERα transcripts rapidly after exposure (Fig.5B, App. p.46). The action of estrogens through ERα in the oviduct around the time of estrus may therefore account for specific compositional changes of the oviduct fluid occuring during this period. Western blotting revealed increased ERα protein during the early luteal phase indicating a translational delay (Fig.5A, App. p.44). Immunoreactive ERα was localized to nuclei of the luminal epithelial cell layer and stroma in cross sections of bovine oviducts during all phases of the estrous cycle and a faint cytoplasmatic staining was visible in the muscular layer surrounding the oviduct (App. p.45). Kimmins & MacLaren (2001) suggest that stromal estrogen receptors as well as progestin receptors may trigger the steroid responsiveness of the epithelium.

The ERβ mRNA was expressed highest in the isthmus as detected both by RT-PCR and western blotting (Fig.5A, App. p.43/44). In the course of the estrous cycle the most intense staining of a double band of approximately 58 and 62 kDa was visible during the luteal stage (App. p.44). In the cow the luteal phase is dominated by high peripheral blood levels of progesterone (Meyer *et al.* 1990). Accordingly, ERβ expression levels were elevated *in vitro* after progesterone treatment, while ERβ mRNA remained unaffected by estradiol (Fig.5B, App. p.46). Therefore, both *in vivo* and *in vitro* data provide evidence for a direct dependency of ERβ on progesterone. The immunohistochemical protein staining for ERβ revealed mainly

nuclear signals in luminal epithelial cells (App. p.45). A moderate cytoplasmatic staining could be revealed in the epithelial cell layer and in the muscle layer, but not in stromal tissue.
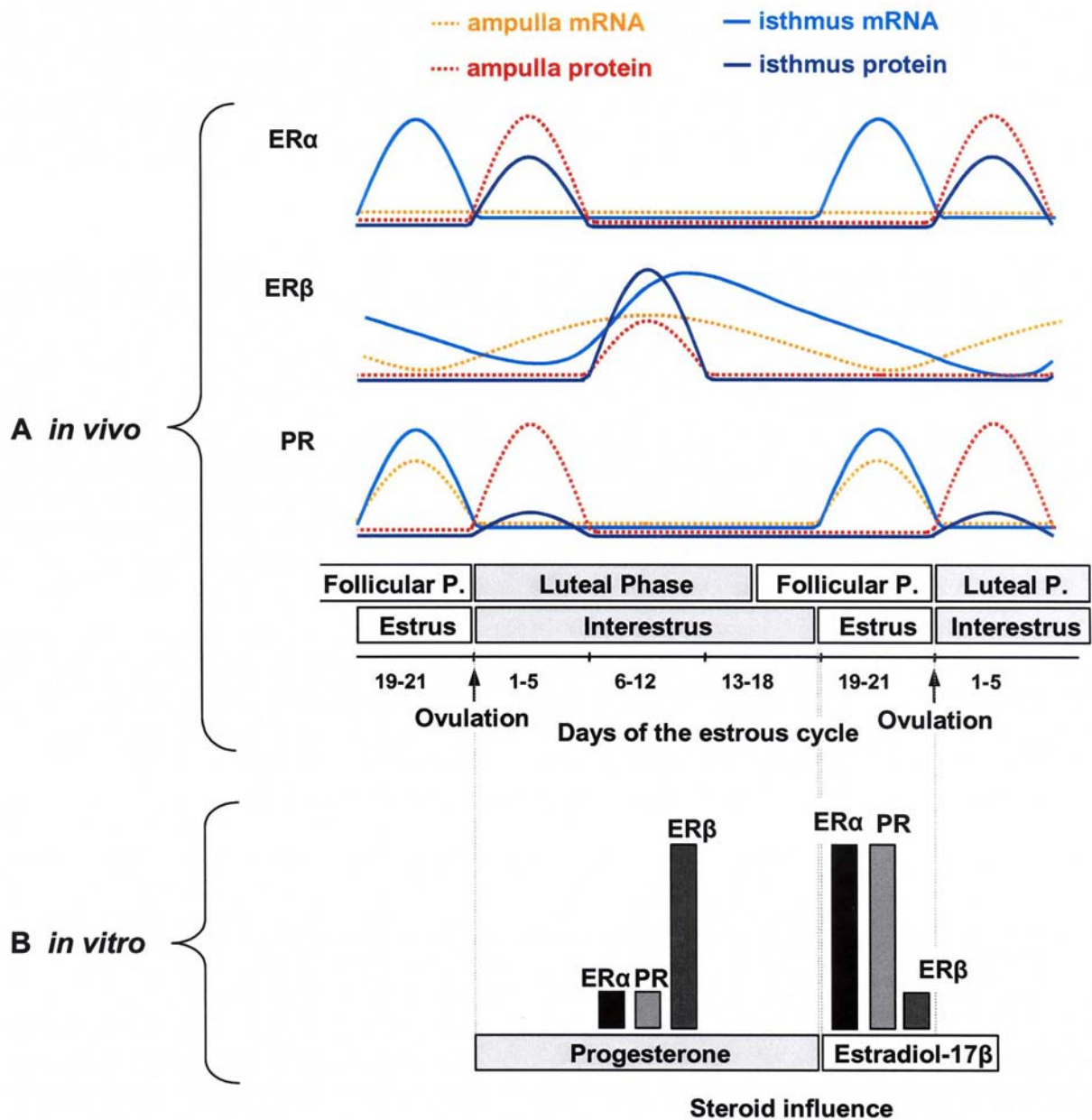

Fig.5: Summarized results of steroid hormone receptor (A) mRNA and protein expression in vivo and (B) in vitro transcript levels after progesterone and estradiol-17β stimulation (Ulbrich et al. 2003).

The predominant presence of ERα has recently been shown for the rat and the human oviduct (Pelletier *et al.* 2000; Taylor & Al Azzawi 2000). In fact, ERβ plays a subordinate role in most parts of the reproductive system with exception of the ovary (Kuiper *et al.* 1996; Wang *et al.* 2000) and the embryo (Kowalski *et al.* 2002), where ERβ is of supreme

importance mediating estrogenic action. Because of a ten-fold higher mRNA expression of ERα versus ERβ (Ulbrich *et al.* 2003) it can be proposed that in the bovine oviduct receptor-mediated actions may be mainly regulated through ERα. Nevertheless the occurrence of two subtypes during the estrous cycle points towards selective time and region specific effects. The hypothesis that both ER subtypes each contribute to different biological functions is supported (Mowa & Iwanaga 2000).

During the estrogen-dominated follicular phase a distinct upregulation of PR transcripts was measured in bovine oviduct epithelium (Fig.5A, App. p.43). Corresponding to this, *in vitro* data showed that estradiol-17β stimulated the expression of PR mRNA (Fig.5B, App. p.46). In contrast, progesterone stimulation resulted in a reduction of transcript numbers, indicating that the oviduct PR was suppressed during progesterone dominance (App. p. 46). 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 estrous cycle. Western blotting analysis of PR revealed three isoforms in the positive control of bovine endometrium (App. p.44). The bands were visible at approximately 116, 92 and 65 kDa molecular weight, corresponding to the known PR isoforms B, A and C, respectively (Haluska *et al.* 2002). 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. PR-B revealed more intense 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. In the isthmus, the PR-A isoform stained moderately during the early luteal phase. The most intense immunohistochemical staining was detected in cell nuclei epithelial cells and of longitudinal and circular muscle layers in early luteal phase oviducts (App. p.45). Intense nuclear staining of the muscular layer surrounding the oviduct provide some evidence for the importance of PR mediating motility (Ulbrich *et al.* 2003). Hunter *et al.* (1999) 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 (Wijayagunawardane *et al.* 1998), 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. Since PR-B was termed as an activator of transcription of progesterone sensitive genes (Vegeto *et al.* 1993) an upregulation of PR-B along the isthmus epithelium could indicate for functional active hormone-receptor complexes which may lead to controlled release of isthmus epithelial-bound sperm probably mediated through relaxation of surrounding oviduct muscular layers.

BOEC were found to be sensitive towards steroid stimulation indicating an analogous situation to *in vivo*. It could be demonstrated that the *in vitro* experiments gave evidence for the elucidation of regulatory mechanisms *in vivo* on the basis of circulating steroids (Fig.5A/B). Therefore, the BOEC culture could serve as a favourable easy and potent model to further study hormone regulations within this part of the female reproductive system.
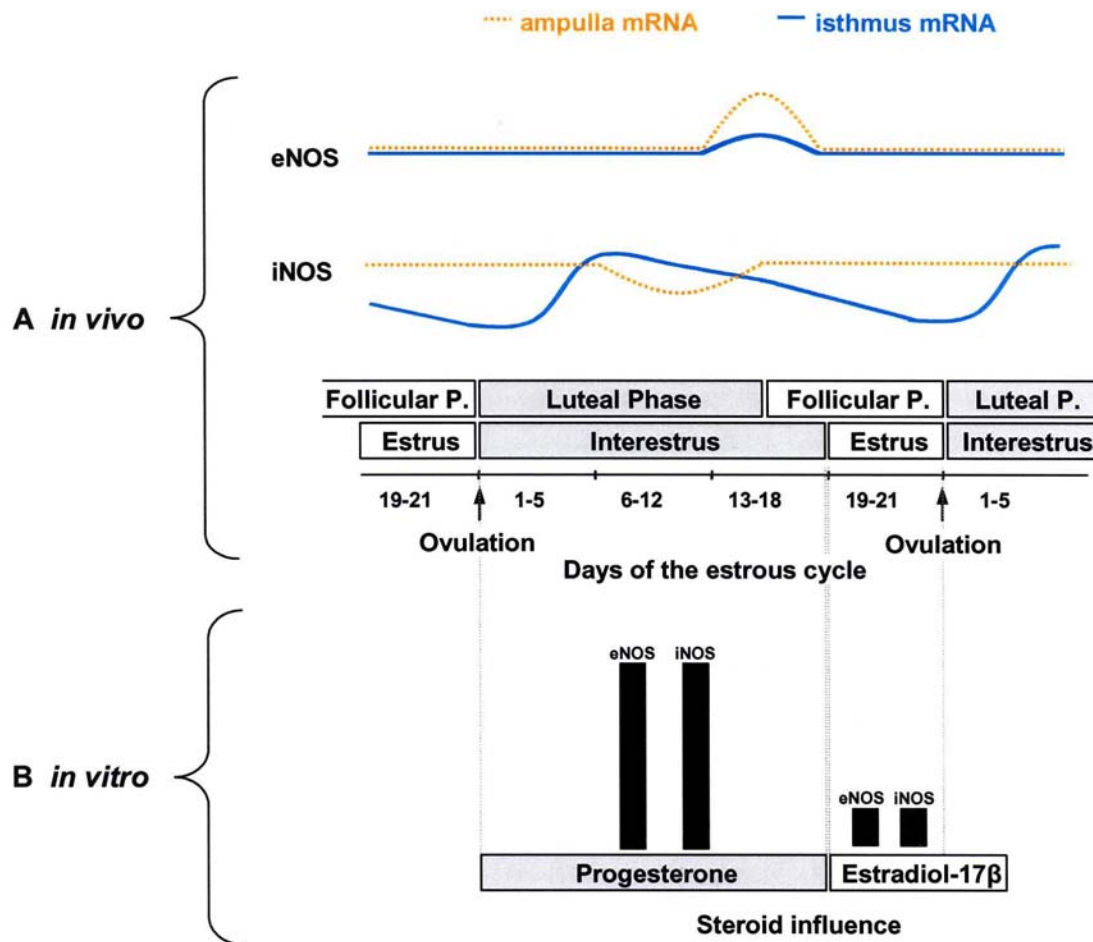

Fig.6: Summarized results of eNOS and iNOS mRNA expression (A) in vivo and (B) after progesterone and estradiol-17β stimulation in vitro (Ulbrich et al. 2005).

Transcripts of eNOS and iNOS were detected in bovine oviduct epithelial cells during the course of the estrous cycle (Ulbrich *et al*. 2005). Subsequently rising levels of eNOS during the estrous cycle (App. p.74) rather provide evidence for a dependency of eNOS on progesterone. This was further demonstrated by the stimulation of BOEC (Fig.6B, App. p.75). Highest transcript amounts for iNOS were detected in the ampulla, with retained high levels throughout the estrous cycle (Fig.6A, App. p.74). At days 0 and 18 there was a significant decrease in the isthmus compared to the ampulla, respectively. At day 3.5 the iNOS expression in the isthmus was again as high as in the ampulla. In agreement with other

studies (Welter *et al.* 2004) endogenous NOS protein targeted epithelial cells as well as cells of the muscle layer of the oviduct in different species.

It is known that the motility patterns of the oviduct show rising frequency and amplitude of motility around estrus (Bennett *et al.* 1988). Therefore, several mediators of contraction could be involved in this regulation influencing or orchestrating the NOS expression in the oviduct, namely estradiol, oxytocin, prostaglandin $F_{2\alpha}$, prostaglandin E_2 and endothelin-1 (Moore & Croxatto 1988; Gilbert *et al.* 1992; Perez *et al.* 1998; Salvemini *et al.* 1993; Rosselli *et al.* 1994). Perez *et al.* (2000) found evidence for increased tubal motility that resulted in accelerated ovum transport into the uterus. Moreover, estradiol treatment caused accelerated ovum transport by increasing the contraction frequency of smooth muscles of the isthmus (Moore & Croxatto 1988). This could be facilitated via ERβ, which is more abundant in the isthmus (Ulbrich *et al.* 2003). Therefore, the endogenous local downregulation of iNOS in the isthmus could persecute the same goal. Our hypothesis therefore is that the downregulation of iNOS at estrus in the isthmus may lead to an increase of oviduct motility by circular smooth muscles and/or ciliary activity (Ulbrich *et al.* 2005).

There is evidence that the accelerated movement of microspheres through the isthmus is due to peristaltic smooth muscle contractions and not due to ciliary activity, supported by the observation that there is a reduced number of ciliated cells in the isthmus (Perez *et al.* 1998). In secretory epithelial cells of the oviduct the nucleus is shifted towards the apical side of the epithelium. Therefore, both eNOS and iNOS protein targeted mostly secretory epithelial cells (App. p.76). NOS protein targeted the supranuclear region towards the oviduct lumen, therefore it can be assumed that NO is released into the lumen. It might be of further interest weather the produced NO is also secreted towards the stroma and myosalpinx. The contribution of NOS might be important for relaxation together with a local exertion during (pro-)estrus facilitating capture, retention and fertilization of the released oocyte and the active transport of the conceptus (Chatterjee *et al.* 1996).

Furthermore, NOS-derived NO has been made responsible for an increased ciliary beating frequency of bronchial airway epithelial cells after stimulation with cytokines (Jain *et al.* 1995). Moreover, through the ability of iNOS to produce large amounts of cytotoxic levels of NO as an inflammatory immune reaction (Guo *et al.* 1995), the downregulation of iNOS in the isthmus at estrous could be an implicit protective mechanismen for advancing sperm and the developing embryo. There is only little evidence for a positive rheotactic movement of sperm against the current of the oviduct fluid towards the uterus (Winet *et al.* 1984).

In this study members of the HA-system were characterized in the bovine oviduct during the estrous cycle (Ulbrich *et al.* 2004). Strong signals of HABP localizing HA were found in the

stroma of the oviduct villi as well as in connective tissue surrounding the oviduct (App. p.57). The relative intensity of HA appeared to be highest in the ampulla and most intense during the mid-luteal phase. The question therefore arose if the action of receptors and/or synthases were the reason for the observed variations. Salamonsen *et al.* (2001) proposed a steroid dependency of HA, and the oviduct fluid was found to contain varying HA concentrations during the estrous cycle (Anderson & Killian 1994). In the present study using bovine oviducts transcripts of HAS2 and HAS3 mRNAs were detected (App. p.55). Although without significant differences neither between ampulla and isthmus nor between the cycle stages, this result demonstrates the potential of the BOEC synthesizing HA.
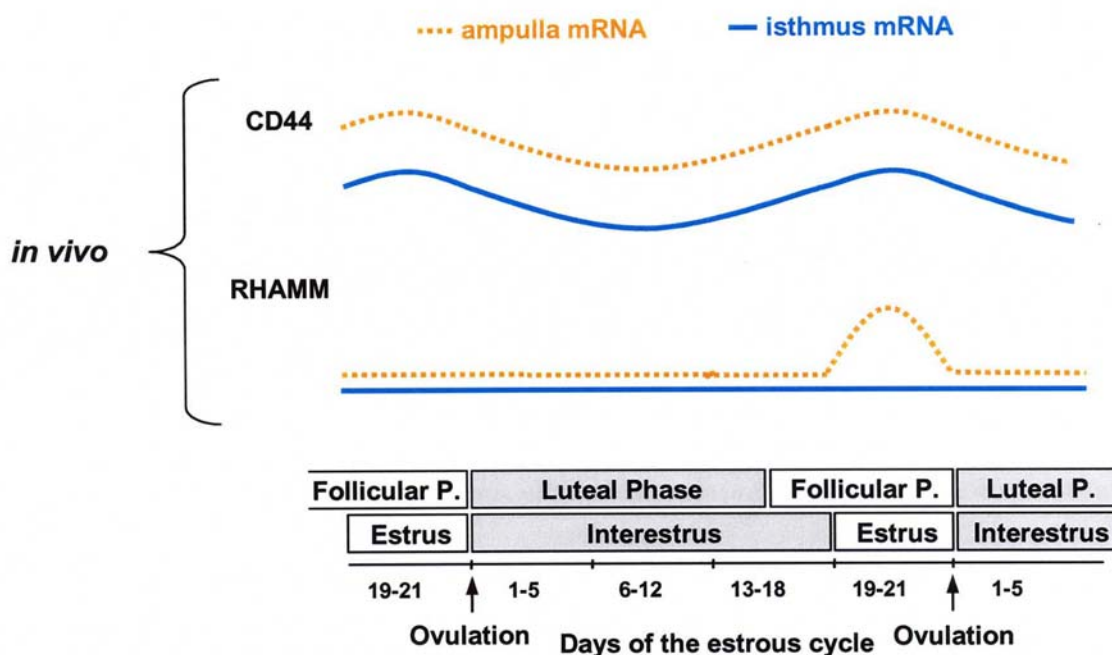

Fig.7: Summarized results of CD44 and RHAMM mRNA expression in vivo (Ulbrich et al. 2004).

RHAMM transcripts were moderately regulated with highest expression during the estrous phase in the ampulla (Fig.7, App. p.55). Since the epithelium of the ampulla is lined much more with ciliated cells than the isthmus especially at estrus, the RHAMM receptor seems to represent an interesting candidate for the local regulation of ciliary beating in this region. The influence of RHAMM in modulating the ciliary beating of airway epithelial cells has been shown before (Lieb *et al.* 2000). Since BOEC express RHAMM, a possible epithelial protein location could be suspected. If this was the case, a direct interaction between HA from the oviductal fluid or as well from the cumulus cells and RHAMM lining the oviduct epithelium could possibly occur.

The CD44 receptor protein was localized to the lamina propria of oviduct villi as well as the transversal and longitudinal muscle layer surrounding the oviduct (Fig.7, App. p.57) throughout the whole estrous cycle with a tremendous difference regarding the location. The mRNA expression was found more than 10-fold higher in the isthmus than in the ampulla (App. p.55). Interestingly, the histological observation of HA pointed towards intense luteal HA staining predominantly in the ampulla controversial to highest CD44 expression at estrus, the latter being most pronounced in the isthmus. Through the ability to bind HA, CD44 is capable of assembling the pericellular matrices and internalizing it (Jiang *et al.* 2002) (Fig.4). The internalization is a multistep process possibly triggered by its molecular weight (Aguiar *et al.* 1999) in which HA is first bound to the cell surface, then internalized, brought into lysosomal compartments and degraded (Underhill 1992). The observed countercurrent regulation in this study could be explained by CD44 mediated internalization of HA as supposed for osteoprogenitor cells (Pavasant *et al.* 1994). Yet concurrent expression patterns have been found for CD44 in the epithelium and HA in the intraluminal fluid (Tienthai *et al.* 2000; Tienthai *et al.* 2003). In the present study it remains difficult to favor a direct interaction between gametes and the oviduct concerning the components of the ECM. The oviduct luminal epithelium is devoid of either HA or CD44 as shown by immunohistochemistry (App. p.57) and we could not confirm recently reported epithelial HA-labeling present in the porcine uterotubal junction (Tienthai *et al.* 2003). Since BOEC express CD44 mRNA either protein expression levels were below detection limit or mRNA was not translated. In the human a direct interaction between CD44 expressed in lateral membranes of the uterine epithelium and proteoglycans expressed on the embryo is doubted as well (Albers *et al.* 1995). Nevertheless the importance of an indirect interaction via the stroma cells can strongly be suggested. Finally, because CD44 additionally binds to fibronectin, collagen and sulphated proteoglycans, the muscular CD44 staining lacking HA staining could be explained.

After selecting cross-species homologues primers, putative HARE could be detected by RT-PCR in the bovine oviduct for the first time (Ulbrich *et al.* 2004). Screening of a complex bovine liver cDNA library enabled the isolation and sequencing of an open reading frame of 1500 bp. The new bovine sequence showed high similarity (86 and 80%) to the published sequences of human and rat, respectively. As demonstrated using semiquantitative RT-PCR analysis the expression of putative HARE varied in different bovine tissues with highest expression in liver, spleen and lymph node (App. p.56). In reproductive tissues the signals for putative HARE transcripts in endometrium and testis were almost equally intense as in liver and demand for further notice. Follicle, oviduct and corpus luteum expressed putative HARE to a much lesser extend (App. p.56). In the bovine oviduct the mRNA expression of putative HARE altered only minorly during the estrous cycle (App. p.55). We can therefore conclude

that the concentration of HA in the oviduct might not be directly correlated with this novel HA-receptor.

In a final approach the effect of HA on BOEC was shown to lead to a tremendous increase of iNOS transcripts *in vitro* (Fig.8). The induction of NOS can be modulated by a variety of growth factors and steroids, depending on the cell type (Sessa 1994). Furthermore, there is evidence that during inflammation HA can exert a stimulating effect on chemokine gene expression (McKee *et al.* 1996). Probable different responses regarding the molecular weight of HA should be taken into account. In this study, HA of high molecular weight ($1.69*10^6$) caused a proinflammatory reaction as seen by the stimulation of iNOS, which is striking, because during inflammation lower molecular weight fragments accumulate (McKee *et al.* 1996). But indeed, the application of exogenous, high molecular HA has been shown to advance bovine IVM (Stojkovic *et al.* 2002). The exact mechanism of action yet has to be determined. It is well-known that the size of HA depends on the present HAS isoform (Spicer *et al.* 1997). As known the oocyte expresses mainly HAS2 (Schoenfelder & Einspanier 2003) which is supposed to synthezise a high molecular weight HA, whereas BOEC express predominantly HAS3. Therefore further studies should be conduced to unravel these uncertainties.
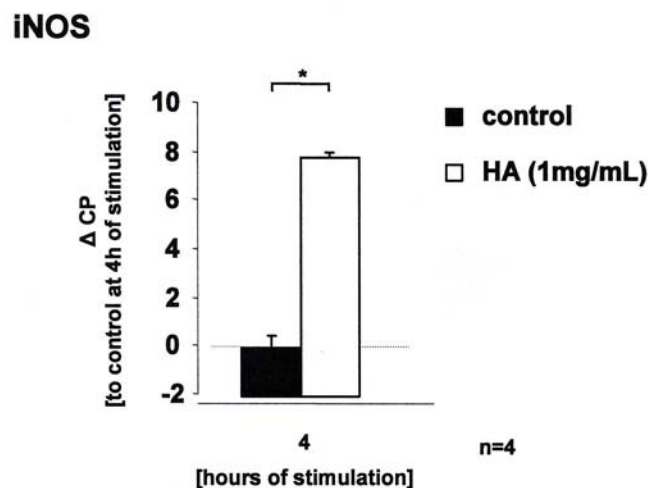

Fig. 8: mRNA expression of iNOS transcripts in a BOEC suspension culture after stimulation with hyaluronan (HA). Data are presented as relative expression of Crossing Points (CP) ± SEM (n=4). Control is set 0 (28.4 CP). One ΔCP signifies a doubling of mRNA.

Nevertheless the following is hypothesized: HA is more pronounced in the ampulla and can therefore exert a stimulating effect on iNOS, triggering a quiescence of oviduct motility especially during estrous in the ampulla. Concomitantly CD44 is more abundantly expressed in the isthmus, leading through binding and internalization of HA to low levels of HA. This

then in turn leads to a local decrease of iNOS in the isthmus at estrus, necessary either for local contractions, cilliary beating or an inhibition of inflammatory immune response towards the gametes. This is supported by estrogens through ERβ as well as RHAMM in the ampulla influencing cilliary beating.

The present work does not account for further occurring regulations of enzymes, which go beyond the analyses of gene and protein expression, namely the differences in enzyme activity of HAS or of both NOS isoforms (Presta *et al.* 1997). Nevertheless the investigation on hand (summarized in Fig. 9) provides important new data for further functional analyses on local regulating mechanisms in the oviduct towards reproductive support.
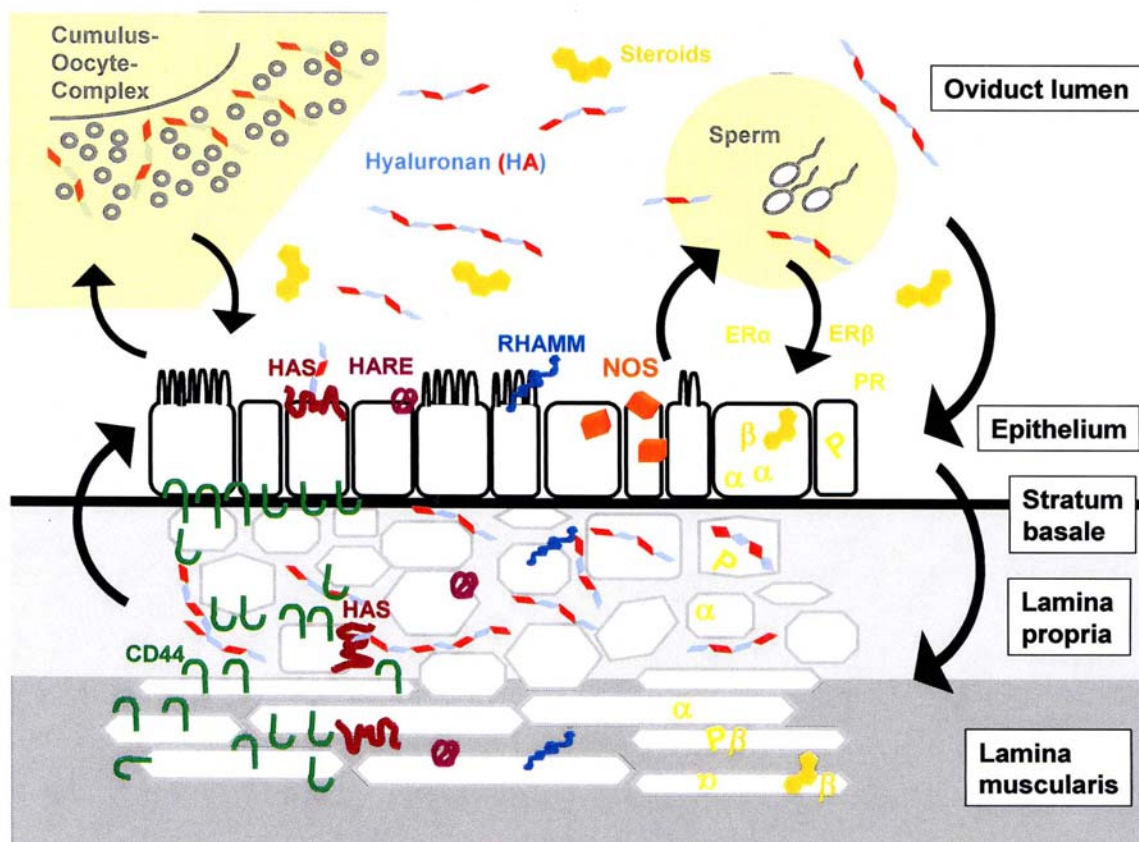

Fig.9: Concluding scheme showing steroid hormone receptors, nitric oxide synthases and hyaluronan turnover in the bovine oviduct. The oviduct is sensitive towards the actions of steroid hormones through the regulation of steroid hormone receptors. The effects of steroid hormones include the synthesis of specific proteins important for the passing gametes (sperm or the cumulus-oocyte-complex). From the oviduct lumen HA acts on target molecules in the epithelium (e.g. NOS) which in turn exert an action either directly towards the gametes or indirectly e.g. via the lamina muscularis (e.g. through regulation of motility).

CONCLUSIONS

In the bovine oviduct obvious cyclic changes of steroid hormone receptor expression *in vivo* were observed and concurrent expression patterns were detected *in vitro*. Taken together, the region specific regulation of the steroid receptors show that the oviduct is differentially sensitive towards the action of steroids. Moreover, possible periods and segments in which the circulating peripheral or local hormones are of special importance can now be addressed. Functional implications include an involvement in distinct transcriptional regulation of steroid sensitive genes. The establishment of a cell culture system that responded to steroid stimulation proved this an adequate *in vitro* model to further investigate the effects of various stimulants.

The results on hand gave evidence for the presence of NOS derived NO in the bovine oviduct. Moreover, a functional HA-system was described thoroughly, indicating a local turnover of HA. Possible regulatory roles for both include the involvement in gene expression and cellular function. Specifically local contractions or relaxation, cilliary beating of epithelial cells or a participation in the regulation of inflammatory immune response of the oviduct epithelium towards gametes might be affected.

To validate the proposed regulatory roles following approaches should include functional assays to review their implications on gamete development and reproductive success. Further investigations should address the question of gamete-induced regulation of oviduct function next to endocrine and paracrine influences. A co-culture system in which the oviduct epithelial cells come into direct contact with gametes could specifically approach detailed questions of this crosstalk.

Considering the increasing relevance of manipulation of fertilization in veterinary and also human reproduction, there is a demanding need for the understanding of the basic scientific mechanisms underlying the outlined processes. This may be beneficial for a reduction of embryonic losses. But additionally, not only for ethical reasons, dealing with early embryonic life definitely requires the most possible detailed knowledge of reproductive biology to prevent precipitated applications resulting in serious phenomenons e.g. the large calf syndrom.

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List of sequence submissions to the EMBL database

Acc.-No. AJ557823   Bos taurus partial mRNA for progesterone receptor (pr gene)

Acc.-No. AJ699400   Bos taurus partial mRNA for inducible nitric oxid synthase (iNOS gene)

Acc.-No. AJ550060   Bos taurus partial mRNA for putative hyaluronan receptor for endocytosis (hare gene)

Acc.-No. AJ586431   Bos taurus partial mRNA for heat shock 70kDa protein 5 (grp78 gene)

Acc.-No. AJ586432   Bos taurus partial mRNA for anterior gradient 2 homologue (agr2 gene)

Acc.-No. AJ586433   Bos taurus partial mRNA for ms4A8B protein

Acc.-No. AJ586434   Bos taurus partial mRNA for complement component 3 (c3 gene)

Acc.-No. AJ586435   Bos taurus partial mRNA for protein disulfide isomerase related protein (erp70 gene)

Acc.-Nos. CK280101 to CK280171: 71 EST genes from the Bovine SSH library oviduct epithelium either oestrus or dioestrus

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APPENDIX

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.
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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

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) denaturing 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

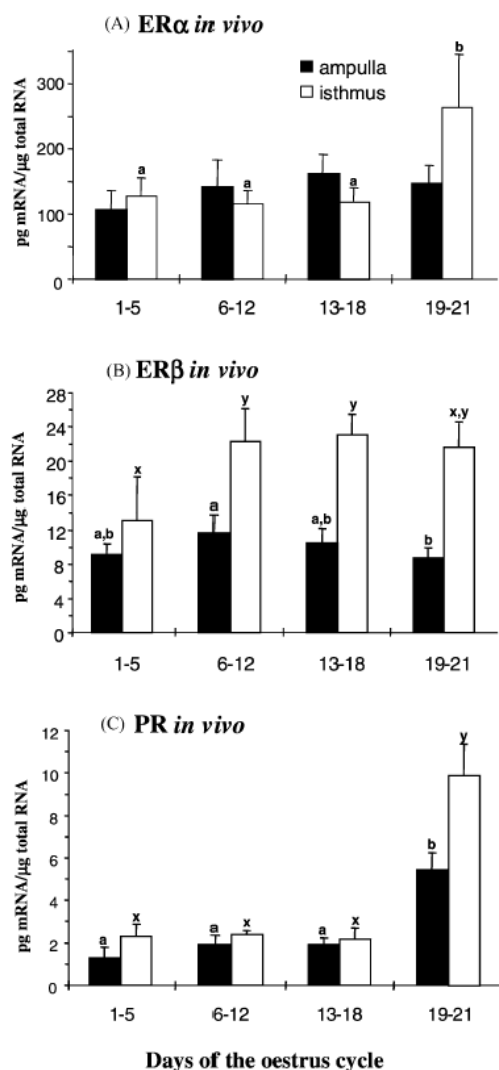In bovine oviduct epithelial cells mRNA transcripts of ERα, ERβ and PR were detected. Obviously, expressions

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
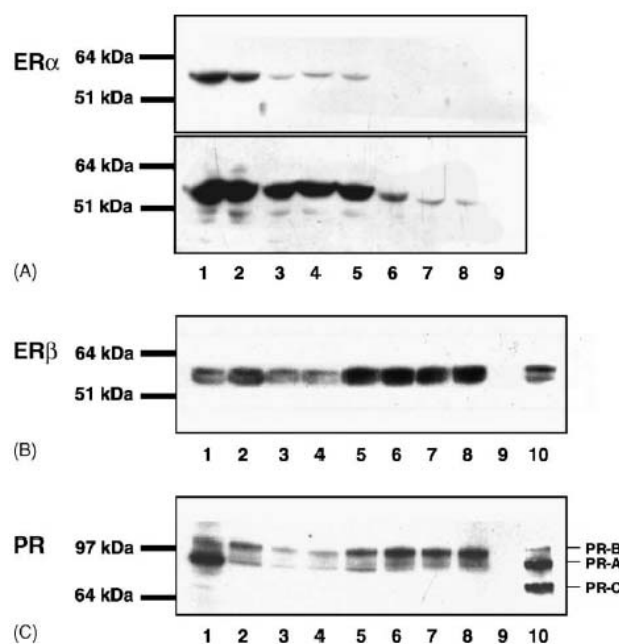
Fig. 2. Western blot analysis of ERα, ERβ and PR during the oestrus cycle. For ERα (A), the membrane was exposed to the X-ray film for varying times (upper part 1 min exposure, lower part 10 min exposure). (A) Lane 1, bovine endometrium; lane 2, ampulla early-luteal phase days 1–5; lane 3, ampulla luteal 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.

Acknowledgements

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Hyaluronan in the bovine oviduct—modulation of synthases and receptors during the estrous cycle

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Abstract

The extracellular matrix (ECM) component hyaluronan (HA) is considered to contribute to the optimal development of the gametes and the embryo. In this study, the regulation and localization of HA, its synthases (HAS 1–3) and the receptors CD44, RHAMM, and putative HARE were investigated in bovine oviducts during the estrous cycle. HA could be demonstrated in the entire lamina propria of the oviduct. Immunoreactive CD44 was found in the muscle layer additionally to the lamina propria. As for HA the luminal epithelium was devoid of CD44. Introducing quantitative real-time RT–PCR, a remarkable cyclic change of CD44 occurred. In addition, CD44 expression was much higher in the isthmus than in the ampulla. RHAMM transcripts were elevated particularly in the ampulla during estrus. A major part of the bovine putative HARE coding sequence was characterized. An analysis of the tissue distribution revealed transcripts not restricted to liver, spleen, and lymph node, providing evidence for a local putative HARE-mediated turnover of HA. These results suggest that in the bovine oviduct the cell surface receptor CD44 in particular might inversely regulate HA during the estrous cycle. The local turnover of HA through synthases and receptors may indicate its participation in the bovine oviduct supporting gamete maturation and early embryonic development.
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Keywords: Bovine oviduct; Hyaluronan; HAS; CD44; RHAMM; HARE

1. Introduction

The oviduct is the site of final gamete maturation and early embryonic development. During this passage local interactions are of significant importance for fertilization and subsequent embryogenesis. Recently, the conducive role of the oviduct towards sperm competence has been shown (Gualtieri and Talevi, 2003). In addition, the maturing cumulus–oocyte complex is considered to interact with the oviduct. Concerning cell–cell contacts, components of the extracellular matrix (ECM) may play important roles. Hyaluronan (HA) is a major component of the ECM and has been demonstrated in the oviductal fluid (Tienthai et al., 2000). In human and porcine oviducts hyaluronan has been localized to the entire lamina propria and surrounding connective tissues (Edelstam et al., 1991; Tienthai et al., 2000). HA forms linear polymers of non-sulphated glucosaminoglycans (GAG) of high-molecular weight (10^4–10^7 Da). It is the only GAG not generally linked to a core protein and due to its highly negative charge it is able to expand dramatically by hydratization. Its biological functions have primarily been associated with matrix structure and plasma protein distribution. More recently, HA has been considered as an important regulator of cell behavior beyond simply being a structural component (Fraser et al., 1997). It has been linked to local cell proliferation and angiogenesis, inflammation, cell recognition, and migration (Laurent and Fraser, 1992).

Exclusively synthesized by HA synthases (HAS) which are located at the plasma membrane (Prehm, 1984), the expanding GAG is extruded through the membrane into the ECM (Weigel et al., 1997). Three highly conserved genes encoding HAS 1, HAS 2, and HAS 3 have been demonstrated in human and mouse (Spicer et al., 1997), each representing differentially expressed HAS isoforms and being therefore functionally disparate. Moreover, it has been shown that the size of HA depends on the present HAS isoform. HA

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synthesis increases specifically during mitosis in actively dividing cells and in particular during in vitro maturation of cumulus–oocyte complexes (Tammi and Tammi, 1991; Schoenfelder and Einspanier, 2003). Several growth factors and cytokines have been demonstrated to trigger either one or more HAS in a tissue and cell specific manner (Kuroda et al., 2001).

Through specific cell surface receptors HA is known to contribute to cellular signaling (Salamonsen et al., 2001). CD44 is suggested to represent the principal cell surface receptor for HA (Aruffo et al., 1990). It belongs to a larger group of hyaluronan-binding proteins (HABPs) termed hyal-adherins. These are considered to be of importance for different events including cell–cell and cell–ECM interactions as well as cell proliferation, adhesion and migration (Lesley et al., 1993). Additionally, CD44 has been implicated in the binding and the presentation of growth factors (Behzad et al., 1994). The binding of HA to the cell surface is a multivalent interaction, since several CD44 molecules are able to bind to the same HA molecule (Underhill, 1992), resulting in an increasing binding affinity. Through the ability to bind HA, CD44 is capable of assembling pericellular matrices as well as internalizing HA (Jiang et al., 2002).

A second receptor for HA-mediated motility RHAMM has been described controversially, either as cell surface receptor for HA with occasional intracellular expression (Hall and Turley, 1995) or, more recently, as an exclusively intracellular protein interacting with microtubules and micro-filaments (Hofmann et al., 1998). Anyhow, the binding of HA to RHAMM is undoubtfully consistent in several reports stimulating cell migration and locomotion via an activation of a signal transduction cascade upon HA binding. Yet, since there is discordance about the cellular localization of RHAMM, a possible alternative HA synthesizing pathway is questioned (Assmann et al., 1999). Concerning reproductive tissues, several reports describe the RHAMM-mediated promotion of cell growth and movement, sperm motility, angiogenesis, and embryonic development (Kornovski et al., 1994; Savani et al., 2001; Stojkovic et al., 2003).

Recently, a third HA receptor has been purified from rat liver, the hyaluronan receptor for endocytosis HARE, characterized by Zhou et al. (2000). The ability of HARE to internalize HA by endocytosis via the clathrin-coated pit pathway was demonstrated in liver sinusoids. Therefore, a responsibility for the clearance of systemic, circulating HA was suggested. Since HARE was found not to be restricted to the liver, a general survey of the bovine tissue distribution of putative HARE was undertaken in the present analysis using RT–PCR.

This study was performed to investigate the local modulation of the HA-system in the bovine oviduct during the normal estrous cycle. A focus was layed on the regulation of synthesizing enzymes and binding receptors using a quantitative real-time RT–PCR approach in addition to the immunolocalization of HA and the predominant receptor CD44. Towards analyzing bovine putative HARE, initial

sequence data were approached. The characterization of selected components of a functional HA-system was aimed to outline their potential contribution to the early reproductive events.

2. Material and methods

2.1. Total RNA extraction and mRNA analysis

Bovine oviducts from healthy cows were collected from the local slaughterhouse within 20 min post-mortem and placed on ice until further treatment. They were grouped depending on the cycle stage referring to Ireland et al. (1980) by examining the color, consistency, number, size of follicles, and corpora lutea: early luteal phase (days 1–5), mid-luteal phase (days 6–12), late luteal phase (days 13–18), and estrous phase (days 19–21). Surrounding tissue was carefully removed from the oviducts. For RNA extraction, about 100–200 mg of oviductal segments were taken distinguishing the ampulla from the isthmus. For examination of HARE in different tissues, 100–200 mg of wet weight fresh tissue was used. Total RNA was extracted as described by Chomczynski and Sacchi (1987) using Trizol reagent (Invitrogen, Karlsruhe, Germany). In a second step the nucleic acids were bound to a spin column and treated with DNAse (RNA-Extraction Kit, Macherey-Nagel, Düren, Germany) to exclude DNA contamination. The elution volume was 50 μl in DEPC treated water, and aliquots were spectroscopically quantified at 260 nm. The integrity of the RNA was determined by the ration of the absorption of 260–280 nm. One microgram of each sample of RNA was reverse transcribed in a total volume of 60 μl: 5× Buffer (Promega), 10 mM dNTPs (Roche, Mannheim, Germany), 50 μM hexameres (Gibco-BRL, Grand Island, USA), 200 U Superscript RT enzyme (Promega). PCR primers were introduced to detect 18S, CD44, HAS 2, HAS 3, RHAMM as depicted in Table 1. To detect putative HARE, new primers were designed referring to the bovine sequences of Gen-Bank accession no. AJ550060. The conventional PCR were performed in a thermal cycler (Biometra, Göttingen, Germany) as previously described (Berisha et al., 2002). Seven microliters of each reaction was subsequently subjected to an agarose gel electrophoresis followed by ethidium bromide staining. All amplified PCR fragments were commercially sequenced to specify the resulting PCR-product (MWG-Biotech, Ebersberg, Germany). The specific melting point of the amplified product carried out within the LightCycler® standard PCR protocol served as verification of the product identity. For each of the following real-time PCR reactions, 1 μl of cDNA was used to amplify specific target genes. Quantitative real-time PCR reactions using the LightCycler® DNA Master SYBR Green I protocol (Roche) were performed as described previously (Pfaffl et al., 2001). Annealing temperatures (AT) and fluorescence acquisition (FA) points for quantification within the fourth

Table 1
Primer sequences and real-time PCR parameters used for the quantification of gene expression

	PCR-product (bp)	Annealing temperature (°C)	Fluorescence acquisition temperature (°C)	Primer sequence	EMBL accession no.
18S	488	60	60	Forward: 5′-AAG TCT TTG GGT TCC GGG-3′ Reverse: 5′-GGA CAT CTA AGG GCA TCA CA-3′	AF176811
HAS 2	144	68	80	Forward: 5′-GGM TGT GTC CAG TGC ATT AGC GGA-3′ Reverse: 5′-CAG CAC TCG GTT CGT TAG RTG CCT-3′	U54804
HAS 3	166	60	84	Forward: 5′-ACA GGT TTC TTC CCC TTC TTC C-3′ Reverse: 5′-GCG ACA TGA AGA TCA TCT CTG C-3′	AJ293889
CD44	221	64	83	Forward: 5′-TAT AAC CTG CCG ATA TGC AGG-3′ Reverse: 5′-CAG CAC AGA TGG AAT TGG G-3′	X62881
RHAMM	249	61	77	Forward: 5′-TGT TGA ATG AAC ATG GTG CAG CTC-3′ Reverse: 5′-CCT TAG AAG GGT CAA AGT GTT TGA-3′	AF310973
Putative HARE	245	62	84	Forward: 5′-ATC ACT GAC TCC ATC CAC ACC C-3′ Reverse: 5′-GGT GTG GAA CTG GCA GTG ACA T-3′	AJ550060

step of the amplification segment are outlined in Table 1. In each PCR reaction 17 ng/μl cDNA were introduced and 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, Roche) compared to a standard curve based on a specific PCR-product. Full-length cDNA sequencing of putative HARE was performed using the SMART-RACE cDNA kit (BD Bioscience Clontech, Palo Alto, USA) introducing total RNA of bovine liver. One microgram of RNA was used for the first strand synthesis of each 5′- and 3′-direction with MMLV-RT enzyme (Promega). Two cDNA libraries were subsequently used for the amplification of specific PCR-products in a touch-down PCR and resulting PCR-products were sequenced commercially (Medigenomix, Martinsried, Germany). The species alignment of the partial putative HARE sequences was done using DNAsis (Version 3.5 Pro).

2.2. Statistical analysis

All data were analyzed by one-way 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 Version 2.03).

2.3. Immunohistochemistry

For histological examination pieces of the oviducts were directly shock-frozen in liquid nitrogen and stored at −80 °C for further investigation. The following procedure of histochemistry has been described elsewhere (Ulbrich et al., 2003). Briefly, about 0.5 cm of each oviduct was embedded in tissue-tek. Serial cross-sections of 7 μm were cut on a cryostat at −27 °C (CM 1850, Leica, Bensheim, Germany), mounted on Poly-L-Lysine coated slides and air-dried. The sections were fixed in either 3.7% paraformaldehyd or in

100% EtOH (−20 °C). The importance of the fixation procedure in preserving HA was taken into account (Lin et al., 1997). For the immunohistochemical procedures, endogenous peroxidases were inhibited by treatment of 1% hydrogen peroxide for 30 min. Non-specific binding was inhibited by incubation of 10% normal goat serum for 1 h. The primary antibody (monoclonal rat-anti-porcine CD44 immunoglobulin IgG1, 1:100 dilution, DPC Biermann, Bad Nauheim, Germany) was allowed to incubate over night at 4 °C in a humidified chamber. After washing off unbound primary antibody, the sections were incubated for 60 min at room temperature with horseradish peroxidase-labeled anti-rat secondary antibody for detection of CD44 (goat-anti-rat IgG, 1:200 dilution, DPC Biermann, Bad Nauheim, Germany). The peroxidase was visualized by applying 0.01% 3,3′-diaminobenzidine hydrochloride (Sigma, Deisenhofen, Germany) and 0.01% hydrogen peroxide in PBS-buffer for 15 min in darkness. The sections were counterstained in Mayer's Haemalaun (Merck, Darmstadt, Germany), dehydrated and mounted on slides for light microscopy (Axioscope, Zeiss, Göttingen, Germany). The specifity of the antibody has been shown by Schoenfelder and Einspanier (2003). In addition, leukocytes from bovine whole blood served as a positive control. The specific immunoreaction was verified by omitting either primary or secondary antibody or incubating with DAB alone. In addition, an isotype matched rat IgG1 (1:100 dilution, Beckman Coulter, Krefeld, Germany) was applied instead of the primary antibody to demonstrate the specificity of the antibody.

For the HA detection a biotinylated hyaluronan acid binding protein from bovine nasal cartilage was used (Calbiochem, Darmstadt, Germany) binding specifically to HA. After fixation the sections were incubated with 5 ng/ml HABP (Calbiochem, San Diego, USA) for 30 min. Streptavidin–fluorescein conjugate (1 μg/ml, Calbiochem, Darmstadt, Germany) or HRP-labeled streptavidin (1 μg/ml, Roche) was used (30 min incubation) for the detection of

biotinylated HABP. For fluorescence microscopy (Axio-scope, Zeiss, Göttingen, Germany) the sections were counterstained with propidium iodid and mounted in anti-fading solution vecta-shield (Lucernachem, Lucerne, Switzerland). Negative controls were performed by excluding either HABP, strepavidin-fluorescein conjugate or HRP-labeled strepavidin, respectively.

Tissue slices of eight oviducts referring to each cycle stage and region were mounted together on a single slide. Two sets of embedded oviducts from 16 different cycling cows were taken under investigation. The antibody staining was reproduced at least twice.

3. Results

A first screening of the components of the HA-system HAS 1, HAS 2, HAS 3, CD44, RHAMM, and putative HARE in bovine oviduct total RNA using conventional RT–PCR led to different amplification signal intensities as shown in Fig. 1. No amplification results were obtained for HAS 1 (bovine follicular cells served as positive controls), so this target gene was not considered in further observations (data not shown). To further characterize these preliminary results a real-time RT–PCR approach was applied to quantify the gene expression patterns of these compounds in oviductal tissue.

Since the expression of the housekeeping gene 18S-ribosomale RNA was balanced (Figs. 1 and 2A) and revealed no significant differences between the groups, a standardization of the gene specific data generated from the real-time specific approach was not necessary.

The mRNA of both HA synthases HAS 2 and HAS 3 were expressed in the bovine oviduct during the estrous cycle.

Significant differences of both synthases could neither be detected between ampulla and isthmus nor between the cycle stages (Fig. 2B and C). Expression levels ranged from 1 to 75 fg mRNA per μg total RNA. Although no gene regulation was detectable because of high standard errors, individual fluctuations were remarkable. The coefficient of correlation of HAS 2 versus HAS 3 was calculated as 0.87.

CD44 was expressed in the bovine oviduct throughout the whole estrous cycle. The mRNA expression of CD44 was found more than 10-fold higher in the isthmus than in the ampulla. Transcript levels changed during the estrous cycle following an equal distribution in both regions of the oviduct (Fig. 3A). Lowest expression signals were measured during the mid-luteal (days 6–12) and the late luteal phase (days 13–18) (9.3 respectively 7.1 fg mRNA per μg total RNA in the ampulla), the latter being significant different from higher levels during the estrous (days 19–21) (15.5 fg mRNA per μg total RNA in the ampulla) (Fig. 3A). In the isthmus the mRNA expression ranged from 108.8 fg mRNA per μg total RNA during the late luteal phase to 277.8 fg mRNA per μg total RNA during the estrous phase.

Transripts of RHAMM were detected in the bovine oviduct as follows: RHAMM transcripts were highest during the estrous phase in the ampulla (days 19–21) (55.0 fg mRNA per μg total RNA), whereas the up-regulation was moderate in the isthmus (Fig. 3B). During the mid-luteal phase (days 6–12) the mRNA expression was lowest in the isthmus (13.9 fg mRNA per μg total RNA).

After selecting cross-species homologues primers, putative HARE could be detected by RT–PCR in the bovine oviduct for the first time. Screening of a complex bovine liver cDNA library enabled the isolation and sequencing of an open reading frame of 1500 bp, which was subsequently submitted to the EMBL database (GenBank accession no.

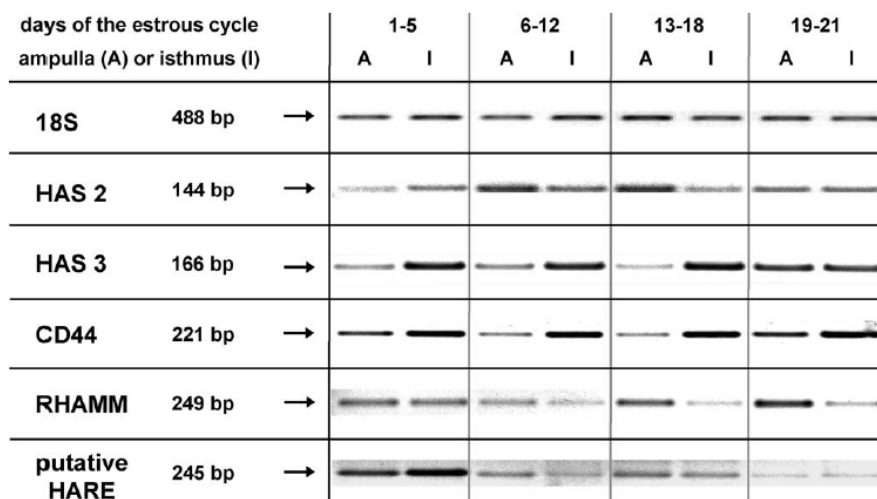| days of the estrous cycle | | 1-5 | | 6-12 | | 13-18 | | 19-21 | |
ampulla (A) or isthmus (I)		A	I	A	I	A	I	A	I
18S	488 bp →								
HAS 2	144 bp →								
HAS 3	166 bp →								
CD44	221 bp →								
RHAMM	249 bp →								
putative HARE	245 bp →								

Fig. 1. mRNA transcripts of selected genes in the bovine oviduct during the estrous cycle exemplified by conventional RT–PCR. One representative out of five independent experiments is shown. The housekeeping gene 18S and the target genes HAS 2, HAS 3, CD44, RHAMM, and putative HARE are shown.
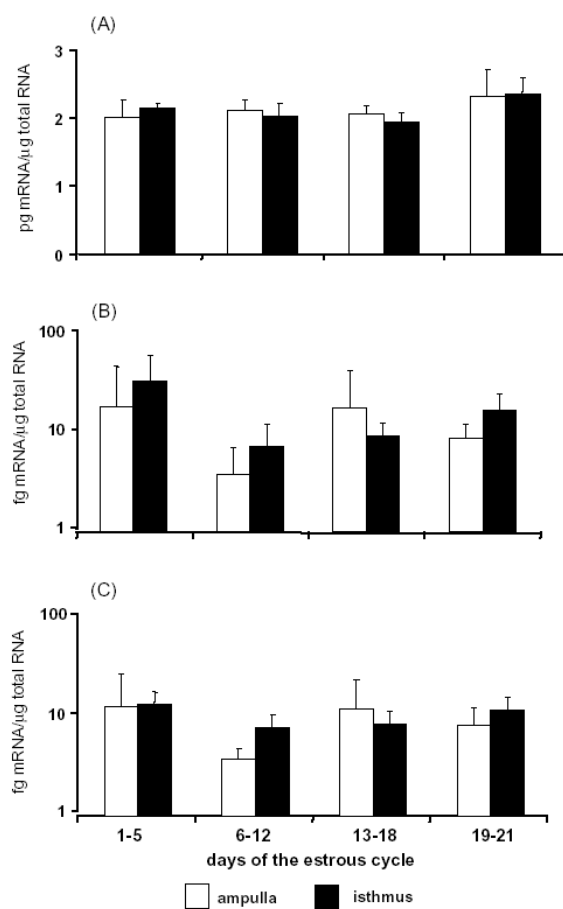
Fig. 2. 18S (A), HAS 2 (B), and HAS 3 (C) mRNA concentrations (real-time RT–PCR) in bovine oviduct epithelial cells during the estrous cycle early luteal phase (days 1–5), mid-luteal phase (days 6–12), late luteal phase (days 13–18), follicular phase (days 19–21) in either ampulla (□) or isthmus (■). Results are depicted as means of mRNA per total RNA ± S.E.M. ($n = 5$). Differences between groups were not significant.

Fig. 3. CD44 (A), RHAMM (B) and putative HARE (C) mRNA concentrations (real-time RT–PCR) in bovine oviduct epithelial cells during the estrous 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 are depicted as means of mRNA per total RNA ± S.E.M. ($n = 5$). Concerning different cycle stages and regions for each factor, values with different superscripts differ significantly ($P < 0,05$).

AJ550060). The sequence showed high similarity to the published human and rat HARE sequence, but also to the human and mouse stabilin-2 sequence as shown in Table 2. The expression of putative HARE varied in different bovine tissues (Fig. 4). Semiquantitative analysis revealed highest expression in liver, spleen, and lymph node, and minor expression in lung and kidney. In reproductive tissues the signals for putative HARE transcripts in endometrium and testis were almost equally intense as in liver. Follicle, oviduct, and corpus luteum expressed putative HARE to a much lesser extend. In heart muscle, hypothalamus, and placenta samples no specific signals could be detected. In the bovine oviduct the mRNA expression of putative HARE altered only during the early luteal phase in the isthmus (9.6 fg mRNA per μg total RNA) compared to mid-luteal and late luteal phase in this particular region (2.3 respectively 2.1 fg mRNA per μg total RNA) (Fig. 3C).

The localization of HA was realized using biotinylated HABP and fluorescence microscopy of FITC-bound

Table 2
Homology of a long fragment of the bovine putative HARE cDNA sequence compared to human and rat HARE as well as human and mouse stabilin-2 gene

	Bovine putative HARE (%) accession no. AJ55006
Human HARE (AY227444)	86
Rat HARE (AY007370)	80
Human stabilin-2 (NM017564)	86
Mouse stabilin-2 (NM138673)	79

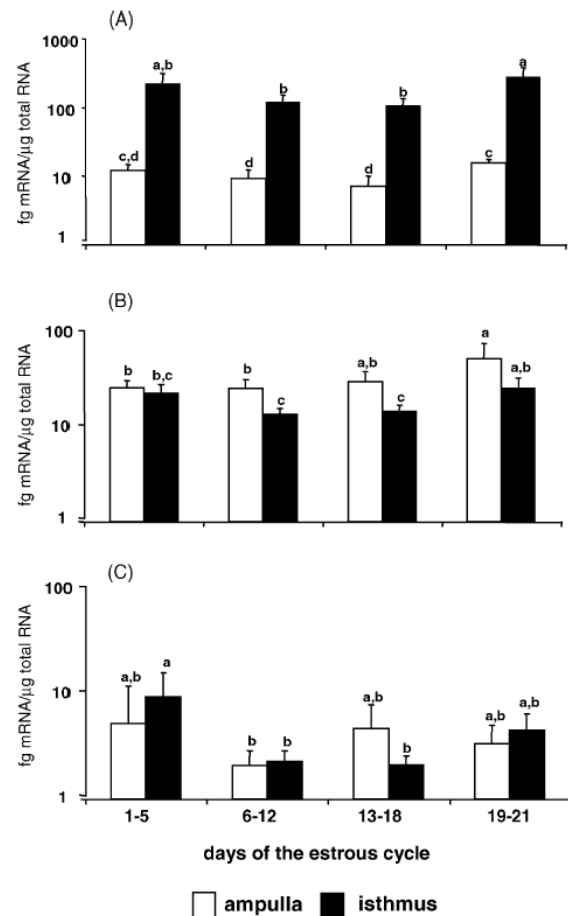The GenBank accession numbers are indicated in parentheses.

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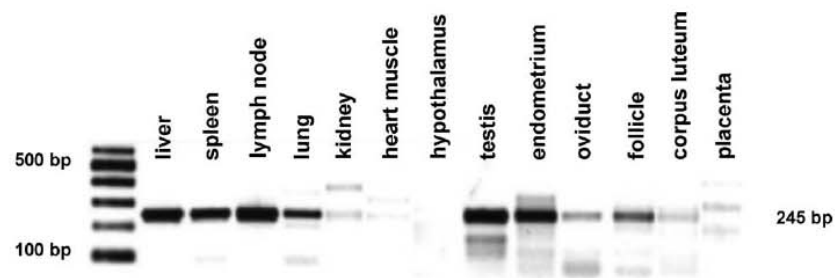

Fig. 4. RT–PCR representing putative HARE mRNA expression in different bovine tissues as indicated. For every tissue one out of three independent experiments is shown.

strepavidin (Fig. 5A and B). Strong signals of HABP were localized to the stroma of the oviductal villi (Fig. 5A and D) as well as to the connective tissue surrounding the oviduct (Fig. 5A and C). Neither the muscle cell layer nor the luminal epithelial cell layer revealed signals of HABP as indicated in Fig. 5A–C. Negative controls as shown in Fig. 5C and D insert showed no staining at all. The relative intensity of the HABP staining appeared to be greater in the ampulla than in the isthmus. Additionally, it seemed that the staining was most intense during early luteal and mid-luteal phases in both oviduct regions investigated (data not shown).

The CD44 receptor protein was localized to the lamina propria of oviductal villi (Fig. 5F). In the lamina propria as well as the transversal and longitudinal muscle layer surrounding the oviduct a positive staining was detected during all cycle stages (Fig. 5E). In both regions the staining seemed equally intense. Occasionally, the basal lamina underlying the luminal epithelial cells was stained indicating the presence of the receptor protein in this area (Fig. 5G). There was no evidence of epithelial staining in any of the slices observed (Fig. 5F). The negative controls as exemplified in Fig. 5F and H showed no specific staining at all.
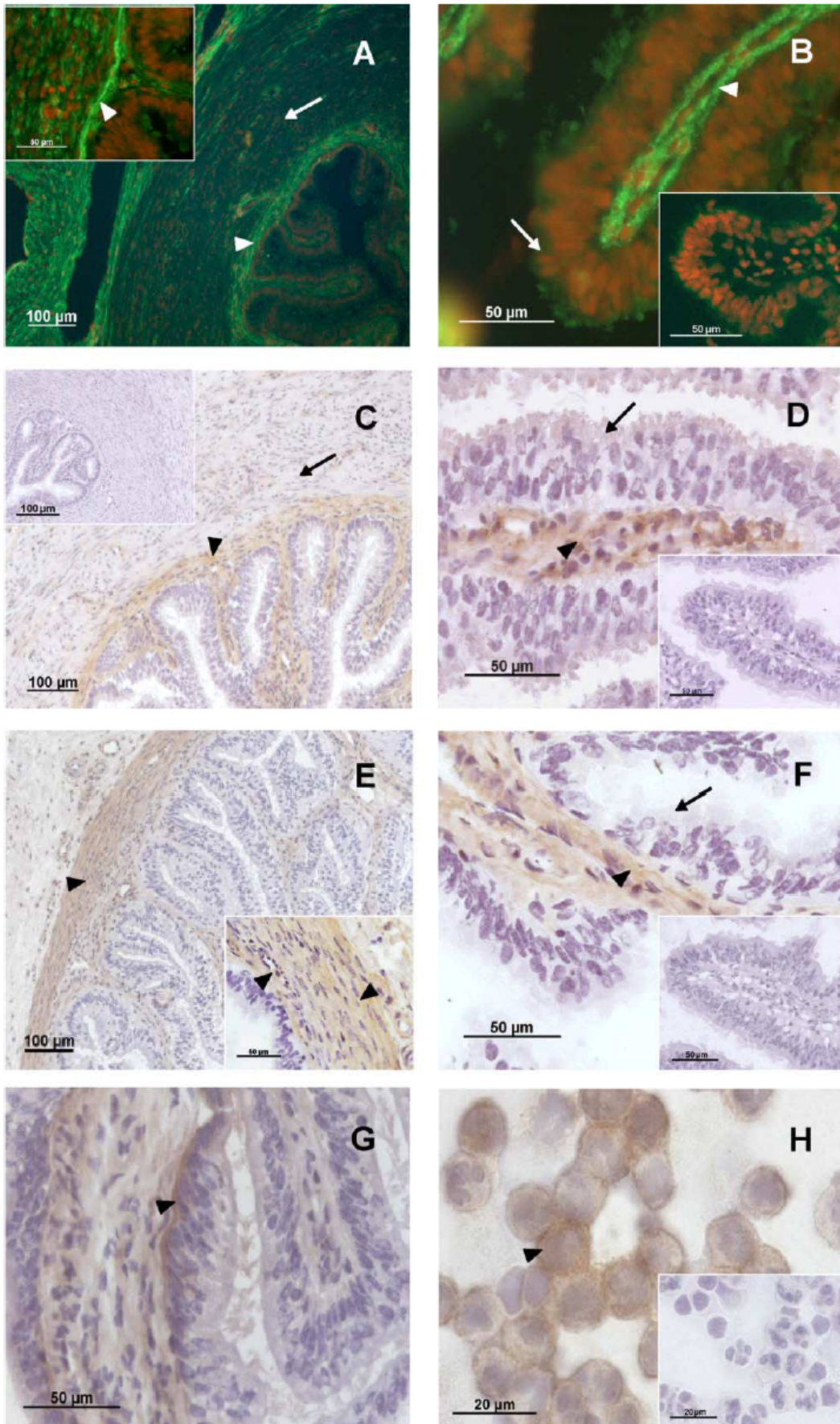
4. Discussion

In this study members of the HA-system were demonstrated in the bovine oviduct during the estrous cycle.

Using biotinylated HABP, HA could be localized to the entire lamina propria and the surrounding connective tissues of bovine oviducts. HA has previously been described in oviducts of human, pig and rodents (Edelstam et al., 1991; Tienthai et al., 2000). In accordance to Edelstam et al.

(1991), the lamina muscularis as well as the luminal epithelium were devoid of HA. We could not confirm additional epithelial HA-labeling present in the porcine uterotubal junction (Tienthai et al., 2003). The intensity of the HA staining seemed highest in the ampulla and additionally, it appeared as if the staining was most intense during the mid-luteal phase. The question therefore arose if the action of receptors and synthases were the reason for the observed variations. Salamonsen et al. (2001) proposed a steroid dependency of HA, and the oviductal fluid was found to contain HA varying during the estrous cycle (Anderson and Killian, 1994). Yet it is not known whether HA originating from HAS in bovine oviduct epithelial cells (BOEC) is de novo synthesized or diffuses from the lamina propria into the lumen. Kimura et al. (2002) have reported the presence of HAS 3 mRNA in pig oviduct epithelium, whereas in the present study using bovine whole oviducts transcripts of HAS 2 and HAS 3 mRNAs were detected. A linear regression was found to be extremely high, pointing towards a coordinated expression level of both HA synthases, which were however unregulated during the estrous cycle. Interestingly, when investigating solely BOEC, HAS 3 was expressed at a much higher level than HAS 2 (unpublished observations). So it may be assumed that there are cell specific differences of the HAS isoforms between epithelium and stroma. The absence of epithelial HA staining would favor a diffusion process from stroma to epithelium, whereas the HAS expression demonstrates the potential of the BOEC synthesizing HA. One must keep in mind that the undoubted accurate quantification of transcript levels using RT–PCR is of limited meaningfulness in the case of enzymes, because it does not provide information about the enzymatic activity. Broader studies, especially the cellular localization of HAS

Fig. 5. Localization of hyaluroran using biotinylated HA-binding protein in serial cryosections of bovine oviducts using either (A and B) strepavidin-FITC (green) or (C and D) HRP-labeled (brown) secondary antibody. Sections A (with enlarged insert) and C (with negative control insert) demonstrate stromal staining. Sections B and D exemplify the stromal staining of oviductal villi. The luminal epithelium appears without specific staining (B, with negative control insert). Using HRP-POD technique, the epithelium is clearly negative (D, with negative control insert). Immunohistochemical localization of CD44 (brown) in serial cryosections of bovine oviducts (E–G). Oviductal stromal and muscular staining is depicted (E, with enlarged insert). Section F demonstrates the stromal staining of oviductal villi (negative control in F—insert). Partially staining of the basal lamina underlying the epithelial layer of the isthmus is found (G). The specificity of the antibody was validated by using bovine leucocytes positive for CD44 (H). An isotype matched rat IgG served as negative control (H—insert). The cell nuclei were counterstained with propidium iodide (red) or haemalaun (blue). Arrowheads indicate specific staining, arrows indicate lack of staining.

isoforms, could unravel further questions. The application of exogenous HA has been shown to advance bovine IVM and embryo culture (Stojkovic et al., 2002) indicating its role in vivo and considering the oviduct as a source of HA.

Bovine oviducts expressed an important HA receptor, CD44, in a region specific manner, namely the isthmus showed a 10-fold higher expression than the ampulla. Interestingly, the histological observation of HA pointed towards intense luteal HA staining predominantly in the ampulla in opposite to highest CD44 expression at estrus, the latter being most pronounced in the isthmus. Through the ability to bind HA, CD44 is capable of assembling the pericellular matrices and internalizing it (Jiang et al., 2002). The internalization is a multistep process possibly triggered by its molecular weight (Aguiar et al., 1999) in which HA is first bound to the cell surface, then internalized, brought into lysosomal compartments and degraded (Underhill, 1992). The observed countercurrent regulation in this study could be explained by CD44 mediated internalization of HA as supposed for osteoprogenitor cells (Pavasant et al., 1994). Yet concurrent expression patterns have been found of CD44 in the epithelium and HA in the intraluminal fluid (Tienthai et al., 2000, 2003).

In the present study, it remains difficult to favor a direct interaction between gametes and the oviduct concerning the components of the ECM, because the oviduct luminal epithelium is devoid of either HA or CD44 as shown by immunohistochemistry. Studies on cultured BOEC demonstrated the presence of CD44 mRNA (own unpublished observation), therefore either protein expression levels were below detection limit or mRNA was not translated. The specificity of the antibody was verified by using bovine leucocytes. In the human a direct interaction between CD44 expressed in lateral membranes of the uterine epithelium and proteoglycans expressed on the embryo is doubted as well (Albers et al., 1995). Nevertheless, the importance of an indirect interaction via the stroma cells can strongly be suggested. Finally, because CD44 additionally binds to fibronectin, collagen and sulphated proteoglycans, the muscular CD44 staining lacking HA staining could be explained.

Since the major part of the oviduct epithelium is covered with ciliated cells, the RHAMM receptor seems to represent an interesting candidate for the regulation of ciliary beating. Towards estrus the RHAMM expression raised specifically in the ampulla, the expression in the isthmus seemed to lag behind. Since the oviductal fluidity and the transport of the oocyte and the embryo happen at estrus in the ampulla while later in the isthmus, a possible involvement of RHAMM could be suspected. In addition, in the ampulla the epithelium is lined much more with ciliated cells than the isthmus especially at estrus, assigning RHAMM a local importance in this region. The influence of RHAMM in modulating the ciliary beating of airway epithelial cells has been shown before (Lieb et al., 2000). Since BOEC express RHAMM (unpublished data), a possible epithelial protein location could be suspected. If this was the case, a direct interaction between HA from the oviductal fluid or as well from the cumulus cells and RHAMM lining the oviduct epithelium could possibly occur. The proposed mechanism of the interference of RHAMM with the cytoskeleton of the cells could modulate as well the activity of the muscle cell layer surrounding the oviduct contributing to synchronized gamete motion.

The identification of a third novel cell surface receptor for HA has been discussed controversially. The intercellular adhesion molecule ICAM-1 had previously been misidentified as liver endothelial cell HA receptor (McCourt et al., 1994; Weigel et al., 2002). Recently, the hyaluronan receptor for endocytosis HARE has been purified from rat liver endothelial cells (Weigel et al., 2003). However in parallel, Politz et al. (2002) proposed two novel genes encoding transmembrane proteins termed stabilin-1 and -2, which are thought to be HA receptor homologues. Stabilin-2 has been designated a fasciclin-like hyaluronan receptor homologue with comparable functions as HARE. In fact, the amino acid sequences between HARE and stabilin-2 are found to be almost identical. Here we demonstrate an enlarged partial bovine sequence of putative HARE (GenBank accession number AJ550060). The bovine sequence shows 86.6% respectively 80.0% homology to the known human and rat HARE sequence (Zhou et al., 2002; Weigel et al., 2003), but also 86.6% respectively 79.9% homology to the human and the mouse stablin-2 mRNA sequence (Politz et al., 2002). Whether the bovine transcript type derives from the HARE or stabilin-2 subtype remains to be unraveled, leaving the correct nomenclature tentative. Nevertheless, the physiological relevances of both appear to be alike. Up to date, HARE has not been detected in tissues other than liver, spleen and lymph node (Weigel et al., 2003). Using a specific RT–PCR approach, we were able to detect high amounts of mRNA transcripts in bovine lung, testis and endometrium, and with a lower abundance in other tissues as well. Especially, the high expression level in testis and endometrium demand for further notice.

During the estrous cycle, minor regulatory transcript changes occurred in the bovine oviduct. We can conclude that HA in the oviduct might not be directly correlated with this novel HA receptor. Nevertheless discovering putative HARE in bovine tissues including reproductive organs points towards distinct local mechanisms of a receptor-mediated turnover of HA. This will be of interest for further studies concerning the bovine ECM.

In this study, an effort to light up the origin of HA and possible local turnover causing regulations in the bovine oviduct was undertaken. Although transcripts were present a direct local regulation of HA through changing HAS expression could not be demonstrated. Nevertheless the conspicuous regulation of CD44 during the cycle and between ampulla and isthmus particularly assigns this HA receptor an important regulatory function. The regulation of RHAMM may contribute to enhanced sensibility of HA during estrus especially important for the local ciliary

beating. Detecting putative HARE, another cell surface HA receptor, in bovine oviducts adds to the presumption that the redundant HA-system may play an important role for reproductive tissue. Further investigations must consider the local varieties of stromal and epithelial cell expression. In conclusion, the results of the present study assign the HA-system an important role during the normal estrous cycle leading towards further implications on gamete development and reproductive success.

Acknowledgements

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Region Specific Expression of Nitric oxide synthases (NOS) in the bovine oviduct during the estrous cycle and in vitro

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Abstract

Nitric oxide synthases (NOS) account for the endogenous production of nitric oxide (NO), a small and permeable bioreactive molecule. NO is known to act as a paracrine mediator during various processes associated with female reproduction. In the present study the mRNA expression of the endothelial (eNOS) and inducible (iNOS) NO-synthases were examined in bovine oviduct epithelial cells during the estrous cycle. In addition, eNOS and iNOS mRNA and protein were localized by in-situ-hybridization and immunocytochemistry, respectively. Furthermore, the effects of exogenously applied estradiol-17β and progesterone on NOS mRNA regulation were studied using a suspension culture of bovine oviduct epithelial cells. eNOS mRNA was low around ovulation (day 0) and gradually increased significantly until proestrus (day 18) in the ampulla. Immunoreactive protein of eNOS targeted predominantly endothelial cells as well as secretory oviduct epithelial cells at proestrus. iNOS mRNA was significantly reduced in the isthmus at proestrus (day 18) and estrus (day 0) compared to retained high levels in the ampulla. Using in-situ-hybridization specific iNOS transcripts additionally were demonstrated in the oviduct epithelium. Immunoreactive iNOS protein was localized to secretory epithelial cells as well as the lamina muscularis. The *in vitro* stimulation showed that both NOS were stimulated by progesterone but not estradiol-17β. The region specific modulated expression of eNOS and iNOS provides evidence for a possible involvement of endogenous produced NO in the regulation of oviductal functions.

Introduction

The oviduct is responsible for the accomodation of the gametes and the early embryo by providing an optimal environment for successful fertilization. In addition, it accounts for the transport of the gametes and the embryo into the uterus. The anorganic free radical nitric oxide (NO) is a small, lable and permeable molecule which is able to pass membranes by diffusion. NO is an important intercellular regulatory molecule and a major paracrine mediator, functioning as a vascular, immune and neuronal signalling molecule [1]. NO is able to diffuse freely through membranes to react with several targets by exerting unspecific immune responses [2]. By bindig to guanylylcyclase, cGMP specific proteinases (PKG), Na-ions and phosphodiesterases can be activated leading toward modulated gene expression [3]. The effect of NO on vasodilatation has been shown thouroughly [4]. NO is supposed to be involved in a variety of different functions as well as reproductive processes such as oocyte maturation, ovulation, implantation, pregnancy maintenance, labor and delivery [5-8]. NO is produced by the conversion of L-arginine to L-citrullin by the enzyme NO-synthase (NOS) with respect to several co-factors in a number of different tissues and cell types. Up to date, three isoforms of NOS, products of separate genes with different molecular weight but apparently similar molecular structure, have been described: neuronal NOS (nNOS) in brain and peripheral nervous system, endothelial NOS (eNOS) as a constitutive NOS mainly in the endothelium and inducible NOS (iNOS) synthesized primarily by activated macrophages, hepatocytes and neutrophiles in several tissue types and organes and upon inflammatory stimulation [9]. Recently, NOS have been identified in the human, bovine and rat oviduct [10-12]. A specific regulatory role of the oviduct function has been subscribed to NOS. In this study, the expression and localization of iNOS and eNOS in the bovine oviduct of days 0, 3.5, 12 and 18 of the estrous cycle was studied in detail. In addition, the effects of the steroid hormones estradiol-17β and progesterone on NOS expression were analyzed in an *in vitro* approach.

Material and Methods

Synchronization of the Estrous Cycle and Collection of Oviduct Samples
Six cyclic Simmental heifers between 18 and 24 months old were cycle synchronized by injecting intramuscularly a single dose of 500 µg Cloprostenol (Estrumate®; Essex Tierarznei, München, Germany) at diestrus. Animals were observed for sexual behavior (i.e., toleration, sweating, vaginal mucus) to determine standing heat, which occurred around 60 h after Estrumate® injection. To confirm physiological ovulation and sexual cycling, animals were checked by ultrasound-guided follicle monitoring starting 48 hrs after Estrumate® application in intervals of 6 hrs. Three animals were slaughtered the morning after standing heat occurred (day 0) and three animals at days 3.5, 12 and 18 after estrus, respectively. Blood samples were taken at day 20 and day 0 of the estrous cycle every 6 to 9 hrs to determine serum luteinizing hormone (LH) levels and just before slaughtering for serum progesterone levels. Animals slaughtered at estrus (day 0) displayed low serum progesterone levels (< 1 ng/mL) and animals at diestrus (days 3.5 and 12) had high serum progesterone levels (>5 ng/mL), respectively. Determination of the LH level, ultrasound monitoring, and evaluation of the ovary state after slaughter revealed that samples from animals slaughtered at estrus were collected between LH surge and ovulation or immediately after ovulation. Oviducts were prepared as described previously [13], but additionally epithelial cells were collected from the ipsi- and contralateral side seperately deviding ampulla and isthmus. All experiments with animals were carried out with permission from the local veterinary authorities.

In vitro cell suspension culture
Four Simmental heifers were slaughtered on day 3.5 of the estrous cycle and BOEC were obtained as described previously [14]. Briefly, the oviducts were squeezed along the ampulla with forceps. The cell sheets were separated mechanically, by repeated passages through syringes and pipetting, and recovered by sedimentation. Cells were cultured in 24-well plates with 800µl TCM-199 supplemented with 2% cow serum from heifers on day 3.5 of the estrous cycle and 0.25 mg/mL gentamicin at a density of 10^6 cells per well at 38°C in a humidified atmosphere of 5% CO_2 in air. BOEC were stimulated with estradiol-17ß (10 pg/mL) or progesterone (10 ng/mL) (both purchased from Sigma, Deisenhofen, Germany) for 6 and 12 hours, respectively. Two animals were additionally used for a short-time stimulation of 2 hours. Cells were collected by centrifugation, washed in buffer solution, dropped into liquid nitrogen and stored at –80°C until further investigation.

Reverse Transcription and Real-time RT-PCR
Total RNA from bovine oviduct epithelial cells *in vivo* and *in vitro* was isolated using Trizol reagent (Invitrogen, Karlsruhe, Germany) according to the manufactor´s instructions. Two-step quantitative real-time RT-PCR using the LightCycler® DNA Master SYBR Green I protocol (Roche, Mannheim, Germany) were carried out as described previously [15]. Briefly, one µg of each sample of RNA was reverse transcribed in a total volume of 60 µL: 5x Buffer (Promega, Madison, USA), 10 mM dNTPs (Roche, Mannheim, Germany), 50 µM hexameres (Gibco BRL, Grand Island, USA), 200 U Superscript RT enzyme (Promega, Madison, USA). The conventional PCR were performed in a thermal cycler (Biometra, Göttingen, Germany) as previously described [16]. Seven µL of each reaction was subsequently subjected to an agarose gel electrophoresis followed by ethidium bromide staining. For each of the following real-time PCR reactions, 1µL of cDNA was used to amplify specific target genes. In each PCR reaction 17 ng/µL cDNA were introduced and amplified in a 10 µL reaction mixture (3 mM MgCl2, 0,4 µM primer forward and reverse each, 1x Light Cycler DNA Master SYBR Green I, Roche, Mannheim, Germany) compared to a standard curve based on a specific PCR product. Primers were adapted to amplify specific PCR-products for 18S rRNA (for: 5`-AAGTCTTTGGGTTCCGGG-3`; rev: 5`-GGACATCTAAGGGCATCACA-3` [365 bp]), eNOS (for: 5´-AGGAGTGGAAGTGGTTCCG-3´; rev: 5´-GCCCCGGTACTACTCTGTCA-3´ [126bp]) and iNOS (for: 5´-ACCTACCAGCTGACGGGAGAT-3´; rev: 5´-TGGCAGGGTCCCCTGTGATG-3´ [316bp]). The predicted size of each PCR product is assigned in parenthesis. The amplified PCR fragments were sequenced once (MWG, Ebersberg, Germany) to verify the resulting PCR product [17]. Thereafter the specific melting point (MP) of the amplified products served as verification of the product identity [18S (MP 88°C, fluorescence aquisition at 80°C), eNOS (MP 93°C, fluorescence aquisition at 87°C) and iNOS (MP 90°C, fluorescence aquisition at 86°C) [15]. The annealing

temperature was 60°C for 18S, 61°C for iNOS and 66°C for eNOS. As negative controls, water instead of cDNA was used. The nucleotide sequence for the partial bovine iNOS cDNA was subsequently submitted to the EMBL database (GenBank accession no. AJ699400). The cycle number (CP) required to achieve a definite SYBR Green fluorescence signal was calculated by the second derivative maximum method (LightCycler software version 3.5.28). The CP is correlated inversely with the logarithm of the initial template concentration.

Data Analysis of Real-time RT-PCR

The CP determined for the target genes were normalized against the housekeeping gene 18S. Resulting data are presented as means of CP (n=3) ± SEM. For statistical analysis the SAS program package release 9.1.3 (2002; SAS Institute, Inc., Cary, NC, USA) was used. The normal distribution of data was tested. In case of significant different groups, a multiple t-test analysis was done with Bonferroni correction. Results were indicated as statistically significant at $p<0.05$.

In-situ-hybridization

Detailed in-situ-hybridization procedure have been described previously [18]. Briefly, formalin-fixed, paraffin-embedded samples were used. Sections were deparaffinised with xylene and immersed in isopropanol. Dried sections were submerged in 2 x saline sodium citrate buffer and preheated at 80 °C. Slides were then washed in distilled water, TBS and permeabilised with 0.05% proteinase K (VWR, Ismaning, Germany) in TBS at room temperature. Sections were relocated in TBS followed by distilled water and post-fixed for 10 min in 4 % paraformaldehyde/PBS. After washing in PBS and distilled water, slides were dehydrated and air-dried. Hybridization was carried out by overlaying the dried sections with the corresponding biotinylated oligonucleotide probe (100 pmol/µl), diluted 1:20 in in-situ-hybridization solution (DAKO, Munich, Germany) and incubating them in a humidified chamber at 38 °C overnight. The sequence of the iNOS antisense oligonucleotides was 5'-TCCAGCATCTCCTCCCAGTA-3'. RNase-free hybridization solution (DAKO, Munich, Germany) contained 60 % formamide, 5 x SSC, hybridization accelerator, RNase inhibitor, and blocking reagents. Subsequently, slides were washed in 2 x SSC (2 x 15 min, preheated to 38 °C), distilled water (2 x 5 min) and TBS (2 x 5 min). Detection of hybridised probes was performed using HRP-labelled ABC kit reagents developed by DAB (DAKO, Munich, Germany) according to the manufacturer's instructions. Negative controls were done exchanging the oligonucleotide probe by the corresponding sense oligonucleotide.

Immunohistochemistry

For the immunohistochemical demonstration of eNOS and iNOS tissue samples were fixed in Bouin's solution for 12 hours as described earlier [19]. The specimen were dehydrated and embedded in paraffin. Serial sections (5 µm) were cut on a Leitz microtome and collected on gelatine/chrom alumn coated slides. To expose antigenic sites dewaxed sections were heated four-times to 95°C in a 600W microwave oven in citrate buffer for 5 min. Endogenous peroxidase activity was then eliminated by incubation with 0.5% (v/v) H_2O_2 solution in absolute methanol for 15 min at 20°C. Nonspecific protein binding was eliminated by incubation with 10% normal goat serum in PBS for 1 hr at 20°C. Sections were then incubated with either polyclonal rabbit antibody against iNOS (upstate, NY, USA) [20] or with rabbit anti-mouse antibody against eNOS (Alpha Diagnostic, TX, USA) [21;22], each at a dilution of 1:200. Incubation was performed at 18 hr at 4°C in a humidified chamber. This was followed by incubating the sections with biotinylated rabbit anti mouse IgG 1:400 (Amersham-Pharmacia) for 1 hr. The sections were then reacted with ABC reagent from a commercial kit (Vector Laboratories, Burlingame, CA). The bound complex was made visible by reaction with 0.05% 3,3-DAB and 0.0006% H_2O_2 in 0.1 M PBS. Sections were viewed unstained or counterstained in Mayer's hematoxylin, dehydrated, cleared, and mounted. Controls were performed by either replacing primary antibody with buffer or non-immune serum, or incubating with DAB reagent alone to exclude the possibility of non-suppressed endogenous peroxidase activity. Lack of detectable staining in the controls demonstrated the specificity of the reactions.

Results

A first screening of eNOS and iNOS mRNA in bovine oviduct total RNA using conventional RT-PCR led to different amplification signal intensities as shown in Fig.1. To further characterize these preliminary results a real-time RT-PCR approach was applied to quantify the gene expression patterns in oviduct epithelial cells.

Transcripts of eNOS and iNOS were detected in BOEC throughout the whole estrous cycle. A statistical analysis of eNOS/iNOS transcript concentrations from ipsi- versus contralateral side revealed no significant differences at any time point ($p=0.07$) in neither ampulla nor isthmus. Therefore ipsi- versus contralateral oviducts were grouped, so that each bar in Fig.2 represents a data set of 6 individual oviducts from 3 different animals.

The eNOS expression in the ampulla was low at estrus (day 0) and increased significantly more than 3-fold until highest levels at proestrus (day 18) (70,7 respectively 245,4 fg mRNA/µg total RNA) (Fig.2A). The same tendency was observed in the isthmus, yet here the differences between days 0 and 18 were not significant.

Highest transcript amounts for iNOS were detected in the ampulla (3680 fg mRNA/µg total RNA at day 18), with retained high levels throughout the estrous cycle (Fig.2B). At estrus (day 0) transcripts in the isthmus were almost 10-fold lower compared to the ampulla (333,8 respectively 2950 fg mRNA/µg total RNA). At day 3.5 the iNOS expression in the isthmus was already again as high as in the ampulla (3246 respectively 2657 fg mRNA/µg total RNA). It declined to an intermediate level at day 12, which was significantly lower than day 3.5 (810,7 fg mRNA/µg total RNA in the isthmus). At proestrus (day 18), there was a significant 3-fold decrease of iNOS in the isthmus compared to the ampulla (1222 respectively 3676 fg mRNA/µg total RNA).

The stimulation of BOEC with estradiol-17β had no effect on the transcript regulation of eNOS (Fig 3A). However progesterone significantly stimulated eNOS transcripts more than 3-fold two hours after application (36,7 respectively 112 fg mRNA/µg total RNA) (Fig.3A). Immunoreactive protein was observed in endothelial cells of blood vessels (Fig. 4). Additionally, supranuclear staining in secretory epithelial cells of the oviduct epithelium was visible at day 18 (Fig.4A). Nuclear and cytoplasmatic staining could be detected as well in the lamina muscularis (Fig.4B).

Estradiol-17β had no effect on the transcript regulation of iNOS in cultured BOEC (Fig.3B). But a significant two-fold upregulation of iNOS was observed 6 and 12 hours (399 respectively 904 fg mRNA/µg total RNA and 368 respectively 883 fg mRNA/µg total RNA) after progesterone stimulation (Fig.3B). The in-situ-hybridization revealed transcripts in the epithelial cells predominantly in the oviductal ampulla at proestrus and estrus (Fig.4G,H). Immunohistochemical analysis revealed a conspicious supranuclear staining in secretory epithelial cells predominantly towards the lumen (Fig.4C). The staining appeared mostly at the top and not in the basal parts of the luminal branching folds (Fig.4E). Additionally, pronounced nuclear staining of the lamina muscularis was observed (Fig.4D,F). The stroma was consistently devoid of iNOS protein (Fig.4C,E).

Discussion

This study demonstrates the presence of the two isoenzymes eNOS and iNOS in the bovine oviduct during the estrous cycle. Moreover, immunoreactive protein of eNOS and iNOS could be located in distinct cell types of the oviduct.

In congruency with our mRNA results for iNOS, the lowest expression of NADPH-diaphorase as a marker for NOS was found in the isthmus at estrus [23]. High expression was noticed during the luteal phase [23]. The presence of NADPH-diaphorase activity in the porcine oviduct has been shown during the estrous cycle. Bryant et al. [11] revealed reduced NO activity during late proestrus by measuring the conversion of L-arginine to L-citrulline in the rat oviduct, and eNOS in the rat oviduct was found most prevalently at proestrus and estrus [24]. The latter result may point towards a direct estradiol effect on eNOS, which was not confirmed in the present study for the cow. Rising levels of eNOS between estrus (day 0), diestrus (day 3.5 and 12) and proestrus (day 18) on both mRNA and protein level rather provide evidence for a dependency of eNOS on progesterone. This could further be supported by the stimulation of NOS expression in cultured BOEC.

Differences concerning the localization of NOS are stated in the currently available literature. NADPH-diaphorase was demontrated predominantly in the oviduct epithelium of rat and pig [11;23] and eNOS protein was consistently present in oviduct epithelial cells of different species [11;12;23]. In the present study we clearly demonstrate the presence of both eNOS and iNOS in oviductal epithelial cells. Previously, iNOS protein was observed in the epithelium of the human and the rat oviduct [10;11], but explicitly not in the pig [23]. These peculiarities might be suggested species differences. In the porcine oviduct eNOS was found to be the predominant isoform [23]. The present results reveal that in the bovine oviduct eNOS might represent the isoenzyme responsible for the constitutive presence of low levels of NO.

NADPH-diaphorase as well as eNOS were demonstrated in the myosalpinx of the rat and pig [11], and iNOS was additionally found in smooth muscles of the human oviduct [10]. The relaxing effect of NO on smooth muscles, possibly controlled by progesterone [25], is well-known, particularly for uterine quiescence during pregnancy [26]. The motility patterns of the oviduct show rising frequency and amplitude of motility around estrus [27]. Therefore, several mediators of contraction could be involved in this regulation influencing or orchestrating the NOS expression in the oviduct, namely estradiol, oxytocin, prostaglandin $F_{2\alpha}$, prostaglandin E_2 and endothelin-1 [28-32]. Prostaglandine $F_{2\alpha}$ ($PGF_{2\alpha}$) can induce the NO-production by NOS in rat oviduct cells [30]. Highest concentrations of $PGF_{2\alpha}$ receptors were found around estrus in the rat [33], and estradiol is known to activate prostaglandin synthase. NO could therefore negatively modulate or antagonize the contractile response of $PGF_{2\alpha}$. Furthermore, NO participates in the release of prostaglandine E_2 (PGE_2) [31]. PGE_2 is increased at estrus [34] and has been made responsible for the relaxation of the oviduct in the presence of progesterone [23]. Subsequently, PGE_2 together with progesterone could then regulate NOS expression [35], which agrees with the *in vitro* findings of this study. Endothelin-1 (ET-1) is high in the ipsilateral oviduct during the follicular and post ovulation stage [34]. Via endothelin receptor beta ET-1 stimulates NO in BOEC [32]. NO reduces the contractile effects of ET-1, and hence the interplay of ET-1 and NO might contribute to the physiogical relaxation of the oviduct.

Using L-NAME (N-nitro-L-arginine methyl ester), a well-known inhibitor of NO-synthases, Perez and co-workers [36] found evidence for increased tubal motility that resulted in accelerated ovum transport into the uterus. Moreover, estradiol treatment caused an increasing contraction frequency of the smooth muscle of the isthmus [28]. This could be made possible via estrogen receptor β, which is more abundant in the isthmus [37]. Therefore, the endogenous local downregulation of iNOS in the isthmus could support similar effects. Our hypothesis therefore is that the downregulation of iNOS at estrus in the isthmus leads to an increase of oviduct motility by circular smooth muscles activity.

There is evidence that the accelerated movement of microspheres through the isthmus is due to peristaltic smooth muscle contractions and not due to ciliary activity, supported by the observation that there is a reduced number of ciliated cells in the isthmus [30]. Moreover, the ciliated epithelial cells were mostly devoid of iNOS protein, whereas the lamina muscularis was pronouncedly stained.

In secretory epithelial cells of the oviduct the nucleus is shifted towards the apical side of the epithelium (F. Sinowatz, personal communication). Therefore, both eNOS and iNOS protein targeted mostly secretory epithelial cells. The supranuclear region towards the oviduct lumen was found to express iNOS, therefore it might be assumed that NO is released into the lumen. It might be of further

interest weather the produced NO is also secreted towards the stroma and myosalpinx. The contribution of NOS might be important for a relaxation together with a local exertion during (pro-) estrus facilitating capture, retention and fertilization of the released oocyte and the active transport of the conceptus [24].

Furthermore, through the ability of iNOS to produce large amounts of cytotoxic levels of NO [38], the downregulation of iNOS in the isthmus at estrus could be an implicit protective mechanismen for advancing sperm and the developing embryo.

The present study does not take into account further regulations of the NOS enzyme activities. Especially the proposed differences in enzyme activity of both NOS isoforms [39] cannot be measured through expression analysis solely. Nonetheless this investigation provides new data on a local NO-regulating mechanism in the bovine oviduct.

In summary the present results hold evidence for the presence of NOS derived NO in the bovine oviduct. The region specific modulated mRNA expression patterns of eNOS and iNOS during the estrous cycle give evidence for a local regulatory system of NO in the bovine oviduct. A different importance of NO in the ampulla and the isthmus region may be deduced. Although functional analyses still remain to be done, the conspicious downregulation of iNOS at estrus in the isthmus demands for further notice. NO might represent another important local factor regulating oviducal functions with possible impact on contractility and immune response. The present findings underline a physiological importance of both NOS in supporting a successful fertilization by regulating the oviduct environment.

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Figure legend

Fig. 1: mRNA transcripts of the housekeeping gene 18S and the target genes eNOS and iNOS in the bovine oviduct during the oestrus cycle exemplified by conventional RT-PCR. One representative out of five independent experiments is shown.

Fig. 2: mRNA expression (real-time RT-PCR) of iNOS and eNOS in bovine oviduct cells during the estrous cycle in either ampulla (■) or isthmus (□). Data are presented as means of mRNA/total RNA ± SEM normalized by 18S. * indicates significant differences between ampulla and isthmus ($p < 0,05$). Different superscript letters indicate significant differences between days of the estrous cycle ($p < 0,05$).

Fig. 3: mRNA expression (real-time RT-PCR) of iNOS and eNOS transcripts in a bovine oviduct epithelial cell suspension culture after stimulation with progesterone or estradiol-17β. Data are presented as means of mRNA/total RNA ± SEM normalized by 18S. * indicates significant differences between control and stimulation ($p < 0,05$).

Fig. 4: Immunohistochemical localization of eNOS (A,B) and iNOS (C-F) in bovine oviducts and in-situ-hybridization of iNOS (G,H) in bovine oviducts (brown staining). Black spearheads point at specific immunopositive secretory epithelial cells. Arrows point at positive cell staining in the lamina muscularis. The black bar indicates 50µm length.
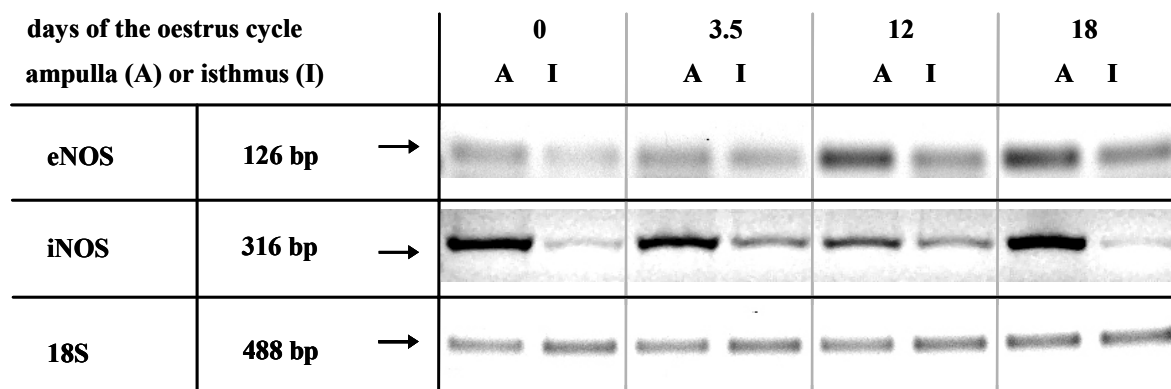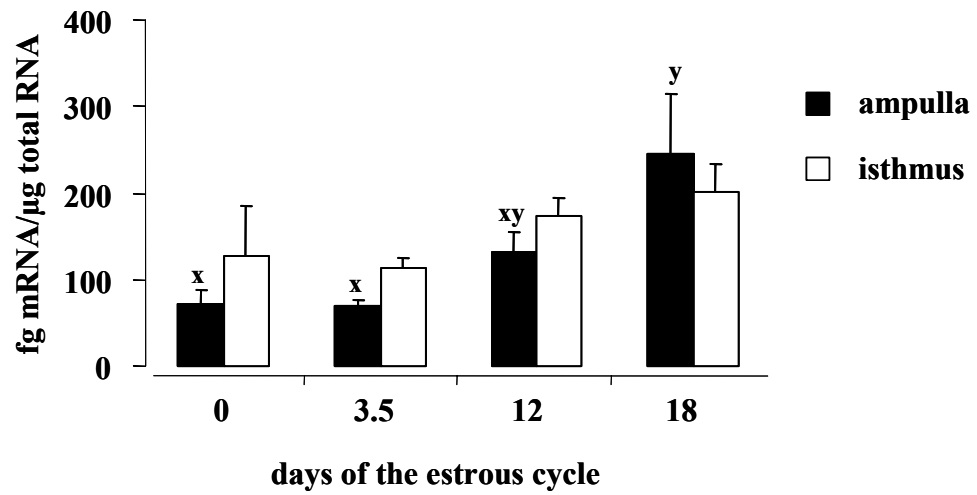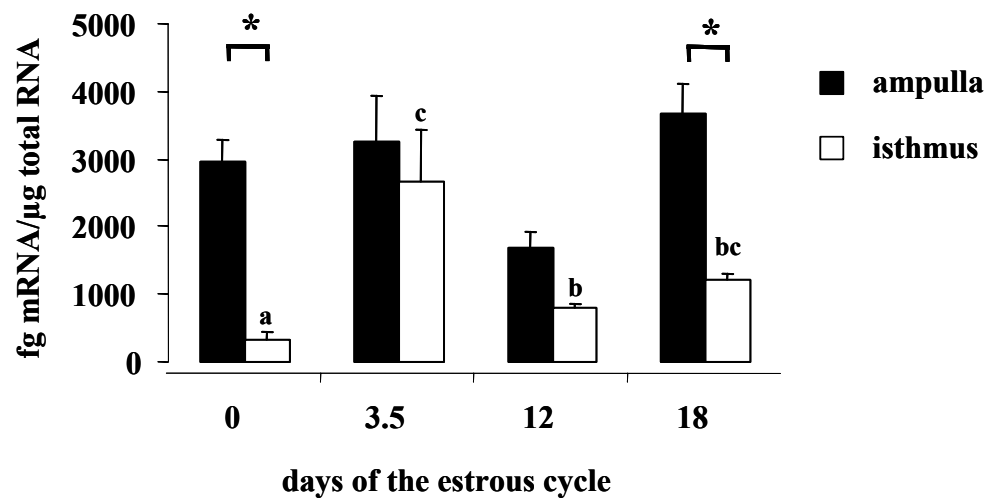
Fig. 1

| days of the oestrus cycle | | 0 | | 3.5 | | 12 | | 18 | |
ampulla (A) or isthmus (I)		A	I	A	I	A	I	A	I
eNOS	126 bp →								
iNOS	316 bp →								
18S	488 bp →								

Fig. 2

(A) eNOS

days of the estrous cycle

(B) iNOS

days of the estrous cycle

Fig. 3

(A) eNOS

(B) iNOS

Fig. 4